THERMAL CHARACTERIZATION OF HOP EXTRACT BY DSC AND HPLC

Miha OCVIRK[12], Polona MEGUŠAR[13], Nataša POKLAR ULRIH[14] and Iztok PRISLAN[15]

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Abstract
One of the main chemical reaction in beer brewing is isomerization reaction of hop α-acids, and these compounds are responsible for the beer bitterness and its characteristic flavour. With its complex chemistry, hop has been the subject of deepened investigations for decades. The aim of this study was to use the differential scanning calorimetry as an alternative technique which can give use further information on thermal behaviour of hop extracts during brewing.
Key words: hop, *Humulus Lupulus* L., differential scanning calorimetry, high pressure liquid chromatography, isomerization

TERMIČNA KARAKTERIZACIJA HMELJNIH EXTRAKTOV Z DSC IN HPLC

Izvleček
Ena izmed najpomembnejših kemijskih reakcij v pivovarstvu je izomerizacija hmeljnih α-kislin v izo-α-kisline. Pivu dajejo značilno grenčico in okus. Hmelj je zaradi svoje kompleksne sestave in številnih kemijskih reakcij med komponentami že desetletja predmet poglobljenih raziskav z namenom razumevanja njegovega obnašanja med varjenjem piva. Namen raziskave je bil z uporabo diferencialne dinamične kalorimetrije in tekočinske kromatografije pridobiti dodatne informacije o obnašanju hmeljnih ekstraktov med simuliranjem procesa varjenja piva.
Ključne besede: hmelj, *Humulus lupulus* L., diferencialna dinamična kalorimetrija, tekočinska kromatografija, Izomerizacija

[12] B.Sc., Institute of Hop Research and Brewing, Cesta Žalskega tabora 2, 3310 Žalec, Slovenia, e-mail: miha.ocvirk@ihps.si
[13] M. Sc., Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, e-mail: polona.megusar@bf.uni-lj.si
[14] Prof., PhD., Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, e-mail: natasa.poklar@bf.uni-lj.si
[15] Assist. Prof., PhD., Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, e-mail: iztok.prislan@bf.uni-lj.si

1 INTRODUCTION

Hops, the inflorescences of the female hop plant (*Humulus lupulus* L.), are a very valuable raw material applied in many industrial branches. Even though they have been reported to have many health benefits (Yayima et al., 2004; Nozawa et al., 2005; Van Cleemput et al., 2009; Colgate et al., 2007, Morimoto-Kobayashi et al., 2016), the most frequent industrial application is still found in the brewing industry. During the brewing process α-acids in hops are converted by isomerization into iso-α-acids as the major bitter components in beer (Steenackers et al., 2015). Instead of adding dried hops directly to the kettle during boiling, hop extracts are used in the modern brewing process which increases the level of utilization of α-acids and isomerization into iso-α-acids (Kostrzewa et al., 2016). The growing importance of hop extracts has generated an increasing interest in improving the quality control of hop extracts by brewing industry.

Different analytical methods are used to characterize compounds from hops, their products, and extracts. High pressure liquid chromatography (HPLC) is traditionally used to analyze hop extracts, especially to determine the amount of α- and β-acids (Ocvirk et al., 2016). Because hop extracts undergo temperature changes during brewing process, it is essential to gain insight into their thermal behavior. Differential scanning calorimetry (DSC) is commonly used to investigate thermal processes of different materials, including material of plant origin (Fernandes et al., 2013; Martinez et al., 1998). DSC allows us to identify glass transition temperatures, investigate the phase transitions, follow crystallization processes and calculate enthalpy changes during thermally induced processes.

The aim of this study was to investigate basic thermodynamic properties of supercritical carbon dioxide hop extracts using DSC, particularly to identify phase transitions. HPLC on the other hand was used as a testing analytical technique to investigate potential isomerization reactions.

