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THE DEVELOPMENT OF A BONDED WEAK ANION-EXCHANGE PACKING SUITABLE FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HOP RESINS

G. E. OTTER and L. TAYLOR
Courage Limited, Southwark Bridge, London SE1 9HS (Great Britain)
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SUMMARY

A weak ion-exchange high-efficiency column packing has been developed specifically for the separation of hop resins. This paper describes the approach to the problems which necessitated the development of a new packing, its preparation and the effects of the operating parameters on the separation of hop materials.

INTRODUCTION

The conversion of the α-acid fraction of hop resins into bitter iso-α-acids (Fig. 1) is of fundamental importance to the brewing industry. This conversion is dependent on pH, temperature and boiling time or alternatively it can be carried out by photolysis. The study of this reaction is complicated by the presence of other resins and conversion products derived from hops. The chromatography of the hop resin components is also complicated by their wide polarity range, which varies from relatively non polar to acidic.

Previous methods of separation have been carried out using an acetic acid gradient on a strong anionic exchanger, *e.g.*, Bio-Rad AG 1-X4[1] or QAE-Sephadex[2].

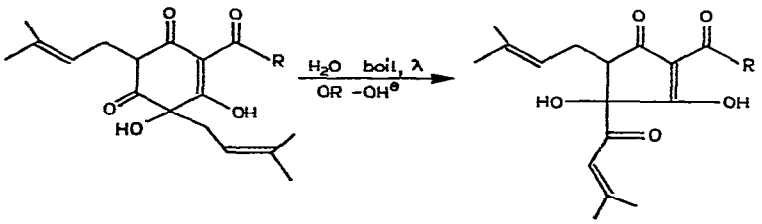


Fig. 1. Scheme showing the conversion of hop resins to iso-α-acid during the hop boiling process. For R = iso-butyl, the α-acid is humulone and the corresponding iso-α-acid is a mixture of *cis*- and *trans*-isohumulone; For R = iso-propyl, the α- and iso-α-acid are cohumulones and *cis*- and *trans*-isohumulones, respectively; For R = *sec.*-butyl, α- and iso-α-acid are adhumulones and *cis*- and *trans*-iso-adhumulones, respectively.

High-performance liquid chromatography (HPLC) would be an obvious way to both speed up the analysis and improve the resolution of these complex mixtures.

First experiments were conducted using a Dupont SAX anion-exchange column which initially gave a reasonable separation, but the high methanol content of the eluent, necessary to keep the resins in solution, caused rapid column deterioration. Dupont AAX which is stable to virtually all solvents was then tried as a replacement for the SAX packing. The result was disappointing in that only partial separation was achieved due to the resins not being sufficiently retained even when the eluent was as weak as 0.0002 *M* formic acid.

This result was surprising since the anionic exchange groups are similar in both packings. However, ambiguities had been found when using the SAX column, viz.:

(1) the pH gradient was successful only when working from low to high pH whereas theoretically the gradient would be expected to run from high to low when used in conjunction with an anion exchange resin;

(2) a separation was not obtained using an ionic strength gradient;

(3) a separation could be obtained using water–methanol mixture without any ions being present.

These factors suggested that the chromatography of hop resins on ion-exchange resins was mainly a partition effect between the backbone matrix and the solvent. Siebert[3], using a range of ion-exchange and partition columns, has formed a similar opinion.

These conclusions are at variance with the previous work recorded in this field, where ion-exchange chromatography was thought to be necesssary. Two notable exceptions were the work of Spetzig[4] who used partition chromatogaphy between chloroform, methanol and water, and the thin-layer chromatographic (TLC) technique of Aitken *et al.*[5,6] which uses a benzene–ether mixture as eluent. Spetzig however found it important to adjust the solvent pH.

Preliminary investigations were carried out both by ourselves and also by Siebert[3] using commercial columns with packings such as ODS, ETH, CN and NH_2. All these columns provided a partial separation, Dupont ETH being the most satisfactory although an ideal result could not be obtained. In each case the main difficulty was that iso-α-acids produced wide and badly tailed peaks.

