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

Khatib, Alfi

CHAPTER 6

β-Cyclodextrin Improves the Stability of Iso-α-acids

Alfi Khatib, Erica G. Wilson, Hye Kyong Kim, Young Hae Choi, and Robert Verpoorte

Division of Pharmacognosy, Section of Metabolomics, Institute of Biology, Leiden University, Einsteinweg 55, PO BOX 9502, 2300 RA Leiden, The Netherlands

ABSTRACT

Iso-α-acids are light and oxygen sensitive compounds derived from hop α-acids. They are prone to decompose during storage and are partly responsible for the off-flavour in ageing beer. Here we report on the stability of iso-α-acids-β-cyclodextrin complexes. The stability of iso-α-acids increases by complexation with β-cyclodextrin. Most of iso-α-acids-β-CD complex does not show degradation after 7 days storage in the presence of light and oxygen. Complexation of individual iso-α-acids and β-cyclodextrin was made using ethanol/water (1:2) at 50 ^{o}C; complexation of iso-α-acids mixtures and cyclodextrin on the other hand was achieved using water at 70 ^{o}C. The complexation was confirmed by shifts in the UV and NMR spectra of the iso-α-acids and β-CD. Integration of NMR signals confirmed the molar ratio of iso-α-acids to β-cyclodextrin to be 1:1 and 1:2 for the *trans*- and *cis*-isomers, respectively.

6.1. INTRODUCTION

Iso-α-acids (IAAs) are considered to be key components of beer, contributing to their taste and to foam stability. However they are very unstable compounds and their degradation products are thought to be partially responsible for the off-flavour characteristic of ageing beer including stale and cardboard flavours which are connected with their oxidative degradation. The compounds that are responsible for these off-flavours are unsaturated aldehydes, such as *trans*-nonen-2-al, formed by the oxidative degradation of isohumulones (Hashimoto and Eshima, 1979). Other compounds are the vicinal diketones which are formed by oxidative decarboxylation of 2-acetohydroxycarboxylic acids. The taste threshold values for these compounds are very low ($<10^{-2}$ mg/l), and even as low as to 5 x 10^{-4} mg/l for *trans*-2-nonenal. In higher concentration it will give beer a very unpleasant resinous taste. Beer is no longer drinkable if the concentration of these compounds is about 1 mg/l (Verzele and De Keukeleire, 1991).

Furthermore, IAAs are sensitive to light, and their degradation is responsible for the light struck flavour of beer (see Fig. 6.1). In order to reduce this, beer is usually bottled in dark-coloured glass. Alternately, light stable reduced-IAAs are used (Hougen, 1963). The light sensitive part of the IAA structure is the acyloin group which contains the tertiary alcohol and the carbonyl group of the side chain at C4. UV light causes bond cleavage leading to **2** and acyl (**3**) radical. The loss of carbon monoxide from the acyl radical and combination with a thiol radical leads to 3-methyl-2-butene-1-thiol (**4**), and dehydrohumulinic acid (**5**). The thiol has an extremely low flavour threshold, concentrations below 10 nanogram/l will already give an offending off-flavour (Benitez *et al*., 1997).

Severeal studies showed that the *cis*-isomers are more stable than the *trans*- isomers (Araki *et al*., 2002; De Cooman *et al*., 2000; Hughes *et al*., 1997). Therefore, the possibility of separating these isomers and using only the *cis*- isomers in the beer production process could be a way of increasing beer stability. A simple and cheap method for the separation of the *cis*- and *trans*-isomers of IAAs using β-cyclodextrin (β-CD) was developed in our laboratory (**Chapter 3**). Additionally, in combination with previously published methods for α-acids purification (Hermans-Lokkerbol and Verpoorte, 1994b) this method allows the production of the individual *cis*- and *trans*-

isomers of the IAA reference compounds in any quantity (**Chapter 4**). This method could replace the rather difficult current isolation method which uses dicyclohexylamine (Maye *et al.*, 1999; Thornton *et al.*, 1993). This allows further studies on the stability of these compounds.

