Contributions to the quality control of two crops of economic importance : hops and yerba mate

Wilson, E.G.

chapter 3

Isolation of Iso- α- Acids (I): Precipitation With Dicyclohexylamine (DCHA)

Erica G. Wilson, Luisa Ugolini and Robert Verpoorte

Isolation of iso-α-acids: general scheme of work

The quality assurance of beer –that is, all the processes involved in the manufacture of a product that consistently meets specifications– requires, among other things, the knowledge of characteristics of the raw material which are vital for the consistency of quality attributes. Quality attributes commonly tested in beer are colour, transparency, flavour, bitterness, foam among others. Although all these characteristics are multifactorial, hops and specifically the iso-α-acids produced during the brewing process have been clearly associated to bitterness and foam.

The isolation of these compounds in preparative-scale amounts could be done in several ways: one was to synthesize them; another was to isolate them from isomerised hop extracts using some kind of preparative chromatography. Alternately, another possibility was to obtain α-bitter acids from hops or hop extracts and then isomerise them or to isomerise α-bitter acids and then separate them.

In 1975, Pfenninger et al. (1975) reported the synthesis of iso-α-acids, having obtained high yields of a 50:50 mixture of *cis*- and *trans*- isomers. However, this solution was not considered satisfactory since these mixtures could in fact be obtained much more cheaply and easily, starting from α-bitter acids isolated from hops and isomerising them.

The isolation of pure individual iso- α- acids was, however, a great challenge, and this had not been successfully achieved applying methods that could realistically be used at a large scale. In 1961, Verzele *et al.,* reported the isolation of gram-scale quantities of all iso-α-acids after 2000 transfers by CCD (counter-current distribution) and in 1979, Schwarzenbach reported the isolation of a wide range of hop-derived substances using buffered silica gel chromatographical systems, though recovery from the system itself was difficult resulting in low yields of pure substances. Further on, individual iso-α-acids were obtained by Verzele *et al.* (1989) using preparative reverse-phase LC. With this method, a successful separation of *cis*-isocohumulone, *trans*-isocohumulone, *cis*-isohumulone, and *trans*-isohumulone was achieved by injecting up to 400 mg iso-α-acids extract. Unfortunately, it did not allow the isolation of any significant amount of neither isomers of iso-adhumulone due to its low initial concentration in the mixture and to insufficient selectivity.

Other preparative and semi-preparative HPLC methods have also been published, a successful one being that of Hughes (1996), who separated all *cis*- and *trans*- isomers by first using a selective precipitation of the *trans*- isomers with dicyclohexylamine (DCHA) from a mixture of iso-α-acids (Thornton *et al.,* 1990, 1993) and then separating the mixtures of isomers by preparative HPLC. The collected fractions then were pooled and purified of mobile phase components but it was possible to obtain 96% pure individual iso-α-acids on a +500 mg -scale.

The weak points of the former methods were either their efficiency in terms of time consumed *vs* their yield, the presence of degradation products due to the amount of manipulation required to recover the obtained iso-α-acids from the separation mixtures or simply, the impossibility of obtaining iso-adhumulone in adequate amounts. Therefore, it was necessary to explore methods other than preparative HPLC to obtain the pure compounds.

A method for the large scale isolation of bitter α-acids from hops had been developed by Hermans-Lokkerbol *et al.* (1994a) using Centrifugal Partition Chromatography (CPC), which could provide the necessary amounts of individual pure α-acids.

With these pure compounds as a starting material, there were diverse strategies that could be implemented to obtain the six iso-α-acids. All of them involved an isomerisation step and a separation step at some point and the preference for one method over others was based on the problems that could be predictably encountered. These basically arose from the following characteristics of the compounds: their similarity as regards solubility (hydrophobicity), acidity (Verzele *et al.*, 1991; Hughes, 2000) and general chemical behaviour, their instability to light, principally, but also in the presence of air and lastly, the low relative abundance of two of them –*cis*- and *trans*-isoadhumulone.

Another question to be considered in this separation was that the resulting pure compounds had to be fit for human consumption in the first place, and additionally any impurities –even when atoxic- could not interfere with the taste or aroma of the pure iso-α-acid.

