APPLICATION NOTE

Liquid Chromatography

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The Qualitative and Quantitative Analysis of α-Acids in Hops and Beers by UHPLC with UV Detection

Introduction

Alpha acids (α-acids) are a class of chemical compounds of primary importance in the production of beer. They are found in the resin glands of the flowers of the hop plant (*Humulus lupulus*) and are normally added to the boil after mashing the grains, providing beers with their aroma and bitter taste.[1]

The α-acids found in hop resins are isomerized to form the iso-α-acids during prolonged boiling in the wort. The degree of isomerization and the amount of bitter taste produced by the addition of hops is highly dependent on the type of hop and the length of time the hops are boiled. Longer boil times will result in isomerization of more of the available α-acids, making the beer more bitter.[2] The α-acid percentages vary within specific varieties of hops, depending on the growing conditions, drying methods, age of hops, climate and other factors. Figure 1 shows the common α-acids and iso-α-acids involved in the beer brewing process.

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Since the quality and quantity of α-acids is so important in consistently providing individual beers with their recognizable taste, it is essential to monitor their amount in hops and beers and to monitor the formation of the iso-α-acids during the beer brewing process. The focus of this application note is to provide an easy, straightforward, and robust analytical method for establishing the type and amount of α-acids in hops pellets, as well as determining the amount of α-acids and iso-α-acids in various beers.

Method conditions and performance data, including linearity and repeatability, are presented.

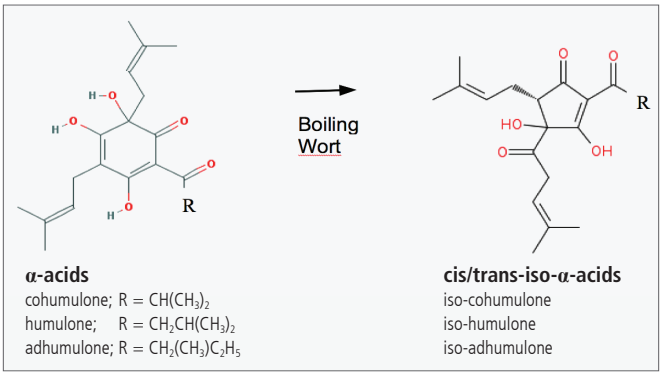

Figure 1. Isomerization path of hop α-acids to iso-α-acids during brewing, showing the representative α-acids.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer Altus™ UPLC® System was used, including the Altus A-30 Solvent/ Sample Module, integrated vacuum degasser, A-30 column heater, and Altus A-30 UV detector. For spectral confirmation, Altus-30 SQ and PDA (photodiode array) detectors were used. All instrument control, analysis and data processing was performed using the Waters® Empower® 3 Chromatography Data Software (CDS) platform.

Method Parameters

The LC method parameters are shown in Table 1.

Table 1. LC Method Parameters.

HPLC Conditions	
Column:	PerkinElmer Brownlee™ SPP C18 2.7 µm, 3.0 x 100-mm (Part# N9308410)
Mobile Phase:	Mobile Phase A (MPA): 0.1% Phosphoric Acid (H_3PO_4) plus 0.2 mM Disodium EDTA Mobile Phase B (MPB): Acetonitrile (ACN) Solvent mix: 35 % MPA/ 65% MPB (isocratic)
Analysis Time:	6 min.
Flow Rate:	1.0 mL/min.
Pressure:	4200 psi/280 bar (maximum)
Oven Temp.:	40 °C
Detection:	270 nm
Injection Volume:	4 µL
Sampling (Data) Rate:	5 pts./sec

Solvents, Standards and Samples

All solvents, reagents, and diluents used were HPLC-grade or ACS grade and filtered via 0.45-µm filters. For all dilutions, 8:2 methanol/0.1% trifluoroacetic acid (TFA) was used.

The α-acids (ICE-3, Hops Extract Standard) and iso-α-acids (DCHA-Iso, ICS-I3 Standard) were obtained from the American Society of Brewing Chemists (ASBC), St. Paul, MN.[3] ICE-3 was reported to contain 13.88% cohumulone and 30.76% humulone/adhumulone (N+adhumulone). ICS-I3 was reported to contain 62.3% iso-α-acids, including trans-isocohumulone, trans-isohumulone and trans-isoadhumulone, all in dicyclohexylamine salt form.

The samples included three different hop pellets (English East, Kent Goldings, American Simcoe and New Zealand Galaxy) and four different beers from a colleague's private stock (English Bitter, India Pale Ale (Double IPA), Extra Special Bitter and Flanders Red.

