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Short communication

Isolation of individual hop iso-α-acids stereoisomers by β-cyclodextrin

Alfi Khatib [a,b,c,*], Erica G. Wilson [b], Moses Supardi [b], Robert Verpoorte [b]

[a] Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, University Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
[b] Division of Pharmacognosy, Section of Metabolomics, Institute of Biology, Leiden University, Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands
[c] Department of Food Science and Technology, Bogor Agricultural University, IPB Darmaga, Bogor, Indonesia

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ABSTRACT

Individual iso-α-acids that are responsible for the bitter taste of beer need to be isolated because these acids are required as reference standards in quantitative analysis and when studying the parameters which effect the quality of beer. However, these pure compounds are very expensive, due to inefficient isolation methods. In this study a new isolation method has been developed, in order to reduce the isolation cost. 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, v/v) as a solvent at a temperature of 50 °C for 30 min. The molar ratio of iso-α-acids sample to β-cyclodextrin for complexation was 1:1. Precipitation time varied between 9 h and 2 days, depending on the iso-α-acid. Release of the guest from the cyclodextrin complex was successfully accomplished by elution with methanol.

1. Introduction

Hop cones, the female flowers of *Humulus lupulus* L. are used in brewing, most importantly because they contain 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 *trans*-/*cis*-stereoisomers (Alderweireldt, Verzele, Anteunis, & Dierckens, 1965; Koller, 1969; Verhagen, 1988).

IAAs in beer can be measured by bitterness unit (BU) analysis (Verhagen, 1988). However, this method cannot 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 analyse individual bitter acids (Harms & Nitzche, 2001; Raumschuh et al., 1999). Other methods, such as gas chromatography (GC) (Martinez & Willemsen, 2002) and capillary electrophoresis (McLaughlin, Weston, & Hauffe, 1996; Royle, Ames, Hill, & Gardner, 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, Menneer, Walters, & Marinova, 1997), foam formation (Bamforth, 1985), and flavour stability (De Cooman, Aerts, & Overmeire, 2000; De Cooman et al., 2001).

Despite the obvious need for these compounds, a complete set of pure individual IAAs are not commercially available, although standards of iso-α-acids are available as mixtures, as managed by the International Subcommittee for Isomerized Hop Acid Standards (ISIHAS). This is due to the difficulty in separating these compounds with economically viable methods and also due to their instability. Several publications describe preparative methods (Hughes, 1996; Thornton et al., 1993; Ting & Goldstein, 1996; Verzele & Steenbeke, 1989) which can separate *trans*- from *cis*-isohumulone. The TLC method that has been reported by Aitken, Bruce, Harris, and Seaton (1968) could separate *trans*- from *cis*-isohumulone. The reference compounds have also been prepared by photoisomerisation of humulone (Clarke & Hildebrand, 1965; Sharpe & 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-acid DCHA salts has been used widely as a reference compound and is commercially available. However, the *cis*-isomers are not available to date.

Hermans-Lokkerbol and Verpoorte (1994) 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

* Corresponding author. Address: Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, University Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia. Tel.: +603 8946 8540; fax: +603 8942 3552.
E-mail address: alfikhatib@hotmail.com (A. Khatib).

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isolated can be isomerised to yield pure *trans*-/*cis*-IAA pairs, the remaining problem being the separation of the *trans*- and *cis*- isomer of each pair.

β-Cyclodextrin (β-CD), had been used to stabilise hop oils and iso-α-acids (Hughes & Simpson, 1994). Also, decrease in the antibacterial activity of *trans*-isohumulone in the presence of β-CD has been reported (Simpson & Smith, 1992). In a later paper, Simpson and Hughes (1995) reported the encapsulation of iso-α-acids in β-CD and the enhance stability of the complex. This led us to test the possibility of using β-CD as a means of separation of *cis*- from *trans*-isomers. Moreover, cyclodextrins have been extensively used to separate isomers, functional groups, and enantiomers of other natural products (Aboul-Enein, El-Awady, & Heard, 2000; Breinholt, Lehmann, & Varming, 1999; Bressolle, Audran, Pham, & Vallon, 1996; Krupcik et al., 2004; Kuramoto, 1986; Salvador, Herbreteau, Dreux, Karlsson, & Gyllenhaal, 2001; Spanik, Lim, & Vigh, 2002; Uemasu & Kushiyama, 2004).

