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

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CHAPTER 3

β-Cyclodextrin to Separate *cis*- from *trans*- Iso-α-Acids

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ABSTRACT

The separation of *cis*-iso-α-acids from their *trans*-isomers can be performed using β-cyclodextrin. The optimum complexation condition to get the purest *cis*-isomers mixture is using water as a solvent with a complexation temperature of 70 ^{o}C. The ratio of the weight of β-cyclodextrin to the volume of water is 1:8, and the molar ratio between total amount of iso-α-acids sample to β-cyclodextrin is 1:1. After 2 days precipitation time, the *cis*-isomers mixture which remains in the supernatant is subjected to ultra filtration in order to remove β-cyclodextrin. The purest *trans*-iso-α-acids mixture is obtained using the same conditions, except that the molar ratio between total amount of iso-α-acids sample to β-cyclodextrin is 4:1. The *trans*-isomers can be released from the β-cyclodextrin complex by methanol elution. This method is promising for large scale production of the stereoisomers which can be applied in hopped beverages.

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3.1. INTRODUCTION

Iso-α-acids (IAAs), which result from the isomerization of hop α-acids during the wort boiling step in the beer brewing process, are the major bittering components of beer (Verzele and De Keukeleire, 1991). They also have foam lacing properties (Bamfort, 1985 ; Bishop *et al*., 1974) and have activity against gram-positive bacteria (Simpson and Smith, 1992).

The IAAs are present as *trans*-/*cis*- pairs, i.e., *trans*-/*cis*-isocohumulone (TICH/CICH), *trans*-/*cis*-isohumulone (TIH/CIH), and *trans*-/*cis*-isoadhumulone (TIAH/CIAH). The *trans*-isomers are less bitter than the *cis*-counterparts (Hughes, 2000; Hughes and Simpson, 1996). The degree of the bitterness of the individual compounds is as follows:

cis-isohumulone > *trans*-isohumulone ≈ *cis*-isocohumulone > *trans*-isocohumulone

The *trans*-isomers were reported to be more prone to oxidation than their *cis*-counterparts, thus they are less stable (De Cooman *et al*., 2001). *trans*-Isomers have a shelf half-life below one year at 12 ^{o}C. A taste panel could already detect the off-flavor due to the degradation of the *trans*-isomers extracted from beer after 15 months although a minimum shelf life of 5 years is specified on the bottle label. The beer was stored in the dark at 12 ^{o}C (De Cooman *et al*., 2000). The lower stability of the *trans*-isomers during processing and storage has also been observed by other researchers (Araki *et al*., 2002; Hughes *et al*., 1997). The decrease of the *trans*-isomers after fermentation was observed but it does not occur during the wort boiling process (Hughes *et al*., 1997).

The degradation of IAAs is caused by the effect of active oxygen yielding off-flavours, including carbonyl compounds. This process is accelerated by metal ions. As a consequence, the beer bitterness decreases with beer age (Kaneda *et al*., 1989; Kaneda *et al*., 1992; King and Duineveld, 1999).

Due to the different behavior of individual IAAs as explained above, developing a low cost method to separate the *cis*-isomers mixture from its *trans*-counterpart could result in an important contribution to increase beer shelf life. Much research has been done for this purpose (Hughes and Simpson, 1996; Maye *et al*., 1999; Thornton *et al*.,

1993; Ting and Goldstein, 1996), but the methods reported are too expensive to be applied commercially for beer or imply the use of very toxic substances.

There was a report on the enhancement of hop oil stability by complexation with β-cyclodextrin (β-CD) (Hughes and Simpson, 1994) though no further details were described. Additionally, a decrease of the antimicrobial activity of *trans*-IAAs in presence of β-CD has been observed (Simpson and 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 the *cis*- from the *trans*- isomers. Cyclodextrins are enzyme-modified starch derivatives which are industrially produced. The present price of the food grade CD is between US$ 5-10/kg for β-CD. β-CD has been on the GRAS list since 1998. It is used as a flavour carrier and protectant at a concentration of 2% in food products (Szente and Szejtli, 2004).

CD consists of six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units linked by α-(1,4) bonds (Del Valle, 2004) to make a truncated cone structure (Fig. 3.1). CD and the guest molecule can form inclusion complexes. The guest molecules move into the cavity of the host (CD) without affecting the host framework structure. Inclusion complexes will be formed if the binding sites of the host and guest molecules are stereoelectronically complementary. There is no covalent bond established between the host and guest, but binding is via a dissociation-association equilibrium in solution (Dobado *et al*., 2004; Szejtli, 1988).