Experimental

Standard Preparation

Two stock solutions, 7.3 mg/mL of ICE-3 standard (α-acids) and 1.4 mg/mL of ICS-I3 standard (iso-α-acids), were prepared as stock solutions by transferring the appropriate weight of each standard into an individual 10-mL volumetric flask. Note: each initial standard contained different amounts of the individual analytes, reflected in the concentrations shown in Table 3. 6 mL of methanol was then added to each flask, which was sonicated for 10 minutes and filled to volume with methanol. The ICE-3 stock solution was filtered through a 0.45 µm nylon filter; however, the ICS-I3 stock solution was not filtered due to potential retention of iso-α-acids on the filter.[3] Both stock solutions were then transferred into clear glass containers and stored under refrigeration until further use. For the working standard, 2 mL of the α-acids stock solution and 6 mL of the iso-α-acids stock solution were transferred into a 25-mL volumetric flask, which was then filled to volume with diluent. Six calibration levels were prepared via serial dilution of the working standard solution. Each standard level was injected in triplicate.

Hops Preparation

Each hop sample, coming in either pellets or coarse powder, was weighed (weight ranged from 0.2 g to 0.5 g) in duplicate and crushed into a fine powder using a mortar and pestle. The powdered samples were transferred into individual 50-mL centrifuge tubes. Following, 6 mLs of diluent was added to each tube, which was then stored at room temperature to soak for four hours. Every hour, each tube was vortex-mixed for 60 seconds. Afterwards, the tubes were sonicated for 15 minutes in water and then filled to the 10-mL mark using diluent. All tubes were then hand-shaken and centrifuged at 7000 rpm for 10 minutes. Each supernatant was transferred into an individual 25-mL volumetric flask. 10 mL of diluent was then added into each centrifuge tube containing the remaining precipitate, followed by vortex-mixing for two minutes and centrifuging for 10 minutes at 7000 rpm. The supernatant from each tube was then added to the corresponding 25-mL flask. Finally, the flasks were filled to volume with diluent, transferred into HPLC vials and injected in triplicate.

Beer Preparation

The four beer samples were prepared by liquid-liquid extraction (LLE). Duplicate 10-mL aliquots of each beer were transferred into individual centrifuge tubes followed by the addition of 0.5 mL of phosphoric acid and 10 mL of trimethylpentane. A blank sample was prepared by substituting 10 mL of water for the 10 mL of beer. The tubes were vortexed for one minute. To diminish the layer of foam that developed between the water and trimethylpentane layers, 1-2 mL of methanol was added to the centrifuge tube. Subsequently, each tube was sonicated in water for 15 minutes, followed by centrifuging for five minutes at 2000 rpm. 5 mLs of clear supernatant was then collected in an evaporating dish and evaporated to dryness under a stream of nitrogen. The residue was re-dissolved in 2.0 mL of diluent, transferred into HPLC vials and injected in triplicate.

Results and Discussion

Using the optimized chromatographic conditions described above, Figure 2 shows the HPLC separation of the level-4 working standard containing three α-acids along with their three isomers, all well resolved in less than four minutes.

Figure 3 shows the overlay of 12 replicate injections of the level-4 working standard containing α-acids and iso-α-acids, demonstrating high reproducibility. The retention time precision for all compounds was < 0.18% RSD.

