Studies of iso-alpha-acids: analysis, purification, and stability.

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CHAPTER 4

Isolation of Individual Hop Iso-α-Acids Stereoisomers by β-cyclodextrin

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ABSTRACT

β-Cyclodextrin has been used for the isolation of *trans*- and *cis*- iso-α-acids. The separation from the mixture of stereoisomers was achieved by complexation using ethanol-water (1:2) as a solvent at a temperature of 50 ^{o}C during 30 minutes. The molar ratio of iso-α-acids sample to β-cyclodextrin for complexation was 1:1. Precipitation time varied between 9 hours and 2 days depending on the iso-α-acid. Release of the guest from the cyclodextrin complex was successfully accomplished by elution with methanol.

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4.1. INTRODUCTION

Hop cones, the female flowers of *Humulus lupulus* L. are used in the brewing most importantly because of the presence of α-acids. These compounds are chemically converted during the brewing process into bitter tasting substances, known as iso-α-acids (IAAs) that are responsible for the characteristic bitter taste of beer. Each α-acid yields a pair of the *trans*-/*cis*-stereoisomers (Alderweireldt *et al*., 1965; Koller, 1969; Verhagen, 1988).

IAAs in beer can be measured by Bitterness Unit (BU) analysis (Verhagen, 1988). However, this method can not be used to determine the individual iso-α-acid content since the UV absorption spectra of these compounds are similar. Nowadays, high performance liquid chromatography (HPLC) coupled with UV spectroscopy or with a mass spectrometer (MS), is routinely used to analyze individual bitter acids (Harms and Nitzche, 2001; Raumschuh *et al.*, 1999; Vanhoenacker *et al.*, 2004). Other methods such as gas chromatography (GC) (Martinez and Willemsen, 2002) and capillary electrophoresis (McLaughlin *et al*., 1996; Royle *et al*., 2001) have also been described. All these methods require pure standard compounds for the quantitative analysis of the individual compounds.

Individual IAAs are also needed when studying the parameters which affect the quality of beer, such as the contribution of these compounds to the final taste of beer (Hughes, 2000; Hughes *et al*., 1997), foam formation (Bamforth, 1985), and stability (De Cooman *et al*., 2000; De Cooman *et al*., 2001).

Despite the obvious need for these compounds they are not commercially available. This is due to the difficulty to separate these compounds with economically viable methods and to their instability. Several publications describe preparative methods (Hughes, 1996; Thornton *et al*., 1990; Thornton *et al*., 1993; Ting and Goldstein, 1996; Verzele and Steenbeke, 1989) which can separate *trans*- from *cis*-isohumulone. The TLC method that has been reported by Aitken *et al*. (1968) could separate *trans*-from *cis*-isohumulone. The reference compounds have also been prepared by photoisomerization of humulone (Clarke and Hildebrand, 1965; Sharpe and Ormrod, 1991). However, the preparation is very tedious, often not reproducible and time consuming. Very stable *trans*-IAAs were prepared by using dicyclohexylamine (DCHA) (Thornton *et al*., 1993). This mixture of *trans*-iso-α- DCHA salts has been

used widely as a reference compound and is commercially available. However, the *cis*-isomers are not available to date.

Herman-Lokkerbol and Verpoorte (1994b) succeeded in isolating pure α-acids from crude supercritical carbon dioxide hop extracts by means of centrifugal partition chromatography (CPC). This method reduces the costs of the isolation of α-acids with the use of lower amounts of solvent and relatively less time in comparison with preparative LC. The individual α–acids thus isolated can be isomerized to yield the pure *trans*-/*cis*-IAAs pair, the remaining problem being the separation of the *trans*- and *cis*- isomer of each pair.