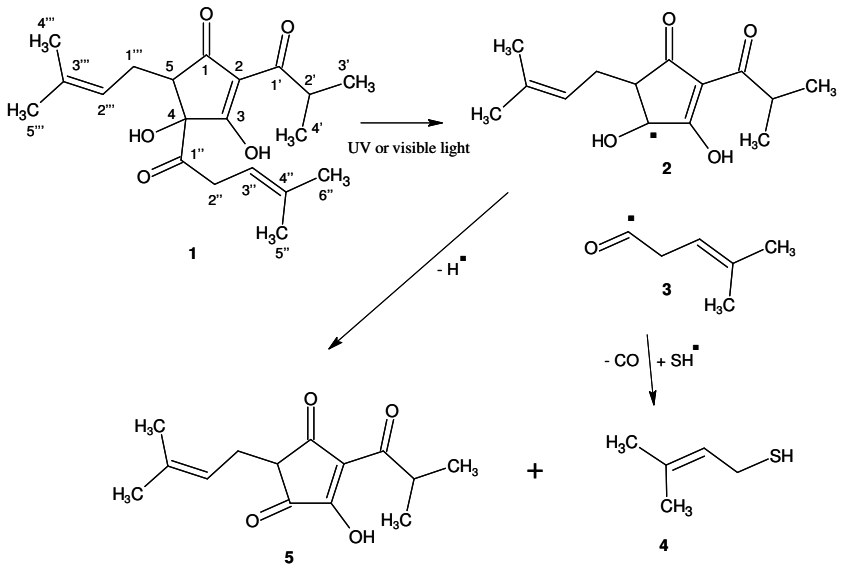


Fig. 6.1. Structural modifications of IAAs leading to the "light struck flavour".

One possibility to increase the stability is by micro encapsulation using CDs. CDs are often used in this way to increase the stability of drugs (Holvoet *et al.*, 2005; Jeon *et al.*, 2005), food (Bhandari *et al.*, 2001; Reineccius *et al.*, 2004), cosmetics (Buschmann, 2002; Jeong *et al.*, 2000) and pesticides (Biebel *et al.*, 2003), and also IAAs (Simpson and Hughes, 1995).

CDs are cyclic oligosaccharides, containing 6 (α-CD), 7 (β-CD), 8 (γ-CD) or more glucopyranose moieties. They have the structure of a truncated cone with a hydrophobic inner cavity, and a hydrophylic outer surface. The interior cavity can house a hydrophobic organic molecule or part of it, producing a CD inclusion complex. As a result of this, the guest molecule trapped inside the CD cavity is protected from oxidation, light and heat induced decomposition (Szente and Szejtli,

2004).

The aim of this study is to explore the stability of IAAs complexed with β-CD and to characterize the formation of IAA-β-CD complexes.

6.2. MATERIALS AND METHODS

6.2.1. Materials

All organic solvents used were purchased from Biosolve Co. Ltd (Valkenswaard, the Netherlands). *Ortho*-phosphoric acid 85% (w/v) and trimethylsilane propionic acid sodium salt (TSP) were obtained from Merck (Darmstadt, Germany). β-cyclodextrin (≥ 98%)_Cavamax® W7 Pharma was purchased from Wacker-Chemie Co. Ltd, (Burghausen, Germany). Ethyl alcohol-d_6 (99.0%) and deuterium oxide (99.9%) were purchased from Euriso-top (Gif-Sur-Yvette, France).

Supercritical CO_2 hop extract and an aqueous solution of its isomerised form in a potassium salts were obtained from Botanix (Paddock Wood, Kent, UK).

6.2.2. Procedure for β- CD complexation

Two different type of samples were complexed to *β*- CD: *trans*- and *cis*- IAAs mixture in potassium salt form, and pure IAAs. Those samples were obtained by following the procedure as described in **Chapter 3** and **4**.

In case of the *trans*-isomers-β- CD complexation, the same method was used as in the separation and isolation without methanol elution of the complex in order to maintain the *trans*-isomers in the complex. Complexation of the *cis*-isomers with β-CD was achieved following the same method but with a molar ratio of 1:4.

