ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 3565−3570

http://pubs.acs.org/journal/acsodf

Quantification of α-Acids in Fresh Hops by Reverse-Phase High-Performance Liquid Chromatography

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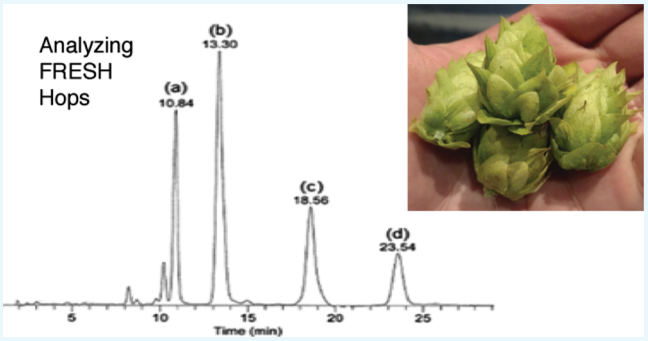
Supporting Information

ABSTRACT: This publication describes a method for the quantification by high-performance liquid chromatography (HPLC) of resinous compounds known as α-acids found in freshly harvested, unprocessed hops. This method provides consistent, efficient, and accurate results as well as the theories and rationale involved in HPLC method development. A system of quality checks was utilized as well as the validation of numerous developmental variables. By starting with a theoretical approach in preparation, extraction, and instrumental techniques and then further developing these practices by experimentation, a reproducible method was developed. Following the validation, fresh cascade hops grown in Sonoma County were analyzed during the 2017 harvest season and found to be within the predicted range specific to this cultivar. This method encompasses the techniques necessary to analyze fresh or dried hops, considering variability between different laboratories.



INTRODUCTION

Hops are used in the production of beer because of their unique chemical compounds, which contribute bitterness, flavor, and aroma. These compounds are produced and contained within a hop's lupulin glands.[1] The first category of compounds found within these glands are a variety of volatile hydrocarbons known as essential oils, which give beer distinct flavor and aroma profiles depending on the types of hops used. These profiles can be described as piney, floral, or citrus. To increase the concentration of these essential oils in a beer, brewers use the process of "dry hopping" where hops are added to cooled beer after fermentation. Adding hops at this point of the brewing process provides a stable environment for the extraction of these oils, minimizing loss because of evaporation. The second category of compounds found within the lupulin glands are resinous amphiphilic molecules known as α-acids and β-acids, which give a bitter taste to the beer. See Figure 1 for α-acid and β-acid molecular structures. This bitterness is attained by boiling these acids in the slightly acidic "wort", which is water with a concentrated amount of unfermented maltose. Bitterness is attributed to the isomerization of α-acids such as humulone to its counterpart isohumulone.[2] Craft breweries report this perceived bitterness in International Bittering Units (IBUs), which is an estimation based on the concentration of α-acids that are isomerized.[2] The IBU calculation requires the weight of hops added along with the percent (weight/weight) of α-acids and the amount of time the mixture is left to boil. β-Acids on the other hand oxidize to give a bitter profile. The oxidation happens at a much slower rate, but the rate increases when left to age in a warm environment. The preoxidized β-acids are insoluble in the wort; therefore, β-acids are not taken into consideration when calculating a beer's IBUs.[3] Overall, these compounds interact with a beer to balance the burning taste of alcohol as

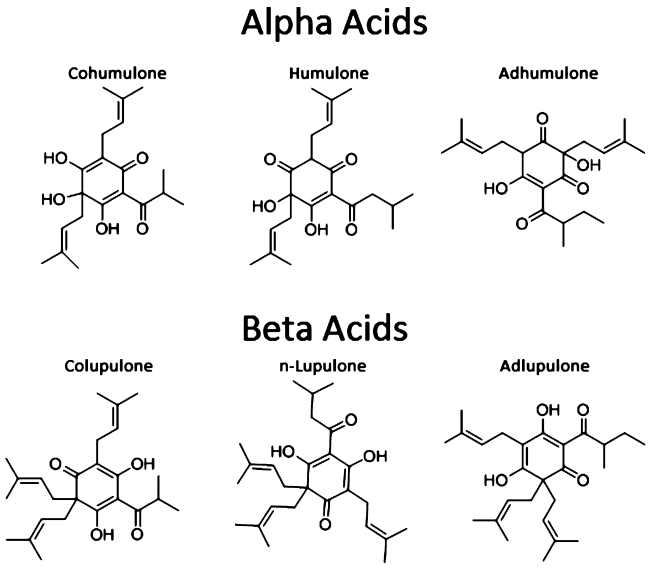


Figure 1. Molecular structures of cohumulone, humulone, adhumulone, colupulone, lupulone, and adlupulone.

