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Identification and quantification α and β-acids of Columbus Hops using Reverse Phase HPLC

Zulfikar*[a] and Antje Labes[b]

Abstract: The present study has demonstrated the HPLC method for identification and determination α and β-acids in Columbus hops. The measurement conditions has been set up included the use of reverse phase C18 column Chromolith, gradient elution with methanol (A) and Acetonitril acidified with phosphate buffer at pH 2.8. The flow rate has been applied of 0.8 mL/min, and run for 25 minutes. In addition, pretreatment sample has been done using SPE with the cartridge of C18 and the separation has been monitored using UV variable detector in the range of 300-340 nm. The results found that a four predominant peaks for α and β-acids, namely cohumulone (6.6 min), the second peak represents a mixture of humulone and adhumulone (8.9 min), the third peak is colupulone (15.9 min) and the fourth is a mixture of lupulone and adlupulone (16.7 min). The quantity of sample Columbus Hops has been determined according to multipoint calibration using ICE2 standard, and it is found that the sample contained 0.269 mg/mL of cohumulone, 0.341 mg/mL of humulone and adhumulone, 0.239 mg/mL of Colupulone and 0.141 mg/mL of mixture of lupulone and adlupulone. In addition, a high precision of measurement performed that is indicated by the value of RSD less than 5%.

Keywords: α and b-acids of Columbus hops; SPE; Reverse Phase; HPLC.

INTRODUCTION

Since ancient times, hops extract (*Humulus lupulus* L.) has been used as an additive agent in the brewing industry to give it a unique taste and aroma. Studies of hop extract have been carried out intensively and have shown the presence of compounds with antimicrobial and antioxidant properties. These results have encouraged and ushered in the use of hop extracts in a variety of fields.

In the food and beverage industry, hop extract is used as an antimicrobial [1] showed that hop extracts with various concentrations of α and β acids inhibited the growth of L. *monocytogenes* in coleslaw, milk, and cottage cheese. Meanwhile, reported the effectiveness of using hops extract as an antifungal in bread making [2]. In addition, also reported that the antimicrobial activity of hops extract was higher in meat marinades at low pH and stored in chilled storage [3].

In the field of health, especially traditional medicine, hops extract has been widely used as a mild sedative and hypnotic [4, 5]. Polyphenolic compounds that have neuroprotective activity against ischemic stroke in rats [6]. This indicates the ability and potential of hops extract as a medicine for various neurodegenerative diseases such as dementia, alzheimers or parkinsons [7].

The major bio-active compounds present in hops extract that play important role in directly or synergistically acts as an antimicrobial, antibacterial, antioxidant and anticancer namely α-acids; humulone, cohumulone, and adhumulone, β-acids; lupulone, colupulone, adlupulone and found a flavonoid as a xanthohumol [8]. The α and β-acids have similar molecule, but the subtle difference in their structures which is enough for them having different polarities.

The big role of extract hops, especially α and β-acids in various industrial fields, requires fast and accurate analysis for the purposes of identification, monitoring and routine analysis. A wide range of methods have been developed for their analyses and determination of alpha and betha acids. Automatic titration wih conductometric detection using reagents of methanol, lead acetate solution, diethyl ether and hydrochloric acid have been demonstrated [9]. Applied spectrophotometric method using wavelength of 355, 325 and 275 nm for the α-acids, β-acids, and the other component, respectively [10, 11]. Currently the use of HPLC is the choice in determining these acids using either electrochemistry [12], spectrophotometry [13, 14], and Mass Spectroscopy detectors [15, 16].

The analysis of the extract hops required a pre-treatment sample commonly using solvent extraction with methanol, ethanol or other solvents followed by microfiltration, as in the standard method of American Society of Brewing Chemists [17] and European Brewery Convention [18]. In addition, the Supercritical Fluid Extraction method was also introduced using Supercritical carbon dioxide is a non-polar solvent where this method has the advantage of less environmental pollution [19].

Focus of this research is to demonstrate a simple method for the quantification of α and β acids including cohumulone, humulone, adhumulone, colupulone, lupulone and adlupulone by reverse phase High Performance Liquid Chromatography (HPLC), utilizing ultraviolet detection. While the pre-treatment sample might be applied using solid phase extraction with C18 cartridge that is similar to its analytical coloumn.

