The Analysis of Sulphur Compounds in Beer

by

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The continued development of new and improved analytical techniques is.....clearly necessary whilst so many of the questions relating to sulphury flavours in beer remain unanswered.

Terry Peppard, *Analytical Measurement of Volatile Sulphur Compounds in Beer*, in *Modern Methods of Plant Analysis, Vol. 7, Beer Analysis*, Springer Verlag, Berlin, 1988

Abstract

A gas chromatographic method for the routine analysis of volatile and semi-volatile sulphur compounds in beer was developed. Various selective and specific detectors, capillary columns and methods of sample preparation were compared. The combination offering the best sensitivity and stability consisted of solid phase microextraction (SPME) as the sample preparation step; a combined polar/non-polar chromatographic column; and a pulsed flame photometric detector (PFPD). All parameters were optimised to achieve maximum sensitivity. The system was linear for the range of sulphur compound concentrations found in beer, and displayed good reproducibility. The calibrated SPME-GC-PFPD system was used to analyse several different varieties of beer from a range of breweries, and to investigate the change in the concentrations of sulphur compounds when beer is subjected to illumination.

Key words: beer, sulphur compounds, lightstruck, gas chromatography, solid phase microextraction, pulsed flame photometric detector

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1 Introduction

The aim of this study is to develop a simple, fast, sensitive and inexpensive routine method of analysis for volatile and semi-volatile sulphur compounds in beer. Sulphur compounds play an important role in food and beverage flavour and are a determining factor of product quality.

Phrases such as "the customer is always right" and "the customer is king" have taken on a new significance with the globalisation of markets, the customer being offered an ever-increasing range of products to choose from. For producers of consumer goods, quality is the number one priority if they are to survive in the increasing competition of the global marketplace. This is no more the case than in the food industry, where top-grade goods of unremitting high quality are the only guarantee of retaining, and more importantly gaining customers.

Over the last decade, in the brewing industry much effort has been put into improving the flavour stability of beer through technological innovation and refinement (especially in Germany owing to the *Reinheitsgebot* of 1516 which allows only the use of water, malt and hops in beer production, thereby ruling out the use of preservatives, such as ascorbic acid or potassium metabisulphite), and eliminating off-flavours which can occur during the fermentation and lagering (storage or maturation) of the beer. In order to be able to do this, analytical methods have to be available to determine where the problems lie, and the influence of technological measures on the compounds responsible.

One area yet to be fully investigated is that of sulphur compounds in beer, despite these substances being of great significance on the grounds of their very low flavour thresholds and, in most cases, unpleasant taste and aroma. One reason for the incomplete study of this area is the lack of a simple, inexpensive, sensitive and accurate method for the routine analysis of these substances.

1.1 Sulphur Compounds in Foodstuffs

The role of sulphur compounds in a wide range of foodstuffs (1) is a very important one, and these substances have been the subject of much research. Volatile sulphur compounds are found in both uncooked and cooked foods. In uncooked foods they are especially important in vegetables of the Allium genus such as leeks, onions and garlic (2), whose overall sensory properties they strongly influence. Propyl propane thiosulphonate, for example, gives onion its characteristic smell. When cruciferous vegetables such as cabbage, broccoli, cauliflower or Brussel sprouts are cooked or injured, volatile sulphur compounds are formed by the enzymatic degradation of involatile sulphur-containing precursors (3). Many fruits also contain sulphur compounds which contribute to their sensory character: for example, Wyllie *et al.* detected 20 sulphur volatiles in the musk melon (4).

In cooked foods the volatile sulphur compounds are usually products of the reactions between the non-volatile sulphur-containing amino acids methionine, cystine and cysteine, and reducing sugars. The mechanisms of these 'non-enzymatic browning' reactions were first elucidated by Louis Maillard in 1912 (5) and have been studied extensively since (6,7). Volatile sulphur compounds formed in this way by the roasting, baking or cooking of food are of great importance in bread, roast beef, coffee and UHT milk. In coffee, for example, furfuryl mercaptan is considered by many researchers to be the single most important odour component (8,9). Other mechanisms for the formation of volatile sulphur compounds from non-volatile precursors are enzymatic degradation and the thermal degradation of thiamine (vitamin B1) (10).

Owing to their high reactivity, the volatile and semi-volatile sulphur compounds formed by these three mechanisms can then go on to react further with other non-sulphur products of the Maillard Reaction, to produce a wide range of sulphur substances (11).

Although sulphur substances contribute in a positive way to the aroma and taste of many foodstuffs, it is not always as a dominant character (as in the afore-mentioned cases of propyl propane thiosulphonate in onions and furfuryl mercaptan in coffee). Often they are an integral part of the overall organoleptic impression. They are also a common cause of off-flavours and smells. For example, during the Ultra High Temperature (UHT) processing of milk many sulphur substances are formed. Four of these (hydrogen sulphide, dimethyl sulphide, dimethyl disulphide and methylmercaptan) are held primarily responsible for the resulting sulphurous 'cooked' off-flavour (12,13). This off-flavour fortunately decreases in intensity or disappears totally after a few days' storage (14).

Substances that are positive contributors to a foodstuff's flavour can also have a detrimental effect when they exceed certain concentrations. A determining factor is the low flavour thresholds of the sulphur compounds (e.g. 5 ng/l in water (15) and 0.01 - 0.02 ng/l in air (16) for furfuryl mercaptan and 1.5 ng/l (17) for propyl propane thiosulphonate) and their powerful sensory characteristics. Owing to these properties, the line between positive aroma contributor and off-smell for volatile sulphur substances is a very narrow one.

In European Pilsener beers, dimethyl sulphide is an important (but not characteristic) taste and aroma contributor to the overall flavour (18,19). A minute increase in the concentration, however, can lead to the beer assuming an off-flavour. Where the exact line between flavour and off-flavour actually lies is dependent on the individual taster. The same phenomenon is observed with furfuryl mercaptan in coffee; the difference in concentration between it having a positive flavour impact and acting as an off-flavour is very small (8).

1.2 Sulphur Compounds in Beer

Much work has been carried out into the occurrence of volatile sulphur compounds and their influences in beer. In 1985 Narziss, Miedaner and Kattein from the Technical University of Munich published an excellent review of the literature to that date on the behaviour of sulphur-containing aroma components during the brewing process (20). There are three possible sources of sulphur substances in beer: sulphate anions present in the brewing liquor (21); malt (22,23,24); and hops (23,25,26,27,28). Malt is the most important of the three sources simply on the grounds of the amount used in comparison to hops. The effect of sulphate anions in the brew water on the concentration of sulphur substances in beer is a matter of some disagreement. Some workers are of the opinion that a correlation exists (21), others claim that the sulphate anion concentration in the brew liquor has no influence (29).

It is possible that non-volatile sulphur substances from the raw materials remain unchanged throughout the brewing process and are subsequently found in the final product, beer. The majority of the volatile and semi-volatile sulphur compounds, however, do not come directly from the raw materials: they are formed by a multitude of reaction pathways and mechanisms, the non-volatile sulphur-containing substances being chemically broken down and converted into smaller, more volatile compounds. Here the brewing yeast plays a very important role, its metabolism determining the sulphur substances formed (30,31,32,33,34,35, 36,37). The strain of yeast used for the fermentation can have a marked effect on the quantities of the sulphur compounds formed (38). A further, but fortunately rare mechanism for the formation of volatile sulphur compounds is through bacterial infection (23,39,40), which leads to spoilage of the beer.

The technology used during the brewing process also has a great influence on the concentration of sulphur compounds found in the final beer (41). Narziss, Miedaner and Zinsberger studied the behaviour of volatile sulphur substances in respect to the technology used during mashing and

wort-boiling in the brewhouse; the wort separation; the pitching and aeration of the wort and the subsequent fermentation; the filling and heat-treatment of the beer. Further influences on the volatile sulphur substances in beer are the storage conditions of the bottled beer, where light is a very important factor.

The first sulphur compound to be fully investigated was dimethyl sulphide, which has since been the subject of many papers and publications. The principal source of dimethyl sulphide (DMS) in beer is the malt: the precursor S-methyl-methionine is produced from methionine during germination of the barley (34,42) which subsequently decomposes to form DMS and the amino acid homoserine (34). This thermal reaction during kilning is desirable as the DMS formed is lost in the kiln exhaust gases. Other mechanisms for the formation of DMS are production by the yeast during fermentation (38,43,44) and contamination of the wort by bacteria (23,40). DMS is a very flavour-active substance: the range of flavour thresholds documented in the literature varies hugely between 30 and 70 ppb, dependent on the sort of beer and the individual taster. In low concentrations DMS gives the beer a 'sweet-malty' flavour (45), a typical flavour component of European Pilsener-type beers. Once a concentration of 70 - 100 ppb is reached the beer develops an exceedingly unpleasant 'cooked vegetable' (cabbage or asparagus) taste and aroma, and on even higher concentrations it takes on a more 'sweetcorn-like' flavour.

Similar in structure to DMS is DES, diethyl sulphide (note: EMS, ethyl methyl sulphide, is not found in beer and is consequently often used as an internal standard for the analysis of sulphur substances in this matrix). DES has been identified in both malt (23,22) and hops (23,46). There is no evidence that DES is formed either during fermentation or by spoilage bacteria. The aroma and taste of DES are similar to DMS, being described as cooked vegetable, onion-like and 'garlicky' (47). The flavour threshold of DES is generally regarded as lying in the range between 1.2 ppb (47) and 2.5 ppb (23).

Two homologous sulphur substances which are reported as both being present in hops (22,23,25,26,27,46) are dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTriS). DMDS is often reported as being present in malt (22,23) and pressure-cooked grain/water mixtures (48). There is only one work, however, which reports DMTriS in boiled, unhopped wort (49). Both DMDS and DMTriS are described as having a flavour profile of cooked vegetable or onion (50), DMDS additionally being described as 'rubbery' (50). The flavour threshold of DMDS is reported as lying between 3 ppb (51) and 7.5 ppb (23), although Baerwald and Niefind reported a threshold of 50 ppb (52). During his work on DMTriS and its formation Peppard (25) noted a flavour threshold of 0.1 ppb, considerably lower than for both DMS and DMDS.

The thiols, or mercaptans, are of great interest to the brewer. Although both methyl and ethyl mercaptan are found in malt (22,48) and hops (27,46,50), the main source of the mercaptans in beer is yeast metabolism during fermentation (30,34,36,41). Methyl mercaptan is described as having a flavour in beer of putrefied egg or cabbage, whilst ethyl mercaptan is described as putrefied leek, onion, garlic and egg (47). The flavour threshold for methyl mercaptan is reported as lying between 2 ppb (30,47) and 3 ppb (23), and for ethyl mercaptan between 1.7 ppb and 5 ppb (47,23,30).

Unlike most other sulphur compounds, sulphur dioxide is not particularly flavour active in beer: its flavour threshold lies at approximately 25 mg/l (53). Sulphur dioxide, however, plays an important role in beer ageing by acting as an anti-oxidant and by binding carbonyl compounds, reducing their negative impact on the flavour of aged beers (54,55,56). An excellent review on aspects of the analysis, role and fate of sulphur dioxide in beer was published by Ilett in 1995 (57).

Other volatile sulphur substances of importance to beer are hydrogen sulphide, mostly formed during fermentation (30,35,38,40) and with its distinctive aroma of rotten eggs (53), and the thioacetates, methyl

thioacetate and ethyl thioacetate. These two substances are also products of the yeast metabolism (38,41). Little work has been done on their sensory properties, the most commonly mentioned descriptions being of cauliflower or cabbage. Arkima *et al.* (58) reported sensory detection thresholds in beer of 27 - 52 ppb for ethyl thioacetate and 270 - 350 ppb for methyl thioacetate. Additional volatile sulphur substances reported in beer are carbonyl sulphide (COS) (59,60), carbon disulphide (CS_2) (22,38,61), methional (62,63) and 3-(methylthio) propylacetate (62). Both carbonyl sulphide and carbon disulphide have also been reported as being formed during pressure cooking of grain/water mixtures, suggesting that they could also be present in malt (48).

The final volatile sulphur substance of interest to the brewing industry is a compound which is found in neither hops nor malt, is not produced by the yeast and is not formed by spoilage bacteria. 3-methyl-2-butene-1-thiol, also known as 'light mercaptan', is formed by the influence of light on beer (64) and is therefore a phenomenon seen only in bottled beer. Under the influence of light, photolysis of iso-α-acids from the hops and of sulphur-containing amino acids leads to a free radical reaction, culminating in the formation of 3-methyl-2-butene-1-thiol (3-MBT). This mechanism was first proposed by Kuroiwa and Hashimoto in 1961 (64) and later confirmed by Gunst and Verzele (65) and Sakuma *et al.* (66). This reaction only affects bottled beer, the darker the bottle the greater the protection against light. Beers sold in green or clear glass bottles are especially susceptible to this phenomenon, 'sunstruck' flavour being formed after less than one hour of direct light influence on the bottle (66,67).

Sunstruck beers are often described as 'skunky' in taste and smell. This is in fact a misnomer: 3-methyl-2-butene-1-thiol is present in none of the anal secretions of the three main species of American skunk (68,69,70,71,72,73). Many sulphur-containing compounds, including 3-methyl-2-butane-1-thiol (i.e. the saturated derivative of 3-MBT), are present in the secretions of skunks' anal glands, giving the animals their distinctive penetrating and pungent smell. The German article (74) listed by

Templar *et al.* (75) as referring to the anal secretions of skunks, in fact deals with the anal secretions of minks, which do not contain 3-methyl-2-butene-1-thiol either.

Meilgaard states that the flavour threshold of 3-methyl-2-butene-1-thiol lies at around 1 µg/l (53). Irwin *et al.* suggested that it is probably more accurate to use an odour threshold rather than a flavour threshold: investigations with a panel of 16 experienced beer tasters gave odour thresholds of 4.4 to 35 ng/l, with an average of 7 ng/l (76). Gunst and Verzele proposed that 3-methyl-2-butene-1-thiol is not the only compound responsible for the sunstruck or light flavour (65). They reported sensory similarities between synthesised 3-methyl-2-butene-1-thiol and the smell of sunstruck beers but came to the conclusion that the two aromas were not identical. Blockmans *et al.* (77) and Haboucha and Masschelein (78) recorded an increase in methyl mercaptan in illuminated beers; Kattein *et al.* (67) found not only an increase in methyl mercaptan concentration in the presence of light but also an increase in the hydrogen sulphide concentration.

A comprehensive review of sulphur compounds in beer was written by Nykänen and Suomalainen in 1983 (79).

1.3 Analysis of Sulphur Compounds in Beer

In view of the importance of volatile sulphur compounds in beer as outlined above, a sensitive method of separation and detection for the analysis of these substances is needed. Several techniques - colorimetric (40,80,81,82,83), titrimetric (84), potentiometric (85) and fluorometric (86) techniques - have been used in the brewing industry for this purpose, but none of them even approach the levels of sensitivity and selectivity required. When the alternative available analytical tools are considered it becomes clear that the method of choice - indeed the only method capable of meeting the demanding analytical requirements - is gas chromatography (GC).

However, although gas chromatography is a very sensitive technique, the analysis of volatile sulphur compounds - owing to their high volatility, which makes separation very difficult, their high reactivity and their very low concentrations in beer - can only be achieved with an optimal combination of sample preparation, separation and detection.

A comprehensive review on the methods available at that time for the analytical control of sulphur compounds in beer was written by Garza-Ulloa in 1980 (87).

1.3.1 Sample Preparation

The analysis of volatile sulphur compounds is not simply a challenge for the chromatographer owing to the difficult separation and the very low concentrations to be detected but also because of the difficulties involved in the handling of the samples to be analysed. There are two major difficulties: the first is simply their volatility. Hydrogen sulphide, for example, has a boiling point of -60°C, sulphur dioxide -10°C and methyl mercaptan +6°C. This means that ideally a sample system should be closed. The second major problem is the high reactivity of sulphur compounds, especially with metals. It has long been known in the brewing industry that copper mash tuns and brew kettles affect sulphur levels in beer, and both Brenner and Walker have carried out work into the influence of metal ions on the concentrations of flavour-active sulphur compounds in beer (35,88). As a result all surfaces that come into contact with the sample during the transfer from the bottle to the GC must be inert.

Owing to the low concentrations of volatile and semi-volatile sulphur compounds present in beer some form of pre-concentration during sample preparation will almost certainly be necessary to allow adequate detection. There are several different methods of sample preparation available to the analyst.

An excellent review of sample preparation methods, in particular static and dynamic headspace techniques, for the analysis of volatile sulphur substances in beer was published by Peppard in 1988 (89).

1.3.1.1 Static Headspace

The simplest form of sample preparation in beer is static headspace (30,34,39,59,90): this inexpensive, simple method can be made more sensitive by the warming of the sample vial (the higher the temperature the more analyte in the headspace). Another relatively simple method of improving the sensitivity of static headspace is to lower the partition coefficients of the analytes in the matrix by adding an inorganic salt to the sample solution (91). This should decrease the solubility of the substances to be analysed. However, work by Nedjma and Maujean on sulphur compounds in brandies (92) surprisingly showed that increasing salt concentrations had no effect on the amounts of thiols in the headspace and only a slight effect on the sulphides and disulphides. The addition of an inorganic salt is rather impractical when applied to beer as it results in a sudden release of carbon dioxide. This causes extreme foaming and the loss of volatile compounds.

The major disadvantages of static headspace are its relative insensitivity, despite the addition of salts and warming of the sample, and that it is only suitable for compounds of high volatility. Work by Burmeister *et al.* in 1992 (59,60) showed that at room temperature, methyl thioacetate (b.p. 98°C) was the least volatile sulphur compound in beer, which could be recorded using static headspace sample preparation in conjunction with a sulphur chemiluminescence detector (SCD). A further disadvantage of static headspace for the analysis of sulphur compounds in alcohol-containing matrices was found by Nedjma and Maujean (92). They recorded a negative linear relationship between the amount of alcohol in the matrix and the amount of the sulphur analytes in the headspace, i.e. an increase in the

alcohol concentration in the matrix gives a decrease in the amount of sulphur-containing compounds in the headspace.

1.3.1.2 Dynamic Headspace

The most commonly used method for the sample preparation of volatile sulphur compounds in beer has been a dynamic headspace technique, purge-and-trap. The use of this method in the brewing field was first described by Leppaenen *et al.* in 1979 (93) and has been used extensively since (38,41,57,61,65,66,88,94,95,96,97).

The purge-and-trap technique involves the volatile compounds in the beer being purged out of the sample by an inert gas, such as nitrogen or helium. The volatiles are subsequently adsorbed onto traps packed with an adsorbent material, such as Tenax, Chromosorb or activated charcoal, or are cold-trapped on a GLC column. After a set collection time the trapped volatiles are purged from the trap by rapid heating and are flushed with an inert gas stream into the GC. Goldstein *et al.* used a variation on this technique (98): the volatile compounds were purged out of the beer sample and the sulphur-containing compounds were trapped on glass wool doped with mercuric cyanide. The sulphur compounds were then extracted from the glass wool with an organic solvent.

A further variation of dynamic headspace is repeated or large volume injection. Larger amounts of the sample headspace are injected into the GC and are trapped in the injector, either using a liner packed with an adsorbent or by cold-trapping. The volatile compounds are then released through rapid heating of the trap (99). This system displays good reproducibility and is quicker than purge-and-trap; it requires, however, a cooled injection block and a supply of liquid nitrogen for cooling.

Dynamic headspace methods, such as purge-and-trap and cold-trapping, provide much better pre-concentration of the analytes than

static headspace, leading to significantly improved sensitivity. Additionally, compounds of lower volatility can be measured using dynamic headspace methods: dimethyl trisulphide (b.p. 239°C) was found by most workers (61,95,100) to be the least volatile sulphur compound in beer that could be analysed with dynamic headspace methods. The disadvantages of dynamic headspace methods are the cost of the equipment, extra analysis time, their susceptibility to leaks, and relative difficulty of use. A further difficulty with purge-and-trap methods is the need for a drying step. Water is transferred into the lines and onto the trap during purging. The drying step can lead to loss of analytes and poorer reproducibility. Cold-trapping methods have the disadvantage of requiring liquid nitrogen for cooling. In summary, dynamic headspace methods are good ways of sample preparation for research purposes but are not very appropriate for the routine analysis of sulphur compounds in beer.

1.3.1.3 Distillation Followed by Solvent Extraction

In the brewing industry another method of concentrating sulphur compounds of lower volatility for GC analysis is steam distillation followed by solvent extraction (31). Steam is bubbled through the beer sample, causing it to heat up and ultimately boil. The volatile compounds evaporate and are subsequently condensed, collected and extracted using organic solvents. This technique has been used as the basis for several research projects (101,102). One method (103) used a column of porous polymer resin to trap the trace flavour components in the distillate obtained by vacuum steam distillation, diethyl ether then being used for extraction from the column.

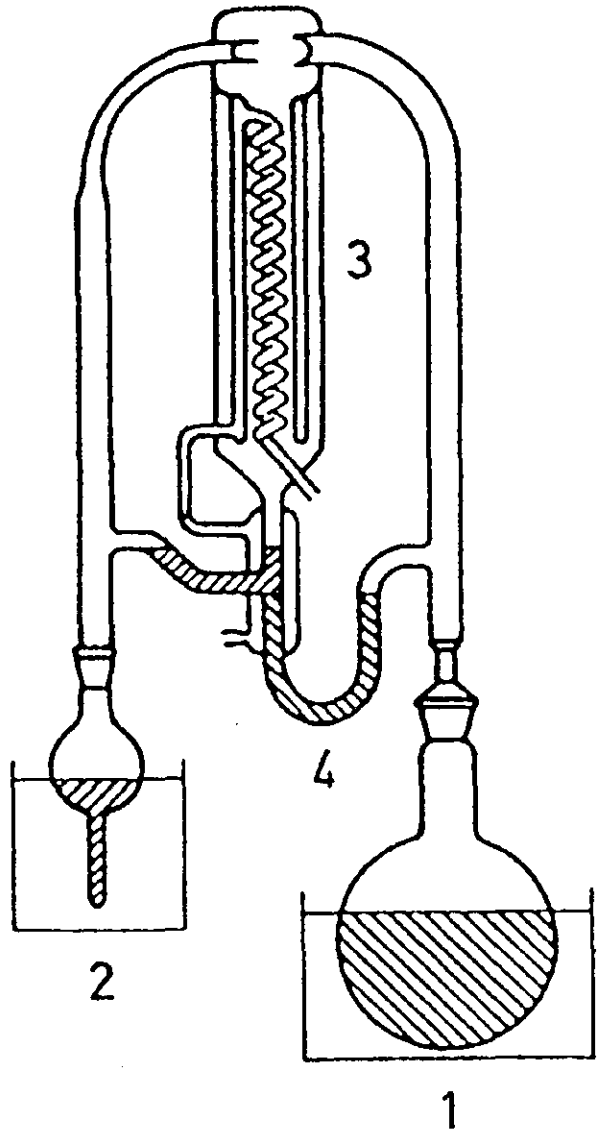
The main disadvantages of steam distillation followed by solvent extraction are that it is very time consuming, requires the use of organic solvents, and that the use of direct heat probably results in the formation of artefacts, leading to the danger of compounds being detected which were not in the original sample. To overcome this last disadvantage

and reduce the amount of direct heat to which the sample is subjected the steam distillation has been carried out under vacuum (104,105). However, the system described by Pickett *et al.* (104,105) is time-consuming and extremely difficult to use. It therefore does not lend itself to a routine method. A further disadvantage of methods which rely on solvent extraction is that they discriminate against more polar compounds, which tend to be more difficult to remove from aqueous solution than less polar compounds.

Direct solvent extraction of beer is seldom used, as emulsions are formed which make phase separation very difficult.

1.3.1.4 Simultaneous Distillation and Extraction

Simultaneous distillation and extraction is a method which has been widely employed in the food industry and aroma industries. The technique was first described by Likens and Nickerson in 1966 (106), the apparatus being shown in Figure 1.



1 = Flask with the aqueous sample in a water bath

2 = Flask with the organic solvent (e.g. pentane) in a water bath

3 = Cooler

4 = Condensate, separated into extract (upper fraction) and water

Fig. 1: Likens & Nickerson simultaneous distillation and extraction apparatus

During simultaneous distillation and extraction both the aqueous sample solution and the organic solvent are warmed in water baths and boil. The vapours are mixed, extraction occurs, the phases are cooled and the condensate fractionates. This is carried on continuously over a

period of anything from an hour to a couple of days. Normally low-boiling organic solvents are used in simultaneous distillation and extraction techniques to allow an easier subsequent concentration step.

Despite its popularity, simultaneous distillation and extraction has several disadvantages. As with steam distillation, thermal influences can be a problem, resulting in the formation of artefacts; organic solvents are required; and polar compounds are difficult to extract from aqueous solutions.

Although the simultaneous distillation and extraction technique has been applied to malt extracts (107) and a modified version of the original Likens-Nickerson apparatus has been used for the analysis of volatile compounds in wine (108,109) there are no publications of its application to beer.

1.3.1.5 Solid Phase Extraction (SPE)

Solid phase extraction is based on the principle of using an adsorbent material to extract organic compounds from aqueous solutions. The solid adsorbent is usually bound to a particulate support, and is contained in a plastic tube or cartridge or is pressed into a disk or membrane. After the adsorbent has been conditioned, the aqueous sample is passed through the cartridge. The compounds of interest are adsorbed and subsequently, following a wash step to remove unwanted interfering compounds, are eluted with an appropriate solvent. Solid phase extraction can be made selective for certain compound groups by the choice of the adsorbent phase and organic solvents. A further advantage of SPE over traditional extraction methods is that very little solvent is required.

Solid phase extraction as a possible method for sample preparation has, however, several drawbacks. Firstly, SPE is restricted to semi-volatile compounds as in an open system the volatile compounds are

easily lost. Additionally, recovery is not always very good, leading to decreased sensitivity of the system as a whole. A further problem specific to beer is that CO_2 in the sample would tend to disrupt the packing of the columns and larger molecules, such as proteins, can plug the columns.

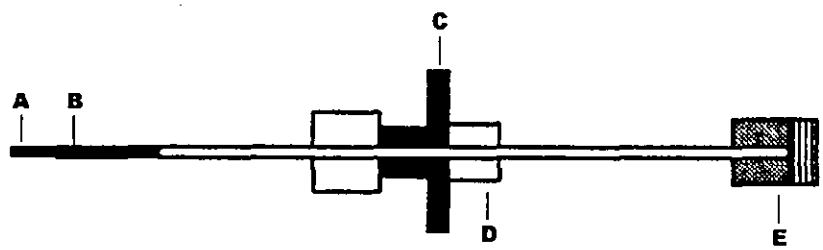
Although SPE has often been used in the brewing industry as a sample preparation technique for the analysis of various involatile compounds in beer using HPLC (110,111,112,113,114,115), there are not very many published applications of its use in conjunction with GC for the analysis of beer (116) or other alcoholic beverages such as cider (117) or wine (118).

1.3.1.6 Solid Phase Microextraction (SPME)

Solid Phase Microextraction (SPME) is a relatively new method, first being described in 1990 (119). SPME is based on the adsorption of compounds onto a coated fused silica fibre mounted on a modified GC syringe. The analytes are concentrated on the surface of the fibre and are then transferred to a GC. In the injection block they are thermally desorbed and subsequently pass onto the column (120,121).

The essential part of an SPME device is the piece of coated fused silica, approximately 1 cm in length. The fused silica is bonded onto a stainless steel plunger and this is fixed in a holder. Two forms of holder are available, one for manual use and one for use with a Varian autosampler. A diagram of the fibre assembly for use with a Varian autosampler is shown in Figure 2. The fibre assembly for manual use is the same except that a spring is placed between the hub and the sealing septum.

Figure 3 shows a diagram of the complete SPME device (i.e. fibre assembly and holder) as used with a Varian autosampler.



A = SPME Fibre

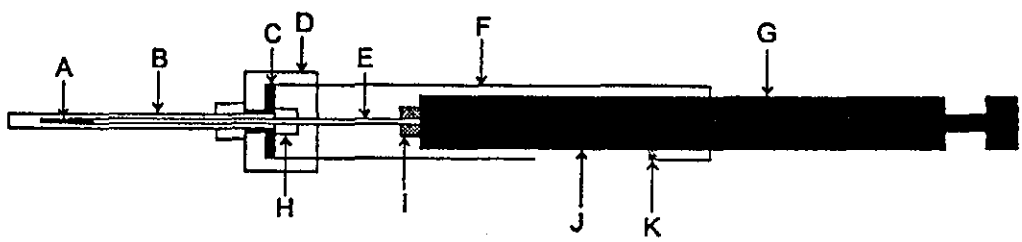
B = Stainless Steel Plunger

C= Needle Ferrule

D = Sealing Septum

E = Colour-coded Screw Hub

Fig. 2: SPME fibre assembly



A = SPME Fibre

B = Septum Piercing Needle

C = Needle Ferrule

D = Retaining Nut

E = Fibre Attachment Needle

F = Barrel

G = Plunger

H = Sealing Septum

I = Colour-coded Screw Hub

J = Slot

K = Retaining Screw

Fig. 3: SPME holder complete with fibre assembly

During piercing of the sample vial septum, the fragile fused silica fibre is protected by the stainless steel sheath of the septum-piercing needle. During sampling the plunger is depressed and the fibre is exposed to the sample - either directly or to the headspace above the sample. After adsorption the fibre is again drawn into the septum-piercing needle and the needle withdrawn from the sample vial.

The SPME device is transferred to the injection port of the GC where the adsorbed compounds are thermally desorbed. The only necessary modification of the injector is the use of a narrower glass injector insert (0.8 mm ID).

SPME fibres became commercially available in 1994, a wide range of different coatings providing different selectivity for various groups of compounds.

The theory of solid phase microextraction has been covered in several publications (121,122). The most extensive discussion on the theory of SPME can be found in the book by Pawliszyn (123), the inventor of the SPME technique. An applications book from the same author is presently in press.

Solid phase microextraction is not an exhaustive extraction technique but is based on an equilibrium between the various phases. The theory varies slightly between direct, liquid sampling and indirect, headspace sampling (124). The amount of analyte which is adsorbed from the sample onto the fibre at equilibrium is determined by the thermodynamics of the extraction process. The time required for equilibrium to be reached is determined by the kinetics of the mass transfer in the system.

Firstly, the thermodynamics: if idealised conditions are assumed (i.e. a three-phase system consisting of an analyte in a simple, aqueous matrix; the fibre coating; and a gaseous headspace phase) the

amount of the analyte adsorbed by the coating on the fibre can be defined by the following equation:

$$(i) \qquad n = C_0.V_F.V_S.K_{FS} / (K_{FS}.V_F + K_{HS}.V_H + V_S)$$

where n = mass adsorbed by the stationary phase on the silica fibre,

C_0 = initial concentration of the analyte in the aqueous solution,

V_F = volume of the stationary phase on the silica fibre,

V_S = volume of the aqueous phase,

K_{FS} = partition coefficient of the analyte between the stationary and aqueous phases,

V_H = volume of the headspace phase,

K_{HS} = partition coefficient of the analyte between the aqueous and headspace phases.

Equation i clearly demonstrates the linear relationship between the mass of the analyte adsorbed by the stationary phase and the initial concentration of the analyte in the aqueous solution. If C_0 is increased, n is increased in direct proportions. Additionally it can be seen from equation i that the position of the fibre in the system has no effect on the amount of analyte adsorbed - as long as the volumes of the three phases are kept constant, the fibre can be placed either directly into the aqueous matrix or in the gaseous headspace phase.

For direct liquid sampling in a closed vial filled with the matrix, i.e. no headspace phase, the term $K_{HS}.V_H$ can be left out of the denominator, giving the following equation:

$$(ii) \qquad n = C_0.V_F.V_S.K_{FS} / (K_{FS}.V_F + V_S)$$

As stated, these two equations only apply for ideal conditions. They can, however, also be approximately applied to more complex systems, for example beer.

For headspace sampling the value of n can never be larger than with direct liquid sampling where no headspace is present because of the extra term, $K_{HS}.V_H$, in the denominator. However, if the volume of the headspace is kept small in relation to the volume of the liquid phase, i.e. $V_S >>> V_H$, this term becomes insignificant and therefore the detection limits of headspace sampling do not differ greatly from those for liquid sampling. Additionally, for many analytes the K_{HS} values are relatively small (Pawliszyn quotes benzene as having a K_{HS} value of 0.26), which reduces the significance of the $K_{HS}.V_H$ term even further. The limiting factor for headspace sampling is effectively the volatility of the analytes.

The stationary phases used in SPME strongly adsorb organic compounds. This leads to high K_{FS} values and good sensitivity. The K values for most compounds, however, are not high enough for exhaustive extraction of the analyte from the aqueous matrix. Therefore an equilibrium is reached between the amounts of analyte in the liquid and stationary phases.

Although the amount of analyte extracted is practically not influenced by the position of the fibre in the system, the kinetics of the extraction *are* dependent on the form of sampling used, i.e. there is a difference in the kinetics of the mass transport between headspace and liquid sampling techniques. The reason that the kinetics of mass transport of the analytes play such an important role, is that SPME is an equilibrium sampling technique.

The theory of the mass transport in direct and headspace sampling systems is based on Fick's second law of diffusion, which describes the mass balance in a one-dimensional dynamic system. The application of Fick's second law to 3-dimensional dynamic systems of mass

balance is covered extensively and in great detail by Pawliszyn in his book (123).

In the direct SPME sampling of aqueous matrices the determining step of the speed of sampling, i.e. the time required for equilibrium to be reached, is the diffusion of the analytes through a static layer of water surrounding the fibre. The molecules have to pass through this static water layer to reach the fibre, and this is a slow process, especially where compounds with large K_{FS} values are involved. This problem can be solved by employing some form of agitation, thereby ensuring that the thickness of the static water layer is kept to a minimum, the final thickness being dependent on the effectiveness of the agitation and the viscosity of the liquid. Many different methods of agitating the sample, including magnetic stirring, sonification and vortex mixing have been employed.

A further drawback of direct liquid SPME sampling is when complex matrices which contain molecules of high molecular mass are sampled, beer for example. The larger molecules (proteins, sugars and polyphenols in the case of beer) are adsorbed onto the fibre but are not desorbed in the injector of the GC, as they are not sufficiently volatile. In effect they are baked onto the fibre, which could lead to artefact formation in the injector port, shortened fibre life-spans and, with all probability, to reduced precision. This problem has been tackled in one publication where a wash step was used after direct liquid sampling (125), but this technique is not easy to incorporate into an automated method.

One way of avoiding both of the above-mentioned problems with direct liquid sampling is to sample the headspace over the liquid phase. The fibre does not come into contact with any high molecular weight compounds in the matrix, and no static water layer is built up around the fibre. There are two disadvantages of headspace sampling. Firstly, the sensitivity is lower than that of direct liquid sampling, as displayed in equations i and ii: this, however, can be made almost negligible by decreasing the volume of the headspace over the liquid to an absolute

minimum. Secondly, the range of molecules extracted is limited to volatile and semi-volatile compounds. High molecular weight compounds are involatile and so do not partition into the headspace and are therefore not adsorbed onto the fibre.

The headspace SPME method appears to be more appropriate than the direct sampling technique for the extraction of volatile compounds, including sulphur compounds, in beer.

The interest in this simple but elegant sample preparation method is large. Despite its recent introduction a huge range of applications has already been recorded, several of them in the area of food analysis (126,127,128,129,130,131,132,133,134,135,136,137,138,139), and more specifically for the analysis of volatile sulphur compounds in food (140,141). There have been two brief reports on the application of SPME for the analysis of beer aroma compounds (142,143) and a more detailed investigation of SPME as an alternative to static headspace for the analysis of some alcohols and esters in beer (144). A Varian SPME Application Note (145) describes the possibility of combining SPME with a pulsed flame photometric detector for the detection of sulphur volatiles in beer, identifying 4 sulphur compounds in American beer samples. Spanish workers recently published two reports on the headspace SPME analysis of volatile sulphides and disulphides in wine aroma (146,147).

A drawback to SPME appears to be poor precision. This was first reported by Aurthur and Pawliszyn in the first publication on SPME (119), stating relative standard deviations ranging from 3% to 25% with a typical value of 10%. Automation of SPME (120,148) has reduced this problem: a precision of typically 5% relative standard deviation for manual operation and approximately 1% for automatic operation has been claimed (121), although one publication on a practical application of SPME reports much higher relative standard deviations of up to 20% (135).

A further possible drawback to SPME could be the effects of the sample matrix. As the SPME technique was primarily developed for the analysis of organic compounds in water samples, the problem of matrix effects did not arise. The only mention in the early literature on SPME was in the 1992 publication by Aurthur *et al.* (120), where the effects of methanol in the aqueous sample were briefly discussed, with the conclusion that matrix effects with less than 1% methanol were insignificant. Reports that 20% methanol in aqueous solution reduced the peak sizes of pesticides after SPME extraction (149,150) led Urruty and Montury (151) to investigate the influences of ethanol on the SPME extraction of pesticides in aqueous solution. They found that variations in the ethanol concentration of the aqueous solutions had no influence on the equilibration time of the system but had a great effect on the amount of analyte extracted. They concluded that ethanol was acting as a 'co-solvent' for the pesticide residues and was thereby influencing their partitioning coefficients.

Ethanol concentration is therefore an important parameter in the analysis of alcoholic beverages with SPME. Various recent investigations on the application of SPME for the analysis of wines (136,137,146,147,152) have confirmed Urruty and Montury's findings. However, Mestres *et al.*, who have been responsible for the most recent publications on this subject (146,147), have a different explanation for the effect of ethanol. They state that the reason for the decrease in the amount of sulphur compounds extracted with increasing ethanol concentration is competition for adsorption onto the fibre. This contradicts Urruty and Montury's conclusion that ethanol acts as a co-solvent.

The use of an internal standard could compensate for the effect of ethanol on extraction. Mestres *et al.* also found that although the absolute peak areas of sulphides and disulphides compounds decreased with increasing ethanol concentration, the S-compound / ISTD ratio remained constant. The recent publication on SPME analysis of alcohols and esters in beer (144) did not consider the effects of alcohol concentration.

The possibility of further matrix effects on the SPME analysis of complex, natural sample matrices such as beer and wine should not be discounted.

A further imaginable problem with SPME is that of 'carryover': if the compounds adsorbed by the SPME fibre are not completely desorbed in the GC injector then they will be carried over to the next extraction, thereby giving distorted results in subsequent analyses.

1.3.2 Separation of Volatile and Semi-volatile Sulphur Compounds

The already-mentioned volatility of the sulphur compounds to be analysed has an effect on the choice of column to be used for the chromatographic separation. Therefore, when selecting a column for this GC method the theory of chromatographic separation has to be briefly considered (153,154,155,156).

Gas chromatography is based on the principle of solutes spending differing amounts of time in a mobile and in a stationary phase, moving from phase to phase in a dynamic equilibrium. The equilibrium - or distribution - constant is known as K_D and can be defined as follows:

(iii) $K_D = C_S / C_M$ where C_S is the concentration in the stationary phase, and C_M is the concentration in the mobile phase

The value of K_D is fixed for the same stationary phase, column temperature and solute.

C_S and C_M can be defined as follows:

(iv) C_S = mass solute in stationary phase / volume stationary phase

(v) C_M= mass solute in mobile phase / volume of mobile phase

Therefore:

(vi) $K_D = \frac{\text{mass solute in stationary phase}}{\text{mass solute in mobile phase}} \times \frac{\text{volume of mobile phase}}{\text{volume stationary phase}}$

(vii) $= k\beta$ where k is the retention or capacity factor, and β is the phase ratio

The phase ratio, ß, is the ratio between the volume of the mobile (i.e. gas) phase in the column and the volume of the stationary (i.e. liquid) phase in the column. For open tubular columns the following approximation is used:

(viii) $\beta \approx \frac{r}{2d_f}$ where r is the column radius, and d_f is the stationary phase film thickness

The retention factor, k, is the ratio between the amounts of a solute in the stationary and mobile phases. This ratio is equivalent to the ratio of the amount of time a solute spends in the stationary and mobile phases. Therefore:

(ix) $k = t'_R / t_M$ where t'_R is the time spent in the stationary phase, and t_M is the time spent in the mobile phase

The retention time, t_R, of a solute can be defined as follows:

(x) $t_R = t'_R + t_M$

The time a solute spends in the mobile phase is proportional to the length of the column (L) and inversely proportional to the average linear velocity of the mobile phase (u):

(xi) $$t_M = \frac{L}{u}$$

From equation xi it can be seen that the time spent in the mobile phase is the same for each solute. This means that the determining factor for the retention time of a solute is the amount of time it spends in the stationary phase.

Chromatographic theory as outlined above can be used in the selection of a column for the separation of highly volatile sulphur compounds. Three separate physical characteristics of a column are critical in raising the time spent in the stationary phase, t'_R, and therefore the retention factor, k: column length, column diameter, film thickness.

To assess the influence of these three parameters on retention, the above equations need to be expressed differently. Substituting iii, viii and ix into vii, the following equation is obtained:

(xii) $$C_S / C_M = (t'_R / t_M) \times (r / 2d_f)$$

and therefore:

(xiii) $$t'_R = (C_S.2d_f.t_M) / (C_M.r)$$

Substituting xi into xiii:

(xiv) $$t'_R = (C_S.2d_f.L) / (C_M.r.u)$$

From equation xiv it can be seen that column length is directly proportional to the amount of time the solute spends in the stationary phase,

i.e. a longer column gives higher retention when the same conditions are used. A decrease in column diameter causes a decrease in the phase ratio (see equation viii) and therefore an increase in k, assuming that the linear flow is kept constant. Equation xiv also shows that increasing the film thickness will also increase retention.

The disadvantage of all these measures to increase retention for highly volatile compounds is that the k values for less volatile compounds are also increased, meaning greatly increased analysis times. This can be compensated for by using a temperature program, as k is inversely proportional to temperature.

The three above-mentioned column parameters do not only influence retention: they also influence column efficiency. Efficiency is often expressed in terms of the number of theoretical plates per metre of column length, or as the height equivalent to a theoretical plate (HETP), which is given in millimetres.

(xv) $H = L / N$ where L is the length of the column (mm), and N is the number of theoretical plates

Therefore the smaller the value of H, the higher the number of theoretical plates and the more efficient the column.

