Solid-Phase Extraction of Hop Acids from Beer or Wort for Subsequent Analysis

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ABSTRACT

A procedure for extracting hop acids from beer or wort was developed. A solid-phase extraction procedure employing a 3.0-ml Bakerbond spe* octyl column was used to absorb the hop compounds. After the concentrated hop compounds were washed, they were desorbed with methanol. UV measurement of either international bitterness units or high-performance liquid chromatography (HPLC) was used to determine the amount of hop acids present. UV analysis could be correlated with the ASBC-recommended procedure for bitterness units. HPLC analysis allowed quantitation of hulupones, humulinic acid, isohumulones, humulones, lupulones, ρ-isohumulones, and tetrahydro-isohumulones. Recovery of isohumulones is better than 90%, and recovery of other specific hop compounds is better than 75%.

Keywords: Hop resins, HPLC, International bitterness units, Solid-phase extraction

A necessary first step in the analysis of hop bitter acids is their separation from the many interfering compounds naturally present in the beer or wort matrix (2). Liquid-liquid extraction procedures have been the mainstay for this separation but usually are cumbersome and require large volumes of solvent (5). Solid-phase extraction procedures have gained in popularity over the last 10 years and have, to a great extent, replaced the conventional liquid-liquid extraction procedures (6). The Institute of Brewing, in a recently completed collaborative study on the estimation of iso-α-acids in beer by high-performance liquid chromatography (HPLC), used an octadecyl solid-phase extraction column (200 mg × 3 ml) for sample preparation. Although the results obtained by the HPLC method were not precise enough to permit adoption as a recommended method, the HPLC method was suggested as an alternative method to the bitterness units procedure because of its advantage in measuring bitterness in terms of iso-α-acid content (4).

The purpose of this research was to develop an improved extraction procedure for the analysis of hop bittering compounds. The octyl column was tested for its suitability for this extraction.

EXPERIMENTAL

Equipment

Bakerbond spe* octyl (C_8) extraction columns (3 ml × 500 mg) were used (7087-03, J. T. Baker, Phillipsburg, NJ). HPLC was performed with a Perkin-Elmer Series 3B pump, an LC-85-B ultraviolet detector, and an LCI-100 integrator (Perkin-Elmer Corp., Norwalk, CT). The analytical column was a Shim-pack CIC-ODS/H (4.6 mm × 25 cm). The solvent guard column was a Shim-pack GRD-ODS (4.0 mm × 25 cm) (Shimadzu Corp., Tokyo, Japan). The column heater was a Croco-Cil standard (C.I.L. Corp., Sainte-Foy-La-Grande, France). The solid-phase extraction system was a J. T. Baker model 10 used with 2.0-ml volumetric flasks (Kimble, Vineland, NJ). An Altech HPLC solvent-filtering apparatus (7326) and nylon 0.45-μm filters (2024) were used (Altech Corp., Deerfield, IL). A Perkin-Elmer Lambda 3B spectrophotometer was used for UV absorbance measurements.

Reagents

The chromatographic solvents used were HPLC-grade methanol (Burdick & Jackson Labs, Muskegan, MI), 85% phosphoric acid (Mallinckrodt Chemical Co., St. Louis, MO), tetrabutylammonium phosphate (Aldrich Chemical Co., Milwaukee, WI), Milli-Q deionized water (Millipore Corp., Bedford, MA), and octanol (Baxter Scientific, McGaw Park, IL). The ASBC α- and β-acid standard (ASBC, St. Paul, MN) and commercial hop extracts were used for calibration and recovery studies.