The separation had thus to be approached in such a way that the few existing differences be enhanced. The differences between the *cis*- and *trans*-iso-α-acids isomers that could be taken advantage of for their separation were basically two: the formation of insoluble salts of only *trans*- isomers with an organic base, dicyclohexylamine (DCHA) as published by Thornton *et al.* (1990,1993) or the use of a technique that could take advantage of their acidic properties, enhancing their different, albeit slight, pKa values. Thus, three methods were attempted, two of which were based on a combination of

CPC and DCHA precipitation, another on the CPC separation. A fourth method was found after most of the work on the first two approaches had been done, as a development of the search for a stabilising agent of the pure isolated iso-α-acids.
The methods are summarised below and shown schematically in Fig.3-1.

DCHA- precipitation based:

I (a) Separation of α-acids by CPC from a hop extract followed by isomerisation to obtain iso-α-acid pairs of stereoisomers, and then DCHA precipitation for *cis* / *trans* separation.

I (b) DCHA precipitation of an isomerised hop extract to obtain an all-*trans*-iso-α-acid mixture and an all-*cis*-iso-α-acid mixture for further separation by CPC.

Isomerisation/CPC based:

II a) Separation of α-acids from a hop extract by CPC, followed by isomerisation and a further CPC separation of all iso-α-acid isomers.

b) Separation of all iso-α-acid isomers from an isomerised hop extract.

Precipitation with agents other than DCHA:

III. Search for alternatives to selective DCHA precipitating agents, including stereo-selective reagents such as β- cyclodextrin.

Among all these approaches, method III proved to be the most successful. However, in the case of methods I(b) and II, a good separation with a reasonable yield of highly pure compounds was obtained using a pH-refining zone CPC technique.

In the case of method (III), the search for alternatives to the use of DCHA –which is toxic and interferes with the taste and aroma even at trace levels - for selective precipitation of *trans*-iso-α-acids, resulted in the discovery of the selective β–CD precipitation of *trans*-iso–α-cids.

This turned out to be the best method, yielding pure *trans*-β-cyclodextrin-iso-α-acid complexes that proved to be more stable than the pure compounds both as solids and in solution.

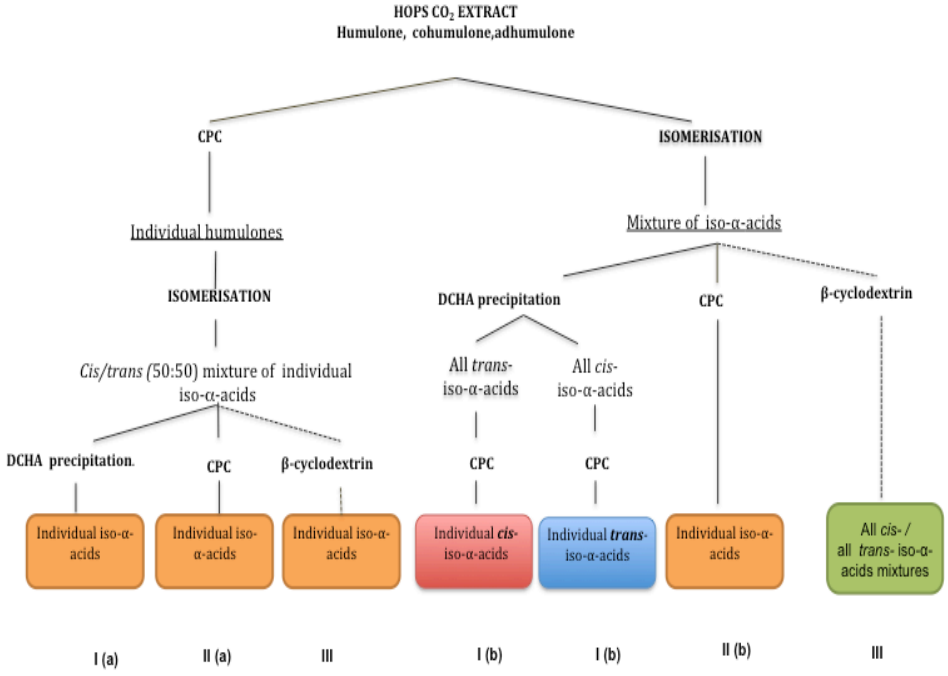


Fig. 3-1 Isolation of iso-α-acids: overview of the scheme of work: method IV was not planned originally but developed during the project.