2 EXPERIMENTAL

2.1 Materials

The experiments were conducted using a six different supercritical CO_2 hop extract from the different hop cultivars produced by Nateco2 (Germany). The extract 1 – ICE3, contained 44.6 % of α-acids and 24.3 % of β-acids. The extract 2 – AHA1, contained 54.5 % of α-acids and 17.2 % of β-acids. The extract 3 – AHA2, contained 27.8 % of α-acids and 8.7 % of β-acids. The extract 4 ICE4#1, contained 48.8 % of α-acids and 24.9 % of β-acids. The extract 5 – ICE4#2, contained 41.9 % of α-acids and 25.8 % of β-acids. The extract 6 – ICE4#3 contained 42.1 % of α-acids and 27.0 % of β-acids.

Extracts 1, 2 and 3 were prepared from the variety Herkules, extract 4 was prepared from the variety Hallertauer Magnum, extract 5 was prepared from the variety Northern Brewer and extract 6 was prepared from the variety Perle.

2.2 Apparatus and procedures

Differential Scanning Calorimetry. DSC measurements were performed with TA Instruments differential scanning calorimeter, model DSC 2500 (TA Instruments, UK) and the data were evaluated using TA instruments TRIOS software. Temperature and cell constant calibration of the instrument were done with Indium reference samples and specific heat capacity - Cp calibration was done with sapphire crystal, provided by TA Instruments. Samples ($m \approx 10$ mg) were placed in hermetically sealed Tzero Aluminium pans and identical empty pan was used as a reference.

Different heating/cooling cycles were used to analyze samples. Hop 1-6 were first cooled to 0 °C, heated to 95 °C, cooled to 0 °C and heated to 95 °C again at rate of 5 °C min^{-1}. Three samples of hop extract ICE3 were prepared and heated/cooled at heating rates of 1, 5 and 10 °C min^{-1}. The observed heat effects were characterized by subtracting baseline, calculating the change in enthalpy as the area under experimental curve and determining transition temperature as the curve peak position.

High pressure liquid chromatography. With intent to determine α-acids and iso-α-acids in extracts, high pressure liquid chromatography was performed with an Agilent 1200 HPLC (Agilent technologies, USA). Prior the analysis, samples were temperate at room temperature and homogenized. The exact amount of 0.5 g was weighted into 50 mL volumetric flask and dissolved in HPLC grade methanol (J.T. Baker, USA) using sonication for 30 s in an ultrasonic bath. 5 mL of the stock solution was pipetted into a 50 mL volumetric flask and diluted up to volume with methanol. The sample solution was than filtered through a 0.45 µm PET filters into 2 mL glass vials. Injection was made on an auto sampler, using methanol, water and ortophosphoric acid (Sigma Aldrich, Germany) as a mobile phase. Separation was performed on a 150 mm long Nucleodur C18 Column (Macherey Nagel, Germany), according to the Analytica EBC method 9.47 (European Brewery Convention, 2010). Iso-α-acids were measured at a wavelength of 270 nm, while α-acids were measured at 314 nm.

3 RESULTS AND DISCUSSION

3.1 Differential Scanning Calorimetry

DSC curves obtained for different hop extracts are shown in Figure 1. Upon first heating all samples undergo several endothermic transitions between 20 °C and 70 °C. Analyzed hop extracts are a condensed form of α-acids, β-acids, fatty acids and other resins found in hops. Even though hop extracts consist of around 70 % of α- and β-acids, the distribution and intensity of peaks do not reflect α- and β-acid composition/content. Therefore the observed endothermic transitions could correspond to thermal behavior of oils and resins. Since resins do not undergo a phase change at temperatures below 100 °C (Bisanda et al., 2003, Walter et al., 1966) it is our strong belief that observed transitions correspond to thermal behavior of fatty acids. Results show that changing the parameters of supercritical CO_2 extraction process influences not only α- and β-acid composition/content but also fatty acid composition/content in hop extracts.

