DUBLIN CITY UNIVERSITY

GUINNESS IRELAND GROUP

# Some Novel Applications of Instrumental Analytical Techniques to the Brewing Industry

Thesis submitted for the award of

Master of Science

by

Brian Daly, B Sc

September, 1996

## DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science in Analytical Chemistry, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

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**Date:** _____

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# Some Novel Applications of Instrumental Analytical Techniques to the Brewing Industry


## ABSTRACT

Modern instrumental analytical techniques play an important role in the brewing industry today They are extensively used for both a quality control/quality assurance function and for research purposes At all stages of the production process, from the assessment of raw materials, through the brewing process, fermentation, maturation, blending of finished beer, packaging and shelf life studies instrumental analytical techniques provide critical data which allow the brewer to understand and keep his process in control Research is ongoing to develop new, more efficient, more sensitive and more selective analytical techniques to achieve a greater understanding of the processes described above and to provide better quality beer at low cost

This thesis describes some such analytical methods which were developed as a result of specific practical needs which arose during normal brewing practice over a number of years

Roasted barley is a most significant ingredient of Guinness Brewing Worldwide stout products A novel analytical method is presented for the analysis of the volatile flavour constituents of roasted barley which is rapid and requires little sample preparation

Secondary coolants are widely used in brewery production plants A sensitive method was developed for the detection of possible coolant contamination in beer The method was based on the addition of a fluorescent dye to the coolant in so that in the event of leakage into beer this contamination could be detected by a simple fluorescence measurement of beer

The development of a gas chromatographic method for the analysis of propylene glycol in beer is presented Propylene glycol is a major constituent of the secondary coolant used at the Guinness Ireland Group, Dublin site It is also naturally present in beer

Two analytical methods are described for the analysis for styrene in beers Styrene is present due to the ability of some yeast strains to decarboxylate phenolic acids present in wort Such yeasts are classified as $Pof^{+}$ yeasts i e produce phenolic-off-flavour Methods are described for the classification of phenolic-off-flavour ($Pof^{+}$) producing yeast strains Formation of styrene during a fermentation was investigated Studies into the kinetics of styrene production were also carried out

## ACKNOWLEDGMENTS

The staff of the Guinness Brewing Worldwide Research Centre in Dublin are acknowledged for their support and contribution to the work reported herein All of the staff contributed to some extent, be it with advice, information, constructive criticism or encouragement The following are particularly acknowledged for their collaboration

Dr Dan Donnelly, Ann-Marie Doyle and June Hurley for the technical support and the microbiological experiments provided with regard to the Pof yeast research carried out in Chapter 4
Gerard Hennigan and Niall McNulty for the pilot scale brew referred to in Chapter 3
Eithne Mooney, Marie Mooney-Hynes and Wendy Costello for the patience with which they listened to my requests for information and the speed at which it was provided
Eamonn Rock of the Public Analysts laboratory, Corn Market, Dublin, for expert advice and collaboration on Chapter 3
Dr Imelda Shanahan and the staff of TMS Environment, Dublin City University, for their collaboration on Chapter 1
Mr Joe Bergin for always being there for me, also to compensate for all those theses where he was not acknowledged

Dr Eddie Collins my industrial supervisor, without whose advice, encouragement, patience and endless hours of proof reading this piece of work would not have been finished His encyclopedic knowledge of the brewing industry proved invaluable
Dr Mary Meaney my academic supervisor for her help

I thank Dr R Letters and Dr F Lynch the past and present Guinness Brewing Worldwide Research managers, for their financial support and permission to engage in this work

# DEDICATION

This thesis is dedicated to all those who gave help, encouragement and advice to the author over the first thirty years of my journey through this life In particular my late father, Pat, my mother Kitty, and my brothers Paul and John

For my son Conor simply for being the person he is

For Bla Tonra for understanding the time and space required to complete this work Also for being a partner who appreciates the time and pain required for growth and most importantly who is willing to meet the challenge

# Analysis for the Volatile Flavour Constituents of Malt and Roasted Barley

## 1.1 INTRODUCTION

### 1.1.1. Production of Malt and Roasted Barley and the Inherent Chemical Changes Induced in the Barley

The brewing process may be divided into five main stages

1 Malting of the barley

2 Mashing and wort production

3 Fermentation of the wort with yeast

4 Storage and maturation of the beer

5 Packaging of the matured beer

This literature review will only deal with item 1 above Furthermore, it will only consider flavour aspects Enzymic aspects of malting, important though they undoubtedly are, will only be mentioned *en passant* In order to discuss the flavour constituents of malt it is necessary to understand how barley is carried through the malting process and exactly what this process involves

Unmalted barley on its own cannot be used for brewing as it does not have the enzyme system which controls the breakdown of the starch to fermentable sugar in the mashing process It is during malting that the enzyme system is developed A further important process in malting and mashing is the breakdown of some of the barley proteins to soluble nitrogenous compounds, such as amino acids These are essential to yeast growth, and the subsequent fermentation stage

In the malting process, the barley is subjected to a chain of processing stages which are described briefly

(i) Cleaning and sorting of the grains Any foreign bodies present must be removed i e dust, weeds, other grains, half grains etc This is achieved by a series of sieves, screens and separators of various types The barley is then graded by size, so that all batches to be malted are of uniform size This is essential to ensure uniformity of the germination

(ii) Storage of raw grain The barley is dried if necessary to a water content less than 14% and is stored in large bins To prevent spoilage during storage, it must be aerated and the temperature and water content must be tightly controlled

(iii) Steeping of the barley In order for the barley to germinate, the water content of the grains must be sufficiently high, since water acts as a carrier of reserve nutrients in the barley grain The grain is steeped for 40 - 60 hours During steeping, the barley is aerated, and oxygen is consumed while carbon dioxide is produced

(iv) Germination During germination, endosperm cell walls, mainly β-glucan, and protein are broken down (dissolution or modification) and the starch in the grains is liberated At the same time, the enzyme complex responsible for the hydrolysis of the starch during mashing (the step after malting of the barley in the brewing process) is generated and/or activated When the desired level of dissolution has been achieved, germination is interrupted by killing the sprouts with heat and reducing the water content of the barley to 3 - 5% This is achieved by drying it in a kiln ("kilning")

(v) Kilning The green malt (malt after germination) is dried in a kiln by passing heated air through the grain bed This process can be divided into two distinct phases

(a) The enzyme activity in the barley is arrested This is accomplished by drying the green malt from a water content in the order of 45% after germination to 3 - 5%

(b) The malt is heated during the second phase, which induces certain chemical reactions to take place between the nitrogenous compounds present and the sugars, to form substances which contribute to the characteristic colour, flavour and aroma of the final beer (see Table 1 1) These chemical reactions are discussed in detail later in this review

(vi) Cleaning of the malt The final stage through which the malt passes before being introduced to the mashing stage is cleaning The rootlets, which sprouted during germination, are removed These rootlets are undesirable for a number of reasons, e g , being lipid-rich they have potential for oxidative rancidity

The malt is now ready for the mashing and wort production stage of the brewing process For the purposes of the present discussion malt may be separated into two groups,

(i) True Malts These are produced by conventional malting processes and have sufficient enzyme activity to break down starch to fermentable sugars They constitute the vast bulk of malts used by the brewing industry

(ii) Special Malts These require special kilning or roasting treatment during which the enzyme activity is destroyed but flavour and colour are enhanced These are used in conjunction with true malts but at a lower usage rate, with the purpose of conveying particular flavour and colour attributes to the final beer

The production of True Malt as described above can be varied in many ways Most malts are first dried and then cured (kilned) at higher temperatures for various specific times, using malts of different degrees of hydration and modification If the curing period is limited then the enzyme activity of the malt is preserved These True Malts are capable of converting their own starch and possibly that of other grain to fermentable sugar

If heat treatment is increased the enzymes are destroyed but both the colour and the flavour of the resulting Special Malts are enriched Variations to the flavour and colour of malt can be achieved by increasing the temperature of kilning before drying is complete This process causes some saccharification and production of caramel flavour in the crystal and cara-pils type Special Malts Variation in kilning procedure thus gives a wide range of malt types, but in general terms higher temperatures give increased flavour to both malt and to beer produced with these These high temperature treated Special Malts include caramel malt, chocolate malt and black malt Table 1 1 gives examples of the range of malts available to the brewer A further product which it is appropriate to include here is roasted barley It is produced by direct kilning of the dried barley grain i e the barley is not allowed to germinate prior to kilning While strictly speaking it is not a malt as described above, similar chemical reactions take place during its kilning/roasting as occur during the kilning of malt

Roasted barley has a different flavour from roasted or black malt as the former starts from unmodified barley, which contains relatively little free ammo acid

or sugar

**Table 1 1** Malt Types

| Malt Type | Colour (EBC*) | Maximum Kilning Temperature°C |
|---|---|---|
| **True Malts** | | |
| Green | - | - |
| White | 2 | 75 |
| Pils | 2 8 | 85 |
| Light | 3 3 | 85 |
| Pale Ale | 5 5 | 95 |
| Mild Ale | 7 | 110-140 |
| Dark Munich | 15-20 | 100-109 |
| **Special Malts** | | |
| (a) Caramelised | | |
| cara-pils | 5-40 | 150 |
| crystal | 75-300 | 150 |
| (b) Roasted | | |
| amber | 55 | 170 |
| brown | 130-500 | 130 |
| chocolate | 1100 | 175 |
| black | 1300 | 220-230 |
| roasted barley | 1400 | 220-230 |

* EBC = European Brewery Convention Colour Units

The chemical reactions and the resulting flavour effects inherent in the kilning of raw barley and/or germinated barley are many and varied These reactions are known also to occur in many other food processes (e g baking, coffee roasting and peanut roasting) Much research has been undertaken over the last two hundred years into this area The reactions differ depending on the temperature and duration of the heating process, and, in the case of malt and barley, the kilning time Several authors[1-14] have reviewed this field in relation to the brewing industry Seaton[2,3] in particular has given an excellent summary of the variety of malts used, their methods of preparation and their contribution to beer flavour He has also described the chemical reactions that occur during kilning and the various flavour characteristics imparted as a result of this treatment

The flavour active chemical compounds of malts and roasted barley may be categorised by their chemical structures and the mechanisms by which they are formed These reaction mechanisms may be broken down into four groups

* Lipid Oxidation
* Strecker Degradation
* Maillard Reactions
* Thermal Degradation

### 1.1.1.1 Lipid Oxidation

Flavour active products occur as a result of the enzymatic oxidation of lipids There are three enzymes responsible for this, lipase, lipoxidase and peroxidase This degradation reaction is seen only in green malt, i e before these enzymes are denatured by kilning Many volatile alcohols, aldehydes, carboxylic acids and lactones are produced by the cleavage of intermediate hydroperoxides of linoleic and linolenic acids present in green malts

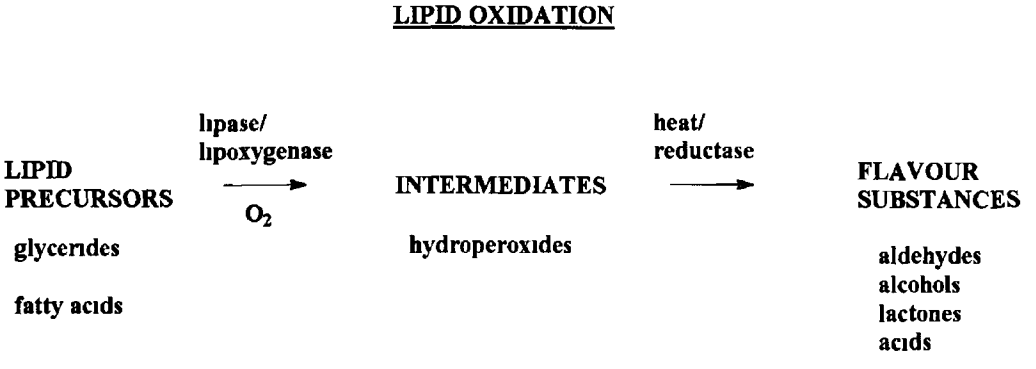



**Figure 1.1.** Lipid Oxidation

### 1.1.1.2 Strecker Degradation

The second group of substances are aldehydes The mechanism by which these are formed is known as the Strecker degradation reaction, which is non-enzymatic in nature The reaction was named in 1948 by Schonberg[15, 16] in honour of Adolph Strecker who reported[17] in 1863 that α-alanine was degraded by alloxan to acetaldehyde and $CO_2$ The Strecker degradation reaction involves the interaction between an α-dicarbonyl compound and an α-amino acid, which yields an aldehyde and an α-amino carbonyl The mechanism is believed to involve formation of a Schiff's base followed by decarboxylation assisted by the presence of the second carbonyl group in the partner molecule (Figure 1 2) The degradation of the acid results in the formation of an aldehyde with one carbon less, $CO_2$ and an α-amino carbonyl An example of this is the reaction between diacetyl (2,3-butanedione) and valine (Figure 1 3), which results in the formation of isobutyraldehyde In model reaction systems a wide variety of carbonyl compounds are capable of initiating this

Strecker degradation The common feature of these structures is the **-CO-(CH=CH)$_n$-CO-** grouping, where n = 0, 1, 2, Table 1 2 gives examples of α-amino acids which undergo this reaction and their reaction products The α-amino ketones formed may dimerise with oxidation resulting in the formation of pyrazines In the example given in Figure 1 3 tetramethylpyrazine is the oxidation product

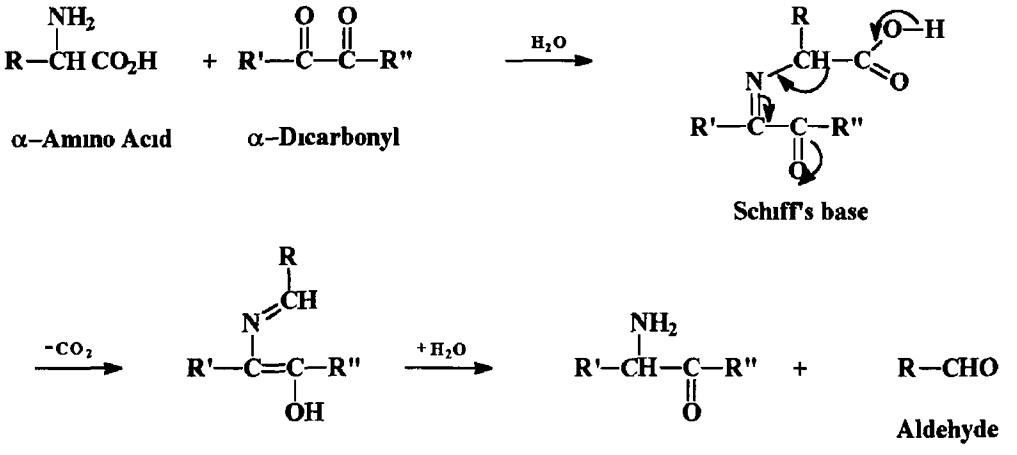



**Figure 1.2.** Mechanism of the Strecker Degradation

In relation to the malting process, the α-amino acids are present as a result of the breakdown of barley protein during germination as described earlier The α-dicarbonyl compounds occur due to the thermal decomposition of sugars during kilning of malt The first stage of α-dicarbonyl formation is the high temperature breakdown (>100°C) of sugars by reversible isomerisation of an aldose sugar to the corresponding ketose via a 1,2-cis-enediol intermediate, as depicted in Figure 1 4 This reaction is known as the Lobry de Bruyn-Alberda Van Echerstein (LBAVE) transformation This enediol is readily dehydrated in a neutral or acidic environment via the sequence of reactions shown in Figure 1 5, with glucose being the aldose sugar illustrated here Hydroxymethylfurfural is by no means the only product of this pathway There are eight aldohexoses which can undergo this reaction Dehydration reactions in the absence of cyclisation give a range of highly reactive α-dicarbonyl compounds

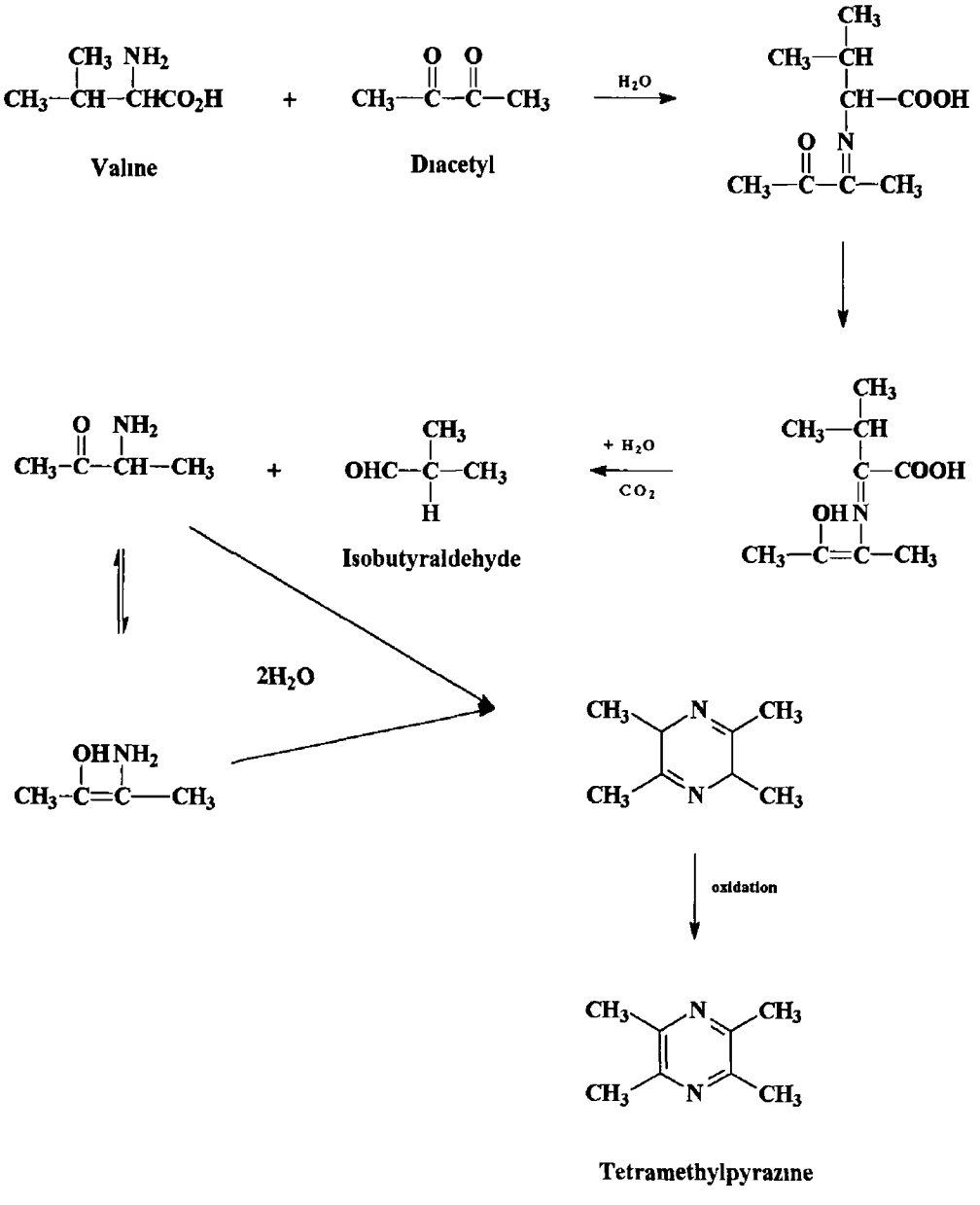
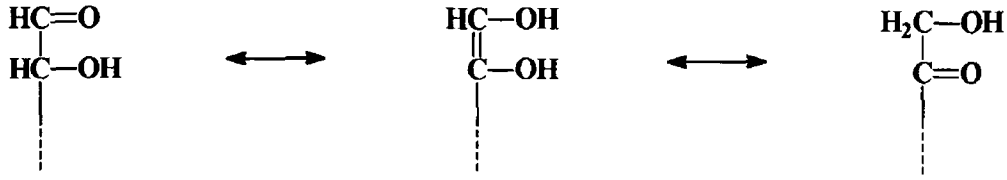



**Figure 1 3.** Reaction between diacetyl and valine




ALDOSE SUGAR 1,2-CIS-ENEDIOL KETOSE

**Figure 1.4.** The Lobry de Bruyn-Alberda Van Echerstein (LBAVE) transformation

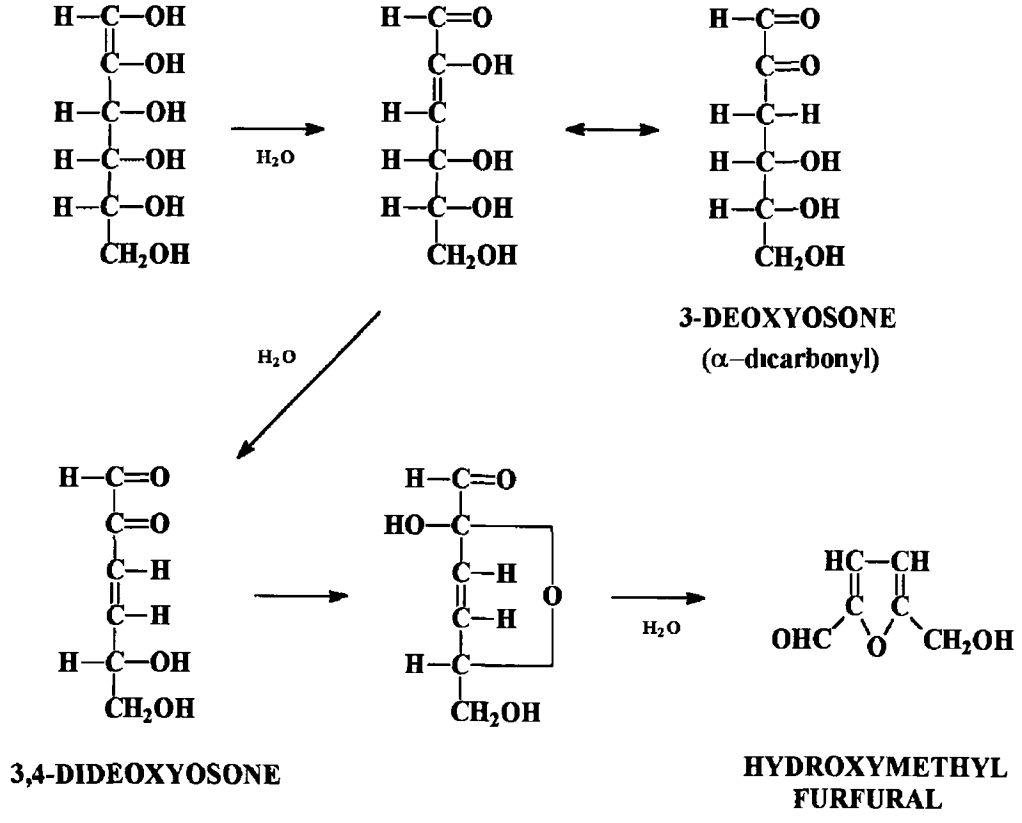



**Figure 1.5.** The dehydration mechanism of a hexose enediol

All of the aldehydes produced by the Strecker degradation are also produced enzymatically from amino acids by transamination with subsequent decarboxylation of the resulting α-ketoacids Each Strecker aldehyde is accompanied in malt by an appreciable amount of the corresponding alcohol, presumably formed by reduction of the aldehyde in the presence of alcohol dehydrogenase (before enzyme denaturation) Model studies[18] with $^{14}C$ labelled glycine have demonstrated that 80-90% of the evolved $CO_2$ is derived from the carbonyl group of the amino acid

**Table 1.2.** Strecker Degradation Substrates and Products

| α-Amino acids | Product |
|---|---|
| valine | isobutyraldehyde |
| leucine | isovaleraldehyde |
| S-methylcysteine | 2-methylthioacetaldehyde |
| methionine | methional |
| phenylglycine | benzaldehyde |
| phenylalanine | 2-phenylacetaldehyde |

### 1.1.1.3. Maillard Reactions

The third series of compounds found in kilned malts and roasted barley are volatile nitrogen, oxygen and sulphur-containing heterocycles They occur as a result of a series of complex, non-enzymatic reactions named after their discoverer Maillard[19] These were systematised by Hodge[6] in 1953, in a scheme which is still believed to be valid despite the fact that much of it is based on speculation and not on hard chemical evidence The Maillard reactions constitute the most important non-enzymatic mechanism for the formation of flavours during the processing of foodstuffs

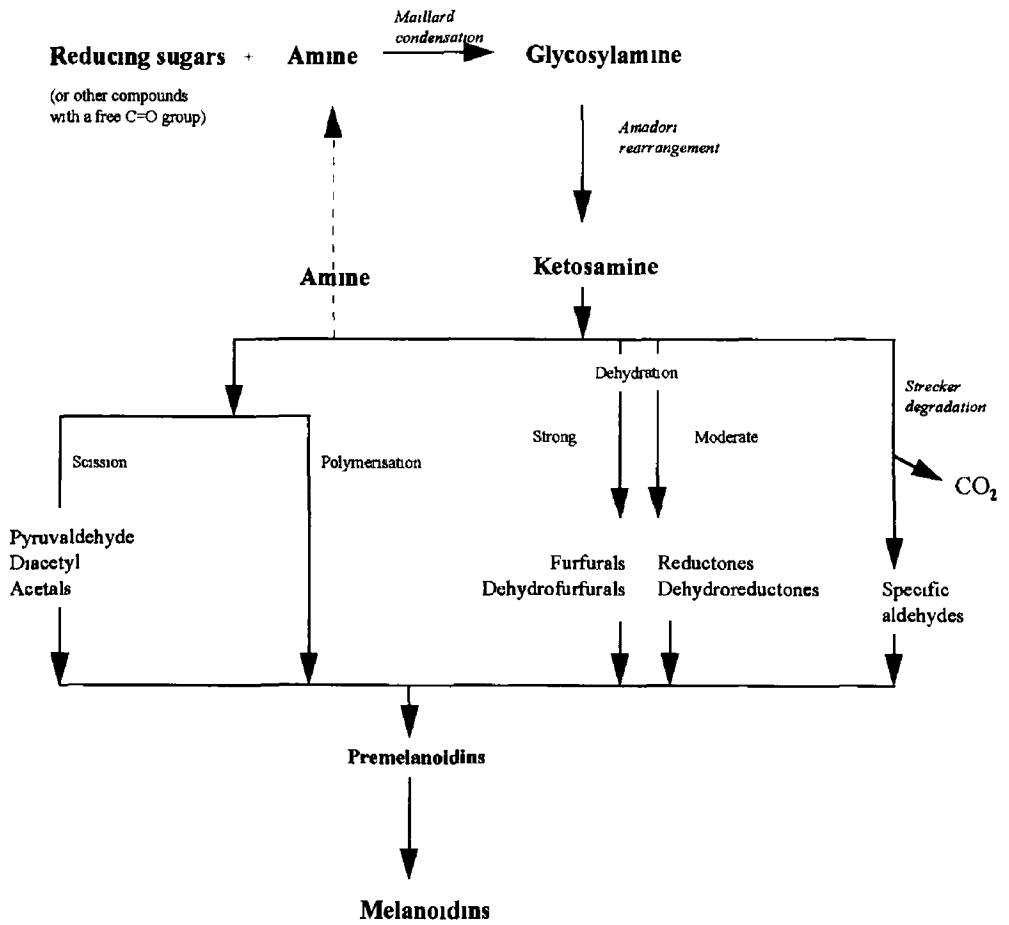



**Figure 1.6** Mechanism for Maillard reactions

The interaction between reducing sugars and α-amino acids at temperatures in excess of ambient gives rise to α-diketone intermediates (reductones) which react further under various conditions to give furans, pyrroles, thiophenes, pyrazines and also non-volatile, polymeric, colour compounds known as melanoidins (Figure 1 6)

For the purposes of this review the reaction is best divided into three stages

(i) glycosylamine formation reactions between reducing sugars and α-amino acids, and subsequent Amadori rearrangement,

(ii) intermediate steps comprising dehydration with formation of oxygen heterocycles and dehydroreductones, and Strecker degradation,

(iii) polymerisation of the intermediate carbonyl compounds to high molecular weight coloured compounds, known as melanoidins

A flow diagram of these stages is given in Figure 1 7 The reversible condensation of the aldose (ketoses undergo a similar reaction) with the amine or amino acid to the aldoxylamine is followed by the irreversible Amadori rearrangement to the ketosamine, or Amadori compound Many of these are crystalline and have been fully characterised by NMR spectroscopy

The intermediate stages of the Maillard reaction involve either 1,2- or 2,3-enolisation of the Amadori compound Low pH favours 1,2-enolisation (Figure 1 8), and leads to formation of the crystalline 3-deoxyosuloses which can be further dehydrated to furfurals

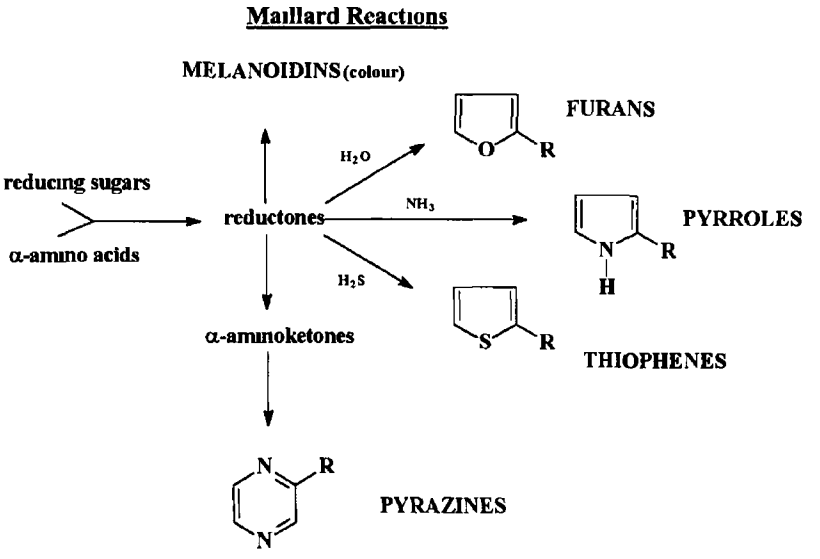



**Figure 1 7** Possible non-enzymatic browning reactions during special malt production

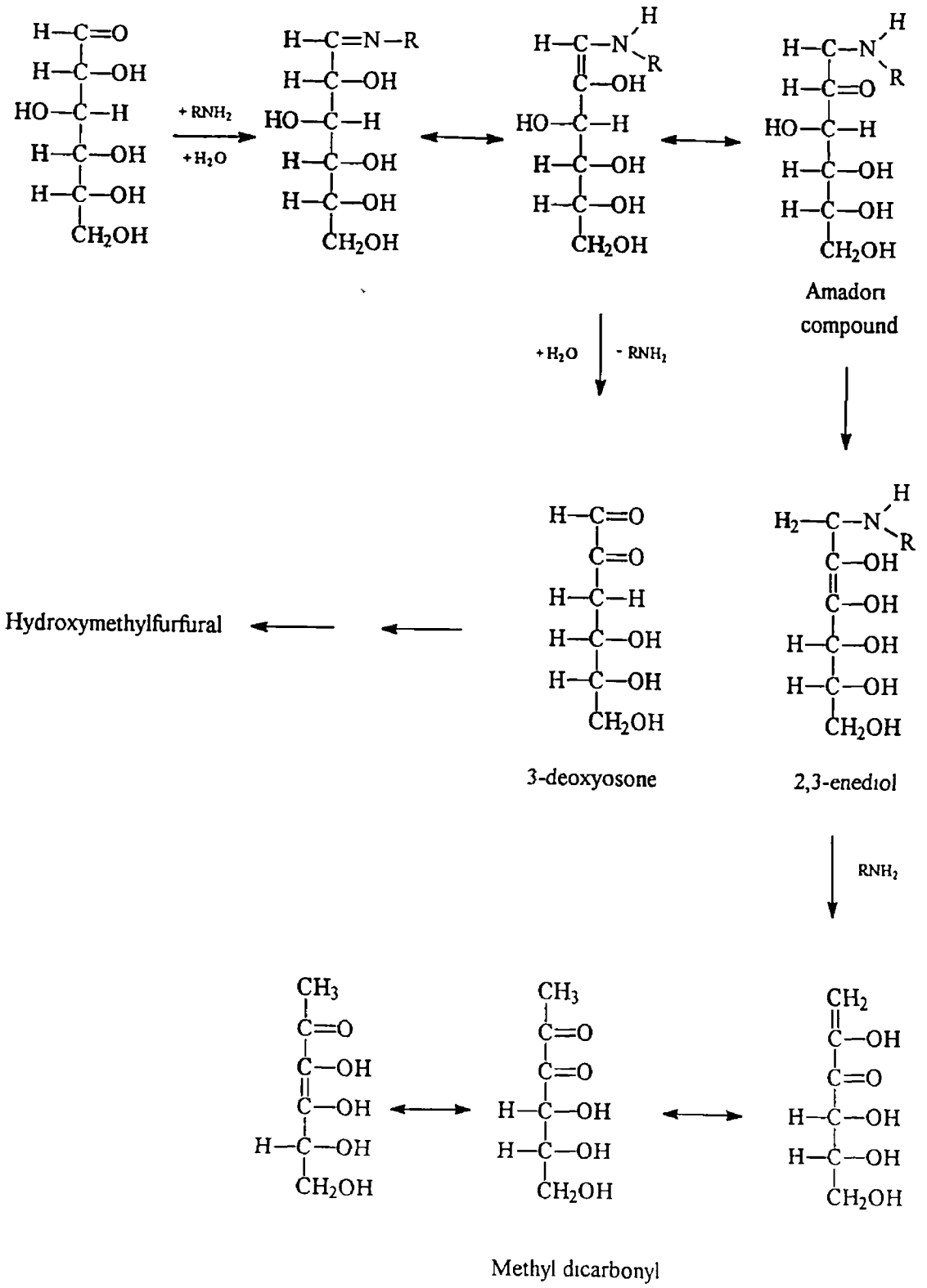



**Figure 1 8** The formation of hydroxymethylfurfural and dicarbonyls in the Maillard reaction

The 3-deoxyosuloses are α-dicarbonyl compounds and, as such, are active in the Strecker degradation Many reactive dicarbonyl compounds are formed as a result of 2,3-enolisation of these 3-deoxyosuloses, such as the 1-deoxy-2,3-diketone derivatives These diketone derivatives have not been isolated, but their cyclodehydration products, such as maltol and isomaltol (Figure 1 9) which have pronounced caramel flavours, are well known Many other volatile heterocyclic compounds are formed by degradation of reductones with or without incorporation of nitrogen or sulphur from amines or hydrogen sulphide

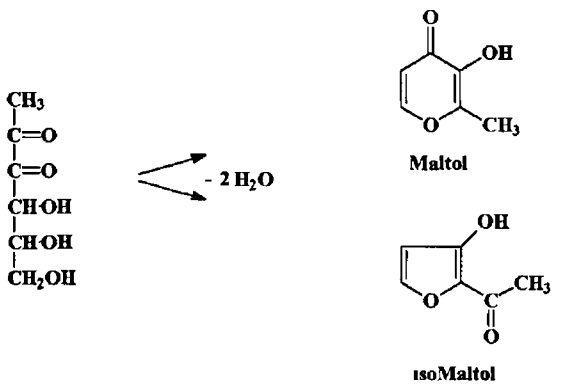
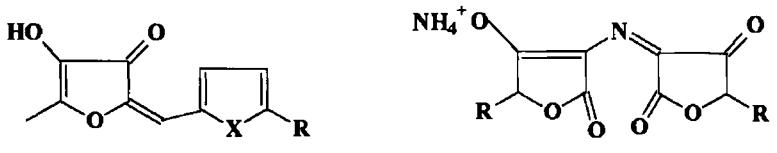



**Figure 1.9.** Formation of maltol and isomaltol

In the final stage of the Maillard reaction, the intermediates condense to form unsaturated, fluorescent, coloured polymers Given that colour formation is one of the primary characteristics of the Maillard reaction it is surprising that little is known of the structure of any chromophore present The lower molecular weight melanoidins (MW < 1,000) are believed to be aldol condensation products of the intermediate carbonyl compounds, with or without the incorporation of nitrogen The coloured compounds shown in Figure 1 10 have been isolated from model reactions Para-quinones may also be formed[20]



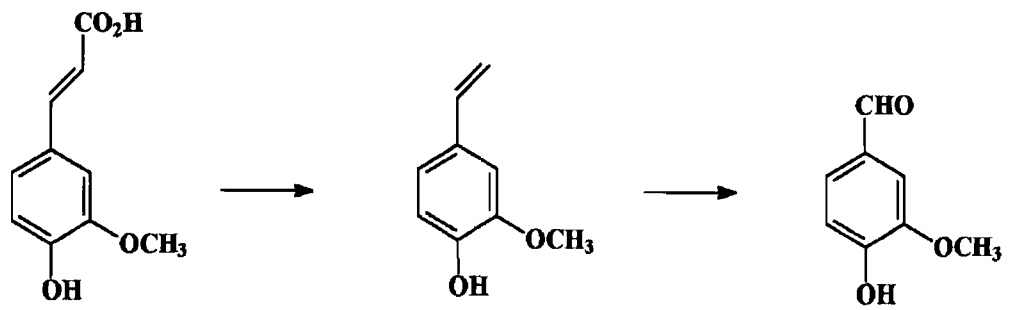

YELLOW/ORANGE RED

**Figure 1.10** Colour compounds from Maillard reactions

### 1.1.1.4. Thermal Degradation

The thermal degradation products can be divided into two groups, phenols and sulphur compounds

The phenols are derived from thermal degradation of phenolic acids present in the husk and cell walls of barley High roasting conditions result in a wide range of compounds with medicinal, smoky, phenolic flavours The degradation of ferulic acid is illustrated in Figure 1 11




