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Isolation of Iso-α-Acids (II): Centrifugal Partition Chromatography

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Introduction

The isolation of the pure individual iso-\(\alpha\)-acids using a combination of a CPC (Centrifugal Partition Chromatography) separation of \(\alpha\)-acids from hop extracts, followed by DCHA precipitation of the trans-iso-\(\alpha\)-acids to separate them from their cis- counterparts was successful but not efficient in terms of the possible yield.

Another possible strategy for this separation was the use exclusively of CPC for the separation of the iso-\(\alpha\)-acids. Though it could appear unlikely that a liquid-liquid chromatographical method could separate stereoisomers with apparently similar physical and chemical characteristics, there were many reports of successful separation of all types of compounds.

In order to develop the method, the chosen approach was to start with the separation of the three isomers of each series (trans- and cis-iso-\(\alpha\)-acids) and then use the acquired experience to attempt the separation of all six isomers.

The method did indeed prove to allow us to obtain gram-scale amounts of both stereoisomers of isoohumulone and isocohumulone, and lower amounts of the isoadhumulone isomers, owing to its low presence in the plant. The highest yields were obtained, however when all cis- or all trans-iso-\(\alpha\)-acid mixtures were injected separately.

The development and optimisation of the system are described below.

4.2 Centrifugal Partition Chromatography (CPC)

A brief description of this technique is provided as a background to the method developed in the laboratory.

Centrifugal Partition Chromatography is a preparative liquid / liquid chromatographic technique based on the partition of the components of a sample between two immiscible liquid phases. This technique that was promoted originally by Ito in 1964, evolved from CCD (counter current distribution) developed by Craig and Post (1949). The latter, though successful in principle, had some disadvantages, such as low reproducibility and long separation times. Preparative LC therefore, not surprisingly replaced it during the 1960s,
greatly due to the improvement of instrumentation and available stationary phases. However, as much as this technique was improved, some basic problems derived from the use of solid supports, such as its relatively low loading capacity which results in tedious, time-consuming procedures or the high back pressure usually generated by low particle size stationary phases needed for high resolution, still prevailed. Additionally, when used to obtain pure, usually minor compounds from a complex matrix such as natural products, retention of other components of the mixture and their late elution could become a real nightmare. Therefore, the development of technology based on liquid/liquid partition continued and in 1970, Ito launched a method based on counter current separation in a continuous mode that finally became commercially available (Ito et al., 1970, 1988, 1992, 1996a, 1996b; Conway, 1996). This method, known as counter current chromatography (CCC) combined the positive aspects of counter current distribution and liquid chromatography. The first method, known as "droplet counter current chromatography" (DCCC) used unit gravity to move the droplets of mobile phase through the column of stationary phase in a tube-like space (Ito, 1970; Tanimura et al., 1970). The commercial instruments introduced produced an unfavourable impression of the CCC technique because though highly efficient, they proved to be time-consuming. However, continuous development led to the much more efficient centrifugal CCC schemes that use a centrifugal force created by a planetary motion of coiled columns (Ito, 1996) that keep the liquid phases in the column, thus providing support-free liquid/liquid chromatography in which the stationary phase occupies between 60 and 80% of the column volume.

Two slightly different applications appeared as Ito continued his work in USA developing what is known as CCC using a variable-gravity field produced by a two-axis gyration mechanism and a rotary seal-free arrangement for the column, while Nunogaki in Japan, with contributions from Ito, developed what was became to be known as a "centrifugal partition chromatograph" (CPC) which uses a constant-gravity field produced by a single-axis rotation mechanism and two rotary-seal joints for inlet and outlet of mobile phase (Foucault, 1998).

Ito went on to invent and improve numerous designs of the two-axis CCC instruments to which the term "counter-current" was reserved (Marchal et al., 2003), achieving an ideal combination of the mode of planetary motion and the geometry of coiled columns on the holder, which led to "high speed CCC" (Ito, 1996) –HSCCC-. The contributions of Ito have resulted in the publication of more than 200 papers containing different applications and theoretical considerations and several books (Foucault et al., 1998).
As for CPC, instead of coils, the "column" consists in a series of channels linked in cascade by ducts and aligned in cartridges or disks in a circle around a rotor. The term CPC was adopted as a generic name for this instrument in the first symposium organized by Sanki in Kyoto in 1986, replacing CCCC because as one of its first users at the time, Dr. Bruening, reflected, it was not really a "counter current" method since the stationary phase was actually fixed to the walls of the channels, that is, it was really immobilised (Marchal et al., 2003).

As a liquid–liquid separation technique, CPC offers distinct advantages for the separation, isolation, and purification of unstable compounds from complex matrixes such as plant material compared to traditional liquid–solid separation methods, as exemplified by normal column chromatography and/or high-performance liquid chromatography (HPLC). CPC does not require the use of a solid support as the stationary phase. Samples are introduced into the system and retained in the liquid stationary phase from which they are eluted according to their affinity with the liquid mobile phase. Thus, once all components of interest are eluted, any highly retained components of the mixture will remain in the stationary phase that is discharged. This is of course, not possible with expensive preparative HPLC columns, which have to be used repeatedly with the risk of contamination of future samples with highly retained analytes.

Any two-phase solvent system may be used; many partition systems can be prepared with non-toxic commonly available solvents. The volume ratio of the stationary phase to the total column (rotor) volume is greater in CPC than in conventional liquid chromatography. Therefore, large quantities of sample materials can be retained in the stationary phase.

Decomposition of valuable sample components, often encountered when using conventional packed chromatographic columns, is virtually non-existent under the mild operating conditions used in CPC. Further advantages of CPC are the low solvent consumption and the fact that the same solvent pair can be used for reversed-phase elution. CPC can readily be adapted for large-scale continuous separations. The entire process is performed in the liquid phases, in a closed system. Environmental problems are minimal, and solvent may be completely recovered and recycled.
Instrumentation

The instrument used for this research was the CPC instrument manufactured by Sanki Engineering Ltd. (Kyoto, Japan). The basic components of the unit are shown in Figure 1. Similarly to most chromatographic systems, CPC has basic components such as a pump for solvent delivery, a valve to control solvent delivery, a preparative sample injector and detector. Additionally, it is equipped with some type of recording system and a fraction collector at the output to collect the eluate. The main component of the instrument is its rotor. The rotor is made by stacking engraved polyphenylene sulfide (PPS) or polychlorotrifluoro-ethylene (DAIFLON) disks, separated by Teflon seal sheets and stainless steel plates. The rotor used has a disk pack with 2136 partition channels for a volume of 230 ml; a rotational speed of a maximum of 2000 rpm and a backpressure of 60 bar. The liquid stationary phase is held in these channels and the
centrifugal field generated by the spinning rotor holds the stationary phase effectively, thus enabling the mobile phase to be pumped through.

**CPC settings**

When using a conventional system, CPC operation involves the initial pumping of the stationary phase solvent into the channel while the rotor is spinning at low speed (~300 rpm), followed by introduction of the mobile phase solvent at the rotation speed required for the separation. Rotating seals at the upper and lower axes of the rotor (Figure 1) allow the passage of solvent into the apparatus. When a steady flow of the mobile phase exiting the instrument is established, the sample is introduced to the channels through the sample loop, and the separation is performed.

The rotational speed of the instrument can be varied. Although higher speeds generally lead to a better resolution, the backpressure in the instrument may increase. The most frequently used rotational speed varies between 800 and 1000 rpm, depending on the specific gravity of the solvents.

