# SYNTHESIS AND CHARACTERISATION OF HOP DERIVED COMPOUNDS AND THEIR APPLICATION IN QUANTITATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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**DECLARATION**

This thesis has not been submitted as an exercise for a degree at this or any other academic institution. The work described within this thesis is based on the author's own work.

Thomas J. Tynan

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CONTENTS

**ABSTRACT**

Synthesis and Characterisation of Hop Derived Compounds, and their Application in Quantitative High Performance Liquid Chromatography.

Thomas J. Tynan.

High Performance Liquid Chromatography (HPLC) has been extensively used in the analysis of hop derived components in beer, but the scope of these methods is limited by the range of attainable reference compounds. The synthesis and purification of a range of relevant components has been described by several authors, but in our laboratory these procedures fail to give compounds of sufficient purity. Modification of published procedures are described for the preparation of humulinic acids, dehydrohumulinic acids, hulupones, and colupulone. These procedures provide facile routes for preparation of these compounds in higher yields than was hitherto reported, and also in sufficient purity for their use as standards in quantitative HPLC analysis.
Although bitterness in beer is largely due to iso-α-acids, other compounds such as hulupones are considered to have similar bitter properties. Alternative routes to hulupones using the readily available and cheap ß-acids extracts as starting material are therefore examined. Oxidation of ß-acids in the presence of sodium sulphite was established as the most feasible route to these compounds.
The structural authenticity of all preparations was confirmed by infra-red, and nuclear magnetic resonance analysis. Purity of the compounds was established using HPLC in conjunction with photodiode array detection.
Developed HPLC methods, and synthesised pure reference standards are used in the quantitative analysis of humulinic acids in hop iso-α-acid extracts before, and after acid hydrolysis. HPLC methods are also developed for the quantitative analysis of hulupone formed from the oxidation of ß-acids solutions.
The stability of all compounds synthesised was investigated so that satisfactory conditions for their long-term storage could be identified.

# SECTION 1

# INTRODUCTION

# 1. INTRODUCTION

## 1.1 Chemistry of Hops

Hops or Humulus lupulus date back to ancient times, often found as decorative plants in medieval gardens. The first use of hops as additives in beer was probably in the twelfth century when German monks, who needed to store beer for long periods of time, realised its preservative power. Unhopped beer is much sweeter and more acidic than hopped beer, and has a strong malt flavour. The distinct "hop flavour" in beer is largely due to bittering components present in the beer. As well as bitterness hops also contribute to the aroma and foam stability of beer. Originally little attention was given to the bittering properties of hops but as the industry grew rapidly in the nineteenth century the hop bitter flavour became a desired characteristic of beer. Today hops are grown all over the world with an annual turn over estimated at over 100,000 tons per year. There are many varieties obtained locally, or by breeding each imparting a unique flavour to beer desired by individual breweries. Varieties like Yakima, Saaz, Bullion, etc., are household names in the brewing industry.

Chemical research on hops started about 100 years ago. The first major step was in 1888 when Hayduck[1] divided hop

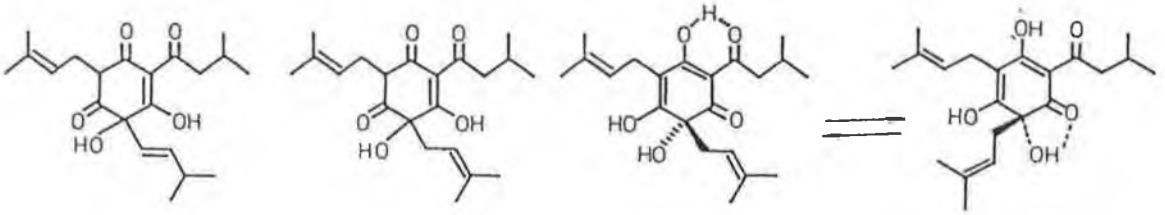
bitter components into two distinct fractions. One fraction, precipitated as a lead salt, and termed the α-acid fraction, the other fraction was a non-lead precipitable fraction termed the ß-acid fraction. These fractions have an appearance of a partly crystallised oil which hardens into a resinous material on exposure to air for long periods. The bitter acids have therefore been called the bitter resins, and even today the distinction between soft resins (hexane soluble), and hard resins (hexane insoluble) is sometimes used as an analytical parameter for hop quality. The chemical composition of hops as it is known today is illustrated in table 1[2].

| Component | Amount (%) |
|---|---|
| α-Acids | 2-12 |
| ß-Acids | 2-10 |
| Essential oils | 0.5-1 |
| Polyphenols | 2-5 |
| Oil and fatty acids | traces to 25 |
| Protein | 15 |
| Cellulose | 40-50 |
| Water | 8-12 |
| Chlorophyll | - |
| Pectins | 2 |
| Salts (ash) | 10 |

Table 1. Chemical composition of hops[2].

α-acids can be easily isolated as a lead-acetate precipitate from a methanolic solution of hop extract. Repetitive recrystallisation of the isolated α-acids as a complex with o-phenylenediamine, from benzene, produces a pure complex. Decomposition of this complex with dilute

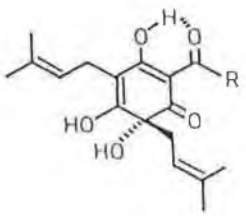
HCl and extraction with ether produces a relatively pure compound now called humulone. Work on the chemistry of α-acids has largely focused on this easily accessible α-acid. The elucidation of the structure of α-acids was carried out in Germany by Wieland[3] and Wolmer[4] in the period 1915-1925. Without the aid of modern analytical equipment they determined the structure of humulone with such accuracy that only minor changes were made at later dates. Using degradation and transformation experiments, such as hydrolysis and hydrogenation, they derived structure (1a) for humulone. Further experiments by Verzele[5] in 1949, Verzele & De Poorter[6] in 1959, and Carson[7] in 1951 led to a modification in the position assigned to the double bond on the isopentenyl side chain of the molecule (1b). The most recent structural modification was in 1970 by De Keukeleire & Verzele[8] and includes enolisation patterns, and the absolute configuration of the chiral center of naturally occurring (-) humulone. (1c)



1a. 1b. 1c.

**Fig.1 Formula of humulone in 1925(1a), 1951(1b), 1970(1c)**

Rigby et al[9]. in 1952 showed the α-acids to be mixtures of homologues and analogues and named the three major ones to as humulone (1), cohumulone (2), and adhumulone (3). Two other naturally occurring α-acids were proposed by Verzele[10] in 1958 and termed pre-humulone (4) and post-humulone (5) (Fig.2). The absolute configuration of all the major α-acids is the same as humulone, and their chemistry studied to date is identical in every respect. Humulone (1) is the only α-acid to be isolated in a crystalline state, lengthy counter-current distribution systems have had little success in isolation of other α-acids.

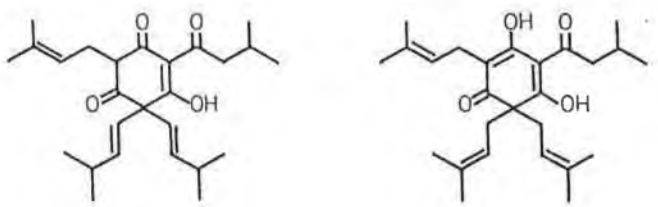


| | | R | % |
|---|---|---|---|
| 1 | humulone | $-CH_2CH(CH_3)_2$ | 35–70 |
| 2 | cohumulone | $-CH(CH_3)_2$ | 20–55 |
| 3 | adhumulone | $-CH(CH_3)CH_2CH_3$ | 10–15 |
| 4 | prehumulone | $-CH_2CH_2CH(CH_3)_2$ | 1–10 |
| 5 | posthumulone | $-CH_2\overset{\dagger}{C}H_3$ | 1–5 |

**Fig.2** α-acid formula and composition of individual homologues and analogues

ß-acids were also obtained in a purified form. Repetitative crystallisations of the resinous material isolated by Hayduck[1] from petroleum-ether leads to a mixture of the major ß-acids lupulone (6) and colupulone (7). The separation of ß-acids from each other is difficult. They can be separated on a gram scale by

counter current distribution methods, but oxidation of the ß-acids in solvents makes this method impractical. Pure ß-acids can be obtained by successive recrystallisation of crude ß-acids mixtures from organic solvents. Colupulone (7) crystallises slightly easier than lupulone (6), and therefore when in excess in a crude mixture it may be obtained pure after a series of recrystallisations.
Wieland[3] in 1925 proposed structure (6a) for lupulone which he based largely on his work with humulone. In 1949 Verzele and Govaert[11] modified the double bond position in this structure (6b), as was done for humulone (1).




(6a) (6b)

**Fig.3** Formula of lupulone in 1925(6a), 1949(6b)

In 1964 Verzele et al.[12] showed that ß-acids exist in two enolic forms (6c) which could co-exist next to each other without equilibration in the absence of acid or base, eg. in iso-octane solution. In 1971 these forms were corrected by Collins et al.[13] (6d)(Fig.4). In contrast humulone (1) was found to occur only in one enolic form. This form appears to be stabilised by hydrogen bond interaction

between the tertiary hydroxyl group on the chiral center and the adjacent carbonyl function. As with α-acids Rigby[9] showed ß-acids to be mixtures of homologues and analogues.

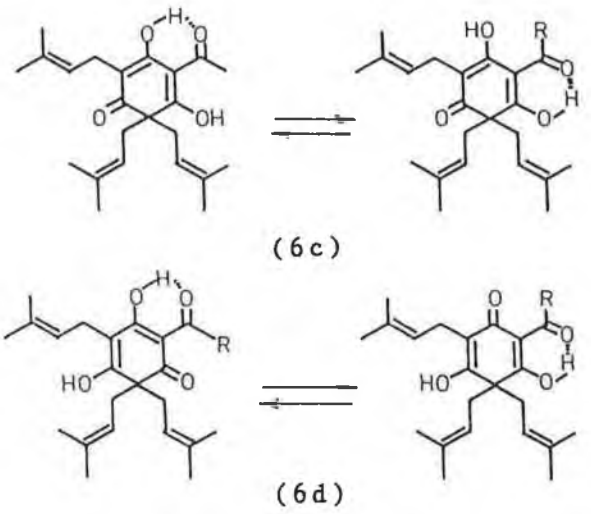
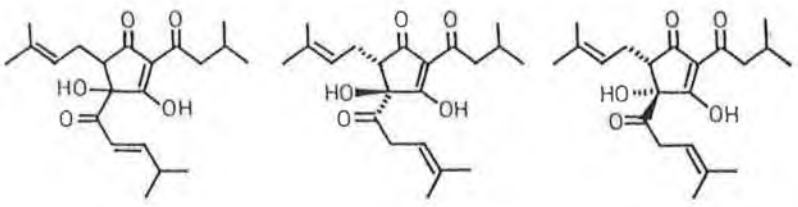



Fig.4 ß-acid formula in 1964(6c), 1971(6d).

The α-acids are not transferred to beer as such, but are transformed chemically during the wort boiling process. Analysis of beer shows that only trace amounts of α- or ß-acids are present. It is now established that the most important reaction in hop chemistry is the production of iso-α-acids by isomerisation under alkaline conditions to produce iso-α-acids. The iso-α-acids are much more soluble in wort beer and are much more bitter than α or ß-acids. Wieland[3] in 1925 was the first to postulate that alkaline fission of humulone proceeded via an intermediate isomer, but he was unable to isolate this intermediate. Windisch et al[14]. in 1927 were the first to study the products resulting from the treatment of humulone (1) with alkaline

buffers and dilute alkaline solutions. The isolated oily product mostly consisted of a compound called iso-humulone (8). In 1947 Verzele and Govaert[15] obtained a crystalline derivative of this oil by hydrogenation. The crystalline product could also be obtained from hydrogenated beer extracts. They assigned the name isohumulone (iso-α-acid) to the oily product, and proposed that this compound was responsible for the bitterness in beer. In 1958 Spetsig[16] showed, using partition chromatography, that each α-acid gave rise to two iso-α-acids. These were later separated on a gram scale using a 2000 transfer counter-current distribution by Verzele et al.[17] in 1965. The isohumulone with the lower partition in a phase consisting of an aqueous buffer and a hydrocarbon could be crystallised from iso-octane and had a melting point of 65°C, and a specific rotation of -7.8° in methanol. This was called trans-isohumulone (9). The second component was obtained as an oil (melting point 18°C) with a specific rotation of +47.6° in methanol, and called cis-isohumulone (10)(Fig.5).




(8) (9) (10)

**Fig.5** Proposed structure of isohumulone in 1925(8), and established trans (9), and cis (10) forms of isohumulone.

De Keukeleire and Verzele[18] established the absolute configuration of the two isohumulones in 1971. By transforming cis- and trans-isohumulone into derivatives which removed the chirality at C4 and then comparing the signs of rotation they showed that C5 in both compounds had the same chirality. Horeau[19] in 1968 had previously shown that C4 of the ring had different chirality in the two compounds, the cis isomer has a 4R,5S form while the trans has a 4S,5S form. The fact that C5 of the ring has the same chirality in both isohumulones proves that the stereospecific step in the isomerisation is not the ring contraction but rather the step which fixes the chirality at C5. A proposed isomerisation mechanism is illustrated in fig. 6[2]. Ionisation of the acid function of humulone deconjugates the double bond system so that ketonisation can occur forming (11). The protonation occurs stereospecifically to give the most stable intermediate (12) with the two large isoprenyl groups in the trans position. The ring contraction occurs non stereospecifically giving a mixture of trans-isohumulone (9) and cis-isohumulone (10). Van Boven[20], in 1971 established that the proportion of the two diastereoisomers formed remains fairly constant over a wide range of pH conditions (25% trans, 75% cis), but in the presence of a bivalent metal more trans-isohumulone is formed. Isomerisation of the three major $\alpha$-acids gives rise to six iso-$\alpha$-acids. Beer contains around 25ppm. iso-$\alpha$-acids, and the bitterness of each individual iso-$\alpha$-acid

is not significantly different (Verzele et al 1970)[21]. The isomerisation of α-acids is carried out industrially and several brands of isomerised product are available to breweries.

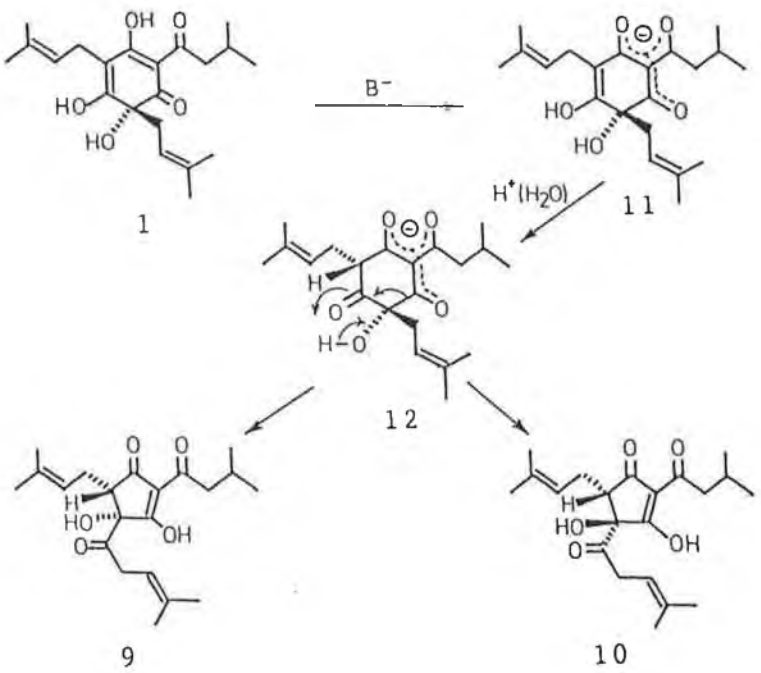



**Fig.6** Proposed mechanism of the isomerisation of humulone leading to trans-isohumulone (9), and cis-isohumulone (10)

Reactions of α-acids in the brewing process are not confined to isomerisations. Humulone has been oxidised in numerous ways, reduced chemically, hydrolysed etc. Some of the reactions have practical importance either because the reaction products may occur in beer or possess desirable bittering potential.

As far back as 1904 reactions involving hydrolysis of α-acids were reported by Lintner & Schnell[22]. As already mentioned hydrolysis of α-acids is strongly catalysed by

alkali. With increasing pH hydrolytic side reactions become increasingly important with the isohumulones being transformed into trans-humulinic (12) and cis-humulinic (13) acids (Fig.7). Since their discovery, a wide range of melting points and specific rotations have been reported for humulinic acid. A number of factors gave rise to this confusion. In earlier studies only one isomer of humulinic acid was isolated and termed humulinic acid A. Anteunis and Verzele[23] in 1962 were the first to present evidence of the occurrence of a second isomer when they succeeded in isolation of pure humulinic acid B. The A and B forms were confirmed as trans and cis forms by Burton et al[24]. in 1964. When humulone (1) is hydrolysed in alkali for a short time iso-$\alpha$-acids are the predominant products. After slightly longer reaction times the main product is a humulinic acid mixture containing about 90% trans form and only 10% cis form, both being partly racemized (Dierckens & Verzele 1969)[25]. The trans form is the more stable and further epimerisation converts most of the cis(+) form into the trans(+) form. Different melting points and optical rotations can be attributed to variable degrees of epimerisation and racemization. Dierckens & Verzele[25] reported that the sodium salt of humulinic acid can easily complex with several chemicals eg. acetone, dioxane, thus adding further to the confusion over the correct melting points and specific rotations. Dierckens and Verzele[25] achieved complete separation of these enantiomorphs via

partial crystallisation of the o-phenylethylamine salt.

The hydrolysis of iso-$\alpha$-acids by alkaline can also lead to the formation of alloisohumulones (14) which occurs by a double bond shift (Fig.7). These also occur in cis and trans forms (Alderweireldt et al.[26], 1965), and can be converted to humulinic acids by the loss of the isohexenoyl side chain. The alloisohumulones are about 50% more bitter than the corresponding iso-humulones, and probably occur in small amounts in beer, but this has yet to be confirmed.