The ability of CD to select the guest allows it to be also used as separation technique. CD has been extensively used to discriminate between isomers, functional groups and enantiomers. It has been applied for HPLC as stationary phase bonded to a solid support or as mobile phase additive in HPLC and capillary electrophoresis for chiral separations (Bresolle *et al*., 1996). It has also been applied in other separation techniques, e.g. thin-layer chromatography (Aboul-Enein *et al*., 2000), gas chromatography (Krupcik *et al*., 2004), supercritical fluid chromatography (Salvador *et al*., 2001), countercurrent chromatography (Breinholt *et al*., 1999), isoelectric focusing (Spanik *et al*., 2002), liquid-liquid extraction (Uemasu and Kushiyama, 2004) and isotachophoresis (Kuramoto, 1986).

The aim of the present study was to determine the optimum conditions for binding *cis*-iso-α-acids (CIM) and *trans*-iso-α-acids mixture (TIM) to β-CD.

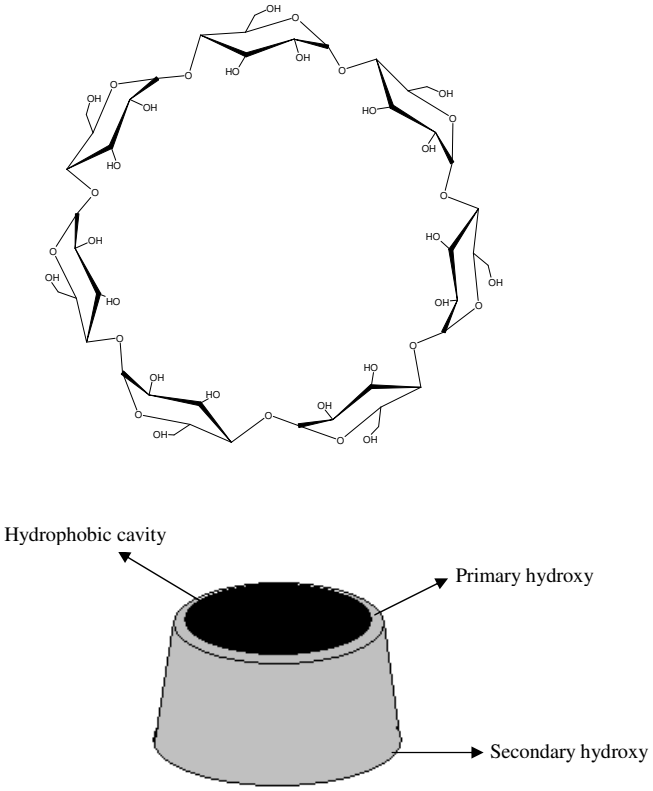

Fig. 3.1. Chemical structure of β-cyclodextrin

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

All organic solvents were purchased from Biosolve Co. Ltd (Valkenswaard, The Netherlands). *Ortho*-phosphoric acid 85% (w/v) was obtained from Merck (Darmstadt, Germany). β-CD Cavamax ® W7 Pharma (purity ≥ 98%) was purchased from Wacker-Chemie Co. Ltd, (Burghausen, Germany) and dimethylsulfoxide-d_6 (99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA).

3.2.2. Isomerized hop extract

Isomerized CO_2 hop extract was in the form of the aqueous solution of the potassium salts of iso-α-acids. It was purchased from Botanix (Paddock Wood, Kent, UK). It contained 1.9, 3.3, 2.0, 6.8, 0.9, and 1.7% (w/v) of TICH, CICH, TIH, CIH, TIAH and CIAH, respectively.

3.2.3. Procedure for complexation

β-CD solution was prepared by dissolving it in ethanol-water (1:2) or water, with different ratios and heated in shaking water-bath to different temperatures (24, 50, 70 or 90 ^{o}C). Isomerized CO_2 hop extract was added to the β-CD solution with different sample loading mode (slow loading or all at once) while continually shaking and keeping the temperature constant. Slow loading was conducted by adding every minutes 100 µl of sample to the β-CD solution, while all at once loading was performed by adding all the sample at the same time to the CD solution.

The mixture was stored at 4 ^{o}C for several days in absence of light. The β-CD complex precipitated as a white-yellow crystalline powder. The first supernatant, containing CIM was collected by filtering through a paper filter (Schleicher & Schuell no.8, Dassel, Germany). The precipitate was washed several times with ethanol:water (1:2) and ethyl acetate. Before HPLC analysis, precipitate was mixed with methanol, vortexed and filtered through 0.45 µm filter (Pall GHP Acrodisc, East Hills, NY, USA). The supernatant was injected into the HPLC without further treatment.

