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GAS CHROMATOGRAPHIC PROCEDURE FOR THE ANALYSIS OF ISO-HUMULONE

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SUMMARY

The gas chromatography of the trimethylsilyl (TMS) derivatives of *cis*- and *trans*-isohumulone on packed columns has been investigated. Difficulties were encountered with the direct injection of the silylation reaction mixtures. These difficulties were surmounted by a procedure wherein the TMS derivatives of isohumulone were extracted from the dimethylformamide–hexamethyldisilazane mixture into isooctane, after saturation of the mixture with ammonium nitrate. This procedure resulted in stable solutions of the TMS derivatives which showed reproducible results on a 1% SE-30 packed column.

INTRODUCTION

Many methods of separation and analysis have been used in the investigation of hop resin constituents, and their transformation products formed in the brewing process[1–5]. Of these techniques, high-pressure liquid chromatography (HPLC) and gas–liquid chromatography (GLC) offer the most attractive possibilities with respect to both separation and quantitation. Retention times and the supplemental utilisation of mass spectrometry provide data suitable for characterisation. However, the development of these two methods for the analysis of hop resin constituents and the hop resin-derived constituents of beer, is not yet at a stage where analyses may be conducted with ease and consistency. Recent publications have described the current state of HPLC as a method for the investigation of hop resin compounds[6–11]. This method, with further development, should become very valuable both for research and routine applications. However, the present discussion will be restricted to GLC.

GLC of hop resin constituents and their transformation products has been investigated by many workers. Dalgliesh and co-workers[12,13] first introduced the GLC of the trimethylsilyl (TMS) derivatives of the humulones, lupulones, isohumulones, hulupones, humulinic acids and humulinones on packed columns. Subsequently Krueger *et al.*[14] used GLC to examine the TMS derivatives of humulinic acid, iso-

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humulone, humulone and lupulone. Lloyd and co-workers[15,16] separated the TMS derivatives of the 4-desoxyhumulones from the TMS derivatives of humulones and lupulones by gas chromatography. In another publication Lloyd[17] discusses further the separation of the TMS derivatives of the humulones, lupulones and desoxyhumulones and the gas chromatography of the TMS derivatives of polyphenols from hops. Murray[18] has described the GLC of the TMS derivatives of the lupulones. Molyneux and co-workers[19,20] have reported the GLC of the TMS derivatives of humulone and colupulone and the application of the method to the analysis of beer samples. The GLC of the TMS derivatives of constituents of hop extracts and isomerised hop extracts was investigated by Di Cesare *et al.*[21]. Butylboronic ester derivatives of hop resin compounds have been examined with GLC by Shaw[22]. Drawert and Beier[23] used GLC to separate the TMS derivatives of the desoxyhumulones, humulones and lupulones in an all-glass system. Laws and McGuinness[24] examined tricyclodehydroisohumulone by GLC after derivatisation with N,O-bis(trimethylsilyl) acetamide (BSA).

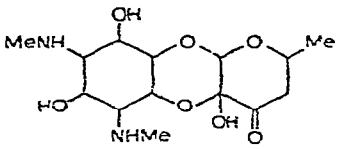
After the initial reports of the GLC of hop resin compounds, Verzele and co-workers[5] undertook a comprehensive study of the GLC of the derivatives of the hop bitter acids with the use of varied derivatising agents, solvents, reaction conditions and GLC conditions. The results of this work showed that the use of packed columns for GLC of solutions of the TMS derivatives of hop bitter compounds was only partially successful.

Indeed, in this publication, Verzele *et al.* stated that several groups had indicated trouble in duplicating the earlier published results. These problems had also been emphasised by Lloyd[17]. Verzele *et al.* overcame some of the problems, attributed to the inherent instability of the TMS derivatives, with the use of wide-bore glass capillary columns, on-column injection, and the use of hydrogen as a carrier gas. However, even with these conditions, it was not possible to obtain consistent results. Most derivatives were found to decompose slowly on the column with the production, after a peak on the chromatogram, of an unstable base line which returned only slowly to the original position. The flame-ionization detector response was unstable and suffered from non-linearity due to silica deposits at the flame tip. Part of this difficulty was attributed to the introduction of a large excess of hexamethyldisilazane (HMDS) with each injection. Lloyd[17] also found that it was impossible to obtain identical responses even with identical injections of the same silylated sample. This was attributed to the derivatives being very liable to slight decomposition at the moment of injection. Verzele and co-workers[5] attempted to improve the derivatisation and injection methods but were unsuccessful in obtaining consistent results.