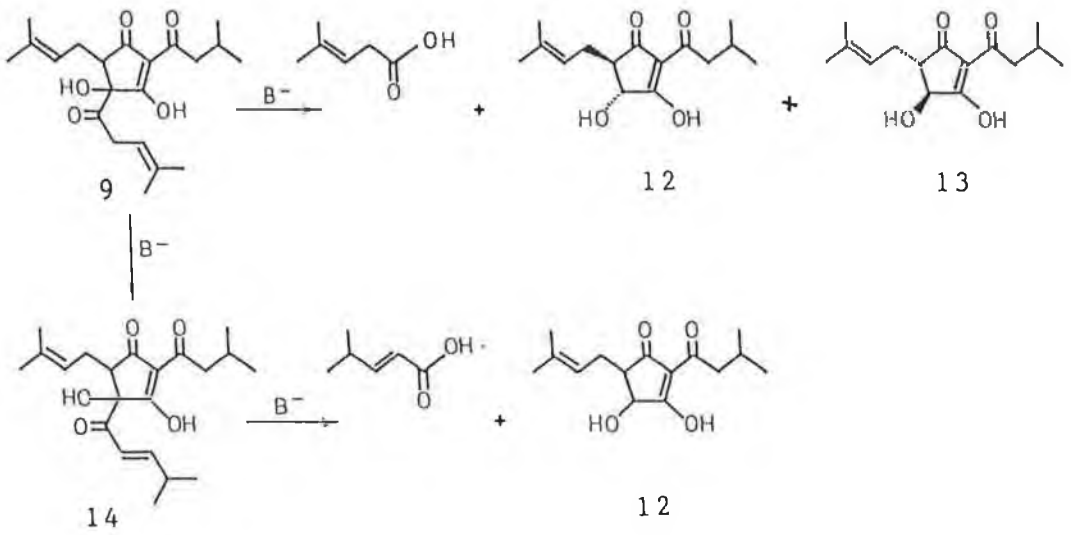
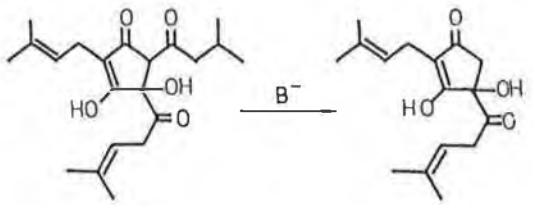



**Fig.7** Alkaline degradation scheme for isohumulone (8) leading to trans-humulinic (12), cis-humulinic acid (13), and alloishumulones (14).

Work by Verzele and Van Boven[20] in 1971 shows that hydrolysis only occurs through the cis-isohumulone (10) form. In the trans form (9) the iso-hexenoyl side chain is protected from base attack by steric hindrance. To react trans-isohumulone it has therefore to be reconverted to

humulone (1) and then reisomerised.

A recent development in hop chemistry has been the isolation of anti-isohumulone (15) and its deacylated derivative (16) from isomerised hop extracts (Fig.8). With these compounds ring contraction is in the opposite direction to that in the usual isomerisation, and are produced in less than 1% in brewing circumstances.




(15) (16)

**Fig. 8** Formula for anti-isohumulone (15) and its deacylated derivative (16)

While it was appreciated in the early considerations of the preservative value of hops, that the ß-resin fraction made some contribution, the potential role of ß-acids derived products in beer bitterness was largely neglected. Because of their low solubility at the pH of wort it was presumed that the ß-acids were precipitated during the wort boiling process and removed as spent hops. The ß-acids have now come under consideration with a view to assessing their value as a source of bittering compounds, which could be incorporated into beer.

Probably the most useful reaction in ß-acids chemistry is

their oxidation. Most of the work to date has utilised colupulone (7). It has been oxidised with hydrogen peroxide in alkaline medium[28] leading to lupuloxinic acid (17) and lupulenol (18)(Fig.9). To date these compounds have not been found in beer. Oxidations have been carried out with sodium or ammonium persulphate by Byrne et al.[29] in 1970, and Reiniger et al.[30] in 1973, up to fifty products were isolated and identified. They were all cyclized structures with furan or pyran nuclei often coupled to the original six membered carbocyclic system of colupulone. These compounds were found not to be bitter. Van Den Bossche et al.[31] in 1972 isolated fourteen compounds by CCD after oxidising colupulone in air, including several peroxides and also five membered compounds (Fig.9). These five membered ring compounds are of importance as they may be potentially bitter. One of these compounds, hulupone (19) has been found in ppm amounts in beer by Spetsig et al.[32] in 1957 and Brohult et al.[33] in 1959. Considerable oxidation of hops occurs during storage before the hops are used for brewing. The lupulones are oxidised more rapidly than the humulones. Because of this considerable attention has been given to the synthesis, and isolation of hulupones. Wright[34] in 1961 oxidised colupulone (7) in the presence of sodium sulphite obtained a 30% yield of cohulupone (20).

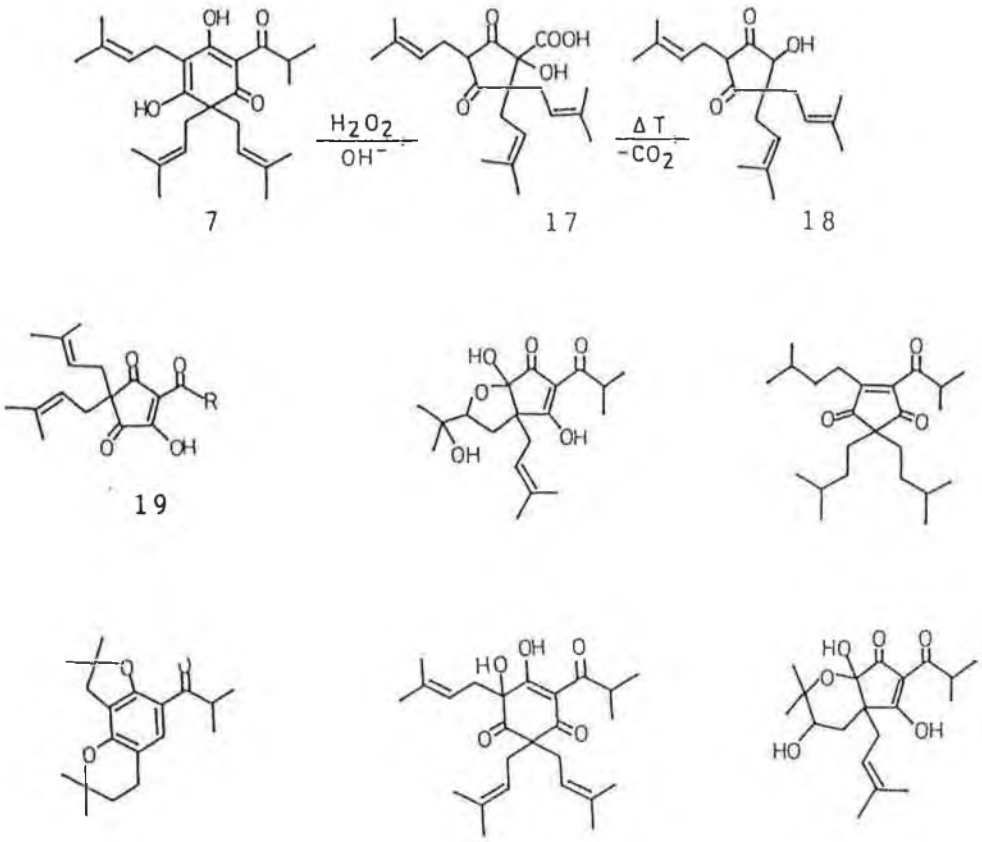
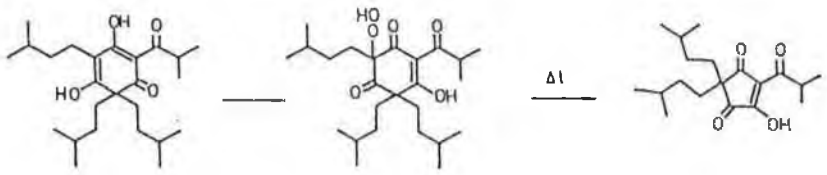



**Fig.9** Oxidation products of ß-acids

Because of the large number of complex products from the autoxidation of colupulone Stevens et al.[35] directed their attention to the oxidation of hexahydrocolupulone (21) This avoided the possible complicating effect of air oxidation of double bonds. From the hexahydrocolupulone a peroxide (22) is formed in yields of over 80%. (Fig.10). Thermal decomposition of the peroxide leads to tetrahydrohulupone (23) in low yields. Siaens et al.[36] in 1977 obtained a 45% yield for this decomposition using CO(II) acetate as catalyst.



(21) (22) (23)

**Fig.10** Oxidation of hexahydrocolupulone (21)

(Tetrahydro)hulupones (23) only arise by some reactions of the peroxide molecule (22). It was shown by Sandra and Verzele[37] in 1975 that 4-hydroxy-4-methyl-2-pentenoic acid lactone (24) is an important by-product of this reaction and only occurs in oxidation mixtures obtained from compounds which produce hulupones. From this observation a reaction pathway to cohulupone (20) was proposed[37] (Fig.11).

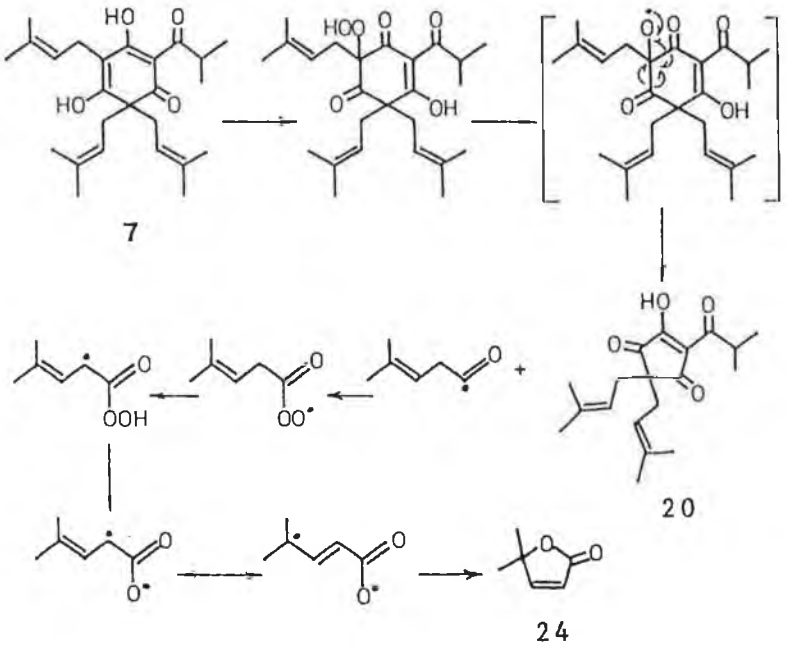


**Fig.11** Proposed reaction mechanism from ß-acids to hulupone

When beer is exposed to sunlight it develops an odour and an off flavour known as "sun struck flavour". The hop acids are very sensitive to visible and u.v. light. Irradiation of humulone (1) at wavelengths greater than 300nm readily affords trans-isohumulone (9) in a totally regio- and stereo-specific fashion. Viriot et al.[38] have adapted this process for the large scale production of beer bitter substances. However at shorter wavelengths the iso-$\alpha$-acids display photoreactivity leading to a rapid development of the so-called "sun struck flavour". Kuriowa et al.[39] have shown that this is due to the formation of 3-methyl-but-2-ene-1-thiol (25). This arises from a photolysed isopentenyl radical and a thiol radical for which there are sources in beer. In 1987 Bondeel et al.[40] examined the behavior of trans-isohumulone (9) upon direct excitation. A major photo product was found to be dehydrohumulinic acid (26) (Fig.12). It occurs as a mixture of two tautomers and represents a rare case of exocyclic enolization in five-numbered hop compounds. Bondeel et al.[40] proposed that dehydrohumulinic acid is formed as a result of $\alpha$-cleavage of the carbonyl group in the side chair at C-4, together with formation of a radical precursor to 3-methyl-but-2-ene-1-thiol (25).

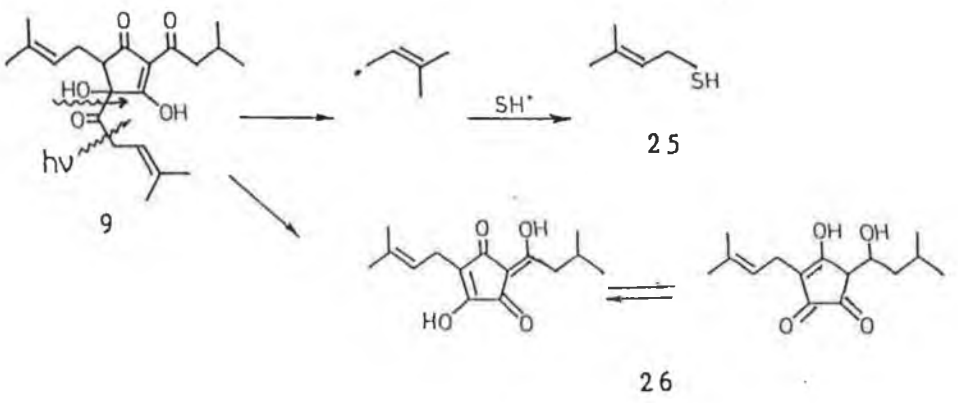



**Fig.12** Photodegradation of trans-isohumulone (9) giving dehydrohumulinic acid (26), and 3-methyl-but-2-ene-1-thiol (25).

Organic chemists has devoted considerable attention to the total synthesis of hop bitter acids. The first synthesis of α-acids and ß-acids was reported by Riedl[41] in 1951 (Fig.13). Starting with phloroglucinol (27), he formed phloroisovalerophenone (28) by a condensation reaction with isopentyl acid chloride. Further reaction with 1-bromo-3-methyl but-2-ene in sodium ethoxide leads to desoxy-humulone (29). This can be oxidised to racemic humulone
(1). The overall yield in this sequence of reactions is as low as 5%. This synthetic α-acid could be isomerised leading to synthetic iso-α-acids. The α-acids and therefore the iso-α-acids are racemic, whereas the hop derived substances are optically active. Ashurst et al.[42] in 1966 reported a direct synthesis of iso-α-acids in a complex series of reactions and again the yield was very

poor. Donnelly and Shannon[43] (1970) described the preparation of tetrahydro-isohumulone (30) by acylation of the diisopentylphloroglucinol (31) followed by oxidation in the presence of lead acetate and rearrangement with alkali. In 1975 Pfenninger et al.[44] improved the yield in all stages of the reaction outlined by Riedl[41], and also claimed the process to be commercially viable. However it is generally accepted that the cost of raw materials alone in a synthetic process like this far exceeds the price of $\alpha$-acids derived from hops. Humulinic acids and their derivatives have also been the subject of synthesis. Howard and Slater[45] oxidised humulinic acid (12) with bismuth oxide to give dehydrohumulinic acid (26). Dihydrohumulinic acid (32) has been obtained by hydrogenation of humulinic acid using Adams catalyst[46]. Oxidation of dihydrohumulinic acid with bismuth oxide affords isohumulinic acid (33)[47]. Burton et al.[24] synthesised cis-humulinic acid (10) from dehydrohumulinic acid (26) using sodium borohydride. Other reactions include synthesis of hulupone (19) by alkylation of dehydrohumulinic acid.[48]

Riedl also obtained ß-acids by total synthesis.[49] Treatment of 2 acylcyclohexane 1,3,5,triones with sodium ethoxide and 1-Bromo-3-methyl-but-2-ene (35) gave mixtures of mono, di, and tetra-alkenyl derivatives together with the required trialkenyl product. For the separation, chromatography is usually necessary, and the overall yields

of ß-acids are low (5-30%)[50]. Collins et al.[13] in 1971 treated 2-isobutyrl-cyclo-hexane-1,3,5-trione (36) in ether with liquid ammonia and then with 1-Bromo-3-methyl-but-2-ene, cohulupone (20) was isolated in 60% yield.

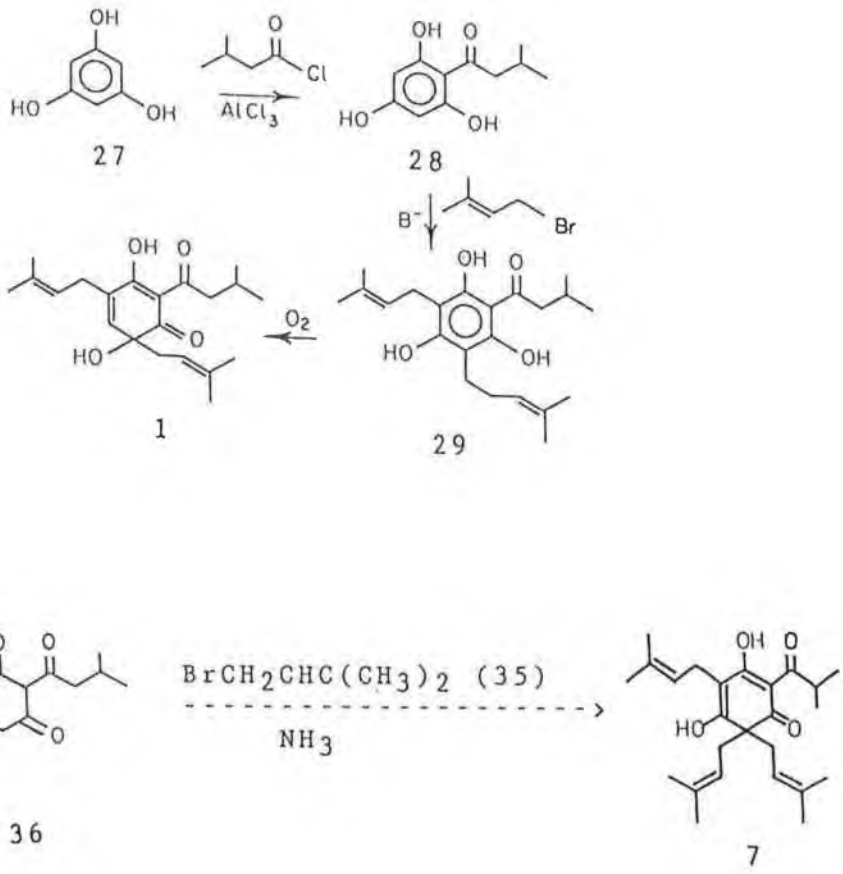



Fig. 13 Total synthetic route to humulone (1) as described by Riedl[41] in 1951, and synthetic route to ß-acids as described by Collins et al.[13] in 1971.

## 1.2 Analysis of Hop Components and Derivatives

From the point of view of brewing technological requirements bitter substances are the most important substances in hops. As discussed they consist of a wide range of chemical substances with divergent properties. A complex multiplicity of analytical methods exist, but they can be broadly divided into three groups, boiling methods, non-specific methods, and specific methods. By the boiling method it is sought to detect the utilisable bitter substances as a total complex. The method of Bishop et al.[51] in 1968 for example estimates the "total realizable bitterness" and is based on extensive isomerisation of α-acids by boiling at pH 7.5 and thereafter measuring the spectrophotometric extinction of an iso-octane extract. Non specific methods include methods based on that of Wollmer where the total resin (hard and soft) could be determined[4]. The specific methods are applicable to the analysis of all hop products and allow the fate of individual bitter substances, whether α- or ß-acids, to be followed during hop processing. Originally the precipitation of lead salts as proposed by Wollmer[4] in 1925 was used as a "specific" gravimetric method, but it can lead to low results especially in old hop extracts. A polarimetric method developed by Salac et al[52]. in 1943 was valued for a considerable time, and was based on the optical activity of α-acids. Today, probably the most

widely used method is the relatively rapid conductometric titration of α-acids. However, not only the α-acids, but also, depending on the solvent used for the analysis, soft resins, hard resins, and in some circumstances even non-bitter substances capable of forming lead salts are detected.

