Applications of High-Performance Liquid Chromatography in the Control of Beer Bitterness[1]

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ABSTRACT

Isocratic high-performance liquid chromatography (HPLC) on reverse-phase columns was used to measure α- and β-acids in hops and hop products and also to measure iso-α-acids in stout worts and beers. For fresh hops and for hexane extracts of hops, the contents of α-acids measured by HPLC were similar to the lead conductance values, but for aged hops marked differences were found. The effects of different conditions on isomerization of α-acids in hop extracts boiled in different production-scale and laboratory-scale vessels were also studied. Decreases in α-acids were shown to follow first-order reaction kinetics, and the rates were influenced by temperature, pH, and the concentrations of divalent cations. Utilization of α-acids was higher in a production-scale superbarometric kettle at elevated temperatures than when worts were boiled at 100° C, cohumulone always being utilized preferentially. A rapid spectrophotometric method did not give an accurate measure of utilization because of variations in the amounts of non-iso-α-acid bittering substances present in iso-octane extracts of worts and beers. Tasting trials, however, indicated that the results of the rapid spectrophotometric method related to palate bitterness better than did measurements of iso-α-acids.

Keywords: α-Acids, β-Acids, High-performance liquid chromatography, Iso-α-acids, Isomerization, Utilization

The potential value of high-performance liquid chromatography (HPLC) as a technique for investigating the use of hops in brewing was recognized (31) long before purpose-built stationary phases became available commercially. It was claimed that the facility with which individual congeners of iso-α-acids, α-acids, and β-acids were separated offered new scope in the control of both beer quality and the economic usage of hops. Since those exploratory studies, various workers (16,23,32,35,41,44) have developed the analytical methodology to its current state of utility, and stemming from this point further progress in the application of microbore HPLC and fast HPLC is anticipated. At the moment, the conventional columns of choice for analyzing hop bittering substances contain bonded hydrocarbon reverse-stationary phases. For reverse-phase columns, elution with a mobile phase composed of water, an organic modifier, and the means to neutralize the weak ionic properties of hop resins is usual. Once calibrated, modern HPLC systems can be operated automatically with high precision and reproducibility for the quantitative analysis of identified solutes. The restricted availability of pure reference hop compounds is, however, an obstacle to the universal adoption of this method by an industry already well served by alternative methods of analysis. Moreover, to be totally acceptable as a tool for routine quality control any HPLC system should satisfy a number of criteria (16), and many systems in use involve some compromise of ideals. Such shortcomings notwithstanding, several productive applications have been described (6,11,23,30,35).

Practical guidance is available (43) to the most economical usage of hops and hop products during hop boiling. Factors such as the pH and gravity of the wort, dosage rate of α-acids, presence of divalent cations, duration and temperature of the boil, and the degree of dispersal of the α-acid source all have important influences on the rate of transformation to iso-α-acids. Wort quality can also depend on agitation during boiling to promote break formation (15). Modern wort-boiling practices are designed, therefore, to afford economies in materials, energy, and processing through the use of hop extracts, hop pellets, heat recovery systems, whirlpools, and other features of large-scale plant that contribute to efficiency (19). In making changes from traditional wort-boiling methods, however, it is essential that no loss in beer quality ensues and that real savings can be realized. Since the early 1960s, it has been a widespread custom among brewers to measure both the efficiency of hop usage (utilizaton) and the bitterness of the final product by inexact, though convenient, methods (13). Through the use of HPLC, the possibility exists now for rapidly obtaining precise information on the rates of isomerization of α-acids in worts and on the extent of competing side reactions under different boiling conditions.

The present account describes results obtained in experimental trials conducted in a plant-scale superbarometric kettle equipped with an external heater (12), a pilot-scale continuous high-temperature wort-boiling system (19), and a laboratory-scale vessel, in which the isomerization of α-acids was studied. The objective was to assess the effects of some brewing variables on the yield and quality of bitterness in stout wort and beer, as judged by both established control measurements and by HPLC.

EXPERIMENTAL

Equipment

HPLC was performed with two systems. The first system

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consisted of a Waters M-6000 pump, U6K injector, and M-440 absorbance detector (Waters Associates Inc., Milford, MA) to which was linked a model 3390A integrator (Hewlett-Packard). The second system comprised two Waters M-510 pumps, automated gradient controller M-680, U6K injector, M-481 LC spectrophotometer and a Chromatopac C-R3A integrator (Shimadzu Corp., Kyoto, Japan). Ancillary equipment included a 25-μl injection syringe (Hamilton no. 802), column heater (HPLC Technology, Macclesfield, England), filter apparatus (Millipore XX 1004700), and syringe with Swinny stainless filter holder (Millipore X 3001200). The analytical chromatographic columns were Nucleosil $^{10}C_{18}$, 30 cm × 4 mm i.d. (Macherey-Nagel, Duren, West Germany) protected by precolumns (Waters 84550) packed with Bondapak C_{18}/Corasil.

Reagents and Chemicals

HPLC-grade methanol was obtained from Lab-Scan (Dublin, Ireland), orthophosphoric acid (85%) from BDH Chemicals Ltd. (Poole, England), and deionized water from the Elgastat R01 system (Elga, High Wycombe, England). For internal standards 2,6-dibutylphenol and *p*-nitroanilide of myristic acid were obtained from Alltech Associates, Carnforth, England. For external standards, noncommercial samples with known contents (approximately 75% by wt) of α- and β-acids were provided by Hopstabil GmbH, Wolnzach, West Germany and an "extra-pure" sample of isomerized-α-acids (approximately 25% by wt as potassium salts) was provided by Pauls Hop Products, Reigate, England. All other chemicals were from BDH Chemicals Ltd.

Chromatographic Conditions

Four different solvent systems were used for different purposes:

1) For α- and β-acids in samples from hops and hop products, the mobile phase consisted of an 80:19.75:0.25 volume ratio of methanol, water, and orthophosphoric acid.

2) For iso-α-acids in samples from beer, the mobile phase consisted of a 70:29.75:0.25 volume ratio of methanol, water, and orthophosphoric acid.

3) For iso-α-, α-, and β-acids in samples prepared from worts or sediments, the mobile phase consisted of a 75:24.75:0.25 volume ratio of methanol, water, and orthophosphoric acid.