Similar problems have been encountered in the GLC analyses of the TMS derivatives of other hydroxy- and keto-substituted compounds. These problems in the cases of the GLC analysis of the TMS derivatives of the antibiotics neomycin, chloramphenicol and lincomycin were detailed by Margosis[25]. These difficulties were the formation of precipitates, serious tailing due both to the solvent and excess silylating reagent, and rapid electrode contamination leading to malfunction of the detector system. In the case of lincomycin[25,26] these problems were avoided by adding water to the mixture of excess BSA, pyridine and the TMS derivative of the antibiotic, and extracting with cyclohexane to remove the TMS derivative. This was only possible

due to the unusual stability of the silyl ether under aqueous conditions. Similar problems were encountered with the GLC analysis of the TMS derivative of chloramphenicol[27]. These problems were avoided by evaporation of all solvent and excess silylating reagent and reconstitution in the inert solvent cyclohexane. However, this is a tedious procedure and could introduce losses. The GLC analysis[28,29] of the TMS derivatives of neomycin was only successful on packed columns when all metal and PTFE contact with the silyl derivatives was eliminated thus minimising degradation or adsorption due to this cause. However, it is significant that the silylating reagent, trimethylsilyldiethylamine, was injected with the reaction mixture and the presence of excess trimethylsilyldiethylamine produced chromatograms indicative of the presence of decomposition products.

Spectinomycin (I) has been derivatised using HMDS in dimethylformamide (DMF) to yield the tetrasilyl derivative, presumably with the TMS groups situated at the three hydroxyls and the enolised keto oxygen[30]. Use of BSA instead of HMDS in this method resulted in a GLC trace containing several peaks. Verzele *et al.*[5] has shown, similarly, that the use of BSA to derivatise isohumulone (II) produced a complex mixture of derivatives whereas the use of HMDS produced a solution containing relatively fewer components.



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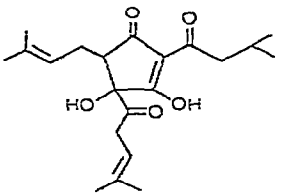

II

Similar difficulties have also been found with the GLC analysis of the TMS derivatives of vitamin A and related compounds[31], and vitamin D[32]. In particular, the TMS derivatives of the 9-*cis*- and 13-*cis*-isomers of vitamin A partially rearrange into each other[31] in the injection port of the gas chromatograph. Also, anhydrovitamin A cannot be analysed quantitatively because it undergoes isomerisation on the column. The TMS derivatives of vitamin D were found to be unstable[32] and difficulty was found in reproducing results. In both these cases the reaction mixtures containing solvent, silylating reagent, and TMS derivatives were injected onto the column.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 402 high-efficiency gas chromatograph with a flame ionization detector was used. This was equipped with a glass column, U-shaped, 1.37 m × 4 mm I.D. and packed with 1% SE-30 on Gas-Chrom Q, 100–120 mesh. Column temperature was programmed from 197° to 275° at 2°/min. The detector was set at 240°. The flash heater was not used. The carrier gas was nitrogen at 40 p.s.i. (35 ml/min) with hydrogen at 40 p.s.i. (35 ml/min) and air at 40 p.s.i. (300 ml/min).

Reagents

DMF and HMDS of silylation grade were obtained from Pierce (Rockford, Ill., U.S.A.). Isooctane was purified by standing overnight over pellets of sodium hydroxide followed by distillation. Tetracosane was obtained from Koch-Light (Colnbrook, Great Britain). Analytical reagent ammonium nitrate was obtained from BDH (Melbourne, Australia). Reagent containing 2 mequiv. of *tert.*-butyldimethylchlorosilane and 4 mequiv. of imidazole was obtained from PCR (Gainesville, Fla., U.S.A.).

Isohumulone preparation and derivatisation procedure

Pure *cis*- and *trans*-isohumulone were prepared from a mixture of the two isomers by an HPLC procedure[33]. *Cis*-isohumulone (25.90 mg, 0.072 mmole) was placed in a stoppered test tube under an atmosphere of nitrogen and DMF (200 μl) and HMDS (130 μl, *ca.* 0.8 mmole) were added. The solution was left standing at room temperature for 16 h. Ammonium nitrate (excess) was added and the mixture was extracted with isooctane (4 × 3.5 ml). The combined isooctane extracts were left standing overnight at room temperature under nitrogen, after which tetracosane (12.95 mg, 0.038 mmole) was added and the solution was filtered. This solution was examined by GLC.