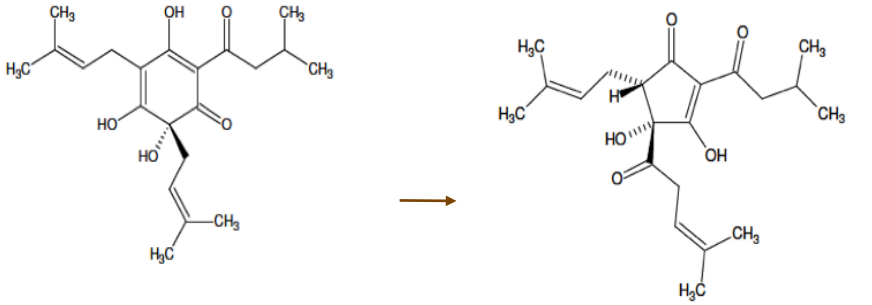
3.2 Isolation of iso-α-acids: DCHA precipitation - based method (I a)

The objective of our project was not simply to isolate the six individual iso-α-acids, but to obtain them in a 100 gram-scale with purity ≥95%. Furthermore, the compounds had to be apt for human consumption, meaning that any remaining impurities had to be atoxic and additionally not produce any distortion in the organoleptic properties of the compounds, especifically in their taste or aroma.

The available starting materials were either a CO_2 hop extract containing approximately 50% α-acids (mostly β-acids and hard resins, tannins, waxes and other minor compounds made up the remaining 50%) or an isomerised hop extract (30% potassium salts of all iso-α-acids in methanol).

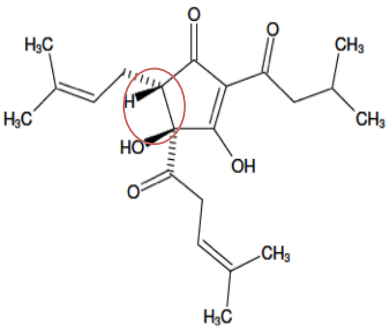
Two methods were devised, indicated as (I-a) and (I-b) in the general scheme of work (*see Fig. 3-1)*. In the first case, we took advantage of the possibility of obtaining pure α-acids from the CO2 extract using a reliable and predictable Centrifugal Partition Chromatography (CPC) method (Hermanns-Lokkerbol et al., 1994a).

All methods included the following processes at one time or another: isomerisation of α-acids, precipitation with DCHA and CPC separation.



Humulone *Cis- isohumulone*

+



Trans- isohumulone

Isomerisation of humulones to iso-humulones
It occurs in various conditions:

- pH > 7
- High temperatures.
- Presence of divalent cations, e.g. Mg^{+2}
- UV radiation of methanolic solutions

The conditions in which the isomerisation occurs will condition the *cis/trans* ratio. Each humulone yields to two diasteromeric isohumulones, *cis*-isohumulone and *trans*-isohumulone, depending on the spatial arrangement of the tertiary alcohol function at C(4) and the prenyl side chain at C(5).

Fig. 3-2 : Isomerisation of humulone to cis- and trans- isohumulone

As explained in Chapter 2, α–acids undergo isomerisation under a variety of experimental conditions to yield pairs of stereoisomers, the *cis/trans*- iso-α-acids. Among these conditions, heating at high pH or heating in presence of ions that act as catalysts such as Mg^{2+}, favour the isomerisation, though the ratio of epimers will vary according to the conditions (Koller, 1968).

Alternatively, irradiation of a methanolic solution of α-acids with UV light produces photoisomerisation which proceeds in fully regio- and stereo- selective ways and forms exclusively *trans*-isomers (Verzele, 1991) (Fig. 3-2). Interconversion of *cis*- into *trans*- and vice versa, as well as conversion of iso-α-acids into their parent compounds is feasible. The *cis*-isomer is the more stable epimer in view of the least steric hindrance between the two large vicinal side chains.