4) For separating iso-α-acids from non-iso-acid bittering substances in iso-octane extracts of beer, a gradient system was used in which the mobile phase changed linearly from a 50:49.75:0.25 volume ratio to one of 99.75:0:0.25 for methanol, water, and orthophosphoric acid in either 30 or 60 min.

For all systems the following applied: injection volume, 5–25 μl; flow rate, 2 ml/min; column temperature, 25° C; detector sensitivity, 0.02 absorbance units full strength. With system 1 the detector wavelength was 315 nm, otherwise 280 nm was used throughout.

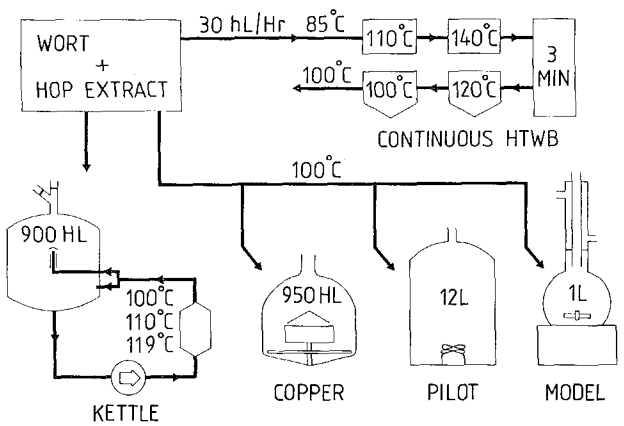

Fig. 1. Schematic representation of superbarometric kettle, conventional copper, pilot-scale copper, model wort-boiling system, and continuous high-temperature wort-boiling system (HTWB).

Standards

The solid concentrated α- and β-acid standards were stable when stored at 2° C in darkness. The liquid iso-α-acid concentrate deteriorated slowly, so its content was assayed (28) periodically and adjustments were made accordingly. Standard mixture A was prepared containing 50–200 mg/L of α- and β-acids, and 100 mg/L of the *p*-nitroanilide of myristic acid dissolved in methanol. Standard mixture B contained 50 mg/L total iso-α-acids and 400 mg/L of 2,6-dibutylphenol dissolved in methanol. The ratios of peak areas for each component of interest in A or B to the area of the appropriate internal standard were measured daily as a check on the state of preservation of the standards. Standard mixtures A and B were stored in darkness at 2° C and were freshly remade from the concentrates on evidence of deterioration. Internal standards were added to wort and beer samples as a check on injection reproducibility and detection response.

Calibration

Each HPLC system was calibrated using known loadings (0.1–1.5 μg) of standard substances. The contents of iso-α-acids, α-acids, and β-acids in test samples were calculated by multiplying the areas of the corresponding peaks by response factors that were checked twice daily.

Wort Boiling

In experimental trials, batches (approximately 900 hl) of high-gravity (15–22° Plato) worts from both transferred mash and infusion brews were boiled with dispersible hexane extract of hops (330–360 mg/L α-acid) at different temperatures (100, 110, and 119° C) in a superbarometric kettle fitted with an external heater (12,19) and at 100° C in a conventional copper kettle (Fig. 1). Worts hopped with extract were also processed continuously at either 130, 135, or 140° C through a pilot-scale high-temperature wort-boiling system (HTWB) (19) at a rate of 30 hl/hr with a residence time of 3 min. For some experiments, 40% of the α-acid input was provided by pellets of milled hops. Control beers were produced either from production worts boiled at 100° C with leaf hops in conventional copper kettles or from pilot-brewery worts also boiled with leaf hops at 100° C (Fig. 1).

Model Boiling System

An all-glass reaction vessel fitted with a reflux condenser was used in laboratory-scale experiments (Fig. 1). The contents of the vessel (500 ml) were heated on a laboratory stirrer/hot plate (Stuart Scientific Co., Croydon, England) and were stirred either at maximum or at half-maximum speed with a magnetic stirring bar.

Analysis of Worts

Hot samples of worts or other reaction mixtures were cooled quickly to 25° C. For the measurement of total iso-α-, α-, and β-acids, wort samples (2.0 ml) were diluted to 10.0 ml with methanol and then set aside at 25° C to precipitate high-molecular-weight materials. For the measurement of soluble iso-α- and α-acids, cooled wort samples (30 ml) were first centrifuged at 13,000 × *g* for 30 min at 25° C to sediment break. Samples (2.0 ml) of the clarified worts were then diluted with methanol as before. Sedimented break was resuspended in 20 ml of methanol and set aside for 30 min. All samples were centrifuged (6,000 × *g* for 15 min), and the clear supernatants obtained were analyzed by HPLC using solvent system 3, after filtration through a 0.45-μm filter when necessary.

Analysis of Beers

Centrifuged samples of beer (2.0 ml) to which was added 0.43 mg of 2,6-dibutylphenol were diluted with methanol and centrifuged

as in the treatment of wort samples. Thereafter, the samples were analyzed by HPLC using system 2. Samples of the centrifuged beers were also extracted with iso-octane by the well-known Brenner method (5,34). Absorbance readings on the iso-octane extracts were converted to milligrams per liter of iso-α-acids by a factor appropriate to stout beers. The Brenner method was also used selectively for measurements on worts. In some cases, iso-octane extracts of beers or worts were used directly for HPLC using either solvent system 2 or solvent gradients.

Analysis of Hops and Hop Products

The lead conductance values for leaf hops and milled hop pellets were determined on toluene extracts according to European Brewery Convention standard methods (7). For hop extracts, the Ganzlin modification of the Wöllmer method was used (8). For HPLC, portions (1.0 ml) of toluene extracts of leaf hops or milled hops or samples of hop extract (100 mg) were dissolved in 100 ml of methanol containing 10 mg of the *p*-nitroanilide of myristic acid.

Flavor Testing

The relative bitterness of different beers was decided by the two-glass test (9) using a 25-member trained tasting panel on three successive days. The general flavor evaluation of beers was determined by triangular tasting (10) and by a routine sampling-room panel of 65 members.

RESULTS AND DISCUSSION

Chromatographic Separation of Hop Acids

Gradient elution of hop acids from a column of Nucleosil $^{10}C_{18}$ (Fig. 2) adequately separated iso-α-acids into the three principal components, although the cis and trans isomers were not resolved. Moreover, adhumulone was not separated from humulone, and adlupulone was not separated from lupulone. The gradient system, however, was not considered appropriate for routine analysis. Isocratic elution with the solvent system 2 gave good resolution of the iso-α-acids, which were all eluted in 10 min. Beer samples were quantitated by calibrating the system with the iso-α-acid standard. The sums of the integrated peak areas were linearly related for sample loadings up to 1.5 μg, the correlation coefficient for 10 different samples being 0.9982. For five repetitions of the same iso-α-acid standard sample, the coefficient of variation was 0.6%. Similar precision and reproducibility was found for injections of the internal standard of up to 5 μg.

