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Enantioseparation of Isoxanthohumol in Beer by Hydroxypropyl-γ-cyclodextrin-Modified Micellar Electrokinetic Chromatography

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Chiral resolution of isoxanthohumol (IX) in beer samples was performed by hydroxypropyl-γ-cyclodextrin-modified micellar electrokinetic chromatography. The optimum running conditions were found to be 20 mM phosphate buffer (pH 7.0) containing 45 mM hydroxypropyl-γ-cyclodextrin and 100 mM sodium dodecyl sulfate with an effective voltage of +20 kV at 20 °C using direct detection at 210, 295, and 370 nm. IX was detected in 12 beer samples and the total levels of (+)- and (−)-IX ranged from 0.15 to 1.4 mg/L. But the amount of xanthohumol (XN) was below the detection limit (0.017 mg/L). Each level of (−)-IX was almost the same as that of (+)-IX, suggesting that IX was a racemic mixture in these beer samples. The racemization of IX in beer could be attributed to the production of a racemic mixture from XN during boiling and to the fact that IX enantiomers were easily interconverted.

KEYWORDS: Enantioseparation; isoxanthohumol; xanthohumol; beer; micellar electrokinetic chromatography

INTRODUCTION

In beer making, hops (*Humulus Luplus* L.) is an important source of phenolic compounds. In hop cones, the most abundant prenylated calcone is xanthohumol (XN), which accounts for 80−90% of the prenylated flavonoids in hop (*1*). During the brewing process of beer, XN is converted into the corresponding isomeric prenylflavanone isoxanthohumol (IX), as shown in **Figure 1**. XN and prenylflavanones have received much attention in recent years as cancer chemopreventive and/or estrogenic agents reviewed previously (*2*, *3*). XN has been identified as a strong cancer chemopreventive agent (*4*). IX and XN have been reported to have antiangiogenic activity to immortalized human microvascular endothelial cells (*5*).

On the other hand, IX was found to be weakly estrogenic (*6*), but 8-prenylnaringenin (8-PN) has been identified as the most potent phytoestrogens currently known (*7*−*10*). It is assumed that 8-PN is formed nonenzymatically during drying, storage, and extraction of hop, and levels in beer are generally very low (*11*). Possemiers et al. (*12*) found that in one-third of fecal samples, IX was converted to 8-PN. They also found that *Eubacterium limosum* was capable of this conversion (O-demethylation). A conversion efficiency of 90% was achieved after strain selection. Nikolic et al. (*13*) reported that IX can be metabolized by human liver microsomes to 8-PN. Recently, Guo et al. (*6*) found that CYP1A2, a cytochrome P450 enzyme, catalyzed the O-demethylation of IX to 8-PN. This suggests that IX is an intermediate of 8-PN production in the human body.

XN and IX in beer samples and/or hop extracts have been analyzed by high-performance liquid chromatography (HPLC) (*14*, *15*), HPLC with mass spectrometry (MS) (*16*−*18*), capillary electrochromatography (*19*), capillary electrophoresis (CE) with MS (*20*) and noneaqueous CE (*21*). Stevens et al. (*16*) found that, during beer making, XN was largely cyclized to IX and that further losses of XN in beer were due to precipitation, adsorption to insoluble proteins and adsorption to yeast cells

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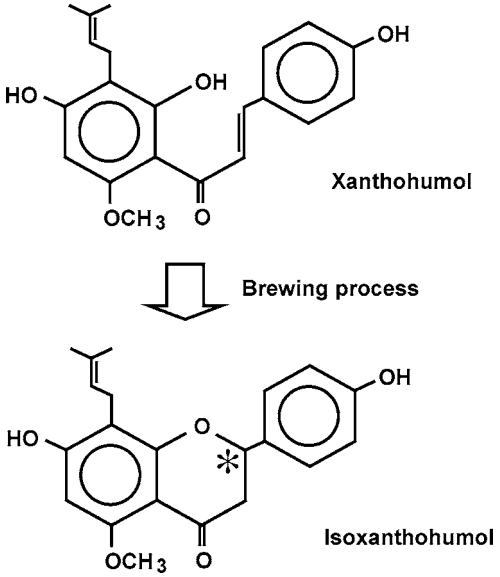

Figure 1. Structural formulas of XN and IX. The asterisk identifies an asymmetric carbon.

during fermentation. In fact, in most commercial beers, the concentration of XN is less than 0.1 mg/L and the concentration of IX is in the range 0.04−3.44 mg/L (*2*, *3*). On the other hand, Wunderlich et al. (*14*) found that the use of dark malts in the brewing process had a positive effect on XN recovery.