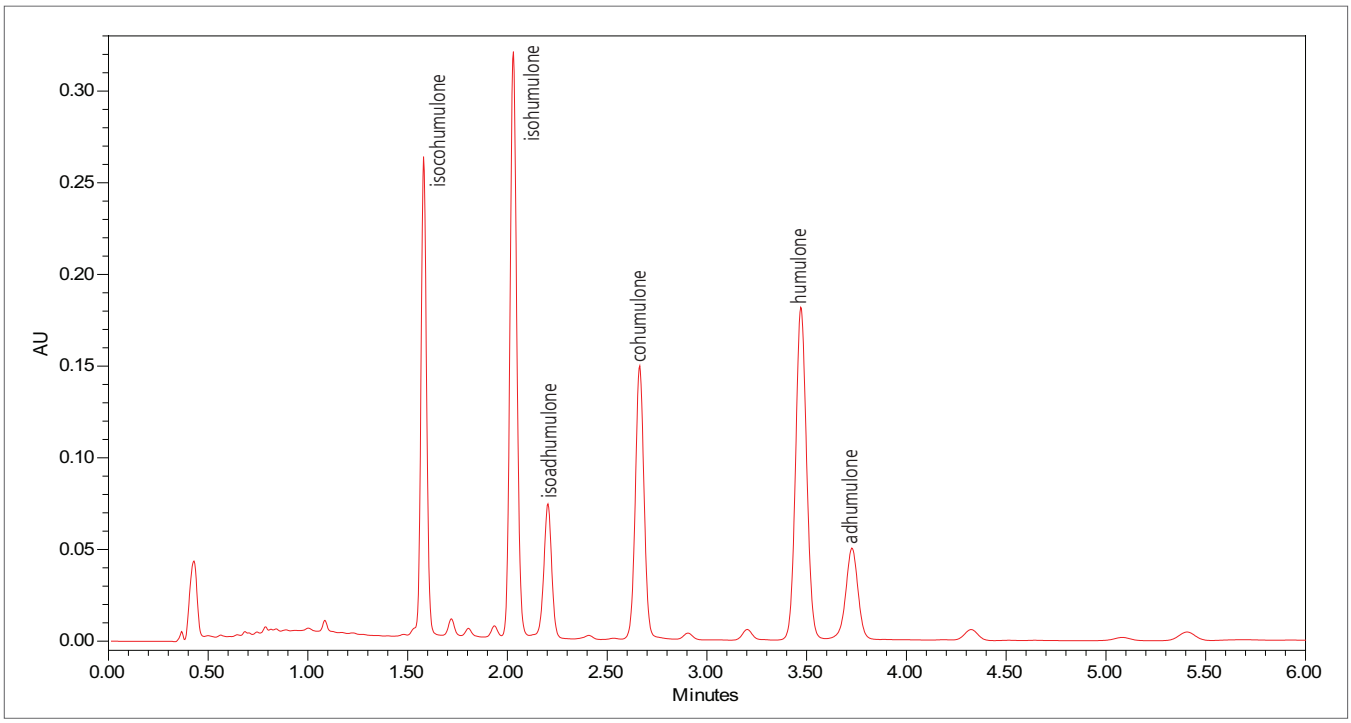

Figure 2. Chromatogram of the level-4 working standard, containing α-acids and iso-α-acids; by UV at 270 nm.

