INVESTIGATION OF THE CONGENERS RESPONSIBLE FOR NUTTY/CEREAL AROMA CHARACTER IN NEW MAKE MALT WHISKY

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ABSTRACT

The nutty and cereal aromas of new make malt whisky are important sensory characteristics of certain distillery malts of commercial value for blending; however there is a lack of understanding regarding the volatile congeners which contribute to these complex sensory characters. The work described in this thesis aimed to improve knowledge of the chemical origins of nutty and cereal aromas in immature spirit in order to aid process control of these characters during manufacturing.

Two aroma extraction methods were compared; liquid-liquid extraction (LLE) using dichloromethane and solid-phase extraction (SPE) with LiChrolut EN sorbent. New make spirit samples from industry (n=5) were evaluated by a trained whisky sensory panel using Quantitative Descriptive Analysis (QDA). Four were noted for their nutty/cereal character, the other served as a non-nutty control. Gas Chromatography-Olfactometry/ Mass Spectrometry (GC-O/MS) was used to try and identify compounds in chromatogram regions coincident with nutty/cereal descriptors. Using LLE extracts, 14 such regions were identified. LiChrolut EN SPE proved to be more selective (19 nutty/cereal odour active regions). 2,5-dimethylpyrazine (known to impart a nutty/cereal character in other food systems) was one noted congener, which was only detected using the more selective SPE method.

The gross volatile compositions of the 5 spirit samples were remarkably similar, suggesting that congeners present at low concentration but with low odour thresholds are likely responsible for nutty/ cereal characters. One analytical difference of note was that the nuttier samples contained higher concentrations of long-chain esters. Thus, ethanolic Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCI-MS) was used to analyse the headspace concentrations of a test set of 14 whisky aroma volatiles above a series of aqueous ethanolic solutions differing in concentrations of alcohol (5-40% ABV) and ethyl hexadecanoate (0-500 mg/L). Ethanol had a significant solubilising effect ($p < 0.0001$) on headspace volatile concentrations of all the aroma compounds, whilst the ethyl hexadecanoate concentration had a selective effect of reducing headspace concentrations of the more hydrophobic aroma compounds (Log $P > 2.5$). We propose that nutty and cereal characters are imparted by relatively polar aroma compounds, whose

characters are emphasised by the selective incorporation of hydrophobic aroma compounds into the interior of micelle-like structures formed by long chain esters (typified here by ethyl hexadecanoate).

Some distillers have reported that manipulation of the lipid concentrations in wash offers a method of controlling the nutty/oily character of new make spirit. A batch of fermented wash sourced from industry was spiked with varying concentrations of oleic (18:1) and linoleic (18:2) acids and (laboratory) distilled at two different temperatures, using a D-optimal experimental design to evaluate the impacts of each factor. Nutty ($p = 0.0203$) and oily ($p = 0.0034$) aroma characteristics were scored as significantly stronger in distillates of wash spiked with 100 µg/mL each of oleic and linoleic acids, as compared to the control. GC-O/MS of distillate extracts once again determined several odour active regions relevant to the nutty/cereal characters and concentrations of some compounds could be correlated with nutty/cereal QDA scores.

New make spirit samples from 35 individual malt whisky distilleries were extracted using the LiChrolut SPE method and analysed by GC-MS. Analytical concentrations of 'candidate' nutty-cereal compounds ($n = 20$) were used to model sensory QDA data for the 35 spirit samples (nutty, oily, cereal and feinty characters) using Principal Components Analysis (PCA). Significant positive correlations with nutty were seen for 7 compounds (using ANOVA). These included the Maillard products 2-furanmethanol ($p < 0.0001$), 2-methylpyrazine ($p < 0.0013$) and 2,5-dimethylpyrazine ($p < 0.0004$). The PCA overlay bi-plot showed clustering of certain higher alcohols near to the nutty aroma descriptor (methionol, pentan-1-ol, 2-phenyethyl alcohol).

Nutty and cereal characters of whisky are of complex origin and likely originate from multiple congeners in a synergistic mixture. This work has shown that processes of particular importance to the expression of this character in new make spirit are lipid oxidation and Maillard chemistry. The conditions for these reactions are to be found during malt kilning and distillation. Whilst these processes are where nutty/cereal compounds are likely to be formed, other distillery parameters such as the mashing protocol, length of fermentation (both determine the supply of key precursors such as fatty and amino acids) and the spirit cut of the distillation govern the chemical composition of the final spirit.

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PUBLICATIONS

Boothroyd, *E. L., Robert S. T. Linforth* and David J. Cook (2012) "Effects of Ethanol and Long-Chain Ethyl Ester Concentrations on Volatile Partitioning in a Whisky Model System." *Journal of Agricultural and Food Chemistry*, **60**(40): 9959-9966.

Boothroyd, E. L., David J. Cook, Frances Jack and Barry Harrison (2012) "The impact of increased wash fatty acid levels on the nutty / cereal aroma volatile composition of new make malt spirit." *Distilled Spirits: Science and Sustainability* Graeme M. Walker, Robert Fotheringham, Ian Goodall and Douglas Murray. Nottingham University Press: 167-174 (Proceedings of the Worldwide Distilled Spirits Conference held in Glasgow, September 2011)

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ABBREVIATIONS

ANOVA	Analysis of Variance
APCI-MS	Atmospheric Pressure Chemical Ionisation-Mass Spectrometry
C16	Ethyl Hexadecanoate Ethyl Ester
DCM	Dichloromethane
DF	Detection Frequency
EE	Ethyl Ester
FAME	Fatty Acid Methyl Ester
GC-MS	Gas Chromatography – Mass Spectrometry
GC-O	Gas Chromatography – Olfactometry
LLE	Liquid-Liquid Extraction
LRI	Linear Retention Indices
m/z	Mass/Charge Ratio
OAA	Odour Active Area
OPA	Odour Port Analysis
PCA	Principal Component Analysis
QDA	Quantitative Descriptive Analysis
SD	Standard Deviation
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
TIC	Total Ion Chromatogram
TMSH	Trimethylsulfonium hydroxide
UFA	Unsaturated Fatty Acid
VP	Vapour Pressure

1 INTRODUCTION

The aim of this research was to identify congeners which contribute to the nutty and cereal aroma character in new make spirit. The term 'congener' is often used in the distilling industry to specifically describe a volatile compound which is formed during whisky production and has a significant impact on the aroma profile of the spirit.

In this introductory chapter, the processes of new make malt spirit manufacture will be summarised, with particular attention to the procedures which could potentially give rise to reactions known to produce compounds with nutty and cereal aroma character. Also discussed will be odour impact compounds which exhibit these qualities in other food systems, as well as the various methods of aroma extraction, detection and theory which will be used in the subsequent results chapters.

1.1 SCOTCH WHISKY LEGISLATION AND PRODUCTION

There are two main types of Scotch whisky, grain whisky and malt whisky. Grain whisky uses only unmalted cereals in its production, whereas malt whisky is produced from barley which has undergone malting. According to the Scotch Whisky Act (1988), whisky is a spirit made from the distillation of a mash of cereals which has been "saccharified" by the malt enzymes, fermented with yeast, and distilled to an alcoholic strength of "less than 94.8% by volume". Maturation must take place in wooden casks and last no less than 3 years. The Scotch Whisky Act (1988) also stipulates that the "distillate has an aroma and taste derived from the raw materials used". As the only raw materials used for whisky production are cereals, water and yeast, where new make spirit is concerned, the only flavours whisky can develop must originate from the naturally occurring compounds found within these three ingredients. It is these precursors which become the reactants in various chemical reactions which take place during the whisky manufacturing process. Figure 1.1 presents a schematic flow diagram of the

main processes of malt and grain whisky production.

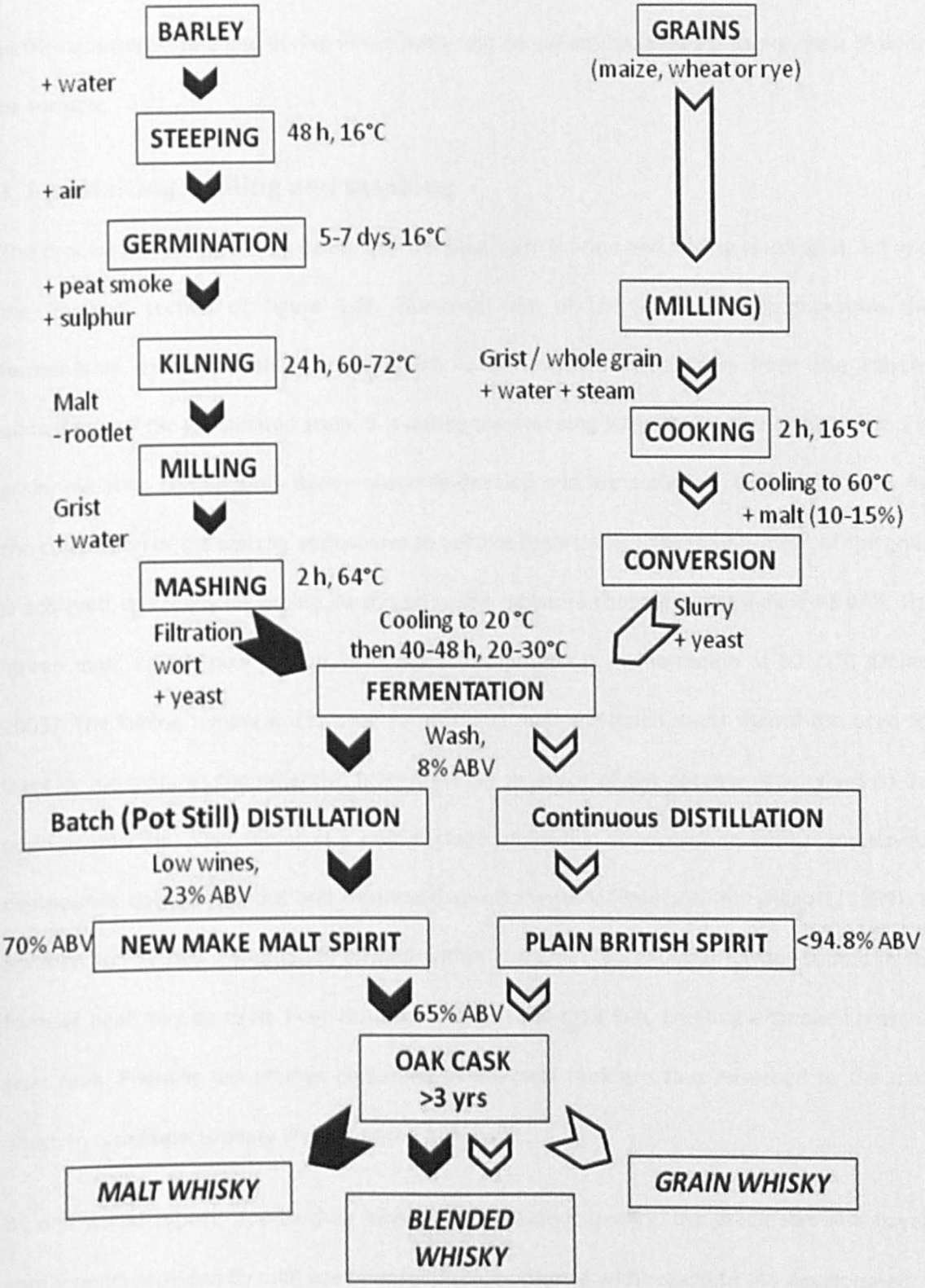


Figure 1.1 Schematic flow diagram showing the production processes for malt, grain and blended whiskies (Russell et al., 2003).

Each processing stage has the potential to impact on the flavour of the spirit. In this section the main processes involved in the production of malt whisky will be summarised, paying particular interest to those during which nutty and cereal aroma compounds are most likely to be formed.

1.1.1 Malting, milling and mashing

The process of malting barley comprises steeping, germination and kilning (see Figure 1.1 and the 'malting' section of Figure 1.2). The main aim of the maltster is to maximize the fermentable extract of the barley, which is derived almost entirely from the starchy endosperm of the germinated grain. It is during the steeping stage that water is taken into the grain, initiating germination. Barley enzymes develop and are activated, which will allow for the conversion of the starchy endosperm to soluble sugars. Once full modification of the grain is achieved, it is ready for kilning. At this point the moisture content of the malt is 46-47%. The 'green malt' as it is now known, is kilned at temperatures in the region of 60-72°C (Dolan, 2003). The kilning temperatures used for distilling malt are much lower than those used for lager or ale malt, as the objective is to preserve as much of the enzyme activity within the grain as possible. Malt kilning is a crucial stage of flavour development, with many flavour compounds being produced and destroyed simultaneously (Paterson and Piggott, 1989). In addition to the new compounds formed within the grain, an external flavour source in the form of peat may be used. Peat can be burned in the malt kiln, creating a smoke known as peat reek. Phenolic compounds contained in the peat reek are thus adsorbed to the malt, which in turn imparts peaty aromas to the final spirit.

As one would expect, due to their inherent malty odour quality, the precursors and flavour components provided by malt are of particular significance with regard to the development of nutty and cereal aroma volatiles. Indeed, malted barley extracts themselves have been found to contain an array of compounds which were found to exhibit nutty aroma qualities (Farley

and Nursten, 1980). Contained within each malt grain are reactants such as lipids, proteins, sugars and amino acids which, when heated in the presence of moisture, give rise to a multitude of compounds with roasted and toasted aromas. When amino acids and reducing sugars interact (within malt, foremost examples of these are proline and maltose, respectively) under such conditions, they start a cascade of reactions. The chemistry taking place is known as the Maillard reaction. This reaction is of great importance with regard to compounds which have nutty and cereal aroma attributes and is discussed in more depth in Section 1.2.1. Once the malt has been ground to grist, it is transferred to a mash tun (Figure 1.2) where it is mixed with hot water (64.5 °C) at a grist to water ratio of 1:4. The mash is thoroughly mixed by rotating paddles and it is allowed to stand before the resulting wort is collected and another volume of increasingly hotter water (70 °C) is added. This is repeated with separate waters of 80 and 90°C (Bamforth, 2005). In this way starch is gelatinised which makes it accessible for degradation to sugar by the malt enzymes. The resulting maltose-rich liquor is cooled to around 20°C before being pumped into the washback (Figure 1.2) for fermentation by yeast.

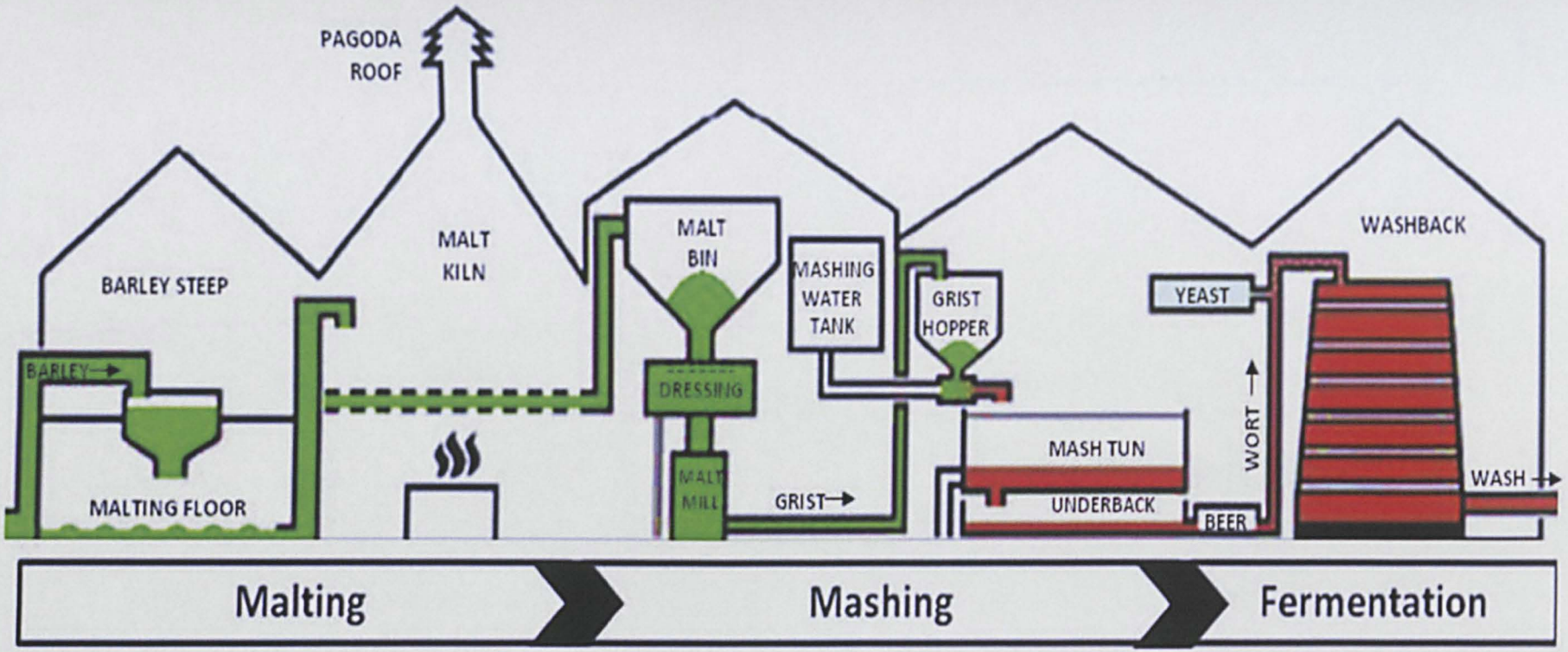


Figure 1.2 Simple cross-section diagram of a malt whisky distillery showing the processes of malting, mashing and fermentation (Butler, 2008).

1.1.2 Fermentation

Hybrid strains have been specifically developed from both the brewing strain *Saccharomyces cerevisae* and *Saccharomyces diastaticus* (wild type) in order that the yeast is able to withstand the high ethanol concentrations. Thus, many malt distilleries employ a mixture of brewer's and distiller's yeast due to their belief that this results in a higher spirit yield than distiller's yeast alone. As the yeast metabolise the sugars (principally glucose, maltose and maltotriose) in the wort to produce ethanol, a range of flavour compounds are formed as byproducts (Jack, 2003). Among these byproducts are important flavour precursors amino acids, fatty acids and esters (de Rijke and ter Heide, 1983). Figure 1.3 is a schematic diagram which illustrates how these compounds react and are formed during fermentation.

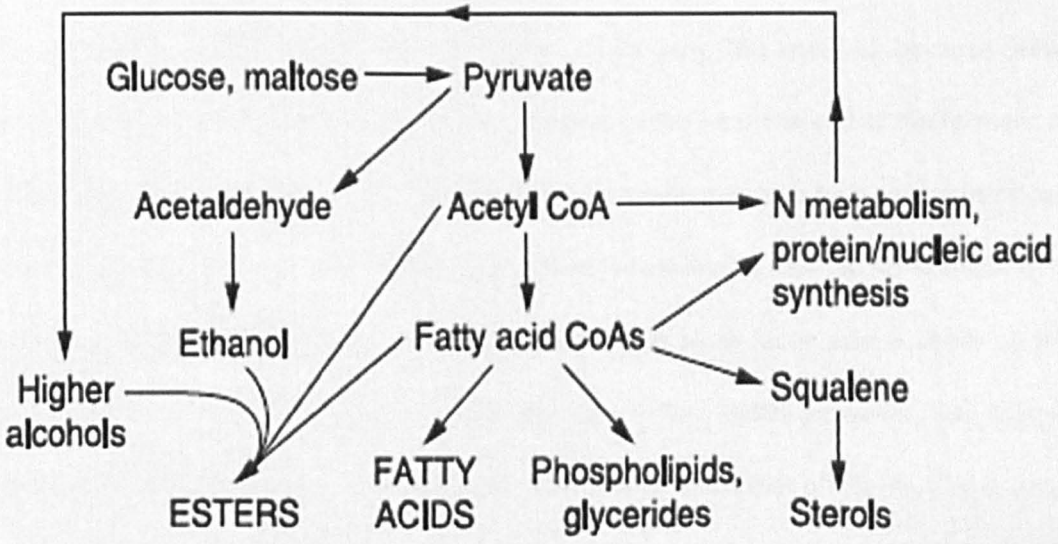


Figure 1.3 Flow diagram to show how alcohols, esters and fatty acids are produced as by-products during fermentation. Key intermediates include acetyl CoA and fatty acid CoA (Campbell, 2003).

Since fermentation is considered to be the process during which the majority of key whisky aroma volatiles are formed, certain fermentation parameters have the ability to significantly influence the final spirit character. For example, if the distiller is creating a fruity spirit, then they may choose a yeast strain which has proven ability to produce high levels of esters, which are considered key congeners for this character. Saerens *et al.* (2008) noticed that a higher

level of UFAs in the wort during fermentation resulted in an overall reduction of ethyl esters synthesised. Ester production relies on the recycling of acetyl Coenzyme A (acetyl CoA as shown in Figure 1.3), so when this esterase activity is inhibited by the increased uptake of fatty acids, less esters are formed. Washbacks can be made from either stainless steel or wood (Figure 1.2). Whilst the former is more hygienic as it can be thoroughly washed between fermentations, the potential microbial contamination which is offered by the yeast and bacteria inevitably present in a wooden washback may be beneficial for the production of desirable congeners. Wanikawa *et al.* (2000) showed that lactic acid bacteria can produce lactones (described as having 'fatty' and 'sweet' aromas) by synthesising unsaturated fatty acids (UFAs) during fermentation. Furthermore, higher amounts of the UFA-derived hydroxy fatty acids (the intermediate reaction product) were formed when both brewer's and distiller's yeast was used than compared to distiller's yeast on its own. This could be because brewing yeasts, while they are slower to ferment, remain more active near the end of the fermentation (Paterson and Piggott, 1989). The duration of a fermentation may also be a contributing factor and can range from 40 to as long as 70 h, with 40-48 h considered normal. An example of why a longer fermentation may be implemented would be to allow lactic acid bacteria to thrive during the latter stages of fermentation (Geddes and Riffkin, 1989). However, this desire for bacterially derived congeners is often balanced with the concern that off-flavours may develop in the wash due to uncontrolled microbial contamination. At the end of the fermentation, the resulting wash has an ethanol content of around 8-10% alcohol by volume (ABV).

1.1.3 Malt Whisky Distillation

Batch or pot distillation using copper stills is used for the production of highly flavoured malt spirit, whilst continuous distillation in a Coffey still is favoured for grain spirit manufacture, where a subtler flavour is preferred, as it is grain whisky which is used as the base for blended whiskies.

Malt distillation has numerous functions which, aside from ethanol concentration, include the removal of solids and with regard to the effect on flavour; selection and elimination (governed by cut points) and formation (thermal reactions). An example of elimination is the removal of undesirable sulphurous compounds as a result of their interaction with the copper still surface which gives rise to solid copper sulphates, which are left behind in the bottom of the still. Of particular interest are the products arising from thermally induced reactions such as Maillard and lipid oxidation that take place at the still surface. Thus how the still is heated may have direct effects on the flavour profile of the whisky. It is less common now for distilleries to use a direct heat source (liquid petroleum gas or coal) as charring occurs at the base of the still, especially when particulate matter are transferred along with the wash. This requires an agitator to be used during distillation and the still to be cleaned more often, hence for efficiency many distillers now use indirect firing in the form of oil or steam coils, kettles or pans.

Figure 1.4 shows an example of wash and spirit stills in a distillery. Each still is comprised of three principal parts: the pot, the bulbous base of the still; a swan neck and a lyne arm (in Figure 1.4 this is angled downwards); and a condenser which cools the spirit vapours so they can be collected.

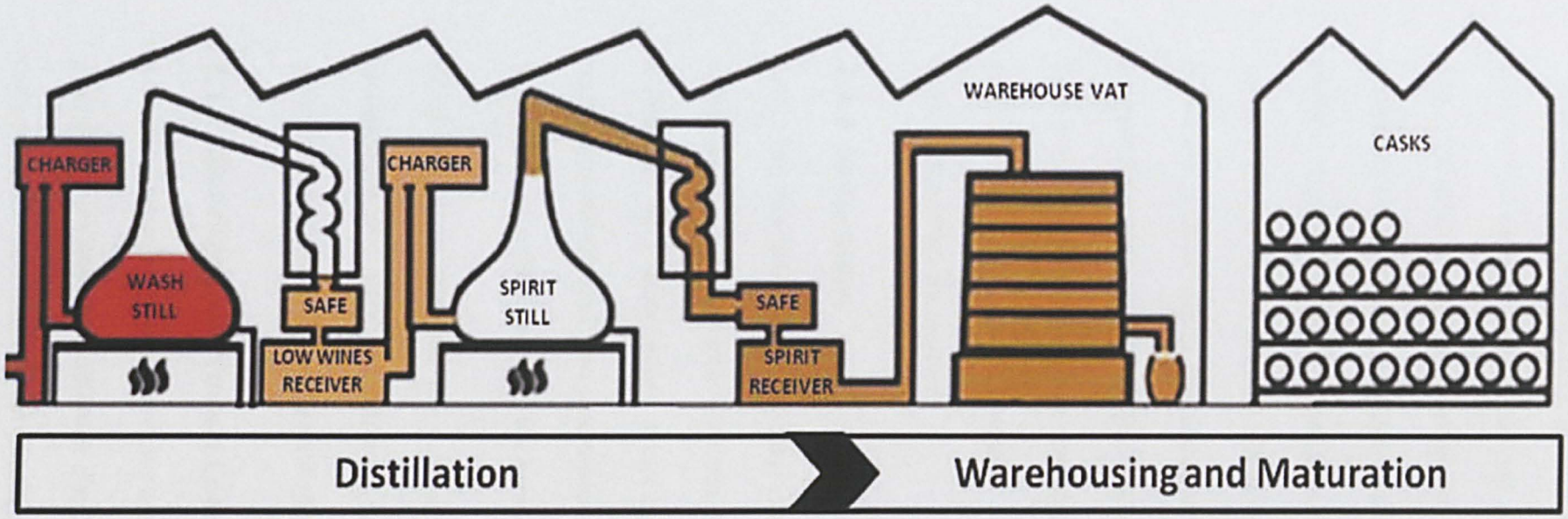


Figure 1.4 Simple cross-section diagram of malt whisky distillation showing the processes of double distillation, the filling of casks and maturation (Butler, 2008).

Firstly, the wash still is charged with the fermented wort (8-10% ABV) and boiled for 5-6 hr until the low wines that are condensed and collected in the receiver are approximately 23% ABV. This liquor is then transferred into the spirit still and heated once more. The spirit distillation is divided into three sequential fractions known as the foreshots, the 'heart' or middle cut, and the feints. The foreshots (85% ABV) contain the majority of the highly volatile compounds and are not deemed worthy of collection as a potable spirit and are collected in the feints receiver. When the ethanolic content is reduced to 75% ABV and considered potable, the middle cut starts to be collected in the spirit receiver as new make malt spirit. The third and final fraction, the feints, consists of the heavier, less volatile and unpleasant-smelling aroma compounds the feints. These are transferred to the feints receiver, where along with the foreshots, they are recycled.

1.1.4 Maturation

Before the new make spirit (70% ABV) can be transferred into oak casks for maturation, it is diluted to a cask strength of 60-65% ABV. Once inside the cask, there the spirit will remain for a minimum of 3 years before it can be called Scotch. Wood-derived congeners will be transferred into the spirit and simultaneously subtractive reactions take place where the spirit meets the cask surface to reduce the harshness of the original spirit.

Whilst it may be said that the maturation process is of considerable importance in the development of a more acceptable flavour, ultimately the final flavour is governed by the starting composition of the newly distilled spirit.

1.1.5 Blending and Product Consistency

A combination of grain and malt spirit are used to create a blended whisky – more than 10 grain distilleries and up to 100 malt distilleries may be used to create the perfect blend. In comparison to single malt or grain whiskies, blended products have a smoother flavour and are therefore more popular with the buying public. Take into account the fact that blended

whiskies account for more than 90% of the market share (Jack, 2003), and that Scotch whisky exports worldwide continue to achieve in excess of £4bn for the UK economy, and one begins to realise the value of a consistent blended product.

Each distiller has a specific spirit aroma character which they strive to achieve with every batch they produce. However, as with any industry, there may be fluctuations in the aroma quality of the spirit produced. If the new make is destined for a blended whisky, then this is less of a problem as this can be rectified by skilful blending after maturation, or by substitution of another spirit with a similar aroma quality. Indeed, such is the necessity for uniformity of the final product, should a distillery find they have produced an excess of spirit with a particular flavour but are lacking in another, and this cannot be remedied by blending, then they may swap some of their surplus spirit with that of another distillery which produces spirit of the character required.

Whisky is considered a premium product, so there is an expectation from the consumer that the bottle of whisky they purchase will have the same aroma quality as the last. Therefore it would be advantageous for the distiller to guarantee consistency by controlling the production of key flavour congeners responsible for desirable aromas throughout the manufacturing process. Indeed, research has shown congeners which contribute nutty and malty flavours are considered desirable by consumers in deluxe brand whiskies (Lee *et al.*, 2001).

1.2 IMPORTANT REACTIONS FOR NUTTY/CEREAL AROMA VOLATILE DEVELOPMENT

Where the production of nutty/cereal aroma compounds in foods and beverages are concerned, there are two chemical reactions of particular significance: the Maillard reaction and lipid oxidation. The Maillard reaction is a complex cascade of reactions, giving rise to the formation of furan compounds, Strecker aldehydes and nitrogen-heterocyclics. Complex lipids and fatty acids can be oxidized enzymically or chemically to produce highly odorous low molecular weight compounds such aldehydes and ketones.

1.2.1 Maillard Reaction

The Maillard reaction is initiated by the condensation process occurring between a free amino group and the carbonyl group of a reducing sugar. In the processing of malts, the amino acid proline is abundant therefore providing the amino group. The carbonyl group may be donated by a simple sugar found in the malt, such as maltose (Tressl *et al.*, 1983). As shown in Figure 1.5, the direct product of an amino group and a reducing sugar is an unstable Schiff base (e.g. N-glycosylamine) which then partially isomerizes to a more stable amino ketone such as 1-amino-1-deoxy-2-ketose or 2-amino-2-deoxy-1-aldose, which are known as the Amadori and Heynes rearrangement products, respectively (Rizzi, 1994).

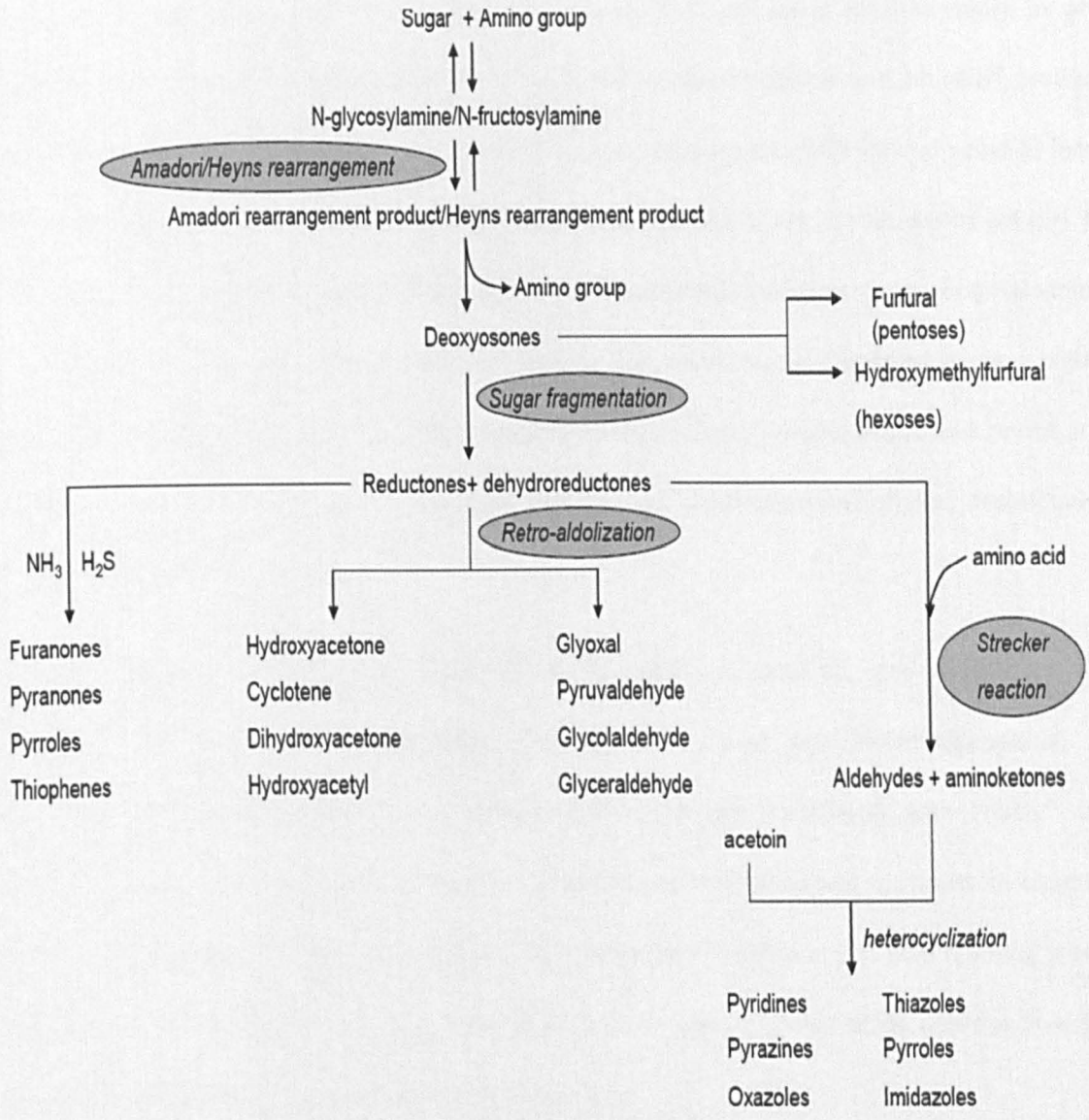


Figure 1.5 Schematic diagram of potential Maillard reaction pathways governed by variables such as temperature, pH, moisture and the availability of reactants. Interaction of precursors with unstable intermediates adds to the complexity of this reaction (Van Boekel, 2006).