For the isocratic separation of α- and β-acids in extracts from hops, the mobile phase used most frequently was solvent system 1. The main components of interest were well separated in a run time of 15 min. For sample loadings of α-acids up to 0.9 μg, linear regression analysis of 12 samples gave a correlation coefficient of 0.9962, and the coefficient of variation for triplicate samples was 2.3%. Results for injections of β-acids and for the internal standard (*p*-nitroanilide of myristic acid) indicated similar degrees of reproducibility and precision.

The analysis of methanolic solutions of uncentrifuged worts required quantitation of iso-α-, α-, and β-acids, for which isocratic separation was less than ideal. As a compromise between those solvent compositions best suited for either isomerized acids or for α- and β-acids, the chosen mobile phase was solvent system 3. As shown in Figure 3, resolution of iso-α-acids was sacrificed, whereas the separation of β-acids was much greater than necessary. Nevertheless, it was possible to process samples more quickly and with greater reproducibility by this system than by the more cumbersome elution with solvent gradients. For 26 samples of standards, the retention times of individual peaks varied by only 0.3–0.4%, and the correlation coefficients for the area measurement calibrations prepared for loadings up to 1.21 μg iso-α-acids, 1.65 μg of α-acids, and 1.0 μg of β-acids dissolved in methanolic solutions of unhopped wort varied from 0.9986 to 0.9991. Typically, the reproducibility of area measurements was less than for the two other isocratic systems; for example, 10 sample repetitions gave a coefficient of variation of 3% for iso-α-acids.

α- and β-Acids in Hops and Hop Extracts

The lead conductance values (LCV) measured on toluene extracts of fresh leaf bitter hops tended to be slightly higher than the contents of α-acids measured by HPLC (Table I). For 18 samples analyzed, the average contents of α-acids was only 0.4% lower than the average LCV. In contrast, the contents of α-acids measured in aged (2–4 years) hops were considerably less than the LCV results, and only traces of β-acids were present as expected (29,42). Aroma hops contained more β- than α-acids, and in hop extracts the proportion of α- to β-acids varied according to the hop variety used for extraction. Extracts from Brewer's Gold hops

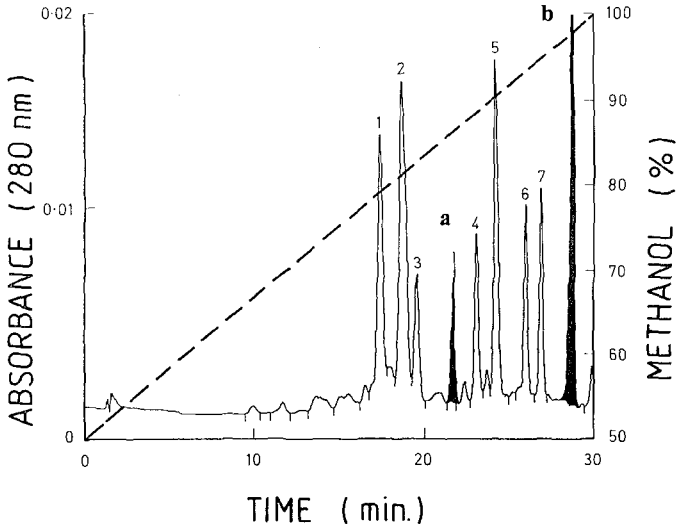

Fig. 2. Gradient-elution reverse-phase high-performance liquid chromatography of mixture containing commercial isomerized hop extract and commerical hexane hop extract and internal standards. Peaks are numbered: **1,** iso-cohumulone; **2,** iso-humulone; **3,** iso-adhumulone; **4,** cohumulone; **5,** humulone/adhumulone; **6,** colupulone; and **7,** lupulone/adlupulone. Internal standards are: **a,** 2,6-dibutylphenol; **b,** *p*-nitroanilide of myristic acid.

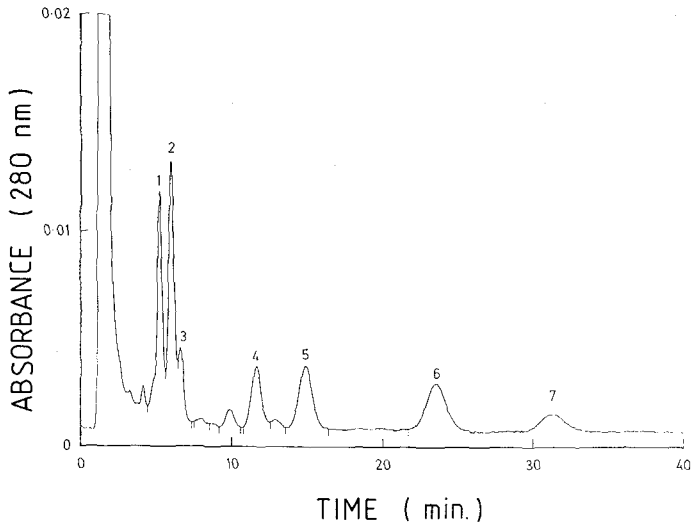

Fig. 3. Isocratic-elution reverse-phase high-performance liquid chromatography with solvent system 3 of methanol-diluted wort sample, taken after boiling in the kettle with hop extract for 75 min. Peaks numbered as in Fig. 2.

usually contained a higher proportion of β-acids than extracts of Northern Brewer hops, and also a higher proportion of cocongeners.

Throughout the trials, both HPLC and LCV analyses were used to check for deterioration in the commercial hop extracts that were stored at ambient temperature. Over 12 months, however, no evidence of significant losses in α-acids was obtained. For hexane extracts of Brewer's Gold and Bullion hops, the contents of α-acids measured by HPLC accounted for 91–92% of the LCVs.