**Ferulic Acid** **4-vinyl guaiacol** **Vanillin**

**Figure 1.11.** Thermal degradation of ferulic acid to 4-vinyl guaiacol

This reaction gives rise to 4-vinyl guaiacol which has a spicy, clove-like flavour Further thermal degradation may give vanillin which has a characteristic sweet, creamy and buttery flavour

The sulphur compounds derived from malt are dominated by one compound, dimethyl sulphide (DMS) DMS is formed by thermal degradation of the precursor S-methylmethiomne, which is itself produced during barley germination This mechanism was elucidated[21] (Figure 1 12) by a number of brewing research groups in the late '70s and early '80s Other aliphatic sulphur compounds derived from malts include Strecker thioaldehydes derived from S-methylcysteine and methionine, and

small amounts of less volatile methyl sulphides which appear to be reduction products of methanethiol with lipid derived unsaturated aldehydes and ketones

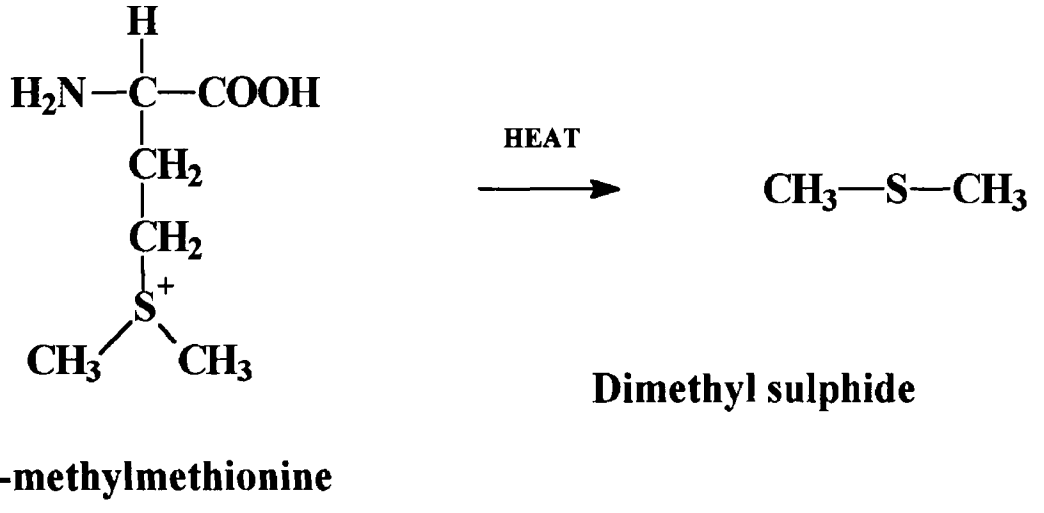



**Figure 1.12.** Thermal degradation of S-methylmethionine

Typical examples of each of the classes of compounds found across the range of malts and roasted barley are given in Table 1 3

### 1.1.1.5. Speciality Malts in Brewing

The variety of malts available give the brewer a wide choice of significantly different flavour and colour characteristics to enhance his final beer product As described earlier, a malster can control the germination, drying and subsequent kilning of barley to give a malt of a desired flavour and colour Malsters did not wait for the chemical mechanisms of malting to be elucidated to attempt control over the malting process but, through innovation and experimentation, developed new malt types The impact of kilning on green malt in terms of flavour and colour changes are most simply described by discussion of the substances formed and the flavour imparted by these substances Table 1 4 gives an outline of these effects

The important features to note with green malt is the dominance of the lipid derived substances, the presence of dimethyl sulphide, and the complete absence of nitrogen heterocycles Green malt is associated with green, grassy, stewed vegetable aroma These result from the presence of the products of lipid oxidation and some Strecker degradation White malt has less of the grassy and sulphidic aroma of the

**Table 1 3.** Typical Flavour Volatiles found in Malt

| **Oxidation products of Lipids** | **Oxygen Heterocyclics** |
|---|---|
| hexanal | 2-pentylfuran |
| *trans*-2-hexenal | furfural |
| *trans, trans*-2, 4-hexadienal | 2-acetylfuran |
| *trans*-2-octenal | 5-methylfurfural |
| *trans*-2-nonenal | furfuryl alcohol |
| *trans*-2-*cis*-6-nonadienal | 3-phenylfuran |
| *trans, trans*-2, 4-nonadienal | |
| *trans*-2-*cis*-4-decadienal | **Nitrogen Heterocyclics** |
| *trans, trans*-2, 4-decadienal | 2-formylpyrrole |
| 1-penten-3-ol | 2-acetylpyrrole |
| *cis*-2-penten-1-ol | pyrazine |
| 1-hexanol | methylpyrazine |
| *cis*-3-hexen-1-ol | dimethylpyrazines |
| *trans*-2-hexen-1-ol | ethylpyrazine |
| 1-octanol | ethylmethylpyrazines |
| 1-nonanol | trimethylpyrazine |
| *trans*-2-nonen-1-ol | 3, 6-dimethyl-2-ethylpyrazine |
| *trans*-2-*cis*-4-decadien-1-ol | 2-furanylpyrazine |
| *trans, trans*-2, 4-decadien-1-ol | indole |
| hexanoic acid | |
| octanoic acid | **Sulphur Heterocyclics** |
| 4-nonanolide | 2-formylthiophene |
| 4-decanolide | 2-methyltetrahydrothiophen-3-one |
| | |
| **Strecker Aldehydes** | **Phenols** |
| isobutyraldehyde | 4-vinylguaiacol |
| isovaleraldehyde | 4-vinylphenol |
| 2-methylthioacetaldehyde | vanillin |
| methional | |
| benzaldehyde | **Sulphides** |
| 2-phenylacetaldehyde | dimethyl sulphide |

green malt but has a trace of caramel aroma due to the presence of oxygen heterocycles It also has a slight nutty flavour due to the presence of nitrogen heterocycles Moving on to pale ale malt, it is found that the lipid derived compounds are lowered as is dimethyl sulphide Conversely, the heterocycles, both oxygen and nitrogen are present in greater quantities

The Special Malts which have been heated to greater temperatures fall into two main groups (Table 1 1) The caramelised group are prepared by roasting green malt Some saccharification occurs during kilning and these malts are characterised by their sweet caramel flavour The oxygen heterocyclic compounds such as maltol, isomaltol and 2-acetylfuran are particularly associated with these caramelised malts The second group is the dry roasted malts The flavour contribution of these malts is much greater than that of the true malts The pyrazines dominate the flavour of the dry roasted malts These compounds impart a burnt, bitter flavour as a consequence of the presence of large amounts of nitrogen heterocycles such as methylpyrazine and 2-furanylpyrazine

Roasted barley is included in this group because, although it is a non-malted product, it gives a similar colour and flavour to the final beer product

This literature review will concentrate on the techniques used to determine the volatile composition of roasted barley since investigative work on it commenced in the early 1960's Roasted barley as utilised in beer production today has its origins in the introduction of a unique product known as "Porter" in London during the 1720's The development of the first porter is attributed to Ralph Harwood[18] of Bell Brewhouse, Shoreditch in 1722 This porter was different from other beers of that time due to the type of malt used in the brewing process It is said to have originated when a brown malt (for brown ale production) which had been subjected to excess firing/kilning was used to brew a beer It remains unclear whether this treatment was intended, accidental, or, indeed, if the story is apocryphal

**Table 1 4** Malt Derived Flavour

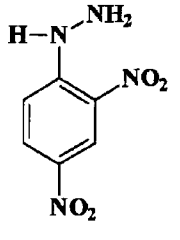
| | Flavour Substances | | | | |
|---|---|---|---|---|---|
| **Malt Type** | **Lipid** | **Strecker** | **Nitrogen Heterocyclics** | **Oxygen Heterocyclics** | **Beer Flavour** |
| Green | +++ | +++ | | | Grassy Sulphidic |
| White | + | +++ | + | ++ | Slightly sulphidic caramel |
| Pale-ale | | +++ | ++ | +++ | Caramel Slightly roast |
| Crystal | | | + | +++ | Caramel Malty, roast |
| Roast barley | | | +++++ | ++ | Roast, burnt Bitter |

This roasted malt enriched the product with a distinctive colour (much darker than any other previously seen) and flavour (roasted and more bitter) as well as improving its microbiological stability, an advantage of incalculable value to brewers To quote Mathias[18] "these improvements were beyond the scope of analysis at that time and owed nothing to a search conducted methodically to implement known principles" Porter became extremely popular throughout the 18th Century and surpassed in production terms all other beer types in the British Isles The specific reasons for this are impossible to explain scientifically, but, as a result of it, the production of roast barley became a primary concern of the brewer

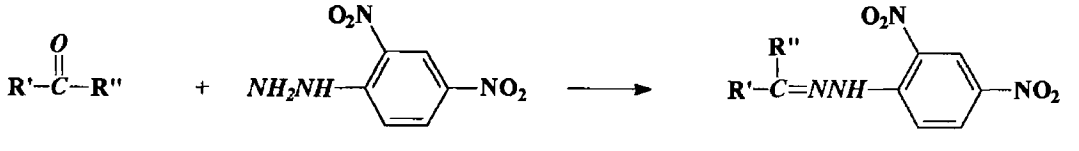
Possibly the most significant innovation in the production of roasted barley came with British Patent No 4112 of 1817[19], the Iron Cylinder Roaster, developed by Daniel Wheeler This was similar to the roaster used for coffee production It was distinct from other roasting techniques in that it roasted slowly enough to retain some fermentable material while giving the correct amount of flavour and colour This new method swept through the industry and the adoption of malt made according to Wheeler's patent marked the beginning of the history of porter/stout as we know it today Roasters of this type were installed adjacent to the Guinness brewery in 1819

## 1.2 Analytical Evaluation of the Volatile Flavour Constituents of Malt and Roasted Barley

As early as 1963 Damm and Kringstad[24] published an investigation into the volatile carbonyl compounds of barley and a variety of malts including green malt, pale malt, dark malt and roasted malt They used thin layer chromatography (TLC) and gas chromatography (GC) to isolate and identify some of the components of malt Melting point and spectrophotometric analysis were used to confirm the identities of isolated components Samples of the various malts for analysis were ground and suspended in water Carbon dioxide was passed through this suspension The carbon dioxide leaving the suspension was passed through a solution of 2,4-dinitrophenylhydrazine (2,4-DNP) Volatile carbonyl compounds present in the carbon dioxide stream were precipitated as 2,4-dinitrophenylhydrazones (2,4-DNPH's) as depicted in Figure 1 13 The carbon dioxide had first been treated with 2,4-dinitrophenylhydrazine to ensure that no carbonyl compounds present in the gas supply were introduced to the sample The roast barley suspension was purged with carbon dioxide in this fashion for 40 hours




**2,4-dinitrophenylhydrazine**




**Carbonyl compound** **2,4-dinitrophenylhydrazone**

**Figure 1 13.** 2,4-Dinitrophenylhydrazine and its reaction with carbonyl compounds

Figure 1 13 illustrates the reaction between carbonyl compounds and 2,4-DNP This derivatisation reaction facilitated the isolation of the carbonyl compounds present in

the carbon dioxide stream The 2,4-DNPH precipitate was filtered and the weight of the carbonyl compounds present was determined The 2,4-DNPH mixture was separated using TLC Separation required rechromatographing the mixture several times until a single band for each 2,4-DNPH was observed on the TLC plate A solution of petroleum ether with 15% v/v ethyl ether was used as the mobile phase The bands were then removed from the plate by removing the portion of the silica stationary phase containing the single band and extracting it from the silica with chloroform Chloroform was removed from the extract by evaporation under reduced pressure The components thus isolated were used for melting point determinations, spectrophotometric determinations and gas chromatographic analysis

The non-volatile carbonyl compounds were recovered from a filtrate of the stripped roast barley suspension by saturating it with ammonium sulphate and extracting with diethyl ether in a liquid/liquid extraction apparatus for 48 hours This extract was treated with the 2,4-DNP reagent and the resulting precipitate was carried through the same analysis steps as described for the volatile carbonyl compounds An 80% v/v benzene, 10% v/v petroleum ether and 10% v/v ethyl acetate solution was used as the mobile phase for TLC

No TLC volatiles were obtained from barley or green malt, but the other malts all contained three common bands and the roasted malt showed a fourth band Ultraviolet-visible (UV-VIS) and infra-red (IR) spectrophotometric analysis demonstrated that these components were in fact mixtures of aldehydes and ketones This was backed up by the gas chromatographic analysis with flame ionisation detection (FID) Gas chromatographic analysis was achieved by regenerating the aldehydes and ketones from the 2,4-DNPH's by heating them with α-ketoglutaric acid in a U-tube which was connected to the inlet valve of the GC in such a way that the carrier could be allowed through the tube before entering the chromatography column Comparisons were made between the chromatograms obtained from 2,4-DNPH derivatives of pure carbonyl compounds with those achieved experimentally

Also, by steam distillation of a roast barley extract, treatment with 2,4-DNP and regeneration of the parent compounds, chromatograms identical to those achieved from the original extract were obtained Compounds identified by gas chromatography were acetaldehyde, propionaldehyde, isobutyraldehyde, isovaleraldehyde, and acetone These were found in varying degrees across the range of malts Data is presented in the publication outlining total carbonyl compound

levels (volatile and non-volatile) over the same range of malts

Shimizu *et al*[25] [32] published a series of eight papers in the late 60's and early 70's on studies on the flavour of roasted barley Many Japanese drink a beverage particularly during the summer months called Mugi-Cha This beverage is prepared from ground roasted barley in a similar fashion as coffee is prepared from coffee beans

In the first of these studies[25] the flavour components of Mugi-Cha (MC) were fractionated using a complex acid/base liquid/liquid extraction technique This type of sample preparation technique was common at that time It involved a series of extraction steps as described in Figure 1 14 to give a sodium carbonate soluble fraction containing stronger organic acids, a sodium hydroxide soluble fraction containing weaker organic acids and a neutral fraction

The MC was extracted with 200 litres of a 1 1 methanol water solution for 24 hours at 50°C, the filtrate was concentrated, acidified with hydrochloric acid and extracted with diethyl ether by the use of a liquid/liquid extractor Acidic and neutral compounds present in the filtrate were carried into the organic layer

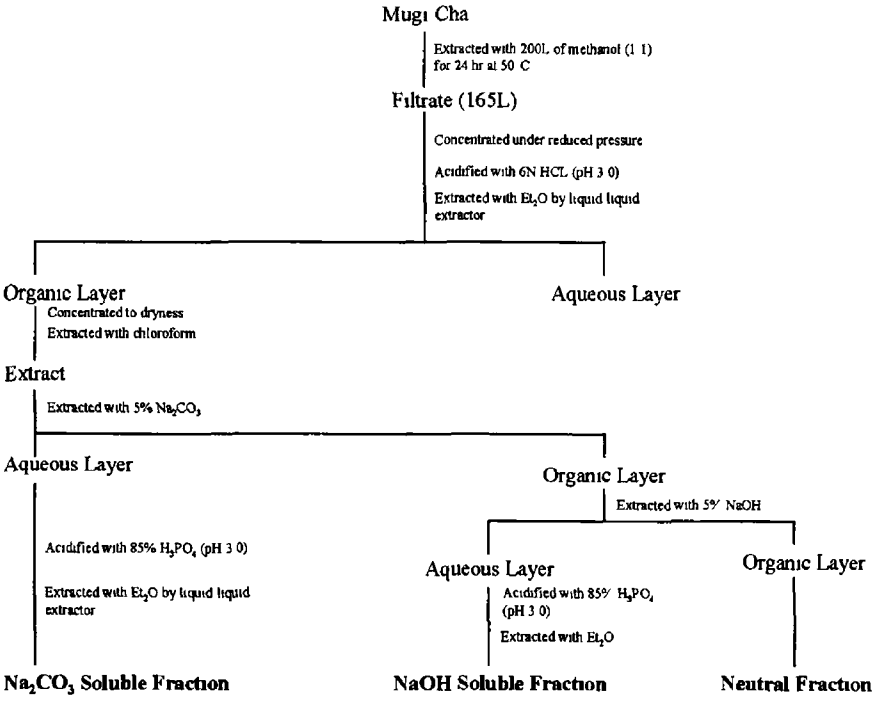


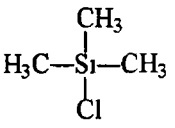

**Figure 1.14** Acid/base liquid extraction of roasted barley

Acidification of the aqueous layer resulted in the acidic species present being converted to non-ionic species which are more soluble in the organic solvent (diethyl ether) while the basic compounds were bound as ionic species in the aqueous layer The organic layer was concentrated to dryness to remove the ether and the residual material was dissolved in chloroform This chloroform solution was then extracted with a 5% w/v sodium carbonate solution and the stronger acidic compounds present were converted to their sodium salts which were more soluble in the aqueous layer The weaker organic acids and the neutral compounds remained in the organic layer The aqueous layer was acidified with 85% w/v phosphoric acid and extracted with diethyl ether by liquid/liquid extractor, to carry the stronger acidic compounds to the organic layer This gave the sodium carbonate soluble fraction

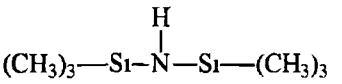
The chloroform layer was treated with a 5% w/v sodium hydroxide solution, and any remaining acidic material present was converted to its sodium salt and carried to the aqueous layer while the neutral compounds present remained in the organic layer The aqueous layer was acidified with phosphoric acid and extracted in the same fashion as the previous aqueous layer This gave the sodium hydroxide soluble fraction containing the weaker organic acids

The constituents of the sodium carbonate and sodium hydroxide fractions were subjected to paper chromatography, column chromatography, gas chromatography and infra-red spectroscopy Trimethylsilane derivatives of the paper and column chromatographic fractions were used for gas chromatographic analysis Silylation reagents are commonly used for GC because the resulting derivatives are generally more volatile, more stable and less polar than the parent compounds Silyl derivatives are formed by the replacement of an active hydrogen in the functional groups -OH, -NH, or -SH Two different silylating reagents, chlorotrimethylsilane and hexamethyldisilazane, were used here The structure for these compounds and the overall reaction is depicted in Figure 1 15 This derivatisation reaction involved reacting between 1 and 10 mg of sample dissolved in pyridine with a mixture of chlorotrimethylsilane and hexamethyldisilane in a 1 $cm^3$ glass tube The tube was sealed and heated on a microburner An aliquot of the supernatant of the TMS reaction mixture was injected onto the GC

Twenty nine components of isolated fractions were identified They are listed amongst the compounds in Table 1 5



**Chlorotrimethylsilane**



**Hexamethyldisilazane**

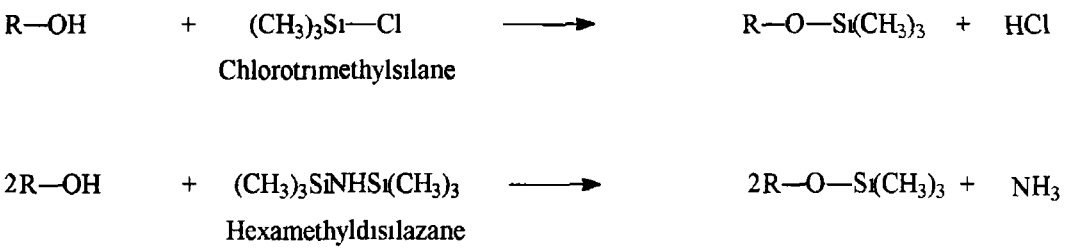



**Figure 1.15.** Derivatisation using silylating agents

The second paper[26] in the series investigated the changes in the composition of roasted barley during the roasting process Barley was roasted continuously from 25°C to 225°C and samples were taken at temperature intervals during the roasting These samples were subjected to a wide variety of analytical techniques which included thermal analysis, chemical analysis of an ether extract, reducing substances, total sugars, total nitrogen, water soluble nitrogen, amino nitrogen, ammoniacal nitrogen, total acid, volatile acid, pH, furfural content, carbonyl content and measurements of UV spectra of ether extracts From the viewpoint of the volatile flavour constituents of roasted barley only some of the data generated was useful The thermogravimetric analysis showed that the weight of the barley decreased with the rise in temperature Remarkable changes of composition were observed in the 150°C - 160°C temperature range, with the roast flavour beginning to appear at this point The pH of a roast barley water extract was more acidic than that of an unroasted barley water extract They suggested that the acidic and neutral fractions of roasted barley appeared to be the most important with regard to roast flavour The increase in the acidity of the barley while roasting was observed to coincide with increases in volatile acid, furfural, carbonyl compounds and total phenol content of the roasting barley No account was taken of the basic pyrazine content of roasted barley, which later publications show to contribute significantly to roast flavour

Volatile acids were assessed as acetic acid by titration of a water extract,

furfural was assessed by extraction with xylene followed by treatment with *para*-bromoaniline and a spectrophotometric measurement at 530 nm, while the total carbonyl content was assessed by treatment with 2,4-DNP and spectrophotometric measurement at 400 nm

The third paper[27] concerned the separation and identification of volatile mono-carbonyl compounds The volatile carbonyl components of MC were isolated by sweeping out the volatile compounds with $CO_2$ and trapping with 2,4-DNP as described earlier The resulting solution contained a mixture of 2,4-DNPH's which included mono-2,4-DNPH's and bis-2,4-DNPH's This solution was extracted using hexane by a liquid/liquid extractor The mono-2,4-DNPH's have less ionic character than the bis-2,4-DNPH's and were carried into the organic layer The hexane extract was concentrated under reduced pressure to give the mono extract The aqueous layer was concentrated to give the bis fraction The mono 2,4-DNPH's fraction was subjected to GC by direct injection and fourteen compounds were detected Of these, twelve compounds were isolated in crystalline form by using column and thin layer chromatography These compounds were identified by their IR spectra, UV-spectra and by the mixed melting points test The compounds identified are listed amongst those in Table 1 5

In the next publication[28] the separation and identification of mono-, di-, and oxo-carboxylic acids was investigated An acidic fraction of MC was prepared using acid/base liquid/liquid extraction This extract was subjected to gas chromatographic analysis Nine compounds were identified They were acetic, propionic, n-butyric, isobutyric, n-valeric, isovaleric, crotonic, n-caproic and n-heptanoic acids The extract was also treated with 2,4-DNP and subjected to further acid/base liquid/liquid extraction to isolate the 2,4-DNPH's of oxo-fatty acids To facilitate gas chromatographic analysis the oxo-acid 2,4-DNPH compounds were converted to their methyl esters by treating the extract with diazomethane Diazomethane is a methylating agent that forms methyl ethers with weakly acidic hydroxy groups such as carboxylic acids, phenols and the enol forms of β-diketones and β-ketoesters Alcohols are not acidic enough to protonate themselves and do not react with diazomethane spontaneously This derivatisation reaction renders the components more volatile and facilitates GC analysis Five oxo-carboxylic acids listed in Table

1 5 were identified by a combination of GC and thin layer chromatography IR spectra of TLC extracts of the 2,4-DNPH's confirmed the identification of glyoxalic, pyruvic, α-ketobutyric, α-ketovaleric and levulinic acids Succinnic and fumaric acids were isolated in the crystalline form and identified by IR spectroscopy

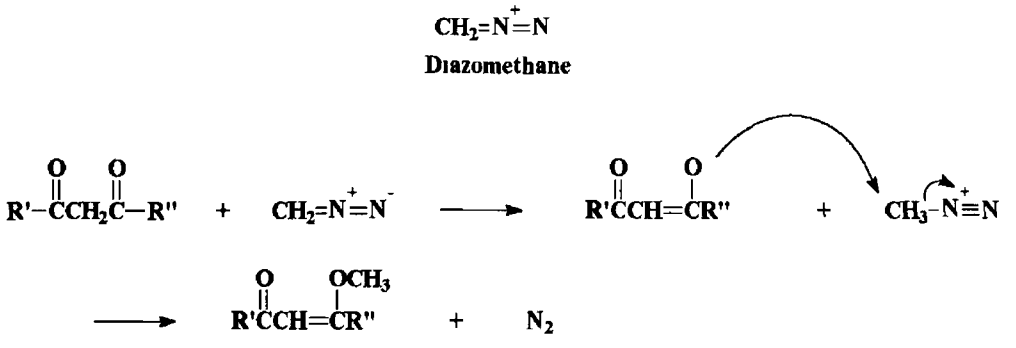



**Figure 1 16.** Derivatisation using diazomethane

Paper five[29] in the series concerned further analysis on a methanol extract of roasted barley Ground roasted barley was immersed in methanol and stirred The methanol extract was separated from the suspension by centrifugation The extract was passed through two ion exchange columns, firstly a cationic Amberlite CG-120 column which removed alkaline compounds from the extract and secondly through an anionic Amberlite CG-400 column which trapped the acidic compounds and allowed neutral compounds to elute This column was washed with a 1 1 methanol water solution to remove colour compounds from the column Finally the acidic compounds were eluted from the column with a 2 N hydrochloric acid solution and were then extracted into diethyl ether using a liquid/liquid extractor The concentrated extract was treated with 2,4-DNP and the 2,4-DNPH's were collected by filtration The filtrate was dissolved in ethyl acetate and extracted with 10% w/v sodium carbonate solution Thus the 2 4-DNPH's of oxo-fatty acids were rendered ionic and carried into the aqueous layer and were separated from the mixture The ethyl acetate fraction was evaporated under reduced pressure, to give a fraction containing weakly acidic 2,4-DNPH's A neutral fraction was prepared from the

methanol extract by passing it through the Amberlite CG-120 and CG-400 columns and reacting the eluent with 2,4-DNP

The 2,4-DNPH's were separated and isolated by column chromatography using silica-gel as stationary phase Stepwise elution was applied by using a series of the following solvents of increasing polarity n-hexane, benzene, ethyl acetate and ethanol Each fraction was further separated by preparative TLC until it showed a single band After development each component was extracted from the TLC stationary phase using ethyl formate and concentrated by evaporation using a rotary evaporator

Each 2,4-DNPH isolated was compared directly with an authentic compound with respect to melting point, the Rf value on TLC, their UV, IR and MS spectra Five compounds not previously known to be present in roasted malt were identified as their 2,4-DNPH's in the weakly acidic fraction by both column and thin-layer chromatography They were 2-pyrrolealdehyde, vanillin, *para*-hydroxybenzaldehyde, protocatechuic aldehyde and syringaldehyde Glyoxal and 5-hydroxymethylfurfural were also identified from the neutral carbonyl fraction

In paper six[30] the acidic fraction as described in the previous paper was subjected to fractionation by column chromatography The components of the prepared fractions containing hydroxyl functional groups were treated with trimethylchlorosilane and hexamethyldisilazane to convert the constituents to their trimethylsilane derivatives, (this derivatisation reaction was described earlier, Figure 1 15) The derivatised fractions were then subjected to gas chromatographic analysis using mass spectrometric detection The compounds present were identified by comparison of retention times and by addition of known standards

Each component corresponding to the main peak of each fraction analyzed by GC-MS as described was trapped using preparative GC The trapped TMS derivatives were hydrolysed to the parent compounds and trimethylsilanol The hydrolysate was extracted with diethyl ether and the extract was evaporated at room temperature under reduced pressure to remove trimethylsilanol The parent compounds were further purified by column chromatography, and by elution with a benzene-ethyl acetate mix The eluent was collected using a fraction collector and each fraction was examined by TLC The TLC bands corresponding to the main components were combined and evaporated This gave the purest possible sample

Identification was achieved by GC-MS, either by direct or indirect injection onto the MS, by IR spectra, melting point determination and NMR spectra The compounds identified from the weakly acidic carbonyl fraction were 5-hydroxy maltol, maltol, 5-methylcyclopent-2-en-2-ol-1-one, phenol, m-cresol, pyrocatechol and resorcinol

The neutral fraction prepared using the ionic exchange columns was studied in paper seven[31] The extract was fractionated using column, thin-layer and gas chromatography and 2,4-DNPH and TMS derivatives were prepared GC-MS and IR spectra were used to identify the components present Three new compounds were identified, furfuryl alcohol, acetylfuran and 3-hydroxypyridine

The acidic fraction prepared from the ionic exchange column described earlier was studied in paper eight[32] The same techniques were applied to this fraction as to the neutral fraction in paper seven Three acids were identified, palmitic, oleic and linoleic acids

Another group of Japanese workers, Wang *et al*[33] [36], also studied the flavour composition of roasted barley This work was carried out simultaneously with that of Shimizu *et al* Initially the production of flavour substances during the roasting of barley was studied[33] They observed that barley produced a favourable aroma when roasted at 160°C for twenty minutes It was also noted that the favourable aroma and browning of the barley occurred at the same time, and that the colour change was a good indicator of the degree of flavour development The levels of $CO_2$ produced during roasting were observed to increase dramatically with increasing temperature, particularly above 150°C Carbonyl compounds were observed by treating an extract of roasted barley with 2,4-DNP and separating them by TLC No specific compound was identified The presence of sulphur compounds was implied as a result of precipitate formation when the extract was treated with mercuric chloride This reaction is described in Figure 1 17

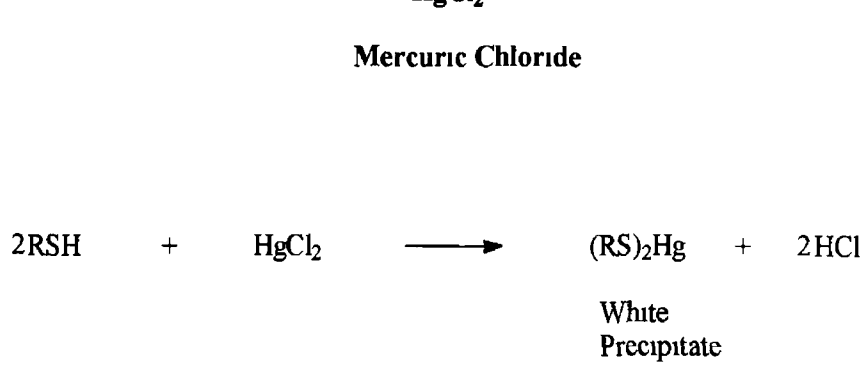



**Figure 1.17.** Precipitation of sulphur containing compounds with mercuric chloride

The authors[34] then carried out some further qualitative studies into the volatile carbonyl compounds present in roasted barley They (a) trapped the volatile carbonyl compounds present in ground roasted barley and (b) trapped the carbonyl compounds formed as barley was being roasted For (a) some ground roasted barley was suspended in water and a stream of nitrogen was bubbled through the suspension while it was maintained at 45°C The nitrogen stream leaving the suspension was passed through a series of tubes containing 2,4-DNP and as before the carbonyl compounds present were precipitated as their 2,4-DNPH's For (b) an air stream was passed through a laboratory scale electric roaster while barley was being roasted, and the air leaving the roaster was also treated with 2,4-DNP

The mixtures of 2,4 DNPH's were isolated by column chromatography and TLC The UV-VIS spectrum of each single TLC band was obtained as was the IR spectrum of a nujol mull Gas chromatographic analysis was achieved by regenerating the parent compounds The compounds successfully identified were furfural, 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, 2,3-pentanedione, ethylglyoxal and pyruvaldehyde

Wang *et al*[35] then turned their attention to the volatile basic components of roasted barley With the same roasting apparatus described in the previous paper, freshly ground raw barley powder was roasted As roasting proceeded the volatile flavour compounds being formed were swept out with air and were trapped as a condensate in an ice water cooled glass bottle followed by a U-tube cooled with dry-ice and ethanol A basic fraction of this condensate was prepared by acid/base liquid/liquid extraction Model studies were also carried out on pyruvaldehyde and

l-leucine and l-isoleucine to investigate possible flavour active products These compounds were mixed in the roasting apparatus and were roasted in the same fashion as the barley The volatiles produced were treated in the same manner as those collected from the roasted barley The concentrates were analyzed by GC using flame ionisation detection and mass spectroscopy To aid identification samples of pure compounds were also analyzed

Compounds positively identified from the roasting barley process were pyridine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2,3,5-trimethylpyrazine and a dimethylpyrazine The formation of 2,5-dimethylpyrazine was demonstrated from the pyruvaldehyde-amino acid model system

Using the same configuration described in the previous paper, the air stream from roasting barley was passed through a solution of mercuric chloride[36] This solution trapped sulphur compounds present, as described earlier The trapped sulphur compounds were regenerated by treating with hydrochloric acid and the resulting vapours were introduced onto a gas chromatographic column via a gas-tight syringe

A volatile acidic fraction was prepared from the condensate prepared as in the previous paper The components of this condensate were converted to their methyl esters by treating the ether extract with diazomethane, (Figure 1 16)

A volatile non-carbonyl fraction was also prepared from the condensate The basic, acidic, and phenolic fractions were eliminated from the ether extract by treatments with hydrochloric acid, sodium hydrogen carbonate and sodium hydroxide The carbonyl compounds present in the neutral fraction were removed by treatment with Girard T reagent

The Girard T reagent is trimethylaminoacetohydrazine chloride It combines with carbonyl compounds to form hydrazone derivatives generating a polar group that renders the substances soluble in water The carbonyl compounds were removed in the aqueous layer

The non-carbonyl fraction was separated into oxygenated and hydrocarbon fractions by column chromatography with silicic acid as stationary phase and elution with pentane and methanol The oxygenated fraction was then subjected to gas chromatographic analysis

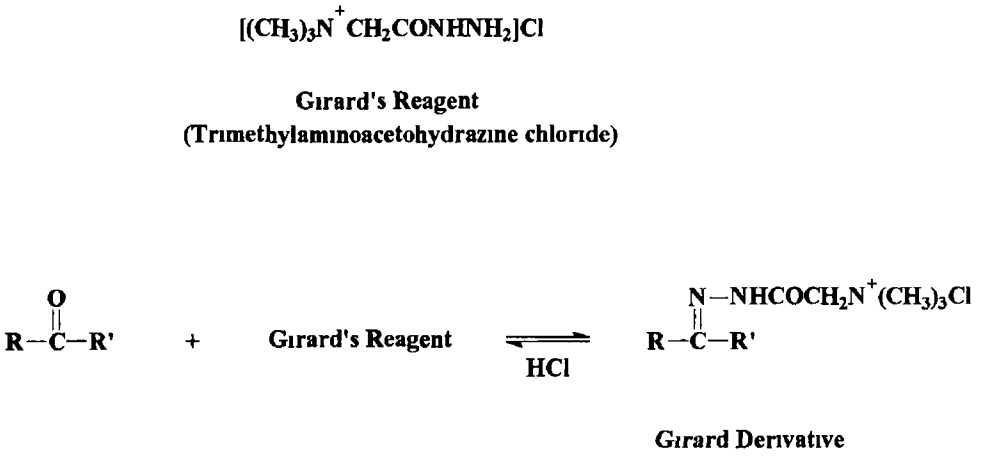



**Figure 1.18.** Derivatisation with the Girard T reagent

GC was carried out using a variety of different stationary phases The only sulphur compound to be identified was dimethyl disulphide A series of fatty acids was identified in the acidic fraction as their methyl esters They were acetic, propionic, butyric, isovaleric, valeric, isocaproic and caproic acid A series of alcohols and ketones were identified as part of the neutral non-carbonyl oxygenated fraction They were ethanol, propanol, butanol, isobutanol, pentanol, hexanol, furfuryl alcohol, γ-octalactone, γ-nonalactone and γ-decalactone

Barwald *et al*[37] studied the volatile constituents of barley and malt using headspace gas chromatography with flame ionisation detection (FID), electron capture detection (ECD) and flame phototometric detection (FPD) Samples were ground and extracted in an extraction column at 60°C with a stream of nitrogen flowing through the column The nitrogen stream was then passed through a series of cooling traps set at -79°C and -185°C, thus any volatile material present was trapped The volatile constituents of these condensates were introduced onto a gas chromatographic column via the static headspace injection technique Using the different detection systems described above the following compounds were identified acetaldehyde, propionaldehyde, isobutyraldehyde, butyraldehyde, valeraldehyde, acetone, diacetyl, ethanol, hydrogen sulphide, methyl mercaptan, ethyl mercaptan, carbon disulphide, dimethyl sulphide, diethyl sulphide and dimethyldisulphide

Collins[38] prepared an extract of roasted barley by steam distillation and also

isolated a basic fraction of the distillate Both were analyzed by GC using FID and ECD detection systems Roast barley was extracted by a system modelling the mashing process used in brewing The extract was prepared by filtration of this mash This extract was then distilled and the distillate was collected A basic fraction of the distillate was prepared by acid/base liquid/liquid extraction and analyzed by gas chromatography Ten compounds which consisted of nine pyrazines and pyridine were identified in this basic fraction

Both the steam distillate and the basic fraction prepared were subjected to headspace gas chromatography The headspace analysis showed forty peaks Four classification reagents were used to treat the extract and the distillate in conjunction with headspace analysis to help confirm identities of the headspace volatiles The classification reagents were hydroxylamine hydrochloride, dimedon (5,5-dimethyl-1,3-cyclohexanedione), bromine and borohydride Hydroxylamine hydrochloride distinguished compounds with a carbonyl group by reacting with the carbonyl group rendering the compound involatile as its oxime Dimedon distinguished aldehydes from simple ketones, as it gives insoluble condensation products with aldehydes but not ketones Bromine was useful for detecting unsaturated linkages Borohydride is a reagent for selective reduction of aldehydes and ketones to alcohols, i e carbonyl compounds were converted to the corresponding alcohols Fourteen compounds were identified in the headspace and ten were identified in the basic fraction The compounds identified are listed amongst those in Table 1 5

Akima *et al*[39] studied the volatile carbonyl compounds in barley and a range of malts A headspace technique similar to the one described by Collins was used Samples were simply ground and suspended in water at 40°C in a sealed container An aliquot was removed from the headspace and injected directly onto the GC Dual FID/ECD was the detection system used No volatile components were found in barley while the principle components found across the range of malts were acetaldehyde, propionaldehyde, isobutyraldehyde, butyraldehyde, isovaleraldehyde and furfural Two diketones were also identified, diacetyl and 2,3-pentanedione Generally speaking they found that dark malts, with the exception of roasted malts, contained more aldehydes than light malts