A way of describing the parameters affecting column performance is the van Deemter equation:

(xvi) $H = A + Blu + C.u$ where A is the Eddy diffusion term,
B is the term representing longitudinal band broadening,
u is the average linear velocity of the carrier gas,
and C is the resistance to mass transfer

As WCOT columns have no packing, the Eddy diffusion term can be disregarded, simplifying equation xvi to the following, known as the Golay Equation:

(xvii) $H = B/u + C.u$

Decreased column diameter and increased column length have a positive effect on column efficiency as well on retention. Increased film thickness, however, can have a negative effect for some compounds. The reason for this is the C term, resistance to mass transfer, in equation xvii. The C term can be divided into C_S and C_M terms (not to be confused with the concentration terms used in equations iii - vi), as shown in equation xviii:

(xviii) $h = B/u + (C_S + C_M).u$

With thick films the C_S term is increased because the probability of a solute diffusing far from the gas-liquid phase interface is much greater than that for a thin film column. Therefore the band of the solute molecules is broadened, giving broader peaks.

Increasing the film thickness for very volatile compounds with a k value of less than 5 results in better resolution because of better retention; for compounds with k values of 5 - 10 a slight improvement in resolution is seen with increased film thickness; for peaks with k values over 10, an increase in retention through thicker films leads to a loss of resolution (154). In short, an increase in film thickness to improve peak resolution for volatile compounds can cause a loss of peak resolution for less volatile compounds.

Hutte *et al.* investigated column selection and optimisation for sulphur compound analyses by GC (157). Their results backed up the above-outlined theory of chromatographic separation. They reported that increasing the thickness of the bonded methyl silicone film resulted in better separation up to a film thickness of 4µm: after this point no improvement in separation was seen. Indeed, peak broadening of less volatile sulphur

compounds was witnessed with films thicker than 4µm. Even with a 4µm film resolution of SO_2 and COS was not obtained at ambient temperatures. The employment of cryo-cooling was successful in solving this problem.

An alternative to capillary columns is to use packed columns, which would certainly allow longer retention times and higher sample capacities for volatile substances because of higher phase ratio (ß) values. These advantages, however, are greatly outweighed by the disadvantages of undefinable different flow paths through the packing material, inconsistent thickness of the stationary phase and temperature gradients across the diameter of the packed columns, all of which contribute to poor resolution and separation (153).

The third possibility is the use of Porous Layer Open Tubular (PLOT) columns, which have a solid adsorption type stationary phase instead of a liquid film, a gas-solid adsorption process replacing the gas-liquid partitioning effect seen with WCOT columns. The problem with PLOT columns, however, is that they are not suitable for use with semi-volatile compounds.

1.3.3 Detection of Volatile and Semi-volatile Sulphur Compounds

The choice of gas chromatographic detector (158) for the determination of volatile sulphur substances (irrelevant of the matrix) is of vital importance (159). This is for several reasons. Firstly, as already stated, these substances are present in very low concentrations in beer, which means the detector has to be very sensitive.

Secondly, the detector of choice must display some form of selectivity for sulphur compounds. This is important because the concentrations of sulphur compounds are often very low in comparison to the concentrations of other volatile compounds present in a matrix. In beer, for example, Meilgaard listed over 850 compounds (160) and Lustig identified

approximately 350 of these substances by gas chromatography (161). The concentrations of many of these substances are significantly higher than those of the sulphur compounds. Therefore sulphur selectivity, or even better sulphur specificity, of the GC detector is very important.

This demand for selectivity rules out the use of universal detectors, such as the flame ionisation detector (FID) and the thermal conductivity detector (TCD). The TCD has to be disqualified on sensitivity grounds alone; the FID, on the other hand, possesses the required sensitivity but gives a response for all compounds that can be ionised in an H_2/air flame. Owing to the complex nature of beer and the resulting number of peaks, the chromatographic demands placed on the column to avoid any co-elution would simply be too high. In addition, this multiplicity of peaks would make identification of the individual compounds very difficult.

The selectivity of the potential detectors has obviously to be for sulphur, thereby disqualifying the nitrogen phosphorus detector (NPD), thermal ionisation detector (TID), photoionisation detector (PID), and electron capture detector (ECD), although some workers have reported the latter detector's sensitivity and specificity for sulphur substances (162,163). Once all these detectors have been disregarded, the list of the possible alternatives is reduced to five: the flame photometric detector (FPD), Hall electrolytic conductivity detector (HECD), mass spectrometric detector (MSD), atomic emission detector (AED) and the sulphur chemiluminescence detector (SCD).

A very good comparison of the FPD, AED and SCD for the analysis of sulphur compounds in food was recently carried out by Mistry, Reineccius and Jasper (164). Steely has also compared the FID, FPD and SCD for the detection of sulphur compounds in milk (165). The sensitivity of the FPD and the AED was compared by David and Sandra (166) and Lee and Wylie (167). The SCD and AED were compared by Eckert-Tilotta *et al.* in 1992 (168). Gaines *et al.* compared the SCD and FPD for the HRGC determination atmospheric sulphur gases (169). A further detector of interest

in the study of volatile sulphur compounds is the olfactory detector or sniffing port. This naturally does not allow quantitative analysis but can be used qualitatively to aid identification of the substances.

The principles of operation, advantages and disadvantages of the GC detectors capable of detecting sulphur compounds are outlined below.

1.3.3.1 Flame Photometric Detector (FPD)

The FPD, a specific detector first introduced in 1966 (170), is without doubt the most commonly-used GC detector for the determination of sulphur substances. The FPD is based on the principle that when sulphur and phosphorus-containing substances are burnt in an H_2/air flame they form S_2 and HPO molecules, respectively, in an electronically excited state. When these electrons fall back to their ground states they emit energy in the form of light. This luminescence is then detected by a photomultiplier tube, a filter being used between the flame and the photomultiplier tube to determine the specificity of the detector (390nm filter for S_2, 526nm for HPO).

The construction of a typical, commercially available FPD is shown in Figure 4 (158).

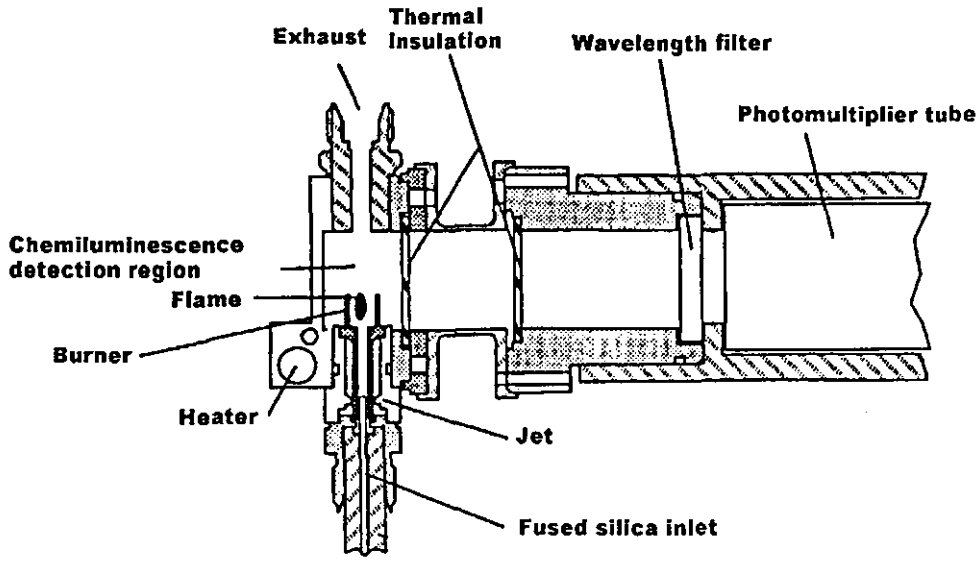


Fig 4: Schematic diagram of a commercial FPD

However, there are several inherent problems connected with the FPD. Firstly, it does not give a linear response for sulphur, i.e. the emission of light is not directly proportional to the concentration of the sulphur atoms present. It is in fact proportional (but only approximately) to the square of the sulphur atom concentration, because it results from S_2. Two quenching effects, due to undesired light absorption in the flame, are also a problem with the FPD. Hydrocarbon-quenching, which is caused by hydrocarbon compound co-eluting with a sulphur-containing compound, and self-quenching, caused by the presence of heteroatoms in high concentrations, which can lead to the photon not being emitted in the first place or even reabsorbed. Further drawbacks to the FPD are the tendency of water to condense on the window of the photomultiplier tube and the dependency of response stability on optimal gas flows. It is obviously impossible to give an exact figure for the sensitivity of the FPD, but the limit of detection is generally regarded as lying in the range 20 - 200 pg sulphur/second (158,159,164).

1.3.3.2 Pulsed Flame Photometric Detector (PFPD)

In the early 1990's Amirav and co-workers at the University of Tel Aviv, Israel, developed a new form of the FPD, the pulsed FPD (PFPD) (171,172,173), and a commercially available version of this detector has recently been launched. The principle of the PFPD is that a flame source and gas rates which cannot sustain a continuous flame are used. The sample is combusted by a propagated ignited flame, a pulse of light is seen, and the flame self-terminates. Selectivity is provided by the appropriate filter and also the added dimension of time as hydrocarbon emission is faster than that of heteroatom species. This means the maximum emission of sulphur and hydrocarbon molecules are separated in time. This should allow not only higher selectivity but also higher sensitivity owing to the reduction of flame background.

A schematic diagram of the Varian PFPD is shown in Figure 5 (174).

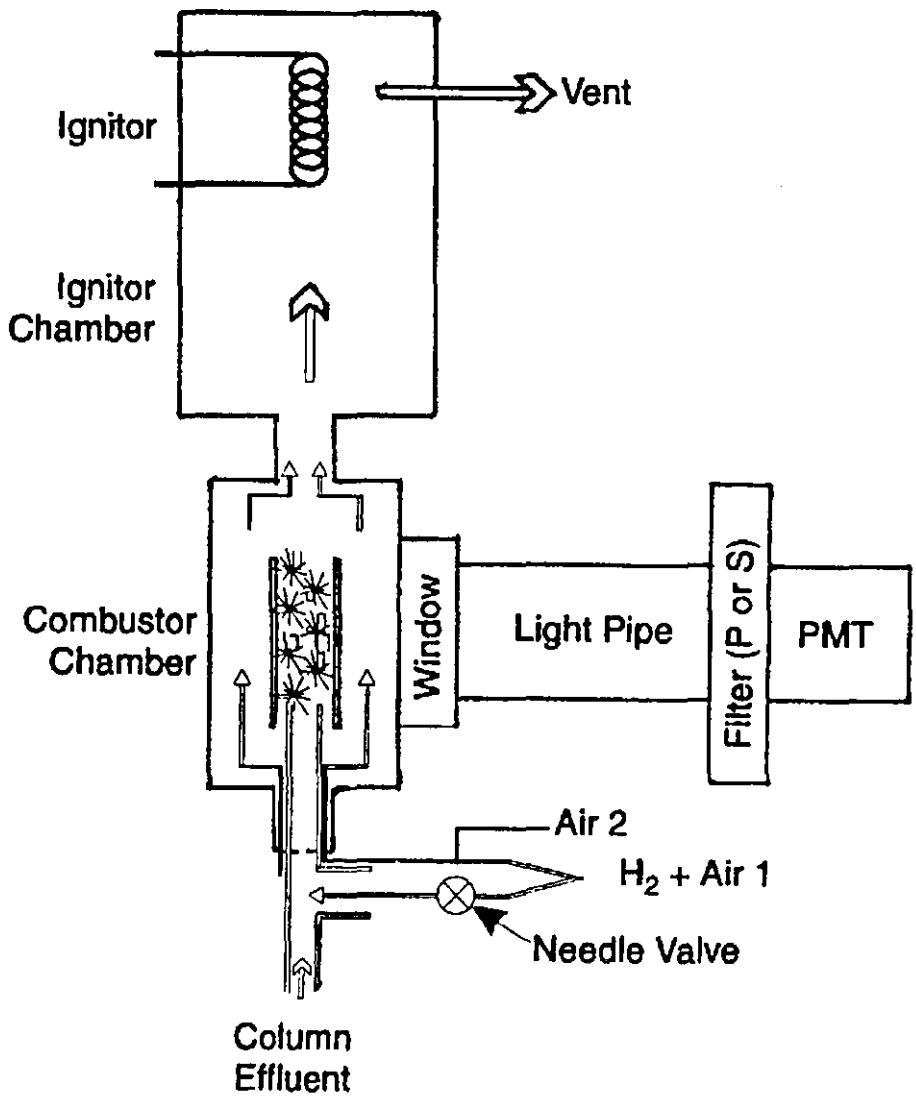


Fig. 5: Schematic diagram of the Varian PFPD

Pulsed flame operation can be divided into four discrete stages. These are shown in detail in Figure 6 (174).

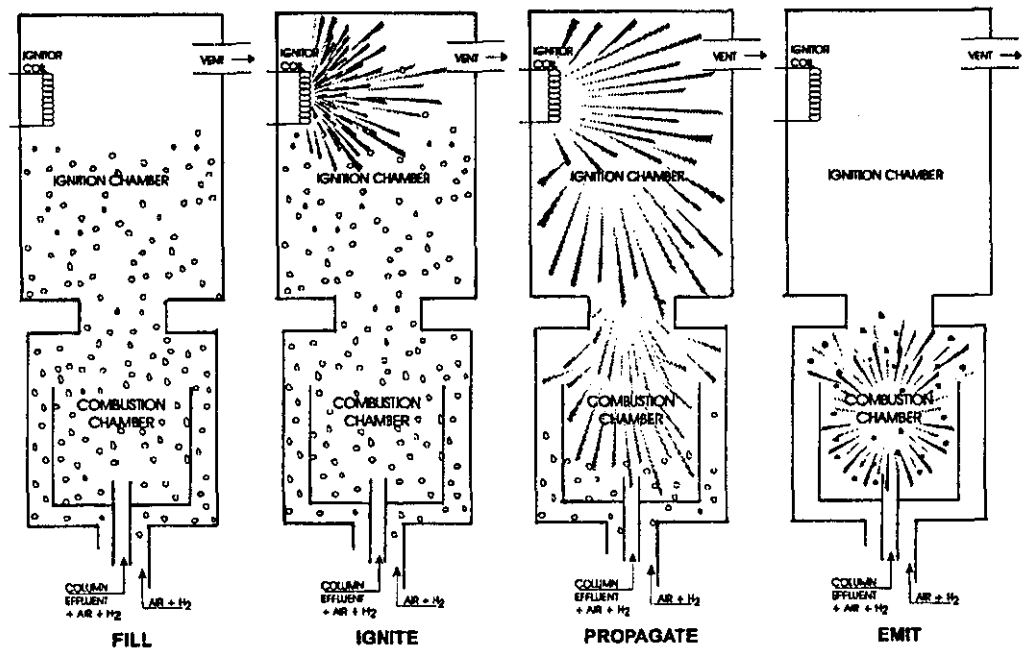


Fig. 6: The four stages of pulsed flame operation in the Varian PFPD

The first stage of pulsed flame operation is the fill stage. The combustion chamber of the PFPD is filled with two different combustible hydrogen/air mixtures, which enter the combustion chamber at two separate points at the bottom of the detector. The first, hydrogen-rich mixture is mixed with the column effluent and flows through the centre of the quartz combustor tube. The second mixture, which contains more air, flows around the outside of the combustor tube and into the ignition chamber. For optimal sulphur sensitivity it is desirable for the combustor to fill slightly faster than the ignition chamber. The rates of the two gas flows are determined by a needle valve, which regulates the first hydrogen/air mixture, and the flow of extra air into the second mixture.

The second stage of flame pulsation is ignition. When the combustible gas mixture reaches the ignition chamber, it is ignited by the continuously heated ignitor coil.

Propagation is the next step. After ignition the flame propagates downward from the ignition chamber into the combustion chamber. When it reaches the bottom of the detector the flame goes out, as all of the combustible material is used up. This process takes less than 5 milliseconds.

The final stage of pulsed-flame operation is emission. The excited atoms and molecules formed by combustion emit their energy in the form of light at different rates (171,175). Hydrocarbon molecules, for example, combust very exothermically and very fast and the emission of excited combustion products such as CH*, C_2* and OH* takes place within a couple of milliseconds of combustion, i.e. within the time it takes for the flame to propagate through the combustor and extinguish. Heteroatom species such as S_2*, HPO* and HNO*, on the other hand, emit at cooler, post-flame conditions and over a much longer time span. S_2* emission, for example, is at its maximum 5 - 6 milliseconds after the emission of CH* and OH* has ceased.

In their first two articles on the PFPD (171,172), Atar, Cheskis and Amirav stated that the origin of the time delay is not clear. They proposed, however, the following 'guide-line' explanation. Hydrocarbon emission from CH* radicals is dependent on the formation of the CH* radical, which is shown in equation xix:

(xix) $C_2 + OH \text{------>} CH^* + CO$

This reaction involves OH radicals, which are very reactive and have very short life-times (< 10^{-4}s). Therefore they are fully consumed during flame propagation. Hydrocarbon emission from C_2* radicals is also very short, owing to the high reactivity of carbon atoms and their subsequent short chemical life-times. The result is that hydrocarbon emission is finished by the time flame propagation through the combustion chamber is complete, i.e. hydrocarbon emission last less than 5 milliseconds.

Sulphur emission, however, is determined mainly by reactions which include atomic hydrogen, which possesses relatively long life-times in hydrogen-rich flames. Reactions involving sulphur can also occur at cooler, post-flame conditions, thereby resulting in emission delay.

The time-dependency of flame emissions can be seen in Figure 7 (176).

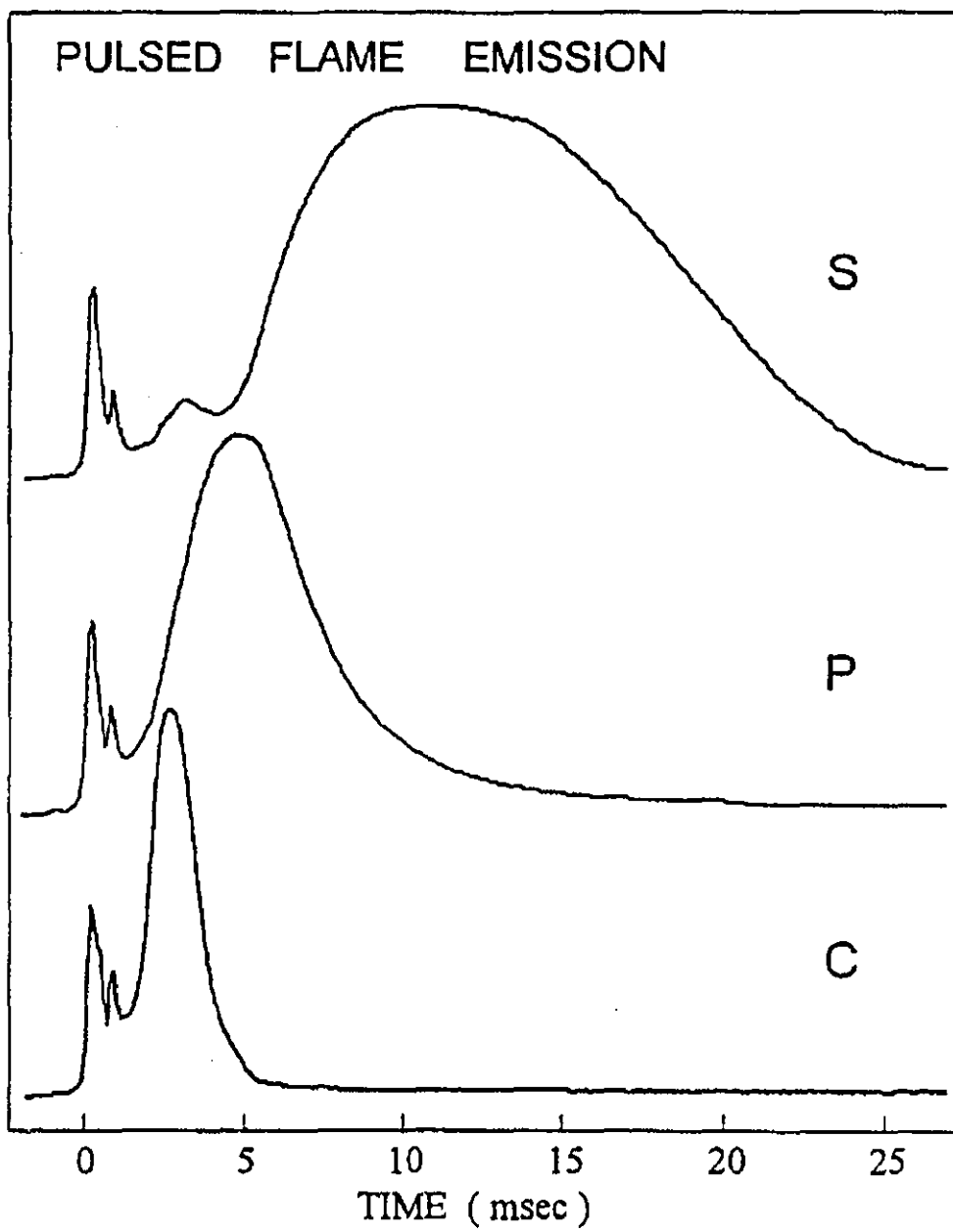


Fig. 7: Time dependency of pulsed flame emissions

Figure 7 effectively demonstrates how the dimension of time allows the molecular emissions of heteroatomic species to be resolved from the background emission, i.e. S_2 emission from CH*, C_2* and OH*. Background noise is reduced and as a result sensitivity is increased. Separation of the emission of interest from the background emission is carried out using an electronic gate. The section of time in which emission is integrated is determined by the gate delay and the gate width. As can be seen from Figure 7, appropriate settings for sulphur emission would be a gate delay of 6 ms and a gate width of 20ms.

The settings of the electronic gate can be used to control selectivity and sensitivity of the PFPD. A good example of this (Figure 8) was provided by Cheskis, Atar and Amirav (172), using a solution containing 1% decane (peak 5 in Figure 8), 1% aniline (peak 4), 3 x 10^{-5} dimethyl methylphosphonate (DMP) (peak 3) and 6 x 10^{-5} tetrahydro- thiophene (THT) (peak 2) in methanol (peak 1). In the first chromatogram the THT peak can hardly be seen. By changing the gate parameters, but using the same filter, either maximum selectivity or maximum detectivity for THT can be obtained, as can be seen in the second and third chromatograms.

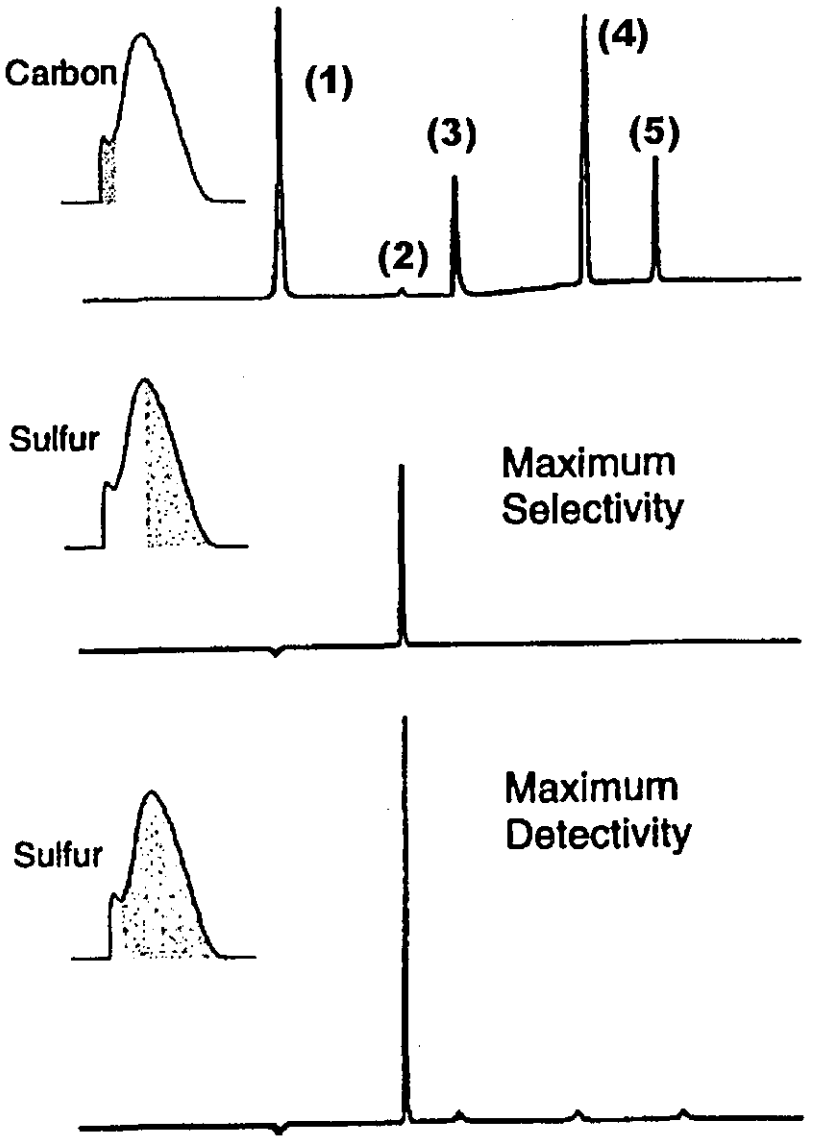


Fig. 8: Dependency of PFPD sensitivity and selectivity on gate settings (172)

Selectivity and sensitivity of the PFPD can also be influenced by the composition of the gas mixtures in the detector. The proportions of air and hydrogen in the flame affect the formation of molecules which emit light: for example, a hydrogen-rich flame favours S_2^* formation, giving better sulphur sensitivity. A flame with proportionally more air and less hydrogen hinders S_2^* formation, giving, for example, better phosphorus sensitivity and selectivity.

An additional cause of higher sensitivity from the PFPD is the concentration in time of the emitted light (174,176). In a conventional FPD the compounds eluting from the column are continually combusted. In the PFPD they are 'accumulated' in the combustion chamber and the accumulated sample is combusted approximately every 300 ms. The emitted signal of the collected molecules is therefore stronger than the signal obtained by continual combustion as in an FPD.

As a complete cycle of filling, ignition, propagation and emission takes approximately 300 ms, a PFPD should pulse approximately 2 - 4 times a second. A further advantage of employing gas flow rates incapable of sustaining a continuous flame is that gas consumption is significantly reduced.

The problem of hydrocarbon-quenching, which is seen with a conventional FPD, can be reduced with the PFPD in two different ways. Firstly, the extra sensitivity allows less sample to be injected to obtain the same response, thereby reducing the amount of hydrocarbons present in the detector and consequently hydrocarbon-quenching. Secondly, slightly increasing the air flow rate reduces quenching whilst giving only small losses in sensitivity (177).

The sulphur response of the PFPD is claimed to be purely quadratic (172). Additionally, the sulphur response is claimed to be equimolar, i.e. the sulphur response is independent of the structure of the sulphur-containing molecule (172).

1.3.3.3 Hall Electrolytic Conductivity Detector (HECD)

The HECD was first developed by Coulson in the mid-sixties (178) and considerably improved by Hall a decade later (179). The HECD can be operated in three different modes, making it selective for compounds containing either sulphur, nitrogen or halogens. The separated compounds from the column enter the detector and are mixed with a reaction gas (air for sulphur compounds) in a nickel reaction cell. The products of the reaction are then mixed with a deionised solvent: for sulphur detection methanol is often used. A conducting solution results, the conductivity of which is then measured.

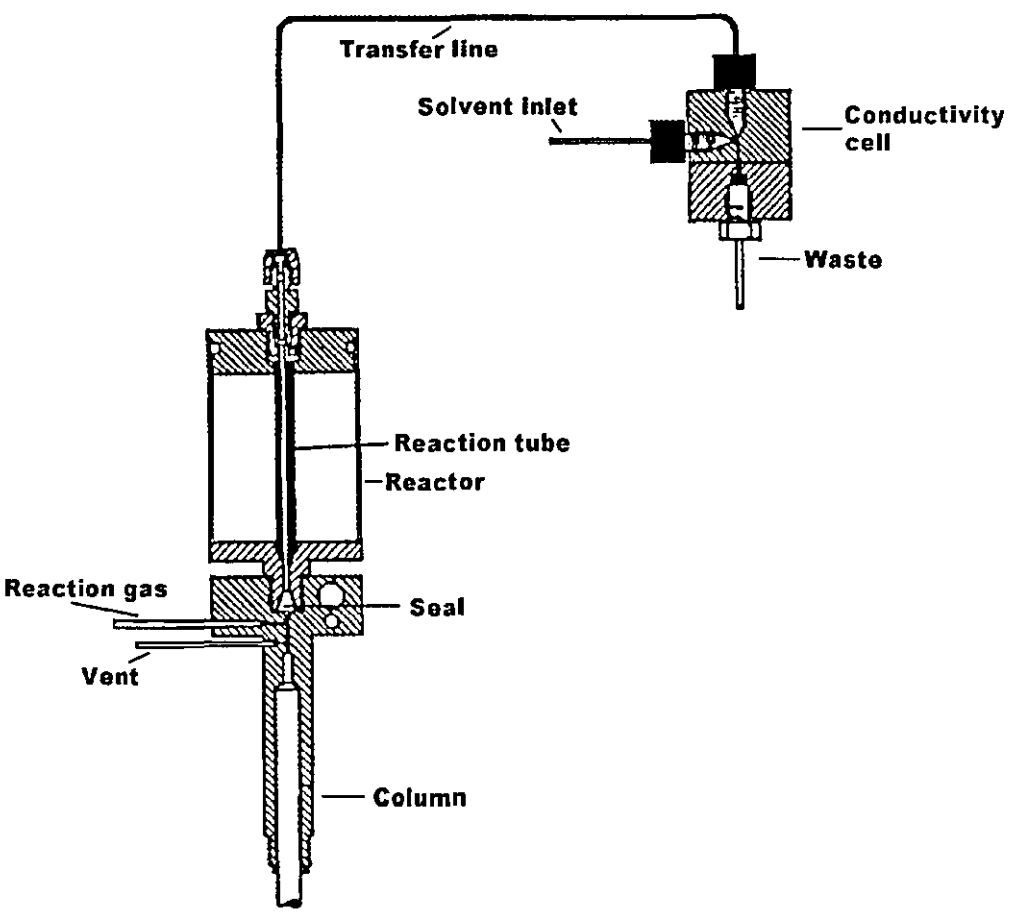


Fig. 9: Diagram of an electrolytic conductivity detector (158)

A comparison between various versions of the HECD and an FPD is described in the literature (180). Although the linearity of the HECD was found to be superior to that of the FPD, the sensitivity of the two detectors was regarded as roughly the same. The HECD suffered from less quenching in the presence of organic hydrocarbons than the FPD.

In comparison to the FPD the HECD is not very easy to use (180), and its performance is dependent on regular and thorough maintenance and cleaning. Another problem is the use of large amounts of organic solvents, but this is solved by the employment of an ion exchanger (158) which cleans up eluent and allows the solvent to be circulated and reused.

1.3.3.4 Mass Spectrometric Detector (MS)

Mass spectrometric detection has the great advantage that it not only allows the quantitative analysis of compounds but also provides qualitative information, enabling the peaks of a chromatogram to be identified (156,158,181). In the Scan mode, where the total ion concentration is simply recorded, the sensitivity of MS detection is not high enough for the low levels of sulphur compounds present. In addition, the MS in scan mode suffers from the same problem as the FID. A multitude of peaks is seen, which puts very high demands on the column to avoid any co-elution and achieve baseline separation.

These problems are solved by the use of the specific ion monitoring mode (SIM), in effect turning the MS from a universal detector into a selective detector. In this mode only those ions which are present in the substances of interest are monitored. This means that fewer peaks are seen, i.e. increased selectivity; and because fewer ions are monitored, the dwell time spent measuring each ion is higher, leading to increased sensitivity and lower limits of detection. The qualitative properties of the MS obviously suffer

in SIM mode as 'incomplete' spectra are delivered, making the library matching process extremely difficult.

The limits of detection for mass spectrometric detectors vary enormously, depending on analyte and the model of MS used. Smaller benchtop MSD's are capable of roughly 10 ng / second in scan mode and 10 pg / second in SIM mode whereas the sensitivities of larger, more powerful machines are probably an order of magnitude better. This means that the sensitivity of the MS lies in roughly the same range as that of the FPD and HECD. The linearity of the MS is approximately one order of magnitude better than that of the HECD (158).

The drawbacks of the MS as a GC detector are the high initial cost, and the need for helium to be used as a carrier gas, which is expensive and causes a small loss of resolving power when compared to hydrogen. However, MSs are continually being reduced both in price and size with no loss in performance. Detectors, which a decade ago would have filled a small room and were very expensive, will now fit easily onto a benchtop next to a GC and cost not much more than the GC itself.

1.3.3.5 Atomic Emission Detector (AED)

The principle of atomic emission spectroscopy has been known for several centuries but it is only in the last 20 years that coupled GC-AES detectors (182,183) have been developed and used. Compounds enter the detector from the column and are cleaved into atoms in a helium discharge chamber. The commonly employed energy sources are microwave induced plasmas (MIP) and inductively coupled plasma (ICP), hence the often used terms MIP-AES and ICP-AES. The atoms are raised to an excited state and subsequently emit light. This light is then separated using a wavelength disperser, usually a diffraction grating. Portion 25 - 40 nm of the spectrum can be monitored at the same time, allowing a number of elements to be analysed simultaneously. The principle of the AED's operation means that it

can be made specific for any element. Sulphur, for example, is measured at 180.7 nm; chromatograms for carbon at 193.1 nm and nitrogen at 174.2 nm can be obtained at the same time if desired.

The Hewlett-Packard G2350A Atomic Emission Detector (Figure 10) is the only commercially-available atomic emission detector for GC. It utilises microwave energy to power the plasma in a re-entrant cavity. The discharge tube is water-cooled, which has the advantage that reactions with the tube walls are reduced, giving fewer interferences and better sensitivity. Helium is used as the plasma gas, and for sulphur-specific detection, hydrogen and oxygen are used as the reagent, or 'scavenger', gases.

The light created in the plasma is focused by a lens onto the entrance slit of a spectrometer. In the spectrometer the light is dispersed into its component wavelengths by a diffraction grating and subsequently focused onto a fixed-position photodiode array.

The sensitivity of the AED is dependent on the element analysed: for sulphur substances it lies around 1 - 10 pg sulphur / second (164). Although the AED has a background correction function, at high levels non-sulphur substances are not always suppressed, which means that the selectivity of the AED is not optimal. This could be a problem in complex matrices, such as beer, as non-sulphur compounds are present in concentrations several orders of magnitude greater than the concentrations of the sulphur substances. The linear dynamic range of the AED is specified by its manufacturers as being approximately in the order of 10^3 - 10^4, depending on the element.

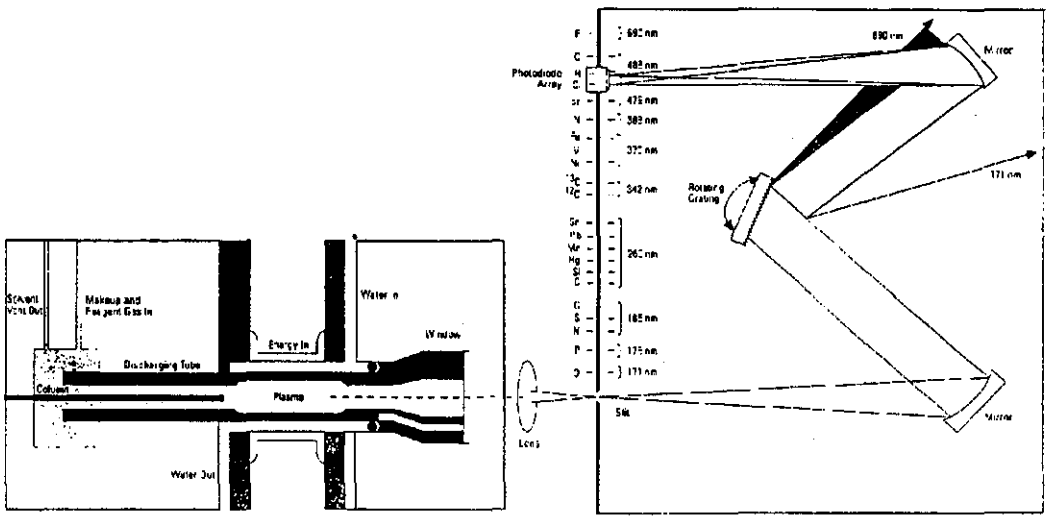


Fig. 10: Diagram of the HP G2350A Atomic Emission Detector

The cost and size of the AED - its two main disadvantages - have both been reduced in the last few years, making the AED a possible choice as a GC detector for the analysis of volatile sulphur substances in beer. Gerbersmann *et al.* (97) carried out GC-AED analysis sulphur compounds in water, beer and coffee samples, but only detected DMS in beer.

1.3.3.6 Sulphur Chemiluminescence Detector (SCD)

The sulphur chemiluminescence detector is a development of the redox chemiluminescence detector (RCD) invented by Nyarady, Barkley and Sievers (184,185). The principle of the SCD is that sulphur compounds entering the detector from the column are combusted in a burner or in an FID flame and converted to sulphur monoxide. The sulphur monoxide is then transferred to a reaction cell under vacuum where it reacts with ozone. The products of this reaction are O_2 and SO_2^*, i.e. sulphur dioxide in an excited state. When the sulphur dioxide falls back to the ground state, it gives out

energy in the form of light with a maximum intensity of 350 nm, which is measured by a photomultiplier tube to provide the signal.

The first sulphur chemiluminescence detector to be commercially available, the Sievers 350 B SCD (Figure 11), consists of a ceramic probe which is placed in an FID and positioned approx. 0.4 cm above the flame jet (164,186). Sulphur compounds are combusted in the hydrogen-rich/air flame of the FID, sulphur monoxide is formed and is instantly transferred via a transfer line under vacuum to the chemiluminescence reaction cell. Here the SO is reacted with ozone produced by a corona discharge from oxygen or synthetic air. Sulphur dioxide in an excited state is formed and this subsequently emits light as it relaxes to the ground state. This light is filtered through a UV band pass filter (300-450 nm) located in front of the cell and is detected by a photomultiplier tube. The whole reaction takes place under vacuum.

The sequence of reactions can be summarised as follows:

(xx) Sulphur compounds + H_2/air => SO + other products

(xxi) $SO + O_3 => SO_2^* + O_2$

(xxii) $SO_2^* => SO_2 + h\nu$

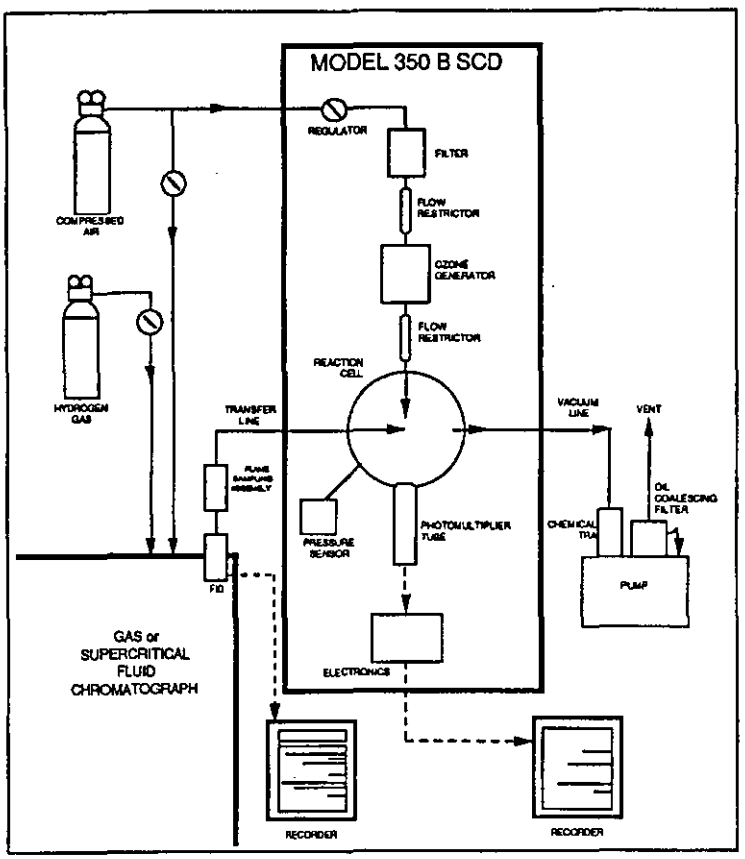


Fig. 11: Schematic diagram of a sulphur flame chemiluminescence detector (Sievers SCD 350 B)

Following the success of the 350 B SCD, Sievers launched a new version of the SCD, the Sievers 355 SCD, utilising a 'flameless' burner and dispensing with the need for an FID. The rest of the detector remained unchanged. The obvious advantage of the flameless burner was that the full amount of the substances eluting from the analytical column entered the burner and not just a sample of the FID combustion gases. The 355 flameless burner is shown in Figure 12 (187).

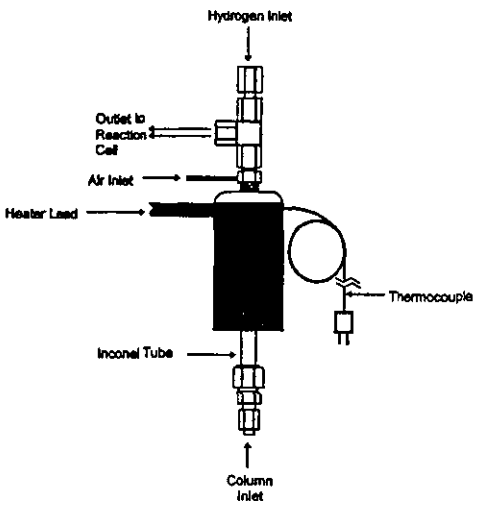


Fig. 12: Sievers 355 SCD Flameless Burner

The detector temperature is set at 800°C. The chemistry happening inside the flameless burner is that sulphur compounds are reduced by hydrogen and then subsequently oxidised with either air or oxygen to sulphur monoxide. This is then transferred to the chemiluminescence reaction cell as in the flame version, resulting in sulphur dioxide in an excited state.

The SCD is linear over four orders of magnitude (188) and the sensitivity is recorded as being approximately 1 - 10 pg sulphur / second, and the newer, flameless burners are claimed to display higher sensitivity. The selectivity of the SCD is very good and the detector shows no response to non-sulphur compounds. Ryerson *et al.* (189) coupled an SCD with a

thermionic ionisation detector, forming a dual system capable of simultaneously detecting sulphur, nitrogen and phosphorus compounds.

Drawbacks to the SCD are its relatively high price (more expensive than an FPD although considerably less expensive than an AED) and the fact that it is not particularly easy to use.

1.3.3.7 Olfactory Detector (Sniffing Port)

Although the sniffing port provides only subjective, qualitative information, it is a very useful supplementary detector (190), especially in the analysis of sulphur compounds where very low concentrations can give a very strong olfactory impression.