6.2.3. Stability test

Stability test of the mixture and pure IAAs were performed in different conditions: without organic solvent (dry), with different organic solvents (chloroform, ethanol and methanol), and β-CD complexes in dry form and in 50% ethanol.

The dry samples were obtained by placing aliquots of sample stock solutions in several vials and evaporating the solvent with nitrogen gas. Equal amounts of freeze dried β-CD complex powder were placed in several vials. The effect of organic solvents was studied by dissolving 1 mg dried IAA sample in 100 µl of the above mentioned solvents. In case of β-CD complexes, 1 mg of freeze dried complex was dissolved in 50% ethanol.

All the samples were transferred to small colourless vials and placed in a room with artificial light (1800 lux) and temperature at 24 °C. The IAAs content of the samples were determined on the first day and 7 days later using HPLC. Dry samples (100 µg) were dissolved in 100 µL of methanol and solutions were diluted 10 times with methanol prior to HPLC measurement. All samples were quantified in triplicate.

6.2.4. HPLC analysis

A Waters HPLC chromatograph consisting of a 626 pump, a 600S pump controller, auto sampler (717 plus) and a photodiode array detector (2996) was used. The column was a Hypersil 5 C18, 250 x 4.6 mm (Phenomenex, Torrance, CA, USA). Mobile phases were filtered with a 0.20 µm hydrophilic polypropylene membrane filter 47 mm type GH Polypro (Pall Corporation, Ann Arbor, MI, USA).

Compounds were eluted isocratically with a mobile phase consisting of acetonitrile-water- H_3PO_4 (50:50:0.01, v/v/v) at a flow rate of 1.5 ml/minute. Baseline separation of all 6 isomers was achieved with a total run time of 25 minutes. Quantitation was carried out using the external standard method. Pure reference compounds are not commercially available, so compounds isolated and identified in our laboratory were used as standards.

6.2.5. NMR measurements

Dry IAA-β-CD complexes were dissolved in 1 ml of ethanol-d_6 and deuterium oxide (1:2) containing 0.01% TSP. ^{1}H NMR was recorded at 25 ^{o}C on a 300 MHz Bruker DPX-300 spectrometer operating at a proton NMR frequency of 300.13 MHz. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 minutes acquisition time. The resulting spectra were manually phased, baseline corrected, and calibrated to TSP

at δ 0.0, all using XWIN NMR (version 3.5, Bruker).

6.3. RESULTS AND DISCUSSION

6.3.1. Formation of iso-α-acids and β-CD complexes

The complexation method as discussed in **Chapter 3** and **4** was applied in this experiment. However, this method only considered the complexation of the *trans*-isomers which precipitate as a yellow crystalline powder after addition of β-CD, the *cis* isomers remaining in the supernatant. In this study, the *cis*-isomers obtained after separation from their *trans*-isomers were treated with β-cyclodextrin using the same conditions as those used for the complexation of the *trans*-isomers except that the molar ratio of samples to β-CD was 1:4. This molar ratio was used under consideration that the complexation did not occur at a molar ratio of 1:1. Complexation did occur if there is an excess of β-CD.

The formation of IAAs-β-CD complex was confirmed by analysing the shift pattern of the UV and NMR spectra of treated and untreated compounds. The UV absorption maximum of the IAAs-β-CD complex were slightly shifted by a partial shielding of the excitable electrons (Szente and Szejtli, 2004).

However, information provided by the UV spectra was insufficient to explain how the IAAs were bound to β-CD. NMR spectra can provide further information about the inclusion. In case of inclusion, the H-3 and H-5 β-CD atoms located in the cavity interior, are shielded by the guest molecule due to hydrogen bonds causing an up field shift of the NMR signals. In most of the cases, the hydrogen atoms on the outer surface, H-1, H-2, H-4 and H-6, will not be affected (Szejtli, 1988).

