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Efficient Formation of Iso-Humulones in Aqueous Hop Solutions at Low Temperatures

In this work, new cognitions on the influence of common parameters for the isomerization of humulones, such as the temperature, pH, storage time, the concentration of humulones, microbial growth and pressure in especially prepared aqueous hop solutions were observed. The results provided an important background for future works regarding the enrichment of humulones and iso-humulones from aqueous solutions by using foam fractionation, in order to produce isomerized and non-isomerized marketable hop products. At low pHs between 2 and 3, higher isomerization rates were observed. Isomerization took place already at low temperatures between 8 and 10 °C, which previously has not been observed. At higher pHs, sufficient isomerization was also induced, but after a duration of ca. 72 hours. Higher isomerization rates occurred at longer storage duration of up to, e.g., 240 hours. Regarding the concentration of humulones in the hop solutions, it was found that the ratio of solved humulones to the formation of iso-humulones remained nearly the same (mostly 50 %, but at least 25 %). The ratio observed over a period of 240 hours continued almost unchanged, except for 144 hours (more than 75 %). Isomerization under the applied conditions took place at normal pressure. A relationship between the concentration of humulones and iso-humulones towards microbial growth could not be established, because microorganisms grew in aqueous hop solutions spiked with a disinfectant, as well as in those without disinfectant.

Descriptors: isomerization parameters, humulones, iso-humulones, aqueous hop solution, extraction

1 Introduction

The nutritious hop ingredients, used in the pharmaceutical and beverage industry, are classified into bitter acids, as well as flavouring and tanning agents. For the pharmaceutical industry, several hop bitter acids are of importance due to their sedative effect on humans [1, 2]. In the beverage industry, hop bitter acids and flavouring agents are mainly used to influence the sensory quality of beer and can be found in the lupulin glands of the hop cone. The tanning agents, playing a decisive role in the shelf-life and flavour-stability of beer [3, 4], are located mainly in the bracts and bracteoles but also in the lupulin. They can also be detected in low amounts in the shaft and stipe [5]. Hop bitter acids are classified into alpha-, beta-, delta and gamma-acids. Alpha-acids, also known as humulones, are the main bitter substances, and beta-acids are the lupulones. As an important physico-chemical characteristic, the humulones are severe water-soluble, whereas the lupulones are not [2].

The isomerization of humulones is mainly responsible for generating the bitter taste of beer. The process takes place during the production of beer and/or hop extracts and depends on several parameters, such as time, pressure, temperature and pH-value. It is, therefore, of importance to take these parameters into careful consideration during beer and hop extract production, as they influence the quality of such products. For example, a longer period of wort boiling and thereby a prolonged influence of temperature increases the isomerization rate. At pH-values higher than those of wort (pH 4.75 ± 0.50), the humulones convert increasingly to iso-humulones. Other influencing factors such as the concentration of tanning agents in wort should be mentioned, but are discussed in details elsewhere [6].

The advantages by using isomerized hop extracts are a shorter wort boiling time and enhanced control over the bitter flavouring of beer [6]. The isomerization during the production of hop extracts can be controlled by extraction with carbon dioxide, taking place at pressures between 60–65 bar and at temperatures of 5 to 15 °C. By staying within these parameter values, the humulones can be extracted. The production of hop extracts becomes more flexible when supercritical carbon dioxide is used ($p \geq 73$ bar; $T > 31$ °C). Isomerized hop pellets are produced by adding magnesium hydroxide in order to catalyse isomerization at higher temperatures (between 45 and 55 °C) during the storage for 10 to 14 days [7].

Besides of methods commonly used for the production of hop extracts (solvent or supercritical fluid extraction), foam fractionation can be applied as an alternative method. The method has advantages such as lower demands on equipments and personnel, and the omission of solvents. Therefore, it can be regarded as more economic and sustainable, especially in comparison to the established methods. By inducing gas into an aqueous solution, bubbles are produced, on which surface active substances contained in the initial solution get concentrated. Thereby, numerous of natural substances and useful proteins could already be successfully enriched, such as carnosic acid from rosemary extract, flavokavins A and B from Kava Kava, curcuminoids from curcuma, or undesirable solanidine alkaloids from potato juice [8–16].