The flow of the mobile phase and temperature of the system are important settings. The pressure limits the flow rate. The temperature should be set as high as possible since it increases solubility of sample, reduces the pressure due to its effect on solvent viscosity and increases resolution. It is limited however by the boiling point of organic solvents.

**Chromatographic phases**

The chromatographic separation is carried out in a biphasic liquid system consisting of two immiscible phases that might be composed of pure organic solvents or water or more frequently of mixtures of these or even aqueous buffers, acid or basic solutions or complexing agents among others. Actually, the composition of these phases is only conditioned by a few factors, i.e., they must be immiscible, not form emulsions, and preferably have a low viscosity to reduce system backpressure. Aside from these physical considerations, the choice will depend basically on solvent/analyte interactions.

The correct choice of solvent system is naturally of utmost importance for a successful CPC separation. Selection of a two-phase (biphasic) solvent system for CPC is similar to choosing the solvents for other chromatographic methods such as for column chromatography or HPLC as recommended by A.P. Foucault (1998). Important criteria are the polarity of the sample components and their solubility, charge state, and ability to form complexes, etc. The critical points in selecting solvent systems for CPC are twofold; one is sample solubility and the other are the partition coefficients of the molecular species that are to be separated, which must differ from each other.
In developing a solvent system for CPC, the following chromatographic parameters have to be optimised in order to get an adequate resolution: efficiency, selectivity and loading capacity. The efficiency of a chromatographic separation strongly affects the resolution, as the latter expresses the separation of compounds achieved in a certain period of time.

The efficiency of a CPC method however, if evaluated by the number of theoretical plates, is still far from that encountered in HPLC. However, we should not forget that the goal when developing a preparative method is the resolution between peaks. Therefore, the parameter that has to be optimised is the selectivity of the solvent system ($\alpha$) that should be as high as possible in order to counterbalance this low efficiency. We must always bear in mind that the objective of these separations is to obtain pure compounds with acceptable yields and this will only be achieved with high resolution (Foucault et al., 1998).

The loading capacity is related of course, to the physical volume of the CPC, but having fixed that, the rotation speed will affect the amount of stationary phase that will remain in the CPC at the operating flow and thus the loading capacity.

**CPC operation**

Any of the liquid phases can be used as a stationary/mobile phase. The solubility of the sample and the compounds to be separated will define which of the two phases will be used as the stationary phase and which will be used to elute these. This is because any of these phases can be loaded into the system and kept immobilised inside the "column" by the centrifugal force generated as it rotates. This rotation is driven by a motor, allowing variable rotating speeds which will affect the amount of stationary phase deposited in the inside of these cells. CPC's have a variable loading capacity, from 200 ml to several litres, according to the "rotor" used.

The mobile phase is pumped through the system continuously, the compounds eluting at different volumes according to the affinity -partition coefficients- for either phase. At the same time according to the density of the liquid phases and to which will be the stationary and which the mobile phase, the direction of the flow must be defined.

**Solvent system choice**

The separation mechanism involved in CPC is a liquid/liquid partition of a solute between two immiscible solvents (mobile and stationary phases). The relative proportion of solute passing into each of the two solvent phases is determined by the respective partition coefficients. The solvent system should ideally result in a partition coefficient
(K) of 1 in relation with the solutes of interest. From a practical viewpoint, \(0.2 < K < 5\) can be used without excessive elution time and band broadening.

Many strategies for solvent system selection have been described in literature. Sorensen and Arlt (1998) created a series of ternary diagrams of solvents on which they based a very useful and successful strategy. In these diagrams the relative proportions of the three solvents and the resulting amount of phases formed (one or two) is displayed. Most systems conform to what they named type 1, comprising one solvent miscible with two other immiscible solvents. A typical example for the type 1 ternary system is chloroform/methanol/water. Very few systems conform to what they named type 0, made with three solvents fully miscible by pairs, but for which a zone exists in the ternary diagram where a biphasic system occurs when mixing them in a suitable ratio. A good example of this type of system is water/dimethylsulfoxide/tetrahydrofuran.

Foucault (1995) suggested a strategy to follow when using ternary diagrams for the selection of solvents:

Most solvent systems are type 1, which means that there is one solvent that is soluble in the other two chosen solvents, that is, it will partition into them in much the same way as we would wish our sample to do so. This solvent, which is usually represented as the ordinate in these diagrams, should completely dissolve the sample. CPC has a preparative goal, and so the final mixture of solvents must be able to dissolve large amounts of sample. Thus, it should contain at least one of the “best” solvents capable of solubilising the sample easily.

We must then partition this solvent between another two solvents in order to build a biphasic system; that is, we should look for a ternary system where our “best” solvent is the solvent 2, represented as mentioned above on the ordinate.

The less polar fraction of the sample will preferentially go into the less polar phase and the more polar fraction will preferentially go into the more polar phase, so the average partition coefficient will stay around 1.

Aside from this, a number of multi-solvent systems were developed. One was developed by Oka (1991), who chose 5 solvents: n-hexane, ethyl acetate, n-butanol, methanol and water, the mixtures of which resulted in binary systems of different polarities and degrees of lipo/hydrophilicity according to their qualitative and quantitative composition. In order to choose the adequate binary phase, the polarity of the compounds of interest in the sample should be matched with the polarity of the binary system.
Another approach using mixtures of n-heptane, n-butanol, methanol and water (HBMW approach) developed by Foucault et al., (1994), who found that mixtures of these solvents could cover a wide range of polarities.

Abbot and Kleiman (1991) proposed a very useful method they called a multisolvent systems approach. This system divides the solvent system into three main groups according to their predominant lipo/hydrophilicity. They classified these groups as lipophilic, intermediate, and polar (hydrophilic) systems as shown in Table 4.1. This system became probably the most used guide when developing CPC solvent systems and was successfully applied by us for a great amount of applications.

All the above methods have been applied very successfully to the separation of neutral compounds. However, if the isomers are basic or acidic, clearly the separation
strategy will have greater chances of success if based on the different solubility of the ionised and non-ionised species that will be achieved at different levels of pH even with slight differences in their pKa or pKb values.

Two different approaches were developed and described for separations of weak acids and bases, displacement chromatography and pH-zone refining chromatography.

4.2.1 pH-zone refining CPC

The use of CPC to obtain pure compounds has many advantages as discussed above. However, when these compounds are extremely similar as regards chemical behaviour and properties as is the case of isomers, their separation in liquid/liquid systems is a real challenge and any differentiating characteristic should be fully exploited in order to help achieve the separation. Thus, when the compounds to be separated are ionisable, the use of a technique based on the ionisability of compounds naturally associated to the difference in hydrophilicity/hydrophobicity of the ionic/molecular species can provide an opportunity to achieve the separation.