Because IX is produced by cyclization of XN, IX has an asymmetric carbon atom. Kofink et al. studied the enantioseparation of IX in standard solution by CE (*22*). CE has been shown to be a very powerful and effective analytical tool for chiral separations in real samples as reviewed previously (*23*−*27*). As compared to HPLC, enantioseparation by CE have the advantages of higher separation efficiency, speed of analysis, and flexibility, allowing the incorporation of various chiral selectors at different concentrations. To our knowledge, the enantiomeric separation of IX in real samples has not previously been studied. The aim of the present study was to develop a method for the chiral separation of IX using hydroxypropyl-γ-cyclodextrin-modified micellar electrokinetic chromatography (HP-γ-CD-modified MEKC). And we also examined the racemization mechanism of IX.

MATERIALS AND METHODS

Chemicals. Sodium dodecyl sulfate (SDS, electrophoresis purity reagent) was obtained from Bio-Rad (Hercules, CA). XN and racemic IX were from Alexis Biochemicals (Lausen, Switzerland). Racemic 8-PN, 2-hydroxypropyl-β-cyclodextrin (2HP-β-CD, average degree of substitution: 7) and heptakis(2,6-di-*O*-methyl)-β-CD were obtained from Sigma (St. Louis, MO). HP-γ-CD (average degree of substitution: 4.2), and HP-α-CD (average degree of substitution: 4.2) were obtained from Aldrich (Milwaukee, WI). γ-CD and other chemicals (analytical grade) were obtained from Wako Pure Chemicals (Osaka, Japan).

Apparatus for CE. Electrophoretic experiments were carried out using a capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany). Samples were injected at a pressure of 50 mbar for 2 s. Separation was performed in a CE/Type S capillary (sulfonated capillary) of 64.5 cm (effective length 56 cm) × 50 μm ϕ (GL Science, Tokyo, Japan). The capillary was kept at 20 °C. The analytes were detected at 210, 295, or 370 nm. The power supply was operated in the constant-voltage mode, at +20 kV, and the substances migrated toward the negative pole. The resulting current was approximately 40 μA.

Apparatus for HPLC. The HPLC system consisted of a Hitachi (Tokyo, Japan) pump model L-6300, a Rheodyne (Cotati, CA) manual injector, a Shimadzu column oven model CTO-10AC, a Shimadzu (Kyoto, Japan) photodiode array detector model SPD-M10AV and a JASCO (Tokyo, Japan) polarimetric detector model OR-990.

Preparation of IX Enantiomers. IX enantiomers were separated by HPLC using a Chiralcel OD-H column (4.6 mm ϕ × 250 mm, Daicel Chemical Industries, Tokyo, Japan) thermostated at 30 °C. Hexane−ethanol (90:10) was used as a mobile phase at a flow rate of 0.8 mL/min. Two fractions, corresponding to the (+)- and (−)-enantiomers of IX, were separately collected. The purities of the (+)- and (−)-IX collected were more than 99.9% enantiomer excess (ee), where ee was defined as the difference between the amount of the two enantiomers in a mixture divided by their total.

Buffer and Sample Preparation for CE. The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 20 mM phosphate buffer (pH 7.0) containing 45 mM HP-γ-CD and 100 mM SDS and was filtered with a 0.22 μm filter before use. Purified water was obtained from a Toray (Mishima, Japan) ultrapure water system. Stock solutions (1000 mg/L) of XN, racemic IX, (+)- and (−)-IX were separately prepared in ethanol, stored at −20 °C and diluted to 100 mg/L before use.

