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Nuclear Magnetic Resonance Spectroscopic Determination of α- and β-Acid Homolog Composition in Hops

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Hexane extracts of hops have been analyzed by nuclear magnetic resonance spectroscopy for percentages of the three major homologs present in the α- and β-acid fractions. A range of hop varieties, including commercial and experimental types, was examined and the homolog composition was utilized in classification of these hops as Continental or Domestic types. Analysis of Cascade hops grown at different locations indicated that the proportions of each homolog are genetically fixed and less prone to environmental variation than α- and β-acid levels. The technique provides a simple, rapid, and accurate means for measuring homolog composition.

The major resin constituents of hops are the α-acid homologs humulone, cohumulone, and adhumulone (**1a, b,** and **c**), together with the corresponding β-acids lupulone, colupulone, and adlupulone (**2a, b,** and **c**). The relative proportions of these homologs are characteristic for each particular hop variety (Rigby, 1956), with the Continental types having lower levels of cohumulone (**1b**) in comparison with the Domestic types. It has been postulated (Rigby, 1972) that high levels of cohumulone are responsible for the unpleasant, harsh bitter flavor imparted to beers brewed with certain varieties of hops and it is known that the three α-acid homologs are utilized to different extents in the brewing process (Howard and Slater, 1957).

Although the heritability of homolog composition has not been definitely established, it is nevertheless essential that such information be obtained for both the male and female parents as well as their progeny produced through a directed hop breeding program. Experimental varieties can thus be screened and appropriate selections made for desirable flavor characteristics and optimum utilization. In addition, it may be desirable to evaluate and identify commercial hop samples, pellets, and extracts using the same criteria.

The large number of samples which would have to be analyzed in such a program requires that a rapid, accurate technique be available for measurement of homolog percentages, preferably needing only a small sample of hop material, since the quantity of hops available from new crosses in the first year of production is strictly limited. Counter-current distribution (Rigby and Bethune, 1953) is accurate, but too time consuming for this purpose. A similar objection applies to GLC analysis of the isopropyl esters of isovaleric, isobutyric, and 2-methylbutyric acids produced by oxidative cleavage of the acyl side chains of the α- and β-acids (Rigby et al., 1960). A rapid method has been developed (Likens and Nickerson, 1971) whereby the acids produced on pyrolysis of a lupulin sample are analyzed directly by GLC, but the adhumulone homologs are not separated from the corresponding humulone or lupulone homologs and the co-fraction value obtained is a combination of contributions from both α- and β-acids. Since the homolog ratios are not constant for both α- and β-acids in a given hop, the analysis provides only an estimate of the relative contribution of the co-homolog in different hop varieties. High-pressure liquid chromatography offers considerable promise as a rapid method for hop analysis but sufficiently well-resolved separations, which would allow determination of individual homolog proportions, have not yet been achieved (Molyneux and Wong, 1973).

We have now developed a rapid, small-scale analytical method which provides a measurement of the homolog composition of both α- and β-acids from a single determination. The technique has been routinely applied to evaluation of new genotypes produced by selective crossing of parents having characteristics desirable in commercial hops.

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EXPERIMENTAL SECTION

Apparatus. Nuclear magnetic resonance (NMR) spectra were obtained using a Varian HA-100 spectrometer. Samples were run as 20% solutions in $CDCl_3$, dried over 4A molecular sieves. Tetramethylsilane (Me_4Si) was used as an internal standard.

Sample Preparation. (*A*) *Lead Salt Method.* The hops (30–40 g) were ground in a Waring Blendor and extracted with benzene (400 ml) for 1 hr. The solvent was removed under reduced pressure and the residue redissolved in methanol (50 ml). α-Acids were precipitated as their lead salts on addition of 4% methanolic lead acetate, leaving β-acids in the methanol solution. The lead salts were collected and washed several times with methanol. α-Acids were regenerated on addition of 6 *N* H_2SO_4 (<2 ml) to a suspension of the lead salts in methanol (100 ml) and isolated by extraction with isooctane. The solution was dried over Na_2SO_4 and the isooctane evaporated to yield the α-acids.