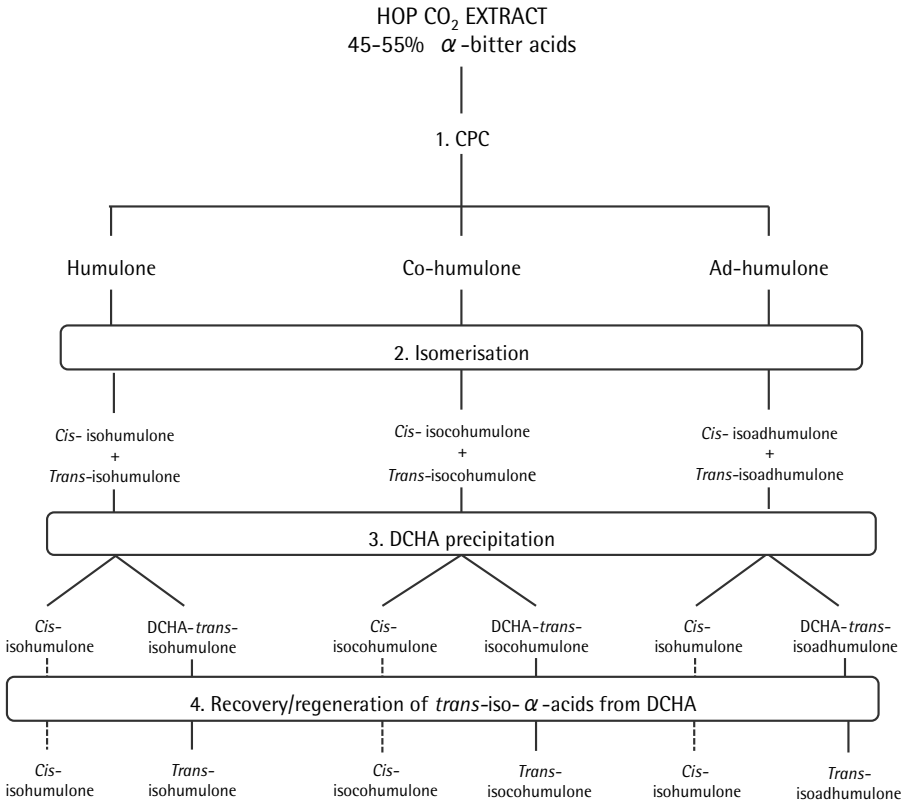


Fig. 3-3 General scheme of work for method I, based on DCHA precipitation of trans-iso-α-acids for separation of isomers.

3.2.2 Dicyclohexylamine precipitation of *trans*-iso-α-acids

In 1990, Thornton *et al.* published a method for the separation of *cis*- and *trans*-isomers of iso-α-acids using DCHA. He discovered that in a strictly anhydrous medium, *trans*-iso-α-acids precipitated in a 1:1 ratio with this organic base to form salts, leaving *cis*-iso-α-acids in solution. The DCHA salts could then be crystallised forming a crystalline solid with the additional advantage of an enormously greater stability as compared to the free iso-α-acids. The iso-α-acids could then be regenerated from the salts with an acid treatment (Thornton *et al.*, 1990,1993). The procedure was standardised and this mixture of DCHA-*trans*-iso-α-acids has been adopted as the official standard for the analysis of iso-α-acids in extracts or beer.

In our experiment, we used this method for the precipitation of *trans*-isomers, though with slight modifications taken from Maye *et al.* (1999). The resulting DCHA-*trans*-iso-α-acids were then treated chemically to regenerate the *trans*-iso-α-acid from the salt, while the *cis*-iso-α-acids were recovered from the reaction media. This was done using Maye's method with minor modifications that were attempted in order to increase the yield of the pure compounds.

The last step was the purification of the compounds to eliminate traces of reagents or solvents and then finally the search for a means of stabilising the extremely unstable iso-α-acids.

Precipitation with DCHA was also used to obtain all *cis*- and all *trans*- iso-α-acid mixtures. For this, an isomerised hop extract (obtained either isomerising the CO_2 hop extract or starting from the isomerised extract directly) was precipitated with DCHA, thus obtaining an all- *cis* –α-acid extract and a solid DCHA-*trans*-iso-α-acid mixture. These all *cis*- or all *trans*- isohumulone samples were then submitted to CPC to obtain the pure compounds.

In this chapter, method (I) that involved DCHA precipitation will be described and the results and attempts to optimise each step of the procedure will be discussed. Fig. 3-3 is scheme of the main steps involved in the procedure.

3.2.3 Materials and methods

The CO_2 hop extract used in the experiments was the kind gift of Mr. Verhagen (Heineken) containing max. 55% of α-acids and 30% β-acids. Dicyclohexylamine (DCHA) was purchased from Fluka, Germany, methanol AR and hexane AR from Biosolve, NL; NaOH, $MgSO_4.7H_2O$, Na_2SO_4 anhydrous p.a. from J.T.Baker were all of analytical grade.

Ethyl acetate, diethylether, triethanolamine, 98% H_3PO_4 and 37% hydrochloric acid were all AR quality and purchased from Biosolve, NL.