3.2.4. Recovery of the iso-α-acids from β-CD by organic solvents

The optimum conditions needed to recover the IAAs from the β-CD precipitate were investigated. For this purpose approximately 3 mg of the precipitate were placed in a 1.5 ml-micro tube and 200 µl of test solvent was added. The solvents tested were methanol, ethanol, ethyl acetate, acetone, *n*-hexane, and water with different pH (pH 1 to 6 adjusted with citric acid, pH 8-12 adjusted by NaOH). After vortexing the supernatant was filtered through 0.20 µm filter and analyzed by HPLC.

3.2.5. Separation of β-CD from iso-α-acids by ultrafiltration

A stirred ultra filtration (UF) cell model 8400 (Amicon, Beverly, MA, USA) was used together with a regenerated cellulose YM1 UF membrane disc with a cut off of 1000 Da and a diameter of 76 mm (Millipore, Bedford, MA, USA). The membrane disc was cleaned from its preservative, sodium azide, by soaking its glossy side down in a beaker with distilled water for one hour, the water was changed three times. The membrane disc was then mounted in an UF cell with the glossy side in contact with the solution- and rinsed by filtering distilled water for 5 minutes using N_2 gas at 55 psi while continuously stirring. The water was then replaced with the sample and the filtering process was performed below 4 ^oC and stopped when about 90% of the sample had passed the membrane. This process was repeated twice using the same membrane. The purity and recovery of the sample before and after UF were verified by HPLC, and the amount of β-CD remaining in the sample was determined by ^1H NMR.

3.2.6. HPLC analysis

The HPLC system used consisted of a Waters system equipped with a 626 pump and 600 S pump controller, a 717 plus auto sampler, and a 2996 photodiode array detector type 2996. A Hypersil 5 µ C18, 250 x 4.6 mm (Phenomenex, Torrance, CA, USA) column was used. The mobile phase was filtered using a 0.2 µm hydrophilic polypropylene membrane filter GH Polypro (Pall Corporation, Ann Arbor, MI, USA) 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/minute allowed the baseline separation of all 6 isomers within a total run time of 25 minutes. Pure IAAs were used as an external standards.

3.2.7. NMR measurements

All samples were taken to dryness with a rotary vacuum evaporator at 40 ^oC, re-dissolved in 1 ml of dimethylsulfoxide-d_6 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 ^{1}H 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).

3.3. RESULTS AND DISCUSSION

The first experiment was carried out using ethanol-water (1:2) as the complexation solvent with the ratio of the weight of β-CD to the volume of solvent ($R_{CD/W}$) of 1:13, complexation temperature (T_i) of 50 ^{o}C and a molar ratio of sample to β-CD ($R_{S/CD}$) of 1:1 (Table 3.1).

After 1 day precipitation at 4 ^{o}C, the supernatant and the β-CD precipitate which was formed were analyzed for IAAs. Surprisingly we found a complete separation of CIM and TIM. The CIM was detected in the supernatant, whereas the TIM was detected in the β-CD precipitate. The TIM could be released from β-CD by methanol elution. While the CIM in the supernatant was free of TIM, the released TIM still contained CIM as impurity (Table 3.1). Optimization of the complexation conditions was therefore necessary especially to increase the purity of TIM. We also considered to lower the amount of ethanol in the complexation solvent, which would reduce costs.

Table 3.1**.** Purity and recovery of CIM and TIM separated using ethanol-water (1:2) as the solvent with $R_{CD/W}$ = 1:13, T_i = 50 ^{o}C, $R_{S/CD}$ = 1:1, slow loading and precipitation time = 1 day. The values between parenthesis represent the standard deviation.

Fractions		CIM	TIM
Purity (%)[1]		99.6 (± 0.2)	75.3 (± 7.8)
Recovery (%, w/w)[2]	ICH[3]	66.6 (± 0.20.5)	NE[4]
	IH[3]	55.1 (± 0.25.2)	NE[4]
	IAH[3]	57.7 (± 0.21.5)	NE[4]

[1] Measured by HPLC, purity of CIM is a percentage of CIM peak area/(CIM+TIM) peak area, and purity of TIM is percentage of TIM peak area/(CIM+TIM) peak area.
[2] Recovery is percentage of the amount of each IAAs obtained after separation compared to the amount of each IAAs in the original sample
[3] ICH = isocohumulone, IH = isohumulone, IAH = isoadhumulone
[4] Not evaluated

The effect of temperature and the ratio of the weight of β-CD to the volume of solvent using a fixed amounts of the guest and β-CD was first studied. Table 3.2 shows the effect of T_i and $R_{CD/W}$ for the purity and recovery of CIM, using only water as the solvent..