β-Cyclodextrin (β-CD), had been used to stabilize hop oils and iso-α-acids (Hughes and Simpson, 1994; Simpson and Hughes, 1995). This, together with the report of a decrease of antibacterial activity of *trans*-isohumulone in presence of β-CD (Simpson and Smith, 1992), led us to consider its application to the separation of the isomers. Moreover, CD have been extensively used to separate between isomers, functional groups, and enantiomers of some natural products (Aboul-Enein *et al*., 2000; Breinholt *et al*., 1999; Bressolle *et al*., 1996; Krupcik *et al*., 2004; Salvador *et al*., 2001; Spanik *et al*., 2002; Kuramoto , 1986; Uemasu and Kushiyama, 2004).

The selectivity of CDs relates to its truncated cone structure. CDs are built up from 6 (α-CD), 7 (β-CD), 8 (γ-CD) or more glucopyranose units (Fig. 3.1). The inner cavity which is hydrophobic can interact with a hydrophobic organic molecule. Complex formation is a dimensional fit between host cavity and guest molecule. Hydroxyl groups can give hydrogen bonding with the guest (Szejtli, 1988).

In the initial experiments in our laboratory to produce the iso-α-acids-CD complexes, it was found that β-CD binds preferentially to the *trans*-isomers (**Chapter 3**). Here we report the optimization of this method for isolation of the individual IAAs. It includes the choice of type of CD, inclusion conditions, precipitation time, and methods to release guest from guest-CD complex.

4.2. MATERIALS AND METHODS

4.2.1. Materials

All organic solvents used were purchased from Biosolve Co. Ltd (Valkenswaard, The Netherlands). *Ortho*-phosphoric acid 85% (w/v) was obtained from Merck (Darmstadt, Germany). Alpha-CD (≥ 98%), β-CD (≥ 99%), and γ-CD (≥ 98%) were purchased from Fluka (Steinheim, Germany).

A supercritical carbon dioxide hop extract was obtained from Botanix (Paddock Wood, Kent, UK).

4.2.2. Isolation and isomerisation of pure individual α-acids

A supercritical liquid carbon dioxide hop extract was subjected to centrifugal partition chromatography using the procedure described by Hermans-Lokkerbol and Verpoorte (1994b). The isolated α-acids (cohumulone, humulone, and adhumulone) were subsequently isomerized to iso-α-acids according to the method described by Koller (1969) with a small modification: $MgSO_4.7H_2O$ (2.15 g) was dissolved in 25 ml water and 30 ml methanol in a 300 ml dark bottle. This solution was heated to 70 ^oC with stirring. A solution of purified α-acids (1.8 g) in 50 ml methanol and 5.35 ml NaOH 1 M was poured slowly into the dark reaction bottle. The reaction mixture was heated to 70 ^oC for 45 minutes under continued stirring. After cooling in an ice bath, the reaction mixture was acidified with 20 ml sulphuric acid 30% and extracted 3 times with 100 ml *n*-hexane. After washing the *n*-hexane phase was washed twice with water, and after drying with sodium sulphate the solvent was removed with a rotating evaporator.

4.2.3. *Affinity of α-.β-, and γ-cyclodextrins for cis- and trans-iso-α-acids*

In order to study the ability of three different CD to bind iso-α-acids, different warm solution of α-. β-, and γ-CD were prepared by adding separately 27 mg, 32 mg, and 36 mg of α-. β-, and γ-CD respectively to 400 µl ethanol-water (1:2) in 1.5 ml-micro tube and heating to 50 ^oC in water bath. The guest compounds were prepared

by dissolving 5 mg of the previously prepared iso-α-acids in 65 µl ethanol. These guest compounds were added drop-wise to 210 µl of CD solution in 1.5 ml-micro tube. The mixtures were vortexed and heating to 50 ^{o}C in water bath during 30 minutes. They were stored at 4 ^{o}C for several days in the dark for 3 days. The supernatants were then checked by HPLC.