Considerable amount of work has been carried out on methods of prefractionation and separation of individual components before using methods of measurement such as spectroscopy. Especially significant among these are Craig counter-current distribution (CCD), thin layer (TLC) and column chromatography, gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

For many years the CCD method served as the standard reference procedure. It allows separation of bitter substances, and can even separate stereoisomers, but it is very time consuming and requires large volumes of expensive solvents. Further more, it is unsuitable for labile compounds such as the many oxygen sensitive hop components. TLC has principally been used when stereoisomers among groups of bitter substances are of interest. Methods developed include those by Aitken et al.[53] in 1968, and Franiau et al.[54] in 1974. A great advantage of this method is to allow a general view of the qualitative composition of various samples to be obtained quickly, but it is of little use in quantitative analysis.

Column chromatography methods have been found to be

extremely labor intensive. A series of such methods have been developed in recent years, e.g. Spetsig[55] 1961, Kleber and Hums[56] 1973. The most useful development in this field has been the use of ion-exchange chromatography (IEC). Ashurst[57] in 1966 studied the auto-oxidation of humulone (1), iso-humulone (8), colupulone (7), and cohumulone (2) using ion-exchange. Humulone gave a mixture of products that were largely insoluble in light petroleum, whereas most of the products obtained by auto-oxidation of colupulone were soluble in that solvent. An ion exchange method on Dowex resin was put forward by Kokubo et al.[58] in 1968. This was modified by Hansen et al.[59] in 1971. He separated ß-acids, a-acids, humulinic acids, iso-$\alpha$-acids, and hulupones by successive elution with increasing acetic acid concentration in methanol\water. This method has been accepted both by the American Society of Brewing Chemists (1976), and by the European Brewery Convention provisionally as an "international method". Other gradient methods have been developed on Sephadex ion-exchange columns by Nikerson et al.[60] in 1978 and Anderegg et al.[61] 1977 are considered improvements on the international method. It has been shown by collaborative trials that either of these methods give results sufficiently reproducible for commercial requirements. With suitable sample preparation these methods can be used equally well for hop and hop products. Drewett et al.[62] used ion exchange chromatography to show the presence of humulinic

acid (12) in beer regardless of the hopping technique employed. Despite the resolution capabilities of ion exchange chromatography it is a very slow and tedious method, and is rarely suitable for rapid in process analysis.

Work on GLC analysis of hop components by Dalgliesh et al.[63] in 1967 has identified problems in its application in the analysis of bitter substances. These include, thermal degradation of the labile derivatives of the hop bitter substances, also silylation, used to make volatile derivatives, often gives more than one derivative from a single component.

Despite the above range of techniques we can see that there is a need for an analytical method that combines speed and accuracy, and capable of resolving individual components in complex mixtures. This problem has largely been solved over the last fifteen years by application of HPLC to the analysis of hop components. Since conventional or low pressure ion exchange separation of hop components was so successful, ion exchange chromatography was the first HPLC technique developed. Vanheertum and Verzele[64] in 1973 described an ion exchange method for the analysis of hop acids in hops and hop extracts. The column consisted of a four metre (1.1mm internal diameter) stainless steel coiled tube packed with fifty micron glass beads coated with a thin film of a basic ion exchanger in its acetate form.

Eluent consisting of potassium acetate-acetic acid solution in methanol/water at pH 6 was pumped through the column at a high pressure 4000psi and at a flow rate of 55ml/hr. At this flow rate separation of ß-acid oxidation products, colupulone (7), trans-isohumulone (9), and humulone (1) was achieved within fifteen minutes as opposed to several hours by conventional ion exchange methods. This work also established that the samples were eluted quantitatively, even the ß-acids which by many other techniques are underestimated due to their oxidation. The relalibility of the HPLC method for $\alpha$-acid determination was also evident when $\alpha$-acid values obtained were compared to those obtained by conductimetric titration. The latter method produces too high $\alpha$-acid percentages because of interference of oxidation products. In general use several problems were found with this HPLC method. The ion exchange materials initially available for HPLC were pellicular layers of ion exchange resin coated on glass beads. The manufacturers instructions cautioned against the use of more than a small percentage of methanol in mobile phases, as this tended to strip ion exchange material from the beads. As all low pressure IEC separations employed 80% methanol, this was a severe limitation. Water with a small percentage of methanol and very low concentrations of salts, or moderate pH, eluted the hop compounds very rapidly with little resolution. The order of emergence of peaks was also substantially different from that reported by workers using

low pressure ion exchange chromatography (iso-α-acids emerged before ß-acids). Siebert[65] in 1975 proposed that the most likely explanation for this was that separation was brought about by adsorption on the glass beads remaining after the ion exchange resin had been stripped off by methanol, and not by an ion exchange mechanism.

Soon after this a new packing material became available. It consisted of a glass bead to which an anion-exchange material was covalently bonded (Permaphase AAX Du Pont). Although this stationary phase could withstand high alcohol concentrations resolution was still poor in comparison to conventional IEC. Another general feature of ion exchange resins was that reproduction of separations between different batches of resin was difficult to obtain. Siebert[65] also tried separation of hop components using a very small macroreticular bead anion exchange resin using potassium acetate buffer in 80% methanol/water. ß-acid were separated in about two hours without the need for gradient elution, and the α-acids were partially resolved into four components. Unfortunately, acetate buffers used in the procedure were very corrosive to stainless steel HPLC pumps.

In 1977 Otter & Taylor[66] reported the development of a weak anionic high efficiency column packing of phenyl diethanolamine bonded silica, specifically for the resolution of hop resin components. Methanol water mixtures with varying concentrations of citric acid were

the most satisfactory eluents, with retention times within two hours. In a subsequent publication[67] they examined the conversion of humulone (1) to iso-humulone (8) using this system. It was noticed that the rate of decrease of humulone (1) was not matched by the increase of the iso-humulone (8) even when differences in yield and extinction co-efficients were taken into account. This suggests the possibility of formation of an intermediate compound. Otter et al.[67] also measured the ratio of humulone (1) to cohumulone (2) in different hop varieties, and concluded that the ratio is a characteristic of variety and largely independent of area and method of cultivation.

A number of publications reporting the use of HPLC for hop analysis have employed liquid solid chromatography (LSC), usually with silica. This is essentially a transfer of TLC technology, developed for hop material into HPLC. Separations reported indicate a high selectivity for hop components, however it was difficult to cause retention of ß-acids. Some very high activity silicas such as Zorbax SIL gave separation of ß-acids from other components, but $\alpha$-acids were not eluted. Still other selectivity problems were caused by differences in solvent strengths required to elute $\alpha$ and iso-$\alpha$-acids. One approach to solving this problem was the use of gradient programming of mobile phase composition. This lead to poor reproducibility due to changes in baseline absorbance as the solvent composition

altered, and also the water content of the mobile phase had a profound effect on retention.

Another general drawback of liquid solid chromatography was peak tailing. Schwarzenbach[68] in 1979 addressed this problem and showed that an acidic environment on the surface of the silica is essential for elution without tailing. To fulfill this requirement he coated the silica gel with a buffer salt, tailing disappeared, and better selectivity and resolution of components was obtained (Fig.14)

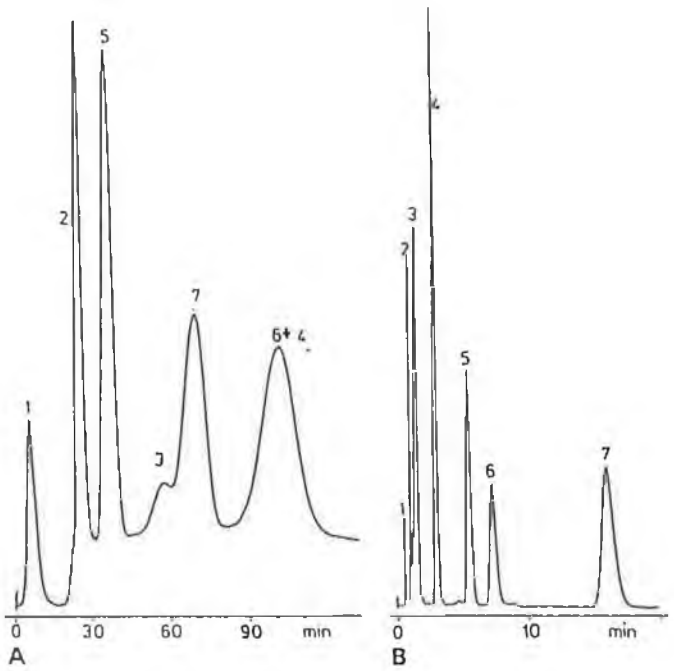



Fig.14 Comparison of IEC and adsorption chromatography on buffered silica gel[68]. **A** Column QAE-Sephadex A-25, mobile phase 80%MeOH in acetic acid to 100% acetic acid. **B** Column LiChrospher SI100,buffered with citric acid sodium citrate,pH 2.8. Mobile phase 10%ethyl ether in hexane. Solvent (1), co-ß-acids (2), oxidised cohumulinic acid (3), cis-co-iso-α-acid (4), co-α-acid (5), trans-co-iso-α-acids (6), cohumulinic acid (7).

With the rapid development of HPLC technology ion exchange, and LSC have been largely replaced by liquid partition chromatography (LLPC). LLPC can be divided into two major categories, normal phase and reverse phase. Normal phase employs a polar stationary phase and a relatively non polar mobile phase, where compounds elute in order of increasing polarity. Reverse phase uses an non polar stationary phase and a polar mobile phase, usually water based, where compounds elute in decreasing polarity order. Spetsig[69] in 1962 was the first to apply reverse phase chromatography to hop components, but it was not until the development of bonded phase packing, where the stationary phase was covalently bonded to a support, that considerable interest was given to reverse phase techniques. The bonded phases have become dominant because of their much greater stability and operational convenience and they permit the use of gradient elution without loss of reproducibility. Siebert[65] in 1975 in analysing the merits of reverse phase chromatography concluded that it was the most suitable HPLC technique for the analysis of the highly polar hop acids and derivatives. Reverse phase separates by polarity and structure and therefore has greater resolving power, capable of resolving classes of compounds such as $\alpha$-acids into individual components. Using a 10u bonded phase Siebert[65] separated iso-$\alpha$-acids into four components. Although base line separation of the components was not achieved, it illustrated the ability of the system to

separate cis and trans isomers.

The versatility of reverse phase HPLC was also examined by Schulze et al.[70] as it applies to hops and hop extracts. The separation of stereoisomers and other closely related compounds were used to demonstrate the powerful capabilities of the technique. Using a μbondapak phenyl column Schulze et al.[70] separated the reaction products from the magnesium catalysed isomerisation of α-acids, these included cis- and trans-isocohumulone and cis and trans isohumulone. No humulinic acid (12) was produced under near neutral conditions in the presence of magnesium. In 1981 Schulze et al.[71] extended this work and examined the formation of trans-humulinic acid (12) from humulone (1) in alkali using a μbondapak C18 column. After 15min reaction isohumulone (8) was observed before any humulinic acid, supporting the proposition that isohumulone is an intermediate in humulinic acid formation. After two hours reaction humulone and isohumulone were nearly depleted with the appearance of cis- and trans-humulinic acids. After three hours trans-humulinic acid became the exclusive humulinic acid product. A second experiment was carried out to see if humulone was formed on conversion of trans-isohumulone to humulinic acid. After nine minutes reaction a small peak for humulone was observed. This work confirmed the proposal by Verzele[20] that trans-isohumulone (9) must be reconverted to humulone and then isomerised to cis-humulinic acid (13). Also over a period of time cis-

humulinic acid will convert to the more stable trans humulinic acid form (Fig.15)

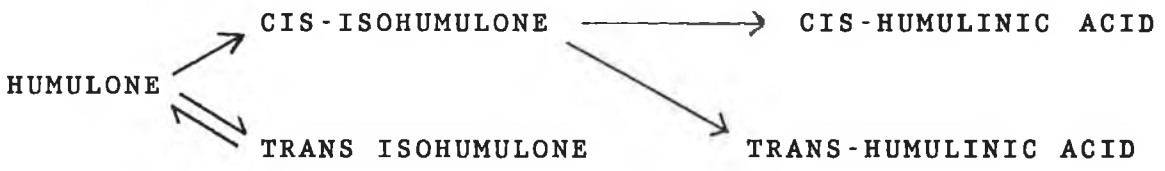



**Fig 15.** Reactions leading to trans-humulinic acid (12)

With the standard reverse phase technique iso-α-acids are in an ionic form while been separated, this can cause them to elute quickly with poor resolution. With paired ion chromatography a large counter ion is added to the solvent system and enters into an equilibrium complex with the ionic species in this case the α-acids. The complexes can then be separated on a reverse phase system as non ionic species according to varying non polar or lipophilic attraction of the complex for the liquid stationery phase. Typical counter ions used include tetra butyl ammonium phosphate (PIC A) for acids, or pentane sulfonic acids for bases. Using a μbondapak C18 with a water methanol mobile phase containing 0.005M PIC A, Whitt & Cuzner[72] in 1979 achieved very good baseline separation of isohumulone (8) and isocohumulone (37), and found that the isohumulone content of total iso-α-acids ranged from 54.2% to 67.7% and depended on the hop variety. This information is important in considering the formulation of hop blends. Also

analysis of foam samples of beer showed that the iso-α-acids were much more concentrated in the foam fraction than in either the liquid fraction or the original beer. This lends support to the theory that iso-α-acids contribute to foam stability.

Verzele and Dewaele[73] (1981) examined the effect of trace metals in HPLC bonded silica gel. A series of hop bitter acids were chromatographed on the commercially available columns. It was proposed that poor resolution and band broadening was largely due to trace metals chelating with hop bitter acids. Without phosphoric acid as an anti-complexing agent, none of the many commercial octadecyl bonded silica gels tested gave satisfactory results. Bonded phase silica gels can be demineralised by boiling with methanolic HCl. On thoroughly demineralised bonded phase good chromatograms were obtained even without phosphoric acid as complexing agent. A demineralised column is commercially available (RSil-$C_{18}$-D), but it has not received universal acceptance, and development work has continued on standard reverse phase columns.

A method for the simultaneous analysis of α-acids, ß-acids and iso-α-acids in hops, hop extracts, was described by Silvester[74] in 1984. By using a high "carbon load" column and inclusion of ortho phosphoric acid in the eluant the effects of metals in silica gel were reduced to a

negligible level for a range of C18 columns. In the analysis of hop components he used a Lichrosorb 5u RP18 column with 15-20% carbon load. Samples were eluted using a gradient system with the initial eluant held for 10min then increased linearly to a more non-polar eluant. Humulinic acid (12) were first identified peaks eluted after 10min. This was followed by two peaks assumed to be the cis (10) and trans (9) forms of isohumulone. Between 20 and 30 min cohumulone (2), humulone (1) and adhumulone (3) eluted, and finally ß-acids eluted after 40min. Iso-$\alpha$-acid samples with components eluting in the first 10min by this method were found to have a high content of haze forming material when used for post fermentation bittering. Although the analysis time is long it provides a simple single analytical method for a wide range of compounds and was therefore an improvement on existing methods.

In the methods up to 1985 separations were achieved using packing material contained in stainless steel columns. The introduction of the radial compression system has greatly improved liquid chromatography in other fields. Buckee[75] in 1985 examined this system in the analysis of hop products and beer. In this system a compression module applies hydraulic pressure to radially compress a polythene cartridge containing the packed material. The wall of the cartridge, when under pressure, is sufficiently pliable to follow the contours of the spherical packing material, and

hence "wall effects" are eliminated and an efficient homogeneous bed is produced which is free from voids and channels. Buckee[75] describes the use of a polythene cartridge packed with spherical C18 material that was treated commercially to remove any residual silanol groups (NOVA-PAK $C_{18}$). Two solvent systems were employed, for α-acids and ß-acids the eluting solvent was methanol/water 85:15 containing 0.025% phosphoric acid and detection was at 313nm. For iso-α-acids 70% methanol in water with 0.005% Pic A reagent as counter ion was used, and detection was at 280nm. Initial studies were carried out on a $C_{18}$ ubondapak as stationary phase but after about 300 to 400 analyses the packing material became contaminated leading to poor resolution of hop components. Using a NOVA-PAK column over 1000 analysis were performed without any significant loss in resolution or precision. NOVA-PAK has a low carbon loading of 7% and this was reported by Buckee[75] as optimum for hop derived component analysis. In contrast Silvester[74] in 1984 used a 20% carbon loading for optimum results. By the method of Bukee separation of α- and ß-acids into their individual components was achieved in 15min, and iso-α-acids in less than 10min. For the majority of the isomerised extracts analysed the total value of for iso-α-acids measured was consistently lower than the value obtained using the European Brewing Commission (EBC) recommended method. In the latter procedure iso-α-acids are eluted from an ion exchange resin

with an acetic acid/methanol gradient. The iso-α-acid fraction is collected and concentrated, and estimated by spectrophomometric measurement at the absorption maximum. However, using the above HPLC system it was shown that peaks representing material other than isohumulones are present on the chromatogram. The EBC method in general over estimates the iso-α-acid content since the other constituents are included in the analysis.

In 1978 The Analysis Committee of the Institute of Brewing appointed a sub-committee with Buckee as chairman to investigate the use of HPLC for the estimation of  and ß-acids in hop extracts, and to establish precision values for the procedure with a view to recommending a standard method. The sub committee decided to test collaboratively the method developed by Buckee[75]. Sample pairs of three hop extracts were sent to seven laboratories for analysis of α-acid and ß-acid content. From the results the sub-committee approved the method for inclusion in "Recommended Methods".

In 1984 two other trials were carried out jointly by Arbeitsgruppe "Hopfenanalyse" (AHA) and the European Analysis Committee. A reverse phase Nucleosil $C_{18}$ column was evaluated for the analysis of hop components. Results were found to be only in agreement if an external rather than an internal standard was used.

Despite the availability of well resolved chromatograms of complex hop and beer extracts identification of unknown components is difficult on the basis of chromatographic data alone. The development of photo diode array (PDA) on line detection has greatly assisted peak identification. The PDA system enables complete ultra-violet absorbance data of each component in a chromatographic run to be obtained. This eliminates the tedious work of collecting and evaporating fractions and bringing compounds off line to a spectrometer for analysis. Moreover, isolating trace amounts of hop acids can be difficult due to their sensitivity to oxidative transformations. Although the information contained in a uv spectra is not as detail as that provided by other spectrometric techniques in practice the uv data can be characteristic of a class of compounds or similar to another known compound. PDA is also valuable in evaluation of peak homogeneity by comparison of uv spectra in different regions of an eluted peak.

Van De Velde & Verzele[76] illustrated the use of PDA detection by identifying hulupone (19) in a commercial beer iso-octane extract. Also identified were two deoxy-α-acids, probably 4-deoxycohulupone (38) and deoxyhumulone (39), both precursors in the biogenesis of α-acids and ß-acids in hops.

