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Inclusion complexes of *trans*-iso-α-acids with β-cyclodextrin: preparation of highly enriched *cis*- and *trans*-iso-α-acids

Separation of *cis*-iso-α-acids from *trans*-iso-α-acids, starting from the commercial isomerised hop extract, was successfully carried out by complex formation of the *trans*-iso-α-acids with β-cyclodextrin. The separation was performed on laboratory scale permitting the quantitative dosage of *cis*-iso-α-acids resp. *trans*-iso-α-acids to 50 L of fermented beer, addition rate 25 mg/L. The methodology consists of two successive complex formation steps with a saturated solution of β-CD in water. The precipitate from the first complex formation step is enriched in *trans*-isomers, which can be recovered from the β-CD inclusion complexes. The collected first supernatant is already enriched in *cis*-isomers, but in order to obtain a higher enrichment in *cis*-isomers in this fraction, it was incubated again with β-CD for a second complex formation step. The *cis*-isomers were then isolated from the second supernatant by solid phase extraction. The final *cis*- and *trans*-isomers fractions were highly enriched in respectively *cis*-iso-α-acids (98 %) and *trans*-iso-α-acids (90 %), as opposed to common isomerised hop extracts with a typical ratio of 70 % *cis*-iso-α-acids and 30 % *trans*-iso-α-acids. The established methodology for the isolation of *cis*-iso-α-acids and *trans*-iso-α-acids from a commercial isomerised hop extract allows highly advanced beer bittering on pilot scale and represents an innovative tool to further investigate both the cis- and trans-specific bitter acids degradation in beer in relation to flavour stability.

Descriptors: hop bitter acids, isohumulones, *cis*-iso-α-acids, *trans*-iso-α-acids, isomerised hop extract, separation, cyclodextrin, inclusion complexes, enrichment

1 Introduction

A key role for the flavour stability of beer is attributed to the unstable iso-α-acids [1–5], the predominant source of bitterness in beer [6]. Indeed, degradation of iso-α-acids has a determining impact on the organoleptic properties of aged beer by imparting a harsh and lingering bitter taste, next to the significant decline in beer bitterness [1–3, 7–11]. Both the quality and intensity changes in beer bitterness during storage have been clearly defined by a series of cyclic *trans*-iso-α-acids transformation products [9, 10, 12], which are suitable markers for beer ageing [13]. Under oxygen-free conditions and in the absence of light, *cis*-iso-α-acids clearly exhibit enhanced stability [3, 4, 14]. Consequently, altering the ratio of *cis*-iso-α-acids to *trans*-iso-α-acids in favour of the *cis*-iso-α-acids in brewing practice opens perspectives for improving the beer bitterness stability. Nevertheless, a higher cis/trans ratio does not necessarilly result in improved flavour stability and the impact of unknown degradation processes of *cis*-iso-α-acids to the taste stability of aged beverages needs further investigation in the future [13]. This is however limited, mainly caused by the difficulty in preparing sufficient amounts of isolated *cis*-isomers. As a result, a methodology for the separation of *trans*- and *cis*-iso-α-acids from a commercial isomerised hop extract is desirable, permitting quantitative dosage of *cis*-iso-α-acids to finished beer at the post-fermentation stage. In addition, the recovered *trans*-iso-α-acids fraction could also be valuable for further brewing-related studies/applications (e. g. the investigation of *trans*-iso-α-acids related degradation products, comparative brewing trials (conventional vs. advanced bittering), etc.).

Pure *trans*-isohumulone has been prepared by photoisomerisation of humulone [15, 16], but this procedure was considered cumbersome and time-consuming. By means of specific complex formation of the *trans*-isomers with dicyclohexylamine (DCHA), various authors succeeded in the separation of *trans*-iso-α-acids from *cis*-iso-α-acids [10, 17–21]. The DCHA salts of the *trans*-isomers crystallise in ethyl acetate and are stable for extended periods at ambient temperature, unlike the pure *trans*-iso-α-acids. Pure *trans*-iso-α-acids can be regenerated from the salt by an acid wash. However, the DCHA-*trans*-iso-α-acids crystals are formed very slowly, requiring too much time for significant amounts of material to be produced. The separation process also demands high levels of *trans*-iso-α-acids in the starting material, i.e. the commercial isomerised hop extract, which is not obvious. Moreover, the *cis*-iso-α-acids, which do not react with DCHA, remain free in solution, however with an extremely low yield and a very low purity [21], as a result of which pure *cis*-isomers are currently not commercially available. On the contrary, the purified, semi-crystalline preparation of co-, n-, and

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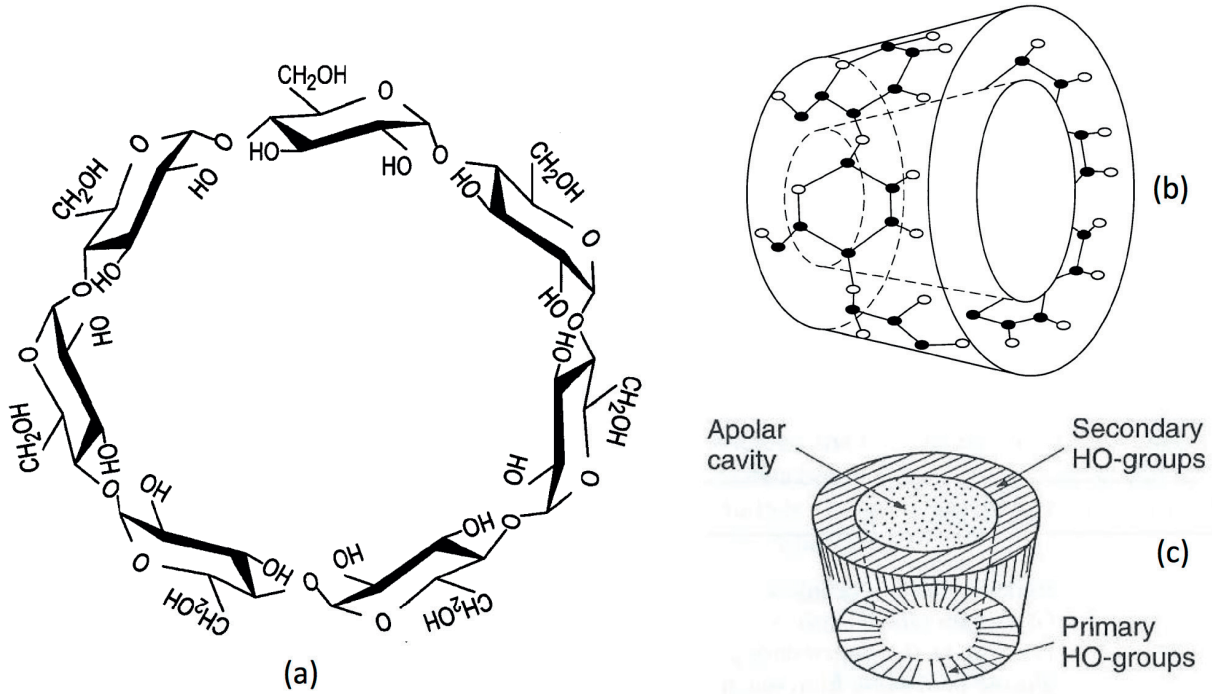

Fig. 1 Chemical structure of β-cyclodextrin [22]: (a) Viewing the structure of β-cyclodextrin from above. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively; (a, b, c) Cyclodextrins are to be regarded as a truncate cone. All secondary hydroxyl groups of the C2 and C3 atoms are located on top of the ring, and all primary hydroxyls on the C6 atoms at the base of the torus. As the latter hydroxyl groups are free to rotate, they partially block the base aperture; (c) Functional structural scheme of cyclodextrins

ad-homologues of *trans*-iso-α-acids in DCHA salt form, derived from CO_2 extracted and isomerised α-acids of hops, is released by the International Subcommittee for Isomerized Hop α-Acids Standards (ISIHAS) as a calibration standard for the LC analysis of iso-α-acids.