4.2.4. Separation of trans- and cis-iso-α-acids using β-cyclodextrin

A β-CD solution was prepared by adding 3.17 g of β-CD to 40 ml ethanol-water (1:2) and heating to 50 ^{o}C in order to dissolve all β-CD. The guest compounds solution were prepared by dissolving 500 mg of the previously prepared iso-α-acids in 6.5 ml ethanol and added drop-wise to 21 ml of the β-CD solution while continually stirring and heating to 50 ^{o}C during 30 minutes. The mixture was stored at 4 ^{o}C for several days in absence of light and the β-CD complex precipitated as a white-yellow crystalline powder. The precipitate was separated by vacuum filtration and washed several times with 50 ml ethanol:water (1:2).

In order to release the *trans*-IAAs from the β-CD complex, the precipitate was treated with different organic solvents: ethanol, ethyl acetate, dichloromethane, *n*-heptane, *n*-hexane or methanol using 2 portions of 50 ml of each solvent. Each eluate was collected in a separate erlenmeyer and with the exception of ethanol, taken to dryness with a rotary evaporator and re-dissolved in 100 ml ethanol for their HPLC analysis. The supernatants that were found to contain essentially pure *trans*- or *cis*-IAAs were pooled. Approximately 50 ml of water were added to each 50 ml pooled supernatant solution and followed by addition of HCl 6M to reach pH 1 while continuously being stirred. The acidified supernatant was extracted 2 times with 100 ml n-hexane using a separatory funnel. The n-hexane phase was washed 2 times with water in separatory funnel in order to remove all dissolved β-CD and HCl. The excess water in n-hexane phase was removed by dry sodium sulfate and subsequently the n-hexane phase was evaporated on rotary evaporator. The concentrates were dissolved in ethanol and stored in a dark bottle at -20 ^{o}C.

The purity and quantity were checked by HPLC and ^{1}H NMR using anthracene as an internal standard. HPLC analysis of the samples was carried out using a binary system consisting of a 1% solution of *ortho*-phosphoric acid (v/v, solvent A) an

acetonitrile/water/*o*-phosphoric acid (81/19/1, v/v/v, solvent B) at a flow rate of 1.0 ml/minute. A 10 minute gradient of 85 to 91% of B allowed the separation of the IAAs.

4.3. RESULTS AND DISCUSSION

4.3.1. Complexation condition

There are many types of complexation techniques with CDs, such as preparation in solution, suspension, kneading, and melting (Del Valle, 2004; Szejtli, 1988). In this study, preparation in solution was applied as both compounds, in supernatant and precipitate were required to be recovered. Water is the most commonly used solvent for complexation reactions. The more solubilised the cyclodextrin in the solvent is, the more molecules become available for complexation. When the guest compound is not readily soluble in water, the complexation process becomes very slow, inefficient or even impossible. In such cases, the use of an organic solvent to increase the dissolution of the guest is desirable. This solvent should not be able to form a complex with the CD and be easily removed by evaporation. Ethanol is a good example of such a solvent (Del Valle, 2004; Szejtli, 1988). In this experiment ethanol was added in order to dissolve iso-α-acids which are not very soluble in water.

Another important parameter for the effectiveness of complexation is temperature. In this study a temperature of 50 ^{o}C was found to be necessary to dissolve the CDs. A higher temperature may cause the degradation of the IAAs.

4.3.2. Affinity of α-.β-, and γ-cyclodextrins for cis- and trans-iso-α-acids

Table 4.1 shows the results obtained in testing the ability of α-, β-, and γ-CD to bind the isolated *trans*-IAAs. The results clearly show that they have a very different behaviour, while α-CD shows little or no complexation, β-CD has a high affinity only for *trans*-IAAs under test conditions. With γ-CD both *cis*- and *trans*- isomers are complexed. Therefore β-CD can advantageously be employed to selectively complex *trans*-IAAs in a solution that contains both *cis*- and *trans*-isomers, yielding a *trans*-

free supernatant that contains the pure *cis*-isomers and a very stable *trans*-α-acid/β-CD complex precipitate.

Table 4.1. Ability of α-, β-, and γ-CDs to bind *cis*- and *trans*-IAAs.