HPLC technology as applied to the analysis of hops and hop derivatives has advanced considerably over the last ten

years. The scope of procedures such as those discussed are generally limited to qualitative or at best semi-quantitative applications, and this is largely due to the unavailability of pure reference standards. Because of the range of mobile phases and elution pHs extinction coefficient cannot be compared from one system to another as has been done in some semi-quantitative analyses of hop derivatives.

Comprehensive application of HPLC to the quantitative determination of hop-derived components in hop extracts, beers and worts demands many reference compounds of a high purity. The synthesis and purification of a range of relevant components has been described by several authors[24,45,48,49,50], but in our laboratory these procedures failed to give compounds of sufficient purity. In this thesis we report modified preparations of humulinic acids (9,10), dehydrohumulinic acids (26,40), and hulupones (19,20). Established procedures were followed as the most feasible route to these compounds, but modifications were introduced in order to obtain compounds sufficiently pure to be used as HPLC standards. The readily available and cheap iso-$\alpha$-acids and ß-acids were used as starting material for these procedures.

# SECTION 2

# RESULTS and DISCUSSION-SYNTHESIS

## 2. RESULTS and DISCUSSION

### 2.1 SYNTHESIS

#### 2.1.1 Preparation of trans-humulinic acid (12) and trans-cohumulinic acid (41).

In the past, published procedures for the isolation of humulinic acids have to a large extent depended on lengthy counter-current distribution (CCD) procedures, such as described by Durant[77] in 1966 and Dierckens et al.[25] in 1969. In the latter procedure, humulinic acids derived from alkaline treatment of α-acids, were separated into their individual components by a 1780-transfer CCD. Although pure compounds can be obtained by this procedure, it is unsuitable for the isolation of large quantities of material. Diffor et al.[78] described the isolation of humulinic acid from hydrolysed iso-α-acids by a simple extraction procedure. The product obtained was impure with a melting point of 81°C in contrast to 93°C by CCD procedures[25]. Because of the simplicity of this method, and the availability of iso-α-acids in large quantities, we have examined this method with a view to improving product purity and yield.

Initial repetition of the procedure as described by Diffor et al.[78] gave 3g of beige crystals, with a melting point of 79°C, from 100g of iso-α-acid extract. Further crystallisation of this product from n-hexane failed to

increase the melting point above 85°C with little improvement in colour even with activated carbon treatment. In the original procedure[78] an iso-α-extract was hydrolysed by adjusting to pH 13 and refluxing for 15 minutes. We find this procedure gives incomplete hydrolysis of the iso-α-acids. Complete hydrolysis was obtained by using an excess of 4M NaOH with refluxing for 15 minutes. On acidification, the humulinic acids formed a water insoluble layer which was separated from the aqueous solution. By the procedure of Diffor et al.[78] extraction of the hot acidified aqueous solution with hexane was proposed, but we found this method to be less efficient and difficult to perform. It was also established that crystallisation of the humulinic acid extract from hexane at -35°C instead of -4°C gave a much higher yield of crude humulinic acids, 16g as opposed to 4g. An important feature of this recrystallisation is that although the greater proportion of humulinic acids precipitated from solution, oils and waxes remained in solution, and were therefore removed by filtration. The product at this stage was a mixture of trans-humulinic acid (12) and trans-cohumulinic acid (41), with some contamination from oily products. Cohumulinic acid was found to be much more soluble in hexane at room temperature than trans-humulinic acid. Three successive recrystallisations at room temperature incorporating activated carbon treatment gave pure trans-humulinic acid (12). From 100g of iso-α-acid extract 28g of product was

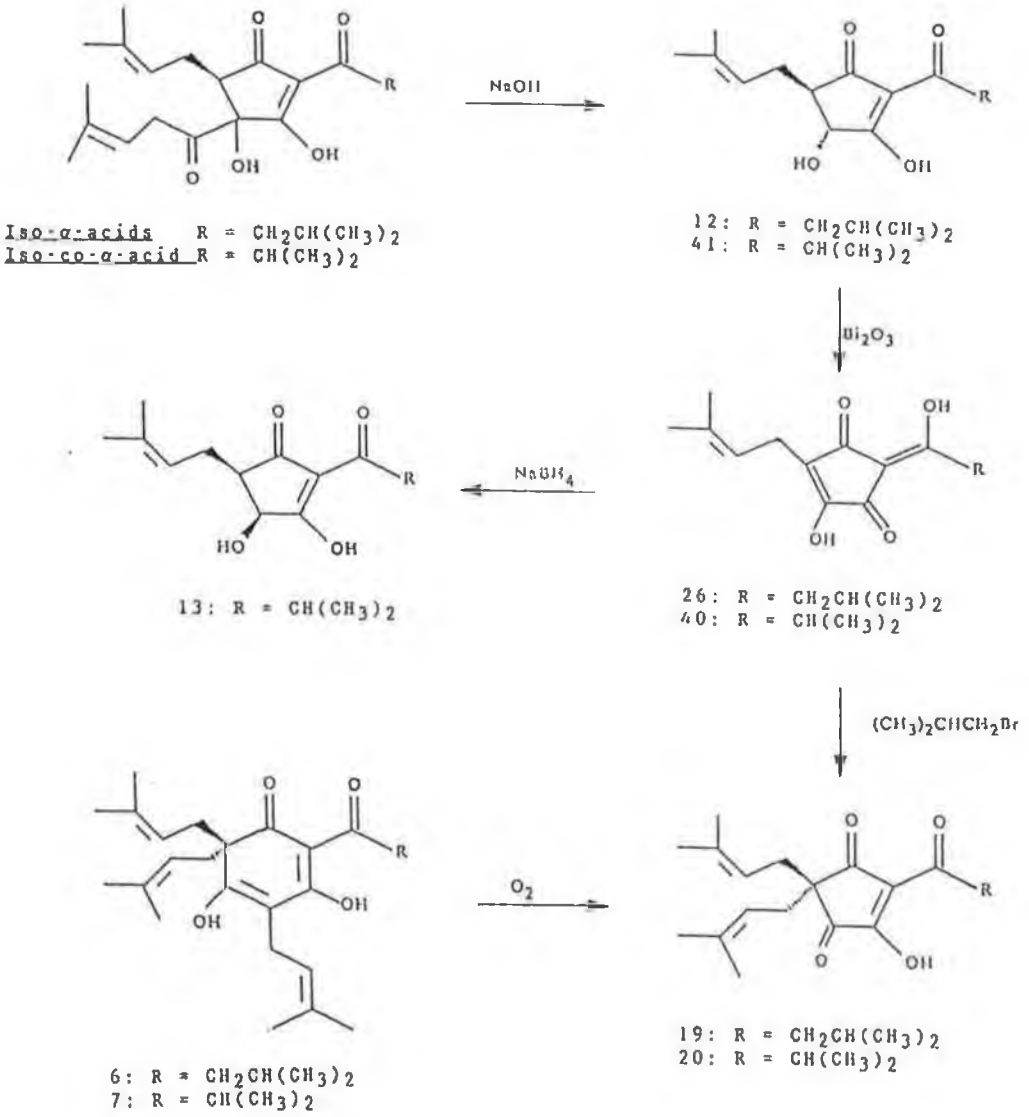
obtained, a 2.5 fold increase in yield compared to the published method.[78] The melting point of this product was 93°C, in agreement with product isolated by CCD procedures[25]. The product isolated by Diffor[78] may contain a large proportion of trans-cohumulinic acid (41) and iso-humulone (8), and possible other undefined hydrolysis side products.

In some reactions hydrolysis produced a small amount of side product with low solubility in hexane. This material was found to be insoluble in carbon tetrachloride and therefore could be removed by crystallisation with this solvent.

By the above method it was not possible to obtain trans-cohumulinic acid (41) without contamination from trans-humulinic acid (12). Several solvents, and combinations of solvents, including $CCl_4$, DMSO, methanol/water, were tried at a range of crystallisation temperatures without success. As our overall aim was to obtain a HPLC pure reference standard, isolation of a mixed reference standard was the most feasible alternative. As one component (trans-humulinic acid) of this standard was available in pure form, the mixed reference standard would be a suitable standard for quantitative HPLC analysis. This standard was obtained by concentration of combined mother liquors after the isolation of trans-humulinic acid (12), and precipitation at -35°C. Crystallisation at room temperature in a minimum volume of hexane gave a pure mixed

reference standard, calculated by HPLC to have a cohumulinic acid (41) to trans-humulinic acid (12) ratio of 65:35 respectively.

**The importance of the above modified procedure is that it not only provides pure reference standards but also provides access to a large quantity of pure starting material for synthesis of other reference standards. (Scheme 1).**




Scheme 1. Reaction pathway from iso-α-acids to: trans-humulinic acid (12), trans-cohumulinic acid (41) dehydrohumulinic acid (26), dehydrocohumulinic acid (40), hulupones (19), cohulupone (20), and cis-humulinic acid (13), and the conversion of ß-acids to hulupones (19,20).

2.1.2 Preparation of Dehydrohumulinic acid (26) and Dehydrocohumulinic acid (40)

Oxidation of trans-humulinic acid (12) with bismuth oxide in acetic acid to form dehydrohumulinic acid (26), has previously been outlined by Howard & Slater[45]. Modifications to this procedure resulted in a reaction that proceeded in a straight forward manner yielding chromatographically pure product. Reaction variables, including amounts of reactants and solvent were examined with a view to increase the yield of product. The quantity of acetic acid used as solvent was found to have a significant effect on yield. Reactions with greater than 10ml acetic acid per 1g of humulinic acid consistently gave lower yields of product. In the procedure of Howard & Slater[45] 40ml/g was used with a yield of 30%. Lowering the acetic acid to 10ml/g gave an increased yield to 40%. Crystallisation of crude product from propan-1-ol increased the melting point from 129°C to 135°C. This value was in agreement with the published value[45]. However, HPLC analysis of this product showed the presence of a number of minor impurities accounting for 5% of the total peak area. These impurities were removed by a further recrystallisation from hexane although no change in melting point was observed. Howard & Slater[45] purified the reaction product by precipitation from aqueous ethanol, we found that the quality of product varied with the amount of water used for precipitation, and also on the precipitation

temperature. These problems were not encountered using propan-1-ol or hexane.

A mixed reference standard of dehydrohumulinic acid (26) and dehydrocohumulinic acid (40) was obtained from the oxidation of trans-humulinic acid (12) and trans-cohumulinic acid (41) mixed reference standard. The ratio of the two components obtained was identical to that of the starting humulinic acids. Unlike the humulinic acids this ratio remained constant after successive recrystallisations from hexane or propan-1-ol.

### 2.1.3 Preparation of Cis-humulinic acid (13)

Under the alkaline conditions used for the hydrolysis of iso-$\alpha$-acids cis-humulinic acid (13) was converted to the more stable trans-humulinic acid (12). A synthetic procedure for the cis isomer was therefore required. A procedure by Burton et al.[24] was followed whereby dehydrohumulinic acid was reduced stereospecifically with sodium borohydride to give cis-humulinic acid (13). On completion of the reaction an oily product was isolated from an ethereal extract of the acidified reaction mix, which was found to be unstable in air. Recrystallisation of this product from hexane was required immediately on isolation to prevent further oily products forming, otherwise it was impossible to obtain crystalline cis-humulinic acid (13). Recrystallisation from n-hexane,

rather than cyclohexane as reported[24], allowed crystallisation at -5°C with subsequent higher first crop recovery of product. Second crop material was found to be of poor quality and difficult to crystallise. No improvement in yield of cis-humulinic acid (13) over that published was obtained, but the melting point of 73.5-74°C was slightly higher than the 72-73°C previously published[24]. Cis-humulinic acid (13) isolated by CCD procedures had a melting point of 74°C[25].

2.1.4 Preparation of Hulupone (19) and Cohulupone (20)

Of the compounds synthesised hulupones proved to be the most difficult to obtain in a chromatographically pure form. Following the procedure of Wright[48] hulupone (19) was obtained by alkylation of dehydrohumulinic acid (26) in a 54% yield. HPLC analysis of this product showed it to have a purity of less than 80%. The major modification to the published procedure was a lowering of the reaction temperature from 0°C to -10°C. This significantly decreased the formation of impurities, and increased yield. It was essential to start with very pure and anhydrous dehydrohumulinic acid (26) as otherwise impurities were carried through to the final product. Also, on forming the sodium salt of hulupone during the work up procedures, it was washed with water and hot hexane. Efficient washing at this stage removed the greater proportion of oily and waxy

impurities. Distillation of the isolated product using a "Kugelrohr" short path distillation apparatus proved to be an efficient procedure for the final purification of hulupone. In our laboratory hulupone distilled at 140°C at 0.3mm Hg, Wright[48] reported a distillation temperature of 100°C at $10^{-4}$mm Hg. Modifications to Wright's[48] procedure increased the yield from 54% of impure product to 67% of a chromatographically pure product, giving 8.5g of pure hulupone from 10g of dehydrohumulinic acid.

On a number of occasions the distilled hulupone while hot was washed from the receiving flask with n-hexane. It was found that this hexane could not be removed completely from the hulupone by rotary evaporation even at 90°C and 0.5mm Hg. The resulting liquid was very fluid in comparison to hulupone and its distillation range was from 100°C to 140°C at 0.3mm Hg. Fractions taken over this distillation range all contained hexane, and no pure product was obtained. A similar effect was observed with diethyl ether. As a very broad distillation range was observed this effect is not a simple azeotropic effect. Verzele et al.[25] observed that humulinic acids formed stable complexes with a number of solvents and this possibility also exists with hulupone.

If the isolated hulupone is to be used for flavour assessment care must be taken not to distill it at too high a temperature. Distillations in excess of 160°C gave a "burned" odour to the distilled product.

Starting with the dehydrohumulinic acid (26) and

dehydrocohumulinic acid (40) mixed reference standard a mixed reference standard of hulupone (19) and cohulupone (20) was also synthesised by the same modifications to the procedure of Wright[48]. The product distilled at the same temperature and pressure as the pure hulupone standard, giving a similar yield of 65%.

### 2.1.5 Isolation and Purification of ß-acids

Hop ß-acid fraction consists of a thick oily mixture of ß-acids, waxes and other resins, and also residual components found in α-acid extracts. Initial attempts at the isolation of ß-acids from a crude ß-acid extract were based on the method of Verzele[79] in which the ß-acids extract was dissolved in hexane and washed with sodium carbonate to remove α-acids. Subsequent extraction into NaOH, acidification, and re-extraction left a ß-acid mixture. Recrystallisation from hexane improved purity of this mixture, but HPLC analysis showed the presence of α-acids. Extractions by this procedure were difficult due to oils, waxes and other resins forming emulsions and poor interfaces at phase splits. An alternative procedure was therefore devised, in which the crude ß-acids was refluxed in a potassium carbonate solution for several hours. This converted all the α-acids to iso-α-acids. Waxes and other resins separated from the aqueous solution and were easily removed. Lowering the pH of the aqueous solution to pH 8

results in separation of iso-α-acids and ß-acids, the ß-acids been precipitated from solution. However the ß-acids invariably formed a viscous mass which made filtration impossible. It was therefore found to be more practical to maintain the aqueous solution hot (80°C) after adjusting to pH 8, and allow the ß-acids to form a dense fluid layer in a separating funnel. Recrystallisation of this fluid ß-acid layer from n-hexane gave a ß-acid mixture which was predominantly colupulone (7) and lupulone (6). ß-acids are very susceptible to oxidation, and to prevent this all crystallisations were carried out under a nitrogen atmosphere. Obtaining individual ß-acids in a pure form can prove difficult. The only practical method of separation on a preparative scale is crystallisation and this is only successful on a mixture containing a decided excess of the desired ß-acid. The crude material we used had a slight excess of colupulone (7). Multiple recrystallisations were required in order to obtain a pure sample of colupulone (7), with a 90% loss of starting ß-acids mixture. Isolated colupulone (7) had a melting point of 94°C and this was in agreement with reported values,[50] although values up to 97°C have been reported[79]. Colupulone (7) synthesised by Collins et al.[13] using a total synthetic procedure also had a melting point of 94°C. Recrystallisation procedures for ß-acids were tried with a wide range of solvents, but none were found that would selectively crystallise one of the ß-acids from a mixture.

As well as been unselective, recrystallisation procedures were generally restricted by oxidation products preventing crystallisation of ß-acids.

2.1.6 Hulupone (19) and cohulupone (20) from ß-acids

In the isolation of $\alpha$-acids from hops a waste ß-acids fraction is also isolated. The possibility of utilising this fraction to produce flavour enhancing components exists, and is of economic importance. The production of hulupones from ß-acids has the most potential in this area, and with this in view synthesis of hulupones from ß-acids has been examined in hexane, methanol, and alkaline solutions. Oxidation under alkaline conditions was the first method examined. A similar method was described in a British Patent in 1964[80] for the large scale production of hulupones from ß-acids. The method involved bubbling oxygen through an alkaline solution of the ß-acids at 90°C and subsequent isolation of a crude hulupone mixture. Several problems were found with the method. Firstly, after the oxidation stage the pH of the solution was lowered to pH 7 to precipitate unreacted lupulones to leave a solution consisting mainly of hulupone (19), cohulupone (20) and lupuloxinic acid (17). Invariably the lupulone precipitated as an oil. Further reduction of the pH to 4.5 and boiling the solution converts lupuloxinic acid (17) to the insoluble lupulenol (18). The lupulenol (18) however,

formed an emulsion and could only be removed by centrifugation. According to the patented procedure[80], after removal of the lupulenol (18) the solution was used to enhance the bitter flavour of beer. HPLC analysis of this solution determined the concentration of hulupone (19) to be approximately 0.15%, together with many undetermined oxidation products. The expected concentration of hulupone (19) according to the published procedure was approximately 0.9%. The poor yield we obtained was most likely due to using air instead of pure oxygen to oxidise the ß-acids. The working up procedure, used for hulupone (19) synthesised by alkylation of dehydrohumulinic acid (26), was applied to this solution. A mixture of hulupone (19) and cohulupone (20) (0.43g) was obtained from 10g of ß-acids. HPLC analysis showed this hulupone sample to contain one major impurity. Due to the inadequate removal of lupuloxinic acid (17) and lupulenol (18) from the oxidised mixture either would be a possible impurity. They were therefore synthesised, and HPLC analysis confirmed the presence of 15% lupulenol (18) in the hulupone (19), cohulupone (20) mixture.

The purification procedure of forming the sodium salt and subsequent acidification and extraction was successful in removing all impurities except lupulenol (18). It would therefore be a worthwhile addition to the patented procedure,[80] and would eliminate the addition of many unknown compounds to fermented beer.

A solution of colupulone (7) in hexane was aerated for twelve hours and the resulting yellow solution extracted with sodium bicarbonate, and worked up in a similar manner to the isolation of hulupone (19). A low yield of cohulupone (20) (100mg) was obtained from the auto-oxidation of 10g of colupulone (7). It had a purity of approximately 75%, and this was not improved by reconversion to the sodium salt and repeated work up. The method is therefore not specific enough for the industrial production of hulupones, nor is it suitable for the isolation of chromatographically pure standards.