Among the many contributions made to counter current chromatography, Ito introduced the concept of pH-zone-refining mode in CCC as a variant of displacement chromatography (Weisz et al., 1994; Ito et al., 1995,1996), based precisely on the principles outlined above. It proved to be very useful for the separation and/or purification of organic compounds which are weak acids or bases and thus ionisable according to the pH of their environment, for example, amino acids (Weisz et al., 1994), Amaryllis alkaloids (Ma et al., 1994; Ito, 1996) Vinca alkaloids (Ito, 1996) and structural (Ma et al., 1994; Ito, 1996) and stereoisomers (Denekamp et al., 1994;Ito et al., 1995). In the case of ionisable compounds, the ionic species is more hydrophilic than the non-ionised form, so that their partition in a biphasic phase can be greatly modified with changes in the pH of one or both of these phases within the ± 1 range of the pKa of the analyte. In pH- zone refining mode, a mixture of acidic compounds is injected in the organic stationary phase that contains an acid that is stronger than all the compounds to separate, for example. This stronger acid is known as retainer acid. The acids are transported along the column when extracted from this stationary phase by the mobile phase which being a basic aqueous solution (the displacer) can ionise and thus solubilise the weak acids. Pure products are thus eluted in the effluent as salts. The system is used in the descending mode, if, as is usually the case, the density of the aqueous mobile phase is greater than the density of the organic stationary phase. The idea is quite clear: ionisation of the acid compounds is suppressed in presence of the strong acid that thus
“retains” them in the lipophilic organic stationary phase. The mobile phase is an aqueous basic solution that can be programmed to run as such or as a pH gradient; the compounds in the stationary phase will be displaced into the mobile phase as they become ionized according to their pKa, eluting in order of decreasing pKa as they form salts with the eluter. A very useful characteristic of displacement chromatography also shared with pH-zone refining chromatography, is the release of the components of the mixture from the stationary phase in contiguous clearly defined blocks arranged according their pKa/pKb values and also partition coefficients. In a block, the concentration of the different species (and thus the pH) is constant and fixed by the acid–base and partition equilibrium constants. The overlap between composition (in principle, should be pure), leading to much higher yields due to absence of overlapping.

As to solvent choice, Ito proposed a system (Ito et al., 1996) based on the standard HSCCC technique, which allows an easy way to start the selection of pH zone-refining CPC system as shown below in table 4.2.

<table>
<thead>
<tr>
<th>Solvent 1</th>
<th>Hexane/Ethyl Acetate / Methanol / Water</th>
<th>lipophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 : 0 : 5 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 : 1 : 5 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 : 2 : 5 : 5</td>
<td></td>
<td></td>
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<tr>
<td>7 : 3 : 5 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 : 4 : 5 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 : 5 : 5 : 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent 2</th>
<th>Methyl-tert-butylether/ Butanol/ Acetonitrile/ Water</th>
<th>hydrophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 0 : 0 : 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 : 0 : 1 : 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 : 0 : 3 : 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 : 0 : 2 : 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 : 2 : 3 : 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 : 2 : 1 : 5</td>
<td></td>
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</tbody>
</table>

The systematic search of a solvent system is firstly defined from the partition coefficient of the solute in a base solvent (K base) as ideally determined to give value \(<\) 1. Secondly, equal amounts of acid, typically TFA, are added to the solvent system for low
pH values. A partition coefficient greater than 1 is expected to provide the best conditions for separation. If K base is not small enough the procedure should be repeated using a less polar system. If K acid is not large enough, a more polar solvent system should be attempted.

How well do these tests represent what will happen later on in a CPC system? In our experience these tests were very time-consuming and did not, perhaps predictably, necessarily have much relation with the behaviour of the sample in the analysis conditions. This applied especially to stability and to resolution. Therefore our work was mostly carried out by a trial and error approach based on our own experience in liquid/liquid separations for selectivity and the use of solvent-systems suggested in literature.

The visualisation (monitoring) of the eluting fractions using a detector such as UV, however might be quite difficult, especially when the compounds to separate all have a similar UV absorption wavelength. Spraul et al. (1997) used NMR monitoring for example, for the separation of amino acids. However, if fractions are collected at relatively low volume intervals, it is possible to use HPLC analysis of the fractions even if it is quite time-consuming.

### 4.3 CPC separation of iso-α-acids

The idea of separating cis/trans isomers by liquid/liquid chromatography, that is, using a technique based on the partition of compounds between two immiscible phases, seemed extremely challenging in principle. It was necessary to work on the differences between cis- and trans- isomers in the design of the solvent system. As mentioned before, the two major differences between the cis/trans series were the capacity of the latter to form insoluble complexes with DCHA and secondly, their pKₐ values. Other differences such as their relative hydrophobicity were also considered.

Hughes (1996) evaluated the hydrophobicity of iso-α-acids according to two parameters, i.e., their partition coefficient in octan-1-ol and aqueous phosphate buffer (pH 5.0) and their behaviour in a reverse-phase HPLC column. According to this, trans-iso-α-acids had a slightly lower partition coefficient than cis-iso-α-acids (1.99 vs. 2.23), indicating that the latter were more lipophilic. In the case of the reverse-phase HPLC capacity factor, measured with a C18 stationary phase and methanol:0.025% H₃PO₄ (30:70), the lipophilicity increased in the following sequence: cis-isocohumulone < trans-isocohumulone < cis-isohumulone < trans-isohumulone < cis-isoadhumulone < trans-isoadhumulone. This difference in capacity is quite large considering that it ranges from 2.93 to 5.83, that is, practically double (see Table 2-5).
The other difference that could be exploited was their relative acidity. There was no complete conclusive information on individual pKₐ values. According to Verzele (1991) the pKₐ of cis-isohumulone, trans-isohumulone and cis-isocohumulone are approximately 3.0 (De Keukeleire et al., 1992), so that the difference between the Kₐ of each acid is probably quite insignificant in water. Simpson (1993) determined a pKₐ of 3.1 for trans-isocohumulone, though he found remarkably different values according to the method used for its determination. He associated this different ionisation behaviour to the formation of covalent hydrates in water. A strategy based on difference of acidity alone could not solve the problem but, considering the difference in solubility between the non-ionised/ionised species, it could be very useful to combine both characteristics and thus separate the compounds.

**Different approaches used for the separation**

Initially, a conventional CPC separation was attempted and several solvent systems were tested. Although some success was achieved as will be shown, it was clear that using mixtures of organic solvents and water was insufficient.

**Effect of DCHA**

Considering the first obvious difference between stereoisomers, DCHA was added to the mobile phase in an attempt to delay the elution of trans-isomers, which, if complexed with DCHA to form the more lipophilic DCHA-trans-iso-α-acid complexes would be easily separated from their cis-counterparts. However this did not help at all because the formation of these complexes does not occur readily, but rather in certain specific conditions (anhydrous medium) and requires time.

These compounds seemed to be the right candidates for the type of CPC separation described above, known as pH-refining zone CPC. Several authors have reported very good results with this method, for example, Spraul et al. (1997) who separated a mixture of three amino acids or in the case of basic samples, the separation of Catharantus indole alkaloids (Renault et al., 1999). Other CCC pH-zone refining applications were mentioned above.

The separation of each series of iso-α-acids from isomerized α-bitter acid extracts was attempted to begin with. Thus, initially, mixtures of the cis-iso-α-acids and mixtures of trans-iso-α-acids obtained by the DCHA precipitation method similar to that described in Chapter 3, but using an isomerised hop acid mixture. Later on, when it became possible to obtain all trans-iso-α-acids and all-cis-iso-α-acid mixtures easily
following the method described in Chapter 5, the individual iso-α-acids were separated with better yields and purity.

Initially based on the systems of solvent choice described above, three different solvent mixtures were tested. Attempts to optimise each one were made until finally one biphasic system consisting of hexane: methanol: water (10:5:5) was found to successfully separate mixtures of all three cis- and trans iso-α-acids separately. Once these methods were optimized, the separation of all iso-α-acids from isomerised extracts was attempted.