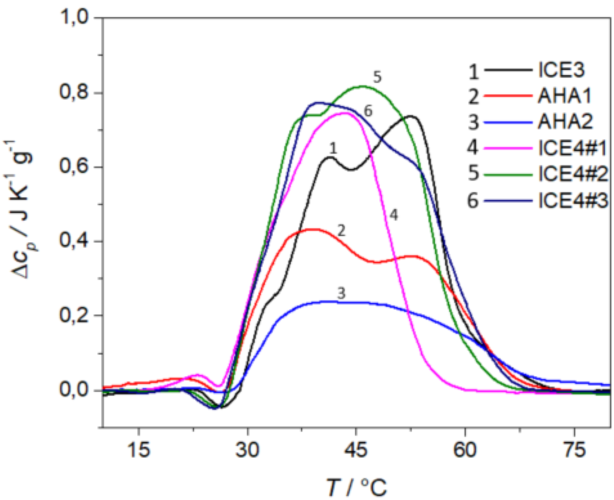

Figure 1*: Thermally induced changes in heat capacity of hop extracts 1- 6 during the first heating. Samples were first cooled to -20 °C and then heated to 95 °C at 5 °C min*$^{-1}$

To further investigate thermal behavior of hop extracts, two cooling and another two heating runs were performed for all samples. Figure 2 shows the results of just first cooling and second heating runs since the shapes of second cooling and third heating thermograms remained unchanged (data not shown). Cooling curves exhibit two exothermic transitions at around 44 °C and 27 °C whereas second heating thermograms show two endothermic transitions at around 50 °C and 30 °C.

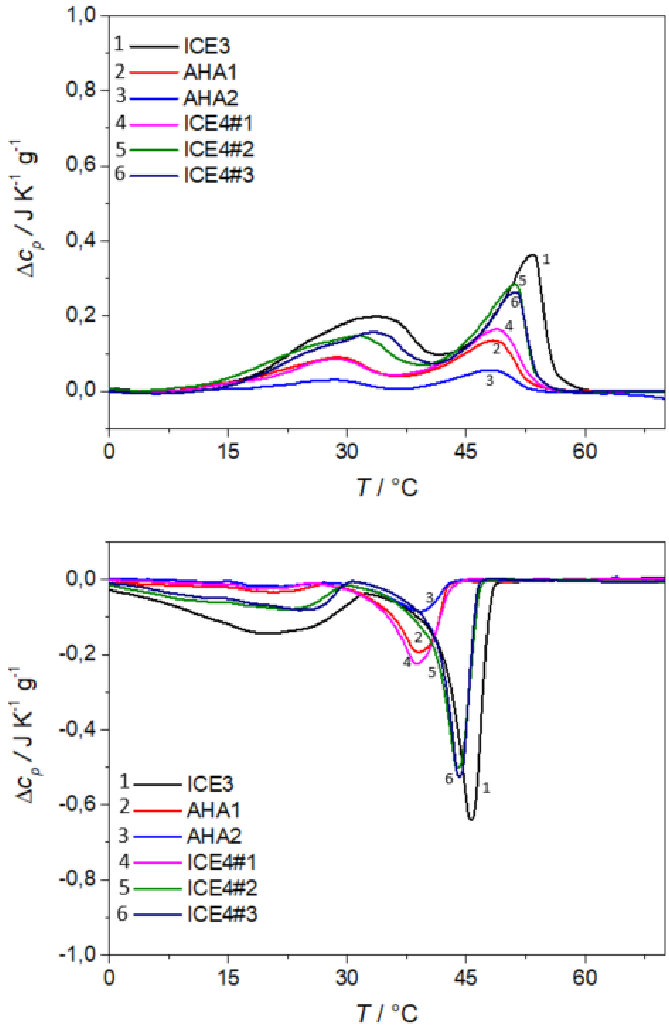

***Figure 2**: Thermally induced changes in heat capacity of hop extracts 1 - 6 during cooling (top) and second heating (bottom). After heating the samples to 95 °C (Figure 1), the samples were cooled to -20 °C at 5 °C min^{-1} (top) and then heated to 95 °C at 5 °C min^{-1} (bottom).*