The change of chemical shift value ($\Delta\delta$) can be used to predict the complex stability and the depth of inclusion (Lyng *et al*., 2005). In the case of the isocohumulones-β-CD complex NMR spectra, the H-3 and H-5 signals of β-CD were only weakly shifted (Table 6.1) implying that no inclusion of IAAs into the cavity of β-CD occurred. A similar result was observed with all other IAAs (data not shown). The shift produced by the *trans*-isomers complexation with β-CD is greatest for the anomeric proton, H-1, on the outer surface of β-CD. This means that the *trans*-isomer

interacts with the outer surface of β-CD. The shifts of *trans*-isocohumulone protons are also weak (Table 6.2), it shows that the complexation of the *trans*-isomer to β-CD is not through the binding of these protons. The binding to β-CD could be through hydroxyl or keton groups. In case of the *cis*- isomer, no large shifts were observed for protons on the surface of β-CD (Table 6.1). However, shifts occurred for H-5, H-2'', and H-1''' of *cis*-isocohumulone (Table 6.2) implying that there is an interaction between these protons and the surface of β-CD.

Table 6.1. Effect of the complexation on the chemical shift of some β-CD protons measured by ^{1}H NMR.

Proton position	Δδ (ppm) of β-CD signals[1]	
	TICH-β CD[2] complex	CICH-β CD[3] complex
1	0.07	0.02
3	0.03	0.02
6	0.04	0.02
5	0.03	0.03
2,4	0.03	0.02

[1] $\Delta\delta$ is the change of chemical shift, calculated by subtraction of $\delta_{\beta\text{–CD}}$ to $\delta_{\beta\text{–CD complex}}$.
[2] TICH is *trans*- isocohumulone.
[3] CICH is *cis*-isocohumulone.

Table 6.2. Effect of the complexation on the chemical shift of some isocohumulones protons measured by ^{1}H NMR.

Proton position	Δδ (ppm) of iso cohumulones signals[1]	
	TICH-β CD[2] complex	CICH-β CD[3] complex
3",2'''	0.02	0.03
2"	0.03	0.06
5	0.03	0.08
1'''a	0.03	0.09
1'''b	0.04	-
6''	0.04	0.03
5'''	0.04	0.03
5''	0.03	0.03
4'''	0.04	0.03
3',4'	0.03	0.04

[1] $\Delta\delta$ is the change of chemical shift, calculated by subtraction of δ_{guest} to $\delta_{\beta\text{–CD complex}}$.
[2] TICH is *trans*- isocohumulone.
[3] CICH is *cis*-isocohumulone.

The IAAs could not be released from the β-CD complex by elution with non polar solvents such as *n*-hexane or chloroform. It could only be released by the polar solvent methanol as discussed in **Chapter 3** and **4**. If inclusion occurs, the guest is expected to be easily released by eluting with non polar solvents because the cavity of β-CD is also non polar (Del Valle, 2004). Moreover, IAAs have a good solubility in choloroform and *n*-hexane. The fact that only methanol can release IAAs, supports the hypothesis that real inclusion does not occur in the β-CD complexation, but another interaction between guest and β-CD occurs.

Possible proton-proton interactions between isocohumulone to β-CD were studied using NOESY NMR. But no NOE's between the protons of the guest and β-CD could be observed. This is in agreement with the very small Δδ values in Table 6.1 and 6.2 which are also indicative for weak binding. Most likely there is a relatively large distance between protons of guest and β-CD. It also confirmed that no guest inclusions occur into the cavity of β-CD. However, the data obtained in this study can not explain how IAAs are bound to the outer surface of β-CD. Further study is necessary to identify the mode of interaction.

The molar ratio of IAA/β-CD for the *trans*- and *cis*-isomers was determined by integration of the ^{1}H NMR signals, a 1:1 and 1: 2 ratio for the *trans*- and *cis*-isomers was found, respectively.

6.3.2. Stability test

The stability studies of IAAs in light and air were carried out according to the conditions described above during 1 week at 24 °C. Different degradation products were observed depending on the storage conditions. In HPLC these degradation products have different retention times and UV absorption spectra. Although we did not determine the structure of the degradation products observed in the HPLC chromatograms, the stability tests still can be performed by measuring the amount of IAAs in the samples by HPLC using a standard calibration curve.