This work deals with new data of isomerization parameters in aqueous hop solutions, in particular with regard to pH, storage

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time, and temperature. Other parameters, such as the concentration of humulones and pressure, are also discussed for their influence on the isomerization process, as well as the effect of dissolved humulones and iso-humulones on microbial growth. The hop solutions which contained the bitter substances in either solid or dissolved form were prepared for the subsequent use of foam fractionation with the purpose to enrich and to extract the nutritious ingredients. The results of the foam fractionation trials are the subject of a following publication. It should be noted that breweries which brew in compliance with the German purity law are not allowed to use isomerized hop products. However, hop extracts produced from aqueous solutions containing isomerized humulones are of interest for breweries not abiding the German purity law, because of their economic value.

2 Materials and Methods

2.1 Hop samples

Hop cones used for the preparation of aqueous hop solutions were vacuum-packed and obtained from HVG Hopfenverwertungsgenossenschaft e.G. (Wolnzach, Germany). Their humulone content was between 6 and 8% in average. The hop standards were purchased from Labor Veritas (Zurich, Switzerland). The calibration extract (standard) assigned with ICE-2 contained 14.45 % of co-humulone, 34.94% of n- and ad-humulone (in total: 49.39 % humulones), 12.92 % of co-lupulone and 12.02 % of n- and ad-lupulone (in total: 24.94 %). The calibration extract assigned with DCHA-Iso, ICS-I2, contained 64.3 % of iso-humulones.

2.2 Analyses by High Performance Liquid Chromatography (HPLC)

Before measuring humulones and iso-humulones by HPLC, the samples taken from the hop solution had to be prepared. The liquid hop samples were measured according to EBC method 7.8 under slightly modified conditions. It was, for example, renounced to apply 40 ml of 0.1 N hydrochloric acid when the samples were already acidulated, and also to dilute the supernatant (2 ml) with methanol [17, 18]. HPLC conditions were as follows: Nucleodur column, 5–100 C18 cc, 125 × 4 mm, by Macherey-Nagel GmbH & Co KG (Düren, Germany). The flow rate was adjusted to 1.4 ml/min by a preparative pump (Gynkotek Model 480 – Germering, Germany). The temperature was at 25 °C. Iso-humulones were detected at 270 nm (UV Detector LDC/Milton Roy, Riviera Beach, Florida, USA) and the humulones and lupulones at 314 nm (UV Detector Merck/Hitachi, Darmstadt, Germany) [17, 18]. As eluent A, methanol (gradient grade, Roth, Karlsruhe, Germany) was used. Eluent B was a mixture of methanol (750 ml), bidistilled water (240 ml), and o-phosphoric acid (10 ml, 85 % purity, Roth, Karlsruhe, Germany). The following gradient for the eluent B was applied: 0–12 min (100 %), 270 nm; 12–25 min (100 %), 314 nm; 25–40 min (65 %), 314 nm; 40–44 min (100 %), 314 nm. The measurements were repeated three times to obtain the standard mean value.

2.3 pH-value and temperature

The pH, measured using a pH meter, was adjusted by 0.1 M hydrochloric acid (Roth, Karlsruhe, Germany). A standard thermometer was used to determine the temperature.

