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

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chapter 5

Isolation of Iso-α-Acids (III): Complexation with β-cyclodextrin

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5.1 Introduction

The isolation of iso-α-acids as pure reference compounds (>95% purity) free of taste-distortioning or toxic impurities was definitely a great challenge as outlined in the previous chapters. It was not impossible, however, as shown by the success achieved with any of the approaches described before that allowed the isolation of all isomers, albeit in low amounts or with a relatively unattractive yield / effort / time ratio.

There was a further problem to be addressed: how could these ultra-pure compounds be preserved once isolated? Free iso-α-acids are oils that are extremely unstable. They can be kept in methanolic solutions as potassium salts or as crystalline dicyclohexylamine (DCHA) salts in the case of *trans*- isomers (Thornton *et al.*, 1990,1993). These solutions were clearly out of the question, so that it was necessary to find some way of preserving these compounds, once again, in a odourless, tasteless and human-friendly environment. Considering the property of these isomers to react with the basic DCHA, other amines were considered, but it is a known fact that all of these compounds have a disagreeably strong odour, so were therefore dismissed.

Given the fundamental role of iso-α-acids in beer quality attributes, a great deal of research has been done on their stability, especially in the aqueous/ethanolic environment of beer, since they are recognized as one of the components which has a major responsibility in determining the period of aptitude for consumption of bottled or canned beer.

The three degradation reactions of iso-α-acids that occur in beer -photooxidation autoxidation and proton catalysed cyclisation have been described in Chapter 2. It is clear that of these reactions, only the latter affects *trans*-iso-α-acids, while the other two reactions affect both stereoisomers.

Another two important observations can be made about these reactions: the first is that in all cases, they involve the isohexenoyl chain on C(4). Secondly, because proton-catalysed cyclisation only affects *trans*-stereoisomers, these are definitely less stable than their *cis*- counterparts, resulting in the suggestion of an all-*cis* -iso-α-acid mixture as a way of achieving a consistent bitterness in beer or of course, the use of reduced iso-α-acid derivatives such as tetrahydro-iso-α-acids (De Cooman *et al.,* 2000).

5.1.2 Stabilisation of iso-α-acids: β-cyclodextrins

Given the instability of iso-α-acids, and though a great deal of the last cited papers had not been published at the time of our experiments, the presence of oxidation compounds was clearly traceable in the HPLC chromatograms obtained when monitoring the different processes throughout isomerisation and recovery of the pure compounds, as a number of peaks with lower retention times.

It was thus necessary to find some way to stabilise the isolated iso-α-acids as mentioned above. Among the different excipients used in the food and pharmaceutical industry to disguise solubilise or stabilise compounds, some of the most well-known are the series of cyclodextrins, α-, β- or γ - cyclodextrins.

Cyclodextrins (CDs) are, generically, cyclic oligosaccharides that consist of (-1,4)-β-α-D-glucopyranose units linked in such a way that a somewhat lipophilic central cavity is formed while the outer surface is hydrophilic with different degrees of hydrogen-bonding capacity (Losftsson *et al.*, 1996). Cyclodextrins were discovered by Viliers in 1891 (Viliers, 1891) appearing as the result of starch degradation by cycloglycosyl transferase amylases (CGTases) produced by various bacilli, among them *Bacillus macerans* and *B. circulans*, they were developed by Schardinger (1911) and finally by Pringscheim (Sicard *et al.*, 1987). Depending on the exact reaction conditions, three main CDs can be obtained: α-, β-, and γ-cyclodextrin, that consist of six, seven, or eight units, respectively (Le Bas *et al.*, 1987). Cyclodextrins with five or less glucopyranose units do not exist since steric factors or tension in the ring render them unstable (Fig. 5-2). Cyclodextrins with more units, i.e., 9, 10, 11, 12 or 13 glucopyranose units (δ -ε -ζ-η-θ, respectively) have been described, but of these, only δ-CD has been well characterized (Loftsson et al, 1997).