The stability of dry β–CD complexes of individual IAAs improved dramatically if compared to that of dry IAAs (Fig. 6.2). The stability of dry β–CD complex is also better to that of IAAs in chloroform, ethanol or methanol solutions. The labile part of the IAAs structure is constituted by the tertiary carbon centres and double bonds

which are prone to oxidation and UV catalysed degradation (Benitez *et al*., 1997; Verzele, 1986).

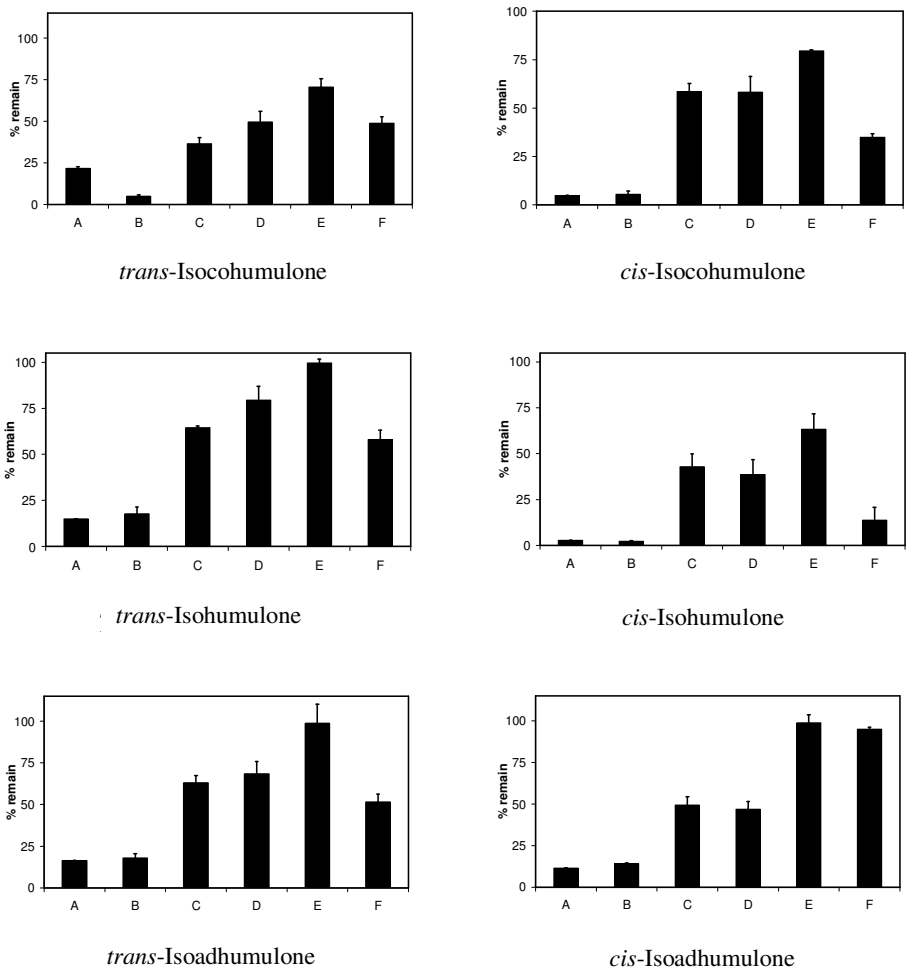


Fig. 6.2. Stability of individual iso-α-acids : dry (A), dissolved in chloroform (B), methanol (C), ethanol (D), β-CD complex (E), and β-CD complex in 50% ethanol (F) measured on 7 days storage. The samples were in colourless vials and placed in a room with artificial light (1800 lux) and temperature at 24 ^{o}C.