Chromatographic Conditions

The HPLC chromatographic conditions were slightly modified from the procedure described by Ono et al (7), and the paired ion chromatography reagent of Buckee (3) was used. The HPLC was operated in the solvent program mode. Mobile phase A consisted of 725 ml of methanol with 275 ml of H_2O containing 2.95 g of tetrabutylammonium phosphate and 17 g of 85% phosphoric acid. It was mixed and filtered through a 0.45-μm nylon filter. Mobile phase B was methanol. The column temperature was 50°C and the flow rate was 1.5 ml/min. Gradient conditions were 3 min at 90% A, 7 min to 50% A, with curve 1 hold for 5 min at 50% A, then 10 min to 0.1% A, with curve 1 hold for 3 min, then a return to equilibrium for 4 min at 90% A. A 20-μl full-loop injection was used. The detector was operated at 275 nm. The integrator was in the area percent mode. The various classes of hop compounds can be seen in Figure 1, starting with the oxidation or Ono S fraction (8), followed by the iso-α-acids and then the α- and β-acids. In addition to the known

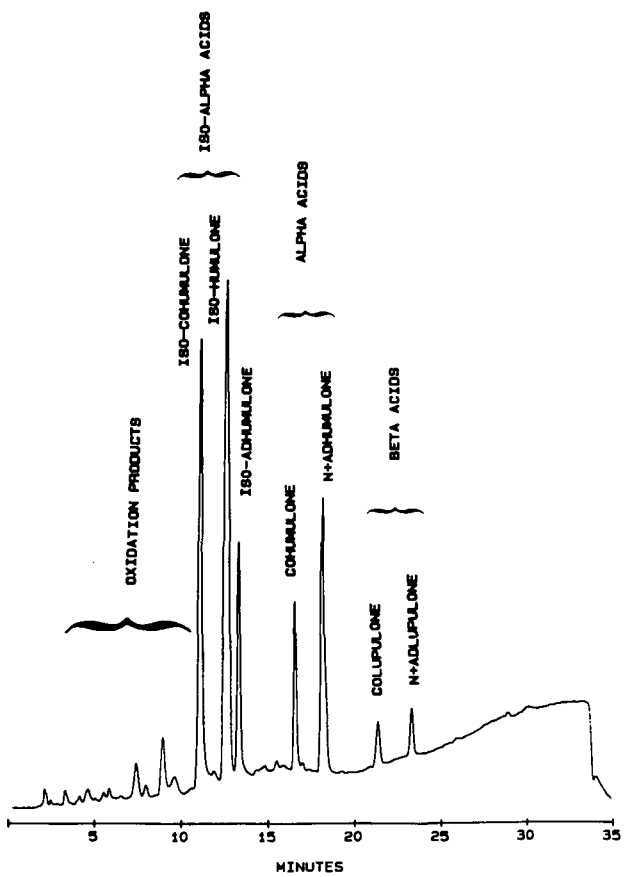

Fig. 1. A beer chromatogram with additional spiked α- and β-acids.

TABLE I
High-Performance Liquid Chromatography Reproducibility After Replicate Injection of α- and β-Acids Mixture

	Cohumulone		N+ Adhumulone		Colupulone		N+ Adlupulone	
Statistic	Retention Time (min)	Peak Area ($\times 10^6$)	Retention Time (min)	Peak Area ($\times 10^6$)	Retention Time (min)	Peak Area ($\times 10^6$)	Retention Time (min)	Peak Area ($\times 10^6$)
Number	6	6	6	6	6	6	6	6
Mean	16.08	2.30	17.60	5.14	20.67	2.24	22.8	1.81
Variance	0.0004	0.00211	0.0008	0.00437	0.009	0.00619	0.0006	0.00115
SD	0.020	0.014	0.027	0.021	0.0301	0.008	0.024	0.011
cv	0.13	0.63	0.16	0.41	0.15	0.35	0.11	0.59

TABLE II
Effects of Solid-Phase Extraction Column Conditioning[a]

Methanol Wash (ml)	Deionized Water Wash (ml)	Sample Eluate[b] A (275 nm)
0	10	0.288
0.5	10	0.277
2.0	10	0.281
10.0	10	0.290
0	0	0.273
0	1	0.280
0	3	0.279
0	10	0.278
2.5	0	0.637
2.5	1	0.270
2.5	10	0.267

[a] Beer volume 20 ml, pH 2.5.
[b] Diluted 1:20.