Received: January 2, 2019
Accepted: January 28, 2019
Published: February 18, 2019

DOI: 10.1021/acsomega.9b00016
ACS Omega 2019, 4, 3565−3570

well as the sweetness of residual maltose. Hops are also known to contribute to antimicrobial activity and retention of the beer's foamy head.

With craft beer becoming more popular within the alcoholic beverage industry as well as with home brewers, demand for hops and the testing of their chemical makeup have increased. Hops may be purchased in bulk on an industrial level or in gram quantities for small batch home brewers. Consumers also have the option of choosing between packaged hops in the natural cone state or a condensed pelletized form. While this has increased the availability of hops globally, brewers lose a large amount of essential oils because of evaporation as the hops must be dried to 10% moisture content before being packaged to prevent spoilage.[4] This has shaped a seasonal tradition in Sonoma County of producing "wet hopped" beers where brewers work with growers to make beer using hops freshly harvested from the bine. With the utilization of fresh hops in the brewing industry, an accurate method for testing α-acids with considerations of a fresh cone's higher moisture content was needed.

The focus of this publication is to describe a method for the quantification of α-acids (cohumulone, humulone, and adhumulone) in freshly harvested hops by reverse-phase high-performance liquid chromatography (HPLC), utilizing ultraviolet detection and an external standard. This method advances previously described methods[5,6] by considering the unique composition of fresh hops, implementing a system of quality checks, maintaining chromatographic separation while reducing the retention range, and reducing solvent consumption. Also, common problems an analyst may encounter with suggested solutions are communicated throughout this publication. This methodology was applied in the analysis of fresh hops during the 2017 Sonoma County harvest season.

MATERIALS AND METHODS

Instrumentation. The chromatographic system used is a Thermo Finnigan Surveyor HPLC; a binary MS Pump Plus was utilized with an A/S Lite Plus Autosampler injecting 10 μL per sample and a PDA Plus detector operating at a wavelength of 233 nm. The average maximum absorbance wavelength of all four samples was found by analyzing standards with a variable wavelength detector operating at the range of 200–600 nm. Xcalibur 2.0.7 software was used to run the sequences and automate the integration of chromatograms. A Phenomenex Luna, C-18, 5μ, 150 × 4.6 mm column was used to provide sufficient separation and accurate quantitation. Each injection had a 30 min run time with a flow rate of 0.8 mL/min.

Eluents used were (A) methanol/0.17% formic acid in water (85:15 v/v %) and a gradient of (B) 100% water. See Table 1 for gradient parameters. This gradient of increased water concentration was necessary to separate α-acids (analyte peaks a and b) because of their similar chemical properties and therefore similar selectivity values. After the elution of both α-acid peaks, the gradient was returned to 100% mobile phase (A) to decrease the retention range and tailing of the later eluting β-acids (analyte peaks c and d). A typical chromatogram of a standard and sample injections is shown in Figures 2

Table 1. Gradient Parameters of Mobile Phase (A) and Water (B)

time (min)	A %	B %	mL/min
0.00	100.0	0.0	0.8
7.99	100.0	0.0	0.8
8.00	90.0	10.0	0.8
13.00	100.0	0.0	0.8
30.00	100.0	0.0	0.8