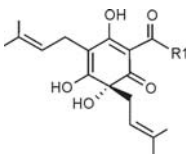
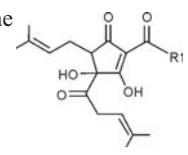
2.4 Preparation of aqueous hop solutions

For the subsequent use of foam fractionation, the aqueous hop solutions (assigned in following with HS) were prepared as follows: Defined amounts of hop cones (a) were added to defined volumes of tap water (b). The ratio a/b varied between 1 and 10 % (w/w). To evaluate the pH-dependency, the pH was adjusted between 2.0 ± 0.2 and 6.0 ± 0.2. The samples were stored at 10.0 ± 2.0 °C for up to 600.0 hours (in the dark at normal pressure). The hop solutions were daily stirred and periodically measured. The microbial growth was measured according to the MEBAK method [19]. Hop solutions were produced without the addition of additives, except for the influential investigation of the concentration of humulones and iso-humulones on the microbial growth, for which mono iodide acetic acid (0.05 % v/v) was added as disinfectant. The hop solutions were prepared at least in triplicates under the same conditions.

3 Results and Discussion

The results presented herein discuss the isomerization of humulones at conditions yet unknown. The hop cones contained ca. 600 mg of humulones (related to 10.0 g initial weight, Table 1). Therefore, with 6 % of humulones, the hop samples can be categorized as aroma hop with medium to high bitter value [20]. As can be seen from Table 1, iso-humulones were not detected in the initial hop samples.

Table 1 **Amount of humulones and iso-humulones in 10.0 g of hop cones.**

Substance		R1	Amount (mg)
Humulone 	Co-Humulone	$CH(CH_3)_2$	178.1 ± 17.8
	n-Humulone	CH_2-$CH(CH_3)_2$	432.7 ± 43.3
	Ad-Humulone	$CH(CH_3)$-C_2H_5	
Iso-humulone 	Co-Isohumulone	$CH(CH_3)_2$	n.d.
	n-Isohumulone	CH_2-$CH(CH_3)_2$	n.d.
	Ad-Isohumulone	$CH(CH_3)$-C_2H_5	n.d.

n.d. not detectable

Table 2 Overview of the produced hop solutions stored at 10.0 ± 2.0 °C

Name	Initial weight of hop (g) – a	Volume of tap water (ml) – b	Ratio a/b (%)	Storage time (h)	Initial pH	Additives
HS-1	400.0	4000.0	10.0	600	5.0	No
HS-2.1	400.0	4000.0	10.0	300	6.0	No
HS-2.2	400.0	4000.0	10.0	300	6.0	Disinfectant**
HS-3.1	400.0	4000.0	10.0	240	6.2	No
HS-3.2	400.0	4000.0	10.0	240	2.2	No
HS-3.3	400.0	4000.0	10.0	240	2.0	No
HS-R-1	0.6*	18.0	3.33	144	2.0	Methanol***
HS-R-2	1.0*	80.0	1.25	456	6.0	Methanol****

* Standard ICE-2;
** mono iodide acetic acid (0.05% v/v);
*** 2 ml;
**** 20 ml

The single hop solutions and their characteristics are listed in Table 2. The solutions HS-1 to HS-3.3 contained 400.0 g of hop cones and 4,000.0 ml of tap water. The reference hop solutions (HS-R-1 and -2) contained 0.6 g and 1.0 g of the ICE-2 extract, respectively. They were added with 18.0 ml and 80.0 ml of tap water, respectively. Methanol was added to the reference hop solutions for enhanced dilution of the extract. The ratio of hop cones to tap water was 10 % for the hop solutions, and 3.33 % and 1.25 % for the reference samples, respectively.

3.1 Temperature and storage time

As it is known from the boiling of wort during the brewing process, the temperature parameter plays an important role in the isomerization of humulones. The temperature dependency was evaluated using HS-1 due to its similar pH to that of wort (pH = 5.0, Table 2), so that the pH influence can be excluded. At temperatures of 10.0 ± 2.0 °C, an isomerization of dissolved humulones takes partially place after a storage time of 120 hours (Fig. 1). Iso-humulones were formed in amounts of ca. 100 mg at up to 500 hours. It is known from the boiling of wort that with longer boiling time the isomerization of humulones is increased. As could be expected, a longer storage duration of the sample has led to higher amounts of solved humulones. Due to progressively more dissolved humulones, also higher amounts of iso-humulones were formed.