The experimental work leading to the separation of the compounds in these three cases is described below.

4.4 Isolation of cis-iso-α-acids

4.4.1 Materials

Iso-α-acids extract was obtained from Heineken Brewery, Zouterwoude, Netherlands. The extract contained 20% iso-α-acids mixture (iso-cohumulone, iso-humulone, iso-adhumulone). Ethyl acetate, ethanol, n-hexane, methanol and toluene were purchased from Biosolve Co. Ltd. (Valkenswaard, The Netherlands). Trifluoracetic acid and diclyclohexylamine (DCHA) were purchased from Sigma Aldrich (St. Louis, USA) and anhydrous sodium sulphate, hydrochloric acid, o-phosforic acid and sulphuric acid were obtained from Merck (Darmstadt, Germany).

4.4.2 CPC instrument conditions

The CPC experiment was conducted using a modular Sanki (Kyoto, Japan) Centrifugal Partition Chromatograph (type LLN). It consists of a power supply (model SPL), a centrifuge (model NMF), a loop sample injector plus flow direction (model FLU-V), supported with a 5.0 ml loop and a triple head constant flow pump (model LBP-V). CPC apparatus was connected to UV/Vis 200 detector (Linear Instruments, Reno, NV, USA) and used Panasonic pen-recorder (Model VP-67222 A) to record the graphic of absorbance. Fractions were collected by means of LKB 17000 Minirac fraction collector. In all experiments 6 CPC cartridges were used, total internal volume was 125 ml. The pressure was limited to 60 bars.

4.4.3 Preparation of sample of cis-iso-α-acids

Approximately 20 ml of the isomerized hop extract were treated with H2SO4 30% in excess until appearance of yellowish oil corresponding to free iso-α-acids. This was
extracted with toluene (3 x 20 ml) and the toluene fractions were pooled and dried with anhydrous Na₂SO₄. After removal of toluene with a Rotavapor (model Buchi, Switzerland) the residue was weighed and analysed by HPLC.

To separate cis-iso-α-acids from their trans-counterparts, the DCHA precipitation method was used (Thornton et al., 1993). For this, 16 g of yellowish oily iso-α-acids were dissolved in 80 ml ethyl acetate and 8.79 ml of DCHA were added and stirred for approximately 1 hour. The solution was dried with anhydrous Na₂SO₄ and vacuum filtered into a brown bottle. The solution was stored at 4°C for three days in the darkness for total precipitation.

After this time, the sample was vacuum filtered to separate the DCHA-trans-iso-α-acids from their cis-counterparts in the supernatant.

To obtain the cis-iso-α-acids, HCl 6M was added to the supernatant, giving a white precipitate. The solution was extracted with toluene (3x20 ml) and the resulting solution was dried with anhydrous Na₂SO₄ and taken to dryness with a Rotavapor. Before injecting the sample it was analysed by HPLC to determine its quali- and quantitative composition.

4.4.4 Preparation of two-phase solvent system and sample solution

The solvent systems for the CPC experiments were prepared as follows:

- System I: Appropriate volumes of toluene and water for a (3:1) proportion were mixed and stirred for 1 hour and then left to decant. The phases were separated and the aqueous lower phase was used as the mobile phase while the upper organic phase was used as the stationary phase.

- System II: Appropriate volumes of toluene, ethanol and water (50:12:13) were mixed and stirred in a 2000 ml flask for 1 hour and left to decant. The phases were then separated and the aqueous lower phase was used as the mobile phase while the upper phase was used as the stationary phase.

- System III: Equal volumes of hexane, ethyl acetate, methanol and water were mixed to give a (1:1:1:1) ratio. After stirring for 1 hour, the mixture was left to settle for 2 h in order to achieve a full saturation of the aqueous phase. The stationary phase was the upper organic layer and the mobile phase was the aqueous lower layer.

- System IV: a mixture of hexane-methanol-water (10:5:5) was stirred in a 2000 ml flask for 1 hour and left to decant until two phases were clearly formed. These were then separated using a separating funnel. The lower layer was used as the mobile phase, while the upper layer was used as the stationary phase.
In all cases, between 0.01 and 2% of trifluoroacetic acid (TFA) was added as a retainer to the upper layer - stationary phase. Initially, a sufficient amount of ammonium hydroxide was added to the aqueous (lower) layer to achieve a concentration of 0.05% as an eluter. In further experiments the amount of ammonium hydroxide was increased during the elution up to 0.1%.

4.4.5 Separation procedure

CPC was run in the descending mode with organic phase as the stationary phase and the aqueous layer as the mobile phase. The CPC column was first entirely filled with organic phase. A 5 ml sample (containing approx. 500 mg of cis-iso-α-acids) was injected and the mobile phase was pumped into the system at a flow rate of 2 ml/min and a centrifugation speed of 900 rpm at 23 °C.

The eluent from the outlet of the column was continuously monitored with an UV detector and registered using a pen-recorder. The eluent was collected in the fraction collector at 3 minutes intervals (9.0 ml / tube). The pH was monitored manually.

4.4.6 HPLC analysis of fractions

Fractions collected from the CPC were analysed using a Waters HPLC consisting of a 626 pump, a 2996 PDA detector, a 717 plus autosampler and Waters Millenium data processing software.

The chromatographic system consisted of a Hypersil C18 column (150 x 416 mm-5) and 10 µL samples were eluted with a linear gradient of two solvents: A: 1% H₃PO₄ in water and B: acetonitrile-water-H₃PO₄ (19:81:1) from 15% to 9% A in 9 minutes, followed by isocratic elution for the following 10 minutes. Samples were prepared by dissolution in solvent B and detection wavelength was set at 279 nm.

4.4.7 Sample Recovery

The fractions that contained pure compounds (98%) were pooled and transferred to an Erlenmeyer flask. Hydrochloric acid 6M was added in excess to precipitate the iso-α-acids. These were then extracted from the water phase using 3 x 30 ml of toluene. Toluene extracts were pooled, dried with anhydrous Na₂SO₄, vacuum, filtered and taken to dryness using a Rotavapor (model Buchi, Switzerland).

The resulting residue was weighed and redissolved in methanol and an aliquot was analysed by HPLC to evaluate its purity.
4.4.8 Results

Several biphasic systems were evaluated for their use, based on papers published by other researchers (Foucault and Chevolot, 1998) and taking into account the chemical characteristics of these compounds, that is, their lipophilicity, their acidic nature and also their emulsifying properties.

As mentioned above, the fact that the isolated compounds were to be free of toxic residues also limited the use of some solvents.

4.4.8.1 Toluene–water (1:3)

At the beginning of our research we had attempted to use the system developed for the separation of a mixture of α-acids by Hermans-Lokkerbal et al. (1997). As such, this system had not worked at all in the preliminary trials for the iso-α-acids, even though the fact that the separation of α-acids with this system used triethanolamine and H₂PO₄ implied that it was a type of displacement chromatography, based on their differences of pKa values (Foucault, 1998). Though no separation was achieved with this system, in view of its stability, we decided to use it, but adapting it to the pH-zone refining concept. For this, several concentrations of TFA were added to the stationary phase as a retainer and ammonium hydroxide to the mobile phase as an eluter.