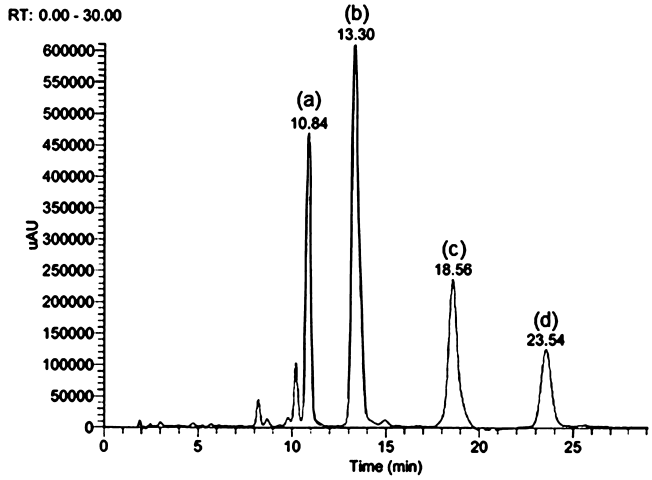


Figure 2. Example chromatogram of a typical standard injection.

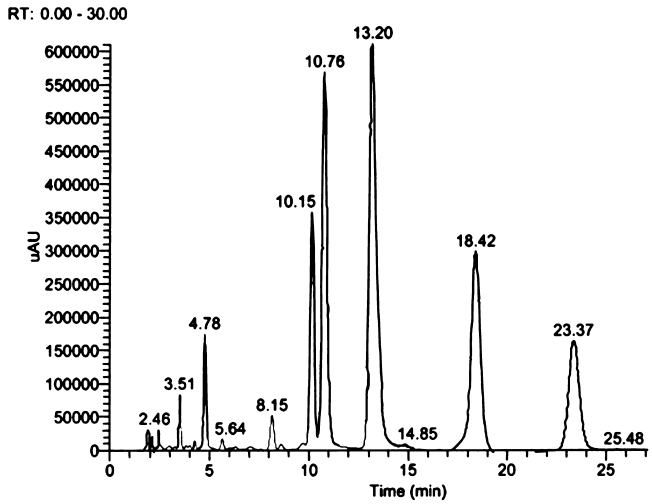


Figure 3. Example chromatogram of a typical sample injection.

and 3, respectively. For this specific system, an instantaneous jump was introduced from 0 to 10% (B) and 90% (A) at t = 8.00 min, followed by a linear gradient back to 100% (A). The gradient returned to 100% (A) at t = 13.00 min. The jump in water concentration was done earlier than the normal elution time of the first analyte peak to allow a sufficient amount of time for the increased water gradient to reach the compounds when moving through the system at 0.8 mL/min. The gradient parameters may need to be adjusted depending on the specific gradient delay volume which is defined as the total volume between the point of gradient mixing and column inlet.

Reagents. The reagents used consisted of HPLC grade methanol, peroxide-free diethyl ether, 98% formic acid, and 0.1 M hydrochloric acid. Millipore Milli-Q water was used. The external standard used is known as the International

DOI: 10.1021/acsomega.9b00016
ACS Omega 2019, 4, 3565–3570

Calibration Extract (ICE-3), which has reported concentrations of α-acids and β-acids. This standard is from The American Society of Brewing Chemists located in St. Paul, MN.

Chromatographic Mobile Phase—Methanol/0.17% Formic Acid in Water (85:15 v/v %). The main eluent used is a mixture of methanol and formic acid in water. To accurately adjust the pH of the mobile phase, it is necessary to adjust the water portion in its aqueous state with 98% formic acid until reaching a pH of 2.6. Adjusting the pH in an aqueous solution will increase the preparation's reproducibility compared to adjusting a solution of highly concentrated organic solvent.[7] Formic acid was the acidifying agent of choice because of its pK_a value of 3.74 at 25 °C with a UV cutoff at 210 nm.[8] Phosphate is also an applicable buffer option. A pH of 2.6 was used because of the analyte pKas which ranged from 4.25 to 5.93 to avoid variability in spacing and resolution.[9,10] See Table 2 for a summary of chemical properties for all six analytes.[11] After the pH adjustment, we combined 85 parts of methanol with 15 parts of the water−formic acid mixture. It is important to ensure that the mobile phase is mixed well, filtered, and degassed.