An alternative for this inefficient isolation method with DCHA is proposed by using β-cyclodextrin (β-CD). In figure 1, the characteristic structural feature of β-CD is depicted. Owing to its molecular structure, which has a hydrophilic exterior and a hydrophobic inner cavity (see fig. 1 (c)), cyclodextrins are able to form water-soluble inclusion complexes with a large number of organic molecules enclosed in the apolar cavity through a host-guest interaction [22, 23]. For this reason it has found wide applications for the separation of various compounds, such as optical isomers (D- and L-phenylalanines) [24], structural isomers (o-, m- and p-nitrophenols) [24, 25], cholesterol, free fatty acids, flavanoids and terpenoids [26], vitamin E [27], and fish oil [28].

With regard to hops, β-CD was found to stabilise hop oils in order to avoid degradation or evaporative losses [29], and more recently the preparation of xanthohumol/cyclodextrin complexes has been reported [30]. *Simpson* and *Smith* [31] were the first to report complex formation between β-CD and *trans*-iso-α-acids to explain the antagonising effect of β-CD on the antibacterial action of *trans*-iso-α-acids. Simpson and *Hughes* [32] reported the encapsulation of iso-α-acids in β-CD and the enhanced stability of the complex. According to *Wilson* et. al. [33], β-CD exhibits a surprisingly high affinity for *trans*-iso-α-acids and a much lower affinity for *cis*-iso-α-acids. *Khatib* et. al. [34, 35] succeeded to obtain either *cis*- or *trans*-isomers from a mixture of iso-α-acids in an isomerised CO_2 hop extract using β-CD.

The aim of the present study was to obtain the necessary amounts of highly enriched *cis*-iso-α-acids resp. *trans*-iso-α-acids appropriate for post-fermentation beer bittering on pilot scale (min. 50 L of beer). The separation of *cis*-iso-α-acids from *trans*-iso-α-acids in an isomerised hop extract via selective inclusion complex formation of the *trans*-iso-α-acids with β-CD, was therefore further upscaled and optimised.

2 Materials and methods

2.1 Chemicals

All chemicals used in this study were of analytical grade unless specified otherwise. Methanol (99.9 %, for HPLC gradient grade) and acetonitrile (99.9 %, for HPLC gradient grade) were purchased from Acros Organics (Geel, Belgium). Ethanol (LiChrosolv® for HPLC) and ethyl acetate (LiChrosolv® for HPLC) were obtained from Merck (Darmstadt, Germany). Phosphoric acid (85 %) was acquired from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q purification system (Synergy 185, Millipore S.A., Molsheim, France). β-cyclodextrin hydrate (99 %) was purchased from Acros Organics (Geel, Belgium). Nitrogen (N28) was obtained from Air Liquide (Liège, Belgium).

2.2 Isomerised hop extract

The isomerised hop extract used in all experiments was a hop concentrate made from a CO_2 extract of hops, and was purchased from Botanix (Paddock Wood, Kent, UK). The iso-α-acids were present as the potassium salts in aqueous solution at a concentration of 19 % (w/w), as was determined by HPLC analysis (see table 1).

2.3 Final procedure for inclusion complex formation

Initially, a saturated aqueous β-cyclodextrin (β-CD) solution (1.85 g/100 mL) was prepared by dissolving β-CD in water, while heated (50 °C) and stirred in a water bath. Inclusion complexes of *trans*-iso-α-acids with β-CD were then prepared by adding dropwise, under continuous stirring, and in the absence of light, 26.2 mL of the isomerised hop extract solution (equal to 5.00 g of total iso-α-acids or 1.21 g of *trans*-iso-α-acids) dissolved in 68.8 mL methanol, to 435 mL of the saturated aqueous β-CD solution. After complete addition of the iso-α-acids to the β-CD solution, the molar ratio β-CD to *trans*-iso-α-acids was 2:1. The methanol concentration of the reaction mixture was then adjusted to 40 % (v/v) with addition of 240 mL of methanol. The reaction mixture was flushed with nitrogen, closed, and cooled to ambient temperature over 16 h under continuous stirring in the dark. Subsequently, the reaction mixture was stored in the absence of light at 0 °C for 5 days. During cooling down to 0 °C and further cold storage, the β-CD complexes precipitated as white-yellow fine powder. The precipitate, filtered off by vacuum filtration through filter paper (Schleicher & Schuell nº 8, Dassel, Germany) in a Büchner funnel, contained almost exclusively *trans*-isomers, which were recovered from β-CD with methanol (see paragraph 2.4). The supernatant was collected, and the remaining iso-α-acids (enriched in *cis*-isomers) were isolated and concentrated in methanol by solid phase extraction (see paragraph 2.5). In order to obtain a higher proportion of *cis*-isomers in the supernatant derived from the first complexation step, this fraction was incubated again with β-CD as described below.

Table 1 Concentrations of iso-α-acids in the commercial isomerised hop extract (Botanix, Paddock Wood, Kent, UK). Mean value of triplicate HPLC analyses

	Isomerised hop extract g/100 g
Total iso-α-acids	19.11
Total *cis*-iso-α-acids	14.44
cis-isocohumulone	4.78
cis-isohumulone	7.92
cis-isoadhumulone	1.74
Total *trans*-iso-α-acids	4.67
trans-isocohumulone	1.57
trans-isohumulone	2.55
trans-isoadhumulone	0.55
T/C ratio (%)[a]	32
C/T ratio (%)[b]	309
Enrichment[c] (% cis[d]/% trans[e])	76/24

[a] T/C ratio = the ratio of the amount (g) of total *trans*-iso-α-acids to the amount (g) of total *cis*-iso-α-acids, multiplied by 100;
[b] C/T ratio = the ratio of the amount (g) of total *cis*-iso-α-acids to the amount (g) of total *trans*-iso-α-acids, multiplied by 100;
[c] Enrichment is the percentage of *cis*- respectively *trans*-iso-α-acids present in the isomerised hop extract;
[c] % cis = the ratio of the amount (g) of *cis*-iso-α-acids to the total amount (g) of iso-α-acids, multiplied by 100;
[d] % trans = the ratio of the amount (g) of *trans*-iso-α-acids to the total amount (g) of iso-α-acids, multiplied by 100.

The first supernatant (enriched *cis*-iso-α-acids in 150 mL of methanol – see 2.5) was combined with 435 mL of saturated aqueous β-CD solution (1.85 g/100 mL) (molar ratio β-CD to *trans*-iso-α-acids 5:1), and the methanol concentration was adjusted to 30 % (v/v) with the addition of 40 mL of methanol. The second complex formation step was performed similarly to the first complex formation step. From the second supernatant, most enriched in *cis*-iso-α-acids, the *cis*-isomers were isolated and concentrated in methanol by solid phase extraction (see paragraph 2.5). The second precipitate obtained, was discarded.

2.4 Recovery of *trans*-iso-α-acids from β-CD

The β-CD precipitate from the first complex formation step was the most enriched in *trans*-isomers, which were recovered from β-CD with methanol. Therefore, 250 mL of methanol was added to the precipitate. The mixture was then flushed with nitrogen, immediately closed, and stirred at ambient temperature in the dark. After 16 hours of stirring, β-CD, insoluble in methanol, was filtered off by vacuum filtration through filter paper (Schleicher & Schuell nº 8, Dassel, Germany) in a Büchner funnel. The supernatant was referred to as the '*trans*-isomers fraction'.

2.5 Isolation of iso-α-acids from the supernatant by solid phase extraction (SPE)

Solid phase extraction (SPE) was used to isolate, concentrate and enrich the remaining iso-α-acids, mainly *cis*-isomers, from the supernatants respectively collected in the first and second inclusion complex formation steps as described in paragraph 2.3. For one inclusion complex formation experiment, two SPE-cartridges were used, each containing 25 g of LiChroprep® RP-18 (Merck, Darmstadt, Germany). The cartridges were initially equilibrated with respectively 50 mL of ethyl acetate and 50 mL of methanol. Then, 50 mL of water and 25 mL of methanol/water [40 % (v/v)] were washed through each column, after which the supernatant, divided up proportionally over the two cartridges, was added. The solvent, β-CD and impurities passed through the cartridges while the iso-α-acids retained on the stationary phase. After loading all the supernatant, the cartridges were washed with 50 mL of Milli-Q water adjusted to pH 2.80 with phosphoric acid (85 %) in order to remove further impurities. In the final step, the retained iso-α-acids were eluted with 150 mL of methanol. After the first complex formation step, this methanol fraction was reincubated with β-CD for a second complex formation step (see 2.3), after which this fraction was referred to as the '*cis*-isomers fraction'.