Wagner[40] also investigated the volatile carbonyl compounds in barley and

malts using similar analytical systems as those described by Arkima His results contradict those of Akima as he found that barley and green malt contain volatile carbonyl compounds Collins also came to this conclusion The compounds he found m barley, green malt and kilned malt were acetaldehyde, propionaldehyde, isobutyraldehyde, acetone, butyraldehyde, isovaleraldehyde, methylethylketone, valeraldehyde and capronaldehyde

Kavanagh *et al* [41] conducted a study into the pyrazine content of a green malt, a lightly kilned malt (75°C) and a heavier kilned malt (120°C) The green malt was processed for analysis by a reduced pressure steam distillation of a ground sample suspended in water followed by an extraction into methylene chloride A basic fraction of this extract was then prepared The malts kilned at 75°C and 120°C were prepared for analysis by extracting a sample of each with methylene chloride The methylene chloride extract of each malt was then separated into acid, base and neutral fractions The fractions were analyzed using GC-MS and the compounds found are listed in Table 1 5

To quote the authors "little or no pyrazine material was found in the green malt" Pyrazines were found in the kilned malts, with the same compounds found in both but in greater quantities in the higher temperature kilned malt This is evidence to suggest that formation of pyrazines occurs primarily during kilning

The authors also conducted a study into the α-amino-mtrogen and reducing sugar levels as samples of malt were roasted One sample was roasted to a maximum of 70°C and the other to a maximum 120°C In both cases an initial increase occurred in α-amino-nitrogen and sugar levels In the latter stages of the 120°C roasted sample marked decreases were also observed

Tressl *et al* [42] examined the thermal decomposition products of phenolic acids present in roasted barley Roller milled barley was taken into a reaction tube and mixed with an equal volume of sand The mixture was heated at 200°C for 2 hours in a current of nitrogen The nitrogen stream exiting the reaction tube was passed through three receivers, one air cooled receiver, and two cooled with an acetone dry-ice mixture Samples of standards of the phenolic acids were treated in the same fashion but with ether in the cold traps A basic fraction of the aqueous roasted barley condensate was prepared The extracts were concentrated on a kieselguhr

column and the concentrates were divided into eight fractions of increasing polarity by absorbtion chromatography on kieselgel Further separation was achieved using preparative-GC and final examination was by GC and GC-MS Seventeen compounds were identified in roasted barley and are listed amongst others in Table 1 5

Tressl and the same group of co-workers[43] also conducted an investigation into the nitrogen containing aroma compounds of malt, worts and beer Dark malts were suspended in water at 50 - 60°C and were homogenised for 5 minutes An extract was centrifuged out of this suspension A basic fraction was prepared by acid/base liquid/liquid extraction into a pentane ether mixture which was concentrated on a kieselguhr column This extract was then fractionated by column chromatography and each fraction was concentrated on kieselguhr Preparative-GC and GC-MS were used for purposes of compound identification Twenty pyrazine compounds, thirteen pyrroles and two thiazols were identified in dark malts (Table 1 5)

Sakamura *et al*[44] conducted a study into the bitter diketopiperazines in roasted malts A chloroform extract of ground roasted barley was prepared and further fractionation using TLC was carried out Five fractions were isolated and analyzed by IR, MS and GC-MS An amino acid analyzer was also used to aid identification Model reactions were also carried out between leucine, isoleucine, valine and proline to aid identification by the aforementioned analytical techniques and to assess the bitterness of the diketopiperazines Five diketopiperazines were identified and are listed in Table 1 5

Harding and Wren[45] generated an aqueous extract of roasted barley by heating it in water (similar to mash conditions) and filtering to obtain an extract A basic fraction of this extract in diethyl ether was obtained by liquid/liquid extraction This extract was analyzed by GC and GC-MS Identifications were achieved by the use of reference MS spectra Twenty-three basic heterocyclic compounds were identified, and are listed in Table 1 5

Von Kossa[46], a co-worker with Tressl on an earlier paper, and Bahri conducted further studies into the aroma substances of malts In particular the lipid

oxidation products of lightly kilned malts were investigated Using the same sample preparation steps as described in their earlier paper, thirty-two lipid oxidation products were identified (Table 1 5) The paper also reviewed the formation of phenols in wort and beer, and the formation of furans, pyrroles, pyrazines in malt Possible mechanisms of formation were suggested

Farley and Nursten[47] examined green and kilned malt for their volatile constituents using two different sample preparation techniques The first technique involved preparing an extract from a commercially available malt extract using the Likens-Nickerson[56] concurrent steam distillation extraction apparatus This apparatus is a special distillation unit which allows simultaneous condensation of a steam distillate and an immiscible extraction solvent The authors developed this apparatus for the detection of hop oil constituents in brewing products, but the technique is useful for most steam distillates Solutes occurring at parts-per-billion level were demonstrated by the authors to have been concentrated up to 32,000 times in a single operation Pentane was the solvent used This pentane extract was concentrated using a fractionating column The extract was then injected onto a GC For their second technique the same commercially available malt extract was purged at 40°C using a stream of nitrogen The nitrogen stream was then passed through activated charcoal traps Volatile compounds escaping from the malt extract in the nitrogen stream were trapped on the charcoal Vapours from the charcoal traps were introduced onto the gas chromatograph by cold trapping and injecting as follows A kieselguhr trap was immersed in solid carbon dioxide, and was connected to the analytical column of the GC via the heated injection port The front end of the kieselguhr trap was connected via PTFE tubing to the charcoal trap The charcoal trap was heated and the vapours generated were passed via a nitrogen stream onto the cold kieselguhr trap where they condensed This cold trap was then rapidly heated with a microburner as a result of which the vapours were regenerated The vapours were then swept on to the analytical column

Both the pentane extract and the vapours collected on the charcoal trap were analyzed by GC with MS, sulphur specific and nitrogen specific detection Sensory evaluation of the components as they eluted from capillary GC column was carried out by use of a sniffer port Thirty three compounds were identified by GC-MS, fourteen of these by trapping and reinjection using preparative-GC, and a further six

by use of charcoal traps The use of charcoal traps avoided the problem of large solvent peaks, therefore peaks usually unseen due to interference from solvent peaks were visible and identifiable The compounds identified are listed in Table 1 5

Tressl and another group of co-workers[48] carried out some model studies by reacting ammo acids with reducing sugars Products formed from these model reactions were prepared and analyzed in the same fashion as described by Van Kossa Protein/maltose and cysteine/methyl glyoxal were the ammo acid/reducing sugar systems of main interest here Comparisons were drawn between the reaction products of the model reactions and the compounds found in roasted barley Novel compounds characterised here included 6-acetyl-1,2,3,4-tetrahydropyridine and 6-acetyl-2,3,4,5-tetrahydropyridine which were isolated both from model reactions and roasted barley

Tressl, Helak and Rewicki[49] followed this work with a paper describing the elucidation of the structure of a new previously unknown nitrogen containing compound This compound was seen in both the reaction products of the proline/maltose model system and the extract derived from roast barley Samples from roast barley were worked up to allow analysis by GC, GC-MS, NMR and IR spectroscopy The compound was demonstrated to be 8-oxo-1,2,3,3a,5,6,7,8-octahydro-cyclopenta[d]pyrrolo[2,1-b]-[1,3]oxazine They gave this compound the name "malzoxazine" and claimed it as the first naturally occurring 2,3-dihydro-4H-1,3-oxazine

Narziss, Meidaner and Koch[50, 51] published two interesting papers concerning research they carried out into the volatile substances of malting and brewing In the first of these publications they investigated the nitrogen containing compounds of a series of malts, worts and beers The malts analyzed are of interest here Pale, dark, caramel and roasted malts were assessed Samples for analysis were prepared by vacuum distillation of cold water mashes The weakly basic nitrogenous heterocycles were isolated by acid/base liquid/liquid extraction followed by their separation on a cationic ion-exchanger, and elution with hydrochloric acid The eluate was made alkaline and extracted with pentane/diethyl ether The nitrogenous heterocycles were identified by GC-MS The compounds identified are detailed in Table 1 5

In their second publication Narziss *et al* [51] considered the effects of malting and mashing parameters on the nitrogen heterocyclic content of malts, worts and beers With relation to malts they studied the effects of (i) germination time, (ii) germination moisture levels, (iii) germination temperature, (iv) drying temperature and kilning temperature The analytical techniques used to assess the level of nitrogen heterocyclic compound levels during the course of these studies were those described in the earlier publication Results were presented in the publication as total nitrogen heterocyclic compound content for each of the studies undertaken

Wittman and Eichner[52] described an analytical technique for the quantitative determination of deoxyfructosazines in malted barley These deoxyfructosazines are Amadori compounds (intermediates in the Maillard reaction) The Amadori compounds were extracted from malt by homogenising milled malt in a 70% v/v ethanol water solution, centrifuging the solution and decanting the supernatant The supernatant was then concentrated on a rotary evaporator, and the Amadori compounds, amino acids and other nitrogen compounds were bound using a strongly acid cation exchanger These compounds were then eluted with trichloroacetic acid The Amadori compounds present were derivatised to their respective oximes by treatment with hydroxylamine as depicted in Figure 1 19 This reaction was described earlier as a classification test

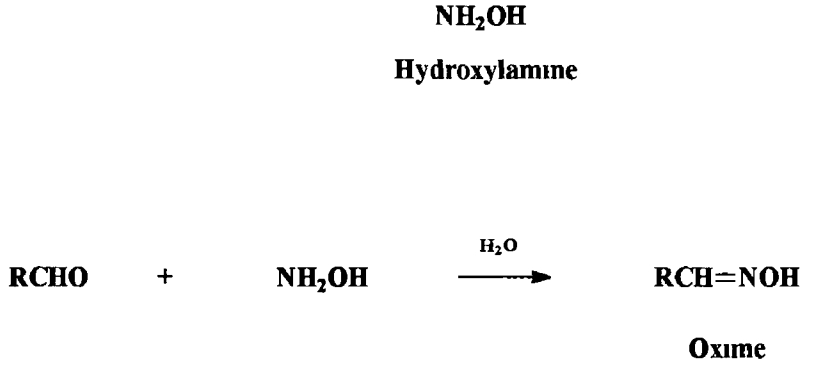



**Figure 1.19** Derivatisation by conversion to an oxime

This reaction is a condensation reaction, where hydroxylamine reacts with compounds containing a carbonyl group The net result is that water is eliminated from the two molecules and an unsaturated nitrogen containing derivative is formed

The products of this reaction are called oximes These oximes were then silylated by a procedure described earlier and these silylated derivatives were identified and quantified by capillary GC Eleven fructosazines were identified and are listed in Table 1 5 Amino acids were analyzed using an amino acid analyzer

Beal and Mottram[53] published a paper entitled "Aroma Characteristics of Malted Barley by Free-Choice Profiling" Free choice profiling is a sensory technique chosen by the authors to evaluate the changing sensory characteristics of malt during kilning Malted barley from a production run of crystal malt was sampled at five minute intervals during the final thirty minutes of roasting Samples were prepared for GC and GC-MS analysis by two different sample preparation techniques For the first technique a volatile extract was prepared for each malt sample using the Lickens-Nickerson apparatus The extract was then concentrated on a Kuderna-Danish apparatus A series of dilutions were prepared from this concentrated extract The extracts were assessed by GC-MS and GC coupled to a sniffer port The column eluent was evaluated by nose Correlations were then drawn between the known peaks and their aroma The second technique was a dynamic headspace technique The malt was milled in a domestic coffee mill Oxygen-free nitrogen was passed through the sample for one hour and the volatiles were swept onto a trap packed with TenaxGC (a porous polymeric adsorbent) After collection the volatiles were thermally desorbed from the TenaxGC directly onto the front of an analytical GC column and were analyzed by GC-MS A sniffer port was also used here to correlate known peaks and their aromas Results were presented for 2-, and 3-methylbutanal profiles during the course of roasting

Table 1 5 consists of a list of the twenty-three classes of volatile flavour active compounds found in barley, malt and roasted barley, as a result of the research described above These twenty-three classes contain two hundred and seventy-seven different compounds

It is interesting to note the evolution of sample preparation methods and analytical techniques over the course of this literature review The early sample preparation work consisted of labour intensive, time consuming and sometimes expensive sample extraction, often followed by further acid/base liquid/liquid

extraction Derivatisation reactions were also incorporated to isolate compounds of interest based on functional group The analytical techniques utilised were non-specific, non-selective techniques such as TLC, column chromatography, UV and IR spectroscopy Some gas chromatography with packed columns and flame ionisation detection was also utilised in this early research

As technology improved and new analytical techniques and equipment came on stream the approach to the analysis of the volatile flavour constituents altered accordingly Sample preparation methods have moved away from the extraction techniques described above Purge and trap techniques have come to the fore for flavour research in recent times This allows rapid isolation of volatile components of solid or liquid samples on a porous polymer These volatile materials are then introduced to a gas chromatograph via a thermal desorption unit The combination of sophisticated electronics/computer based gas chromatographic instruments using capillary column technology have dramatically improved the flavour chemists ability to detect and quantify the volatile flavour constituents of many species The advent of selective detection systems, electron capture, nitrogen detector, sulphur chemiluminescence detector and MS detection have also had a major impact

The most modern analytical systems for volatile flavour analysis would incorporate a purge and trap system for sample preparation, a thermal desorption unit to introduce the sample to a gas chromatograph, a gc with capillary column technology, and finally a range of detection systems as described above The gc capillary column system would be configured to allow heart cutting i e have the capability for column switching

**Table 1.5.** Volatile Compounds found in Barley, Malt and Roasted Barley

| COMPOUNDS | REFERENCES |
|---|---|
| *1 ACIDS* | |
| Benzoic | 25 |
| *meta*-Toluic | 25 |
| *para*-Toluic | 25 |
| Salicylic | 25 |
| Cinnamic | 25 |
| *meta*-Hydroxybenzoic | 25 |
| *para*-Hydroxybenzoic | 25 |
| Gentisic | 25 |
| Vanillic | 25 |
| Protocatechuic | 25 |
| Syringic | 25 |
| *para*-Coumaric | 25 |
| Ferulic | 25 |
| Caffeic | 25 |
| 2-Furoic | 25 |
| Acetic | 28, 36 |
| Propionic | 28, 36 |
| Butyric | 28, 36 |
| *Iso*-butyric | 28 |
| Valeric | 28, 36 |
| *Iso*-valeric | 28, 36, 47 |
| Crotonic | 28 |
| Caproic | 28, 36 |
| Heptanoic | 28 |
| Succinic | 28 |
| Fumaric | 28 |
| Glyoxalic | 28 |
| Pyruvic | 28 |

| COMPOUNDS | REFERENCES |
|---|---|
| α-Ketobutyric | 28 |
| α-Ketovaleric | 28 |
| Levulinic | 28 |
| Palmitic | 32 |
| Oleic | 32 |
| Linoleic | 32 |
| *Iso*-caproic | 36 |

## *2 ALCOHOLS*

| | |
|---|---|
| Ethanol | 36, 37, 38, 47 |
| Propanol | 36 |
| Butanol | 36 |
| *Iso*-butanol | 36 |
| Pentanol | 36, 38, 46, 47 |
| Hexanol | 36 |
| Phenethanol | 45, 47 |
| 1-Penten-3-ol | 46 |
| 2-Penten-1-ol | 46 |
| Hexanol | 46 |
| 3-Hexen-1-ol | 46 |
| 2-Hexen-1-ol | 46 |
| Heptanol | 46 |
| 2-Hepten-1-ol | 46 |
| 1-Octen-3-ol | 46 |
| Octanol | 46 |
| 2-Octen-1-ol | 46 |
| Nonanol | 46 |
| 2-Nonen-1-ol | 46 |
| 2,6-Nonadien-1-ol | 46 |
| 2-Methylpropanol | 47 |
| 2-Butanol | 47 |

| COMPOUNDS | REFERENCES |
|---|---|
| 3-Methylbutanol | 47 |
| 2-Pentanol | 47 |
| Benzyl alcohol | 47 |
| ***3 ALDEHYDES*** | |
| Acetaldehyde | 24, 27, 37, 38, 39, 40, 47 |
| Propionaldehyde | 24, 37, 38, 39, 40, |
| *Iso*-butyraldehyde | 24, 27, 34, 37, 38, 39, 40, 47 |
| *Iso*-valeraldehyde | 24, 34, 38, 39, 40, 47 |
| Formaldehyde | 27 |
| 2-Methylbutanal | 27, 34, 38, 47 |
| 2-Pyrrolealdehyde | 29 |
| *para*-Hydroxybenzaldehyde | 29 |
| Syringaldehyde | 29 |
| Protocatechuic aldehyde | 29 |
| Glyoxal | 29 |
| Ethylglyoxal | 34 |
| Pyruvaldehyde | 34 |
| Butyraldehyde | 37, 39, 40 |
| Valeraldehyde | 37, 38, 39, 40, 46 |
| Hexanal | 38, 40, 46, 47 |
| 2-Hexanal | 46 |
| 2-Heptenal | 46 |
| 2,4-Hexadienal | 46 |
| 2-Octenal | 46 |
| Nonanal | 46 |
| *trans*-2-Nonenal | 46 |
| 2 4-Heptadienal | 46 |
| 2,4-Nonadienal | 46 |
| 2,6-Nonadienal | 46 |
| 2,4-Decadienal | 46 |

| COMPOUNDS | REFERENCES |
|---|---|
| 2,4,7-Decatrienal | 46 |
| 2,4-Undecadienal | 46 |
| 3-Methylpentanal | 47 |
| Benzaldehyde | 47 |
| Phenylacetaldehyde | 47 |
| 2-Butenal | 47 |
| 2-Methylbutenal | 47 |
| 2-Phenyl-2-butenal | 47 |
| 5-Methyl-2-phenyl-2-hexenal | 47 |
| ***4 AROMATIC ETHERS*** | |
| Anisole | 25 |
| Phenetole | 25 |
| ***5 AROMATIC HYDROCARBON*** | |
| Toluene | 47 |
| ***6 DIKETOPIPERAZINES*** | |
| Cyclo(L-Phe-L-Pro) | 44 |
| Cyclo(L-Leu-L-Pro) | 44 |
| Cyclo(L-Pro-L-Pro) | 44 |
| Cyclo(L-Val-L-Pro) | 44 |
| Cyclo(L-ILe-L-Pro) | 44 |
| ***7 ESTERS*** | |
| *Iso*-amyl acetate | 47 |
| Diethyl phthalate | 47 |
| Ethyl nicotinate | 50, 51 |

| COMPOUNDS | REFERENCES |
|---|---|
| ***8 FRUCTOSAZINES*** | |
| Fru-Ala | 52 |
| Fru-Gly | 52 |
| Fru-Val | 52 |
| Fru-Leu | 52 |
| Fru-Ile | 52 |
| Fru-Ser | 52 |
| Fru-Thr | 52 |
| Fru-Asp | 52 |
| Fru-Pyr | 52 |
| Fru-Glu | 52 |
| Fru-Phe | 52 |
| ***9 FURANS*** | |
| Maltol | 25, 30 |
| ısoMaltol | 25 |
| 5-Methylfurfural | 27,42, 45, 46, 47 |
| 2-Furfural | 27, 34, 38, 39, 42, 45, 46, 47 |
| 5-Hydroxymethylfurfural | 29 |
| 5-Hydroxymaltol | 30 |
| Acetylfuran | 31, 42, 45, 46, 47 |
| Furfuryl alcohol | 31, 36, 45, 46, 47 |
| Dıhydro-5-methyl-2(3H)-furanone | 45 |
| 3-Furfural | 46 |
| 2-Pentylfuran | 46, 47 |
| 3-Phenylfuran | 46, 47 |
| 5-Methyl-2-vınylfuran | 46 |
| Propıonalfuran | 46, 47 |
| 1-(2'-Furyl)-propan-1,2-dıone | 46 |
| 2,2'-Dıfuryl | 46 |

| COMPOUNDS | REFERENCES |
|---|---|
| 2,2'-Difurylmethane | 46 |
| 5-Methyl-2-furyl-2'-furylmethane | 46 |
| Bis-(5-methyl-2-furyl)propan-1,2-dione | 46 |
| Furfurylmethylsulphide | 46 |
| Furfurylmethyldisulphide | 46 |
| 1-Furfurylpyrrole | 46 |
| 2-Methylfuran | 47 |
| 2-Ethylfuran | 47 |
| 2,5-Dimethylfuran | 47 |
| 2-Butylfuran | 47 |
| 2-Methyl-5-acetylfuran | 47 |
| 2-Vinylfuran | 47 |
| 2-Methyl-5-vinylfuran | 47 |
| 2-Methyltetrahydro-3-furanone | 47 |
| 2,5-Dimethyltetrahydrofuran | 47 |

## *10 KETONES*

| | |
|---|---|
| Acetone | 24, 27, 37, 38, 40, 47 |
| 2-Butanone | 27, 38, 40, 47 |
| 3-Pentanone | 27 |
| 2-Methyl-3-pentanone | 27 |
| 5-Methylcyclopent-2-en-2-ol-1-one | 30 |
| 2,3-Pentanedione | 34, 38, 39, 47 |
| 2,3-Butanedione (diacetyl) | 37, 38, 39, 47 |
| 2-Cyclopentenone | 42 |
| 2,5-Hexanedione | 45 |
| 2-Heptanone | 46 |
| 2-Octanone | 46 |
| 3,5-Octadienone | 46 |
| 2-Pentanone | 47 |
| 1-Phenyl-2-propanone | 47 |

| COMPOUNDS | REFERENCES |
|---|---|
| Damascenone | 47 |
| ***11 LACTONES*** | |
| γ-Octalactone | 36 |
| γ-Nonalactone | 36 |
| γ-Decalactone | 36 |
| 2,5-Dimethyl-2-cyclopenten-1-one | 42 |
| 4-Butanolide | 42 |
| 2-Buten-4-olide | 42 |
| 3-Methyl-2-butene-4-olide | 42 |
| 2,3-Dimethyl-2-butene-4-olide | 42 |
| 2-Butene-4-olide | 42 |
| ***12 OXAZINES*** | |
| Malzoxazine | 46, 48, 49, 50, 51 |
| ***13 OXAZOLES*** | |
| Trimethyloxazole | 50, 51 |
| 4,5-Dimethyloxazole | 50 |
| 5-Hexyl-2,4-dimethylethyloxazole | 50 |
| Benzoxazole | 50, 51 |
| 2-Methylbenzoxazole | 50, 51 |
| 2-Phenyloxazole | 50, 51 |
| ***14 PHENOLS*** | |
| Guaiacol | 25, 42 |
| Catechol | 25 |
| Resorcinol | 25, 30 |

| COMPOUNDS | REFERENCES |
|---|---|
| 2,6-Dimethoxyphenol | 25 |
| *ortho*-Hydroxybenzyl alcohol | 25 |
| 4-Ethylguaiacol | 25 |
| Phenol | 25, 30, 42 |
| 4-Methylguaiacol | 25 |
| Vanillin | 25 |
| *meta*-Cresol | 25, 30 |
| Pyrocatecol | 30 |
| *para*-Cresol | 42 |
| 4-Ethylphenol | 42 |
| 4-Vinylguaiacol | 42, 47 |
| 4-Vinylphenol | 42 |

## *15 PYRAZINES*

| | |
|---|---|
| 2-Methylpyrazine | 35, 38, 41, 43, 45, 50, 51 |
| 2,5-Dimethylpyrazine | 35, 38, 41, 43, 45, 46, 50, 51 |
| 2-Ethyl-5-methylpyrazine | 35, 38, 41, 43, 45, 50 |
| 2,3,5-Trimethylpyrazine | 35, 38, 41, 43, 45 |
| Diethylmethylpyrazine | 35 |
| Pyrazine | 38, 45, 50, 51 |
| 2,3-Dimethylpyrazine | 38, 41, 43, 45, 46, 50, 51 |
| 2-Ethyl-3-methylpyrazine | 38, 46, 51 |
| 2,5-Dimethyl-3-ethylpyrazine | 38, 41, 43, 45, 46, 50, 51 |
| 2,6-Dimethylpyrazine | 41, 43, 45, 46, 50, 51 |
| 2-Ethyl-3,5-dimethylpyrazine | 38, 41, 43, 46, 50, 51 |
| 2-Ethyl-6-methylpyrazine | 43, 45, 46, 50 |
| 2-Ethyl-3-methylpyrazine | 43, 50 |
| 2-Ethyl-5,6-dimethylpyrazine | 43, 45, 46 |
| 6,7-Dihydro-5H-cyclopentapyrazine | 43, 45, 46 |
| 2-Methyl-6,7-dihydro-5H-cyclopentapyrazine | 43, 45, 46 |
| 5-Methyl-6,7-dihydro-5H-cyclopentapyrazine | 43, 45, 46, 50, 51 |

| COMPOUNDS | REFERENCES |
|---|---|
| Tetramethylpyrazine | 43, 46, 50, 51 |
| 5-Methylcyclopentapyrazine | 43, 46 |
| 2-Furylpyrazine | 43, 46 |
| 2-(2'-Furyl)-methylpyrazine | 43, 46 |
| 2-(2'-Furyl)-dimethylpyrazine | 43, 46 |
| Acetylpyrazine | 45 |
| Ethylpyrazine | 46, 50, 51 |
| 2-Methoxypyrazine | 50, 51 |
| 2-Propylpyrazine | 50 |
| 2-Pentylpyrazine | 50 |
| 2-Betylpyrazine | 50 |
| 3-Acetylpyrazine | 50, 51 |
| 2,5-Dimethyl-3-(3-methylbutyl)-5-methyl-5-propenylpyrazine | 50 |
| 2-Vinylpyrazine | 50, 51 |
| 2,3-Diethylpyrazine | 50, 51 |
| 2,6-Diethylpyrazine | 50, 51 |
| 3-Isobutyl-2,5-dimethylpyrazine | 50 |
| 2,3-Dimethyl-5-(2-methylpropyl)-iso propenylpyrazine | 50 |

## *16 PYRAZOLES*

| | |
|---|---|
| Pyrazole | 50, 51 |

## *17 PYRIDINES*

| | |
|---|---|
| 3-Hydroxypyridine | 31 |
| Pyridine | 35, 38, 45, 50, 51 |
| 2-Methylpyridine | 45, 50, 51 |
| 2-Acetylpyridine | 45, 50, 51 |
| 6-Acetyl-1,2,3,4-tetrahydropyridine | 48 |

| COMPOUNDS | REFERENCES |
|---|---|
| 6-Acetyl-2,3,4,5-tetrahydropyridine | 48 |
| 2,6-Dimethylpyridine | 50, 51 |
| 3-Methylpyridine | 50, 51 |
| 2-Ethylpyridine | 50 |

### *18 PYRROLES*

| | |
|---|---|
| Pyrrole | 43, 46, 50, 51 |
| 2-Methylpyrrole | 43, 46, 50 |
| 2-Formylpyrrole | 43, 46, 50 |
| 2-Acetyl-5-methylpyrrole | 43, 46 |
| 2-Formyl-5-methylpyrrole | 43, 46 |
| 1-Acetylpyrrole | 43, 46 |
| 1-Furfurylpyrrole | 43, 46 |
| 1-Methyl-2-formylpyrrole | 43, 46 |
| 1-Ethyl-2-formylpyrrole | 43 ,46 |
| Indole | 43, 46, 50, 51 |
| 2-Acetylpyrrole | 46, 47, 50, 51 |

### *19 PYRROLIDINES*

| | |
|---|---|
| n-Acetylpyrrolidine | 50, 51 |

### *20 PYRROLIDONES*

| | |
|---|---|
| 2-Pyrrolidone | 43, 46 |
| 1-Methyl-2-pyrrolidone | 43, 46, 51 |

### *21 PYRROLIZINES*

| | |
|---|---|
| 5-Acetyl-2,3-dihydro-1H-pyrrolizine | 50, 51 |

| COMPOUNDS | REFERENCES |
|---|---|
| 5-Formyl-6-methyl-2,3-dihydro-1H-pyrrolizine | 50, 51 |
| 5-Acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine | 50, 51 |

## *22 ALIPHATIC SULPHUR COMPOUNDS*

| | |
|---|---|
| Hydrogen sulphide | 33, 37, 47 |
| Methyl mercaptan | 37 |
| Ethyl mercaptan | 37 |
| Carbon disulphide | 37 |
| Dimethyl sulphide | 37, 47 |
| Diethyl sulphide | 37 |
| Dimethyl disulphide | 36, 37, 47 |
| Methional | 47 |
| Dimethyl sulphoxide | 47 |
| Methyl methanethiosulphonate | 47 |

## *23 THIAZOLES*

| | |
|---|---|
| 2-Acetylthiazol | 43, 50, 51 |
| 4-Methyl-5-hydroxy-ethylthiazole | 43 |
| Thiazole | 45, 50, 51 |
| 4-Methylthiazole | 45, 50, 51 |
| 5-Methylthiazole | 45 |
| 2,4-Dimethylthiazole | 45 |
| 2,5-Dimethylthiazole | 45 |
| Benzothiazole | 50, 51 |
| 4,5-Dimethylthiazole | 50 |
| 5-Ethylthiazole | 50 |
| 5-Ethyl-4-methylthiazole | 50 |

Key for Diketopiperazines and Fructosazines

| | | |
|---|---|---|
| Phe | = | Phenylalanine |
| Pro | = | Proline |
| Leu | = | Leucine |
| Val | = | Valine |
| ILe | = | *Iso*-leucine |
| Ala | = | Alanine |
| Gly | = | Glycine |
| Ser | = | Serine |
| Thr | = | Threonine |
| Asp | = | Aspartic acid |
| Pyr | = | Pyrrolidone carbonic acid |
| Glu | = | Glutamic acid |
| Fru | = | Fructose |

## 1.3 Direct Thermal Desorption of Roasted Barley

### 1.3.1. Introduction

The identification of the volatile flavour constituents of malt, roasted barley and other solid materials, e g coffee, vanilla beans, is of great interest to flavour chemists The information derived from studies of this nature allows a greater understanding of the chemical reactions and the mechanisms which give rise to flavour i e the roasting of coffee/barley The information is also used for varietal differentiation, conformation of country of origin, product quality and suitability for use

Various sample preparation methodologies have been discussed in the literature review Headspace techniques in particular have shown promise because of their simplicity, ease of automation, efficiency and the ability with which the instrumentation associated may be coupled with other analytical instrumentation (particularly GC-MS systems) When a food is enclosed in a sealed vessel, its flavour volatiles will partition into the headspace of the container, and the many individual compounds comprising the flavour will eventually attain gas phase equilibrium concentrations This is influenced by the solubility of the flavour compounds in the food matrix, their vapour pressures, and temperature For gas chromatographic analysis an aliquot of this headspace may be injected directly onto a GC This technique is described as static headspace analysis It has two major shortcomings, low sensitivity and detection threshold capabilities often higher than organoleptic significance

A variation on this technique described as dynamic headspace analysis may overcome these limitations There are two approaches In both cases the sample is placed in a sealed container with an inert gas (helium or oxygen-free nitrogen) flowing through it The container may be heated Any material which volatilises is drawn off in the gas The sample never achieves gas phase equilibrium but is continually purged in this fashion

At this point the techniques diverge For the technique known as purge and trap thermal desorption the gas stream leaving the sealed container is passed through a thermal desorption tube containing a porous polymer e g TenaxGC which traps the volatile materials present in the gas The volatile material is introduced via a thermal

desorption unit to a gas chromatograph In this unit the tube is ballistically heated in a stream of inert carrier gas to out-gas volatile flavour The volatile flavours are trapped cryogemcally at sub-ambient temperatures and then introduced at high temperature onto a GC

The second technique is known as direct thermal desorption The sample of interest is placed directly into a glass lined stainless steel desorption tube and treated in the same fashion as the porous polymer thermal desorption tubes above

Hartman *et al* [54, 55] have successfully applied this direct thermal desorption technique to the analysis of the volatile flavour constituents of vanilla beans The advantages of this technique include the absence of the organic solvent extraction step which eliminates worker exposure/disposal costs Also there is no solvent peak which allows quantitation of compounds which heretofore were masked The technique is easily automated and involves very little sample preparation

The work described attempts to apply the direct thermal desorption technique to roasted barley

## 1.3.2. MATERIALS AND METHODS

### 1 3.2 1 Instrumentation

A PE 8420 series gas chromatograph with a flame ionisation detector coupled to a Spantech TD4 single shot thermal desorption unit was used throughout the study Perkin-Elmer Turbochrom V 4 data-handling software was used to analyze the data

### 1 3.2 2 Materials

Methyl propyl ketone, acetaldehyde, acetone, dimethyl sulphide, butyraldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, 2-methylbutyraldehyde, hexanal, heptanal, octanal, 2,3-pentanedione, diacetyl, furfural, trans-2-hexenal, 5-methyl furfural, benzaldehyde, phenol, pyrazine, ethylpyrazine, 2-methylpyrazme, 2,5-dimethylpyrazine and 2-acetylfuran were supplied by Fluka, The Old Brickyard, New Road, Gillingham, Dorset, England 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine were all supplied by the Aldrich Chemical Company, The Old Brickyard, New Road, Gillingham, Dorset, England Ethanol was supplied by Hayman Ltd , 70 Eastways Industrial Park, Witham, Essex CM8 3YE, England Helium, hydrogen, liquid carbon dioxide and air were supplied by Air Products Ltd , Unit 950 Western Industrial Estate, Killeen Road, Dublin 12, Ireland All were of a purity suitable for gas chromatography Roast barley was supplied by Guinness Ireland Group, St James's Gate, Dublin DMCS treated glass wool was supplied by Chrompak U K Ltd , Unit 4, Indescon Court, Millharbour, London E14 9TN

### 1.3.2 3 Equipment

Analytical balance accurate to 4 decimal places Liquid tight syringe 5 µl 100 $cm^3$ volumetric flasks Perkin-Elmer thermal desorption tubes and accessories A domestic coffee mill was used to mill the roasted barley

### 1 3.2 4 Surrogate Internal Standard Solution

**1 3 2 4 1** The stock solution for methyl propyl ketone (MPK) was 0 5% w/v in ethanol

### 1 3.3 Sample Preparation

10 g of roasted barley was milled in the coffee mill for 30 seconds, or until a uniform powder was observed A Perkin Elmer thermal desorption tube was sealed at one end with a wire gauze followed by a plug of DMCS treated glass wool on the inside of the tube A 0 1 g portion of the milled roasted barley was weighed out and placed into the tube This was followed by another plug of glass wool to keep the roasted barley in place mid way down the tube and also to stop it from spilling from the tube 1 µl of the 0 5% MPK solution was added via a liquid tight syringe as a surrogate internal standard The tube end was then fixed with a wire gauze and spring It was then ready for thermal desorption

### 1 3 4 Thermal Desorption

The Spantech TD4 thermal desorption unit was programmed to automatically raise the contents of the tube to 200°C while purging the tube with carrier gas (helium) The volatile constituents of the roasted barley were swept in the helium onto a solid carbon dioxide generated cold trap set at -30°C where they condensed This process was allowed to continue for 2 minutes The sample was transferred to the chromatography column by flash heating the cold trap to 300°C, with an injection time of 45 seconds and a transfer line temperature of 200°C

### 1.3 5 Gas Chromatographic Method

A Perkin-Elmer 8420 series gas chromatograph coupled to a flame ionisation detector (FID) was used, with a Chrompak CP-SIL 5CB (50 m x 0 32 mm id WCOT, 1 2 µm film thickness, with a coating efficiency of >80%) chromatography column The oven temperature was held at 30°C for 15 minutes and ramped to 100°C at 2°C/minute The temperature was then ramped to 200°C at 5°C/minute and

then to 250°C at 10°C/minute with a 15 minute hold at 250°C This resulted m a total run time of 90 minutes

The carrier gas used was helium Injector and detector temperatures were 250°C

### 1 3.6. Identification of Compounds Present in Roasted Barley

Compounds were identified by comparison of retention times using commercially available reference standard solutions They were injected onto thermal desorption tubes containing only glass wool and the surrogate standard The tubes were then treated in the same fashion as the sample tubes Additions of these standards were also made to tubes containing roasted barley to help confirm the identity of some compounds

## 1.4. RESULTS AND DISCUSSION

The chromatogram shown in Figure 1 20 was obtained after optimising the operating parameters of the Spantech TD4 thermal desorption unit for this analysis Two-hundred and twenty five peaks were integrated using the Turbochrom data-handling system Twenty five compounds were tentatively identified by matching retention times of peaks for standards and peaks from the chromatogram They were acetaldehyde, ethanol, acetone, dimethyl sulphide, isobutyraldehyde, diacetyl, butyraldehyde, isovaleraldehyde, valeraldehyde, methyl propyl ketone (surrogate internal standard), pentanal/2,3-pentanedione, pyrazine, hexanal, 2-methylpyrazine, furfural, *trans*-2-hexenal, heptanal, 2,5-/2,6-dimethylpyrazine, ethylpyrazine, 2,3-dimethylpyrazine, 5-methylpyrazine/benzaldehyde, phenol, 2,3,5-trimethylpyrazine and octanal The classes of compounds identified as being present included alcohols, aldehydes, ketones, pyrazines and a sulphur containing compound

Limitations were encountered with the analytical equipment and the technique Roasted barley has a moisture content of 5 - 7% This factor must be taken into account when one considers the instrumentation being used Moisture released while heating the sample is carried onto the cryogenic trap where ice could form, possibly blocking it This is not good practice as the water can damage the glass-lined cold trap by expansion while freezing When the trap is heated the ice will be converted to water which will be introduced to the GC injection port and onto the analytical column This can give rise to bad chromatography It may also extinguish the FID flame Most modern systems have a moisture removal system located between the sample and the cryogenic trap to eliminate this problem, but the Spantech TD4 did not This restricted the sample size to 0 1 g Hence sensitivity was reduced

The roast barley was heated at 200°C for 2 minutes to volatilise the flavour volatile material from the sample This regime was chosen to overcome the sensitivity limitations described above Given that roast barley is produced by heating to 225°C there is the possibility that this heating period, however short, may have induced some chemical reactions which gave rise to flavour compounds In order to investigate this possibility the same sample in its original tube was thermally desorbed 4 times The assumption was made that all volatile material was removed