As the reaction rate increases with higher temperatures, the Maillard reaction, as a manner of flavour formation, is particularly influential during thermal processes such as distillation and during the kilning of the malt (Jack, 2003). Maillard reaction pathways are also driven by pH, for example in alkaline conditions (> pH 7) the Schiff base compound and Amadori product undergo chain fragmentation, forming 2- and 3-carbon compounds which further react to form melanoidins (Hayashi and Namiki, 1986). Alternatively, at acidic (< pH 5) and neutral pH (pH 7), the reaction progresses much more slowly; the Amadori product eliminates the original amine to yield either 3- or 1- isomeric forms of deoxyosone respectively. Deoxyosones are highly reactive intermediates (Figure 1.5) which undergo dehydration by monosaccharides xylose and glucose to produce the furan compounds furfural and hydroxymethylfurfural, respectively (Rizzi, 1994).

Strecker degradation, is another pathway of the Maillard reaction, and allows for the formation of the Strecker aldehydes. Examples of these are 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, compounds which are associated with "nutty" and "malty" aromas, and have been shown to contribute to the perceived nuttiness in cheddar cheeses (Avsar *et al.*, 2004). Strecker aldehydes are formed from an amino acid reacting with a dicarbonyl compound (reductones). Some examples of parent amino acids and the Strecker aldehydes derived from them are shown in Table 1.1.

Table 1.1 Examples of parent amino acids which take part in Strecker degradation as part of the Maillard reaction.

Parent Amino Acid	*Strecker aldehyde*
valine	2-methylpropanal
isoleucine	2-methylbutanal
leucine	3-methylbutanal
methionine	methional
phenylalanine	phenyl acetaldehyde

Following on from the Strecker reaction, in the later stages of the Maillard reaction, nitrogen (N), sulphur (S) and oxygen (O) heterocyclic compounds are formed. It is the nitrogen heterocyclics such as, pyrroles, pyridines and pyrazines which are said to exhibit 'cereal', 'nutty', 'roasted' and 'bready' aromas (Vernin and Vernin, 1982; Jack, 2003). Pyrazines with an alkyl substitution are often described as having nutty/grassy aromas, whereas a thiol group creates a pyrazine with nutty/biscuit-like aroma. Thiazoles have a green, vegetable type aroma, however with increasing substitution of the aromatic ring nutty, roasted and meaty notes become more dominant (Varnam and Sutherland, 1994).

From the Maillard reaction alone, three groups of flavour compounds from which nutty/ cereal flavours may originate have been identified: furan compounds, Strecker aldehydes and the nitrogen heterocyclics.

1.2.2 Lipid Oxidation

Alongside the Maillard reaction, and also reacting with its products and precursors is another key flavour forming reaction: the enzymic and chemical oxidation of fatty acids and lipids (Paterson and Piggott, 1989). Fatty acids are the building blocks of lipids, and both are degraded on heating, to form secondary carbonyl products such as aldehydes and ketones (Lingnert *et al.*, 1983). These secondary lipid oxidation products are often characterized by low aroma thresholds, hence their formation, even in small amounts, is likely to impact on the overall aroma of the spirit (Farmer and Mottram, 1994; Belitz *et al.*, 2004). In the barley grain the most abundant fatty acid is linoleic acid (18:2). Malt contains the lipoxygenase enzymes which convert linoleic acid to 9- and 13-hydroperoxide intermediates. The hydroperoxides undergo further reactions resulting in trihydroxy fatty acids, which can in turn become the precursors for the production of aldehydes such as hexanal and trans-2-hexenal.

1.3 THE EXTRACTION AND DETECTION OF AROMA VOLATILES IN FOODS AND BEVERAGES

As mentioned by Augusto and his colleagues (2003), difficulties associated with the extraction of flavour compounds are that they are often present in low concentrations, yet substances may be perceptible at less than 1 ng/L by the human olfactory system. Therefore, the analytical method used must be capable of very high levels of sensitivity if detection and subsequent quantification are to be achieved. Another issue to consider when extracting flavour congeners is the likelihood of complex blends of compounds (Augusto *et al.*, 2003) which may have "synergistic" or "masking" interaction effects (Jack, 2003). Choosing a suitable isolation procedure is fundamental to success in the chromatographic resolution of key aroma compounds and for complex systems such as whisky, modern up-to-the-minute techniques are required (Augusto *et al.*, 2003). Another aspect for consideration when sensory properties are to be evaluated is that for the purposes of consistency, the extract isolated from the matrix should represent the aromas perceived in the original sample (Plutowska and Wardencki, 2008).

1.3.1 Extraction of Aroma Volatiles from Foods and Beverages

Direct solvent and solid-phase techniques were among the first to be used for the extraction of flavor volatiles.

1.3.1.1 Solvent Extraction

Solvent extraction (SE), or liquid-liquid extraction (LLE) is often made use of in the initial stages of most isolation strategies (Poisson and Schieberle, 2008). Where sensory analysis of the extract is concerned, the solvent chosen must not have so strong an odour that it masks the aroma being analysed. The use of LLE may be advantageous when the compounds are not of sufficient volatility to be detected in the headspace, especially when present in low concentrations (Augusto *et al.*, 2003). However, used as the only method of isolation, LLE can

yield very complex extracts resulting in the co-elution of a number of peaks in GC-O, making the identification of specific aroma compounds very difficult (d'Acampora Zellner *et al.*, 2008).

1.3.1.2 Solid Phase Extraction

Campo *et al.* (2007) successfully extracted four ethyl esters in whisky, wine and brandy using selective solid-phase extraction (SPE) and quantitatively analysed using multidimensional GC-MS. Careful method optimisation of the SPE and the injection of a large volume resulted in high percentage recoveries for the spiked compounds and a good limit of detection was achieved. By choosing the appropriate SPE column and optimising the conditions to suit the elution of group of analytes required, then SPE can be a useful method of extraction, especially for the recovery of semi-volatiles. Ferreira *et al.* (2003) used polymeric LiChrolut EN resin [poly(styrene-divinylbenzene), PSDVB] with DCM elution to extract trace polar compounds sotolon, maltol and free furaneol from wine. As we expect that the volatile compounds we are interested in are also polar and likely to be present in low amounts (such as pyrazines), this particular sorbent will be used for solid-phase extractions of the spirit samples. A drawback of using a selective SPE sorbent to specifically isolate a group of compounds is that quantification of a compound extracted by SPE may not be representative of the whole concentration within the foodstuff or beverage. If you require an exhaustive extraction for quantification purposes it may be wise to choose solvent extraction over SPE.

1.3.1.3 Solid Phase Microextraction (SPME)

Less time consuming than previous methods, SPME requires minimal sample prep and allows for direct, solvent-less analysis of a foodstuff or beverage. In a SPME vial there are three phases; the sample, the headspace above it and the fibre itself. SPME is based on the partitioning of the volatiles in the headspace on to the fibre and thus relies upon complete equilibrium for successful analysis. Akiyama (2003) and his colleagues compared the effectiveness of dynamic headspace with solid-phase microextraction to characterize the

volatile compounds present during the grinding of roasted coffee beans with that of a "conventional" static SPME method using ground coffee. Both GC-MS and GC-O were used. Four "nutty-roast" compound volatiles were recorded, of which three were pyrazines. It was concluded that the dynamic SPME method afforded higher adsorption to the fibre, as reflected in the higher levels of sensory response values (CharmAnalysis – see Section 1.3.2.2) for that technique (Akiyama *et al.*, 2003). It has been used extensively and to good effect for the analysis of whisky (Fitzgerald *et al.*, 2000; Demyttenaere *et al.*, 2003; Caldeira *et al.*, 2007; Câmara *et al.*, 2007). As made evident in each of the articles mentioned here, SPME requires the user to ensure full optimisation of the running parameters in order to obtain accurate and reproducible results.

1.3.1.4 Fractionation Methods

From the literature studied, two methods of fractionation are favoured in the characterization of flavour volatiles. One is instigated by manipulation of the pH to yield a neutral/basic fraction (extracted at high pH) and an acidic fraction (low pH) (Avsar *et al.*, 2004); the other is fractional chromatography, where the extract is separated by means of increasing polarity (Bonvehi, 2005). Fractionation is of notable use in the analysis of complex mixtures of compounds, such as whisky, which can contain over eighty different flavour congeners. Poisson & Schieberle (2008) actually used both of these fractionation techniques to ensure the isolation of only the "most odour-active" volatile compounds in American Bourbon whisky. In doing so, they were able to identify 45 flavour congeners, among which 13 were identified in whisky for the first time.

It is recommended that for the best isolation of flavour volatiles a combination of extraction techniques should be employed: tailored to the specific extraction of the compounds of interest, taking into account their chemical properties.

1.3.2 Detection of Aroma Volatiles in Foods and Beverages

1.3.2.1 Gas Chromatography-Olfactometry (GC-O)

Gas chromatography-olfactometry is an invaluable technique for the detection of aroma-active flavour volatiles. On leaving the GC column, the eluent is split between two detectors: one is a mass spectrometer (MS), the other is the human olfactory system. By sniffing at a specially designed nose port, the assessor can perceive the odour compounds leaving the column while the MS simultaneously detects them. Due to this synchronized method of odour and peak detection, it is possible to correlate the odours being perceived with the compounds being detected on the GC-MS chromatogram.

There are certain requirements which must be met for successful GC-O analysis. Firstly, the laboratory must be free of all foreign odours and sounds and kept at constant temperature. The panellists must be adequately trained in order to achieve reliable and consistent results. Due to the differences in pressure between the MS (under vacuum) and the odour port (atmospheric), there may be slight delay with respect to the port. This can be resolved by fitting a restrictor just before the MS, decreasing the pressure between the "interface and the flow splitter" (Plutowska and Wardencki, 2008). The individual assessing the odours may feel discomfort owing to the drying of the mucous membranes of the nose during long periods of analyses. This can be overcome by shortening the length of time any one assessor is sat at the port, or by the addition of moist air to the eluent escaping from it. Often the concentration of the compound in the sample is inversely proportional to its relative importance within the sample aroma. For example, a large peak in GC may not necessarily mean that compound will exhibit a strong odour intensity at the port, and often aroma volatiles present in low concentrations are readily perceived at the odour port, yet not easily visualised as a peak in the GC chromatogram. The reason for this discrepancy is that all volatile compounds have specific aroma thresholds which relate to how well they bind to the olfactory receptors in the

nose. Often measured in ppb (parts per billion) or ppm (parts per million), once the level of the compound reaches that threshold value, it can be sensed, in spite of what trace concentrations the compound is actually present. The GC-MS however, has a fixed lower limit of detection, below which it cannot resolve peaks.

1.3.2.2 Sensory Techniques used to measure perception in GC-O Analyses

The two quantitative methods primarily used in GC-O analysis are detection frequency methods and dilution to threshold methods. Detection frequency methods are sometimes known as Surface Nasal Impact Frequency (SNIF). This method, pioneered by Pollien and his colleagues in 1997, uses a minimum of six assessors who analyse the same sample at one dilution level. The percentage of people who sense the odour compound at a given retention time is recorded. Therefore those aromas smelled by everyone are recognised as having the most influence on the sample odour. Similar to the concept of probability, the olfactometric indices employed by this approach are 0 and 1. 0 signifies no odour perceived by any of the evaluators at that retention time, and 1 is used to express when all of the evaluators sensed the odour. Advantages of such a technique are that it is simple, no prior training of the panellists is required, it has good repeatability, and the least time-consuming method for GC-O aroma quantification (Pollien *et al.*, 1997). A limitation of this approach, however, is that it does not take into account the intensity of the odour, therefore as long as an odour compound is present above its threshold concentration, (able to be sensed by all evaluators) regardless of its concentration, the results of the analysis may be the same. Ferrari *et al.* (2004) used the principles of NIF (Nasal Impact Frequency) in their analysis of the main odour components of freshly distilled cognac. This study may be of relevance to the topic of research as it is newly distilled whisky which will be provided for aroma analysis. Of the 150 volatiles identified by GC-MS, around a fifth (34) were shown to have a substantial impact on overall cognac aroma.

In the analysis of odours of alcoholic beverages, dilution to threshold methods are most frequently used. In all dilution analysis methods, a series of increasing dilutions of the sample is prepared and analysed by GC-O.

Aroma Extract Dilution Analysis (AEDA) developed by Grosch (1993), is a popular method and has been used to assess odour activity in red wine (Aznar *et al.*, 2004; Escudero *et al.*, 2007; Poisson and Schieberle, 2008) and bourbon whiskey (Poisson and Schieberle, 2008). It can provide both qualitative and quantitative data relating to the compound's "odour potential". This information is based on the ratio of concentration within the sample and the airborne aroma threshold (Delahunty *et al.*, 2006). AEDA is achieved by using a series of two to ten-fold dilution levels. The evaluators record under which dilution level the compound can still be sensed whilst providing, where possible, a description of the smell (Plutowska and Wardencki, 2008). Odour Activity Values (OAVs) represent the ratio of the concentration of a given substance in the sample to the sensory detection threshold. Thus, AEDA measures the highest level of dilution at which the odour being investigated can be still be sensed.

In addition to the above, 'CharmAnalysis' can also measure the duration of time during which the odour is perceived, allowing for the determination of specific chromatographic peaks (Acree *et al.*, 1984). This is achieved by combining all the instances when a smell is perceived becoming a graph which, like a chromatogram, consists of peaks that can be integrated to produce peak areas or "charm values". The size of the charm value corresponds with the level of contribution of that peak to the overall aroma.

1.3.2.3 Atmospheric Pressure Chemical Ionisation – Mass Spectrometry (APCI-MS)

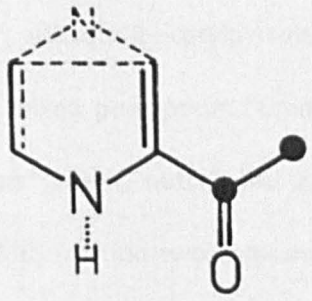
Another name for the APCI-MS is the MS-Nose, so called because it allows for the real time analysis of trace aroma volatiles (< 10ppb) and can be used in a wide variety of applications. A simple instrument, it consists of an inlet, where the liquid or gaseous sample is introduced,

and an ionising source which can be the form of the corona charge. Once the molecule is ionised, detection is by a quadrupole mass spectrometer which is maintained under vacuum. It is ideal for measuring aroma release in-vivo, for example by sampling the nasal breath of the participant while they drink or eat. Unlike GC-O where the compound is fragmented into component parts, APCI uses a soft ionisation technique, whereby the molecule (R) is protonated to become (RH^+). Of particular note is the option to use ethanol as a protonator instead of water (Aznar *et al.*, 2004; Tsachaki *et al.*, 2006) which allows for the more accurate measurement of aroma volatile concentrations in ethanolic systems such as that of a model of a whisky (Boothroyd *et al.*, 2012). This technique is used in Chapter 5 for this purpose.

1.3.3 Nutty/cereal aroma volatiles in alcoholic beverages and other food flavour systems

Various nutty flavour volatiles have been determined in other food flavour systems. Meilgaard (1982) puts forward some aroma volatiles which he states are responsible for caramelised, roasted and cereal flavours in beer; long chain aldehydes, and oxygen, nitrogen and sulphur-heterocyclics which are also known to arise from lipid oxidation and Maillard chemistry. He cites aliphatic alcohols, esters and aldehydes as having the potential to contribute nutty, green and grassy flavour notes to beer. Wanikawa *et al.* (2002) sought to identify aroma compounds in new malt whisky spirit associated with a "green note", using multidimensional gas chromatography with olfactometric analysis. During the sensory analysis of the GC-O four aromas were described as "nut", yet no other information is given other than a chromatogram demonstrating approximately when the corresponding "nut" peaks were eluted from the column. However, although they profess to have identified two aldehydes and three alcohols which may contribute to the "green" aroma, they do not show proof of any reference standards used to justify this claim, and the extraction method is described incompletely, deeming it unrepeatable and thus its validity cannot be proven.

Tressl *et al.* (1983) discussed the lipid and in particular the Maillard products which they cited as compounds responsible for the flavours in malt. Notable was the similar molecular arrangement of 5- and 6-membered monosubstituted nitrogen heterocyclics which exhibited the same cereal, nutty and bready aroma characters (Figure 1.6). The 6-membered compound with both nitrogens is 2-acetylpyrazine, with only 1 nitrogen it is 2-acetylpyridine, and as the 5 membered ring (1 nitrogen) it is 2-acetylpyrrole. Descriptors are popcorn/hazelnut, popcorn/fatty and musty/nutty respectively, which is in agreement with the finding of Tressl *et al.* (1983).



CORNY, NUTTY, BREADY

Figure 1.6 Diagram showing the similar molecular configurations of the various 5 and 6-membered monosubstituted nitrogen heterocyclic compounds found in malt, all of which are associated with 'corny, nutty, bready' aromas (Tressl et al., 1983). The 6-membered, 2N compound is 2-acetylpyrazine, the 6-membered, 1N compound is 2-acetylpyridine, and the 5-membered 1N molecule is 2-acetylpyrrole.

Avsar *et al.* (2004) sought to determine the nutty flavour characteristics of young and matured cheddar cheeses. The cheeses were extracted using solvent extraction and distilled under high vacuum. The extracts of cheeses with and without nutty flavour were analysed using dynamic headspace GC-O. Of the five nutty flavour volatiles identified in the neutral/basic fraction of the nutty cheddar cheese extracts, four were pyrazines; tetramethylpyrazine ("nutty"); 2-isopropyl-3-methoxypyraz-ine ("dirty, nutty"); 2,3-diethyl-5-methylpyrazine ("sweet, nutty"); 2-isobutyl-3-methoxypyrazine ("nutty").

In his detailed investigation into the aromatic compounds of roasted cocoa, Bonvehi (2005) was able to identify a plethora of nutty flavour compounds. The sample preparation method consisted of a combination of methods. Firstly reduced pressure steam distillation, followed by simultaneous steam distillation and solvent extraction, after which the distillates were fractionated by their polarities using adsorption chromatography on 3 g of silica gel 60 (activity II-III, Merck) using a column (200 x 0.9 mm i. d.). Solvents of increasing polarity (varying ratios of pentane/DCM) eluted 6 fractions. Over one hundred aroma compounds were successfully detected and identified by GC-MS. Among them, those described by the word "nutty" accounted for eight. In the fourth fraction, pyrrole was described as "nutty, sweet", and 2-acetyl-5-methylfuran as "strong, nutty", whereas 2-acetylpyrrole and 2-acetyl-1-methylpyrrole were associated with somewhat of a mixed perception: "bread, walnut, licorice". The fifth fraction saw linalool oxide depicted as "sweet, nutty" and 2, 3-diethylpyrazine as "nutty, hazelnut, cereal, meaty". Also in the fifth fraction two compounds were described as "malt, roasted nuts": 3-hydroxy-2-methyl-4-pyrone and pyrrole-2-carboxaldehyde. From these last three compounds we can see that the aroma compounds described as having nutty character are often also described as having "cereal" or malty flavours. The sixth and final fraction, depicts a further six compounds as having a "nutty" or "roasted nuts" aromatic quality. All of which are highly polar pyrazine compounds (Bonvehi, 2005). This information is in accordance with other sources with respect to nutty/ cereal flavour characteristics, therefore lending support to the theory that I am looking to isolate highly polar compounds. As with the manufacture of whisky, Bonvehi (2005) refers to the key role of the Maillard reaction in the formation of important flavour volatiles such as the heterocyclic compounds which occur during the roasting process.

1.3.4 Correlation of Sensory and Chemical Data

The statistical comparison of sensory and chromatographic data allows for the significance testing of the relationships between the two. This chemometric evaluation is a vital part of flavour congener identification, proving whether a correlation exists, linking the chemical analysis with the sensory. Lee *et al.* (2001) used multivariate chemometric analyses to distinguish between the aroma characteristics of 40 blended Scotch whiskies, from "Deluxe" to "multiple retailer" brands. A sensory panel of 26 were trained to use specific descriptors, agreed upon by using reference standards. Analysis of variances (ANOVA) and a Principal Component Analysis (PCA) were implemented to determine the most significant characteristics for discrimination between the whiskies, of which "nutty" was significant. Furthermore, both the "nutty" and "malty" attributes were considered to be characteristics mainly perceived in the "Deluxe" blends. This study therefore supports the notion that these particular features of whisky are highly regarded.

1.3.5 Overview of Research

The main aim of the research undertaken was to identify compounds which contribute to the nutty and cereal aroma character on new make spirit. The more that is understood regarding the identity and possible origins of flavour congeners which impart these specific spirit characters, the more this should enable the distiller to control these characters during manufacture and thus maintain a consistent product. This thesis aims to increase our understanding of this area, in particular with regard to the aroma compounds which could be responsible for nutty and cereal character in new make spirit.

The first objective of the research was to develop an extraction method which would isolate and concentrate odour active volatile compounds associated with these attributes in immature whisky so that they could be detected by both panellists and by gas chromatographic methods (described in Chapters 3 and 4). Statistical significance tests were applied to instrumental and

sensory data relating to two spirits of low and high nutty/cereal character, in order to ascertain differences between the concentration of the nutty and cereal odour compounds as confirmed by Gas Chromatography-Olfactometry. One of the recurrent differences noted in chemical composition of the distillates, the disparate levels of ethyl esters, led to the exploration in Chapter 5 of the physicochemical properties of a large ethyl ester and its ability to affect partitioning of other aroma compounds at various ethanolic concentrations by measurement of headspace volatiles. Chapter 6 describes the laboratory scale distillations that were undertaken to test the hypothesis that a lipid-rich fermented wash will produce spirit with nutty and oily aroma character, with further GC-O to elucidate the odour activity of compounds. In Chapter 7, a large sample set of new make spirits are extracted and analysed by the method previously developed, and multivariate statistical analysis was applied to show trends between congener composition and the sensory scores of the various distillates.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 New Make Malt Spirit Samples

Five new make spirit samples (A-E; 60-70% ABV) were sourced from Scotch malt distilleries and provided by the Scotch Whisky Research Institute, Riccarton, Edinburgh, UK. Spirits A, B, C and D had been scored as having a 'nutty' character by the SWRI panel, whilst E was said to have minimal nutty character and thus served as the control for 'non-nutty'. Thirty new make malt spirit samples were obtained in order to perform the multivariate statistical analysis carried out in Chapter 7. In addition to these, an immature malt spirit sample which had been produced using an undisclosed proportion of chocolate malt, was received from an industrial source.

2.1.2 Standards and chemicals

The following authentic compounds (> 97 % purity) were obtained from Sigma Aldrich (Poole, Dorset, UK): 2-acetylfuran, ethyl-L-lactate, pyrazine, 2-furanmethanol, 2-furaldehyde (furfural), 2-methylpyrazine, 2,5-dimethylpyrazine, 2-acetylthiazole, benzaldehyde, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, β-damascenone, 1-octen-3-ol. Ethyl hexadecanoate was obtained from Alfa Aesar (VWR, Lutterworth, Leicestershire, UK). Dichloromethane (DCM) ENVISOLV GC-MS grade, Fluka Analytical (Sigma Aldrich, Poole, Dorset, UK) Methanol (Fisher Scientific, Loughborough, Leicestershire, UK).

2.1.3 Other Consumables

SPE Cartridges that were used for the work undertaken in Chapter 4 were LiChrolut EN (500 mg/ 6mL; Merck KGaA, Darmstadt, Germany) and Strata-X (200 mg/6 mL; Phenomenex, Torrance, CA).

2.2 METHODS

2.2.1 Liquid-Liquid Extraction (LLE)

New make spirit (100 mL) was spiked with an internal standard (2-acetylthiazole; 10 µg/mL) , diluted with water (400 mL) and twice extracted with dichloromethane (200 mL) using a 1 L separation funnel. The two dichloromethane fractions were combined and concentrated to 1 mL under a stream of nitrogen whilst being heated in a 40 °C water bath. All five new make spirits (A-E) were extracted in triplicate using this method.

2.2.2 Solid-Phase Extraction (SPE)

New make spirit (5 mL), was diluted with 25 mL water. The mixture was shaken and allowed to equilibrate for a minimum of 4 hours before application onto the SPE column.

The LiChrolut EN Column (Merck KGaA, Darmstadt, Germany) with a sorbent bed of 500 mg was placed in the vacuum manifold (Phenomenex, Torrance CA), conditioned with 8 mL methanol and equilibrated with 8 mL aqueous solution in 10% ethanol. The diluted spirit was loaded on to the column and was allowed to fully saturate the sorbent bed for 1 minute before a gentle vacuum was applied. Once the sample had been loaded, care was taken not to allow the bed to run dry until after the wash step, at which water (5 mL) was applied to the column. The sorbent bed was then dried by applying the vacuum for 30 minutes (10 kPa). Elution was with dichloromethane (6 mL). This method was adapted from Ferreira *et al.* (2003) who used it to extract the highly polar compounds sotolon, maltol and furaneol from wine.

2.2.3 Drying and Concentration of Solvent Extracts

When it was noted that there were globules of water floating on the dichloromethane extract, anhydrous magnesium sulphate (Sigma Aldrich; Poole, Dorset, UK) was used to dry the extract before the concentration step. The solvent was then decanted into a conical flask, where it was heated in a water bath at 37 °C. Thus, DCM extracts were concentrated down to 1 mL

under a stream of nitrogen. This method of concentration was used for both LLE and SPE solvent extracts.

2.2.4 Ethanolic Atmospheric Pressure Chemical Ionisation - Mass Spectrometry (APCI-MS)

When working with a system where ethanol is present in such high levels, the highly volatile nature can have an adverse effect on the detection of other less abundant volatiles. It is therefore advantageous to remove this competing element by introducing ethanol directly into the source, using it as an ion protonator in place of water. This method was developed by Aznar *et al.*(2004).

Headspace concentrations were measured on a Platform LCZ APCI mass spectrometer fitted with an MS Nose interface (Micromass, Manchester, UK) and with a modified source designed to operate at high and differing ethanol concentrations as described previously (Aznar *et al.*, 2004). The flow of ethanolic vapour to the source was controlled using a mass flow meter (Aalborg, Orangeburg, U.S.A.) and was adjusted depending on the ethanol concentration in the sample (0-200 mL/min). The APCI source was operated with a corona voltage of 15 kV. All analyses were performed in selected ion mode, whereby the protonated ion ($[M+H]^+$) of each volatile compound was specifically monitored to increase the sensitivity of measurement. Sampling flow rate (2 mL/min) was measured using a VeriFlow 500 gas flow meter (Humonics Inc., Folsom, CA). The heated transfer line (Hillesheim, Waghausel, Germany) was maintained at 170 °C.

2.2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

Solvent extracts were analysed using a ThermoScientific Trace GC Ultra with a DSQ II mass spectrometer and AS 3000 Autosampler (Thermo Electron Corporation, Altrincham, Cheshire, UK). Compounds were separated on a Zebron WAX column (30 m x 0.25 mm i.d., 1.0 µm film thickness) starting at an oven temperature of 40 °C (1 min hold) followed by a ramp to 250 °C

at 8 °C min^{-1}. The Helium carrier gas flow rate was 1.5 mL/min and injection (1 µL) was splitless. The mass spectrometer was operated in full scan mode over the range *m/z* 35-250. Identification and quantification of compounds was achieved using the Qual and Quan Browser applications of Xcalibur Software (Thermo Electron Corporation, Altrincham, Cheshire, UK). Concentrations were calculated by use of an internal standard 3-heptanone (0.1 µg/mL).

2.2.6 Gas Chromatography-Olfactometry (GC-O)

Gas chromatography-olfactometry is an invaluable technique for the detection of aroma-active flavour volatiles. On leaving the GC column, the eluent is split between two detectors: one is a mass spectrometer (MS), the other is the human olfactory system. By sniffing at a specially designed nose port, the assessor can perceive the odour compounds leaving the column while the MS simultaneously detects them. Due to this synchronised method of odour and peak detection, it is possible to correlate the odours being perceived with the compounds being detected on the GC-MS chromatogram. A splitter was fitted to the DB-WAX column, so half of the flow was diverted to the odour port, connected to the oven by a heated mass transfer line set at 180 ^{0}C. In preliminary GC-O runs with experienced assessors, the temperature gradient of 8 ^{0}C min^{-1} was deemed too fast to distinguish between odours, therefore 4 ^{0}C min^{-1} was used instead. The assessors were not told which extract they would be detecting. As the runs were 52 minutes long, 2 assessors were required for each run. After approximately half the run time had elapsed (26 minutes), the second assessor took over from the first. Run halves were overlapped wherever possible to avoid one assessor missing a section of the chromatogram. The assessors were asked to record the retention time at which they perceived an odour, an appropriate descriptor, the intensity of that odour (on a scale of 1-3) and its duration. Emphasis was placed on the intensity and duration of the smell as the main aim of the experiment was to distinguish the odour active areas of the chromatogram.

2.2.7 Sensory Analysis of Distillates

New make spirit samples were prepared for sensory evaluation by dilution to 20% ABV to reduce the pungency of ethanol. Trained panellists scored each spirit sample using Quantitative Descriptive Analysis (QDA) (Stone *et al.*, 1974). Sensory attributes focused on in this work were those relating to nutty, cereal, oily and feinty characters. The attribute 'feinty' is an attribute associated with the heavy, fusel alcohols which exit the still at the end of the distillation, otherwise known as the feints. Mean scores from the panel were calculated and ANOVAs were carried out for each attribute to determine whether there were any significant differences between the spirits.

3 LIQUID-LIQUID EXTRACTION AND GAS CHROMATOGRAPHY-OLFACTOMETRY OF NEW MAKE SPIRITS

3.1 AIM

To extract and concentrate volatile aroma compounds from new make spirit samples, and use Gas Chromatography-Olfactometry to identify compounds or areas of odour activity which might contribute to the nutty/cereal character of the spirit.

3.2 INTRODUCTION

Liquid-liquid extraction, (LLE) otherwise known as Solvent Extraction (SE) is often made use of in the initial stages of aroma compound isolation strategies, with the aim that the extract should be representative of the whole sample. Liquid-liquid extraction works on the basis that the affinity of analytes for the extracting solvent is greater than the affinity they have for the aqueous phase, hence the ability to isolate them from an aqueous/ethanolic sample such as whisky or wine. Dichloromethane was used here because it is moderately polar organic solvent which can extract aroma compounds of varying polarity which makes it ideal for a whole spirit extraction. Where sensory analysis of the extract is concerned, the solvent chosen must not have so strong an odour that it masks the aroma being analysed (Plutowska and Wardencki, 2008). The use of LLE may be advantageous when the compounds are not of sufficient volatility to be detected in the headspace, especially when present in low concentrations (Augusto *et al.*, 2003).

In this chapter, Gas Chromatography – Olfactometry (GC-O) was used to screen extracts of new make spirit samples in order to identify odour-active areas of the chromatogram with cereal/nutty related aroma characters (for the GC-O methodology used see section 2.2.6). Five new make spirit samples, known to differ in respect of sensory nutty/cereal character, were supplied by SWRI for extraction and analysis. Within the odour-active regions that were

identified, any major qualitative and quantitative differences between the composition of compounds in the nutty and non-nutty control spirit, in particular those which could contribute to the perception of nutty/cereal character compound were investigated.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Shop-bought blended 8 year old Bell's whisky was used for method development and extraction efficiency experiments. New make spirit samples from anonymous distilleries were then used in further experiments.

3.3.2 New Make Spirit Samples

Five new make spirit samples (A-E) were obtained from the Scotch Whisky Research Institute. These had been rated by the SWRI sensory panel using Quantitative Descriptive Analysis to profile the flavour characteristics of the 5 spirit samples. Various attributes were scored using a scale of 0-3 and the average score determined across the panel (Table 3.1). Full scores for these attributes and others can be seen in Appendix 1.

Table 3.1 Mean sensory scores from SWRI panel (n = 15).