The sniffing port is normally used parallel to a conventional detector, the gas flow being split at the column outlet. Half of the flow is then transferred to a nose cone: here it is mixed with a humidified air stream to prevent 'drying out' of the olfactory epithelium in the operator's nose. The operator identifies the aroma of the individual substances as they elute and records his/her impressions either directly onto the chromatogram from the integrator or by using a tape recorder.

A sniffing port is inexpensive and provides useful information for the identification of the sulphur compounds present in the matrix: this is especially important when an off-smell in the beer is involved. The disadvantages of a sniffing port are that it only provides subjective, qualitative information and requires extensive operator training and time.

1.3.4 Methods Already Available for the Analysis of Sulphur Compounds in Beer

In the brewing industry in the last 30 years much work on the analysis of volatile sulphur substances in beer has been carried out. The only method which has shown itself to be capable of quantifying the low concentrations present has been gas chromatography.

The most commonly used detector has been the FPD, the first GC applications of this detector in the brewing field being work by Drews, Baerwald and Niefind in 1969 (191). Owing to advances in its technology and despite its inherent lack of linearity the FPD is still the detector of choice today. The introduction of capillary columns in the early 1970's allowed huge leaps in separating and resolving power to be achieved. However, despite improved limits of detection the only volatile sulphur compound in beer which can be analysed to some level of accuracy using GC-FPD and static headspace, i.e. without any form of pre-concentration step, is dimethyl sulphide (DMS).

Most breweries carry out analysis of DMS (192) and its precursor (DMSP) (193) in malt as part of their raw material quality control programmes. Unfortunately collaboration trials between different laboratories reveal large deviations (normally in the range 15 - 30 % relative standard deviation) between the absolute values obtained (194). Possible reasons for these discrepancies are the use of different columns and the subsequent differences in the quenching effects; the lack of linearity of the FPD and the resulting need for logarithmic calibration curves to be created; different methods of calibration; and the dependency of the FPD signal on the exact setting of the detector gas flows. For the purpose of raw material control, however, GC-FPD analysis of DMS is adequate; the in-laboratory accuracy and reproducibility are good and the method allows a brewery to set criteria for the acceptance or rejection of malt deliveries. In addition, the analysis of DMS can provide important information on the coefficient of evaporation

during the wort-boiling and the presence of bacterial infections in the wort. However, the analysis of DMS alone does not provide detailed information on the yeast metabolism during the alcoholic fermentation.

For an FPD to be able to detect the sulphur compounds which are present in the lower ppb range some form of pre-column concentration is required. Most methods used in the brewing industry utilise purge and trap methods (38,41,58,61,65,66,88,93,94,95,96,97), which allow measurement in the sub-ppb area. Pre-concentration systems such as purge and trap, however, are not very suitable for routine laboratory analysis owing to the costs involved, problems with reproducibility and that they are not particularly easy to use. As a result, although much research work has been carried out in the brewing sector on the purge and trap plus GC-FPD analysis of sulphur compounds in beer, no such method has established itself in the routine laboratory.

Chemiluminescence detection of sulphur compounds following gas chromatographic separation has also been used in recent years: Owades and Plam briefly reported sulphur chemiluminescence detection of sulphur compounds in beer in 1988 (195); in 1992 Burmeister *et al.* used a GC-SCD system with two different sampling systems (59,60); Dercksen *et al.* employed two novel methods of sample preparation in conjunction with GC-SCD, one involving in-bottle purging and on-column trapping and desorption (100), the other using an ingenious membrane extraction system (196). The SCD used in all these studies was the original flame version from Sievers Research. However, although the SCD is more sensitive than an FPD, the sensitivity of the first flameless version was still not sufficient to supply information on more than a handful of sulphur compounds in beer and required some form of dynamic sampling. In the United States, the American Society of Brewing Chemists has conducted collaborative trials of DMS determination in beer using a chemiluminescence detector with a view to accepting the method for inclusion in the ASBC "Methods of Analysis" (197). GC-SCD has also been used to investigate sulphur compounds in hop oils used in the brewing process (198).

1.4 Applications of the Method in the Brewery

The scope of applications in the brewery for a simple, sensitive, reproducible routine method for the analysis of volatile sulphur compounds is huge. The method could be employed in every step of the brewing process, from the selection of raw materials through brewing and fermenting to the bottling and canning of the final product. A good overview of the brewing process has been published by Narziss (199).

1.4.1 Raw Materials

1.4.1.1 Hops

The method could be used to investigate the influence of the choice of hops on the flavour of the final beer. Not only could the effects of the different hop strains be studied but also the growing conditions of the hops and the methods used to dry and store them. In addition to analysis of beer headspace, analysis of the hops themselves could be carried out.

1.4.1.2 Malt

The malt used for brewing has a great influence on the final concentrations of sulphur compounds in beer. Firstly, the variety of the barley used for malting plays a significant role in determining the final sulphur content. Then the various technological parameters relevant to the malting process are all important factors (200,201): the steeping of the barley grain and the subsequent germination; the length, intensity and method of the kilning. With the appropriate assay method, all these influences could be studied, allowing better understanding of the effects of the barley and its malting on the sulphur compound concentrations in beer.

1.4.1.3 Brewing Liquor

The question of whether the sulphate concentration in the brewing liquor influences the concentration of volatile sulphur compounds in the final beer is disputed (21,29). Trials using brewing liquors with differing sulphate concentrations followed by analysis of the beer could allow this dispute to be settled.

1.4.2 Brew-house

In the brew-house, where mashing of the malt followed by the boiling of the hopped wort takes place, there are many possibilities to influence the final concentration of the volatile sulphur compounds in the beer.

1.4.2.1 Milling

The method could be used to investigate the effect of the process of malt grinding, or milling. Although milling is a purely mechanical process, it is very significant for the biochemical reactions involved in the following production step, mashing. Different systems of milling and grinding could be compared: for example, to investigate whether modifications to prevent damage to the acrospire - apparently beneficial to the flavour stability of the beer (202,203) - also affect the sulphur substances.

1.4.2.2 Mashing

During the mashing process proteolysis occurs in addition to the degradation of starch (199,204). Some of the most important products of this breakdown of proteins are the amino acids. As several of these amino acids contain sulphur (199), which can then be converted into volatile

sulphur compounds by the metabolism of the yeast during fermentation, the influence of the mashing process with regard to the sulphur content in beer needs to be examined.

1.4.2.3 Lautering and Wort-boiling

Once the mash has been filtered using either a lauter (or clarifying) tun or a mash filter, it is boiled in a wort or brew kettle and the hops are added (199). There are several factors at this point which could have an influence on the sulphur content: the extent of the evaporation of volatile compounds, the species and amount of hops used and the form of the hops used, i.e. either natural hop cones, hop pellets or liquid hop extract.

1.4.2.4 Wort-cooling and Trub Removal

Wort-cooling and trub removal are the last steps before the pitching of the wort and the starting of the fermentation stage; they do not necessarily take place in the brew-house itself but are usually classified as brew-house procedures (199). A variety of methods (whirlpool separation, filtration or sedimentation) can be used to remove the hot trub, a insoluble mixture of lipids, bitter substances, polyphenols, minerals and other organic substances. The boiled and hopped wort is then cooled to its pitching temperature (4 - 12°C for bottom-fermenting beers and 12 - 18°C for top-fermenting beers). Once the wort has been cooled the cold trub is removed, again by filtration, flotation, sedimentation or centrifugal separation. As the amounts of hot and cold trub remaining in the wort are considered to have repercussions on the fermentation (199) it is conceivable that the concentrations of sulphur substances are also affected. A further important process which occurs during wort-cooling is the binding of oxygen - both physically and chemically - by the wort. Chemical binding, which takes place at wort temperatures greater than 40°C, is not so important for the fermentation. Physical binding, on the other hand, occurring at temperatures

lower than 40°C, is very important for the propagation of the yeast. As insufficient physical uptake of oxygen will affect the fermentation and therefore could influence the formation of volatile sulphur substances, adequate aeration of the wort is required. This is carried out at 6 - 10°C. The extent of the influence of wort aeration on the fermentation in respect to sulphur compounds in the final beer offers scope for study.

1.4.2.5 Other Brew-house Considerations

Other factors in the brew-house which need to be taken into consideration are the materials used in the construction of the mash tuns, wort kettles and lauter tubs, their design and the layout of the piping connecting the various vessels. The materials used are important because of their ability to bind sulphur substances - it is a long-known fact in the brewing industry that copper vessels greatly influence the sulphury properties of a beer (41); the design of the vessels and connecting piping is also important because it is believed that centrifugal forces could put the organic molecules under such physical strain that they are chemically altered, leading to sulphury off-flavours. This phenomenon was witnessed in a Bavarian brewery using a centrifugal separator for trub removal followed by a powerful pump, an onion-like off-flavour being the result (205). (Olsen *et al.* believed the sulphur compound responsible for this onion-like off-flavour to be 2-mercapto-3-methyl-1-butanol (206)).

1.4.3 Fermentation

The pitching of the wort, i.e. the adding of yeast to the sludge-free, aerated, cooled wort, is the start of the fermentation part of the brewing process (199). Owing to the myriad of biochemical reactions occurring during the yeast metabolism, this step is especially important with regard to the concentrations of volatile sulphur compounds in beer (40).

1.4.3.1 Yeast

The yeast is the most important single factor in the fermentation, determining not only the speed and degree of fermentation but also the formation of by-products - including by-products from the breakdown of sulphur-containing proteins and amino acids (207) - and the elimination of proteins, bitter substances and tannins. To a large extent this determines the aroma, colour, bitterness, palatability and foam of the final beer (23,34,35,40,199). It is also widely believed that beers brewed with lower original gravity - for example light beer - have a more sulphury aroma and taste owing to the yeast having less extract to ferment: this theory could be examined with the appropriate method.

Each individual strain of yeast has its own properties and will affect the course of the fermentation (38,41,199). The choice of yeast strain with respect to the formation of volatile sulphur compounds has been reported in the literature (199). The amount of yeast used during pitching will also have an effect on the course of the fermentation. Additional important factors concerning the yeast are its storage conditions, management, 'washing' and the number of times the yeast is used (199).

1.4.3.2 Fermentation Conditions

Owing to the importance of the yeast metabolism on the production of sulphur substances, it is clear that any parameters influencing the fermentation will influence the sulphur content of the beer (199,208). This means that the effects of the temperature and pressure at which the fermentation is carried out need to be examined. Due to the scarcity of tank capacity and for economical reasons, more and more breweries are attempting to increase productivity by speeding up, or 'forcing', the fermentation process; this is achieved by raising both the temperature and pressure at which the fermentation is carried out. In the future, the economic pressures on the breweries to 'cut corners' will be increased rather than

reduced and therefore the effects on beer quality - especially with regard to off-smells and off-flavours - need to be extensively studied. Also in recent years a trend towards the employment of cylindrical conical tanks (CCTs) instead of horizontal tanks has been seen, the high cylindrical form of the CCTs giving capacity and subsequently economical advantages. The disadvantages of CCTs, however, are the higher static pressures involved and greatly reduced surface-to-volume ratio (199), which leads to poorer evaporation of volatile by-products of the fermentation. This in turn may result in higher concentrations of these compounds in beer and thereby increasing the danger of off-flavours.

1.4.4 Maturation

Once the primary or main fermentation is completed (determined by the slowing of the fermentation of the fermentable extract to less than approx. 0.4% in 24 hours and the flocculation of yeast cells) the young beer can be pumped from the fermentation vessel into a storage tank, where the secondary or after-fermentation (199) can take place. The aims of the secondary fermentation are to reduce the extract to a minimum; to saturate the beer with carbon dioxide; to fine or clarify the beer by allowing the remaining yeast and other substances to settle; to mature the beer, giving it a more rounded taste. The secondary fermentation is carried out at very low temperatures (near the freezing point of the beer) and normally over a time span of several weeks. As sulphur compounds are considered to make a large contribution to the 'young' or unripe taste of the beer after the primary fermentation (209), the maturation step is of great relevance for the present work.

1.4.4.1 Maturation Conditions

Owing to the length of the maturation process, economic and space pressures again lead to the temptation to compact this step. This can be done not only by simply shortening the maturation time but also by not cooling the beer down to its optimal maturation temperature of -1.5 - -2.0°C, and passing the young beer through a powerful cooler between the primary and secondary fermentation processes, thereby allowing the lower secondary fermentation temperature to be reached in a matter of minutes rather than days. The effects of bowing to these pressures on the quality of the beer need to be closely examined, and this of course means studying the behaviour of the volatile sulphur compounds.

1.4.4.2 One-Tank (Unitank) or Two-Tank Process

Traditionally the primary fermentation is carried out in a vessel in the fermentation cellar and then the young beer is pumped for the secondary fermentation to another vessel in the storage or stock cellar: this is described logically enough as the two-tank process. As already mentioned, however, the use of cylindrical conical tanks (CCTs) has become more widespread: in a CCT it is possible to carry out both the primary and secondary fermentations in one tank, the yeast collected in the cone at the bottom of the CCT being pumped out at the end of the primary fermentation. Here again the surface-to-volume ratio plays an important part in the elimination of the volatile sulphur compounds which are thought to play a part in determining the 'ripeness' of the beer (199).

1.4.5 Filtration and Filling

By the filtration and filling of beer the main concern is to clarify the beer, stabilise it physically and minimise the uptake of oxygen by the beer as far as possible (199). It would be interesting to look at the relationship between the concentration of oxygen in the beer and the concentrations of the volatile sulphur substances present.

With a method for the analysis of volatile sulphur compounds it could be determined whether the beer container - bottle, can or keg - has an influence. Additionally the effects of pasteurisation and flash pasteurisation could be studied - this is of importance when considering that the Maillard reaction between reducing sugars and amino acids (including sulphur-containing amino acids) is a temperature-dependent series of reactions (5).

1.4.6 Ageing

The effects of ageing and temperature on the flavour stability of beer have been comprehensively examined (161), but no extensive studies have been carried out into the behaviour of volatile sulphur compounds during the ageing process. Increases in the concentration of methional (161,210,211), 2-acetyl thiophene (161) and 3-methyl-3-mercapto-butyl-formate (212), and decreases in the concentration of sulphur dioxide (161), which acts as an anti-oxidant, during beer ageing have been reported. Articles on the formation of volatile sulphur compounds from the Maillard reaction during beer staling have also been published (213,214).

The benefits of investigating the time dependent change of sulphur-containing compounds in beer were outlined in a recent paper on the flavour impact of aged beers (211).

1.4.7 Light Influences

There has been a large number of publications on off-flavours in beer due to the influence of light: Templar *et al.* recently published an excellent review of the findings of these articles (75). Another good review was published in 1991 (215). Although the general mechanism of the formation of the lightstruck flavour in beer is known (64,65,66), a routine method sensitive enough to analysis the compound presumed responsible for the lightstruck off-flavour - 3-methyl-2-butene-1-thiol - would allow much more detailed study of the causes of the problem to be carried out.

Possible solutions to the lightstruck problem could be tested for their effectiveness. Effects of changes to the brewing process to provide a beer more stable to light could be investigated. The amount of protection against light provided by specially-developed glass bottles could be evaluated. The wavelengths of light primarily responsible for the lightstruck flavour could be determined, giving important information, for example, for the design of light-resistant bottles or the ideal lighting conditions in supermarket shelves.

1.5 Aims of Present Study

The aim of the present work is to develop a simple, fast, sensitive and inexpensive routine method of analysis for volatile and semi-volatile sulphur compounds in beer. This will be done by firstly comparing the sensitivity of the different detectors described earlier, in order to determine which would be the most suitable on which to base the method.

Secondly, the theory of chromatographic separation will be applied to find a capillary column which is capable of separating all the sulphur compounds detected, a column capable of separating very volatile compounds whilst at the same time providing acceptable analysis times for heavier compounds. As a simple routine method is wanted, the separation

should take place at conventional GC oven temperatures without the use of cooling agents.

Different methods of sample preparation to extract the analytes from the sample matrix and concentrate them prior to injection into the GC will also be considered.

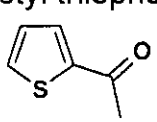
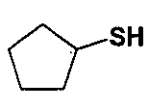
The method of choice will subsequently be used to investigate the amounts of sulphur compounds in various different beers. Particular interest will be paid to the compound, or possibly compounds, which is/are responsible for the formation of the lightstruck flavour in beers which have been subjected to illumination.

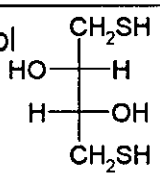
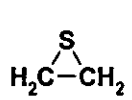
2 Experimental

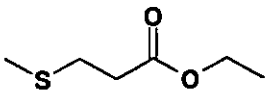
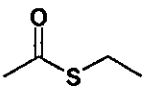
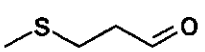
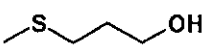
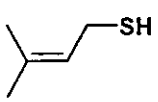
2.1 Chemicals

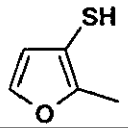
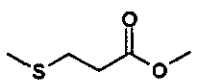
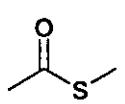
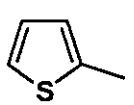
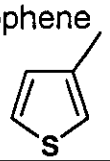
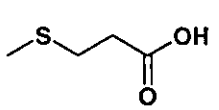
The sulphur compounds used for peak identification and calibration of the system were obtained in the highest purity available. The chemicals were purchased from Fluka Chemie AG (Buchs, Switzerland); Aldrich Chemie AG (Steinheim, Germany); Merck KGaA (Darmstadt, Germany); Lancaster Synthesis (Mühlheim am Main, Germany); Oxford Chemicals (Hartlepool, UK); Gueldenhaus Distillery (Bremen, Germany); Newchem Inc. (Parkton, MD21120, USA); Bio-Rad (Hercules, California, USA). The CAS numbers of the compounds studied, their structures, abbreviations and suppliers are listed in Table 1 below.

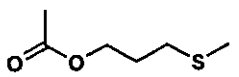
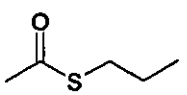
Table 1: List of all chemicals used

Compound	Abbreviation	CAS number	Supplier
2-Acetyl thiophene 	2-AcThPh	88-15-3	Fluka Chemie AG
Affi-Gel 501			Bio-Rad
1-Butanethiol $CH_3CH_2CH_2CH_2SH$	1-BuSH	109-79-5	Aldrich Chemie AG
Carbon disulphide S=C=S	CS_2	75-15-0	Fluka Chemie AG
Cyclopentylmercaptan 		1679-07-8	Aldrich Chemie
Dichloromethane CH_2Cl_2		75-09-2	Merck KGaA

Compound	Abbreviation	CAS number	Supplier
Diethyl disulphide $CH_3CH_2SSCH_2CH_3$	DEDS	111-81-6	Lancaster Synthesis
Diethyl sulphide (Ethyl sulphide) $CH_3CH_2SCH_2CH_3$	DES	352-93-2	Fluka Chemie AG
Dimethyl disulphide CH_3SSCH_3	DMDS	624-92-0	Fluka Chemie AG
Dimethyl sulphide CH_3SCH_3	DMS	75-18-3	Fluka Chemie AG
Dimethyl trisulphide CH_3SSSCH_3	DMTriS	3658-80-8	Oxford Chemicals
Dimethyl tetrasulphide $CH_3SSSSCH_3$	DMTetraS	5756-24-1	Oxford Chemicals
Dithiothreitol 		27565-41-9	Lancaster Synthesis; Aldrich Chemie
Ethanethiol (Ethyl mercaptan) CH_3CH_2SH	EtSH	75-08-1	Fluka Chemie AG
Ethanol CH_3CH_2OH	EtOH	64-17-5	Gueldenhaus distillery, Bremen, Germany
Ethylene sulphide (Thiirane) 	Thiirane	420-12-2	Aldrich Chemie
Ethyl methyl sulphide (Internal standard) $CH_3CH_2SCH_3$	EMS	624-89-5	Aldrich Chemie

Compound	Abbreviation	CAS number	Supplier
Ethyl-3-(methylthio)propionate 		13327-56-5	Lancaster Synthesis
Ethyl thioacetate (Thioacetic acid S-ethyl ester) 	EtSAc	625-60-5	Lancaster Synthesis
1-Hexyl mercaptan $CH_3(CH_2)_5SH$	HexSH	111-31-9	Lancaster Synthesis
Methanethiol (Methyl mercaptan) CH_3SH	MeSH	74-93-1	Fluka Chemie AG
Methional (3-methylthiopropanal, 3-(methylthio)propionaldehyde 		3268-49-3	Aldrich Chemie
Methionol (3-methylthiopropanol) 		505-10-2	Aldrich Chemie
2-Methyl-1-butanethiol $C_2H_5CH(CH_3)CH_2SH$	2-MeBuSH	1878-18-8	Aldrich Chemie
3-Methyl-1-butanethiol $(CH_3)_2CHCH_2CH_2SH$	3-MeBuSH	541-31-1	Aldrich Chemie
3-Methyl-2-butene-1-thiol (light mercaptan) 	3-MBT	5287-45-6	Newchem Inc. Parkton, MD 21120, USA

Compound	Abbreviation	CAS number	Supplier
2-Methyl-3-furanthiol 	MeFuSH	28588-74-1	Aldrich Chemie
Methyl 3-(methylthio) propionate 		13532-18-8	Lancaster Synthesis
1-Methyl-1-propanethiol, 2-Butanethiol, sec.-Butylmercaptan $C_2H_5CH(CH_3)SH$	1-MePrSH	513-53-1	Aldrich Chemie
2-Methyl-2-propanethiol, tert.-Butylmercaptan $(CH_3)_3CSH$	2-MePrSH	75-66-1	Aldrich Chemie
Methyl thioacetate (Thioacetic acid S-methyl ester) 	MeSAc	1534-08-3	Lancaster Synthesis
2-Methyl thiophene 	2-Methph	554-14-3	Aldrich Chemie
3-Methyl thiophene 	3-Methph	616-44-4	Aldrich Chemie
3-(Methylthio) propionic acid 		646-01-5	Lancaster Synthesis

Compound	Abbreviation	CAS number	Supplier
3-(Methylthio) propyl acetate 	3-MeSPrAc	16630-55-0	Oxford Chemicals
1-Pentanethiol $CH_3(CH_2)_4SH$	1-PeSH	110-66-7	Aldrich Chemie
1-Propanethiol $CH_3CH_2CH_2SH$	1-PrSH	107-03-9	Aldrich Chemie
2-Propanethiol $(CH_3)_2CHSH$	2-PrSH	75-33-2	Aldrich Chemie
1-Propyl thioacetate 	PrSAc	2307-10-0	Lancaster Synthesis

2.2 Methods

2.2.1 Sulphur Chemiluminescence Detection

Gas chromatograph:	Hewlett-Packard 5890 (Hewlett-Packard GmbH, Waldbronn, Germany)	
Detector:	Sievers 355 sulphur chemiluminescence detector with flameless burner (Sievers Instruments, Inc., Boulder, Colorado, USA)	
Burner temperature:	800°C	
Burner gases:	Hydrogen:	100 ml/min
	Air:	40 ml/min
	Oxygen (instead of air):	5 ml/min

Detector pressure:	~ 17 torr
Injector temperature:	200°C
Column:	Supelco SPB-1, 30m x 0.32mm x 4µm (Supelco, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany)
Carrier gas:	Hydrogen, ca. 40 ml/min
Sample preparation:	Static headspace, equilibrated at 50°C and with added NaCl, 1 ml injected
Integrator:	Hewlett-Packard 3396A (Hewlett-Packard GmbH, Waldbronn, Germany)

2.2.2 Atomic Emission Detection

The analyses with the AED were carried out by Bernard Rothweiler, Hewlett-Packard Waldbronn, Germany.

Gas chromatograph:	Hewlett-Packard 6890 (Hewlett-Packard GmbH, Waldbronn, Germany)
Detector:	Hewlett-Packard G2350A Atomic Emission Detector (Hewlett-Packard GmbH, Waldbronn, Germany)
Detector temperature:	250°C
Detector reagent gases:	Hydrogen and oxygen
Detector wavelength:	181 nm
Carrier gas:	Helium
Injector temperature:	230°C
Column:	HP-17, 30m x 0.25mm x 0.25µm (Hewlett-Packard GmbH, Waldbronn, Germany)

Sample preparation:	Static headspace, equilibrated at 62°C for 15 min, 1 ml injected
Headspace autosampler:	Hewlett-Packard 7694 HSS (Hewlett-Packard GmbH, Waldbronn, Germany)
Data processing:	Hewlett-Packard Chemstation (Hewlett-Packard GmbH, Waldbronn, Germany)

2.2.3 Pulsed Flame Photometric Detection

Gas chromatograph:	Varian 3800 (Varian GmbH, Darmstadt, Germany)	
Detector:	Varian Pulsed Flame Photometric Detector (Varian GmbH, Darmstadt, Germany)	
Detector temperature:	210°C	
Detector gases:	Air 1:	16.9 ml/min
	Air 2:	9.8 ml/min
	Hydrogen:	10.3 ml/min
Detector voltage:	600 V	
Detector gate delay:	6 ms	
Detector gate width:	20 ms	
Column:	10m x 0.25mm x 0.5µm DB-Wax (J&W Scientific Inc., Folsom, California, USA) connected to 60m x 0.25mm x 0.5µm VA-1 (Varian GmbH, Darmstadt, Germany)	
Carrier gas:	Hydrogen:	2.7 ml/min
Injector:	Varian 1079, split/splitless	
Split program:	Split initially off, on after 0.8 minutes at 10:1	
Injector temperature:	250°C	

Data processing:	Varian Star Workstation (Varian GmbH, Darmstadt, Germany)

2.2.4 Columns used

The chemical composition of the liquid phases of the various GC capillary columns used are listed below:

SPB-1:	100% polydimethylsiloxane
VA-1:	100% polydimethylsiloxane
DB-Wax:	polyethylene glycol
OV-1701:	14% cyanopropyl-phenyl, 86% polydimethyl siloxane
HP-17:	50% phenyl, 50% polydimethylsiloxane
Optima Delta-3:	methyl/phenyl-silcone (exact phase composition unknown)

2.2.5 SPME

The SPME fibres were all purchased from Supelco (Supelco, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Before use the fibres were conditioned in the injector of the GC. The conditioning parameters were as follows:

7µm PDMS:	320°C, 4 hours
100µm PDMS:	250°C, 1 hour
85µm Polyacrylate:	300°C, 2 hours
65µm Carbowax/DVB:	250°C, 30 minutes
75µm Carboxen/PDMS:	280°C, 30 minutes

The SPME extractions and injections were carried out with a Varian 8200 CX Autosampler with SPME III agitation modifications (Varian GmbH, Darmstadt, Germany). A heated sample carousel was used.

For the comparison of the various fibres the following experimental conditions were used: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

2.2.6 Steam Distillation followed by GC-MS for the Identification of Sulphur Compounds

100ml of beer acidified with H_2SO_4 (2.5 ml) was steam distilled. The clear distillate was extracted with 1ml dichloromethane; NaCl (22g) was used to aid the extraction. 0.5µl of the organic phase was injected into the GC-MS system.

Gas chromatograph:	Hewlett-Packard 5890 Series II (Hewlett-Packard GmbH, Waldbronn, Germany)
Detector:	Hewlett-Packard 5972 Mass Selective Detector (Hewlett-Packard GmbH, Waldbronn, Germany)
Interface temperature:	280°C
Carrier gas:	Helium
Column:	10m x 0.25mm x 0.5µm DB-Wax (J&W Scientific Inc., Folsom,

	California, USA) connected to 60m x 0.25mm x 0.5µm VA-1 (Varian GmbH, Darmstadt, Germany)
Injector temperature:	230°C
Autosampler:	Hewlett-Packard MS 7673 (Hewlett-Packard GmbH, Waldbronn, Germany)
Data processing:	Hewlett-Packard MS-Chemstation (Hewlett-Packard GmbH, Waldbronn, Germany)

2.2.7 Covalent Chromatography followed by GC-MS for the Identification of Sulphur Compounds

A variation on the covalent chromatography sample preparation method published by Full and Schreier (216) was used.

4ml of an agarose gel, containing a phenyl mercuric chloride group to reversibly bond thiols (Affi-Gel), was mixed with 250ml of beer with continual stirring for a period of 30 minutes. The mixture was then centrifuged and the residue containing the Affi-Gel was poured into an empty SPE cartridge. The cartridge was effectively packed, forming an Affi-Gel column. This column was then washed with 20ml pentane/dichloromethane (2:1 v/v) to remove any interfering compounds from the beer matrix. The thiols from the beer were then displaced from the column using an excess of 11mM dithiothreitol solution (42mg dithiothreitol in 25ml pentane/ dichloromethane, 2:1 v/v). 1µl of the eluent was injected into the GC-MS system in both scan and SIM modes. The GC-MS system used is described in section 2.2.5. The following ions were monitored in SIM mode: 43, 45, 47, 48, 60, 61, 62, 66, 69, 75, 76, 79, 80, 90, 94, 102, 104, 111, 122, 126.

2.2.8 Dynamic Headspace Extraction (DHSE) followed by GC-MS for the Identification of Sulphur Compounds

The DHSE-GC-MS analyses used in an attempt to identify sulphur compounds present in beer were carried out together with the chromatographic services company Mplus at the University of Bremen, Germany.

The method was based on work carried out by Goldstein *et al.* (98). Three different variations of the sample preparation step were used. The chromatographic system remained the same for each variation:

Gas chromatograph:	Varian 3400 (Varian GmbH, Darmstadt, Germany)
Detector:	Finnigan ITS 40 Mass Spectrometer (Finnigan MAT, Bremen, Germany)
Interface temperature:	225°C
Source temperature:	200°C
Carrier gas:	Helium
Column:	10m x 0.25mm x 0.5µm DB-Wax (J&W Scientific Inc., Folsom, California, USA) connected to 60m x 0.25mm x 0.5µm VA-1 (Varian GmbH, Darmstadt, Germany)
Injector temperature:	250°C
Data processing:	Finnigan MS software (Finnigan MAT, Bremen, Germany)

2.2.8.1 Variation 1

In the first attempt with DHSE, helium was purged through 250ml of illuminated beer at a speed of 100ml/min for 30 mins. Orbo™ 826 filters - mercuric acetate coated glass fibre - were placed in the gas flow.

This was repeated 5 times. The filters were transferred to a dilute solution of hydrochloric acid which also contained 5mg dithiothreitol, to prevent the oxidation of the thiols. The filters were shaken in the solution. Dichloromethane was then used to extract any organic compounds in the solution. The dichloromethane extract was analysed with the GC-MS system described above.

2.2.8.2 Variation 2

The method was adapted further: helium was purged through 1l of illuminated beer at a speed of 50ml/min for 1 hour. The helium was passed through a GC injector liner which contained 2 rolled-up Orbo™ 826 filters. This was carried out twice. Directly after purging, the injector liner was placed in a GC injection block. After the start of the GC run the injector was rapidly heated to 250°C.

2.2.8.3 Variation 3

The final variation was a combination of the first two. Helium was purged through 1l of illuminated beer at a speed of 50ml/min for 3 hours. This was repeated 3 times. Five Orbo™ 826 filters to trap thiols and other sulphur-containing compounds were placed in a glass tube in the gas flow. After purging, the filters were placed in 5mg dithiothreitol and extracted with 2ml dichloromethane. After evaporation to a final volume of 200µl the dichloromethane extract was analysed by GC-MS.

2.2.9 SPME-GC-PFPD/MS for the Identification of Sulphur Compounds

The SPME-GC-PFPD/MS analyses for identification purposes were carried out by Jim Yano at Varian Chromatography Systems, Walnut Creek, California, USA.

Gas chromatograph:	Varian 3400
Detectors:	Varian PFPD Varian Saturn MS
Column:	10m x 0.25mm x 0.5µm DB-Wax connected to 60m x 0.25mm x 0.5µm DB-1
SPME fibre:	75µm Carboxen/PDMS
SPME adsorption:	30 minutes, unheated
SPME desorption:	3 minutes, 250°C, 0.8mm injector liner

2.3 Method Selected

The final method selected after the completion of method development is described below.

2.3.1 Sample Preparation: SPME

Autosampler:	Varian 8200CX with SPME III (Varian GmbH, Darmstadt, Germany)
Fibre:	75µm carboxen/PDMS (Supelco, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany)

Sample:	9ml of sample + 1ml internal standard 95% water / 5% ethanol solution
Adsorption conditions:	32 minutes adsorption time 45°C adsorption temperature Agitation
Desorption conditions:	0.8mm injector liner 250°C injector temperature 3 minutes desorption time Split-splitless injection, split initially off, on after 0.8 minutes

2.3.2 Chromatographic Separation

Gas Chromatograph:	Varian 3800 (Varian GmbH, Darmstadt, Germany)
Injector:	Varian 1079, split/splitless (Varian GmbH, Darmstadt, Germany)
Column:	10m x 0.25mm x 0.5µm DB-Wax (J&W Scientific Inc., Folsom, California, USA) connected to 60m x 0.25mm x 0.5µm VA-1 (Varian GmbH, Darmstadt, Germany), in that order
Carrier gas:	Hydrogen
Carrier gas flow:	2.7 ml/min, constant flow
Oven program:	7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/ min, increased to 235°C at 22°C/min, held for 6 mins

2.3.3 Detection: PFPD

Detector:	Varian PFPD (Varian GmbH, Darmstadt, Germany)
Detector temperature:	210°C
Detector voltage:	600°C
Detector gate width:	20ms
Detector gate delay:	6ms
Detector trigger level:	200mV
Detector gas flows:	air1 = 16.9 ml/min air2 = 9.8 ml/min hydrogen = 10.3 ml/min
Peak calculation:	square root of peak height (quadratic PFPD response)

3 Results and Discussion

3.1 Method Development

The development of the method was to be carried out by looking at the three different stages of analysis - sample preparation, separation and detection - independently, and then combining the best alternatives to ideally form a simple, fast, sensitive and inexpensive routine method.

3.1.1 Detection

The first stage of the work was to compare the different selective and specific GC detectors for sulphur compounds.

3.1.1.1 Sievers Sulphur Chemiluminescence Detector

At the start of the project the detector considered most likely to satisfy the requirements of sensitivity, selectivity and robustness was the sulphur chemiluminescence detector from Sievers Research. Although relatively expensive - approximately three times the cost of an FPD - the Sievers SCD cost about a quarter of the price of the atomic emission detector, the HP 5921A from Hewlett-Packard. Neither the more recent, more sensitive and (relatively) less expensive version of the AED, the HP G2350A, nor the pulsed FPD were commercially available at the start of the project.

The decision to use the Sievers SCD was backed up by the encouraging results being achieved in the milk (165), gas (159,217) and brewing (59,60,100,196,197) sectors with the flame version of this detector (188) in the early 1990s.

3.1.1.1.1 Results

The Sievers sulphur chemiluminescence detector was assessed over a period of one year.

The selectivity of the Sievers sulphur chemiluminescence detector was excellent: no non-sulphur peaks were seen despite the presence of large numbers of organic substances in relatively high concentrations in beer headspace (218,219). The stability, robustness and the sensitivity of the detector, however, failed to live up to expectations.

The SCD displayed a susceptibility to leaks, mainly in the area of the T-piece and the column inlet (see Figure 12, section 1.3.3.6). These leaks had a marked effect on the sensitivity of the detector. The fragility of the ceramic burner tubes meant that attempts to achieve leak-free seals often resulted in the tubes breaking. In addition, the heating block of the burner displayed a tendency to crumble, leaving the heater wires partly uncovered. This led to uncertain temperature conditions in the burner itself.

When the burner tubes were replaced initially a significant improvement in sensitivity was seen (also, however, an increase in background noise and sensitivity to column bleed at higher column temperatures). This improvement in sensitivity unfortunately was very short term, the sensitivity decreasing with each temperature-programmed run until a relatively insensitive but stable level was reached: this process occurred over a very short time span as can be seen from the two chromatograms in Figures 13 & 14 obtained within 1½ hours of each other.

Figure 13 shows good sensitivity but as the temperature in the GC oven was raised the signal rose and went off-scale. As the detector temperature was kept constant at 800°C, and would therefore not have been be influenced by raising the GC oven to a temperature of 230°C, the assumption can only be that the new detector tubes had displayed great sensitivity towards the column bleed.

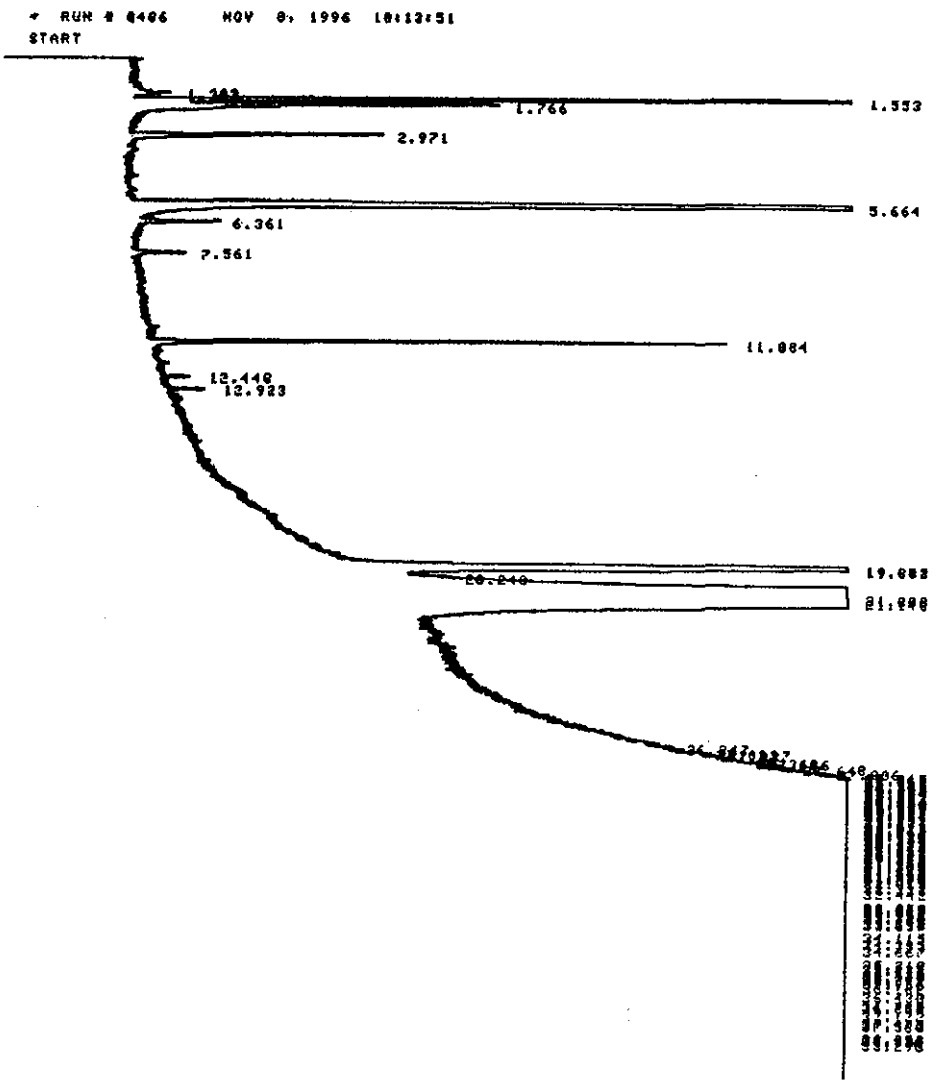


Fig. 13: Chromatogram of a beer sample analysed with the Sievers 355 SCD with new burner tubes. Column: Supelco SPB-1, 30m x 0.32mm x 4µm. Details of the experimental conditions are listed in 2.2.1

The chromatogram in Figure 14 shows that 1½ hours later, with the same burner tubes, the increase in signal with the increase in GC oven temperature was not seen. The sensitivity of the detector, however, had also greatly decreased over this time, as a comparison between Figures 13 and

14 clearly displays. The experimental conditions and the scales in both chromatograms were the same.

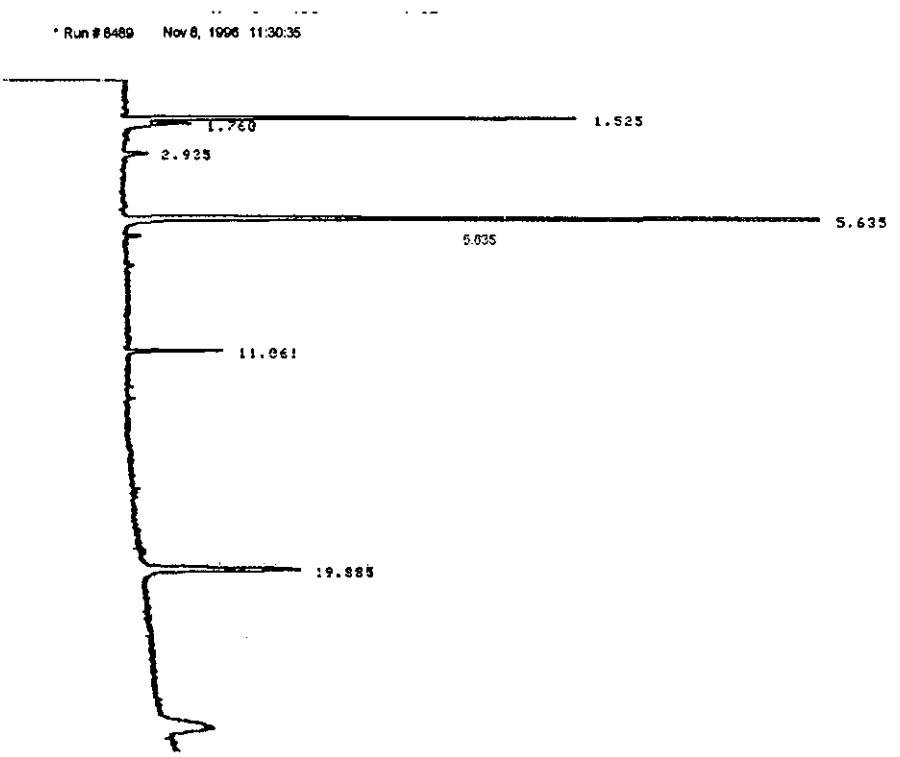


Fig. 14: as Fig. 13, 1½ hours later. Column: Supelco SPB-1, 30m x 0.32mm x 4µm. Details of the experimental conditions are listed in 2.2.1

The use of oxygen instead of air in both the flameless burner (the flow being adjusted accordingly) and for the production of ozone in the detector did not give significant improvements in sensitivity.

The occasions where the Sievers SCD displayed its real potential for sensitive and selective detection were very, very seldom and

extremely difficult to repeat. Figure 15 shows one of the few such chromatograms.