α-Acid Isomerization in a Model System

The course of α-acid isomerization was investigated initially in a simple model system (Fig. 1); stirred suspensions of α-acids (305–330 mg/L) added as dispersible hop extract in either brewing water, buffer, or wort, were boiled under reflux at 100°C for various times. Some results obtained for suspensions in 0.1*M* KH_2PO_4 buffer (pH = 5.5) are shown in Figure 4. Analysis by isocratic HPLC system 3 of samples taken at intervals indicated a progressive decrease in α- and β-acids, whereas the contents of iso-α-acids increased. Linear regression analysis of the logarithms of α-acid contents plotted against time for duplicate experiments yielded a straight line ($r = 0.9958$), indicating that the decrease in α-acids was a first-order reaction (4,36). A rate constant and a reaction half-life were then calculated from the slope of the line obtained.

The disappearance of β-acids was on occasion erratic, being greatly accelerated by vigorous stirring (1,4). When suspensions were stirred vigorously, β-acids were decreased to about 4% of their initial concentration on refluxing for 5 hr. Accompanying the marked losses in β-acids, the yields of iso-α-acids were also diminished and the presence of unknown components more polar than iso-α-acids appeared in chromatograms. The appearance of iso-α-acids did not match the disappearance of α-acids, the yield of iso-α-acids relative to the consumption of α-acids after two half-lives being an indicator of the extent of competing side reactions (4,33,38,43). The results obtained for a series of experiments are given in Table II; from these the extent to which isomerization depended on both pH and the content of divalent cations can be seen. The half-life values were lengthy for α-acids measured in phosphate buffers in the absence of divalent metal ions but were similar to those measured earlier by others (18,36). These results also reemphasize the primary importance of pH in determining both the rate of conversion and yield of iso-α-acids as noted by others (18,36). Equally important in the context of brewing is the catalytic influence of divalent ions (18). For a sample of wort brewed in a pilot-scale brewery to an original gravity of 11° Plato, the contents of magnesium and calcium were insufficiently high to promote rapid isomerization as compared with the reaction in brewing water, even though the wort pH was not too unfavorable.

TABLE I
Analyses of Lead Conductance Values, α-Acids, and β-Acids in Hops, Hop Pellets, and Hop Extracts

Sample Types	Lead Conductance Value (%)	α-Acids[a] (%)	β-Acids[a] (%)
Fresh leaf bitter hops	7.3	7.3	4.7
	10.4	10.6	6.7
	11.6	11.5	7.2
	12.0	11.7	4.1
	13.0	12.7	3.4
	14.3	13.1	3.8
Aged leaf bitter hops	3.0	1.3	trace
	3.2	1.3	trace
	4.0	1.8	trace
Aroma hop pellets	2.5	2.5	3.0
	2.7	2.6	3.3
	3.1	3.2	3.2
Kettle extracts	20.9	20.4	9.6
	33.9	30.6	30.6
	34.0	34.1	18.7

[a]Determined by high-performance liquid chromatography.

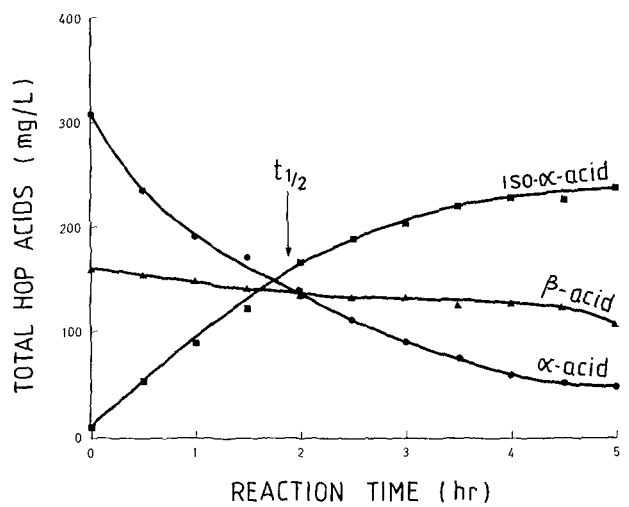

Fig. 4. Contents of total α-acids, total β-acids, and total iso-α-acids in a mixture containing 0.1*M* KH_2PO_4 buffer (pH = 5.5) and commercial hexane hop extract (1.5 g/L) boiled at 100°C in the model wort-boiling system. $t_{1/2}$ = Reaction half-life of α-acids.

TABLE II
Isomerization of α-Acid in Model System

Medium	pH	α-Acid Half-Life (min)	Iso-α-Acid[a] Yield (%)	Calcium Content (mg/L)	Magnesium Content (mg/L)
KH_2PO_4 buffer	7.0	101	92	0	0
	5.5	112	73	0	0
(0.1*M*)	4.0	132	57	0	0
Brewing water	6.8	21	97	114	12
Pilot brewery wort (11° Plato)	5.5	46	82	19	70

[a](Increase in iso-α-acids/decrease in α-acids) × 100, measured after two half-lives.

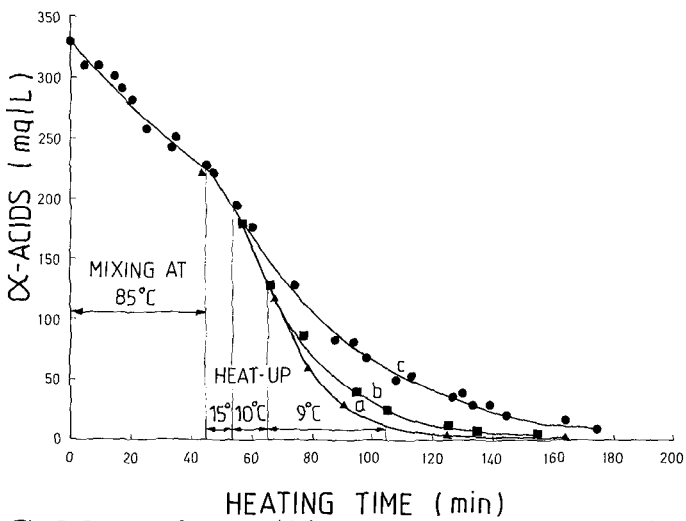

Fig. 5. Contents of total α-acids in worts bittered with hop extract during boiling cycles in superbarometric kettle at different pressures. Symbols: ● = two worts boiled at 100°C (atmospheric pressure) after mixing at 85°C; ■ = wort boiled at 110°C (500 mbar excess pressure); ▲ = wort boiled at 119°C (900 mbar excess pressure). Curves **a, b,** and **c** were calculated on a computer according to first-order reaction kinetics.