Cyclodextrins have a ring-like structural formula, but as a consequence of the ^{4}C1 conformation, all primary hydroxyls are situated on one of its edges while secondary hydroxyls are on the other edge. The ring is actually a conical cylinder, which is frequently described as a doughnut or wreath-shaped truncated cone. The inside of this cylinder, which is referred to as the cavity, is lined by the hydrogen atoms and the glycosidic oxygen bridges of the glucopyranoside units and the non- bonding electron pairs of these glycosidic oxygen bridges are directed toward the inside of the cavity producing a high electron density resulting in some Lewis base characteristics (Szejtli, 1998). The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution (Loftsson *et al.*, 1997).

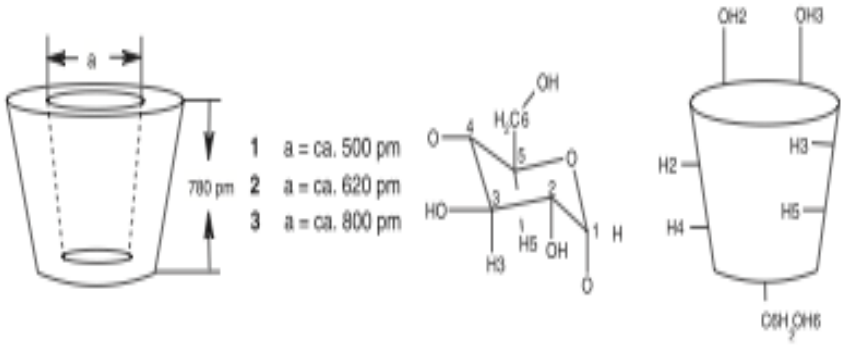

Fig 5-1: Schematic view of native CDs, sizes and orientation of most important atoms and hydroxyl groups groups (Dodziuk, 2006)

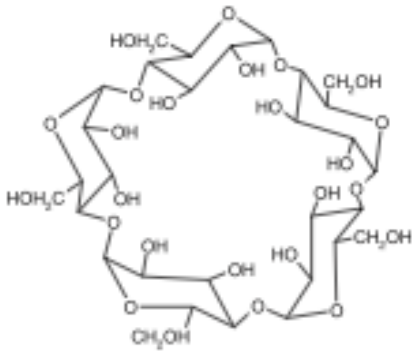

Fig. 5-2 Upper view of β-cyclodextrin (Szejtli, 1996)

Below, in Fig. 5-4, the disposition of primary and secondary hydroxyls on the different edges of the α-CD molecule can be observed. Primary hydroxyl groups are on the narrower rim of the truncated cone, while secondary hydroxyl groups remain on the wider rim.The characteristics of the three "natural" CDs are quite different and not necessarily predictable.

Table 5-1: Characteristics of main CDs (Duchene, 2011).

Cyclodextrin (CD)	α	β	γ
Glucopyranose units	6	7	8
Molecular weight	972	1135	1290
Central cavity diameter (ext/int) (Å)	5.3/4.7	6.5/6.0	8.3/7.5
Water solubility (at 25°C -%)	14.5	1.85	23.2

Another interesting characteristic of CDs is their ability to simultaneously change the direction of hydrogen bonds, in a movement known as a "flip-flop", dramatically modifying the configuration of the inner cavity (Zabel *et al.*, 1986). This finding provides the basis for the theory sustained by several authors, that claims that CDs are actually not rigid at all and that precisely this non-rigidity allows them to accomodate such a diversity of molecules (Dodziuk, 2006). The other application, which will be dealt with in this paper, is its *molecular recognition* property, which allows it to form complexes selectively with certain molecules that might fit into the inner part of the ring or cavity (Dodziuk, 1996).

The complexation process in an aqueous solvent involves the displacement of at least one molecule of water from the relatively hydrophobic CD cavity, the removal of the polar hydration molecules from the apolar guest molecule, its entry into the vacant CD cavity and stabilisation by weak but numerous *van der Waals* forces. The hydration of the exposed part of the guest is restored and integrated with the hydration shell of the host macrocycle (Dodziuk, 2006). Complexation can also occur in solid phase.

Once the (guest) molecule is included, a great change in the physicochemical properties of guest (and host) molecules occurs, since they are are temporarily enclosed in a microenvironment. These properties are, among others: solubility enhancement of highly insoluble guests, stabilisation of labile guests against the degradative effects of oxidation, visible or UV light and heat, control of volatility and sublimation, physical isolation of incompatible compounds, chromatographic separations (Del Valle, 2004).