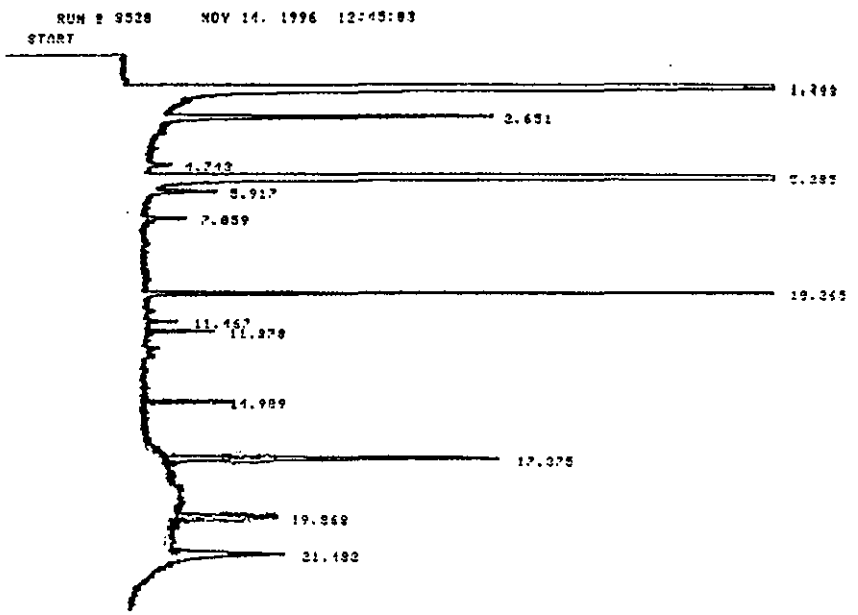


Fig. 15: A rare example of a sensitive Sievers 355 SCD chromatogram. Column: Supelco SPB-1, 30m x 0.32mm x 4µm. Details of the experimental conditions are listed in 2.2.1

Although various columns, chromatographic conditions and sampling methods were assessed, no real conclusions could be made owing to the instability of the detector system.

3.1.1.1.2 Discussion

Although the Sievers SCD displayed excellent selectivity, its inherent instability and lack of robustness gives rise to very strong doubts about its suitability as a routine detector for the daily analysis of volatile sulphur compounds.

The suggestion from Sievers Inc. that the lack of sensitivity could be caused by a high background owing to large amounts of sulphur compounds in either the analytical gases (i.e. carrier and / or reaction gases for the burner) or the gas supply system was refuted by the use of gas filters in the gas supply lines. After this measure failed to give any improvement the step - at considerable expense - of replacing the complete gas supply system was taken. This also failed to provide any improvement, emphasising that the instability and lack of sensitivity of the detector were *not* caused by problems with the gas supply.

The reaction gas flow rates were also investigated but no evidence was found to suggest that they were the cause of the instability and poor sensitivity. The use of helium or nitrogen as carrier gas instead of hydrogen also did not have a beneficial effect.

The main problem with the detector is that it appears that the chemistry of the reactions occurring in the combustion tubes in the burner is not fully understood. While some SCD experts claim that the ceramic tubes in some way act as a reaction catalyst (220), the manufacturers insist that this is not the case and that the tubes simply act as a reaction chamber and have no catalytic function. However, this contradicts the report that the tubes can be 'poisoned' by hydrogen (187). Also, it is conceivable that the decrease in sensitivity with each run following the instalment of new burner tubes was caused by bleed from the analytical column, coating the tubes. Silicon dioxide bleed would be reduced in the burner and it is possible that the resulting silicon then forms a layer on the tubes. This theory is backed up by other Sievers users (221) who have seen no decrease in sensitivity with

new tubes and isothermal oven programs at low temperatures. However, as soon as temperature programs are used and the column is subjected to higher temperatures, significant loss of sensitivity is seen. Again, these findings appear to contradict the official view of Sievers Inc. that the tubes have no catalytic function.

Further evidence that the instability problems lie with the surface chemistry of the combustion tubes is provided by the observations that these problems did not occur with the FID-based Sievers 350 B SCD (59,60,100,159,165,188,196,197,217), nor with the FID-based version of the Sievers 355 SCD utilising a FID-SCD interface as described by Beens and Tijssen (222). In these systems any substances liable to interfere with the performance of the ceramic combustion tubes were burned in the FID. Unfortunately the coupling of the SCD with an FID means a loss in sensitivity of a factor of 7 (222).

All capillary columns bleed at higher temperatures, especially the thick film columns needed to achieve separation of highly volatile compounds, and until the reaction chemistry of the combustion in the burner tubes is fully understood and this problem is addressed, the Sievers SCD cannot be recommended for routine analysis of volatile sulphur compounds in beer.

The decision to reject the Sievers SCD as a suitable detector for the determination of volatile sulphur compounds in beer was backed up by the discussion at a users' meeting for all analysts in Germany who used the Sievers detector. All users needing the low levels of detection specified by Sievers Research Inc. complained of not being able to obtain the promised sensitivity, many noting the same drop in sensitivity after the first few runs with new burner tubes. Further evidence of the Sievers SCD not being able to live up to its specifications was the willingness of the company licensed to sell Sievers products in Germany to give a full refund on the detector's original purchase price.

3.1.1.2 Hewlett-Packard Atomic Emission Detector

Following the rejection of the Sievers SCD, an alternative method of detection was needed. The two possible options were the Atomic Emission Detector (AED) or the newly-developed Pulsed Flame Photometric Detector (PFPD). The AED had established itself as a popular sulphur-selective detector, being used for a wide range of applications (4,97,164,223,224,225,226). The PFPD on the other hand was a very new detector which had only very recently been launched commercially. Few applications of the PFPD have been published (176,227,228,229).

Hewlett-Packard launched a newer, more sensitive (230) and less expensive version of the AED, the G2350A, during the time when the Sievers SCD was being tested. This, together with the increasing acceptance of the AED as a routine detector, influenced the decision to first assess the G2350A as a specific detector for the analysis of volatile sulphur compounds in beer.

The G2350A AED used for the analysis of volatile sulphur compounds in beer was located at the Hewlett-Packard analytical centre in Waldbronn, Germany. It was connected to a HP 6890 GC and controlled by HP G2360AA GC-AED software. A Hewlett-Packard 7694 Headspace Sampler was used for sample preparation and introduction. The analyses were carried out together with Mr Bernard Rothweiler from Hewlett-Packard, Waldbronn, Germany.

3.1.1.2.1 Results

1 ml of beer headspace was injected into the GC. Sodium sulphate was added to the beer sample to increase the concentration of volatile compounds in the headspace, and the sample equilibrated at 62°C using the headspace sampler. The sulphur trace chromatogram measured at

181 nm showed a total of over 20 peaks (Figure 16). This large number of peaks was unexpected.

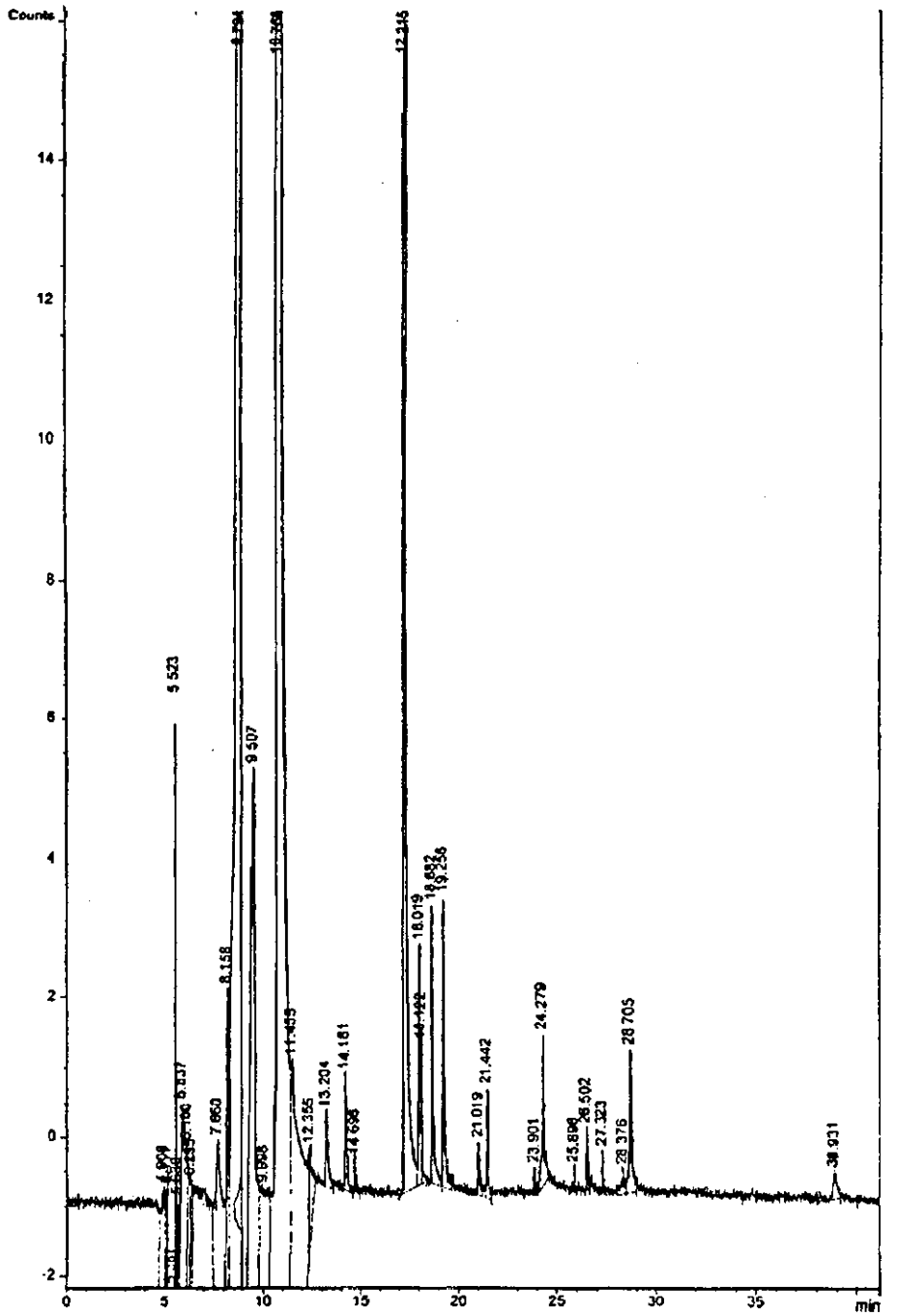


Fig. 16: AED sulphur trace (181 nm) of beer headspace. Column: HP-17, 30m x 0.25mm x 0.25µm. Details of the experimental conditions are listed in 2.2.2

After studying the carbon trace chromatogram (Figure 17), measured at 193 nm, it became clear that not all the peaks shown on the sulphur trace were sulphur compounds.

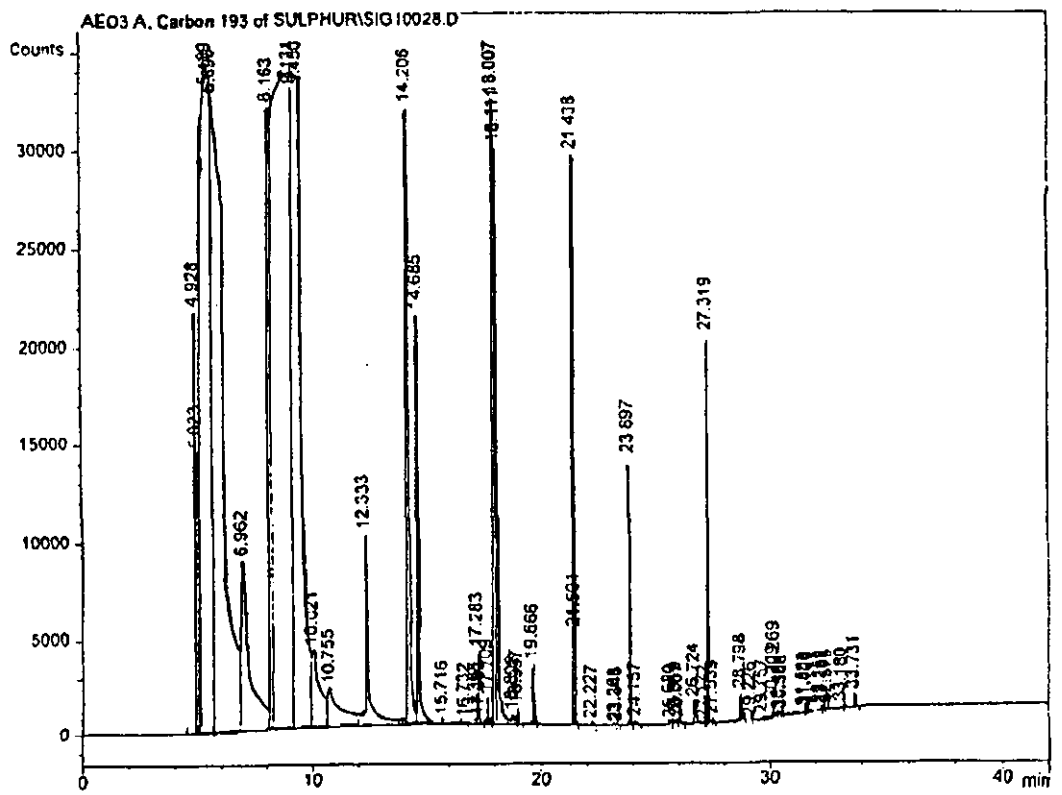


Fig. 17: AED carbon trace (193 nm) of beer headspace. Column: HP-17, 30m x 0.25mm x 0.25µm. Details of the experimental conditions are listed in 2.2.2

Several of the sulphur trace peaks were corresponded to the larger carbon peaks - ethanol, higher alcohols, fatty acids and esters which are found in beer (160,161) - from the carbon trace. From Figures 16 & 17 it can be seen that this is the case with the peaks at 5.523, 8.794, 9.507, 14.181, 14.698, 18.101, 18.122, 21.442 and 27.323 minutes.

3.1.1.2.2 Discussion

The appearance on the sulphur trace of the several of the alcohols and esters which are present in beer headspace shows that the specificity of the AED is not complete. However, it is possible to tune the specificity of the AED using the software, giving much better selectivity against carbon with little loss in sensitivity. Unfortunately it was not possible to optimise the selectivity with the software in the 2 days available for the analysis of the samples.

Even when the cross-selectivity is taken into consideration, the sulphur trace at 181 nm still shows at least 10 sulphur compounds. The sensitivity of the AED appeared to be comparable to that of the Sievers SCD. According to Hewlett-Packard specifications (231) the minimum detectable level for sulphur is 2 pg/sec; the selectivity over carbon is listed as 10000.

Despite not being fully optimised the HP G2350A AED proved to be a very sensitive detector which would be capable of measuring volatile sulphur compounds in beer headspace at very low concentrations. Further optimisation of the detector parameters would be required to assess whether sensitivity and selectivity could be increased further. Another advantage of the G2350A AED is that it could also be used as a specific detector for a variety of other elements. Despite the very promising results obtained with the HP G2350A AED, for financial reasons it was decided to assess the considerably less expensive pulsed flame photometric detector (the AED was approximately 7 times more expensive than the PFPD) before a final choice of detector was made.

3.1.1.3 Varian Pulsed Flame Photometric Detector

The Pulsed Flame Photometric Detector (PFPD) was developed by Amirav and co-workers in Israel in the early 1990's, as outlined in chapter 1.3.3.2 (171,172,173). At the start of the project the PFPD was not available commercially, first coming onto the market in 1995. There are two versions of the PFPD available: one from OI Analytical, the other from Varian. The Varian PFPD, which was used, is in design identical to the improved PFPD design described by Amirav and Jing in 1995 (173).

The Varian PFPD was connected to a Varian 3800 GC. Originally for a two-week trial period a PFPD was connected to a Varian 3400 CX GC, but the absence of a constant carrier gas flow function did not allow constant conditions in the detector. Therefore it was decided to wait until the new Varian 3800 GC with electronic gas control was launched onto the market.

The carrier gas was hydrogen. In the sulphur mode a BG-12 deep-violet glass filter is the filter of choice. A narrow bore (2 mm ID) quartz combustor tube was used (figure 5). The GC and PFPD parameters were optimised using a 4 m x 320 µm DB-1 column and a test solution containing 20 ng/µl each of n-dodecanethiol and methylparathion.

3.1.1.3.1 Results

3.1.1.3.1.1 Optimisation of Detector Parameters

The optimal detector conditions were determined experimentally: the values recommended in the Varian PFPD manual were taken as the initial starting points for each parameter to be optimised. The object of the optimisation process was to maximise the selectivity of sulphur over carbon and the detectivity of the detector. Detectivity (174) can be

defined as the minimum detectable quantity (MDQ) of sulphur, expressed in units of weight/time, e.g. femtograms sulphur per second. The term 'detectivity' is preferred to 'sensitivity' by Amirav (232) because it takes into account the signal-to-noise ratio. This is important with the PFPD as the 'sensitivity' of the detector could simply be increased by increasing the voltage of the photomultiplier tube. However, the noise would increase proportionally to the signal and would not give an effective improvement in the minimum amount detectable. Detectivity is defined using equation xxii (174):

xxii $$D_S = [W_S / (1.5 \times W_h)] \times \sqrt{(2 \times N/H)}$$

where D_S = detectivity for sulphur (pg/sec), W_S = weight of sulphur (pg), W_h = width of sulphur peak at half height (sec), N = baseline noise (mm), H = height of sulphur peak (mm).

Firstly, the optimum detector voltage was determined. All the chromatographic and detector parameters were kept constant and the PFPD voltage was increased in 10 volt steps, starting at 560V. The resulting chromatograms can be seen in Figure 18.

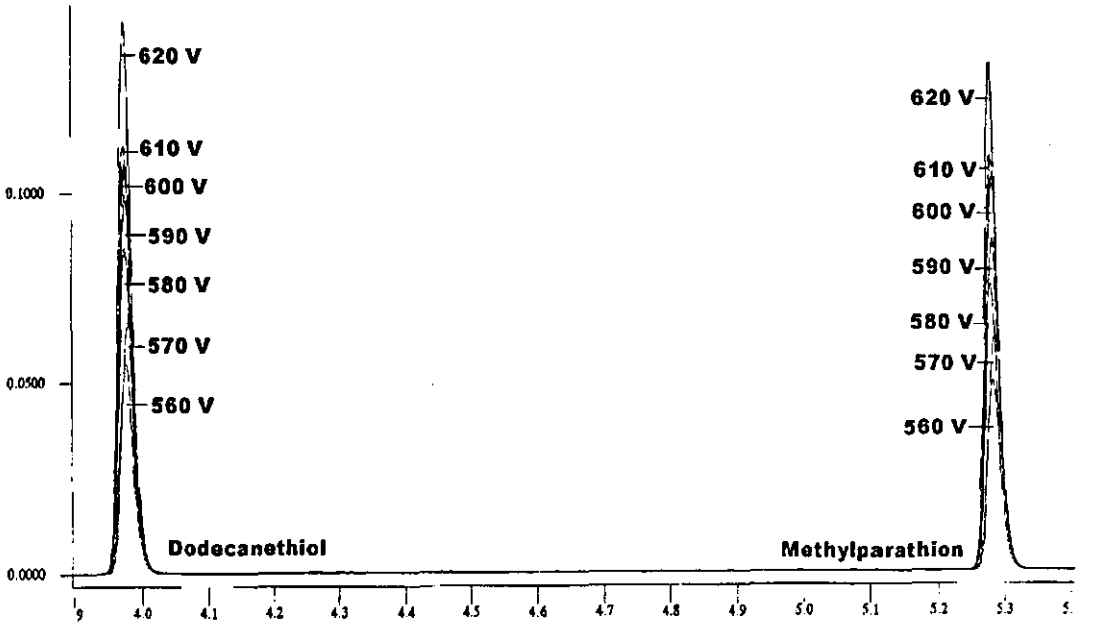


Fig. 18: A comparison of various PFPD voltage settings. Injection of 1µl of test solution containing 20 ng/µl each of dodecanethiol and methylparathion. Chromatographic conditions: column = DB-1, 4m x 0.32mm x 0.25µm; constant column flow = 2.7 ml/min hydrogen; column oven = 80°C isothermal; 1 µl injected, split ratio 20:1. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; temperature = 200°C; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; range = 10; attenuation = 1

A linear relationship between signal intensity and detector voltage is clearly displayed. As expected the highest voltage, of 620V, gave the largest peak areas for both dodecanethiol and methylparathion. The baseline at 620V, however, was very noisy in comparison with the baselines obtained at other voltage settings. This increase in baseline noise would lead to a significant loss of detectivity. As the difference in signal response between the detector settings of 600V and 610V was minimal, a voltage setting of 600V was considered to be optimal.

The next detector parameter to be considered was the gate delay. The recommended value (174) for sulphur detection is 6 ms. This setting was compared to a gate delay setting of 5 ms. The chromatograms are shown in Figure 19.

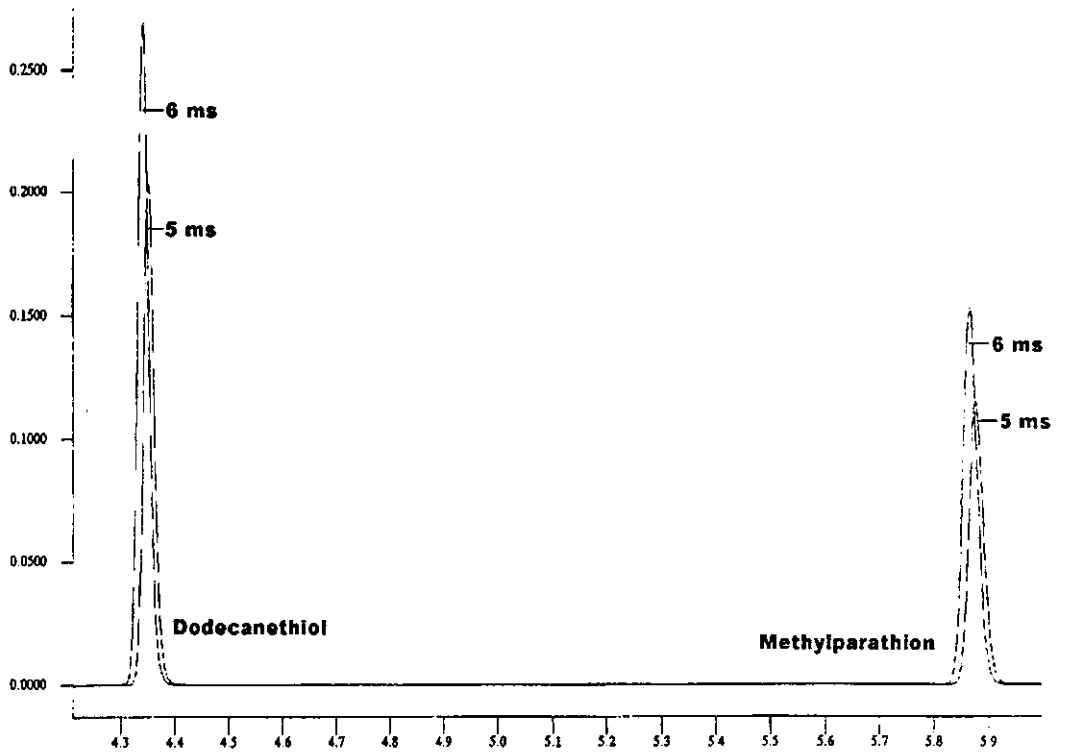


Fig. 19: A comparison of different PFPD gate delay settings. Chromatographic conditions: column = DB-1, 4m x 0.32mm x 0.25µm; constant column flow = 2.7 ml/min hydrogen; column oven = 80°C isothermal; 1 µl injected, split ratio 20:1. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; temperature = 200°C; detector voltage = 600V; gate width = 20.0 msec; trigger level = 200 mV; range = 10; attenuation = 1

The peak area for the 5 ms gate delay was not only smaller, as can be seen in Figure 19, but also the baseline was noisier, giving poorer detectivity. A gate delay of 6 ms was considered to be the optimum value, confirming the recommendation made in the Varian PFPD manual.

The optimisation of the PFPD gate width at first seemed to give unexpected results. As can be see from Figure 20 the narrower gate width of 10 ms actually gave the larger signal response. The response with the 20 ms gate width - the value recommended by Varian - was lower.

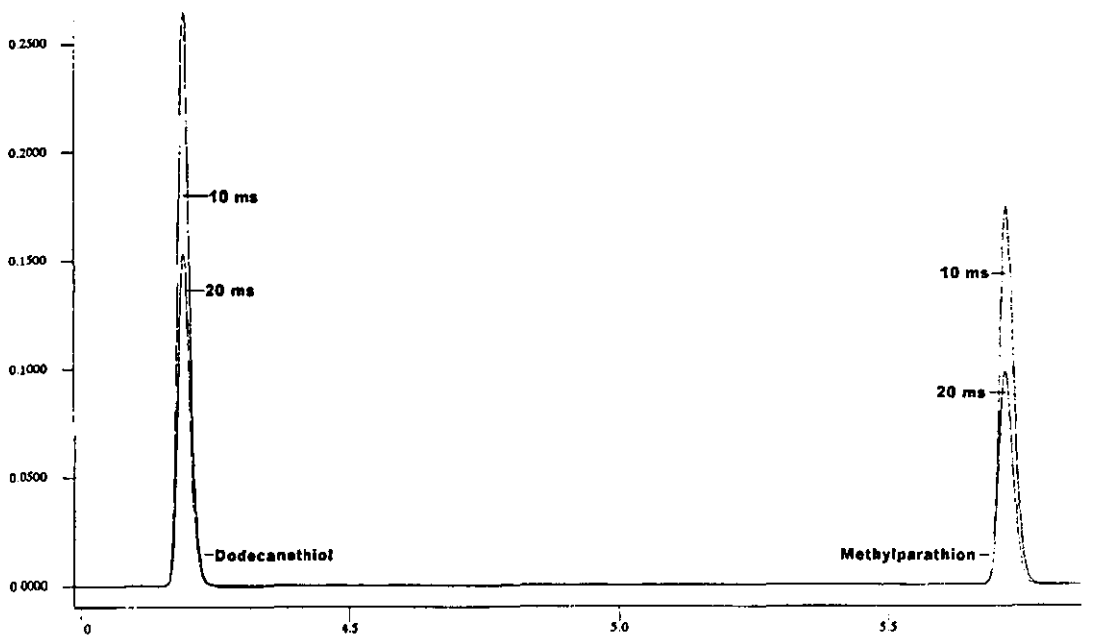


Fig. 20: A comparison of different PFPD gate width settings. Chromatographic conditions: column = DB-1, 4m x 0.32mm x 0.25µm; constant column flow = 2.7 ml/min hydrogen; column oven = 80°C isothermal; 1 µl injected, split ratio 20:1. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; temperature = 200°C; detector voltage = 600V; detector gate delay = 6 ms; trigger level = 200 mV; range = 10; attenuation = 1

This seemingly paradoxical result is a result of the gated amplifier giving the emission integral divided by the gate width (232). Remembering that the pulsed flame emission curve displays its maximum at approximately 10 ms (Figure 7, section 1.3.3.2), and taking into account a gate delay of 6 ms, it is logical that the average signal is higher with the 10 ms gate width and the total signal higher with the 20 ms gate. Therefore the observation that the 10 ms gate width signal is higher, as seen in figure 20, can be explained by the theory. The reason that a 20 ms gate width is preferred is that the noise is increased only as the square root of the gate width but is normalised twice. For a gate width of 20 ms the noise is a factor of 0.7 lower than that of the 10 ms gate width but the signal is reduced by less than a factor of 0.7. The result is a slight gain in detectivity with a 20 ms gate.

The practical effects of this can be seen in Figure 21, which shows a small section of two SPME-GC-PFPD chromatograms of an identical beer sample. The peaks near the limit of detection of the system are larger with a 10 ms gate width, but so is the background noise. The better detectivity, despite a lower average signal and therefore lower peak area, is clearly seen to be provided by the 20 ms gate.

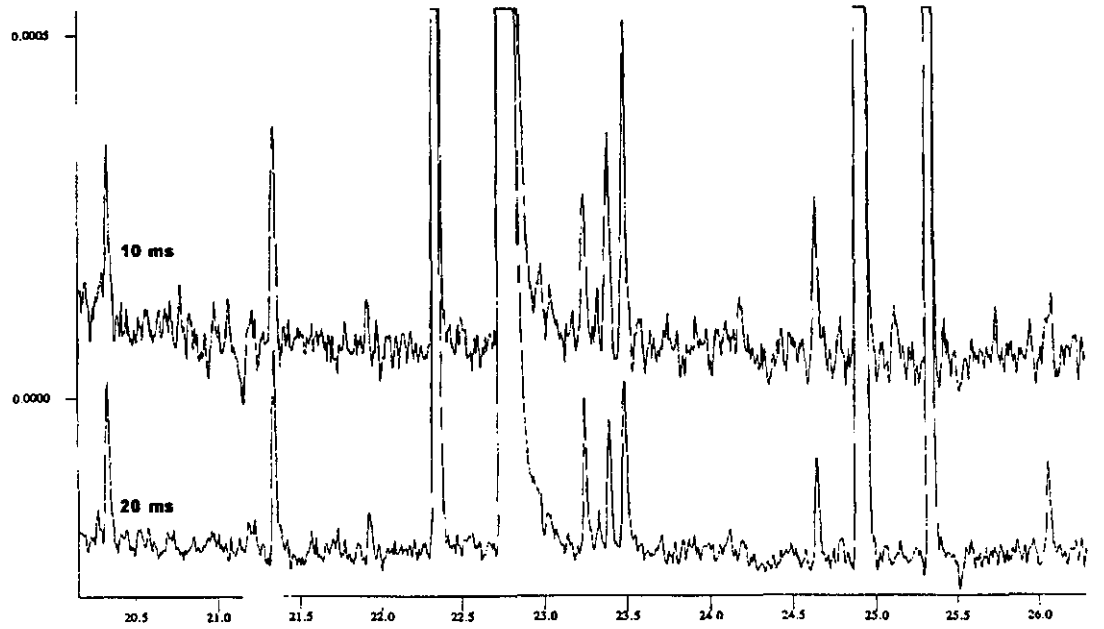


Fig. 21: A comparison of gate widths of 10 ms and 20 ms, beer sample with SPME-GC-PFPD. SPME conditions: carboxen fibre; absorption = 36 mins, agitation; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; temperature = 200°C; detector voltage = 600V; gate delay = 6.0 msec; trigger level = 200 mV; range = 10; attenuation = 1

The detector flow rates were not very simple to optimise. Three gases are used in the detector (hydrogen, air 1 and air 2), giving innumerable possible combinations of gas flows. Additionally, the ratio of the hydrogen / air mix flows which pass through and which bypass the combustor has to be optimised by the appropriate setting of the needle valve. As the carrier gas used is hydrogen, to give the best possible chromatographic

separation, this also has to be taken into consideration, hence the importance of electronic pressure control to allow the carrier gas flow to be kept constant during the run.

The gas flows recommended in the Varian PFPD Operators Manual (174) were very close to the experimentally-determined optimum values, i.e. air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min. The hydrogen carrier gas flow was kept constant at 2.7 ml/min during the whole run. The optimum needle valve setting is that recommended in the handbook, i.e. the needle valve should be opened just so far that the combustor refills slightly faster than the ignitor (Figure 5, section 1.3.3.2). If the needle valve is not opened far enough the combustor refills too slowly, the flame does not propagate into the combustor and the 'tick-tock' effect is seen. Tick-tock is when the flame only propagates into the combustor on alternate pulses, leading to a very noisy background.

The final variable parameter is the detector operating temperature. The PFPD Operator's Manual recommends a detector temperature of 200°C for maximum sulphur response. Tests with the test solution between temperatures of 200°C and 250°C showed no great differences in response. The difference in response for a beer sample analysed with the SPME-GC-PFPD system at two different PFPD operating temperatures is shown in Figure 22. The chromatograms show a 10 minute section of the run.

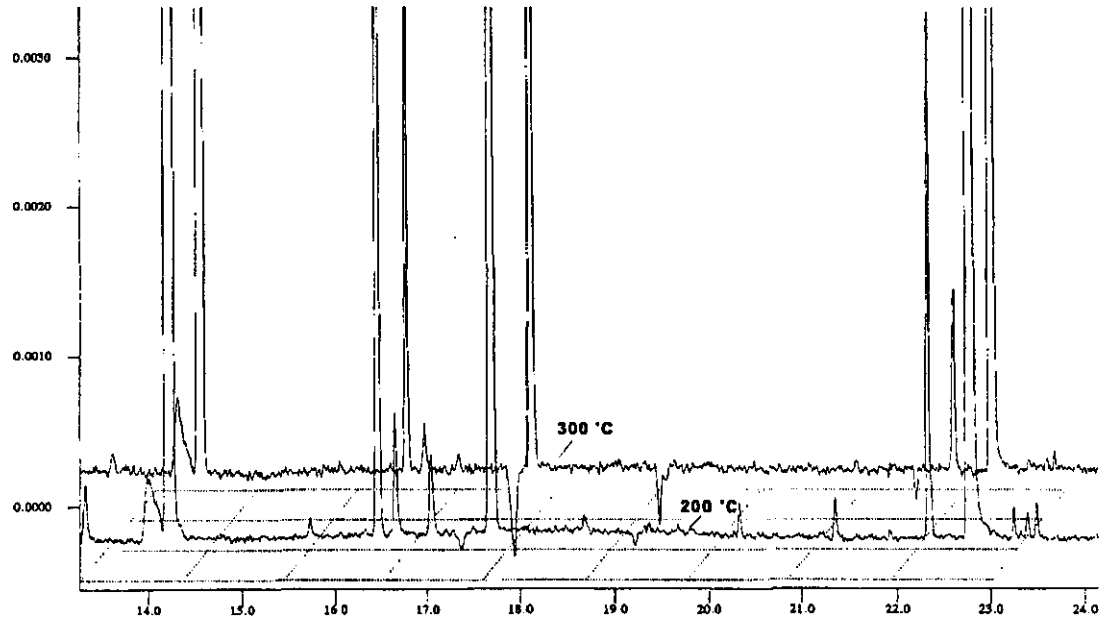


Figure 22: A comparison of PFPD operating temperatures, beer sample with SPME-GC-PFPD. SPME conditions: carboxen fibre; absorption = 36 mins, agitation; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; detector voltage = 600V; gate delay = 6.0 ms; gate width = 20 msec; trigger level = 200 mV; range = 10; attenuation = 1 .

From Figure 22 it can be seen that the sensitivity with a PFPD operating temperature of 300°C is markedly lower than the sensitivity obtained with a PFPD temperature of 200°C. Additionally, the background noise is greater at the higher detector temperature.

To avoid the possibility of peak broadening at detector temperatures of 200°C and below (174) a PFPD operating temperature of 210°C was selected.

After the completion of the optimisation process the detectivity of the PFPD was measured using the Varian PFPD test solution and compared to the specifications stated by Varian.

The test solution contained 20 ng/µl each of n-dodecanethiol and methylparathion, $C_{12}H_{26}S$ and $C_8H_{10}NO_5PS$ respectively. The percentage by mass of sulphur in dodecanethiol = 32/202 x 100 = 15.84%. The percentage by mass of sulphur in methylparathion = 32/263 x 100 = 12.17%. Therefore in a 20ng/µl solution there is 3.17 ng/µl and 2.43 ng/µl sulphur present for dodecanethiol and methylparathion. If a 1µl sample with a split of 1/20 is injected then the dodecanethiol peak contains 158.4 pg of sulphur and the methylparathion peak 121.7 pg of sulphur.

The detectivity of the PFPD with the optimised parameters was found to be 0.7 pg of sulphur per second. The calculated detectivity was checked with by injecting low concentrations of the test solution at high splits. 0.1 µl of the test solution was injected at a split ratio of 1:100, giving sulphur concentrations of 3.2 pg and 2.4 pg for dodecanethiol and methylparathion respectively. The resulting chromatogram is shown in Figure 23.

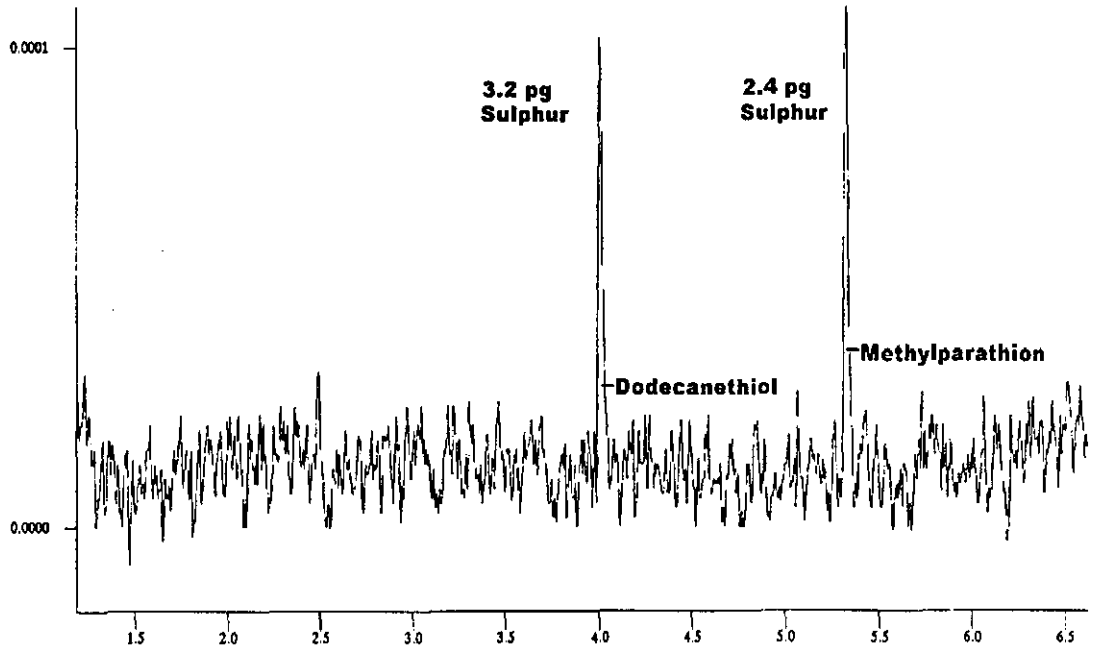


Fig. 23: Chromatogram of sulphur-containing compounds in low concentrations. Chromatographic conditions: column = DB-1, 4m x 0.32mm x 0.25µm; constant column flow = 2.7 ml/min hydrogen; column oven = 80°C isothermal; 1 µl injected, split ratio 20:1. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; temperature = 200°C; detector voltage = 600V; detector gate delay = 6 ms; detector gate width = 20 ms; trigger level = 200 mV; range = 10; attenuation = 1

The element selectivity of the detector could not be accurately judged from the test solution. No peaks from the pentadecane and tributylphosphate present in the test solution were seen, however, indicating that cross-selectivity would probably not be a problem. The selectivity of the PFPD for sulphur over carbon is declared by Varian to be 10^6 (174).

3.1.1.3.1.2 Equimolar Sulphur Response of the PFPD

In their paper on the PFPD, Cheskis, Atar and Amirav (172) claimed that the sulphur response of the PFPD is equimolar, i.e. it is independent of the structure of the sulphur-containing molecule and dependent purely on the absolute mass of sulphur. This claim was checked using a solution containing 23.26 mg/l dimethyl sulphide (DMS), 28.50 mg/l ethyl methyl sulphide, 33.76 mg/l methyl thioacetate, 38.96 mg/l ethyl thioacetate in iso-octane. The solution was so prepared that the amount of each of the four compounds contained exactly 12 mg/l of sulphur. 1 µl of this solution was injected with a split ratio of 1:48, meaning that exactly 250 pg of sulphur per compound were injected onto the column. The resulting chromatogram can be seen in Figure 24.

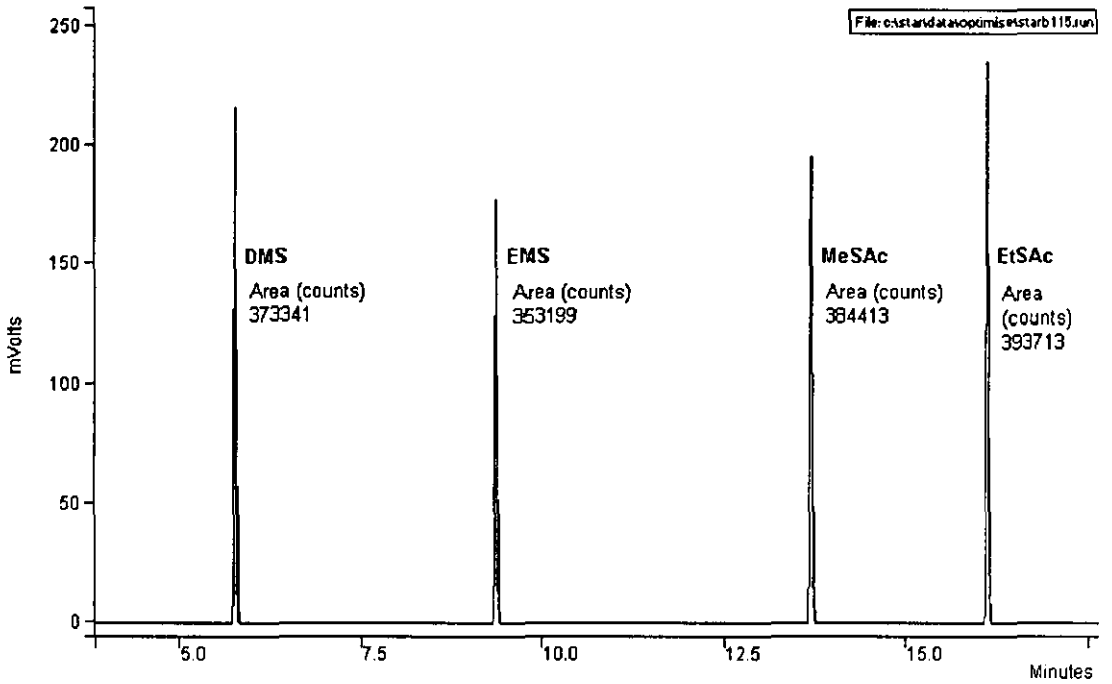


Fig. 24: Chromatogram of dimethyl sulphide, ethyl methyl sulphide, methyl thioacetate and ethyl thioacetate, all containing exactly 250 pg of sulphur. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: air 1 = 17.0 ml/min, air 2 = 10.0 ml/min, hydrogen = 13.0 ml/min; detector voltage = 600V; gate delay = 6.0 ms; gate width = 20 msec; trigger level = 200 mV; range = 10; attenuation = 1. Liquid injection of 1µl of solution, split 1:48.