Gas chromatography and calculations

Six replicate analyses were made of the isooctane solution in the range of volumes 0.6–2.4 μl (equivalent to 1–3 8 μg of *cis*-isohumulone). Peak areas of *cis*-isohumulone and tetracosane were measured by two methods for each injection. The methods used were the measurement of width at half height by height of the peaks and by tracing on tracing paper and weighing the cut-out peaks. The average of the two methods for each injection was taken as the peak area. It was found that for six replicate analyses within the sample range of 1–3.8 μg of *cis*-isohumulone there was a relative standard deviation of 1.1% in the percentage of *cis*-isohumulone in the injected sample. Similar results were obtained for *trans*-isohumulone.

RESULTS AND DISCUSSION

Initially in our laboratories GLC of the TMS derivatives of *cis*- and *trans*-isohumulone and mixtures of the two isomers were investigated on packed columns following the reported procedures[12,13]. It was confirmed that inconsistent results are obtained when solutions containing the silylated sample, the derivatising reagent and solvent are injected. Therefore, it did appear that packed-column GLC of reaction solutions of silylated isohumulones was not completely satisfactory.

On consideration of these results and those of previous workers[5,17] it appeared that injections of the original reaction mixture containing excess silylating reagent, solvent, and the TMS derivative was likely to cause difficulties due to decomposition or rearrangement. This could be due in some cases to reaction between excess silylating reagent and the TMS derivative. It was felt that unless some method was found to remove excess silylating reagent, solvent and any precipitate from the reaction mixture it could not be certain whether inconsistent results were due to the inherent instability of the TMS derivatives in the solution as injected or due to reactions and rearrangements occurring in the injection port. Of course, some compounds, especially

those with several keto groups are likely to form several enol ethers and thus several peaks may appear on the gas chromatogram even though no degradation has taken place.

In the investigation of the derivatisation and GLC of the isohumulones several possibilities were considered in an effort to overcome these difficulties. An aqueous or alcoholic procedure to remove excess silylating reagent after derivatisation could be used, as was employed by Margosis[25,26] for the antibiotic alcohol, lincomycin. However, in the case of enol ethers such as those present in the TMS derivatives of isohumulone this is not possible due to the known ease of hydrolysis of keto–enol ethers[34,35]. The TMS derivatives may be isolated, for example by column chromatography, as carried out by Wolfrom *et al.*[36] for the separation of the TMS derivatives of carbohydrate intermediates. However, these methods are lengthy and losses of derivatives are likely. It is possible to remove solvents and reagents by evaporation of the derivatisation solution[27] to dryness and reconstitution in an inert solvent. However, losses of the TMS derivatives are possible during the evaporation procedure, especially if the solvent used during derivatisation is high-boiling and is present in a large excess.

Another alternative is to use a silylating reagent which produces a derivative which is not easily hydrolysable under aqueous conditions. Such a reagent is *tert.*-butyldimethylchorosilane and imidazole in DMF as developed by Corey and Venkateswarlu[37] for the derivatisation of prostaglandin intermediates. These workers reported reaction with alcohols, the resultant *tert.*-butyldimethylsilyl ethers being stable in the presence of aqueous or alcoholic bases. Workers at Applied Science Labs.[38,39] have extended the use of this reagent to compounds containing keto groups. However, these reactions needed higher temperatures for satisfactory derivatisation. Attempts in our laboratories to apply this method to *cis*-isohumulone using a range of reaction temperatures failed to produce any significant level of derivatisation.