2.6 HPLC analysis of iso-α-acids

The HPLC method for the complete separation of iso-α-acids as described by *De Cooman* et. al. [3] and *Jaskula* et. al. [36], was applied for initial quality control of the isomerised hop extract as well as for evaluation of the *trans*- and *cis*-isomers fractions. Prior to HPLC analysis, these fractions were diluted and filtered through a 13 mm syringe filter (0.20 µm PTFE) (Alltech Associates, Deerfield IL, USA). HPLC separations of iso-α-acids were performed on a Hitachi liquid chromatograph (Merck, Darmstadt, Germany), consisting of a programmable HPLC pump (L-7100) with a quaternary low-pressure gradient system, a diode array detector (L-7450A),

an interface module (D-7000), a solvent degasser (L-7612), an autosampler L-7200 with 100 µL sample loop, a Compaq Deskpro 2000 (Merck HPLC System Manager D-7000 software, version 2.1), and an Alltima 5-µm C18 column (150 mm x 4.6 mm i.d., Alltech Associates, Deerfield, IL, USA). Chromatographic conditions were as follows: Eluent A was Milli-Q water adjusted to pH 2.80 with phosphoric acid (85 %), containing 10 mg·L^{-1} EDTA. Eluent B was HPLC-grade acetonitrile. Isocratic elution was performed using 48 % (v/v) eluent A and 52 % (v/v) eluent B. The analysis time was 35 min, the flow rate was 1.8 mL·min^{-1}. The oven was adjusted to ambient temperature. UV detection of the iso-α-acids was performed at 270 nm. A dicyclohexylamine (DCHA)-iso-α-acids ICS-I1 complex with known composition [66.5 % (w/w) iso-α-acids; Labor Veritas, Zürich, Switzerland] was used as an external standard for quantification of iso-α-acids. Calculation of the *trans*-/*cis*-iso-α-acids ratio (T/C ratio) and the *cis*-/*trans*-iso-α-acids ratio (C/T ratio) is based on the concentrations of *trans*- and *cis*-isocohumulone, *trans*- and *cis*-isohumulone, and *trans*- and *cis*-isoadhumulone, as measured by HPLC:

$$\text{T/C ratio (\%)} = \frac{[\textit{trans}\text{-isocohumulone}] + [\textit{trans}\text{-isohumulone}] + [\textit{trans}\text{-isoadhumulone}]}{[\textit{cis}\text{-isocohumulone}] + [\textit{cis}\text{-isohumulone}] + [\textit{cis}\text{-isoadhumulone}]} \times 100$$

$$\text{C/T ratio (\%)} = \frac{[\textit{cis}\text{-isocohumulone}] + [\textit{cis}\text{-isohumulone}] + [\textit{cis}\text{-isoadhumulone}]}{[\textit{trans}\text{-isocohumulone}] + [\textit{trans}\text{-isohumulone}] + [\textit{trans}\text{-isoadhumulone}]} \times 100$$

3 Results and discussion

3.1 Preparation of β-CD/*trans*-iso-α-acids inclusion complexes

3.1.1 Preconditions for complex formation

In this study, β-CD complex formation was performed in solution because both the β-CD and the iso-α-acids had to be recovered after complexation. Complex formation in water is most common [22], and the more solubilised the β-CD is in water, the more molecules are available for complexation. On account of the low solubility of β-CD in water (1.85 g/100 mL), a warm (50 °C) saturated aqueous solution of β-CD was prepared. A temperature of 50 °C was necessary to dissolve the β-CD in water, but temperatures higher than 50 °C were not applied in order to avoid degradation of the iso-α-acids when added to the solution.

Complex formation in solution is a very rapid process, however with poorly water-soluble guest molecules in aqueous solution, either a long reaction time is needed, or the guest has to be dissolved in some organic solvent to obtain a high complexation degree, because both the β-CD and the guest molecule need a certain solubility in solution to increase the probability that they meet and interact. The choice of solvents is rather limited as most form stable complexes with β-CD, except for small molecular and strongly hydrophilic solvents such as methanol and 2-methoxyethanol. Ethanol for example, cannot be completely removed from the isolated complex, as it is bound to the cyclodextrin cavity, either as a solvent-cyclodextrin complex, or as a ternary complex, beside the guest molecule [22]. As iso-α-acids are very poorly soluble in water[1], β-CD complexes of *trans*-iso-α-acids cannot be prepared without the use of an organic solvent. In all experiments described below, 5.00 g of iso-α-acids (26.2 mL of the isomerised hop extract, containing 19.11 g of iso-α-acids per 100 mL) were initially dissolved in methanol (68.8 mL) before addition to the aqueous β-CD solution. Moreover, ionisation of the guest molecules (iso-α-acids) should be avoided as they become rather poor complexing agents in the ionised state. Dissolved in methanol, the iso-α-acids (pKa 3-3.5) are not ionised when added to the neutral aqueous β-CD solution, but they will become (partly) ionised once present in the reaction medium. It is however unclear to what extent the pKa values of the iso-α-acids and, consequently their degree of ionisation, are affected in a methanol solution (40 % (v/v)) compared to pure water.

3.1.2 Method development for preparation of β-CD/trans-iso-α-acids inclusion complexes

The procedure for the preparation of β-CD/*trans*-iso-α-acids inclusion complexes in solution is very straightforward. The first experiment was carried out in 40 % (v/v) methanol with the molar ratio of β-CD to *trans*-iso-α-acids equal to 2:1. In accordance with *Szejtli* [22], who reported the preparation of flavour complexes by adding aroma substances (essential oil) under vigorous stirring to a saturated aqueous solution of β-CD at 50 °C, the iso-α-acids, dissolved in methanol, were added to the neutral saturated aqueous β-CD solution at 50 °C. The temperature of the medium was chosen as a compromise since the stability of the cyclodextrin complex strongly depends on the temperature, and dissociation can be considered to be complete above 80 °C [22]. Addition of the isomerised hop extract dissolved in methanol was completed dropwise in view of the enrichment[2] of both the *cis*-isomers and *trans*-isomers fractions, as well as of the recovery[3] of iso-α-acids. Although addition of the extract to the β-CD solution all at once was not tested in this study, Khatib et. al. [35] observed a lower enrichment and recovery when doing so, compared to slow addition. With intense stirring under slow cooling to ambient temperature overnight, precipitation of the inclusion complexes from the 40 % (v/v) methanolic solution occurred. In the presence of organic molecules, the β-CD solubility generally decreases owing to complex formation. In the first experiment (for results see table 2), the supernatant was separated by filtration after 1 day of precipitation at 0 °C. After filtration and drying of the isolated β-CD complexes, a white-yellow fine powder was obtained, referred to as the β-CD precipitate. The supernatant and the β-CD precipitate were analysed for their iso-α-acids content by HPLC (see 2.6). Before analysis, the remaining iso-α-acids were first isolated and concentrated from the collected supernatant by SPE (see 2.5). The β-CD precipitate was analysed as methanol fraction, after the iso-α-acids were eluted from the β-CD with 100 % methanol (see 2.4).

As depicted in table 2, separation of *cis*- and *trans*-isomers was obtained after a first complex formation step of *trans*-iso-α-acids

[1] Solubility of iso-α-acids in hot wort: 120 mg/L [37, 38]
[2] Enrichment of *cis*-iso-α-acids in the *cis*-isomers fraction resp. *trans*-iso-α-acids in the *trans*-isomers fraction
[3] Recovery is the percentage of the amount (g) of iso-α-acids obtained in each fraction after separation with β-CD compared to the amount (g) of iso-α-acids in the original reaction mixture (start)

with β-CD. Mainly *cis*-iso-α-acids were detected in the supernatant of the complexation medium, referred to as '*cis*-isomers fraction', whereas most *trans*-iso-α-acids were detected in the β-CD precipitate, referred to as '*trans*-isomers fraction'. Elution of *trans*-isomers from the β-CD complexes using methanol was successful. However, the enrichment of both the *cis*- and *trans*-isomers fractions could still be improved. In the *trans*-isomers fraction, 89 % of the iso-α-acids were *trans*-iso-α-acids. Likewise, 89 % of the iso-α-acids present in the *cis*-isomers fraction were *cis*-iso-α-acids.