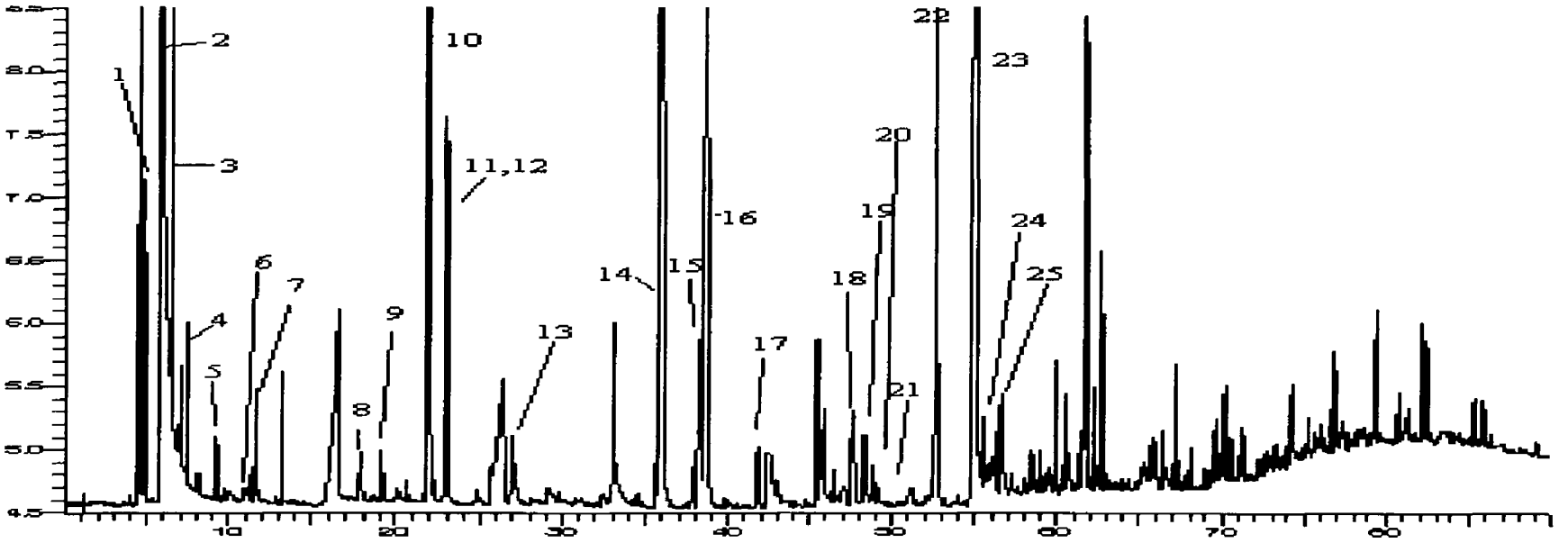



**Figure 1.20** Chromatogram for the direct thermal desorption of roasted barley Peaks 1, acetaldehyde, 2, ethanol, 3, acetone, 4, dimethyl sulphide, 5, isobutyraldehyde, 6, diacetyl, 7, butyraldehyde, 8, isovaleraldehyde, 9, valeraldehyde, 10, methyl propyl ketone (surrogate standard), 11 and 12, pentanal and 2,3 pentanedione, 13, pyrazine, 14, hexanal, 15, 2-methylpyrazine, 16, furfural, 17, *trans*-2-hexenal, 18, heptanal, 19, 2,5 and 2,6-dimethylpyrazine, 20, ethylpyrazine, 21, 2,3-dimethylpyrazine, 22, 5-methylpyrazine and benzaldehyde, 23, phenol, 24, 2,3,5 trimethylpyrazine and 25, octanal

during the first analysis of the tube Only four compounds were found to be present in significant quantities in the later analysis i e greater than 10% their size of the first analysis These peaks corresponded to ethanol, hexenal, furfural and phenol

## 1.5. CONCLUSION

Roasted barley was assessed for its volatile flavour active constituents using direct thermal desorption followed by gas chromatographic analysis with flame ionisation detection Twenty-five compounds were tentatively identified over five classes of organic compounds Necessary requirements were pointed out with relation to instrumentation in order to successfully use the direct thermal desorption technique to solids with significant moisture levels

This technique has potential for monitoring the roasting of coffee, peanuts and barley Pyrazines are recognised as the volatile compounds contributing most to the roasted aromas of cooked foods[57] Correlation of pyrazine quantity with sensory and other analysis indicate the possibility of monitoring the roasting process Off-flavour development and flavour-fade are also areas of potential Flavour-fade in stored roasted peanuts, is generally believed to occur as a result of masking of pyrazines and other flavour compounds by low-molecular-weight aldehydes from lipid oxidation[58] This technique would allow monitoring of these compounds

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# DETECTION OF COOLANT CONTAMINATION IN BEER BY FLUORESCENCE SPECTROSCOPY

## 2 1 INTRODUCTION

The use of coolants, both primary and secondary, is necessary in modern brewing plants A wide variety of secondary coolants[1] (industrial methylated spirits, ethanol, glycols and mixtures of these) are utilised at many stages throughout the production process e g water chilling, wort cooling etc At the time of writing, this brewing company utilises different secondary coolants in various areas of production

Potential problems as a result of secondary coolant usage may arise, and require special attention Two of these concern leakages, which are of particular interest in this work

The first is "external" leakage of coolant Continuous checks are required at valves, flanges, plate heat exchangers etc , to ensure that coolant leakage onto floors etc, is not occurring Since secondary coolant solutions are usually colourless, food permitted dyes e g Eurocert green, are often added to bulk coolant, to aid simple visual detection of an "external" leakage The concerns in this instance are of a plant maintenance, safety/good housekeeping and financial nature

The second area of concern is of extreme importance from a product quality point of view This is the possibility of an "internal" leakage of secondary coolant into product, e g through pinholes developing in plate heat exchangers While engineers in brewing plants may monitor the levels of secondary coolant in coolant storage vessels, this is not sufficient to detect small "internal" leakages Also, dyes such as those described above are not suitable for use in darker products i e ales and stouts The detection of this type of leakage is more complex than can be met by simple addition of dyes to coolant, but rather necessitates sensitive analytical techniques The fundamental requirement of such an analytical technique is that it is sensitive enough to detect trace amounts of coolant in beer

At the present time this brewing company utilises gas chromatography to monitor possible contamination of beer products by secondary coolant Industrial methylated spirits based coolant is made up of an aqueous solution of approximately 2% methanol w/v and 45% ethanol w/v Methanol is assayed using a static

headspace gas chromatography[2] technique which has a limit of detection of 2 ppm Gas chromatography is not suitable for monitoring ethanol as a secondary coolant in any form This is due to the fact that ethanol is a product of fermentation and is present in such large concentrations in beer products that it would be impossible to establish whether coolant leakage was occurring at low levels Propylene glycol is used as a secondary coolant as an aqueous solution at 28% w/v While also a product of fermentation, it is present at background levels of only 10 - 40 ppm in stouts Gas chromatography can, therefore detect increases above these background levels Analysis for propylene glycol[3] involves a sample concentration step and an extraction step followed by gas chromatographic analysis, with a detection limit of 5 ppm

These techniques are labour intensive, specific to particular coolant compounds, require trained personnel and relatively expensive instrumentation It would be advantageous to have the capability to distinguish these coolant compounds by source i e as a result of fermentation or contamination due to leakage

The most desirable analytical technique from a quality assurance point of view, would be universal (apply to all coolants), rapid, inexpensive, simple, and easily applicable for routine analysis It should also be of comparable sensitivity to the gas chromatographic techniques described which are capable of detecting a 1 10,000 coolant product (1 part coolant to 10,000 parts beer) contamination This corresponds to 2 ppm methanol of a 2% w/v methanol in the case of IMS based coolant, or 20 ppm of a 28% w/v propylene glycol based coolant

One possible approach is that of Kavanagh *et al*[4] His technique involves the addition of a fluorescing substance (in this case sodium fluorescein) to the bulk coolant and subsequent monitoring of the product background fluorescence Any increase in the background fluorescence of product would indicate contamination by coolant Kavanagh *et al* worked exclusively with lager products

The object of this study was to investigate the wider applicability of this technique to stouts and ales as well as lagers, and also to ascertain whether the darker colour of these products would interfere with fluorescein detection

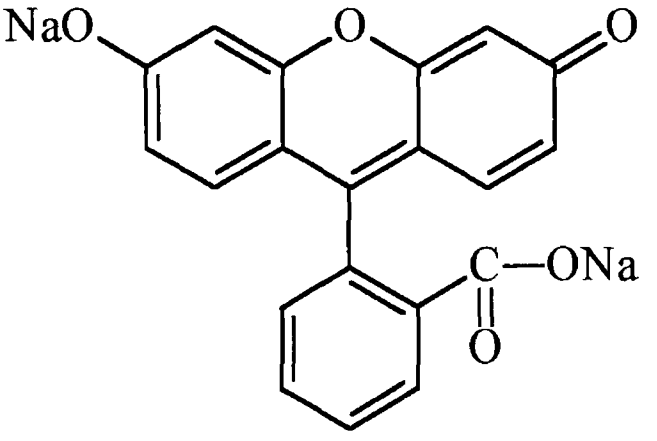


**Figure 2 1** Structure of sodium fluorescein

### 2 1 1 Principle of Fluorescence Spectroscopy

A simplified definition[5] of fluorescence is that it is the immediate emission of light from a molecule or atom following the absorption of radiation In solution at normal temperatures, fluorescence occurs at longer wavelengths than the absorbed light Current theories of the absorption and emission of radiation by matter combine the classical and quantum theories of optics with the quantum mechanical theory of atomic and molecular structure

Light is a form of electromagnetic radiation (energy), the propagation of which is regarded as a wave phenomenon It is characterised by a frequency ($\nu$), and has a wavelength ($\lambda$) and, *in vacuo*, a constant velocity (c) These are related in the following manner

$$\nu = c/\lambda \qquad (1)$$

When light enters matter two things may happen to it

(a) It may pass through the matter with little absorption taking place

(b) On its passage through the matter, the light may be absorbed, either entirely or in part

For fluorescence to take place, light must first be absorbed, therefore only case (b) is of interest here The absorption of light involves a transfer of energy to the medium It is a highly specific phenomenon, and radiation of a particular energy can only be absorbed by characteristic structures According to the quantum theory, energy from light is absorbed in integral units called quanta The energy of a quantum is given by the expression

$$E = h\nu \tag{2}$$

or

$$E = h\, c/\lambda \tag{3}$$

where h is Planck's constant, c is the velocity of light, ν the vibration frequency, and λ the wavelength

Each molecule possesses a series of closely spaced energy levels and can pass from a low energy level to a higher one by absorbing an integral quanta of light which is equal to the energy difference between the two energy states In a liquid solution only a small proportion of molecules absorb and are promoted to an excited state, and are then capable of fluorescing or undergoing photochemical change This leap to an excited state corresponds on a sub-atomic level to electronic changes within the molecule The absorption of a quantum of light by a molecule raises an electron to a higher energy level Just as light absorption accompanies transfer of an electron to a higher state, so does the reverse transition from the excited state to the ground state lead to a release of energy In fact several types of energy release may occur These include resonance radiation, the Raman effect, phosphorescence and fluorescence Fluorescence as the form of energy release of interest in this work

Assuming the molecule is in the excited state, the associated electronic change yields a band spectrum of absorption which comprises the whole series of transitions, electronic, vibrational and rotational If the molecule does not decompose as a result of the increase in energy and if all the energy is not dissipated by subsequent collisions with other molecules, then after a short period of time τ, which is characteristic of the atom or molecule, the electron returns to the lower energy level, emitting a photon in the process This radiation is called fluorescence The

emitted radiation has a greater wavelength or a lower energy than the light which is absorbed (Stokes Law) This implies that the electron, after being raised to a higher energy level by the absorbed radiation, falls firstly to an intermediate energy level, with a loss of some energy Subsequently the electron falls to the lower energy level from whence it was originally excited emitting radiation of lower energy than that which induced fluorescence Almost all fluorescence in solution at ordinary temperatures, is of the Stoke type

All absorbing molecules have the potential to fluoresce Most compounds do not, however, because their structure provides alternative processes for the utilisation of the electronic excitation energies of the molecules These processes are referred to as "quenching" The most intense and useful molecular fluorescence behaviour is found in compounds containing aromatic rings While certain aliphatic and alicyclic carbonyl compounds as well as highly conjugated double bonded structures also fluoresce, their numbers are small in comparison with the number of fluorescent compounds containing aromatic systems Most unsubstituted aromatic hydrocarbons fluoresce in solution, the efficiency increasing with the number of rings and their degree of condensation It is found experimentally that fluorescence is particularly favoured in rigid molecules The influence of rigidity has also been invoked to account for the increase in fluorescence of certain organic chelating agents when they are complexed with a metal ion For example, the fluorescence intensity of 8-hydroxyquinoline is much less than that of the zinc complex[6]

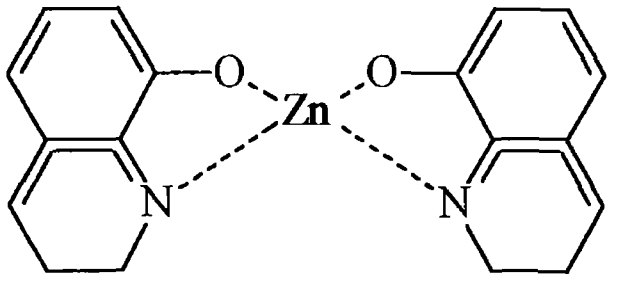


**Figure 2.2.** Structure of [8-hydroxy quinoline]$_2$ zinc complex

The quantum yield of fluorescence is the percentage of the absorbed energy which can be re-emitted as fluorescence This is designated as Φ, where

$$\Phi = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} \quad (4)$$

Simply in terms of molecular fluorescence it is the ratio of molecules that fluoresce to the total number of excited molecules Highly fluorescent molecules such as fluorescein, have quantum efficiencies that approach unity under some conditions Non fluorescent species have efficiencies that are essentially zero Some values for quantum yields of fluorescence (Φ) are given for certain compounds under specific conditions in Table 2 1

**Table 2 1** Quantum Yields of Fluorescence (Φ) in Solution

| Compound | Solvent | $\Phi^{(\text{ref 7})}$ |
|---|---|---|
| Sodium Fluorescein | Water, pH 7 | 0 65 |
| Sodium Fluorescein | 0 1 N NaOH | 0 92 |
| Rhodamine B | Ethanol | 0 97 |
| Phenol | Water | 0 22 |
| Chlorophyll B | Methanol | 0 10 |

Upon withdrawal of the exciting light, the fluorescent intensity of a solution of fluorescent molecules decays with a rate characteristic of a first-order process, exactly like radioactive decay

The relationship between the change in fluorescence with time and the original fluorescence is expressed in the following manner

$$I = I_o e^{t/\tau} \quad (5)$$

where $I$ = fluorescence intensity at time t,

$I_o$ = maximum fluorescence intensity during excitation,

t = time after removing the source of excitation,

$\tau$ = average lifetime of the excited state

The average lifetime of the excited state of some compounds is given in Table 2 2

**Table 2.2.** Average Lifetime of the Excited State ($\tau$) of some Compounds

| Compound | $\tau$ seconds | Reference |
|---|---|---|
| Dinitrophenyl histidine | $5 \times 10^{10}$ | 8 |
| Fluorescein anion | $5\ 1 \times 10^{9}$ | 9 |
| Quinine | $4 \times 10^{8}$ | 10 |
| Chlorophyll | $3 \times 10^{8}$ | 10 |
| Anthracene | $2\ 5 \times 10^{7}$ | 10 |
| Naphthalene | $3\ 3 \times 10^{5}$ | 11 |

This data demonstrates the speed with which fluorescence decreases after excitation

Just as fluorescence will create a problem when measuring absorption spectophotometrically, so absorption raises certain problems during fluorometric assay Obviously, light must be absorbed before fluorescence can occur However, it is also apparent that when absorption is so great as to make the solution opaque, no light will pass through to cause excitation At intermediate concentrations, even though the light penetrates the solution, it is not evenly distributed along the light path The non-uniform distribution of the fluorescence in a solution of strong absorption presents a problem for detection A plot of fluorescence against concentration yields some interesting trends in terms of fluorophore concentration and angle of detection of fluorescence

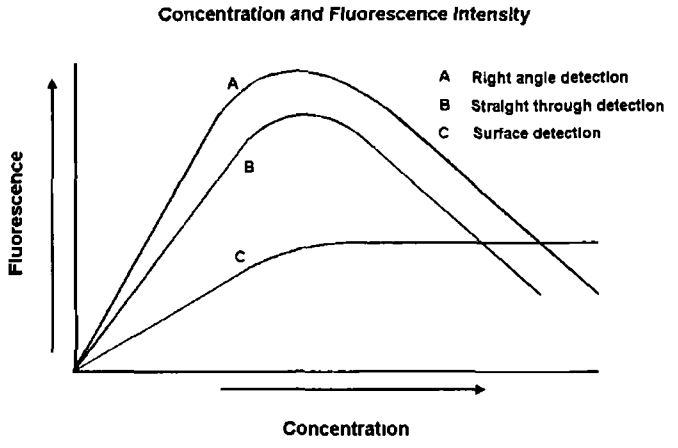


**Figure 2.3.** Fluorescence versus fluorescent yield

Right angle detection is the convention for measuring fluorescence as the graph demonstrates that this orientation collects greater amounts of fluorescent light

The analyst must resort to the end of the scale where fluorescence is proportional to concentration The intensity of fluorescence (emitted in all directions) is equal to the intensity of the absorbed light multiplied by the quantum efficiency of fluorescence[12]

$$F = [I_o (1 - 10^{\varepsilon cd}] [\Phi] \quad (6)$$

where F = total fluorescence intensity, quanta per second

$I_o$ = intensity of exciting light, quanta per second

c = concentration of solution

ε = molecular extinction coefficient

Φ = quantum efficiency (yield) of fluorescence

When the solutions used are so dilute that the amount of light absorbed is very small, then equation 6 is reduced to

$$F = [I_o (2\ 3\ \varepsilon cd)] [\Phi] \quad (7)$$

It is apparent that at such low concentrations when the exciting wavelength and intensity are kept constant, the detector response will be directly proportional to the concentration of fluorophore A general rule is that a linear response will be obtained until the concentration of the fluorescent substance is sufficiently great so as to absorb significant amounts of the exciting light A solution having an absorbence of 0 500 in the spectrophotometer would be expected to lower the apparent fluorescence to a great extent Bowen and Wokes[13] state that to obtain a linear response, the solution must absorb less than 5% of the exciting radiation

Sodium fluorescein is the disodium salt of fluorescein, see Figure 2 1, and it is freely soluble in water whereas fluorescein is not This solubility in water gives it much wider applicability as a fluorescent marker for water based systems than fluorescein It has an intense yellow/green fluorescence which disappears when the solution is made acid but reappears when the solution is again made neutral or alkaline It shows an absorption maximum in water at 493 5 nm and a fluorescent maximum at 520 nm Its fluorescent properties make it useful in a diverse number of applications[14-16], including use as a protein label[17 21], an immuno-fluorescent label[22,23] and as an immuno-histological stain Sodium fluorescein is also approved by the FDA for externally applied drugs and cosmetics[24]

## 2 2 MATERIALS AND METHODS

### 2.2.1 Instrumentation

Fluorimeter Perkin Elmer LS 50B Luminescence spectrophotometer coupled with Perkin Elmer FLDM fluorescence/luminescence data manager software was used throughout for fluorescence measurements A sartorius ultrasart cell 50 filtration unit was used for some filtration work

### 2 2 2 Materials

Buffer solution 150 g Potassium bicarbonate ($KHCO_3$) and 65 g potassium carbonate ($K_2CO_3$) per litre deionised water, both were supplied by BDH Laboratory Supplies, Poole, Dorset, England Whatman No 1 filter paper 1 cm silica cuvettes Excelo 10 $cm^3$ volume tubes Polyclar R was supplied by International Speciality Products, Guilford, Surrey, UK Sodium fluorescein was supplied by BDH Laboratory Supplies, as above A 0 0135% w/v sodium fluorescein in deionised water was used throughout to simulate coolant unless otherwise stated

### 2.2.3. Methods

#### 2 2.3 1 Fluorescence Spectroscopic Analysis

A 25 $cm^3$ portion of beer was degassed and filtered through No 1 Whatman filter paper 5 $cm^3$ of beer and 5 $cm^3$ buffer solution were mixed Fluorescence emission was measured in a 1cm silica cuvette at 520 nm with excitation at 487 nm, immediately after mixing buffer with beer

#### 2.2 3.2. Sighting Experiment

A range of Guinness Ireland products was analyzed for fluorescence, both with and without a fluorescein spike Two levels of addition were used corresponding to 1 2,500 (that is 1 part coolant to 2,500 parts beer) and 1 5,000 of a 0 0135% fluorescein w/v solution, this being the concentration of fluorescein in

coolant recommended by Kavanagh *et al* This gave a fluorescein concentration of 54 ppb and 27 ppb respectively Seven lagers, two ales and two stouts were analyzed

### 2.2.3.3. Stout Background Fluorescence

Two stout products, stouts 1 and 2, were diluted progressively over a colour range from an original colour of 122 European Brewing Convention colour units (EBC) for stout 1 and 222 EBC for stout 2 to 10 EBC The background fluorescence at selected intermediate colours was determined Both stouts were spiked with 1 5,000 coolant and again diluted in the same fashion to 10 EBC and the fluorescence at intermediate colours was determined

Colour was measured according to the EBC method[25 26] i e the sample was diluted to less than 0 8 absorbance units at 430 nm and the following calculation was carried out,

$$\text{Colour EBC} = A_{430} \times D \times 25 \quad \text{where}$$

$A_{430}$ is the absorbance at 430 nm (using a 1 cm cuvette)
D is the dilution factor

### 2 2 3 4 Typical Background Stout Fluorescence

The fluorescence of 9 separate stout products (31 samples in all) was measured at a colour of 50 EBC These samples were taken at random during normal production runs over a two month period

### 2.2.4 Calibration

Three stout products that were deemed to cover the complete range of stouts were taken and additions in the range 0 to 154 ppb fluorescein were made Fluorescence was measured after adjustment to 50 EBC

### 2 2 5 Enhancement of Fluorescence in Stouts

Attempts were made to overcome inhibitors of fluorescence in stouts by the following,

#### 2.2.5.1. Treatment with Polyclar R

It was hoped to remove fluorescence inhibiting material Stout 1, a control and spiked with fluorescein at 48 ppb (1 2,800 coolant) had its colour adjusted to 50 EBC Both were then treated with Polyclar R (12 5 g/L) for 30 minutes After filtration fluorescence was measured

#### 2.2.5.2. Ultrafiltration

This was known to reduce colour Stout 1 was spiked at 1 10,000 and 1 5,000 coolant Both of these and a control were filtered through a 100,000 molecular weight cut off filter (nominal) in a Sartorius Ultrastart cell 50 filtration unit The fluorescent intensity of all 3 samples was then measured

### 2 2 6 Stability of Fluorescein

#### 2 2 6 1 Stability in Beer

Stout 1 was spiked with simulated coolant levels (containing fluorescein) of 1 2,500 and 1 5,000 Control and spiked samples, at original colour and adjusted to 50 EBC, were measured for fluorescence immediately and after 3 days storage (at 4°C in the dark and at 20°C on the bench)

A lager was also treated in this way to investigate differences if any due to the darkness of the stout The lager was dosed at 1 10,000 and stored under 4 conditions of lighting ,

- in clear glass on window ledge,
- in brown glass on window ledge,
- in clear glass on laboratory bench,
- in the dark

### 2.2.6.2 Stability in Presence of Dyes

Coolant systems generally contain food permitted dyestuffs in order to enable detection of leakages at pumps, glands etc It was important to ascertain whether these dyes (Eurocert Green, E142 and Arigran Tartrazine, E102) interfered with or inhibited fluorescence in any way Stout 1 containing 27 ppb fluorescein was dosed at 1 100 with coolant containing each of these dyes and fluorescence was measured at 50 EBC

### 2.2.6.3. Stability in Aqueous Solution

A freshly prepared stock solution (135 ppm) of fluorescein was made up and divided into two lots One aliquot was stored in the dark for 7 days and the other was exposed to direct sunlight on a window ledge for the same time The fluorescence of each of these solutions was measured after dilution to 2 7 ppb fluorescein

## 2 3 RESULTS AND DISCUSSION

### 2 3 1 Sighting Experiments

**Table 2.3.** Fluorescence of Various Products with and without Fluorescein

| Product | FLUORESCENCE[A] | | |
|---|---|---|---|
| | NO ADDITION | 1 · 5,000[B] | 1 . 2,500[B] |
| Lager 1 | 126 | 670 | 1,000[C] |
| Lager 2 | 118 | 714 | 1,000[C] |
| Lager 3 | 119 | 705 | 1,000[C] |
| Lager 4 | 116 | 811 | 1,000[C] |
| Lager 5 | 113 | 650 | 1,000[C] |
| Lager 6 | 118 | 668 | 1,000[C] |
| Lager 7 | 115 | 678 | 1,000[C] |
| Ale 1 | 99 | 445 | 615 |
| Ale 2 | 104 | 453 | 750 |
| Stout 1 | 10 | 24 | 36 |
| Stout 2 | 2 | 3 | 5 |

A Fluorescence was measured at 520 nm emission, following excitation at 487 nm, fluorescence is measured in arbitrary units

B Ratio of simulated coolant product, with a dosage of 0 0135% w/v (135 ppm) fluorescein in coolant

C Saturation/Off-scale

All of the above were Guinness Ireland Group Products

### 2 3.1.1. Lagers

The background level for lagers was shown to be slightly above 100, but increased 5-fold with a simulated contamination level of 1 5,000 (1 part coolant to 2,500 parts beer) and reached saturation at a level of 1 2,500 Assuming linearity over this range, contamination levels of 1 10,000 and 1 20,000 would give fluorescence values which are significantly different from the highest background level of 126, i e greater than or equal to twice this background level

**Table 2.4.** Fluorescence of Lagers

| Sample | Fluorescence |
|---|---|
| Backround | 126 |
| 1 10,000 | 398 |
| 1 20,000 | 262 |

### 2.3.1.2. Ales

The ales showed a similar trend but the fluorescein addition did not produce as large an increase as in the case of the lagers However the increased levels would be significant enough to allow detection of 1 10,000 and 1 20,000 coolant contamination e g in the case of Ale 1

**Table 2.5.** Fluorescence of Ales

| Sample | Fluorescence |
|---|---|
| Backround | 99 |
| 1 10,000 | 272 |
| 1 20,000 | 180 |

### 2.3 1 3 Stouts

Both the background fluorescence and the increases following fluorescein addition to stouts were much lower than those for ales and lagers, particularly in the case of stout 2 relative to stout 1 One interesting difference between these stouts was their colour Stout 1 (120 EBC) gave a higher fluorescence than stout 2 (200 EBC), see Appendix A, Table 1

### 2.3.2. Stout Background Fluorescence

Upon dilution of stouts 1 and 2 background fluorescence increased in each case and reached a maximum in the region 40 - 50 at a colour of approximately 25 EBC See Figure 2 4 (raw data contained in Appendix A, Table 1)

The comparison between spiked (1 5,000) and unspiked samples of stouts 1 and 2 showed that the spiked sample was significantly above the background levels for both See Figures 2 5 and 2 6 (raw data Appendix A, Tables 2 and 3) Although the differences between spiked and unspiked were much less compared to those for ales and lagers, they were sufficient to encourage further investigation

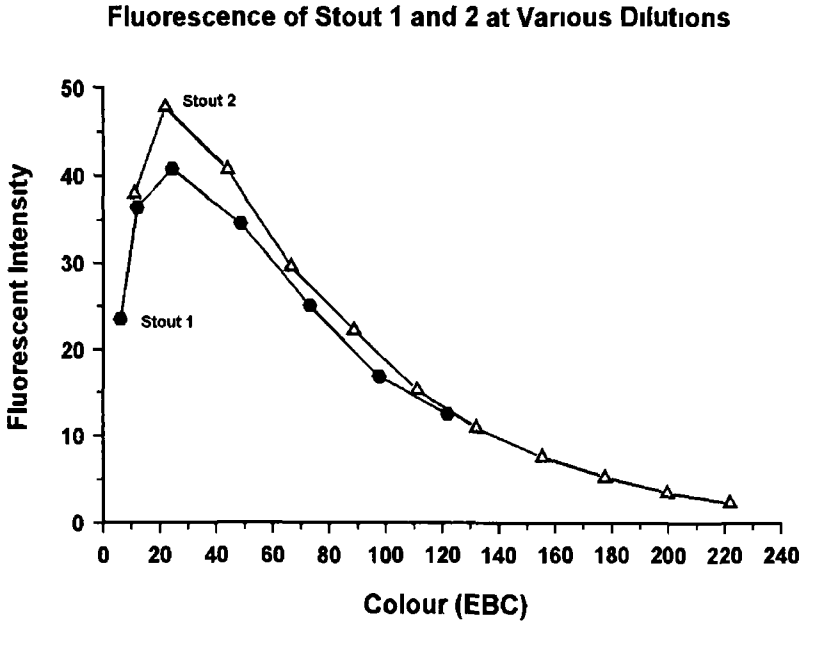


**Figure 2 4** Fluorescence of stout 1 and 2 at various dilutions

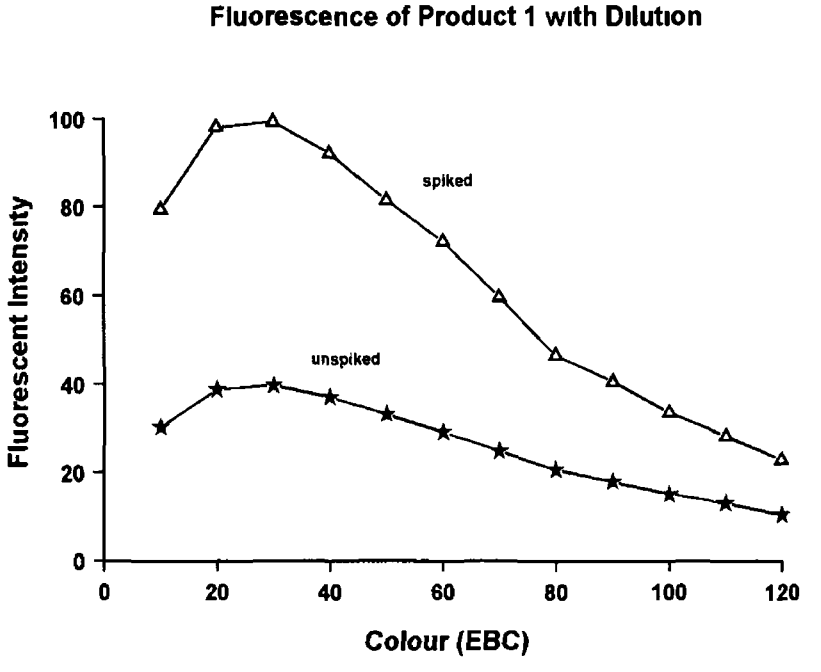


**Figure 2 5.** Fluorescence of product 1 with dilution

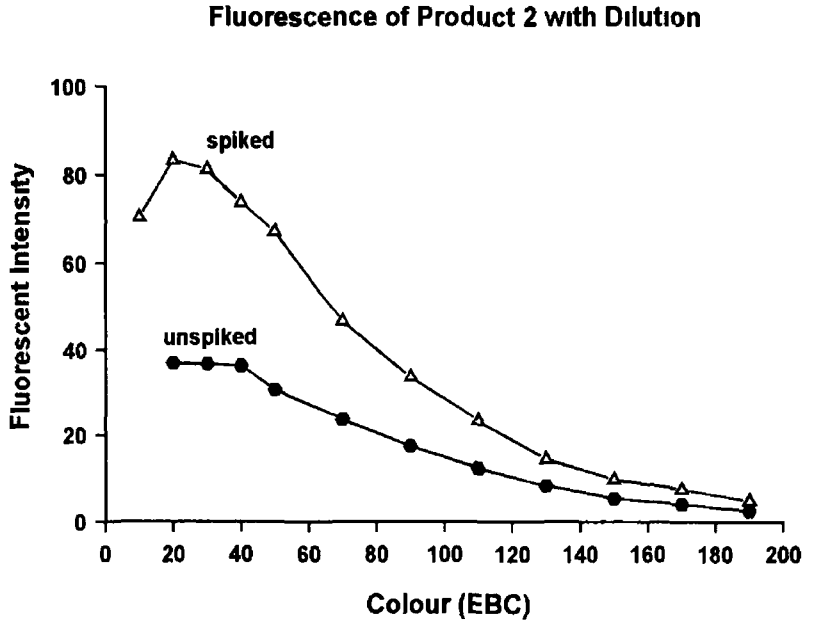


**Figure 2.6.** Fluorescence of product 2 with dilution

### 2.3.2.1. Consistency of Stout Fluorescence

For convenience for dilution purposes a colour of 50 EBC was decided upon as the target colour for fluorescence measurements of stout.

The raw data are given in Appendix A, Table 4. A summary is given here. The results for stout 3 stood out and are highlighted for comparison purposes.

**Table 2.6.** Consistency of Stout Fluorescence

| | **Stout 3** | **Other Stouts*** |
|---|---|---|
| No. of Samples | 6 | 25 |
| Mean | 55 | 38 |
| Range | 45 - 66 | 31 - 44 |
| Standard Deviation | 8 | 4 |

* including stouts 1 and 2

From Table 4 (Appendix A) stout 3 showed a higher fluorescence than the other stout types. The reason for this is probably due to the fact that of the stouts examined it has the lowest proportion of roast material (which contributes melanoidins, the compounds responsible for most of the colour in beer) in the grist. Thus, assuming roast components are inhibitory to fluorescence, they will have least effect in stout 3 (because at colour 50 EBC stout 3 has an original gravity* of 24, whereas stout 1 has an original gravity of 16).

* Original gravity of a beer is the specific gravity of the wort from which the beer was produced by fermentation with yeast. The recommended method[7] of calculation involves distilling off the alcohol, returning both the distillate (spirit) and the residual matter separately to the original volume and determining their specific gravities. Reference to official tables of the specific gravities of spirit by Thorpe and Brown gives the portion of original gravity lost producing this amount of alcohol. This gravity added to the specific gravity of the residual matter gives the original gravity.