Wright[48] reported a procedure for the oxidation of ß-acids in methanol in the presence of sodium sulphite. This procedure was followed with the modification that air was used instead of pure oxygen. To compensate for this the reaction time was extended from 3 to 12 hours. Also, the procedure was modified to include a purification step which included reformation of the sodium salt, extraction and distillation. A yield of 30% hulupones was obtained after purification and distillation, and this yield was similar to that reported by Wright.[48] HPLC analysis showed the compound to be a mixture of hulupone (19) and cohulupone (20) in a ratio of 65:35. Other impurities in the mixture accounted for less than 1% of the chromatogram area. The method is therefore very suitable for isolation of a mixed reference standard of hulupones. Isolation of a pure cohulupone (20) sample by this method would also be

feasible provided a ß-acid sample with a large excess of colupulone (7) was available. This procedure gives approximately the same yield as that reported for the oxidation in alkaline conditions, without the need for high temperatures and inadequate precipitation procedures. It may therefore be suitable for the large scale synthesis of hulupones.

### 2.1.7 Lupuloxinic acid (17) and Lupulenol (18)

In addition to the above compounds, which were synthesised as HPLC reference standards, lupuloxinic acid (17) and lupulenol (18) were synthesised in order to verify their appearance as impurities in hulupones derived from ß-acids. Their synthesis followed a method outlined by Howard and Pollock.[28] Lupuloxinic acid (17) was obtained by oxidising ß-acids using hydrogen peroxide. Initial attempts at the synthesis failed, due to the production of hulupone (19) and cohulupone (20) and other oxidation products. Degassing of the ß-acids solution and oxidation under nitrogen was necessary in order to produce lupuloxinic acid (17) in the yield and purity reported.[28] It was found that lupuloxinic acid (17) could be readily decarboxylated in acid solution at room temperature. It was therefore important to maintain a low temperature (10°C) during the acid precipitation of the product. Complete decarboxylation of lupuloxinic acid (17) was

obtained by heating in toluene resulting in the formation of lupulenol (18) in an 81% yield.

## 2.2 STRUCTURAL AUTHENTICITY

### 2.2.1 Nuclear Magnetic Resonance.

N.m.r. spectroscopy was used as the main criterion in establishing structural authenticity of standard and mixed reference standard compounds. A feature of the $^{1}H$ n.m.r spectrum of cis-humulinic (13) and trans-humulinic acid (12) is the overlap of peaks in the 2.4 to 3.0ppm region. This region of the spectrum was assigned by a combination of decoupling experiments, COSY n.m.r. (Fig.17), and comparison with related spectra. Cis-humulinic acid (13) and trans-humulinic acid (12) possess a chiral centre at C-5 of the ring system (Fig.16), which complicates the splitting patterns for protons in the isopentenyl chain attached to C-5. Proton assignment is further complicated by overlap of peaks from the isopentenyl and acyl side chain in the 2.4 to 3.0 ppm region of the spectrum.

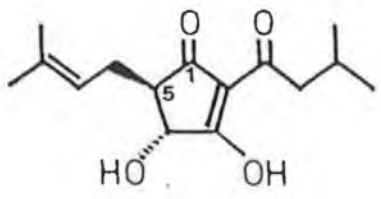



**Fig 16. Humulinic acid (12)**

In trans-humulinic acid (12) the C-5 proton was confirmed on two grounds to have a chemical shift of 2.9 ppm. COSY n.m.r. shows this peak to be coupled to the C-4 proton at 4.04 ppm, and secondly, an analogous peak was absent from dehydrohumulinic acid (26) where the C-5 proton is absent. Two protons at 2.7 ppm were only coupled to the CH septet of the acyl chain and therefore are due to the $CH_2$ of this chain. Two broad peaks remain in the 2.4 to 3.0 ppm region with an integration value of one proton each. Decoupling at 5.2 ppm (C=CH of iso-pentenyl) had an effect on both these peaks, although the effect was more remarked on the peak with the higher shift value. This coupling was also observed by Cosy n.m.r.. It was therefore established that peaks at 2.65 and 2.48 ppm were due to the $CH_2$ protons of the isopentenyl chain. The difference in shift values for these two protons is largely due to the presence of the chiral centre at C-5. The non-equivalency of these protons may be further exaggerated by one of them coming under the influence of the anisiotropy cone of the ring carbonyl group. The two methyl protons on the isopentenyl chain of humulinic acid were found to be non-equivalent with one set giving a singlet at approximately 12Hz lower field than the other (recorded at 270 MHz). Although these $CH_3$ groups are cis and trans to a proton it is unlikely to have an effect as these $CH_3$ protons in dehydrohumulinic acid (26) give rise to a singlet. The most likely factor contributing to the non equivalence is therefore the chirality of C-5. The

proton at C-4 gave a sharp doublet with a coupling constant of 4.4Hz. This points to a dihedral angle of 120°C between the C-4 and C-5 protons so confirming the trans configuration.

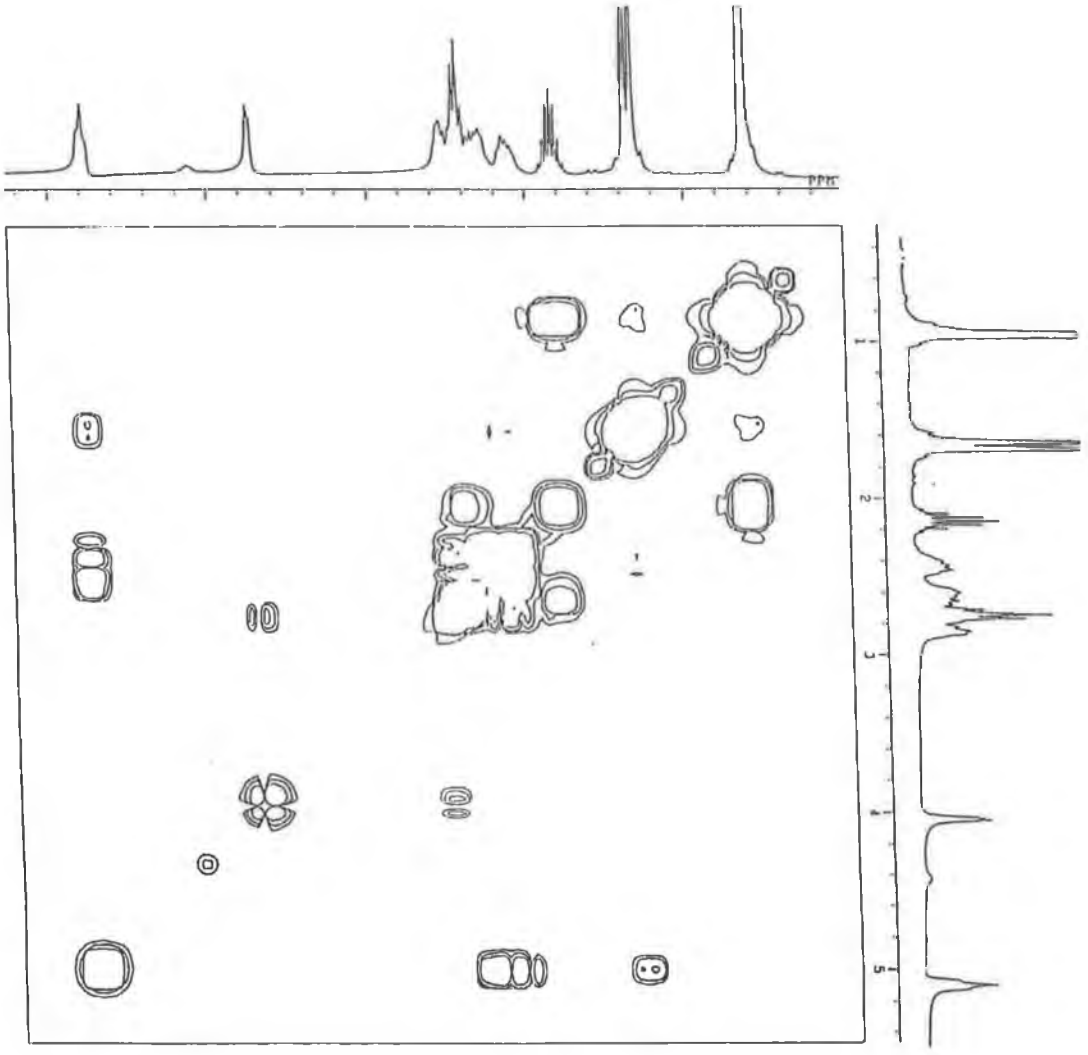


**Fig.17** C.O.S.Y. N.m.r spectrum of trans-humulinic acid (12)

Cis-humulinic acid (13) gave a similar spectrum to the trans isomer (12), except that the C-4 and C-5 proton signals are at slightly lower field. This simplifies the spectrum as the C-5 proton signal is unobscured. The C-4 proton has a coupling constant of 7.4Hz and this points to a dihedral angle of $0^{o}$ between the C-4 and C-5 protons, and so is in a cis configuration.

The spectrum of dehydrohumulinic acid (26) was less complicated than that of the humulinic acids, mainly due to the removal of the chirality at C-5.

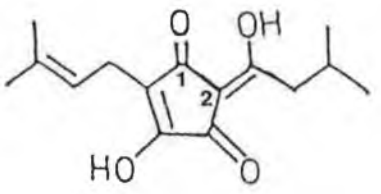



**Fig. 18** Dehydrohumulinic acid. (26)

The methyl protons on the isopentenyl group, in contrast to the humulinic acids, gave a single peak. The $CH_2$ group in the 2-acyl group gave rise to a doublet approximately 0.2ppm upfield of the corresponding signal in humulinic acid. Scoolery's constants and the ß-shift for a hydroxyl[81] predict that methylene protons adjacent to a carbonyl would be less shielded than when adjacent to enolized carbonyl by about 0.28ppm. The observed shift is as expected for methylene protons adjacent to an enolized

carbonyl, but is lower than predicted due to the carbon double bond at C-2. The acyl carbonyl is therefore likely to be enolized, in contrast to the situation in humulinic acid. As expected the signal for the $CH_2$ group of the isopentenyl chain is 0.5ppm lower field than in humulinic acid due to deshielding by the 5C-double bond, and it is split as a sharp doublet due to coupling to the adjacent olefinic proton. No signals were observed for C-4 or C-5 protons confirming the required oxidation had taken place. Bondeel et al.[40] in 1987 published $^1H$ n.m.r. data for dehydrohumulinic acid (26), and the reported chemical shift values were identical to those obtained for our pure standard. However, from decoupling experiments we have established that the peak at 3.04 ppm is due to the $CH_2$ on the isopentenyl side chain, while that at 2.6 ppm is due to the $CH_2$ on the acyl side chain, not the contrary as reported[40].

The $^1H$ n.m.r. spectrum of hulupone (19) (Fig.19) confirmed that a second iso-pentenyl chain had been added at C-5, as integration values for all relevant protons were doubled in comparison to dehydrohumulinic acid (26). Like the humulinic acids it appears from chemical shift values that the $CH_2$ on the acyl group (2.9ppm) is attached to a non-enolized carbonyl. The signals for the two $CH_2$ of the isopentenyl group are distributed between two peaks between 2.4 and 2.6 ppm as was for the humulinic acids, but the

ratio of protons in the two peaks is 3:1. Twelve protons from the isopentenyl $CH_3$ groups gave a singlet as was obtained before in the absence of the chiral centre at C-5.

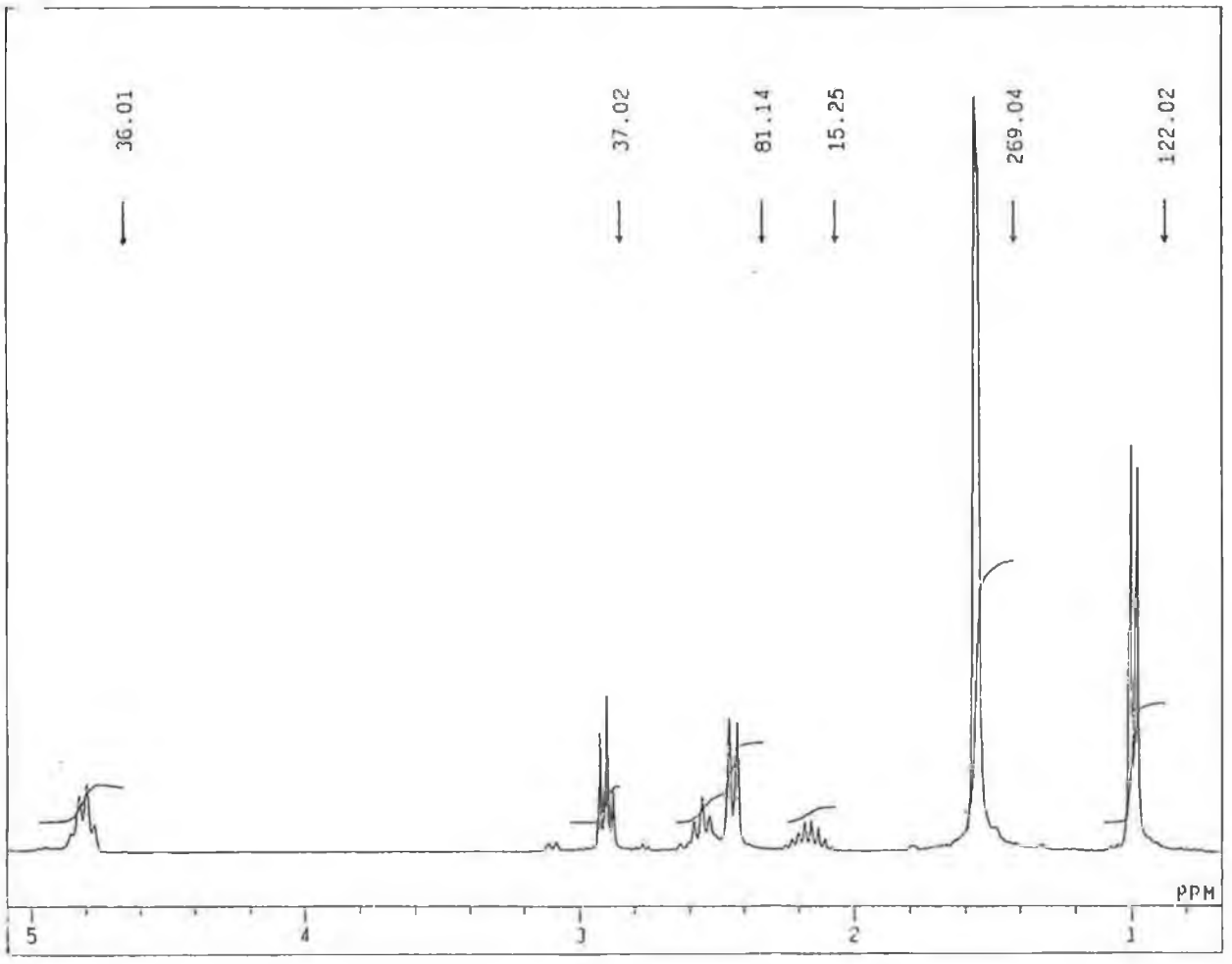



**Fig. 19** $^1H$ N.m.r (270MHz) Spectrum of hulupone (19).

The spectrum of colupulone (20) combined many of the features of the spectra of the other standards. The iso-pentenyl group at C-6, attached to an unsaturated ring carbon gave an identical spectrum as the isopentenyl group in dehydrohumulinic acid (26). The two isopentenyl groups at C-4, attached to a saturated carbon atom gave a spectrum almost identical to that of the two isopentenyl groups in hulupone (19). The main difference was that the $CH_2$ in this group gave rise to two multiplets of equal intensity rather than the 3:1 ratio in hulupone (19). The CH of the acyl group gave rise to a septet as expected, but a feature of this signal not previously appreciated as spectra were recorded at lower frequency, was the presence of two other sets of septets (Fig.20). The total intensity of the septets was equivalent to one proton, and they had a ratio of 0.27:0.6:0.13. This indicates that the acyl group exists in three different forms. Chelated enolic structures could possibly account for these forms (Fig.20). Only two OH signals of a combined intensity of 1H were observed in the 6-8ppm region. These were in a ratio of 0.73:0.26 and as they appear sharp and at high frequency they are unlikely to be involved in chelation.

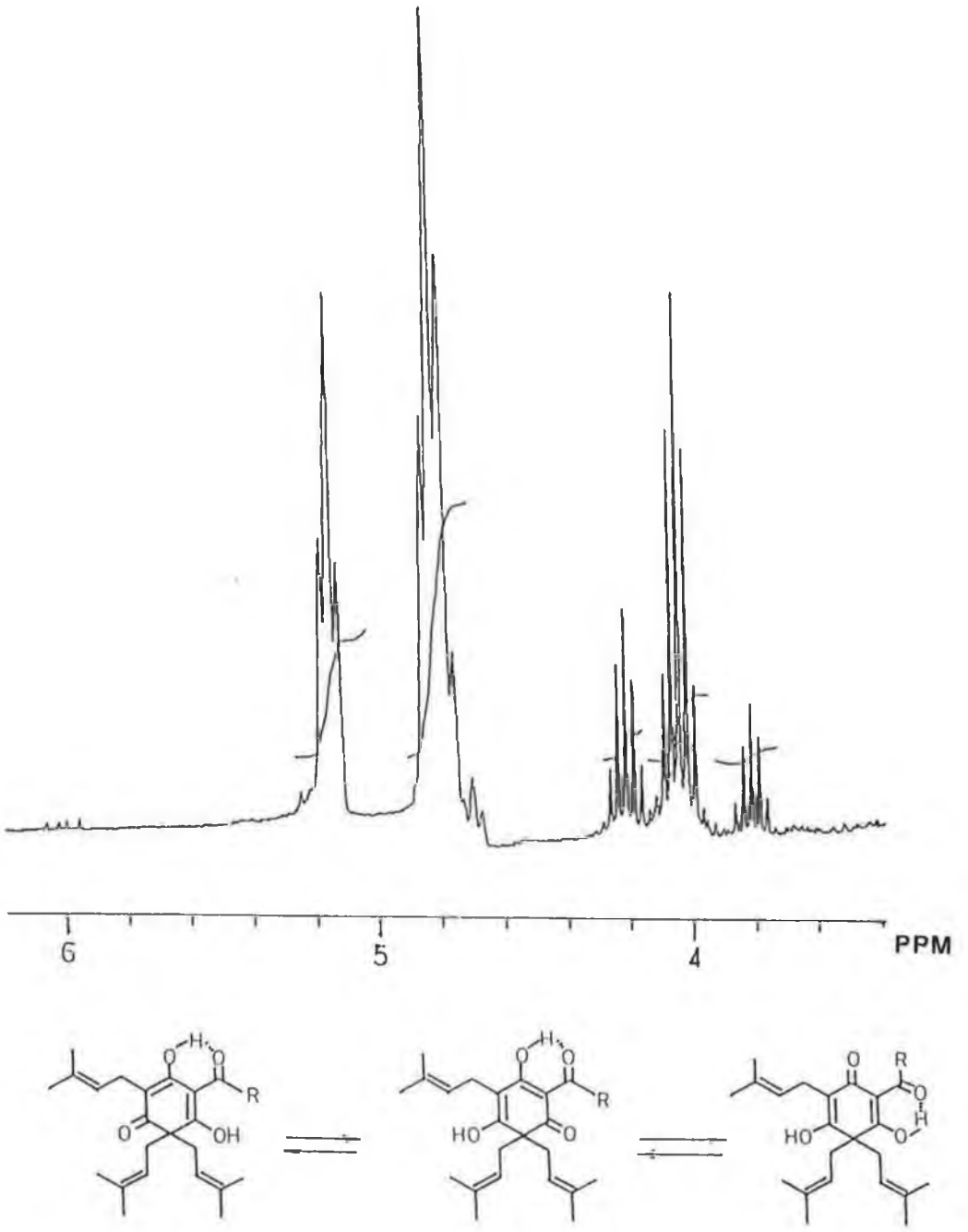



**Fig. 20** $^{1}H$ N.m.r (270MHz) Spectrum of colupulone (7) from 3.5ppm to 6ppm, showing three septets for the acyl side chain CH proton, and proposed enolic forms accounting for these septets.