These columns are expensive and the trial of different commercial products involves a high capital outlay. Furthermore the pellicular materials, though capable of giving separations had insufficient theoretical plates for the resolution required.

Consideration of these factors suggested that an attempt be made to develop a suitable stationary phase which could be packed in the laboratory. This approach would be considerably cheaper and less laborious than the "hit and miss" method of trying to decide which commercial product might be suitable.

It was clear from the preliminary work that a large number of theoretical plates would be required in any satisfactory column and on this assumption it would be necessary to use 5-μm "totally porous" silica as the support. To utilise the potential of this material it would also be necessary to use "on column injection" and an "infinite diameter"[7] column. An infinite diameter column is one in which the diameter is sufficiently wide to prevent any of the sample touching the walls whilst traversing its length. Such a column has been described by Cox *et al.*[8].

EXPERIMENTAL AND RESULTS

Pure hop resin components were kindly supplied by Dr. D. R. Laws of the Brewing Research Foundation. All the analyses were conducted on a Dupont 830 High-Performance Liquid Chromatograph with the injection and detector couplings modified to accept the columns which were constructed as follows.

Column construction

A 15-cm length of straight $^1/_4$ in. O.D. × 4.6 mm I.D. tube, as sold for gas chromatography, was cleaned by soaking in 50% phosphoric acid then washing with a sequence of water, methanol, acetone, dichloromethane and isooctane.

Two $^1/_4$ in. diameter discs of 8-μm stainless steel cloth were inserted into a $^1/_4$–$^1/_{16}$ in. drilled-out Swagelok coupling; this was then connected to an extension tube, consisting of 24 × $^1/_4$ in. I.D. stainless-steel tubing, by means of a Swagelok coupling.

The support material, 5-μm Lichrosorb SI 60 (Merck), was suspended in acetone and filled as a thick, but mobile paste into the column and extension tube. The upper end of the extension tube was connected to the Dupont 830 solvent supply line via the septumless injector. The instrument reservoir was filled with acetone, the pump set to maximum pressure (3,500 p.s.i.), and at least 400 ml of acetone passed through the column to pack and consolidate the silica gel into the column.

The extension tube was then removed and the top $^1/_4$ in. of the silica gel carefully scraped away so as to leave a flat surface; a disc of stainless-steel cloth was then forced down on to the surface and glass wool rammed into a tight wad above this.

The column was then activated by passing 100 ml each of methanol, isopropanol, dichloromethane and isooctane in succession through the column. All these solvents had been previously dried by passage through an activated silica column, except for dichloromethane when basic activated alumina was used.

The column was conditioned before use by passing the required eluent through the column until the detector base line became smooth and level. The finished column had a theoretical plate count of 3,980 when measured using 2-phenyl-2-propanol.

Separation of hop resins on silica gel

The silica gel column was used to investigate the possibility of separating the hop resins. It was found that any solvent mixture with an elution strength[9], E°, greater than 0.30 would elute the resins from the column. However, every solvent mixture tried produced peaks which were badly tailed and unacceptable. This experience was confirmed using TLC, when only solvents based on benzene or toluene gave sharp spots without tailing. Benzene and toluene are ultraviolet (UV) absorbing and cannot therefore be used with a HPLC UV detector. This tailing effect is not uncommon in the silica gel chromatography of highly polar compounds and the only surprising point is that the relatively non-polar benzene does give a good separation.

One way of preventing the tailing of highly polar compounds is to separate them as ion pairs[10–16]. For instance, if an acidic mixture is to be separated, the addition of a quaternary ammonium compound will result in the suppression of the polar character of the acids.

In silica gel chromatography the most polar component of the eluent (water)

is retained on the silica gel whilst the less polar compounds remain in the mobile eluent. Whether the ion pairs are retained on the polar surface of the silica or in the non-polar phase depends on the pH.