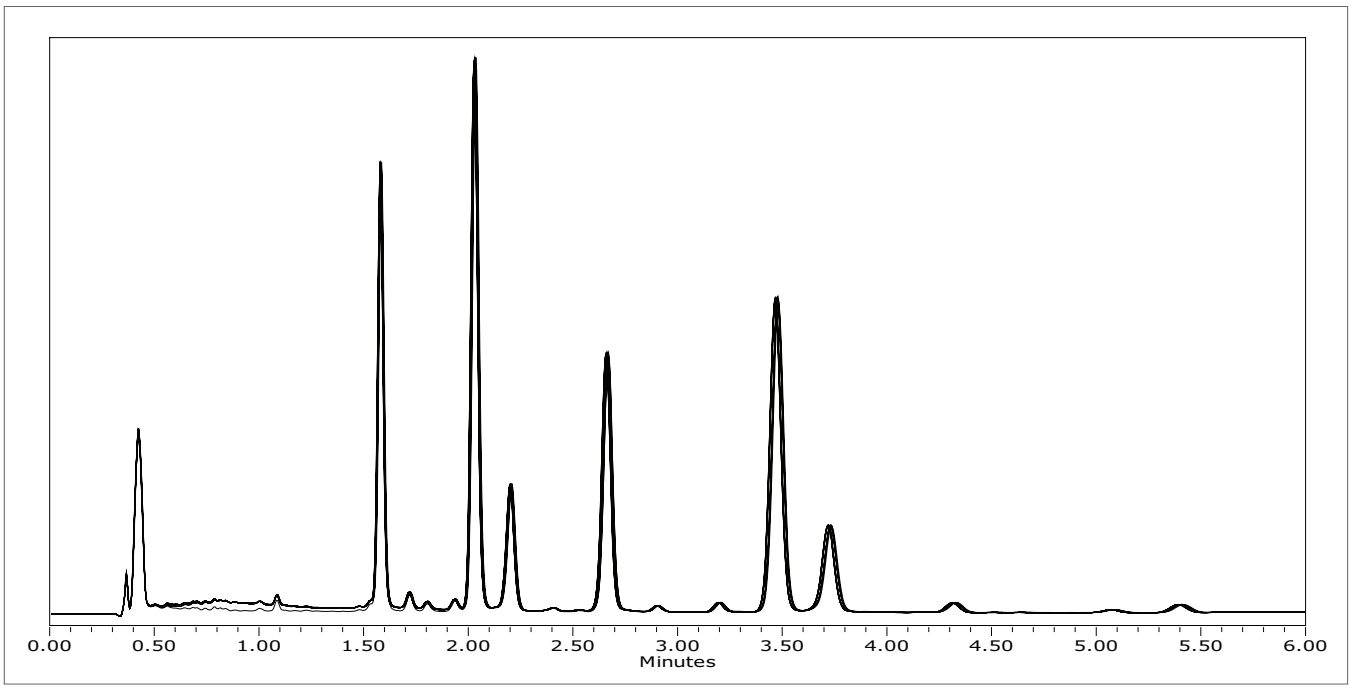

Figure 3. Overlay of 12 replicates of level-4 working standard, by UV at 270 nm.

A summary of the method performance, including %RSD, USP tailing, USP plate count and USP resolution is presented in Table 2.

The calibration set was based on a series of working standard dilutions. The individual analyte concentrations are shown for each level in Table 3.

Table 2. Method performance.

Peak Name	RT (min)	%RSD	Mean USP Tailing	Mean USP Plate Count	USP Resolution
isocohumulone	1.582	0.150	1.04	15645	-----
isohumulone	2.031	0.165	1.06	17172	7.98
isoadhumulone	2.203	0.164	0.98	15551	2.59
cohumulone	2.662	0.176	1.00	17986	6.13
humulone	3.472	0.176	1.02	18893	8.97
adhumulone	3.725	0.177	0.96	18431	2.40

Figure 4 shows the calibration results for the six analytes (α-acids and iso-α-acids). All six analytes had an exceptional linear fit, with R^2 values > 0.9999 (n = 3 at each level).

Table 3. Concentrations of α-acids and iso-α-acids in working standard at each concentration level.

Compound	Level 1 (µg/mL)	Level 2 (µg/mL)	Level 3 (µg/mL)	Level 4 (µg/mL)	Level 5 (µg/mL)	Level 6 (µg/mL)
isocohumulone	3.64	7.28	14.6	21.8	36.4	72.8
isohumulone	5.35	10.7	21.4	32.1	53.5	107
isoadhumulone	1.40	2.79	5.59	8.38	14.0	27.9
cohumulone	4.39	8.77	17.5	26.3	43.9	87.7
humulone	7.48	14.9	29.9	44.9	74.8	149
adhumulone	2.24	4.49	8.98	13.5	22.4	44.9
Total iso-α-acids	10.4	20.8	41.6	62.3	103	207
Total α-acids	14.1	28.2	56.4	84.6	141	282