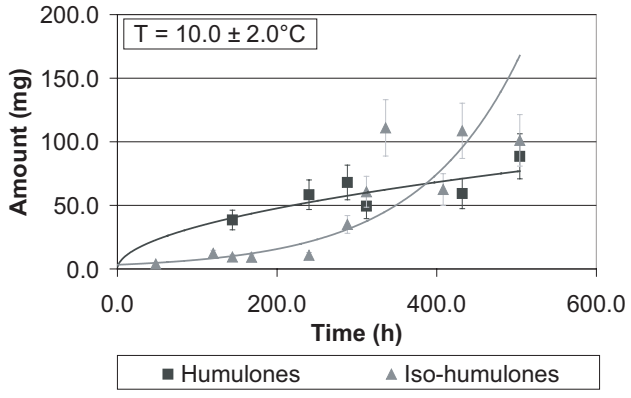


Figure 1 Change of the dissolved amounts of humulones and iso-humulones in dependence of storage time in the hop solution HS-1 at a starting pH of 5.0; the lines indicate the trend (humulones: $R^2 = 0.67$, iso-humulones: $R^2 = 0.86$)

The verification experiments, performed with the reference hop solution HS-R-2, confirmed the above stated results. Several dependencies of temperature on the isomerization are already known. Isomerization can be induced at 40 °C, but only when magnesium hydroxide has been added previously to catalyse the isomerization at higher temperatures. Lower temperatures between 5 and 15 °C, or above 31°C, can have an influence on the extraction of humulones (taking place at pressures above 60 bar) [7]. Considering the influence of the boiling temperature on the isomerization during beer production, then about 30–50% of humulones are isomerized after 90 min [6]. The duration of 120 hours observed in our experiments is considerably longer compared to that of wort boiling. However, isomerization can be generated at lower temperatures and, thus, it is generally not necessary to boil the aqueous solution in order to induce the process.

3.2 pH-value and storage time

To investigate the pH-dependency on the isomerization of humulones, some of the hop solutions were acidulated. The initially fixed pHs decreased in every hop solution, which is exemplarily shown in Figure 2 for HS-1. In this sample, the pH initially set at 5.0 decreased by 0.2 units between 72 and 96 hours due to the highest amount of dissolved hop bitter acids within that period (before and afterwards, lesser amounts were dissolved).

Therefore, the solubility of humulones and in turn the isomerization taking place afterwards is pH dependent. Figure 3 shows the amount of humulones initially determined in the hop cones [A] and, for comparison, the humulones and iso-humulones in the solutions that were prepared with hop cones. From that, it can be deduced that isomerization occurs in acidic and neutral milieu. At pH 2.0 ± 0.2 the highest amount of humulones was dissolved (Figure 3, C), and isomerization occurred to a greater extent com-

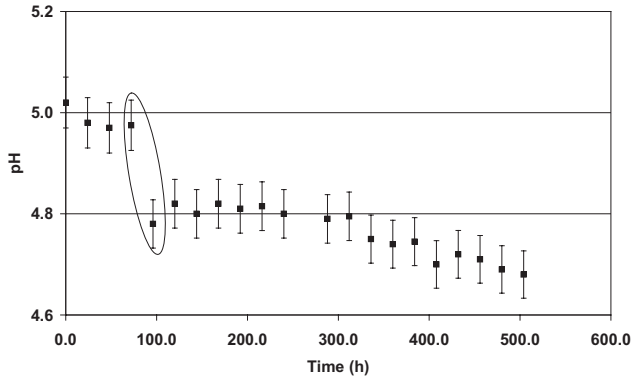
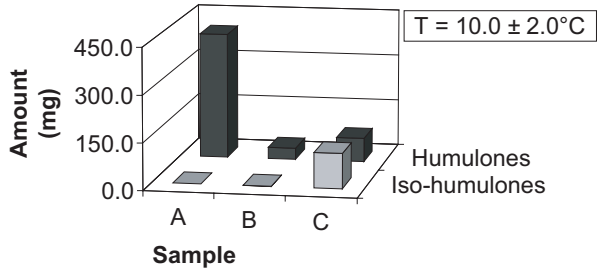