	A	B	C	D	E
Mean nutty score	0.82	0.94	0.72	0.69	0.51
Mean cereal score	1.10	0.88	0.94	0.76	0.37

See Appendix 1 for the full sensory scoring of the 5 new make spirits.

A, B, C and D had been scored as having a 'nutty' character by the SWRI panel, whilst spirit E was said to have minimal nutty character and thus served as our control or 'non-nutty' sample. Sample B was scored highest for nutty character, hence the GC-O chromatograms and olfactograms for samples B and E were compared in detail in order to try and identify

significant differences between them which might be driving the perceived nuttiness in sample B.

3.3.3 Solvent Extraction and Concentration of the Spirit Sample

Spirit (100 mL) was diluted with 400 mL water and subsequently extracted with two successive aliquots of dichloromethane (200 mL) in a 1 L separating funnel. The two dichloromethane extracts were combined and concentrated under a stream of nitrogen whilst being heated in a water bath at 37 °C. The combined dichloromethane extracts were concentrated down to 1 mL and transferred to a glass vial ready for GC analysis. Each spirit was extracted in triplicate.

3.3.4 Gas Chromatography-Olfactometry Analysis

Spirit extracts were analysed on ThermoScientific Trace GC Ultra with a DSQ II mass spectrometer and AS 3000 Autosampler (Thermo Electron Corporation, Altrincham, Cheshire UK). Compounds were separated on a Zebron WAX column (30 m x 0.25 mm i.d., 1.0 µm film thickness; Phenomenex, UK). Chromatographic conditions were as specified in section 2.2.6. Sequence of extracts analysed was randomised to remove any incremental order effects.

For GC-O work, a splitter was fitted downstream of the DB-WAX column, so that approximately half of the flow was diverted to the odour port which was connected to the oven by a heated and insulated transfer line (Hillesheim, Waghausel, Germany) maintained at 180 °C.

An initial screening oven temperature gradient of 8 °C min^{-1} was judged too fast for adequate separation or for panellists to distinguish between odours, therefore a ramp rate of 4 °C min^{-1} was used for odour port work. This entailed a chromatographic run-time of around 52 min. To minimize the adverse effects of this on panellist fatigue, 2 assessors were used to sniff each run. After approximately half the run time had elapsed, the 2nd assessor took over from the first. Run halves were overlapped wherever possible to avoid one assessor missing a section of the chromatogram. The assessors were asked to record the retention time at which they perceived an odour, to generate an appropriate aroma descriptor, and to record the perceived

intensity of that odour and, wherever possible, its duration. Emphasis was placed on the intensity and timing of the smell as it was important to distinguish the odour active areas within the chromatogram. Each spirit sample was assessed by 9 panellists (5 males and 4 females).

3.4 RESULTS AND DISCUSSION

3.4.1 Gas Chromatography of New Make Spirits

The most striking observation when looking at the chromatograms of spirit extracts for both nutty (sample B) and control (sample E) samples (Figure 3.1) is their similarity. At this stage, it was concluded that differences in aroma between the spirit samples must therefore be caused by compounds able to impart aroma at very low levels, or by subtle differences in the ratio of major components to one another. Aroma compounds differ substantially with regard to their aroma thresholds and it is quite feasible that peaks which are insignificant or obscured on a total ion chromatogram can have significant odour impacts.

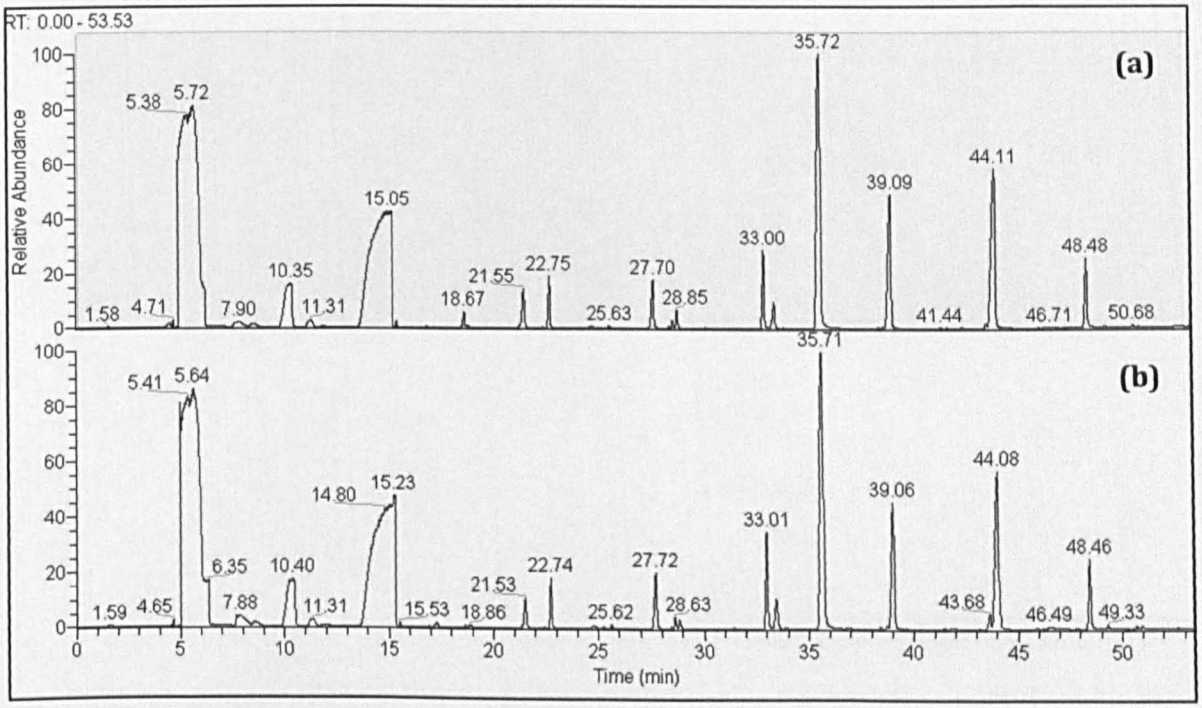


Figure 3.1 Total Ion Chromatograms of LLE extracts used for GC-O analysis: (a) spirit sample B (b) spirit sample E.

With the exception of furfural, the major peaks in the chromatogram were organic acids, esters and alcohols (Table 3.2). Although these quantitatively significant compounds may have a role to play in the overall aroma profile of the whisky, the odour descriptors typically associated with them do not suggest a direct role in the nutty and cereal characters that were of interest in this research. To try and characterise these it was necessary to use GC-Olfactometry.

Table 3.2 Major peaks in sample chromatogram W01 (Spirit B) as shown in Figure 3.1(a). Linear retention indices were calculated using an alkane series standard (C8-C21).

Retention Time (RT)	*Linear Retention Indices (LRI)*	*Compound*	*Known odour character**
5.00-6.35	960-1010	DCM (O/L)	n/a (solvent)
10.35	1130	2-methyl-1-propanol	bitter
11.31	1160	isoamyl acetate	banana
13.70-15.40	1200-1240	fusel alcohols: 2-methylbutan-1-ol 3- methylbutan-1-ol 1-pentanol	balsamic, alcohol bitter, harsh fruity
18.67	1375	ethyl-(L)-(-)-lactate	fruity
21.55	1460	ethyl octanoate	sweet, fruity
22.75	1500	furfural	woody, almond
27.70	1670	ethyl decanoate	fruity
28.85	1705	diethyl butanedioate	fruity
33.00	1850	2-phenethyl acetate	honey, apple
33.48	1870	ethyl dodecanoate	grape
35.72	1950	phenylethyl alcohol	roses
39.09	2090	octanoic acid	fatty acid
44.11	2215	decanoic acid	fatty acid
48.48	27.82	dodecanoic acid	Dry, metallic

* (Ferreira *et al.*, 2001; Lee and Noble, 2003; Cullere *et al.*, 2004; Venkateshwarlu *et al.*, 2004)

3.4.2 Gas Chromatography-Olfactometry of liquid-liquid extracts

In spite of the clear sensorial differences between the two samples, their similarity in major peak composition (Figure 3.1) hints at the potential significance of low-concentration constituents. For example, a compound present at the ppb level can have a larger impact on aroma than that of another one present at ppm levels; in this regard it is the ratio of concentration to odour threshold which is important. Also, it is likely that a mixture of compounds present at trace levels interact to produce each whisky's characteristic aroma, especially as a whisky can contain more than 80 aroma compounds (Jack, 2003).

The 'non-nutty' control (E) and nutty (B) extracts were analysed by GC-O as described in Section 2.2.6. As we were able to recruit 9 assessors, detection frequency methods were used. It has been shown when compared with other GC-O quantitation methods (outlined in Chapter 1) detection frequency gives a similar profile with regard to relationships of odour intensity to that of the overall sensory analysis of the headspace (van Ruth and O'Connor, 2001).

Panellists were asked to record when and how intensely they perceived an aroma. Significant areas of odour activity were assessed according to how many panellists recorded an aroma at a particular retention time. Table 3.3 summarises the regions in both chromatograms of spirit B and IH where 5 or more people recorded that they were able to perceive an odour at approximately the same retention time (RT). For clarification, the odours are classed as sharing the same RT if a) the observations are recorded as occurring within 0.5 seconds of one another and/or b) the panellists could perceivably be describing the same odour.

It should also be noted that when an aroma compound is present in the extract at a level above its odour threshold then it is likely that it will be picked up on by a similar number of panellists for both samples. Hence the odour port analysis (OPA) described in this chapter was used more as a tool to highlight the odour active regions of the chromatogram than in an

attempt to quantify or compare the sensory impacts of specific compounds on the characters of the two spirits.

Table 3.3 Odour active areas as identified by Gas Chromatography-Olfactometry of the spirit extracts B and E as extracted by LLE with dichloromethane.

		Detection Frequency (%)		*Panellists' Descriptors*		*Information sourced from Literature*		
OAA	**LRI**	**B**	**E**	**B**	**E**	**Possible Compound**	**Lit. LRI (DB-Wax)**	**Lit. Descriptors**
1	930-940	78	67	popcorn, chocolate, solventy/green, sweet, malty, marzipan, ethanol/sweet	baileys, solvent/alcohol, nutty, caramel, sweet chocolate, glue	2/3-methyl butanal (96-17-3 / 590-86-3)	926/ 936	Green, almond, strong burnt, malty, cocoa
2	990-1000	100	100	Bubblegum, solventy, chocolate, malty, methyl butanal/musty	malt, chocolate, sweaty aldehyde, animal feed, savoury	2,3-butanedione (431-03-8)	*983*	Buttery, caramel, cream, fruit, spirit
3	1030-1040	89	89	sl. fruity, buttery diacetyl, golden syrup, toffee, sweet/toffee popcorn, chocolate, caramel	buttery, caramel toffee, sulphury, vanilla, toffee popcorn, toffee butterscotch	2,3-pentanedione (600-14-6)	1041	Buttery, nutty, cheese
4	1080	89	67	tutti frutti, sweet almond estery, fruity, cape gooseberry, cheesy, chocolate/coffee, ethanol/sweet	nuts/chocolate, bubblegum, sweaty/off, sl. fruity, gooseberries & glue, alcohol	2-methylpropanol (78-83-1)	1085	Solvent-like, bitter, glue, alcohol
5	1160	89	67	pear drops, hept tanone, juicy fruit, refreshers	pear drops, heptanone, banana, refreshers	isoamyl acetate (123-92-2)	1147	Pear drops, banana

6	1230-1280	100	100	musty/green/malty (alcoholic), savoury, alcoholic, sweaty, faecal, meat, solventy, sulphur, wheat, yeasty, cherry brandy, malty/savoury/sweaty, almond, sulphur, wheat/grass, acidic, nutty, biscuit, marzipan, burnt toffee, glue, plastic/ phenolic	cinnamon, dark/roasty chocolate, solventy sl. caramel, sweaty/nutty, solvent, animal feed, sweaty cheese, rot, alcoholic/fruit/sweet chocolate, marzipan/benzaldehyde/tutti frutti, strong plastic/phenolic, solventy green, adhesive, sharp, pineapple, fruity	Fusel alcohols: A. 2-methyl-1-butanol (137-32-6); B. 3-methyl-1-butanol (123-51-3); C. 1-pentanol (71-41-0)	A. 1206; B. 1230; C. 1244	A. malty, balsamic, wine, ripe onion, buttery; B. pungent, balsamic, alcohol, fruity, malty, ripe onion, burnt, cheese, bitter, harsh; C. fruity, green, sweet, pungent
7	1420-1430	89	100	Meat/chicken, sulphury, musty, cooked/nutty/roasted, rotten veg, metallic	oil, faecal, spoiled food/sulphur, musty sulphurous, savoury/ sulphury, eggy, metallic, garlic	2-nonanone	1401	Fatty green earthy
						2-ethyl-5-methyl pyrazine (13360-64-0)	1419	Coffee bean, nutty
8	1460-1470	100	100	Roasted, green (fresh peas), warm barley, roasted/ soapy, leathery, peapods, metal, adhesive, walnut	strong chemical unpleasant, popcorn but greener, weak green, roasted/ burnt/ nutty, mushrooms/oily, wet tweed, fresh peas from pod, solvent, nuts	1-octen-3-ol (3391-86-4)	*1463*	mushroom, earthy
9	1490-1510	100	89	earthy/must/potato, pea-pods type, potato, potato/ cooked, potatoes/ chips, malt/maltesers, cooking, roasting, wet grass, potatoes/veg cooking	cooked veg/potatoes, savoury becoming stale, musty, malty/savoury, pasta/wheat, roasting, wet grass, vegetables	furfural	1485	Bread, almond, sweet
10	1570-1580	78	89	new books, putty/plasticine, feints, sl. Creamy, aldehyde/green, fruity, nail varnish, hint of cheese, new car, sweet, baking sweet	new carpet, marzipan/almond, perfumed soap/cosmetics, sweet/ bready, meaty, new car smell, aldehyde/metallic/ cucumber, green/ grassy, bakewell tart,	benzaldehyde	*1572*	Bitter almond

					new shoes/books			
11	1600	56	67	faeces, plasticine, sweaty/musty, berries, unpleasant	waxy, musty/savoury, sweaty, feet, sweaty gym kit, cheesy	methyl-propanoic acid (79-31-2)	1588	rancid butter, cheese
12	1630	56	78	Cucumber, green, fishy, watermelon, sweets	metal, floral, cucumber, watermelon, raw courgette, almond, mint	3,3-diethoxy-1-propanal (16777-87-0)	nk	nk
						benzoin ethyl ether (574-09-4)	nk	nk
13	1650	67	44	27.3-27.44 Popcorn, socks/musty Earthy, feet	27-27.3 musty, nutty, dog biscuits, popcorn	butyric acid (107-92-6)	1650	cheesy, sharp, acetic, butter, fruit
14	1670	89	78	Cheese, socks, musty, savoury/ earthy, vomit, cheddar, parmesan, musty	socks, cheese, sick/cheesey, savoury, cheesy feet, parmesan cheese	ethyl decanoate	1630	waxy, fruity, apple, grape
15	1680-1700	89	78	corny, cereal-like, stale, nutty, musty, socks, burnt toffee, malt (brewery), musty/ popcorn, grass, baking	cheese, popcorn, smoky/ rubbery, musty, nutty, honey, savoury baking	2-furanmethanol (98-00-0)	1686	burnt sugar, fermented, creamy, caramellic note
16	1710-1740	100	100	corny/cheesy,green, yeasty, peanut, blue cheese/feet, sweaty/musty/unpleasant, roasting, chinchilla cage/must/ musk	bready, spring onions/ savoury, yeasty, boullion/savoury/meaty, grain, animal musk	propyl decanoate (30673-60-0)	nk	Fatty, green, woody, oily
17	1750-1770	67	44	plastic, metallic, potato, clean, metal, malt/wheat, meaty, plants	yeast & baking, cheesy yeasty, corny, metallic, malt, solventy/ green	methionol	1745	Meaty, sulphurous, vegetable

OAA: odour active areas. Detection Frequency: the proportion of assessors (n=9) who recorded perceiving an odour at this time, expressed as a percentage. nk: data not known

Once odour active areas had been identified, (Table 3.3) using the panellist descriptors as a guide, any regions which could be relevant to nutty/cereal aroma were further investigated. I will now proceed to review each of these nutty/cereal odour active areas: their panellist-derived descriptors and the compounds which appear to be underlying them. The differences in concentrations between these compounds in the spirit samples were also sought in order to ascertain whether there was any correlation with the sensory data.

3.4.2.1 2-methylbutanal and 3-methylbutanal

Although the aroma of these simple Strecker aldehydes were unmistakably detected by the panellists for the malty/solventy aromas they were experiencing early in the run (LRI 930-940), we were unable to obtain a clear mass spectral identification under the present chromatographic conditions. This was due to the abundance of highly volatile compounds of low molecular weight which yielded similar sized ion fragments (*m/z* 86 and under) and were also found in the first section of the chromatogram. In the GC-O study undertaken by Acena *et al.* (2010), a WAX column was used to elucidate the compounds responsible for the aroma of pistachio nuts. The authors reported that the LRI of 2/3-methylbutanals was 936. Hence these compounds were tentatively identified by their odour and their linear retention indices. Formed during the Maillard reaction, these Strecker aldehydes result from the degradation of amino acids isoleucine and leucine (2- and 3-methylbutanal respectively).

Beal and Mottram (1994) identified 2- and 3-methylbutanals as being the largest contributor to the malty character of malted barley, with the levels of both compounds increasing dramatically during the roasting process. These compounds are therefore likely to be contributors to the 'cereal' character of spirits, although a specific role in nuttiness is less probable.

3.4.2.2 2,3-butanedione and 2,3-pentanedione

Sharing similar molecular structures, the vicinal diketones 2,3-butanedione and 2,3-pentanedione are both known to exhibit buttery and creamy aromas. These compounds are considered to have particular importance in the aroma of alcoholic beverages as they have such low odour thresholds; in beer this is 0.15 µg/mL and 0.9 µg/mL, and in whisky 0.020 µg/mL and 0.078 µg/mL for 2,3-butanedione and 2,3-pentanedione respectively (Salo *et al.*, 1972). Therefore it is not surprising that the same high proportion of panellists were able to perceive both aromas in each spirit extract; all 9 panellists noted malty aromas for 2,3-butanedione in extracts B and E, and 8 recorded buttery, toffee aromas for 2,3-pentanedione (LRI 990-1000; 1030-1040, Table 3.3). Both compounds were tentatively identified by their odour and linear retention indices, as I was unable to resolve them from the solvent peak at the beginning of the chromatogram.

2,3-butanedione, or diacetyl as it is more commonly known, is a fermentation byproduct of the synthesis of valine. Yeast produces alpha-acetolactate as a precursor to valine, which when it escapes the cell, is spontaneously decarboxylated into diacetyl. Formation of the pentanedione is analogous to this process, with precursor aceto-alpha-hydroxybutyrate and amino acid isoleucine being the substances of interest here (Nykänen and Suomalainen, 1983). Reduction of these diketones by the yeast cell can also take place, to produce acetoin and 2,3-pentanediol. In the brewery, it is advantageous to keep the levels of diacetyl produced during fermentation as low as possible, especially when the finished product is to have a lighter flavour, as it can impart an undesirable buttery note. However in the distillery, the aroma complexity of the finished product means that should the level of diacetyl within the spirit exceed the threshold, it would have less of a sensory impact than it would in a light beer. Another way in which diacetyl may be formed is during non-enzymatic browning, or the Maillard reaction (Hollnagel and Kroh, 1998).

3.4.2.3 Fusel alcohols

As can be seen from Figure 3.1 and Table 3.2, fusel alcohols such as 3-methyl-1-butanol, 2-methyl-1-propanol and 1-propanol (LRI 1230-1280) are abundant in whisky. They are characterized by an 'alcoholic' aroma; pungent, whisky-like, malty and burnt are among their descriptors in the literature. Highly volatile, these compounds are known to have a marked impact on the overall spirit aroma, and are formed during fermentation as a product of amino acids which have been decarboxylated and deaminated (Nykänen, 1991). When these compounds undergo electron ionization in the MS, they fragment to similar m/z ions. Due to their molecular similarities the three alcohols also elute at the same point on the chromatogram, resulting in an overloaded peak which made it difficult to resolve one alcohol from another. Therefore the fusel alcohols have been quantified collectively, resulting in a relatively high concentration 400 – 500 µg/mL and the large amount of variation shown by the standard deviation error bars (Figure 3.2). There were no significant relationships between the combined fusel alcohol levels and the nutty/cereal sensory scores.

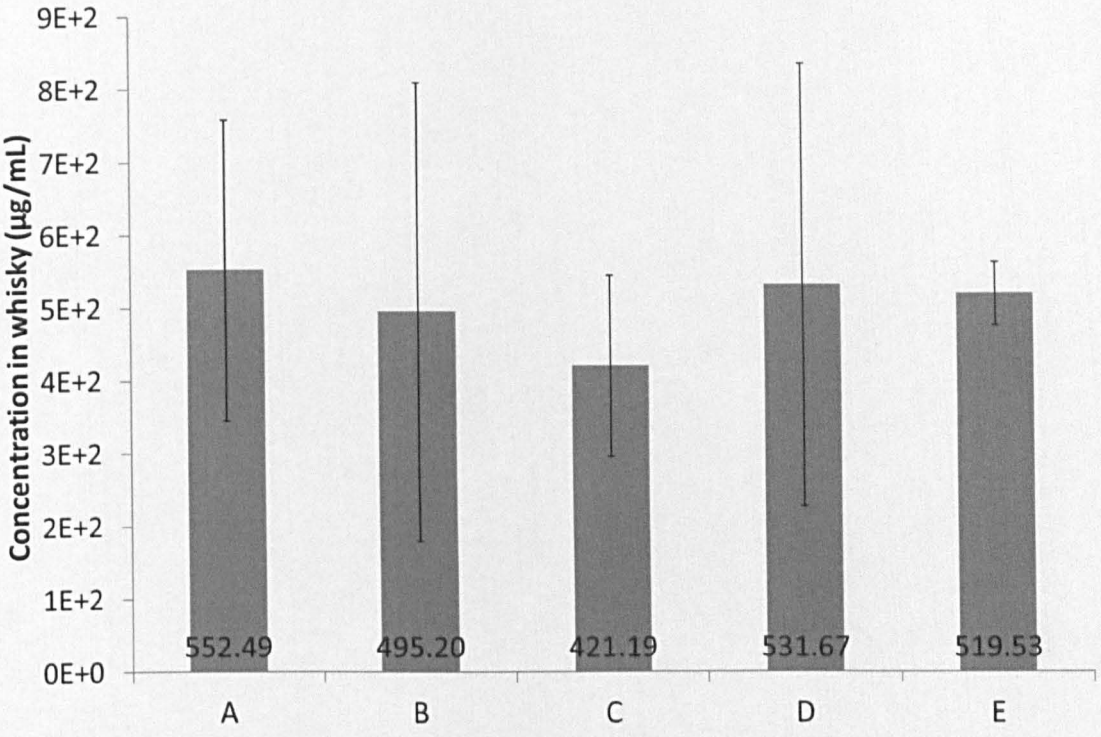


Figure 3.2 Combined concentration of fusel alcohols extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

3.4.2.4 Methionol

Methionol and methional are derived from methionine and produced during fermentation as by-products of the yeast metabolism. These sulphur compounds have very low odour thresholds, 0.005 and 0.0002 µg/mL respectively (Belitz *et al.*, 2004), therefore a small amount of either compound can have a significant effect on the overall aroma.

In the chromatographic region where methionol falls, 6 panellists were able to detect an odour in the nutty spirit B in comparison to only 4 in the control spirit E. This is in agreement with levels seen in Figure 3.3: we see significantly higher methionol concentration in the nutty spirits A-D than the control spirit E.

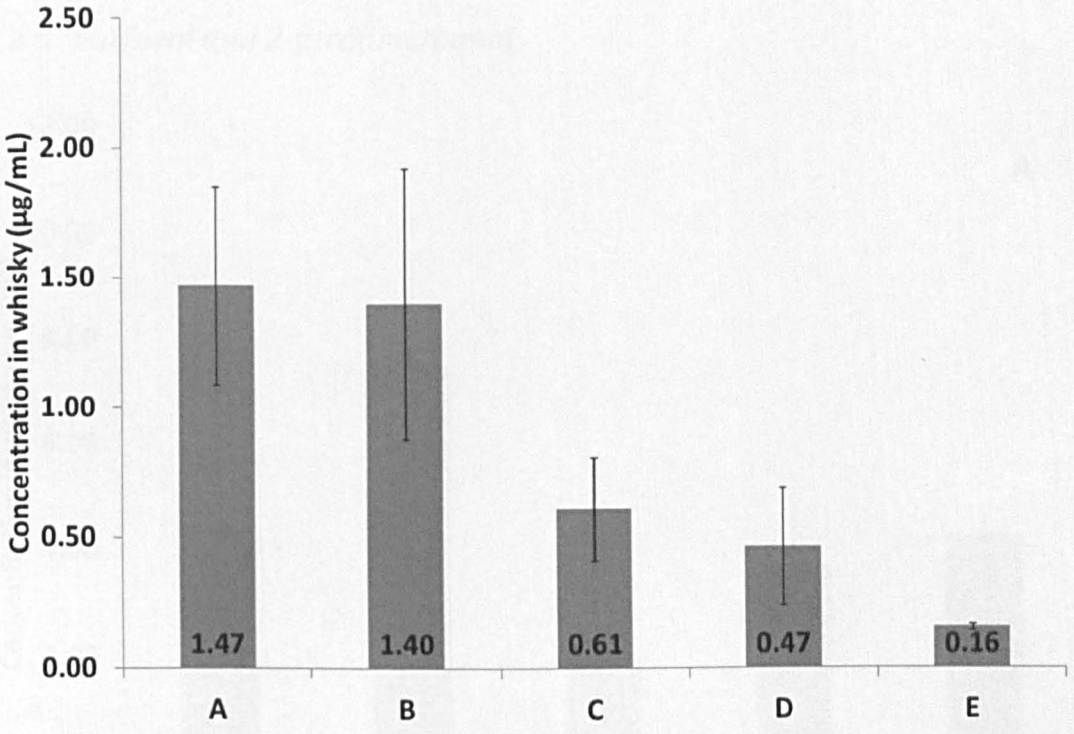


Figure 3.3 Concentration of methionol extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

Methionol levels were shown to have a significant correlation with both the nutty ($p = 0.0124$) and cereal ($p = 0.0134$) scores for the spirits. Panellist descriptors for this region (LRI 1750-1766) included 'yeasty', 'meaty', 'malty' and 'solventy/green'.

3.4.2.5 Furfural and 2-furanmethanol

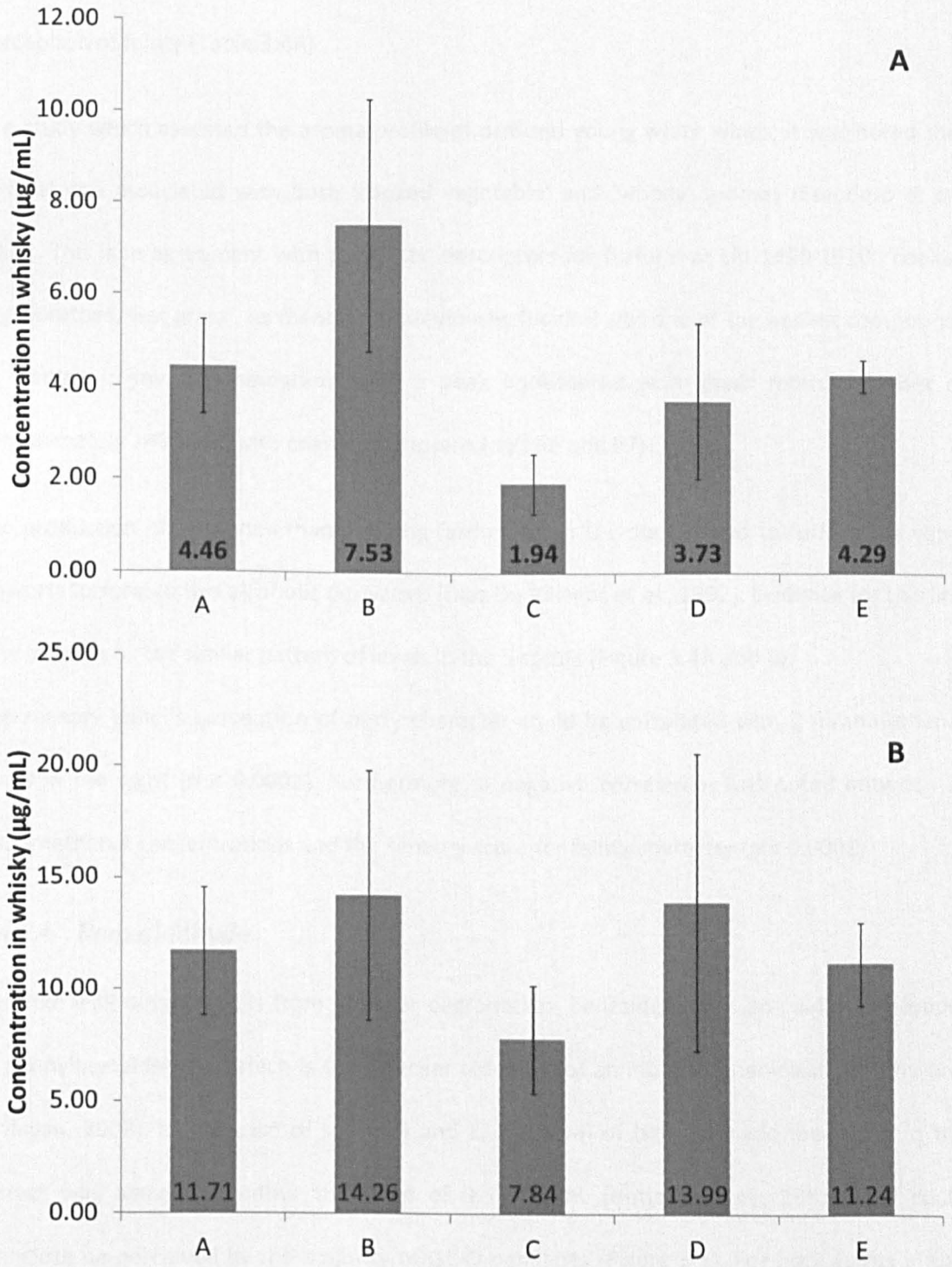


Figure 3.4 Concentration of (A) furfural and (B) 2-furanmethanol extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

Furfural levels were shown to have a statistically significant positive relationship ($p = 0.0075$) with perceived oily aroma and to negatively correlate ($p = 0.0078$) with the sensory panel's perception of feinty (Table 3.4A).

In a study which assessed the aroma profile of oxidized young white wines, it was noted that furfural was associated with both 'cooked vegetable' and 'woody' aromas (Escudero *et al.*, 2002). This is in agreement with panellists' descriptors for furfural at LRI 1490-1510; 'cooked veg/potatoes, wet grass'. As mentioned previously, furfural was one of the easiest compounds to identify in my chromatograms with a peak unobscured at a linear retention index of approximately 1485 and with characteristic ions (*m/z* 96 and 97).

The production of 2-furanmethanol during fermentation is closely linked to furfural, as yeast converts furfural to this alcoholic derivative (Diaz De Villegas *et al.*, 1992). Evidence for this link may be seen by the similar pattern of levels in the 5 spirits (Figure 3.4A and B).

The sensory panel's perception of nutty character could be correlated with 2-furanmethanol levels in the spirit ($p < 0.0001$). Furthermore, a negative correlation was noted between 2-furanmethanol concentrations and the sensory score for feinty character ($p < 0.0001$).

3.4.2.6 Benzaldehyde

Another MRP which results from Strecker degradation, benzaldehyde is an oxidation product of phenylacetaldehyde, which is the Strecker aldehyde of amino acid phenylalanine (Chu and Yaylayan, 2008). In the case of spirits B and E, the level of benzaldehyde measured in the extract was above the odour threshold of 0.35 µg/mL (Buttery *et al.*, 1981), and could therefore be perceived by the majority of GC-O panellists (Figure 3.5). For both spirits in this region (LRI 1570-1584), panellists commented 'new' smells e.g. 'new books/car/shoes', as well as the 'marzipan/almond' aroma which is characteristic of benzaldehyde.