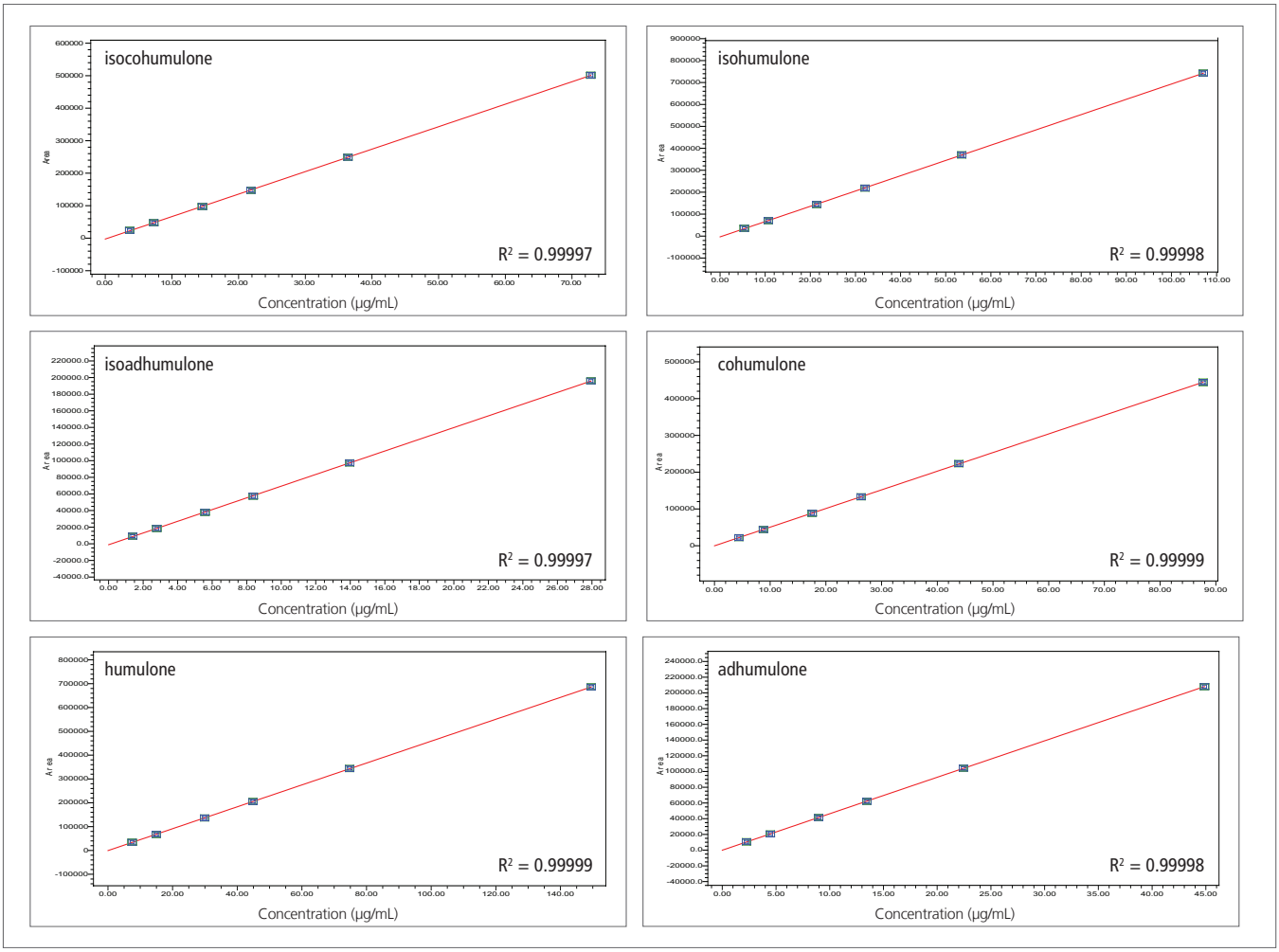

Figure 4. Results of 6-level calibration set for α-acids and iso-α-acids.

Table 4 presents the estimated limits of quantitation and detection (LOQ, LOD) for all tested α-acids and iso-α-acids. These limits were derived using the signal-to-noise (s/n) results obtained during calibration, using an average of three replicates per level.

As can be seen, the LODs and LOQs for the three iso-α-acids are quite similar. Also, they are over twice as low as those of the three α-acids.

By applying our chromatographic conditions, we were able to separate all six α-acids/iso-α-acids that were analyzed.

Figure 5 shows the UV spectrum and corresponding mass spectrum for the peaks eluting at 3.47 and 3.73 minutes. The UV spectra of both peaks are very similar and, as the peak at 3.47 minutes is known to contain humulone, this suggests that the peak at 3.73 minutes is also related to humulones. The mass spectra of both peaks are identical and match the expected M-H mass of humulone and adhumulone (negative ion mode). Based on the above, as well as the elution order of isohumulone and isoadhumulone, it is likely that the peak eluting at 3.47 minutes is humulone and the peak eluting at 3.73 minutes is adhumulone. Having access to pure individual standards of humulone and adhumulone would help to confirm the identity of these peaks.

Table 4. Calculated estimated LOQ and estimated LOD for α-acids and iso-α-acids.

Compound	LOD (µg/mL) (s/n ≥ 3/1)	LOQ (µg/mL) (s/n ≥ 10/1)
cohumulone	0.09	0.30
humulone	0.13	0.42
adhumulone	0.19	0.46
isocohumulone	0.04	0.14
isohumulone	0.05	0.17
isoadhumulone	0.06	0.20