On the other hand, the recovery of iso-α-acids in the *cis*-isomers fraction was unsatisfactory. Only 0.97 g of the iso-α-acids originally added to the reaction mixture (5.00 g) were found in the *cis*-isomers fraction after concentration of the supernatant by SPE, which is a recovery of only 19.4 %. When loading the SPE-cartridges with the supernatant, the solvent (40 % (v/v) methanol) passing through the stationary phase had been collected and analysed for its iso-α-acids content. Clearly, early elution of the iso-α-acids with the passing solvent was perceived since 2.62 g or 52.4 % of the original iso-α-acids were found in the eluted SPE fraction (see table 2). This is due to the large volume of the 40 % (v/v) methanol solution, and consequently the too high hydrophobicity of the supernatant loaded onto the SPE-cartridges. Early elution of iso-α-acids was overcome by evaporation of the methanol from the supernatant under reduced pressure prior to SPE, because then a less hydrophobic and smaller volume of supernatant was loaded. The results in table 3 (see next page) bear evidence that the objective to retain all iso-α-acids on the stationary phase of the SPE-cartridges to elute in the *cis*-isomers fraction is met by this action.

The solvent that now passed through the SPE-cartridges was totally free of iso-α-acids, and could thus be discarded. By applying the described methodology, including lowering the supernatant

Table 2 **Recovery of *cis*- and *trans*-iso-α-acids in the different fractions after separation from the isomerised hop extract with β-CD without volume reduction. First complex formation step: 40 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 2:1; precipitation time: 1 day at 0 °C. Mean values of duplicate experiments**

Without volume reduction	Start	Solvent passed through SPE-cartridges	*cis*-Isomers fraction	*trans*-Isomers fraction	Loss
	Recovery[a]				
Total iso-α-acids					
g	5.00	2.62	0.97	0.85	0.56
% (w/w)	100. 0	52.4	19.4	17.0	11.2
Total *cis*-iso-α-acids					
g	3.79	2.33	0.86	0.09	0.51
% (w/w)	100.0	61.5	22.7	2.37	13.4
cis-isocohumulone [% (w/w)]	100.0	69.6	12.8	1.92	15.7
cis-isohumulone [% (w/w)]	100.0	57.1	28.1	2.85	12.0
cis-isoadhumulone [% (w/w)]	100.0	59.5	25.0	1.43	14.1
Total *trans*-iso-α-acids					
g	1.21	0.29	0.11	0.76	0.05
% (w/w)	100.0	24.0	9.09	62.8	4.13
trans-isocohumulone [% (w/w)]	100.0	32.5	6.51	54.7	6.27
trans-isohumulone [% (w/w)]	100.0	18.1	9.97	69.6	2.37
trans-isoadhumulone[% (w/w)]	100.0	27.8	12.4	54.0	5.80
T/C ratio (%)[b]	32	12	13	844	10
C/T ratio (%)[c]	313	803	782	12	1020
Enrichment[d] (% cis[e]/% trans[f])	76/24	89/11	89/11	11/89	91/9

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

Table 3 **Effect of reducing the volume of the supernatant by evaporation under reduced pressure prior to solid phase extraction on the recovery of *cis*- and *trans*-iso-α-acids in the different fractions after separation from the isomerised hop extract with β-CD. First complex formation step: 40 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 2:1; precipitation time: 1 day at 0 °C. Mean values of duplicate experiments**

With volume reduction	Start	Solvent passed through SPE-cartridges	*cis*-Isomers fraction	*trans*-Isomers fraction	Loss
	Recovery[a]				
Total iso-α-acids					
g	5.00	0.0	3.71	0.80	0.49
% (w/w)	100. 0	0.0	74.2	16.0	9.80
Total *cis*-iso-α-acids					
g	3.79	0.0	3.32	0.09	0.38
% (w/w)	100.0	0.0	87.6	2.37	10.0
cis-isocohumulone [% (w/w)]	100.0	0.0	86.5	1.90	11.7
cis-isohumulone [% (w/w)]	100.0	0.0	88.6	2.83	8.63
cis-isoadhumulone [% (w/w)]	100.0	0.0	86.5	1.55	12.0
Total *trans*-iso-α-acids					
g	1.21	0.0	0.39	0.71	0.11
% (w/w)	100.0	0.0	32.2	58.7	9.09
trans-isocohumulone [% (w/w)]	100.0	0.0	38.3	52.0	9.64
trans-isohumulone [% (w/w)]	100.0	0.0	26.9	64.5	8.56
trans-isoadhumulone[% (w/w)]	100.0	0.0	39.1	50.8	9.96
T/C ratio (%)[b]	32	–	12	789	29
C/T ratio (%)[c]	313	–	851	13	345
Enrichment[d] (% cis[e]/% trans[f])	76/24	–	89/11	11/89	78/22

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

volume prior to SPE, total recovery of iso-α-acids throughout the whole separation procedure became 90.2 %, and the recovery of iso-α-acids in the *cis*-isomers fraction increased from 0.97 g to 3.71 g, or from 19.4 % to 74.2 % (compare tables 2 and 3). From the 3.79 g of *cis*-iso-α-acids initially present in the reaction mixture, 3.32 g or 87.6 % were found in the *cis*-isomers fraction (see table 3). As such, significant enrichment in *cis*-iso-α-acids was obtained and the % cis/% trans ratio changed from 76/24 for the added isomerised hop extract to 89/11, meaning that 89 % of the iso-α-acids present in the *cis*-isomers fraction were *cis*-iso-α-acids. On the other hand, on average 0.74 g of the 1.21 g of *trans*-iso-α-acids initially added to the reaction mixture (60.3 %) were recovered in the *trans*-isomers fraction owing to inclusion complex formation with β-CD (see tables 2 and 3). Because of the low amount of *trans*-isomers present in the original isomerised hop extract (4.67 g of *trans*-iso-α-acids in 19.1 g of iso-α-acids or 24 %), on average only 16.5 % of the initial iso-α-acids are finally found in the *trans*-isomers fraction. Therefore it is more expedient to look at the recovery of *trans*-iso-α-acids in the *trans*-isomers fraction. From the iso-α-acids recuperated in the *trans*-isomers fraction (on average 60.8 %), 89 % were *trans*-iso-α-acids. In conclusion, good separation of the *cis*- and *trans*-iso-α-acids was obtained, however further experiments were carried out to increase the enrichment and recovery of both the *cis*- and *trans*-isomers fractions by optimisation of the complexation conditions, as will be discussed in section 3.4.

3.2 Recovery of *trans*-iso-α-acids

From the results depicted in tables 2 and 3, it was concluded that almost exclusively *trans*-iso-α-acids bind to β-CD, which needed to be released by elution with an appropriate solvent to obtain them separated. In this study, only methanol was tested, and gave complete recovery of the *trans*-iso-α-acids from the β-CD/

trans-iso-α-acids inclusion complexes. This is in full agreement with the observations by Khatib et. al. [34, 35], who tested several aqueous solvents with different pH, but only methanol gave good recovery. It was concluded from our experiments that elution of the *trans*-iso-α-acids from the β-CD/*trans*-iso-α-acids inclusion complexes was complete after 16 hours in methanol (data not shown). Recuperation of β-CD for use in a new experiment was also tested and found feasible.

3.3 Affinity of β-CD for *trans*-iso-α-acids

Tables 2 and 3 show the results obtained in testing the ability of β-CD to selectively complex *trans*-iso-α-acids in a solution containing iso-α-acids. Notwithstanding the fact that β-CD also binds small amounts of *cis*-isomers, in particular the high affinity of β-CD for *trans*-iso-α-acids under the test conditions is demonstrated. β-CD can thus be used to yield a supernatant enriched in *cis*-isomers and a β-CD/*trans*-iso-α-acids inclusion complex precipitate, and this is in contrast with α-CDs that show very little or no complexation, and γ-CDs that complex both *cis*- and *trans*-isomers, according to Khatib [39].