$^1H$ N.m.r. data of mixed reference standards e.g. (26) and (40), (19) and (20) was also obtained. These spectra enabled the ratio of components in the mixture to be obtained, as the $CH_3$ of the 2-acyl side chain of the co-homologues were at approximately 0.2 ppm lower field than that for the pure reference standards. This was due to the deshielding effect of the carbonyl and ring double bonds. For all mixed standards the ratio of methyl protons area at approximately 0.98 ppm to methyl proton area at 1.16 ppm was in agreement with HPLC analyses of the ratio of homologues in mixtures. In the co-homologues the septet from the acyl group was moved to lower field (ca.2.2 ppm to 3.5 ppm) as expected from its proximity to the carbonyl and ring double bonds.

### 2.2.2 IR analysis

Infra-red analysis was also used to confirm structural authenticity of standard compounds. As all these compounds have common functional groups they all exhibit expected characteristic absorbances, but with slight changes in frequency depending on the environment. Only minor differences were observed in the spectra of trans-humulinic (12) acid and cis-humulinic acid (13). The trans isomer had a sharper free OH peak at 3420 $cm^{-1}$ than the corresponding peak at 3380 $cm^{-1}$ in the cis isomer. The

carbonyl peak in the trans isomer was at 1686 $cm^{-1}$ while that of cis-humulinic acid was at 1700 $cm^{-1}$. All standards had characteristic C-$CH_3$ bending vibrations in the 1350 $cm^{-1}$ to 1395 $cm^{-1}$ region. Also common were typical methylene and OH, bending vibrations at 1440 $cm^{-1}$ and stretching vibrations from 2800 $cm^{-1}$ to 3000 $cm^{-1}$. In comparison to the humulinic acids, dehydrohumulinic acid (26) has a broad OH peak in the 3400$cm^{-1}$ to 3000$cm^{-1}$ region, typical of a hydrogen bonded OH. It also has a much more intense C=C stretching vibration and this is consistent with the formation of an additional C=C bond on oxidation of humulinic acid. Hulupone (19) had an additional peak at 1570 $cm^{-1}$ possibly due to ring olefin stretching, or to carbonyl stretching of the enolic form of the ß-diketone in the molecule. The latter is supported by the very broad OH stretch over the 2600 $cm^{-1}$ to 3800 $cm^{-1}$ region. This type of peak is characteristic of chelated intramolecular hydrogen bonding.

In order to ascertain the type of hydrogen bonding in these molecules IR spectra were run at various concentrations in carbontetrachloride. For trans-humulinic acid (12), and trans-cohumulinic acid (13) the sharp OH peak observed in the solid spectrum at 3420 $cm^{-1}$ was absent from the spectrum of a saturated solution in $CCl_4$, and a broad peak from 3200 to 3600 $cm^{-1}$ was observed with a small shoulder peak at 3540 $cm^{-1}$. On dilution this shoulder peak increased to give a sharp peak typical of a free OH, while

the broad peak decreases in intensity, and was removed completely in very dilute solution. This broad peak is likely due to intermolecular hydrogen bonding. The appearance of free OH peak on dilution was also observed for spectra of dehydrohumulinic acid (26) (Fig.21). However a dilution was reached where no further change in the spectrum was observed. At this stage a free OH peak and a broad OH peak over the range 2800 $cm^{-1}$ to 3400$cm^{-1}$ remained. This broad peak, characteristic of a chelated OH, had previously been obscured by C-H peaks and hydrogen bonded OH peak, and confirms $^{1}H$ n.m.r. data suggesting dehydrohumulinic acid exist as a chelated form. Hulupone (19) and colupulone (7) gave no change in their spectra on dilution, and both maintained a very broad OH peak consistent with chelate formation.

IR was particularly useful in differentiating between lupuloxinic acid (17) and lupulenol (18), the former having an additional carbonyl peak at 1720$cm^{-1}$

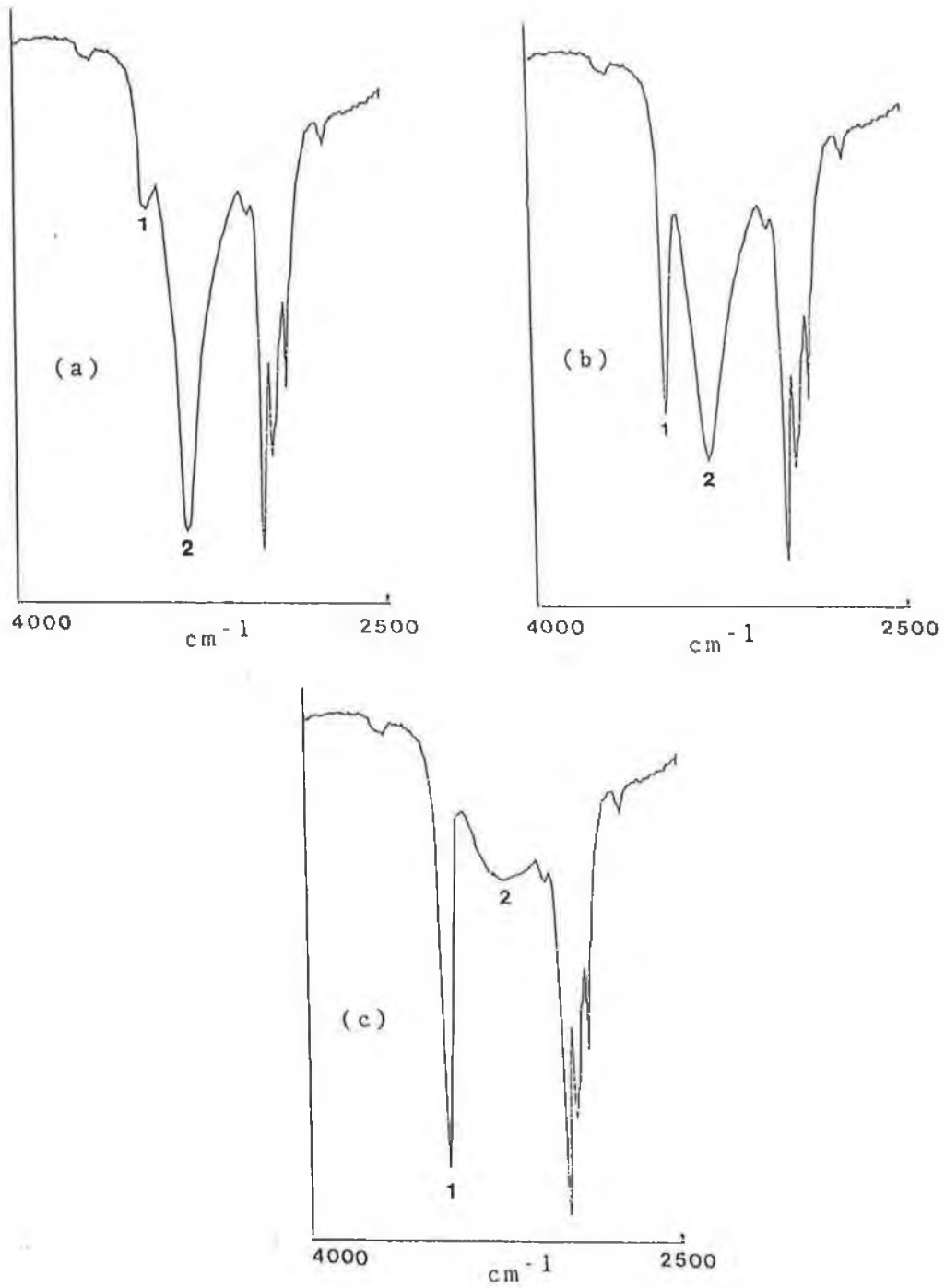



**Fig. 21** IR. spectra of dehydrohumulinic acid (26) in carbon tetrachloride as, (a) a saturated solution, (b) 1 in 5 dilution of of (a), and (c) a 1 in 20 dilution of (a).
" Peak 1 = free OH" "Peak 2 = Hydrogen bonded OH

# SECTION 3

# RESULTS AND DISCUSSION - ANALYSIS

## 3. RESULTS and DISCUSSION - ANALYSIS

### 3.1 HPLC analysis of reference standards.

HPLC was the main criteria in the determination of product purity. A number of HPLC systems were developed each depending on the nature and number of components in the sample and on the range of suitable columns available. Attempts at separation of reference standard compounds on a range of columns including uBondapak C18, Pye Unicam RP18, Nucleosil C18, and Nova Pak C18 proved the latter column to be the most suitable. Using this column simple isocratic systems were developed for the analysis of pure and mixed reference standard compounds with a water\acetonitrile mobile phase.

Hulupones (19,20) proved to be the most difficult compounds to chromatograph not only because of their co-elution with other components, but also because of peak tailing. This tailing could be eliminated by using a Nova Pak C18 column, and the inclusion of ortho-phosphoric acid at a concentration of 1% in the mobile phase. This gave a pH of about 1.6, a lower pH than the column manufacturers would recommend for a C18 column. Two possible reasons for this tailing effect may exist, firstly chelation of the sample with metals in the column packing as proposed by Verzele et al.[73] When included in the mobile phase phosphoric acid acts as an anti-complexing agent, and therefore eliminates the tailing, as was observed. Secondly as hulupone (19)

was the only compound to show a major tailing effect it may be related to its lower pka value relative to other standards.

It was not possible to separate cis- (13) and trans-humulinic acid (12) on our system, nor has any published procedure achieved this. Using this same system ß-acids gave retention times of over 30 minutes. For routine analysis of ß-acids mixtures and standards a less polar mobile phase was used so that an acceptable analysis time could be obtained. Good resolution of lupulone (6) and colupulone (7) was obtained with an analysis time of less than 15 minutes. This system also gave separation of the other reference standard components but their resolution was reduced significantly. A third system was developed to give resolution of the components in hop extracts, in particular separation of humulinic acids (12,41) from other components. Because of the large number of compounds present in hop extracts a gradient elution system was necessary. This eluted humulinic acids (12,41) and other polar compounds first, which allowed the remaining components to be washed from the column.

Although a nova-pak C18 column was found to be the most suitable column for separation of standard components, after about 100 analysis column resolution and selectivity deteriorated, and extensive washing did little to improve it. Other columns such as a nucleosil C18, Pye Unicam RP18

were unusable after 50-100 analyses. This may be due to complexing of sample components with column packing, or alternatively it may be due to removal of octadecyl groups from the packing. The latter explanation is more likely as column deterioration was not accompanied by an increase in column back pressure.

HPLC analysis confirmed that all standards and mixed standards synthesised were of a high purity and suitable for quantitative HPLC analysis. Reference standards of trans-humulinic acid (12), cis-humulinic acid (13), and dehydrohumulinic acid (26) were obtained at a purity greater than 98.5%, while hulupone (19) was obtained at a purity greater than 97%. Analysis of purified colupulone (7) showed its purity to be a greater than 99%. Mixed reference standards were also obtained in high purity, with the humulinic acids (12,41) and dehydrohumulinic acids (26,40) at greater than 98% purity. A hulupone (19) and cohulupone (20) mixed reference standard at 97.5% purity was obtained by alkylation of dehydrohumulinic acids (26,40). This mixed reference standard was also obtained from ß-acid oxidation, and gave a slightly higher purity of 98.5%. HPLC chromatograms of a composite of a number of purified samples with their corresponding Photo diode array (PDA) Uv spectra are illustrated in fig.22.

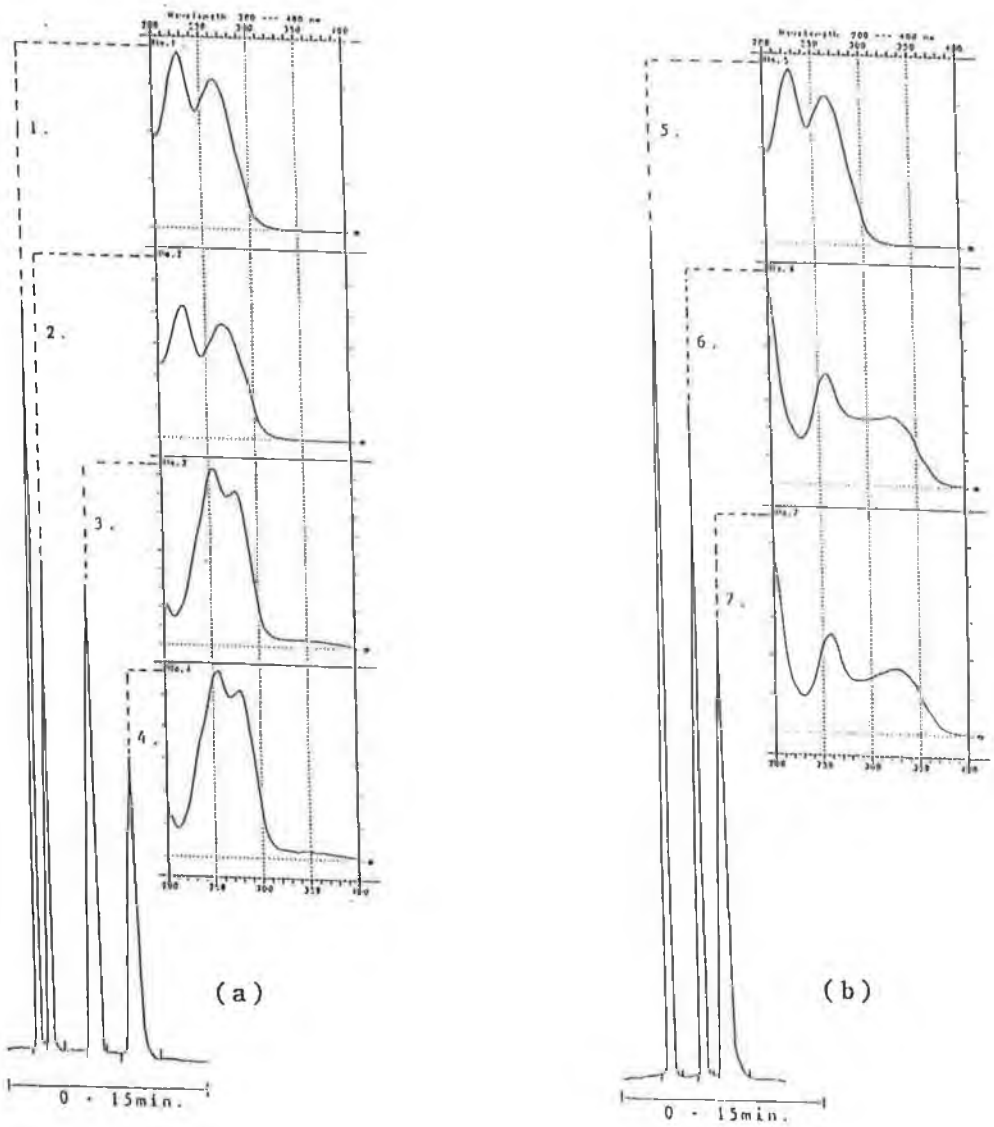



Fig. 22a. HPLC and PDA analysis of a composite of purified samples of: 1.co-humulinic acid (41), 2.trans-humulinic acid (12), 3.dehydrocohumulinic acid (40), 4.dehydrohumulinic acid (26).
Fig. 22b. HPLC and PDA analysis of a composite samples of: 5.cis-humulinic acid (13), 6.cohulupone (20), 7.hulupone (19).
Mobile phase: $CH_3CN:H_2O:H_3PO_4$ 55:45:1. Column NOVA-PAK $C_{18}$,5u.

PDA detection was used to confirm peak homegenity for all chromatographed compounds. Uv data in different regions of an eluted peak were examined and compared to uv data obtained from a standard spectrometer, giving further verification of peak homogeneity and of product purity. PDA detection was particularly useful in the analysis of compounds such as hulupones (19,20) that gave rise to tailing. In the case of hulupones the peak tail gave the identical uv spectrum as the centre of the main peaks, showing that tailing was not due to an underlying impurity.

From the uv absorption data of our standard compounds the effect of solvent and pH on absorption characteristics may be observed. Also, absorption data differs greatly between these compounds under the same conditions. For instance, the molar absorbtivity of hulupone (19) in water:acetonitrile:phosphoric acid (45:55:1) or in methanol is approximately half that of dehydrohumulinic acid (26) or humulinic acid (12) at 254nm in the same solvents. Clearly quantitative analyses by peak area may lead to large errors, and highlights the need for pure reference standard compounds. The λmax of these compounds ranges from 254nm for humulinic acids (12,41) to 330nm for ß-acids making detection of individual components in mixtures difficult using mono-chromatic detectors, but ideally suited to PDA detection.

Because of the large effect of pH on the retention of

hulupone (19) on HPLC columns its uv spectra was examined over a range of pH's from 10.7 to 0.02. (Fig.23). From pH 10.7 to pH 4 very little change appears in absorption characteristics. From pH 4 to 0.55 the absorbances at 255nm and 320nm decrease in intensity with a gradual shift in the absorbance at 255nm to approximately 258nm. From a pH of 0.55 to pH 0.02 the λmax is shifted to 276nm with the peak at 258nm absent. These protonation effect was reversible giving an identical sequence of spectra in reverse order. These spectra show that hulupone (19) can exist as a ratio of different protonated forms depending on pH. Chemical or structural differences in these forms could account for peak tailing observed in chromatograms.