Figure 2 Change of pH in dependence of storage time in the hop solution HS-1



	A	B	C
Iso-humulones	n.d.	0.40	112.14
Humulones	403.40	36.54	76.25

Figure 3 Dissolved amounts of humulones and iso-humulones after 240.0 hours of storage (in mg per 1000 ml hop solutions; based on the hop cones weighted in); A is the theoretical maximum amount of solvable humulones, B amount of humulones and iso-humulones at pH 6.0 ± 0.2, and C the amounts at pH 2.0 ± 0.2

pared to sample B (neutral pH of 6.0 ± 0.2). This observation has been documented in the literature, but under boiling conditions [6]. When wort is brought to boil during beer production, the pH decreases along with increasing amounts of dissolved humulones, and higher isomerization rates occur at low pH-values. In our experiments, isomerization can take place sufficiently at relatively low temperatures of ca. 8 °C and at the pH of 2.

To verify these results, the experiments were repeated at the same conditions using the ICE-2 standard. The solubility of humulones and the formation of iso-humulones in dependence of storage time and pH are shown in Table 3. The pH of HS-R-1 was initially set at pH 2.0 and this of HS-R-2 at pH 6.0 (both stored at temperatures of 10.0 ± 0.2). The different initial weights of hop cones in the reference hop solutions (cf. with Table 2) have caused the different amounts of dissolved humulones and in turn their isomerization. After 24 hours, 83.7 mg of humulones could be detected in HS-R-1 and an isomerization appearing firstly at pH 2.0. The amounts of humulones and iso-humulones increased with prolonged storage time, while the pH decreased slightly. This observation is similar as to that for the HS-R-2 sample, for which the initial hop weight was higher than for HS-R-1. The higher amounts of humulones have caused in turn the increased formation of iso-humulones, along with a slightly decreased pH (5.89 ± 0.02), after the storage time of 192 hours.

3.4 Concentration of humulones and storage time

The concentration dependency was evaluated using the hop solutions HS-2.1 and HS-2.2. Table 4 shows the change of amounts of humulones and iso-humulones in solution over storage time for both samples. Figure 4 shows exemplarily the course of humulones in solution for HS-2.1 (duration: 300.0 hours, pH 6.0, no disinfectant) and simultaneously the formation of iso-humulones.

Table 3 Amounts of dissolved humulones and iso-humulones in the reference hop solutions HS-R-1 and HS-R-2 at starting pHs of pH 2.0 and pH 6.0, stored at 10.0 ± 2.0 °C.

	HS-R-1				HS-R-2			
Time (h)	Amount Humulones (mg) – A	Amount Iso-humulones (mg) – B	Ratio B/A (%)	pH	Amount Humulones (mg) – A	Amount Iso-humulones (mg) – B	Ratio B/A (%)	pH
0	n.d.	n.d.	ø	2.0 ± 0.02	n.d.	n.d.	ø	6.04 ± 0.02
12	(-)	(-)	ø	2.0 ± 0.02	(-)	(-)	ø	(-)
24	83.7 ± 8.4	11.1 ± 1.1	13.2	1.95 ± 0.02	498.1 ± 25.0	n.d.	ø	5.99 ± 0.02
48	73.5 ± 7.5	11.0 ± 0.9	15.0	1.90 ± 0.02	(-)	(-)	ø	(-)
72	(-)	(-)	ø	(-)	568.4 ± 22.0	30.4 ± 2.8	5.3	(-)
96	71.9 ± 7.3	9.6 ± 0.9	13.3	1.90 ± 0.02	(-)	(-)	ø	(-)
144	140.9 ± 7.3	16.1 ± 1.3	11.4	1.84 ± 0.02	(-)	(-)	ø	(-)
192	(-)	(-)	ø	(-)	394.8 ± 39.5	34.9 ± 2.2	8.8	5.89 ± 0.02
456	(-)	(-)	ø	(-)	403.6 ± 40.4	39.2 ± 1.4	9.7	5.84 ± 0.02