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METHODS

Chemical, standard and sample

The chemicals used are analytical grade including methanol, Acetonitril, Ether, Phosphate buffer containing sodium dihydrogenphosphate and phosphate aqueous solution (Merck, Germany) and Purified water was obtained from a PURELAB purification system. For the analysis of α and β-acids, there is no individual standard solution available, so the standard solution used is the international calibration standard hop extract of EBC which contains the compounds cohumulone, Adhumulone + humulone, colupulone, Adlupulone+lupulone has been accurately determined. While, the sample used is a hop flower extract from Columbus hops USA.

The standard solution has been prepared by dissolving 96.2 mg ICE-2 extract which contained Cohumulone 14.45% Adhumulone + Humulone 34.94% and Colupulone 12.92% Adlupulone + Lupulone 12.02% in methanol, and it is then diluted to final volume of 25.00 mL. The solution was then filtered through a 0.2 µm nylon filter and used as stock solution as well as coded as Standard 5. The 10 mL of stock solution is diluted to be 20 mL which is coded as Standard 4. This solution is then diluted again so that the concentration is halved. This process is repeated and found the final concentration of Standard 1 solution is 1/16 times that of standard 5 or stock solution.

Columbus hops sample was weighted of 1000 mg and extracted with solvent containing 100 mL ether, 20 mL methanol and acidified by few drops of 0,1M HCl, then sonicated for 30 min at 25 ^{o}C using an ultrasonic bath (Ultrasonic Cleaning, RND). The sample of 10 mL is then loaded on a C18 solid-phase extraction (SPE) cartridge (Waters) after conditioning with 5 mL methanol and 5 mL water. The SPE cartridge was then washed with 5 mL of water and the fraction of interest was eluted with 5 mL of methanol. Finally, a portion of the extract was diluted using 45 mL methanol and filtered with a 0.20 µm PTFE membrane filter before introduced to the column HPLC.

Instrumentation and measurement conditions

The chromatographic system used is An Agilent 1200 HPLC series (Hewlett-Packard, Waldbronn, Germany) which is equipped by vacuum degasser, a quaternary pump, an autosampler, a column thermostat system, and UV detector. ChemStation software has been installed and used to control the HPLC. While the Chromatographic separation was achieved with a Chromolith Reverse Phase C18 end capped column (250 × 4.6 mm, 3 µm).

An autosampler has been used to introduce 10 mL of standard or sample solutions to the reverse phase C18 column that was maintained temperature at 25 °C. The separation process was carried out in reverse phase with gradient elution. The eluent used are methanol 45% as an eluent A and Acetonitrile 52% as an eluent B conditioning on pH 2.8 using phosphate buffer pH 2.8 consisting of sodium dihydrogenphosphate and phosphate aqueous solutions. The gradient elution started with the composition of eluent 48% methanol A and 52% acetonitrile (B), while the flow rate was set at 0.8 mL/min using pressure pump on the range of 10-180 Bar. Furthermore, the polarity eluent was lowered by increasing gradually the concentration of acetonitrile and reaching 100% within 25 minutes. The separated analytes were recorded using a variable wavelength UV detector in the range of 300 to 340 nm with the slit width set at 2 nm.

RESULT AND DISCUSSION

Separation α and β-acids

Analysis of the separation of α and β acids samples by comparing the chromatograms of the samples and standard ICE 2 solutions. Separation is carried out by injecting 10 µL the standard solution and followed by the sample solution. The use of HPLC with an RP C18 column and a gradient eluent consisting of methanol and acetonitrile which was acidified with phosphate buffer pH 2.8 showed excellent separation performance. It is indicated that the chromatogram of the ICE-2 standard shows four predominant peaks for α and β-acids. The first α-acid peak, from the injection point, is given by cohumulone (6.5 min). The second peak represents a mixture of humulone and adhumulone (8.8 min). Similarly for β-acids the first peak represents colupulone (15.8 min) and the second represents a mixture of lupulone and adlupulone (16.6 min). For the second and fourth peaks, the two compounds cannot be separated and they coeluate. This is due to the similarity of the hydrophobicity of humulone with adhumulone and lupulone with adlupulone. Only four chromatograms found, these are shown on Figure 1 [20]. The use of pH 2.8, which might reduce the variability in spacing and increase the resolution, considering that the two analytes have a pKa ranging from 4.25 to 5.93. [20, 21]