TABLE III
Effects of Sample Degassing[a]

	HPLC[b]		
Condition	Sample Eluate Iso-α-Acids Peak Area ($\times 10^6$)	Sample Eluate α-Acids Peak Area ($\times 10^4$)	UV Spec[c] Sample Eluate A (275 nm)
Not Degassed	3.55	5.82	0.307
Degassed with foam	3.37	4.94	0.264
Degassed with one drop of octanol	3.61	7.60	0.274
Degassed with 1 ml of octanol	2.90	7.44	0.259

[a] Beer volume 20 ml, pH 2.5.
[b] High-performance liquid chromatography.
[c] UV solid-phase extraction column. Eluate diluted 1:20.

TABLE IV
Effects of Beer pH Adjustment[a] After Addition of 85% H_3PO_4 to Sample

85% H_3PO_4 (μl/100 ml)	pH Measured	Sample Eluate[b] A (275 nm)	Sample Eluate Peak Area ($\times 10^6$)
0	4.3	0.129	1.32
100	3.0	0.266	3.51
200	2.4	0.289	3.68
1,000	2.0	0.304	3.66

[a] Volume applied to solid-phase extraction column was 20 ml.
[b] Diluted 1:20.

peaks identified on the chromatogram, other small unknown peaks also were present. For purposes of total area summation, the small unknown peaks were included with the peak areas for each class of known compounds. The reproducibility of the HPLC system was verified by six injections of a purified Galena hop extract dissolved in methanol. The results are shown in Table I. The use of peak areas resulted in coefficients of variation of less than 1%. Retention times also had coefficients of variation of less than 1%.

RESULTS AND DISCUSSION

Sample Preparation

The various stages in sample preparation were evaluated, i.e., column conditioning, sample addition, sample washing, and sample elution. Except for the parameters being varied, the following preparation scheme was followed. First, 2 ml of methanol was passed through the spe* C_8 column, followed by 2 ml of deionized water. Next, 20–40 ml of sample at pH 2.5 was applied. The column then was washed with 6 ml of acidified water (0.2 ml of H_3PO_4 per 100 ml), followed by 2 ml of a mixture of methanol, H_2O, and H_3PO_4 (50:50:0.2). The sample then was eluted with acidified methanol (3 × 0.6 ml) (0.1 ml of H_3PO_4 per 100 ml) and diluted to 2 ml with methanol. Finally, the effects of each procedural modification were measured either by diluting the eluate 1:20 with methanol and measuring the absorbance at 275 nm or by injecting 20 μl onto the HPLC and comparing peak areas.

Most manufacturers of solid-phase extraction columns recommend column conditioning before sample application. The suggested solvents for conditioning an octyl column are methanol followed by deionized water. Various combinations of methanol and deionized water wash volumes were examined. When the methanol volume was varied from 0 to 10 ml, followed by a 10-ml deionized water wash, all extracts had similar UV absorbance. Deionized water washing alone ranging from 0- to 10-ml volumes produced identical results. Methanol washing when followed by as little as 1 ml of deionized water washing produced similar results. Methanol conditioning alone resulted in more than a twofold increase in the UV absorbance. The reason for this increase was examined using HPLC. The increase was found to be primarily attributable to increases in void volume components or eluents and to a slight increase in oxidation products. If the methanol was not removed by drying or washing, problems arose in international bitterness unit (IBU) measurements. In spite of the fact that the column conditioning variables had no effect on the column performance, it was decided to retain the standard conditioning as part of the procedure. The data are shown in Table II.

Sample Additions

The effects of CO_2, antifoam, pH, and sample amount were examined. Beers must be degassed before being applied to the octyl column, because a nondegassed beer takes three to four times longer to pass through the column. Also, the UV absorption of the eluate is higher because of increases in void volume and oxidation product peak areas, whereas the iso-α-acids and α-acid peak areas are decreased (see Table III). If the foam head is not collapsed, losses in iso-α-acids and α-acids occur because of their retention in the foam. One drop of octanol per bottle of beer is sufficient for fast collapse of the foam. (However, if

1.0 ml of octanol is used, hop acid losses occur.) Degassing was accomplished by beaker-to-beaker transfers of the beer 20 times followed by one drop of octanol, then a few transfers to assure complete foam collapse.