To regenerate the β-acids, the methanol filtrate was acidified with 6 *N* HCl and extracted with hexane. The hexane solution was washed with water and dried over Na_2SO_4. After removal of the solvent, the residue was dissolved in benzene and chromatographed on a silicic acid column (100 mesh) with ethyl acetate–hexane (15:1, v/

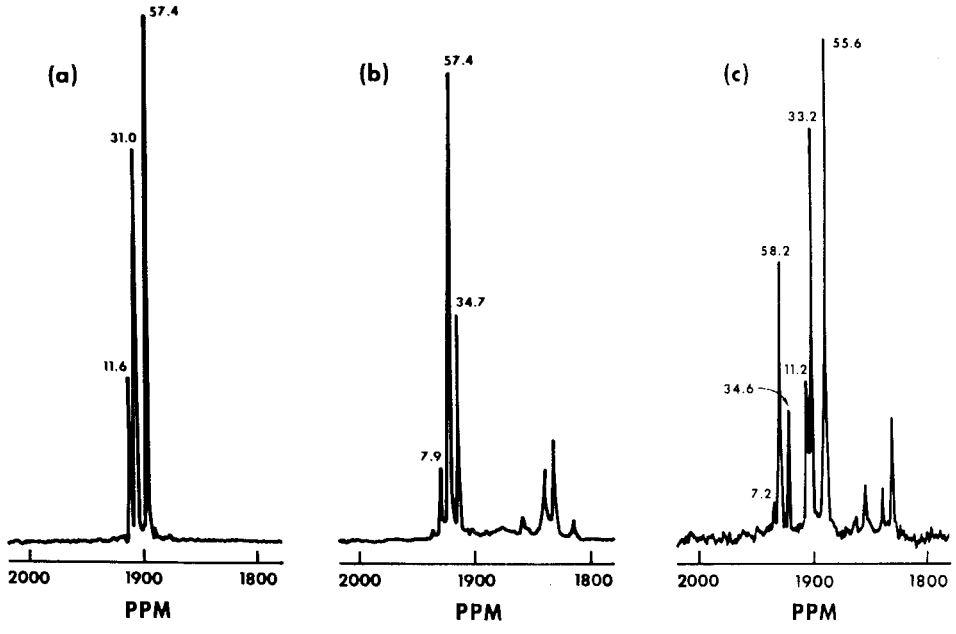


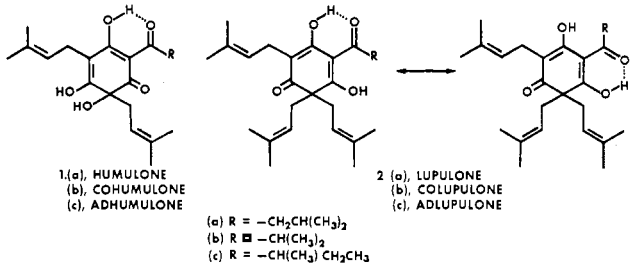
Figure 1. 100-MHz NMR spectra of (a) α-acids purified via lead salts, (b) β-acids purified chromatographically, and (c) benzene extract (α- and β-acids). Each fraction was isolated from the same sample of Cluster hops.

v).The β-acids fraction was collected and evaporated to give pure β-acids.

(*B*) *Direct Extraction Method.* Ground hops (1–2 g) were extracted with benzene (50 ml) or hexane for 30 min and the solution dried over Na_2SO_4 for 4 hr. Either benzene or hexane could be used for the extraction, although the hexane extract was found to give an NMR spectrum with less background due to interfering substances. The solvent was evaporated and the residue redissolved in $CDCl_3$ for NMR analysis without further purification.