The investigation of ion pair formation

For the successful chromatography of ion pairs the substances to be separated must be capable of partitioning between the less polar phase and the more polar phase; this can be found by measuring k',

$$\text{where } k' = \frac{\text{amount of solute in polar phase}}{\text{amount of solute in non-polar phase}}$$

If k' has a value between 1 and 10, chromatography is possible[17]. To test this possibility some iso-α-acid was dissolved in dichloromethane and shaken with aqueous buffers containing quaternary *n*-butylamine hydroxide, the layers allowed to separate and the absorbance of each layer measured:

$$k' = \frac{\text{absorbance polar phase}}{\text{absorbance non-polar phase}}$$

The value of k' exceeded 1 only when the pH was above 7.0.

The implication of this is that the separation using ion pairs on silica gel was only likely to occur at a pH above 7.0. This would lead to the following problems.

(1) Due to the relatively high pH, the α-acids might be converted to iso-α-acids during the separation.

(2) The absorption maximum of α-acid shifts from 275 to 360 nm at pH's above 4.5 whereas the iso-α-acid maximum remains at 275; thus a multiple-wavelength detector would be required.

From these considerations it was concluded that silica gel is unsuitable for the separation of hop components.

The hop resins were preferentially distributed into the organic phase at acidic pH irrespective of whether they were in the free state, or as ion pairs. These circumstances are indicative of a reversed-phase partition technique which coincides with the conclusion from the previous work. The only case where iso-α-acids are satisfactorily separated by partition chromatography is in TLC on silica gel using benzene. Attachment of the benzene to the silica and the use of an aqueous eluent appeared to be the obvious technique to pursue. The bonding of phenyl groups to silica is readily achieved by reaction of the silica with a phenylchlorosilane. It was decided therefore to examine the possibility of using reversed-phase partition chromatography on a phenyl bonded phase.

Preparation of a phenyl silica support (Fig. 2)

Lichrosorb SI 60 (5 g) of 5-μm particle size was refluxed with conc. HCl for 2 h, filtered on a glass sinter, washed with water until neutral and dried overnight at 110°. The dry silica was suspended in anhydrous toluene and 25 g of phenyldichlorosilane and 1 ml anhydrous pyridine added. The mixture was refluxed for 8 h and