Complex formation is the result of the combined action of several factors. True β-CD inclusion complexes, formed by substitution of the included water by the guest molecule, are not established by a covalent bond, but are due to the dissociation-association equilibrium of host and guest in solution. The stability of the complex, referring to this dissociation-association equilibrium in solution, is largely determined by the hydrophobic character of guest molecule substituents that are in an appropriate position for complex formation. Besides polarity of the guest compound, the inclusion complex formation is also dependent on the size and shape of the enclosed compound. It is obvious that geometrical compatibility with the cavity of the host molecule is the predominant factor for a guest compound to penetrate. Complex formation does not necessarily occur with the complete molecule. Only certain functional groups or side chains may penetrate into the carbohydrate cavity. Orientation of the included molecules is such that maximum contact between the hydrophobic part of the guest and the apolar cyclodextrin cavity is achieved [22, 31]. The presence of hydrophobic side chains which can penetrate into the cavity may explain why iso-α-acids are able to interact with β-CD. Since *cis*-iso-α-acids and *trans*-iso-α-acids have common structural features, a similar affinity to β-CD would be expected. However, the affinity of *cis*-iso-α-acids for the β-CD molecule is significantly lower and a definite preference for *trans*-iso-α-acids is perceived. This can only be explained on the basis of the different relative configuration of the tertiary hydroxyl at C4 and the prenyl side chain at C5 in the molecular structures of the *cis*- and *trans*-iso-α-acids [40]. The isohexenoyl side chain at C4 and the prenyl side chain at C5 have a relatively unobstructed position in *cis*-iso-α-acids, but the proximity of these side chains in *trans*-iso-α-acids provide a region of high hydrophobicity, presumably leading to better affinity for the hydrophobic β-CD cavity and increased strength of hydrophobic interactions. Besides, our experiments also demonstrate that from the three *trans*-isomers, *trans*-isohumulone shows the highest affinity to β-CD as the recovery of this isomer from the β-CD precipitate is more than 10 % higher than for the other *trans*-isomers (see tables 2 and 3). This must undoubtedly be attributed to the different side chain at C2 (see fig. 2). Nevertheless, Khatib [39] speculated that the β-CD/*trans*-iso-α-acids complex precipitate, is not a true inclusion complex. Based on NMR investigations, he assumed that the *trans*-isomers interact with the outer surface of the β-CD molecule. However, the observation that both *cis*- and *trans*-isomers are complexed with g-CD, and completely not with α-CD [39], leads one to suspect that complex formation is related to the size of the CD-cavity, determined by the amount of glucose units in the CD-ring, and thus also to hydrophobic interactions with this inner cavity.

3.4 Factors affecting recovery and enrichment of the *trans*- and *cis*-isomers fractions

Distinct separation between *trans*- and *cis*-iso-α-acids was obtained in the first series of experiments. In order to obtain a higher proportion of *cis*-iso-α-acids in the '*cis*-isomers fraction' and of *trans*-iso-α-acids in the '*trans*-isomers fraction' in combination with an acceptable recovery, the methodology was further optimised. The effect of the precipitation time on the enrichment and recovery was studied. Besides, a different medium for complex formation was evaluated.

3.4.1 Cold precipitation time

As the complexation proceeds and the reaction mixture is cooled to 0 °C, the solubility of the β-CD/*trans*-iso-α-acids inclusion complexes decreases. The time needed for total precipitation depends on the complex formation and its stability [22]. Table 4 (see next page) shows the effect of the precipitation time at 0 °C for the separation of iso-α-acids from an isomerised hop extract, applying the methodology as described in section 3.1. After 1, 3 and 5 days at 0 °C, both the supernatant (referred to as '*cis*-isomers fraction') and β-CD precipitate (referred to as '*trans*-isomers fraction') were analysed for their iso-α-acids composition.

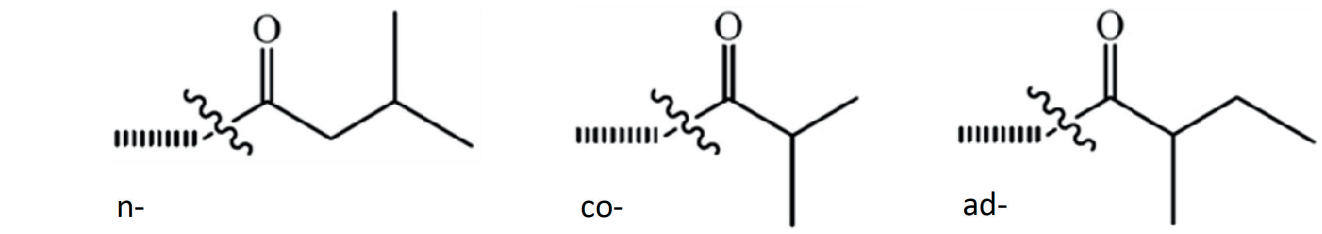

Fig. 2 Representation of the acyl side chain at C2 of the five-membered ring of iso-α-acids. This side chain is a 3-methylbutanoyl group in iso-n-humulone, a 2-methylpropanoyl group in isocohumulone, and a 2-methyl-butanoyl group in isoadhumulone

Table 4 **Effect of the precipitation time (days at 0 °C) on the recovery of *cis*- and *trans*-iso-α-acids in the different fractions after separation from the isomerised hop extract with β-CD. First complex formation step: 40 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 2:1. Mean values of duplicate experiments**

		Precipitation time at 0 °C					
		1 day		3 days		5 days	
	Start	*cis*-Isomers fraction	*trans*-Isomers fraction	*cis*-Isomers fraction	*trans*-Isomers fraction	*cis*-Isomers fraction	*trans*-Isomers fraction
	Recovery[a]						
Total iso-α-acids							
g	5.00	4.02	0.67	4.06	0.72	3.99	0.70
% (w/w)	100. 0	80.4	13.4	81.2	14.4	79.8	14.0
Total *cis*-iso-α-acids							
g	3.79	3.48	0.06	3.55	0.07	3.51	0.04
% (w/w)	100.0	91.8	1.58	93.7	1.85	92.6	1.06
cis-isocohumulone [% (w/w)]	100.0	92.2	1.24	93.7	1.62	93.4	1.08
cis-isohumulone [% (w/w)]	100.0	91.4	1.87	93.6	2.22	91.7	1.19
cis-isoadhumulone [% (w/w)]	100.0	92.2	1.18	93.9	1.16	93.6	0.58
Total *trans*-iso-α-acids							
g	1.21	0.54	0.61	0.51	0.65	0.48	0.66
% (w/w)	100.0	44.6	50.4	42.1	53.7	39.7	54.5
trans-isocohumulone [% (w/w)]	100.0	51.3	43.5	47.0	47.7	46.2	48.1
trans-isohumulone [% (w/w)]	100.0	38.6	56.5	35.8	60.4	32.0	61.9
trans-isoadhumulone[% (w/w)]	100.0	53.2	41.9	49.9	47.9	47.6	47.0
T/C ratio (%)[b]	32	16	1017	14	929	14	1650
C/T ratio (%)[c]	313	644	10	696	11	731	6
Enrichment[d] (% cis[e]/% trans[f])	76/24	87/13	9/91	87/13	10/90	88/12	6/94

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

After 1 day of cold precipitation, total recovery of iso-α-acids from the reaction medium was as high as 93.8 %, of which respectively 4.02 g of iso-α-acids, or 80.4 % of the initial content, is found in the *cis*-isomers fraction, and 0.67 g of iso-α-acids, or 13.4 % of the initial content, is found in the *trans*-isomers fraction. Total recovery was highest after 3 days (95.6 %), and slightly decreased again to 93.8 % at the 5th day of precipitation. Higher recoveries were thus obtained compared with the earlier experiments (see table 3) due to a diminished loss throughout the experimental procedure. The percentage of *cis*-iso-α-acids recovered in the *cis*-isomers fraction was high (91.8–93.7 %), with an optimum (93.7 %) after 3 days of precipitation. Nevertheless, for the determination of the optimal precipitation time, the highest enrichment of both the *cis*- and *trans*-isomers fraction was aimed at, in combination with a high recovery. Therefore, the optimal precipitation time was not considered 3 days, as the amount of *trans*-iso-α-acids, detected in the β-CD precipitate (*trans*-isomers fraction), gradually increased from 50.4 % of the initial content after 1 day at 0 °C up to 54.5 % during 4 additional days of precipitation. Likewise, the level of *trans*-iso-α-acids in the *cis*-isomers fraction decreased with increasing precipitation time (from 44.6 % of the initial content after 1 day of cold storage, up to 39.7 % after 5 days), resulting in 88 % *cis*-iso-α-acids after 5 days of precipation. The amount of *cis*-iso-α-acids that had formed inclusion complexes with the β-CD molecules, was also the lowest after 5 days of precipitation (0.04 g of *cis*-iso-α-acids or 1.06 % of the initial content). The highest enrichment in *trans*-iso-α-acids was thus also found after 5 days of cold storage, where the *trans*-isomers fraction contained 94 % of *trans*-iso-α-acids. The drawback is that only half of the original *trans*-iso-α-acids were complexed by β-CD in the complex formation step, i.e. 0.66 g of the 1.21 g of *trans*-iso-α-acids initially present in the reaction medium, equal to a recovery of only 54.5 %. This means that the experiment has to be repeated in order to obtain a substantial amount of *trans*-iso-α-acids of high enrichment (at least 94 %), taking also the low amount of *trans*-iso-α-acids in the original commercial iso-α-acids extract (only 24 % of the iso-α-acids present in the extract are *trans*-iso-α-acids) into account.