3.2.4 CPC

Preparative chromatography was carried out using a modular Sanki (Kyoto, Japan) Centrifugal Partition Chromatograph (type LLN) with an HPLC pump (model LKB-V) and a UV/Vis detector (Linear Instruments, Reno, NV, USA), a 5 ml loop injector (Rheodyne) and a pen-recorder (Panasonic Model VP-67222 A). Fractions were collected by means of a LKB 1700 Minirac fraction collector. In all experiments 6 cartridges were used, with a total internal volume of 125 ml.

The method used to obtain the individual α-bitter acids was that proposed by Hermans-Lokkerbol *et al.* (1994a), with a few modifications that reduced the run-time and improved separation. Thus, a two-phase solvent system consisting of 3 parts of a 3% (0.15M) solution of triethanolamine taken to pH=8.5 with 98% H_3PO_4, to 1 part of toluene was prepared. This mixture was stirred with a magnetic stirrer during 1 hour and allowed to stand overnight to allow a good separation in two phases; the organic phase was used as the stationary phase and the alkaline aqueous solution as the mobile phase.

The rotation speed of the CPC was set at 800 rpm, the flow at 3.5 ml/min and the temperature at 23°C. It was used in descending mode, and the system was loaded in the traditional manner by running the organic phase through the instrument in ascending mode to displace all aqueous solvent from the system, followed by a switch to the descending mode to load the mobile phase, allowing the displacement of the amount of stationary phase necessary to achieve equilibrium. In these conditions, the volume of remaining stationary phase was approximately 80 ml.

Samples were prepared by dissolving up to 1.5 g of CO_2 hop extract in approximately 3.5 ml of stationary phase. This solution was centrifuged and the supernatant was filtered through a 45- micron disposable Nylon filter. This sample was injected once the system was equilibrated and run during 4 hours; 10 ml fractions were collected and analysed by HPLC.

This CPC separation was repeated as many times as necessary to obtain approximately 4 g of α-acids.

3.2.5 Isomerisation

Koller´s alkali-magnesium catalysed isomerisation method was used.

3.2.5.1 Method

A sample of 3.0 g of α-acids was dissolved in 8.75 ml of NaOH 1M and 83 ml of methanol. This solution was added to a solution of 42 ml of 8.6% $MgSO_4.7H_2O$/ H_2O and 50 ml of H_2O.

This mixture was kept at 70°C in a water-bath for approximately 20 minutes, and then cooled to room temperature. The resulting solution was acidified to pH = 2 with 200 ml of H_2SO_4 2M and extracted with 3 portions of n-hexane which were collected in an Erlenmeyer and washed with a saturated NaCl solution. The hexane solution was thoroughly dried with anhydrous Na_2SO_4, filtered and taken to dryness at reduced pressure and temperature. All this process was carried out using antiactinic glassware in order to protect the iso-α-acids from the light.

3.2.6 Preparation of DCHA–trans-iso-α-acid salts

Two methods were used, the first being that described by Thornton *et al.* (1993) and then a similar method but with slight modifications reported by Maye *et al.* (1999).

3.2.6.1 Method I

Approximately 4.0 g of iso-α-acids were dissolved in 20 ml of ethyl acetate and 2,20 g of dicyclohexylamine (DCHA) (Sigma-Aldrich, Germany) were added to the iso-α-acid solution. The addition of DCHA produced a colour change (from dark orange to dark green) and was exothermic. The solution was allowed to cool for one hour at room temperature and left to stand for approximately 3 days at 7°C or until no further precipitation was observed in an antiactinic container in the dark. The precipitation was aided by gently scratching the walls of the container with a glass rod. The crystals were filtered, washed with ice-cold ethyl acetate and dried under a nitrogen stream in the dark.

3.2.6.2 Method II (Maye *et al.*, 1999)

Approx. 4.0 g of iso-α-acids were dissolved in 30 ml diethyl ether and 1,25 g of DCHA were added to the solution. The solution turned a dark amber colour. After stirring for 3 h at room temperature, the solution was placed in an antiactinic flask and stored at -20°C overnight. The crystals were filtered by Buchner filtration, washed with cold acetone and dried under a nitrogen stream.