Types of complexes. If inclusion complexes are formed with CD, it means that a molecule or a lipophyllic part of a molecule is included in the CD cavity. Other types of interaction have been described or are suspected to occur, in which a part of the molecule interacts with the external part of a CD molecule (Loftsson et al, 2003).

Furthermore, depending on the size of the guest and its structure, one or two (or more) CD molecules might react with one guest molecule (2:1) or two guest molecules with one CD molecule (1:2). In general, the most common type of inclusion complexes occur in a 1:1 ratio while non-inclusion complexes have different ratios (Zhao et al, 2002).

The inclusion complexes form stable crystalline substances that can be isolated by filtration. When these substances are dissolved, an equilibrium between the guest-CD complex and the guest (D) molecules is established, expressed by the complex stability constant, *K*a, which reflects the stability of the inclusion complex in a non-disassociated form. The dissociation is governed by a thermodynamic equilibrium (Szetli, 1998).

$$CD + D \leftrightarrow CD.D \qquad \textit{and} \qquad K_{1:1} = \frac{[CD.D]}{[CD][D]} \quad ; \quad \textit{Eq. 5.1}$$

In this case $K_{1:1}$ refers to the *K* or complex stability constant for a guest:host relationship of 1:1; CD is the cyclodextrin molecule and D, the host molecule. *K* is also known as the affinity constant, referring to the affinity of the guest molecule for the CD cavity, association constant, binding constant (affinity of the guest molecule for the CD cavity), stability constant (stability of the inclusion complex in a nondissociated form), association constant, or binding constant. The higher the *K* value, the more stable the inclusion, and the less dissociation that occurs. The value of K is thought to depend on various factors, being stronger when there is a good fitting of the guest molecule inside the cavity and a good size complementarity between guest and CD cavity (Gabelica *et al*., 2002). Depending on their respective size, the guest molecule will enter the CD cavity at the narrow side (primary hydroxyl groups) or at the wide side (secondary hydroxyl groups)

Production of complexes. There are various methods for preparing CD complexes: co-precipitation, slurry and dry-mixing (Hedges, 1998). The most common laboratory scale method is co-precipitation. There are other methods such as co-evaporation (and variations of this such as Spray-drying or freeze-drying), kneading, seal and dry, all of which vary basically in the amount of water added to the CD and guest, time and means of eliminating the solvent. More recent methods include the supercritical carbon dioxide method or microwave treatment among others (Duchêne, 2011).

Cyclodextrins and iso-α-acids

In the case of our project, the use of cyclodextrins to complex iso-α-acids was considered because there were a number of conditions which seemed to indicate that they could be good candidates as guest molecules: their low solubility in water and lipophyllicity and the possibility of increasing their stability if protected from oxygen and light. There were no records of the use of cyclodextrins for iso-α-acids, though Hughes *et al.* (1995) had attempted to stabilise hop oils (α-bitter acids) with cyclodextrins. An interesting observation was that of Simpson *et al.* (1992), who described the decrease of antibacterial activity of an isomerised hop extract when β-cyclodextrin was added to its aqueous solution.

Thus, with two purposes in mind, that of finding a possible preservative for free iso-α-acids and the possibility of the use of one of the cyclodextrins for the separation of these isomers, we begun a series of experiments testing different procedures and cyclodextrins with isomerised hop extracts and pure iso-α-acids (*trans/cis* isocohumulone; *trans/cis* isohumulone and *trans/cis* isoadhumulone).

Of the available cyclodextrins, β-cyclodextrin was chosen as a starting point due to the estimated hydrodynamic volume of iso-α-acids on one hand, but also because it was at the time, the only cyclodextrin to have been given a GRAS grade, apart from its reduced toxicity and low cost. All major pharmacopoeias (United States, Japan and EC) have a β-cyclodextrin monograph.

The method chosen for preparation of the complexes was co-precipitation as described by Hedges (1998). In general terms, it consists in the preparation of an aqueous solution of cyclodextrin to which a solution of the guest molecule is added while stirring. It is important to choose conditions such that the complex is insoluble, so that after a period of time - that can vary between hours and days- all the CD complex precipitates and can be separated by filtration or centrifugation. This method works best with β-cyclodextrin because of its low solubility and it is necessary to heat the aqueous solutions to around 60ºC to help it dissolve. It is often necessary to add an organic solvent to dissolve the guest molecule. In this case, iso-α-acids were dissolved in ethanol.