A comparison of the areas of the peaks gave a relative standard deviation of 4.01%, which, because of the quadratic nature of the sulphur response of the PFPD, translates to a sulphur amount relative standard deviation of 1.99%. This result backs up the claim that the sulphur response of the PFPD is equimolar and independent of the structure of the sulphur-containing molecule.

3.1.1.3.1.3 Reproducibility of the PFPD

The solution described in section 3.1.1.3.1.2 to examine the equimolarity of the PFPD sulphur response was injected another 12 times to examine the reproducibility of the detector. Liquid injections were used so as to allow the reproducibility of the detector alone to be investigated, without any errors being brought in by the SPME. The results of the 13 injections can be seen in the Table 2.

Table 2: Peak areas of 4 sulphur compounds repeatedly injected with GC-PFPD. Conditions as in Figure 24.

	Compound: DMS	Compound: EMS	Compound: MeSAc	Compound: EtSAc	Peak Area RSD (%)	Sulphur Concentration RSD (%)
Run 1	427319	373809	331470	399641	9.21	4.5
Run 2	395213	347028	323893	423482	10.51	5.12
Run 3	373341	353199	384413	393713	4.01	1.99
Run 4	426673	370793	379479	382139	5.57	2.75
Run 5	448285	363416	402887	452430	8.74	4.28
Run 6	405933	362985	347065	382560	5.88	2.9
Run 7	429636	402393	365560	412242	5.82	2.87
Run 8	377575	358140	384216	414012	5.23	2.58
Run 9	453561	378026	420810	437634	6.67	3.28
Run 10	359687	323488	346224	368302	4.84	2.38
Run 11	444885	394076	350053	444454	9.67	4.72
Run 12	458880	372787	414822	378695	8.46	4.14
Run 13	445968	370235	408679	365018	8.22	4.03
Peak Area Mean	418997	366952	373813	404177	5.47	2.07
Peak Area RSD (%)	7.64	5.24	8.33	6.92		
Sulphur Concentration RSD (%)	3.75	2.59	4.08	3.4		

Table 2 displays two important results. Firstly, that the sulphur concentration relative standard deviations for the equimolar response of the PFPD lie between 1.99% and 5.12%, confirming the result from section 3.1.1.3.1.2 that the sulphur response of the PFPD is equimolar and is independent of the structure of the sulphur-containing molecule. Secondly, the sulphur concentration relative standard deviations for the four compounds over the 13 injections lie between 2.59% and 4.08%, showing that the PFPD itself displays good reproducibility.

3.1.1.3.2 Discussion

In trials using the test solution the Varian PFPD proved itself to be a sensitive and stable detector, suitable for the detection of sulphur compounds in low concentrations. The detectivity specifications listed by Varian were achieved and even surpassed. The experimentally-determined optimal detector settings were an operating temperature of 210°C; a detector voltage of 600V; a gate width of 20ms; a gate delay of 6ms. The optimal detector gas flows, with a hydrogen carrier gas flow of 2.7 ml/min, were 16.9 ml/min for air 1; 9.8 ml/min for air 2; 10.3 ml/min for hydrogen. All of these values differed only very slightly from those provided by Varian in the PFPD Operator's Manual.

The first tests with a PFPD connected to a Varian 3400 CX GC underlined that a constant carrier gas flow is very important to maintain stable conditions in the detector. Therefore it is advisable to always use the PFPD in conjunction with a GC which is capable of providing constant carrier gas flow rates.

On the grounds of the very promising results obtained with the test solution, and the low cost of the detector in comparison to the other possible alternative of the Hewlett-Packard Atomic Emission Detector, it was decided that the Varian PFPD in conjunction with a Varian 3800 GC would be used for the detection of sulphur compounds in beer.

3.1.2 Sample Preparation

The second stage of the work was to consider different methods of sample preparation which could complement and enhance the sensitivity of the detector. Some form of selectivity for the extraction of sulphur compounds from the beer matrix would also be desirable.

3.1.2.1 Solid Phase Microextraction (SPME)

The most promising method of sample preparation appeared to be solid phase microextraction, which is explained in more detail in section 1.3.1.6. At the time this study was started SPME was not in wide use owing to its novelty. However, it appeared, theoretically at least, to offer good sensitivity and simplicity of use without any of the disadvantages found with other sample preparation techniques.

Additionally, Varian was the only company licensed to manufacture and sell an SPME autosampler. As the PFPD from Varian had proved itself to be the best detector on which to base the method, it seemed the logical step to combine it with an adapted Varian autosampler to try out the new and potentially very promising SPME technique.

A Varian 8200/SPME autosampler was borrowed from Varian, Germany, for test purposes. This autosampler is capable of agitating the fibre during adsorption, thereby allowing equilibrium to be reached more rapidly. Adsorption and desorption times are controlled using the software. The software can set whether the sample is directly sampled or only its headspace is measured. Either 2 ml or 10 ml sample vials can be used with the 8200/SPME autosampler.

3.1.2.1.1 Results

The most important parameter to be decided when using SPME is which fibre provides the best extraction and selectivity for the compounds of interest. Miller and Stuart used five different fibres to compare traditional static headspace to SPME-sampled headspace for the analysis of volatile flavour components (233). Clark and Bunch compared the performance of four SPME fibres for the analysis of flavour additives to tobacco products (234). A comparison of 6 different SPME fibres for the investigation of wine bouquet components with SPME-GC-MS was published by De la Calle Garcia *et al.* in 1997 (137).

Several different SPME fibres from Supelco, the only company licensed to manufacture SPME fibres, were tested. The experimental conditions were identical for each fibre (see section 2.2.5)

3.1.2.1.1.1 SPME Fibre: 7µm PDMS

The 7µm polydimethylsiloxane (PDMS) fibre was suggested by Supelco as being suitable for mid- to non-polar semi-volatiles. In the literature there are very few applications which use the 7µm PDMS fibre (137).

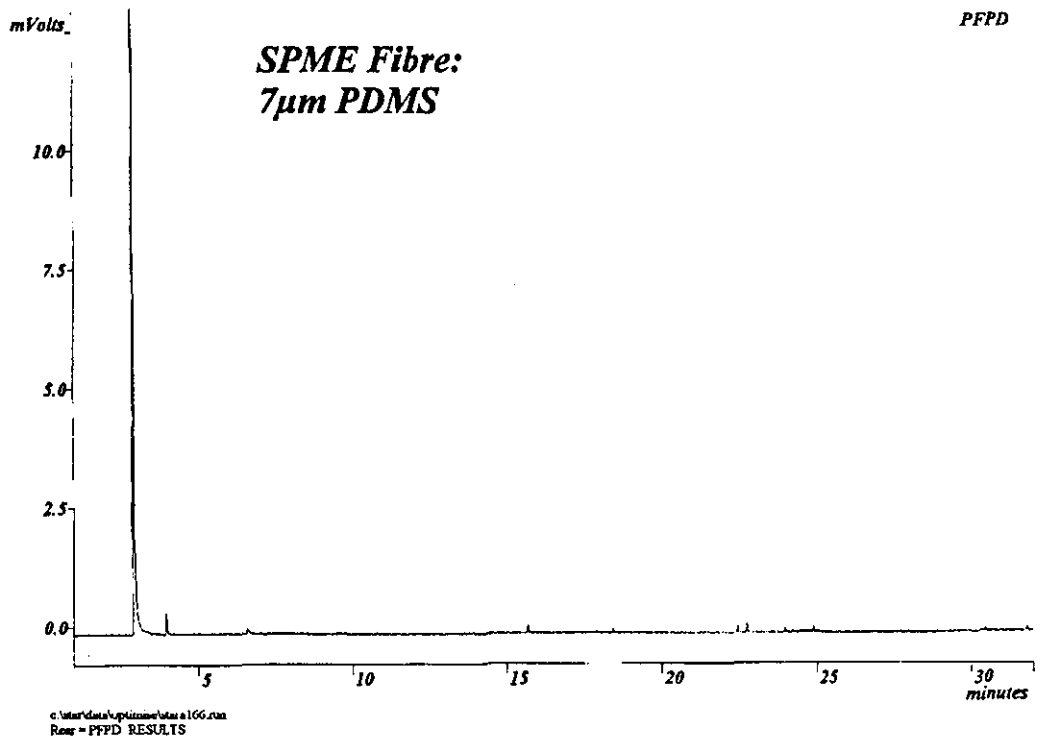


Fig. 25: Beer sample extracted with a 7µm PDMS SPME fibre. SPME conditions: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

As can be seen from the above chromatogram, very few sulphur-containing compounds were extracted from the beer headspace with the 7µm PDMS fibre. This fibre is thus not suitable for the analysis of volatile and semi-volatile sulphur compounds in beer.

3.1.2.1.1.2 SPME Fibre: 100µm PDMS

The thicker, 100µm, PDMS fibre coating is recommended for volatile compounds and has been successfully used in many studies of the application of SPME in the field of flavour and fragrance (127,128,129,131,132,135,137,138,140,141,142,146,151,152,233,234,235).

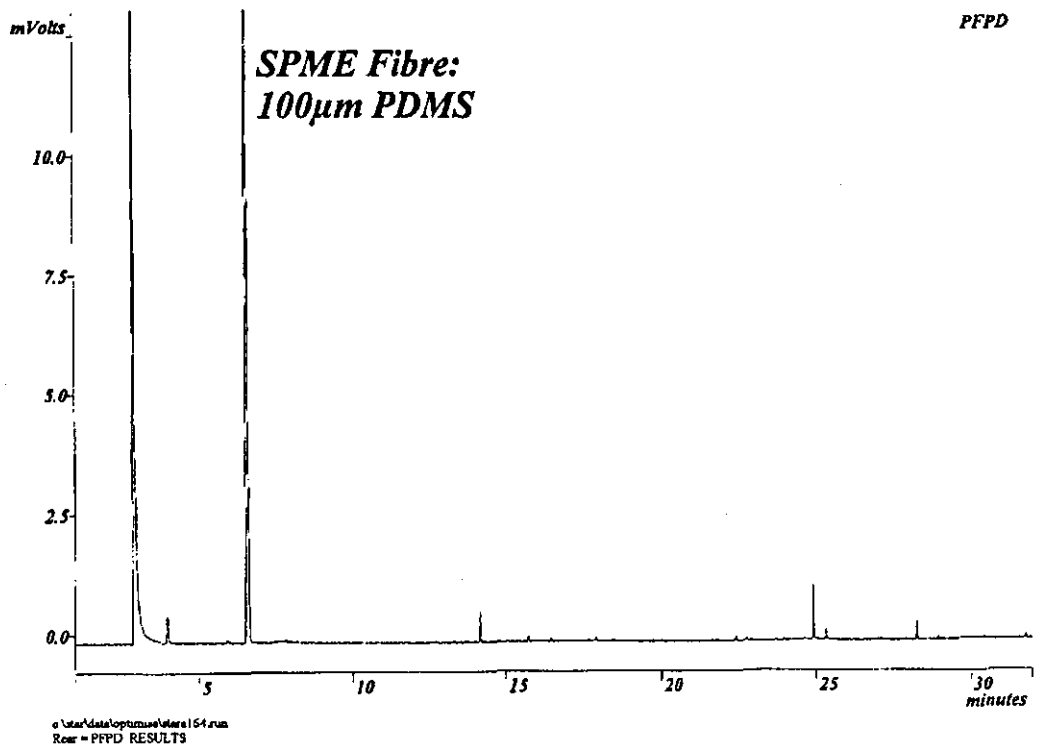


Fig. 26: Beer sample extracted with a 100µm PDMS SPME fibre. SPME conditions: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

Although the 100µm PDMS fibre showed higher extraction than the 7µm PDMS fibre, it appeared that the PDMS coating was also not a very efficient coating for the extraction of volatile and semi-volatile sulphur compounds from beer headspace.

3.1.2.1.1.3 SPME Fibre: 85µm Polyacrylate

The polyacrylate coating is relatively polar and therefore suitable for extracting polar semi-volatile compounds. As with the 100µm PDMS fibre, the 85µm polyacrylate fibre has often been used for flavours and fragrances (125,128,132,136,137,138,142,144,146,152,234)

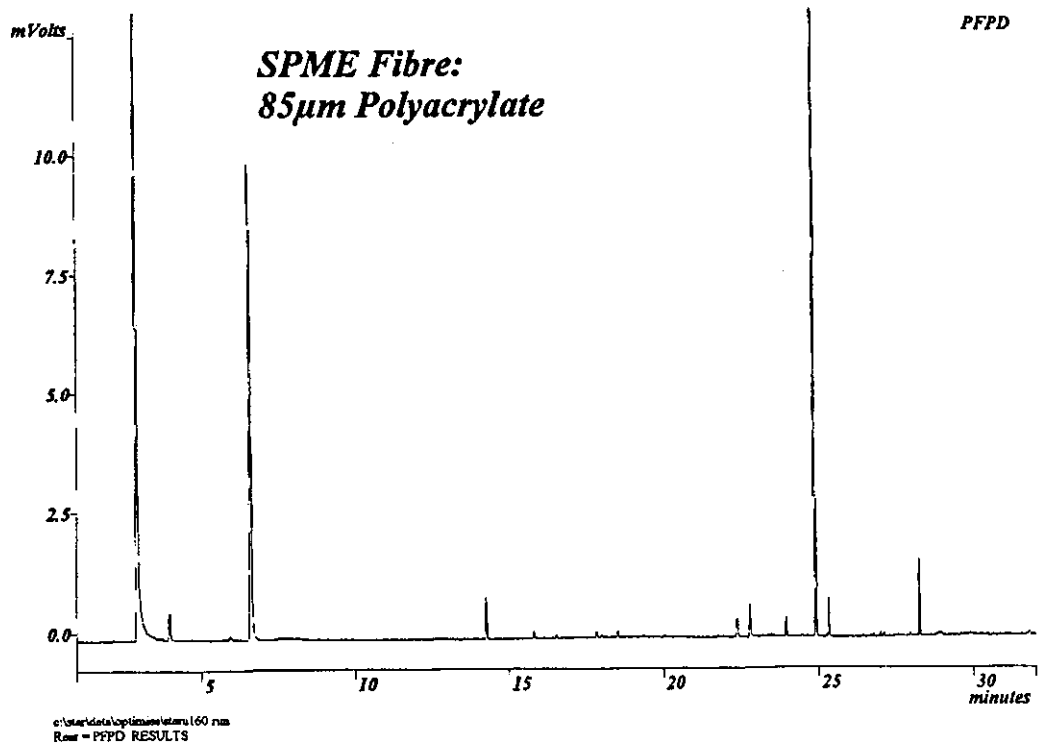


Fig. 27: Beer sample extracted with a 85µm polyacrylate SPME fibre. SPME conditions: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

As can be seen from the chromatogram in Figure 27, the 85µm polyacrylate was an improvement for the extraction of sulphur compounds on the PDMS coatings. Approximately 15 peaks of a size sufficient to be evaluated were detected, mostly semi-volatile compounds as can be seen from the retention times.

3.1.2.1.1.4 SPME Fibre: 65µm Carbowax / Divinylbenzene

The carbowax/divinylbenzene coating is reported to be suitable for the extraction of polar compounds. Few applications of its use in the field of flavour and aroma have been published (137,233,234,236).

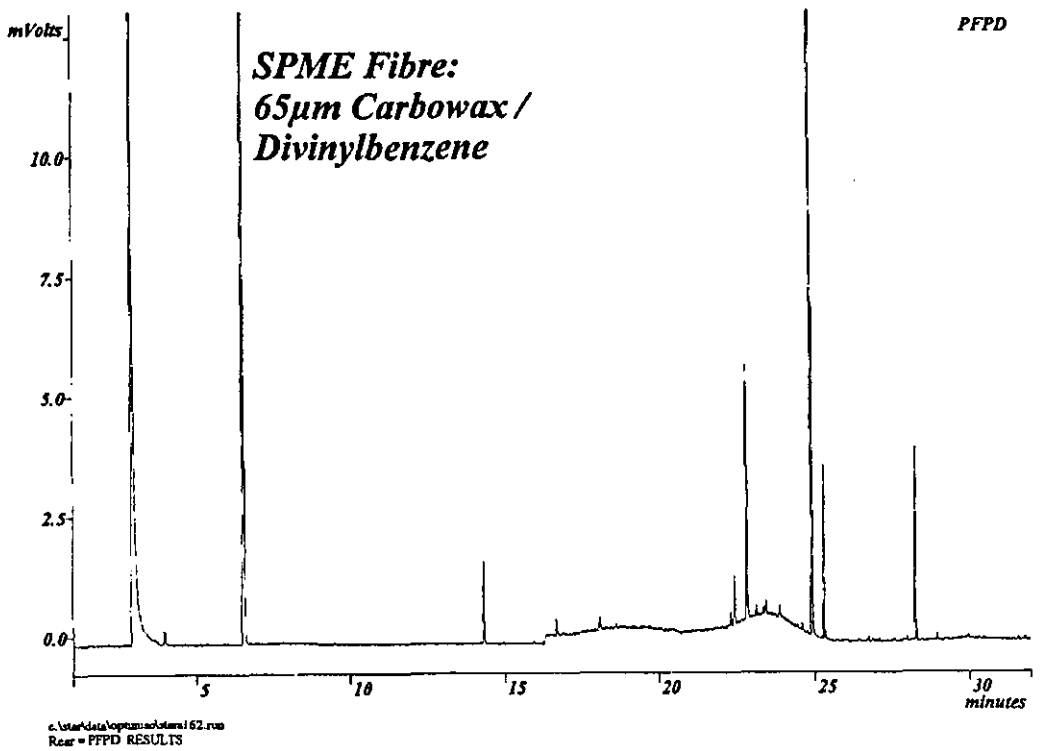


Fig. 28: Beer sample extracted with a 65µm carbowax/divinylbenzene SPME fibre. SPME conditions: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The 65µm Carbowax/divinylbenzene SPME fibre extracted a similar number of sulphur compounds from the beer as the 85µm polyacrylate coating. The area of the peaks obtained with the Carbowax/ divinylbenzene coating was greater.

3.1.2.1.1.5 SPME Fibre: 75µm Carboxen / PDMS

Supelco recommends the 75µm carboxen/PDMS coating for gases and low molecular weight analytes. Supelco describes carboxen as a 'carbon molecular sieve' coating (237). There are very few publications which report the use of the carboxen coating for the extraction of flavour and aroma components (145,147,233).

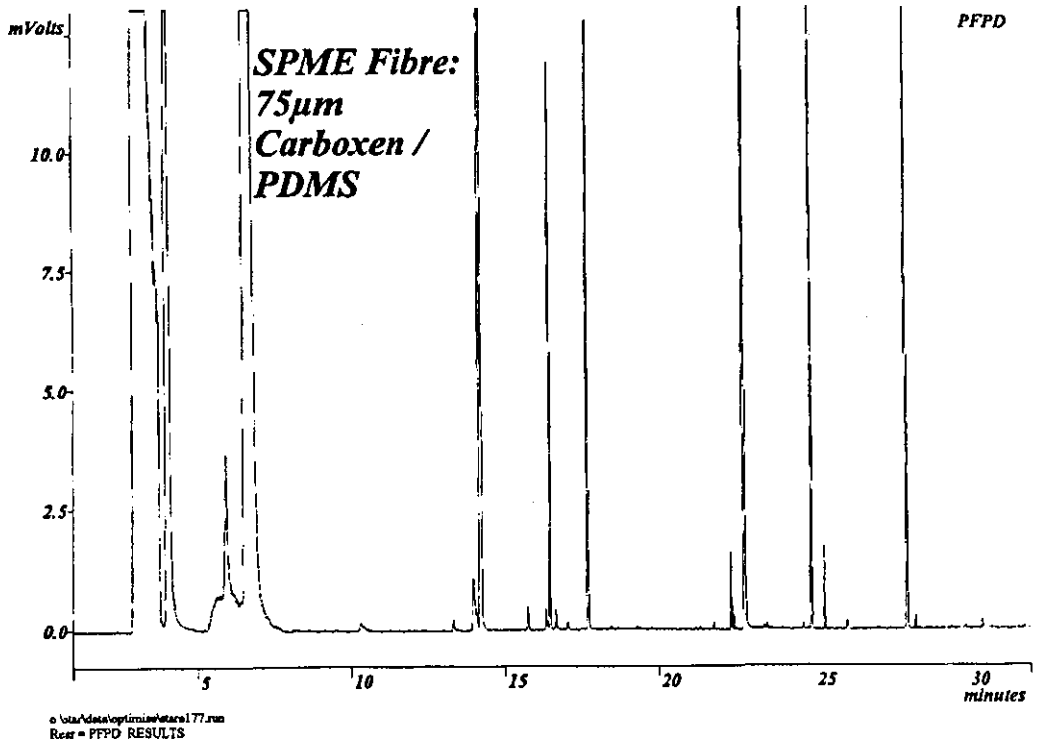


Fig. 29: Beer sample extracted with a 75µm carboxen/PDMS SPME fibre. SPME conditions: adsorption = headspace, 36 mins with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The carboxen/PDMS coating gave excellent extraction of both volatile and semi-volatile sulphur compounds from the beer sample, as can be seen from the chromatogram in Figure 29. The carboxen/PDMS fibre was clearly the best of the five tested SPME coatings. Two authors report that the price for the high extraction efficiency of the carboxen/PDMS fibre is poorer repeatability (147,238).

3.1.2.1.1.6 Adsorption Conditions

The adsorption conditions with the 75µm carboxen/PDMS SPME fibre were investigated and optimised.

Headspace SPME sampling and not liquid sampling was used because the higher molecular weight involatile compounds in beer, such as proteins and sugars, would be adsorbed to the fibres during liquid SPME sampling. These compounds would then be 'baked' onto the fibre during desorption in the GC injector, considerably shortening the lifetime of the fibre. Although it is possible to add a wash step after liquid sampling to remove any larger compounds, as described by Verhoeven *et al.* (125), the inclusion of such a wash step does not lend itself to automation and could conceivably cause losses in accuracy and precision.

The first adsorption parameter to be investigated was the length of the adsorption process. A carboxen/PDMS SPME fibre was exposed to six identical beer samples for differing lengths of time. The increase in area of the individual peaks was plotted against time. The peaks are identified in the following diagrams by their retention times (RT) as the compounds had yet to identified. The results can be seen in Figures 30 - 34, which are sorted according to the maximum peak areas to provide a clearer overview of the effect on minor and major compounds.

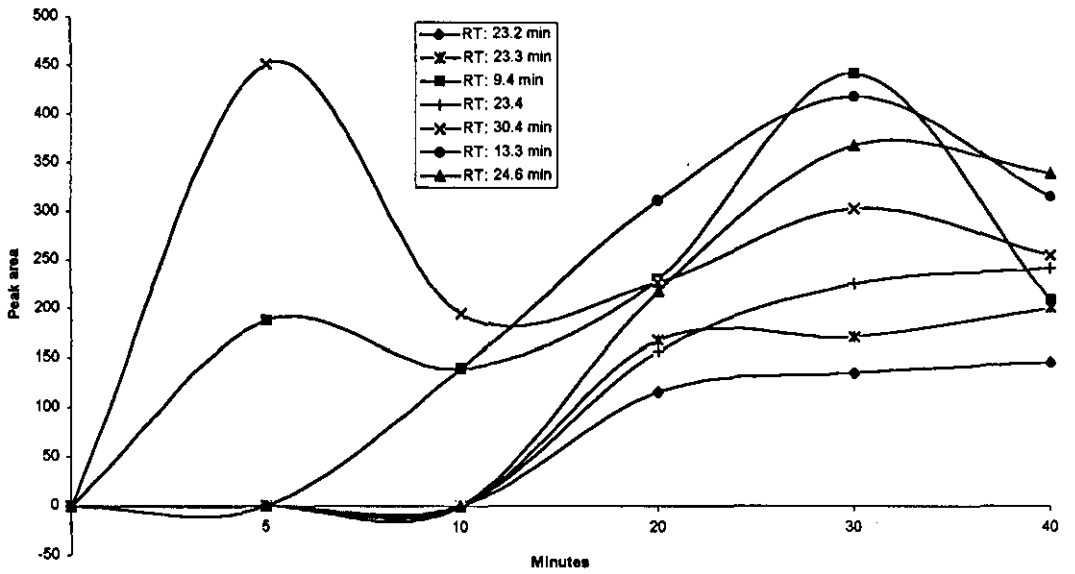


Fig. 30: Extraction time profiles (max. peak area 100 - 500) for various sulphur compounds with the SPME-GC-PFPD system. SPME conditions: adsorption = headspace, 5, 10, 20, 30 and 40 minutes with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

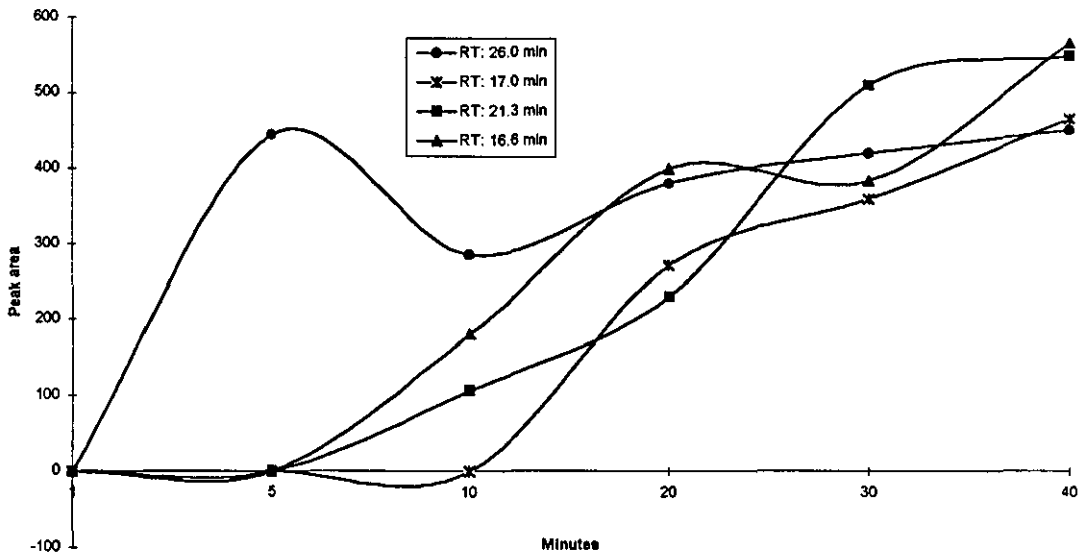


Fig. 31: Extraction time profiles (max. peak area 300 - 600) for various sulphur compounds with the SPME-GC-PFPD system. SPME conditions: adsorption = headspace, 5, 10, 20, 30 and 40 minutes with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

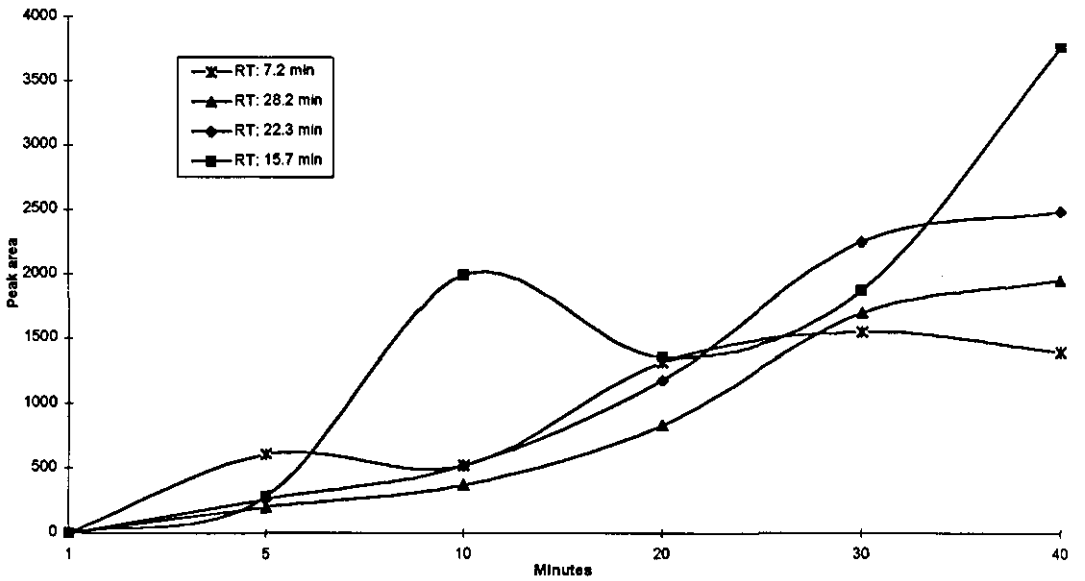


Fig. 32: Extraction time profiles (max. peak area 1000 - 4000) for various sulphur compounds with the SPME-GC-PFPD system. SPME conditions: adsorption = headspace, 5, 10, 20, 30 and 40 minutes with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

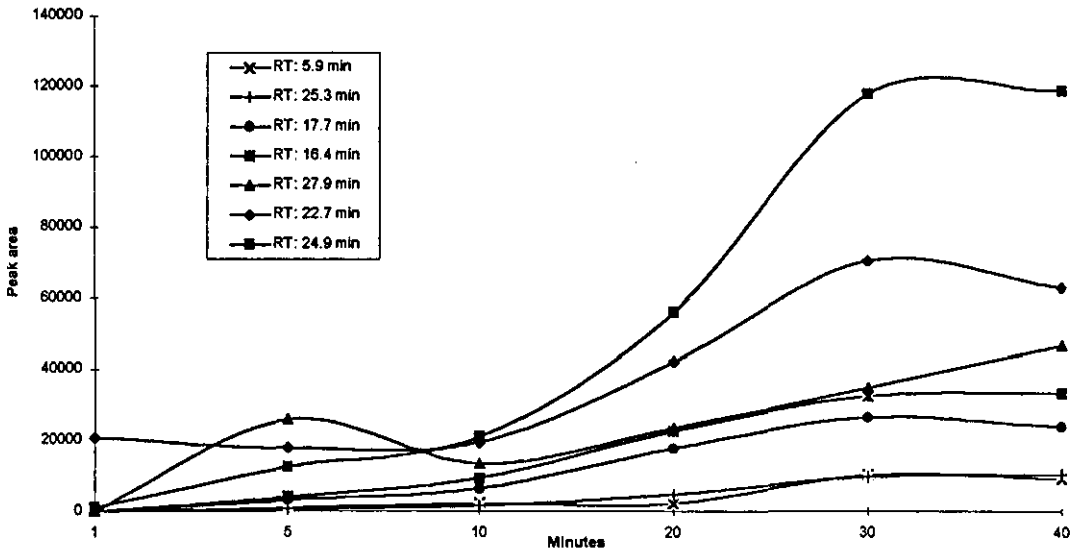


Fig. 33: Extraction time profiles (max. peak area 10000 - 130000) for various sulphur compounds with the SPME-GC-PFPD system. SPME conditions: adsorption = headspace, 5, 10, 20, 30 and 40 minutes with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

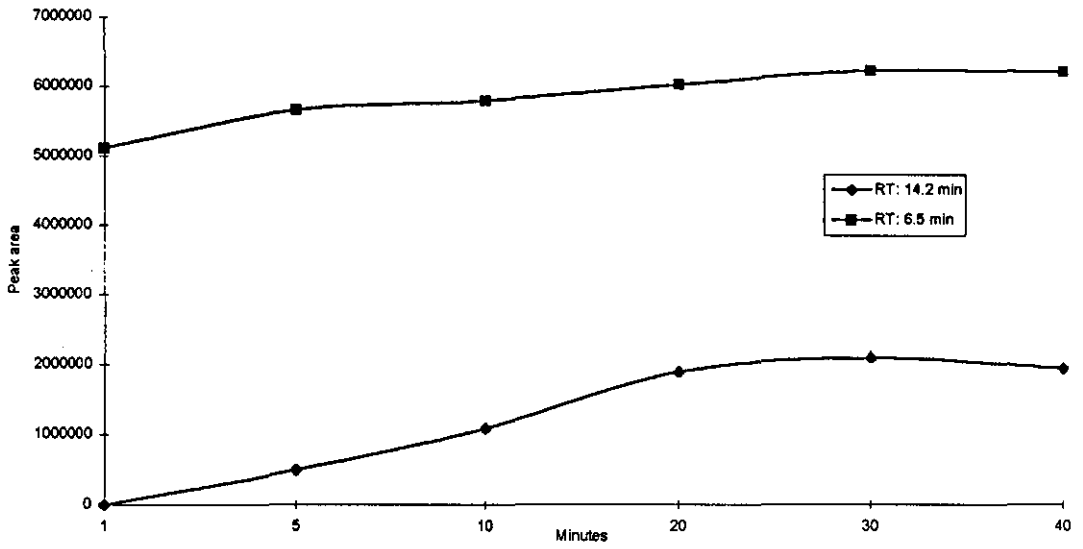


Fig. 34: Extraction time profiles (max. peak area 1900000 - 6200000) for various sulphur compounds with the SPME-GC-PFPD system.SPME conditions: adsorption = headspace, 5, 10, 20, 30 and 40 minutes with agitation at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

Figure 34 displays the extraction time profiles for the two largest peaks in the PFPD chromatogram: equilibrium is clearly reached after 30 minutes. The peaks shown in the extraction profile in Figure 33 behave similarly, equilibrium generally being reached after approximately 32 minutes. This finding is backed up, with a few exceptions, by the extraction profiles of the peaks with lower areas, as demonstrated in Figures 31, 32 & 33. Therefore the most suitable adsorption time for the extraction of sulphur

compounds from beer with a carboxen/PDMS SPME fibre was selected to be 32 minutes.

The effect of agitation on the extent of the adsorption was investigated by injecting an identical beer sample with and without agitation. The resulting chromatograms can be seen in Figure 35.

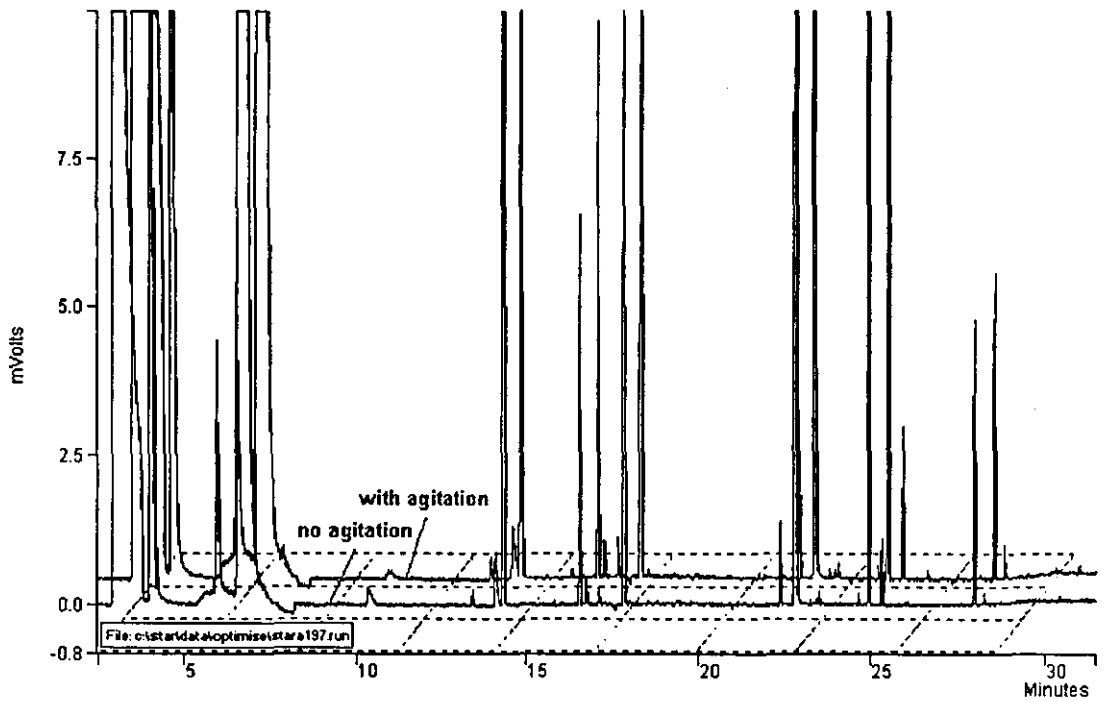


Fig. 35: Comparison of beer samples with SPME-GC-PFPD. SPME extraction with and without agitation. SPME conditions: adsorption = headspace, 36 mins at room temperature; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The differences between the two chromatograms shown in Figure 35 are very small. On closer inspection it can be seen that extraction with agitation provides a slight sensitivity advantage near the limit of detection in comparison to the extraction without agitation. Although not substantial, the improved sensitivity with agitation could be significant as the minor compounds are diagnostically interesting: sulphur compounds possess very low flavour thresholds and it is possible that they could be flavour active in concentrations very near the limit of detection of the method.

The next adsorption condition to be investigated was the effect of heating the sample carousel. A heatable carousel from Varian became available towards the end of the project.

The warming of the sample carousel up to 45°C gives an increase in sensitivity of a factor of approximately 2, as is shown in Figure 36. Higher temperatures were not tried: at temperatures in excess of 45°C the probability of artefacts being produced through the Maillard reaction is too high to be risked (239).

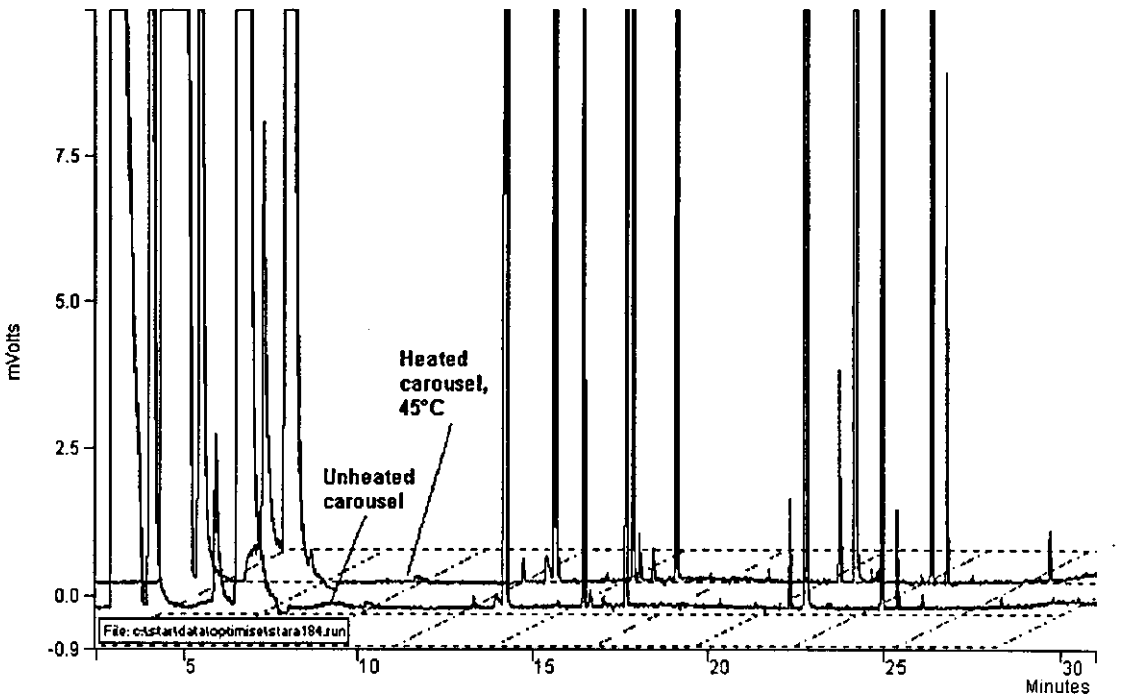


Fig. 36: Comparison of beer samples with SPME-GC-PFPD. SPME extraction with and without heating of the sample carousel. SPME conditions: adsorption = headspace, 36 mins with agitation; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

3.1.2.1.1.7 Desorption Conditions

The GC injector liner used was a specially-designed 0.8mm SPME liner. The reason for this is that a narrower bore liner produces much higher linear flow rates in comparison to the conventional wider bore (2-5 mm) liners. As a result, the desorbed analytes are swept more quickly from the injector onto the column, giving sharper peaks and better separation (123).

The injector temperature was set at 250°C, allowing the desorption of semi-volatiles whilst not being too hot to cause artefact production, and to extend the lifetime of the fibre (123).

The desorption of the analytes from the SPME fibre was carried out in the split/splitless mode. As the linear flow through the narrow liner is high, a short split off time of 0.8 minutes was considered sufficient. At 0.8 mins the split was opened with a ratio of 10:1, which was reduced to 1:1 after 2 minutes to save carrier gas.

3.1.2.1.1.8 Carryover

The possible problem of carryover, i.e. not all of the compounds adsorbed onto the fibre being desorbed in the injector and consequently being 'carried over' into the next run, resulting in inaccurate results, was investigated. Firstly a blank run was made: the headspace of a water sample was analysed using a clean carboxen/PDMS SPME fibre and the usual adsorption and desorption conditions. Subsequently a beer sample was analysed. A blank run was then again carried out. The chromatograms of the three runs, shown in Figures 37, 38 & 39 were compared.