3.2.7 Regeneration of *trans* -iso-α-acids

Pure *trans*- iso-α-acids were obtained by displacement of DCHA from the complex in acid medium. This was done by dissolving the salts in dichloromethane (CH_2Cl_2) and stirring with HCl 6M during 20 minutes; when no further precipitation was visible, the mixture was placed in a separatory funnel and allowed to separate into the two phases. The organic phase was decanted and washed with 2 portions of H_2O. The organic phase was dried with anhydrous Na_2SO_4 and taken to dryness with a Rotavapor. The dry residue was dissolved in warm ethyl acetate, filtered and allowed to cool for recrystallisation.

3.2.8 Recovery of *cis*-iso-α-acids

The filtrate from (3.1/3.2) was placed in a separatory funnel and extracted with 3 x 30 ml of Cl_2CH_2. The CH_2Cl_2 fractions were rinsed with water, dehydrated by addition of anhydrous Na_2SO_4 and taken to dryness with a Rotavapor. As usual all this process was carried out protected from the light.

3.2.9 HPLC system

The fractions obtained from the CPC of the hop extract and all steps involving isomerisation, were analysed using the following methods:

3.2.9.1 α-bitter acids

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. Samples of 20 μl were injected onto a Hypersil C18 column (150 x 416 mm -5) and eluted with a mobile phase of 7.46 g of triethanolamine in 350 ml of H_2O, (taken to pH= 6.00 with o-phosphoric acid) and methanol (35:65). Detection wavelength was 280 nm (Hermans-Lokkerbol *et al.,* 1994b).

3.2.9.2 Iso α- acids

Isomerisation of the α- acids was controlled using the same HPLC instrument as detailed above, a Hypersil C18 column (150 x 416 mm-5) and gradient elution with two solvents: A: 1% H_3PO_4 in water and *B:* acetonitrile-water-H_3PO_4 (19:81:1).
Samples were eluted with a linear gradient from 15% to 9% 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.

3.3 Results and discussion

The CO_2 SFE hop extract was submitted to CPC in order to obtain the three pure α-acids: humulone, cohumulone and adhumulone. This extract contained approximately 45% of α-acids, of which 45% were humulone and cohumulone while the remaining 10% were adhumulone. In the conditions detailed above, and injecting 1.5 g of extract, a yield of around 0.15 g (10%) of humulone and 0.17g (11%) of cohumulone could be isolated, and only about 0.06-0.08 g (4-5%) of adhumulone –in all cases approx. 95% pure. In order to obtain the compounds, 10 ml fractions were collected and every other fraction was analysed by HPLC using the above detailed method. Those above 95% purity were pooled, acidified to pH ≅ 2 with HCl 6M, placed in a separatory funnel and extracted with 3 portions of CH_2Cl_2. The pooled organic extracts were washed with an additional portion of HCl 1M, dried with anhydrous Na_2SO_4 and taken to dryness. All this process required careful protection from light, heat and air. This process was repeated till a critical mass of approximately 3 g of iso-α-acids was obtained to work with to continue to the following steps since higher amounts decreased the degradation of the compounds due to easier handling.

Each individual α-acid fraction was then isomerised using Mg-catalysed isomerisation in alkaline medium described above. The process was similar to that described by Koller, but a step directed to remove the magnesium from the obtained iso -α- acids at the end of the process, consisting in the addition of sulphuric acid, as indicated by Maye *et al.* (1999) was added in order to release the isohumulonates from their magnesium salts. Free iso-α- acids react more readily with DCHA (*trans* iso-α-acids).

The iso-α- acids thus obtained were treated with DCHA according to the process described above. After this, the DCHA-*trans*-iso-α-acids precipitate was separated by filtration and dried under a nitrogen stream. The method used for regeneration of the DCHA salts was similar to that described by Hughes (1996). *Cis*-iso-α-acids were recovered from the supernatant as described above and all free iso-α-acids were redissolved in pure EtOH and preserved in vials protected from the light at -18°C.

As can be observed in the Table 3-1, it was possible to obtain isolated iso-α-acids using this method, albeit with very low yields, if considering the initial content of α-acids in the hop extract. Up to this point of the process, the total yield calculated from the starting point, that is, the hop extract was below 1% for *trans*-iso-α-acids and around 5 % for *cis*-iso-α-acids. Moreover, though the purity was quite acceptable, the types of impurities were not, since remnant DCHA was clearly visible in NMR purity studies carried out and this had to be removed. In the case of the *cis*-iso-acids, the

situation was more complex because impurities were degradation products formed during the DCHA precipitation process, due basically to the time needed for the completion of the precipitation of the *trans*-isomers. These could not be fully removed with the extraction.