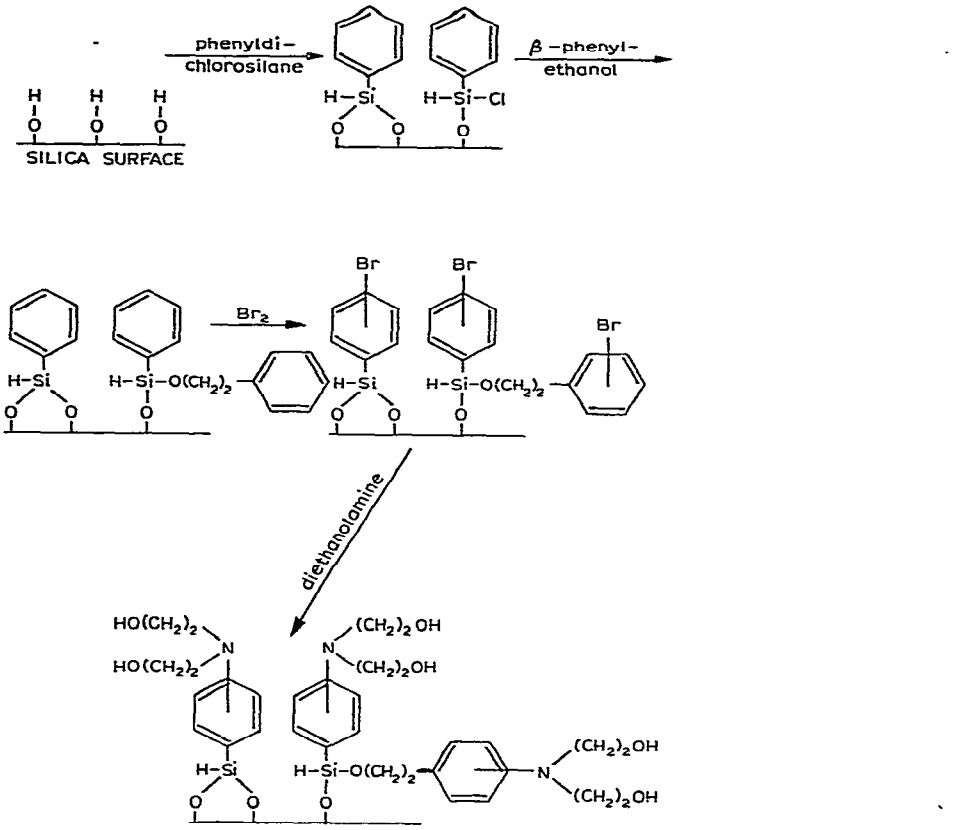


Fig. 2. Scheme showing the reactions occurring at the silica surface involved in the preparation of phenyl and phenyl diethanolamine bonded phases.

allowed to stand overnight, then filtered on a glass fibre filter paper and washed with dry toluene.

Any residual chloro groups were removed by suspending the phenyl silica in phenylethanol and boiling under reflux for 1 h. The phenyl silica was filtered on glass paper, washed with methanol, water and acetone and then dried at 80°.

A number of solvents have been recommended for the dispersion of silica into a slurry prior to packing[8,18–20]. It is our experience that the best results are obtained with phase bonded silicas when the solvent is chemically similar to the bonded phase.

The column was packed as described for silica except that the phenyl silica was suspended in benzene and 400 ml of benzene passed through the column, followed by isooctane, dichloromethane, isopropanol, methanol and water.

Chromatography on phenyl silica

A mixture of the pure hop resin components humulone, colupulone, photo-isohumulone, humulinic acid and hulupone were used to investigate the separation characteristics of the column. These compounds were too strongly retained on the column for satisfactory separation using methanol–water, and acetonitrile–water mixtures.

Chromatography of ion pairs using quaternary *n*-butylammonium hydroxide in buffered methanol–water mixtures did produce a separation and there was little distortion of the peaks. Trials with different basic compounds to form ion pairs showed that the best results were obtained using 0.01 *M* diethanolamine at a pH of 7.0–8.0 in a 55% methanol–water mixture (55:45) (Fig. 3). A lower pH resulted in the hop materials being too strongly held on the column. The use of a phenyl column had overcome the problem of the distorted peaks, but, unfortunately, the objections to the high pH were still present.

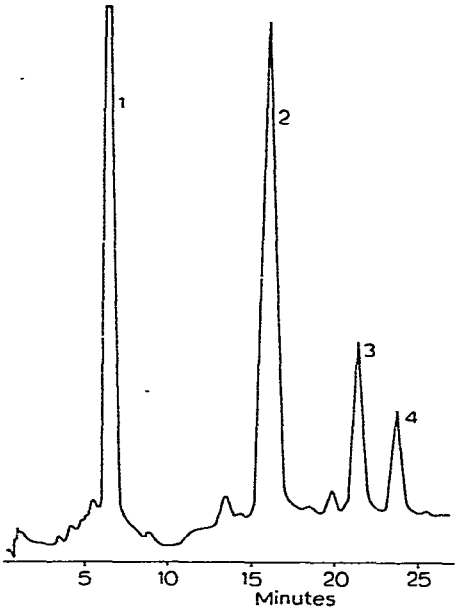


Fig. 3. The resolution of authentic hop resin components on phenyl silica bonded phase. 1 = humulinic acid; 2 = *trans*-isohumulone (prepared by photolysis); 3 = humulone (α-acid); 4 = colupulone (β resin).