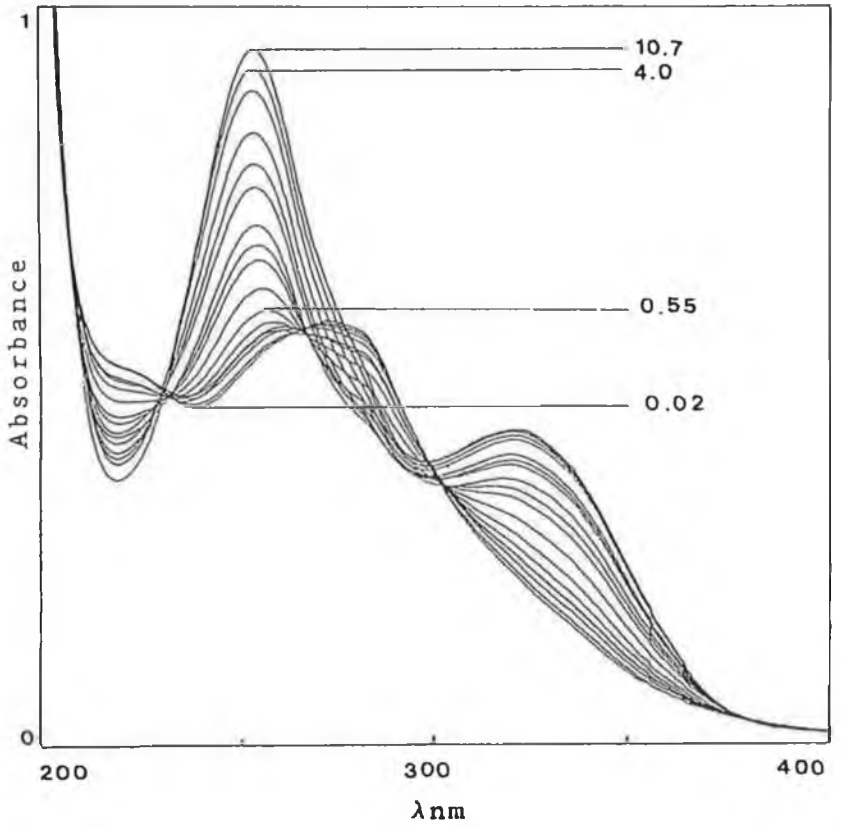



**Fig. 23** Uv spectra of hulupone (19) at pH 10.7 to pH 0.02

## 3.2 Quantitative Analysis

### 3.2.1 Determination of trans-humulinic acid (12) and trans-cohumulinic acid (41) in isomerised hop extracts

Iso-α-acids consist of a large number of components and it is not feasible to obtain resolution of every component on a HPLC column. However these components have a broad spectrum of polarities and chemical properties which make gradient elution practical. The gradient HPLC method developed was successful in resolving humulinic acids (12,41) from other components in a hop extract, and modifying this gradient would give resolution in other areas of the chromatogram if required. Humulinic acids (12,41) were quantified in an isomerised hop extract before and after hydrolysis. Using standard addition of a mixed reference standard containing trans-humulinic acid (12) and trans-cohumulinic acid (41), isomerised extract was found to contain 0.34% of trans-humulinic acid (12) and 0.07% trans-cohumulinic acid (41)(Fig.24a). Although the peak area for the trans-cohumulinic acid (41) was very small it was identified by PDA uv analysis as well as by standard addition. Hydrolysis was performed in methanol giving solubility to all the components in the reaction mixture, and eliminates losses in extraction procedures. Reaction temperature was at 70°C in contrast to 100°C in the standard procedure, and the reaction time was increased from 20 to 45min to compensate for this. After hydrolysis

the concentration of trans-humulinic acid (12) increased to 9.2% and that of trans-cohumulinic acid (41) to 6.3% (Fig.24b). This was slightly lower than we obtained from our procedure for the isolation of standard humulinic acid, which gave a total of 11% trans-humulinic acid (12) and 9.7% trans-cohumulinic acid (41).

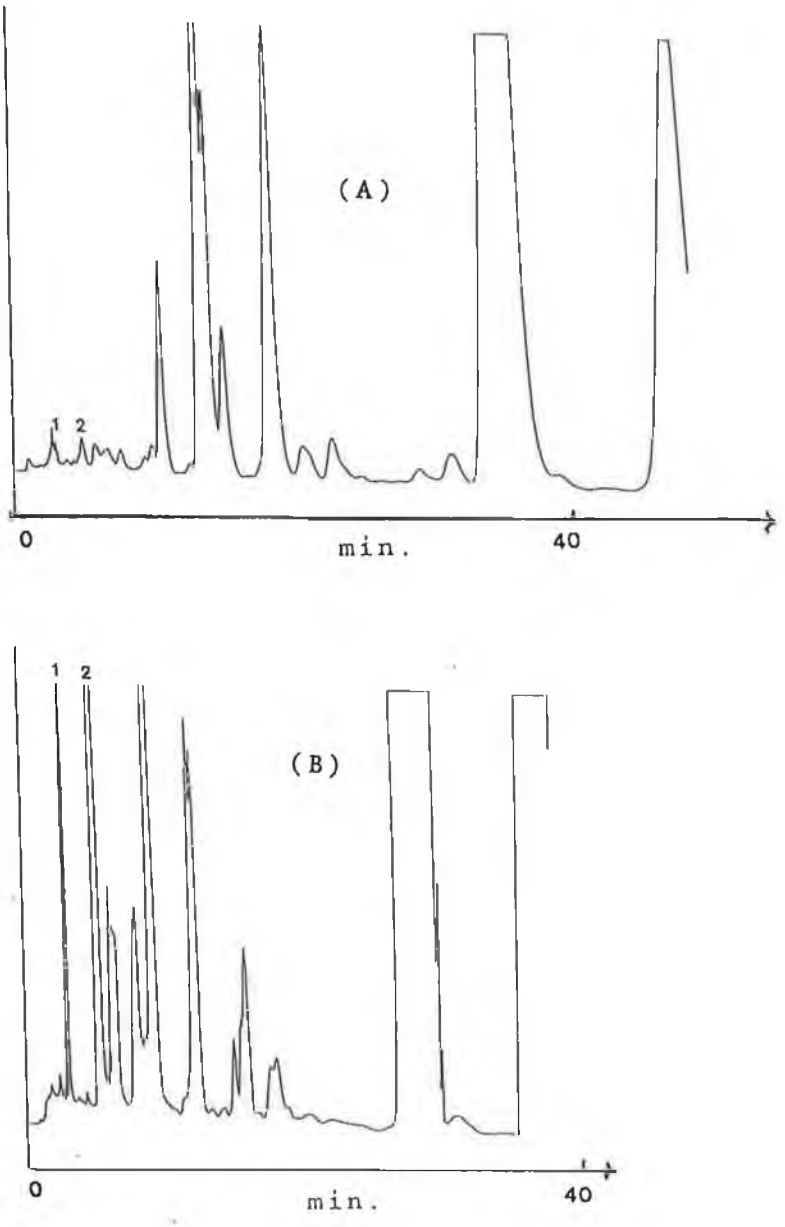



**Fig.24** (A) HPLC of isomerised hop extract, (B) HPLC of hydrolysed hop extract. Column:NOVA-PAK C18,
(1) Co-humulinic acid, (2) Humulinic acid.

### 3.2.2 Determination of cohulupone (20) from ß-acid oxidation

Synthesised reference standards, and HPLC methods developed were applied to the quantitative analysis of cohulupone (20) formation from ß-acids in a hexane solution. On dissolving pure colupulone (7) sample in hexane a chromatogram of the solution showed the presence of a single pure compound (fig.25a). Analysis after two hours stirring shows a large increase in the number of components in the solution (Fig.25b). The largest at 9.81min, was determined by HPLC standard addition methods to be cohulupone (20), at a concentration of 0.005 %. After eight hours the solution had turned from colourless to a dark yellow. HPLC analysis showed an increase in cohulupone (20) concentration to 0.025% with a large increase in other colupulone degradation products, and a corresponding decrease in colupulone (7)(Fig.25c). At the end of two days stirring no colupulone (7) could be detected in the hexane solution (Fig.25d). Cohulupone (20) concentration was decreased to 0.007%, showing that it too was unstable in hexane solutions. A small amount of solid material had precipitated from the hexane solution over the two day period. This was consistent with the findings of Wright[48] in that cohulupone (20) is oxidised in air, changing gradually to a resin with a lower carbon content than cohulupone (20), and insoluble in light petroleum.

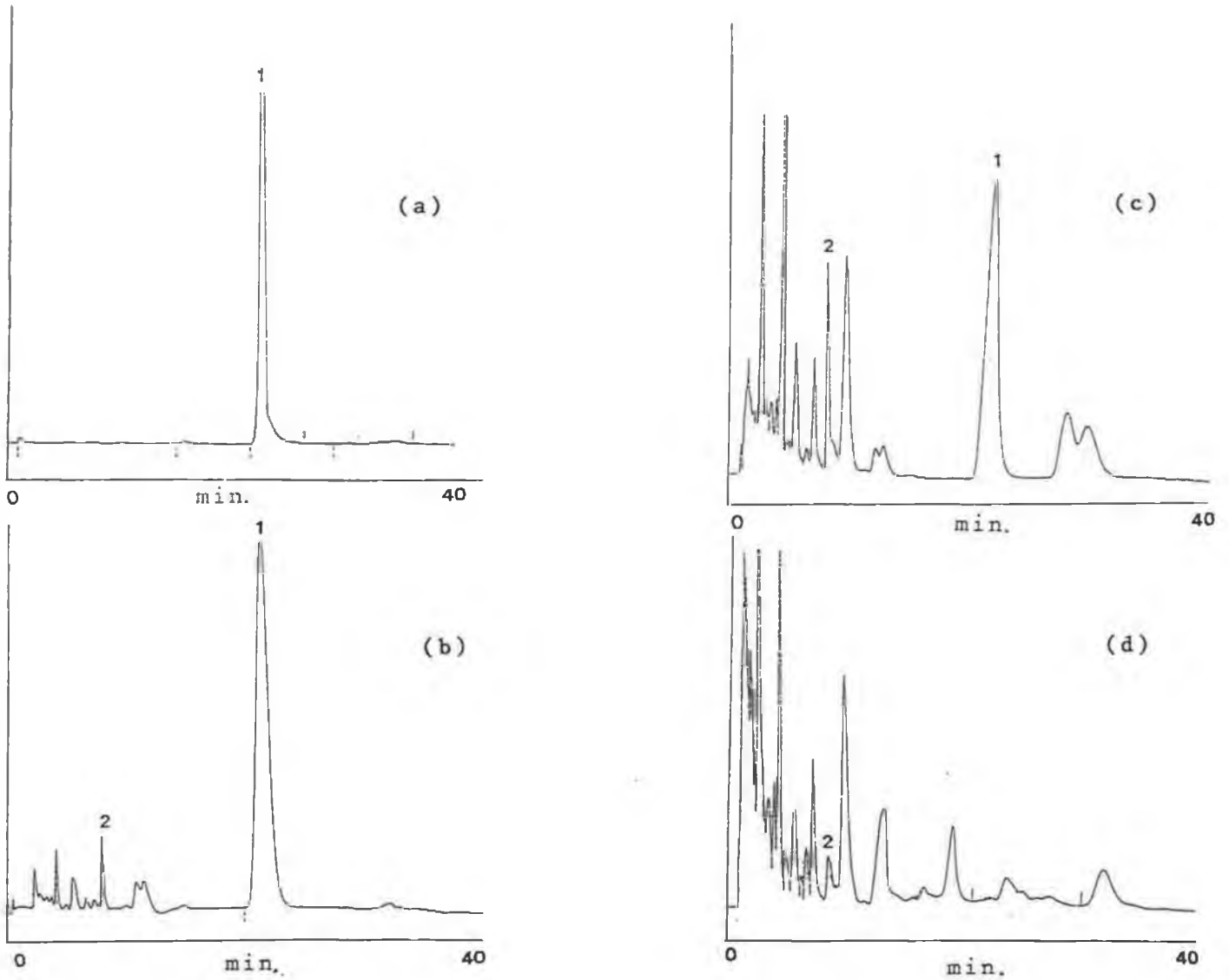



Fig. 25 HPLC analysis of cohulupone (7) in hexane (a) at time 0.
(b) after 2hrs., (c) after 8 hrs., (d) after 48hrs.
"Peak 1 = colupulone. "Peak 2 = cohulupone.

### 3.3 Reference Standard Stability

The humulinic acids (12,41) and dehydrohumulinic acids (26,40) when assayed by HPLC after a six month storage period at room temperature showed no signs of degradation. Hulupones (19,20) were found to be unstable under certain storage conditions. HPLC of solutions in methanol, hexane, or aqueous acetonitrile showed detectable impurities after two days at room temperature. Hulupones (19,20) stored at room temperature under nitrogen were stable for up to two weeks. Storage at -35°C, under nitrogen, and under anhydrous conditions increased stability to at least six months. Solutions of ß-acids in hexane, or methanol showed detectable impurities after 5h. at room temperature. Pure ß-acids stored at room temperature were stable for up to two weeks, and increased to a minimum of two months by storage in an oxygen free desiccator at room temperature. It was also noted that final crystallisation of ß-acids from methanol/water rather than from hexane gave increased stability.

# SECTION 4

# EXPERIMENTAL

## 4. EXPERIMENTAL:

### 4.1 Instrumentation.

HPLC analysis was carried out using a "Waters 510" liquid chromatograph system equipped with "Waters 990" Photodiode array detector (PDA).

$^1H$ N.m.r and COSY n.m.r. analysis was performed on a "JOEL GX-FT-270" spectrometer for samples in deuteriochloroform.

UV analysis was carried out on "Bausch & Lombe" spectronic 2000 spectrophotometer. For extinction coefficient determinations approximately 50mg of sample was dissolved in 100ml of HPLC grade methanol. From this solution 1 in 50 dilutions were made in the analysis solvent. The molar extinction coefficients were calculated at the $\lambda_{max}$ of each peak in the uv spectrum and expressed as $mol^{-1}dm^3cm^{-1}$.

Infra-red analysis (IR) was carried out using a Perkin Elmer 983G IR spectrometer. Solid samples were run as KBr discs and liquids as films between sodium chloride plates. Also spectra of solutions of varied concentrations in carbon tetrachloride were examined. The volume of solution analysed was increased to compensate for dilutions, ensuring a constant number of sample molecules were analysed in all cases.

Elemental Analysis were provided by the micro analytical laboratory University College Dublin.

Melting point (mp.) were obtained on a Buchi 535 mp. apparatus.

## 4.2 SYNTHESIS.

### (4.2.1) Pure reference standards:

**Trans humulinic acid (12)**

Isomerised hop extract (300g) containing approximately 100g of iso-$\alpha$-acids was added slowly with stirring to 4M NaOH (300ml) at 60°C. The resultant oily mixture was refluxed for 20 min. After cooling to 70°C the pH of the solution was adjusted to pH 2 with concentrated HCl. While still hot the solution was allowed to separate into two layers. The lower aqueous layer was discarded and the upper oily layer was dissolved in n-hexane (1000ml). This solution was cooled to -35°C and was held at that temperature for 12h. The yellow oily precipitate obtained (49g) was recrystallised three times at room temperature from n-hexane, and each recrystallisation incorporated decolourisation with activated carbon. This procedure yielded trans-humulinic acid as a white crystalline solid (28g). Mp. 92.5-93.0°C: (Found: C,67.55; H,8.36. $C_{15}H_{22}O_4$ requires C,67.64; H,8.32%). $\lambda_{max}$ [$H_2O$:$CH_3CN$:$H_3PO_4$ (45:55:1)], 266.5(9241.19), 226.5(11067)nm. $\lambda_{max}$ methanol, 266.3 (14979.5), 251.3(18985.3), 219.5(5067.62)nm. $\lambda_{max}$ 0.1M NaOH, 250.1(22755.9)nm. IR $\nu_{max}$ (in KBr) 3420, 2984, 1686, 1584, 1436, 1383, 1369, 1252, 1112, 937, 559 $cm^{-1}$. $\delta_H$(270 MHz), 0.98(6H,d,J6.6Hz,$Me_2$CH), 1.67(3H,s,MeC=C), 1.71(3H,s,MeC=C), 2.2(1H,m,$Me_2$CH), ca.2.57(2H,m,CHC$H_2$CH),

2.75(2H,m,$CHCH_2CO$), 2.9(1H,m,$CH_2CH$), 4.04(1H,d,J4.77, CHC(OH)HCOH), 5.15(1H,m,CH=C), 10.0(1H,br,OH):

**Dehydrohumulinic acid. (26)**

Trans-humulinic acid (20g) was added to a suspension of bismuth trioxide (32g) in glacial acetic acid (200 ml) and heated under reflux for 5h. The cooled solution was filtered free of bismuth metal, and poured into 200ml of 2M HCl. The precipitated product was isolated and washed with hot water. After drying over phosphorous pentoxide it was recrystallised from propan-1-ol, followed by a recrystallisation from n-hexane incorporating activated carbon treatment. The product was finally dried at 80°C over phosphorous pentoxide. This procedure yielded dehydrohumulinic acid (26) as a yellow crystalline solid. (8g, 40% yield). Mp. 134.5-135.0°C: (Found: C,68.51; H,7.55. $C_{15}O_4H_{20}$ requires C,68.20; H,7.60%). $\lambda_{max}$ [$H_2O$:$CH_3CN$:$H_3PO_4$ (45:55:1)], 277.9(18990.0), 254.1 (21288.4)nm. $\lambda_{max}$ methanol, 276.3(14281.5), 255.0 (19404.8), 220.0(6897.6)nm. $\lambda_{max}$ 0.1M NaOH, 299.7 (25186.2), 220(6897.57)nm. IR $\nu_{max}$ (in KBr) 3203, 2958, 1714, 1622, 1428, 1363, 1210, 1156, 825$cm^{-1}$.
$\delta_H$(270 MHz), 0.98(6H,d,J6.4Hz,$Me_2CH$), 1.72(6H,s,$Me_2C$=C), 2.11(1H,m,$Me_2CH$), 2.60(2H,d,J7.0Hz,$CHCH_2CO$), 3.04(2H,m, $CHCH_2C$=CO), 5.23(1H,m,CH=C), 7.8(1H,br,enol OH), 12.4(1H,br,enol OH).

Cis-humulinic acid. (13)

Dehydrohumulinic acid (2.2g) was dissolved in ethanol (90ml) and added dropwise to a solution of sodium borohydride (1.65g) in water (30ml). The reaction temperature was maintained at 0°C during the addition and for a further two hours at room temperature. 200ml of water was added, and the resulting solution acidified with HCl. The acidified solution was extracted with hexane (3x100ml) and a white solid was recovered on concentration of the hexane extract. Recrystallisation from hexane gave cis-humulinic acid (700mg, yield 32%). **Mp.** 73.5-74.0°C. (**Found**: C,67.72; H,8.41. $C_{15}H_{22}O_4$ **requires** C,67.64; H,8.32%). $\lambda_{max}$ [$H_2O$:$CH_3CN$:$H_3PO_4$ (45:55:1)], 266.5 (9186.2), 226.0(11004)nm. $\lambda_{max}$ methanol, 266.3(14771.1), 251.3(19009.1), 219.5(5051.4)nm. $\lambda_{max}$ 0.1M NaOH, 250.1 (22701.6)nm. IR $\nu_{max}$ (in KBr) 3380, 2980, 1700, 1686, 1610, 1594, 1440, 1250, 1145, 910, 750$cm^{-1}$.
$\delta_H$(270 MHz) 0.98(6H,d,J8.8Hz,$Me_2CH$), 1.59(3H,s,**Me**C=C), 1.66(3H,s,**Me**C=C), 2.17(1H,m,$Me_2$**C**H), ca.2.58(2H,m, CH**C**$H_2$CH), 2.80(2H,m,CHC$H_2$CO), 3.12(1H,m,$CH_2$**C**H), 4.37(1H,d,J7.4,CHC(OH)HCOH), 5.1(1H,m,**C**H=C).