The objective we had pursued was (fortunately) only partially accomplished since only the *trans*-isohumulones precipitated as an apparent CD-complex while the *cis*-isohumulones remained in solution, allowing a complete separation (purity≈95%) of *cis/trans* isomers. The procedure is described below and the results and considerations on the possible structure of the formed complex are discussed.

5.2 Experimental

5.2.1. Materials

Ethyl acetate and ethanol were purchased from Biosolve Co. Ltd. (Valkenswaard, The Netherlands); o-phosphoric acid 85% (w/v) from Merck (Darmstadt, Germany). α-CD (>98%), β-CD (>99%), and γ-CD (>98%) were purchased from Fluka (Steinheim, Germany). The supercritical carbon dioxide hop extract was obtained from Botanix (Paddock Wood, Kent, UK).

5.2.2. Isolation and isomerisation of pure individual α-acids

A supercritical carbon dioxide hop extract was subjected to CPC using the procedure described by Hermans-Lokkerbol and Verpoorte (1994). The isolated α-acids (cohumulone, humulone, and adhumulone) were subsequently isomerised, according to the method described by Koller (1969) with a small modification. Approximately 2.15 g of $MgSO_4 .7H_2O$ was dissolved in 25 ml water and 30 ml methanol in a 300-ml dark bottle. This solution was heated to 70 °C with stirring. A solution of the pure 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 °C for 45 min while stirring continuously. After cooling in an ice bath, the reaction mixture was acidified with 20 ml H_2SO_4 30% and extracted with 3 x 100 ml of n-hexane. The resulting hexane extracts were pooled, washed with 2 x 20 ml of water, dried with anhydrous $Na_2 SO_4$ and taken to dryness with a rotary evaporator.

5.2.3 Preparation of iso-α-acid extract sample

An isomerized CO_2 hop extract from Botanix (Paddock Wood, Kent, UK) containing the potassium salts of 1.9, 3.3, 2.0, 6.8, 0.9 and 1.7% (w/v) of *trans*-isocohumulone, *cis*-isocohumulone, *trans*-isohumulone, *cis*-isohumulone, *trans*-isoadhumulone and *cis*-isoadhumulone respectively was used without previous treatment.

5.2.4 Preparation of β-cyclodextrin inclusion complex

A β-CD solution was prepared by adding 1,8 g (equivalent to 1,58 x 10^{-3} mols) of β-CD to 18 ml ethanol: water (1:2, v/v) and heating to 50 °C, in order to dissolve the β-CD. The samples were prepared by dissolving approximately 0.5 g of iso-α-acids (equivalent to 1,57 x 10^{-3} mols, considering an average MW=326da) of the previously prepared iso-α-acids in 6.5 ml ethanol and added dropwise to 18 ml of the β-CD solution, while continually stirring at 50 °C for 30 min. The yellow coloured iso-α-acid solution

changed colour to an off-white opaque suspension. The mixture was stored at 4 °C for 3 days in the absence of light after which an off-white crystalline precipitate appeared, leaving a transparent colourless supernatant.

The precipitate was separated by vacuum filtration and washed several times with 50-ml aliquots of ethanol: water (1:2, v/v).

A sample of the supernatant was analysed by HPLC with no previous treatment, while the precipitate was dissolved in water and then analysed by HPLC with the same method.

5.2.5 HPLC method

The iso-α-acids content of all samples was tested using a Waters HPLC instrument consisting in a 626 pump, a 2996 PDA detector, a 717 plus autosampler and Waters Millenium data processing software. In all cases, 20μL of sample were separated using a Hypersil C18 column (150 x 416 mm -5 μ) and gradient elution with two solvents: A - 1% H_3PO_4 in water and *B:* acetonitrile-water-H_3PO_4 (19:81:10). 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 iso-α-acids were quantified at 279 nm.

5.2.6 Recovery of *cis*-iso-α-acids

5.2.6.1 The supernatant was transferred to an Erlenmeyer flask and the ethanol was evaporated with a nitrogen stream. The resulting aqueous solution was extracted with 2 x 100 ml portions of ethyl acetate. The organic layers were collected, dehydrated with anh. Na_2SO_4 and taken to dryness. The resulting yellow oil was redissolved in ethanol and stored at 4ºC in the dark. All the process was carried out protected from the light.