For the ion-exchange chromatography of hop resins a high acetic acid concentration is required in the eluent. It follows therefore that if the ion pairing moiety is transferred from the liquid to the stationary phase, *i.e.*, to produce an ion-exchange support, then the pH necessary for elution should be lowered. Hopefully the partitioning effect of the phenyl silica would be retained along with the ion pairing effect of the diethanolamine. The attachment of the diethanolamine to the phenyl groups on the silica means that a weak ion-exchanger had been produced. Weak ion exchangers exert their ion-exchange potential in acid solution so that if the partitioning effect of the phenyl group is damaged the support should still act as an ion exchanger, in direct analogy with the traditional ion-exchange chromatography of hops.

The preparation of phenyl diethanolamine silica support (Fig. 2)

Phenyl silica prepared as described above was suspended in carbon tetrachloride and a few drops of pyridine added to act as a halogen carrier. The mixture was heated on a steam bath and a solution of bromine in carbon tetrachloride added drop-wise

until HBr ceased to be evolved and the mixture was a deep red-brown colour. The mixture was refluxed for a further 1 h then filtered on a glass sinter and washed with carbon tetrachloride until the bromophenyl silica was a pale yellow colour and the filtrate was colourless.

The bromophenyl silica was suspended in diethanolamine and a change in colour from yellow to white and a rise in temperature indicated that a reaction had started; the mixture was refluxed for 2 h then filtered, washed with water, then with formic acid–water mixture (20:80), water, methanol and acetone.

The white phenyl diethanolamine silica was packed in the same manner as was silica gel, except that the acetone was removed by washing with methanol and 0.01 *M* formic acid.

Chromatography on phenyl diethanolamine silica

Initially samples were run on the column with the support in the formate form, however, analysis time was far too long. Different anions were therefore tried and the most successful proved to be citrate.

The effect on retention of pH. The separation is shown in Fig. 4. This shows that below pH 2.0 hulupone is not eluted and that the lower the pH the better the separation of iso-α-acids; humulinic acid, humulone and colupulone are virtually unaffected by pH.

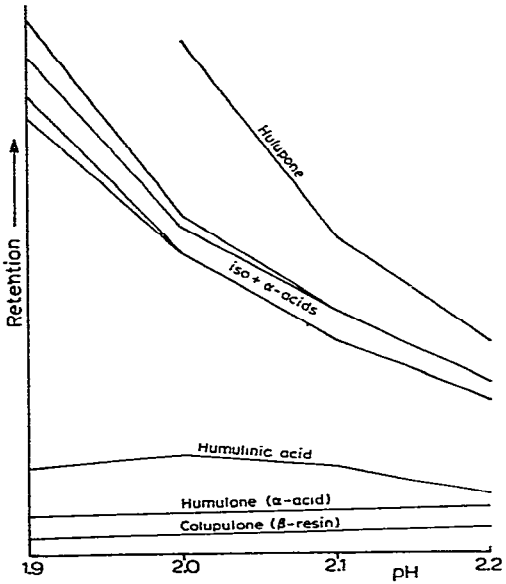
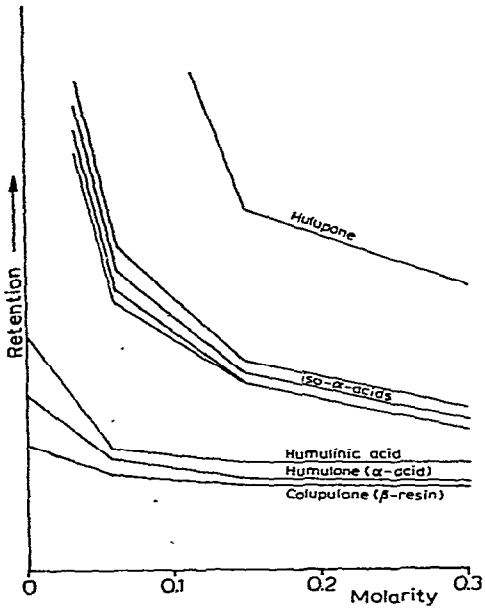


Fig. 4. Dependence of hop resin component retention on pH. Methanol concentration, 62.0%; citric acid, 0.06 *M*; pH adjusted by the addition of KOH.