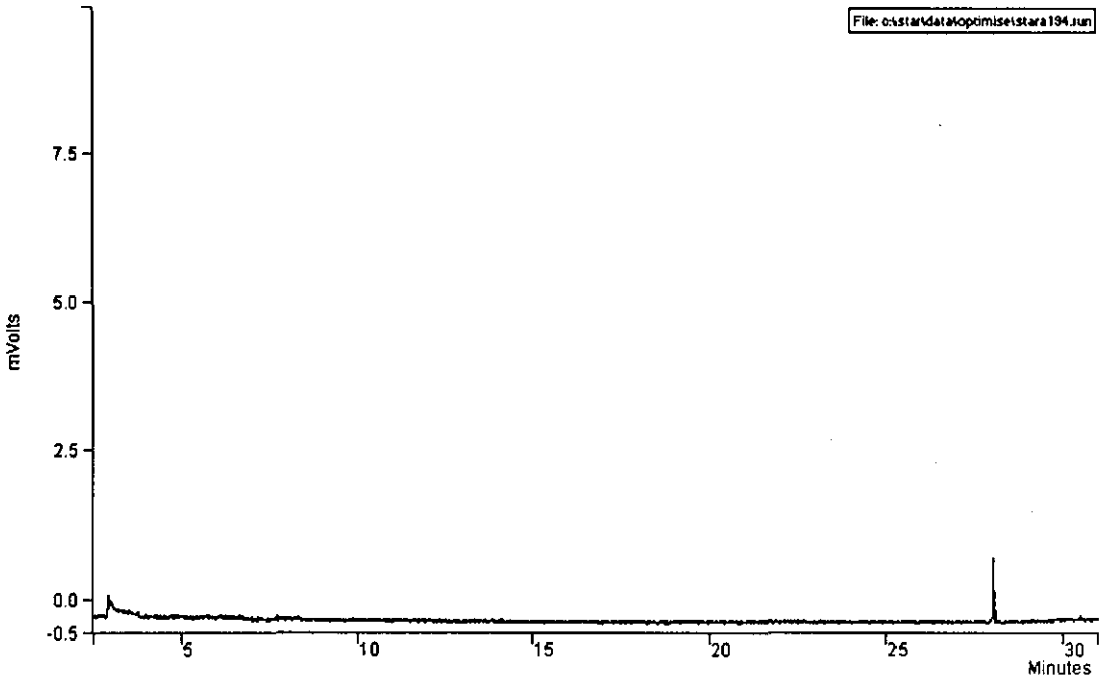


Fig. 37: Blank run before analysis of a beer sample with SPME-GC-PFPD. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

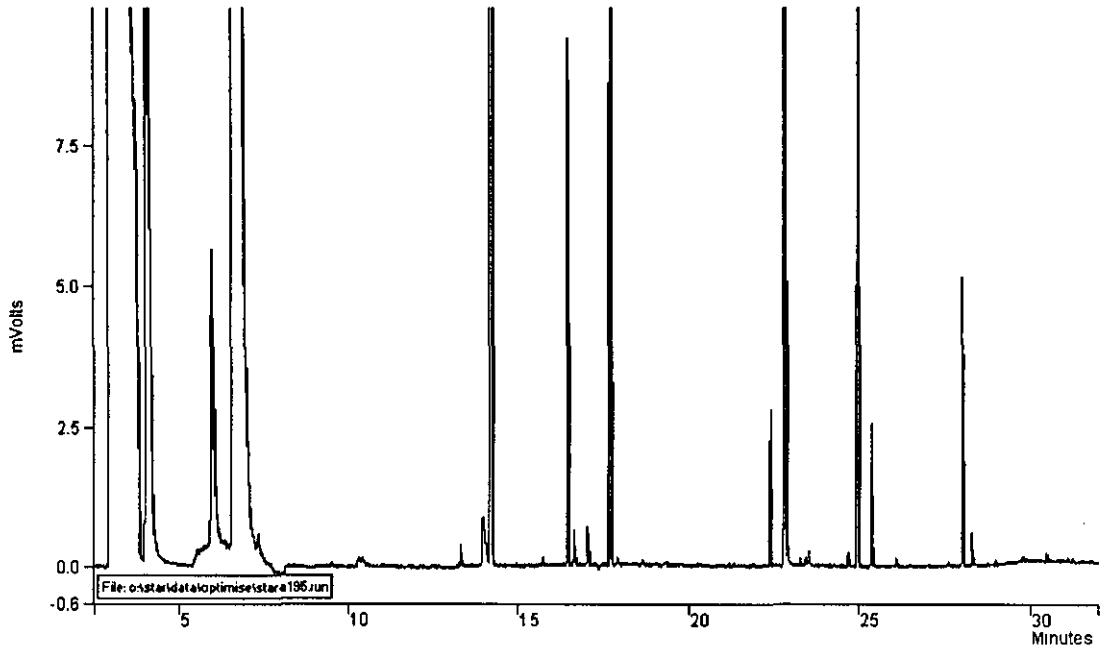


Fig. 38: Beer sample with SPME-GC-PFPD. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

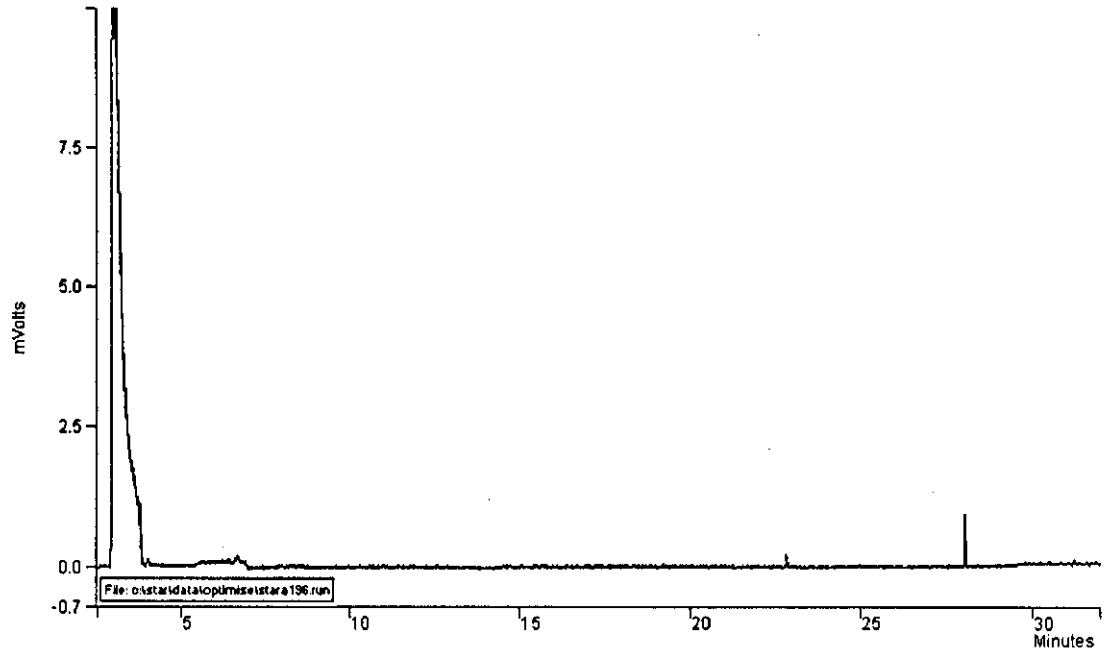


Fig. 39: Blank run after analysis of a beer sample with SPME-GC-PFPD. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The three chromatograms in Figures 37, 38 & 39 show that carryover is not a problem, with one exception: a large amount of the compound which elutes at 2.5 mins appears to be carried over from run to run. A very small amount of the compound with a retention time of 22.8 mins is also carried over: this, however, will not make a significant difference to the accuracy of the system. The peak at approximately 28 minutes was present in the blank run with the clean fibre, so cannot be considered to be carryover.

A desorption time of 3 minutes was considered sufficient to thermally clean the fibre of all compounds.

3.1.2.1.1.9 Comparison Between Autosampling and Manual Sampling

For all of the above investigations a Varian 8200/SPME autosampler was used. To study the effect of the autosampler itself, a beer sample was extracted using a manual sampling apparatus. During manual sampling the sample was warmed to 45°C and stirred with a magnetic stirrer.

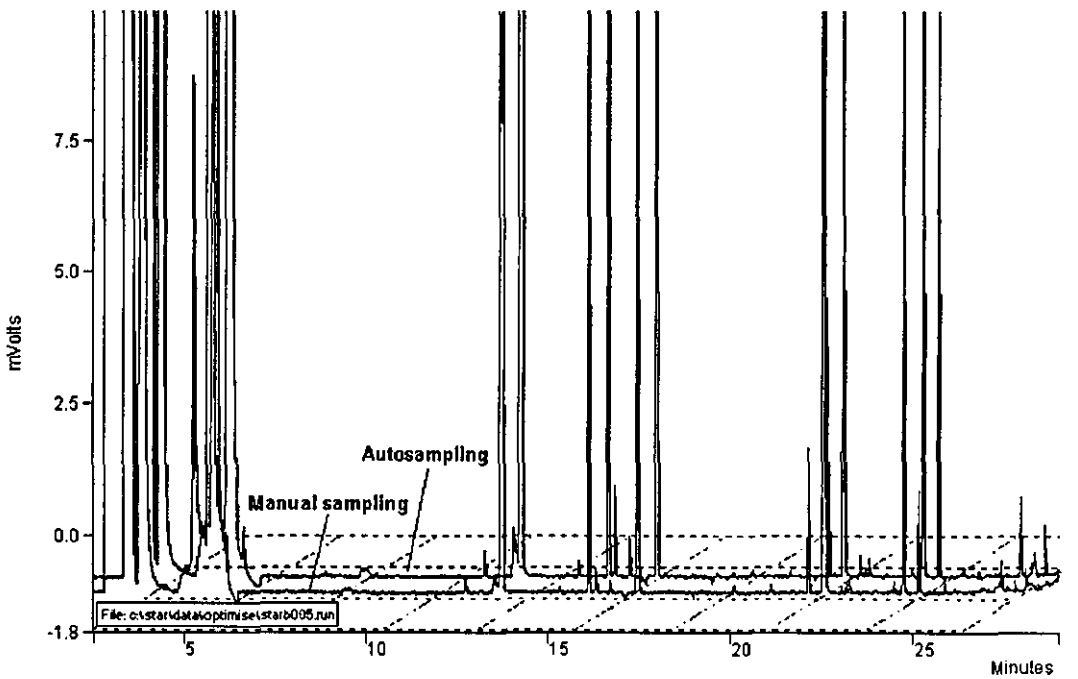


Fig. 40: Comparison of beer samples extracted by SPME manual sampling and autosampling and analysed with GC-PFPD. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The differences between manual sampling and autosampling are small. Autosampling appears to give slightly better sensitivity for less volatile compounds as can be seen from Figure 40.

A conceivable problem with the autosampler used, the Varian 8200CX with SPME III, is that the sample carousel is heated as a complete unit, i.e. it is not possible to temperate individual samples before analysis, with the result that a sample could be subjected to a temperature of 45°C for a period of up to ten hours before being analysed. This could promote thermal reactions in the beer, such as the Maillard reaction and the Strecker degradation, which may have an influence on the concentrations of the sulphur compounds determined. Additionally, the longer equilibration time could result in greater concentrations of semi-volatile sulphur compounds being extracted in relation to the internal standards.

The effects of this problem on reproducibility were experimentally determined and are discussed in detail in section 3.2.3.

3.1.2.1.1.10 Matrix Effects

As outlined in section 1.3.1.6, one possible disadvantage of SPME is that of matrix effects. Of particular importance for the SPME analysis of alcoholic beverages is the influence of ethanol on the extraction step. In several recent studies on the application of SPME for the analysis of wines, a negative effect of the ethanol content on the amount of analyte extracted has been reported (136,137,146,147,152).

The effect of ethanol was investigated by adding 3 standards - ethyl methyl sulphide (EMS), propyl methyl thioacetate (PrSAc) and hexanethiol (HexSH) - to the following matrices: water; water + 5% ethanol (EtOH); alcohol-free beer (AfB); AfB + 5% EtOH; beer (5% EtOH). AfB is normal pilsener beer which has been subjected to vacuum distillation to remove the ethanol. During this process other volatile compounds are also removed from the beer. The non-volatile components of the matrix remain unchanged. Such a 'deodorised' matrix has already been used for the calibration of a headspace SPME system: in 1998 Jia *et al.* used a combination of vacuum rotary evaporation and solvent extraction to produce a 'deodorised' orange juice for calibration purposes (139).

The final concentrations of the standards in the solution sampled were 5 ppb for EMS and 2.5 ppb for both PrSAc and HexSH. EMS eluted at 9.5 minutes, PrSAc at 19.0 minutes and HexSH at 20.2 minutes. The SPME-GC-PFPD chromatograms of the 3 standards in 5 different matrices are shown in Figures 41 to 45.

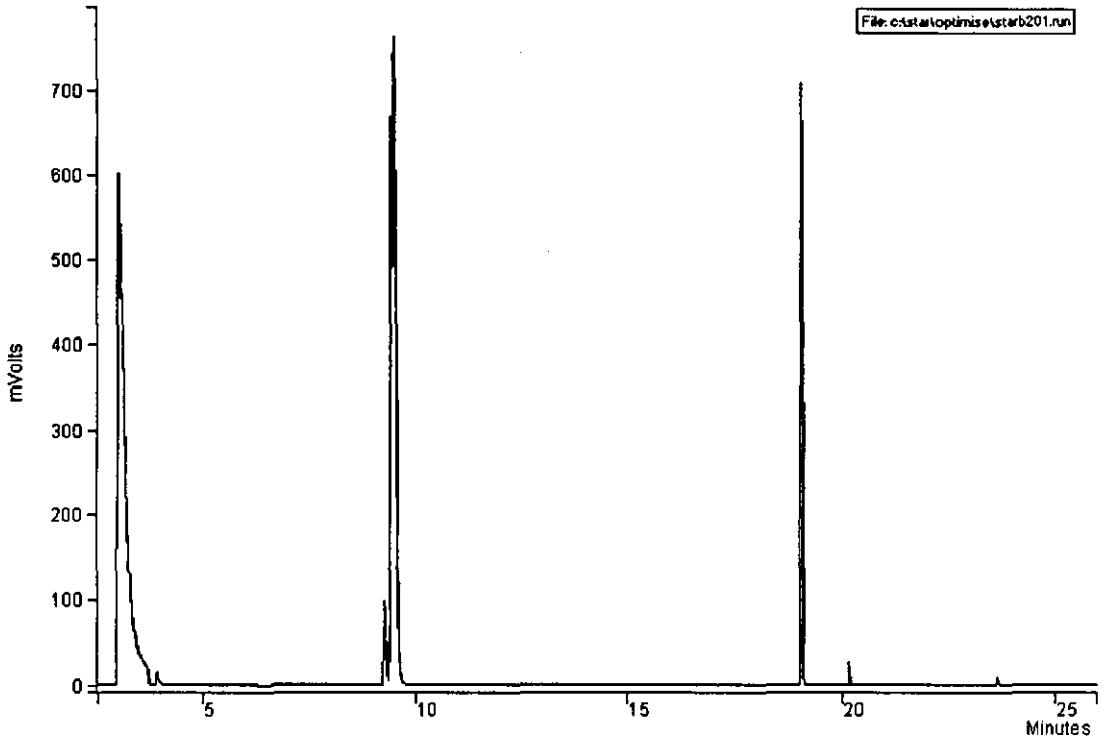


Fig. 41: SPME-GC-PFPD chromatogram of EMS (RT: 9.5 mins), PrSAC (RT: 19.1 mins) and HexSH (RT: 20.2 mins) in water. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

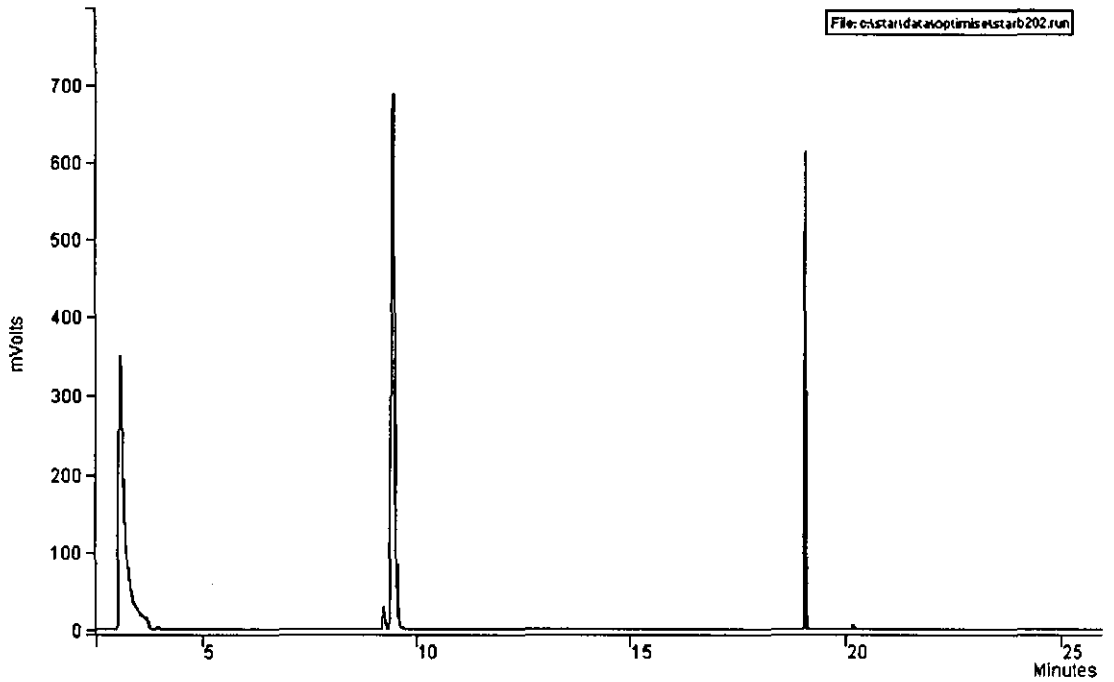


Fig. 42: SPME-GC-PFPD chromatogram of EMS (RT: 9.5 mins), PrSAC (RT: 19.1 mins) and HexSH (RT: 20.2 mins) in water + 5% ethanol. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

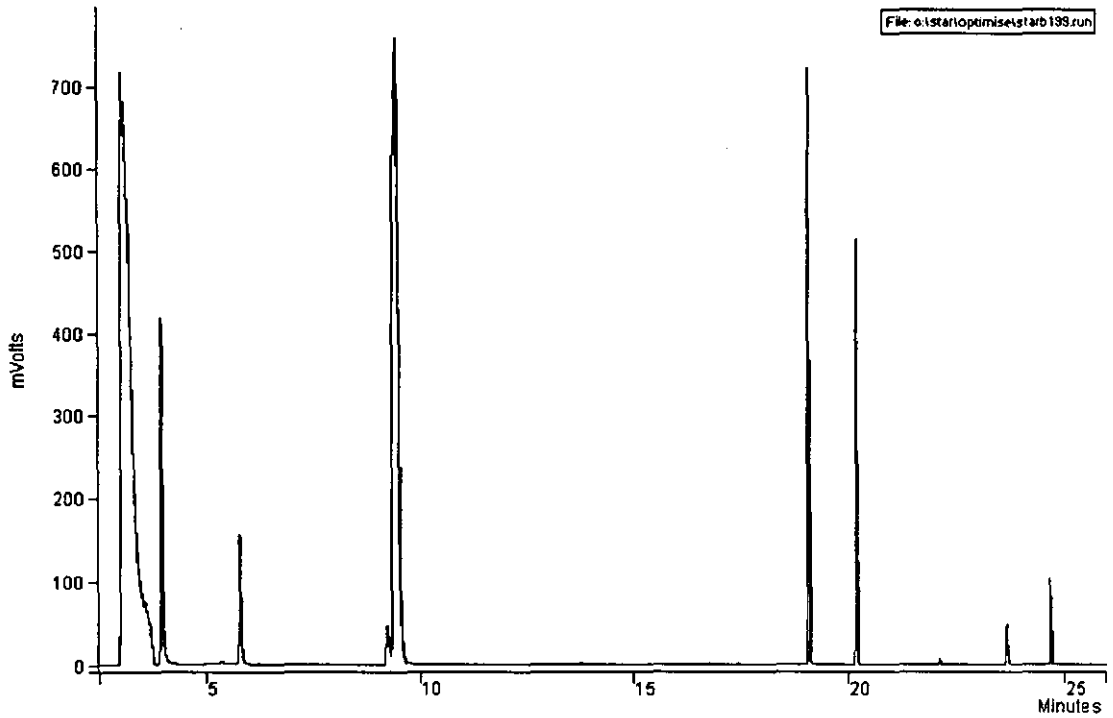


Fig. 43: SPME-GC-PFPD chromatogram of EMS (RT: 9.5 mins), PrSAC (RT: 19.1 mins) and HexSH (RT: 20.2 mins) in alcohol-free beer. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

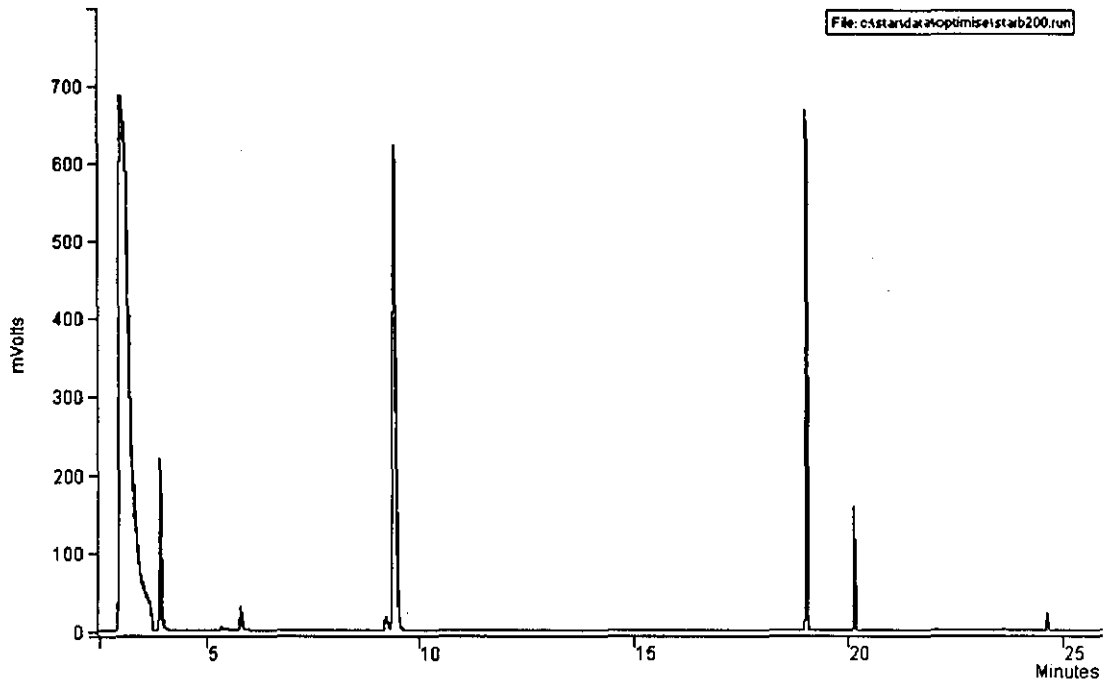


Fig. 44: SPME-GC-PFPD chromatogram of EMS (RT: 9.5 mins), PrSAC (RT: 19.1 mins) and HexSH (RT: 20.2 mins) in alcohol-free beer + 5% EtOH. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

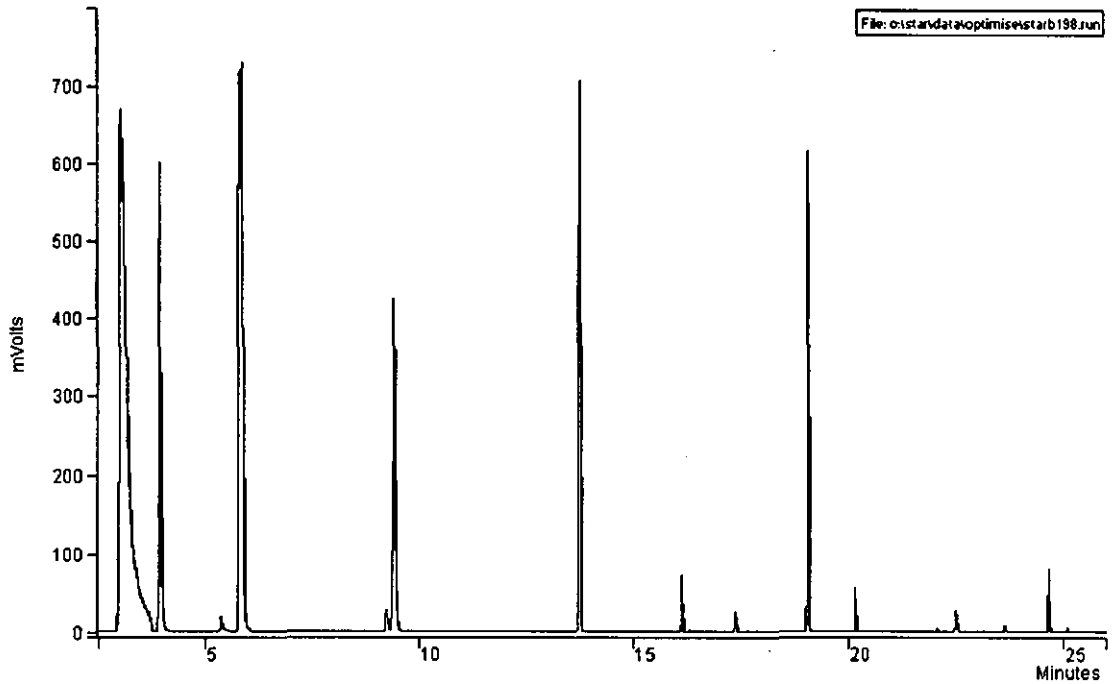


Fig. 45: SPME-GC-PFPD chromatogram of EMS (RT: 9.5 mins), PrSAC (RT: 19.1 mins) and HexSH (RT: 20.2 mins) in beer (approx. 5% ethanol content). SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The peak areas and their relative standard deviations are listed in Table 3.

Matrix	Standard		
	EMS	PrSAc	HexSH
Water	5758400	1867863	40303
Water with 5% EtOH	3649140	1028349	9919
AfB	5094138	1222000	761966
AfB + 5% EtOH	2959221	1340675	237307
Beer (5% EtOH)	1572160	1217428	83434
Peak Area RSD (%)	39.32	21.3	123.08
Sulphur Concentration RSD (%)	18.03	10.14	49.36

Table 3: peak areas obtained with SPME-GC-PFPD of 3 standards in various matrices.

From Table 3 it can be seen that the ethanol concentration has a great effect on the amount of the standards extracted by SPME. When the differences in peak area between alcohol-free beer with 5% ethanol added and normal beer, which also contains 5% ethanol, are looked at, it appears that other matrix effects apart from the ethanol effect play an important role in 'retaining' sulphur compounds in the matrix.

Different compounds appear to be influenced to different extents by matrix effects: the extraction of propyl thioacetate by SPME is influenced by the alcohol content to a much lesser extent than ethyl methyl sulphide. Extraction of hexanethiol is affected the most by differing ethanol concentrations.

3.1.2.1.2 Discussion

The results of the various test and investigations clearly show that solid phase microextraction is an elegant, simple and effective method of sample preparation for the analysis of volatile and semi-volatile sulphur compounds in beer.

The best fibre for this purpose was shown to be the 75µm Carboxen/PDMS fibre.

The ideal adsorption time was experimentally determined to be 32 minutes. Agitation of the sample during adsorption and heating of the sample carousel to 45°C also gave increased sensitivity.

Carryover was shown not to be a problem for any of the sulphur-containing compounds with the exception of the very volatile compound eluting at 2.6 minutes.

The use of an autosampler instead of manual sampling gave a slight improvement in sensitivity for semi-volatile compounds. Reproducibility of manual sampling and autosampling was not compared but other authors (120,148) have reported much better reproducibility with autosampling. Autosampling naturally has the advantage that samples can be analysed overnight and at weekends.

The study of matrix effects showed that this could be a problem when using SPME for the analysis of complex matrices, especially when an organic solvent (ethanol in the case of alcoholic beverages) is present in high concentrations. When beers with similar levels of alcohol are analysed the matrix effects can be considered to be negligible. However, when beers with greatly differing ethanol concentrations are analysed, the possibility of matrix effects causing distortion of the results cannot be ignored. A possible solution would be to add ethanol to the samples to achieve identical ethanol

concentrations: this would, however, have a negative effect on the sensitivity of the system.

The lifetime of the fibres was not directly investigated: the main reason for a fibre having to be replaced was mechanical bending of the fibre by the autosampler. A possible reason for this is that the release of carbon dioxide from the beer into the headspace causes the thin vial septa - required for SPME to prevent damage to the needle - to swell and distort. It is probable that the needle contacts the 'bulge' of the septum and is deflected, resulting it being bent and the fibre being broken. Supelco claims that fibres should have a lifetime of at least 100 injections: fibres used in this study have been used for over 250 injections before being destroyed by the autosampler.

3.1.2.2 Steam Distillation

Steam distillation has previously been used in the brewing industry for the preparation of samples for aroma analysis (101,102,161). Although SPME looked very promising, a beer sample was steam distilled and analysed with the GC-PFPD to assess the viability of steam distillation as an alternative method of sample preparation to SPME.

3.1.2.2.1 Results

A steam distillate of beer was prepared as described in section 2.2.6 and subsequently extracted with dichloromethane. 0.5µl of the organic phase was injected into the GC-PFPD system. The resulting chromatogram can be seen in Figure 46.

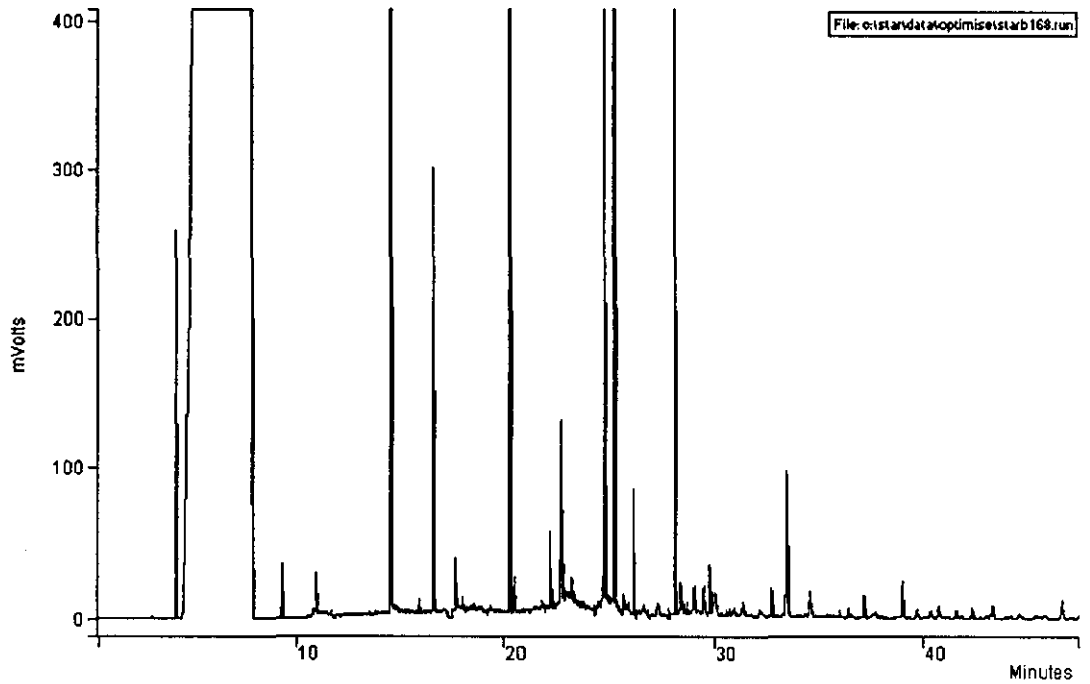


Fig. 46: GC-PFPD chromatogram of a beer sample prepared by steam distillation followed by solvent extraction. Chromatographic conditions: column = Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm); constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 20 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min. Liquid injection.

3.1.2.2.2 Discussion

The chromatogram in Figure 46 shows that steam distillation is an effective method of concentrating sulphur compounds for injection into the GC-PFPD system, especially for semi-volatile compounds. Steam distillation followed by solvent extraction suffers from the problem that the sampling system is not a closed system and so loss of volatile sulphur compounds is to be expected. Additionally, this form of sample preparation is energy- and labour-intensive and can lead to the formation of artefacts due to thermal influences. Therefore, despite its effectiveness in extracting volatile and semi-volatile compounds from the beer matrix, steam distillation followed by solvent extraction cannot be regarded as a serious alternative to SPME.

3.1.3 Chromatographic Column Selection

The demands on a chromatographic column for the SPME-PFPD system are great. The column must be capable of separating highly volatile sulphur compounds. At the same time it must not have too high a retention capacity, as this would prevent semi-volatile sulphur compounds from eluting within a reasonable analysis time and would require intensive heating of the column. For example, thick film columns give good separation of volatile compounds. The thickness of the film, however, means that less volatile compounds are retained much longer. The only way to force elution of the less volatile compounds is to intensively heat the column to a higher temperature in the oven. This, however, leads to increased column bleeding, a phenomenon to which thick film columns are particularly susceptible, and therefore also to decreased column stability and lifetime.

Several different columns were assessed for their ability to give good separation over the large range of boiling points and polarities. A prerequisite for all columns was the optimal setting of the carrier gas flow, as only gas flows very near the lowest point of the Van Deemter curve give the best possible theoretical efficiency.

3.1.3.1 Results

After the installation of the following columns the oven program was optimised for each of the individual columns. The chromatograms were then studied to see which column was the most appropriate for the analysis of volatile and semi-volatile sulphur compounds in beer. In each chromatogram the peak suspected to correspond dimethyl sulphide (DMS) is labelled to allow comparison of the performances of the various columns.

3.1.3.1.1 VA-1, 60m x 0.25mm x 1µm

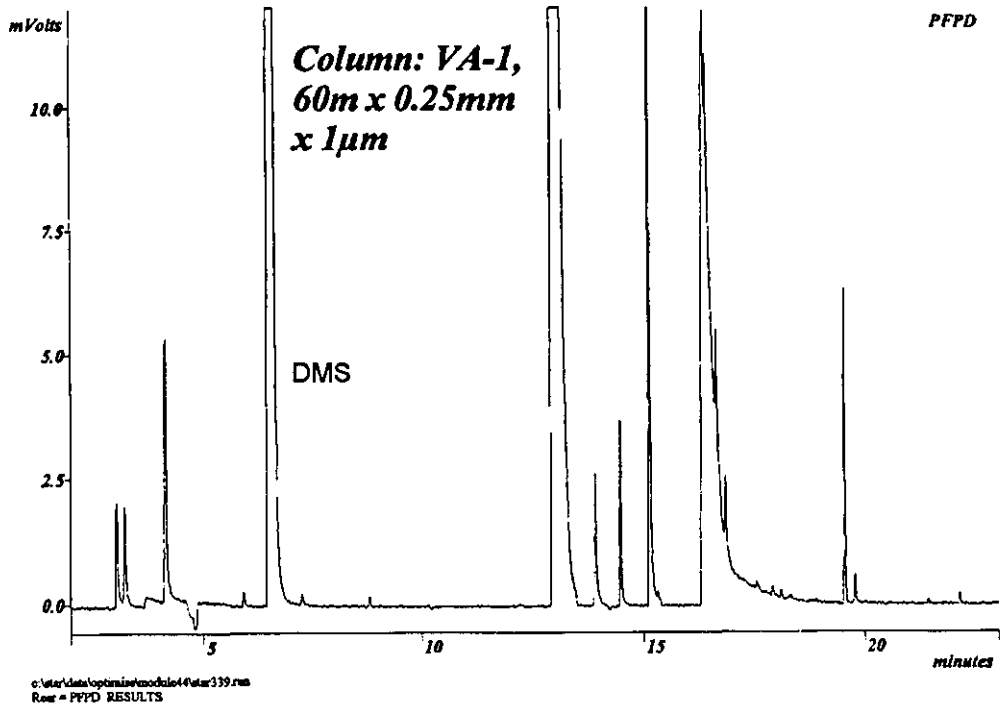


Fig. 47: Beer sample analysed with SPME-GC-PFPD. Column: VA-1, 60m x 0.25mm x 1µm. Chromatographic conditions: injector temperature = 260°C; constant column flow = 2.7 ml/min; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The VA-1, with a 100% dimethylpolysiloxane (DMPS) film, appeared to be a good choice as the Supelco SPB-1, also 100% DMPS, had given good results on the rare occasions that the Sievers SCD had worked. The SPB-1 used, however, had a 5µm film, and first tests with the SPME-PFPD system showed that less volatile molecules were retained much too highly on the column. Therefore, a 60m x 0.25mm VA-1 with a 1µm film was examined, to reduce the retention capacity.

As can be seen from the chromatogram in Figure 47, the 1µm VA-1 appeared to give good resolution of the highly volatile sulphur compounds whilst allowing all the heavier compounds to be eluted in a reasonable run time of 20 minutes. The only problem was the unresolved group of peaks at approx. 17 minutes. Changes to the gas flow rates and temperature program did not improve the resolution of this group, one inference from this being that the compounds concerned were simply too polar to be separated on a very non-polar column.

3.1.3.1.2 DB-Wax, 30m x 0.25mm x 0.5µm

In an attempt to separate the polar group which could not be separated on the non-polar VA-1, a polar wax (polyethylene glycol) column was used.

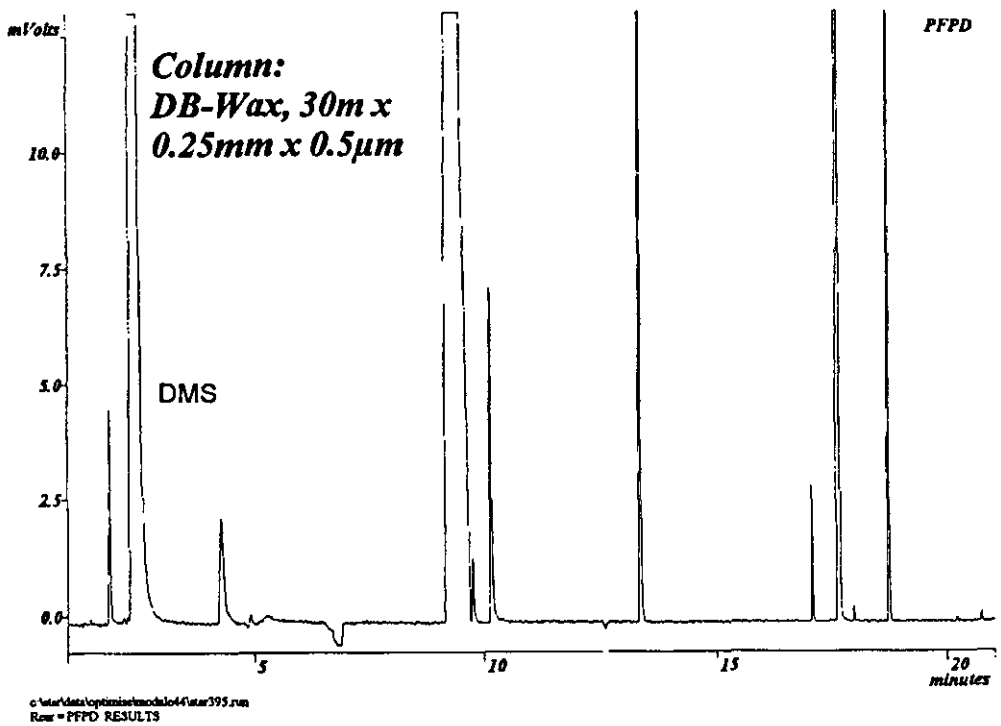


Fig. 48: Beer sample analysed with SPME-GC-PFPD. Column: DB-Wax, 30m x 0.25mm x 0.5µm. Chromatographic conditions: injector temperature = 260°C; constant column flow = 1.7 ml/min; column oven = 5 mins at 30°C, increased to 110°C at 9°C/min, increased to 220°C at 15°C/min and held for 6 mins. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The separation of the polar group of compounds was achieved with the wax column: however, the highly volatile compounds were not well separated. The separation of these compounds could probably be improved by increasing the film thickness, thereby giving more retention. However, the relative instability of wax films means that thicker polar films lead to greatly increased column bleeding. Therefore increasing the thickness of the polar wax film should be regarded as a last alternative.

3.1.3.1.3 OV-1701, 50m x 0.20mm x 0.5µm

After the non-polar and polar columns failed to give the required separation, a low to mid polarity column, the OV-1701 with a methylpolysiloxane phase substituted with 14% cyanopropyl-phenyl groups, was selected. The aim behind this choice was that the column would give similar separation to the VA-1 whilst at the same time the slightly increased polarity of the phase would allow the polar group of peaks to be resolved.

A relatively thick film of 0.5µm and a column length of 50m were chosen to give more retention. Additionally a smaller column internal diameter of 0.20mm was chosen, to give more efficiency and therefore better separation.

It was therefore quite a surprise that the performance of the OV-1701 was far worse than that which could be expected, as shown by the chromatogram in Figure 49.

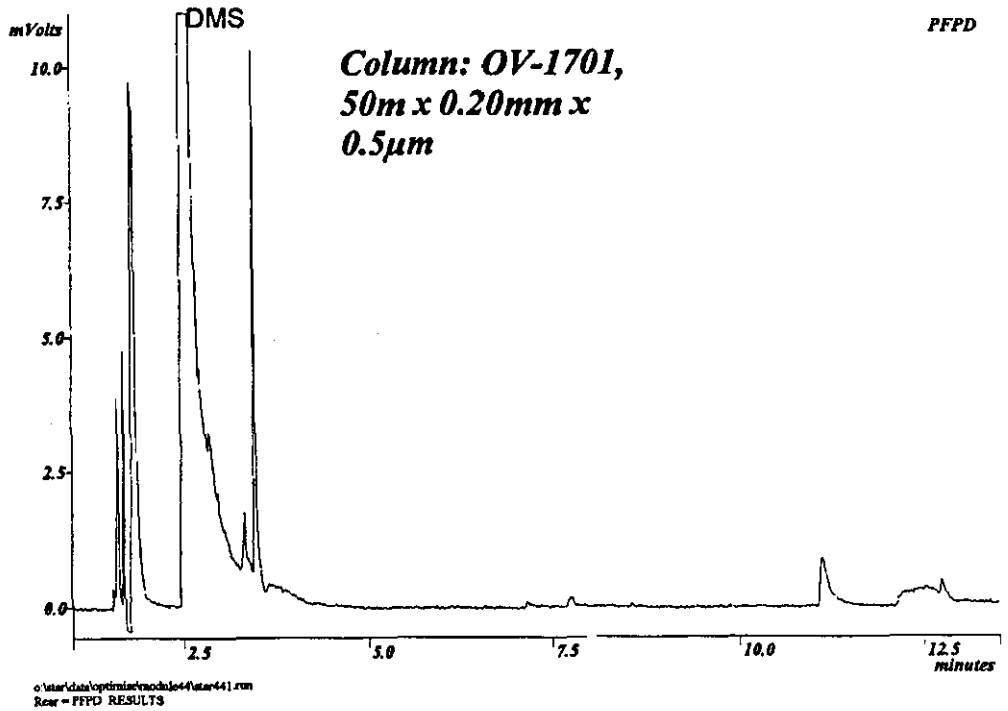


Fig. 49: Beer sample analysed with SPME-GC-PFPD. Column: OV-1701, 50m x 0.20mm x 0.5µm. Chromatographic conditions: injector temperature = 260°C; constant column flow = 1.8 ml/min; column oven = 5 mins at 32°C, increased to 220°C at 15°C/min and held for 20 mins. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

No possible explanations for the failure of the OV-1701 to live up to expectations could be found. The column was not used above its recommended maximum temperature of 260°C. The manufacturers of the column used, CS Chemie, Germany, do not list a recommended minimum temperature: however, manufacturers of similar phases (Hewlett-Packard and J&W Scientific) state a recommended minimum temperature of -20°C. Therefore this could not be the cause of the disappointing performance of the OV-1701. The OV-1701 is not suitable for the separation of volatile sulphur compounds in beer.