Several differences can be observed when comparing thermograms of first and second heating thermograms of the same sample. All second heating runs show two transitions. First transition with a broad and endothermic peak occurs at lower (≈30 °C) temperatures while the second one with a sharper peak occurs at higher temperature (≈ 50 °C). The number of transitions, corresponding changes in heat capacity and onset temperatures are lower than in the first heating runs. Because the transitions in the first heating run differ from transitions in the second heating run and the third heating run is the same as second, we may conclude that thermal transitions observed during the first heating runs are not reversible whereas thermal transitions observed during the second and subsequent heating runs are reversible. It has been shown that solid-liquid transition for fatty acids and size of dispersed particles depend on parameters during supercritical CO_2 extraction (Kokot and Knez, 1999). We speculate that thermal transitions observed in the first heating run are result of phase transitions/particle formation during supercritical CO_2 extraction whereas thermal transitions observed during cooling/second heating curves correspond to solidification/melting of fatty acids.

The transition enthalpies accompanying all observed transitions from heating and cooling experiments were calculated and the results are shown in Table 1. Transition enthalpies corresponding to the first heating scan, $\Delta H_{i,\mathrm{h1}}$, are much higher that transition enthalpies corresponding to the second heating scan, $\Delta H_{i,\mathrm{h2}}$. Furthermore, the extracts ICE3, AHA1 and AHA2 which were obtained from the same variety of hops yielded significantly different transition enthalpies. This confirms our observations that parameters of supercritical CO_2 extraction influence thermal behavior of hop extracts. Similarly to the observed discrepancies in peak temperatures (Figure 2), transition enthalpies corresponding to cooling scan, $\Delta H_{i,\mathrm{c}}$**,** are not the same as $\Delta H_{i,\mathrm{h2}}$. These observations may indicate that solidification and melting of fatty acids is kinetically governed process (Desgupta et at., 2016).

Table 1*: Calculated enthalpies of observed thermally induced changes in hop extracts. Three enthalpies were calculated for each sample corresponding to first melting curve, cooling curve and second melting curve*

i	**Sample**	**$\Delta H_{i,\mathrm{h1}}$ (1st heating) / J g^{-1}**	**$\Delta H_{i,\mathrm{c}}$ (cooling) / J g^{-1}**	**$\Delta H_{i,\mathrm{h2}}$ (2nd heating) / J g^{-1}**
1	ICE3	15,7±0,2	-6,4±0,2	6,5±0,2
2	AHA1	11,6±0,2	-2,0±0,2	2,8±0,2
3	AHA2	7,2±0,2	-0,9±0,2	0,6±0,2
4	ICE4#1	13,4±0,2	-1,9±0,2	2,9±0,2
5	ICE4#2	18,7±0,2	-4,2±0,2	4,9±0,2
6	ICE4#3	18,5±0,2	-3,7±0,2	4,5±0,2

3.2 HPLC

HPLC was used to determine the exact α- and β-acids contents in hop extracts before and after heating. Chromatograms in Figure 1 show that heating has only minor impact on α- and β-acids levels. After first cycle of heating and cooling, relative % of α-acids was decreased from 44.46 to 42.39 %, while the relative % of β-acids was decreased from 24.28 to 22.07 %. The final relative % of α and β-acids in investigated extract was 41.82 % and 20.69 % (Table 2).