The Effect of Temperature on α-Acid Decreases in Different Wort-Boiling Systems.

As one measure of the isomerization reaction, the rates of decrease of α-acids were examined in worts (15–22° Plato) treated in different boiling systems. In large-scale trials, α-acids (330 mg/L) in the form of predispersed hop extract were added to wort (650 hl) held at 80–85° C for 30–75 min before the vessels (copper or kettle) were fully charged. Immediately after charging the vessels, worts were drawn off to the collection vessel of the continuous HTWB system when required, and the remainder of the brews were heated to the target temperatures. In this way, 18 different hop-extract brews were processed through the kettle, four were boiled in the conventional copper, and four were processed through the HTWB.

In Figure 5 the total contents of α-acids in samples drawn at intervals from the kettle during the boiling of two worts at 100° C, one wort at 110° C, and one wort at 119° C are plotted. The curves were calculated with a computer program according to the known effects of temperature on first-order reaction kinetics (1,36). The program also accounted for changes in both wort volume and rates of temperature rise during kettle-filling and heating phases. The closeness of fit of the data points to the calculated curves indicates that the decreases in α-acids were predictable by classical reaction kinetics throughout the course of the boiling cycle. For nine worts with a pH mean of 4.8, a calcium content mean of 48 mg/L, and a magnesium content mean of 175 mg/L, the half-lives of α-acids averaged 30.1 min when boiling was at 100° C. Neither the rates of change of reactants nor the yields of products from the individual congeners were significantly different. Invariably the nine worts contained unisomerized α-acids (17–37 mg/L) after boiling for 120 min.

By boiling at 110° C, the average half-life of α-acids in six trials was decreased to 16.1 min, and less than 5 mg/L of α-acid remained unchanged after boiling for 90 min. Whereas the average half-life of α-acid in three worts boiled at 119° C was only 9.8 min, the overall rates of transformation were not greatly different from those achieved at 110° C because of the long heating up period required at the higher temperature. Nevertheless, only traces of α-acids remained in worts processed for 60 min at 119° C.

In accordance with the claims for the relative efficiency of continuous HTWB systems (19), treatment of worts at 130–140° C for only 3 min decreased the α-acid contents to 40–80 mg/L. During collection of the hot worts after processing, their contents of α-acids continued to decrease but always remained greater than in kettle-boiled worts after 60 min in the wort receiver.

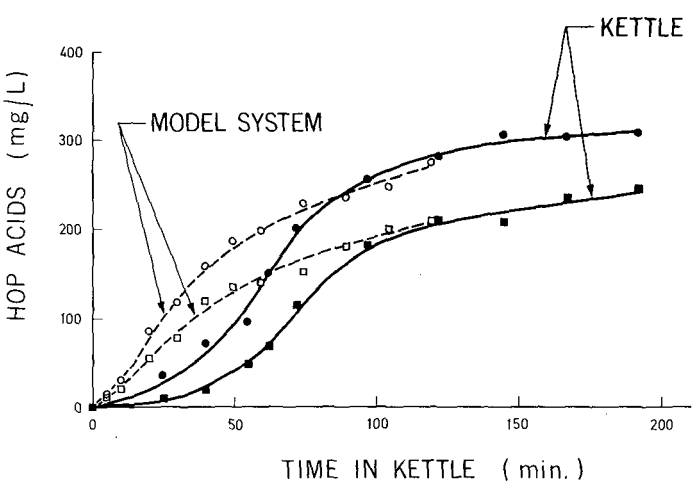

Fig. 6. Decreases in total α-acids (circles) and increases in total iso-α-acids (squares) in worts boiled at 100° C with hop extract in the model system and in the kettle. In the kettle the temperature was raised from 85 to 100° C in 75 min.

Formation of Iso-α-Acids in Worts

For all worts examined, the decreases in α-acids during boiling with hop extract were always greater than the corresponding increases in iso-α-acids throughout the boiling period. For two different worts boiled at 100° C, either in the model system or in the kettle (Fig. 6), the yields of iso-α-acids produced relative to the α-acids consumed were 76 and 80%, respectively, after 120 min of boiling time. Despite the similar yields of iso-α-acids in the two worts after boiling, the contents of iso-α-acids remaining in the cooled and centrifuged worts differed widely. For the wort boiled in the model system, the concentration of iso-α-acids decreased to 149 mg/L, which corresponded to a utilization of 49%, whereas the iso-α-acid content in the kettle-boiled wort decreased to 80 mg/L, a utilization of 24%. The differences in utilization were ascribed to the high pH of the wort in the model system (pH = 5.5) compared with that of the kettle-boiled wort (pH = 4.8). In separate experiments conducted in the model system, the pH of wort samples was adjusted after boiling to a range of values (3.5–5.5), and the contents of soluble iso-α-acids were determined after cooling and the removal of break. On average, each decrement in pH of 0.1 units decreased utilization by 1%.

Measurement of Utilization

The calculated values for utilization obtained for centrifuged samples of eight high-gravity worts that had been boiled at 100° C in the kettle are given in Table III. Values for apparent utilizations calculated from Brenner values and LCV inputs indicated little variability between the different brews and did not show substantial differences were achieved by operating the kettle in either the central riser mode or as a whirlpool. In contrast, values for real utilization calculated from the measurement by HPLC of soluble iso-α-acids and α-acid input indicated wide variations between brews, particularly for those processed through the central-riser kettle configuration. By subtracting the calculated contributions of iso-α-acids from the total absorbances of the iso-octane extracts used in Brenner measurements, the proportional absorbances attributable to non-iso-α-acid bittering substances (NIBS) were calculated for each wort. The higher than average proportion of NIBS in trial 12 wort was attributable to the presence of unisomerized α-acids, in keeping with its reduced boiling time of only 90 min. An alternative explanation was required for the presence of high contents of NIBS in trial 3 and trial 18 worts, seemingly at the expense of true iso-α-acids.

Analyses of the soluble contents of four worts after boiling in the copper at 100° C are given in Table III and indicate variations similar to those for worts boiled in the kettle. One wort (trial 21) was outstanding in containing a much lower than average content of NIBS and a high content of iso-α-acids. It is significant that for one special trail (trial 24), in which the contents of the copper were flushed with nitrogen during filling, the real utilization value was 32.1% and the content of NIBS was 27%.