Table 2. Chemical Properties of the Six Analytes

compound	analyte peak	fully protonated species (%) @ pH 2.6	net atomic charge	molecular weight (g/mol)	pK_a
cohumulone	1, (a)	97.8	−0.014	348.4362	4.25
humulone	2, (b)	99.4	−0.004	362.4630	4.85
adhumulone		97.9	−0.014	362.4630	4.26
colupulone	3, (c)	100.0	0.000	400.5554	5.93
n-lupulone	4, (d)	100.0	0.000	414.5820	5.92
adlupulone		99.8	−0.001	414.5822	5.38

The mobile phase pH should be at least 1−2 units from the compound analyte pK_a.[9,10] Utilizing ARChem's physiochemical calculator SPARC at the pH of 2.6, all analytes are theoretically calculated to be completely protonated, which is ideal for chromatography purposes. Refer to Figure 1 for the analyte neutral structures. With a high percent of nonionized single species compounds, molecular charge is decreased, in turn reducing compound retention variability.

Note that an isocratic method is an option if a binary pump is not available. However, the analyst will more than likely need to increase the concentration of water within the mobile phase, which will produce increased run times and increased peak tailing. The aqueous pH should be adjusted to 2.6 regardless of which method the analyst chooses.

PROCEDURE

Standard Solutions: ICE-3 Standard (1.0 mg/mL). A single-point calibration of ICE-3 in methanol with a concentration of 1.0 mg/mL was utilized for quantification. Standard stability, when stored in cool conditions and protected from light, was found to be 24 h.[12] To ensure accuracy, working standard injections should have an analyte peak % relative standard deviation (RSD) of <3.0% for both standard injections of n = 5 (first standard injections) and n = all (bracketing standard injections included). See eq 1 for the calculation of working standard suitability. To ensure standard precision, it is suggested to prepare this solution in duplicate. With known concentrations of both the working and check standards, the analyst can confirm standard accuracy. Standard suitability can be calculated with eqs 2 and 3, and the calculated check standard concentration should be within 3.0% of the actual concentration.

First, the response factor (RF) of α-acids from the working standards (n = all) should be calculated. Next, the RF value should be used to calculate the check standard's concentration by system response and compare that value to the recorded concentration from the raw data recorded.

Sample Preparation. The hop samples used in this study were donated by members of the Nor-Cal Hop Growers Alliance with farms located throughout Sonoma County, California. Before extracting the samples, the moisture content was analyzed by a gravimetric method.[5] This technique is necessary as a form of pretreatment by removing all volatile and semivolatile components as well as being utilized in the final w/w % calculations for precise quantitation. The extraction process of freshly harvested hops utilizes liquid−liquid extraction to separate analyte molecules from contaminants. Because of the amphiphilic characteristics of α-acids and β-acids, diethyl ether is used as the extracting solvent with a 5:1 ratio of methanol. After the acids have been extracted from the plant matter, 0.1 M HCl is added to separate analytes and contaminants. The 0.1 M HCl solvent not only filters out the polar contaminants but also displaces them to the bottom layer because of its higher solvent density (Figure 4). This is a preferred extraction technique making analytes easily accessible to aliquot from the top layer. After analyzing both top and bottom layers, the extraction efficiency was found to be acceptable with negligible α-acids and no β-acids in the bottom layer. It is suggested to extract samples in duplicate in order to minimize sample homogeneity variances.

Figure 4. Picture of sample liquid−liquid extraction layering.

Suggested Sequence Order. The suggested sequence order is as follows: zero injection, blank, five working standards, one check standard, and up to six sample injections for every bracketing standard. When first attempting this method, it is suggested to run two blanks after both a standard and sample to confirm that there are no carryover peaks that will affect the accuracy or system suitability of the sequence. If carryover peaks of the analytes are present in either of the blanks, a prolonged syringe wash of 100% methanol may be necessary. If interfering peaks continue to be a problem, a blank after every standard and sample injection to flush the system is suggested. If the carryover peaks are below 0.1% of the area of any respective analyte peak, the carryover may be considered negligible.

DOI: 10.1021/acsomega.9b00016
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RESULTS AND DISCUSSION

Chromatography. Under these separation conditions, the first eluting analyte peak is (a) cohumulone, followed by (b) coeluting adhumulone and humulone, followed by (c) colupulone, and last (d) coeluting adlupulone and lupulone. These chromatograms offer desired and repeatable separation between peaks and are obtained within 30 min for a relatively low retention range. In this method, a possible source of error can be attributed to the lack of resolution between two α-acid peaks. The ideal resolution between peaks is >1.5 and can be calculated with eq 4.[13] Several unidentified components were observed. However, they did not interfere with analyte peaks, so accuracy was retained. With the narrow peaks observed, a high signal-to-noise ratio was obtained. The variability of sample injections was analyzed by calculating the average difference between the duplicate injections and determined to be 0.30% with a standard deviation of 0.26% and a maximum difference of 0.83%.