Table 5 **Recovery of *cis*- and *trans*-iso-α-acids in the different fractions after separation from the isomerised hop extract with β-CD when using ethanol as organic solvent. First complex formation step: 40 % (v/v) ethanol in water; molar ratio β-CD to *trans*-iso-α-acids: 2:1; precipitation time: 1 day and 5 days at 0 °C. Mean values of duplicate experiments.**

		Precipitation time at 0 °C					
		1 day			5 days		
	Start	*cis*-Isomers fraction	*trans*-Isomers fraction	Loss	*cis*-Isomers fraction	*trans*-Isomers fraction	Loss
	Recovery[a]						
Total iso-α-acids							
g	5.00	4.48	0.38	0.14	4.28	0.54	0.18
% (w/w)	100. 0	89.6	7.62	2.78	85.6	10.8	3.61
Total *cis*-iso-α-acids							
g	3.79	3.64	0.04	0.11	3.60	0.06	0.13
% (w/w)	100.0	96.0	1.06	2.90	95.0	1.58	3.43
cis-isocohumulone [% (w/w)]	100.0	96.3	0.99	2.67	94.6	1.49	3.91
cis-isohumulone [% (w/w)]	100.0	95.7	1.17	3.09	95.2	1.78	3.02
cis-isoadhumulone [% (w/w)]	100.0	96.2	0.89	2.87	95.4	1.16	3.44
Total *trans*-iso-α-acids							
g	1.21	0.84	0.34	0.03	0.68	0.48	0.05
% (w/w)	100.0	69.4	28.1	2.48	56.2	39.7	4.13
trans-isocohumulone [% (w/w)]	100.0	72.3	25.0	2.73	59.9	35.3	4.78
trans-isohumulone [% (w/w)]	100.0	66.0	31.5	2.53	52.5	43.8	3.71
trans-isoadhumulone[% (w/w)]	100.0	72.9	24.7	2.43	60.1	34.7	5.16
T/C ratio (%)[b]	32	23	850	27	19	800	38
C/T ratio (%)[c]	313	433	12	367	529	13	260
Enrichment[d] (% cis[e]/% trans[f])	76/24	81/19	11/89	79/21	84/16	11/89	72/28

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

3.4.2 Reaction medium for complex formation

The β-CD molecule used for complexation of the *trans*-iso-α-acids, is a cyclic oligosaccharide which has the 'generally recognised as safe' or GRAS status, regulated by the U.S. Food and Drug Administration. However, the formation of β-CD/*trans*-iso-α-acids inclusion complexes was carried out using methanol/water solutions [40 % (v/v)] and both the *cis*- and *trans*-isomers fractions were finally obtained in methanol. Although the use of organic solvent is indispensable for the formation of β-CD inclusion complexes with iso-α-acids as the guest molecules on the one hand, and the release of the iso-α-acids from the inclusion complexes on the other, methanol should be avoided for further application (e. g. addition of in particular the *cis*-isomers fraction in beer processing). Consequently, it was investigated whether ethanol could replace methanol as organic solvent for separation of *trans*-iso-α-acids from the isomerised hop extract with β-CD.

Complex formation was tested in ethanol/water [40 % (v/v)] instead of methanol/water [40 % (v/v)] mixtures, and subsequent elution of iso-α-acids from the SPE-cartridges as well as the release of the *trans*-isomers from the β-CD precipitate was carried out with ethanol. The results of duplicate experiments with cold precipitation (0 °C) of the β-CD/*trans*-iso-α-acids inclusion complexes for 1 day and 5 days, are depicted in table 5.

The results in table 5 clearly show that, with 1 day of cold storage, enrichment in *trans*-isomers obtained with ethanol as the solvent is highly comparable to the experiments performed with methanol (see table 4, 1 day of precipitation), the *trans*-iso-α-acids thereby accounting for 89 % of the iso-α-acids present. However, far less *trans*-iso-α-acids were recovered in this fraction when using ethanol (0.34 g of *trans*-iso-α-acids or 28.1 % of the initial amount) compared to methanol (0.61 g of *trans*-iso-α-acids or 50.4 % of the initial amount). The majority of *trans*-iso-α-acids remained in the *cis*-isomers fraction, i.e. 69.4 % of the initial content vs. 44.6 %

when using methanol as the solvent (see respectively tables 5 and 4, 1 day of precipitation), and thus markedly less complex formation is observed between the *trans*-iso-α-acids and β-CD in 40 % (v/v) ethanol/water mixtures. A reasonable explanation for this observation is that ethanol forms stable complexes with β-CD [22]. Another possible explanation can be that ethanol is a too good apolar solvent for the non-ionised *trans*-iso-α-acids so that they have less affinity for the cavity of β-CD. As a result, less guest molecules, i.e. *trans*-iso-α-acids, are accommodated in the cavity of β-CD, demonstrating once again that successful complex formation between *trans*-iso-α-acids and β-CD is the result of the concerted action of several factors. As a result, also a supernatant less enriched in *cis*-isomers is obtained. The proportion of *cis*- to *trans*-iso-α-acids in the *cis*-isomers fraction is still 81/19 when using ethanol against 87/13 when using methanol (see respectively table 5 and 4, 1 day of precipitation). Generally, the use of ethanol leads up to a higher total recovery (97.2 %) compared to methanol (93.8 %) due to a reduced loss of iso-α-acids during the whole experiment.

When extending the precipitation time at 0 °C up to 5 days while using ethanol as the solvent, the amount of *trans*-iso-α-acids forming inclusion complexes with β-CD clearly increased, from 28.1 % after 1 day to 39.7 % after 5 days of precipitation (see table 5). In comparison, with methanol as the solvent, up to 54.5 % of the *trans*-iso-α-acids resided in the cavity of β-CD after 5 days at 0 °C (table 4). In addition, the *trans*-isomers fraction did not really enrich in *trans*-iso-α-acids because also slightly more *cis*-iso-α-acids were complexed with β-CD with longer precipitation time, i.e. 1.58 % of the initial content after 5 days against only 1.06 % after 1 day (see also table 5). Accordingly, after 5 days of precipitation with ethanol as the solvent, the *trans*-isomers fraction was less enriched in *trans*-iso-α-acids as with methanol: the % cis/% trans ratio was respectively 11/89 (table 5) and 6/94 (table 4).

On the other hand, the enrichment in *cis*-iso-α-acids in the *cis*-isomers fraction slightly improved with increasing the precipitation time up to 5 days (table 5). The proportion of *cis*- to *trans*-iso-α-acids in the *cis*-isomers fraction was 81/19 after 1 day and 84/16 after 5 ays of precipitation. However, still 56.2 % of the *trans*-iso-α-acids remained in the supernatant when using ethanol as the solvent (see table 5, 5 days of precipitation), compared to only 39.7 % when using methanol (see table 4, 5 days of precipitation). Hence, also the enrichment in *cis*-iso-α-acids in the *cis*-isomers fraction was lower when using ethanol in the complexation medium instead of methanol: the % cis/% trans ratio in this fraction was 84/16 (table 5) respectively 88/12 (table 4) after 5 days at 0 °C. In conclusion, the results show that 40 % (v/v) methanol/water as the reaction medium for complex formation was giving a markedly higher yield in *trans*-iso-α-acids and a higher enrichment for both the *cis*- and *trans*-isomers fractions after 1 complexation step, compared to the ethanol/water mixture, which explains the choice for methanol as the solvent in all other experiments.