The pH of the applied sample also is important for good iso-α-acid recovery. Table IV shows the effect of pH adjustment. Adjusting the pH of the sample to approximately 2.5 gave the best recovery of iso-α-acids and was easily accomplished by adding 200 μl of 85% phosphoric acid per 100 ml of beer. Wort may be adjusted to pH 2.5 by adding 400 μl of 85% phosphoric acid per 100 ml of wort.

Good recoveries were obtained when 20- to 120-ml aliquots of beer were applied to the octyl column (Fig. 2). Normally, either 20- or 40-ml sample aliquots were applied.

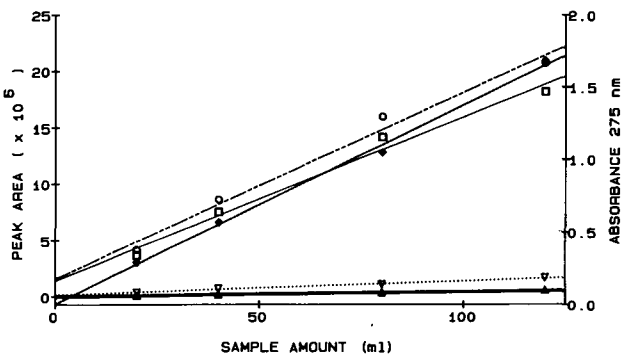

Fig. 2 Standard regression plots of the high-performance liquid chromatography peak area of the various classes of hop compounds with increasing sample applied to the octyl column. ○ = Total peak area, $r = 0.992$; □ = iso-α-acids peak area, $r = 0.991$; △ = α-acids peak area, $r = 0.999$; ▽ = oxidation products peak area, $r = 0.996$; and ◆ = UV absorbance at 275 nm of a 1:20 dilution, $r = 0.998$.

TABLE V
Effects of Column Wash Acidification Variables[a]

Water Wash (H_2O + 0.2 ml H_3PO_4/100 ml)	Water Wash Reapplied and Eluted from Octyl Column Iso-α-Acids Peak Area (× 10^5)
First 5 ml	0
Second 5 ml	0
Fourth 5 ml	0
40 ml	0.99
20 ml (no H_3PO_4)	9.93

[a] Beer volume 40 ml, pH 2.5.

TABLE VI
Effects of Column Wash Solvent Volume Variables[a]

Wash Volume[b] (ml)	Sample Eluate Iso-α-Acids Peak Area (× 10^6)
0	3.66
2	3.64
4	3.52
10	3.66

[a] Beer volume 20 ml, pH 2.5.
[b] Methanol/H_2O/H_3PO_4 (50:50:0.2).

TABLE VII
Effects of Wash Solvent Composition Variables[a]

Solvent Wash Composition (Methanol/Water/H_3PO_4)	Solvent Wash Reapplied and Eluted from Octyl Column Iso-α-Acids Peak Area (× 10^6)	Sample Eluate Iso-α-Acids Peak Area (× 10^6)
2.0 ml of 40:60:0.2	0	6.76
2.0 ml of 50:50:0.2	0	6.62
2.0 ml of 60:40:0.3	0	6.54
2.0 ml of 70:30:0.2	1.34	6.17

[a] Beer volume 40 ml, pH 2.5; acid water wash 6.0 ml.