The most satisfactory result was obtained using 2% TFA in the stationary phase and 0.015 % ammonium hydroxide in the mobile phase. Lowering the amount of TFA had no beneficial effect on the separation, while increasing the amount of TFA apparently produced instability in the system that led to bleeding of the stationary phase. The amount of ammonium hydroxide in the mobile phase affected the retention time, without a noticeable increase in the selectivity. While low ammonium hydroxide concentrations resulted in longer CPC runs, high ammonium hydroxide concentrations reduced the CPC run-time. Increasing ammonium hydroxide above 0.05% ammonium hydroxide reduced resolution drastically. As regards the loading capacity of the system, the injection of a 200 mg sample required a 5- hour runtime, while a 500 mg sample could be separated in 8 hours.

Unfortunately, this system did not produce a satisfactory separation of cis-isocohumulone and cis-isoadhumulone as shown by the large overlapping (white) peak area in Fig. 4-2. The more sample injected, the bigger overlapping area was produced.

In order to improve this, we decided to add ethanol into the mobile phase. The idea behind this was to improve selectivity of the system, as it could dissolve partially in both components of the mobile phase, exactly as we would like our sample to do (Foucault and Chevolot, 1998) reducing the extreme differences in polarity of the biphasic system.
Additionally, ethanol had been reported to improve resolution of the iso-α-acids in a selective HPLC system (Hughes, 1996).

Fig. 4-2 The chromatogram of the separation of A=200 mg, B=500 mg iso-cis-α-acids in CPC system toluene: water (1:3), with 2% TFA and 0.015% ammonia, flow rate 2.0 ml/min, 900 rpm. Coloured areas correspond to pure: 1=cis-isocohumulone, area 2=cis-isohumulone, area 3=cis-is adhumulone. All other areas are overlapping.
Several experiments were done to find an adequate solvent composition. It was found that toluene-ethanol-water (50:2:13) gave the best resolution. Lower proportions of ethanol made no difference to the results obtained using toluene-water (1:3), whereas more ethanol led to the instability of the system.

As for the retainer and eluter, the concentrations that gave the best results were 2% TFA in the stationary phase and 0.05% NH₄OH in the mobile phase. A flow rate of 2.0 ml/min and 900 rpm could be used in this case. This system slightly improved the separation decreasing the overlap between cis-isocohumulone and cis-isoadhumulone.

The role of ethanol in this system was predicted to increase the selectivity and this did in fact occur. However, even in such low amounts, the compounds were found to elute following a peak shape rather than the typical block shape characteristics of pH-zone refining CPC so that the yield of pure (>95%) compounds was relatively low (Fig. 4-3).

4.4.8.2 Hexane-ethyl acetate-methanol-water (1:1:1:1)

Given the poor results obtained with the previous methods, other biphasic systems were tested. In particular, this solvent had been reported by Denenkamp et al. (1994) to provide a good CCC separation of two stereoisomers of 1-methyl-4-methoxymethylcyclohexane-carboxylic acids using TFA and octanoic acid at a concentration of 0.02% as retainer acids and ammonium hydroxide 0.025% as an eluter. In Abbot and Kleiman’s table of solvents it was classified for moderately hydrophobic substances.
Similarly to the previous example, the CPC separation was carried out in the reverse displacement mode so that the upper organic phase was used as the stationary phase while the lower aqueous phase was used as the mobile phase. In this case, we decided to use TFA and not octanoic acid, since the latter has a high boiling point and is very lipophilic, so that it would be difficult to eliminate if it eluted with the compounds. Trifluoroacetic acid concentration was added to the organic stationary phase at concentrations between 0.02 and 2% ammonium hydroxide was added to the aqueous mobile phase at concentrations between 0.02 and 0.05%. Using a flow rate of 2.0 ml/min and 800 rpm, 500 mg of the sample were injected into the CPC system obtaining the chromatogram shown in Fig. 4.4.

![Chromatogram](image)

*Fig. 4–4 CPC chromatogram of 500 mg iso-α-acids in n-hexane: ethyl acetate: methanol: water (1:1:1:1), 0.5% TFA, 0.1% NH$_3$OH, flow rate 2.0 ml/min, 800 rpm. All the iso-α-acids eluted together without any separation.*

Although a rectangular peak shape was formed, HPLC analysis of the fractions showed that all cis-iso-α-acids eluted together. Several attempts were made to improve the separation, that is, different combinations of concentrations of the retainer and eluter, and proportions of the 4 solvents, but no satisfactory result was obtained.

Moreover, it was observed that any increase of the TFA concentration above 2% of the stationary phase volume decreased the system stability as could be observed by the bleeding of the stationary phase. Changing hexane for heptane, another popular choice (Foucault et al, 1994), did not, predictably improve the resolution of the iso-α-acids.
4.4.8.3 Hexane: Methanol: Water (10:5:5)

Among the changes that were attempted with the purpose of improving the resolution of the previous biphasic system, the total removal of ethyl acetate was considered. The same eluter and retainer were used. This system was chosen because of its extremes of lipo/hydrophilicity: hexane and methanol are sparsely miscible providing a very selective solubilisation of the non-ionised iso-α-acids in the non-polar hexane phase, and providing a good media for their ammonium salts which are readily soluble in methanol-water. The idea was thus to increase the difference in solubilising power of the two phases: only non-ionised species would be soluble in the TFA-rich hexane layer, while the aqueous methanolic mobile phase would fully dissolve the ionised iso-α-acids when the amount of ammonium in this solution provided the required pH. In this way, the partitioning of the non-ionised/ionic species should be more pronounced. Different concentrations of the eluter and retainer were tested and once more a 2% of TFA proved to be the best option. Starting with 0.1 % ammonium hydroxide produced the co-elution of acid impurities and cis-isocohumulone in the first 30 minutes. Furthermore, cis-isocohumulone was found to elute throughout the whole chromatographic run contaminating both cis-isohumulone and cis-isoadhumulone. The best solution was found by making a 2-step gradient of ammonium hydroxide: the first peaks corresponding to the acid impurities were eluted with a lower ammonium hydroxide concentration (0.05%; pH=10.3). Once these eluted, the concentration of ammonium hydroxide had to be increased to 0.1% in order to elute all three cis-iso-α-acids. This resulted in the best separation yet (Fig. 4.5).

As shown in figure 4.5, the modification introduced by addition of 0.1% ammonium hydroxide after 1.5 hour CPC run produced a rectangular peak which allowed the separation of iso-α-acids in a defined area. Although some overlapping between cis-isocohumulone and cis-isohumulone still could not be avoided, the selectivity was improved as noted by the increasing amount of collected fraction.

Particularly in the case of cis-isoadhumulone and cis-isohumulone and the less acids impurities (6), no overlapping occurred. This sharp peak border of cis-isoadhumulone indicated how pH zone refining based CPC system occurred in this system.

The loading capacity of this system also proved to be quite high, since a sample of 1.0 gram of cis-iso-α-acids mixture produced a peak with almost double the height but with the same width.
Fig. 4–5 The chromatogram of the separation of 500 mg iso-\(\text{cis-}\alpha\)-acids in CPC system \(n\)-Hexane: methanol: water (10:5:5), with 2% TFA and 0.05% ammonium hydroxide for the first 1.5 hours followed by addition 0.1% ammonium hydroxide, flow rate 2.0 ml/min, 700 rpm. Area 1=TFA peak, area 2= strong acid impurities 3=\(\text{cis-}\)-isocohumulone, area 4=\(\text{cis-}\)isohumulone, area 5= \(\text{cis-}\)iso adhumulone.