Table 3-1: Percentage yield and purity (M/M) of individual iso-α-acids obtained in the DCHA precipitation process.

	TIH(%)	CIH(%)	TIC(%)	CIC(%)	TIA(%)	CIA(%)A
Yield	0.10	5,00	0.09	5.00	N/A	N/A
Purity (%)	97	95	95	95	N/A	N/A

TIH: trans-isohumulone;CIH: cis-isohumulone;TIC: trans-isocohumulone; CIC: cis- isocohumolone; TIA: trans-isoadhumulone; CIA: cis-isoadhumulone

In order to improve the yield, a breakdown of the process and the yield of each step was analysed in order to determine which steps were the least efficient. The results can be observed in Table 3-2.

Table 3-2: Yield and purity of individual iso-α-acids obtained in the whole process starting from the hop extract, using the DCHA precipitation method

STEP	Compound	Yield (%)	Compound	Yield(%)	Compound	Yield(%)
1.CPC	Humulone	7-10	Isocohumulone	8-11	Adhumulone	2-3
2.Isomerisation	TIH + CIH	94	TIC + CIC	92	TIA +TIC	90
3.DCHA precipitation	DCHA- TIH	94	DCHA-TIC	94	N/A	N/A
	CIH	50	CIC	50	N/A	N/A
4.Regeneration/ Recovery of Isomers	TIH	<10	TIC	<10	N/A	N/A
	CIH	≈50	CIC	50	N/A	N/A
Total process	TIH	<1(≈0,88)	TIC	<1(≈0,85)	N/A	N/A
	CIH	50	CIC	50	N/A	N/A

TIH: trans-isohumulone;CIH: cis-isohumulone;TIC: trans-isocohumulone; CIC: cis-isocohumolone; TIA: trans-isoadhumulone; CIA: cis-isoadhumulone

Clearly, the step where most of the isomers were lost was recovery of the iso-α-acids from the DCHA-*trans*-complexes and the degradation suffered by the *cis*-isomers during the DCHA-precipitation process. Additionally, the total cycle could last about 4 days, broken up in 3 hours per CPC separation and approximately 3 days for the DCHA precipitation.

Having determined the critical points in the process, a great deal of effort was put into trying to solve them. Some of these are summarised below:

- CPC run-time was decreased and the yield per run doubled by submitting the hop extract to a clean-up previous to the CPC process: β-acids were eliminated by extraction with an alkaline solution. The sample injected thus was practically 90% α-acids.

- The DCHA precipitation method was that described by Thornton *et al.* (1990,1993). The method described by Maye *et al.* (1999) was attempted, since it reportedly decreased precipitation time to one day, but very slight improvements were achieved in *cis*- iso-α-acid purity.

- Efforts to improve the recovery of *trans*-α-acids from the DCHA complex were also made, but the choice of solvents and reagents was restricted by their toxicity and other conditions such as boiling point considering that they had to be quickly removed to preserve the thermally unstable iso-α-acids. Thus the use of hexane instead of isooctane or dichloromethane did not improve the yield and recrystallisation-using acetone instead of ethyl acetate- was not viable. Additionally a further purification step had to be implemented for both isomers: the 50% of impurities in *cis*-iso-α-acids had to be removed perhaps using C18 solid-phase extraction cleanup processes (to remove more polar degradation products, while all remaining DCHA had to be removed from the *trans*-iso-α-acids, using ion-exchange solid phase extraction for example. In the case of *trans*-iso-α-acids the major impurities were, as could be expected, *cis*-iso-α-acids.

The most critical point, however, i.e., recovery of *trans*-isomers could not be appreciably improved.

A possible approach seemed to be to submit the DCHA-iso-α-acids to solid-phase extraction on an ion-exchange cartridge. This might have been possible because the DCHA salt is destabilised in the aqueous medium of the HPLC solvent used to analyse the DCHA salts. The proof of this is that the retention times obtained when injecting free *trans*-iso-α-acids and DCHA-*trans*-iso-α-acids are identical, a fact that had been observed by Hughes (1999).

Even then, the problem of the *cis*-isomers had yet to be solved. Progress made in the other methods, i.e., the development of a CPC system to separate the iso-α-acids led us to take the decision to suspend the work with this method.