Linearity. During method development, the response linearity was tested over a large concentration range of 0–3.0 mg/mL. Excellent linearity was provided for all four analyte peaks. Also, in each sequence, a three-point calibration curve was applied to prove the reliability of utilizing the single-point 1.0 mg/mL working standard for quantification. With a standard concentration ranging from 0.5463 to 1.6389 mg/mL, the responses for both α-acid and β-acid analyte peaks produced adequate linearity (R^2 = 0.997 or higher) with a forced intercept of zero. See Figure 5 for an example calibration curve portraying the response because of the variable concentration of α-acid peak 2, coeluting adhumulone and humulone.

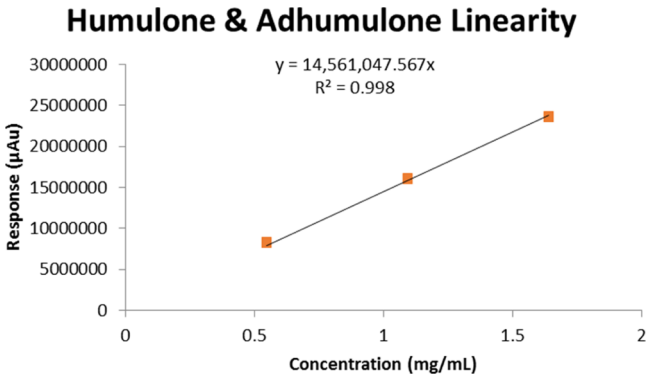

Figure 5. Three-point calibration curve of coeluting peaks humulone and adhumulone from a standard concentration range of 0.5463–1.6389 mg/mL.

Example Calculations. See eq 5 to calculate moisture content and eq 6 to calculate a sample's acid concentration.

Sample and Standard Calculations. During this study, actual samples were collected and stored at 4 °C for roughly 12 h prior to being analyzed. Excel was used for all calculations with intermediates left unrounded throughout and reported results rounded to two decimal places. For this sequence, the working standard % RSD was 2.3% for n = 5 and 1.9% for n = all. The standard agreement was 100.05%. Refer to the Supporting Information for sample calculations. Results for the α-acid concentrations of hops are reported in both their freshly harvested form and packaged conditions of 10% moisture content. β-Acids are calculated by the same procedure. As previously mentioned, the effect of β-acid on overall bitterness contributes a small fraction to that of its counterpart. Therefore, β-acids are not included in the calculation of IBUs and are not reported on most hops that are packaged for sale.

Limitations. Sample size, sample collection, and lab error were the main limitations during this study. Samples were collected from a single hop plant for every time point analyzed. Although this showed how a single plant matured during harvest, it did not consider the variability between plants on a farm. Also, there was variability in how each sample was collected; ideally you would want to create a homogeneous sample by collecting hops throughout the bine. Last, lab error was a considerable limitation, although measures were taken to reduce variability and error. When working with biological samples, there is concern regarding homogeneity. To limit this, extracting larger sample sizes multiple times would be necessary.

Discussion. This method contributes to previous methods[5,6] with its consideration of an evolving brewing industry and its use of fresh "wet" hops, enabling brewers to report a beer's perceived bitterness. This method is applicable from an industrial standpoint for hop growers and brewers alike who want to fully inform their consumers about their product. This publication along with the Supporting Information has used the procedure of analyzing fresh hops step by step and made it accessible for chemists with minimal analytical liquid chromatography experience. It has provided the option of utilizing numerous pharmaceutical grade quality checks to ensure precise quantitation.