The effect on retention of ionic strength. This effect is shown in Fig. 5. The retention of all the resin components is affected by citrate ion concentration, humulone, colupulone and humulinic acid being better separated at low and iso-α-acids and hulupone at relatively high ionic strength.



g. 5. Dependence of hop resin component retention on the ionic strength of citric acid. Conditions: 2.0% methanol–water; pH adjusted to 1.90.

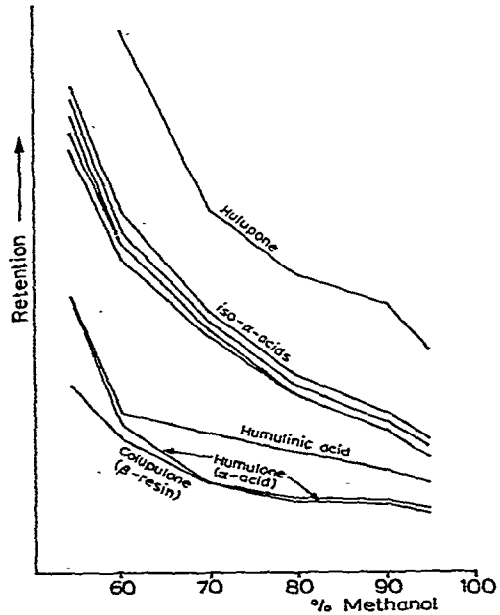


Fig. 6. Dependence of hop resin component retention on the methanol concentration in the eluent. Conditions: citric acid, 0.06 *M*; pH 1.90.

TABLE I

PARAMETERS FOR THE SEPARATION OF HOP COMPONENTS

Separation	*Mobile phase*	*Flow-rate (ml/min)*	*Pressure (p.s.i.)*	*Temp. (°C)*
Hops, methanol extract	0.001 *M* Citric acid in 66.0% methanol–water	0.85	825	60
Iso-α-acids	0.1 *M* Citric acid (pH 1.75) in 53.6% methanol–water	0.85	825	60
Hulupones	0.1 *M* Citric acid (pH 2.0) in 70.0% methanol–water	0.85	825	60
α-Acids (humulinic acid and β-resin absent)	0.001 *M* Citric acid in 53.6% methanol–water	0.85	825	60

The effect on retention of the methanol–water ratio. From Fig. 6 it can be seen that α-acid retention is affected strongly by the methanol concentration, being poorly separated from β-acids when the concentration is above 70% and poorly separated from humulinic acid when below 60%. It follows therefore that to separate the α-acids from β-resins and humulinic acid a methanol concentration between 60 and 70% would be required. The iso-α-acid isomers are best separated below 60% methanol. When the methanol concentration falls below 50% the peaks become very wide probably due to low solubility in the eluent mixture. This information was correlated and used as a guide for the optimisation of conditions for the separation of hop components (Table I). Typical chromatograms are shown in Fig. 7.

Phenyl triethanolamine

A phenyl triethanolamine strong anion exchanger column was also prepared and investigated. Similar results to the diethanolamine support were obtained with no advantages and a slightly poorer resolution.