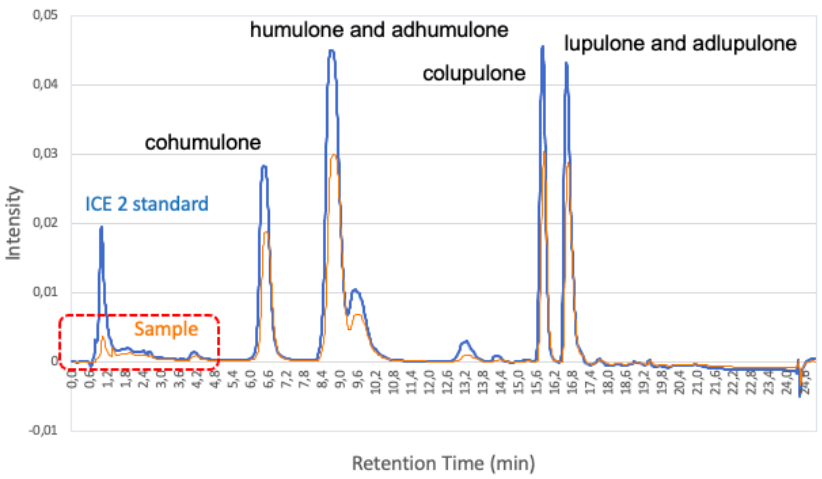

Figure 1. Separation of a and b acids of the ICE2 standard and Columbus Hops sample

On the separation of the Columbus hops samples, the results showed that the chromatogram had the same pattern as the standard pattern. However the retention time of the sample is slightly slower than the standard. A comparison of the retention times is presented in Table 1.

Table 1. Retention time of α and β acids on ICE2 standard and Columbus hops

Source	Retention time (minutes)			
	Cohumulon	N+Adhumulon	Coulupulon	N+Adlupulon
ICE2 Standard	6.5	8.8	15.8	16.6
Columbus Hops	6.6	8.9	15.9	16.7

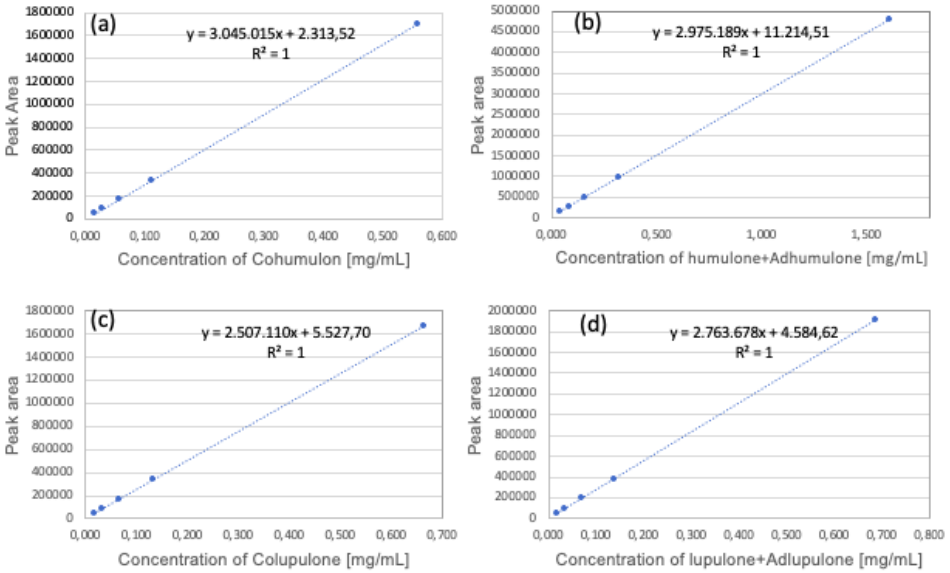

Figure 2. Calibration curve for cohumulone (a), humulone+adhumulone (b), colupulone (c) and calibration curve for lupulone+adlupulone (d)

The time consumed for the present method in identifying α and β acids is better than the results where the retention time of alpha acid is above 10 minutes and β acids is greater than 19 minutes [20]. In addition, the samples were quite well separated indicating that some impurities appeared and separated by retention time on the range of 1.1 - 4.5 minutes. However, the impurities signal did not interfere with analyte peaks, so accuracy was retained. Several compounds that appeared before the cohumulone compound signal were iso-a-acids, this signal was very sharp when detected using UV at a wavelength of 270 nm [22]. Finally, this method also has a minimum baseline resolution of the other components in the mixture which is shown its selectivity parameter.

Calibration, LOD and LOQ

Calibration curves were prepared using standard ICE2 solutions for each concentration ranges of 0.014 - 0.556 mg/mL, 0.040 – 1.608 mg/mL, 0.017 – 0.663 mg/mL, and 0.017 - 0.668 mg/mL for cohumulone, humulone and adhumulon, colupulon and lupulone and adlupulone respectively. Those standard were then injected in duplicate for each concentration. Based on the mean peak area versus, it is found the calibration curves as simplified in the following Figure 2.