3.1.3.1.4 Optima Delta-3, 30m x 0.25mm x 0.25µm

The chromatographic column manufacturer Macherey & Nagel recently launched a new column, the Optima Delta-3. Described as having a cross-linked methyl/phenyl-silicone phase, this column was claimed to possess 'unique autoselective properties'. A 30m x 0.25mm x 0.25µm version of this column was obtained from Macherey & Nagel for test purposes. The resulting chromatogram can be seen in Figure 50.

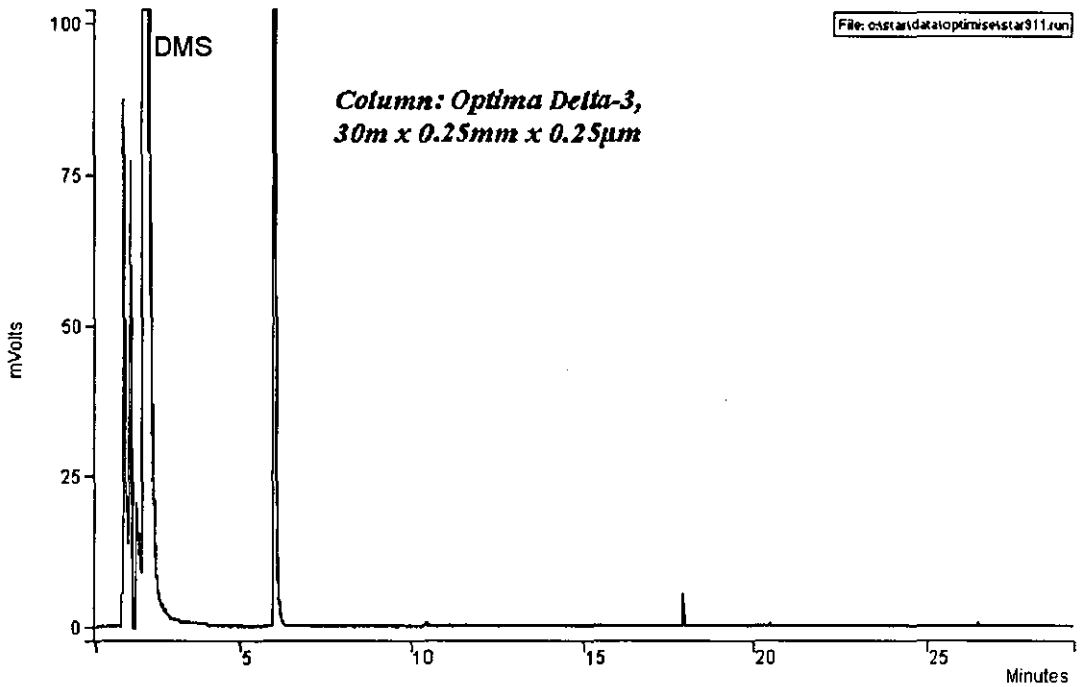


Fig. 50: Beer sample analysed with SPME-GC-PFPD. Column: Optima Delta-3, 30m x 0.25mm x 0.25µm. Chromatographic conditions: injector temperature = 240°C; constant column flow = 2.6 ml/min; column oven = 10 mins at 33°C, increased to 180°C at 10°C/min, increased to 230°C at 30°C/min and held for 3 mins. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The specifications of the Optima Delta-3 obtained for test purposes were admittedly not ideal for the separation of volatile sulphur compounds: however, the poor separation shown in Figure 50 did not give much promise that a longer column with a thicker film would be capable of providing the required separation. The Optima Delta-3 was discounted as a possible chromatographic column for the separation of volatile sulphur compounds in beer.

3.1.3.1.5 Combined DB-Wax (10m x 0.25mm x 0.5µm) and VA-1 (60m x 0.25mm x 1µm)

A further possibility was to combine the 60m non-polar VA-1 with a shorter piece of wax column, giving separation similar to that seen with the VA-1 alone whilst at the same time allowing the polar group to be resolved. The result can be seen in the chromatogram in Figure 51.

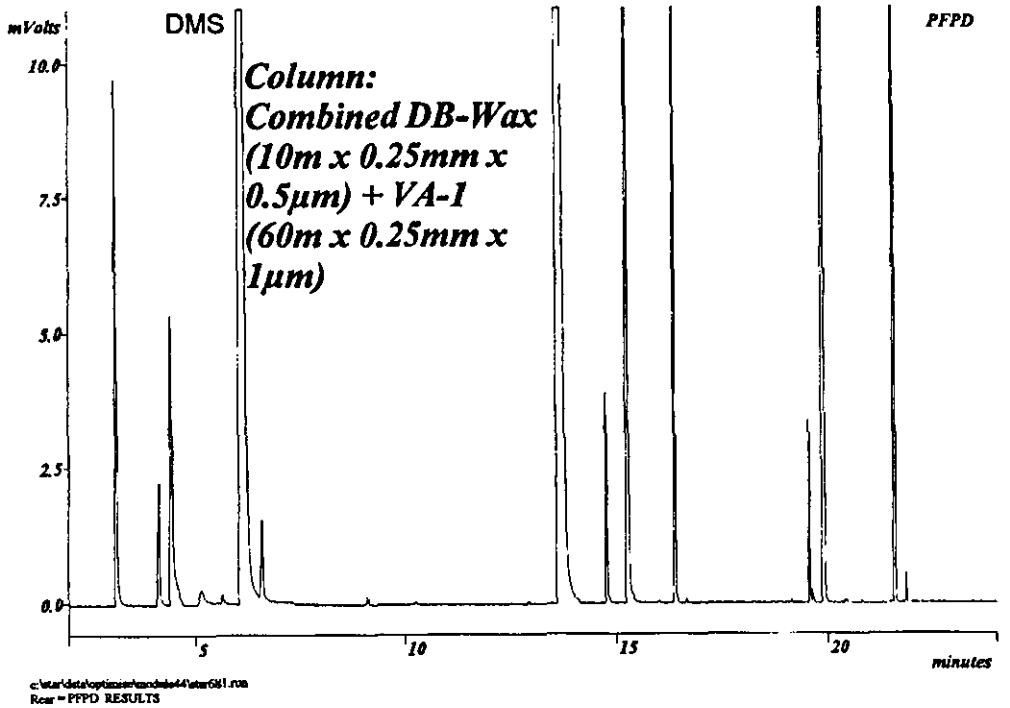


Fig. 51: Beer sample analysed with SPME-GC-PFPD. Column: combined DB-Wax (10m x 0.25mm x 0.5µm) + VA-1 (60m x 0.25mm x 1µm). Chromatographic conditions: constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

The combination of the two columns joined together with a glass press-fit fitting gave the best separation of all the columns tested. The polar group of compounds which were not separated on the VA-1 was sufficiently resolved on the combined column. Experimental tests showed that it is important that the 10-metre piece of polar wax column is placed before the VA-1. When the piece of polar column is placed after the

non-polar column, the group of apparently polar peaks, which was not resolved on the non-polar VA-1 alone, remains unresolved.

3.1.3.2 Discussion

The aim of separating very volatile sulphur compounds whilst obtaining acceptable times of analysis for heavier, semi-volatile compounds was difficult to achieve with conventional single columns.

Neither the non-polar VA-1 nor the polar DB-Wax provided the desired separation, the former failing to separate an apparently polar group of compounds, the latter not giving sufficient separation of the very volatile compounds. The application of chromatographic theory to the problem suggested the use of the OV-1701. The combination of the mid-polarity methylpolysiloxane / 14% cyanopropyl-phenyl phase and the dimensions of the column (50m x 0.20mm x 0.5µm) was considered theoretically ideal to provide the required separation. The column unfortunately inexplicably failed to live up to the expectations. The 'unique autoselective properties' of the Optima Delta-3 did not prove sufficient to provide the required separation either.

The best solution was found to be a combination of a short piece (10m) of polar wax column joined to a longer (60m) non-polar column with a 100% dimethylpolysiloxane phase. All peaks appeared to be well resolved, with the possible exception of the first peak, the form of which gave the impression that it could be a combination of two or more compounds co-eluting.

3.2 Method Verification

Once the optimal combination of sample preparation, chromatographic column and conditions, and detector had been selected, it was necessary for the method to be verified. The peaks needed to be identified, the analytical system calibrated and the linearity, precision and limits of detection determined.

3.2.1 Identification of Volatile and Semi-volatile Sulphur Compounds in Beer

The identification of the individual sulphur compounds detected was difficult, owing to their low concentrations and the relatively high concentrations of other, non-sulphur compounds present in beer.

3.2.1.1 Identification with GC-MS

Ideally GC-MS would be used to detect the sulphur compounds and identify them. However, that was not possible with the GC-MS systems available, hence the need for a sensitive method based on a specific detector.

The first GC-MS tested was a HP5890 Series II connected to a HP 5972 Mass Selective Detector, a set-up which is used in the Beck & Co GC laboratory for the routine analysis of aroma compounds in beer and beer wort (see section 2.2.6).

3.2.1.1.1 Steam Distillation followed by GC-MS

A sample prepared by the routine method of steam distillation followed by solvent extraction with dichloromethane (for method see section 2.2.6) was injected into the system in both SCAN and SIM modes. With both modes the only sulphur compound detected was dimethyl sulphide, present in beer at a concentration of approximately 60 ppb. The MS was simply too insensitive to detect any other sulphur compounds.

3.2.1.1.2 Covalent Chromatography followed by GC-MS

A further attempt to identify the sulphur-containing compounds in beer using the above-described GC-MS system was undertaken. Instead of steam distillation, a variation on a method of sample preparation published by Full and Schreier in 1994 was used (216). The method is described by the authors as being selective for thiols and is based on covalent chromatography. The experimental details are described in section 2.2.7.

1µl of the eluent was injected into the GC-MS system described in section 2.2.6. The scan chromatogram can be seen in Figure 52.

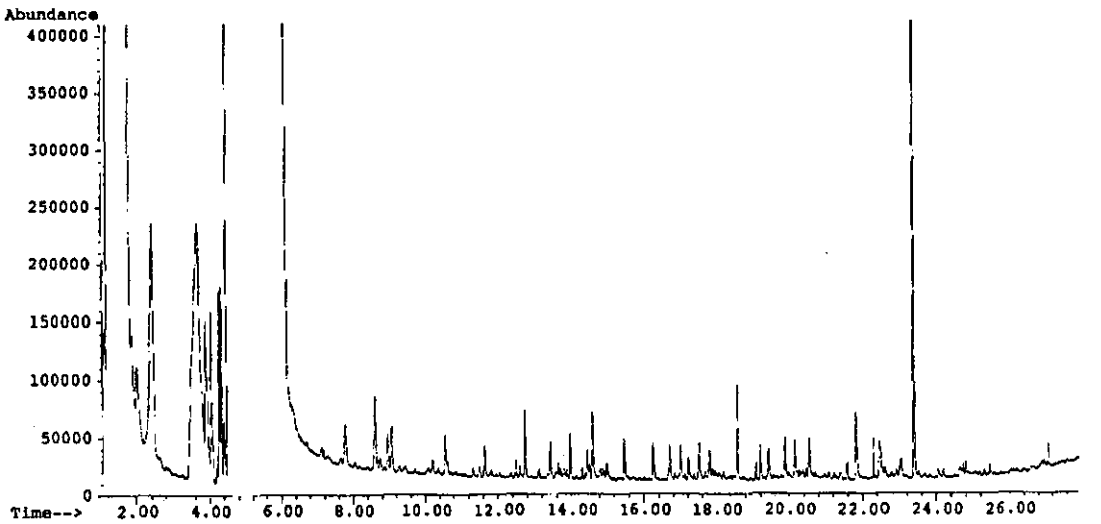


Fig. 52: GC/MS scan chromatogram of pentane/dichloromethane eluent from Affi-Gel column. Column: DB-Wax. Experimental details are listed in section 2.2.6.

Most of the peaks in the chromatogram in Figure 52 were identified as aliphatic and aromatic hydrocarbons: none of them could be identified as either thiols or other sulphur compounds. The sample was again analysed, this time in SIM mode. The ions monitored (see section 2.2.7) were chosen because they are the main ions in the mass spectra of sulphur compounds which are listed in the literature as being possibly present in beer (53). The SIM chromatogram can be seen in Figure 53.

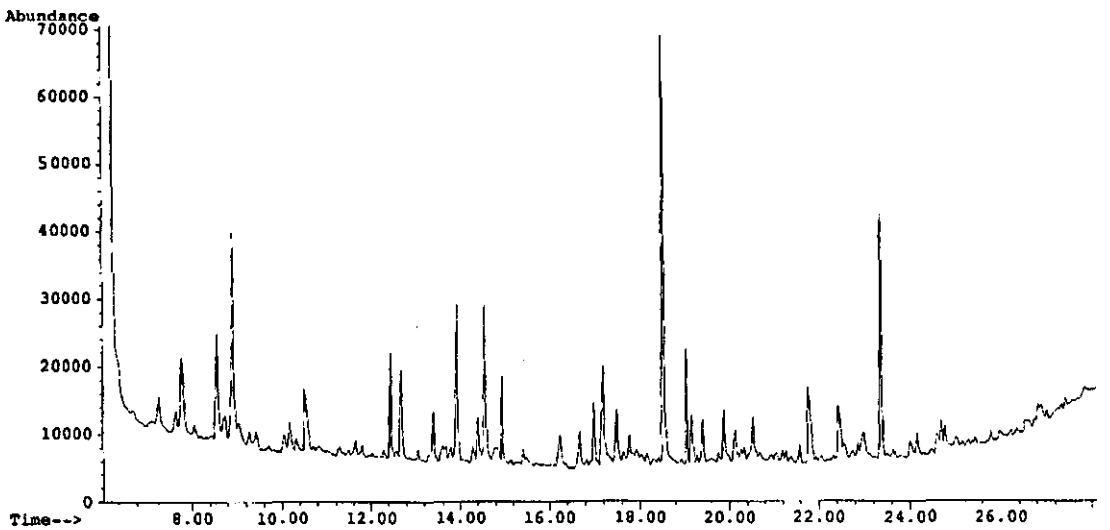


Fig. 53: GC/MS SIM chromatogram of pentane/dichloromethane eluent from Affi-Gel column. Column: DB-Wax. Experimental details are listed in section 2.2.6.

The SIM chromatogram failed to give any evidence of thiols or other sulphur-containing compounds in the pentane/dichloromethane Affi-Gel column eluent.

The attempt at identification was repeated using dithiothreitol from a different manufacturer but the result was unfortunately the same.

3.2.1.1.3 Dynamic Headspace Extraction (DHSE) followed by GC-MS

A further method for the identification of the volatile and semi-volatile sulphur compounds in beer was developed together with Mplus, a Bremen-based company specialising in instrumental analysis, especially GC-MS.

The method was based on work carried out by Goldstein *et al.* (98), which itself was based on a method for the determination of methyl

mercaptan in air (240). The GC-MS analysis was carried out using a Finnigan ATS 40 MS at the University of Bremen.

Three variations of the sample preparation step were used. No sulphur compounds were detected with the GC-MS following the first (2.2.8.1) or second (2.2.8.2) variations. The third variation (2.2.8.3) was slightly more successful, dimethyl sulphide being positively identified in the dichloromethane extract. However, no other sulphur compounds were identified.

The attempts to identify sulphur-containing compounds in beer with DHSE were not pursued further.

3.2.1.1.4 SPME-GC-MS

The final method in the attempt to identify sulphur compounds in beer with mass spectroscopy employed SPME for sample preparation. Preliminary tests with the Beck & Co system consisting of a HP5890 Series II connected to a HP 5972 Mass Selective Detector, however, allowed no sulphur compounds to be identified owing to the insensitivity of the detector. Varian Chromatography Systems, USA, kindly offered to attempt the identification using SPME sample preparation and simultaneous PFPD/MS detection.

The analyses were carried out by Varian in Walnut Creek, California. A PFPD identical to the one at Beck & Co in Bremen and a Varian Saturn mass spectrometer in both EI and CI modes were used. SPME was carried out manually as an SPME-autosampler was not available.

The simultaneous PFPD/MS detection allowed the following peaks to be positively identified: hydrogen sulphide, methyl mercaptan, carbon disulphide and methyl thioacetate. No further compounds could be identified owing to insufficient sensitivity.

3.2.1.2 Identification using Retention Times

The only remaining possible method of identification was by retention time. Identification by retention time does not, however, allow compounds to be positively identified. A further problem with this method of identification is that sulphur compounds are very reactive and it is subsequently difficult to obtain pure reference standards. Additionally, the discriminatory nature of the SPME extraction step means that it is possible to obtain two or more peaks of a similar size from a supposedly 'pure' reference standard.

The compounds listed in Table 4 below were injected and their retention times compared to the retention times of the unknown peaks in the SPME-GC-PFPD chromatogram using the 10m x 0.25 x 0.5µm DB-wax / 60m x 0.25 x 1µm VA-1 column.

Table 4: Reference standards analysed with SPME-GC-PFPD system to identify unknown compounds using retention times. Conditions as in section 2.3.

Compound	Abbreviation	Retention Time on DB-Wax / VA-1 column (mins)	Identified in beer using SPME-GC-PFPD?
2-Acetyl thiophene	2-Acthph	25.78 [1]	No
1-Butanethiol	1-BuSH	- [1]	No
Carbon disulphide	CS_2	6.25	Yes
Cyclopentylmercaptan		18.16	No
Diethyl disulphide	DEDS	20.44	Yes
Diethyl sulphide (Ethyl sulphide)	DES	13.05	No

Compound	Abbreviation	Retention Time on DB-Wax / VA-1 column (mins)	Identified in beer using SPME-GC-PFPD?
Dimethyl disulphide	DMDS	15.37	Yes
Dimethyl sulphide	DMS	5.78	Yes
Dimethyl trisulphide	DMTriS	22.12 [2]	Trace
Dimethyl tetrasulphide	DMTetraS	22.12 [2]	Trace
Ethanethiol (Ethyl mercaptan)	EtSH	5.35	Yes
Ethylene sulphide (Thiirane)	Thiirane	9.28 [3]	Trace
Ethyl-3-(methylthio)propionate		24.52	No
Ethyl thioacetate (Thioacetic acid S-ethyl ester)	EtSAc	16.16	Yes
Methanethiol (Methyl mercaptan)	MeSH	3.92	Yes
Methional (3-methylthiopropanal, 3-(methylthio)propionaldehyde)		21.65	Trace
Methionol (3-methylthiopropanol)		24.66	Yes
2-Methyl-1-butanethiol	2-MeBuSH	16.35	Yes
3-Methyl-1-butanethiol	3-MeBuSH	- [1]	No
3-Methyl-2-butene-1-thiol (light mercaptan)	3-MBT	17.89	Yes [4]
2-Methyl-3-furanthiol	MeFuSH	- [1]	No

Compound	Abbreviation	Retention Time on DB-Wax / VA-1 column (mins)	Identified in beer using SPME-GC-PFPD?
Methyl 3-(methylthio) propionate		23.45	No
1-Methyl-1-propanethiol	1-MePrSH	12.38	No
2-Methyl-1-propanethiol	2-MePrSH	8.9	No
Methyl thioacetate (Thioacetic acid S-methyl ester)	MeSAc	13.79	Yes
2-Methyl thiophene	2-Methph	16.37 [5]	No
3-Methyl thiophene	3-Methph	16.75	Yes
3-(Methylthio) propionic acid	Me-3-MeSH-prop	30.4	No
3-(Methylthio) propyl acetate	3-MeSPrAc	25.32	Yes
1-Pentanethiol	1-PeSH	- [1]	No
1-Propanethiol	1-PrSH	9.28	No
2-Propanethiol	2-PrSH	7.35	No

[1] = reference standard too impure to allow identification

[2] = DMTriS and DMTetraS appear to co-elute

[3] = possible co-elution with EMS

[4] = present in beer subjected to light

[5] = possible co-elution with 2-MeBuSH

To understand why DMTriS and DMTetraS co-eluted, the two reference standards were examined using the GC-MS system described in section 2.2.6. The resulting mass spectra showed that both reference standards were in fact primarily DMTriS. This observation was backed up by the identical retention times obtained with the GC-MS for both standards.

3-Methylthiophene has not been previously reported as being present in beer. Suggett, Moir and Seaton first reported its presence in hops in 1979 (27,28,50). Additionally, there are no reports in the literature of 2-methyl-1-butanethiol being found in beer. Both of these compounds could be identified in beer for the first time because of the excellent sensitivity of the SPME-GC-PFPD system.

3.2.2 Calibration of the SPME-GC-PFPD system

Once as many as possible of the peaks obtained with the SPME-GC-PFPD system had been identified, the system was calibrated using reference standards. When the equimolar behaviour of the PFPD is considered, it would theoretically be possible to calibrate the system with just one sulphur compound and apply the obtained response factor to the other sulphur compounds. This approach, however, does not take into account the differences in extraction of the compounds from the matrix by SPME. Therefore, reference standards were used for each identified peak to achieve maximum accuracy.

3.2.2.1 Choice of Internal Standards

Despite the equimolar sulphur response of the PFPD, internal standards are required owing to the discriminatory nature of SPME. Three different internal standards were considered to cover the range of the functional groups and molecular masses of the sulphur compounds present in beer: ethyl methyl sulphide (EMS); 1-propyl thioacetate (PrSAc);

1-hexanethiol (HexSH). To investigate the dependency of the extraction step on the molecular masses, functionality of the sulphur compounds, and hence the need for different internal standards, a beer sample was repeatedly analysed by the SPME-GC-PFPD system described in section 2.3. This was carried out with 3 different beer samples: six replicates of one sample and five replicates of the other two samples were analysed. The peak areas of the three internal standards and of the following compounds were evaluated: methyl mercaptan (MeSH); ethyl mercaptan (EtSH); dimethyl sulphide (DMS); methyl thioacetate (MeSAc); ethyl thioacetate (EtSAc); 3-methylthiophene (3-Methph); methionol; methyl propyl thioacetate (MeSPrAc). An unidentified peak with a retention time of 22.5 minutes (RT22.5) was also evaluated.

The sulphur relative standard deviations (RSD) were calculated for the three internal standards and each of the above-mentioned analytes. In addition, the sulphur RSDs of the analyte peak areas divided by the peak areas of each of the three internal standards were calculated. These results are summarised in Tables 5 and 6.

Table 5: Sulphur RSDs (%) for the three internal standards using three different beer samples

	Internal Standard		
	EMS	**HexSH**	**PrSAC**
Sample 1	8.42	15.05	0.76
Sample 2	7.74	14.0	1.30
Sample 3	13.6	13.4	2.24
Mean	**9.92**	**14.2**	**1.43**

Table 6: Sulphur RSDs (%) for analytes

Sulphur Compound		No ISTD	ISTD: EMS	ISTD: HexSH	ISTD: PrSAc
MeSH	Sample 1	6.56	13.0	14.0	6.95
	Sample 2	4.45	8.00	12.3	5.26
	Sample 3	3.92	8.30	16.7	2.10
	Mean	**4.98**	**9.77**	**14.3**	**4.77**
EtSH	Sample 1	8.97	15.1	11.7	9.31
	Sample 2	8.72	9.99	11.9	8.87
	Sample 3	5.58	9.18	15.4	3.92
	Mean	**7.76**	**11.4**	**13.0**	**7.37**
DMS	Sample 1	5.16	5.07	20.0	5.40
	Sample 2	4.60	3.19	12.0	4.29
	Sample 3	3.09	10.7	16.2	3.77
	Mean	**4.28**	**6.31**	**16.1**	**4.49**
MeSAc	Sample 1	6.66	15.1	13.2	6.47
	Sample 2	9.34	11.3	17.1	9.77
	Sample 3	5.28	11.4	18.0	6.00
	Mean	**7.09**	**12.6**	**16.1**	**7.41**
EtSAc	Sample 1	6.82	12.2	12.8	7.30
	Sample 2	2.82	8.18	13.8	2.55
	Sample 3	5.99	12.0	18.0	6.70
	Mean	**5.21**	**10.8**	**14.9**	**5.52**
3-MeThPh	Sample 1	2.82	6.82	16.5	3.07
	Sample 2	8.71	4.49	11.7	8.28
	Sample 3	7.95	16.3	16.5	9.84
	Mean	**6.49**	**9.22**	**14.9**	**7.06**
RT22.5	Sample 1	11.9	10.7	17.7	11.8
	Sample 2	21.4	20.2	13.1	20.8
	Sample 3	21.7	7.76	34.8	20.2
	Mean	**18.3**	**12.9**	**21.9**	**17.6**

Sulphur Compound		No ISTD	ISTD: EMS	ISTD: HexSH	ISTD: PrSAc
Methionol	Sample 1	28.4	35.3	16.0	27.9
	Sample 2	18.3	19.1	15.1	18.5
	Sample 3	20.1	24.5	13.6	20.3
	Mean	**22.3**	**26.3**	**14.9**	**22.2**
MePrSAc	Sample 1	18.4	25.9	7.28	18.2
	Sample 2	8.08	12.4	18.1	8.73
	Sample 3	5.62	13.9	11.6	6.65
	Mean	**10.7**	**17.4**	**12.3**	**11.2**

The results suggest that the best internal standard for nearly all the sulphur compounds is 1-propyl thioacetate (PrSAc). For DMS and 3-Methph either PrSAc or EMS looks suitable as the internal standard. EMS appears to be the best choice for the unknown compound with a retention time of 22.5 minutes (RT22.5). For methionol the most appropriate internal standard appears to be HexSH. Methionol is the sulphur compound with the highest sulphur RSD from those examined.

These results give only a first indication of the most appropriate internal standards for the compounds which are to be calibrated. A correlation of the sulphur compounds to the internal standard amounts extracted during the calibration process will give more definite information on which internal standard is best suited for which sulphur compound.

3.2.2.2 Calibration Procedure

The SPME-GC-PFPD system was calibrated using known concentrations of reference standards. The concentrations of the compounds were selected to cover the range of concentrations found in beer. In addition, the compounds were sorted into groups to avoid problems with interference effects owing to impurities in the reference compounds. For example, if the DMS reference standard, calibrated at concentrations of 6, 30 and 60ppb, contained 1% DMDS, which is calibrated at 0.1, 0.5 and 1ppb, the error for DMDS would be very large if both substances were calibrated together. Contamination of the reference standards by other sulphur compounds were noted: using this information the following groups of compounds to be calibrated were drawn up to minimise the risk of interference effects:

Group 1	
Compound	**Concentrations (ppb)**
DMDS	0.1; 0.5; 1.0
EtSAc	0.2; 1.0; 2.0
3-Methph	0.01; 0.05; 0.1
3-MeSPrAc	4; 20; 40

Table 7: concentrations of compounds in calibration group 1

Group 2	
Compound	**Concentrations (ppb)**
MeSAc	1; 5; 10
Methionol	50; 250; 500

Table 8: concentrations of compounds in calibration group 2

Group 3	
Compound	**Concentrations (ppb)**
CS_2	0.05; 0.25; 0.5
2-MeBuSH	0.05; 0.25; 0.5
DEDS	0.05; 0.25; 0.5

Table 9: concentrations of compounds in calibration group 3

Group 4	
Compound	**Concentrations (ppb)**
MeSH	1, 5, 10
DMS	6, 30, 60

Table 10: concentrations of compounds in calibration group 4

Group 5	
Compound	**Concentrations (ppb)**
EtSH	0.25; 1.25; 2.5
DMTriS/DMTetraS	0.25; 1.25; 2.5
Me-3-MeSHprop	0.5; 2.5; 5

Table 11: concentrations of compounds in calibration group 5

Group 6	
Compound	**Concentrations (ppb)**
3-MBT	0.8; 4; 8

Table 12: concentrations of compound in calibration group 6

The standard addition method of calibration was employed, the compounds were calibrated using 3 different concentrations across a range of one order of magnitude. The reference standards were added to alcohol-free pilsener, which was used to simulate the beer matrix. Alcohol-free pilsener is produced by vacuum extraction of a normal pilsener beer, which results in an almost complete removal of volatile compounds. To compensate for matrix effects (see section 3.1.2.1.1.10), especially the effect of the ethanol concentration on the partitioning coefficients of the sulphur compounds, ethanol was added to bring the alcohol content of the alcohol-free beer up to 5% (v/v). The calibration samples were then analysed using the method detailed in section 2.3. The square root of the height of the peaks was used as the basis for the calculations to compensate for the quadratic response of the PFPD.

3.2.2.3 Results

Calibration curves were obtained for each of the compounds to be calibrated. Figures 54 & 55 show examples of the calibration curves for a single compound (DEDS). Figure 54 shows the calibration curve obtained using the heights of the peaks. Figure 55 shows the calibration curve obtained using the square root of the height of the peaks to compensate for the quadratic response of the PFPD and give a straight line.

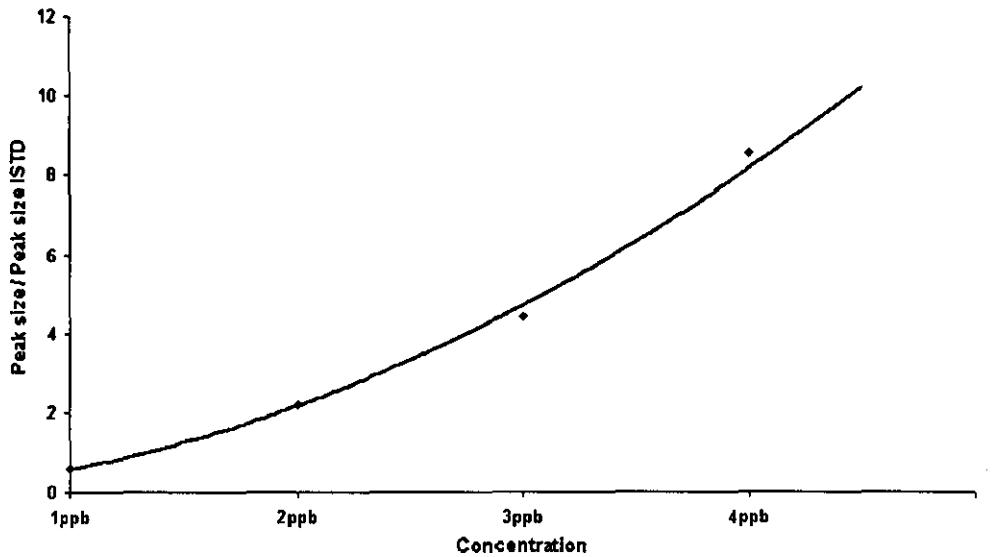


Fig. 54: Calibration curve for DEDS obtained using peak heights. Experimental conditions as listed in section 2.3.

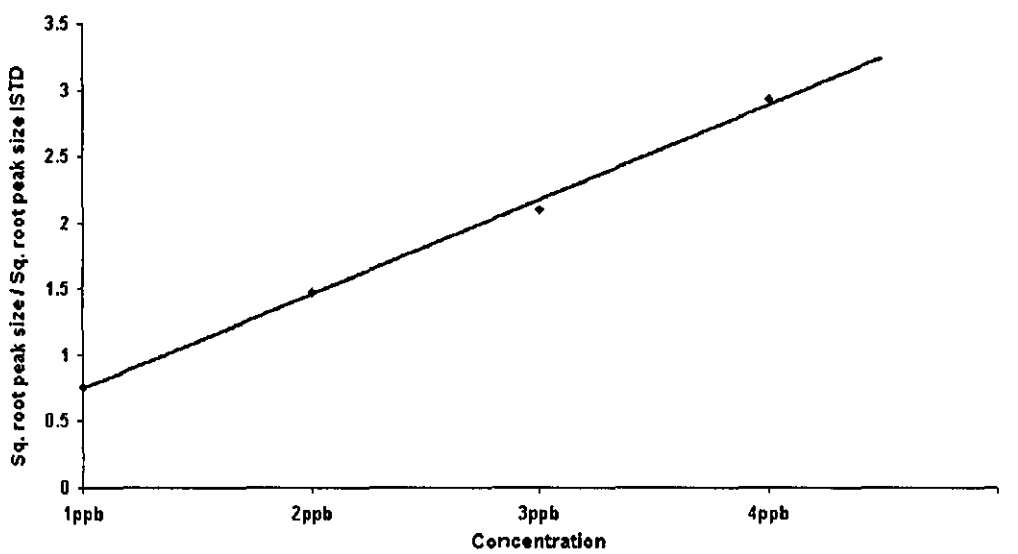


Fig. 55: Calibration curve for DEDS obtained using square roots of the peak heights. Experimental conditions as listed in section 2.3.

The calibration curves of the 15 calibrated compounds were calculated by comparison with each of the three internal standards, to ascertain which gave the best curve fit. The correlation coefficients for the 45 calibration curves can be seen in Table 13 below.

Analyte	Internal Standard		
	EMS	PrSAc	HexSH
MeSH	0.986855	0.909761	0.040871
EtSH	0.983745	0.986848	0.981133
DMS	0.997165	0.950252	0.125339
CS_2	0.995347	0.990932	0.989839
MeSAc	0.996983	0.999924	0.997151
DMDS	0.999716	0.994924	0.999603
EtSAc	0.999863	0.998388	0.997819
2-MeBuSH	0.997971	0.999328	0.976935
3-MeThPh	0.999388	0.999313	0.992642
3-MBT	0.995989	0.998085	0.993591
DEDS	0.999648	0.998382	0.987572
DMTriS/DMTetraS	0.993797	0.993224	0.929742
Me-3-MeSHprop	0.999049	0.998564	0.941982
Methionol	0.997032	0.999928	0.996249
3-MeSPrAc	0.996412	0.999825	0.989776

Table 13: Correlation coefficients of the 15 calibrated sulphur compounds calculated using the three different internal standards

The correlation coefficients in Table 13 clearly show that the response of the system is linear over one order of magnitude, which covers the range of concentrations of the respective sulphur compounds in beer. The correlation coefficients for some analytes show that linearity is very poor when HexSH is used as the internal standard. This is not a problem of detector response but an indicator of HexSH as an internal standard. This problem will be discussed in section 3.2.2.3.1.

The most appropriate internal standards for the analytes were considered using the data in Table 13 and the results in Table 6. There appeared to be no single major factor in the suitability of an internal standard for a specific compound. The best correlation coefficients for most of the thiols and sulphides were obtained with EMS. However, the sulphur RSDs of the peak areas of the thiols divided by the areas of the three possible internal standards (Table 6) suggested that PrSAc was the better internal standard. A criterion which appeared to be as important as functionality of the analytes was volatility. Finally it was decided to use EMS as the internal standard for the volatile thiols and sulphides. PrSAC would be used for all the other analytes.

Table 14: Allocation of internal standards to sulphur compounds for analysis using the SPME-GC-PFPD system described in section 2.3

Compound	Internal Standard
MeSH	EMS
EtSH	EMS
DMS	EMS
CS_2	EMS
MeSAc	PrSAc
DMDS	EMS
EtSAc	PrSAc
2-MeBuSH	PrSAc
3-MeThPh	PrSAc
3-MBT	PrSAc
DEDS	PrSAc
DMTriS/DMTetraS	PrSAc
Me-3-MeSHprop	PrSAc
Methionol	PrSAc
3-MeSPrAc	PrSAc

3.2.2.3.1 Unsuitability of 1-Hexanethiol as an Internal Standard

Mainly on the grounds of the results shown in Table 6 in section 3.2.2.1, 1-hexanethiol was initially chosen as the internal standard for methionol. The first analyses of beer samples, however, showed that 1-hexanethiol was not stable in the 95% water / 5% alcohol solution used for internal standards. After only 24 hours at a temperature of 4°C, the 1-hexanethiol had decomposed to a small fraction of its original concentration. The stability of 1-hexanethiol in the 100% ethanolic stock solution was, on the other hand, excellent. An 100% ethanolic internal standard solution could not be used, however, as it would have greatly increased the matrix effects detailed in section 3.1.2.1.1.10. Therefore propyl thioacetate was employed as the internal standard for methionol.

The difference between a freshly-made aqueous internal standard solution and a two-week old aqueous internal standard solution can be seen in Figures 56 and 57. In the second chromatogram (Figure 57) the size of the 1-hexanethiol peak (retention time 20.2 minutes) has decreased so significantly that it cannot be seen in the chosen chromatogram scale. The reason for this decrease is unknown. A possible explanation could be that 1-hexanethiol bound strongly to the glass walls of the flask which contained the standard solution.

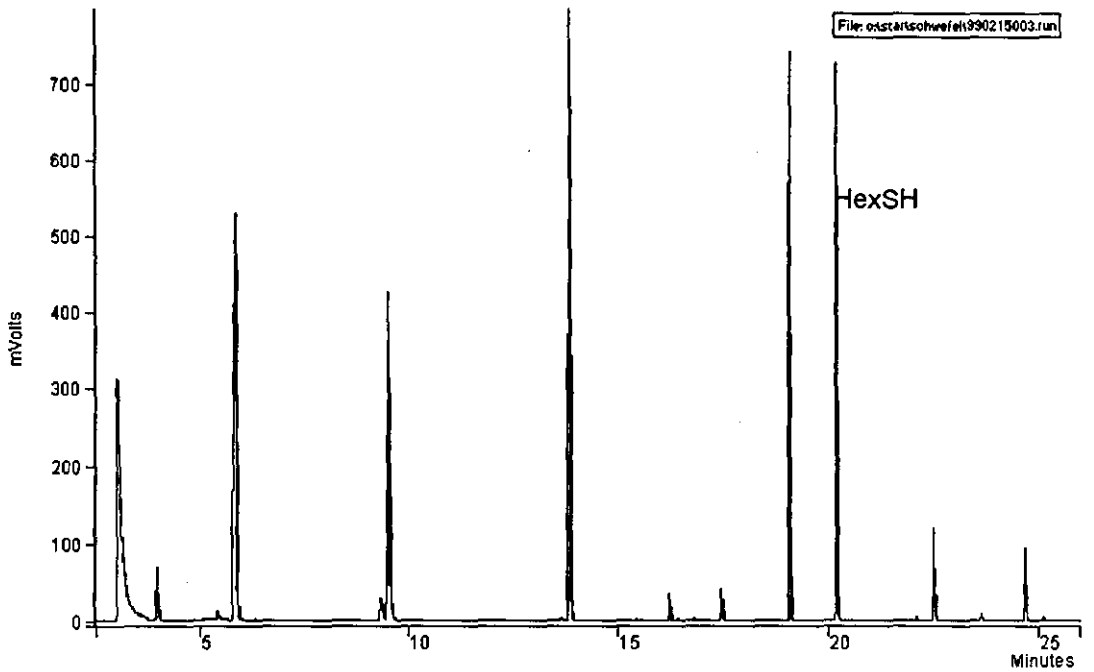


Fig. 56: Beer sample with added fresh aqueous internal standard solution containing 2.5ppb HexSH (RT 20.2 minutes). SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

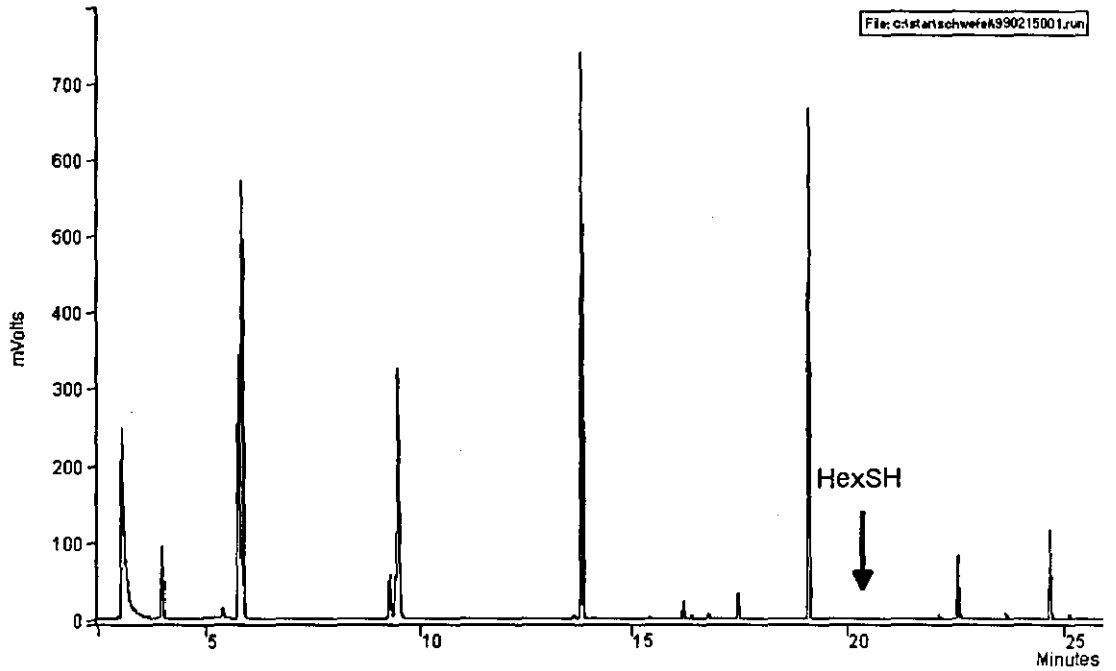


Fig. 57: Beer sample with added two-week old aqueous internal standard solution containing 2.5ppb HexSH (RT 20.2 minutes). SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

3.2.2.3.2 Calibration of 3-Methyl-but-2-ene-1-thiol

3-methyl-but-2-ene-1-thiol (3-MBT), the sulphur compound considered to be mainly responsible for the lightstruck taste in beers subjected to illumination, is not commercially available. Because of the stench the compound causes, the decision was taken not to attempt the synthesis in the laboratory.

A calibration of 3-MBT can be achieved using the equimolar response of the PFPD. As the PFPD response is independent of the structure of the sulphur-containing molecule and dependent purely on the absolute mass of sulphur, 3-MBT could be calibrated by comparison to any other sulphur compound. The problem with this approach, however, is the discriminatory nature of SPME. To neutralise differences in the extraction step as far as possible, 3-MBT could be calibrated using the response factor of a homologue.

Fortunately, in the final stages of this study, a reference standard for 3-MBT was synthesised in the USA for a British company dealing in sensory kits for the identification of off-flavours in beer. 0.5g of the 3-MBT was purchased from the British company and used to calibrate the SPME-GC-PFPD system. During the calibration with 3-MBT, however, it became clear that the purity of the reference standard was lower than the 95% declared by the American laboratory which had carried out the synthesis. This was probably due to the long transportation the compound had been subjected to. Figure 58 shows the chromatogram with the highest concentration of the reference compound (8ppb).