### 2.3 3 Calibration

On the basis of the information contained in Appendix A, Table 5, three stout products were chosen to cover all stouts for calibration purposes In consideration of background fluorescence at 50 EBC and stout type, they were

(a) A finished product, Stout 1

(b) Stout 2, which gave a similar fluorescence to stout 1 at 50 EBC but required a greater degree of dilution to attain this colour

(c) Stout 3, which had a greater fluorescence at 50 EBC than other finished products

**Table 2 7** Calibration for Three Stout Products

| **Product** | **Regression Coefficient** | **Slope** |
|---|---|---|
| Stout 1 | 0 988 | 1 44 |
| Stout 2 | 0 997 | 0 887 |
| Stout 3 | 0 972 | 1 21 |

The fluorescein concentration range for these calibrations was 0 - 154 ppb

These figures indicate a good degree of linearity for the three stout products The slopes indicate a significant relationship between fluorescence and added fluorescein, the order being

Stout 1 > Stout 3 > Stout 2

Having decided that a doubling of background is required to establish contamination, the fluorescein concentrations and corresponding coolant product ratios, with 0 0135% w/v fluorescein in the coolant, are

**Table 2.8.** Detection Levels in Various Stout Products

| Product | Fluorescein (ppb) | Coolant Product |
|---|---|---|
| Stout 1 | 29 | 1 4,655 |
| Stout 2 | 45 | 1 3,000 |
| Stout 3 | 41 | 1 3 277 |

Clearly, the sensitivity of the fluorescence procedure for coolant contamination detection is significantly lowered in the case of stouts compared with lagers or ales, by a factor of approximately 5 To double the background fluorescence for lagers a 1 20,000 contamination level of coolant containing 0 0135% w/v fluorescein is sufficient (see Table 2 4) But for stouts at least a 1 5,000 contamination level is required to achieve doubling of the background level The simplest way to overcome this reduced sensitivity would be to increase the fluorescein addition to the coolant i e an increase from 0 0135% w/v to 0 04% w/v would give detectability of about 1 10,000 in stouts, which is required to meet gas chromatographic procedures currently in use for propylene glycol and methanol in our laboratory That is at a detection level of 2 ppm methanol, and having established an approximate backround level of 20 - 30 ppm for propylene glycol in stout

### 2 3 4 Enhancement of Fluorescence in Stouts

#### 2.3 4.1 Treatment with Polyclar R

Treatment with Polyclar R was carried out to attempt to remove fluorescence inhibiting material Polyclar R is in use within the brewing industry to remove haze causing polyphenols from beer

**Table 2.9.** Treatment with Polyclar R

| Sample | Fluorescence (50 EBC) |
|---|---|
| Stout 1 | 33 |
| Stout 1 + Polyclar R | 36 |
| Stout 1 Spiked | 121 |
| Stout 1 Spiked + Polyclar R | 41 |

The use of Polyclar R was counter-productive It did not change the background fluorescence of the stout, but did apparently remove most of the added fluorescein

**2 3.4 2. Ultrafiltration**

As ultrafiltration is known to remove colour by reducing high molecular weight melanoidin levels, this offered the possibility of enhancing fluorescence in stouts As a consequence of the ultrafiltration step the colour of Stout 1 was reduced from 120 EBC to 31 EBC i e to an ale-like colour (the specification for Ale 1 is 20 - 24 EBC)

**Table 2 10.** Treatment by Ultrafiltration (at original colour)

| Sample (at original colour) | Fluorescence |
|---|---|
| Stout 1 | 122 |
| Stout 1 + 1 10,000 spike | 136 |
| Stout 1 + 1 5,000 spike | 157 |

Background fluorescence rose from 10 to 122 units, the latter being in the ale/lager region (see Table 2 3) When spiked stout 1 was analyzed in this way the

results were disappointing Although the overall fluorescence increased, the differences between control and spiked samples did not increase proportionally

When the same stout was diluted to 50 EBC, spiked in the same fashion and treated by ultrafiltration, the proportionate increase in fluorescence values were greater than those observed at original colour

**Table 2.11.** Treatment by Ultrafiltration (at 50 EBC)

| **Sample (at 50EBC)** | **Fluorescence** |
|---|---|
| Stout 1 | 31 |
| Stout 1 1 10,000 spike | 56 |
| Stout 1 1 5,000 spike | 78 |

But by comparison with the results from simply diluting stout 1 with and without a spike to 50 EBC and measuring fluorescence (raw data Appendix A, Table 2), this procedure offered no advantage

Filtration through a lower molecular weight filter was attempted but was impractically slow and was not pursued i e took over 2 hours to filter 20 $cm^3$ of beer

### 2 3 5. Stability of Fluorescein

#### 2 3 5.1 Stability in Stout

It was important to assess the stability of fluorescein in stout in the event of a coolant leakage coupled with a delay in measurement of fluorescence

**Table 2 12.** Fluorescein Stability in Stout

| Sample | Fluorescence | | |
|---|---|---|---|
| | Day 0 | Day 3 | |
| | | 20°C Storage ( on Bench ) | 4°C Storage ( in Dark ) |
| **Original colour** | | | |
| Stout 1 | 10 | 11 | 11 |
| Stout 1 + 1 5,000 | 20 | 21 | 23 |
| Stout 1 + 1 2,500 | 35 | 34 | 37 |
| **50 EBC** | | | |
| Stout 1 | 38 | 36 | 37 |
| Stout 1 + 1 5,000 | 75 | 72 | 78 |
| Stout 1 + 1 2,500 | 122 | 115 | 121 |

No significant change in the fluorescence of control or spiked stouts at the different temperatures over the 3 days was noted

### 2.3 5 2. Stability in Lager

A more detailed experiment for lager showed slightly different results to those for the stouts

**Table 2 13.** Fluorescein Stability in Lager

| Lighting | Sample | Fluorescence | | | |
|---|---|---|---|---|---|
| | | **Day 0** | **Day 1** | **Day 2** | **Day 5** |
| (i) Window/ Clear Glass | Control | 82 | 73 | 48 | 42 |
| | Spiked | 371 | 387 | 350 | 224 |
| (ii) Window/ Brown Glass | Control | 82 | 69 | 69 | 63 |
| | Spiked | 371 | 460 | 460 | 444 |
| (iii) Bench/ Clear glass | Control | 82 | 72 | 62 | 63 |
| | Spiked | 371 | 458 | 450 | 442 |
| (iv) Dark | Control | 82 | 71 | 74 | 66 |
| | Spiked | 371 | 452 | 485 | 438 |

The samples stored in the least desirable conditions, i e in the clear glass on the window ledge, showed a downward trend over the five days Under the other 3 storage conditions fluorescence was relatively stable, showing slight decreases in the case of the control samples and slight increases in the spiked samples

In the context where a 100% increase in fluorescence is to be taken as an indication of contamination, the changes seen here were not significant with the possible exception of the least desirable conditions On this evidence, while not necessarily acceptable from a quality control point of view, a delay of 3 days in fluorescence measurement is acceptable, from an analytical viewpoint, provided that the samples are taken in dark bottles and are preferably stored in the dark prior to analysis

### 2 3.5.3. Stability in the Presence of Dyes

It was essential to assess the dyes which enable "external leakage" detection of coolant for interference with this fluorescent technique
The following results were obtained for this portion of the experiment

**Table 2.14** Fluorescein Stability with Dyes

| Sample | Fluorescence (50EBC) |
|---|---|
| Stout 1 | 35 |
| (Solution A)<br>Stout 1 + 27 ppb Fluorescein | 71 |
| Solution A + 1% Eurocert<br>Green (E142) | 80 |
| Solution A + 1% Arigran<br>Tartrazine (E102) | 70 |

No interference from dyes was noted

### 2.3.5.4. Stability in Aqueous Solutions

The fluorescence of the aqueous solution deteriorated on exposure to light This demonstrates that although the coolant (if dosed) is enclosed in a dark system, it should be monitored for fluorescence regularly

**Table 2.15** Fluorescein Stability in Aqueous Solutions

| Fluorescein Solution* | Fluorescence |
|---|---|
| Initial (Day 0) | 245 |
| Stored in light (Day 7) | 48 |
| Stored in dark (Day 7) | 207 |

* aqueous 2 7 ppb solution

Overall it is important that samples, whether coolant or beer, should not be unduly exposed to light before analysis This is easily achieved by collecting samples in dark bottles and storing them in a dark place or covering them with tinfoil prior to analysis

## 2 4 CONCLUSION

The work described here has confirmed that the fluorescence technique is suitable for lagers It has further been demonstrated that it is applicable to ales Stouts show slightly reduced sensitivity when compared to lagers and ales It is possible to overcome this reduced sensitivity for stouts by a combination of increasing fluorescein addition to bulk coolant to 0 04% w/v and of dilution of the stout to 50 EBC (prior to analysis) This will bring leakage detection rates for stouts into the same order of magnitude as those for lagers and ales i e a leakage of 1 10,000 (coolant product) by this technique The detection rates for this technique are comparable with the gas chromatographic techniques used to monitor IMS and propylene glycol based coolants

Some lagers are treated with Polyclar R or similar materials to remove haze causing polyphenols Polyclar R binds polyphenols by strong hydrogen bonding Excess Polyclar R and bound polyphenols are removed by filtration It has been demonstrated here that Polyclar R will remove fluorescence from beers dosed with fluorescein (although at levels of Polyclar R exaggerated in comparison to those actually used in practice) It is recommended that if this technique were used the lager should be analyzed for coolant contamination prior to Polyclar R treatment only

Although fluorescein is not in use as a food additive, it is extensively used in the cosmetic industry and is of low toxicity Its $LD_{50}$ oral in rat is 6 7 g/kg, similar to that of ethanol which is 7 06 g/kg At the dosage level of 0 04% w/v recommended for stout, 1,884 pints of coolant itself would have to be consumed by a 10 stone man to reach the $LD_{50}$ of fluorescein

In summary this technique is universal, unlike the gas chromatographic techniques which are compound specific It is rapid, straight-forward, and simple to carry out, similar to a routine colour measurement In terms of detectability rates, it is equally as good as the gas chromatographic techniques A particular advantage is its applicability to any liquid coolant (including those based on ethanol), that does not interfere with fluorescence

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# Development of a Gas Chromatographic Technique for the Determination of Propylene Glycol in Beer and Beer-Like Products

## 3 1 INTRODUCTION

Propylene glycol (PG) is a clear, viscous, colourless liquid, with a boiling point of 187°C and will set to glass at -60°C It is highly hygroscopic and has a low order of toxicity[1 2] in humans When taken internally PG is harmless, probably due to the fact that its oxidation products, pyruvic acid and acetic acid[3], are also harmless

In the food industry PG is used as a solvent[4], humectant, and preservative, in the manufacture of products that come into contact with food, such as plasticisers for food wraps, as a solvent for food processing and as a lubricant for food machinery It is a softening agent, spreader, emollient, intermediate, drug vehicle, and preservative in the preparation of cosmetics and pharmaceuticals Aqueous solutions are effective antifreeze mixtures (Figure 3 1) and are preferred in refrigeration units in breweries, dairies and packing houses, where a coolant/heat transfer solution of low toxicity is important At the time of writing PG is extensively used at Guinness Ireland Group's Dublin site as a secondary coolant Propylene glycol is also a naturally occurring by-product of fermentation[5] Hardwick and co-workers[6] demonstrated that yeast alcohol dehydrogenase (ADH) catalyses the reduction of methyl glyoxal (pyruvaldehyde) to acetol, and that cell free extracts (CFE) of brewers yeast will interconvert acetol and propylene glycol The specific enzyme responsible for this is as yet unknown (Figure 3 2) Both diacetyl and 2,3-pentanedione undergo similar reactions

While there is no direct legislation relating to propylene glycol levels in beer, a concern for the brewer is to ensure that PG does not contaminate his product via leakage from refrigeration units used throughout the production process Even though, as noted earlier, PG has a very low toxicity, such contamination would be unwelcome as it would indicate a breakdown of product integrity Contamination of this nature could occur e g through pinholes developing in plate heat exchangers, and is extremely difficult to detect In reality, it is necessary to monitor product for its PG content continuously in order to confidently guarantee that coolant contamination is not occurring

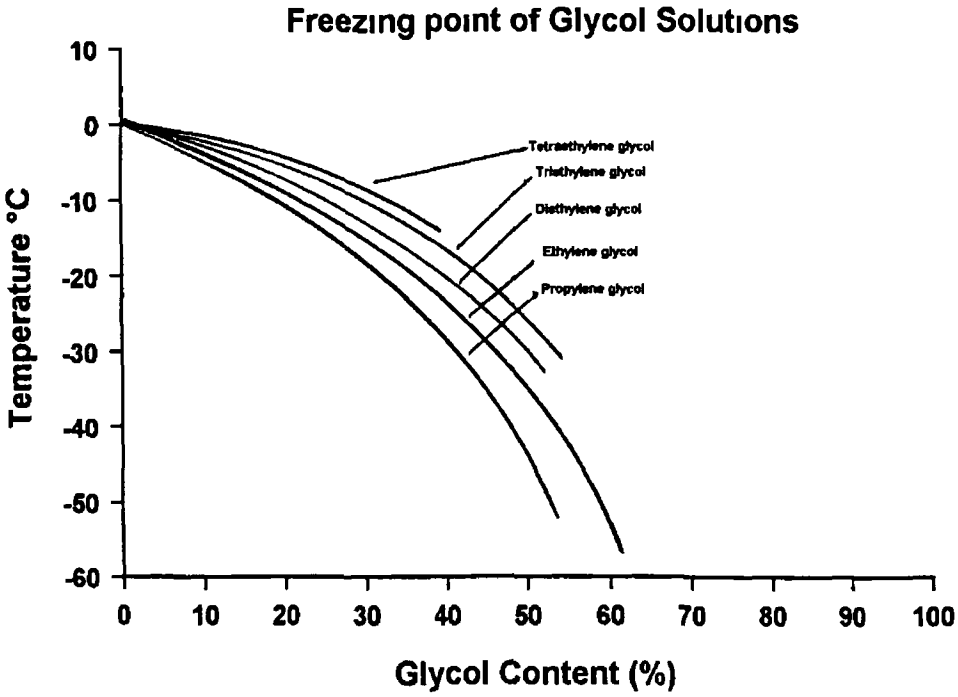


**Figure 3.1.** Freezing points of aqueous glycol solutions [7]

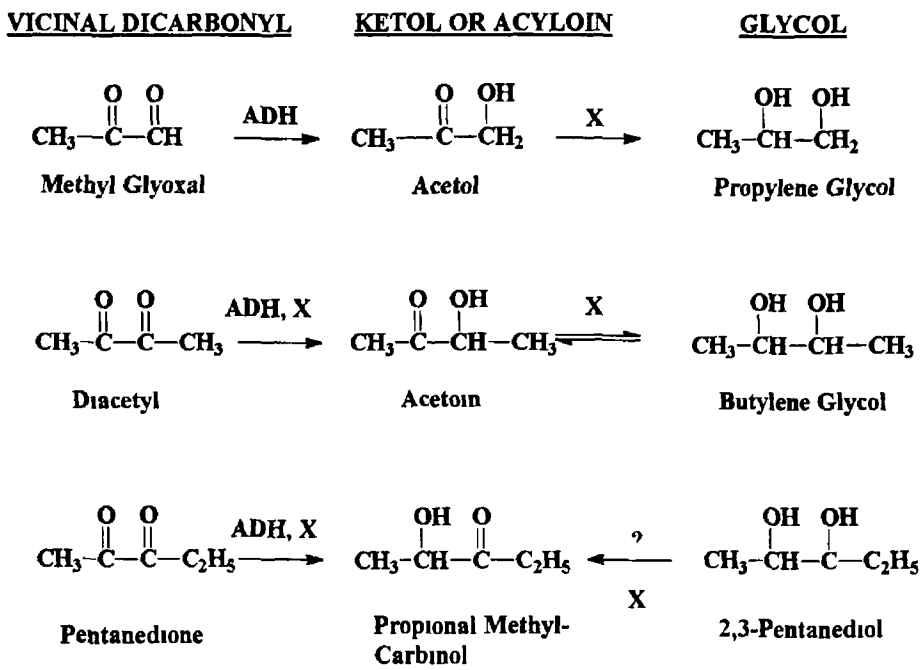


**Figure 3.2.** Summary of the interconversions of vicinal diketones-related compounds by brewers yeast enzymes Alcohol dehydrogenase, ADH, unidentified enzymes, X

In order to monitor product for contamination by PG based coolant, it is necessary to have an analytical technique available which is accurate, precise, sensitive (with a low limit of detection) and selective for PG The technique should also lend itself to routine use in a Quality Control laboratory i e be easily automated with minimal sample work-up prior to analysis

The objective of this work was to develop an analytical technique for the analysis of PG in beer and beer-like products and which would possess those qualities described The approach taken initially was to compare two slightly differing sample preparation techniques, both of which utilised gas chromatography for sample quantitation The first of these techniques, developed at this laboratory, consisted of a sample preconcentration followed by solvent extraction, with a split injection onto the gas chromatograph The second was based on that described by Williamson and co-workers[5] and is a direct extraction of beer, followed by a splitless injection onto a gas chromatograph Several solvents (ethanol, acetonitrile and ethyl acetate) as well as two potential internal standards, ethylene glycol and pinacol (2,3 dimethyl-2,3-butanediol), were assessed for suitability, see Figure 3 3

Propylene glycol has been assayed for in a variety of matrices by differing analytical techniques In foods such as noodles, fish, smoked produce, soft drinks, ice cream, margarine and tobacco casing, enzymatic[9,10,11] techniques have been utilised A homogenised portion of the sample of interest is treated with glycogen dehydrogenase This enzyme cleaves the propylene glycol molecule, releasing a $H^+$ ion for each propylene glycol molecule In the presence of added $NAD^+$, a molecule of NADH is formed which can be quantitatively detected via spectrophotometric analysis at 340 nm, thus by a simple calibration of NADH, propylene glycol may be quantitatively determined Alternatively, without addition of $NAD^+$ a pH meter has been used by Montoya *et al* to determine $H^+$ while analysing tobacco casing Where glycerol might be present, glycerol kinase may be added prior to the glycerol dehydrogenase to denature it A flow injection analysis system[12] coupled with refractive index detection has been reported for the analysis of ethylene glycol and propylene glycol in aircraft deicing fluids

High performance liquid chromatography has been used by Nagel *et al* [13] for analysis of propylene glycol as a humectant in meats The technique applied here required some sample work-up prior to analysis, the meat samples requiring homogenisation, centrifugation followed by treatment with an ion exchange column

to remove interfering ions such as NaCl and proteins i e a Dowex 50 $H^+$ column coupled to an Amberlite IR 45 CP OH column Analytical separation was achieved using a Bio Rad Aminex HPX-87 HPLC column with detection by differential refractive index detector using 0 013N $H_2SO_4$ as mobile phase Another HPLC technique was used by Nitsche and Hueber[14] to optimise the conditions for biodegradation of glycols in the presence of a nutrient containing peptone, beef extract and salts, in a laboratory activated sludge, the method was also used to determine glycols in surfactant Aqueous samples were filtered and analyzed using an Hypersil ODS HPLC chromatography column with water as a mobile phase using refractive index detection The method was shown to resolve peaks for glycerol, propylene glycol, diethylene glycol, propan-2-ol and triethylene glycol, all of which are constituents of detergents

Gas chromatographic techniques have also been utilised to assay a wide variety of matrices for propylene glycol These include synthetic culture flavour concentrates, a variety of commercial foods, industrial pollutants (hydrolysis products of the corresponding glycol nitrates used as military propellants), margarine and mayonnaise, in human serum and urine, rat and mouse plasma as well as anchovies

Waldrat and Linsay[15] assayed synthetic culture flavour concentrates for acetaldehyde, dimethyl sulphide, acetic acid, diacetyl and propylene glycol by direct injection of the aqueous based concentrate using acetone as an internal standard A stainless steel packed column was used with Porapak Q as stationary phase, with a short pre-column packed with Porapak R The column was operated isothermally at 155°C using flame ionisation detection (FID) Kaplan *et al*[17] studied the biodegradability of propylene glycol, diethylene glycol, triethylene glycol and trimethylolethane, when challenged by activated or anaerobic sludge microorganisms The biodegradation of the substrates described was monitored by a gas chromatographic technique The levels of propylene glycol in fatty foods i e margarine and mayonnaise, was tackled by Yoshida *et al*[18] Samples were suspended in hot water, the resulting solution was eluted through an Extrelut 20 column to remove fat (which would not adhere to the column packing) and the analyte was eluted with water The aqueous solution was analyzed by a packed gas chromatographic system using a column packed with (i) Chromosorb 101 or (ii) packed with Tenax GC FID detection being used in both of the above Houze *et al*[19] studied human serum and urine in cases were propylene glycol had been consumed

orally Samples were vortexed followed by centrifugation to remove sedimentable proteins A portion of the supernatant was treated with phenylboronic acid in methanol for fifteen minutes The organic layer was dried over sodium sulphate An aliquot of this organic layer was introduced onto a wide bore DB5 (non-polar) capillary chromatography column with FID detection Ferrala *et al* [20] investigated rat and mouse plasma by direct injection of protein free supernatant onto a DBwax (polar) capillary chromatography column

A number of studies[22 24] have been carried out into broad areas within which propylene glycol falls Castle *et al* [22] have investigated the migration of glycols from regenerated cellulose film into food while Dagnall *et al* [23] have published on the determination of trace organics in food and Blake *et al* [24] have published on the determination of stabilisers in food It is beyond the scope of this work to examine these in detail

# Development of an Analytical Technique for Propylene Glycol Analysis in Beer and Beer-Like Products.

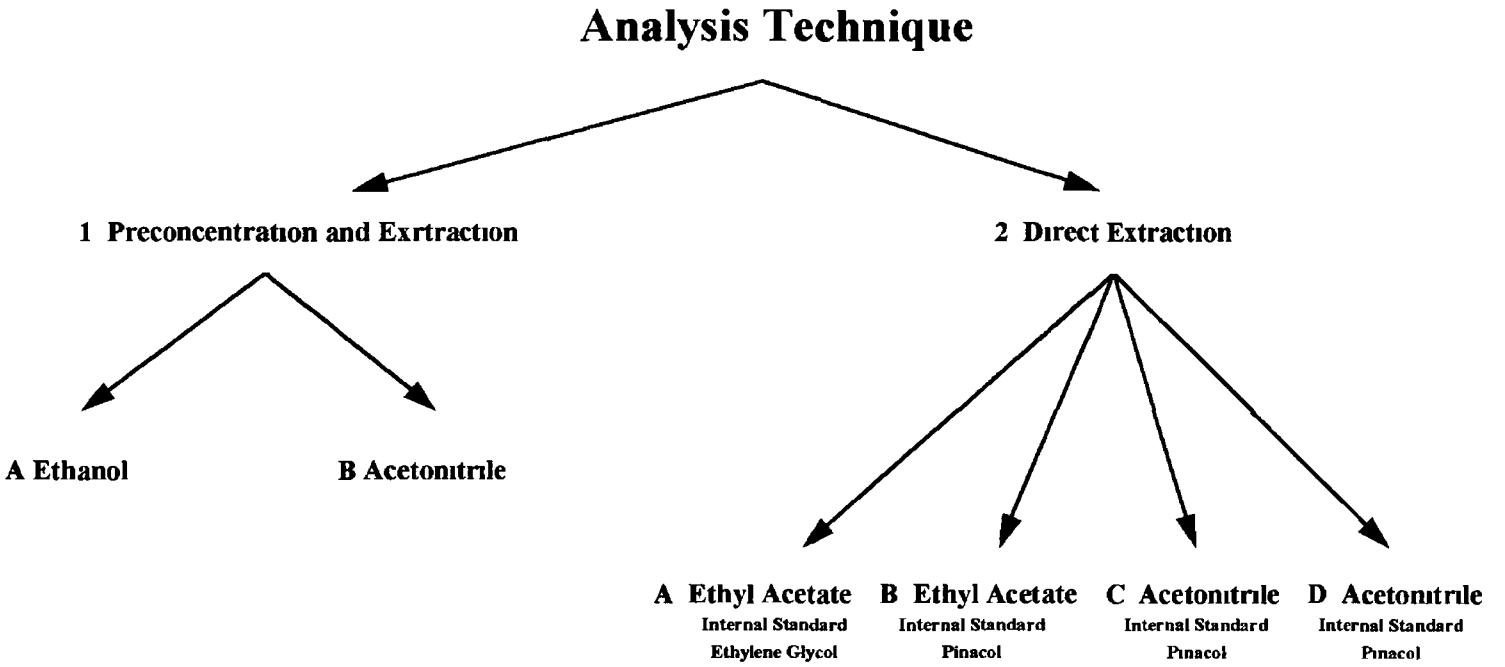


**Figure 3.3** Overview of the sample preparation techniques assessed for propylene glycol analysis

## 3.2. MATERIALS AND METHODS

### 3.2.1. Instrumentation

Perkin Elmer 8410 series gas chromatograph with a split/splitless injector coupled to a flame ionisation detector (FID) Hewlett Packard HP5890 Series II gas chromatograph with split/splitless injector coupled to a Hewlett Packard HP5971 MS detector Shimadzu GC-9A gas chromatograph with a AOC spht/splitless automated injector coupled to an FID PAAR DMA46 density meter

### 3.2.2. Materials

Analar-grade granular ammonium sulphate, ethyl acetate, ethylene glycol (EG), propylene glycol (PG) and pinacol (2,3-dimethyl-2,3-butandiol) were supplied by Aldrich Chemical Co , The Old Brickyard, New Road, Gillingham, Dorset, England Ethanol as absolute alcohol was supplied by Hayman Ltd , 70 Eastways Industrial Park, Witham, Essex CM8 3YE, England HPLC grade acetonitrile was supplied by Lab Scan Ltd , Unit T26, Stillorgan, Co Dublin, Ireland Silicone anti-foam was supplied by BDH, Merck Ltd , Broom Rd, Poole, Dorset BH12 4 NN, England Air, Hydrogen and Helium used for chromatography were supplied by Air Products Ltd , Western Industrial Estate, Dublin 12 Stout malt, whole barley and stout yeast were supplied by Guinness Ireland Group, Dublin, Ireland

### 3.2.3. Equipment

Corex centrifuge tubes (25 $cm^3$) A vortex mixer, and a centrifuge capable of 3,000 x g Rotary evaporator Fifty litre fermentation vessel Analytical balance accurate to 4 decimal places Graduated cylinders 25 $cm^3$ Excelo 10 $cm^3$ volume tubes (graduated) Pear shaped flasks (50 $cm^3$) B 19/26 Automatic pipettes, 1 $cm^3$ pipette, 200 µl pipette, both positive displacement Liquid tight syringe 5 µl Whatman anatop 10 plus 0 2 µm filters No 1 Whatman filter papers Glass funnels 2" diameter

### 3 2.4. Standard Solutions

**3.2.4.1.** The stock solution for PG was 1% w/v in deionised water

**3.2.4.2** The Pinacol internal standard stock solution was 0 4% w/v in deionised water and the working solution was 0 04% w/v in deionised water

**3.2.4.3.** The EG internal standard solution was 0 5% w/v in deionised water

### 3.2.5 Sample Preparation Techniques

**3.2 5 1 Technique 1a:** Sample Pre-concentration Followed by Ethanol Extraction (EG internal standard)

A 25 $cm^3$ portion of stout to which 1 $cm^3$ of 0 5% w/v EG and 1 drop silicone anti-foam had been added (200 ppm) was slowly evaporated to 2 $cm^3$ under vacuum using a rotary evaporator This 2 $cm^3$ concentrate was then added to 8 $cm^3$ of ethanol in an Excelo tube which was stoppered and then vortexed for 1 minute The solution was allowed to stand for 30 minutes and filtered through a No 1 Whatman filter paper 1 µl of this solution was injected onto the gas chromatographic column

**3 2.5 2. Technique 1b.** Sample Pre-concentration Followed by Acetonitrile extraction (EG internal standard)

As for 1a above except that acetonitrile was used in place of ethanol

**3 2 5 3 Technique 2a.** Ethyl Acetate Extraction (EG internal standard)

If necessary, samples were degassed at room temperature A 10 $cm^3$ portion of stout was added to a 25 $cm^3$ Corex tube which contained 10 g of ammonium sulphate An addition of 0 4 $cm^3$ of EG internal standard solution (200 ppm) was followed by 5 $cm^3$ of ethyl acetate The sample tube was capped and vortexed for 1 minute The resultant emulsion was broken down by centrifugation at 3020 x g for 15 minutes A portion of the ethyl acetate layer was transferred to a 2 $cm^3$ vial and sealed tightly 1 µl of this solution was injected onto the gas chromatographic

column

**3.2.5.4. Technique 2b:** Ethyl Acetate Extraction (Pinacol internal standard)

As for 2a above except that 150 µl of 0 04% w/v pinacol was used in place of EG as internal standard

**3 2 5.5 Technique 2c** Acetonitrile extraction (Pinacol internal standard)

As for 2a except that acetomtrile was used as solvent and 150 µl of 0 04% w/v pinacol was used as internal standard

**3.2.5.6. Technique 2d :** Direct Acetomtrile extraction (Pinacol internal standard)

A 5 $cm^3$ portion of stout was placed into a 10 $cm^3$ Excelo tube and 75 µl of 0 04% w/v pinacol was added An aliquot of 5 $cm^3$ of acetomtrile was placed on top of this and the Excelo tube was capped and shaken vigorously This solution was centrifuged at 3020 x g for 10 minutes The supernatant was decanted and 2 $cm^3$ was placed into a 2 $cm^3$ gas-tight vial 1 µl of this solution was injected onto the gas chromatographic column

### 3.3. Analysis for Propylene Glycol during the Course of a Stout Fermentation

A high gravity stout fermentation was set up, 50 L of wort was pitched at 3 5 g/L of stout yeast The grist for mashing consisted of 75% stout malt and 25% whole barley The wort was hopped with 100% hop extract and RMX addition rate was 7% The wort was fermented with stout yeast, initially at a wort temperature of 20°C rising to 25°C over the course of the fermentation

Samples were drawn over the course of the fermentation and analyzed for (a) their present gravity using the PAAR DMA46 density meter and (b) prepared for analysis for propylene glycol using sample preparation technique 2b described above The analysis for propylene glycol was carried out using the instrumentation described in chromatographic system 4

## 3 4 Chromatographic Systems

A Perkin-Elmer 8410 series gas chromatograph coupled to a flame ionisation detector (FID) was used for chromatographic systems 1 and 2, with a Chrompak CP-WAX 58CB (25 m x 0 32 mm WCOT, 0 2 μm film thickness, with a coating efficiency of > 80%) gas chromatography column

The GC-MS chromatographic system (chromatographic system 3) consisted of a HP5890 Series II GC coupled to a HP5971 MS detector Two different gas chromatographic columns were used in this system, a HNU Nordian NB-351 (25 m x 0 2 mm x 0 2 μm df) and a Jones Chromatography DB1701 (30 m x 0 25 mm x 1 μm df)

A Shimadzu GC-9A gas chromatograph coupled to an FID was used for the analysis of the propylene glycol formed during the course of a fermentation The samples were introduced to the GC via Shimadzu AOC autosampler using splitless injection A Jones chromatography DB1701 GC column was used for analytical separation

**3.4 1. Chromatographic System 1.** Sample Preparation Technique 1 (pre-concentration followed by ethanol or acetonitrile extraction)

The oven temperature was held at 80°C for 10 minutes and ramped at 6°C/minute to 180°C The temperature was held at 180°C for 5 minutes This resulted in a total run time of 31 6 minutes Both the injector and detector were held at 250°C A split flow of 30 $cm^3$ was maintained

**3.4 2. Chromatographic System 2** Sample Preparation Technique 2 (ethyl acetate or acetomtrile extraction)

The oven temperature was held at 65°C for 5 minutes and ramped to 140°C at 5°C/minute, the temperature then being ramped to 215°C at 10°C/minute with a hold time of 10 minutes This results in a total run time of 32 5 minutes Both the injector and detector temperature were held at 250°C The splitless mode of injection was used, with the split opening after 0 5 minutes with a flow of 60 $cm^3$/minute Peak identifications were made by comparison of retention times with those of

known solutions under identical conditions

### 3 4 3 Chromatographic System 3· GC-MS Analysis

Samples were assessed using two differing capillary chromatography columns on a Hewlett Packard HP5890 Series II gas chromatograph coupled to a HP5971 MS detector

#### 3 4.3.1. Column 1

A HNU Nordian NB-351, 25 m x 0 2 mm x 0 2 μm df This is similar in polarity to a Carbowax 20 m stationary phase Helium was used as carrier gas at a pressure of 11 psi The oven temperature was held at 60°C for 5 minutes and ramped at 8°C/minute to 150°C The temperature was ramped from 150°C to 240°C at 15°C/minute and held at this temperature for 15 minutes This resulted in a total run time of 37 3 minutes The injector was held at 260°C and the output to the detector at 50°C A split flow of 30 $cm^3$ was maintained

#### 3.4 3 2 Column 2

A Jones Chromatography DB1701, 30 m x 0 25 mm x 1 μm df Helium was used as carrier gas at a pressure of 5 psi The oven temperature was held at 50°C for 5 minutes and ramped at 6°C/minute to 115°C The temperature was ramped from 115°C to 270°C at 30°C/minute and held at this temperature for 10 minutes This resulted in a total run time of 31 minutes The injector was held at 280°C and the output to the detector at 50°C The splitless technique for injection was employed here

### 3 4.3 Chromatographic System 4. Analysis for Propylene Glycol during the Course of a Stout Fermentation

The chromatographic conditions described in 3 4 3 2 were used here The FID temperature was held at 280°C

**Table 3.1.** Details of Chromatographic Systems Used

| | Chromatographic Systems | | | |
|---|---|---|---|---|
| | **1** | **2** | **3 1** | **3 2** |
| **Gas Chromatograph** | PE8410 | PE8410 | HP5890 Series II | HP5890 Series II |
| **Detector** | *FID* | *FID* | *MS* | MS |
| **Carrier Gas** | Helium | Helium | Helium | Helium |
| **Carrier Pres(psi)** | 15 | 15 | 11 | 5 |
| **Column** | Chrompak<br><br>CP WAX 58 CB<br>25 m x 0 32 mm WCOT<br>0 2 µm film thickness | Chrompak<br><br>CP WAX 58 CB<br>25 m x 0 32 mm WCOT<br>0 2 µm film thickness | HNU Nordian<br><br>NB 351<br>25 m x 0 2 mm WCOT<br>0 2 µm film thickness | Jones Chromatography<br><br>DB1701<br>30 m x 0 25 mm WCOT<br>1 µm film thickness |
| **Injector Temp °C** | 250 | 250 | 260 | 280 |
| **Detector Temp °C** | 250 | 250 | 50 | 50 |
| **Temp Programme** | 80°C for 10 min | 65°C for 5 min | 65°C for 5 min | 50°C for 5 mm |
| | 6°C/min to 180°C | 5°C/min to 140°C | 8°C to 150°C | 6°C/min to 115°C |
| | 180°C for 5 min | 10°C/min to 215°C | 15°C/min to 240°C | 30°C to 270°C |
| | | 215°C for 10 min | 240°C for 15 min | 270°C for 10 min |
| **Injection Type** | Split | Splitless | Split | Splitless |
| | 30 $cm^3$/min | | 30 $cm^3$/min | |
| **GC Run Time** | 31 6 minutes | 32 5 minutes | 37 3 minutes | 31 minutes |
| **Amount Injected** | 1 µl | 1 µl | 1 µl | 1 µl |

### 3.4.4. Calibration

Calibration was achieved by the method of standard additions Fixed amounts of PG were spiked into stout (20, 40, 60, 80 ppm) and these spiked samples were treated by each technique as previously described The relative peak areas (PG peak area/internal standard peak area) obtained at each concentration were plotted against the added concentration

Calibration curves were constructed by subjecting the data points to the least squares method for regression The correlation of added concentration to area ratio was evaluated to simulate the practical situation of an unknown sample for analysis, and the confidence interval for a predicted value of concentration at the 95% confidence level was calculated for each technique A response factor, which because of the correlation calculation described above (correlation of added concentration to area ratio as opposed to correlation of area ratio to added concentration) is equivalent to the slope of the best fit line from the least squares method was calculated for each of the techniques described

### 3.4.5. Calculations

The concentration of PG was determined by multiplying the relative peak area of an unknown by the response factor (RF) determined from the method of standard additions

Concentration in mg/L = (compound peak area/internal standard peak area)x(RF)

## 3 5. RESULTS AND DISCUSSION

### 3.5.1. Sample Preparation

Sample Preparation Technique (SPT) 1 (sample preconcentration followed by ethanol or acetonitrile extraction), is labour intensive and time consuming When using a rotary evaporator it is necessary to continually monitor the sample being concentrated in order to avoid bringing the sample to dryness and to prevent sample loss due to splashing If the sample should dry out (see Appendix B, Calibration Raw Data SPT 1a, 20 *ppm* standard) the absolute amounts extracted for both PG and EG are lower compared to all other cases The rate of sample concentration may be limited by the day-to-day variations in the system vacuum, by the water pressure at the water vacuum pump and the condition of the pump itself It is not possible to automate this sample concentration step, therefore this SPT only allows a low sample throughput, typically 1 hour per sample per rotary evaporator

On the other hand SPT 2 (direct extraction into ethyl acetate or acetomtrile) allows a large number of samples to be carried through the sample preparation step simultaneously i e 10 samples at one time This number of samples takes approximately 1 hour to process The SPT is neither time-consuming nor labour intensive

On this basis a preparation technique of type 2 is the more desirable for the routine analysis for propylene glycol in product throughout the brewing process right up to and including the final packaging

### 3.5.2. Calibration Results

Calibrations based on each of the 6 sample preparation techniques coupled to specific gas chromatographic techniques outlined in Table 3.1 were carried out as per the Materials and Methods section (3.2.5). Chromatographic system 1 was used for SPT 1 and chromatographic system 2 for SPT 2. From this point forth, technique refers to the SPT coupled with the gas chromatographic system described in Table 3.1. The regression calculations were made for Concentration of propylene glycol (ppm) on Area Ratio to simulate the practical situation of a beer sample for analysis (subject to the experimental conditions). The raw data are given in Appendix B.

The following table (Table 3.2) gives a summary of the analysis of variance (ANOVA) assessment for each technique:

**Table 3.2.** ANOVA Data for Each Technique

| **Regression Data** | **Technique** | | | | | |
|---|---|---|---|---|---|---|
| | **1a** | **1b** | **2a** | **2b** | **2c** | **2d** |
| Intercept | -22.81 | -19.99 | -11.95 | -12.49 | -9.78 | -1.95 |
| Slope | 147.50 | 113.39 | 41.12 | 50.35 | 19.83 | 12.53 |
| Regression Coefficient | 0.9794 | 0.9740 | 0.9983 | 0.9988 | 0.9941 | 0.9630 |
| Standard Error of the Residuals | 6.38 | 7.17 | 1.83 | 1.55 | 3.44 | 8.03 |
| Confidence Interval +/- (ppm)* | 14.71 | 16.52 | 4.22 | 3.57 | 7.94 | 18.52 |

* This is the confidence interval for a predicted value of propylene glycol calculated using the standard error of the residuals at 95% level.

The limit of detection was calculated for each technique as twice the signal to noise ratio. For practical purposes this was 2 ppm for all techniques.