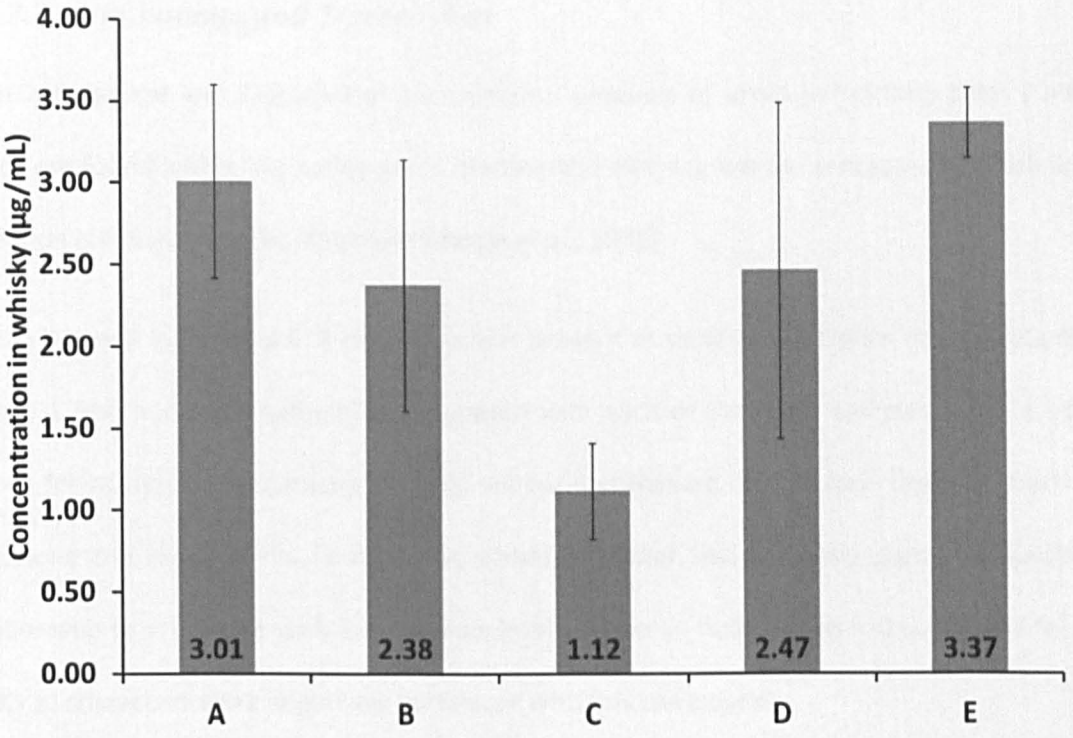


Figure 3.5 Concentration of benzaldehyde extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

As shown in Table 3.4, benzaldehyde spirit concentration was shown to have a significantly positive relationship with the cereal score ($p = 0.0009$) and, similarly to the other important MRP furfural, benzaldehyde exhibits a significant negative correlation with sensory attribute feinty ($p = 0.0001$).

Table 3.4 Statistically significant (p value > 0.05) of odour-active whisky volatiles extracted using LLE; statistical analyses was done for sensory panel descriptors and compound concentrations.

Sensory Scores	*2-furan-methanol*	*benz-aldehyde*	*2-nonanone*	*furfural*	*methionol*	*1-octen-3-ol*
Nutty	< 0.0001 +				0.0124 +	0.0004 -
Cereal		0.0009 +	0.0408 +		0.0134 +	0.0053 -
Oily			0.0006 -	0.0075 +		0.0001 +
Feinty	< 0.0001 -	0.0001 -	0.0049 -	0.0078 -	0.0177 -	

3.4.2.7 2-nonanone and 1-octen-3-ol

Both 2-nonanone and 1-octen-3-ol are oxidation products of unsaturated fatty acids (UFAs) which are found within the barley grain. Malting and mashing are the processes in which lipid oxidation is known to occur (Kaukovirta-Norja *et al.*, 1993).

As can be seen in Figure 3.6, 2-nonanone was present at significantly higher concentration in whisky E (the non-nutty sample) as compared with each of the other samples which scored higher for nutty/cereal character. In fact, whisky E contained around four times as much 2-nonanone the other spirits. Statistically, cereal character had a weakly significant positive relationship ($p = 0.0408$) with 2-nonanone levels, whereas both oily ($p = 0.0006$) and feinty (0.0049) characters were negatively correlated with this compound.

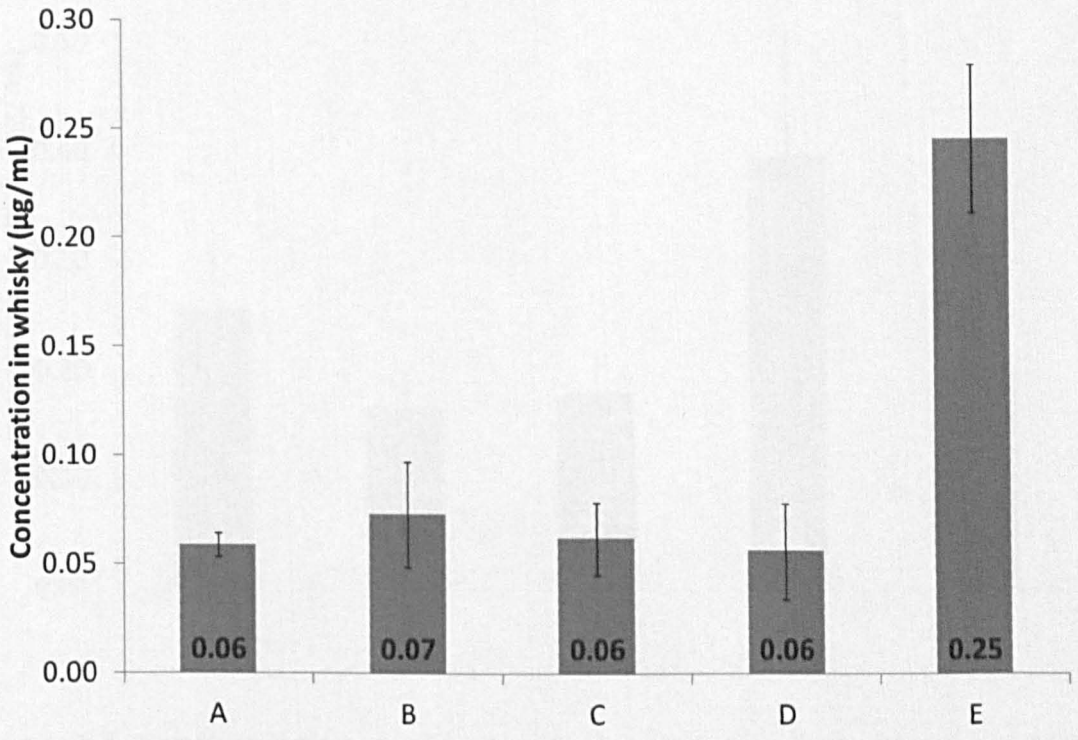


Figure 3.6 Concentration of 2-nonanone extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

The descriptors noted in this chromatographic region (LRI 1420) are sulphur-like; "meat/chicken", "musty", "eggy", "spoiled food/sulphur", as well as one person commenting

that they could smell a "cooked/nutty/roasted" note (Table 3.3). Thus it is unlikely that these descriptors relate to this compound, but more likely a sulphur-containing compound with a very low odour threshold.

Formed during the oxidation of the fatty acid linoleic acid, 1-octen-3-ol is known to have a very distinctive 'mushroom' aroma. Therefore its identification came as no surprise when the descriptors being recorded at this region of the chromatogram (LRI 1460-70) were 'mushrooms/oily', 'weak green', 'roasted/ burnt/ nutty. Further confirmation of this assignment was made by comparison with the literature LRI value for 1-octen-3-ol of 1429 (Aceña *et al.*, 2010).

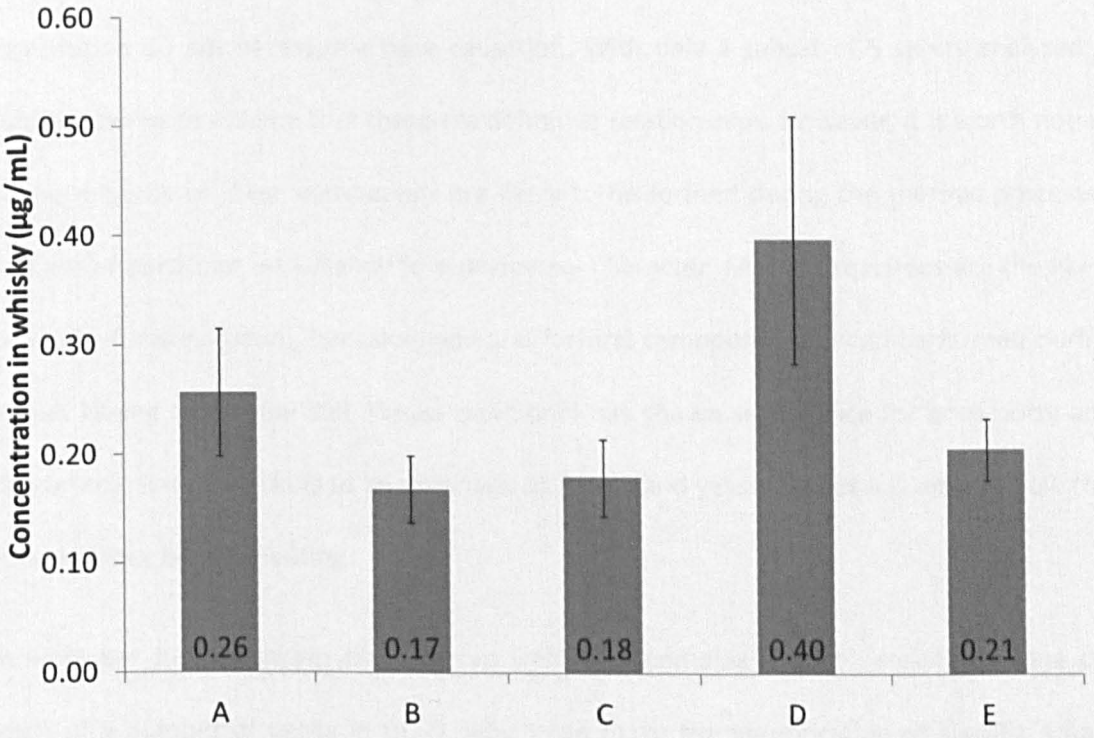


Figure 3.7 Concentration of 1-octen-3-ol extracted from the new make spirits by LLE. Error bars represent standard deviation of triplicate extracts.

Statistically, 1-octen-3-ol showed significant negative relationships for both nutty ($p = 0.0004$) and cereal ($p = 0.0053$) characters (Figure 3.7). However, in keeping with the 'oily' odour

descriptors mentioned for this compound, there was a significant positive relationship with the oily score ($p = 0.0001$).

3.5 CONCLUSIONS

In this GC-O study, 17 odour active areas were identified, 6 of which have shown statistical significance with potential to contribute to the nutty/cereal character of spirit; 2-furanmethanol and methionol showed a positive correlation with nutty scores; benzaldehyde, 2-nonanone and methionol all showed a positive relationship with spirit cereal character; whilst furfural and 1-octen-3-ol levels correlated positively with perceived oily character. It should be noted that the correlations between the sensory scores and compound quantification do not necessarily belie causation. With only a subset of 5 spirits analysed it would be unwise to assume that these are definitive relationships. However, it is worth noting that the majority of these compounds are likely to be formed during the thermal processes which are of particular importance to nutty/cereal character. Maillard reactions are the likely source of 2-furanmethanol, benzaldehyde and furfural compounds and can be formed during the malt kilning and in the still. Whilst methionol has shown significance for both nutty and cereal aroma, it is more likely to be described as meaty and yeasty, as yet it is unclear how this compound may be contributing.

This work has further shown that LLE can yield very complex extracts, resulting in the co-elution of a number of peaks in GC-O, which can make the identification of specific aroma compounds almost impossible (d'Acampora Zellner *et al.*, 2008). Although an attempt was made to significantly slow down the rate of elution, the complexity of the extract is also likely to have affected the panellists' sensitivity to single odours, as their ability to distinguish one aroma from another is likely to have been reduced. In order to overcome these problems, a more selective extraction method was sought, and is described in Chapter 4.

4 METHOD DEVELOPMENT OF SOLID PHASE EXTRACTION OF NEW MAKE SPIRITS AND GAS CHROMATOGRAPHY-OLFACTOMETRY OF SPIRIT EXTRACTS

4.1 AIMS

To develop a suitable solid phase extraction (SPE) method for the recovery of polar nutty/cereal compounds from new make spirit. To use Gas Chromatography-Olfactometry (GC-O) to determine differences in the odour compound profiles of the spirit extracts, due both to differences between individual spirits and as a result of the applied LLE and SPE techniques.

4.2 INTRODUCTION

The chemical complexity of malt spirit is well documented; a whisky can contain more than 80 volatile compounds (Steele *et al.*, 2004), two thirds of which have been identified by means of GC-O studies (Jack, 2012). Whilst these congeners are broadly categorized into chemical families, each have individual physical and chemical characteristics which govern their behaviour during any specific extraction protocol and in order to isolate them effectively this must be taken into account.

In the previous chapter, the medium polarity solvent dichloromethane was shown to extract a broad range of compounds. However, the concern was that while some volatiles were being lost as a result of the exhaustive concentration by nitrogen blow down, others became so abundant in the chromatograms that it was thought that they would potentially be masking smaller peaks underneath – such that the LLE chromatograms at a first glance were incredibly similar, in spite of noted differences in nutty/ cereal aroma character of the new make spirit samples. Hence a more selective extraction procedure was required. LLE relies on the only on the affinity of the analyte for the solvent, whereas solid phase extraction (SPE) uses liquid-solid partitioning with analytes being bound to active sites on the surface of a solid sorbent. This

affords more selectivity as both the sorbent and the solvent can be chosen to specifically extract the analytes of interest. With a wide selection of sorbents now available in handy disposable cartridges, SPE has quickly become one of the most popular and effective ways to extract aroma compounds from alcoholic beverages, especially wine (Wada and Shibamoto, 1997; Lopez *et al.*, 2002; Cullere *et al.*, 2004). Ferreira and his colleagues (2000) compared the extraction ability of various SPE sorbents alongside more traditional LLE solvent methods for the extraction of divergent compounds from a synthetic wine solution (12% ABV). Overall it was a polymeric SPE sorbent that was found to be the most effective at extracting compounds, with DCM performing the best of the solvents.

Due to their known nutty and cereal properties it was particularly important to isolate the relatively polar pyrazines. It was therefore decided to choose an extraction sorbent which had been proven to be effective for polar hydrophilic compounds. LiChrolut EN (Merck KGaA, Darmstadt, Germany) is a styrene-divinyl benzene co-polymer which exerts primarily hydrophobic interactions to extract both polar and non-polar compounds from the matrix. It is highly cross-linked, with greater specific area (700-1200 m^2g^{-1}) than silica-based resins, and is therefore proven to have better extraction capacity. Originally developed by environmental scientists for the isolation of trace polars in water (Loos and Niessner, 1998), LiChrolut EN sorbent has now found popularity amongst flavour scientists for having a high retention and recovery for a range of highly water-soluble (polar) analytes in aqueous matrices such as wine (Lopez *et al.*, 2002; Ferreira *et al.*, 2003; Mendes *et al.* 2012), spirits (Campo *et al.*, 2007) and other beverages (Jurado-Sanchez *et al.*, 2007). Strata-X is another SPE sorbent which has numerous retention mechanisms with reversed phase selectivity. It was used here as a prefilter, to see if we could use it to remove interfering hydrophobics before then applying the residue to the LiChrolut column.

4.3 METHODS

4.3.1 LiChrolut EN SPE

The following method was adapted from Ferreira *et al.* (2003) who used it to extract highly polar compounds such as sotolon, maltol and furaneol from wine.

For preliminary experiments such as this, commercially bought matured whisky (Bell's) was used instead to conserve the new make malt whisky samples for later experiments. The whisky had previously been diluted to 5%, 10% and 15% ABV in 30 mL aliquots ready for loading onto the column. Samples were then stored at different temperatures before sampling (minimum 4 h). Temperature was also varied over 3 levels: 4 °C (cold room) 12 °C and room temperature (approx. 20°C). The LiChrolut EN Column (Merck KGaA, Darmstadt, Germany) with a sorbent bed of 500 mg was placed in the vacuum manifold (Phenomenex, Torrance CA), conditioned with 8 mL methanol and equilibrated with 8 mL aqueous solution in 10% ethanol. The diluted spirit was loaded on to the column and was allowed to fully saturate the sorbent bed for 1 minute before a gentle vacuum was applied. Once the sample had been loaded, care was taken not to allow the bed to run dry until after the wash step, at which water (5 mL) was applied to the column. The sorbent bed was then dried by applying the vacuum for 30 minutes (10 kPa). Elution was with dichloromethane (6 mL).

4.3.2 Strata-X pre-filtration with LiChrolut SPE

Here a Strata-X solid phase extraction cartridge was used as a filter prior to using the LiChrolut SPE cartridge. This was in order to see whether it was possible to reduce the high levels of compounds such as esters, acids and alcohols which are present in the liquid-liquid extracts of the spirits (Chapter 3), whilst still retaining lower level more polar/ hydrophilic compounds that might be of interest in the present study. It was postulated that the over abundance of some volatiles may be masking these low level polar compounds, so they cannot be easily distinguished on a gas chromatogram. This may be especially true of those Maillard

heterocyclics which have very low odour thresholds and can thus make a large impact on the aroma without being present in large amounts. Therefore by employing Strata-X, a mixed mode cartridge that retains a range of hydrophobic compounds, before final adsorption and elution from the LiChrolut EN cartridge, it was thought some of the overabundant compounds would be filtered out, and we may see more low level polar compounds coming to the fore in the chromatograms. Firstly the Strata-X SPE cartridge (200 mg/6 mL; Phenomenex, Torrance CA) was conditioned with methanol (6 mL) and equilibrated with aqueous ethanolic solution (6 mL; 12 % ABV) ready for the diluted spirit sample to be loaded. New make spirit sample B (30 mL) which had been diluted to 12 %ABV was loaded on to the Strata-X cartridge and allowed to fully saturate the sorbent bed for 1 minute before a gentle vacuum was applied. The solution that passed through the cartridge was collected (~30 mL) and it was this Strata-X load eluate that was to be used as the load sample for the LiChrolut EN cartridge (500 mg/6 mL; Merck KGaA, Darmstadt, Germany). A schematic diagram of the two parallel SPE methods outlining this is shown in Figure 4.1. The Strata-X cartridge was then washed with water (6 mL) and the after 10 minutes drying with the vacuum (10 kPa) the Strata-X cartridge was eluted with methanol (6 mL). The LiChrolut EN (Merck KGaA, Darmstadt, Germany) SPE method was carried out as described in Section 2.2.2, using the Strata-X Load 'residue' or eluate as the load sample instead of the diluted spirit. As described in the previous SPE method (section 4.3.1), a vacuum manifold (Phenomenex, Torrance CA) was used. The protocol was done in triplicate. DCM extracts (~6 mL) were concentrated to 1 mL by N_2 gas in a water bath heated to 37 °C (section 2.2.3).

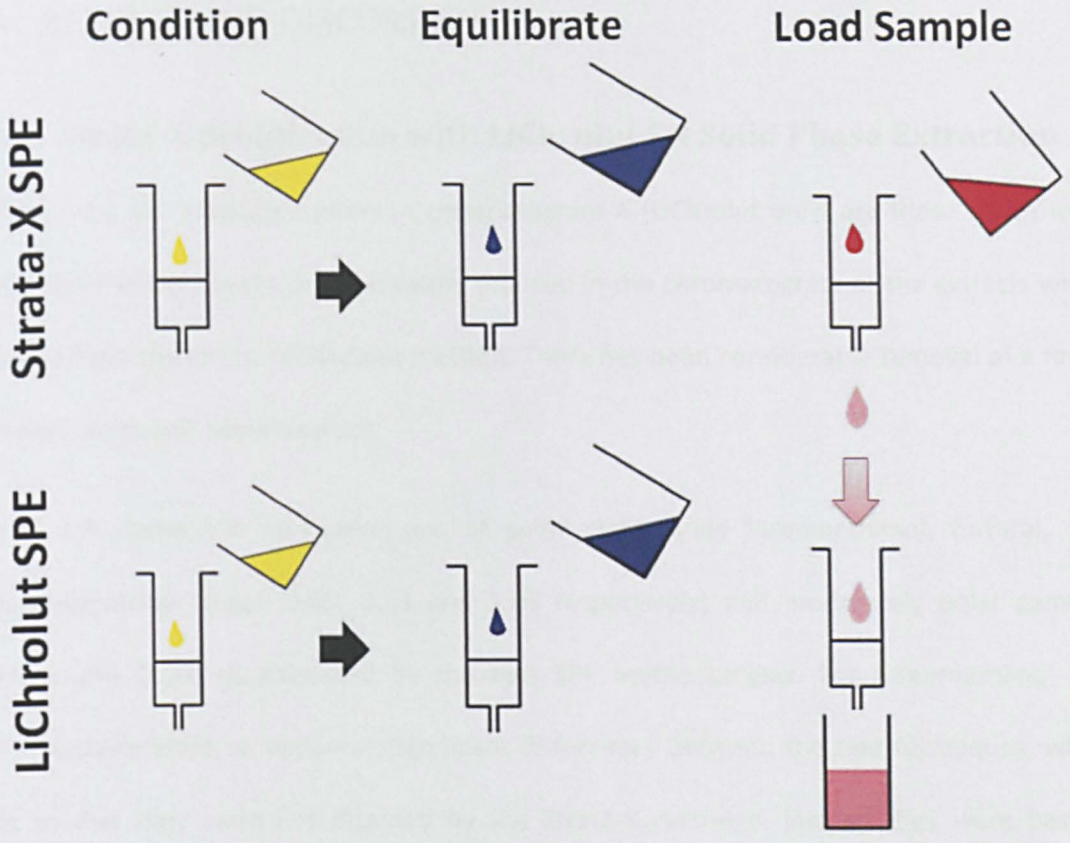


Figure 4.1 Schematic diagram to show the solid phase extraction of nutty new make spirit B using Strata-X and LiChrolut SPE prefiltration experiment.
Cartridges were conditioned with methanol (yellow), equilibrated with 12% ABV aqueous ethanolic solution (blue) and the sample of whisky diluted to 12% ABV (red) was loaded on to the Strata-X cartridge, the eluate from which was applied directly to the LiChrolut cartridge.

4.3.3 GC-MS and GC-Olfactometry Methods

Both Strata-X/LiChrolut sample extracts of spirit B were analysed by gas chromatography using the shorter run length (Section 2.2.5). LiChrolut EN SPE extracts A-E were analysed by gas-chromatography and samples B and E were analysed by GC-O for direct comparison between the two techniques. For full gas chromatography parameters please refer to Section 0 in the Materials and Methods Chapter.

4.4 RESULTS AND DISCUSSION

4.4.1 Strata-X prefiltration with LiChrolut EN Solid Phase Extraction

In Figure 4.2 the annotated peaks in chromatogram A (LiChrolut only) are those which were noted to be either absent or significantly reduced in the chromatogram of the extracts which resulted from the Strata-X/LiChrolut method. There has been considerable removal of a range of esters, acids and some alcohols.

Figure 4.3 shows the concentrations of polar compounds furanmethanol, furfural, 2,5-dimethylpyrazine (LogP 0.45, 0.83 and 1.03 respectively) and moderately polar gamma-nonalactone (2.08) as extracted by the two SPE methodologies. For furanmethanol and furfural, there were no apparent significant differences between the two techniques, which tells us that they were not retained by the Strata-X cartridge, instead they were passing through into the eluate which was used to load the LiChrolut cartridge, where they were retained and eluted. Similar levels of 2,5-dimethylpyrazine were extracted by the two SPE methods, and slightly more gamma-nonalactone was extracted by LiChrolut, suggesting some of the compound was retained on the Strata-X column.

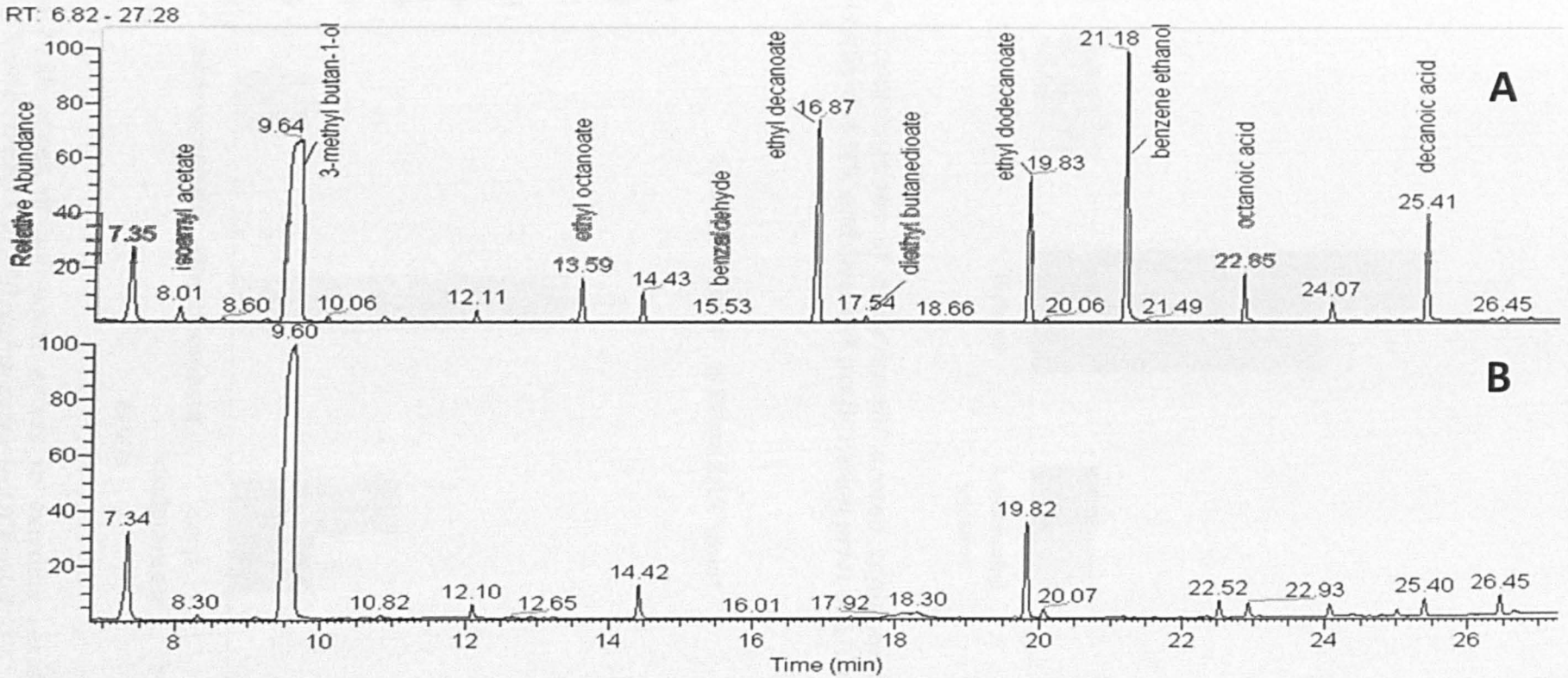


Figure 4.2 Gas chromatograms of concentrated dichloromethane extracts of spirit B which resulted from (A) normal LiChrolut SPE (B) Strata-X prefiltration prior to LiChrolut SPE

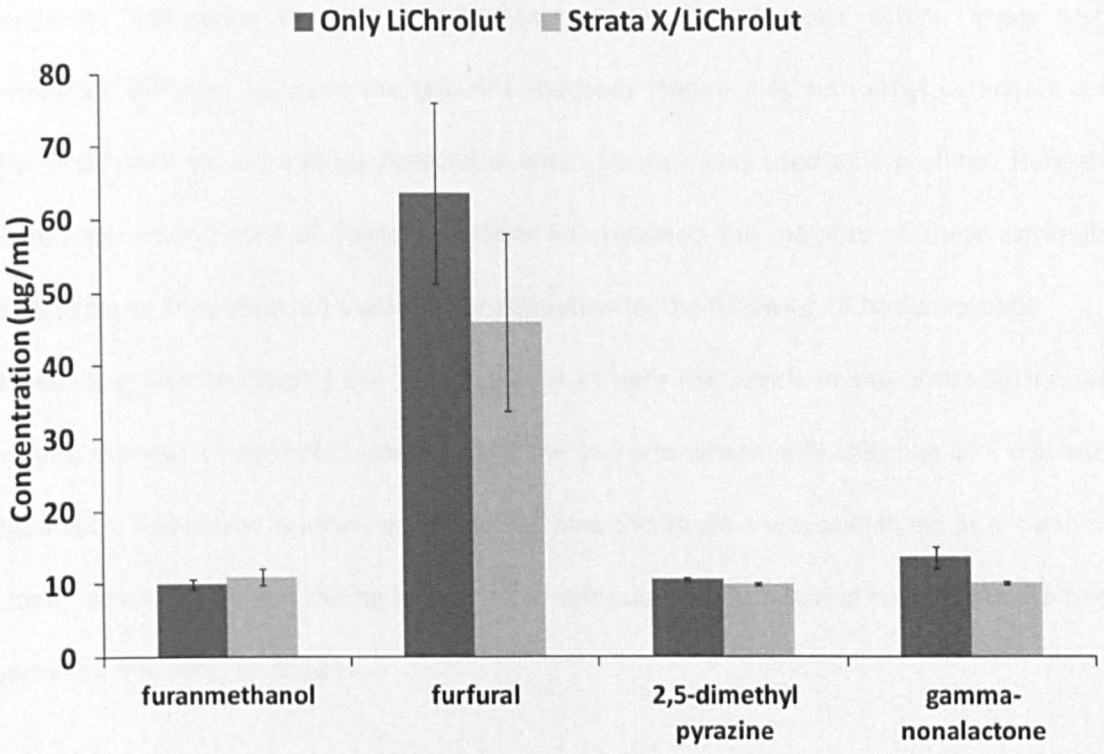


Figure 4.3 Concentrations of nutty/cereal aroma compounds as extracted using only LiChrolut SPE and Strata-X prefiltration prior to LiChrolut SPE

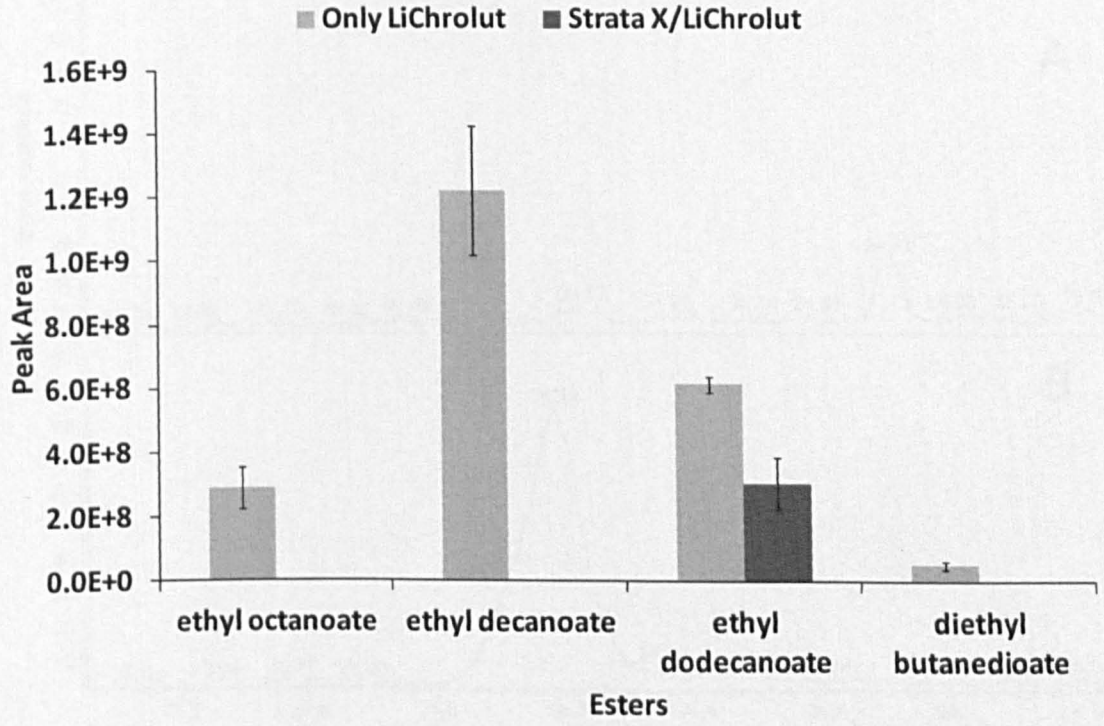


Figure 4.4 Peak areas of non-polar esters in extracts resulting from only LiChrolut SPE and Strata-X prefiltration prior to LiChrolut SPE

Conversely, comparing the peak areas obtained of the non-polar esters, levels were significantly different between the two SPE methods (Figure 4.4) with ethyl octanoate and ethyl decanoate not even being detectable when Strata-X was used as a prefilter. Here the hydrophobic interactions of Strata-X sorbent has retained the majority of these non-polar compounds, so they were not available for extraction by the following LiChrolut sorbent.

Interestingly, vanillin (LogP 1.05) was detected at very low levels in the Strata-X/LiChrolut samples, but was completely undetected in the extracts where only LiChrolut SPE was used (Figure 4.5). This serves as a further example of how the Strata-X was able to act as a 'clean up' sorbent, removing the interfering hydrophobic compounds which would have otherwise been concealing this small polar peak.