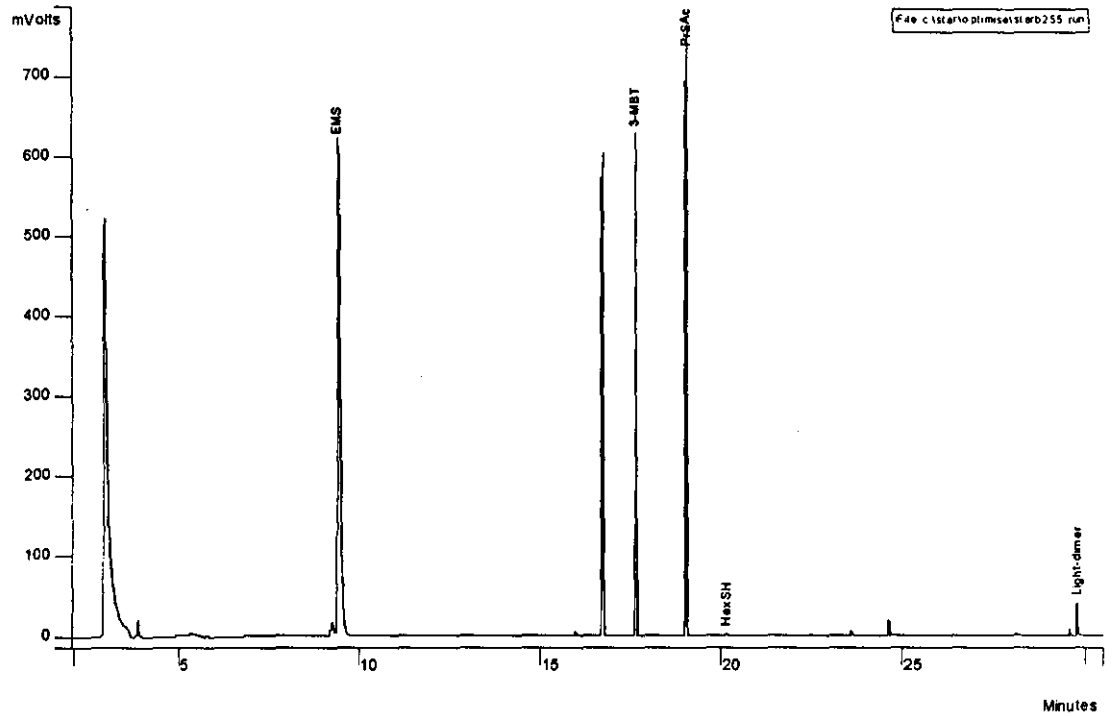


Fig. 58: Calibration chromatogram: 8ppb 3-MBT (RT 17.89 minutes) in alcohol-free beer. SPME conditions: adsorption = headspace, 36 mins with agitation at 45°C; desorption = 3 mins. Chromatographic conditions: constant column flow = 2.7 ml/min hydrogen; column oven = 7 mins at 32°C, increased to 110°C at 7°C/min, increased to 190°C at 11°C/min, increased to 235°C at 22°C/min and held for 6 mins; injector = 250°C. PFPD conditions: temperature = 210°C; PMT voltage = 600 V; gate delay = 6.0 msec; gate width = 20.0 msec; trigger level = 200 mV; Air 1 = 16.9 ml/min; air 2 = 9.8 ml/min; hydrogen = 10.3 ml/min.

As can be seen from the chromatogram in Figure 58, there is a large amount of another sulphur-containing compound (apart from the three internal standards) present, eluting at 16.75 minutes. This retention time corresponds to the compound identified using reference standards as 3-methylthiophene. Additionally, a compound is seen at 29.77 minutes - this could conceivably be a polymer of 3-MBT, which are known to be formed when 3-MBT is exposed to UV light (241).

The 3-MBT reference standard was analysed using the GC-MS system described in section 2.2.5, to identify the impurities. A library search found the main sulphur-containing impurity to be 3-methylthiophene. The best match found for the spectrum of the compound at 29.77 minutes was 3-MBT, backing up the theory that this impurity was a polymer of 3-MBT.

Following the calibration, a beer sample in a green bottle was subjected to six hours of controlled illumination using a specially-designed device. The sample was subsequently analysed using the SPME-GC-PFPD system described in section 3.2. The resulting chromatogram was evaluated using the response factors for both 3-MBT and 2-methyl butanethiol (as a reduced form and isomer of 3-MBT). Using the 3-MBT response factor a value of 1.0697ppb was obtained; using the 2-MeBuSH response factor a value of 0.0924ppb was obtained. The second value correlates much better to the published values of 3-MBT in beers subject to illumination: it was therefore decided to use the response factor for 2-MeBuSH to calculate 3-MBT values.

3.2.3 Determination of the Reproducibility of the Calibrated SPME-GC-PFPD System

Once the SPME-GC-PFPD system had been calibrated, the reproducibility of the method was determined. The reproducibility check was so designed as to allow the examination of the possible problem of the sample carousel not being able to heat the samples individually (see section 3.1.2.1.1.9).

Eight samples were analysed. The first four samples were each placed on the carousel exactly 45 minutes before they were extracted. The last four samples were all placed on the carousel at the same time as the first sample, i.e. they remained on the heated carousel for periods of between 4 and 7 hours. The chromatograms were evaluated using the

calibration described in section 3.3.2. The results of the eight analyses can be seen in Table 15. All values are in ppb.

Sample	Analytes										
	MeSH	EtSH	DMS	CS_2	MeSAc	DMDS	EtSAc	2-MeBuSH	3-MeThPh	Methionol	3-MePrSAc
1	3.490	0.470	57.552	0.137	10.466	0.161	0.866	0.065	0.015	193.585	4.708
2	3.080	0.469	60.137	0.137	10.606	0.149	0.816	0.052	0.012	118.712	4.044
3	3.287	0.442	55.468	0.131	10.174	0.154	0.841	0.062	0.015	144.779	5.183
4	3.489	0.484	55.774	0.126	10.824	0.166	0.834	0.063	0.016	177.701	4.423
5	3.271	0.485	60.245	0.144	10.631	0.156	0.829	0.053	0.012	196.097	4.757
6	3.897	0.468	59.788	0.140	10.547	0.148	0.819	0.060	0.013	192.209	4.319
7	4.036	0.462	64.102	0.177	9.125	0.199	0.813	0.056	0.013	283.309	4.623
8	2.975	0.445	59. 25	0.164	9.620	0.178	0.788	0.059	0.014	210.490	5.170
Mean	3.440	0.468	59.039	0.144	10.249	0.164	0.826	0.059	0.014	189.610	4.654
S.D.	0.348	0.016	2.611	0.016	0.548	0.016	0.021	0.004	0.001	45.357	0.370
Rel. S.D. (%)	10.1	3.35	4.42	11.3	5.35	9.76	2.59	7.48	10.1	23.9	7.96

Table 15: Results of a beer sample analysed eight times using the SPME-GC-PFPD method described in section 2.3, with relative standard deviations for the individual sulphur compounds.

The relative standard deviations for the sulphur compounds are mostly under 10%, i.e. the method shows very good reproducibility. The RSD for CS_2 is slightly higher but at 11.3% is still acceptable. The RSD of 23.9% for Methionol is unacceptable. A possible reason for this poor precision could be the lack of an appropriate internal standard (see section 3.2.2.3.1) for methionol.

The above results are for all of the samples in the reproducibility check, regardless of the time they spent on the heated carousel before extraction and injection. In Table 16 the results of the first four samples (each with 45 minutes equilibration time before extraction) are listed. In Table 17 the results of samples 5 - 8 (4 - 7 hours on the heated carousel before extraction) are listed.

Sample	Analytes										
	MeSH	EtSH	DMS	CS_2	MeSAc	DMDS	EtSAc	2-MeBuSH	3-MeThPh	Methionol	3-MePrSAc
1	3.490	0.470	57.552	0.137	10.466	0.161	0.866	0.065	0.015	193.585	4.708
2	3.080	0.469	60.137	0.137	10.606	0.149	0.816	0.052	0.012	118.712	4.044
3	3.287	0.442	55.468	0.131	10.174	0.154	0.841	0.062	0.015	144.779	5.183
4	3.489	0.484	55.774	0.126	10.824	0.166	0.834	0.063	0.018	177.701	4.423
Mean	3.336	0.466	57.233	0.132	10.518	0.157	0.839	0.060	0.014	158.694	4.590
S.D.	0.169	0.015	1.856	0.005	0.236	0.007	0.018	0.005	0.002	29.029	0.416
Rel. S.D. (%)	5.08	3.32	3.24	3.49	2.24	4.21	2.15	8.05	11.1	18.3	9.06

Table 16: Results of a beer sample analysed four times using the SPME-GC-PFPD method described in section 2.3, with relative standard deviations for the individual sulphur compounds. Each of the samples was equilibrated at 45°C for 45 minutes before extraction.

Sample	Analytes										
	MeSH	EtSH	DMS	CS_2	MeSAc	DMDS	EtSAc	2-MeBuSH	3-MeThPh	Methionol	3-MePrSAc
5	3.271	0.485	60.245	0.144	10.631	0.156	0.829	0.053	0.012	196.097	4.757
6	3.897	0.468	59.786	0.140	10.547	0.148	0.819	0.060	0.013	192.209	4.319
7	4.038	0.482	64.102	0.177	9.125	0.199	0.813	0.056	0.013	283.309	4.623
8	2.975	0.445	59. 25	0.164	9.620	0.178	0.788	0.059	0.014	210.490	5.170
Mean	3.545	0.470	60.846	0.156	9.981	0.170	0.812	0.057	0.013	220.526	4.717
S.D.	0.438	0.016	1.913	0.015	0.634	0.020	0.015	0.003	0.001	36.882	0.306
Rel. S.D. (%)	12.3	3.34	3.14	9.77	6.35	11.6	1.88	5.08	5.19	16.7	6.48

Table 17: Results of a beer sample analysed four times using the SPME-GC-PFPD method described in section 2.3, with relative standard deviations for the individual sulphur compounds. The samples were equilibrated at 4 - 7 hours before extraction.

The results in Tables 16 and 17 were compared using a *t*-test (242). The means of the two sets of results were compared for each compound. The calculated values of *t* can be seen in Table 18.

	Analytes										
	MeSH	EtSH	DMS	CS_2	MeSAc	DMDS	EtSAc	2-MeBuSH	3-MeThPh	Methionol	3-MePrSAc
Value of *t*	-0.890	-0.365	-2.711	-3.036	1.588	-1.227	2.305	1.029	0.894	-2.635	-0.492

Table 18: Calculated *t* values obtained by comparing the means of the results in Tables 16 and 17

The null hypothesis is adopted that the time the sample equilibrates before sampling has no effect on the concentrations of the sulphur compounds found. The critical value of $|t|$ (6 degrees of freedom) for a confidence interval of 95% (242) is 2.45. The values of *t* for most compounds are less than the critical value: there is no evidence that the equilibration time on the heated sampling carousel has an effect on the determined concentrations of these compounds. The exceptions are DMS, CS_2 and methionol. The calculated *t* values for these three compounds are greater than the critical value, and the null hypothesis is rejected.

A further result from Tables 16 and 17 is that there seems to be a tendency for the RSDs for the less-volatile sulphur compounds to be better after a longer equilibration time at 45°C. The RSDs for the volatile sulphur compounds, however, are slightly poorer after the longer equilibration times.

Although the inability of the Varian 8200CX autosampler with SPME III to warm the samples individually does not have a significant effect on the concentrations of most of the analytes, it does give cause for concern. An SPME autosampler capable of heating each sample individually is currently being launched by Varian.

When the ranges of concentrations at which the analyses are carried out are considered, the reproducibility of the SPME-GC-PFPD method is - with the exception of methionol - satisfactory.

3.2.4 Limits of Detection of the Calibrated SPME-GC-PFPD System

The limits of detection (LOD) of the system were very difficult to determine owing to the effects of the sample matrix. The SPME-GC-PFPD system is most sensitive when 100% aqueous samples are analysed. The sensitivity decreases with increasing ethanol concentration. The limits of detection therefore vary from sample to sample. Also, because of the discriminatory nature of SPME, the limits of detection vary from compound to compound. Therefore it is impossible to define exact limits of detection for the SPME-GC-PFPD system.

A very approximate idea of the LODs for certain compounds in a standard European Pilsener beer (alcohol concentration approximately 5%) can be gained by studying the chromatograms of the analysis of such samples. The SPME-GC-PFPD system appears to be most sensitive for 3-methylthiophene, the LOD lying between 1 - 5 ppt. For several other compounds, such as 3-methyl-but-2-ene-1-thiol (the 'lightstruck' thiol), DMDS, 2-MeBuSH, EtSAc and CS_2, the LODs lie between 10 - 60 ppt.

4 Some Applications of the Method

The potential of the SPME-GC-PFPD method for the analysis of volatile and semi-volatile sulphur compounds in beer developed in this study could best be demonstrated by showing possible applications.

4.1 Concentrations of Sulphur Compounds Analysed in a Selection of Beers

4.1.1 Procedure

Fresh samples of various different beer varieties from a range of breweries were purchased from supermarkets and analysed using the SPME-GC-PFPD system described in section 2.3.

4.1.2 Results

Table 19: Beers analysed with SPME-GC-PFPD

Sample	Sulphur Compounds (all values in ppb) (Retention Times / min)											
	MeSH (3.92)	EtSH (5.35)	DMS (5.78)	CS_2 (6.25)	MeSAc (13.79)	DMDS (15.37)	EtSAc (16.16)	2-MeBuSH (16.35)	3-MeThPh (16.75)	3-MBT (17.89)	Methional (24.66)	3-MeSPrAc (25.32)
Pilsener Beer												
Brewery A, Pils 1	3.074	0.560	70.52	0.167	1.193	0.306	0.688	0.049	0.026	0.000	356.0	4.286
Brewery A, Pils 2	3.470	0.679	63.76	0.163	11.56	0.225	0.751	0.042	0.020	0.000	188.1	3.986
Brewery B	5.633	0.786	58.41	0.327	15.59	0.308	1.703	0.058	0.020	0.000	32.42	9.828
Brewery C	2.784	0.066	56.20	0.181	3.169	0.317	0.000	0.063	0.027	0.000	725.4	8.095
Brewery D	4.931	0.371	61.66	0.227	10.17	0.202	0.880	0.058	0.027	0.000	1140.0	13.79

Sample	Sulphur Compounds (all values in ppb) (Retention Times / min)											
	MeSH (3.92)	EtSH (5.35)	DMS (5.78)	CS_2 (6.25)	MeSAc (13.79)	DMDS (15.37)	EtSAc (16.16)	2-MeBuSH (16.35)	3-MeThPh (16.75)	3-MBT (17.89)	Methionol (24.66)	3-MeSPrAc (25.32)
Pilsener Beer (cont)												
Brewery E	3.643	0.264	56.02	0.159	7.282	0.321	0.763	0.092	0.022	0.000	400.3	8.373
Brewery F	3.949	0.235	52.88	0.141	8.3473	0.190	0.374	0.047	0.018	0.000	746.4	22.67
Brewery G	3.978	0.337	53.80	0.145	6.018	0.173	0.618	0.045	0.015	0.000	758.7	9.689
Brewery H	3.962	0.158	58.23	0.146	2.099	0.218	0.253	0.069	0.019	0.000	262.2	6.515
Brewery J	3.398	0.248	46.12	0.162	5.043	0.266	0.412	0.071	0.021	0.000	1053.7	11.44
Brewery K	3.752	0.287	67.39	0.150	5.433	0.216	0.325	0.052	0.015	0.000	423.9	5.643
Brewery L	3.582	0.252	59.16	0.197	5.477	0.249	0.300	0.051	0.024	0.000	474.3	5.894
Brewery M	3.646	0.303	61.26	0.180	4.323	0.173	0.353	0.050	0.017	0.000	1119.8	7.199
Brewery N	4.148	0.139	59.09	0.152	2.608	0.352	0.110	0.060	0.056	0.031	774.0	10.48
Brewery P	4.760	0.706	54.84	0.372	12.16	0.226	0.726	0.041	0.016	0.000	568.5	7.105
Lager Beer												
Brewery B	3.019	0.581	59.18	0.398	11.88	0.247	1.085	0.042	0.021	0.000	454.8	9.427
Brewery Q	4.133	0.346	57.03	0.206	5.044	0.276	0.338	0.076	0.022	0.000	128.5	1.446
Brewery R	5.781	0.561	68.33	0.284	5.666	0.350	0.720	0.071	0.021	0.000	229.3	3.460
Brewery S	5.253	0.243	63.57	0.201	14.12	0.373	0.523	0.094	0.026	0.000	239.1	3.227
Bock Beer												
Brewery B	3.537	0.634	64.49	0.187	11.10	0.176	1.402	0.043	0.011	0.000	576.7	18.51
Brewery P, Bock 1	8.275	0.804	69.93	0.219	19.86	0.399	2.457	0.073	0.019	0.000	803.6	15.21
Brewery P, Bock 2	7.781	0.656	69.37	0.325	18.16	0.298	1.810	0.059	0.017	0.000	829.7	16.58
Brewery R	9.599	0.943	91.65	0.507	8.555	0.667	1.751	0.102	0.029	0.000	1038.4	13.07

Sample	Sulphur Compounds (all values in ppb) (Retention Times / min)											
	MeSH (3.92)	EtSH (5.35)	DMS (5.78)	CS_2 (6.25)	MeSAc (13.79)	DMDS (15.37)	EtSAc (16.16)	2-MeBuSH (16.35)	3-MeThPh (16.75)	3-MBT (17.89)	Methionol (24.66)	3-MeSPrAc (25.32)
Alcohol-free Beer												
Brewery A	1.633	0.099	5.112	0.068	0.0549	0.081	0.000	0.000	0.000	0.000	206.2	1.163
Brewery B	3.261	0.078	39.49	0.206	2.062	0.878	0.073	0.030	0.008	0.000	44.42	0.000
Wheat Beer												
Brewery T	3.589	0.184	47.78	0.267	4.288	0.170	0.289	0.066	0.031	0.000	2621.0	136.9
Brewery U	6.648	0.231	82.56	0.208	13.42	0.298	0.149	0.060	0.027	0.000	2730.5	88.27
Yeast Beer												
Brewery B	5.428	0.565	64.84	0.157	14.09	0.317	1.399	0.051	0.023	0.000	405.7	12.75
Export Beer												
Brewery B	3.978	0.528	61.59	0.172	11.27	0.221	1.270	0.066	0.012	0.000	597.1	18.90
Dark Pilsener Beer												
Brewery A	2.901	0.429	52.98	0.125	10.16	0.152	0.752	0.067	0.011	0.000	223.0	5.371
Non-alcoholic Malt Beer												
Brewery B	1.553	0.000	41.99	0.104	0.913	0.345	0.000	0.033	0.008	0.000	60.82	0.000

4.1.3 Discussion

It can be seen from Table 19 that the largest differences in the concentrations of sulphur compounds were beer variety dependent and not brewery dependent. For example, the concentrations of sulphur compounds in pilsener beers from different breweries were similar. This can be visually observed in Figure 59 from the chromatograms of 3 Pilsener beers from 3 different breweries.

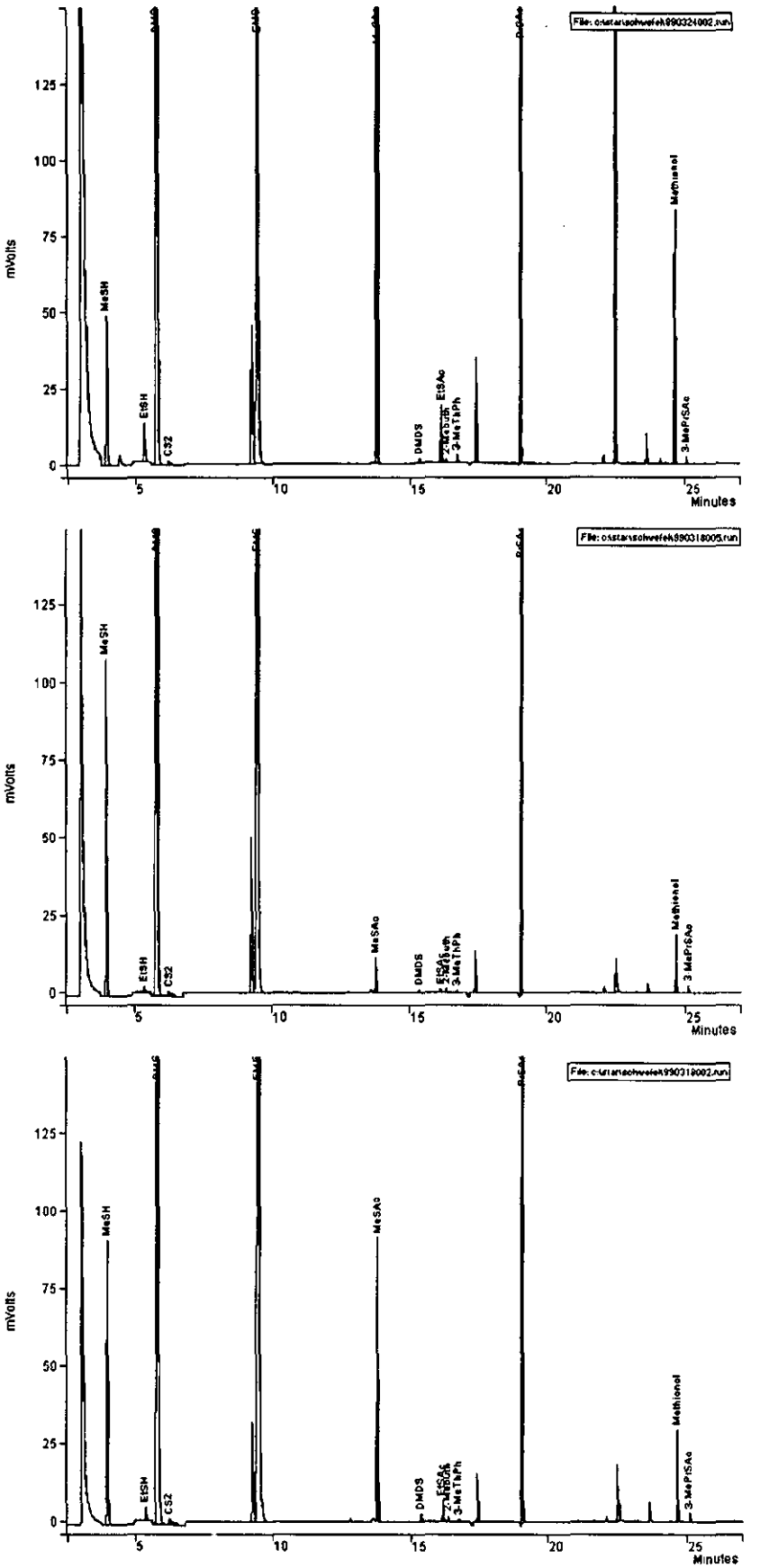


Fig.59: Pilsener beers from 3 different breweries (Top = A; Middle = H; Bottom = E) analysed using SPME-GC-PFPD as described in section 2.3

As can be seen from the chromatograms of three Pilsener beers from three independent breweries in Figure 59, the concentrations of sulphur compounds in beers of a single variety were similar. Owing to the quadratic nature of the PFPD response (172), the size of the peaks can be misleading. It should be remembered when comparing peaks that the sulphur concentration is proportional to the square root of the peak area. The MeSAc peak (13.79 mins) in the chromatograms in Figure 59 is a good example of this. The amounts of MeSAc found in Pilseners A, H and E were 11.9ppb, 2.10ppb and 7.28ppb respectively. Although Pilsener A contained approximately six times the amount of MeSAc than Pilsener H, the peak was the square of this factor larger, i.e. approximately 36 times larger. This can be clearly seen in Figure 59. Equally, Pilsener E contained approximately 3½ times as much MeSAc as Pilsener H: the MeSAc peak was 12 times larger.

The brewery dependent differences can be partly explained when information about the various production processes of the individual breweries is available. For example, the beers of Breweries A, B and P all displayed the highest concentrations of methyl and ethyl thioacetate (with the exception of alcohol-free beers), two sulphur substances produced by the yeast during fermentation. The Breweries A, B and P all use the same yeast strain, suggesting that the concentrations of some sulphur compounds are dependent on the yeast culture used for fermentation.

The differences between beer varieties were much larger. This is demonstrated by the concentration of 3-MeSPrAc in various types of beer. In the wheat beers over 85ppb 3-MeSPrAc was determined, whereas the Pilsener beers contained less than 15ppb and the alcohol-free beers less than 2ppb 3-MeSPrAc. Figure 60 shows the chromatograms of 3 different beer varieties.

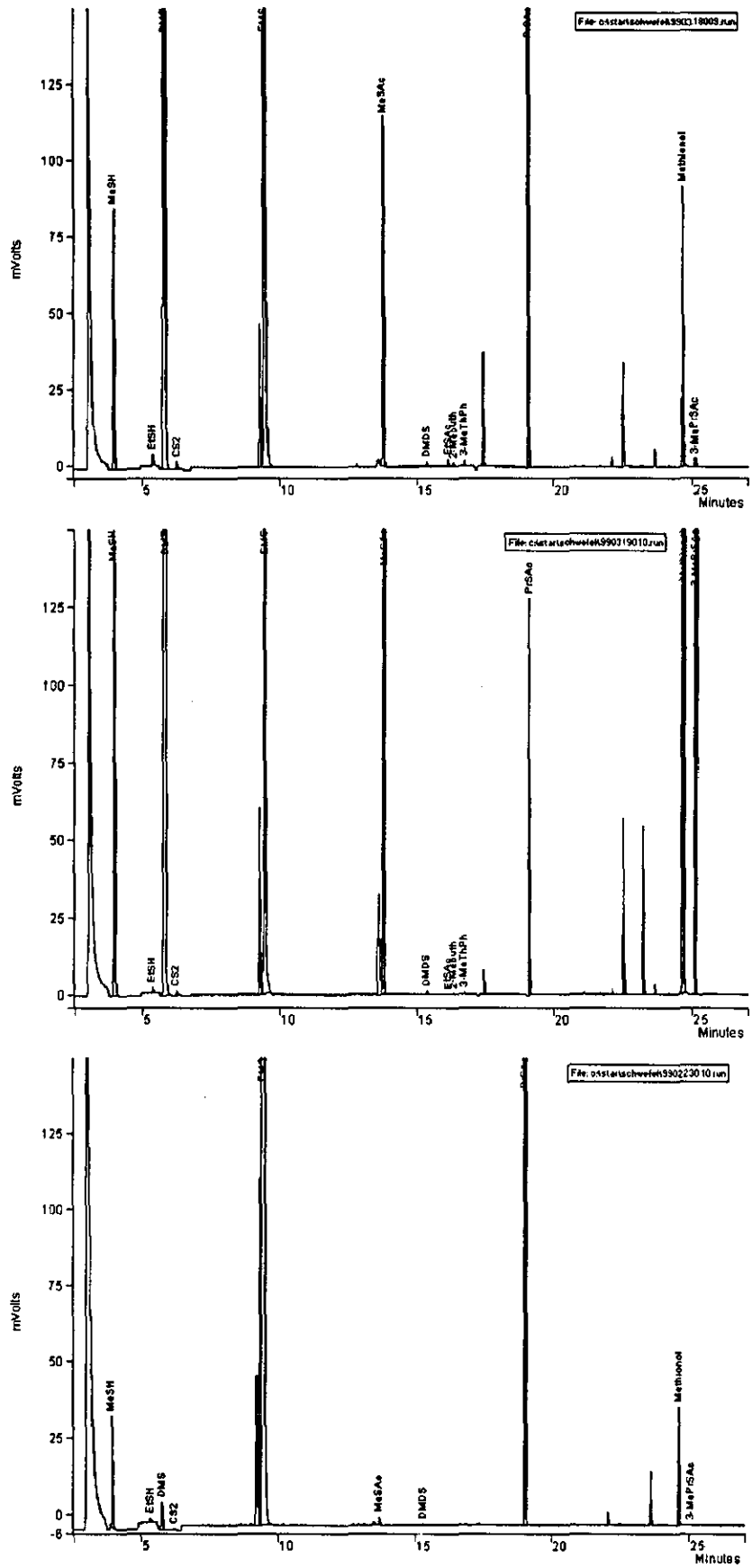


Fig.60: 3 beer varieties (Top = Pilsener L; Middle = wheat beer T; alcohol-free beer A) analysed using SPME-GC-PFPD as described in section 2.3

The chromatograms in Figure 60 demonstrate that the concentration of sulphur compounds differ greatly from beer variety to beer variety. Table 20, containing the mean values of the determined sulphur compounds for each beer variety, depicts these differences numerically.

Table 20: Mean values for the concentration of sulphur compounds in different beer varieties

Sample	Sulphur Compounds (all values in ppb) (Retention Times / min)											
	MeSH (3.92)	EtSH (5.35)	DMS (5.78)	CS_2 (6.25)	MeSAc (13.79)	DMDS (15.37)	EtSAc (16.16)	2-MeBuSH (16.35)	3-MeThPh (16.75)	3-MBT (17.89)	Methionol (24.66)	3-MeSPrAc (25.32)
Pilsener Beer	3.914	0.359	58.624	0.191	7.414	0.249	0.550	0.057	0.023	0.002	601.59	9.000
Lager Beer	4.547	0.433	62.025	0.272	9.177	0.312	0.667	0.071	0.023	0.000	262.90	4.390
Bock Beer	7.298	0.759	78.859	0.310	14.412	0.385	1.855	0.069	0.019	0.000	811.71	15.845
Alcohol-free Beer	2.447	0.089	22.303	0.137	1.058	0.480	0.037	0.015	0.004	0.000	125.31	0.582
Wheat Beer	5.119	0.208	15.168	0.238	8.855	0.234	0.219	0.063	0.029	0.000	2675.74	111.59
Yeast Beer	5.428	0.565	64.842	0.157	14.090	0.317	1.399	0.051	0.023	0.000	405.68	12.746
Export Beer	3.978	0.528	61.591	0.172	11.271	0.221	1.270	0.066	0.012	0.000	597.11	18.903
Dark Pilsener	2.901	0.429	52.976	0.125	10.157	0.152	0.752	0.067	0.011	0.000	222.99	5.371
Non-alc. Malt Beer	1.553	0.000	41.992	0.104	0.913	0.345	0.000	0.033	0.008	0.000	60.82	0.000

A further interesting result of the analyses of a selection of beers can be seen in Table 19. In the Pilsener beer from Brewery N 0.031ppb 3-MBT, the so-called light thiol, was determined. The beer was packaged in a green bottle, which are known to be more susceptible to light influences than brown bottles (75). The assumption is that the beer had been subjected to direct light between being filled and being purchased in the supermarket.

4.2 The Influence of Light on Beer

The influence of light on beer has been the subject of many publications (64,65,66,67,75,76,78). Although the general mechanism of the formation of the main sulphur compound believed to be responsible for the 'lightstruck' or 'sunstruck' off-flavour, 3-methyl-but-2-ene-1-thiol (3-MBT), has long been known (64,65), the phenomenon of the 'skunky' (incidentally a misnomer, as detailed in section 1.2) off-flavour in beers subjected to light is far from being fully understood. The extremely low flavour thresholds of sulphur compounds and the lack of accurate and simple methods of analysis at such low concentrations are the principal reasons for this.

4.2.1 Procedure

To investigate the changes in the concentrations of sulphur compounds on illumination, samples of beer from a single production batch were subjected to defined light conditions for various lengths of time and then analysed with the SPME-GC-PFPD method described in section 2.3. Fresh samples of German Pilsener beer, in green bottles, were illuminated using a specially-designed device. Both bottles with the normal front, back and neck labels (i.e. the usual packaging as purchased by the consumer) and bottles devoid of all labels were studied. The bottles were stood on revolving plates and illuminated with Osram Eversun L80W/79 UV lamps for 2, 4, 6, 8 and 24 hours. Control samples were kept in the dark. The illuminated samples and the control samples were subsequently analysed using SPME-GC-PFPD.

4.2.2 Results

During the analysis of the 24 hour light sample (without label) the injector block septum started to leak, making it impossible for the chromatogram to be evaluated. The results of the remaining analyses can be seen in Table 21.

Table 21: bottled beer samples (with and without labels) subjected to different degrees of illumination

Sample	Sulphur Compounds (all values in ppb) (Retention Times / min)											
	MeSH (3.92)	EtSH (5.35)	DMS (5.78)	CS_2 (6.25)	MeSAc (13.79)	DMDS (15.37)	EtSAc (16.16)	2-MeBuSH (16.35)	3-MeThPh (16.75)	3-MBT (17.89)	Methionol (24.66)	3-MeSPrAc (25.32)
Without labels												
Control (no light)	2.699	0.359	59.348	0.063	10.073	0.100	0.631	0.040	0.010	0.000	210.20	4.925
2 hours light	2.793	0.406	63.713	0.119	10.166	0.222	0.813	0.037	0.037	0.031	276.08	6.188
4 hours light	3.316	0.478	67.505	0.100	10.732	0.194	0.741	0.051	0.042	0.084	167.32	4.682
6 hours light	2.665	0.364	55.455	0.184	9.722	0.164	0.684	0.042	0.041	0.105	157.70	4.315
8 hours light	3.587	0.388	60.883	0.072	10.21	0.209	0.740	0.071	0.079	2.302	246.75	4.909
With labels												
Control (no light)	2.849	0.385	61.527	0.101	10.465	0.177	0.716	0.013	0.013	0.000	255.98	4.743
2 hours light	2.874	0.445	62.588	0.209	10.180	0.161	0.728	0.048	0.021	0.024	236.77	4.809
4 hours light	3.432	0.470	67.052	0.105	10.787	0.183	0.707	0.047	0.031	0.062	141.92	4.441
6 hours light	3.479	0.435	65.461	0.113	9.445	0.208	0.733	0.053	0.037	0.093	261.68	4.077
8 hours light	3.533	0.426	63.097	0.094	10.799	0.143	0.687	0.050	0.036	1.051	132.30	3.971
24 hours light	4.665	0.443	66.114	0.095	10.352	0.177	0.649	0.049	0.066	1.862	121.16	3.887

4.2.3 Discussion

The results in Table 21 confirm that 3-methyl-but-2-ene-1-thiol (3-MBT) is produced when beer is subjected to illumination. The concentration of 3-methylthiophene also increases upon illumination. This phenomenon has not previously been described in the literature. From the results in Table 21 it can also be seen that the beer labels provide a certain degree of protection against light influences.

Figure 61 shows the chromatograms of three beers (without labels) which were subjected to varying degrees of illumination. The top chromatogram was of the control sample which had been kept in the dark before analysis. The middle and bottom chromatograms were of samples which had been illuminated for four and eight hours respectively. The increase in the size of the 3-MBT (17.89 mins) and 3-methylthiophene (16.75 mins) peaks can be clearly seen. Additionally, a slight increase in the size of the MeSH peak witnessed. The peak at 20.2 minutes seen in the second and third chromatograms was 1-hexanethiol, which had at first been used as an internal standard (see section 3.2.2.3.1) and was present in the standard solution used for these two samples. This peak can be ignored.

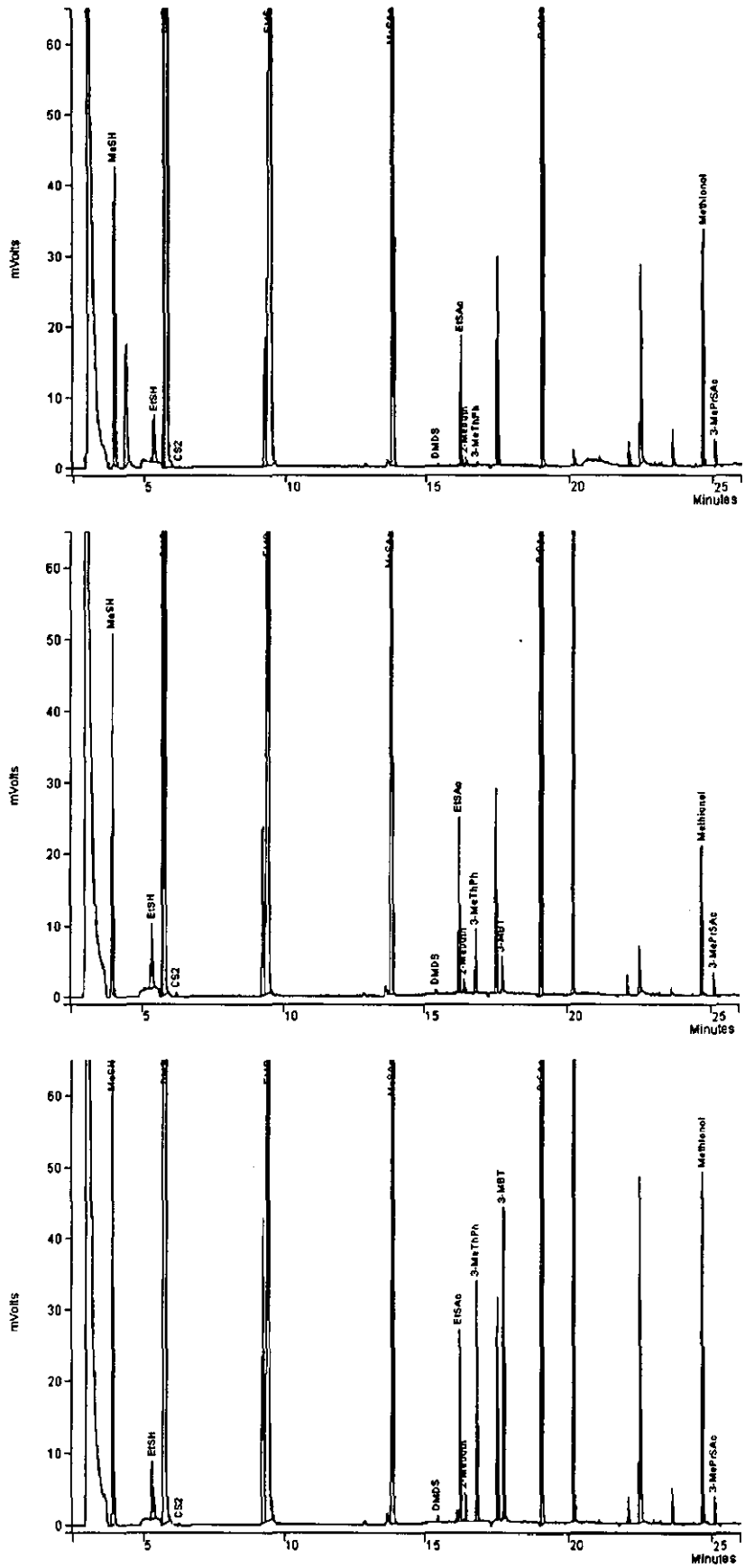


Fig. 61: Chromatograms of 3 beer samples subjected to varying degrees of illumination. Analysed using SPME-GC-PFPD as described in section 2.3

The results of the illumination experiment demonstrate the ability of the SPME-GC-PFPD system to analyse sulphur compounds in the ppt region. Because of the sensitivity of the method 3-methylthiophene has been identified in beer for the first time and is shown to be a good indicator of the extent of light influences on a beer. The system is a useful tool to study the development of 3-MBT, MeSH and 3-methylthiophene levels during the illumination of beer, and provides an excellent opportunity to allow the phenomenon of the lightstruck flavour in beer to be more fully understood.

5 Conclusion

The present work has developed a simple, fast, sensitive and inexpensive routine method of analysis for volatile and semi-volatile sulphur compounds in beer. The method development was based on a comparison of various selective and specific GC detectors, capillary columns and methods of sample preparation.

The best detector proved to be the recently-developed pulsed flame photometric detector (PFPD). Other detectors investigated included the sulphur chemiluminescence detector (SCD) and the atomic emission detector (AED). The SCD showed excellent specificity for sulphur compounds but proved to be instable and unable to achieve high sensitivity for more than very short periods. The brief investigation of the AED indicated that the detector was extremely sensitive and displayed acceptable specificity, which could be improved by tuning of the software. However, the AED was rejected as a routine detector for the analysis of sulphur compounds in beer on the grounds of its high price. The PFPD showed excellent stability, sensitivity and specificity and is reasonably priced. The PFPD parameters were subsequently optimised to achieve maximum detectivity.

Various chromatographic columns were tested. The separation of sulphur compounds over a wide range of boiling points and polarities was found to be possible, without the need for cryo-cooling, by using a combined column consisting of a 10m length of polar wax column connected to a 60m non-polar VA-1 column.

Because of the low levels of volatile and semi-volatile sulphur compounds in beer, solid phase microextraction (SPME) of the sample headspace was used as a concentration step. A study of the various, commercially-available fibres showed that a 75µm carboxen/PDMS coating was optimal. The SPME adsorption and desorption parameters were

optimised and SPME manual sampling and autosampling were compared. The effect of the sample matrix, in particular the influence of ethanol, on the extraction of trace amounts of sulphur compounds was investigated.

The SPME-GC-PFPD system displayed good linearity over the calibration range of one order of magnitude and excellent sensitivity. The reproducibility of the system was good, the relative standard deviations for most compounds being under 10%. The sensitivity of the system allowed two compounds which had previously been unreported in beer to be identified. 3-methylthiophene, which has been previously found in hops, and 2-methyl-1-butanethiol were both determined in beer in ppt levels.

The potential of the SPME-GC-PFPD method was demonstrated by determining the concentrations of sulphur compounds in samples of different kinds of beers from a range of breweries. Additionally, the change in the levels of sulphur compounds when beer is subjected to illumination was investigated. Not only were increases in the concentrations of 3-methyl-but-2-ene-1-thiol (3-MBT) and methanethiol witnessed, but the newly-identified sulphur compound 3-methylthiophene also showed an increase in concentration during illumination.

Further work could be carried out to complete the identification of all the sulphur compounds in beer, but techniques will need to concentrate the sample even further to allow identification using GC-MS. Additionally, the phenomenon of increased 3-methylthiophene concentrations during the illumination of beer, the kinetics of its formation and the proposal of possible reaction mechanisms provide scope for further research.

This study has shown that the combination of solid phase microextraction, gas chromatography and pulsed flame photometric detection provides a simple, fast, sensitive and inexpensive routine method of analysis for volatile and semi-volatile sulphur compounds in beer. The SPME-GC-PFPD system will be used a routine method of analysis at Brauerei Beck & Co. to provide a better understanding of the role of sulphur

compounds in beer and the changes in their concentrations during ageing and illumination.

6 References

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