Clearly by comparison of the statistical data outlined in Table 3 2, techniques 1a, 1b and 2d are very significantly less satisfactory statistically than the other three techniques Techniques 2a, 2b and 2c are statistically satisfactory i e with regression coefficients > 0 99, but 2b is the technique demonstrating the lowest standard error of the residuals and also the regression coefficient closest to 1 It has the tightest confidence interval for a predicted value of concentration (ppm) based on one observation of area ratio at the 95% level of confidence

From a statistical viewpoint SPT 2b coupled with chromatographic system 2 has been demonstrated to be the most satisfactory sample analysis technique

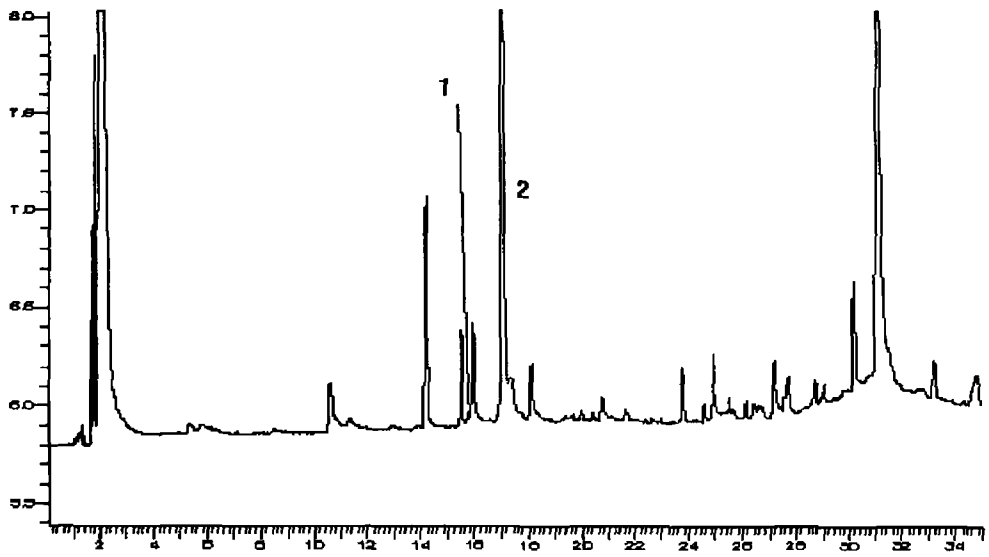


**Figure 3.4.** Full chromatogram for technique 1a (ethanol extraction of stout for propylene glycol analysis) Peaks 1, propylene glycol, 2, ethylene glycol

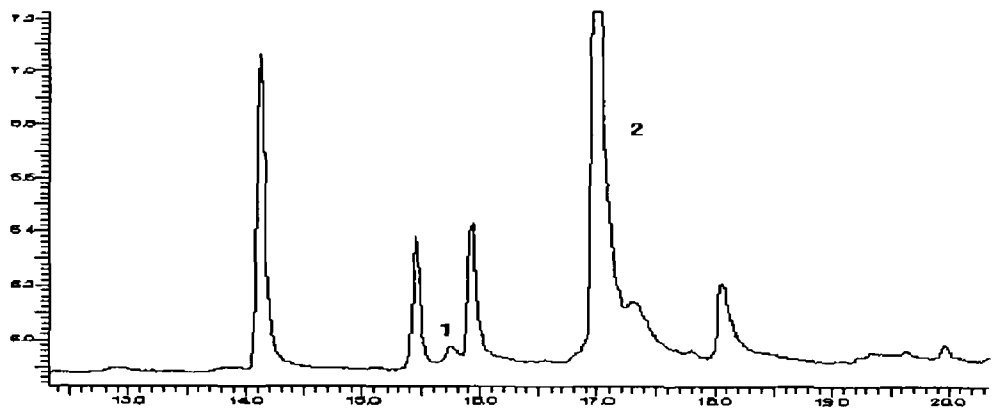


**Figure 3 5** Expanded chromatogram for technique 1a Peaks 1, propylene glycol, 2, ethylene glycol

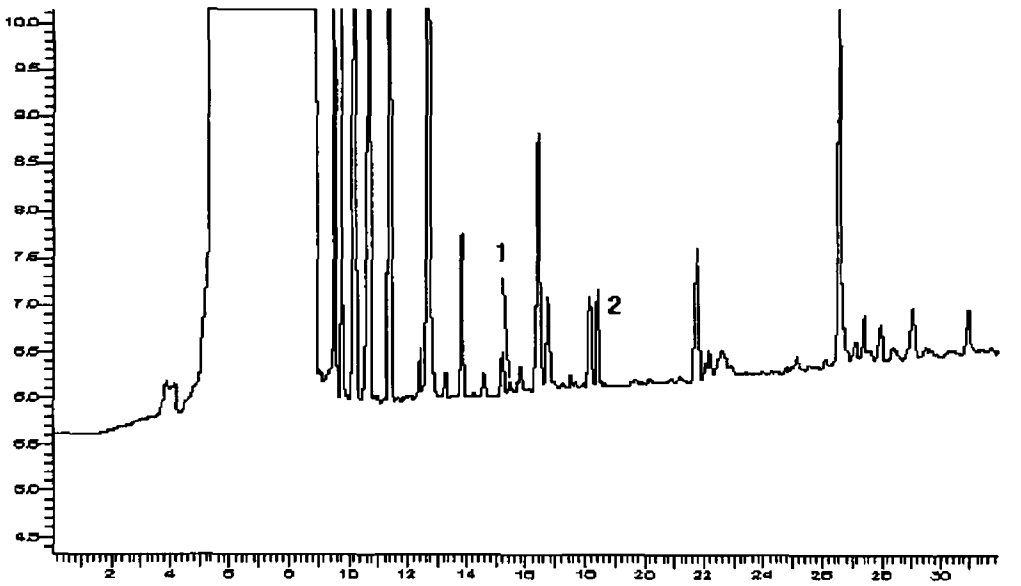


**Figure 3.6.** Full chromatogram for technique 2b (ethyl acetate extraction of stout for propylene glycol analysis) Peaks 1, propylene glycol, 2, ethylene glycol

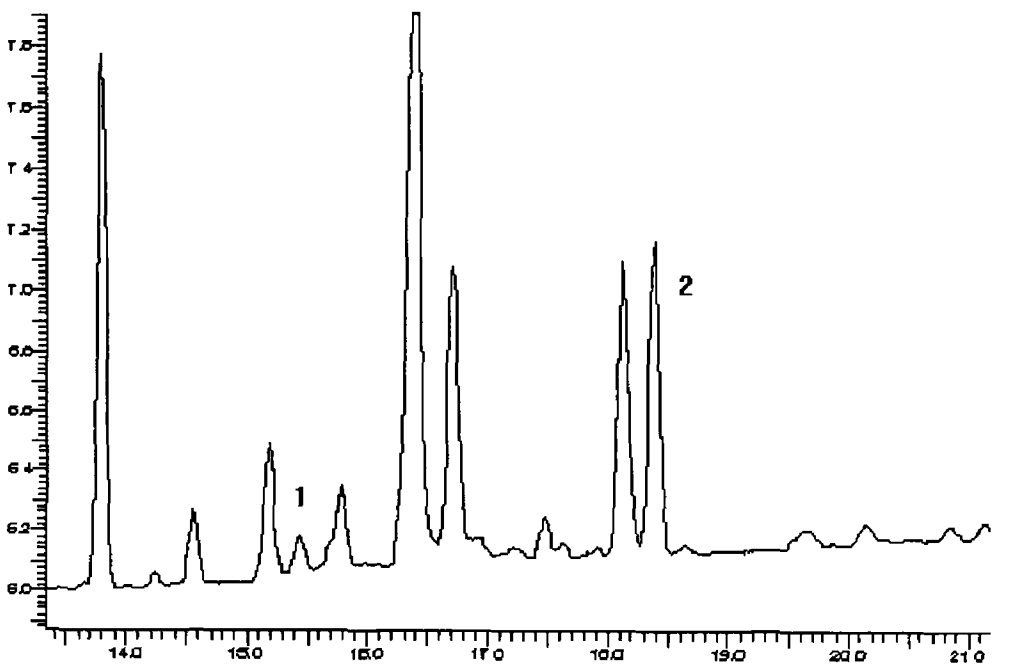


**Figure 3.7.** Expanded chromatogram for technique 2b Peaks 1, propylene glycol, 2, ethylene glycol

### 3 5 3 Suitability of Internal Standards

A stout product was subjected to SPTs 1a, 1b, and 2a described (as these were representative of all six SPTs embracing both internal standards investigated, ethylene glycol and pinacol), without the addition of internal standard, to investigate what interferences might occur as a result of peaks coeluting with the internal standard The investigation was carried out on both the polar column CP-Wax 58CB and the semi-polar DB1701 column, i e chromatographic systems 2 and 3 2 from Table 3 1

#### 3 5 3 1 Ethylene Glycol with the polar column

**Table 3 3.** Suitability of Ethylene Glycol as an Internal Standard

| AVERAGE VALUES | SAMPLE PREPARATION TECHNIQUE | | |
|---|---|---|---|
| | **1a** | **1b** | **2a** |
| EGCE * | 0 86 | <0 2 | 1 16 |
| EG (STD) ** | 22 3 | 15 3 | 4 69 |
| EGCE/EG(STD) % | 3 87 | <1 31 | 24 73 |

Raw data are given in Appendix B

*EGCE - Peak areas for peaks co-eluting with ethylene glycol These values were obtained by injecting the same preparation (repeated 5 times) and taking the average of the 5 sets of results, of peaks detected where the EG was expected to elute

** EG (STD) - Peak areas for ethylene glycol These values were taken from the Precision analysis (3 7 5 ) for the technique in question using EG as internal standard, and are an average area for EG over ten analyses

Given that there are peaks co-eluting with ethylene glycol in the stout samples analyzed which show varying degrees of interference, ethylene glycol is not suitable as an internal standard with techniques 1a and 2b for the polar column

The same trend was observed for these techniques using the medium polarity DB1701 column

### 3.5.3.2. Pinacol

In a similar exercise to 3 7 3 1 comparisons were made between chromatograms of stout samples prepared by SPTs 2b, 2c and 2d where pinacol was used as an internal standard, with chromatograms of samples not containing internal standard In the cases of SPT 2b and 2c no peaks were observed to co-elute with the pinacol on either the polar or medium polarity columns

Technique 2d demonstrated some unusual chromatographic effects, baseline resolution of the peaks on either side of the pinacol was not achieved Also the peak shape and retention time of many peaks in the chromatogram, including that of pinacol were variable from run to run and consistency was never achieved The fact that the prepared sample for technique 2d was a 50 50 acetonitrile water mix may have had some influence here This effect was not noted during the calibration and precision stages for technique 2d, but the effect could not be eliminated and may have been a result of a deterioration of the column due to water contamination
On this basis pinacol is ideal for use with techniques 2b and 2c but not with technique 2d

### 3 5.4. Recovery

SPTs 1a, 2a, 2b and 2c only, were assessed for their ability to recover the analyte from beer It was decided earlier during the course of the project that the aforementioned techniques showed the most promise Each SPT was assessed for recovery from two differing points of view

### 3.5.4.1. Recovery using A.O.A C (Association of Official Analytical Chemists) definition

Both a fortified (with 20 ppm propylene glycol) and unfortified sample of beer was analyzed by each of the techniques A figure for recovery was then generated for each technique according to the equation described by the A O A C , see Equation 1, Appendix B

**Table 3.4.** AOAC Recovery Analysis

| **Propylene Glycol** | **TECHNIQUE** | | | |
|---|---|---|---|---|
| | **1a** | **2a** | **2b** | **2c** |
| **Average % Recovery** | 89 | 157 | 77 | 89 |

Raw data are given in Appendix B

The AOAC definition of "Recovery" is "The fraction of analyte added to a test sample (fortified or spiked sample) prior to analysis, which is measured (recovered) by the method, when the same method is used to analyze both the fortified and unfortified samples" The techniques investigated show % Recovery levels of >70%, with technique 2a at 157%, this figure may be as a result of coelution

### 3 5 4.2 Recovery as Extraction Efficiency

Secondly the peak areas for internal standard and analyte resulting from each technique (data generated for precision analysis, Results and Discussion, 3 7 5 ) were compared with peak areas of solutions containing the concentration of both analyte and internal standard assuming 100% extraction from beer were achieved

**Table 3 5.** Recovery by Comparison of Peak Areas

| Technique | (Precision analysis peak area/Simulated solution peak area) % | |
|---|---|---|
| | **Propylene Glycol** | **Internal Standard** |
| **1a** | 77 | 99 (ethylene glycol) |
| **2a** | 5 7 | 1 6 (ethylene glycol) |
| **2b** | 10 | 56 (pinacol) |
| **2c** | 15 | 63 (pinacol) |

The figures in Table 3 5 demonstrate the effectiveness of recovery of the analyte from its matrix into the extraction solvent Since technique 1a is essentially a concentration step rather than an extraction, high recovery levels are expected here, and that is what the figures show The poor recovery for technique 2a is a result of the ineffectiveness of ethyl acetate as an extraction solvent for propylene glycol and ethylene glycol (internal standard) under the experimental conditions used The figures for technique 2b and 2c are quite low, in each case pinacol (the internal standard) recovery, was a factor of at least four times greater than that of propylene glycol

### 3.5.5 Precision

A stout sample was subjected to each technique 10 times to investigate the precision of each technique

The areas of the peaks for propylene glycol and ethylene glycol as well as the concentration of propylene glycol (based on the calibrations described earlier) were then compared statistically

**3 5.5.1. Technique 1a:** Pre-concentration and Ethanol Extraction (EG internal standard)

**Table 3.6.** Precision Data for Technique 1a

| | EG Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 22 3 | 3 45 | 23 8 |
| **STD Dev** | 2 96 | 0 62 | 3 16 |
| **% RSD** | 13 27 | 18 04 | 13 32 |

**3.5.5.2 Technique 1b:** Pre-concentration and Acetonitrile Extraction (EG internal standard)

**Table 3.7** Precision Data for Technique 1b

| | EG Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 15 32 | 3 37 | 26 12 |
| **STD Dev** | 1 76 | 0 58 | 2 81 |
| **% RSD** | 11 51 | 17 17 | 10 76 |

**3.5 5 3 Technique 2a·** Ethyl Acetate extraction (EG internal standard)

**Table 3 8** Precision Data for Technique 2a

| | EG Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 4 69 | 1 60 | 14 04 |
| **STD Dev** | 0 25 | 0 09 | 0 67 |
| **% RSD** | 5 29 | 5 54 | 4 74 |

**3 5.5.4 Technique 2b:** Ethyl Acetate Extraction (Pinacol internal standard)

**Table 3 9** Precision Data for Technique 2b

| | Pinacol Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 7 65 | 1 74 | 11 47 |
| **STD Dev** | 0 25 | 0 10 | 0 59 |
| **% RSD** | 3 21 | 5 97 | 5 11 |

**3.5.5.5. Technique 2c:** Acetonitrile Extraction (Pinacol internal standard)

**Table 3 10.** Precision Data for Technique 2c

| | Pinacol Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 6 64 | 3 47 | 10 49 |
| **STD Dev** | 0 32 | 0 35 | 0 88 |
| **% RSD** | 4 91 | 10 19 | 8 42 |

**3.5.5 6 Technique 2d:** Acetonitrile Extraction (Pinacol internal standard)

**Table 3.11.** Precision Data for Technique 2d

| | Pinacol Area | PG Area | Conc (ppm) |
|---|---|---|---|
| **Average** | 2 14 | 1 94 | 11 31 |
| **STD Dev** | 0 48 | 0 64 | 2 31 |
| **% RSD** | 22 37 | 32 92 | 20 42 |

Raw data are given in Appendix B

**Table 3 12.** Summary of Precision Data for the Analysis Techniques

| | Technique | | | | | |
|---|---|---|---|---|---|---|
| | **1a** | **1b** | **2a** | **2b** | **2c** | **2d** |
| **Average Concentration (ppm)** | 23 8 | 26 12 | 14 04 | 11 47 | 10 49 | 11 31 |
| **Standard Deviation** | 3 16 | 2 81 | 0 67 | 0 59 | 0 88 | 2 31 |
| **% RSD** | 13 32 | 10 76 | 4 74 | 5 11 | 8 42 | 20 42 |

Reviewing the data in Table 3 12, it is noteworthy that techniques 2a, 2b, and 2c give not only significantly lower propylene glycol results, but also very significantly lower repeatability errors than SPT 1a, 1b and 2d

Sample preparation techniques 2a and 2b show relative standard deviation (%RSD) levels significantly lower than those for the other techniques at 4 7 and 5 11% respectively It is desirable to have a technique which has a relative standard deviation of ~5% or less Technique 2a meets this objective and technique 2b is borderline The higher figures for propylene glycol found with techniques 1a and 1b are a reflection of the wider confidence intervals demonstrated in their calibration statistics outlined in Table 3 2

An investigation of the lower propylene glycol figures associated with techniques 2a - 2d was carried out by GC-MS, the conclusions of which are discussed in the Results and Discussion 3 7 6

### 3.5 6 GC-MS Analysis

A series of samples of significance to the analysis of propylene glycol in final stout product, were assessed by GC-MS using both a polar and medium polarity column i e chromatography systems 3 4 3 1 and 3 4 3 2 The samples were as follows

**Table 3.13.** Samples for GC-MS Analysis

| Sample No. | Sample Preparation Technique | Sample Details |
|---|---|---|
| 1 | 2b | Ethyl acetate extract of Stout |
| 2 | 2b | Ethyl acetate extract of RMX (2% and 4% in water)* |
| 3 | 2b | Ethyl acetate extract of TM67 (No RMX)* |
| 4 | 2b | Ethyl acetate extract of GFE (2% in water)* |
| 5 | 1a | Ethanol extract of Stout (concentrated) |

*These are raw material preparations utilised in the production of stout products, as distinct from Sample No 1 and 5 which are finished product

In some cases the above extracts were concentrated by a factor of 10 1 for GC-MS analysis This was achieved under reduced pressure by rotary evaporation at 30°C When the Palisade Bench Top/PBM software utilised here for reliability ranking retrieves a reference spectrum during a search, two reliability numbers are reported

1 Class I reliability, indicating the probability that the unknown is the same compound as, or a stereoisomer of the reference

2 Class IV reliability, indicating the probability that the unknown is a compound with structural differences from the reference that have little or no appreciable impact on the mass spectrum

Class IV reliabilities always are higher than class I reliabilities A class I reliability cannot be higher than approximately 70%, even if the match is correct This is due to the non-unique nature of mass spectra and the variations imposed by experimental differences Class IV reliabilities for a correct match often will be over 90%

The software also calculates a "percent contamination" figure A high "percent contamination" combined with a high match reliability indicates that the unknown is a mixture

During the course of the discussion of the GC/MS data the reliability ranking parameters are given in parenthesis after the peak name i e propylene glycol (class IV, class I, % contamination)

### 3 5.6 1 GC-MS with Polar NB-351 Column

Examining the data generated with the polar NB-351 column, propylene glycol was not present in the ethyl acetate extracts from SPT 2b of TM67, RMX and GFE By injection of a propylene glycol (72, 41, 6) standard the peak was observed at 13 91 minutes No peak was observed in these samples at +/- 0 3 minutes of 13 90 A peak at 13 61 minutes in the RMX sample was identified as 5-methyl-2-furancarboxaldehyde (83, 53, 4) and a peak at 13 58 in the TM67 was identified as 1,3-butanediol (79, 48, 10) No peak appeared within +/- 1 minute of an expected propylene glycol peak in the GFE sample

The ethyl acetate extract of Stout by SPT 2b gave a peak for 2,3-butanediol (86, 56, 4) at 13 58 minutes and propylene glycol (68, 37, 15) at 13 83

Duplicate analysis of an ethanol pre-concentration extract by SPT 1a showed differing results for each analysis The first sample showed a peak at 13 62 for 1,4-benzenediol (71, 35, 10) and propylene glycol (79, 48, 10) at 13 83 A second peak appeared as a shoulder on the front end of the propylene glycol peak and was identified as cyclo-pent-2-en-1,4-dione (68, 37, 20)

The second GC/MS analysis of another extract of this type identified the peak at 13 63 as 5-methyl-2-furancarboxaldehyde (24, 5, 50), the fronting shoulder peak at 18 82 as 4-H-pyran-4-one (58, 27, 30) and propylene glycol (74, 42, 13) at 13 94

The fronting peak on the propylene glycol was not observed with the ethyl acetate extract of Stout This indicates an overlapping co-elution problem as a result

of this SPT i e SPT 1a, pre-concentration followed by ethanol extraction and chromatographic analysis with a polar column The % contamination figures indicate the possibility of exact co-elution of an unknown with propylene glycol as a result of ethyl acetate extraction (SPT 2b), as the figures for % contamination for propylene glycol are slightly elevated for the beer samples over the standard injections

### 3.5.6.2. GC-MS with Medium Polarity DB1701 Column

Analysing the same TM67, RMX and GFE samples (SPT 2b) on the medium polarity DB1701 column confirmed the above observations with the polar NB-351 column By injection of a standard solution, propylene glycol (72, 41, 6) was observed to elute at 14 02 minutes With regard to these samples no peaks were observed to elute within +/- 0 3 minutes of 14 minutes

In the ethyl acetate extract of stout, SPT 2b, a peak was identified as propylene glycol (79, 48, 8) eluting at 14 04 minutes This peak was observed to stand alone, with good peak shape and with no other interfering peaks

Injection of the ethanol extract of stout gave a peak at 14 02 which was identified as propylene glycol (74, 42, 15), this peak was also observed to stand alone, with good peak shape and with no other interfering peaks i e no peak within +/- 0 5 minutes

**Table 3 14.** Summary of GC/MS Results

| Sample Type | Sample Preparation Technique | GC/MS System | |
|---|---|---|---|
| | | NB-351 Propylene Glycol | DB1701 Propylene Glycol |
| Stout | 2b | + | + |
| RMX | 2b | - | - |
| TM67 | 2b | - | - |
| GFE | 2b | - | - |
| Stout | 1a | + | + |

\+ Present, identified by GC-MS

\- Not present

### 3.5 6 3 Summary of GC/MS Data

GC-MS analysis of stout samples prepared by SPT 1a, with separation being achieved using a polar column (NB-351), has been shown not to be capable of completely resolving the PG peak from two possible fronting co-eluants, cyclo-pent-2-en-1,4-dione/4-H-Pyran-4-one This trend has also been found with the polar CP WAX 58CB column with an FID detector

On the other hand the same GC/MS system used to analyze stout samples produced by SPT 2b does not demonstrate this fronting co-elution problem This may be as a result of differences in selectivity of the solvents used for extraction in these SPTs, i e ethanol and ethyl acetate respectively The possibility of an exact co-elutant for both SPTs with the polar NB-351 has also been demonstrated

This GC-MS work accounts for the higher results for propylene glycol in stout for techniques 1a, 1b over 2a, 2b, and 2c at 14 71, 16 52 and 4 22, 3 57 and 7 94 ppm respectively

GC-MS analysis of stout samples produced by both SPT 1a and SPT 2b, with separation being achieved using a semi-polar column (DB1701), has shown clear distinct peaks for PG in both cases This is the most desirable chromatographic setup for the analysis for PG whether using SPT 1a or SPT 2b

Propylene Glycol was present in final stout product but not in RMX, GFE or wort (with no RMX added) This indicates that the PG is formed (as per the scheme given in the introduction ) either during fermentation or further processing of the stout

### 3.5.7. Formation of Propylene Glycol during the course of a Stout Fermentation

The levels of propylene glycol formed during the fermentation along with the present gravity for the samples analyzed are given in Figure 3 8 The raw data are given in Appendix B

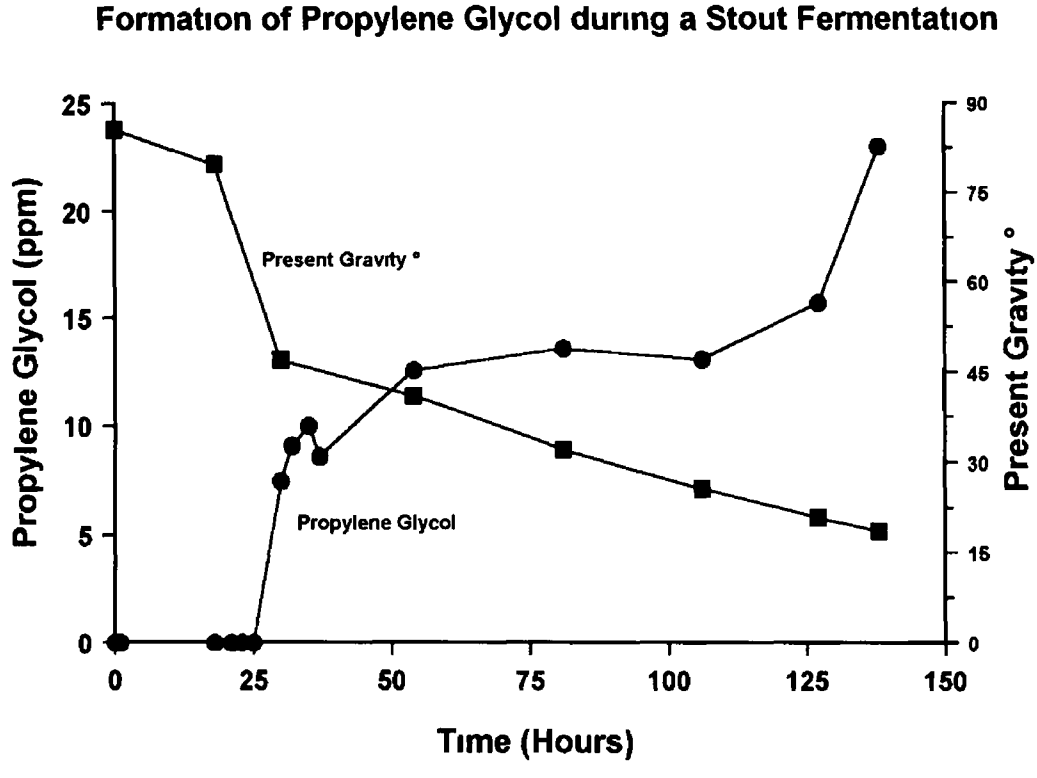


**Figure 3 8.** Propylene glycol formation during fermentation

Propylene glycol was first detected in the vessel after twenty-five hours of fermentation From this time it rose steadily to a maximum value of 23 ppm at 0 5° from completion of the fermentation step The initial increase after 25 hours coincided with a large decrease in the gravity of the fermenting wort After diluting the fermented beer to its packaging gravity one would expect propylene glycol levels in the order of 10 ppm This is borne out by results given earlier for calibration and precision of SPT 2b

## 3.6. CONCLUSION

The use of an ethyl acetate extraction, salting out with ammonium sulphate, coupled with splitless gas chromatography using a semi polar DB1701 chromatography column has been shown to have the most potential for the analysis for propylene glycol in beer

Of the sample preparation techniques investigated it is the most easily automated and gave the most statistically satisfactory calibration and precision data Chromatographic separation on a DB1701 column and has been shown not to suffer from chromatographic interferences and gives completely resolved peaks of interest

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# AN INVESTIGATION INTO STYRENE IN BEER

## 4.1. INTRODUCTION

Some yeast strains used for the fermentation of beers and wines contain an enzyme activity which has been shown[1-5, 10] to transform phenolic acids present in wort and must into their corresponding vinyl phenols. These phenolic compounds can impart a phenolic or pharmaceutic taint at higher than threshold levels to beers and wines. In beers these are referred to as phenolic off-flavours (Pof)[8]. Yeast strains which demonstrate this capability are described as $Pof^{+}$, some yeast strains which exhibit a weak decarboxylating activity are denoted $Pof^{+/-}$. This activity may be variable depending on the yeast strain. Strains of Saccharomyces cerevisiae normally used in brewing are $Pof^{-}$, but some brewing strains have been found to posses $POF^{+/-}$ characteristics[46], therefore paradoxically such phenolic off-flavours are appreciated as an integral characteristic of some beers. Certain brewers use $Pof^{+}$ yeast strains to give their beers a phenolic, spicy, clove-like flavour, e.g. German weissbiers.

This conversion of phenolic acids to vinyl phenols has been demonstrated to be a non-oxidative decarboxylation[6,7] of the phenolic acid. Two different pathways have been postulated for the conversion (see Figure 4.1). On the basis of stereochemical considerations Chatonnet *et al.*[9] proposed that only pathway B is possible.

**Decarboxylation of Phenolic Acids to Vinyl Phenols.**

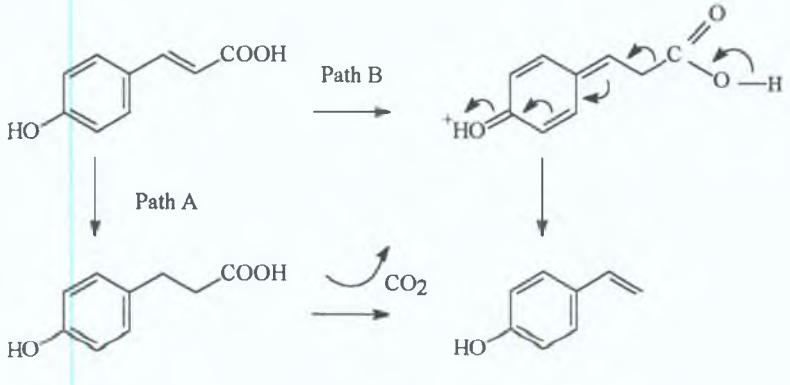


**Figure 4.1.** Reaction pathways for vinylphenol synthesis by S. cerevisiae. Path A, by a phenylpropanoic acid intermediate; path B, direct decarboxylation.

When $Pof^{+}$ yeast strains are used in brewery fermentations, the presence of phenolic off-flavours in beer is indicative of infection. Over the years there have been many attempts to develop analytical techniques for the quantitation of (i) phenolic acids in wort and beer[10-18], and (ii) the decarboxylation products of these phenolic acids by $Pof^{+}$ and $Pof^{+/-}$ yeasts in beers and other matrices[19-25].
A table of the most abundant phenolic acids in worts and beers and their decarboxylated products is given below.

**Table 4.1.** Enzymatic Decarboxylation Products of Phenolic Acids by S. cerevisiae

| Phenolic Acid | Corresponding Phenol |
|---|---|
| 3,4,5-Trihydroxybenzoic | Pyrogallol |
| 4-Hydroxybenzoic | Phenol |
| 3,5-Dihydroxybenzoic | Pyrocatechol |
| 4-Hydroxy-3methoxybenzoic | Guaiacol |
| 4-Hydroxy-3,5-dimethyloxybenzoic | Syringol |
| Cinnamic | Styrene |
| 4-Hydroxycinnamic | 4-Vinylphenol |
| 4,5-Dihydroxycinnamic | Vinylcatechol |
| 4-Hydroxy-3-methoxycinnamic | 4-Vinylguaiacol |
| 4-Hydroxy-3,5-dimethoxycinnamic | Vinylsyringol |
| 2-Methoxycinnamic | 2-Methoxy-vinylphenol |
| 3,4-Dimethoxy-2-phenylpropan-1-oic | 3,4-Dimethoxy-vinyl phenol |

Much of the work in this area has concentrated on 4-hydroxy-3-methoxycinnamic acid (ferulic acid) and its decarboxylated derivative 4-vinylguaiacol (4VG). This is because of the low flavour threshold of 4VG and the clove-like flavour it imparts to beer. The threshold has been given as 0.3 ppm in Mexican

beers[47] and 0.31 ppm in extra strong stouts[48].

This project investigated a related decarboxylation i.e. the conversion of cinnamic acid to styrene, the objective being to develop a rapid analytical technique for the determination of styrene in beers and worts. The technique was used to analyze a variety of beer products for styrene and investigate styrene formation during the course of fermentation with a $Pof^{+/-}$ strain. Studies of the kinetics of styrene synthesis from cinnamic acid by $Pof^{+}$ and $Pof^{+/-}$ yeast strains are also reported.

In 1956 it was established[26] that a mutant of S. cerevisiae produced styrene from cinnamon in hot cross buns. Hope[27] developed a technique for the detection of $Pof^{+}$ wild yeasts utilising cinnamic acid as the basis of a medium for yeast growth. The cinnamic acid was shown to inhibit $Pof^{-}$ yeast growth (brewing yeast) permitting growth of $Pof^{+}$ wild yeast contaminants. Because of the highly pungent nature of styrene the presence of contaminant yeast strains is easily qualitatively detected by sniffing.

**Table 4.2.** Styrene Levels in Various Dairy Products

| Product | Styrene (ppb) | Reference |
|---|---|---|
| **Yogurt** | 3 - 220 | 28,40,41 |
| **Yogurt** | 100 | 35 |
| **Sour cream** | 4 - 246 | 28,40,41 |
| **Butterfat** | 22 - 59 | 28,40,41 |
| **Milk** | 14 - 17 | 28,40,41 |
| **Cottage Cheese** | 6 - 9 | 28,40,41 |

Polystyrene is used for packaging a wide variety of food products including dairy products. Residual styrene monomer may be leached into food in small amounts from this packaging and adversely affect sensory properties of the food. It is for this reason that the analysis for styrene in various matrices has been exhaustively examined[28-32]. Methods for isolation of styrene in dairy products have

involved solvent extraction[33], distillation[34-36], azeotropic distillation with methanol followed by extraction with hexane[37], and headspace[35,38,39] methods Typical concentrations of styrene in dairy products are given in Table 4 2

The influence of fat content, alcohol, acidity or citrus fruit aroma of foodstuffs on extent of migration of styrene from packaging have also been reported[42,43]

There is little information in the literature regarding the styrene content of beers Renner *et al*[44], using a purge and trap technique demonstrated the presence of styrene in a pilsner type beer at 100 ppb, while Tressl *et al*[45], using a liquid/liquid extraction followed by GC-MS found styrene at 70 ppb also in a pilsner type beer product

### 4.1.1. Enzyme Theory

Enzymes are highly specialised proteins, which demonstrate remarkable catalytic power This catalytic activity is generally far greater than that of synthetic catalysts They have a high degree of specificity for their substrates and accelerate specific chemical reactions without the formation of by-products They cannot change the equilibrium point of the reactions they promote, nor are they used up or permanently changed by these reactions

Catalysts lower the activation energy barrier of chemical reactions The activation energy is the amount of energy required to bring all of the molecules in 1 mmol of a substance to the transition state At this point there is an equal probability for them to undergo reaction to form product or fall back to a pool of unreacted molecules The rate of any chemical reaction is proportional to the concentration of the transition state species The catalyst, in this case an enzyme E, achieves this by combining transiently with the reactant A to produce a new complex EA whose transition state has a much lower activation energy than the transition state of A in the uncatalysed reaction The enzyme reactant complex EA, then reacts to form the product P, releasing the free enzyme, which can combine with another molecule of A and repeat the cycle

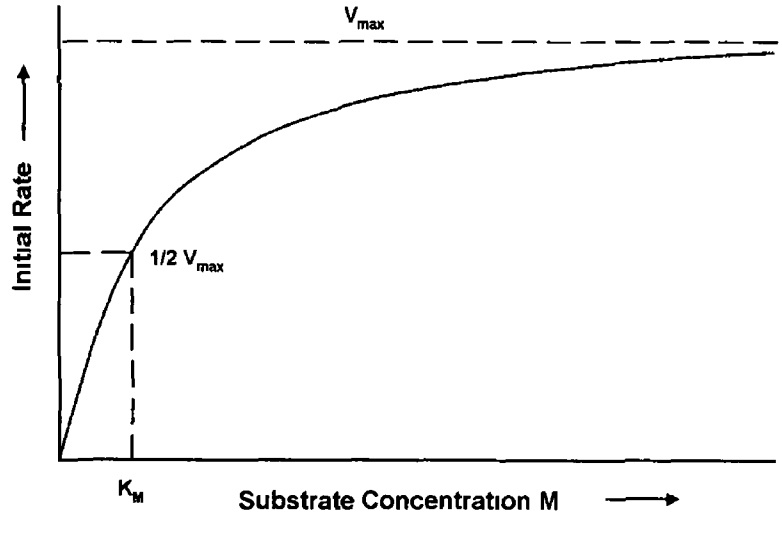


**Figure 4 2** Effect of substrate concentration on the initial rate of an enzyme catalysed reaction

In order to examine the kinetics of the enzyme that catalysed phenolic acid to vinyl phenol conversion an understanding of the effects of varying the substrate concentration on the initial rate of reaction when the enzyme concentration is held constant is important At very low concentrations of substrate the rate of the reaction is very low, but it will increase with an increase in the substrate concentration But, as the increases in substrate concentration get larger it is noted that the initial rate of reaction increases by smaller amounts Finally a point will be reached beyond which there are only small increases in the reaction rate with increasing substrate concentration Regardless of how high the substrate concentration is raised beyond this point, the reaction rate will approach but never quite reach a plateau At this plateau, called the maximum rate ($V_{max}$), the enzyme is saturated with its substrate and can function no faster This is demonstrated in Figure 4 2 This saturation effect is exhibited by nearly all enzymes

This concept was expanded into a general theory of enzyme action by Leonor Michaelis and Maud Menten in 1913 By examining Figure 4 2 it is difficult to estimate from the closer and closer approach of the reaction rate to the $V_{max}$ exactly what substrate concentration is required to attain $V_{max}$ However, because the curve expressing this relationship has the same general shape for all enzymes, Michaelis and Menten defined a constant, $K_M$, that is useful in establishing the precise relationship between the substrate concentration and the velocity of the enzyme catalysed reaction $K_M$, the Michaelis-Menten constant, is defined as the concentration of a specific substrate at which a given enzyme yields one half its maximum velocity

The characteristic shape of the substrate-saturation curve for an enzyme can be expressed mathematically by the Michaelis-Menten equation

$$v_o = V_{max}[S] / K_M + [S] \qquad (1)$$

where $v_o$ = initial rate at substrate concentration [S]

$V_{max}$ = maximum rate

$K_M$ = Michaelis-Menten constant of enzyme for particular substrate

This equation was derived by Michaelis-Menten starting from the basic hypothesis that the rate-limiting step in enzymatic reactions is the breakdown of the enzyme-substrate (ES) complex to form the product and the free enzyme If $K_M$ and $V_{max}$ is known, then it is possible to calculate the reaction rate of an enzyme at any given concentration of its substrate

The key element in the Michaelis-Menten equation is $K_M$, which is characteristic for any given enzyme for a specific substrate under defined conditions of pH and temperature It is difficult to obtain an accurate value from the earlier plot for $V_{max}$, as it is only approached but never actually attained A more accurate value of $K_M$ can be obtained by plotting the data in a different manner, called a double reciprocal or Lineweaver-Burk plot This is achieved by a simple algebraic transformation of the Michaelis-Menten equation giving equation 2

$$\frac{1}{v_o} = \frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

For enzymes obeying the Michaelis-Menten relationship exactly, a plot of $1/v_0$ against 1/[S] yields a straight line This line will have a slope of $K_M/V_{max}$, an intercept of $1/V_{max}$ on the $1/v_0$ axis and an intercept of $-1/K_M$ on the 1/[S] axis