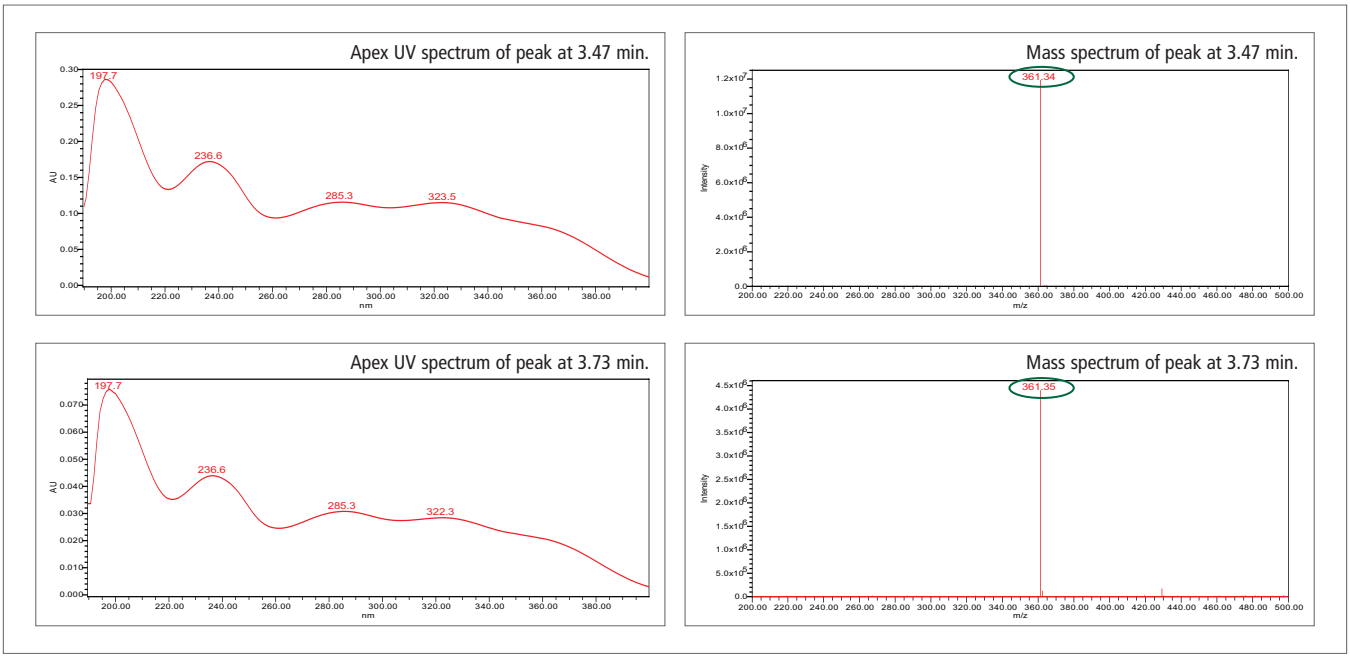

Figure 5. The UV spectra and corresponding mass spectra (neg. ion mode) of the peaks eluting at 3.47 min. and 3.73 min.

Figure 6 shows the chromatograms for each of the three different hops samples. Comparing the chromatograms, there are clear differences between each hops sample. Also, of note is that they all show very low iso-α-acids content, compared to the α-acids, as expected.