Beer samples (pilsner) were purchased from a local market. Fifteen grams of the beer sample was applied to a Sep-Pak Plus tC18 cartridge (Nihon Waters, Tokyo, Japan). The cartridge was washed with 10 mL of 10% (v/v) ethanol and was dried under vacuum by aspiration. The adsorbed materials were eluted with 5 mL of ethanol. The eluent was centrifuged at approximately 1200*g* for 5 min and the supernatant was obtained. The pellet was reextracted twice with 2 mL of acetonitrile. The supernatants were combined and were added to 63 mL of water and was applied to a Sep-Pak Plus tC18 cartridge again. The cartridge was washed with 10 mL of 20% (v/v) ethanol and was dried under vacuum by aspiration. The adsorbed materials were eluted with 5 mL of ethanol and concentrated to dryness under rotary vacuum evaporation. The resulting residue was dissolved in 0.5 mL of 50% (v/v) ethanol and was analyzed by the above HP-γ-CD-modified MEKC method.

Isomerization of XN in Boiling Water. Fifteen microliters of 600 mg/L XN in 50% (v/v) ethanol was added to 60 μL of purified water, and the mixture was left to stand at 100 °C for 0−120 min. Seventy five microliters of ethanol was added to the samples after the samples were cooled with ice water bath, and then the mixtures were analyzed by the HP-γ-CD-modified MEKC method.

Racemization of IX enantiomer in boiling water. Fifteen microliters of 500 mg/L (+)-IX in 50% (v/v) ethanol was added to 60 μL of purified water, and the mixture was left to stand at 100 °C for 0−120 min. Seventy-five microliters of ethanol was added to the samples after the samples were cooled in an ice water bath, and then the mixtures were analyzed by the HP-γ-CD-modified MEKC method.

Calculation of Resolution. The resolution (*R*s) of an enantiomer was calculated by using the following equation:

$$Rs = 2(t_2 - t_1)/(w_1 + w_2)$$

where t is the migration time and w is the width of the peak at the baseline.

RESULTS AND DISCUSSION

Factors Affecting Chiral Separation. As demonstrated by Terabe et al. (*28*, *29*), the introduction of micelles, which serve as a pseudostationary phase, into the separation electrolyte results in the separation of neutral species along with ionic species. That is, the distribution coefficient of the solute between the pseudostationary phase and the surrounding aqueous phase determines the relative migration order. Chiral compounds have been separated by micellar electrokinetic chromatography (MEKC) by adding cyclodextrin (CD) to the buffer solution as reviewed (*30*−*33*). CDs and their derivatives have been most widely used in CE for the separation of enantiomers of many compounds. The effect of the type of CD on the enantioseparation of IX was investigated by MEKC using a background electrolyte (BGE) containing separately 45 mM HP-α-CD, 2HP-

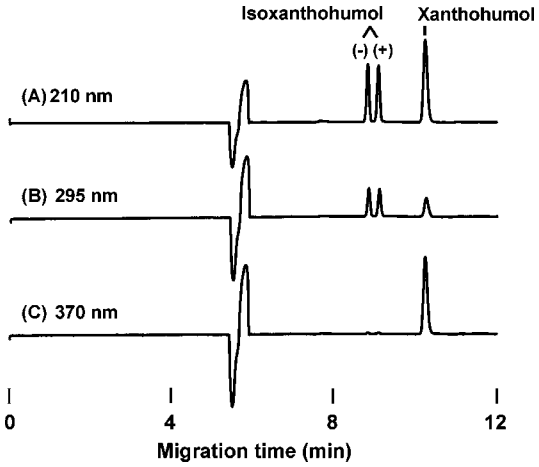

Figure 2. Electropherograms of a standard solution of XN and IX. The running conditions were 20 mM phosphate buffer (pH 7.0) containing 45 mM hydroxypropyl-γ-cyclodextrin and 100 mM sodium dodecyl sulfate with an effective voltage of +20 kV at 20 °C using direct detection at 210, 295, and 370 nm. Standard solution contained 100 mg/L XN and 100 mg/L racemic IX.

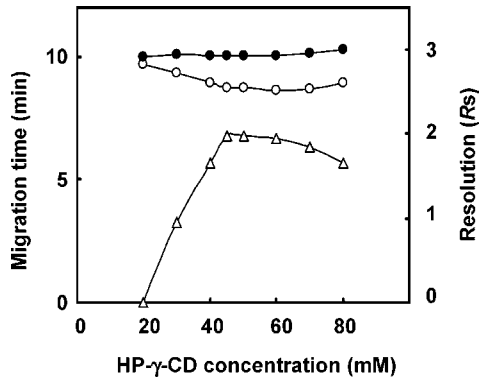

Figure 3. Effect of HP-γ-CD concentration on the resolution and migration time of IX. The BGE was composed of various concentrations of HP-γ-CD containing 100 mM SDS and 20 mM phosphate buffer (pH 7.0): (△) resolution (*Rs*) of IX; (○) migration time of (–)-IX; (●) migration time of XN.