During the study of the 2017 Sonoma County hop harvest, results were collected for hops used in numerous "wet" hopped beers in breweries throughout Sonoma County. This study attempted to limit as many variables between multiple farms as possible by analyzing hops of the same cultivar and origin that were planted in the same year. The Northern California Hop Growers Alliance also utilized universal growing conditions among the members participating in this study such as the average distance between rhizomes, daily water intake, and soil amendments. Problems associated with small-scale hop cultivation include knowing when to harvest as well as knowing the chemical makeup of those hops. Without access to a lab, harvesters judge the maturity and ideal harvest time of a hop plant based on the cone moisture content and perceived aromas.[14] The goal of this research is to develop a method for analyzing fresh hops as well as comparing the chemical profile and peak harvest time differences between hops grown in Sonoma County to those grown in Yakima, WA. See Table 3 for a result summary including moisture content and calculated α-acid concentrations at harvest as well as in packaged conditions. These results generally confirmed that the α-acid concentrations found within the cascade cultivar are between 5 and 7% as far south as the 38th parallel where Sonoma County lies. These data give legitimacy to small hop farmers in Sonoma County, which in turn increased the acreage of hop plants in the area as well as instilled confidence in the quality of the product for consumers. From these results, a direct correlation between the cone moisture content and α-acid concentration is not observed. Parts of Sonoma County received rainfall multiple times over the course of this study in addition to having varying humidity levels. Both of these factors significantly affect the hop moisture content. This explains the irregular observations in moisture content during the 2017

DOI: 10.1021/acsomega.9b00016
ACS Omega 2019, 4, 3565–3570

Table 3. Summary Results of Sonoma County Cascade Hop Samples from the 2017 Harvest Season

Sonoma County cascade hop sample							literature
location	farm	year planted	date harvested	moisture content %	alpha acid % (w/w) at 10% moisture content	alpha acid % (w/w) at harvest	predicted alpha acid % (w/w) of the cascade cultivar
Sonoma	BiRite	2015	8/11/2017	66.08	5.98	4.39	5.0−7.0
			8/18/2017	60.32	5.87	3.93	
			8/25/2017	68.13	7.19	5.45	
		2016	8/11/2017	66.97	6.79	5.06	
			8/18/2017	62.65	6.09	4.24	
			8/25/2017	67.12	7.03	5.24	
Sebastapol	warm springs wind	2016	8/11/2017	63.54	7.55	5.33	
			8/18/2017	64.16	6.78	4.83	
			8/25/2017	70.35	8.97	7.02	
	Redwood Hill	2016	8/11/2017	41.52	3.49	1.61	
			8/18/2017	58.34	5.43	3.52	
			8/25/2017	65.01	5.39	3.89	
Cloverdale	Eric	2016	8/18/2017	69.84	5.38	4.17	
Santa Rosa	Cassius	2017	8/11/2017	64.58	1.76	1.26	
	Fogbelt Brewing Co.	2016	8/11/2017	73.81	5.36	4.39	
			8/18/2017	61.35	6.22	4.24	
			8/18/2017	71.08	6.89	5.44	wild cultivar

harvest season. It should be noted that a significant amount of variability of α-acid concentration was observed depending on the date samples were harvested from that plant. This affirms that the time of harvest is an important factor in hop cultivation. It is also worth noting that the one sample harvested in the same year it was planted had the lowest concentration of bittering acids, suggesting that a hop plant needs one full season to fully mature.

Previous methods, such as Hops 14[5] from the ASBC and HPLC analysis of α- and β-acids in Hops from the Journal of Chemical Education,[6] focus on analyzing processed hops in a pelletized matrix. With this method, fresh hops can be analyzed and the moisture content can be used to quantify the acids as well as predict concentrations after processing. This is an important factor for everybody involved in the brewing industry. The preparation of mobile phase was improved upon by adjusting the aqueous portion to a specific pH giving chromatograms consistent retention for each analyte peak. This is necessary when quantifying compounds by improving the peak shape for undeviating integration. All the calculations needed to observe system suitability such as peak resolution and standard precision are included in this method along with suggested criteria based off of suggestions from the United States Pharmacopeia ⟨621⟩. Also, sample and standard calculations are left in a broad format for labs without the specific glassware used in this method. This procedure was made to be user-friendly and rugged across all labs in order to reduce time in method development as well as reducing costs of lab supplies.