Sample Washing

Sample washing is required to remove nonhop and interfering compounds from the column; both water and methanol and water mixtures were used. Table V shows the results of water washing experiments. The addition of 0.2 ml of 85% phosphoric acid per 100 ml of water did not affect removal of nonhop compounds but did retard the premature elution of iso-α-acids from the column. Deionized water alone removed many more iso-α-acids. After 5 ml of acid water washing, most of the water-removable nonhop interfering compounds were removed. Bloomfield and Moir (2) used a solution of methanol, water, and phosphoric acid (50:50:0.2) to wash a C_{18} column. Washing with this combination and varying the amount from 0 to 10 ml did not affect iso-α-acid recovery (see Table VI). When various combinations of methanol and water were tried, no deleterious effect was observed unless the methanol concentration exceeded 60% (Table VII). The solvent washing composition of Bloomfield and Moir was selected with a 2.0-ml wash volume.

Sample Elution

Both eluant composition and elution volume portions were examined in eluting the sample from the octyl column. The addition of 0.1 ml of 85% phosphoric acid per 100 ml of methanol resulted in better recovery than did straight methanol. Further addition of phosphoric acid to 1.0 ml/100 ml methanol did not improve recoveries (see Table VIII). For ease in sample handling and calculations, a 2.0-ml final elution volume was preferred. Three successive and separate applications of 0.6 ml each of the eluting solvent gave the best overall recovery of the total range of components. This also resulted in the least amount of iso-α-acid that could subsequently be eluted with another 0.6-ml portion (see Table IX).

TABLE VIII
Effects of Solvent Composition on Elution[a]

Eluate Solvent Composition	Sample Eluate Iso-α-Acids Peak Area (× 10^6)	Further Elution[b] Iso-α-Acids Peak Area (× 10^6)
Methanol	6.84	0.360
Methanol + 0.1 ml of H_3PO_4/100 ml	6.92	0.273
Methanol + 1.0 ml of H_3PO_4/100 ml	6.79	0.274

[a] Beer volume 40 ml, pH 2.5, using 3 × 0.6 ml elution portions.
[b] Further elution at 0.6 ml.

TABLE IX
Effects of Eluting Sequence Variable[a]

Elution Sequence Volume (ml)	Sample Eluate Iso-α-Acids Peak Area (× 10^6)	Further Elution[b] Iso-α-Acids Peak Area (× 10^6)
1.8	7.09	0.130
1.2 + 0.6	7.17	0.105
0.9 + 0.9	7.22	0.103
0.6 + 0.6 + 0.6	7.16	0.097

[a] Beer volume 40 ml, pH 2.5.
[b] Further elution at 0.6 ml.