β-CD, 2,6-di-*O*-methyl-β-CD, 6-O-glucosyl-β-CD, 2-hydroxyethyl-β-CD, γ-CD or HP-γ-CD. Of these CDs, only HP-γ-CD was found to effectively resolved IX enantiomers ($Rs = 1.9$) as shown in **Figure 2**. The (−)-isomer moved faster than the (+)-isomer. IX was partially enantioseparated by using a BGE with 2HP-β-CD ($Rs = 0.6$), but not with other CDs. According to Terabe et al. (*34*), in CD-MEKC, an analyte is distributed among three phases, the micellar phase, the CD phase and the aqueous phase excluding the micelles and CD, although the CD phase is not a true phase. As HP-γ-CD is electrically neutral, it migrates at the same velocity as the bulk solution. The distribution of the analyte between the micelles and the nonmicellar aqueous phase including HP-γ-CD directly affects the resolution. HP-γ-CD added to the micellar solution reduced the partitioning of the analyte to the micelles by increasing the fraction of the analyte in the non-micellar aqueous phase. This indicates that the (−)-isomer forms stronger diastereomer complexes with HP-γ-CD than the (+)-isomer.

The migration time of IX enantiomers decreased with increasing HP-γ-CD concentration of the BGE up to 60 mM and then increased gradually (**Figure 3**). The migration time of XN, however, was not affected by the HP-γ-CD concentration. The resolution of IX enantiomers showed a maximum at 45 mM HP-γ-CD. Wren and Rowe (*35*) developed a theoretical model relating mobility to the concentration of a CD selector. They suggested that the maximum resolution can be obtained at the optimum CD concentration, $C_{opt} = (K_A K_B)^{-1/2}$, where K_A and K_B are the formation constants for inclusion complexes of CD with enantiomers A and B, respectively.

Increasing the SDS concentration from 50 to 200 mM increased the migration time of IX as well as XN. The resolution (*Rs*) increased significantly with increasing SDS concentration of the BGE up to 100 mM and then decreased gradually. This result is not attributable to an increase in ionic strength because the electroosmotic flow was not changed significantly over the whole SDS concentration range. The partition of the solute between the CD and the micelles depends on the SDS concentration. This suggests that the longer migration time resulted from an increase in the ratio of the micellar phase to the aqueous-CD phase.

Varying the pH from 6.0 to 8.0 had no effect on the migration times of IX and XN. The resolution of IX was kept constant with increasing pH up to 7.0 and then tended to decrease. This might result in the dissociation of hydroxyl groups of IX at higher pH.

Lowering the capillary temperature from 50 to 20 °C increased the migration times of both XN and IX and increased the resolution (*Rs*) of IX, probably as a result of increasing the buffer viscosity. According to Heuermann and Braschke (*36*), the increase in the *Rs* value with a decrease in temperature might be explained by a decrease in rotational and/or vibrational energy, increasing the fixation of the enantiomers to the inside or to the rim of CD and thus, increasing the enantioselectivity. The longer migration time at lower temperature would increase the chance of interaction between the analyte enantiomers and HP-γ-CD.

Therefore, the optimum BGE conditions, i.e., the conditions giving both high resolution and short migration time, were found to be 45 mM HP-γ-CD and 100 mM SDS in 20 mM phosphate buffer (pH 7.0) with an effective voltage of +20 kV at 20 °C.