5.2.6.2 The white crystalline powder was dissolved in a mixture of EtOH: water (80:20) and stored in a dark brown bottle protected from the light.

5.2.7 ^{1}HNMR analysis

All samples were taken to dryness with a rotary vacuum evaporator at 40 ºC, re-dissolved in 1 ml of dimethylsulfoxide-*d6* containing 0.03% TMS and transferred to an NMR tube. 1H NMR was recorded at 25 ºC on a 300 MHz Bruker DPX-300 spectrometer operating at a proton NMR frequency of 300.13 MHz. Each 1H NMR spectrum consisted of 64 scans requiring 5 minutes acquisition time. The resulting spectra were manually

phased, baseline corrected, and calibrated to TMS at δ 0.0, all using XWIN NMR (version 3.5, Bruker).

5.3 Results and discussion

Samples of the redissolved off-white precipitate obtained in the procedure described in 5.2.4 both with pure individual iso-α-acids and the iso-α-acid extract were analysed in the conditions detailed above using a mixture of iso-α-acids as a reference sample.

Unexpectedly, the resulting chromatogram showed that only *trans*-iso-α-acids were present as can be seen in Fig. 5-7, while the chromatogram of the supernatant revealed the presence of cis-iso-α-acids exclusively.

Of the three types of natural cyclodextrins, α-CD, β-CD and γ-CD, only β-CD was found to "include" the *trans*-iso-α-acids selectively, since α-CD did not include any of the two stereoisomers (0.2%) and γ-CD included both (99.8%).

As discussed above, the complexation method that was chosen was the precipitation method since it was the one most fitted for our case and adequate for laboratory-scale production. The temperature and time of exposition was later optimised in order to increase the yield (purity) of *cis*-iso-α-acids obtained and the total time required for the process (Khatib *et al.*, 2010). The purity of the *trans*-iso-α-acids obtained in this case was of 75.8%, the impurities being due to the presence of *cis*-iso-α-acids. It was possible to improve this by rinsing the white precipitate with ethanol, achieving a purity of≥ 95%. The presence of the *cis*-isomers was attributed to their interaction with the hydrogen-bonding external surface of β-CD
particles. Clearly the separation of the *trans/cis* stereoisomers of the iso-α-acids had been achieved in presence of β-cyclodextrin, with the selective formation of an inclusion complex with the *trans*-iso-α-acids that precipitated, leaving (in these condtions) the *cis*-iso-α-acids in solution.

The ^{1}HNMR analysis of the compounds recovered from the supernatant was performed to confirm their identity, as well as the white solid precipitate suspected to be the *trans*-iso-α- acids. In both cases the NMR spectral data confirmed the presence of the compounds.