Figure 2 shows that the multiple point calibration curve shows its linearity as shown by the regression equation, namely y = 3.045.015x + 2313.52, y = 2.975.189x + 11214.51. y = 2.507.110x + 5527.70 and y = 2.763,678x + 4584.62, for cohumulone (a), humulone+ adhumulone (b), colupulone (c) and lupulone+adlupulone (d) respectively. This is also supported by the distribution of data which fit to the equation of the line and it is indicated by the correlation factor (R^2) = 1 for α and β acids standard.

Performance measurement is done by evaluating the accuracy and precision of measurement results. The accuracy was assessed by standard addition method, a precisely prepared ICE2 standard solution was spiked into a sample Columbus hops. It is done in duplicate and found that the recovery percentages at low concentration around 98 %, with a correspondingly low relative standard deviation of < 1 %. In addition, limit of detection (LOD) and limit of quantification (LOQ) are also reasonable high. LOD was determined based on the standard deviation of y-intercepts of regression lines according to the equation of LOD = 3.3 (Sy/S). Similar model was also used for the calculation of LOQ that is can be determined based on the standard deviation of y-intercepts of regression lines following the equation of LOQ = 10 (Sy/S). These results are summarized on the following Table 2.

Table 2. Limit of Detection and Quanitification based on calibration curve

α and β acids	Average of Intercept	SD	Slope	LOD	LOQ
Cohumulone	2313.52	139.58	3045.02	0.05	0.46
Humulone+Adhumulone	11214.51	229.81	2975.19	0.08	0.77
Colupulone	5527.70	220.76	2507.11	0.09	0.88
Lupulone+Adlupulone	4584.62	173.81	2763.68	0.06	0.63

Table 3. The performance of Quantitative measurement a and b acids on real sample Columbus Hops

Component	Quantity Sample mg/mL	RSD Sample	% Standard	% Sample	% α acids standard	% β acids Sample
α acids						
Cohumulone	0.269±0.001	0.34	14.45	18.27	49.39	41.28
Humulone+Adhumulone	0.341±0.016	4.55	34.94	23.01		
β acids						
Colupulone	0.239±0.001	0.42	12.92	16.24	24.94	25.71
Lupulone+Adlupulone	0.141±0.002	1.26	12.02	9.47		

Determination of Columbus hop sample

Real sample analysis was performed using the Columbus hop sample. The elution results in the solid phase extraction process were diluted with methanol to reach a final volume of 45 mL. Then the sample solution was filtered using a 0.20 µm PTFE membrane, and the results were ready to be injected into the HPLC.

Sample quantification was measured in duplicate and the results obtained were 0.269±0.001 mg/mL cohumulone, 0.341±0.016 mg/mL of humulone and adhumulone, 0.239 ±0.001 mg/mL of Colupulone and 0.141±0.002 mg/mL of mixture of lupulone and adlupulone. In addition it is high precision measurement that is indicated by the value of RSD less than 5 %. The result is simplified on Table 3.

Table 3 also shows the amount of α-acid and β-acid is different, the total percentage of α-acid in columbus hops is smaller compared to standard ICE2, this causes that Columbus is less bitter. The relative amount of β-acid does not differ, so that both hops have the same strong aroma. This difference is related to the unique characteristics of hops, where the composition of α-acid and β-acid varies greatly. The average α-acid and β-acid weight percentage varies among the varieties of hops but is typically between 3 and 15% with β-acid concentration between 2 and 8%.

CONCLUSION

The HPLC method described are suitable for identification and determination α and b-acids in Columbus hops. The procedures are sufficiently rapid for separating and monitoring standard and sample with running times around 25 minutes and the flow rate has been set up 0.8 mL/min. A simple pretreatment sample has also demonstrated using SPE with the cartridge of C18 similar to the analyte column. The α and β-acids namely Cohumulone, mixture of humulone and adhumulone, colupulone and mixture of lupulone and adlupulone have been separated. Multipoint calibration calibration approach has excellent performances for determining sample. Which shown by its linearity in wide range, minimum error and high precision measurement that is indicated by the value of RSD less than 5%. α-acid and β-acid in Columbus hops has been determined that are 0.269 mg/mL cohumulone, 0.341 mg/mL of humulone and adhumulone, 0.239 mg/mL of Colupulone and 0.141 mg/mL of mixture of lupulone and adlupulone. Finally, it can be concluded that the present HPLC method is quite simple and can be used for routine commercial analysis and quality control purposes.

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