Enantioseparation of IX in Beer Samples. XN and racemic IX were subjected to the CE method using the above optimum conditions. XN and IX have maxima in UV spectra around 370 nm and at 295 nm, respectively. The detection limits, defined as a signal-to-noise ratio of 3, at 370 nm for XN and at 295 nm for the IX enantiomers were 0.5 and 1 mg/L, respectively. Linearity ($r^2 > 0.999$) was demonstrated in the range 2−200 mg/L by standard curves (6 points) at 370 nm for XN and was demonstrated in the range 3−200 mg/L by standard curves (5 points) at 295 nm for (+)- and (−)-IX. The reproducibility of five consecutive determinations was evaluated at 20 mg/L for XN and 10 mg/L for (+)- and (−)-IX. Good reproducibilities of peak areas (RSD < 1.8%) and migration times (RSD < 0.32%) were obtained for XN and the (+)- and (−)-IX. When a standard solution (45 μL) containing 100 mg/ L racemic IX and 100 mg/L XN (final concentration: 0.15 mg/L (−)-IX, 0.15 mg/L (+)-IX and 0.3 mg/L XN) was added to a beer sample (15 mL) containing 0.630 mg/L (−)-IX and 0.628 mg/L (+)-IX, recoveries of XN and IX enantiomers were between 97 and 100%. As reported previously (*37*), the sulfonated capillary gave reproducible migration times and rapid analyses.

The (+)- and (−)-IX contents in 12 beer samples, as measured by the proposed CE method, were very similar (**Table 1**), suggesting that IX was racemic in the beer samples. **Figure 4** shows a representative electropherogram of beer sample A. Electropherograms for the other beers were similar. The amount of XN was below the detection limit (0.017 mg/L).

Table 1. Concentrations of (–)- and (+)-IX in Beer Samples

	IX concn (mg/L)		
beer	(–)-IX	(+)-IX	total
A	0.688 ± 0.008[a]	0.699 ± 0.014[a]	1.39
B	0.626 ± 0.006	0.622 ± 0.004	1.24
C	0.527 ± 0.011	0.551 ± 0.007	1.08
D	0.524 ± 0.008	0.529 ± 0.009	1.05
E	0.520 ± 0.004	0.531 ± 0.004	1.05
F	0.507 ± 0.013	0.509 ± 0.008	1.02
G	0.486 ± 0.009	0.503 ± 0.004	0.989
H	0.358 ± 0.010	0.362 ± 0.007	0.720
I	0.356 ± 0.014	0.358 ± 0.019	0.714
J	0.306 ± 0.005	0.306 ± 0.003	0.612
K	0.251 ± 0.005	0.259 ± 0.006	0.510
L	0.077 ± 0.001	0.081 ± 0.003	0.158

[a] Mean ± standard deviation, $n = 3$.

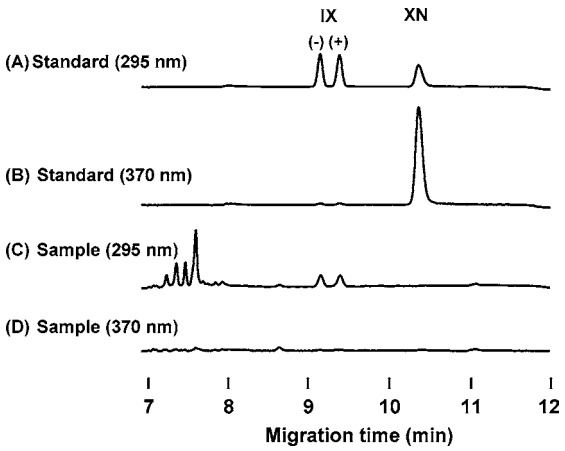

Figure 4. Electropherograms of a representative beer sample. The BGE was composed of 45 mM HP-γ-CD containing 100 mM SDS and 20 mM phosphate buffer (pH 7.0).

Racemization Mechanism of IX. Stevens et al. (*16*) studied the conversion of XN into IX in boiling water containing 10% sucrose, where XN was fully dissolved, by using a HPLC/MS analysis. They found that the decrease of XN was in very good balance with the formation of IX in boiling water and the half-life of xanthohumol was 30 min. We studied the enantioseparation of IX, which was produced from XN in boiling water (**Figure 5**). The decrease in concentration of XN correlated well with the increase in that of IX, which was a racemic mixture. In this experiment, the half-life of XN was approximately 70 min, which was significantly longer than that reported by Stevens et al. (*16*). Because the sensitivity of the proposed MEKC method was significantly lower than that of HPLC/MS method, our experiment was carried out at a concentration of 120 mg/L XN in water where XN was not dissolved. This suggests that the longer half-life of XN was due to its not being fully dissolved.