Table 3.2. Effect of complexation temperature (T_i) and ratio of the weight of β-CD to the volume of solvent ($R_{CD/W}$) to the purity and recovery of CIM after separation using water as the solvent with $R_{S/CD}$ = 1:1, slow loading, precipitation time =1 day. The values between parenthesis represent the standard deviation.

T_i		24 °C				
$R_{CD/W}$		1:2	1:4	1:8	1:13	1:18
Purity (%)[1]		98.3 (± 0.3)	98.9 (± 0.1)	99.7 (± 0.1)	99.3 (± 0.1)	98.7 (± 0.5)
Recovery (%, w/w)	CICH[2]	1.2 (± 0.1)	3.8 (± 0.1)	38.7 (± 2.1)	66.2 (± 4.6)	75.7 (± 13.7)
	CIH[2]	1.2 (± 0.1)	3.4 (± 0.1)	36.5 (± 2.0)	62.6 (± 4.9)	68.2 (± 15.3)
	CIAH[2]	0.8 (± 0.1)	3.4 (± 0.1)	33.4 (± 0.8)	62.9 (± 3.1)	81.5 (± 7.4)
T_i		50 °C				
$R_{CD/W}$		1:2	1:4	1:8	1:13	1:18
Purity (%)		99.8 (± 0.1)	99.9 (± 0.1)	99.8 (± 0.2)	99.7 (± 0.1)	99.2 (± 0.3)
Recovery (%, w/w)	CICH[2]	39.1 (± 1.4)	58.3 (± 1.5)	78.3 (± 1.0)	92.2 (± 5.1)	100.0 (± 16.3)
	CIH[2]	37.4 (± 1.8)	57.8 (± 1.0)	75.7 (± 0.5)	86.1 (± 5.9)	98.3 (±14.6)
	CIAH[2]	24.2 (± 0.7)	41.0 (± 1.0)	65.8 (± 1.9)	84.9 (± 2.8)	100.0 (± 4.0)
T_i		70 °C				
$R_{CD/W}$		1:2	1:4	1:8	1:13	1:18
Purity (%)		99.9 (± 0.1)	99.8 (± 0.1)	99.8 (± 0.1)	99.6 (± 0.1)	99.5 (± 0.2)
Recovery (%, w/w)	CICH[2]	46.6 (± 1.8)	70.6 (± 4.0)	88.9 (± 7.1)	96.9 (± 1.9)	100.0 (± 0.2)
	CIH[2]	39.9 (± 12.2)	70.2 (± 3.8)	89.7 (± 5.6)	92.9 (± 3.8)	98.3 (± 2.1)
	CIAH[2]	21.8 (± 11.7)	46.7 (± 1.1)	73.6 (± 0.6)	92.1 (± 2.9)	100.0 (± 0.2)
T_i		90 °C				
$R_{CD/W}$		1:2	1:4	1:8	1:13	1:18
Purity (%)		99.8 (± 0.1)	99.9 (± 0.10)	99.7 (± 0.1)	99.7 (± 0.1)	99.4 (± 0.2)
Recovery (%, w/w)	CICH[2]	34.5 (± 4.7)	48.1 (± 1.7)	83.6 (± 5.3)	93.9 (± 8.3)	100.0 (± 0.7)
	CIH[2]	32.4 (± 4.7)	46.2 (± 1.5)	79.8 (± 5.7)	87.2 (± 8.3)	95.0 (± 1.0)
	CIAH[2]	20.8 (± 2.5)	31.5 (± 0.8)	66.9 (± 4.6)	83.0 (± 0.1)	100.0 (± 1.7)

[1] Measured by HPLC, purity is calculated as a percentage of CIM peak area/(CIM+TIM) peak area
[2] CICH = *cis*-isocohumulone, CIH = *cis*-isohumulone, CIAH = *cis*-isoadhumulone

These results showed that the elimination of ethanol from the complexation media is possible under optimum complexation conditions, giving better purity and recovery of CIM at lower solvent volume compared to that of the ethanol-water method. The optimum condition was established as: T_i = 70 °C, $R_{CD/W}$ = 1:8 and $R_{S/CD}$ = 1:1 using 1 g β-CD and 1.6 ml of sample extract (contain 266 mg of total IAAs). In these conditions the purity of CIM was determined as 99.8% which is not much different

from the purity obtained by complexation with $R_{CD/W}$ =1:2 and T_i = 70 ^oC (99.9%), but with a higher recovery. At $R_{CD/W}$ = 1:18 ml the recovery is highest but the purity is lower and therefore can not be considered as the optimum complexation condition. The lower volume of supernatant has the additional advantage of easier handling and storage.