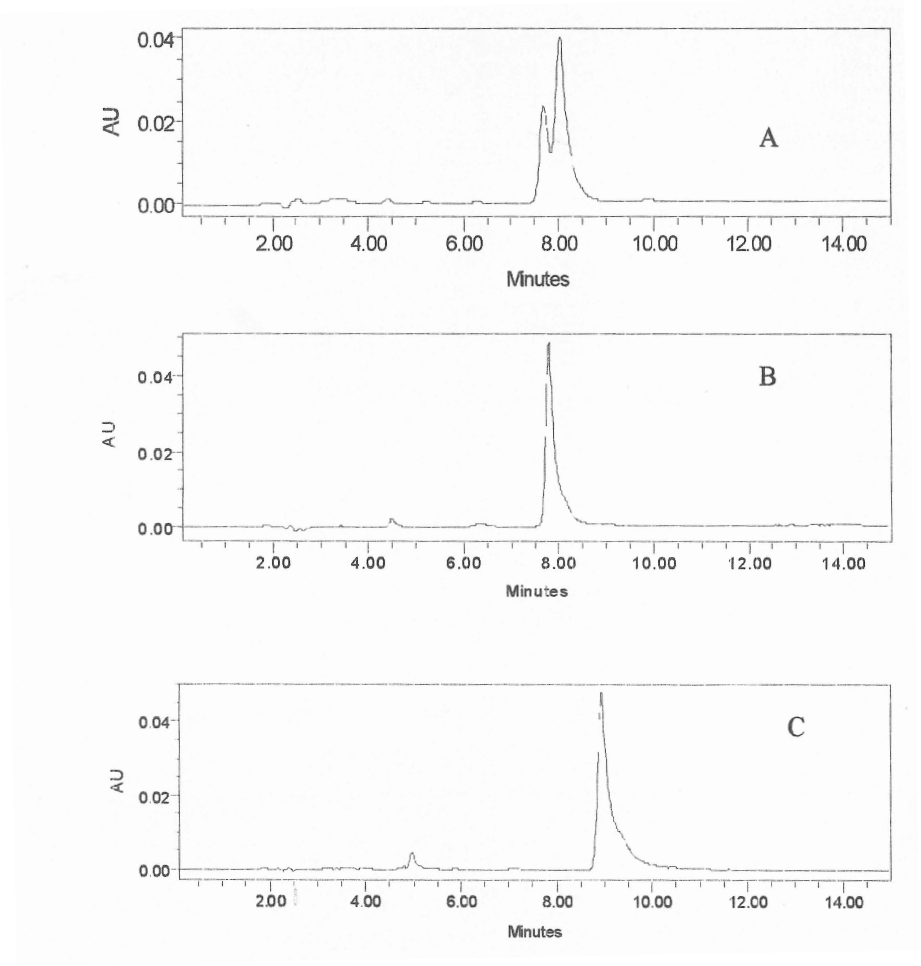

Fig. 5-3 HPLC chromatograms of pure isocohumulone: trans-isocohumolone: 7.9 min; cis-isocohumulone: 8.4 minutes (A); β-CD-isocohumulone precipitation (solid) (B); isocohumulone β-CD precipitation (supernatant)(C)

There were several clear indications of the existence of the complex:

1- The precipitation of white crystals which left a supernatant that contained only *cis*-iso-α-acids.
2 -The stability of the *trans*-iso-α-acids in the white crystals: as has been mentioned above, *trans*-iso- α-acids are extremely unstable in presence of light and oxygen. These crystals showed a remarkable stability when stored in a colourless traslucid bottle during 30 days showing that β-CD was exerting a protective effect on the molecules.

In view of these findings, and considering that the labile part of the molecule is the isohexenoyl side-chain on C (4), we came to the conclusion that the *trans*-iso-α-acids formed an inclusion complex in which they were parcially included in the β-CD cavity, in such a way that the 3 possible types of degradation suffered by these molecules were impeded. It is possible that the prenyl side-chain attached to C5 could eventually enter the cavity also, since in *trans*-iso-α-acids, these two chains are in a *cis*- configuration. This would also explain why *trans*-isomers were included selectively in β-CDs while both isomers were included in γ -CD that has a larger cavity. The hydrogen-bonding between the primary hydroxyls on outer ridge of the β-CD molecules with the C3 and C4-OH of the *trans*-iso-α-acids was stronger and thermodinamically more stable than that established with *cis*-iso-α-acids, so that in a 1:1 ratio of iso-α-acid to β-CD the former competed favourably achieving a practically 100% inclusion.

The increase of stability of the *trans*-isoacid: β-cyclodextrin crystals was not, however, observed when dissolved in water, so that while the solid forms of the *trans*-iso-α-acids were stable and resulted in a good way of storing these compounds, their aqueous solutions were extremely unstable. A similar phenomenon had been observed by Szente *et al.* (2004) when exposing aqueous solutions or suspensions of aldehyde-type flavouring complexes with β-CD to UV irradiation. They observed that the protective effect of molecular encapsulation against light-induced alteration was only 15–25% of that of the experimental data obtained in the solid state. The authors explained this to be due to the partial release of the entrapped flavours upon contact with water, following the dissociation of the inclusion complexes in aqueous systems and their subsequent rapid degradation when unprotected.

Apart from the observation of effects (increased stability, minor shifts in UV maximum and HPLC retention times) after treatment of a sample with a CD, it is important to obtain more detailed information that might help to determine whether a true complex has been formed and characteristics such as its stoichiometry and structure. There are a number of studies that can be performed, such as scanning

electron microscopy, circular dichroism, infrared spectroscopy (both IR and FT-IR), but perhaps the one that provides most information is ^{1}HNMR. Samples are dissolved in adequate solvents, so that the information obtained refers to the behaviour of the product in solution, not to the solid. The inclusion of the guest in the CD cavity produces changes in the chemical shift values of the CD protons—more specifically, H3 and H5 located inside the cavity, or H6 on the cavity rim—indicating the formation of an inclusion complex and providing, additionally, an idea of the penetration of the guest molecule in the cavity. Changes in the chemical shifts of the guest molecule are also naturally observed (Duchêne, 2011).