Conversion of (+)-IX enantiomer to (−)-IX enantiomer was also studied. As shown in **Figure 6**, (+)-IX decreased with boiling time and the decrease correlated well with the increase of (−)-IX. Conversion of (−)-IX enantiomer to (+)-IX enantiomer, like that of (+)-IX enantiomer to (−)-IX enantiomer, was also observed. Caccamese et al. (*38*) found that naringin, a flavanone glycoside of grapefruit, was practically all in the form of the (*2S*)-epimer in small grapefruit (diameter 1.5 cm) but became nearly completely racemic in mature grapefruit. Uchiyama et al. (*39*) reported that (*2S*)-hesperidine, another flavanone glycoside, was easily racemized under boiling conditions. These racemizations have seemed to occur via ring opening. However, in **Figure 6**, XN was not detected even though the conversion of XN to IX was slow. This suggests that the IX enantiomers were easily interconverted and that the conversion occurred through any intermediates, not XN.

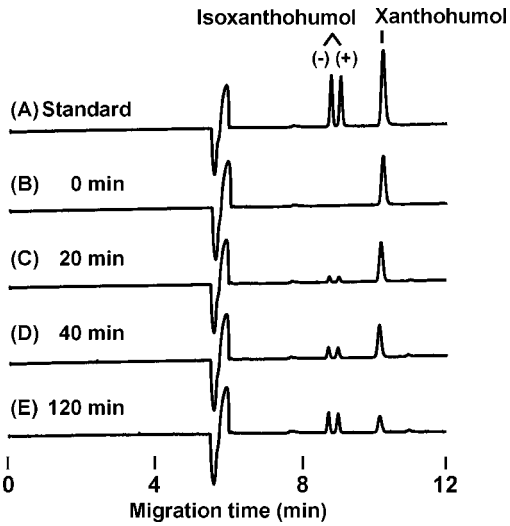

Figure 5. Isomerization of XN to IX in boiling water. XN (120 mg/L, 75 μL) in 10% ethnol solution (v/v) in each vial was heated at 100 °C for 0–120 min. Seventy five microliters of ethanol was added to the sample vials after cooling the vials, and then the mixtures were analyzed by the HP-γ-CD-modified MEKC method with detection at 210 nm.

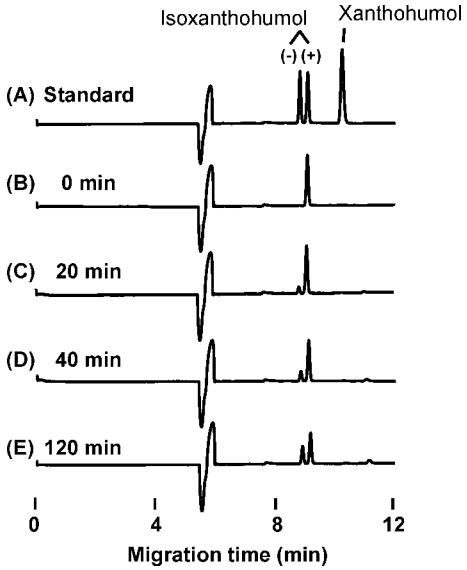

Figure 6. Conversion of (+)-IX to (–)-IX in boiling water. (+)-IX (100 mg/L, 75 μL) in 10% ethnol solution (v/v) in each vial was heated at 100 °C for 0–120 min. Seventy five microliters of ethanol was added to the sample vials after cooling the vials, and then the mixtures were analyzed by the HP-γ-CD-modified MEKC method with detection at 210 nm.

Schaefer et al. (*9*) found that (*2S*)-8-PN exhibited an overall higher affinity for both estrogen receptors α and β than did (*2R*)-8-PN. IX is converted to 8-PN by intestinal microbiota (*12*) and human liver microsomes (*6*, *13*). Further studies are needed to clarify the chiral selectivity of this conversion.

In conclusion, a HP-γ-CD-modified MEKC for the enantioseparation of IX was developed. The concentration of IX in beer samples ranged from 0.15 to 1.4 mg/L. But XN was not detected (less than 0.017 mg/L). The levels of (−)- and (+)-IX were almost the same, suggesting that IX was racemic in these

beer samples. The racemic mixture of IX in beers could be attributed to two facts: first, the racemic mixture of IX was produced by XN during boiling, and second, the IX enantiomers were easily interconverted.

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