Therefore, in summary, this line of work was discontinued for the following reasons:

- The total yield of compounds with the required purity (>95%) was below 2%, major losses occurring during the regeneration of DCHA-*trans*- iso-α-acids.
- The obtained compounds had remnant DCHA, which had to be removed owing to its toxicity, aroma and flavour. Thus some sort of ion exchange based purification step had to be considered.
- DCHA precipitation proved to be a difficult process with very significant variations in the yield and time needed for total precipitation.
- The whole process was time consuming and required a skilled operator if losses due to light and oxygen exposition were to be avoided.

In the case of the *cis*-iso-α-acids, the problems that were identified were:

- Degradation during DCHA precipitation was impossible to avoid.
- Degradation products had to be removed, involving a further time-consuming process that led in itself to further degradation.
- Another problem was adhumulone. Because of the low yield in the CPC separation, owing to a poor separation but also to its low presence in hops, its isolation was not attempted in this phase. The idea was to attempt this later on when all the process had been optimised.

It was therefore considered too inefficient for our preparative purposes and at this point more promising results were being achieved with methods based on combinations of DCHA precipitation and CPC or CPC by itself as described in the following chapters.

On the other hand, a positive aspect of this isolation method was that it allows the production of pure **individual DCHA-***trans*-iso-α-acids that are not to date available as reference compounds. What is used at present is the mixture of

the three DCHA-*trans*-iso-α-acids. The individual *cis*-iso-α-acids could also, with some adjustments in the process be available in solution as reference compounds.

.....

- REFERENCES

- **Bamforth CW (2000).** Brewing and brewing research: past, present and future. J Sci Food Agric, 80: 1371–1378.
- **Briggs DE, Boulton CA, Brookes PA, Stevens R** (2004). Brewing: Science and Practice; Woodhead Publishing: Cambridge UK (ISBN 1855734907)
- **Hermans-Lokkerbol ACJ, Verpoorte R** (1994a). Preparative separation and isolation of three α-bitter acids from hop, Humulus lupulus L., by centrifugal partition chromatography. J Chromatogr A, 664, 45-53.
- **Hermans-Lokkerbol ACJ, Verpoorte R** (1994b). Development and validation of a high-performance liquid chromatography system for the analysis of hop bitter acids. J Chromatogr A, 669, 65-73.
- **Hughes PS** (1996). Preparative regime for the purification of bitter acids derived from hops (Humulus lupulus, L.), J Chromatogr A, 731, 327-330.
- **Hughes PS** (2000). The Significance of Iso-α-Acids for Beer Quality. Cambridge Prize Paper. J Inst Brew W6, 5
- **Koller H** (1968). Magnesium ion catalysed iosmerization of humulone: a new route to pure isohumulones, J Inst Brew, 75, 75-179
- **Maye, JP, Mulqueen S, Weis, S, Xu J, Priest M** (1999). Preparation of Isomerized alpha-Acid Standards for HPLC Analysis of Iso-alpha-Acids, Rho-Iso-alpha-acids, Tetrahydro-Iso-alpha-acids, and Hexahydro-Iso-alpha-Acids. J Am Soc Brew Chem,57(2):55-59.
- **Pfenninger HB, Schur G, Vaterlaus BP, Sigg T, Wild J** (1975). Proceedings of the European Brewery Convention, Nice, 159-169.
- **Schwarzenbach R** (1979). HPLC of Hop Acids on Buffered Silica Gel Systems. J. Am. Soc. Brew. Chem, 37:0180.
- **Simpson WJ** (1993). Ionization Behaviour of Hop Compounds and Hop-Derived Compounds. J Inst Brew, 99, 317-326.
- **Thornton HA, Kunlandai J, Hawthorne DB, Kavanagh TE** (1990). Preparation of a *trans*-isohumulones standard. J Inst Brew, 96:367.
- **Thornton HA, Kunlandai J, Bond M, Jontef MP, Hawthorne DB, Kavanagh TE** (1993). Preparation of *trans*-iso-alpha-acids and use of their dicyclohexylamine salys as a standard for iso-alpha-acids analysis. J Inst Brew, 99:473-477

- **Verzele M, Anteunis M, Alderweireldt F** (1965). Isohumulones. J Inst Brew, 71:232-239.
- **Verzele M, Steenbeke G, Verhagen L, Strating J** (1989). Preparative liquid chromatography of hop and beer bitter acids. J Chromatogr, 484, 361–368.
- **Verzele M, De Keukeleire D** (1991). Developments in food science 27: Chemistry and analysis of hop and beer bitter acids (pp. 203–205).