Whereas the ratios of iso-α-acid congeners in the worts (Fig. 3) corresponded closely to the ratios of α-acid congeners in the original hop extract, invariably the dominant iso-α-acid in cooled and centrifuged worts was isocohumulone. Accordingly, the average utilization for cohumulone in all trials was 27%, whereas that for humulone/adhumulone was only 18%, confirming other claims that cohumulone is utilized preferentially (26,37).

NIBS

Apart from the occasionally high proportion of NIBS in worts bittered entirely with hop extract, high proportions (80%) of NIBS were invariably found in worts prepared with either hop pellets or hops, particularly when the α-acid source was less than fresh. In Figure 7 the HPLC gradient-elution profile of an iso-octane extract of wort prepared with one-year-old hop pellets and hop extract is shown. Apart from the numbered peaks of iso-α-acids, numerous other substances are present, some of which were identified tentatively as being hulupones or humulinic acids.

These, and many other substances (2,13,17,25) are known to be formed by oxidative routes from hop resins during the aging for hops. It has been shown that the formation of some oxidation products can be accelerated at elevated temperatures (38,39,43).

As shown in Table IV, the contents of NIBS did not vary greatly in worts boiled with hop extract over a wide range of temperatures. Nevertheless, the presence of NIBS, some of which were α-acids, distorted the calculation of utilizations based on Brenner values. The highest value for real utilization of α-acid was obtained for wort boiled for 90 min at 110° C.

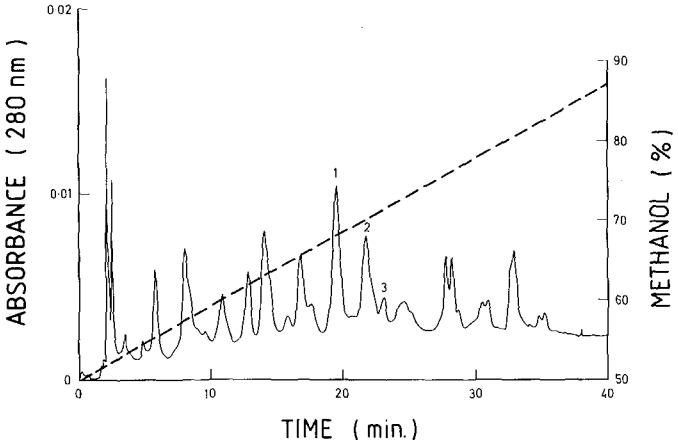

Fig. 7. Gradient-elution reverse-phase HPLC of an iso-octane extract from a wort boiled at 100° C in the kettle with hop-extract and milled hop pellets. Numbered peaks as in Fig. 2. Non-numbered peaks are non-iso-α-acid bittering substances (NIBS).

Using the model system, worts containing high proportions (80%) of NIBS and low contents of iso-α-acids (25 mg/L) were produced from hop extract by vigorous stirring at low temperatures (80–85° C) for 6 hr. Throughout this period, which was intended to simulate the early stages of the boiling cycle in the kettle, α-acids decreased from 330 to 7 mg/L in accordance with expected first-order reaction kinetics, but β-acids disappeared abruptly. These results agree with the findings of others (3,22) on model systems and add to the claim that oxidation during the early stages of boiling in production systems decreases the yield of iso-α-acids (27,37).

Effect of Boiling Time and Break Formation on Soluble Iso-α-Acids

Whereas α-acids from dispersible hop extracts were rapidly transformed to iso-α-acids on boiling wort in the kettle at 100° C (Fig. 6), prolonged boiling (120 min) maximized the contents of iso-α-acids in the clarified worts (Fig. 8). In contrast, prolonging the mixing phase at 85° C from 45 to 75 min had a deleterious effect on real utilization because of increased production of NIBS. The value of prolonged boiling was particularly evident for brews in which 40% of the α-acid input was supplied by pellets. The presence of hop particles apparently promoted the removal of iso-α-acids during cooling, a capacity that diminishes when boiling is prolonged (40). Similarly, the presence of particulate solid matter in the form of break was held responsible for the low values for real utilization (Tables III and IV) obtained with high-gravity worts of low pH (mean = 4.8) boiled only with extract. Using the model system, utilizations of 43, 45, 47, and 57%, respectively, were obtained for worts with gravities of 20, 15, 10, and 6° Plato and with pH values averaging 5.5. Although utilization was increased to 59% in a 12° Plato wort by decreasing the α-acid addition rate

TABLE III
Analyses of Worts Obtained After Boiling with Hop Extract at 100°C, Performed After Cooling and Centrifuging Samples

System	Trial Number	Iso-α-Acids (mg/L)	α-Acids (mg/L)	Real Utilization[a] (%)	Brenner Value (mg/L)	NIBS[b] (%)	Apparent Utilization[c] (%)
Central riser kettle	1	67	trace	18.6	73	45	20.1
	2	70	7	19.4	77	45	21.2
	3	42	trace	11.7	78	67	21.4
	10	91	4	27.6	82	33	23.5
Kettle/Whirlpool	11	75	7	22.7	73	38	21.0
	12	70	27	21.2	88	52	25.4
	18	60	2	18.2	72	50	20.8
	19	76	6	23.0	73	37	21.0
Copper	20	78	6	23.6	84	44	23.3
	21	91	trace	27.6	74	26	20.6
	22	61	10	18.5	77	52	21.4
	25	86	trace	26.0	89	42	24.7

[a] Calculated from high-performance liquid chromatography measurements. Input of α-acids: trials 1–3 = 360 mg/L, trials 10–25 = 330 mg/L.
[b] Percentage of absorbance of iso-octane extract of wort attributable to non-iso-α-acid bittering substances (NIBS).
[c] Calculated from Brenner values and lead conductance values. Input of α-acid = 360 mg/L.