Since none of the above procedures for isolating the TMS derivatives from extraneous material appeared satisfactory it was decided to attempt a method of solvent extraction of the TMS enol ethers from the reaction mixture. The TMS derivative of *cis*-isohumulone was formed using HMDS in DMF at room temperature overnight. Equivalent results were obtained with reaction for two hours at 50°. The DMF solution of TMS derivatives was then extracted with isooctane to remove the TMS derivatives of the *cis*-isohumulone (DMF and isooctane are practically immiscible). GLC examination of the isooctane extract indicated one major component, presumably the TMS derivative of *cis*-isohumulone. However, it was found that extraction of the TMS derivative from the DMF solution was not complete, necessitating a salting-out procedure. Salts such as ammonium nitrate and sodium nitrate are known to be very soluble in DMF[40]. The DMF solution containing the TMS derivative was saturated with crystalline ammonium nitrate and then extracted with isooctane. Several extractions resulted in complete removal of the TMS derivative from the DMF into the isooctane. The isooctane solution was allowed to stand overnight under nitrogen and then filtered. GLC analysis of this solution on a packed column (1% SE-30) indicated a single major component with no serious tailing on either the solvent or derivative peak (Fig. 1). Subsequent injections were reproducible with respect to tetracosane added as an internal standard. None of the objectionable characteristics of gas chromatograms obtained by injection of TMS derivatisation

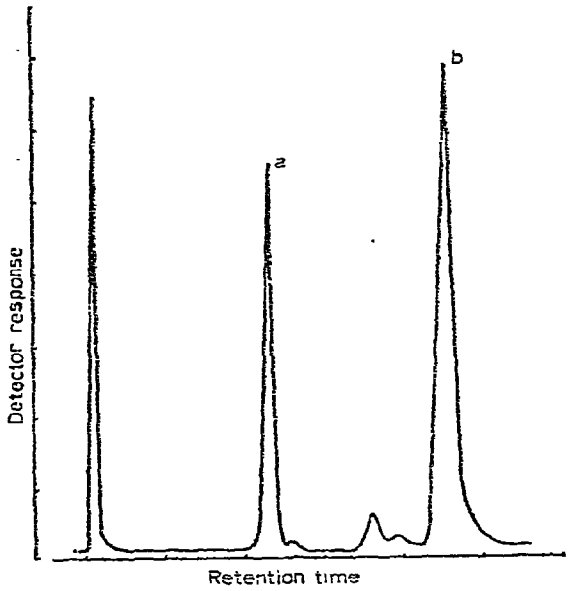

Fig. 1. Gas chromatogram of *cis*-isohumulone as its trimethylsilyl derivative. a = tetracosane, b = *cis*-isohumulone derivative.

reaction mixtures were found. The isooctane solution of the TMS derivative was found to be stable for over two months at 0° under nitrogen. Using this procedure, one microgram of *cis*-isohumulone may be detected and six replicate analyses of a solution of the TMS derivative of *cis*-isohumulone in isooctane showed a relative standard deviation of 1.1%.

Investigation by GLC of the isooctane extract immediately after extraction showed the presence of one major component and two components present in much smaller amounts. On standing overnight the minor components disappeared and the major component was enhanced with respect to the internal standard. Possibly this change is due to the initial formation of three enol ethers in DMF which are converted with time into the most stable of the three. It is intended to investigate these compounds by mass spectrometry and this will be the topic of a subsequent publication. It is possible for three bis-TMS ethers to be formed and the change from the polar solvent, DMF, to the non-polar solvent, isooctane, may result in an equilibration to the most stable enol ether. The behaviour of such enol ethers is of interest due to the possibility of analogous enolic changes of isohumulones in beer. Verzele and co-workers[41] have postulated that after the addition of isohumulones to the beer medium an enol equilibration may explain changes of bitterness with time. The confirmation of the structure of these TMS derivatives must await investigation using gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy.

Preliminary investigations have shown that the same extraction procedure can be applied to the derivatisation of *trans*-isohumulone and a mixture of isohumulones from an isomerised hop extract. In addition, other hop resin components such as xanthohumol may also be derivatised and subjected to the extraction procedure

with good results. Lloyd[17] previously reported that it is possible to obtain GLC peaks for some polyphenols from hops. In our laboratories it was found in all cases that, to obtain consistent results, it was necessary to use the ammonium nitrate–isooctane extraction procedure described above.

Investigations are now in progress to quantitate this procedure by use of an internal standard and extend it to other hop resin components likely to be present in hop extracts. The increased resolution of a glass capillary column as reported by Verzele *et al.*[5,42,43] may be necessary to fully resolve the constituents of some isomerised hop extracts.

The application of this extraction procedure to the investigation of the TMS derivatives of antibiotics[25], vitamin A[31] and vitamin D[32] may avoid some of the previously reported difficulties.

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