(-) no sampling; n.d. not detectable; ø incalculable

Table 4 **Amounts of humulones and iso-humulones, and the isomerization ratio in aqueous hop solutions in dependence of storage time at 10.0 ± 2.0 °C**

	HS-2.1			HS-2.2		
Time (h)	Amount Humulones (mg) – A	Amount Iso-humulones (mg) – B	Ratio B/A (%)	Amount Humulones (mg) – A	Amount Iso-humulones (mg) – B	Ratio B/A (%)
48	63.0 ± 6.3	28.1 ± 2.2	44.6	79.9 ± 8.0	40.9 ± 2.0	51.2
72	86.3 ± 8.6	45.3 ± 3.6	52.3	130.1 ± 13.0	67.6 ± 3.4	52.0
144	28.5 ± 2.9	32.2 ± 2.6	113.0	97.7 ± 9.5	81.0 ± 4.1	82.9
168	69.7 ± 6.9	38.3 ± 3.1	54.9	84.8 ± 8.5	67.3 ± 3.4	79.4
192	323.1 ± 32.3	128.2 ± 10.3	39.7	356.3 ± 35.6	123.9 ± 6.2	34.8
240	234.3 ± 23.4	119.9 ± 9.6	51.2	181.5 ± 18.2	102.9 ± 5.2	56.7

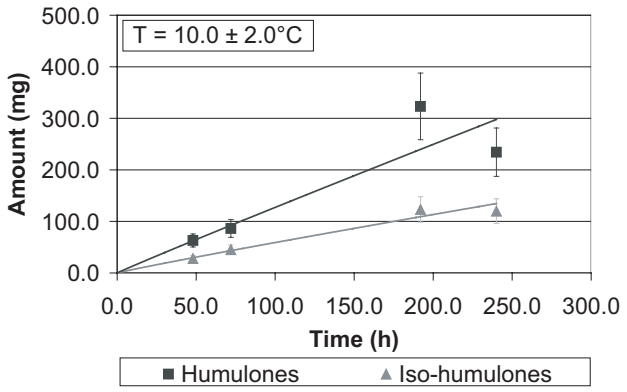


Figure 4 **Change of dissolved amounts of humulones and iso-humulones in the hop solution HS-2.1 at starting pH of 6.0; the lines indicate the trend (humulones: $R^2 = 0.92$, iso-humulones: $R^2 = 0.98$)**

It is indicated, as has been observed previously, that the longer the storage time is the more humulones are dissolved which in turn can isomerize to more amounts of iso-humulones. In Table 4 the ratio between the amounts of iso-humulones (B) and humulones (A) over the storage time is also given. It can be seen, for example, that about 50 % of dissolved humulones were isomerized after 72 hours. After 168 hours more than 75 % of humulones were isomerized, and after 192 hours still about 35 %. This concludes that isomerization proceeds inconsistently, but in all cases with levels of more than 25 %. A specific relationship between the isomerization of humulones and their initial concentration cannot be established.

These observed isomerization rates are partially lower than those occurring in the production of isomerized hop products and during boiling of wort, in which more than 50 % of dissolved humulones can isomerize [6]. The verification experiments basically confirmed the obtained results with the difference that isomerization rates in the reference solutions HS-R-1 and HS-R-2 were 15 % maximum (Table 3), which is about 5-times lower compared to the rates observed in the hop solutions (80 %, Table 4).

3.5 Pressure

Pressure when applying supercritical carbon dioxide for the production of isomerized hop extracts can have an influence on the isomerization of humulones. To obtain higher isomerization rates, the pressure must be varied to above 73 bar [7]. In our experiments, all hop solutions were stored at normal pressure, at which, therefore, isomerization can take place. This fact is known from the boiling of wort, which also occurs under normal pressure. Thereby, however, the temperature is the main factor for the isomerization of humulones and pressure can only be regarded as an indirect parameter.