3.5 Second complex formation step to enhance the enrichment in *cis*-iso-α-acids in the *cis*-isomers fraction

From all previous results it is clear that after one single complex formation step, complete separation of *cis*- and *trans*-isomers was not yet achieved. In the *trans*-isomers fraction, containing primarily *trans*-iso-α-acids (94 %) that were eluted from the β-CD precipitate with methanol, still around 6 % of *cis*-iso-α-acids were present after 5 days of precipitation at 0 °C (table 4). Nevertheless, the enrichment in *trans*-isomers was considered already adequate for application in further (brewing) experiments to study the behaviour of *trans*-iso-α-acids in the absence of *cis*-isomers. When compared with the use of a conventional commercial isomerised hop extract, the portion of *trans*-isomers in the sample will already change from 24 % to 94 %, with a likewise increase of the T/C ratio from 32 to 1650. On the other hand, the *cis*-isomers fraction obtained after the first complexation step, is not yet sufficiently enriched in *cis*-iso-α-acids for the intended purpose, although more *cis*-isomers relative to *trans*-isomers are yet found in comparison with a conventional commercial iso-α-acids extract (88 % vs. 76 % of *cis*-iso-α-acids, respectively). Therefore, aiming at a higher enrichment, the *cis*-isomers fraction derived from the first complex formation step, was incubated a second time with β-CD in order to form inclusion complexes between the β-CD molecules and residual *trans*-iso-α-acids. The effect of the second complex formation step on the enrichment and the recovery of iso-α-acids in the 2nd *cis*- and *trans*-isomers fractions as a function of the precipitation time at 0 °C, is presented in table 6.

In this additional complex formation step, the molar ratio β-CD to *trans*-iso-α-acids changed from 2:1 to 5:1 since the same saturated solution of β-CD in water (1.85 g/100 mL) was used to perform the second complexation step, whereas the proportion of *trans*-iso-α-acids in the medium already decreased from the initial 24 % to 12 % as a result of the first complex formation step (see table 4, 5 days of precipitation). Therefore, the concentration of methanol/water in the medium was decreased from 40 % (v/v) to 30 % (v/v), so that the affinity of the remaining *trans*-iso-α-acids would not be higher for the reaction medium than it is for the hydrophobic β-CD cavity.

When performing the additional complex formation step, a supernatant highly enriched in *cis*-iso-α-acids was produced, referred to as '2nd *cis*-isomers fraction'. After 3 days of precipitation at 0 °C, the proportion of *cis*-iso-α-acids relative to *trans*-iso-α-acids in this *cis*-isomers fraction was 96/4 and further increased to 98/2 with 2 more days of precipitation (see table 6). The amount of *trans*-iso-α-acids that complexed in the β-CD precipitate clearly increased with longer precipitation time. After 5 days of precipitation, around 79.2 % of the residual *trans*-iso-α-acids were accommodated in the β-CD cavity (see also table 6). As a result, nearly complete separation of *trans*- and *cis*- iso-α-acids was observed in the 2nd *cis*-isomers fraction. In this highly enriched *cis*-isomers fraction, containing 98 % of *cis*-iso-α-acids after 5 days of precipitation, 3.03 g of the 3.51 g of *cis*-iso-α-acids present at the start of the second complexation step were recuperated, which is a reasonable recovery of 86.3 % (table 6). Therefore, the additional complex formation step with a 5-day precipitation time was found to be optimal to achieve a highly enriched 2nd *cis*-isomers fraction. The *trans*-isomers fraction derived from this second complexation step however, was not retained since the enrichment was unacceptable. After two successive complex formation steps, the proportion of *cis*-iso-α-acids relative to *trans*-iso-α-acids in the 2nd *trans*-isomers fraction was 43/57

after 5 days at 0 °C, against 6/94 after only one complex formation step (see respectively tables 6 and 4). Presumably, next to *trans*-iso-α-acids, also *cis*-iso-α-acids (8.26 %) formed inclusion complexes with β-CD at this point because of the excess of β-CD in the reaction medium. Khatib et. al. [35] also observed complexation of *cis*-isomers in the excess of β-CD. Therefore, the highly enriched *trans*-isomers fraction (94 %) was realised in the first complex formation step, as stated earlier in this section. The 2nd complexation step was not further optimised, because it was only carried out to improve the enrichment of the *cis*-isomers fraction.

3.6 Yield of highly enriched *cis*- and *trans*-iso-α-acids

Applying the above described methodology for separation of *cis*- and *trans*-iso-α-acids, relatively high yields were observed, as summarised in table 7 (see next page).

Clearly, the *trans*-iso-α-acids percentage in the 1st *trans*-isomers fraction was 94 % (table 7). The average yield however was only 54.5 %. The 2nd *trans*-isomers fraction containing 31.4 % of the original *trans*-iso-α-acids was discarded due to the high proportion of *cis*-isomers (% cis/% trans 43/57). The 1st *cis*-isomers fraction still contained 92.6 % of the *cis*-iso-α-acids originally present in the reaction medium. After two successive complex formation steps, the *cis*-iso-α-acids percentage in the 2nd *cis*-isomers fraction was 98 %, and the average yield was 80 % (79.9 %, table 7). From one experiment, starting with 3.79 g of *cis*-iso-α-acids and 1.21 g of *trans*-iso-α-acids in the mixture (5 g of iso-α-acids), 0.66 g of *trans*-iso-α-acids and 3.03 g of *cis*-iso-α-acids were respectively isolated for further use. Our developed separation methodology was repeated and the respective fractions combined, until the desired amounts of highly enriched *cis*- resp. *trans*-iso-α-acids were obtained for addition to e. g. 50 L of finished beer at the post-fermentation stage (addition rate 25 mg of *cis*- resp. *trans*-iso-α-acids/L of beer).

Table 6 **Recovery of *cis*- and *trans*-iso-α-acids in the different fractions derived from a second complex formation step. Second complex formation step: 30 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 5:1, precipitation time: 3 days and 5 days at 0 °C. Mean values of duplicate experiments.**

		Precipitation time at 0 °C					
		3 days			5 days		
	Start 2nd step	2nd *cis*-Isomers fraction	2nd *trans*-Isomers fraction	Loss	2nd *cis*-Isomers fraction	2nd *trans*-Isomers fraction	Loss
	Recovery[a]						
Total iso-α-acids							
g	3.99	3.26	0.50	0.23	3.10	0.67	0.22
% (w/w)	100. 0	81.7	12.5	5.76	77.7	16.8	5.51
Total *cis*-iso-α-acids							
g	3.51	3.13	0.18	0.20	3.03	0.29	0.19
% (w/w)	100.0	89.2	5.13	5.70	86.3	8.26	5.41
cis-isocohumulone [% (w/w)]	100.0	89.5	4.59	5.94	87.2	7.67	5.13
cis-isohumulone [% (w/w)]	100.0	89.3	5.16	5.57	86.3	8.44	5.23
cis-isoadhumulone [% (w/w)]	100.0	88.3	6.33	5.39	85.2	8.10	6.70
Total *trans*-iso-α-acids							
g	0.48	0.13	0.32	0.03	0.07	0.38	0.03
% (w/w)	100.0	27.1	66.7	6.25	14.6	79.2	6.25
trans-isocohumulone [% (w/w)]	100.0	27.8	66.2	5.95	16.0	77.4	6.64
trans-isohumulone [% (w/w)]	100.0	26.9	66.8	6.34	14.3	79.3	6.40
trans-isoadhumulone[% (w/w)]	100.0	25.5	67.8	6.74	13.3	81.0	5.73
T/C ratio (%)[b]	14	4	178	15	2	131	16
C/T ratio (%)[c]	731	2408	56	667	4329	76	633
Enrichment[d] (% cis[e]/% trans[f])	88/12	96/4	36/64	87/13	98/2	43/57	86/14

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

Table 7 **Recovery of *cis*- and *trans*-iso-α-acids in the different fractions after separation from the isomerised hop extract with β-CD in two successive complex formation steps. First complex formation step: 40 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 2:1; precipitation time: 5 days at 0 °C. Second complex formation step: 30 % (v/v) methanol in water; molar ratio β-CD to *trans*-iso-α-acids: 5:1; precipitation time: 5 days at 0 °C. Mean values of duplicate experiments.**