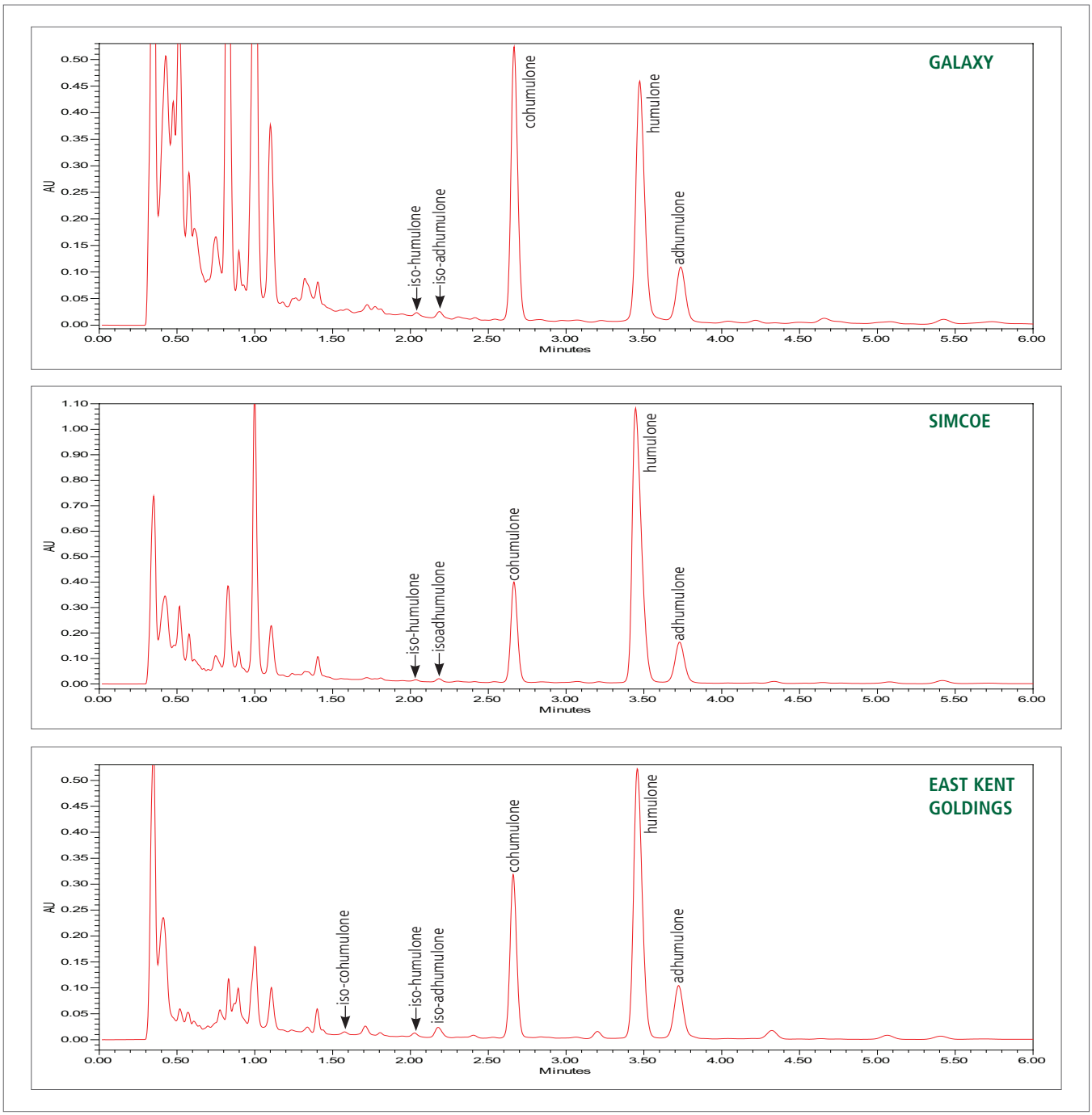

Figure 6. Chromatograms of the three analyzed hop species.

The quantitative results of the three analyzed hops samples are presented in Table 5, reflecting an average of two sample preparations and three injections per sample. As expected, the concentrations of α-acids vary from hop to hop species. Although the proportions of α-acids in both Simcoe and East Goldings hops are similar, the Simcoe hop contains about twice the overall amount of α-acids. In addition, the concentration of iso-α-acids in each of the hops is very low or not detected, which is to be expected as the iso-α-acids only arise as part of the brewing process.

Table 5. Quantitative results for α-acids and iso-α-acids in the four different hops samples.

Compound	Concentration (mg/g) in 3 species of Hops		
	Galaxy	Simcoe	East Kent Goldings
cohumulone	15.9	11.1	9.12
humulone	19.7	45.4	21.5
adhumulone	4.67	6.93	4.62
isocohumulone	ND	ND	NQ
isohumulone	0.18	NQ	0.17
isoadhumulone	0.28	0.25	0.46
Total α-acids	40.3	63.5	35.2
Total iso-α-acids	0.46	0.25	0.63

ND = not detected; NQ = detected, but not quantitatable

Figure 7 shows the chromatograms of the four different beer samples, with all six analytes well resolved. The amount of α-acids in Double IPA beer is similar to the iso- α-acids, while in Extra Special Bitter, the α-acid amounts are much lower in comparison. Of particular note is the apparent absence of α-acids in English Bitter and in Flanders Red beers.