CONCLUSIONS

This method is an example of how freshly harvested hops can be analyzed. This procedure is preferred over previous methods because of its considerations of sample moisture content, accuracy, efficiency, and system variability. Accurate quantification is ensured with the application of multiple calibration standards, a check standard, and an efficient sample extraction method. Run times have been reduced to below 30 min while also decreasing the flow rate for an efficient use of solvent. Also, variability in peak retention has been reduced by using a mobile phase with a specific pH. This method addresses common problems with HPLC method development and explains general solutions. This makes it an applicable method for less-experienced analysts and helps the user develop their own method for their specific system.

% RSD of working standards is calculated as follows:

$$\%\ \mathrm{RSD} = \frac{\mathrm{SDn}}{\mathrm{mean}} \times 100 \tag{1}$$

where SDn is the standard deviation of working standard areas, mean is the average area response of working standards, and 100 = conversion into a %.

RF of working standards (n = all) is calculated as follows:

$$\mathrm{RF_{Cal}} = \frac{\mathrm{Avg\ area_{Cal}}}{([\mathrm{Cal}] \times P_{\mathrm{Cal}})} \tag{2}$$

where Avg $\mathrm{area_{Cal}}$ is the mean peak area of calibration standards (n = all), [Cal] is the concentration of calibration standard in (mg/mL) from recorded weight and dilutions, P_{Cal} is the potency of the standard substance as a decimal as reported by the ASBC. 0.1344 for cohumulone, peak 1, 0.3344 for n ± humulone, peak 2, 0.1320 for colupulone, peak 3, and 0.1054 for n ± lupulone, peak 4.

Agreement between working and check standards is calculated as follows:

$$\%\ \mathrm{agreement} = \frac{\mathrm{area_{Chk}}}{(\mathrm{RF_{Cal}} \times [\mathrm{Chk}])} \times 100 \tag{3}$$

where $\mathrm{area_{Chk}}$ is the peak area of check standard, $\mathrm{RF_{Cal}}$ is the RF of calibration standards (n = all), and [Chk] is the concentration of check standard in (mg/mL) from recorded weight and dilutions.

Resolution between two peaks is calculated as follows:

DOI: 10.1021/acsomega.9b00016
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$$R_s = \frac{2(t_2 - t_1)}{W_1 - W_2} \quad (4)$$

where t_1 is the retention time of peak 1, t_1 is the retention time of peak 2, W_1 is the baseline width of peak 1, and W_2 is the baseline width of peak 1.

% Moisture content of wet hops is calculated as follows:

$$\%\ \text{moisture content} = \frac{W_{wet} - W_{dry}}{W_{wet}} \times 100 \quad (5)$$

where W_{wet} is the weight in grams of wet hops before drying, W_{dry} is the weight in grams of dried hops, and 100 is the conversion into a %.

% w/w of α- and β-acids in hops at 10% moisture content is calculated as follows:

$$\text{acid},\ \%\ \text{w/w} - \frac{A_{smp}}{A_{Cal}} \times \frac{W_{Cal} \times P_{Cal}}{W_{smp}} \times \frac{DV_{smp}}{DV_{Cal}} \times 100 \times 0.9 \quad (6)$$

where A_{smp} is the sample average peak area of cohumulone, (ad)humulone, colupulone, and (ad)lupulone; A_{Cal} is the mean peak area of cohumulone, (ad)humulone, colupulone, and (ad)lupulone in the working standard chromatograms; W_{Cal} is the weight of the working standard solution in g; P_{Cal} is the potency of the standard substance as a decimal as reported by the ASBC. 0.1344 for cohumulone, peak 1, 0.3344 for $n \pm$ humulone, peak 2, 0.1320 for colupulone, peak 3, and 0.1054 for $n \pm$ lupulone, peak 4, DV_{Cal} is the dilution factor of standard solution in mL (500), DV_{smp} is the dilution factor of sample solution in mL (1000), W_{smp} is the weight of the total sample weight extracted in g, 100 is the conversion into a %, and 0.9 is the moisture content conversion.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00016.

Standard operating procedure for the quantification of α- and β-acids in fresh hops with sample calculations and recording sheets (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Thanks are given to Dow Pharmaceutical Sciences for donating HPLC equipment for analysis as well as the Nor-Cal Hop Growers Alliance for donating loose cascade hops. We also greatly appreciate the Sonoma State University Chemistry Department for their expertise and funding.

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DOI: 10.1021/acsomega.9b00016
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