Hulupone (19)

Dehydrohumulinic acid (10g) after drying over phosphorous pentoxide was dissolved in dry ether (40ml) and methanol

(10ml), and added dropwise to 23ml of sodium methoxide (25%$^{w}$/w soln.) at -10°C. This solution was stirred at-10°C for 2h. and then 15.3g of 4-bromo-2-methyl-2-butene in 25ml. of ether was added dropwise maintaining the temperature at -10°C. The solution was stirred at 0°C for 1h. and at room temperature for a further 12h. Ether (20ml) was then added and the solution filtered. The filtrate was washed sequentially with dilute HCl and water, and finally dried over magnesium sulphate. Evaporation of the ethereal solution gave a yellow oil. The oil was dissolved in hexane and mixed with a saturated sodium bicarbonate solution. The resulting solid was recovered by filtration and washed successively with water and hot hexane. After dissolving this solid in methanol and acidifying, it was extracted into n-hexane. The organic solution was dried (sodium sulphate) and evaporated to give a yellow oil. "Kugelrohr" distillation of this oil (0.4mbar/140°C) gave pure hulupone (8.5g, yield 67%), which was stored under anhydrous and oxygen free conditions at -35°C. (**Found**: C,72.19; H,8.50. $C_{20}H_{28}O_4$ **requires** C,72.26 H,8.51%). $\lambda_{max}$ [$H_2O$:$CH_3CN$:$H_3PO_4$ (45:55:1)] 256.5(9968.6), 324.9(6417.9)nm. $\lambda_{max}$ methanol, 317.5 (4635.9), 258.5 (8789.5)nm. $\lambda_{max}$ 0.1M NaOH, 323.5 (9164.6), 252.5 (10584.9)nm. IR $\nu_{max}$ (liquid film) 3120, 2980, 1750, 1700, 1640, 1570, 1440, 1380, 1150, 1075, 825cm$^{-1}$.
$\delta_H$(270 MHz) 1.00(6H,d,J6.6Hz,$Me_2CH$), 1.57(12H,s,2$Me_2C$=C),

2.15(1H,m,$Me_2CH$), ca.2.50(4H,m,2$CHCH_2CO$), 2.91(2H,d, J6.9Hz,$CHCH_2CO$), 4.83(2H,m,2CH=C), 12.7(1H,br,enol OH).

**Colupulone (7):**

A sample (400g) of a crude ß-acid extract was added to water (1000ml) at 90°C. Potassium carbonate (20g) was added slowly, and the solution boiled for 3h. to convert any α-acids to iso-α-acids. The solution was allowed to stand hot for 10 min. Waxes and other resins formed a dense surface layer. The lower layer was removed and adjusted to pH 8. This solution was allowed to stand in a conical vessel at 80°C for 12h. A lower layer of ß-acids formed, and could easily be run off as a fluid layer. This ß-acid fraction was dissolved in an equal volume of n-hexane, and maintained under nitrogen at -35°C for 12 hours. The oily crystals obtained were crystallised from methanol/water (9:1) to give 35g of beige crystals. Two further recrystallisations from n-hexane were required to obtain a "pure" ß-acid mixture (28g) of lupulone (6) and co-lupulone (7) in a ratio of 20:80 respectively. After a further five successive recrystallisations from n-hexane a pure sample of colupulone (2.5g) was obtained. Mp. 94°C-94.5°C: (Found: C,75.15; H,9.00. $C_{25}H_{36}O_4$ requires C,74.96; H 9.06%). $\lambda_{max}$ [$H_2O$:$CH_3CN$:$H_3PO_4$ (45:55:1)], 335.1 (9249.4), 275.6(6313.5), 223.8(11964.7)nm. $\lambda_{max}$ methanol, 340.5(10176.6), 206.7(13796.9), 223.0(10154.5)nm. $\lambda_{max}$

0.1M NaOH, 353.9(18322.2), 222.3(20066.2)nm. IR $\nu_{max}$ (in KBr) 3236, 2926, 1660, 1595, 1520, 1450, 1370, 1350, 1200, 1175, 1150, 1100, 815, 430$cm^{-1}$.

$\delta_H$(270 MHz), 1.14(6H,d,J7.3,2Me terminal of acyl group), 1.58(12H,d,J4.4,2$Me_2$ gem-isopentenyl), 1.79(6H,d,J2.0,$Me_2$ 4-isopentenyl), ca.2.6(4H,m,2$CH_2$ gem isopentenyl), 3.2(2H,d,J7.33,$CH_2$ 4-isopentenyl), {3.8(0.13Hsp) 4.0(0.6H, sp) 4.2(0.27H,sp) CH of acyl group}, 4.83(2H,m,:CH$_2$ gem isopentenyl), 5.2(1H,m,:CH of 4-isopentenyl), {6.6(0.26H,s) 7.15(0.73H,s) OH unchelated}, 18.1(1H,s,OH chelated). (sp = septet).

### (4.2.2) Mixed reference standards

Trans-humulinic acid (12) and Trans-cohumulinic acid (41).

A mixture of trans-humulinic acid (12) and trans-cohumulinic acid (41) was obtained from a combination of mother liquors left after the recrystallisation of trans-humulinic acid (12). The combined mother liquors were first evaporated to one third their volume, and the product precipitated at -35°C was crystallised and recrystallised from n-hexane to give a mixture of trans-humulinic acid (12) and trans-cohumulinic acid (41)(15g) in a ratio of 3.5:6.5 respectively.

Mp.78°C-79°C. $\delta_H$(270 MHz), Identical to trans-humulinic

acid (12), but with additional cohumulinic acid (41) acyl group peaks at 1.22(d,J6.7,$Me_2CH$), and 3.53(m,$Me_2CH$)ppm.

Dehydrohumulinic acid (26) and Dehydrocohumulinic acid (40)

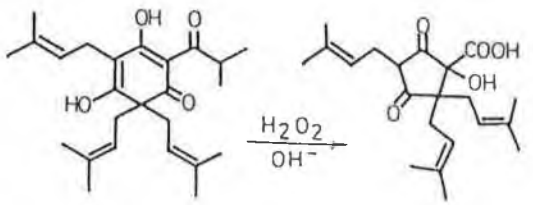
A mixture of dehydrohumulinic acid (26) and dehydrocohumulinic acid (40) was prepared from a mixture of trans-humulinic acid (12) and trans-cohumulinic acid (13) by oxidation with bismuth oxide in a similar manner to the procedure described previously for dehydrohumulinic acid (26). Dehydrohumulinic acid (26) and dehydrocohumulinic acid (40) were obtained in a ratio of 3.5:6.5 respectively. Mp. 127°C-128°C. Yield 40%. $\delta_H$(270 MHz), Identical to dehydrohumulinic acid (26), but with additional dehydrocohumulinic acid (40) acyl group peaks at 1.17(d,J6.9, $Me_2CH$), and 3.5(m,$Me_2CH$)ppm.

Hulupone (19) and Cohulupone (20)

Using a mixture of dehydrohumulinic acid (26) and dehydrocohumulinic (40) as substrate for the reaction outlined previously for the synthesis of hulupone (19) the products were hulupone (19) and cohulupone (20) in a ratio of 3.5:6.5 respectively. Yield 67%. Boiling point 140°C at 0.4mm Hg. $\delta_H$(270 MHz), Identical to hulupone (19), but with additional cohulupone (20) acyl group peaks at 1.21(d,J6.7,$Me_2CH$), and 3.53(m,$Me_2CH$)ppm.

## (4.2.3) Synthesis of Lupuloxinic acid (17)

Reaction



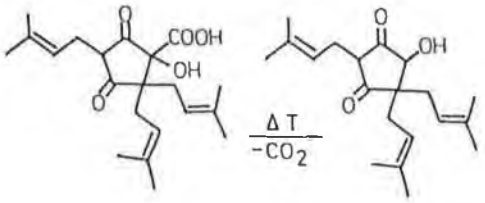

7 17

To a degassed solution of ß-acids (1g) in 10% aqueous sodium hydroxide (50ml) was added 30% hydrogen peroxide (4ml). The solution was maintained under nitrogen and at room temperature for 5 hours. The resulting gel was stirred into iced 10% sulphuric acid (100ml), and maintained at 0°C for 12 hours. Lupuloxinic acid (17) precipitated as an oily solid and was isolated. It was then dissolved in hot ethanol (3ml) and water was added until the first sign of precipitation was evident in the hot solution. On cooling to room temperature lupuloxinic acid precipitated as a white solid, which was dried over phosphorous pentoxide, and finally washed with hexane to give 0.5g of product (ca.53% yield).

**Mp.**107°C-108°C. (**Found**: C,70.39; H,8.58. $C_{22}H_{32}O_5$ **requires** C, 70.18; H,8.60%). IR $\nu_{max}$ (in KBr) 3360, 2920, 2650, 1720, 1590, 1430, 1380, 1348, 1150, 1030, 850$cm^{-1}$.

## 4.2.4 Synthesis of Lupulenol (18)

Reaction




17 18

A suspension of lupuloxinic acid (0.25g) in toluene (2ml) was heated slowly to boiling. At just over 100°C carbon dioxide was evolved, and this temperature was maintained for five minutes. On cooling to 0°C lupulenol (18) precipitated and was filtered off, washed with hexane and dried to give 0.18g of product (Yield 82%). **Mp.** 118°C-118.5°C: (**Found**:C,76.15; H,9.55 $C_{21}H_{32}O_3$ **requires** C,75.90; H,9.70%). **IR** $\nu_{max}$ 3390, 2920, 2675, 1570, 1430, 1375, 1120, 1000, 820$cm^{-1}$.

## 4.3 Oxidation of ß-Acids

### 4.3.1. Oxidation of ß-acids under alkaline conditions

ß-acids (10g) were dissolved in 1% aqueous potassium hydroxide (400ml), and the resulting solution heated to 90°C and aerated for five hours. Gaseous carbon dioxide was then bubbled through the reaction mixture to reduce the pH to 7, giving precipitation of unreacted ß-acids, which were removed by filtration. The pH of the reaction mixture was further reduced to 4.5 using sulphuric acid, and it was then boiled for 10min to convert lupuloxinic acid (17) to lupulenol (18). The lupulenol formed an emulsion which was removed by centrifugation leaving an aqueous hulupone solution. HCl was added to reduce the pH to 1.5 and the solution was extracted with hexane. Hulupones (19,20) were isolated from the hexane solution by forming their sodium salt, acidifying and extracting as previously described. Distillation at 140°C/0.3mm gave a yield of 0.43g, (5.3%) of a mixture of hulupone (19) and cohulupone (20).

### 4.3.2. Isolation of hulupone from air oxidation of ß-acids in hexane.

Pure colupulone (7) (1g) was dissolved in hexane (30ml) and air was bubbled through the solution for 5hrs. The resulting yellow solution was shaken with a saturated solution of sodium bicarbonate. A small amount of oily

solid was isolated and washed successively with water and hot hexane. It was then dissolved in methanol, acidified and extracted into n-hexane (10ml). This solution was evaporated to give an oil (100mg) and found by HPLC analysis to have the same retention time as cohulupone (20), Yield 12.5%

### 4.3.3. Oxidation of ß-acids in methanol in the presence of Sodium Sulphite.

Pure ß-acids (20g) were dissolved in methanol (500ml), and anhydrous sodium sulphite (40g) added. The stirred solution was aerated for 12h. at room temperature. Solids were removed by filtration. The filtrate was evaporated to give a solid residue which was washed with hot hexane. The remaining solid was dissolved in hot methanol, acidified with dilute HCl, and extracted into n-hexane. The hexane solution was mixed with a saturated sodium bicarbonate solution, and the resulting solid was washed with water and hot hexane. After dissolving the solid in methanol it was acidified and extracted into n-hexane. The yellow oil obtained after removal of solvent was distilled at 145°C/0.4mm. This oil (4.9g) was found by HPLC to be a mixture of hulupone (19) and cohulupone (20) in a ratio of 20:80 respectively. Yield 30.4%

## 4.4 Application of HPLC in Quantitative Analysis of hop components.

### 4.4.1 Chromatographic conditions

Method 1

Column: NOVA-PAK $C_{18}$, 5u, 15cm.

Mobile phase 1: Acetonitrile:water:Phosphoric acid. 55:45:1.

Flow rate: 1.0 ml/min.

Method 2

Column: NOVA-PAK $C_{18}$, 5u, 15cm.

Mobile phase 2: Acetonitrile:water:Phosphoric acid. 80:20:1.

Flow rate: 1.0 ml/min

Method 3

Column: NOVA-PAK $C_{18}$, 5u, 15cm.

Mobile phase 3: Solvent A = 72:24:4 Water:Methanol:THF containing 1% sodium acetate

Solvent B = Methanol containing 1% sodium acetate

Flow rate: 1.0 ml/min

## (4.4.2) Quantitative analyses of humulinic acids in isomerised hop extract before and after hydrolysis.

Method : Standard addition HPLC analysis

Chromatographic Conditions

Column and mobile phases were as described in Chromatographic method 3 (4.4.1). Only solvent A was pumped through the column for the first 15min giving elution of humulinic acids and other highly polar compounds. This was followed by a gradient of 0% B (100% A) to 60% B over 15min and 60% B was maintained for a further 20min to elute remaining components from the column.

Standard Preparation

Trans-humulinic acid (12) was added to a mixed reference standard of trans-humulinic acid (12) and trans-cohumulinic acid (41) so as to obtain a standard with a 50:50 mixture of these two compounds. A 0.01mg/ml solution of this standard (Soln A) was used for standard additions to unknown samples.

Sample Preparation: isomerised extract.

Hop extract (1.0g) was dissolved in 100ml of methanol. A 1 in 10 dilution of this gave a working solution of 1µg/ml

(Soln B). For analysis of the neat extract a 1 in 2 dilution of solution B was made, and 20µl injected into the HPLC column (Fig.24).

A sample spiked with a known amount of humulinic acids (12,41) was made by diluting solution B by a factor of two using the standard solution A. 20µl of this solution was analysed by HPLC.

Preparation of hydrolysed hop extract.

Isomerised hop extract (1g) was dissolved in methanol (50ml), adjusted to pH 13 with 4M sodium hydroxide, and made up to a final volume of 100ml with methanol. This solution was refluxed for 45min. and cooled. 2ml of this solution was acidified with HCl (conc.), and made up to a final volume of 40ml with methanol. 20µl of this solution was analysed by HPLC (Fig.24).

Analysis results

| Sample | Cohumulinic acid (41) concentration | Humulinic acid (12) |
|---|---|---|
| Neat extract | 0.07% | 0.34% |
| Hydrolysed extract | 6.3% | 9.2% |

## (4.4.3) Quantitative analysis of cohulupone formed during the autoxidation of ß-acids in Hexane.

Method: HPLC external standard analyses.

Chromatographic condition

Column and mobile phase were as described in method 1.(4.4.1)

Sample Preparation

A freshly crystallised and HPLC pure sample of colupulone (7) (1.0g) was dissolved in HPLC grade hexane (100ml), and aerated by stirring. Samples (1µl) were taken initially and after 2h, 8h, and 2 days, and analysed without further preparation by HPLC (Fig.25).

Standard Preparation

38.5mg of mixed reference standard of hulupone (19) and cohulupone (20) was dissolved in hexane (100ml) to give a standard of 25mg/100ml of cohulupone (20). 1µl of this standard was injected onto the HPLC column, and the area obtained for cohulupone (20) was compared directly with the area of cohulupone (20) in samples.

Analysis results

| Time (hrs) | 0 | 2 | 8 | 48 |
|---|---|---|---|---|
| % cohulupone | 0 | 0.005 | 0.025 | 0.007 |

## CONCLUSION

This thesis was successful in its primary objective, in that synthetic and purification procedures were established for a range of hop derived compounds, so that they could be used as standards in quantitative HPLC analysis. Using iso-α-acids as starting material chromatographically pure standards of trans-humulinic acid, cis-humulinic acid, dehydrohumulinic acid, and hulupone were obtained in higher yields and purity than hitherto reported. It was not possible to isolate single pure standards of the "co" homologues of these compounds, however mixed reference standards of the pure compounds and their "co" homologues were obtained. These standards when used in conjunction with a pure reference standard were suitable for use in quantitative analyses. A purification procedure was developed for ß-acids giving chromatographically pure colupulone. This procedure included isomerisation of α-acid impurities followed by selective precipitation of ß-acids at pH 8. Separation of individual ß-acids was only achieved by multiple recrystallisation of crude ß-acids containing a decided excess of the required ß-acid. Oxidation of ß-acids produces a large spectrum of compounds the most important of which been the potentially bitter hulupones. Low yields of hulupone were obtained from ß-

acid oxidation in alkali solutions or in hexane. The most feasible route to hulupones was the oxidation of ß-acids in methanol in the presence of sodium sulphite. This procedure may be suitable to the large scale synthesis of hulupones.

We have used our reference standard compounds, and developed HPLC methods to quantify humulinic acids in hydrolysed hop extracts, and also to quantify hulupone formation from ß-acids in hexane solutions.

The availability of our reference standards and other similar quality standards, will extend the usefulness of HPLC as an analytical tool for the many analyses needed by breweries.

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**Appendix:**

**Quantitative HPLC Calculation .**

**Quantitative analysis of humulinic acid in isomerised hop extract before and after hydrolysis. (Ref: Section 4.4.2)**

| Component | Component area before hydrolysis | Area after standard addition | Component area after hydrolysis |
|---|---|---|---|
| *Trans-humulinic acid | 6069 | 24032 | 164220 |
| *Trans-cohumulinic acid | 1251 | 19400 | 114221 |

*Average peak area of two analysis.

$$\% \text{ w/w component} = \frac{\text{Area Component}}{(\text{Area component after standard addition}) - (\text{area component before hydrolysis})}$$

**Quantitative analysis of cohulupone formed during the autoxidation of ß-acids in Hexane (Ref: Section 4.4.3)**

| | Reaction Time \ hour | | | |
|---|---|---|---|---|
| | 0 | 2 | 8 | 48 |
| *Cohulupone Sample Area | 0 | 10722 | 53716 | 16083 |

*Cohulupone standard area (0.025% solution) = 53612

*Average of three analysis.

$$\% \text{ w\v hulupone} = \frac{\text{Area sample x 0.025}}{\text{Area standard.}}$$