In addition, 5-methylfurfural and ethyl benzoate were not present at a detectable levels in the extracts resulting from the pre-filtration with Strata X and subsequent LiChrolut (data not shown).

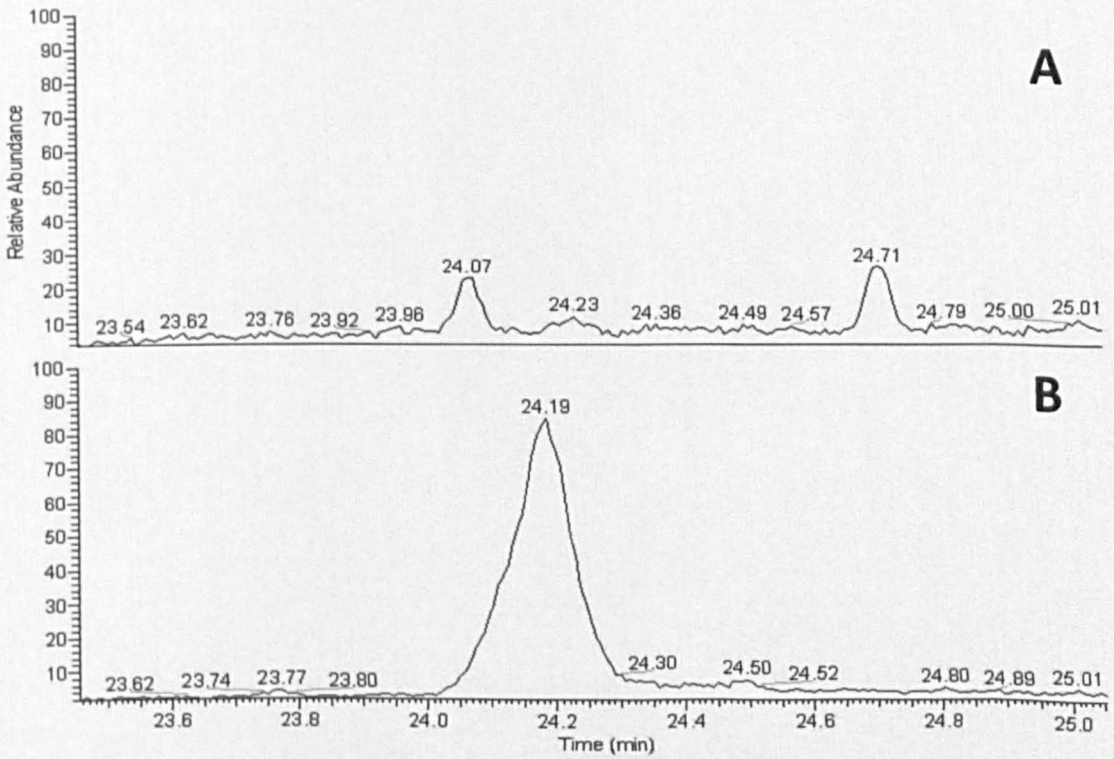


Figure 4.5 Gas chromatograph showing Vanillin compound peak (m/z 151, 152) which was undetected in (A) only LiChrolut SPE but was present in extract (B) Strata-X as a prefilter to LiChrolut SPE.

In summary, using Strata-X as a prefilter was useful for the removal of esters prior to compound adsorption to and elutions from the LiChrolut column, however with the exception of vanillin, no compounds of particular interest were revealed that were not already seen in the LiChrolut. Indeed, due to the mixed mode retentions of Strata-X, compounds with a high concentration such as furfural were retained by the Strata-X column. Therefore it was decided that the LiChrolut SPE method alone was suitable for the detection of the polar compounds of interest and would be used for all future SPE work.

4.4.2 LiChrolut SPE: Comparison of LLE and SPE Methods

In Figure 4.6 the decrease in the peak levels of SPE (B) when compared to LLE (A) is shown for chromatograms of solvent extracts of spirit B. The main compounds affected are the esters, alcohols and acids which have been labelled.

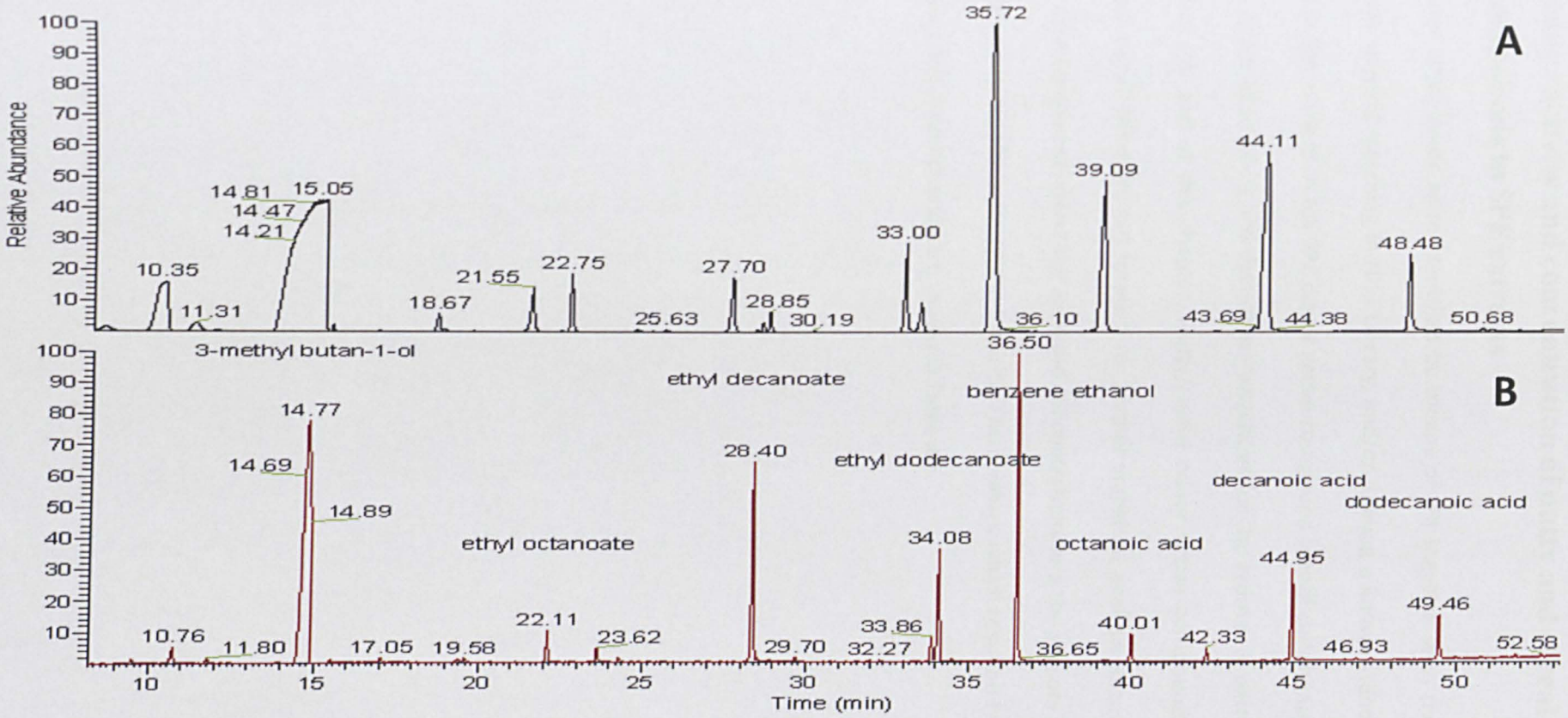


Figure 4.6 Gas chromatograms of concentrated (A) LLE and (B) SPE extracts of spirit B run using the GC-O method (RT 8.19-53.54). The major peaks (acids, alcohols and esters) which were decreased in the SPE extracts are labelled.

4.4.3 Odour activity and concentration of nutty and cereal character compounds in SPE extracts

Odour active compounds were identified by means of LRI together with the corresponding odour, mass spectral matching in NIST Library, and/or running a known standard in the same sequence as the extracts. A full SPE odour active compound identification table with this data as well as odour descriptors and detection frequencies for the aromas of extracts B and E can be found at the end of this chapter. Nutty/cereal odour active compounds identified and quantifiable by GC-MS were put forward for further statistical analysis to see whether there were any correlations between their analysed concentrations and the sensory scores for nutty, cereal, oily and feinty character of the 5 spirits. The p values which resulted from the ANOVAs for the odour active compounds are shown in Table 4.1.

Table 4.1 Statistically significant correlations (p value > 0.05) between odour-active whisky volatiles extracted using SPE and whole spirit sensory descriptors nutty, cereal, oily and feinty.

	*furfural**	*2-acetylfuran**	*benz-aldehyde**	*5-methyl-furfural**	*1-methyl-pyrrole-2-carb-aldehyde**	*2-furan-methanol**	*propan-1-ol*	*methionol*	*gamma-nonalactone*
Nutty	< 0.0001 +	0.0040 -	ns	0.0403 -	ns	< 0.0001 +	0.0046 -	0.0151 -	< 0.0001 +
Cereal	ns	< 0.0001 +	< 0.0001 +	< 0.0001 +	< 0.0001 +	ns	0.0016 +	< 0.0001 +	< 0.0001 +
Oily	0.0141 -	0.0022 -	0.0002 -	ns	0.0289 -	< 0.0001 -	ns	ns	0.0010 -
Feinty	< 0.0001 -	< 0.0001 -	< 0.0001 -	< 0.0001 +	< 0.0001 -	< 0.0001 -	0.0002 -	< 0.0001 -	< 0.0001 -

***Maillard reaction products**
ns: not significantly correlated

The statistical significances of the odour active compounds will now be discussed in relation to their concentrations in the spirit and their differences in odour activity.

Spirit concentrations are shown graphically, with concentrations measured in all 5 spirit samples extracted by LLE and SPE. In most cases, odour activity is shown in a table format detailing the detection frequency and odour descriptors generated by GC-O analysis. Due to the different number of panellists used in the LLE and SPE GC-O work, it was decided to express detection frequency (or 'NIF') as a percentage to facilitate comparison between the two techniques.

Where compounds are grouped it is according to the region of the chromatogram in which they were present.

4.4.3.1 Furfural and 2-furanmethanol

Regardless of extraction method, the levels of furfural (Figure 4.7) and 2-furanmethanol (Figure 4.8) in the spirits exhibited a similar pattern, with the highest concentration in spirit B and lowest in spirit C, however less significance can be attributed to the differences in the LLE extracts due to high inter-sample variation shown by the large error bars. Being that they are derived from the same pathway, from pentose sugars, this is not unsurprising. The statistical ANOVA analysis reflects this similarity, as both furfural and 2-furanmethanol levels as extracted by SPE were shown to have a statistically significant positive correlation (both $p < 0.0001$) with perceived nutty aroma and to negatively correlate with the sensory panel's perception of feinty (both $p < 0.0001$) and oily (furfural $p = 0.0141$; furanmethanol $p < 0.0001$; Table 4.1).

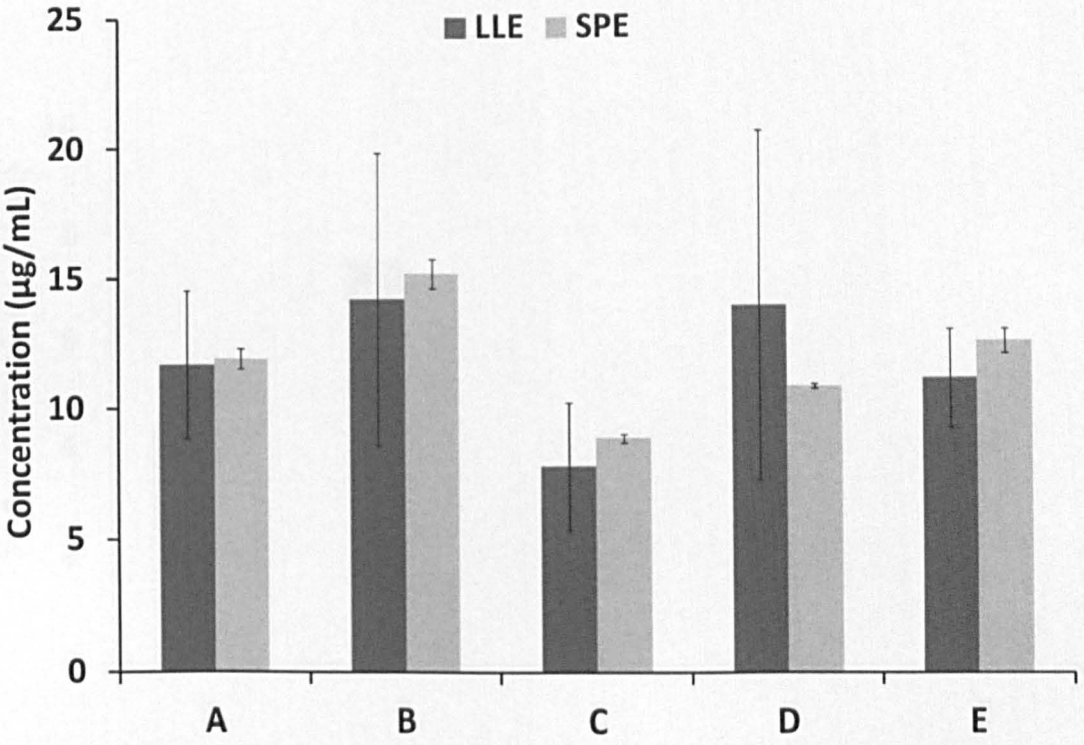


Figure 4.7 Furfural concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

Furfural odour quality descriptors vary greatly between panellists and it is therefore difficult to say what its' defining odour characteristics are. In their studies on the perception of whisky flavour reference compounds, Lee and her colleagues (2000) found furfural was described as having a 'grainy' character at 20-30 mg/L in Scotch whisky, however the majority of the panellists described furfural as "marzipan (coconut, cake mix, almond, nutty, walnut oil)". Other descriptors used included grassy, hay-like, sweet, oily and woody. Identification in the chromatogram does not prove as challenging however, as it is readily quantified by GC-MS, being a prominent peak in the chromatogram found near LRI 1485 (Cullere *et al.*, 2004).

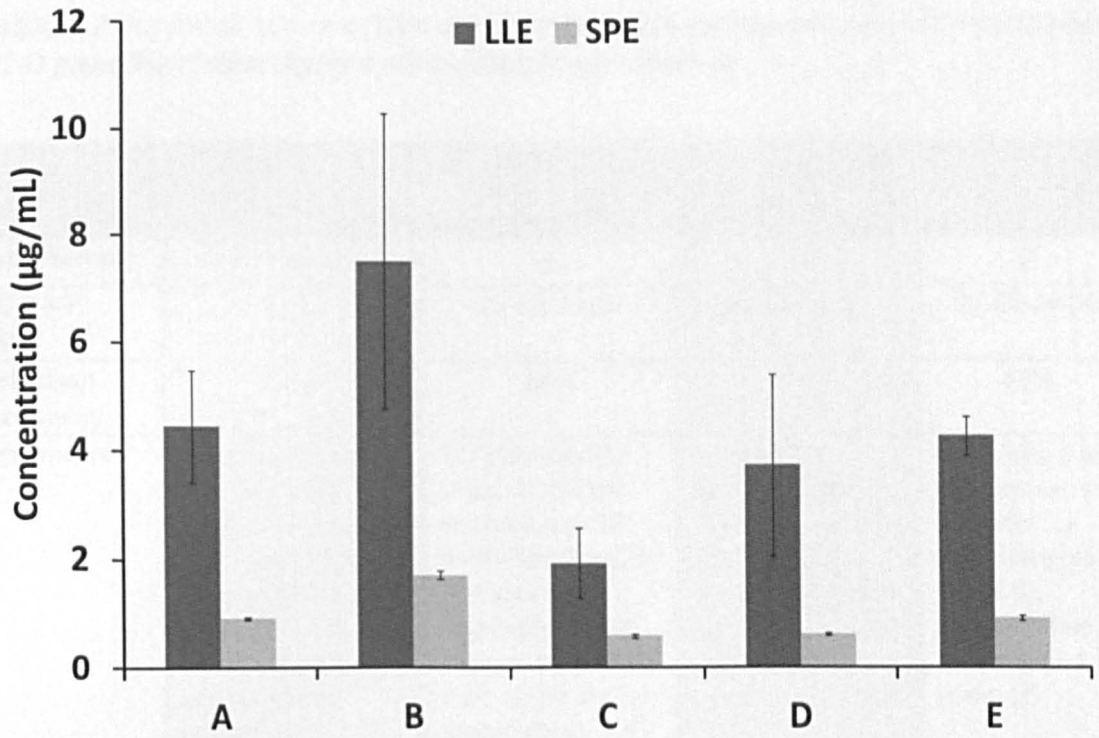


Figure 4.8 2-furanmethanol concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

Odour activity seems reduced in furfural SPE extracts compared to furfural LLE extracts' based on GC-O descriptors and detection frequency (Table 4.2). In the LLE extract, 'musty' or 'potato' descriptors were common, however in SPE 'peanut' and 'fatty' notes are apparent. These descriptors could originate from unsaturated aldehydes coincident with the furfural peak, that are not detectable by the mass spectrometer. For example, in literature (E,E)-2,4-heptadienal has been known to have an LRI of 1480 (Buttery *et al.*, 1981). There is also a transition of aroma which is more obvious in the SPE extract of spirit B; the 'peanut/fatty' odour becomes 'burnt' and 'mocha coffee' – this change could be due to another compound occurring simultaneously, or could simply be a function of the fall in peak concentration itself at that retention time.

Table 4.2 Furfural odour active areas of the chromatogram and corresponding GC-O panellists' descriptors with detection frequency

LRI 1480-1510	*LLE*		*SPE*	
Spirit Sample	**B**	**E**	**B**	**E**
GC-O RT (min)	22.57-22.97	22.50-23.00	22.90-23.43	22.90-24.50
Detection Frequency	**100%**	**89%**	**86%**	**60%**
Descriptors	Pea-pods (1) potato (1/2) potato/ cooked, nice (2-3) earthy/must/potato (3-2) wet grass (3) ? (1) cooking (3) potatoes/ veg cooking (3) potatoes/ chips (2) malt/malt-esers (1) roasting (3)	? (1) savoury (1) chips (2) cooked veg/potatoes (2) malty/savoury (2) wet grass (3) vegetables (2.5) savoury becoming stale (1) musty (1) pasta/wheat (1.5) roasting (3) lasagne (1.5)	Peanuts (3), cooking sweet things (2), fatty (2), fatty (3) becomes burnt (2) ? (0.5) unpleasant, pungent (1) ? (2) ? (2)	milky/egg-nog (1), new carpet (2), fatty (2) becomes green veg (1.5), mocha coffee (1) nutty (1-1.5) green (3)

LRI: Linear Retention Indices
RT: Retention Time

Table 4.3 2-furanmethanol odour active areas of the chromatogram and corresponding GC-O panellists' descriptors.

LRI 1680-1700	**LLE**		**SPE**	
Spirit Sample	**B**	**E**	**B**	**E**
GC-O RT (min)	28.10-28.60	28.40-28.57	29.25-29.50	29.30-29.43
Detection Frequency	**89%**	**78%**	**100%**	**100%**
Descriptors	Cereal-like (1.5) corny (2) grass (1) ? (2) musty (3) nutty (3) socks (3-0.5) malt (brewery) (1.5) stale (1.5) musty/ popcorn (3) baking (2) nutty (3)	Smoky/rubbery (1.5) nutty (2) cheese (1) musty popcorn (3) savoury baking (2) musty (3) honey (2)	Sweaty feet (1-2) acrid (1) floral (2) musty old sewer (3) nutty/ savoury (3) baked nutty (2-3) popcorn (3)	Cheesy (1) old musty museum (3) burnt (2) cheesy (1) nutty (2) popcorn (3)

Known descriptors for 2-furanmethanol are musty, bready and caramellic which agrees with the odours reported in this region (Table 4.3). The extract concentration was above the odour threshold of 2400 ppb (Takeoka *et al.*, 2008) which explains why the majority of the panellists were able to perceive it. In fact, panellists' perception of the odour of this compound increased from 89% and 78% for LLE extracts B and E, to 100% for both B and E SPE extracts. This could be due to the fact that this area of the chromatogram was less overcrowded with other peaks than the LLE extract due to the selectivity of the LiChrolut SPE method, therefore panellists could more clearly perceive 2-furanmethanol.

For furfural and furanmethanol, where a difference between detection frequencies exists (not including furanmethanol in SPE extracts where all panellists detected this odour), a larger proportion of panellists recorded nutty/cereal aromas in the nuttier spirit B than the control spirit E. Together with the significant correlations with nutty scores, this adds weight to the hypothesis that these compounds are of significance to this attribute.

4.4.3.2 Benzaldehyde (LRI 1575) and 2-acetylfuran (LRI 1542)

Being moderately polar benzaldehyde and 2-acetylfuran were well extracted by dichloromethane during LLE, and being of medium volatility not as easily lost when blowing down with N_2 (LogPs are 1.71 and 0.80; vapour pressures are 1.01 and 0.95 respectively) therefore more of these compounds could be extracted using large volume LLE than SPE.

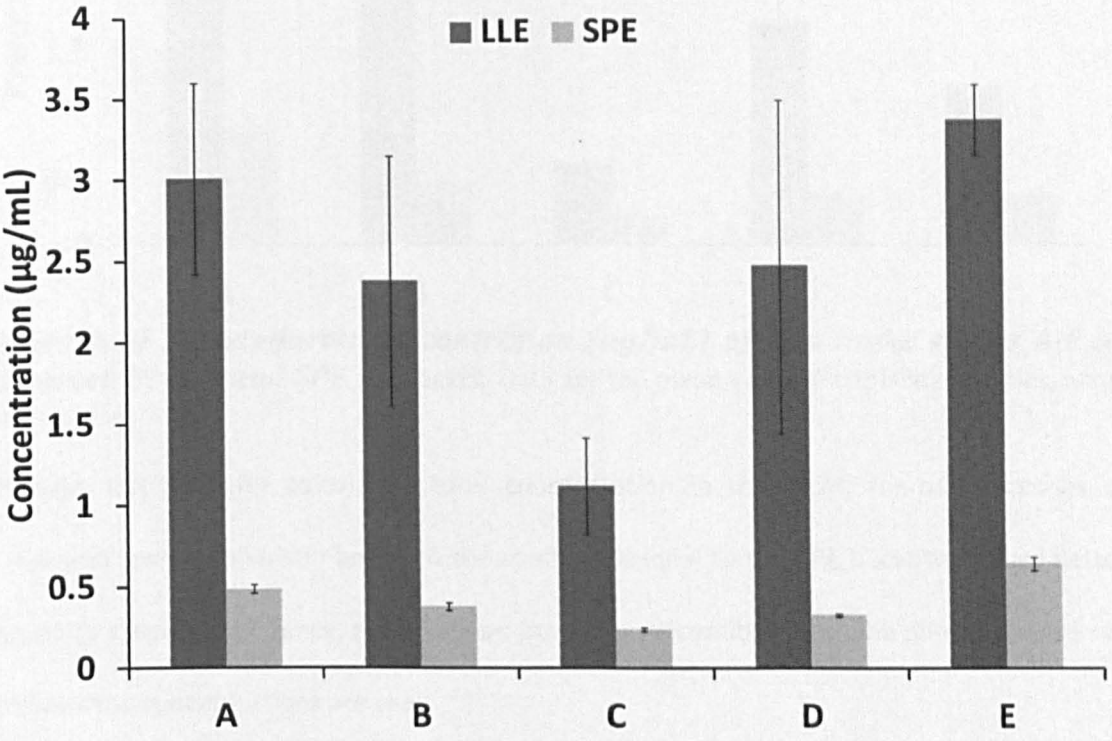


Figure 4.9 Benzaldehyde concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

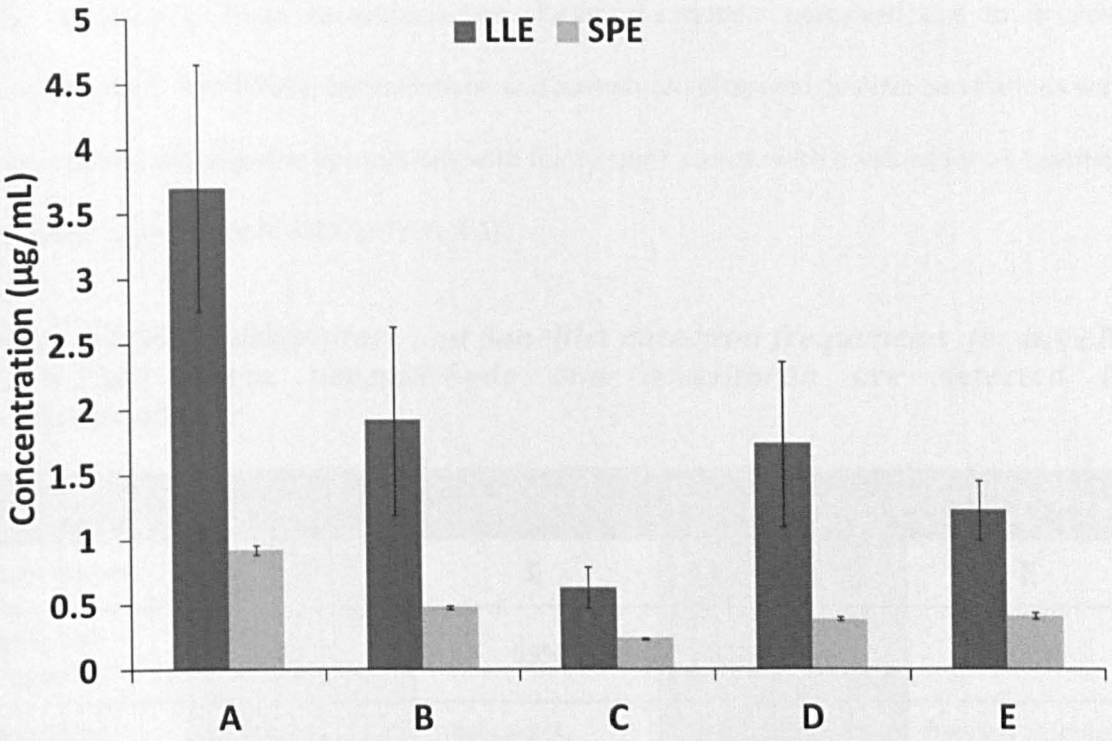


Figure 4.10 2-acetylfuran concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

Although not ideal for calculating total concentration in the spirit, for our purposes of compound level comparison between the spirits, it is ideal to use SPE because it gives better extract to extract consistency, therefore we can be more confident that the differences we see between the concentrations are real.

Odour activity in the LRI range 1540-1580 where acetyl furan and benzaldehyde are found is shown in Table 4.4. A whole host of different GC-O descriptors were generated for this region. Across the two extraction techniques, panellists recurrently described the aromas of 'new' things such as cars, books or carpet. Although not traditionally associated with benzaldehyde's characteristic almond/marzipan character, one can see how these aromas may be related. 2-acetylfuran is described as having a "sweet, almondy, nutty, brown and toasted with a milky undernote", which could correspond to the creaminess perceived in the LLE GC-O of extract B. In the LLE extracts, more of these bready/baking and green aromas are described than in the SPE extracts where solventy and leather descriptors were reported. In this way, we can see

how reduction of these compounds has altered the odours perceived due to lowered concentrations. Statistically, benzaldehyde and acetylfuran displayed positive correlations with cereal aroma and negative correlations with feinty spirit scores, with p values for all reaching maximum significance (< 0.0001; Table 4.1).

Table 4.4 Odour descriptors and panellist detection frequencies for the LRI 1540-1580 where benzaldehyde and acetylfuran are detected in chromatograms.

LRI 1540-1580	*LLE*		*SPE*	
Spirit Sample	**B**	**E**	**B**	**E**
Detection Frequency	**78%**	**89%**	**86%**	**100%**
Descriptors	new books, putty/ plasticine, feints, creamy, aldehyde/ green, fruity, nail varnish, hint of cheese, new car, sweet, baking	new carpet, marzipan/ almond, perfumed soap/ cosmetics, sweet/ bready, meaty, new car smell aldehyde/ metallic/ cucumber, green/ grassy, bakewell tart, new shoes/books	carrot, plastic, new car smell, solventy, sweet, leather shoe, oily, paper/new, new car, putty/plastic	fresh peas, pine, new things, mallow, leather, paper, solventy/ new car, dentist/air freshener, minty, fruity fragrant

4.4.3.3 5-methylfurfural (LRI 1612)

Considerably more 5-methylfurfural was isolated using SPE than LLE (Figure 4.11). Indeed there was not any interest in this region of the chromatogram previously, owing to a lack of nutty descriptors in the LLE GC-O analysis where cucumber and mint odours were most prevalent (Table 4.5). However, in the SPE extract nutty and green aromas which were indicative of 5-methylfurfural were now detected by the panel. As 5-methylfurfural has a fairly high odour threshold of 1.3 µg/mL (Takeoka *et al.*, 2008), and the concentration was below this in the LLE extracts, its odour was not detectable by the panellists. Statistical analysis of 5-

methylfurfural concentration in the SPE extracts showed a positive relationship between the levels of 5-methylfurfural and cereal (<0.0001) and feinty (<0.0001) sensory scores.

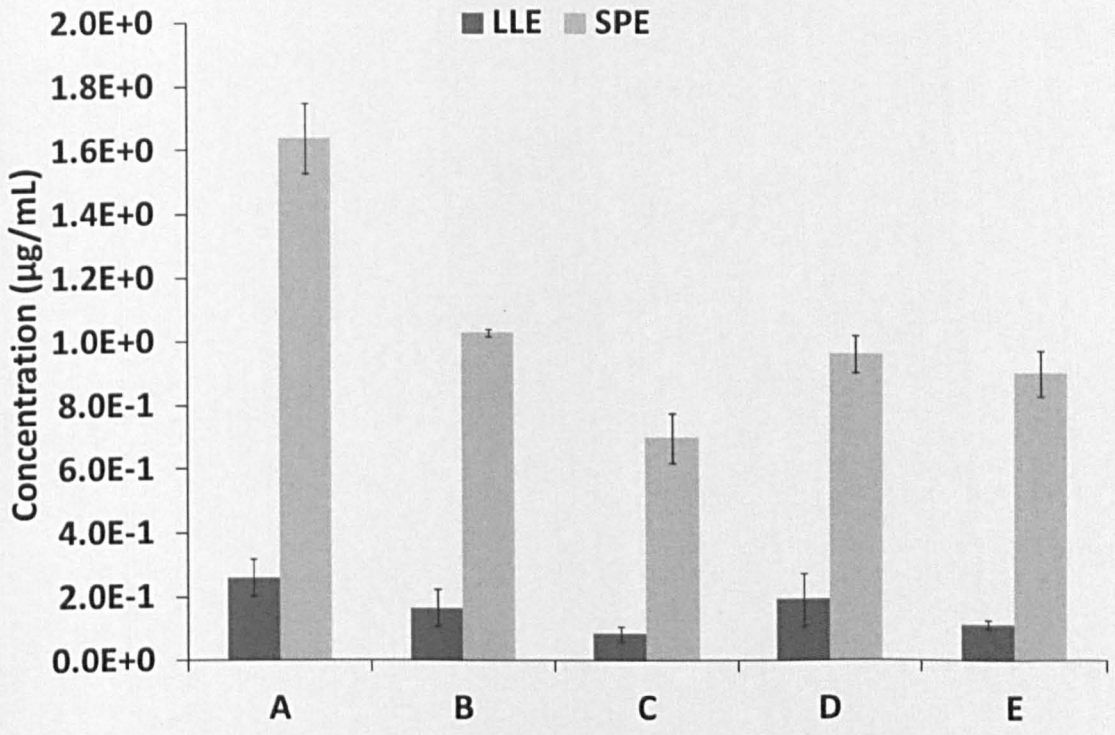


Figure 4.11 5-methylfurfural: concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

Table 4.5 Odour descriptors and panellist detection frequencies for the LRI 1630 where 5-methylfurfural (LRI 1612) was detected.

LRI 1630	**LLE**		**SPE**	
Sample	**B**	**E**	**B**	**E**
Detection Frequency	**56%**	**78%**	**71%**	**60%**
Descriptors	cucumber, green, fishy, watermelon, sweets	metal, floral, cucumber, watermelon, raw courgette, almond, mint	fusel alcohol, nutty, green leaves, onion, green	?, body lotion, mossy/ peaty, coconut

4.4.3.4 Fusel alcohols: 3-methyl-1-butanol, 2-methyl-1-propanol and 1-propanol (LRI 1230-1280)

As a result of the increased selectivity of LiChrolut SPE, levels of fusel alcohols were significantly decreased in the SPE extracts as compared to the LLE extracts (Figure 4.12). This reduction was desirable as it was thought the previously overloaded fusel alcohols peak was possibly overshadowing areas of the chromatogram where low level compounds of interest may be occurring. As with other compounds analysed, smaller variation between replicates can be seen in the SPE extracts, which could be because there was less concentration required - once the 6 mL solvent extract was obtained it was blown down to 1mL under a stream of nitrogen before GC-MS analysis. In LLE however, the volume to be concentrated down to 1mL was ~400 mL, hence the compounds that were not lost during this exhaustive process were concentrated 400 fold. However, both techniques extract a high concentration of these alcohols (>100 µg/mL), resulting in a wide peak in the range spanning the LRI 1230-1280, hence 100% of panellists were able to perceive malty, chocolate, fruity and solventy aromas at this time.