Fig. 4–6 Plot of HPLC results obtained from the analysis of the fractions collected from the CPC separation of 0.5 g of \(\text{cis-}\)-iso-\(\alpha\)-acid mixture. (CIC: \(\text{cis-}\)-isohumulone; CICH: \(\text{cis-}\)isocohumulone; CIAH: \(\text{cis-}\)iso adhumulone).
4.4.9 Conclusions

This method allowed a good separation of all cis-iso-α-acids. The biphasic system made up of hexane: methanol: water (10:5:5) with 2% TFA in the stationary phase as a retainer and 0.5% ammonium hydroxide during the first 1.5 hour approximately (until elution of acid impurities) followed by an increase to 1.5% ammonium hydroxide clearly worked as pH-zone refining as can be appreciated by the elution of the compounds as rectangular blocks. This was particularly useful for the up scaling of the separation since the peak size was almost doubled and although the increase in yield was not linear, it was still possible to obtain very pure compounds (> 95%) in just over 3 hours (Fig. 4-5).

A summary of the yield and purity of cis-isohumulones isolated with each different method can be observed in Fig. 4-7. As can be observed, duplication of sample size (columns 3 and 4) was not reflected in a duplication of the yield of pure compounds. This was due, mostly, to the capacity of the CPC instrument used in this case, rather than limitations in the method itself.

![HPLC chromatogram of CPC sample, A= cis-iso-α-acids mixture, and CPC collected fraction B=cis-isocohumulone (95%), C=cis-isohumulone (97%), D=cis-isoadamullone (98%) obtained with the system detailed in 4.4.8.3.](image-url)
Another interesting conclusion about the method is that it allowed a confirmation of the acidity of the cis-isomers. The order of elution: cis-isocohumulone, cis-isohumulone and cis-isoadhumulone confirmed that both the hydrophobicity and the acidity of this series of compounds decreases in that order.

4.5 Separation of individual trans-iso-\(\alpha\)-acids

Having optimised the method for the separation of cis-iso-\(\alpha\)-acids we proceeded to apply the same method to mixtures of trans-iso-\(\alpha\)-acids. As the method used to separate trans-iso-\(\alpha\)-acids from their counterparts involved the formation of DCHA-trans-iso-\(\alpha\)-acids, separation was tested with both complexed and free trans-iso-\(\alpha\)-
acids, the advantage of the former being the possibility of avoiding the time-consuming and yield-reducing elimination of DCHA. On the other hand, injecting DCHA-free samples had the advantage of avoiding the possible presence of this very toxic compound in the compounds.

4.5.1 Materials (see 4.4.1)

4.5.2 CPC instrument conditions (see 4.4.2)

4.5.3 Preparation of sample (DCHA-trans-iso-α-acids)
To obtain a pure iso-α-acids fraction, approximately 20 ml extract 20% iso-α-acids mixture was precipitated with an excess of H₂SO₄ 30% until the appearance of the yellowish oil of iso-α-acids. After extraction with toluene (3 x 20 ml) the resulting fraction was dried with anhydrous Na₂SO₄ and taken to dryness with a rotary evaporator (Buchi, Switzerland). The resulting residue was weighed and analysed by HPLC as described in Chapter 3.

Separation of trans- iso-α-acids from their cis-counterparts was done using the DCHA precipitation method (Thornton et al., 1993). For this, 16 grams of yellowish oil iso-α-acids were dissolved in 80 ml ethyl acetate after which 8.79 ml of dicyclohexylamine (DCHA, Sigma–Aldrich, Germany) were added and stirred approximately 1 hour. The solution was dried with anhydrous Na₂SO₄ and vacuum - filtered into a brown bottle. The solution was stored at 4°C for three days for total precipitation. Sample DCHA-trans-iso-α-acids were obtained by vacuum-filtration and an aliquot was analysed by HPLC to verify its composition. One part of these DCHA-trans-iso-α-acids were dissolved in mobile phase and submitted to CPC.

4.5.4 Preparation of trans-iso-α-acids sample
Free trans-iso-α-acids were obtained by removing the DCHA from the salts obtained in as described formerly (see 3.2.6.1/2). The DCHA-trans-iso-α-acids were dissolved in methanol and HCl 6M was added until no further precipitation was observed. This mixture was extracted with toluene (3x20 ml) and the extracts were pooled, dried with Na₂SO₄ anhydrous and taken to dryness with a Rotavapor (model Buchi, Switzerland). The residue was weighed and an aliquot was analysed by HPLC (see Chapter 3) to verify its content.
4.5.5 Preparation of two-phase solvent system and sample solution
The solvent system was prepared as in section 4.2.1.5 System IV: n-hexane; methanol: water (10:5:5). Appropriate amounts of ammonium hydroxide (0.05-0.1%) and TFA (2%) were added to the mobile and stationary phase respectively.
The sample solution was prepared by dissolving 500 mg of DCHA-trans-iso-α-acids or 500 mg of trans-iso-α-acids in 5 ml of the stationary phase.

4.5.6 CPC separation procedure (See 4.4.5)

4.5.7 HPLC analysis of CPC fractions (See 4.2.1.6)

4.5.8 Recovery of trans-iso-α-acids
The contents of the tubes containing similar compounds were transferred to an Erlenmeyer flask. Individual trans-iso-α-acids were precipitated from the mobile phase adding HCl 1 M until a pH of 3.5. The compound was extracted from the water phase with toluene (3x20ml). The toluene extracts were pooled, dried with anhydrous Na2SO4, vacuum-filtered and taken to dryness with a rotary evaporator. (Buchi, Switzerland) connected to pump model Divac 2,4 L

4.5.9 Results
The separations achieved for 500 mg DCHA-trans-iso-α-acids and 500 mg free trans-iso-α-acids are shown in fig. 4.9 and 4.11 respectively.
As regards the yield, separation of DCHA-trans-iso-α-acids (calculated as free iso-α-acids) afforded 85 mg trans-isocohumulone, 115 mg trans-isohumulone, and 40 mg trans-isoadhumulone while the injection of 500 mg of trans-iso-α-acids yielded 90 mg trans-isocohumulone, 125 mg trans-isohumulone, and 50 mg trans-isoadhumulone (Fig 4-13).
The difference in selectivity could be appreciated in the improved separation between trans-isocohumulone and trans-isohumulone as compared to the DCHA-trans-iso-α-acids sample. On the other hand, the overlapping area between trans-isohumulone and trans-isoadhumulone increased in both cases.
**Fig. 4-9** CPC chromatogram of separation 500 mg DCHA-trans-iso-α-acids in CPC system n-Hexane: methanol: water (10:5:5), with 2% TFA and 0.05% ammonium hydroxide for the first 1.5 hours followed by addition of 0.1% ammonium hydroxide, flow rate 2.0 ml/min, 900 rpm. Area 1=TFA peak, area 2= strong acid impurities 3=trans-iso-cohumulone, area 4=trans-iso-humulone, area 5= trans-iso adhumulone

**Fig. 4.10**: Plot of HPLC results obtained from the analysis of the fractions collected from the CPC separation of 500 mg DCHA- trans-iso-α-acid mixture. (TIC: trans-isocohumulone; TIH: trans-iso-humulone; TIAH: trans-iso-adhumulone).
Fig. 4–11 The CPC chromatogram of the separation 500 mg trans–iso-α-acids in system n-Hexane: methanol: water (10:5:5), with 2% TFA and 0.05% ammonium hydroxide for the first 1.5 hours followed by addition 0.1% ammonium hydroxide, flow rate 2.0 ml/min, 900 rpm.