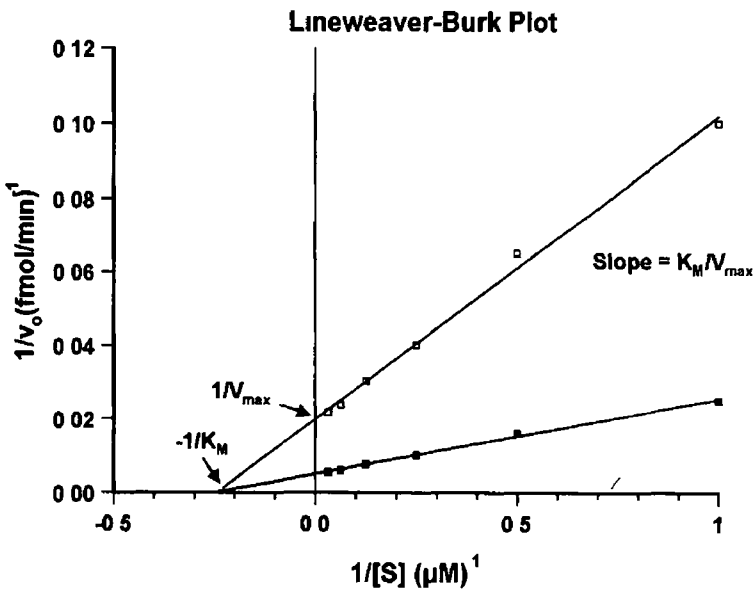


**Figure 4 3.** Double reciprocal/Lineweaver-Burk plot

The kinetic studies carried out in this work utilised the Lineweaver Burk plot to ascertain the $K_m$ and $V_{max}$ for the enzyme systems being studied

## 4.2. MATERIALS AND METHODS

### 4 2.1. Instrumentation

A PE 8420 series gas chromatograph with flame ionisation detector coupled to a Spantech TD4 single shot thermal desorption unit was used throughout the study A PE 8700 series gas chromatograph with flame ionisation detector coupled to a PE HS-101 headspace autosampler was also used throughout the study Perkin-Elmer Turbochrom V 4 data-handling software was used to analyze the data A Beckman Model J-21B centrifuge was used throughout

### 4.2.2 Materials

Analar-grade granular ammonium sulphate was supplied by Aldrich Chemical Co., The Old Brickyard, New Road, Gillingham, Dorset, England Styrene, Methyl Propyl Ketone and Isobutanol were supplied by Fluka, The Old Brickyard, New Road, Gillingham, Dorset, England Cinnamic acid (*trans*) was supplied by Sigma-Aldrich Company Ltd , Fancy Road, Poole, Dorset, BH17 7NH, England Ethanol was supplied by Hayman Ltd , 70 Eastways Industrial Park, Witham, Essex CM8 3YE, England Glucose and silicone anti-foam were supplied by BDH, Merck Ltd , Broom Rd , Poole, Dorset BH12 4NN, England Tenax TA was supplied by Perkin Elmer Ltd , Maxwell Road, Beaconsfield, Bucks HP9 1QA, England Helium, hydrogen, oxygen and air were supplied by Air Products Ltd , Unit 950 Western Industrial Estate, Killeen Road, Dublin 12, Ireland All were of a purity suitable for gas chromatography Lager wort was obtained from the lager plant, and stout wort from the brewhouse at Guinness Ireland Group, St James's Gate, Dublin Malt Extract and Agar Technical No 3 were supplied by Unipath Ltd , Basingstoke, Hampshire, England Yeast Extract and Peptone were supplied by DIFCO, Detroit, Michigan, U S A Zinc was supplied as zinc sulphate by Koch-Light Ltd , Coinbrook, Bucks , England

### 4.2.3. Equipment

Analytical balance accurate to 4 decimal places Automatic pipettes, 1 $cm^3$

pipette, 200 µl pipette 100 µl pipette, all positive displacement Liquid tight syringe 5 µl 250 $cm^3$ Erlenmeyer flasks, 250 $cm^3$ centrifuge pots, 100 $cm^3$ Duran flasks, 100 $cm^3$ Volumetric flasks Quickfit Dreschel bottle head Perkin-Elmer thermal desorption tubes and accessories Gas flowmeter capable of delivering 100 $cm^3$/min Helium McCartney bottles (22 $cm^3$ volume) Incubator, capable of maintaining 28°C overnight 15 L Fermentation Vessels

### 4.2.4. Standard Solutions

**4.2.4.1** The stock solution for methyl propyl ketone (MPK) was 0 5% w/v in ethanol

**4.2.4.2** The working standard solution for headspace analysis was 0 025% MPK/0 2% isobutanol w/v in 4% ethanol i e take 5 $cm^3$ of 4 1 into a 100 $cm^3$ volumetric flask with 0 2 g of isobutanol and 4 $cm^3$ of ethanol and make up to the mark with distilled water

**4.2 4 3** The working solution for cinnamic acid was 1% w/v in ethanol

### 4.2.5. Sample Preparation Techniques

#### 4.2.5.1 Purge and Trap-Thermal Desorption Technique

A 125 $cm^3$ portion of the sample for analysis was placed into a 250 $cm^3$ Dreshel bottle to which 1 drop of a 10% silicone anti-foam solution and 2 5 µl of 0 5% w/v MPK solution were added The flask was then immersed into a water bath at a controlled temperature of 50°C +/- 1°C The Dreschel head was placed into the flask and secured The gas flowmeter was attached to the inlet end of the Dreschel fitting and the thermal desorption tube to the outlet end with the grooves on the tube closest to the outlet Helium was allowed to bubble through the beer and exit the vessel via the sampling tube at a flowrate of 50 $cm^3$/minute for 30 minutes The tube was then removed from the apparatus and capped It was then preflushed with helium at 50 $cm^3$/minute for five minutes in preparation for purge and trap gas chromatographic analysis

If dilution was deemed necessary, then the sample was diluted with distilled water at 4% w/v ethanol After analysis the tubes were reconditioned for further usage as outlined in Appendix C

#### 4.2 5 2 Headspace Technique

A 5 $cm^3$ portion of sample for analysis was placed into a 22 $cm^3$ headspace vial containing 3 5 g of ammonium sulphate 100 µl of the headspace working standard solution was placed into the vial, which was then sealed in readiness for incubation and headspace GC analysis If dilution was deemed necessary, then the sample was diluted with distilled water at 4% w/v ethanol

### 4.2 6. Experimental Techniques

#### 4.2.6.1 Styrene Production during the Course of a Fermentation

Two 15 L fermentations were set up to mimic the experimental conditions in a production environment Stout worts were pitched with a stout yeast known to be $Pof^{+/-}$ at a rate of 2 2 g/L Oxygenation was carried out for twenty minutes, fermentation was allowed to proceed at a temperature of 24°C for three days with a sample being taken every 2 hours over 8 hour periods for styrene analysis, pH and PG

The fermentations were staggered to achieve a wide time profile of the fermentation The samples were centrifuged immediately and stored cold to ensure fermentation had stopped

#### 4 2.6.2 Method for the Qualitative Determination of Phenotype by Plating the Specific Yeast Strain in a Cinnamic Acid Rich Medium

A loopful of yeast was inoculated in duplicate into 10 $cm^3$ of sterile hopped lager wort, in a McCartney bottle One culture was supplemented with 100 µl of cinnamic acid (1% w/v ethanol), i e to give a level of 100 ppm in the culture The samples were incubated at 28°C for 24 hours and the aroma was assessed at this time The incubated wort samples were also analyzed by headspace gas

chromatography to determine relative amounts of styrene produced

Contaminant (wild) yeasts were selectively plated to achieve pure colonies where necessary, prior to the above test Known $Pof^+$ and $Pof^-$ yeast strains served as controls

#### 4.2.6.3. Method for the Study of the Kinetics of Styrene Production by Various $Pof^+$, $Pof^{+/-}$ and $Pof^-$ Yeast Strains

A loopful of the yeast strain was aseptically transferred from an MYGP agar slope from the Guinness Brewing Worldwide culture collection (make-up of this MYGP media is given in Appendix C) to a sterile 250 $cm^3$ conical flask containing 100 $cm^3$ of lager wort supplemented with zinc (0 2 ppm) This solution was allowed to incubate while shaking at 24°C for two days

From this flask, 2 $cm^3$ of culture was aseptically transferred to each of sixteen 250 $cm^3$ conical flasks containing 100 $cm^3$ of the zinc supplemented lager wort and incubated while shaking at 24°C for one day Yeast cells were harvested by centrifugation at 3020 x g for fifteen minutes, combining the contents of two flasks into one 250 $cm^3$ sterile centrifuge pot The supernatant was poured off and each yeast pellet was resuspended in 50 $cm^3$ of sterile distilled water at 25°C in a 100 $cm^3$ Duran flask The required level of cinnamic acid was added and the flasks were shaken and incubated at 25°C After incubation the samples were centrifuged at 3020 x g for fifteen minutes to remove the yeast Supernatants were refrigerated at 4°C until analyzed

Total yeast counts were determined using a haemocytometer

### 4.2.7 Chromatographic Methods

#### 4.2.7.1. Purge and Trap Gas Chromatographic Method

A Perkin-Elmer 8420 series gas chromatograph coupled to a flame ionisation detector (FID) was used, with a Chrompak CP-SIL 5CB (50 m x 0 32 mm id WCOT, 1 2 µm film thickness, with a coating efficiency of >80%) chromatography column The oven temperature was held at 30°C for 15 minutes and ramped to 100°C at 2°C/minute The temperature was then ramped to 200°C at 5°C/minute and then to 250°C at 10°C/minute with a 15 minute hold at 250°C This resulted in a total run time of 90 minutes

The carrier gas used was helium, the injector and detector temperatures were 250°C and a split flow of 30 $cm^3$/minute was maintained at the gas chromatograph injection port The sample was introduced onto the GC from the sample tube via the Spantech TD4 thermal desorption unit The volatile material was removed from the sample tube by desorbing at 250°C for 10 minutes onto the cold trap set at -30°C The sample was transferred to the chromatography column by flash heating the cold trap to 300°C, with an injection time of 45 seconds and a line temperature of 200°C

#### 4 2.7.2. Headspace Gas Chromatographic Method

A Perkin-Elmer 8700 series gas chromatograph coupled to both a flame ionisation detector (FID) and an electron capture detector (ECD) was used, with a Chrompak CP-WAX 57CB (60 m x 0 25 mm id WCOT, 0 42 µm film thickness, with a coating efficiency of > 80%) chromatography column An isothermal run at 55°C, with a run time of 44 minutes was employed

The carrier gas was helium, with nitrogen used as make-up for the ECD, the injector temperature was 105°C, FID temperature was 225°C and ECD temperature was 110°C A post column split ratio of 3 1 FID ECD was maintained

The sample was introduced onto the GC via a Perkin-Elmer HS-101 headspace autosampler The sample contained in a 22 $cm^3$ sample vial was incubated for 40 minutes at 35°C prior to injection The needle temperature was 60°C, the transfer line was at 105°C with a vial pressurisation time of 0 5 minutes, an inject time of 0 27 minutes and a withdrawal time of 0 1 minutes

## 4.3 RESULTS AND DISCUSSION

### 4.3 1 Calibration

#### 4 3.3 1 Purge and Trap

Styrene was calibrated for by the purge and trap sample preparation technique coupled with thermal desorption/gas chromatography by making additions of 0 - 80 ppb styrene to beer and then carrying the beer through the steps outlined in Materials and Methods sections 4 2 5 1 and 4 2 7 1 The calibration curve obtained is shown in Figure 4 4 The raw data are given in Appendix C

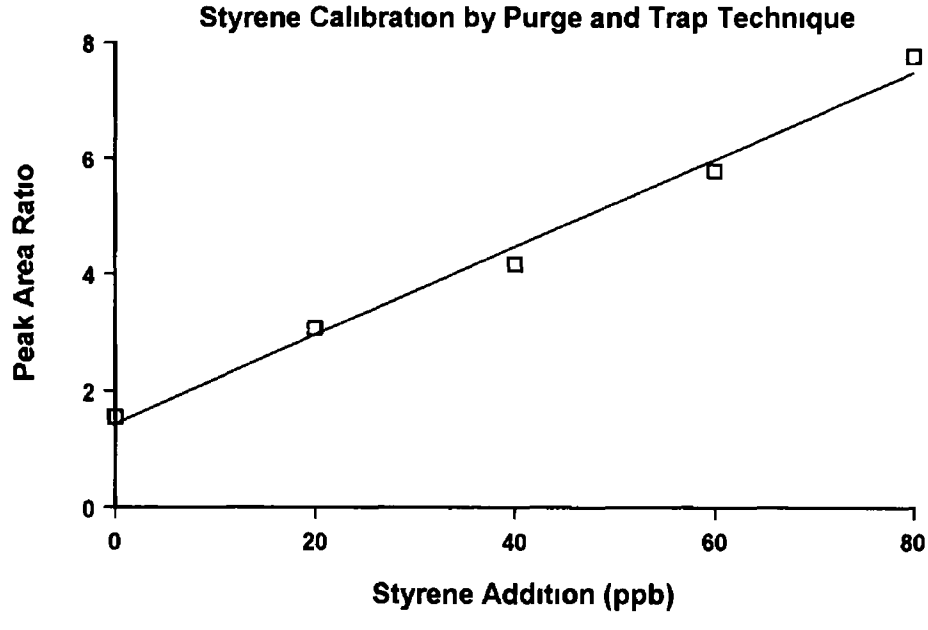


**Figure 4 4** Calibration by purge and trap of styrene

The correlation coefficient for the regression of Styrene addition (ppb) on Area ratio was 0 9950 with a confidence interval at the 95% level of +/- 10 07 ppb The detection limit of styrene for this technique, calculated at a signal noise ratio of 2, was 12 ppb

### 4.3 1.2 Headspace Analysis

Styrene was calibrated for by the headspace sample preparation technique/gas chromatography by making additions of 0 - 80 ppb to beer and then carrying the beer through the steps outlined in Materials and Methods sections 4 2 5 2 and 4 2 7 2 The calibration curve obtained is shown in Figure 4 5 The raw data are given in Appendix C

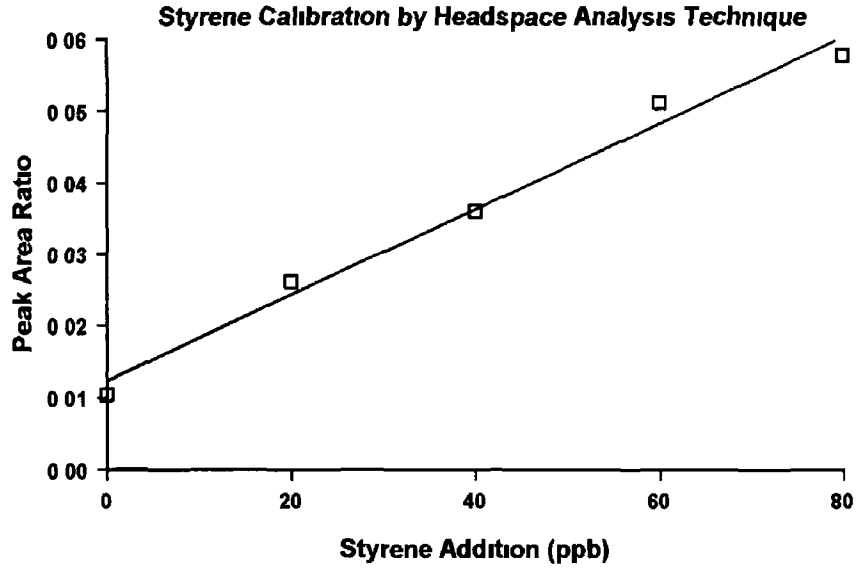


**Figure 4 5** Calibration by headspace analysis of styrene

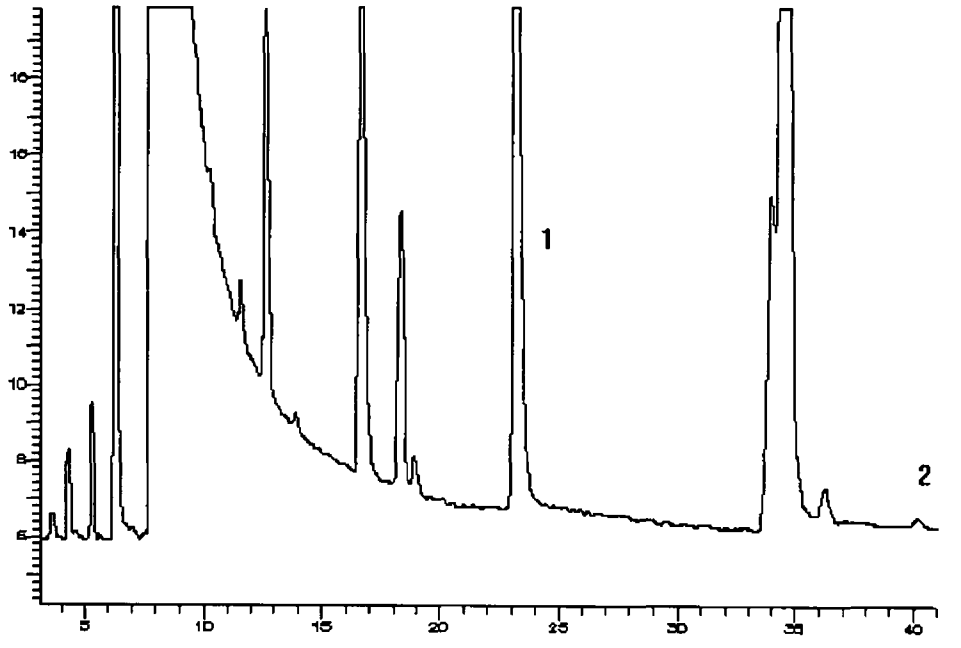


**Figure 4.6.** Chromatogram for the headspace analysis for styrene Peaks 1, n-butanol (internal standard), 2, styrene

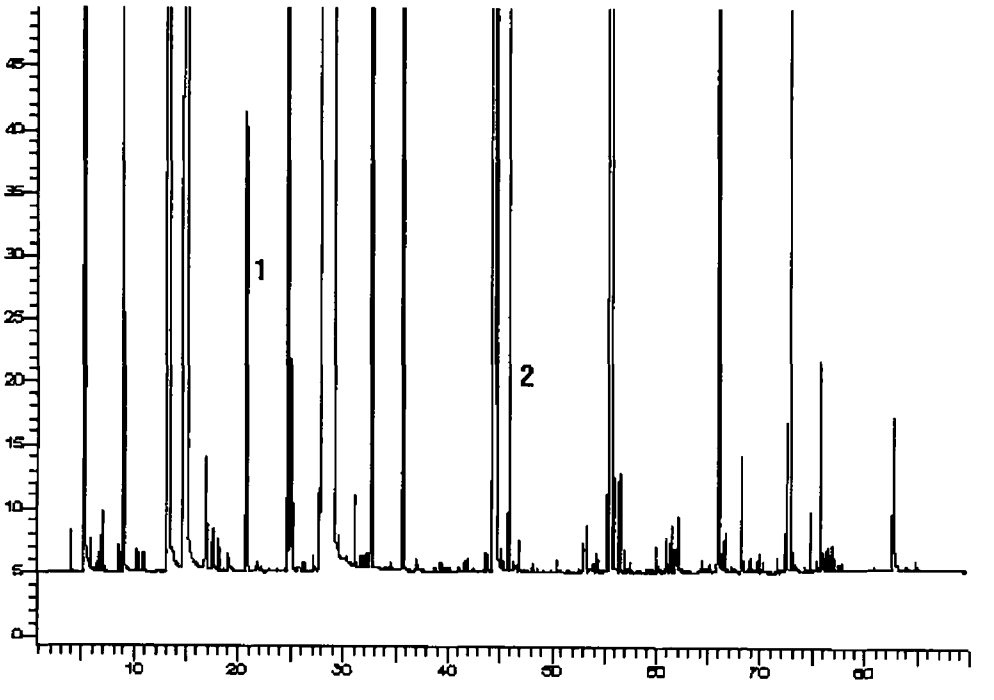


**Figure 4.7** Chromatogram for the purge and trap analysis of styrene Peaks 1, n-butanol (internal standard), 2, styrene

### 4.3.2. Styrene Levels in Beers

Results for analysis of commercially available beers are given in Table 4 3 Names of individual products are not listed here, but a description of beer type is given All of these beers were analyzed by the purge and trap sample preparation technique/thermal desorption/gas chromatography

#### 4.3.2 1 Styrene levels in Non-alcoholic beers (NAB's), Lagers, Ales and a Weissbier

**Table 4.3** Analysis of NAB's, Lagers, Ales and a Weissbier for Styrene

| Product | Type | Styrene (ppb) | Product | Type | Styrene (ppb) |
|---|---|---|---|---|---|
| **A** | NAB | 21 | **H** | NAB | <1 |
| **A** | NAB | 17 | **I** | NAB | <1 |
| **A** | NAB | 16 | **J** | NAB | <1 |
| **A** | NAB | 24 | **K** | NAB | <1 |
| **A** | NAB | 31 | **L** | LAGER | <1 |
| **A** | NAB | 27 | **M** | LAGER | <1 |
| **A** | NAB | 40 | **N** | LAGER | <1 |
| **A** | NAB | 17 | **O** | LAGER | <1 |
| **B** | NAB | 9 | **P** | LAGER | <1 |
| **C** | NAB | <1 | **Q** | LAGER | <1 |
| **D** | NAB | <1 | **R** | LAGER | <1 |
| **E** | NAB | <1 | **S** | ALE | <1 |
| **F** | NAB | <1 | **T** | ALE | <1 |
| **G** | NAB | <1 | **U** | WEISS | 31 |

NAB = Non-alcoholic beer

Lager = Lager > 3 4% Alcohol by volume

Ale = Ale > 3 4% Alcohol by volume

Weiss = Weissbier > 3 4% Alcohol by volume

The techniques presently used for non-alcoholic/low-alcoholic beer production include cold contact fermentation, restricted fermentation, immobilised yeast technology or alternatively dealcoholisation of regular beer by evaporation or reverse osmosis

While the actual production techniques used for the beverages analyzed here are not known, product A has consistently shown levels of styrene of approximately 25 ppb and product B has a level of 9 ppb styrene This is indicative of the use of a $Pof^{+/-}$ yeast strain during production or contamination in a $Pof^{-}$ production environment by a $Pof^{+}$ or $Pof^{+/}$ yeast strain None of the other NAB products analyzed had styrene present

As expected neither the lager or ale products contained styrene, $Pof^{-}$ yeasts are used for fermentation here The weissbier contained 31 ppb styrene resulting from the $Pof^{+/}$ yeast traditionally used in its production

#### 4.3.2.2. Stout Products Available on the Irish Market

**Table 4.4.** Analysis of Stout Products for Styrene

| Product | Type | Pack | Styrene (ppb) |
|---|---|---|---|
| A | STOUT | KEG | 22 |
| A | STOUT | KEG | 23 |
| A | STOUT | KEG | 27 |
| A | STOUT | KEG | 26 |
| B | STOUT | CAN | <1 |
| B | STOUT | KEG | <1 |
| B | STOUT | KEG | <1 |
| B | STOUT | KEG | <1 |
| C | STOUT | CAN | 26 |
| C | STOUT | CAN | 20 |
| C | STOUT | BOTTLE | 29 |
| C | STOUT | KEG | 39 |
| C | STOUT | KEG | 26 |
| C | STOUT | BBT | 19 |

Three different stout products available on the Irish market were analyzed for styrene Products A and C were consistently > 20 ppb (this was found to be the case across all packaging types), while product B did not contain styrene

Contamination is unlikely to be a factor here given the consistent levels of styrene found over the time scale of the samples analyzed, therefore the conclusion must be that the yeast strains used during fermentation are $Pof^{+/}$ for products A and C

#### 4.3.2.3. Styrene Levels in Overseas Produced Stout Products

A range of stout products from overseas markets was analyzed for their styrene content

**Table 4.5.** Styrene in Overseas Stout Products

| Product | Type | Pack | Styrene (ppb) |
|---|---|---|---|
| **D** | STOUT | BOTTLE | 10 |
| **E** | STOUT | BOTTLE | 7 |
| **F** | STOUT | BOTTLE | <1 |
| **G** | STOUT | BOTTLE | 19 |

Product F is the only product of those analyzed with no styrene present Clearly a $Pof^{-}$ yeast strain is used for its production The other products show varying levels up to 20 ppb styrene Each of products D, E and G must be brewed using a yeast strain containing $Pof^{+/}$ characteristics In certain circumstances this may impinge on the flavour profile of these products given their ability to produce other vinyl phenols with low flavour thresholds e g 4-vinyl guiacol

### 4.3.3. Styrene Production during a Stout Fermentation

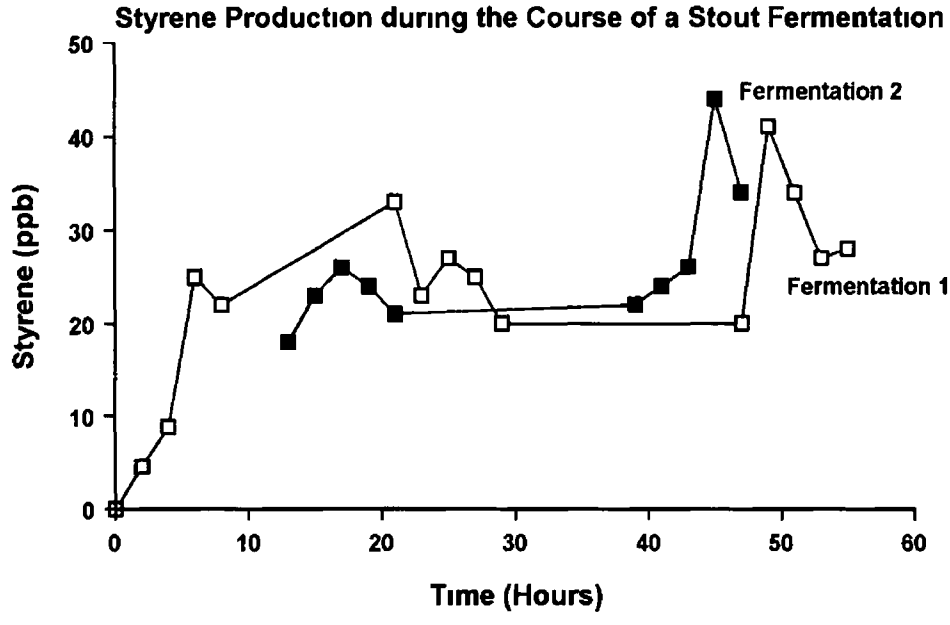


**Figure 4.8.** Formation of styrene during the course of a stout wort fermentation with a $Pof^{+/}$ yeast strain

Samples for this investigation were analyzed by the purge and trap sample preparation technique/thermal desorption/gas chromatography The raw data are given in Appendix C Data were collected from two staggered fermentations, the objective being to gather the most information possible over the course of a three day fermentation There is good overlapping agreement between the two fermentations SF1 (Styrene Fermentation 1) and SF2 as Figure 4 8 above demonstrates

Styrene has been shown to reach levels of up to 20 ppb in the first ten hours of fermentation and from that point on varies between 20 ppb to 44 ppb A simple explanation for this variability in styrene content is not obvious at this point, except that it is likely that styrene is continually being formed and removed from the system with the $CO_2$ evolving during the course of fermentation Another possibility is that $O_2$ present at the beginning of fermentation promotes styrene formation

Investigations into the kinetics of styrene formation may shed further light on this

### 4.3.4 Determination of Phenotype of a Range of Yeast Strains

Several series of known yeast strains as well as some unknown wild yeasts from the Guinness culture collection were plated out in a cinnamic acid rich medium as described in the Materials and Methods section 4 2 6 2 , to investigate their ability to produce styrene The samples were assessed by olfactory analysis and headspace gas chromatography

**Table 4 6** Phenotype of a Variety of Yeast Strains

| Yeast Strain | Yeast Type | Nose | Styrene |
|---|---|---|---|
| **A** | Known/Wild | +++ | 32 ppm |
| **B** | Known/Wild | +++ | 28 ppm |
| **C** | Known/Stout | + | 2 ppm |
| **D** | Known/Lager | - | <1 ppb |
| **E** | Unknown/Wild | +++ | 47 ppm |
| **F** | Unknown/Wild | - | <1 ppb |
| **G** | Known/Wine | - | <1 ppb |
| **H** | Known/Wine | +++ | 35 ppm |
| **I** | Known/Wine | +++ | 62 ppm |
| **J** | Known/Wine | +++ | 30 ppm |
| **K** | Known/Wine | - | <1 ppb |

| | |
|---|---|
| + | Weak presence of Styrene |
| +++ | Strong presence of Styrene |
| - | Styrene not present |

Yeast strains A and B were carried through the procedure as positive controls and produced large amounts of styrene, whereas yeast strain D a Pof$^-$ lager yeast produced no styrene This test will easily distinguish a Pof$^+$ from a Pof$^-$ yeast strain, both by smell and headspace gas chromatography The stout yeast strain C is used to ferment one of the stout products described earlier producing levels of styrene of approximately 20 ppb This yeast has given a positive result here, but not to the same extent as the other Pof$^+$ strains Therefore this yeast strain should be described as Pof$^{+/}$

Yeast strain E is a strain isolated from a fermentation plant in a brewery, and demonstrates the problems associated with wild yeast strain contamination It is clearly Pof$^+$

A wild yeast strain F, isolated from a brewery, has been shown to be Pof$^-$ These test results demonstrate the ease with which an unknown wild yeast strain can be classified quickly with regard to its ability to decarboxylate phenolic acids present in wort to vinyl phenols, and hence the extent to which it will taint beer with phenolic off flavours

Conflicting reports exist regarding the ability of wine yeast strains to decarboxylate phenolic compounds Chatonnet *et al* [9] stated as a result of their own studies and using the work of Gramatica *et al* [7] as further evidence, that wine yeast strains are incapable of decarboxylating cinnamic acid to form styrene They also directly contradict the conclusions of Dubois[49] An investigation using the technique described here on 5 yeast strains G - K used for wine production shows that three of these strains are clearly Pof$^+$ and decarboxylate cinnamic acid to styrene

### 4 3.5 Study of the Kinetics of Styrene Production by Yeast Strains A and C

Varying concentrations of cinnamic acid were added to sterile distilled water at 25°C containing the yeast strain of interest, as per Materials and Methods section 5 5 The levels of styrene produced in each case was determined by headspace sample preparation technique/gas chromatographic analysis The data obtained in the case of each yeast strain are presented below as a Lineweaver-Burk plot

### 4.3 5.1 Lineweaver-Burk Plot for Yeast Strain A Data

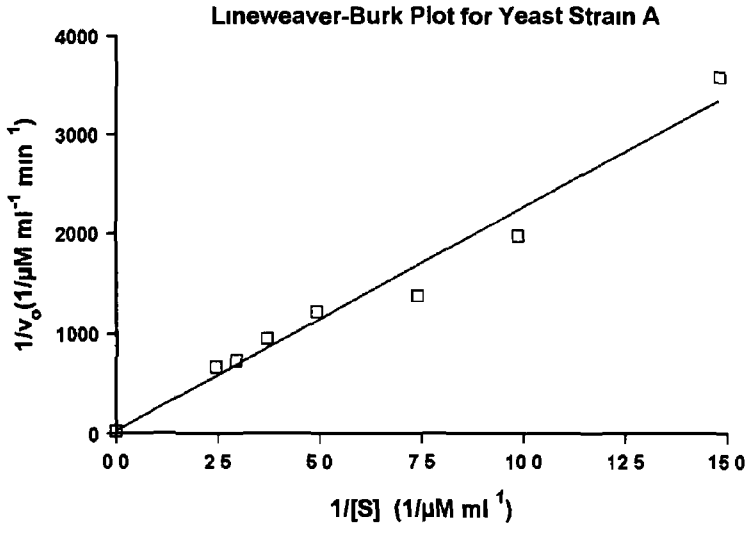


**Figure 4 9** Formation of styrene by yeast strain A with increasing amounts of cinnamic acid

The raw data are given in Appendix C

### 4.3.5.2. Lineweaver-Burk Plot for Yeast Strain C Data

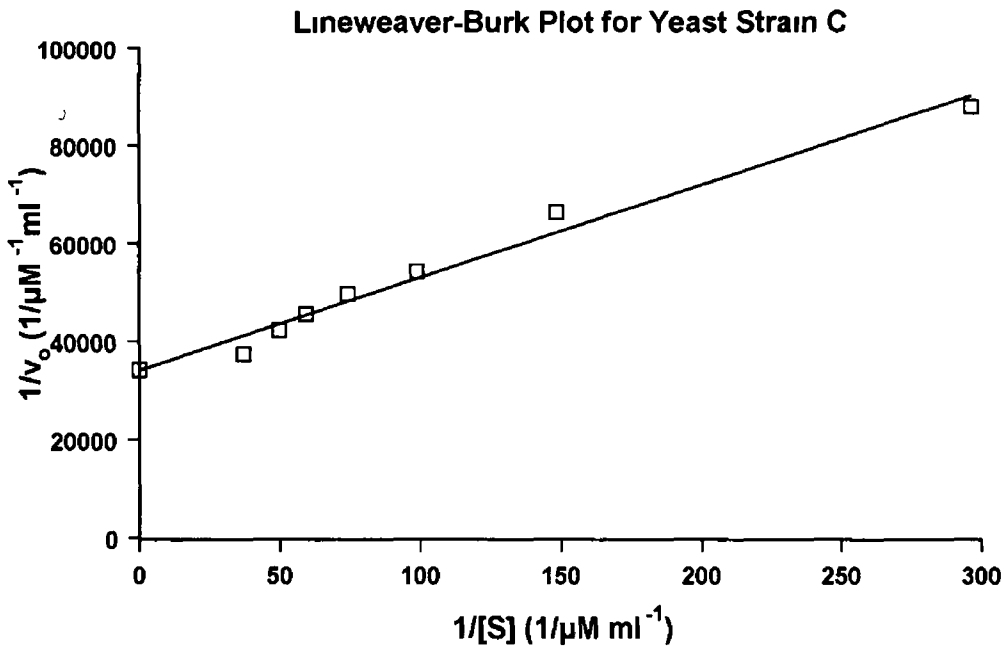


**Figure 4.10.** Formation of styrene by yeast strain C with increasing amounts of cinnamic acid

The raw data are given in Appendix C Given that the enzyme responsible for the phenolic acid to vinyl phenol conversion is intracellular, the convention for studies of this nature is to utilise cell free extracts of the yeast strain of interest This allows unrestricted access between the enzyme and its substrate (cinnamic acid in this case) The enzyme reaction rate is several orders of magnitude slower than the rate of transport of substrate across the cell wall Therefore, the rate limiting step for the conversion of phenolic acid to vinyl phenol is the enzyme-substrate reaction

The emphasis for this study was to mimic "live" fermentation circumstances in brewing The objective being to investigate the reaction kinetics of a Pof$^{+/}$ yeast strain used in the brewing industry and compare it with similar kinetic data for a Pof$^{-}$ yeast strain On this basis whole yeast cells were used throughout this work

The Lineweaver-Burk plots obtained from the data generated are shown on the previous page This data came from within the linear range of the enzymatic phenolic acid to vinyl phenol conversion

**Table 4.7.** Kinetic Parameters for Both Yeast Strains

| | **Yeast Strain A** | **Yeast Strain C** |
|---|---|---|
| **$K_m$ ($\mu M^{-1}$)** | 9 22 | 5 55E-3 |
| **$V_{max}$ ($\mu M^{1}$ ml min)** | 4 13E-2 | 2 93E-5 |
| **Slope or $K_m/V_{max}$ (min)** | 223 | 189 |
| **Correlation Coefficient** | 0 9801 | 0 9880 |

With comparison of the kinetic data generated and outlined in Table 4 7 above, it is possible to draw some conclusions with regard to the kinetic properties of the two yeast strains studied By standardisation of the cell number for both strains, in the same environment, it has been demonstrated that the maximum velocity ($V_{max}$) for yeast strain A is several orders of magnitude greater than that for yeast strain C At this reaction rate the enzyme(s) responsible for the decarboxylation activity of the yeast strains (cinnamate decarboxylate) is saturated with substrate (cinnamic acid) and can function no faster The concentration of cinnamic acid at which the given enzyme(s) yield half its maximum velocity (the $K_m$) has also been demonstrated to be several orders of magnitude greater for yeast strain A over yeast strain C Interestingly the slope for both Lineweaver-Burk plots are similar Thus it appears that the kinetic parameters are proportionally different, which may indicate a simple case of yeast strain A containing proportionally more enzyme than yeast strain C

The enzymes responsible for the decarboxylation of cinnamic acid may be isozymes Isozymes catalyse the same biochemical reaction but will differ in their primary structure and hence their kinetic behaviour

In summary this work has demonstrated that when equal numbers of yeast strain A and yeast strain C cells are present and in contact with the same quantity of cinnamic acid yeast strain A will produce greater quantities of styrene

Further studies are required to ascertain why this is the case These studies should involve production of cell free extracts of both yeast strains followed by isolation and identification of the enzyme of interest A study of the type described

above could then be carried out to investigate the kinetic parameters of these isolated enzymes

## 4.4. CONCLUSION

Two analytical techniques were demonstrated for the analysis of styrene in aqueous solution. The first technique consisted of a purge and trap sample preparation technique coupled to a gas chromatograph using a non-polar gas chromatography column. This technique had a detection limit of 40 ppt. The second technique utilised a static headspace sample preparation technique coupled to a gas chromatograph using a polar chromatography column. This technique had a detection limit of 12 ppb. Sample preparation for the purge and trap technique was labour intensive and time consuming. The thermal desorption unit used allowed manual operation only, and did not have multiple sample introduction capability. The chromatographic run time was ninety minutes.

The sample preparation for the headspace technique was rapid, and was also easily automated for multiple sample injection i.e. up to 20 samples could be placed in an autosampler and run overnight, whereas the purge and trap instrument does not allow this. The chromatographic run time was 42 minutes. Both techniques were used during the course of this work.

A series of commercially available beers were analyzed for their styrene content. One non-alcoholic product was found to consistently contain levels of approximately 20 ppb styrene, a weissbier was found to have levels of 31 ppb while no styrene was detected in any of the lagers or ales analyzed. Two of the three stout products available on the Irish market showed levels of styrene of between 20 and 30 ppb. This was shown to be consistent across keg, can and bottle. The only conclusion that may be drawn from this is that all of the above beers containing styrene were fermented with $Pof^{+/-}$ yeast strains. An investigation into overseas stout products, which are all manufactured to the same specification, demonstrated that three of the four analyzed were fermented using a $Pof^{+/-}$ yeast strain.