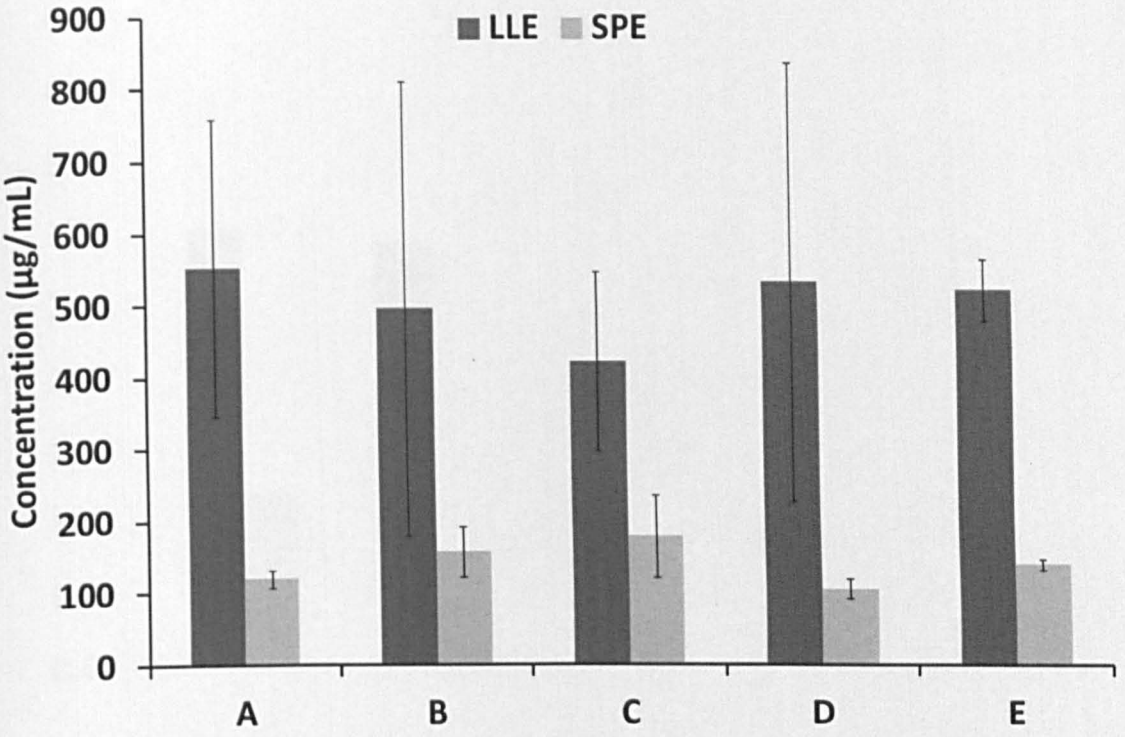


Figure 4.12 Fusel alcohols: concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

4.4.3.5 Methionol (LRI 1746)

Odour descriptors for the LRI 1750-1770 where methionol was found varied from SPE to LLE and from spirit B to E. Methionol is associated with 'meaty, 'sulphurous' and 'vegetable' aromas and it is these sorts of descriptors that some panellists recorded for this region in the LLE solvent extract, writing 'meaty', 'plants', 'yeasty'; however terms which could relate to cereal aroma were also used; 'malt/wheat' and 'corny'. These kinds of aromas are greatly reduced in the SPE extract, where the panel reported 'green/solventy, waxy, tea leaves' in extract B, and in E 'fire, balsam/cosmetics waxy'. Overall, there was less methionol extracted by SPE than was seen in the LLE extracts (Figure 4.13), so in turn its odour activity could have been decreased.

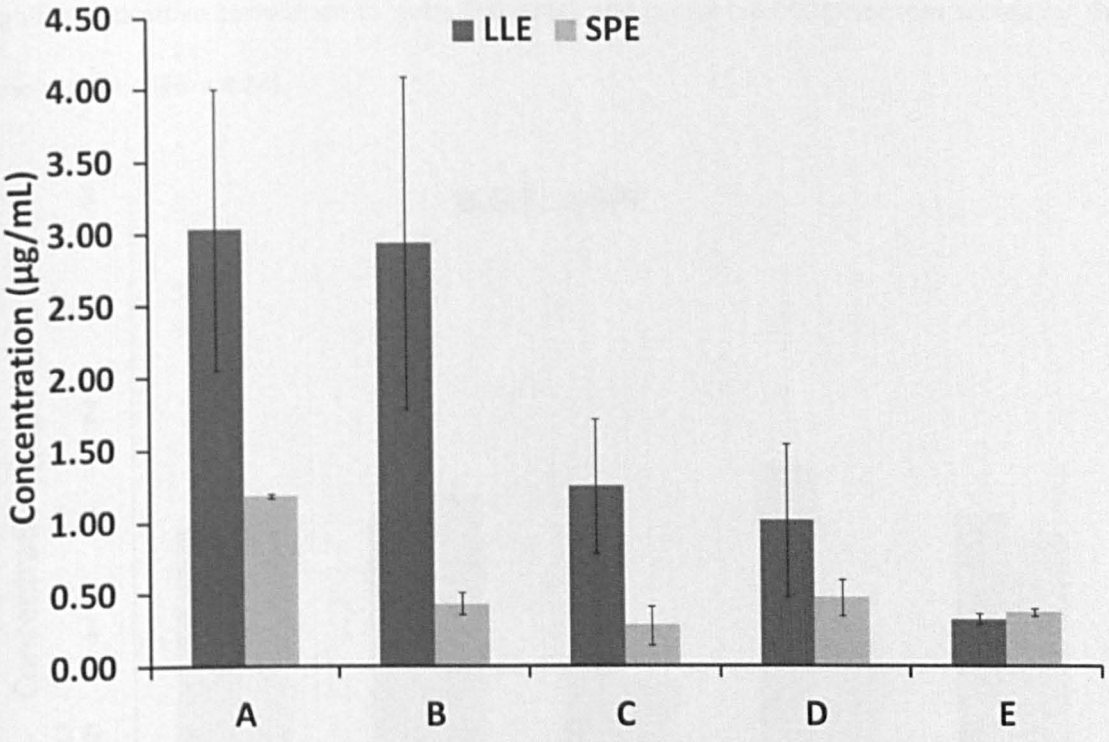


Figure 4.13 Methionol concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD.

It may be that another compound is responsible for the 'waxy' aromas, as it appears that methionol was present below its odour threshold of 1-3 µg/mL in the SPE extracts of B and E. As in the LLE samples, methionol concentrations showed a significant positive correlation (<0.0001) with cereal character score.

4.4.3.6 Gamma-nonalactone (LRI 2088)

Not previously perceived as odour active in the LLE extracts, at LRI 2040 in the SPE extracts panelists used the descriptors 'sweet/popcorn/ caramel' (extract spirit B) an d 'sweet/cinnamon/roasting nuts' (extract spirit E). Gamma-nonalactone, which peaks at LRI 2088 in the chromatogram was identified as the probable source of these aromas. Furthermore, 100% of panellists picked up on this aroma in SPE extracts B and E, compared to a mere 22% in the LLE extracts (2 in a group of 9). The lactone was also found to have a

significant positive correlation to nutty (<0.0001) and cereal (<0.0001) sensory scores for the whole spirit (Figure 4.14).

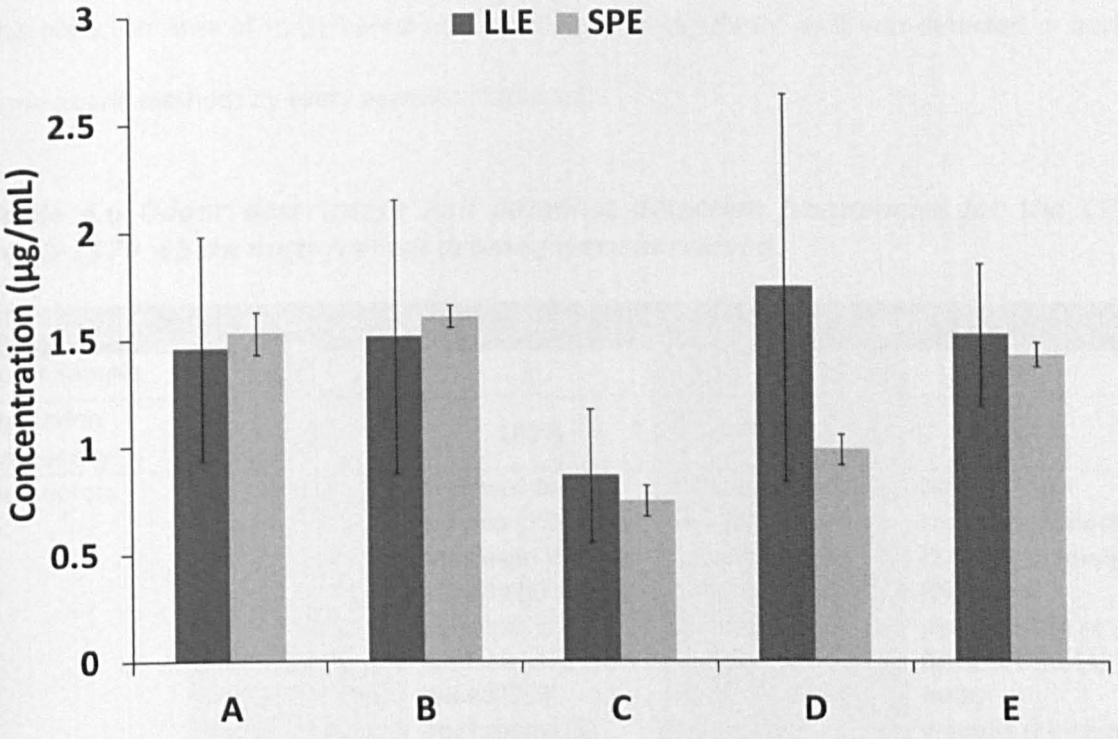


Figure 4.14 Gamma-nonalactone concentration (µg/mL) of new make spirits A-E as extracted by LLE and SPE methods. Data are the mean value of triplicate samples, error bars are ±SD

4.4.3.7 Nutty/cereal odour active area – unidentified compound (LRI 1460-1470)

This particular area of nutty/cereal odour activity was significant as it was detected in both samples and methods by every panellist (Table 4.6).

Table 4.6 Odour descriptors and panellist detection frequencies for the LRI 1460-1470 where nutty/cereal aromas were perceived.

LRI 1460-1470	LLE		SPE	
Spirit Sample	B	E	B	E
Detection Frequency	**100%**	**100%**	**100%**	**100%**
Descriptors	Roasted (2) adhesive (0.5) metal (2) **pea pods (1)** leathery (2) **green (fresh peas) (3) warm barley (2) nuts walnut? (3) roasted/ soapy (2)**	**popcorn? but greener (1.5) fresh peas from the pod (3) nuts (1)** strong chemical unpleasant (1) wet tweed (1.5) mushrooms (3) **roasted/ burnt/ nutty (2-3) solvents (2) weak green (1) ? (3) oily (1)**	**Sweet heptan-2-one (1) nutty/ pyrazine (3) burnt but green (3) earthy compost (2) nutty (2) burnt (2/3)** fusel/ pungent (2-3) **green (3) green/fresh (1) nuts (1)**	Nice (1) ? (2) chemical (2) **nutty (2.5) burnt smell (3) baked pyrazine-like or furfural (3) ? (1) nutty/ walnuts (2) fresh peas in a pod (3)**

At LRI 1461 1-octen-3-ol was detected. However as this compound has a very characteristic mushroom aroma, leading us to believe there are likely other compounds which are the source of these strong nutty/popcorn odours.

A group of compounds which are strongly associated with nutty and cereal character are the nitrogen heterocyclics, of which the pyrazine, pyridines and thiazoles are of special interest, owing to their nutty/cereal aroma qualities. One compound from this group which is characterized by the type of nutty/popcorn aroma descriptors being used here and has a very low odour threshold is 2-acetylpyrroline, which has an LRI of 1333 on a CarboWax column

(Wong *et al.*, 1992). It is suspected that such compounds underly the large ethyl octanoate peak the tail end of which encroaches into this area.

4.4.3.8 2,5-dimethylpyrazine (LRI 1353)

A polar compound well known for its characteristic roasted peanut odour (Przybylski *et al.*, 1983), 2,5-dimethylpyrazine was detected in trace amounts in extracts obtained from LiChrolut SPE (Figure 4.15). This is especially significant as no pyrazines had been identified previously in LLE extracts.

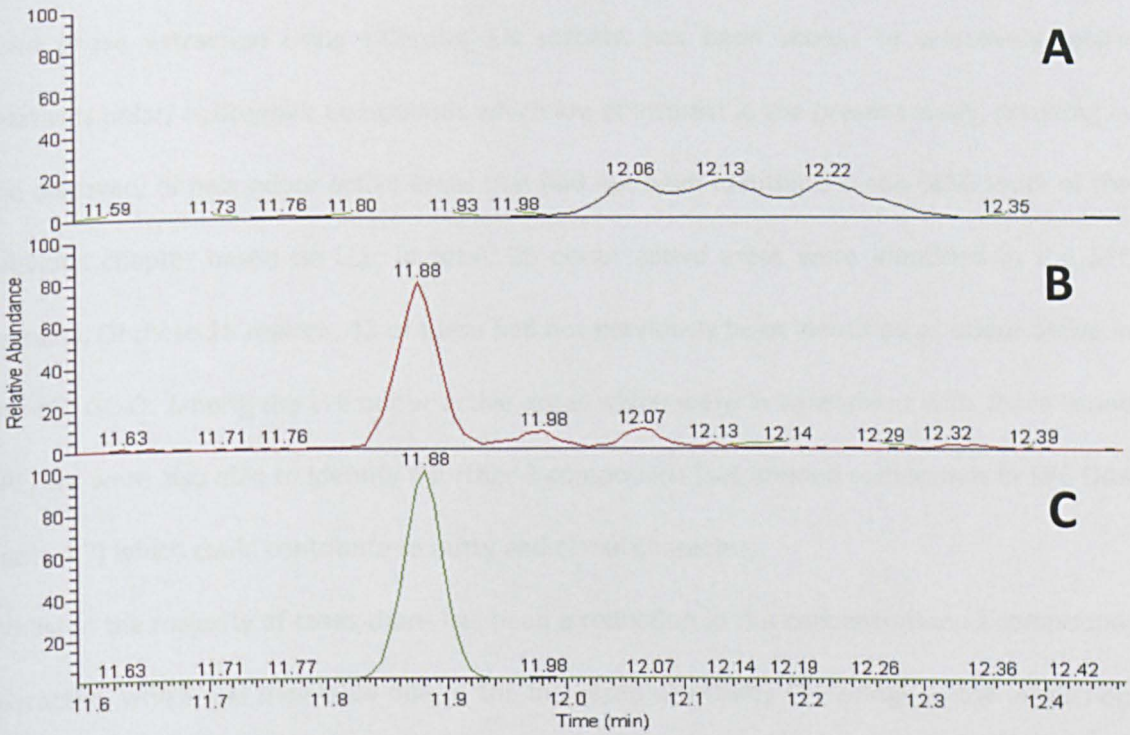


Figure 4.15 GC Chromatograms: 2,5-dimethylpyrazine (m/z 108) is detected in SPE extracts but not in LLE extracts of nutty new make spirit B. (A) LLE, (B) LiChrolut SPE & (C) 2,5-dimethylpyrazine standard.

2,5-dimethylpyrazine is a highly volatile compound, with a vapour pressure of 3.18 mm Hg, therefore a possible reason for there being less of this compound extracted in LLE when compared to SPE, is that it is easily lost to the atmosphere during the somewhat exhaustive process of nitrogen blow down where the solvent sample is concentrated from 400mL DCM to 1mL. In addition, 2,5-dimethylpyrazine may be better retained by the LiChrolut SPE due to Π-

Π (pi-pi) interactions with the polymeric sorbent, as it has been shown to be effective for the extraction of trace level aromatic polars (Jurado-Sanchez *et al.*, 2007). Retention of a polar compound by LiChrolut sorbent is known to increase considerably where there is an alkyl group attached to the aromatic ring - of which this compound has two - and where there is a side without substituents which enables stronger aromatic/Π-Π binding to the polystyrene sorbent (Loos and Niessner, 1998).

4.5 CONCLUSIONS

Solid phase extraction using LiChrolut EN sorbent has been shown to selectively retain relatively polar/ hydrophilic compounds which are of interest in the present study, resulting in the discovery of new odour active areas that had not been identified in the GC-O work of the previous chapter based on LLE. In total, 26 odour active areas were identified in the SPE extracts. Of these 26 regions, 12 of these had not previously been identified as odour active in the LLE GC-O. Among the LLE odour active areas which were in agreement with those found SPE, we were also able to identify a further 3 compounds (see shaded compounds in SPE OAA Table 4.7) which could contribute to nutty and cereal character.

Whilst in the majority of cases there has been a reduction in the concentration of compounds extracted, which was inevitable due to the increased selectivity SPE brings to the extraction, odour activity reported in GC-O studies was not always adversely affected. In fact the opposite was often the case, possibly due to the selective reduction in concentrations of interfering compounds, which meant the aromas coming through to the odour port were better separated and odours in those busier areas of the chromatogram were able to be noted down before the next odour arose.

Less variability was seen between analysed concentrations using the SPE method suggesting that this facilitated a better comparison of volatile compositional differences between spirit samples.

Furthermore there are some practical advantages to using solid phase extraction over liquid-liquid methods; the reduction in the usage of carcinogenic and expensive solvents, the in-built concentration step, the ability to extract several samples simultaneously, not as much of the sample is required – only 5 mL of each spirit was used compared to the 100 mL of spirit required for LLE extraction.

Table 4.7 Gas Chromatography-Olfactometry of spirit extracts B and E as extracted by LiChrolut SPE: Linear Retention Indices (LRIs), panellist detection frequency and aroma descriptors for the most odour active areas* as perceived by panellists.

		Detection Frequency (%)		*Panellists' Descriptors*		*Information sourced from Literature*			
OAA	**LRI**	**B**	**E**	**B**	**E**	**Suspected Compound**	**Lit. LRI (DB-Wax)**	**Lit. Descriptors**	**Method of ID**
1	930-945	71	60	methyl butanal/malty/sweet, sweet cocoa, chocolate, butterscotch, fruity/solventy, sweet	sweet, bread-like, malty/chocolate	2- / 3-methyl butanal (96-17-3 / 590-86-3)	926/ 936	green, almond, strong burnt, malty, cocoa	O, LRI
2	960-990	100	100	solvent/paint, fresh/solvent/floral, bubblegum, solvent/alcohol, choc/praline, nail varnish remover, sweet, paint, sweet, choc/nut/almond, alcohol, solventy	warm cake, nutty, sweet/ethereal, nail varnish remover, solvent, bubblegum/synthetic, sweet, sweets/jelly beans, inky/musty, acetone, dark chocolate	2,3-butanedione (431-03-8)	*983* (Cullere et al., 2004)	buttery, caramel, cream, fruit, spirit	O, LRI
3	1020 - 1030	86	60	sweet, buttery/diacetyl, butter, yeasty, praline/butterscotch, popcorn/caramel	choc/cream, diacetyl/buttery, popcorn/caramel/toffee	1013 2,3-pentanedione (600-14-6)	1041 (Aceña *et al.*, 2010)	buttery, nutty, cheese	NIST, O, LRI
4	**1045 - 1075**	**86**	**100**	**fruity solvent, malty, solventy, putty/sweet, cream soda/fruity, praline/nut, cola/fruity,**	**choc/praline, plastic/pea/ink, nail polish, floral, cola, sweets, solventy,**	**1047 propan-1-ol (71-23-8)**	**1052 (Le Guen et al., 2000)**	**fruity, plastic, pungent, musty**	**NIST, O, LRI, Std**

				sherbert, sweets, faint flower/fruit	**sweeties/chemical, sweet/fruity, strawberries and cream sweets**				
5	1100 - 1120	71	60	heavy putty/musty, rubbery, nail varnish remover, popcorn, sweets, malty/sweet/ chocolate, nut/choc/praline, sulphur, sweet, solventy	nail polish, sweet, chocolate, toffee, fruity/solventy, sweet alcoholic, benzaldehyde/ chocolate	1105 2-methylpropan-1-ol (78-83-1)	1108 (Ferreira *et al.*, 2001)	ethereal, winey	LRI, O, NIST, Std
6	1160 - 1180	71	100	solvent/pineapple/banana, pear drops, sweet/candy, marzipan, almonds	candy floss, pear drops, slightly musty, new things/plastic	1138 isoamyl acetate	1132 (Ferreira *et al.*, 2001)	pear drops, banana	LRI, O, NIST, Std
7	1250 - 1260	100	100	solventy, sweet chocolatey, tutti frutti, cocoa, benzaldehyde, almond/marzipan, solventy/unpleasant, stale/sweaty	sweet/cheesy, alcohol/sweet, musty/sweaty, solventy, chocolate, benzaldehyde	Fusel alcohols: A. 2-methyl-1-butanol (137-32-6); B. 3-methyl-1-butanol (123-51-3); C. 1-pentanol (71-41-0)	A. 1206; B. 1230; C. 1244	malty, pungent, alcohol, fruity, malty, ripe onion, burnt, cheese, bitter, harsh, green, sweet	LRI, O, NIST, Stds
8	**1320**	**86**	**80**	**coke, pyrazine, virkon, citrus, sweet drink, fruity cleaning fluid**	**bathroom cleaner, solventy, lemon washing up liquid, citrus cleaner**	**1321 1,1,3-triethoxypropane (7789-92-6)**	**nk**	**nk**	**LRI, NIST**
9	**1340**	**57**	**80**	**weak mushroom, mushrooms/metallic**	**metal/mushroom, metallic, weak mushroom**	**1-octen-3-one**	**1317 (Cullere *et al.* 2004)**	**mushroom**	**O, LRI**
10	1460 - 1470	100	100	sweet heptan-2-one, green/burnt, nutty/pyrazine, nutty, earthy compost, green/fresh,	pleasant, chemical, nutty/walnuts, nutty, baked pyrazine-like or furfural, burnt smell, fresh peas in a pod	1461 1-octen-3-ol? 2,4-heptadienal?	1465 (Hognatta-dir & Rouseff, 2003)	garlic, earthy mushroom, spicy, rubbery, carrots,	LRI, O, NIST, Std

				fusel/pungent, nuts				herbaceous	
11	1480 - 1500	86	60	peanuts, cooking, fatty becomes burnt, unpleasant/pungent	milky/eggnog, new carpet, fatty becomes nutty, green, green veg, mocha coffee	1485 furfural diethyl acetal 1498 furfural	nk 1485 (Cullere *et al.* 2004)	nk bready, burnt sugar	LRI, O, NIST, Stds
12	1540 - 1570	86	100	carrot, plastic, new car smell, solventy, sweet, leather shoe, oily, paper/new, new car, putty/plastic	fresh peas, pine, new things, mallow, leather, paper, solventy/new car, dentist/air freshener, minty, fruity/fragrant	1542 2-acetylfuran 1575 benzaldehyde	*1499 (Mottram, 1987)* *1572*	balsam, almond bitter almond	LRI, O, NIST, Stds
13	1630	71	60	fusel alcohol, nutty, green leaves, onion, green	? Body lotion, mossy/peaty, coconut	1612 5-methylfurfural	*1560 (Valim et al., 2003)*	caramel, burnt sugar, spicy, acid, coffee	LRI, O, NIST, Std
14	1690 - 1700	100	100	sweaty feet, acrid, floral, musty/old sewer, baked nutty, popcorn, nutty/savoury, nutty/popcorn/cooking, cheesy,sour/putrid, lactone-like	nutty, musty, cheesy, musty/cheesy, popcorn/savoury, yeasty	1668 1-methylpyrrole-2-carboxaldehyde (1192-58-1) 1682 2-furanmethanol	nk 1686	nk burnt sugar, fermented	LRI, O, NIST, Std
15	1720	57	80	metal, fish, off cream, mechanical	woody metallic, plastic, pungent/oily machinery	1720 1-(5-methylfuran-2-yl)propan-1-one (10599-69-6)	nk	green, hazelnut	LRI, O, NIST
16	1740	71	80	mushroom, minty, liquorice	liquorice, floral, sweet, minty	1739 propyl decanoate	nk	woody, oily, waxy, fruity	NIST, LRI

17	1760	86	60	green/solventy, waxy, tea leaves	fire, balsam/cosmetics waxy	1746 methionol	1745 (Cullere *et al*. 2004)	meaty, yeasty	NIST, LRI
18	**1780 - 1800**	**43**	**60**	**waxy, floral**	**coconut lactone, winey, sweet**	**1776 isobutyl decanoate**	**nk**	**nk**	**NIST**
19	**1860 - 1870**	**100**	**100**	**beer, wine, floral, brandy**	**beer, winey, winegums, spice, choc eclairs**	**1864 2-phenethyl acetate**	**1803 (Ong & Acree, 1999)**	**floral, rose, sweet, honey**	**O, NIST**
20	**1900**	**57**	**80**	**chestnuts, cake, almond cinnamon**	**cookie dough, sweet smell/floral note, cough syrup**	**1902 decyl acetate (112-17-4)**	**nk**	**nk**	**NIST**
21	**1930**	**100**	**100**	**floral, sweaty, stale wine**	**sweet/musty/oldclothes, musty/dusty/floral, vinegar, malty**	**2-phenethyl alcohol (100-51-6)**	**nk**	**rose, brandy**	**Std, O, NIST**
22	**2040**	**100**	**100**	**soap, waxy/coconut, sweet/popcorn/caramel**	**banana, coconut, cola cubes, lactone, sweet/cinnamon/roasting nuts**	**2088 gamma-nonalactone**	**1991 (CW-20M; Jennings & Shibamoto, 1980)**	**coconut**	**Std, O, LRI, NIST**
23	**2130**	**29**	**60**	**metal/machinery, old oil, burnt**	**linalool, cola cubes, mechanical**	**2137 ethyl pentadecanoate**	**nk**	**nk**	**NIST**
24	**2160 - 2190**	**100**	**100**	**musty/oily, sweets, old books**	**incense shop, oak, slightly fruity, musty, liquorice**	**2161 phenethyl butyrate**	**nk**	**nk**	**NIST**
25	**2200**	**71**	**40**	**metal, soil**	**metal/coins, old/musty**	**ni**	**ni**	**ni**	**n/a**
26	**2330 - 2350**	**57**	**60**	**soapy, waxy, baking, sweet**	**white choc, vanilla, creamy**	**vanillin**	**2589 (Valim *et al.*, 2003)**	**vanilla**	**LRI, O**

OAA: odour active areas (*60%≤ of panellists perceived a similar aroma within 0.5 sec retention time). Areas in bold are odour active compounds/areas not previously identified in the LLE extracts. ni: not identified, nk: data not known. Method of ID: Identification was on the basis of odour (O), Linear Retention Indices (LRI), NIST library database (NIST), confirmed by a standard (Std).

5 INVESTIGATING THE EFFECTS OF ETHANOL AND LONG-CHAIN ETHYL ESTERS ON AROMA PARTITIONING IN A WHISKY MODEL SYSTEM

5.1 INTRODUCTION

Whisky is a complex physicochemical system containing a range of components that influence both the static (equilibrium) and dynamic partitioning of aroma. A major component which has been shown to play a significant role in the partitioning behaviour of other compounds is ethanol concentration (Aznar *et al.*, 2004). In order for a whisky to be sensory evaluated it is diluted to 23% alcohol by volume (ABV) to reduce pungency (Perry, 1989). The addition of water (and hence the dilution of ethanol content) has a direct impact on the solubility of aroma compounds, (Aznar *et al.*, 2004) many of which will be less soluble at lower alcohol strengths and thus will partition into the headspace more efficiently. This is one reason why some recommend adding a drop or two of water to their malt whisky which may allow the flavour to be fully appreciated. However for sparingly soluble amphiphilic compounds such as long-chain fatty acid ethyl esters, the reverse effect occurs: their solubility is lowered by reducing ethanol content. This has been shown to induce 'structuring' of the solution due to the formation of ethyl ester agglomerates (Conner *et al.*, 1994a; Conner *et al.*, 1994b; Conner *et al.*, 1998a). These micelle-like structures comprise of surface active molecules such as long-chain alcohols, aldehydes and esters (Conner *et al.*, 1998a; Conner *et al.*, 1998b). They have the ability to incorporate other hydrophobic compounds (Figure 5.1) and thus to decrease their concentrations in the headspace, potentially altering perceived aroma (Piggott *et al.*, 1996). Another way in which agglomeration of ethyl esters can occur is by adding them at a

concentration sufficient to saturate the surface of the solution (due to their amphiphilic nature ethyl esters act as surfactants, arranging themselves on the surface of an aqueous solution).

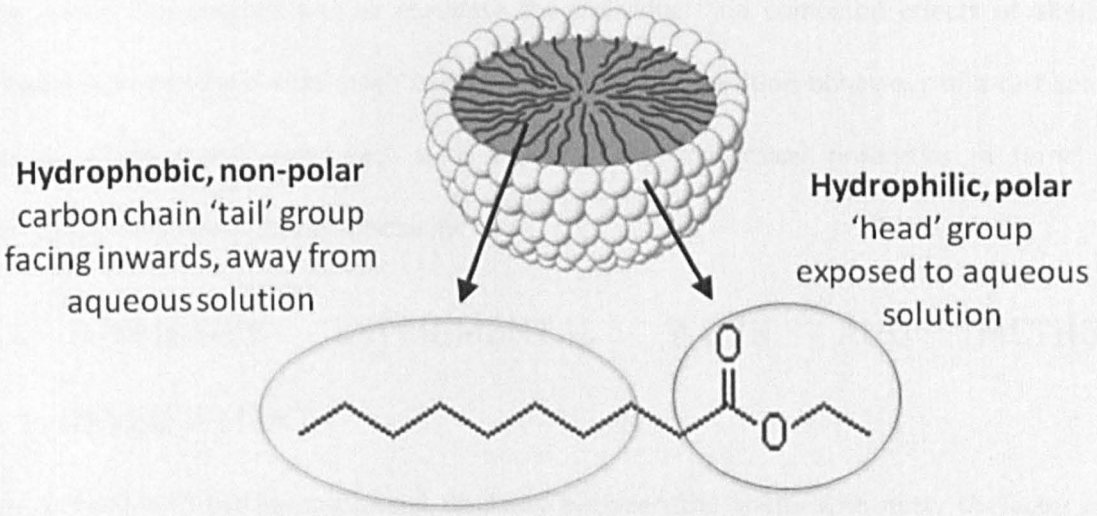


Figure 5.1 Schematic diagram of an agglomerate/micelle with an amphiphilic molecule, ethyl decanoate, which it can incorporate.

In this chapter we investigate the impacts of two surface active components on the headspace concentrations of aroma volatiles above aqueous model systems; ethanol and the large fatty acid ethyl ester, ethyl hexadecanoate.

Due to the analytical difficulties of sensitively analysing aroma compounds in the presence of very high ethanol concentrations, there is a paucity of data in the scientific literature dealing with the partition behaviour of volatile compounds at ethanol concentrations relevant to distilled spirits (40% ABV). In the Samworth Flavour Laboratory at University of Nottingham Food Sciences, a method has been devised known as ethanolic Atmospheric Pressure Chemical Ionisation-Mass Spectrometry (APCI-MS; Aznar *et al.*, 2004). The principle is that ethanol is introduced into the ionisation source via the make-up gas according to the % ABV of a sample, such that the source ethanol concentration is constant across all samples. This is necessary because in high ethanolic systems ethanol acts as a charge transfer reagent in APCI and thus

the ionisation of volatile compounds would otherwise vary according to the accompanying ethanol concentration in the gas phase.

The aim of this chapter was to elucidate the individual and combined effects of altering ethanol and long-chain ethyl ester concentrations on the partition behaviour of a test set of whisky aroma compounds, each with differing physicochemical properties in terms of compound volatility and hydrophobicity.