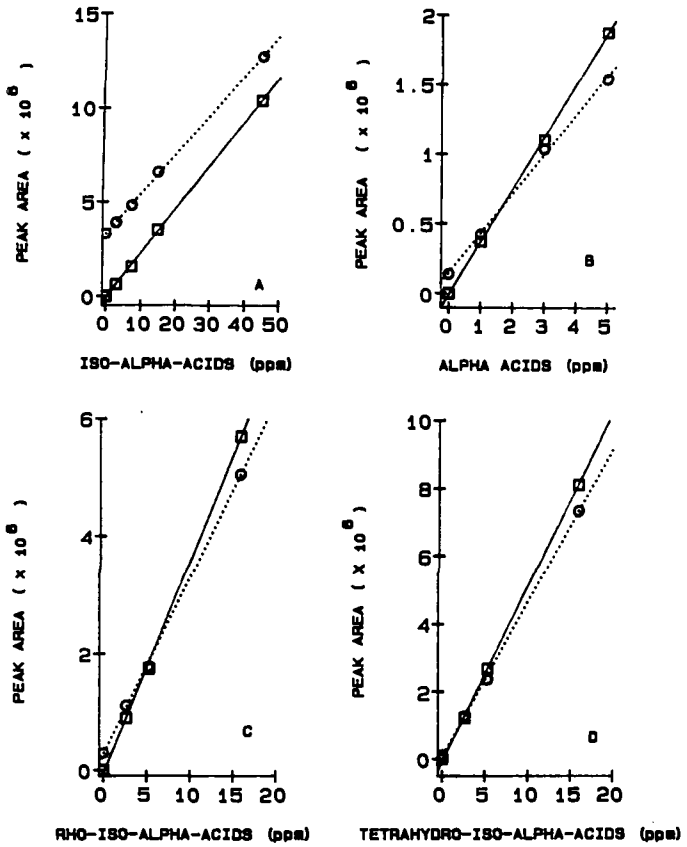

Fig. 3. Standard regression plot of the high-performance liquid chromatography area of beer spiked with hop acids through an octyl column compared with an equal concentration of the hop acid. **A,** Beer + iso-α-acid (O), standard solution (□); $r = 0.999$, recovery 90%. **B,** Beer + α-acid (O), standard solution (□); $r = 0.999$, recovery = 75.5%. **C,** Beer + ρ-iso-α-acid (O), standard solution (□); $r = 0.999$, recovery = 83%. **D,** Beer + tetrahydro-iso-α-acid (O), standard solution (□); $r = 0.999$, recovery = 88.7%.

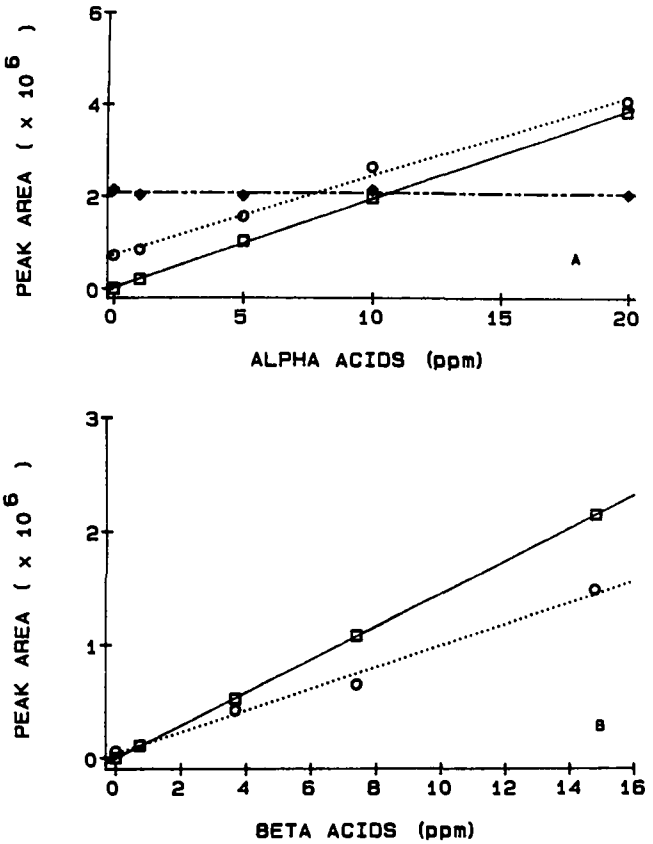

Fig. 4 Standard regression plot of the high-performance liquid chromatography peak area of wort spiked with hop acids through an octyl column compared with an equal concentration of the hop acid. **A,** Wort + α-acids (O), $r = 0.997$; □ = standard solution, $r = 0.999$; recovery = 89%; ◆ = iso-α-acids endogenous in wort. **B,** O = Wort + β-acids, $r = 0.995$; □ = standard solution, $r = 0.999$; recovery = 65%.

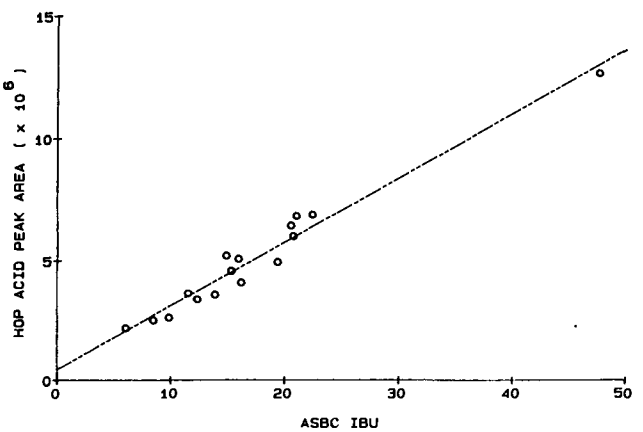

Fig. 5. Standard regression plot of the total high-performance liquid chromatography peak area of 16 competitive beers versus their ASBC international bitterness units (IBU) value. $r = 0.981$.