	Start 1st step	1st *cis*-Isomers fraction = Start 2nd step	1st *trans*-Isomers fraction	Loss 1st step	2nd *cis*-Isomers fraction	2nd *trans*-Isomers fraction	Loss 2nd step
	Recovery[a]						
Total iso-α-acids							
g	5.00	*3.99*	0.70	0.31	3.10	0.67	0.22
% (w/w)	100. 0	*79.8*	14.0	6.20	62.0	13.4	4.40
Total *cis*-iso-α-acids							
g	**3.79**	*3.51*	0.04	0.24	**3.03**	0.29	0.19
% (w/w)	**100.0**	*92.6*	1.06	6.33	**79.9**	7.65	5.01
cis-isocohumulone [% (w/w)]	100.0	*93.4*	1.08	5.52	81.4	7.15	4.76
cis-isohumulone [% (w/w)]	100.0	*91.7*	1.19	7.11	79.2	7.74	4.82
cis-isoadhumulone [% (w/w)]	100.0	*93.6*	0.58	5.82	79.8	7.58	6.21
Total *trans*-iso-α-acids							
g	**1.21**	*0.48*	**0.66**	0.07	0.07	0.38	0.03
% (w/w)	**100.0**	*39.7*	**54.5**	5.79	5.79	31.4	2.52
trans-isocohumulone [% (w/w)]	100.0	*46.2*	48.1	5.70	7.41	35.8	3.17
trans-isohumulone [% (w/w)]	100.0	*32.0*	61.9	6.11	4.60	25.4	2.19
trans-isoadhumulone[% (w/w)]	100.0	*47.6*	47.0	5.40	6.33	38.6	2.87
T/C ratio (%)[b]	32	*14*	1650	29	2	131	16
C/T ratio (%)[c]	313	*731*	6	343	4329	76	633
Enrichment[d] (% cis[e]/% trans[f])	**76/24**	*88/12*	**6/94**	77/23	**98/2**	43/57	86/14

[a] Recovery in % (w/w) = the ratio of the amount (g) of iso-α-acids after separation with β-CD to the amount (g) of iso-α-acids in the original reaction mixture (start), multiplied by 100;
[b] T/C ratio = the ratio of the amount (g) of *trans*-iso-α-acids to the amount (g) of *cis*-iso-α-acids, multiplied by 100;
[c] C/T ratio = the ratio of the amount (g) of *cis*-iso-α-acids to the amount (g) of *trans*-iso-α-acids, multiplied by 100;
[d] Enrichment of *cis*- respectively *trans*-iso-α-acids in the different fractions;
[e] % cis = the ratio of the amount of *cis*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100;
[f] % trans = the ratio of the amount of *trans*-iso-α-acids (g) to the total amount of iso-α-acids (g), multiplied by 100.

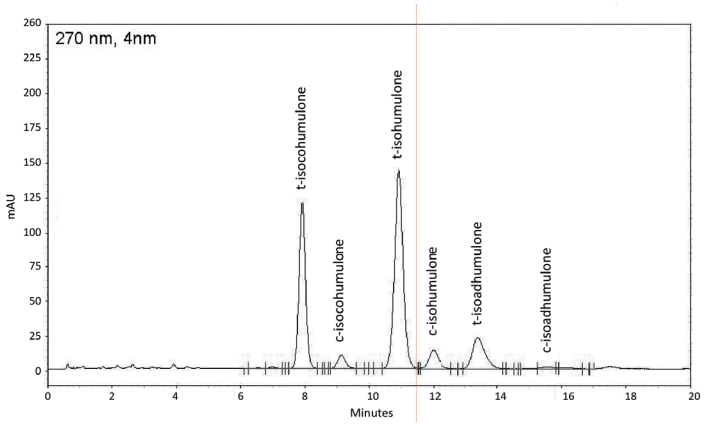

Fig. 3 **HPLC separation of the 1st *trans*-isomers fraction, illustrating the enrichment of *trans*-iso-α-acids (94 %). Preparation according to the described isolation methodology based on selective removal of *trans*-iso-α-acids from the isomerised hop extract by treatment with β-cyclodextrin**

For further use of the combined 2nd *cis*-isomers fractions, respectively 1st *trans*-isomers fractions, enclosing the highly enriched *cis*-, respectively *trans*-iso-α-acids in methanol, all methanol was evaporated under reduced pressure with a rotary evaporator. The concentrates were then redissolved in ethanol and stored at 0 °C in the dark before further application. To illustrate the enrichment of *trans*-, respectively *cis*-iso-α-acids in the bittering preparations available for further brewing experiments, the HPLC separation patterns of the *trans*- and *cis*-isomers fractions are depicted in figure 3 and 4, respectively.

In a previous paper, we reported the use of enriched *cis*- resp. *trans*-iso-α-acids in a study on the storage-induced appearance of staling aldehydes in beer [41]. Aldehyde formation as a function of forced ageing was irrespective of the mode of bittering, in spite of the trans-specific degradation observed during beer ageing. Clearly, other critical factors are related to the flavour

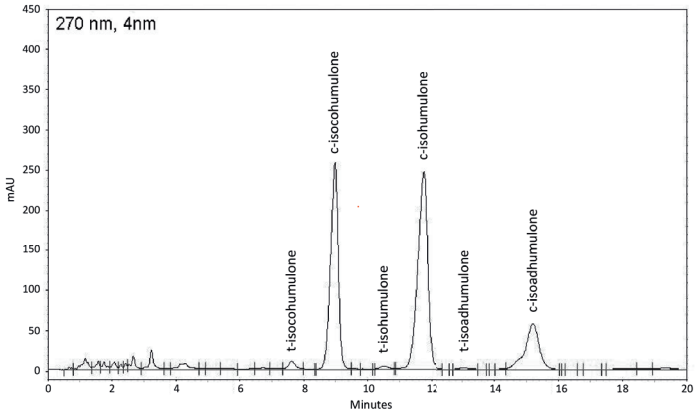

Fig. 4 HPLC separation of the 2nd *cis*-isomers fraction, illustrating the enrichment of *cis*-iso-α-acids (98 %). Preparation according to the described isolation methodology based on selective removal of *trans*-iso-α-acids from the isomerised hop extract by treatment with β-cyclodextrin

instability of beer [42, 43]. Also *Schmidt* et. al. [13] confirmed that specific transformation products of *trans*-iso-α-acids are suitable markers for beer ageing, but a higher cis/trans ratio in a fresh beer sample did not result in improved flavour and bitter taste stability. The impact of unknown degradation processes of *cis*-iso-α-acids to the taste stability of aged beverages clearly needs further investigation in the future [13]. The established methodology for the isolation of *cis*-iso-α-acids from a commercial isomerised hop extract is therefore helpful for researchers to further explore issues related to bitter acid degradation and beer flavour stability.

4 Conclusion

In this paper, it was demonstrated that β-cyclodextrin (β-CD) can successfully be employed to selectively complex *trans*-iso-α-acids in a solution containing both *cis*- and *trans*-isomers. Separation of *cis*-iso-α-acids from *trans*-iso-α-acids starting from a commercial isomerised hop extract was carried out in two successive complex formation steps, yielding a supernatant containing *cis*-isomers nearly free of *trans*-isomers, and a stable β-CD/*trans*-iso-α-acids inclusion complex precipitate. The most enriched *cis*-isomers fraction (98 % of the total iso-a-acids in this fraction) was isolated from the supernatant derived from the second complex formation step. An enriched *trans*-isomers fraction (94 % of the total iso-a-acids in this fraction) was eluted from the precipitate derived from the first complex formation step. The highly reproducible method was based on readily available equipment and was not labour-intensive, exhibiting yields of 80 % of the initial *cis*-iso-α-acids content in the most enriched *cis*-isomers fraction, and 54.5 % of the initial *trans*-iso-α-acids in the *trans*-isomers fraction.

In summary, the presented methodology allows the preparation of sufficient quantities of highly enriched *cis*- resp. *trans*-iso-α-acids fractions, which are both applicable in real brewing practice on pilot scale. This is highly relevant for R&D research in view of future investigations of issues related to bitter acids degradation and beer flavour stability.

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