5.2 FORMATIVE EXPERIMENTAL WORK AND METHOD DEVELOPMENT

When faced with the compositional similarity between the spirits with nutty character and those with minimal nutty character (Figure 3.1), it became increasingly obvious that any significant or recurrent differences which could be discerned between samples would be worth further investigation. A recurrent difference noted between the samples was the levels of ethyl esters; both in the headspace of the 5 spirit samples and in their solvent extracts (Figure 5.2). It is widely known that fatty acid ethyl esters form a significant part of the headspace of distilled beverages. Ethyl acetate, hexanoate, octanoate, decanoate and dodecanoate were shown to make a major contribution to whisky aroma (Salo *et al.,* 1972). Câmara *et al.* (2007) also found that fatty acid ethyl esters , especially octanoate, decanoate and dodecanoate were quantitatively significant in whisky headspace. Thus, their addition or depletion from the headspace is likely to have a significant effect on the overall aroma of the spirit (Conner *et al.,* 1998). These findings are in agreement with the SPME and APCI measurements (data not shown) of headspace volatiles in the new-make spirit samples diluted to 10% ABV, where ethyl esters were notably abundant in the headspace, second only to the alcohols. Of the esters detected in the APCI measurement of the headspace, ethyl octanoate and decanoate were the most abundant. Significantly, it was noted that in the non-nutty

whisky there were much lower levels of ethyl ester present when compared with the nuttier spirits of A, B, C and D.

In order to quantify the concentration of ethyl esters in the spirit, samples were exhaustively LLE extracted into DCM solvent (for full details of the methods see Materials and Methods section 2.2.1). Three replicate extractions were performed on the nutty spirit B and the non-nutty control sample E and the results are shown in Figure 5.2. As previously stated in Chapter 4, whilst liquid-liquid extraction allows for the higher recovery of non-polars such as ethyl esters, it also brings with it a higher level of variation to that seen in SPE extracts. Therefore, although the differences in ethyl ester levels extracted from the spirit were only evidently significant for C6, C8 and C14 esters, in the majority of cases, more esters were noted in the nutty spirit B (Figure 5.2). This data, together with APCI-MS headspace measurements, suggested an overall higher level of key esters hexanoate, octanoate and decanoate in nutty spirit B than compared with control spirit E.

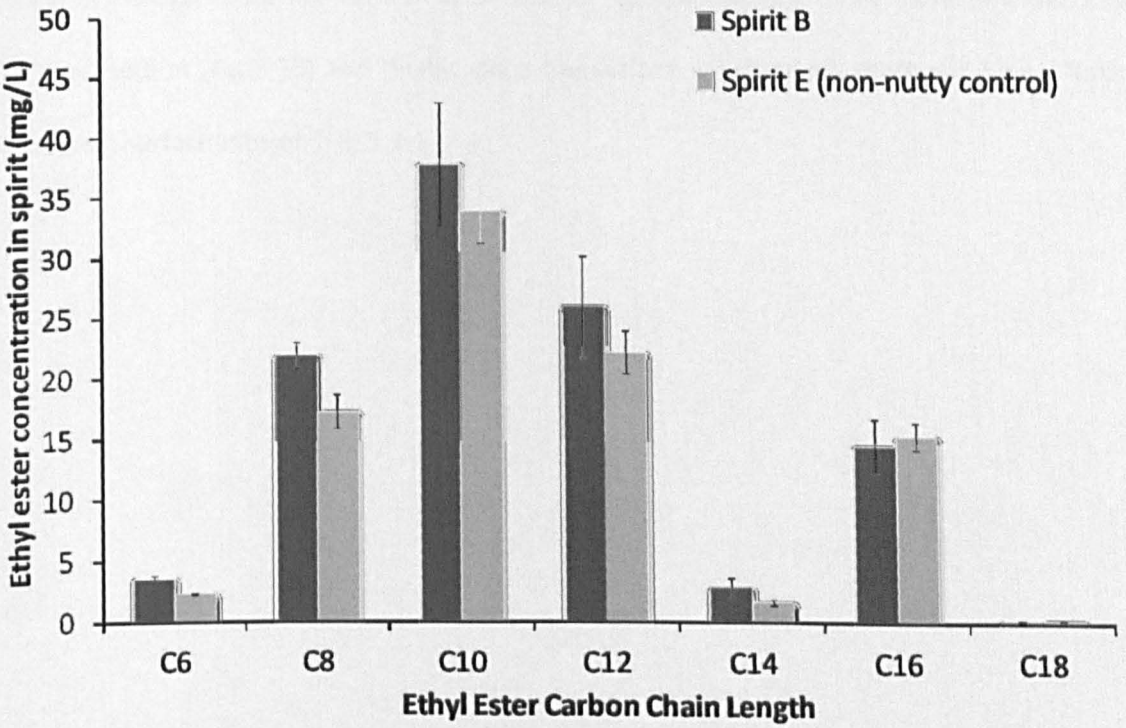


Figure 5.2 Ethyl ester concentrations of nutty spirit B and non-nutty spirit E. Data are the mean ±SD of 3 replicate measurements.

5.3 DETERMINATION OF THE CRITICAL MICELLE CONCENTRATION OF ETHYL HEXADECANOATE IN ETHANOLIC SOLUTION

As already discussed in the introduction to this chapter, agglomeration or formation of micelle structures can occur on dilution of an ethanolic solution. Another way in which agglomeration of ethyl esters can occur is by adding a high enough concentration to saturate the surface of the solution. Due to their amphiphilic nature, ethyl esters act as surfactants, arranging themselves on the surface of the aqueous solution, polar heads submerged, while the non-polar hydrocarbon tail is held above the surface. When more of the amphiphilic compound is added, the surface becomes crowded until they are present at a concentration at which there can no longer be any more compounds arranged on the surface, forcing the surplus to form agglomerates. This is called the critical micelle concentration (CMC). Therefore, in order to discern the point at which the CMC is reached, a graph can be plotted of surface tension against concentration of the surfactant (Figure 5.3). At very low concentration of surfactant, minimal change in surface tension is detected (Fig 5.3a), adding more surfactant decreases surface tension (Fig 5.3b) and finally, once the surface is saturated, there will be no further change in surface tension (Fig 5.3c).

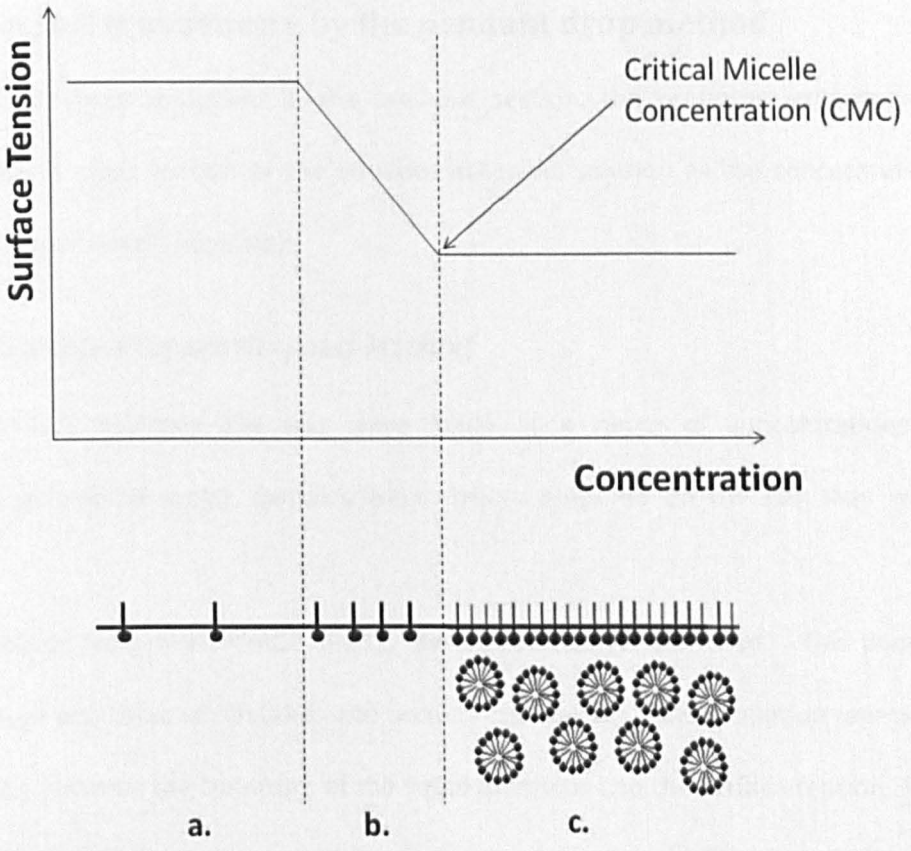


Figure 5.3 The surface tension effects of increasing the concentration of an amphiphile such as an ethyl ester (a-b), until critical micelle concentration is reached and agglomerates are formed (c).

It was this theory of surface saturation and the resulting surface tension gradient and plateau which was the reason for employing surface tensiometry to determine the CMC of ethyl hexadecanoate, the ethyl ester of choice due to its relative odour inactivity, and its likelihood of having the structuring effects of micelle formation.

In total three methods were used to find the range within which the critical micelle concentration of ethyl hexadecanoate in aqueous ethanolic solutions was occurring; surface tensiometry, dynamic light scattering and ethanolic APCI-MS.

5.3.1 Surface tensiometry by the pendant drop method

Using the principles discussed in the previous section, the proposed experiment was to measure the surface tension of the aqueous ethanolic solution as the concentration of the ester was incrementally increased.

5.3.1.1 *Sample Preparation and Method*

Fresh 5% ABV solutions (30 mL) were made at a range of concentrations of ethyl hexadecanoate (0-50 mg/L). Samples were freshly prepared on the day they were to be analysed.

A tensiometer from SINTERFACE (PAT1, Berlin, Germany) was used. The pendant drop methodology was used, which takes into account the Gauss-Laplace equation representing the relationship between the curvature of the liquid meniscus and the surface tension. The system consists of a capillary dosing system to form the drop and a video camera fitted with an objective lens which transfers the real time images to the PC by way of a frame grabber. Prior to forming a drop the atmosphere in the cuve was equilibrated for 20 minutes at 25 °C. The pendant droplet method measured surface tension of each solution for 600 s (1 point every 2.5 seconds). Drop size was 11 mm^3. Density measurements were performed with a DMA 5000 density meter from Anton Paar. The surface tension of each solution was measured in duplicate. All measurements were taken throughout the course of one day.

5.3.1.2 Results and Discussion

It should be noted that there were inherent pitfalls associated with the physical measurement of solutions containing ethanol. Due to their increased volatility, before a droplet could be formed the a small amount of solution (~1 mL) must first equilibrate in the cuvée, the curvature of the droplet can only then be accurately quantified and the measurement recorded. Problems were experienced with equilibration of the sample in the cuvée, which resulted in any measurements of solutions at a greater ethanolic concentration than 5% ABV

proving near impossible, with droplet measurements for the same solution varying greatly. There are a number of reasons why this could have occurred: as surface tension is a function of temperature, temperature fluctuations in the room could have affected the readings (*the tensiometer had a temperature controlled chamber, however the room the tensiometer was housed in was very small with little ventilation and during the summer months when many of the readings were attempted, it was prone to reaching temperatures upwards of 23°C*) the length of time that the sample was left to equilibrate may not have been sufficient or it is possible that there were impurities within the capillary feed system. For the aforementioned reasons, it was believed that the surface tension results that were obtained for the ethyl hexadecanoate concentration range of 0 – 50 mg/L at 5% ABV (Figure 5.4), may be misleading. For example, between 20 – 30 mg/L the surface tension measurement seem to plateau and could possibly be thought of as the range within which agglomeration may have occurred i.e. the surface being saturated thus the tension could not decrease further. However in the tensiometry work undertaken by Taylor and his colleagues (2010) data is shown for surfactants Tween20 and SDS in 12% ethanol, the drop in surface tension measurement to the CMC was much more pronounced at over 10 mN/m.

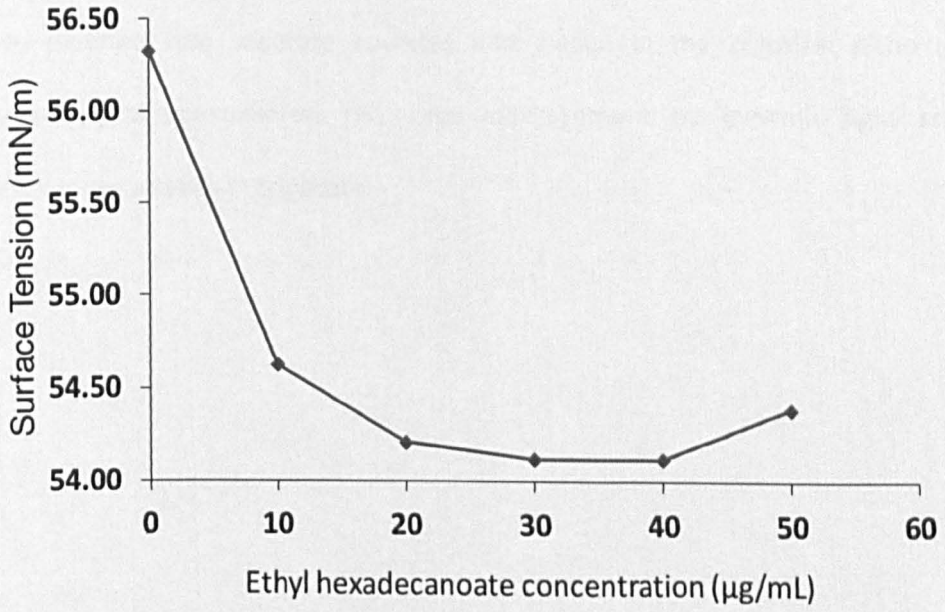


Figure 5.4 Surface tension measurements of ethyl hexadecanoate (0-50 µg/mL) in 5% ABV aqueous ethanolic solutions.

As the decrease that was seen in Figure 5.4 was only 2 mN/m, we can conclude that this was not the result of the CMC being reached, but likely due to variations between measurements due to the problems experienced with ethanol volatility during the pendant drop formation. Something that added further weight to this conclusion, was the fact that previous formative ethanolic APCI measurements had been undertaken within the ranges of 0-20 and 0-30 µg/mL ethyl hexadecanoate solutions (5% ABV) looking at the ratio of polar pyrazine to non-polar ethyl octanoate (data not shown). These concentration ranges proved to be inconclusive in terms of ethyl ester agglomeration as the only significant factor for both compounds was the solubilisation facilitated by ethanol concentration ($p < 0.0001$). This could be seen as further evidence that the CMC was not likely to be within the range of 0-30 mg/L as may have been suggested by the surface tension measurements seen in Figure 5.4.

5.3.2 Particle Size Measurement by Dynamic Light Scattering (DLS)

A 5% ABV solution containing 200 µg/mL ethyl hexadecanoate was made up. As can be seen in Figure 5.5C this solution (unfiltered) was turbid in appearance. Two aliquots of this solution were then passed through 2 filters: (A) Filtered with 0.1 µm (Whatman, ANOTOP25); (B) filtered using 5 µm (Nexstar Pharmaceuticals). These 2 samples and a third unfiltered sample were then pipetted into separate cuvettes and placed in the Zetasizer Nano (Malvern Instruments Ltd, Worcestershire, UK) for measurement by dynamic light scattering. Measurements were taken in triplicate.

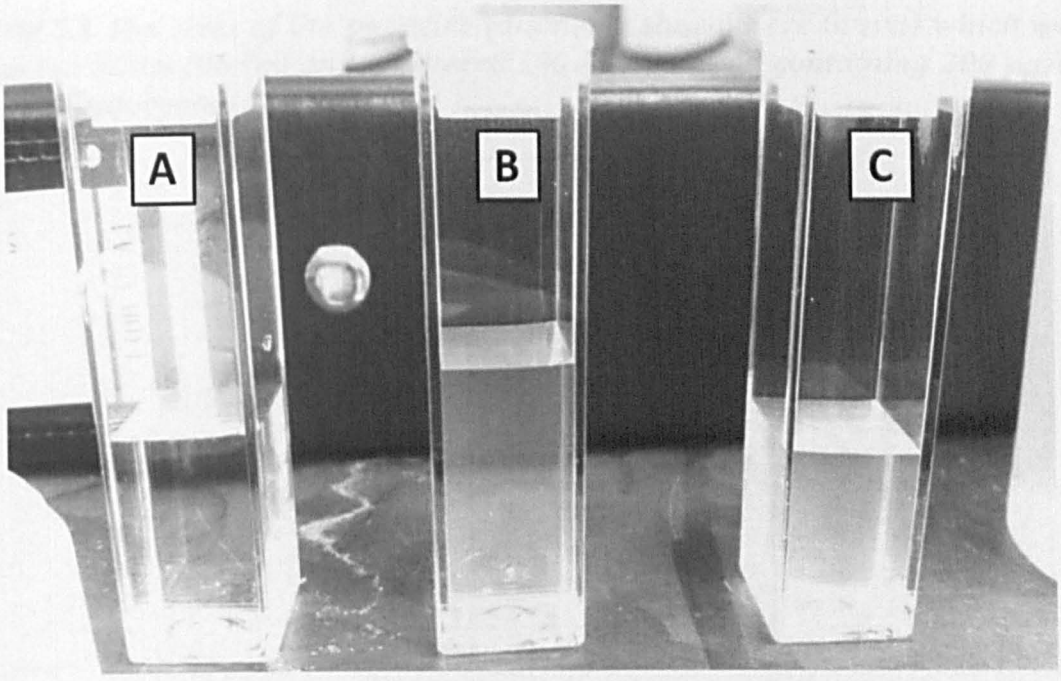


Figure 5.5 Ethyl hexadecanoate (5% ABV; 200µg/mL) solutions in cuvettes ready for DLS measurement. Sample (A) was filtered with 0.1 um (Whatman, ANOTOP25); sample (B) was filtered using 5 µm (Nexstar Pharmaceuticals) and sample (C) was left unfiltered.

As the particles causing the turbidity were removed on using the 0.1 µm filter (Figure 5.5A), but were not removed completely by the 5 µm filter (Figure 5.5B), it could crudely be surmised that the diameter of the particles present in the solution are between 5 and 0.1 µm. This is in agreement with the dynamic light scattering results which show the particle size to be within the range of 0.255 and 1.1064 µm diameter (Table 5.1).

In both the unfiltered and the 5 µm filtered solutions, the peak intensity of particles measured is at 229.3 nm radii (458.6 nm diameter), suggesting the majority are of the same size (Table 5.1). Table 5.1 demonstrates that the filtered sample gave a slightly higher percentage intensity measurement (24.5%) and a sharper peak range in which the particle size could fall, suggesting a higher precision of measurement. For the unfiltered, the distribution of percentage intensity is spread over a larger range of size measurements than compared to the filtered. This could suggest that filtering removes some of the larger particles of debris/dust present in the sample which can skew the results to show a higher proportion of the bigger particle sizes.

Table 5.1 The sizes of the particles (diameter shown here in μm) which were detected in the filtered and unfiltered 5% ABV solution containing 200 μg/mL ethyl hexadecanoate by DLS

	Intensity (%)	
Particle Size (diameter in μm)	5 μm filter	Unfiltered
0.2550	0	0.7
0.2954	5.1	3.8
0.3420	14.4	10.1
0.3960	22.5	16.6
0.4586	**24.5**	**20.3**
0.5312	19.6	19.6
0.6152	10.8	15
0.7124	3.1	8.9
0.8250	0	3.7
0.9554	0	1.2
1.1064	0	0.3

Figure 5.6 shows 5% ABV solutions with various concentrations of ethyl hexadecanoate added from the range of 10-200 μg/mL. What can clearly be seen in these samples is that the level of turbidity within each samples increases with the increase in concentration of the large ethyl ester. This suggested that there may be agglomeration taking place, and it is therefore the micellar structuring which is causing the solutions to become turbid. After this photo was taken (Figure 5.6), these samples were measured by the DLS and what were considered to be the key results are shown in Table 5.2. Here, comparison of the intensity of the particle size distribution for the lower C16 ester concentration levels of 10-50 μg/mL and the higher concentration of 100 and 200μg/mL, showed that there were notable differences in particle size distribution. For the samples containing 10-50 μg/mL C16 ester the largest proportion of the particles (on average 40%) were distributed across the radius size range of 198-229.3 nm. The radii for samples with concentrations of 100 and 200 μg/mL however, were shown to fall within smaller ranges - 82.09-95.07 and 110.1-127.5 nm respectively.

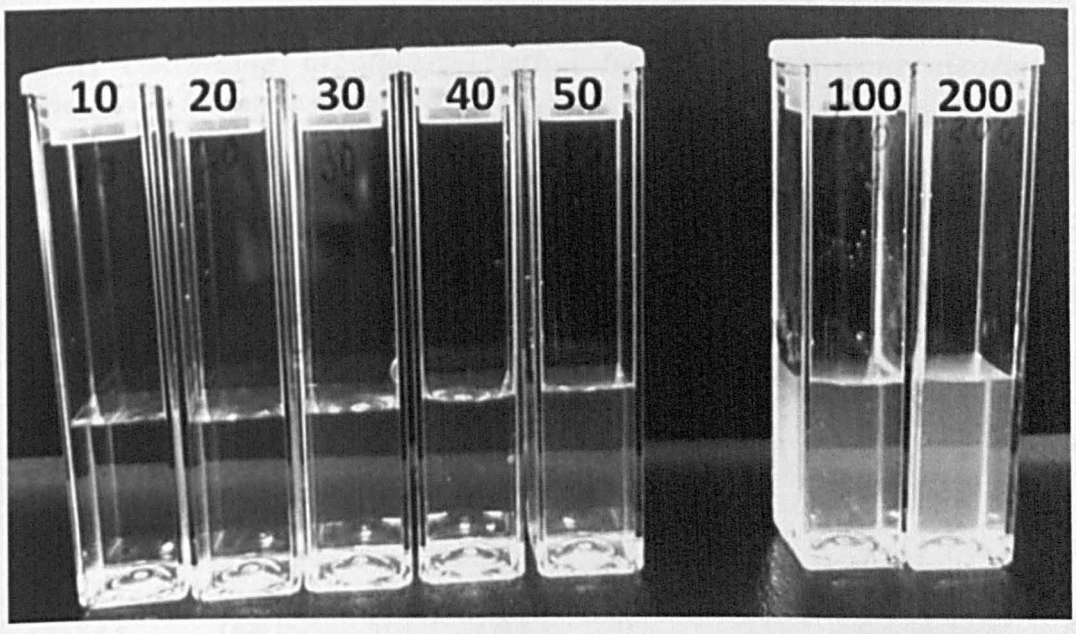


Figure 5.6 Ethyl hexadecanoate (5% ABV; 10-200µg/mL) solutions in cuvettes ready for DLS measurement. Note that turbidity increases with increasing concentration of ethyl hexadecanoate.

In addition to this, a very small percentage of the total distribution of particles (0.5% for 100 and 1.15% for 200 µg/mL) for these concentrations were also seen in the range of 2073-2080 nm (Table 5.2). As these larger particles were not found in the lower concentration samples which showed less turbidity than the higher concentrations, it was postulated that these could be an indication of the formation of agglomerates.

Table 5.2 Intensity measurements of particle size distribution of 5% ABV solutions containing 0-200µg/mL ethyl hexadecanoate concentration, as obtained from DLS measurements.

	Ethyl Hexadecanoate Concentration in 5% ABV Solutions (µg/mL)							
Size (radius; nm)	0 (control)	10	20	30	40	50	100	200
29.39	2.9	0	0	0	0	0	0	0
34.03	2.9	0	0	0	0	0	0.15	0
39.41	2	0	0	0	0	0	1.2	0
45.64	1.9	0	0	0	0	0	3.7	0
52.85	8.2	0	0	0	0	0	7.05	0.15
61.21	18.6	0	0	0	0	0	10.6	2
70.89	14.4	0	0	0	0	0	13.2	6.55
82.09	2.2	0	0	0	0	0	14.5	12.2
95.07	4.3	0	0	0	0	0	14.15	16.8
110.10	8.4	0.4	0.3	0.2	0.7	0.2	12.45	18.65
127.50	10.6	3.2	2	2.7	4.2	3.7	9.7	17.25
147.70	8.3	9.2	8.3	8.4	9.5	10.3	6.65	13.2
171.00	4	15.8	16	14.7	14.7	16.9	3.8	8
198.00	3	20	21.2	19.1	18	20.6	1.7	3.35
229.30	2.9	20	21.3	19.9	18.2	19.9	0.5	0.65
265.60	2.3	16.2	16.6	16.8	15.6	15.4	0.15	0
307.60	1.3	10.1	9.5	11.3	11	9.1	0	0
356.20	0.4	4.3	3.5	5.5	6	3.5	0	0
412.50	0	0.9	0.9	1.4	2	0.5	0	0
477.70	0	0	0.2	0	0.1	0	0	0
553.20	0	0	0	0	0	0	0	0
1790.00	0	0	0	0	0	0	0	0
2073.00	0	0	0	0	0	0	0.05	0
2400.00	0	0	0	0	0	0	0.15	0.15
2780.00	0	0	0	0	0	0	0.3	1
3219.00	0	0	0	0	0	0	0	0

Darker grey shading denotes maximum peak intensities, with lighter grey shading showing the fall in intensity i.e. the peak slope at either side of the maximum.

In order to confirm or disprove this theory, a light microscope was used to visualise these larger particulates which may be responsible for the spikes in intensity at 2400 nm (particle diameter 4800 nm). The only large particles that it was possible to identify under the light microscope in this size range was that of dust (Figure 5.7) which meant that these findings, although interesting were not conclusive regarding the presence of agglomerates .

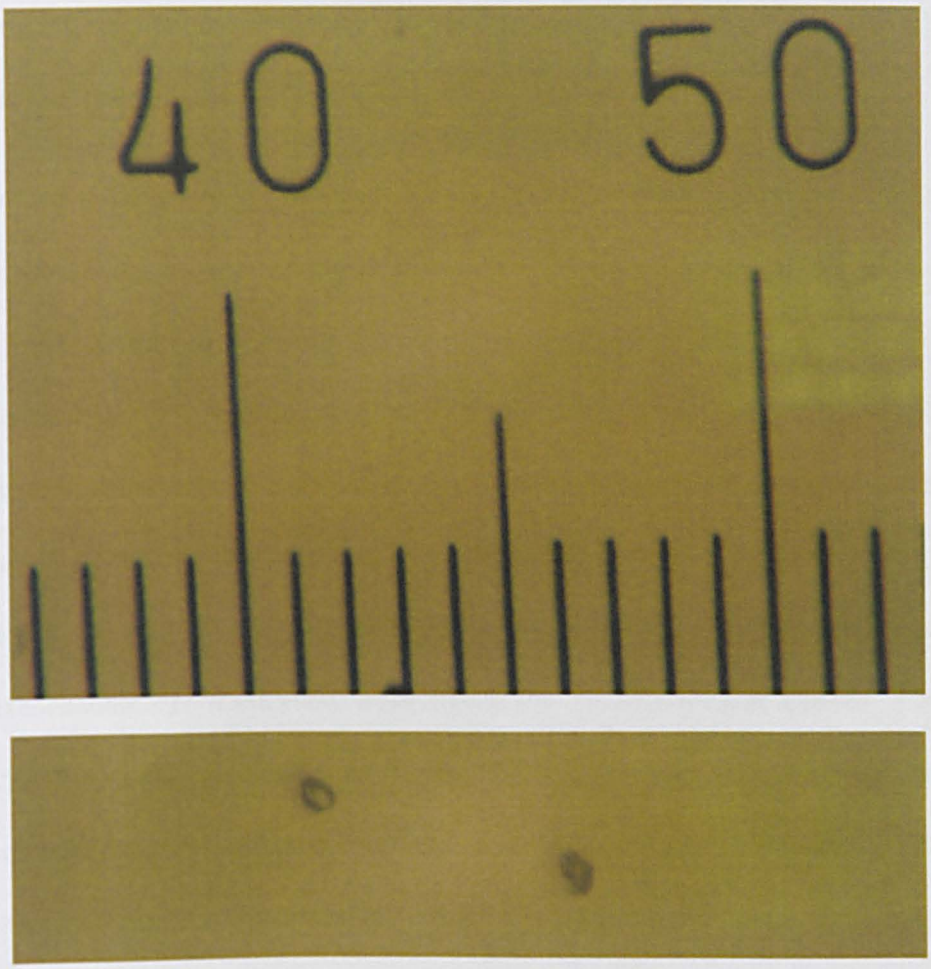


Figure 5.7 Light microscopy of 5% ABV solution containing 200 µg/mL ethyl hexadecanoate. Particles are likely to be that of dust due to their size and shape.

In addition to surface tensiometry and DLS measurements, these samples were also measured on a CPS Disc Centrifuge, however this proved inconclusive due to the incompatibility of 5% ABV aqueous ethanolic solution and therefore did not give repeatable results.

5.3.3 Investigating the range within which the critical micelle concentration of ethyl hexadecanoate is reached in 23% ABV solution using ethanolic APCI-MS

Aqueous ethanolic samples (23% ABV; 40 mL) were spiked with ethyl hexadecanoate at concentrations of 5, 50 and 500 µg/mL Each sample was made fresh on the day of analysis and was contained in a Schott bottle with a stoppered one port lid for sampling at the APCI inlet.

Measurements were taken in triplicate and were randomised and continuous. Further details of the methods used are set out in Section 2.2.

When surface tensiometry and dynamic light scattering measurements proved inconclusive and time consuming, it was decided that ethanolic APCI, with its benefit of real time analyses, could at least be used to elucidate the concentration range of ethyl hexadecanoate within which this agglomeration may be occurring. This was done by assessing the effect it had on the headspace concentrations of β-damascenone, another hydrophobic compound (LogP 4.21) which may be susceptible to agglomeration, at the nosing dilution of 23% ABV.

To discern whether differences between headspace concentrations for the samples at differing ethyl hexadecanoate concentration were significant, an ANOVA was applied. The ANOVA stated that the statistical model had a good fit (r^2=0.97) and that the reduction of the headspace concentration of the hydrophobic ionone was highly significant (p = 0.0019) in relation to the increasing concentration of ethyl hexadecanoate. This is in agreement with what Conner and his colleagues (1994b) found when looking at 23% ABV model whisky solutions; that the headspace concentration of hydrophobic aroma compounds were not influenced by concentration alone, but by the existence of other hydrophobic compounds in the spirit. The fact that the marked decrease in signal came about within the range of 50 – 500 µg/mL suggested that agglomeration may be taking place within this concentration bracket. Thus, on the basis of these results it was decided that 0-500 mg/L be the concentration range that ethyl hexadecanoate would be added to the volatile compound mixtures in the main experiment as described in Section 5.4.

5.4 LONG CHAIN ETHYL ESTER ADDITION: THE EFFECT ON HEADSPACE CONCENTRATIONS OF WHISKY AROMA VOLATILES

5.4.1 Materials & Methods

5.4.1.1 Chemicals

Authentic compounds (> 97 % purity) were obtained from Sigma Aldrich (Poole, Dorset, UK): ethyl-L-lactate, pyrazine, 2-furanmethanol, 2-furaldehyde (furfural), 2-methylpyrazine, 2,5-dimethylpyrazine, 2-acetylthiazole, benzaldehyde, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl octanoate, β-damascenone. Ethyl hexadecanoate was obtained from Alfa Aesar (VWR, Lutterworth, Leicestershire, UK).

New make spirit samples were sourced from industry and provided by the Scotch Whisky Research Institute, Riccarton, Edinburgh, UK.

5.4.1.2 Ethanolic APCI: Sample preparation

Aqueous ethanolic solutions (5-40% ABV) containing ethyl hexadecanoate (0-500 $mg.L^{-1}$) were prepared according to the experimental design as laid out in 5.4.1.4. Samples (40 mL) were prepared in 100 mL flasks with a one port lid serving as an inlet for the APCI sampler. A set of 14 whisky aroma compounds were chosen for their varying physicochemical properties, as well as their aroma characters (pyrazines were chosen as they represented polar nutty/cereal compounds) Each aroma compound was added at a concentration of 50 µg/mL. A graph of the compounds with their Vapour pressures and LogPs can be seen in Figure 5.8.

5.4.1.3 Ethanolic APCI-MS analysis of selected (whisky) volatiles

Headspace congener concentrations were analysed using a Platform LCZ APCI mass spectrometer fitted with an MS Nose interface (Micromass, Manchester, UK) with a modified source designed to operate at high and differing ethanol concentrations as described previously (Aznar *et al.*, 2004). The flow of ethanolic vapour to the source was controlled using

a mass flow meter (Aalborg, Orangeburg, U.S.A.) and was adjusted depending on the ethanol concentration in the sample (0-200 mL/min). The APCI source was operated with a corona voltage of 15 kV.

All analyses were performed in selected ion mode, whereby the protonated ion ($[M+H]^+$) of each volatile compound was specifically monitored to increase the sensitivity of measurement. Volatile compounds were thus analysed in small pre-determined groups whence it had been ascertained that there was no interference beween compounds in respect of the monitored ions. The full experimental design of 20 samples was repeated for each of these groups. Sampling flow rate (set to 2 mL/min) was measured using a VeriFlow 500 gas flow meter (Humonics Inc., Folsom, CA). The heated transfer line (Hillesheim, Waghausel, Germany) was maintained at 170 °C.