3.6 Microbial growth

Because there is evidence that the concentration of humulones and iso-humulones influences the microbial growth [21-23] due to nearly similar amounts of dissolved hop bitter acids, the samples HS-2.1 and HS-2.2 were compared to each other after the addition of a disinfectant (0.05 % v/v mono iodide acetic acid) to one of the samples in order to inhibit the growth of microorganisms. The samples were also stored at temperatures of ca. 8.0 °C. Wild yeasts, moulds, short and long rods were detected in both samples within the storage for up to 336 hours (Table 5). It is known from the literature that microbial growth is induced at this relatively high pH of 6.0 [6]. It seemed further that the amount of disinfectant added to the sample was insufficient, as well as the concentration of humulones and iso-humulones, for inhibiting microbial growth. This observation suggests that the cones used for preparing the normal hop solutions might have been contaminated with microorganisms. Because sterile hop extracts were used for the preparation of the reference hop solutions, microbial growth was not measured in these samples.

4 Conclusion

This work describes new cognitions about the influence of common parameters on the isomerization of humulones, focussing on temperature, pH, storage time, and the concentration of humulones. The pressure and the influence of the concentration of humulones and iso-humulones on microbial growth are also discussed as secondary parameters. Isomerization already took place at low temperatures

Table 5 **Microbial growth in the hop solutions HS-2.1 (without disinfectant) and HS-2.2 (with disinfectant)**

Hop solution	Duration (h)	pH-value	Temperature (°C)	Microorganism count			
				Wild yeasts	Moulds	Short rods	Long rods
HS-2.1	48.0	6.10	8.0	3	2	uncountable	uncountable
	144.0	5.74	8.0	1	7	uncountable	n.d.
	336.0	5.80	10.0	n.d.	3	1000	n.d.
HS-2.2	48.0	6.08	8.0	1	2	uncountable	n.d.
	144.0	5.72	8.0	7	9	uncountable	n.d.
	336.0	5.80	9.0	n.d.	6	1500	n.d.

n.d. not detectable

of ca. 10 °C, which is an observation that has not been examined previously. In acidic milieu (at pH 2), higher isomerization rates were observed than in neutral milieu. However, at higher pHs sufficient isomerization took also place after a storage duration of ca. 72 hours. Therefore, it is recommended to adjust the pH to lower values in order to achieve isomerization within a shorter duration. Higher isomerization rates occurred at a prolonged storage time of hop solutions of up to, e.g., 240 hours. This period is longer than this for the production of beer. However, the boiling process can be avoided to achieve isomerization to a sufficient extent, which can be of concern for saving energy when, e.g., higher volumes of hop solutions should be processed. For our purpose, the solutions containing isomerized humulones were ready for the use with foam fractionation without previous boiling.

As to the concentration of humulones in the hop solutions, it was observed that the ratio of solved humulones to the formation of iso-humulones remained nearly the same (mainly 50 %, but at least 25 %). With increased concentration of solved humulones, higher amounts of iso-humulones were formed. Observing the course of concentration over a period of 240 hours revealed that the ratio remained almost unchanged, except for 144 hours. A relationship between the concentration of humulones and iso-humulones and microbial activity, as known from the literature, could not be established.

In conclusion, isomerization can occur at normal pressure. The process can be influenced positively by decreasing the pH down to 2, by increasing the storage duration for at least up to 24 hours, and by decreasing the temperature down to ca. 8 °C. For the subsequent application of foam fractionation, these observations provided an important background in order to enrich and to extract humulones and iso-humulones with highest efficacy, and thereby to produce isomerized and non-isomerized marketable hop products. In this respect it should be noted that the isomerization conditions observed herein are less labour- and equipment-intensive.

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