RESULTS AND DISCUSSION

The nuclear magnetic resonance spectra of both α- and β-acid fractions exhibit sharp signals at very low field (18–20 ppm), which are due to hydroxyl groups hydrogen bonded to the carbonyl group of the acyl side chains. Each acyl group has a different shielding effect upon this particular proton, resulting in slightly different shifts for each of the three major homologs. Whereas the α-acids exist in solution as a single dienolic form (1) the β-acids exhibit tautomerism (2) (De Keukeleire and Verzele, 1970), giving rise to two sets of signals for each homolog, although the major tautomer accounts for much stronger signals in the set at lower field. A typical NMR spectrum of the low-field region exhibiting these hydroxyl proton signals for both α- and β-acids is shown in Figure 1c.



The presence of these signals in the NMR spectrum was first used by Shannon et al. (1969) to provide a qualitative estimate of the change in relative concentration of different homologs during ripening of the hop cone. Shortly thereafter Kowaka et al. (1970) reported experiments correlating NMR analysis with the accurate but tedious method of oxidative cleavage of the acyl side chains and GLC analysis of the acids produced as their isopropyl esters. Correction factors were determined, relative to the latter method, whereby NMR peak heights could be used to calculate homolog percentages. These factors were obtained for α- and β-acid fractions, purified by formation of their lead salts and chromatographic separation, respectively, thus precluding use of the technique for analysis of numerous samples.

We have found that it is not necessary to perform time-consuming purification steps in order to obtain well-resolved NMR spectra. Extraction of small samples (1–2 g) of hops with benzene, or preferably hexane, provides an extract of α- and β-acids, the 100-MHz NMR spectrum of which yields the homolog percentages for both fractions by application of the correction factors developed by Kowaka et al. (1970). These factors allow the percentages to be calculated from the peak heights (H) of each individual low-field hydroxyl proton signal as follows:

$$\% \text{ humulone} = 100H_H/A$$

$$\% \text{ cohumulone} = 73H_{CoH}/A$$

$$\% \text{ adhumulone} = 65H_{AdH}/A$$

where $A = H_H + 0.73H_{CoH} + 0.65H_{AdH}$. β-Acid homologs are calculated similarly, using the set of signals due to the major tautomers, at ca. 20 ppm:

$$\% \text{ lupulone} = 100H_L/B$$

$$\% \text{ colupulone} = 78H_{CoL}/B$$

$$\% \text{ adlupulone} = 70H_{AdL}/B$$

where $B = H_L + 0.78H_{CoL} + 0.70H_{AdL}$

The low-field regions (18–20 ppm) of the NMR spectra were compared for: (a) α-acids purified via their lead salts, (b) β-acids purified chromatographically, and (c) α- and β-acid fractions extracted directly with benzene (Figure 1). Each of these extracts was prepared from the same sample of the Cluster hop variety. Although the spectrum for the unpurified benzene extract (c) showed more base-line noise, the resolution of the individual peaks and relative peak heights was constant for all three samples. Relative percentages of each homolog are given above the appropriate signal, showing that the values obtained do not vary significantly between the purified samples and the unpuri-

Table I. Cohumulone and Cofraction Analyses for Various Hop Varieties[a]

Variety	% cohumulone NMR	% cohumulone GLC[b]	% cofraction GLC[c]
Hallertau	15	15	20
Fuggle-H	21	21	29
Fuggle-N	22	21	27
Bullion [10a]	28	28	33
Bullion	29	29	34
Cascade	28	28	34
6761-117	29	28	31
6761-61	33	32	36
6769-02	32	32	46
Cluster [L-1]	35	36	43
Talisman	39	38	48

[a] 1972 crop. [b] Analyses provided by F. L. Rigby, John I. Haas Co., Yakima, Wash. [c] Analyses provided by S. T. Likens and G. Nickerson, Oregon State University, Corvallis, Ore.