Further on, the β-cyclodextrin: iso-α-acid complexes were analysed by ^{1}HNMR and the results were published by Khatib *et al.(*2006). Samples were dissolved in ethanol-*d6* and deuterium oxide (1:2). The resulting spectra showed only weak shifts of the H-3 and H-5 signals ,implying that no inclusion of the iso-α-acids in the β-CD cavity occurred, while a shift in the H1 anomeric proton on the surface of the β-CD was observed. Furthermore, no proton-proton interactions (NOEs) between isocohumulone and β-CD were detected using NOESY NMR. This allowed us to conclude that there was very little or no β-CD: iso-α-acid complexation in solution and that if any, the interaction occurred on the surface of the CD particle.

These results, though disappointing, reflected the instability of the aqueous solutions of β-CD:iso-α-acid complex. When a complex is placed in water, two steps are involved in the release of the complexed guest. First, the complex is dissolved. The second step is the release of the complexed guest when displaced by water molecules. An equilibrium will be established between free and complexed cyclodextrin, the guest and the dissolved and undissolved complex. Dissociation of the inclusion complex depends on the value of *K* (*see Eq.5-1)*: if this value is low, the presence of a large number of water molecules in the surrounding environment will result in a concentration gradient that shifts the equilibrium to the left, and the guest has difficulty finding another cyclodextrin to reform the complex and is consequently left free in solution (Del Valle, 2004). In the case of the iso-α-acids,
when unprotected, they rapidly decompose as a result of their reaction with oxygen or light as described above.

Further evidence of this, was the bitter taste perceived in the aqueous solution of the β-CD complexes, obviously due to the free-iso-α-acids. The dry solid had a different behaviour: when placed in the mouth, it had a slight sweet taste, quite soon followed by an "explosion" of a strong prevailing bitter taste, very likely due to the release of the iso-α-acids from the cavity.

A possible interpretation of the β-CD complex using the representation made by De Cooman *et al.* (2000) of *trans*-iso-α-acids is the following:

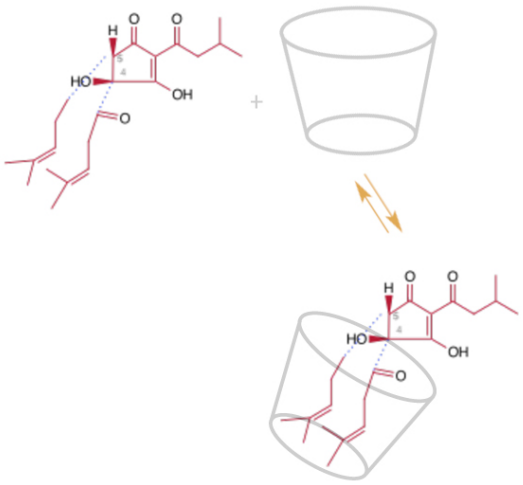

Fig 5-5: Possible structure of β-CD complex and trans-isohumulones.

The C4- and C6-hydroxyls could H-bond to the primary hydroxyls on the outer rim of the β-cyclodextrin molecule, fixing the *trans*-iso-α-acid inside the hydrophobic cavity.

Stability testing of the precipitate obtained from β-CD inclusion of *trans*-iso-α-acids done further on confirmed these results (Khatib *et al.*, 2010a) and conditions for improved yield of the complexation process were achieved (Khatib *et al.*, 2010b).

In conclusion, it was thus clearly established that reaction between β-CD and *trans*- iso-α-acids produced a crystalline solid that was stable both when exposed to light and air. Further tests should be carried out on the solid complexes to fully determine their structure. On the other hand, analysis of solutions of this solid in water or water: ethanol, revealed a rapid decomposition of iso-α -acids, possibly due to the release of the iso-α-acids from the β-CD complex and subsequent degradation in presence of air, light and water.

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