The selectivity of CDs relate to their truncated cone structure. CDs are built up from 6 (α-CD), 7 (β-CD), 8 (γ-CD), or more glucopyranose units. 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. Here we report the optimisation 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.

2. Materials and methods

2.1. Materials

All organic solvents used were purchased from Biosolve Co. Ltd. (Valkenswaard, The Netherlands). Orthophosphoric acid 85% (w/v) was obtained from Merck (Darmstadt, Germany). α-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).

2.2. Isolation and isomerisation of pure individual α-acids

A supercritical carbon dioxide hop extract was subjected to centrifugal partition chromatography, using the procedure described by Hermans-Lokkerbol and Verpoorte (1994). The isolated α-acids (cohumulone, humulone, and adhumulone) were subsequently isomerised to iso-α-acids, according to the method described by Koller (1969) with a small modification. $MgSO_4{\cdot}7H_2O$ (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 °C 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 °C for 45 min under continuous stirring. After cooling in an ice bath, the reaction mixture was acidified with 20 ml sulphuric acid 30% and extracted three 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 rotary evaporator.

2.3. 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, v/v) and heating to 50 °C, in order to dissolve the β-CD. The guest compounds solution was prepared by dissolving 500 mg of the previously prepared iso-α-acids in 6.5 ml ethanol and added dropwise to 21 ml of the β-CD solution, while continually stirring and heating to 50 °C for 30 min. The mixture was stored at 4 °C for several days in the 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 aliquots of ethanol:water (1:2, v/v).

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 two portions of 50 ml of each solvent. Each eluate was collected in a separate Erlenmeyer flask and, with the exception of ethanol, taken to dryness with a rotary evaporator and re-dissolved in 100 ml ethanol for 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 (6 M) to reach pH 1 while continuously being stirred. The acidified supernatant was extracted twice with 100 ml *n*-hexane, using a separating funnel. The *n*-hexane phase was washed twice with water in a separating funnel, in order to remove all dissolved β-CD and HCl. The excess water in the *n*-hexane phase was removed by dry sodium sulphate and subsequently the *n*-hexane phase was evaporated using a rotary evaporator. The concentrates were dissolved in ethanol and stored in a dark bottle at −20 °C.

2.4. HPLC analysis

The HPLC system used consisted of a waters system equipped with a 626 pump and 600S pump controller, a 717-plus autosampler, and a 2996 photodiode array detector (Waters Corporation, Milford, MA). A Hypersil 5 μ C18, 250 × 4.6 mm (Phenomenex, Torrance, CA) column was used. The mobile phase was filtered using a 0.2 μm hydrophilic polypropylene membrane filter GH Polypro (Pall Corporation, Ann Arbor, MI) and helium sparged.

Isocratic elution with a mobile phase containing acetonitrile:water: H_3PO_4 (50:50:0.01, v/v/v) at a flow rate of 1.5 ml/min allowed the baseline separation of all six isomers within a total run time of 25 min. The purity of the isolated compounds was calculated by comparing the peak area of the isolated compound to the total peak area of all compounds in the sample.

2.5. ^1H NMR analysis

The ethanolic solutions of each isolated individual iso-α-acids were diluted in ethanol to a concentration of about 1.0 mg/ml. They were mixed with 1.00 mg of anthracene as internal standard (all in triplicate). These samples were evaporated using a vacuum centrifuge and re-dissolved in 1 ml of $CDCl_3$ for NMR analysis. ^1H NMR spectra were recorded in CDC_{l3}, using a Bruker DPX 300 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). For each sample, 64 scans were recorded with the following parameters: 32 K data points, pulse width of 4.0 ms and relaxation delay of 1 s. FID signals were Fourier transformed with a line broadening factor of 0.5 Hz. For quantitative analysis, peak area was used after baseline correction.