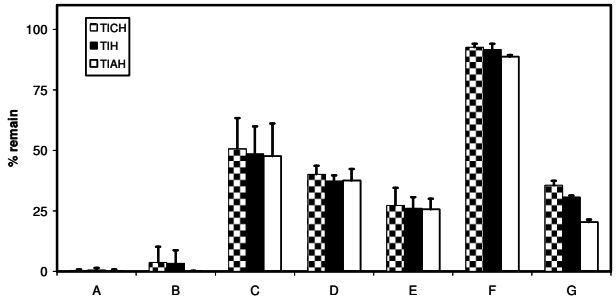
Dry β–CD can apparently protect the compounds from oxidation and light. However, the stability of the β–CD complex in water/ethanol was poor. The explanation of this phenomenon is still unclear. One assumption is that oxygen is dispersed in solution and in direct contact with the IAAs causes degradation. Because IAAs are not protected in the cavity, but bind to the outer surface of β–CD, oxygen and light can attack the compounds in solution. In the dry complex only few IAAs molecules are in direct contact with the air. Most are inside the solid β–CD complex particles.

The stability of the IAAs mixture is shown in Fig. 6.3. The test was also conducted by using water as a solvent since the IAAs mixture was complexed as potassium salt and can dissolve in water. The stability of dry β-CD complex of the mixture is very good, neither the *trans*- nor the *cis*-isomers suffered any degradation. Mostly the stability of the β-CD complex of the mixture is similar to pure IAAs-β-CD complex, although small differences can be observed. For example pure *cis*-isoadhumulone-β-CD complex in 50% ethanol is much more stable than in the mixture in the same condition. Several factors could cause this difference such as pH, as stability for pure IAAs and mixture of IAAs was determined at a pH around 5 and 7 respectively. The IAAs mixture was obtained commercially and is a potassium complex with pH 7, where the pure IAAs were isolated as acids and they have pH 5 in solution.

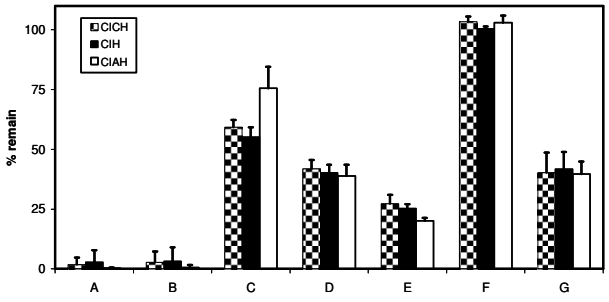
As mentioned, previous studies showed that the *cis*-isomers are more stable than the *trans*-isomers (Araki *et al*., 2002; De Cooman *et al*., 2000; Hughes *et al*., 1997). These studies were conducted by measuring the stability of IAAs in beer. However, the results in Fig. 6.2 show the opposite. The *trans*-isomers are more stable than the *cis*-isomers. The explanation of this contradiction maybe that the conditions are different from the conditions in our study. Moreover, IAAs in previous studies were not pure compounds but mixtures of all six IAAs.

The findings reported here are promising, especially those related to the β–CD complex and their possible use as stable reference compounds. Pure IAAs can easily be recovered from the complex by elution with methanol elution as mentioned in **Chapter 4**. Moreover, the higher stability of *trans*- and *cis*-IAAs mixtures as β–CD complexes opens the possibility of developing hop beverages containing selected IAAs. However, further study will be necessary to fully evaluate the effect of light,

oxygen, humidity, and heat on the stability of the β–CD complexes, both in dry complex and in solution.



trans-Iso-α-acids



cis-Iso-α-acids

Fig. 6.3. Stability of iso-α-acids mixture: dry(A), dissolved in chloroform (B), methanol (C), ethanol (D), water (E), β-CD complex (F), and β-CD complex in 50% ethanol (G) measured on 7 days storage. The samples were in colourless vials and placed in a room with artificial light (1800 lux) and temperature at 24 ^{o}C. TICH = *trans*-isocohumulone, TIH = *trans*-isohumulone, TIAH = *trans*-isoadhumulone, CICH = *cis*-isocohumulone, CIH = *cis*-isohumulone, CIAH = *cis*-isoadhumulone.

6.4. CONCLUSION

This study confirmed that individual IAAs can make complexes with β-CD. The complexation occurs through the binding of IAAs to the outer surface of β-CD, not through inclusion in the cavity. The molar ratio of IAAs to β-CD is 1:1 and 1:2 for the *trans*- and *cis*-isomers respectively. The resulting dry IAA β–CD complexes are stable even in presence of light and oxygen. However, the dissolved complexes are less stable.