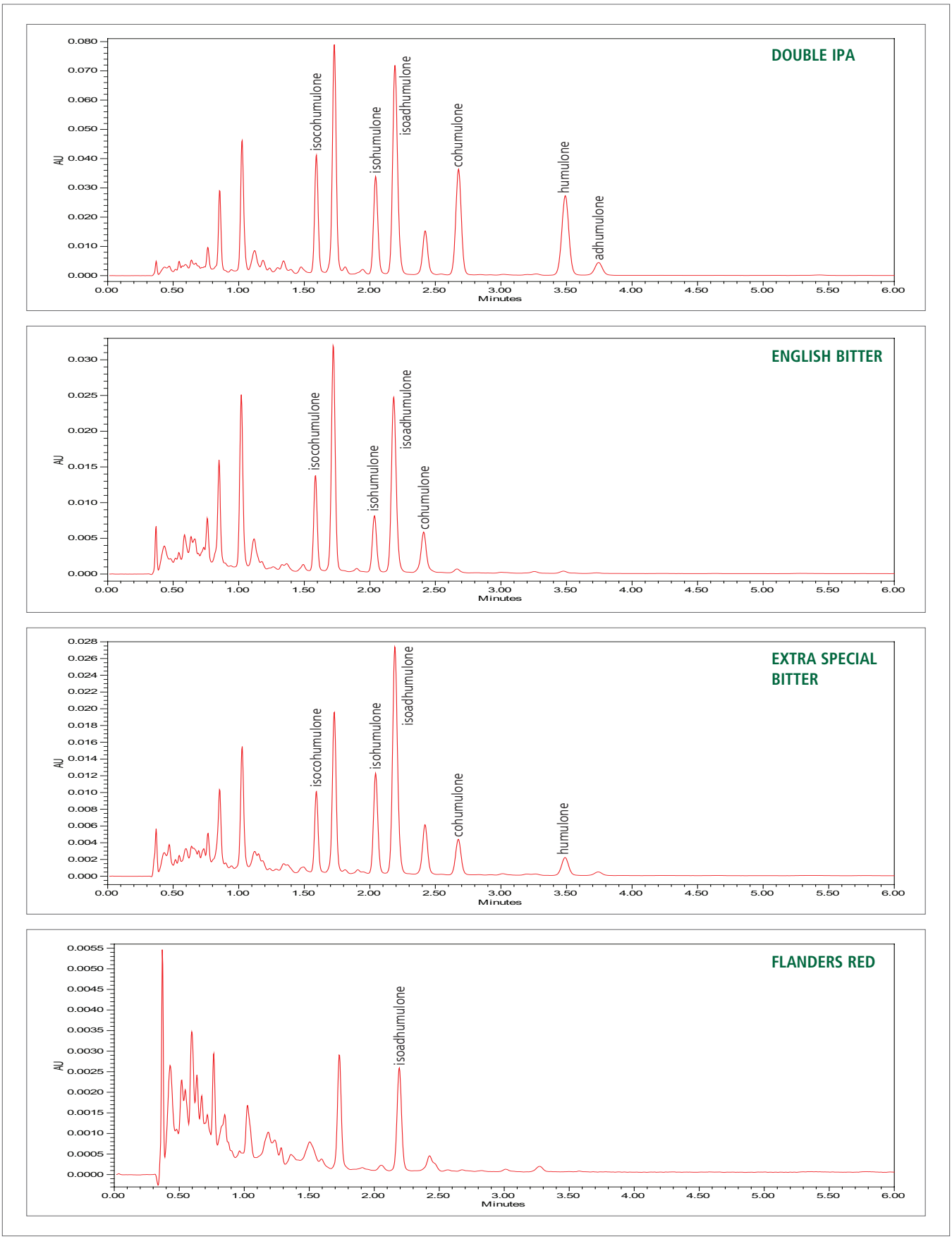

Figure 7. Chromatograms of the four analyzed beers.

Table 6. Quantitative results for α-acids and iso-α-acids in four different beers.

Compound	English Bitter	Double IPA	Extra Special Bitter	Flanders Red 2014
	µg/mL	µg/mL	µg/mL	µg/mL
cohumulone	NQ	5.51	0.66	ND
humulone	ND	5.90	0.50	ND
adhumulone	ND	1.02	ND	ND
isocohumulone	0.90	2.94	0.74	ND
isohumulone	0.69	3.00	1.13	ND
isoadhumulone	2.03	6.94	2.57	0.23
Total α-acids	ND	12.4	1.16	ND
Total iso-α-acids	3.62	12.9	4.44	0.23

NQ = detected, but not quantitatable; ND = not detected

The results for the analysis of the four tested beers are presented in Table 6 and reflect an average of two sample preparations and three injections per sample. The concentration and proportion of α-acids and iso-α-acids differ markedly from beer to beer. Compared to the concentrations found in hops, the concentrations of iso-α-acids are much higher. This was to be expected, as these acids increase markedly during the brewing process.[5]

Conclusion

The results obtained confirm the applicability of this method for the efficient, routine, and robust chromatographic analysis of the six investigated α-acids including their isomers. All compounds are completely separated in under six minutes by UHPLC® using UV detection. The results showed excellent retention time repeatability as well as exceptional linearity over the tested concentration ranges. Thereupon and considering the very lower LOQ levels for these α-acids and their corresponding isomers, this application can be considered very effective for the monitoring of these acids during beer production.

References

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