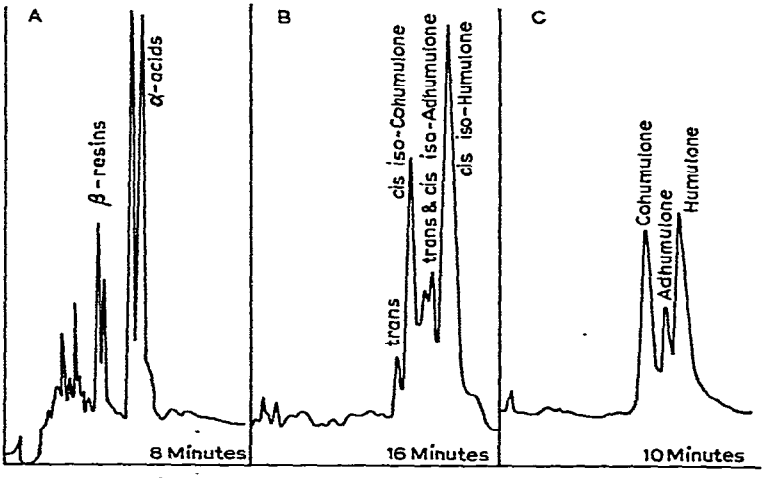


Fig 7 Chromatograms obtained from typical hop resin materials A, Methanol extract of fresh hops; B, separation of the iso-α-acids in a commercial isomerised hop extract; C, separation of α-acids into their component humulones

CONCLUSIONS

A totally porous weak ion-exchange silica (phenyl diethanolamine silica) has been developed which has useful properties for the resolution of hop components by HPLC. This support is relatively cheap, fairly easy to prepare and gives a better separation than any of the commercial columns tried. The column is efficient having approx. 5000 theoretical plates for a 15-cm length and has the advantage that it can separate mixtures containing both acidic and relatively non-polar compounds.

The conditions for the chromatographic separation of hop resins have, to a large degree, been clarified. Reverse-phase partition is the major effect necessary for the separation of α-, β- and humulinic acids, ion pairing is important for the resolution of the iso-α-acids.

It has been shown that silica gel cannot be satisfactorily used as a support in the HPLC of hops without modification, unless an aromatic eluent such as benzene, or toluene, is used. These eluents are not suitable for use with a UV detector, but could be of value with a fluorescence detector.

The reason for the suppression of the polar character of iso-α-acids by aromatic hydrocarbons is not understood, but speculatively could be a result of π bond interaction between the benzene ring and the conjugated carbonyl groups in the hop resin molecules.

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REFERENCES

1 G. L. Hanson, D. Hetzel and R. Miller, *Amer. Soc. Brew. Chem., Proc.*, (1971) 246.
2 G. E. Otter, D. J. Silvester and L. Taylor, *J. Inst. Brew., London*, 78 (1972) 57.
3 K. J. Siebert, *J. Amer. Soc. Brew., Chem.*, 34 (2) (1976) 79.
4 L. O. Spetzig, *J. Inst. Brew., London*, 70 (1964) 440.
5 R. A. Aitken, A. Bruce, J. O. Harris and J. C. Seaton, *J. Inst. Brew., London*, 73 (1967) 528.
6 R. A. Aitken, A. Bruce, J. O. Harris and J. C. Seaton, *J. Inst. Brew., London*, 74 (1968) 436.
7 J. H. Knox and J. F. Parcher, *Anal. Chem.*, 41 (1969) 1599.
8 G. B. Cox, C. R. Loscombe, M. J. Slucutt, K. Sugden and J. A. Upfield, *J. Chromatogr.*, 117 (1976) 269.
9 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 194.
10 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
11 B.-A. Persson, *Acta Pharm. Suecica*, 5 (1968) 343.
12 G. Schill, R. Modin and B.-A. Persson, *Acta Pharm. Suecica*, 2 (1965) 119.
13 B. L. Karger, S. C. Su, S. Marchese and B.-A. Persson, *J. Chromatogr. Sci.*, 12 (1974) 678.
14 B. L. Karger and B.-A. Persson, *J. Chromatogr. Sci.*, 12 (1974) 521.
15 J. H. Knox and J. Jurand, *J. Chromatogr.*, 103 (1975) 311.
16 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
17 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, 1974, p. 255.
18 J. J. Kirkland, *J. Chromatogr. Sci.*, 10 (1972) 593.
19 R. Majors, *Anal. Chem.*, 44 (1972) 1722.
20 T. J. N. Webber and E. H. McKerrell, *J. Chromatogr.*, 122 (1976) 243.