Area 1=TFA peak, area 2= strong acid impurities 3=trans-isocohumulone, area 4=trans-isohumulone, area 5= trans-isoadhumulone, area 6=less strong acid impurities.

Fig. 4–12: Plot of HPLC results obtained from the analysis of the fractions collected from the CPC separation of 500 mg trans–iso-α-acid mixture. (TIC: trans-isocohumulone; TIH: trans-isohumulone; TIAH: trans-isoadhumulone).
As can be observed (Fig. 4-10 and 4-12), while there was not an appreciable difference between the separation of DCHA complexed acids and free acids as regards selectivity, the yield of compounds of similar purity was slightly higher in the absence of DCHA. The difference in selectivity could be appreciated in the improved separation between trans-isocohumulone and trans-isohumulone as compared to the DCHA-trans-iso-α-acids sample. On the other hand, the overlapping area between trans-isohumulone and trans-isoadhumulone increased in both cases.

*Fig. 4-13 HPLC chromatogram of CPC samples: A=Injected DCHA-trans-iso-α-acids mixture, and CPC collected fractions B=trans-is-cohumulone (94%); C= trans-isohumulone (98%); D=trans-isoadhumulone (98%)*
4.5.10 Conclusions

The separation achieved for both free trans- iso-α-acids and DCHA-trans- iso-α-acids was quite similar to that of cis-iso-α-acids, though the width of the trans-isocohumulone block was larger than its cis-counterparts (see Fig 4.7). This was due to a decrease in the overlapping of trans-isocohumulone and trans-isohumulone, which had been rather problematic in the case of cis-isohumulones.

The difference between DCHA- and free- trans-iso-α-acids was probably due to the presence of DCHA in the medium, introducing another variable into the already quite complicated setup, since it is a weak base which can partition into both phases and compete with ammonium hydroxide for TFA. But, considering that in these conditions, the DCHA-trans-iso-α-acids salts were destabilized and the individual trans-iso-α-acids were obtained free of DCHA, the time-consuming but above all, low-yield step required to release the DCHA from the iso-α-acids could be avoided.

![Graph](image)

**Fig. 4-14** Yield of trans-iso-α-acids obtained with the injection of a 0.5 g of a mixture of DCHA-trans-iso-α-acids (1) and 0.5 g of a mixture of trans-iso-α-acids. (TIC: trans-isocohumulone; TIH: trans-isohumulone; TIA: trans-isoadhumulone).
The purity of the trans-iso-α-acids can be observed in Fig 4-13. As the major impurities were the other iso-α-acids themselves, purity was calculated using an area-normalisation method.

4.6 Separation of a mixture of all iso-α-acids.

The ultimate goal of our project was to obtain all iso-α-acid isomers in a gram-scale, as pure (circa 95%) compounds, free of toxic or taste/aroma affecting impurities.

The previous experiments successfully allowed the separation of the isomers within the cis- and trans- series with acceptable purity and a yield that was reasonable if related to the amount of iso-α-acids injected. That is, the chromatographic separation was acceptable, but if the whole process was analysed, the yield was very low, conditioned by the DCHA precipitation step - specifically the recovery of trans-iso-α-acids from the DCHA complexes - that yielded very low amounts of compounds (see Chapter 3).

Therefore, it was important to explore the possibility of separating the compounds by CPC, avoiding the time-consuming chemical step.

Experiments carried out on mixtures of the three trans/cis isomers provided two systems that were potential candidates as biphasic solvent systems, i.e. toluene: ethanol: water and hexane: methanol: water.

In principle, the separation of the 6 isomers could be thought to be easy to predict, simply adding the results of the separate cis/trans series of isomers. Considering, however, that we were dealing with liquid/liquid partition chromatography, this hypothesis could very probably not work. Moreover, considering that the compounds were acids and that the separation mechanism relied on the ionisation of each acid (using the pH-zone refining mechanism), the behaviour of each individual acid could very possibly vary in this different environment.

Thus, two systems were tested: (toluene: ethanol: water: 50:12:13) and the second system, which had been successful for both cis and trans-iso-α-acids: (hexane: methanol: water: 10:5:5).

4.6.1 Materials (see 4.4.1)

Iso-α-acids extract were obtained from Heineken Brewery, Zouterwoude, Netherlands. The extract contained 20% iso-α-acids mixture (isocohumulone, isohumulone, isoadhumulone).
4.6.2 CPC instrument conditions (see 4.4.2)

4.6.3 Preparation of sample iso-α-acids
To obtain a pure iso-α-acids fraction, approximately 20 ml extract 20% iso-α-acids mixture was precipitated using an excess of H₂SO₄ 30% until the appearance of the yellowish oily free iso-α-acids. After extraction with toluene (3 x 20 ml) the resulting fraction was dried with anhydrous Na₂SO₄ and taken to dryness using a rotary evaporator (model Buchi, Switzerland). The resulting residue was weighed and an aliquot was analysed by HPLC as described in Chapter 3.

4.6.4 Preparation of biphasic solvent system (see 4.4.5-System II and IV)

4.6.5 Separation procedure
CPC was run in the descending mode with organic phase as the stationary phase and the aqueous layer as the mobile phase. The CPC column was first entirely filled with organic phase. A 5 ml sample (containing approx. 500 mg of cis-iso-α-acids) was injected and the mobile phase was pumped into the system at a flow rate of 2 ml/min and a centrifugation speed of 900 rpm at 23 °C. The eluent from the outlet of the column was continuously monitored with an UV detector and registered using a pen-recorder. The eluent was collected in the fraction collector at 3 minutes intervals (9. ml / tube). The pH was monitored manually.

4.6.6 Analysis of CPC fractions (See 4.4.5)

4.6.7 Sample Recovery
The fractions that contained pure compounds (98%) were pooled and transferred to an Erlenmeyer flask. Hydrochloric acid 6M was added in excess to precipitate the iso-α-acids. These were then extracted from the water phase using 3 x 30 ml of toluene. Toluene extracts were pooled, dried with anhydrous Na₂SO₄, vacuum, filtered and taken to dryness using a Rotavapor (model Buchi, Switzerland). The resulting residue was weighed and redissolved in methanol and an aliquot was analysed by HPLC to evaluate its purity.
4.6.8 Results

4.6.8.1 Toluene: ethanol: water (50:2:13)

The results obtained with this system are displayed below in Fig. 4-15. Three blocks consisting of the pairs of cis/trans isomers were obtained. That is, the system was unable to separate the pairs of stereoisomers, which eluted in equimolar mixtures. The yield of each iso-\(\alpha\)-acid (purity>90%) was: 60 mg isocohumulone (12%), 100 mg isohumulone (20%) and 24 mg isoadhumulone (4.8%) of the 500 mg injected sample.

