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

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Summary

Hop (*Humulus lupulus* L.) is a climbing herbaceous plant belonging to the family of the Cannabaceae. The female cones of hop are added to beer, providing taste and flavour and contributing to the stability of foam (Verzele and De Keukeleire, 1991; Hughes, 2000; Bamforth, 1985). The main constituents of hop related to these properties are generically known as α-acids. The major ones are humulone, cohumulone and adhumulone. During the brewing process, these acids are isomerized, resulting in the formation of three pairs of *trans*-/*cis*-iso-α-acids, (*trans*-/*cis*-isocohumulone, *trans*-/*cis*-isohumulone, and *trans*-/*cis*-isoadhumulone).

The aim of this thesis project was to develop a method for the analysis (**Chapter 2** and **7**) as well as the purification of iso-α-acids (**Chapter 3-5**). Moreover, the stability of iso-α-acids need to be improved (**Chapter 6**) since these compounds degrade easily in the light and in the presence of oxygen.

Two different methods of purification were explored in this project. The first method (**Chapter 3**) was developed to produce an isomerized hop extract free of *trans*-iso-α-acids. This extract contained only the mixture of *cis*-isocohumulone, *cis*-isohumulone, and *cis*-isoadhumulone. The *trans*-isomers need to be separated from its *cis*-counterparts since the *trans*-isomers were reported to be more prone to oxidation than their *cis*-counterparts, therefore they are less stable. The *trans*-isomers are also reported to be less bitter than the *cis*-counterparts. This extract could find applications in beer brewing to increase beer stability. As discussed in **Chapter 3**, a method to separate *cis*-iso-α-acids mixtures from their *trans*-isomers was developed using β-cyclodextrin (β-CD). It was found that 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-CD to the volume of water is 1:8, and the molar ratio between total amount of iso-α-acids sample to β-CD 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 uncomplexed β-CD. 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-CD is 4:1. The *trans*-isomers can finally be released from the β-CD complex by methanol elution. This method is promising for large scale production of the stereoisomers.

The second purification method (**Chapter 4**) was developed to produce the pure individual iso-α-acids. The pure individual iso-α-acids are required as standard compounds for analysis and also needed in brewery research when studying the parameters which affect the quality of beer. Extensive studies on the properties of the different iso-α-acids are hampered by the fact that pure compounds are difficult to obtain because the existing isolation methods are not capable to meet the demands qualitatively nor quantitatively. The isolation using preparative HPLC is time consuming and expensive. Other isolation methods using photo isomerization and DCHA salts only yield the *trans*-isomers. Therefore, there is a need to find an efficient large scale separation method which can produce high quality standards of individual iso-α-acids. To overcome this problem we used centrifugal partition chromatography (CPC) combined with β-CD complexation.

Herman-Lokkerbol and Verpoorte (1994b) succeeded in isolating pure individual α-acids from crude supercritical carbon dioxide hop extracts by means of CPC. This method reduces the costs of the isolation of α-acids by consuming lower amounts of solvent and requiring relatively less time in comparison with preparative LC. The individual α–acids thus isolated can be isomerized to yield the pure *trans*-/*cis*-iso-α-acids pair. The remaining problem to produce pure individual iso-α-acid is the separation of the *trans*- and *cis*- isomer of each pair. β-CD can be used for this purpose (**Chapter 4**). Complete separation of *trans*- from *cis*-iso-α-acids was successfully performed by β-CD using ethanol/water (1:2) as solvent. Temperature was maintained at 50 ^{o}C during 30 minutes for complexation. The molar ratio of iso-α-acids / β-CD for complexation is 1:1 and the time required after complexation for precipitation varied between 9 hours and 2 days depending on the type of iso-α-acid. Release of the guest from the cyclodextrin complex was successfully accomplished by elution with methanol. The purity of the isolated compounds is above 95% by HPLC and the recovery is around 50%, which might be improved by further optimization of the process. The confirmation of the chemical structures of the isolated compounds was performed by ^{1}H- , ^{13}C-, and two-dimensional NMR (COSY, HSQC, HMBC) together with electrospray ionisation mass spectrometry spectra as discussed in **Chapter 5**.

Another goal of this project is to develop an analytical method for qualitative research on iso-α-acids using HPLC and NMR. In **Chapter 2**, the HPLC system is

developed to be applied when pure iso-α-acids are used as reference standards. Previously, the HPLC methods used only dicyclohexylamine (DCHA) salt of the *trans*- iso-α-acids complex as the standard instead of pure *trans*-isomers. In order to get the pure iso-α-acids, we have developed a simple method for the isolation of individual iso-α-acids as discussed in **Chapter 4**. This method allowed us to use both *trans*- and *cis*-isomers in free form instead of in DCHA salts complex for HPLC analysis. High resolution of all 6 isomers was achieved with a total run time of 25 minutes. The UV spectra of these pure compounds using this system are quite different. This behavior may have implications for the quantitative iso-α-acids determinations by means of LC-UV and allows to recognize different individual iso-α-acids.