Compounds		% binding to cyclodextrin [1]		
		α-CD	β-CD	γ-CD
Isocohumulone	*trans*-	0.2	98.5	97.4
	cis-	0.3	2.0	98.4
Isohumulone	*trans*-	0.1	98.8	99.2
	cis-	0.2	0.7	99.5
Isoadhumulone	*trans*-	0.2	97.3	96.1
	cis-	0.7	2.5	95.8

[1] % Binding was determined from HPLC analysis of concentration of *trans*- and *cis*-IAAs in starting solution and supernatant. Thus % binding = 100 - (area after treatment/area before treatment) x 100.

4.3.3. Rate of complexation

The solubility of the β-CD-guest complex decreases as the complexation reaction proceeds and the mixture is cooled. The time needed for total precipitation depends on the complex formation and its stability. Table 4.2 shows the ratio of the concentrations in which the *trans*- and *cis*-isomers were found in the supernatant as a function of time (precipitation at 4 ^{o}C) showing that after about 24 hours an equilibrium is reached.

4.3.4. Recovery of trans-iso-α-acids

The binding of guest molecules to cyclodextrins is a dynamic equilibrium. Thus, guest molecules can be released by decreasing the stability of the complex (Szejtli, 1988). There are four industrial methods of releasing guest molecules from the complexes: heating, acid resolution, hydrolytic enzyme decomposition, and extraction with organic solvent (Matsunaga *et al.*, 1984). The latter method was applied in the following experiment.

Table 4.2. Ratio of *trans*- to *cis*-IAAs in the supernatant during precipitation of the β-CD complex.

Time (hours) / Guests	% *trans* to *cis* [1]						
	0	0.5	9	12	24	48	96
Isocohumulone	39.9	13.6	1.1	1.1	1.1	1.3	1.3
Isohumulone	38.3	11.0	5.9	4.8	3.8	3.0	3.0
Isoadhumulone	40.2	25.1	6.2	3.8	2.9	2.9	3.0

[1] Analysed using HPLC, % *trans* = (area of *trans*/area of *cis*) x 100.

After repeatedly washing the precipitates with the same solvent used in the complexation experiments (ethanol/water, 1:2), the complexes were eluted with several organic solvents: ethanol, ethyl acetate, dichloromethane, *n*-heptane, *n*-hexane, and methanol. Of these, methanol proved to be the best solvent, followed by ethanol in a very much lower degree (about four fold less effective). All other non-polar solvents proved to be practically unable to extract the iso-α-acid molecules from the β-CD complex.

The purity of the isolated *trans*-isomers fractions obtained by elution with methanol was determined by means of HPLC and ^{1}H NMR. It was found that all of the isolated *trans*-isomers fractions had a purity above 95%. The impurities observed are believed to originate from degradation reactions that occur during the complexation process and from the ability of β-CD to also bind small amount of the *cis*-isomers.

Recovery of IAAs isolated by this method is around 50% determined by weighing of the purified compounds. The remaining compounds are thought to be eluted in the washing step of the complexation process in which ethanol/water (1:2) is used. In this step some of β-CD precipitate was dissolved in this solvent.

The chemical structure of isolated iso-α-acids has been confirmed by NMR and mass spectrometry (**Chapter 5**).

4.4. CONCLUSION

α- and γ-CD do not separate the stereoisomers. Complete separation of *trans*- from *cis*-IAAs was successfully performed only by β-CD using ethanol/water (1:2) as a solvent. Temperature was maintained at 50 ^{o}C during 30 minutes for complexation. The molar ratio of IAAs/β-CD for complexation is 1:1 and the time required after complexation for precipitation varied between 9 hours and 2 days depending on IAAs.

A further experiment on the effect of different organic solvents on the release of the guest from the complex showed that methanol is the most powerful organic solvent to destabilize the complex. Ethanol, ethyl acetate, *n*-heptane, and *n*-hexane have a very much lower ability to release the guest. The purity of the isolated compounds is above 95% and the recovery is reasonable (around 50%), which might be improved in further optimization of the process.