This optimum condition was used for further experiments to determine the rate at which the sample should be added to the solvent. As shown in Table 3.3, this rate affects the efficiency of the complexation process. Adding all the sample to the CD solution at once resulted in lower purity and recovery compared to slow loading

Another factor that was considered is the molar ratio of the sample to β-CD. The higher $R_{S/CD}$ reduces the purity of CIM in the supernatant but increases the purity of the TIM-β-CD precipitate (Table 3.4). To obtain the most pure CIM supernatant, $R_{S/CD}$ = 1:1 is the optimum condition; but for highest purity of TIM, $R_{S/CD}$ = 4:1 ml is the best.

Table 3.3. Effect of sample loading to the purity and recovery of CIM after β-CD separation with $R_{CD/W}$, T_i = 70 ^oC, $R_{S/CD}$ = 1:1, precipitation time = 1 day and different sample loading. The values between parenthesis represent the standard deviation.

Sample loading mode		Slow	All at once
Purity (%)		99.8 (± 0.1)	99.7 (± 0.1)
Recovery (%, w/w)	CICH[1]	88.9 (± 7.1)	71.7 (± 5.6)
	CIH[1]	89.7 (± 5.6)	72.0 (± 6.7)
	CIAH[1]	73.6 (± 0.6)	62.3 (± 8.6)

[1] CICH = *cis*-isocohumulone, CIH = *cis*-isohumulone, CIAH = *cis*-isoadhumulone

Table 3.4. Effect the molar ratio of sample to β-CD ($R_{S/CD}$) to the purity and recovery of CIM and TIM separated with $R_{CD/W}$ = 1:8, T_i = 70 ^oC, precipitation time = 1 day, slow loading. The values between parenthesis represent the standard deviation.

$R_{S/CD}$	Purity (%)	
	TIM (precipitate)	CIM (supernatant)
1:1	72.7 (± 5.1)	99.8 (± 0.1)
2:1	91.7 (± 2.6)	92.9 (± 0.6)
3:1	96.5 (± 1.0)	89.2 (± 0.4)
4:1	98.7 (± 0.6)	87.3 (± 0.5)
5:1	98.6 (± 0.3)	84.7 (± 0.4)
6:1	98.1 (± 0.1)	84.0 (±0.1)

The effect of precipitation time was examined and the results are shown in Table 3.5. Both purity and recovery were slightly increased after 2 days at 4 ^{o}C storage and the recovery was stable after 3 days but were slightly decreased at the fourth day. Therefore, 2 days was considered as the optimum precipitation time.

After precipitation there was a considerable amount of β-CD in the CIM containing supernatant. This supernatant can potentially be used for human consumption as β-CD is not toxic. However, in order to obtain pure IAAs, it is necessary to separate the remaining β-CD, taking into account that any treatment used should not include the use of chemicals toxic or inconvenient for human consumption.

Experiments involving the extension of the precipitation time to 1 month or reducing the solubility of β-CD by adding ethanol to the supernatant were made but β-CD still remained (>30%). Considering the very different dynamic volume of the iso-α-cids and β-CD, ultrafiltration (UF) with a membrane with a cut off of 1000 Da was tested. After UF, the supernatant was free of β-CD. This technique is promising to be applied on a large scale since it has low operating costs (US$ 1-10/1000 l filtrate) (Cheryan, 1986).

Table 3.5. Effect of precipitation time to the purity and recovery of CIM at different precipitation times after complexation with $R_{CD/W}$ = 1:8, T_i = 70 ^{o}C, $R_{S/CD}$ = 1:1, and slow loading. The values between parenthesis represent the standard deviation.