Besides iso-α-acids, there are a lot of compounds in beer that influence quality. Therefore there is a need for a broad-spectrum analytical method that allows for the determination of a wide array of compounds in beer including iso-α-acids. Some analytical methods using HPLC, GC and capillary electrophoresis are only able to analyze a certain groups of compounds. There is also a possibility that some compounds are retained in the column. To overcome this problem NMR spectrometry was applied. The quantitative information provided by NMR is better than that provided by all other methods mentioned as it is the only one in which the intensity of the signals is directly proportional to the molar concentration. It means that the relative amount of all compounds can be directly compared, eliminating the need for calibration curves for each individual compound. However, despite the undoubted attraction of NMR, the complexity of the ^{1}H NMR spectra is a drawback for its use in the analysis of beer. The complexity of the resulting spectra makes any attempt to identify the components difficult. However, we are able to overcome this problem by two dimensional *J*-resolved NMR spectrometry (**Chapter 7**). In the J-resolved spectra the spin – spin couplings are shown along the second axis. These additional variables largely improve the resolution of the NMR spectrum of a mixture and therefore the overlapping signals in ^{1}H NMR spectra were fully resolved. It was successfully applied to the analysis of 2-butanol extracts of beer. Principal component analysis based on the projected *J*-resolved NMR spectra showed a clear separation between all of the six brands of pilsner beer evaluated in this study. The compounds responsible for the differentiation were identified as nucleic acid derivatives (adenine, uridine and

xanthine), amino acids (tyrosine and proline), organic acids (succinic and lactic acid), alcohols (tyrosol and isopropanol), cholines and carbohydrates. Unfortunately, the presence of iso-α-acids can not be detected by this method. Further study is required to optimize the method for the detection of the iso-α-acids.

Iso-α-acids are very unstable compounds and their degradation products are thought to be partially responsible for the off-flavour characteristic of ageing beer including stale and cardboard flavours which are connected with their oxidative degradation. Complexation of iso-α-acids to β-CD was conducted in order to improve the stability of iso-α-acids (**Chapter 6**). The formation of iso-α-acids -β-CD complex was studied by analysing the shift pattern of NMR spectra of treated and untreated compounds. The NMR spectrum were only weakly shifted implying that no inclusion of iso-α-acids into the cavity of β-CD occurred. This was also confirmed by NOESY NMR. No NOE interaction between the protons of the guest and β-CD could be observed. Most likely iso-α-acids are bound non-covalently to the outer surface of β-CD with a relatively large distance between protons of guest and β-CD.

The stability of dry β– CD complexes of individual iso-α-acids improved dramatically if compared to that of uncomplexed and dry iso-α-acids. The stability of dry β– CD complex is also better to that of iso-α-acids in chloroform, ethanol or methanol solutions. Dry β–CD complex can apparently protect the compounds from oxidation and light. However, the stability of the β–CD complex of individual iso-α-acids in water/ethanol was poor. The explanation of this phenomenon is still unclear. One assumption is that oxygen dispersed in solution is in direct contact with the iso-α-acids and causes degradation, because iso-α-acids are not protected in the cavity, but bind to the outer surface of β–CD. In the dry complex only few iso-α-acids molecules are in direct contact with the air because most are trapped in the solid β–CD complex particles.

Summarizing the conclusions, we have shown that β-CD is effective to separate *cis*-isomers from *trans*-isomers and at the same time increases the stability of the complexed compounds. Separation of total *cis*-isomers from *trans*-isomers using β-CD is promising for a large scale production of a high quality hop product that can be applied to obtain a more stable beer.

The pure individual iso-α-acids can now be produced from pure α-acids, obtained from a CPC separation, followed by isomerization and β-CD separation of the two isomers. These compounds can be used as reference standard for analytical purposes to replace the DCHA-salts of *trans*-iso-α-acids. It also opens the possibility to use pure iso-α-acids in brewery research, since it is easy to isolate these compounds on a preparative scale. An HPLC system using these pure individual iso-α-acids as calibration standard was developed as well.

The application of 2D J resolved NMR has been developed and seems promising to detect a wide range of compounds in beer although it can not detect iso-α-acids. Therefore the optimization of the iso-α-acids detection is required.