Styrene production with a $Pof^{+/-}$ yeast strain was monitored during the course of two staggered stout fermentations. Styrene levels rose to 20 ppb in the first ten hours and varied between 20 and 40 ppb for the duration of the fermentation.

The phenotype of a range of yeast strains was determined by a test similar to that described by Hope[27]. The styrene produced was quantified by the headspace technique. The $Pof^{+}$ yeast strain A produced 32 ppm styrene under the experimental conditions while the yeast strain D did not produce styrene. In comparison yeast

strain C produced 2 ppm styrene Two wild yeast strains isolated as contaminants in two separate breweries were carried through this test Wild yeast strain E produced 47 ppm styrene while wild yeast strain F did not produce styrene

Yeast strain E is $Pof^{+}$, while yeast strain F is $Pof^{-}$ The stout yeast strain which produced only 20 ppb in beer produced 2 ppm styrene under the conditions described is $Pof^{+/}$ This test allows a rapid, quantitative assessment of the phenotype of wild yeast strains

An investigation into wine yeast strains also demonstrated that three of the five strains analyzed here were $Pof^{+}$, contradicting reports in the literature regarding the ability of wine yeast strains to decarboxylate phenolic acids

Studies into the kinetics of styrene production by $Pof^{+}$ yeast strain A and $Pof^{+/}$ yeast strain C demonstrated that yeast strain A has a greater affinity for cinnamic acid and also a greater reaction than that of the yeast strain C Further studies are necessary to isolate the enzyme(s) responsible for this decarboxylation activity

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# APPENDICES

# APPENDIX A. DETECTION OF COOLANT CONTAMINATION IN BEER AND BEER-LIKE PRODUCTS

**Table 1.** Fluorescence of Stouts at Various Dilutions

| Product No | Colour EBC | Fluorescence |
|---|---|---|
| Stout 1 | 122 | 12 5 |
| | 97 2 | 16 9 |
| | 73 2 | 25 1 |
| | 48 8 | 34 6 |
| | 24 4 | 40 8 |
| | 12 2 | 36 5 |
| | 6 1 | 23 5 |
| Stout 2 | 222 | 2 3 |
| | 199 8 | 3 4 |
| | 177 6 | 5 2 |
| | 155 4 | 7 6 |
| | 132 2 | 10 9 |
| | 111 0 | 15 3 |
| | 88 8 | 22 3 |
| | 66 6 | 29 6 |
| | 44 1 | 40 7 |
| | 22 1 | 47 8 |
| | 11 1 | 38 0 |

**Table 2** Stout 1 Spiked and Unspiked

| Colour EBC | Fluorescence | |
|---|---|---|
| | Unspiked | Spiked 1 5,000* |
| 135 | - | - |
| 120 | 10 3 | 22 8 |
| 110 | 13 0 | 28 1 |
| 100 | 14 9 | 33 4 |
| 90 | 17 7 | 40 4 |
| 80 | 20 5 | 46 4 |
| 70 | 24 7 | 59 4 |
| 60 | 29 0 | 72 0 |
| 50 | 33 1 | 81 4 |
| 40 | 37 0 | 92 0 |
| 30 | 39 6 | 99 3 |
| 20 | 38 8 | 98 2 |
| 10 | 30 2 | 79 3 |

* Coolant Product (with 0 0135% w/v Fluorescein in coolant)

**Table 3** Stout 2 Spiked and Unspiked

| Colour EBC | Fluorescence | |
|---|---|---|
| | Unspiked | Spiked 1·5,000* |
| 190 | 2 6 | 4 9 |
| 170 | 4 1 | 7 5 |
| 150 | 5 4 | 9 8 |
| 130 | 8 4 | 14 6 |
| 110 | 12 4 | 23 3 |
| 90 | 17 5 | 33 6 |
| 70 | 23 7 | 46 7 |
| 50 | 30 6 | 67 2 |
| 40 | 36 2 | 73 8 |
| 30 | 36 7 | 81 3 |
| 20 | 36 9 | 83 4 |
| 10 | - | 70 6 |

* Coolant Product (with 0 0135% w/v Fluorescein in coolant)

**Table 4** Fluorescence of Various Stout Products at colour 50 EBC

| Stout Type | Onginal Colour | Fluorescence at 50 EBC |
|---|---|---|
| 1 | 128 | 38 9 |
| | 120 | 36 7 |
| | 123 | 35 6 |
| | 122 | 41 2 |
| | 135 | 33 1 |
| | | 35 2 |
| 2 (Maturation) | 171 | 40 2 |
| | 169 | 34 0 |
| | 170 | 37 1 |
| | 169 | 35 9 |
| 2 (Fermentation) | 218 | 43 5 |
| | 221 | 31 4 |
| | 209 | 34 1 |
| | 200 | 36 1 |
| | 208 | 35 7 |
| | 219 | 32 4 |
| | 201 | 34 3 |
| 3 | 162 | 65 5 |
| | 150 | 52 8 |
| | 128 | 53 7 |
| | 146 | 44 7 |
| | 157 | 50 8 |
| | 161 | 60 7 |
| 4 | 222 | 44 0 |
| | 210 | 40 8 |
| 5 | 194 | 41 2 |
| 6 | 133 | 39 5 |
| | 140 | 39 4 |
| | 130 | 42 5 |
| 7 | 140 | 39 3 |
| 8 | 180 | 34 0 |

**Table 5.** Calibration Results

| Fluorescein (ppb) | Fluorescence (50 EBC) | | |
|---|---|---|---|
| | **Stout 1** | **Stout 2** | **Stout 3** |
| 0 | 37 8 | 34 2 | 60 7 |
| 6 75 | 44 5 | 38 8 | 67 8 |
| 13 5 | 60 9 | 44 6 | 74 4 |
| 18 0 | 53 3 | 44 8 | 83 2 |
| 27 0 | 74 4 | 54 9 | 86 5 |
| 54 0 | 93 0 | 87 2 | 144 |
| 108 | 175 | 124 | 165 |
| 135 | 244 | 154 | 241 |
| Regression coefficient | 0 988 | 0 997 | 0 972 |
| Slope | 1 44 | 0 887 | 1 21 |
| Intercept | 32 6 | 32 7 | 60 0 |

# APPENDIX B. DEVELOPMENT OF A GAS CHROMATOGRAPHY TECHNIQUE FOR THE DETERMINATION OF PROPYLENE GLYCOL IN BEER AND BEER-LIKE PRODUCTS

## 1 CALIBRATION RAW DATA

1 1 **Technique 1a.** Sample Pre-concentration and Ethanol Extraction (EG internal standard)

| Concentration Added (ppm) | Ethylene Glycol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 16 7229 | 2 2953 | 0 1373 |
| 0 | 13 5667 | 2 2191 | 0 1636 |
| 20 | 8 5765 | 2 0287 | 0 2365 |
| 20 | 18 0686 | 5 8034 | 0 3212 |
| 40 | 22 2722 | 10 2812 | 0 4616 |
| 40 | 23 5483 | 11 0882 | 0 4709 |
| 60 | 20 5820 | 11 5951 | 0 5634 |
| 60 | 16 4917 | 10 0629 | 0 6102 |
| 80 | 14 2389 | 9 2299 | 0 6482 |
| 80 | 22 4194 | 14 4713 | 0 6455 |

### 1.1.1 Ethylene Glycol (internal standard) area statistics

Average area 17 65

Standard Deviation 4 72

% RSD 26 74 %

### 1.1.2. Regression Statistics

| | |
|---|---|
| Multiple R | 0 9794 |
| R Square | 0 9593 |
| Adjusted R Square | 0 9542 |
| Standard Error | 6 3780 |
| Observations | 10 |

### 1 1 3. Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 7674 56 | 7674 56 | 188 66 | 0 0000 |
| **Residual** | 8 0000 | 325 43 | 40 68 | | |
| **Total** | 9 0000 | 8000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -22 81 | 5 00 | -4 56 | 0 0018 | -34 34 | -11 29 |
| **Slope** | 147 50 | 10 74 | 13 74 | 0 0000 | 122 74 | 172 27 |

**1.2. Technique 1b.** Sample Pre-concentration and Acetonitrile Extraction (EG internal standard)

| Concentration Added (ppm) | Ethylene Glycol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 16 1990 | 2 7509 | 0 1698 |
| 0 | 13 6004 | 2 6248 | 0 1930 |
| 20 | 10 2794 | 4 4030 | 0 4283 |
| 20 | 15 4009 | 5 4406 | 0 3533 |
| 40 | 14 1635 | 7 3615 | 0 5198 |
| 40 | 13 6153 | 6 4585 | 0 4744 |
| 60 | 11 3193 | 7 5833 | 0 6699 |
| 60 | 14 2029 | 11 0681 | 0 7793 |
| 80 | 12 6868 | 9 7293 | 0 7669 |
| 80 | 16 4504 | 15 3907 | 0 9356 |

**1 2 1. Ethylene Glycol (internal standard) area statistics**

| | |
|---|---|
| Average area | 13 79 |
| Standard Deviation | 1 99 |
| % RSD | 14 40 % |

**1.2.2. Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9740 |
| R Square | 0 9487 |
| Adjusted R Square | 0 9423 |
| Standard Error | 7 1645 |
| Observations | 10 |

### 1.2 3. Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 7589 36 | 7589 36 | 147 86 | 0 0000 |
| **Residual** | 8 0000 | 410 64 | 51 33 | | |
| **Total** | 9 0000 | 8000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -19 99 | 5 43 | -3 68 | 0 0062 | -32 51 | -7 47 |
| **Slope** | 113 39 | 9 33 | 12 16 | 0 0000 | 91 89 | 134 89 |

**1 3 Technique 2a** Ethyl acetate extraction (EG internal standard)

| Concentration Added (ppm) | Ethylene Glycol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 4 3106 | 1 3688 | 0 3175 |
| 0 | 4 1079 | 1 2933 | 0 3148 |
| 20 | 4 5058 | 3 3024 | 0 7329 |
| 20 | 4 3246 | 3 5422 | 0 8191 |
| 40 | 4 3945 | 5 3469 | 1 2167 |
| 40 | 4 2920 | 5 4329 | 1 2658 |
| 60 | 4 0367 | 6 9372 | 1 7185 |
| 60 | 3 9542 | 6 7435 | 1 7054 |
| 80 | 4 4897 | 10 3778 | 2 3115 |
| 80 | 4 3892 | 9 8007 | 2 2329 |

**1 3 1. Ethylene Glycol (internal standard) area statistics**

| | |
|---|---|
| Average area | 4 28 |
| Standard Deviation | 0 19 |
| % RSD | 4 39% |

**1.3.2 Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9983 |
| R Square | 0 9967 |
| Adjusted R Square | 0 9962 |
| Standard Error | 1 8299 |
| Observations | 10 |

### 1 3 3 Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 7973 21 | 7973 21 | 2381 | 0 0000 |
| **Residual** | 8 0000 | 26 79 | 3 35 | | |
| **Total** | 9 0000 | 8000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -11 95 | 1 21 | -9 86 | 0 0000 | -14 74 | -9 16 |
| **Slope** | 41 12 | 0 84 | 48 80 | 0 0000 | 39 17 | 43 06 |

**1 4 Technique 2b·** Ethyl acetate extraction (Pinacol internal standard)

| Concentration Added (ppm) | Pinacol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 7 9412 | 1 8967 | 0 2388 |
| 0 | 7 5659 | 1 8324 | 0 2422 |
| 20 | 7 6812 | 5 1056 | 0 6647 |
| 20 | 8 0397 | 5 4140 | 0 6734 |
| 40 | 8 0614 | 8 0218 | 0 9951 |
| 40 | 7 7946 | 8 2314 | 1 0560 |
| 60 | 8 1432 | 11 6746 | 1 4337 |
| 60 | 7 9972 | 11 6066 | 1 4513 |
| 80 | 8 0565 | 15 1291 | 1 8779 |
| 80 | 8 4313 | 15 1087 | 1 7920 |

**1.4.1 Ethylene Glycol (internal standard) area statistics**

| | |
|---|---|
| Average area | 7 97 |
| Standard Deviation | 0 25 |
| % RSD | 3 08% |

**1 4 2 Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9988 |
| R Square | 0 9976 |
| Adjusted R Square | 0 9973 |
| Standard Error | 1 5503 |
| Observations | 10 |

### 1.4.3. Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 7980 77 | 7980 77 | 3320 50 | 0 0000 |
| **Residual** | 8 0000 | 19 23 | 2 40 | | |
| **Total** | 9 0000 | 8000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -12 49 | 1 03 | -12 08 | 0 0000 | -14 88 | -10 11 |
| **Slope** | 50 35 | 0 87 | 57 62 | 0 0000 | 48 34 | 52 37 |

1 5. **Technique 2c:** Acetonitrile extraction (Pinacol internal standard)

| Concentration Added (ppm) | Pinacol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 8 3489 | 4 5241 | 0 5419 |
| 0 | 5 6424 | 3 0883 | 0 5473 |
| 20 | 6 4999 | 9 6606 | 1 4863 |
| 20 | 6 9142 | 11 4600 | 1 6575 |
| 40 | 6 3301 | 15 5569 | 2 4576 |
| 40 | 6 1675 | 14 2495 | 2 3120 |
| 60 | 6 3338 | 21 5324 | 3 3996 |
| 60 | 6 9475 | 24 6180 | 3 5434 |
| 80 | 6 7742 | 32 8559 | 4 8502 |
| 80 | 6 6172 | 28 4778 | 4 3036 |

**1 5 1 Pinacol (internal standard) area statistics**

| | |
|---|---|
| Average area | 6 65 |
| Standard Deviation | 0 71 |
| % RSD | 10 7% |

**1 5 2 Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9941 |
| R Square | 0 9882 |
| Adjusted R Square | 0 9867 |
| Standard Error | 3 4419 |
| Observations | 10 |

### 1.5.3. Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 7905 23 | 7905 23 | 667 29 | 0 0000 |
| **Residual** | 8 0000 | 94 77 | 11 85 | | |
| **Total** | 9 0000 | 80000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -9 78 | 2 21 | -4 42 | 0 0022 | -14 89 | -4 68 |
| **Slope** | 19 83 | 0 77 | 25 83 | 0 0000 | 18 06 | 21 60 |

1 6. **Technique 2d** Acetonitrile extraction (Pinacol internal standard)

| Concentration Added (ppm) | Pinacol Area | Propylene Glycol Area | Area Ratio |
|---|---|---|---|
| 0 | 2 5680 | 1 9323 | 0 7525 |
| 0 | 2 7414 | 2 8623 | 1 0441 |
| 20 | 1 6529 | 2 5386 | 1 5358 |
| 20 | 2 0566 | 3 2452 | 1 5779 |
| 40 | 2 3019 | 6 1026 | 2 6511 |
| 40 | 2 6219 | 6 3941 | 2 4387 |
| 60 | 2 6017 | 12 2618 | 4 7130 |
| 60 | 2 8078 | 15 1853 | 5 4083 |
| 80 | 2 7537 | 18 759 | 6 8123 |

**1 6 1. Pinacol (internal standard) area statistics**

| | |
|---|---|
| Average area | 2 4562 |
| Standard Deviation | 0 3846 |
| % RSD | 15 66% |

**1 6.2 Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9630 |
| R Square | 0 9274 |
| Adjusted R Square | 0 9170 |
| Standard Error | 8 0337 |
| Observations | 9 |

### 1.6.3. Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 0000 | 5770 44 | 5770 44 | 89 41 | 0 0000 |
| **Residual** | 7 0000 | 451 78 | 64 54 | | |
| **Total** | 8 0000 | 6222 22 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -1 95 | 4 79 | -0 41 | 0 70 | -13 26 | 9 37 |
| **Slope** | 12 53 | 1 33 | 9 45 | 0 0000 | 9 40 | 15 67 |

## 2. INTERNAL STANDARD SUITABILITY RAW DATA

**2 1 Technique 1a** Sample pre-concentration and ethanol extraction (EG internal standard)

| Sample No. | Interfering peak area's |
|---|---|
| 1 | 1 14042 |
| 2 | 0 8452 |
| 3 | 0 7134 |
| 4 | 0 9512 |
| 5 | 0 6914 |

Average area of interfering peak 0 86

**2 2. Technique 1b.** Sample pre-concentration and acetonitrile extraction (EG internal standard)

| Sample No. | Interfering peak area's |
|---|---|
| 1 | < 0 2 |
| 2 | < 0 2 |
| 3 | < 0 2 |
| 4 | < 0 2 |
| 5 | < 0 2 |

Average Area of interfering peaks < 0 2

**2 3 Technique 2a·** Ethyl acetate extraction (EG internal standard)

| Sample No. | Interfering peak area's |
|---|---|
| 1 | 1 4504 |
| 2 | 1 0916 |
| 3 | 1 0007 |
| 4 | 1 1603 |
| 5 | 1 1025 |

Average Area of interfering peaks 1 16

# 3 RECOVERY RAW DATA

## 3.1. AOAC Recovery Analysis

Recovery is described as the fraction of analyte added to a test sample (fortified or spiked sample) prior to analysis, which is measured (recovered) by the method When the same analytical method is used to analyse both the unfortified and fortified samples, %R is calculated as follows

$$\% R = [ ( CF - CU ) / CA ] * 100 \qquad \textbf{Equation 1.}$$

where

CF = Concentration in fortified sample
CU = Concentration in unfortified sample
CA = Concentration of analyte added to fortified sample

**3.1.1 Technique 1a:** Sample preconcentration and ethanol extraction (EG internal standard)

| Sample | Fortified Sample Result (ppm) | % Recovery |
|---|---|---|
| 1 | 46 00 | 93 4 |
| 2 | 43 32 | 80 0 |
| 3 | 44 67 | 86 8 |
| 4 | 46 75 | 97 2 |

Unfortified sample result 27 32 ppm

**3.1.2. Technique 2a:** Ethyl Acetate Extraction (EG internal standard)

| Sample | Fortified Sample Result (ppm) | % Recovery |
|---|---|---|
| 1 | 65 48 | 181 2 |
| 2 | 50 62 | 106 9 |
| 3 | 57 34 | 140 5 |
| 4 | 69 35 | 200 5 |

Unfortified sample result 29 24ppm

**3 1.3. Technique 2b.** Ethyl Acetate Extraction (Pinacol internal standard)

| Sample | Result (ppm) | % Recovery |
|---|---|---|
| 1 | 25 17 | 70 9 |
| 2 | 25 90 | 74 6 |
| 3 | 26 60 | 78 1 |
| 4 | 28 04 | 85 3 |

Unfortified sample result 10 98ppm

**3.1.4. Technique 2c** Acetonitrile Extraction (Pinacol internal standard)

| Sample | Result (ppm) | % Recovery |
|---|---|---|
| 1 | 27 02 | 74 1 |
| 2 | 30 91 | 93 6 |
| 3 | 31 44 | 96 2 |
| 4 | 32 47 | 101 35 |

Unfortified sample result 12 20ppm

## 3 2 Recovery based on Peak Area's

Recovery defined as the peak area for propylene glycol and the internal standard for each sample preparation technique (from the precision analysis described earlier) expressed as a percentage of the peak area for a solution containing the concentration of propylene glycol and internal standard if 100% extraction had occured

**3.2 1. Technique 1a·** Sample preconcentration and ethanol extraction (EG internal standard)

| Injection No. | Propylene Glycol Area | Internal Standard Area |
|---|---|---|
| 1 | 2 5913 | 22 8632 |
| 2 | 2 6572 | 20 6377 |
| 3 | 2 5853 | 22 5406 |
| 4 | 2 6433 | 23 3914 |
| 5 | 2 7990 | 20 9498 |
| Average | 2 6552 | 22 0765 |

**3 2 2. Technique 2a.** Ethyl Acetate Extraction (EG internal standard)

| Injection No | Propylene Glycol Area | Internal Standard Area |
|---|---|---|
| 1 | 27 27 | 280 86 |
| 2 | 29 12 | 307 57 |
| 3 | 28 18 | 299 78 |
| 4 | 27 39 | 295 14 |
| 5 | 28 07 | 296 70 |
| Average | 28 01 | 296 01 |

**3 2.3. Technique 2b:** Ethyl Acetate Extraction (Pinacol internal standard)

| Injection No. | Propylene Glycol Area | Internal Standard Area |
|---|---|---|
| 1 | 17 9073 | 14 3913 |
| 2 | 17 8398 | 14 0648 |
| 3 | 15 5145 | 13 1910 |
| 4 | 15 8014 | 13 2838 |
| 5 | 16 9955 | 13 6181 |
| Average | 16 8117 | 13 7098 |

**3.2.4. Technique 2c.** Acetonitrile Extraction (Pinacol internal standard)

| Injection No. | Propylene Glycol Area | Internal Standard Area |
|---|---|---|
| 1 | 13 8757 | 10 9455 |
| 2 | 13 9260 | 11 1902 |
| 3 | 11 6373 | 10 4943 |
| 4 | 11 8160 | 10 0024 |
| 5 | 12 3414 | 10 0564 |
| Average | 12 7193 | 10 5378 |

## 4. PRECISION RAW DATA

The same stout was analysed for all precision analysis

4 1 **Technique 1a**· Sample preconcentration and ethanol extraction (EG internal standard)

| Sample No. | Ethylene Glycol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| 1 | 22 8093 | 4 0763 | 0 1787 | 27 50 |
| 2 | 21 0196 | 2 8264 | 0 1345 | 20 69 |
| 3 | 17 4485 | 2 1469 | 0 1230 | 18 94 |
| 4 | 19 9765 | 3 4442 | 0 1724 | 26 53 |
| 5 | 20 8851 | 3 6812 | 0 1763 | 27 13 |
| 6 | 22 8775 | 4 1051 | 0 1794 | 27 62 |
| 7 | 28 1960 | 4 0346 | 0 1431 | 22 02 |
| 8 | 20 9948 | 3 0548 | 0 1455 | 22 39 |
| 9 | 24 0385 | 3 5609 | 0 1481 | 22 80 |
| 10 | 24 7987 | 3 5202 | 0 1420 | 21 85 |

| | Ethylene Glycol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| AVERAGE | 22 3 | 3 45 | 23 75 |
| STD DEV | 2 96 | 0 62 | 3 16 |
| % RSD | 13 27 | 18 04 | 13 32 |

**4.2. Techqique 1b** Sample preconcentration and acetonitrile extraction (EG internal standard)

| Sample No | Ethylene Glycol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| **1** | 17 4540 | 4 0048 | 0 2294 | 27 30 |
| **2** | 15 8557 | 3 0588 | 0 1929 | 22 96 |
| **3** | 14 0368 | 2 7484 | 0 1958 | 23 30 |
| **4** | 14 5400 | 3 8342 | 0 2637 | 31 38 |
| **5** | 16 8628 | 3 9217 | 0 2326 | 27 68 |
| **6** | 11 6373 | 2 4111 | 0 2072 | 24 66 |
| **7** | 14 7208 | 3 3209 | 0 2256 | 26 85 |
| **8** | 16 5475 | 4 0596 | 0 2453 | 29 19 |
| **9** | 16 8818 | 3 4086 | 0 2019 | 24 03 |
| **10** | 14 5519 | 2 9162 | 0 2004 | 23 85 |

| | Ethylene Glycol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| **AVERAGE** | 15 31 | 3 37 | 26 12 |
| **STD DEV** | 1 76 | 0 58 | 2 81 |
| **% RSD** | 11 51 | 17 17 | 10 76 |

**4 3. Technique 2a**· Ethyl Acetate extraction extraction (EG internal standard)

| Sample No. | Ethylene Glycol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| 1 | 3 6161 | 1 3616 | 0 3765 | 15 53 |
| 2 | 3 8112 | 1 4649 | 0 3844 | 15 86 |
| 3 | 3 5204 | 1 3375 | 0 3799 | 15 67 |
| 4 | 3 4887 | 1 2847 | 0 3682 | 15 19 |
| 5 | 3 4080 | 1 2755 | 0 3743 | 15 44 |
| 6 | 3 7824 | 1 4522 | 0 3839 | 15 84 |
| 7 | 3 5044 | 1 2729 | 0 3632 | 14 98 |
| 8 | 3 9526 | 1 5077 | 0 3814 | 15 74 |
| 9 | 3 7077 | 1 3427 | 0 3621 | 14 94 |
| 10 | 3 4068 | 1 3427 | 0 3941 | 16 26 |

| | Ethylene Glycol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| AVERAGE | 3 62 | 1 36 | 15 55 |
| STD DEV | 0 19 | 0 08 | 0 42 |
| % RSD | 5 15 | 6 10 | 2 68 |

**4.4. Techqique 2b·** Ethyl Acetate extraction extraction (Pinacol internal standard)

| Sample No | Pinacol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| **1** | 7 1294 | 1 7243 | 0 2419 | 12 22 |
| **2** | 7 7391 | 1 9030 | 0 2459 | 12 42 |
| **3** | 7 8558 | 1 7916 | 0 2281 | 11 52 |
| **4** | 7 4239 | 1 5951 | 0 2149 | 10 85 |
| **5** | 7 6621 | 1 6719 | 0 2182 | 11 02 |
| **6** | 7 7716 | 1 7020 | 0 2190 | 11 06 |
| **7** | 7 8535 | 1 7085 | 0 2175 | 10 99 |
| **8** | 7 4418 | 1 6110 | 0 2165 | 10 93 |
| **9** | 7 8895 | 1 8806 | 0 2384 | 12 04 |
| **10** | 7 7681 | 1 7955 | 0 2311 | 11 67 |

| | Pinacol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| **AVERAGE** | 7 65 | 1 74 | 11 47 |
| **STD DEV** | 0 25 | 0 10 | 0 59 |
| **% RSD** | 3 21 | 6 00 | 5 11 |

**4 5. Techqique 2c·** Acetonitrile extraction extraction (Pinacol internal standard)

| Sample No | Pinacol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| 1 | 6 2591 | 3 4961 | 0 5586 | 11 21 |
| 2 | 6 6882 | 4 0995 | 0 6129 | 12 30 |
| 3 | 6 6712 | 3 5951 | 0 5389 | 10 82 |
| 4 | 7 0604 | 3 4642 | 0 4907 | 9 85 |
| 5 | 6 4991 | 2 9937 | 0 4606 | 9 25 |
| 6 | 7 1050 | 3 6854 | 0 5187 | 10 41 |
| 7 | 6 3608 | 3 0924 | 0 4862 | 9 76 |
| 8 | 7 0008 | 3 8284 | 0 5469 | 10 98 |
| 9 | 6 2414 | 3 0725 | 0 4923 | 9 88 |
| 10 | 6 4771 | 3 3669 | 0 5198 | 10 43 |

| | Pinacol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| AVERAGE | 6 64 | 3 47 | 10 49 |
| STD DEV | 0 33 | 0 35 | 0 88 |
| % RSD | 4 91 | 10 20 | 8 42 |

**4.6 Techqique 2d.** Acetonitrile extraction extraction (Pinacol internal standard)

| Sample No. | Pinacol area | Propylene Glycol area | Area Ratio | Concentration (ppm) |
|---|---|---|---|---|
| 1 | 2 4619 | 2 7051 | 1 0988 | 13 77 |
| 2 | 2 7609 | 2 8300 | 1 0250 | 12 84 |
| 3 | 2 1933 | 1 4201 | 0 6475 | 8 11 |
| 4 | 1 8337 | 1 6170 | 0 8818 | 11 05 |
| 5 | 2 1383 | 2 5918 | 1 2121 | 15 19 |
| 6 | 2 5716 | 1 9605 | 0 7624 | 9 55 |
| 7 | 1 6413 | 1 3390 | 0 8158 | 10 22 |
| 8 | 2 1780 | 1 4464 | 0 6641 | 8 32 |
| 9 | 1 1626 | 1 1004 | 0 9465 | 11 86 |
| 10 | 2 4161 | 2 3512 | 0 9731 | 12 19 |

| | Pinacol Area | Propylene Glycol Area | Propylene Glycol Concentration (ppm) |
|---|---|---|---|
| AVERAGE | 2 14 | 1 94 | 11 31 |
| STD DEV | 0 48 | 0 64 | 2 31 |
| % RSD | 22 37 | 32 92 | 20 42 |

## 5. Formation of Propylene Glycol during Fermentation

| Fermentation Time (Hrs) | Present Gravity ° | Propylene Glycol (ppm) |
|---|---|---|
| 0 | 85 5 | <2 |
| 1 | | <2 |
| 18 | 79 7 | <2 |
| 21 | | <2 |
| 23 | | <2 |
| 25 | | <2 |
| 30 | 46 9 | 7 5 |
| 32 | | 9 1 |
| 35 | | 10 0 |
| 37 | | 8 6 |
| 54 | 40 9 | 12 6 |
| 81 | 32 0 | 13 6 |
| 106 | 25 6 | 13 1 |
| 127 | 20 8 | 15 7 |
| 138 | 18 5 | 23 0 |

# APPENDIX C. AN INVESTIGATION INTO STYRENE IN BEER

## 1 CONDITIONING OF THE THERMAL DESORPTION TUBES PRIOR TO USE FOR PURGE AND TRAP:

**1 1** The tubes are placed in a modified gas chromatograph, where the tube replaces the analytical column The tube is not connected to the detector port The carrier is allowed to flow to atmosphere

**1.2.** The nitrogen carrier flow gauge was set to 15 psi

**1 3** The tubes were subjected to the following temperature regime

Initial Hold 10 mm at Room Temperature (20°C)
20°C - 300°C at 12°C/min
Final Hold 10 min at 300°C

**1.4.** The tubes were allowed to cool to room temperature before removed from the oven

**1 5.** End caps were placed on the tubes to ensure contamination does not occur prior to use

## 2. PREPARATION OF MYGP

The ingredients detailed below were weighed out

| | |
|---|---|
| Oxoid Malt Extract | 3 g |
| Difco Yeast Extract | 3 g |
| BDH Glucose | 10 g |
| Difco Peptone | 5 g |
| Oxoid Peptone Technical No 3 | 20 g |

The above ingredients were placed into a 2 litre flask to which 1 litre of distilled water was added The solution was well mixed and placed in a steamer for 45 minutes to allow the agar to melt

10 $cm^3$ of the resultant solution was dispensed into each McCartney bottle and autoclaved at 121°C for fifteen minutes Bottles being used for slopes were allowed to cool and then swirled to mix in any condensation before being allowed to set in a sloped position on a rack

# 3 CALIBRATION RAW DATA

## 3 1. Purge and Trap Calibration Data

| Concentration Added (ppb) | Methyl Propyl Ketone Area | Styrene Area | Area Ratio |
|---|---|---|---|
| 0 | 225129 | 352529 | 1 5659 |
| 20 | 219319 | 672045 | 3 0642 |
| 40 | 226270 | 943200 | 4 1685 |
| 60 | 218624 | 1264377 | 5 7833 |
| 80 | 189604 | 1471751 | 7 7622 |

### 3.1.1 Methyl Propyl Ketone(internal standard) area statistics

| | |
|---|---|
| Average area | 215789 |
| Standard Deviation | 15027 |
| % RSD | 6 96% |

### 3.1.2 Regression Statistics

| | |
|---|---|
| Multiple R | 0 9950 |
| R Square | 0 9901 |
| Adjusted R Square | 0 9868 |
| Standard Error | 3 6303 |
| Observations | 5 |

### 3 1 3 Analysis of Variance

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 | 3960 | 3960 | 300 | 0 0004 |
| **Residual** | 3 | 39 5 | 13 2 | | |
| **Total** | 4 | 4000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -18 6 | 3 75 | -4 9 | 0 016 | -30 5 | -6 63 |
| **Slope** | 13 1 | 0 75 | 17 33 | 0 0004 | 10 7 | 15 5 |

**3 2. Headspace Analysis Calibration Data**

| Concentration Added (ppb) | Butanol Area | Styrene Area | Area Ratio |
|---|---|---|---|
| 0 | 522399 | 6891 | 0 0132 |
| 0 | 496668 | 3724 | 0 0075 |
| 20 | 425369 | 11263 | 0 0265 |
| 20 | 484720 | 12410 | 0 0256 |
| 40 | 465652 | 16794 | 0 0361 |
| 40 | 485256 | 17436 | 0 0359 |
| 60 | 460426 | 24803 | 0 0539 |
| 60 | 458278 | 22170 | 0 0484 |
| 80 | 494211 | 32776 | 0 0663 |
| 80 | 467656 | 23047 | 0 0493 |

The following averaged figures were used for the calibration

| **Concentration Added(ppb)** | **Area Ratio** |
|---|---|
| 0 | 0 0104 |
| 20 | 0 0261 |
| 40 | 0 0360 |
| 60 | 0 0512 |
| 80 | 0 0578 |

**3 2 1 Butanol(internal standard) area statistics**

| | |
|---|---|
| Average area | 476064 |
| Standard Deviation | 26604 |
| % RSD | 5 59% |

**3 2 2 Regression Statistics**

| | |
|---|---|
| Multiple R | 0 9926 |
| R Square | 0 9852 |
| Adjusted R Square | 0 9803 |
| Standard Error | 4 4425 |
| Observations | 5 |

**3.2 3. Analysis of Variance**

| | df | Sum of Squares | Mean Square | F | Significance F |
|---|---|---|---|---|---|
| **Regression** | 1 | 3941 | 3941 | 200 | 0 0000 |
| **Residual** | 3 | 59 | 20 | | |
| **Total** | 4 | 4000 | | | |

| | Co-efficients | Standard Error | t Statistic | P-Value | Lower 95% | Upper 95% |
|---|---|---|---|---|---|---|
| **Intercept** | -19 7 | 4 67 | -4 21 | 0 02 | -34 5 | -4 81 |
| **Slope** | 0 0006 | 116 3 | 14 13 | 0 00 | 1273 | 2014 |

# 4 RAW DATA FOR STYRENE FERMENTATION 1 AND 2

| Fermentation No | Time Hours | Present Gravity | pH | Styrene (ppb) |
|---|---|---|---|---|
| 1 | 0 | 66 9 | 4 89 | <1 |
| 1 | 2 | 63 9 | 4 78 | 4 6 |
| 1 | 4 | 64 0 | 4 70 | 8 8 |
| 1 | 6 | 63 9 | 4 64 | 25 |
| 1 | 8 | 62 3 | 4 59 | 22 |
| 1 | 21 | 40 0 | 3 99 | 33 |
| 1 | 23 | 34 9 | 3 83 | 23 |
| 1 | 25 | 33 0 | 3 82 | 27 |
| 1 | 27 | 30 1 | 3 77 | 25 |
| 1 | 29 | 24 1 | 3 76 | 20 |
| 1 | 47 | 15 0 | 3 79 | 20 |
| 1 | 49 | 13 8 | 3 85 | 41 |
| 1 | 51 | 13 9 | 3 85 | 34 |
| 1 | 53 | 14 2 | 3 86 | 27 |
| 1 | 55 | | | 28 |
| 2 | 13 | 58 4 | 4 51 | 18 |
| 2 | 15 | 53 5 | 4 36 | 23 |
| 2 | 17 | 52 1 | 4 31 | 26 |
| 2 | 19 | 49 | 4 22 | 24 |
| 2 | 21 | 42 2 | 4 17 | 21 |
| 2 | 39 | 26 0 | 3 90 | 22 |
| 2 | 41 | 24 3 | 3 94 | 24 |
| 2 | 43 | 22 6 | 3 93 | 26 |
| 2 | 45 | 20 6 | 3 92 | 44 |
| 2 | 47 | | | 34 |

# 5 KINETICS RAW DATA

## 5.1. C504 Kinetics Data.

### 5.1.1. Raw Experimental Data

| Cinnamic Acid Added (ppm) | Styrene Produced (ppb) |
|---|---|
| 10 | 1742 |
| 15 | 3162 |
| 20 | 4537 |
| 30 | 5127 |
| 40 | 6608 |
| 50 | 8586 |
| 60 | 9341 |

### 5.1.2 Processed Data for Lineweaver-Burk Plot

| 1/[S] (1/uM $ml^{-1}$) | 1/[Vo] (1/uM $ml^{-1}$ $min^{-1}$) |
|---|---|
| 14 82 | 3582 |
| 9 88 | 1973 |
| 7 41 | 1375 |
| 4 94 | 1217 |
| 3 71 | 944 |
| 2 96 | 727 |
| 2 47 | 668 |

### 5 1 3 Regression Statistics

| | |
|---|---|
| Multiple R | 0 9801 |
| R Square | 0 9608 |
| Adjusted R Square | 0 9526 |
| Standard Error | 222 25 |
| Observations | 7 |

### 5.1.4. Lineweaver-Burk Data

| | |
|---|---|
| $K_m$ | 9 22 $uM^{-1}$ ml |
| $V_{max}$ | 4 13E-2 $uM^{-1}$ ml min |
| $K_m/V_{max}$ (slope) | 223 35 min |

### 5.2. 1511 Kinetics Data:

#### 5.2.1. Raw Experimental Data

| Cinnamic Acid Added (ppm) | Styrene Produced (ppb) |
|---|---|
| 0.5 | 71 |
| 1.0 | 94 |
| 1.5 | 115 |
| 2.0 | 126 |
| 2.5 | 137 |
| 3.0 | 148 |
| 4.0 | 167 |

#### 5.2.2. Processed Data for Lineweaver-Burk Plot

| 1/[S] (1/uM $ml^{-1}$) | 1/[Vo] (1/uM $ml^{-1}$ $min^{-1}$) |
|---|---|
| 296.4 | 87887 |
| 148.2 | 66383 |
| 98.8 | 54261 |
| 74.1 | 49534 |
| 59.3 | 45547 |
| 49.4 | 42162 |
| 37.1 | 37365 |

### 5.2.3 Regression Statistics

| | |
|---|---|
| Multiple R | 0 9880 |
| R Square | 0 9761 |
| Adjusted R Square | 0 9714 |
| Standard Error | 2936 |
| Observations | 7 |

### 5 2 4 Lineweaver-Burk Data

| | |
|---|---|
| $K_m$ | 5 55E-3 $uM^{-1}$ ml |
| $V_{max}$ | 2 934e-10 $uM^{-1}$ ml min |
| $K_m/V_{max}$ (slope) | 189 35 min |