TABLE IV
Means of Analyses of Worts Boiled at Different Temperatures with Hop Extract, Performed after Cooling and Centrifuging

Boiling Temperatures (°C)	Boiling Time (min)	Iso-α-Acids (mg/L)	α-Acids (mg/L)	Brenner Value (mg/L)	NIBS[a] (%)	Real Utilization[b] (%)	Apparent Utilization[c] (%)
100	120	69	4	75	45	19.0	20.7
110	90	87	1	91	42	25.0	25.5
119	60	87	trace	87	49	23.9	24.4
130	3	68	10	76	46	20.6	21.1
135	3	74	7	79	43	22.4	21.9
140	3	71	5	80	46	21.5	22.2

[a] Percentage of absorbance of iso-octane extract of wort attributable to non-iso-α-acid bittering substances (NIBS).
[b] Calculated from high-performance liquid chromatography measurements.
[c] Calculated from Brenner values and lead conductance values.

fourfold, the concentration of iso-α-acids obtained was considerably below requirements. For the standard addition rate (330 mg/L) of α-acids it was found that 51% of the total iso-α-acids produced during boiling was recovered from the hot break by washing with methanol. Another 1.5% of the iso-α-acids originally present in the hot wort was recovered from the cold break. Utilizations of up to 69% were obtained by boiling hop extracts in worts from which hot and cold break had been removed previously in a cycle of boiling and cooling. Clearly, solubility characteristics alone cannot account for the poor utilization of iso-α-acids (14). Moreover, the iso-α-acids recovered from the breaks always contained much more isohumulone than isocohumulone. Our results, therefore, support the proposal that the composition of bittering substances in wort depends on equilibria between poorly soluble molecules in solution and those adsorbed on solid surfaces, on which less polar solutes compete more effectively for available sites (14,24,37,39). Accordingly, the extents to which different hop bitters can be utilized should be related to their retention behaviors on reversed-phase chromatography.

Relationships with Palate Bitterness

To examine relationships between palate bitterness, analytical bitterness, and iso-α-acid contents, 18 different beers were brewed on a pilot scale, varying the hop rate, the age of hops used, and the postfermentative addition rates of isomerized extract. Each beer was analyzed and compared for bitterness by the taste panel with a control beer brewed by traditional methods. In Figure 9 the total absorbances of iso-octane extracts from the beers are represented as the sums of the component absorbance contributions from iso-α-acid and NIBS, the diagonal lines passing through points of specified Brenner value. Brenner values (in mg/L of iso-α-acid) were calculated by multiplying total absorbances by a factor (42.5) derived specifically for stout beers brewed from fresh hops. The data points are plotted as the calculated absorbances attributable to iso-α-acids and to NIBS for each of the 19 beers extracted. Of the 18 experimental beers, six were judged to be significantly less bitter than the control beer, six were more bitter, and six could not be distinguished in bitterness from the control by the two-glass test. In agreement with the proposal that Brenner values are an accurate assessment of relative bitterness, the four beers that had low values (<39 mg/L) were all less bitter than the control (42 mg/L), six beers with high values (>47 mg/L) were all more bitter than the control, and five beers with "normal" values (approximately 40–46 mg/L) were indistinguishable in bitterness from the control. The bitterness of each of the three remaining beers was less than that indicated by their respective Brenner values. Two of the beers were significantly less bitter than the control, although of similar Brenner value (40–42 mg/L). The third beer displayed a Brenner value (49 mg/L) much greater than the control but was not significantly different from the control in palate bitterness.

The results show that the palate bitterness of experimental stout beers was materially underestimated by Brenner measurements only when beers contained unusually high ratios of NIBS to iso-α-acids—between 8 and 18, based on relative absorbance contributions. It is notable that this effect was evident only for three beers for which the hops were aged three years before use. The corresponding ratios of NIBS to iso-α-acids in beers prepared from fresh hops, one-year-old hops, and two-year-old hops were 0.6, 1.5, and 3.9, respectively. Palate bitterness of these beers was in accordance with their Brenner values. As a further check on the usefulness of the Brenner measurement, 15 production beers brewed at approximately two-week intervals from leaf hops and 15 production-scale trial beers brewed over a similar time span from hop pellets and hop extract were examined also. Brenner values for these beers varied from 37 to 45 mg/L, whereas their contents of iso-α-acids varied from 14 to 40 mg/L. Although the 30 beers were not evaluated for flavor as sensitively as were the experimental beers, no evidence of abnormal bitterness was obtained. The wide variation in their iso-α-acids resulted in a variation in the calculated ratios of NIBS to iso-α-acids therein from 0.6 to 3.7, around an average of 2.1. Our results indicate, therefore, that for beers that contain appreciable quantities of NIBS, the Brenner value remains for the moment a better estimate of palate bitterness than does measurement by HPLC of iso-α-acid contents alone. Even the Brenner value, however, overestimated the bitterness of beers brewed from very old hops that contained unusually high contents of NIBS. Whereas some workers (20) regard the presence of NIBS as beneficial, others (39) favor increasing the iso-α-acid content of beer relative to NIBS. Already, considerable progress has been made towards separating and identifying the numerous components of beer that contribute to bitterness (21). Doubtless, further application of HPLC in the measurement of NIBS will

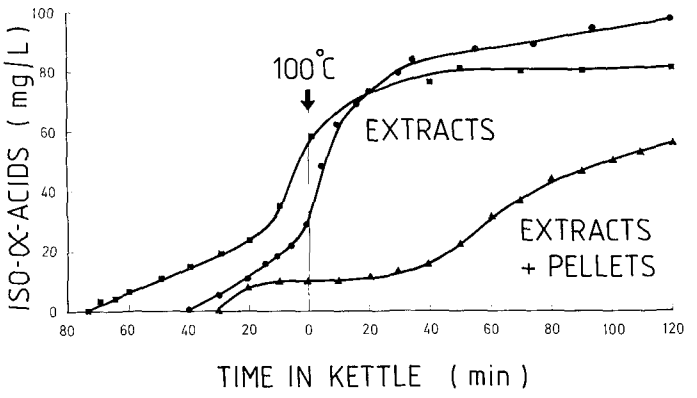

Fig. 8. Contents of soluble iso-α-acids in cooled and centrifuged worts sampled during boiling at 100° C in the kettle with either hop extract or hop extract and hop pellets.