No efforts were made to improve this separation that had an extremely low selectivity. As can be observed, with this system, the pure iso-\(\alpha\)-acids pairs were obtained, with no separation of the cis/trans isomers.

\[ \text{Area of isocohumulone:} \quad \text{Area of isohumulone:} \quad \text{Area of isoadhumulone:} \]

\[ \text{Area:} \quad \text{Area:} \quad \text{Area:} \]

\[ \text{cis:} \quad \text{trans:} \quad \text{cis:} \quad \text{trans:} \quad \text{cis:} \quad \text{trans:} \]

\[ \text{Fig. 4-15 - CPC chromatogram of 500 mg iso-\(\alpha\)-acids with toluene: ethanol: water (50:2:13), 2% TFA and 0.015% ammonia, flow rate 2.0 ml/min, 900 rpm. Area 1=iso-cohumulone (cis+trans), area 2=isohumulone (cis+trans), area 3=isoadhumulone (cis+trans).} \]

4.6.8.2 n-Hexane-methanol-water (10:5:5)

This system, characterised by having a much larger difference in lipophilicity/hydrophilicity of the two phases was attempted in order to try and space out the elution of the cis/trans pairs of iso-\(\alpha\)-acids. It had worked very well for the all-cis and all-trans mixtures and was derived from a frequently used biphasic mixture that
included ethyl acetate. It was similar to the first one tested but without the ethyl acetate which we interpreted was carrying both ionized and non-ionized acids into the mobile phase.

In these conditions, a good, though not complete separation of all 6 isomers was obtained as can be observed in Fig. 4-16.

Fig. 4-16 The CPC chromatogram of 500 mg iso-α-acids in CPC system n-Hexane: methanol: water (10:5:5), with 2% TFA and 0.05% ammonia for the first 1.5 hours followed by addition 0.1% ammonia, flow rate 2.0 ml/min, 900 rpm. Area 1= TFA peak, area 2= strong acid impurities, area 3= trans-iso-cohumulone, area 4= cis-iso-cohumulone, area 5= isohumulone (cis+trans), area 6= cis-isohumulone, area 7= cis-isoadhumulone, 8= weak acid impurities

4.6.7 Sample Recovery

The fractions that contained pure compounds (98%) were pooled and transferred to an Erlenmeyer flask. Hydrochloric acid 6M was added in excess to precipitate the iso-α-acids. These were then extracted from the water phase using 3 x 30 ml of toluene. Toluene extracts were pooled, dried with anhydrous Na₂SO₄, vacuum, filtered and taken to dryness using a Rotavapor (model Buchi, Switzerland). The resulting residue was weighed and redissolved in methanol and an aliquot was analysed by HPLC to evaluate its purity.
4.6.9 Conclusions

Using this last system, it was possible to obtain 3 of the iso-α-acid isomers in a highly pure state: cis-isohumulone, cis-adhumulone and trans-isohumulone. Trans-isoadhumulone which together with cis-isoadhumulone is a minor component, was not able to be collected in any significant amount and trans-isohumulone eluted in a low proportion during one hour approximately, contaminating and decreasing the purity of trans-isocohumulone (from which it was also partly separated) and cis-isocohumulone. In the case of iso-cohumulone, the fractions obtained were only 60% pure, with a 40% of trans-isocohumulone. As for cis-isohumulone, one fraction of 98% purity was obtained -impurified with 2% cis-isoadhumulone and another fraction with around 40% trans-isohumulone as mentioned above.

Thus, the stereoisomer pairs, cis/trans-adhumulone were separated, while cis/trans-isocohumulones were only partially separated. The pair of cis/trans-isohumulones were also only partially separated, due especially to the fact that trans-isohumulone eluted in a wide block, very probably affected by the acidity of cis-isohumulone which supressed its ionisation.

The elution order in pH-zone refining chromatography is related to the acidity in this case of the compounds, that is, their pKa. In the case of similar pKa values, the hydrophilicity of ions will define the order of elution of the compounds. Therefore, the least lipophilic and most acid compounds, corresponding to acid impurities, eluted immediately after the TFA. After this, the six iso-α-acids eluted in order of increasing hydrophobicity and pKa value: trans-isocohumulone > cis-isocohumulone > trans-isohumulone > cis-isohumulone > trans-isoadhumulone > cis-isoadhumulone. This was supported by the variation observed in the pH of the eluent, which, starting from a low pH, increased slightly as the different compounds eluted. The pH value remained constant throughout the elution of each acid.

This order of elution coincided with the relative hydrophobicity calculated by Hughes (1996) and cited in the introduction.

Do these results conform to a true pH- zone refining mechanism? The HPLC plot of the fractions does not exactly coincide with the idea of definite blocks of compounds, especially in the case of trans-isocohumulone that eluted slowly spreading over cis-isohumulone and cis-isocohumulone. In the case of trans-isohumulone and cis-isocohumulone, elution followed a pH zone refining-like separation with a mixing zone as defined by Ito (1996) of 70 and 30% respectively. Cis-isocohumulone and trans-isocohumulone had an even greater mixing zone with a fraction of 90% trans-isocohumulone that could be rechromatographed for purification of the latter.
The problem very likely resides in the closeness of the pKa values of some of the isomers. Ito (1996) suggested that pKa values should differ in at least 0.2 in order to ensure separation, minimising the "mixing zone" and thus increasing resolution. This is not the case for these pairs of acids, at least according to published values.

Below in Fig. 4-19, the purity and yield of the isolated iso-α-acids can be observed. As can be appreciated the fraction corresponding to an approximately 50:50 ratio of cis/trans-humulone must be rechromatographed for a good separation of the stereoisomers, while the fraction corresponding to a 90:10 ratio of cis/trans-isocohumulone could probably be improved with an adjustment of the CPC conditions.
Fig. 4-18. Yield of iso-α-acids and their purity, obtained with the injection of
1) 0.5 g and 2) 1.0 g of an iso-α-acid extract: CIH: cis–iso-humulone; CICH: cis–isocohumulone; CIA: cis–isoadhumulone; TIH; trans–iso-humulone; TICH: trans–isocohumulone; TIA: trans–iso-adhumulone

4.7 Final conclusions

The feasibility of the application of CPC for the separation of iso-α-acids was explored and proved to be successful. The three isomers of the cis-series were well separated, as was the case with the mixture of trans-isomers.

When an isomerised hop extract containing all isomers was injected, it was possible to obtain 11% trans–isocohumulone, 9% cis–isoadhumulone and 2% trans–isocohumulone. A fraction of 22% containing 90% pure cis–isocohumulone was also obtained. It was not possible to improve this separation, though further work on the eluter gradient or the sample loading size could have provided some improvements.
However, the relative yield of each isomer was lower (CPC results) as compared with the yield of the separate cis/trans fractions.

Therefore, if only considering the CPC separation, injecting all cis- or all trans-mixtures was clearly better. However, if the whole process was analysed, i.e., starting from the isomerised hop extracts or even the hop extract, a major loss of compounds occurred during the chemical precipitation needed to separate the cis- and trans-isomers, which as explained in Chapter 2, gave a yield below 2%.

During the CPC trials, however, attempts to find methods other than DCHA precipitation for trans/cis isomer separation resulted in the discovery of the also selective complexation of trans-isomers with β-cyclodextrin, which will be explained in the following chapter, allowing us thus to obtain all cis- and trans-iso-α-acid mixtures easily, which could then be submitted to CPC and obtain excellent yields. In this case, the best situation would be to work with all cis- or all trans- mixtures of iso-α-acids.

The importance of applying CPC is clear: it is cheap since the only expense incurred is the solvent. There is no need to use the extremely expensive preparative HPLC columns that accumulate non-eluted analytes over time, that sooner or later elute, contaminating the compounds of interest. On the other hand, if they do not elute they reduce the loading capacity and resolution of the column and have to be replaced.

Another advantage is that no pre-treatment of the extract is necessary since the impurities elute before or remain in the stationary phase when all compounds of interest have eluted. This saves time, and avoids handling of the very unstable iso-α-acids.

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