Table II. NMR α-Acid Homolog Proportion Analysis for Different Hop Varieties[a]

Variety	Direct extract method % H	Direct extract method % CoH	Direct extract method % AdH	Lead salt method % H	Lead salt method % CoH	Lead salt method % AdH
Hallertau	75	16	9	74	15	11
7006-398	74	18	8	75	16	9
Northern Brewer	68	22	10	68	22	10
Tettnanger	65	22	13	64	21	15
Fuggle	65	24	11	64	22	14
6761-117	54	30	16	54	29	17
21001[b]	58	30	12	58	29	13
Bullion	57	31	12	58	30	12
Cluster (Rivard)	56	33	11	57	31	12
62013[c]	55	34	11	55	34	11
6761-02	51	35	14	51	33	16
Cluster	49	37	14	50	35	15
Talisman	44	44	12	44	43	13

[a] 1972 crop. [b] Released 1972 as new variety Cascade. [c] Released 1974 as new variety Comet.

fied extract. Similar results were obtained using various other hop varieties.

Cohumulone and Cofraction Analyses. In order to ascertain that the GLC vs. NMR correlation factors (Kowaka et al., 1970) were truly applicable, a selection of commercial and experimental hop varieties was analyzed by both methods for cohumulone content. The lead salts of the α-acids were isolated and the acids produced analyzed by GLC. A portion of the same lead salts was treated with dilute hydrochloric acid to regenerate the α-acids and these were analyzed by the NMR method. The results obtained (Table I) correspond within 1% for hop varieties ranging from 15 to 39% in cohumulone content. Humulone and adhumulone contents cannot be compared by the two methods of analysis since the acids produced on pyrolysis are not resolved by GLC.

In addition to cohumulone content, the "cofraction" percentages for the same hop samples were determined by GLC analysis of the acids produced on pyrolysis of lupulin from these hops (Likens and Nickerson, 1971). The higher values obtained, relative to the cohumulone content, reflect the contribution of the isobutyric acid produced by pyrolysis of colupulone, which frequently occurs at a higher level in the β-acids than does cohumulone in the α-acids (Table I).

α-Acid Composition of Different Hop Varieties. Utilizing the same correlation factors, a comparison was made between the rapid direct extract method and the more tedious lead salt isolation method for a number of different commercial and experimental hop varieties. Very little variation in α-acid homolog percentages between the two methods was observed for hop varieties with cohumulone levels ranging from 16 to 44% (Table II). The results are tabulated with the hop varieties being arranged in increasing order of cohumulone content. It is of interest to note that those varieties regarded as having Continental hop character show low cohumulone percentages, whereas the Domestic hop types have cohumulone values greater than 30%.

The results obtained provide evidence that the NMR method of analysis can be used for rapidly classifying hops in terms of cohumulone content. The cohumulone percentages determined for the experimental varieties 21001 and 62013 were utilized in the evaluation of these hops for commercial purposes and both types have now been released as new varieties under the respective names Cascade and Comet (Brooks et al., 1972). The Cascade variety, in accord with its low cohumulone value, is grown for its similarity in aroma and brewing characteristics to those hops imported from Europe, whereas Comet is intended mainly for use as an extract hop and is classified as a Domestic-type hop

Table III. NMR α- and β-Acid Homolog Proportion Analysis of Cascade Hops from Different Locations[a]

Location	% α	% β	α-Acid % H	α-Acid % CoH	α-Acid % AdH	β-Acid % L	β-Acid % CoL	β-Acid % AdL
Moxee 1, WA	4.4	4.8	60.0	29.5	10.5	47.0	44.0	9.0
Moxee 2, WA	5.0	5.7	60.0	29.5	10.5	46.5	45.0	8.5
Grandview, WA	5.0	6.2	61.5	28.5	10.0	49.0	44.0	7.0
Toppenish, WA	5.4	5.9	59.0	31.5	9.5	45.5	46.0	8.5
Morton, ID	6.9	5.6	58.5	31.0	10.5	46.0	46.0	8.0
Post Falls, ID	7.1	6.3	59.0	30.5	10.5	47.0	45.5	7.5
Prosser, WA (virus-free)	4.8	6.9	60.5	28.5	11.0	47.5	44.5	8.0
Prosser, WA (virus-infected)	4.4	6.0	58.5	30.0	11.5	48.5	42.0	9.5

[a] 1974 crop, average of two determinations, direct extract method.

similar to the Cluster variety.