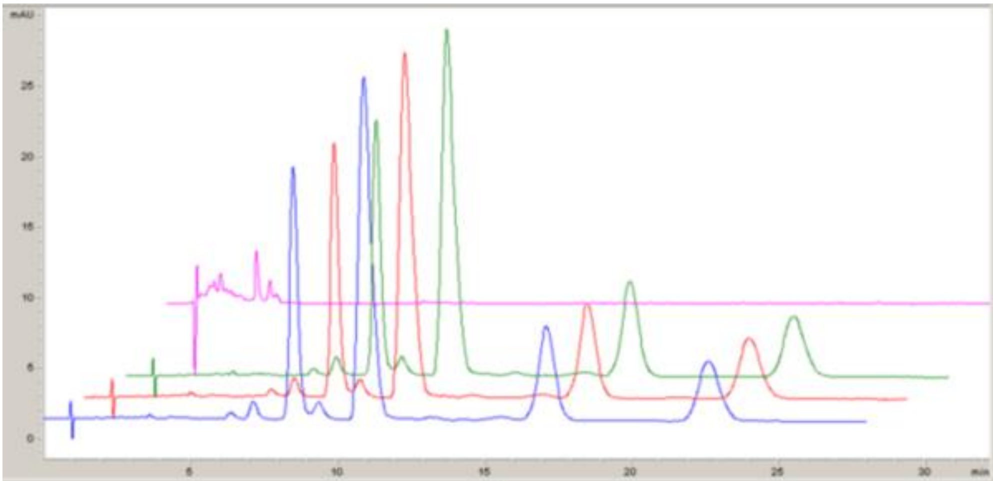

***Figure 3**: HPLC chromatogram of hop extract 1 at the wavelength of 314 nm before heating, after the first and after the second heating to 95°C. The samples were cooled to 20 °C at 5 °C min^{-1} and then heated to 95 °C at 5 °C min^{-1}. Flat line represents the chromatogram of distilled water after heating as a blank*

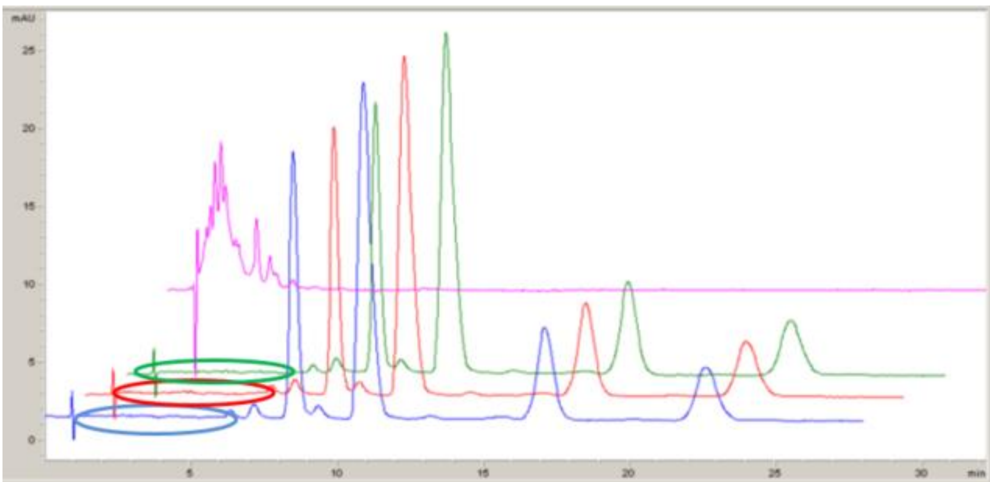

***Figure 4**: HPLC chromatogram of hop extract 1 at the wavelength of 270 nm before heating, after the first and after the second heating to 95°C. The samples were cooled to 20 °C at 5 °C min^{-1} and then heated to 95 °C at 5 °C min^{-1}. Flat line represents the chromatogram of distilled water after heating as a blank*

After processing the chromatograms for determination of iso-α-acids and iso-β-acids, we discovered, that in all three samples, isomerized products of α-acids and β-acids were not detected – marks on Fig.2 (LOD =0,001 mg/L).

***Table 2**: Relative % of α- and β-acids in the hop extract 1 before and after heating*

Extract 1	Rel. %	Rel. % after first heating	Rel. % after second heating
α-acids	44.64	42.39	41.82
cohumulone	13.88	13.44	12.90
n+adhhumulone	30.76	28.95	28.92
β-acids	24.28	22.07	20.69
colupulone	13.44	12.04	11.52
n+adlupulone	10.84	10.03	9.17

4 CONCLUSIONS

We have followed thermally induced transition of different hop extract. Even though hop extracts consist of around 70 % of α-acids and β-acids, it has been shown by HPLC analysis that α-acids and β-acids are not responsible for observed thermal transitions. We believe that observed thermally induced transitions correspond to solidification/melting of fatty acids and that this is kinetically governed process. Further analyses are required to confirm this.

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