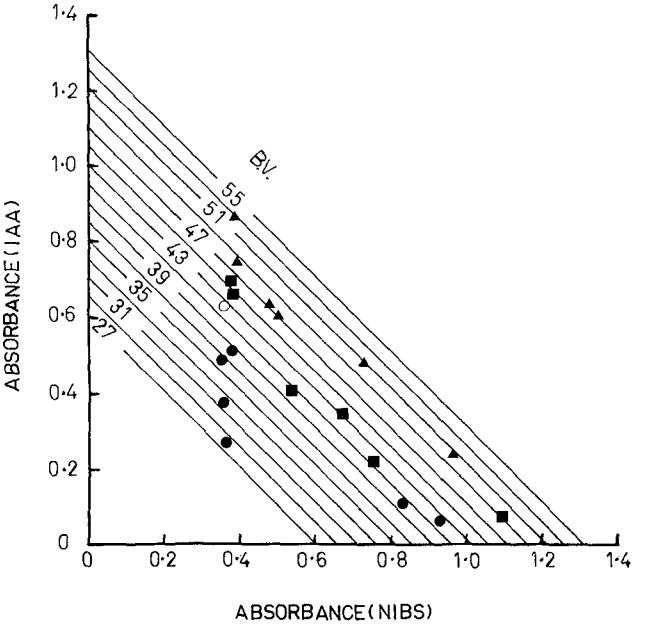

Fig. 9. Relationships between iso-α-acid (**IAA**) contents, contents of non-iso-α-acid bittering substances (**NIBS**), Brenner values (**BV**), and palate bitterness for beers. Symbols denote data for small-scale experimental beers brewed from different combinations of old hops, fresh hops, hop extract, and isomerized hop extract: O = control; ● = beers significantly less bitter than control; ▲ = beers significantly more bitter than control; ■ = beers not significantly different in bitterness from control. The diagonal lines signify Brenner values corresponding to different levels of total absorbance. The axes represent the separate contributions to total absorbance by IAA and NIBS calculated for iso-octane extracts from each beer.

enable progress in quantifying the constitution of beer bitterness (21).

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LITERATURE CITED

1. Anteunis, M., and Verzele, M. *Bull. Soc. Chim. Belg.* 68:102, 1959.
2. Ashurst, P. R., Gramshaw, J. W., and Laws, D. R. J. *Brew. Guild J.* 51(614):579, 1965.
3. Askew, H. O. *J. Inst. Brew.* 70:503, 1964.
4. Askew, H. O. *J. Inst. Brew.* 71:10, 1965.
5. Brenner, M. W., Vigilante, C., and Owades, J. L. *Am. Soc. Brew. Chem., Proc. 1956,* p. 48.
6. Buckee, G. K. *J. Inst. Brew.* 91:143, 1985.
7. European Brewery Convention. Analytica. 3rd ed. 1975. E.47, 6.3.1.
8. European Brewery Convention. Analytica. 3rd ed. 1975. E. 54/2, 6.3.2B.
9. European Brewery Convention. Analytica. 3rd ed. 1975. E.83, 7.15.3.
10. European Brewery Convention. Analytica. 3rd ed. 1975. E.83, 7.15.4.
11. Forster, A. *Inst. Brew. Aust. N.Z. Sect. Proc. Congr. 17th, Perth, 1982,* p. 103.
12. Herrmann, H. *Brew. Dist. Int.* 15(3):32, 1985.
13. Howard, G. A., and Martin, P. A. *J. Inst. Brew.* 70:424, 1964.
14. Howard, G. A., and Slater, C. A. *J. Inst. Brew.* 63:478, 1957.
15. Hudson, J. R., and Birtwistle, S. E. *J. Inst. Brew.* 72:46, 1966.
16. Knudson, E. J., and Siebert, K. J. *J. Am. Soc. Brew. Chem.* 41:51, 1983.
17. Kokubo, E., Kowaka, M., and Kuroiwa, Y. *Am. Soc. Brew. Chem., Proc. 1971,* p. 265.
18. Köller, H. *Tetrahedron Lett.* 40:4317, 1968.
19. Kollnberger, P. *Tech. Q. Master Brew. Assoc. Am.* 21:124, 1984.
20. Kowaka, K., Fukuoka, Y., Kawasaki, H., and Asano, K. *Eur. Brew. Conv. Proc. Congr. 19th, London, 1983,* p. 71.
21. Kowaka, M., and Kokubo, E. *J. Am. Soc. Brew. Chem.* 35:16, 1977.
22. Krauss, G. *Brew. Dig.* 47(4):65, 1972.
23. Lance, D. G., Kavanagh, T. E., and Clarke, B. J. *J. Inst. Brew.* 87:225, 1981.
24. Maule, D. R. *J. Inst. Brew.* 72:285, 1966.
25. Meilgaard, M. *Brygmesteren* 14:159, 1957.
26. Meilgaard, M. *J. Inst. Brew.* 66:35, 1960.
27. Meilgaard, M., and Trolle, B. *Eur. Brew. Conv. Proc. Congr. 6th, Copenhagen, 1957,* p. 27.
28. Mussche, R., and Franiau, R. *J. Inst. Brew.* 80:201, 1974.
29. Nickerson, G. B., and Likens, S. L. *Am. Soc. Brew. Chem., Proc. 1967,* p. 63.
30. Ono, M., Hashimoto, S., Kakudo, Y., Nagami, K., and Kumada, J. *J. Am. Soc. Brew. Chem.* 41:19, 1983.
31. Otter, G. E., and Taylor, L. *J. Inst. Brew.* 84:160, 1978.
32. Schulze, W. G., Ting, P. L., Henckel, L. A., and Goldstein, H. *J. Am. Soc. Brew. Chem.* 39:12, 1981.
33. Shaw, S. J., and Mills, A. K. *Am. Soc. Brew. Chem., Proc. 1967,* p. 45.
34. Sierra, F. *Brew. Dig.* 39(11):54, 1964.
35. Silvester, D. J. *J. Inst. Brew.* 90:319, 1984.
36. Spetsig, L. O. *Monatsschr. Brau.* 17:131, 1964.
37. Spetsig, L. O. *Eur. Brew. Conv. Proc. Congr. 10th, Stockholm, 1965,* p. 416.
38. Verzele, M. *Eur. Brew. Conv. Proc. Congr. 10th, Stockholm, 1965,* p. 400.
39. Verzele, M. *Am. Soc. Brew. Chem., Proc. 1970,* p. 63.
40. Verzele, M., Claus, H., and van Hoey, M. *Wallerstein Lab. Commun.* 28:99, 1965.
41. Verzele, M., Dewaele, C., van Kerrebroeck, M., Strating, J., and Verhagen, L. *J. Am. Soc. Brew. Chem.* 41:36, 1983.
42. Verzele, M., van Dyck, J., and Claus, H. *J. Inst. Brew.* 86:9, 1980.
43. Whitear, A. L. *Brew. Dig.* 44(2):68, 1969.
44. Whitt, J. T., and Cuzner, J. *J. Am. Soc. Brew. Chem.* 37:41, 1979.

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