α- and β-Acid Composition of Cascade Hops. The simplicity and rapidity of the direct extract method provided a means of investigating the consistency of homolog composition for a given hop variety grown in various locations. Six samples of 1974 crop Cascade hops grown in Washington State and two from Idaho were analyzed and the level of all homologs found to be consistent, cohumulone values ranging from 28.5 to 31.5% (Table III). In view of the fact that the α-acid level in the hops showed considerable variation, ranging from 4.4 to 7.1%, the homolog composition would appear to be much less sensitive to growing area or cultural practices than α- or β-acid contents.

Also, two samples of Cascade hops grown under identical conditions, with the exception of one being virus-free and the other virus-infected, were also analyzed. The homolog composition for both of these hops fell within the range found for commercially grown Cascade hops, while the α- and β-acid levels of the virus-infected hops were somewhat lower (Table III). Homolog percentages therefore do not appear to be significantly affected by virus expression whereas the α- and β-acids are known to be decreased.

The results obtained indicate that, at least for Cascade hops, the homolog composition of both α- and β-acid fractions is not significantly affected by location, cultural practice, or disease and is, therefore, a genetically fixed characteristic of the hop variety. This finding supports the value of the facile direct-extraction NMR homolog analysis method as a technique for evaluation of experimental varieties in hop breeding programs and for establishing the varietal authenticity of commercial hop samples.

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Okra Seeds: A New Protein Source

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Okra seed was investigated for the first time for its potential as a seed protein. Chemical and nutritional studies were carried out to evaluate the seed and compare it to other seed proteins such as soya, cottonseed, etc. One variety (Emerald) of okra seeds was used throughout the study. All determinations were carried out on the whole seed including the seed coat and endosperm. The amino acid composition of okra seed was found to be similar to that of soybeans, yet the PER value was higher for okra seed. Rats fed on zein as a source of protein failed to grow. However, when okra or casein replaced zein, the rate of growth and recovery was about the same.

Oilseeds together with legume seeds are the most promising type of crops for protein production. Animal and fish products provide about one-third of the total dietary protein, whereas plant proteins account for 50–75% of the total needs. Cereal grains, oilseeds, and pulses are the three groups of plants which supply most of the protein in the world (Dimler, 1971). Though certain plant proteins are low in some essential amino acids (Watt and Merril, 1963) they are the main source of protein intake in many parts of the world where availability of animal protein is not adequate. So far among seed bearing plants, soybeans and cottonseeds only have been utilized to an appreciable extent for protein isolates and concentrates production.

Okra (*Hibiscus esculentus L.*) (Gobo, Combo, Gumbo, Bamya, or Ocra) is of African origin and was introduced into the United States and West Indies under the Spanish name, gumbo. It is one of the botanical species cultivated for its pod for more than 2000 years. It grows in many parts of the world, India, Malaysia, the Philippines, the Middle East, the Mediterranean region, Central, East, and West Africa, Central America, and in general throughout the tropics (Cooke, 1958).

Okra has been used as a vegetable for its green pods, in the fresh state, canned, or frozen, and no attempt has been made to use its seeds as a source of protein. It belongs in the Malvaceae or Mallow family, as does cotton. Yields as high as 2000 lb of seed per acre have been reported in Louisiana (Clopton et al., 1948; Miller and Wilson, 1949). An okra pod 9 in. long can bear up to 100 seeds. The okra plant grows in soils of medium fertility, well-drained sandy loam, and in a wide range of altitudes and rainfall. It grows both in dry and wet seasons. A plant that is constantly cropped can bear pods and seeds until killed by frost (Tindall, 1968; Spartsis, 1972).

Edwards and Miller (1947) analyzed samples of okra seed meal from which the oil had been extracted with hexane and found the following composition: crude protein, 13.56%; fat, 1.92%; carbohydrate, 31.50%; crude fiber, 8.14%; moisture, 6.69%; ash, 8.19%; CaO, 0.37%; P_2O_5,

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