3. Results and discussion

3.1. Complexation conditions

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

Table 1
Ratio of *trans*- to *cis*-IAAs in the supernatant during precipitation of the β-CD complex.

Guests	Time (h)						
	% *trans* to *cis*[a]						
	0	0.5	9	12	24	48	96
Isocohumulone	39.9 (±1.2)	13.6 (±1.5)	1.1 (±0.5)	1.1 (±1.0)	1.1 (±0.7)	1.3 (±1.0)	1.3 (±0.9)
Isohumulone	38.3 (±1.1)	11.0 (±1.3)	5.9 (±0.6)	4.8 (±1.3)	3.8 (±0.7)	3.0 (±0.5)	3.0 (±0.6)
Isoadhumulone	40.2 (±0.6)	25.1 (±0.4)	6.2 (±0.5)	3.8 (±0.6)	2.9 (±0.4)	2.9 (±0.5)	3 (±0.6)

[a] Analysed using HPLC, % *trans* = (area of *trans*/area of *cis*) × 100%. The values in parentheses are standard deviation.

solution was applied, as compounds, both in the 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 °C was found to be necessary to dissolve the CDs.

3.2. 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 1 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 °C), showing that after about 24 h equilibrium is reached.

3.3. Recovery of trans-iso-α-acids

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

After repeatedly washing the precipitates with the same solvent used in the complexation experiments (ethanol:water, 1:2, v/v), the complexes were eluted with several organic solvents: ethanol, ethyl acetate, dichloromethane, *n*-heptane, *n*-hexane, and methanol (Fig. 1). Of these, methanol proved to be the best solvent, followed by ethanol to 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*-isomer 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 95.0% and no peak signals of β-CD were detected by ^{1}H NMR. 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 amounts of the *cis*-isomers.

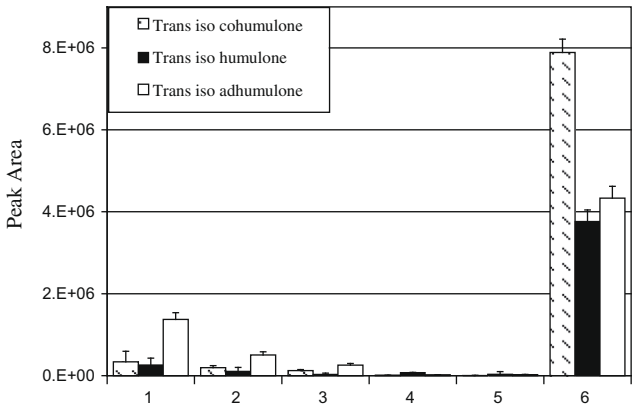

Fig. 1. Release of *trans*-iso-α-acids from β-cyclodextrin complex with organic solvents: ethanol (1), ethyl acetate (2), dichloromethane (3), *n*-heptane (4), *n*-hexane (5) and methanol (6).

Recovery of IAAs isolated by this method is around 50%, determined by weighing 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, v/v) is used. In this step some of the β-CD precipitate was dissolved in this solvent.

The chemical structure of the isolated iso-α-acids was confirmed by NMR and mass spectrometry as has been reported by our group (Khatib et al., 2007).

4. Conclusion

α and γ-CD do not separate the stereoisomers. Complete separation of *trans*- from *cis*-IAAs was successfully performed only with β-CD, using ethanol:water (1:2, v/v) as a solvent. Temperature was maintained at 50 °C over 30 min 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 h and 2 days depending on the 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 destabilise 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 optimisation of the process.

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