Precipitation time (days)		1	2	3	4
Purity (%)		99.8 (± 0.2)	99.9 (± 0.3)	99.8 (± 0.1)	99.9 (± 0.1)
Recovery (%, w/w)	CICH[1]	88.9 (± 7.5)	91.0 (± 5.5)	92.6 (± 2.6)	85.0 (± 4.1)
	CIH[1]	87.1 (± 7.8)	95.1 (± 5.4)	92.0 (± 6.2)	87.4 (± 5.2)
	CIAH[1]	73.7 (± 3.0)	78.2 (± 0.5)	77.7 (± 3.8)	77.7 (± 1.0)

[1] CICH = *cis*-isocohumulone, CIH = *cis*-isohumulone, CIAH = *cis*-isoadhumulone

3.3.1. Recovery and purification of the trans-isomers mixture

As can be concluded from the results in Table 3.4, the purest TIM was obtained with the same condition to that of CIM except $R_{S/CD}$ = 4:1. In this step, TIM binds to β-CD and needs to be released by eluting with the appropriate solvent (Fig. 3.2). Several aqueous solvents with different pHs were tested but only methanol gave good

recovery values. Therefore, care has to be taken when this method is applied to obtain TIM for human consumption.

The TIM solution obtained after elution of the β-CD complex with methanol contained a high amount of β-CD. To remove the β-CD, two methods were applied, UF using a membrane with a cut off of 1000 Da or taking the solution to dryness and re-dissolving it in ethyl acetate. Of these, only the last method proved successful. β-CD is very insoluble in ethyl acetate and can therefore be easily separated by vacuum filtration. The ratio between the isolated *trans*-isomers produced by this method is similar compared to those of original sample.

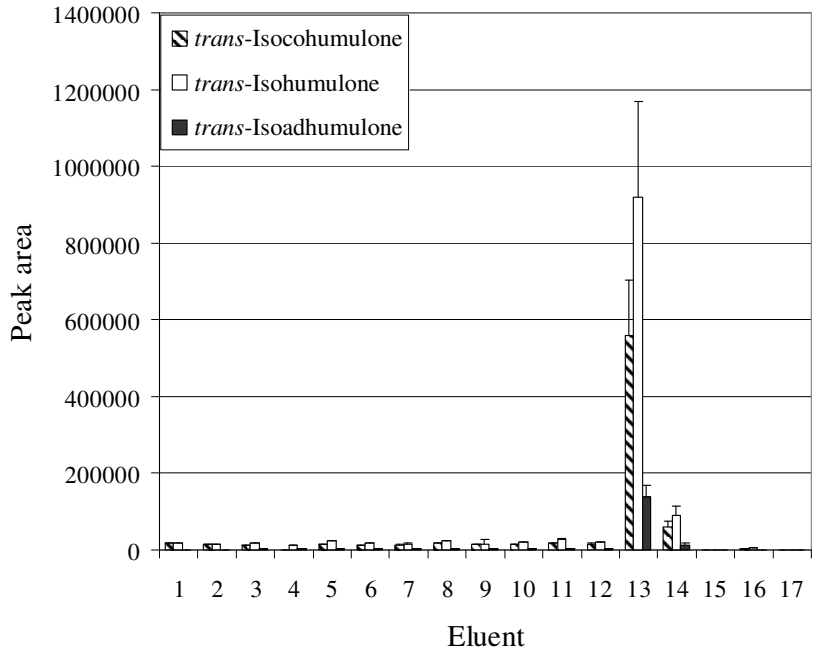

Fig. 3.2**.** Recovery of the *trans*-isomers from β-CD complex using different eluents: citric acid pH 1 (1), citric acid pH 2 (2), citric acid pH 3 (3), citric acid pH 4 (4), citric acid pH 5 (5), citric acid pH 6 (6), water pH 7 (7), NaOH pH 8 (8), NaOH pH 9 (9), NaOH pH 10 (10), NaOH pH 11 (11), NaOH pH 12 (12), methanol (13), ethanol (14), ethyl acetate (15), acetone (16), and *n*-hexane (17).

3. 4. CONCLUSION

The optimum conditions needed to obtain pure *cis*-isomers from a mixture of IAAs is using β-CD dissolved in water in which $R_{CD/W}$ = 1:8, T_i = 70 ^oC, $R_{S/CD}$ = 1:1, slow loading and precipitation time = 2 days. The purest TIM is obtained using the same conditions but with a $R_{S/CD}$ = 4:1. TIM can be recovered from the β-CD complex by methanol elution. Remnant β-CD can be removed from the CIM solution using UF, while the TIM methanol solution must be taken to dryness and re-dissolved with ethyl acetate in order to eliminate β-CD by filtration. These methods for producing either CIM or TIM are promising for a large scale production since β-CD and UF operating is very cost effective.