Recovery Studies

Additions of commercially available hop bittering extracts were used to check their recovery from beer and wort using the sample preparation procedure. Additions to beer included iso-α-acids, α-acids, ρ-iso-α-acids, and tetrahydro-iso-α-acids. Additions of α- and β-acids were made to wort for recovery studies. The manufacturers claim for concentration of the various hop acids was used without further verification. A graphical display of the data is shown in Figure 3. Because the procedure was optimized for the recovery of iso-α-acids from beer, their recovery was the overall best at 90%, whereas α-acids had the lowest recovery at 75.5%. The peak areas for the nonspiked sample were attributable to endogenous hop acids. Linear regression correlation coefficients for both standard injections and spiked beers were better than 0.999. The ASBC hop standard was used to spike wort (Fig. 4). The recovery of α-acids was 89%, whereas β-acids were recovered at 65.5%. This low recovery for the latter is probably due in part to their low solubility. In Figure 4, the iso-α-acid level was plotted to show the consistent recovery of this hop acid with increasing α-acid concentration spikes in the wort sample.

IBU Correlation

A set of 16 commercial beers was examined, and the total HPLC peak area for each was plotted versus the results obtained with ASBC Method Beer 23-A (1). A linear relationship was obtained with a correlation coefficient of 0.981 (Fig. 5). Until an acceptable iso-α-acid standard becomes available, this relationship could be used for the determination of IBU by HPLC.

When the eluate was diluted 1:20 with methanol and the absorbance measured at 275 nm, the readings agreed with the absorbance reading from the ASBC Method Beer 23-A (1). The solid-phase extraction procedure extracts a little more UV-absorbing material so a multiplier of 47 rather than 50 gives the corresponding IBU value (Fig. 6).

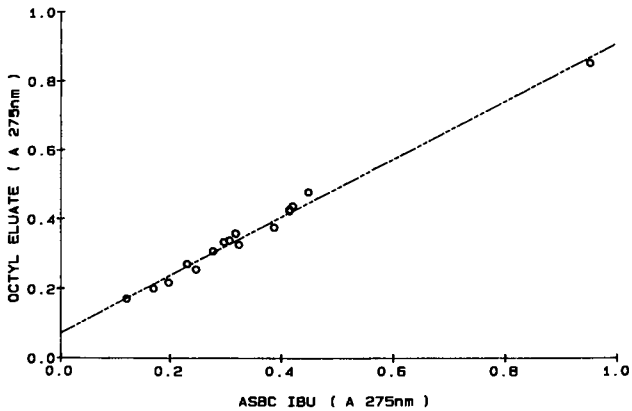

Fig. 6. Standard regression plot of the UV absorbance of a 1:20 dilution of the octyl column eluate of 16 competitive beers versus their ASBC international bitterness units (IBU) absorbance. $r = 0.994$.

C_8 Versus C_{18}

In comparing the C_8 and the C_{18} extraction columns under similar sample application techniques, the C_8 column took 3–6 min while the C_{18} column took 15–30 min for sample application. Also, the HPLC void volume peak areas were larger on the C_{18} extracts. α-Acid recoveries were better on the C_{18} extracts.

CONCLUSIONS

The Bakerbond spe* octyl column may be used for the separation of hop bittering compounds. All steps in sample preparation were examined and a preferred protocol was established. Recovery of the various hop acids was good. Values ranged from 75 to 90%, and all correlation coefficients of spiked samples were better than 0.99. α- And β-acids can now be quantitated using the ASBC standard. Quantitation of iso-α-acids and the other hop acids require standards. Alternatively, quantitation of various hop acids as their IBU contribution may be used.

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