The order of sample analysis was fully randomized across the experimental design. Each of the 20 aqueous ethanolic solutions were sampled at the port for 2 minutes, with a 1 minute gap between each to let the signal return to baseline and for the ethanol make-up gas flow to be adjusted in accordance with the ethanol concentration of the next sample. An external standard of ethyl nonanoate (50 µg/mL in 5% ethanol v/v) was sampled at the beginning, middle and end of the sequence.

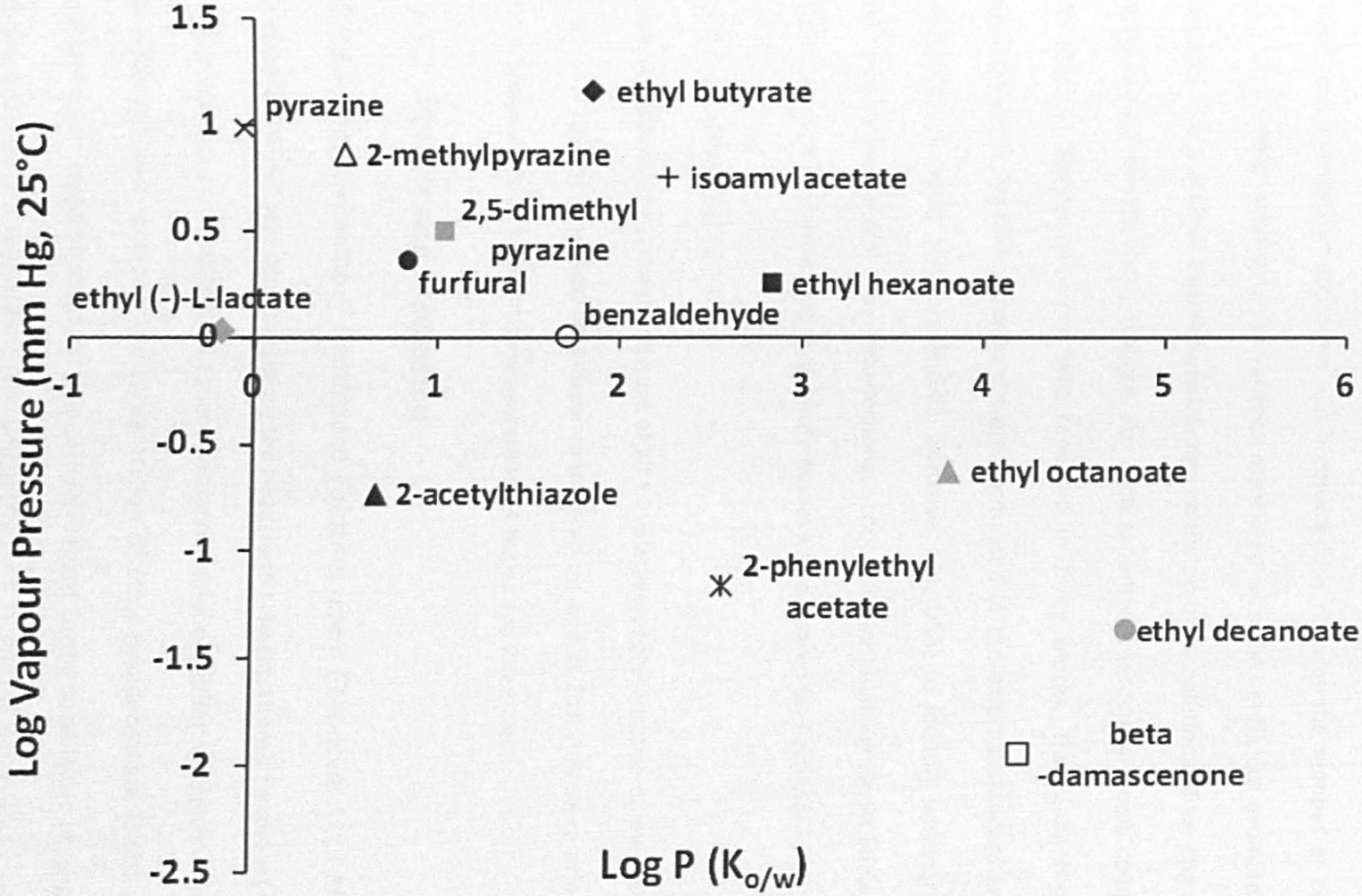


Figure 5.8 Test set of 14 whisky volatiles including those of interest (polar compounds) Log P and VP values are estimated using the modelling software Episuite (EPA 2000-2007)

5.4.1.4 Experimental Design and Statistical Analysis of APCI Intensity Data

A D-optimal response surface design was used (Design Expert Software v. 6.02, Stat-Ease, Mn, USA) as it employs an algorithm which considerably reduces the number of design points required, whilst minimizing the variance associated with the coefficient estimates of specific models. The D-optimal experimental design consisted of 20 solutions of varying ethanol and ethyl hexadecanoate concentrations. For each experiment (group of aroma compounds) the randomised sample sequence was repeated in three blocks. The mean headspace ion concentrations for each compound were normalised to the external standard and modelled using Design Expert software (v.6.02, Statease, Mn, USA) to identify whether the factors (ethanol content and/or ethyl hexadecanoate concentration) had significant impacts upon the headspace ion concentrations. A separate model was prepared for each individual compound.

5.4.1.5 Sensory analysis

Samples (40 mL) containing a mixture of all 14 volatiles, representative of low (50 ug/mL) and high (500 ug/mL) ethyl hexadecanoate concentrations and at 23% ABV were offered up for a brief sensory appraisal by untrained assessors (4 males and 2 females).

5.4.2 Results and Discussion

5.4.2.1 Measurement of Headspace Volatiles using Ethanolic APCI-MS

Ethanolic APCI-MS was used to analyse the headspace concentrations of a test set of 14 whisky aroma volatiles above a series of aqueous ethanolic solutions differing in alcohol content (5-40 % ABV) and with regard to the concentration of ethyl hexadecanoate (0-500 µg/mL). The concentration range investigated was set with regard to the total levels of long-chain ethyl esters analysed in a new make spirit sample (Figure 5.2). For the two samples analysed, total long chain ethyl ester concentrations (C-8 and greater) were of the order of 100 µg/mL. Hence the concentration range of ethyl hexadecanoate selected for the model probably exceeded the

range typically found in new make spirit samples, in order to improve the chances of determining a clear physicochemical effect at higher levels.

Ethyl esters are formed intracellularly by yeast during fermentation from medium chain fatty acids and ethanol. The levels of ethyl esters produced by yeast are governed primarily by the strain used, the conditions of the fermentation and the composition of the medium (Saerens *et al.*, 2008). The aliphatic chain length of ethyl esters in spirits can extend up to 18 carbons (C18; (Nykänen and Suomalainen, 1983). Selected physicochemical and odour properties of long-chain ethyl esters commonly found in whisky are presented in Appendix 3. It is widely known that some of these hydrophobic fatty acid ethyl esters form a significant part of the headspace of distilled beverages. In particular, ethyl octanoate, decanoate and dodecanoate were found to be quantitatively significant in whisky headspace (Câmara *et al.*, 2007), making a major contribution to whisky aroma (Salo *et al.*, 1972). These findings were in accordance with the data for new make spirit samples as seen in Figure 5.2 regarding total ethyl ester concentrations.

The mean headspace ion concentrations monitored for each compound were modelled across the 20-point D-optimal design space using ethanol and ethyl hexadecanoate concentrations as factors. A summary of the modelling data is provided in Table 5.3, which lists aroma compounds in order of ascending hydrophobicity (LogP).

Table 5.3 The effect of ethanol concentration on the partition behaviour of all 14 volatiles. Data have been normalised against the measured intensity at 0 μg/mL ethyl hexadecanoate and 40% ABV for each volatile and are plotted on a logarithmic scale. Log P values for each compound are shown.

		Factor Significance (*P* value)				
Aroma Compound	**Log P**	**Ethanol conc^n**	**C16 ethyl ester conc^n**	**Interact^n between C16 & EtOH**	**Model R^2**	**Interact^n Effect**
ethyl-L-lactate	-0.18	**<0.0001**	0.40	0.69	0.61	
pyrazine	-0.06	**<0.0001**	0.92	0.68	0.85	
2-methyl-pyrazine	0.49	**<0.0001**	**0.018**	**0.0014**	0.82	**salting out effect at low EtOH**
2-acetyl-thiazole	0.67	**<0.0001**	0.91	0.70	0.87	
furfural	0.83	**<0.0001**	**0.0034**	**0.0054**	0.82	**salting out effect at low EtOH**
2,5-dimethyl-pyrazine	1.03	**<0.0001**	0.79	0.92	0.94	
benzaldehyde	1.71	**<0.0001**	0.83	0.18	0.92	
ethyl butyrate	1.85	**<0.0001**	0.54	0.77	0.96	
isoamyl acetate	2.26	**< 0.0001**	0.90	0.31	0.91	
2-phenylethyl acetate	2.57	**<0.0001**	**0.014**	0.23	0.96	**salting out effect at low EtOH**
ethyl hexanoate	2.83	**<0.0001**	0.50	0.46	0.78	
ethyl octanoate	3.81	**<0.0001**	**<0.0001**	**<0.0001**	0.91	**HS reduced on addition of C16**
β-damascenone	4.21	**< 0.0001**	**<0.0001**	**<0.0001**	0.92	**HS reduced on addition of C16**
ethyl decanoate	4.79	**<0.0001**	**<0.0001**	**<0.0001**	0.81	**HS reduced on addition of C16**

5.4.2.2 Influence of ethanol concentration on aroma partitioning behaviour

Perhaps unsurprisingly, ethanol content over the range 5-40 % ABV had a significant impact upon the headspace ion concentrations of all 14 compounds investigated ($P<0.0001$; Table 5.3). To illustrate how this would impact upon the balance of headspace aroma compounds as whisky samples are diluted we plotted the relative headspace concentrations of the 14 aroma compounds (sorted in order of ascending Log P) at 5, 23 and 40 % ABV (Figure 5.9). The data are normalized against the headspace concentration at 40% ABV (=100) in each case and hence increase in all cases as ethanol concentration is lowered. However, there is clearly an exaggerated impact on the headspace concentrations of hydrophobic compounds at lower ethanol concentrations (note that the scale in Figure 5.9 is logarithmic because of this 'orders of magnitude' effect) which would impact upon perceived aroma. This illustration also makes clear that dilution from 40 to 23% ABV had a marked effect on aroma headspace concentrations (represented by the mid-grey band) whilst dilution from 23 to 5% ABV had a progressive but lesser impact (the dark-grey band). Note that the data in Figure 5.9 are for samples containing no ethyl hexadecanoate.

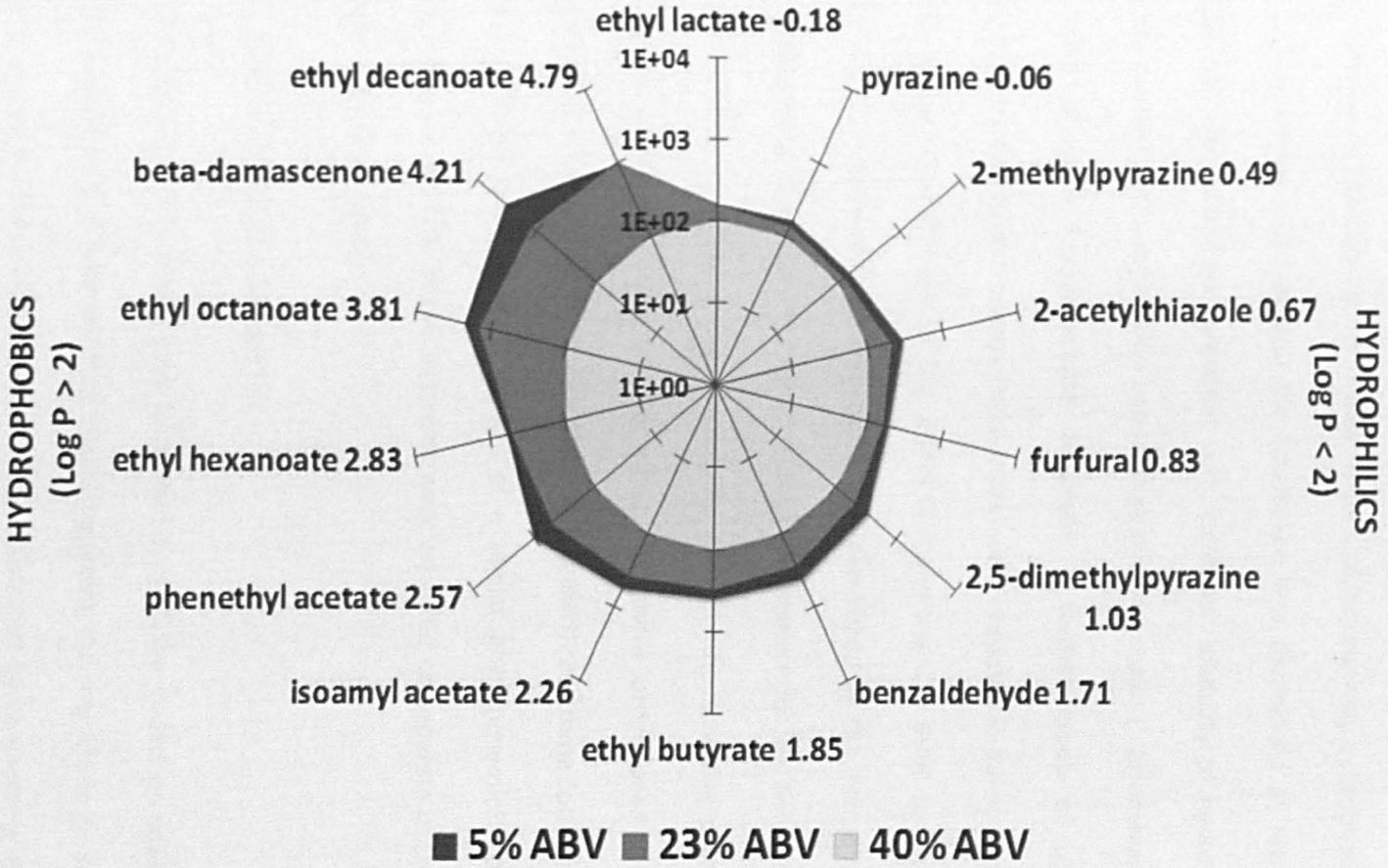


Figure 5.9 The effect of ethanol concentration on the partition behaviour of all 14 volatiles. Data have been normalised against the measured intensity at 0 μg/mL ethyl hexadecanoate and 40% ABV for each volatile and are plotted on a logarithmic scale. Log P values for each compound are shown adjacent to the name.

5.4.2.3 *Influence of ethyl hexadecanoate concentration on aroma partitioning behaviour*

The impacts of ethyl hexadecanoate could be split into two discernible trends; C16 ester concentration was a significant factor in models derived for relatively hydrophobic compounds (Log P > 2.5), whose partition into the headspace was diminished at higher C16 ester concentrations. This would be consistent with increased solubility of hydrophobic aroma compounds due to their incorporation into hydrophobic structures ('agglomerates') formed at higher levels of ethyl hexadecanoate. However, a further group of compounds (2-methylpyrazine, furfural and 2-phenylethyl acetate) were impacted in the opposite sense, such that there was a moderate 'out salting' effect of increasing C16 ester concentrations. This implies that the solubility of these compounds was diminished by the increasing presence of ethyl hexadecanoate. It is relatively easy to think of explanations for this behaviour in terms of changes in solution structure which presumably diminish the solvating power of ethanol towards these molecules, being as the effect was more pronounced at low ethanol concentrations. It is harder to explain why specifically these three aroma compounds showed a weak but significant effect whereas others of a similar physicochemical nature did not. However, it can be said that the experiments were repeated on separate occasions and that the findings were reproducible.

5.4.2.4 *Effects on ethyl ester series*

Whilst Table 5.3 indicates whether each factor had a significant impact on aroma partitioning across the design space, it provides little insight regarding the magnitude or directionality of the effects observed. These trends were further illustrated by considering the ethyl ester series (C4, C6, C8 and C10) which encompass a suitable range of compound hydrophobicities and volatilities (Figure 5.10).

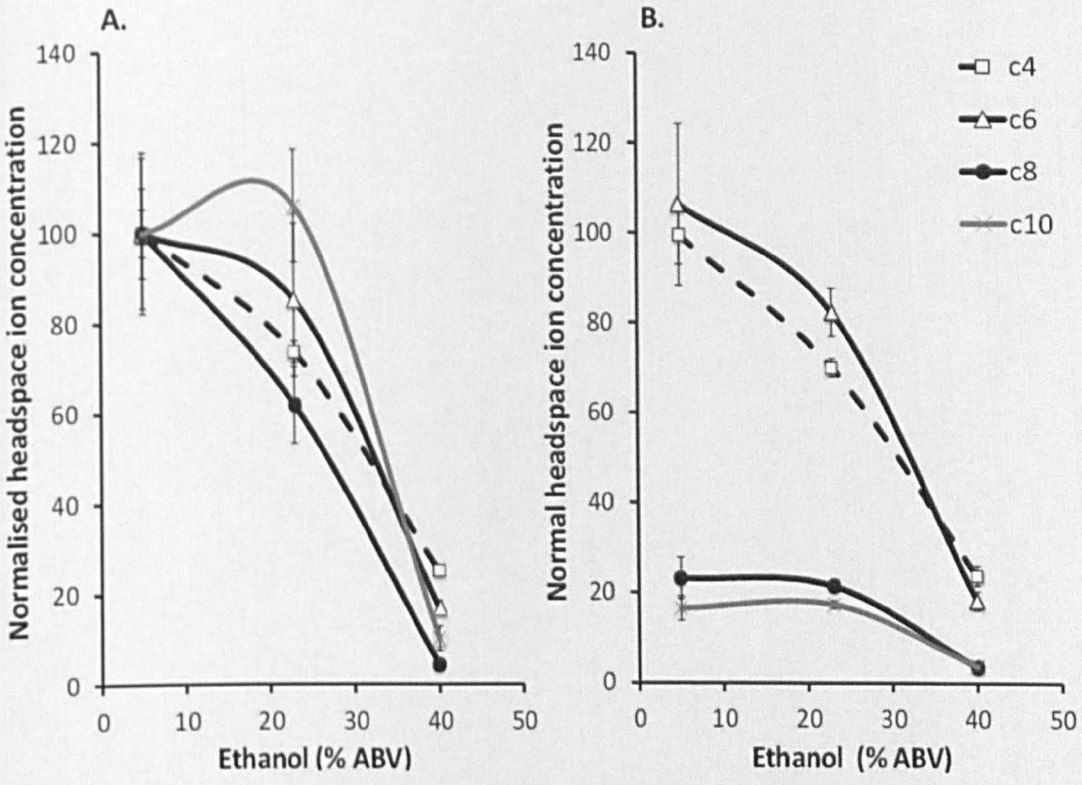


Figure 5.10 Effect of ethanol concentration on the APCI headspace ion intensities of the ethyl ester series (C4, C6, C8 & C10) with (A): no ethyl hexadecanoate and (B): 500 µg/mL ethyl hexadecanoate. Data have been normalised against the 5% ABV sample with no ethyl hexadecanoate for each volatile and are the mean ±SD of at least 3 replicate measurements.

Figure 5.10 depicts the impact of ethanol on the partitioning behavior of this ethyl ester series with no added ethyl hexadecanoate (Figure 5.10A) and at the highest concentration of ethyl hexadecanoate (500 µg/mL; Figure 5.10B). Data have been normalized against the monitored headspace concentration for each series at 5% ABV and 0 µg/mL ethyl hexadecanoate. The solvating effects of ethanol on the ethyl ester homologous series can clearly be observed (Figure 5.10A) with headspace concentrations at 40% ABV being less than a quarter of those at 5% ABV and with the magnitude of effect increasing with ethyl ester chain length. It is notable that at 23% ABV the mean headspace concentration of ethyl decanoate increased slightly relative to that observed at 5% ABV. This trend is likely due to the observed 'structuring' of ethanol-water mixtures as % ABV increases (Conner *et al.*, 1998b; D'Angelo *et al.*, 1994). Aqueous solutions containing up to 15% ABV have been shown to contain ethanol mono-dispersed in water; however, between 20-57% ABV there is a progressive aggregation of

ethanol molecules to form 'pseudo-micelles' which initially (at 23% ABV) lowers the solvating power of ethanol towards the longer chain-length esters such as ethyl decanoate (Conner *et al.*, 1998b). At 40% ABV, present results indicated that there was sufficient ethanol in the system to effectively solubilise each of the ethyl esters in the series investigated. This is consistent with the observation by Conner *et al.* (1998b) that, for ethyl esters, the change from ethanol-rich 'pseudo-micelles' to an ethanolic solution had little effect on headspace partitioning behavior. In other words, by 40% ABV the solution is rich in structured ethanol and is behaving similarly to when it becomes the continuous phase (above 57% ABV ethanol-water mixtures are water monodispersed in ethanol (D'Angelo *et al.*, 1994).

In the presence of ethyl hexadecanoate at 500 µg/L (Figure 5.10B), there was a divergence of behavior between the C4 and C6 ethyl esters which were largely unaffected (relative to Figure 5.10A) and the C8 and C10 esters, whose headspace intensities were significantly reduced (<0.001; Table 5.3). This effect was most apparent at low to intermediate ethanol concentrations and would certainly be evident at the typical whisky 'nosing' concentration (23% ABV).

Figure 5.11 illustrates the impact of varying C-16 ester concentrations on the headspace ion intensities of the ethyl ester series at ethanol concentrations of 5, 23 and 40% v/v (Figures 5.11A to C respectively). The data have been normalized against the headspace ion concentrations with no added C16-ester in each case, to facilitate comparison. Once again, at both 5 and 23% ABV, increasing C16 ethyl ester concentrations lowered the air-liquid partition coefficients of the relatively hydrophobic (C8 & C10) ethyl esters. A concentration of greater than 250 µg/L ethyl hexadecanoate was sufficient to reduce the headspace concentrations of these compounds to less than half of those in the absence of ethyl hexadecanoate. By comparison, the partitioning of the C4 and C6 ethyl esters was not significantly affected by the concentration of ethyl hexadecanoate in the system. Figures 5.11A-C show that as the ethanol concentration was increased to 40% ABV, the impact of ethanol progressively outweighed that

of ethyl hexadecanoate on the C8 and C10 ethyl esters. Ethyl octanoate in particular showed only a small decline in headspace concentration with increasing ethyl hexadecanoate at 40% ABV.

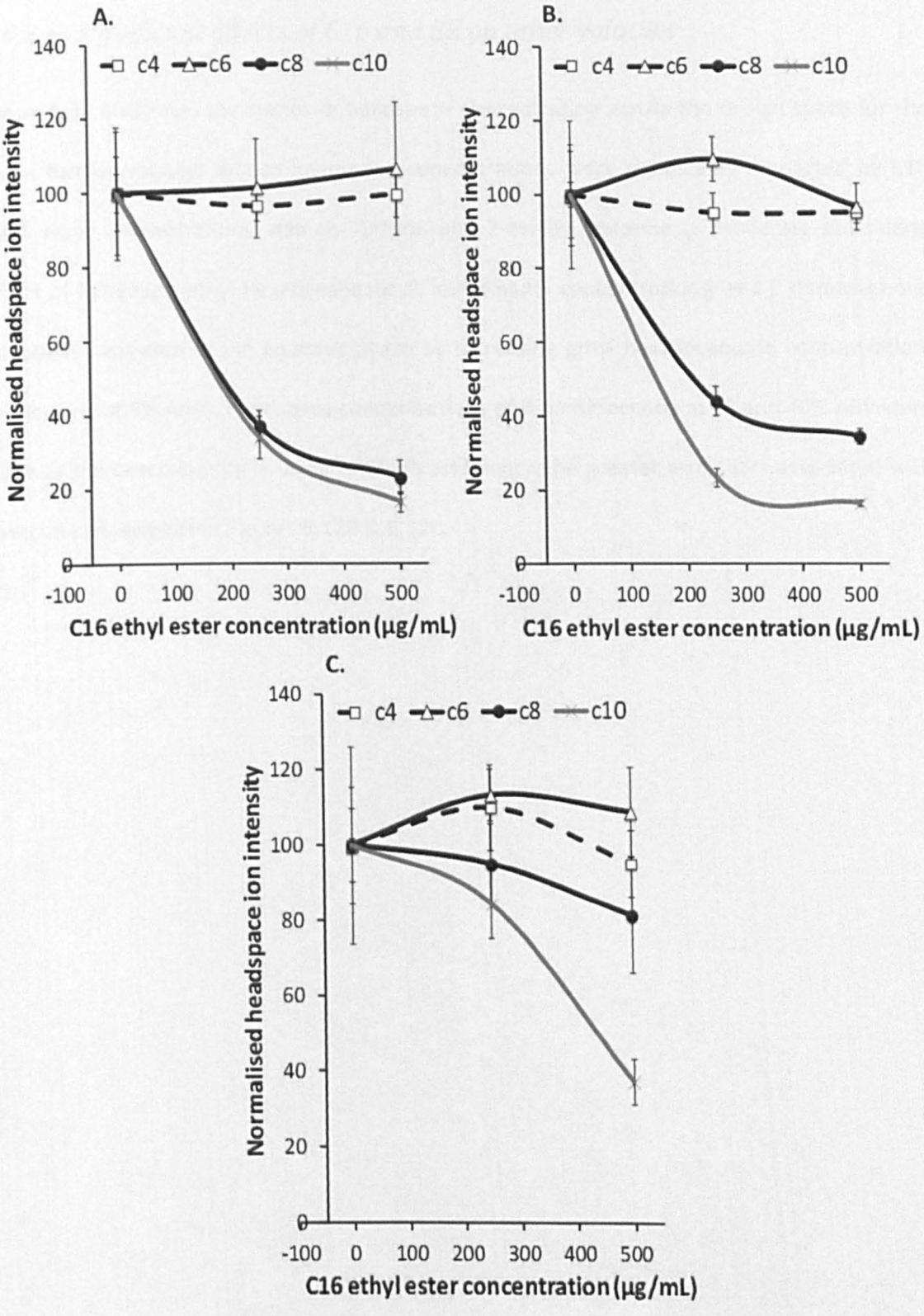


Figure 5.11 Effect of ethyl hexadecanoate concentration on ethyl ester headspace intensity at 5% (A), 23% (B) and 40% ABV (C).

5.4.2.5 Significant effects of C16 and EE on other volatiles

Figure 5.12 illustrates the trends in headspace concentration across the design space for the three further volatiles whose headspace concentrations were significantly impacted by C16 ethyl ester concentrations; namely furfural and 2-methyl pyrazine (a moderate out-salting effect of increasing ethyl hexadecanoate at low ethanol concentrations) and β-damascenone (solubility increased in the aqueous phase by increasing ethyl hexadecanoate concentration, particularly at 5% ABV). Headspace concentrations of β-damascenone at 23 and 40% ABV were close to the detection limits using APCI-MS and hence the greater error bars associated with these measurements in Figures 5.12B & 5.12C.

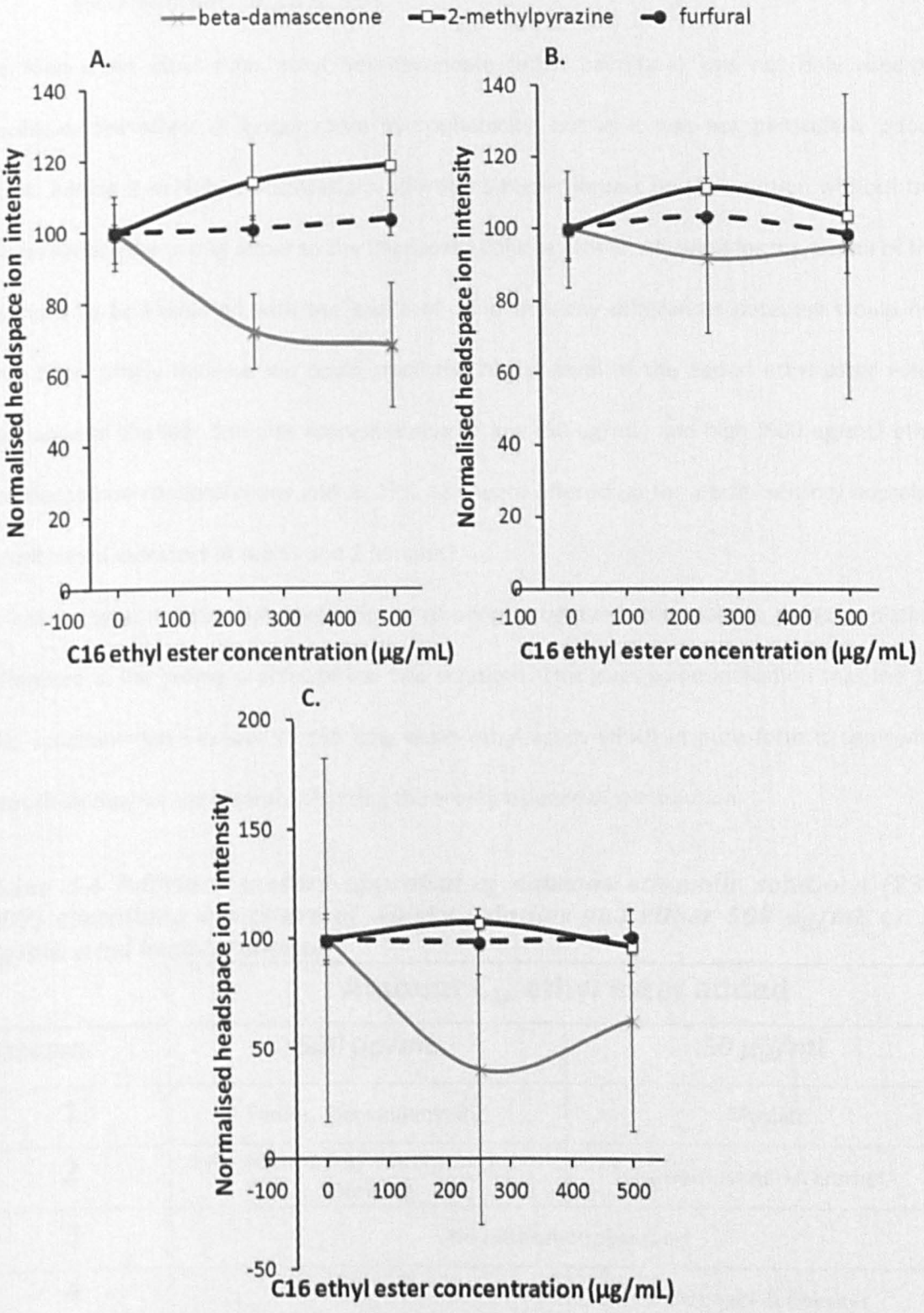


Figure 5.12 The significant effects of ethyl hexadecanoate concentration on headspace intensity of hydrophobic beta-damascenone, polar pyrazine, and mid-polar furfural at 5% (A), 23% (B) and 40% ABV (C).

5.4.2.6 *Informal sensory appraisal of the high & low concentration C16 ethyl ester samples at 23% ABV*

The long chain ethyl ester ethyl hexadecanoate (ethyl palmitate) was not only used to emphasise the effect of longer chain hydrophobicity, but as it was not particularly odour active, adding it in high concentrations afforded a bigger impact on the solution without the accumulation of a strong odour to the headspace volatile profile. Allowing for the aroma of the solutions to be evaluated with the peace of mind that any differences detected would not have been simply because we could smell the higher level of the added ethyl ester when compared to the low. Samples representative of low (50 ug/mL) and high (500 ug/mL) ethyl hexadecanoate concentrations and at 23% ABV were offered up for a brief sensory appraisal by untrained assessors (4 males and 2 females).

As can be seen in Table 5.4, the majority of people surveyed were able to detect a distinct difference in the aroma profiles of the two solutions. This gives some indication that the 10-fold concentration increase of the long chain ethyl ester, which in pure form is somewhat odourless, may be significantly affecting the aroma balance of the solution.

Table 5.4 Informal sensory appraisal of aqueous ethanolic solutions (23% ABV) containing a mixture of whisky volatiles and either 500 µg/mL or 50 µg/mL ethyl hexadecanoate.

	Amount C_{16} ethyl ester added	
Assessor	***500 µg/mL***	***50 µg/mL***
1	Sweet, 'benzaldehydey'	Mustier
2	Less intense, less sweetness; more alcoholic	Sweet, vanilla/caramel
3	No difference observed	
4		Stronger & sweeter
5	Pear drops/sweets	Stronger, more complex, harder to define
6	Coconut stronger	Similar but more peaches

5.5 CONCLUSIONS

Overall the results reported here demonstrate that the aroma balance of whisky, between its hydrophilic and hydrophobic components, can be influenced both by the ethanol content (Figure 5.10) and also by the concentration of long chain ethyl esters, which have the potential to form agglomerates when present above the critical micelle concentration (CMC). These agglomerates form a reservoir for small hydrophobic aroma compounds and reduce their headspace concentrations. Furthermore there was evidence of interactions between the two factors (Figure 5.11), such that the impact of ethyl hexadecanoate concentration was more pronounced at 5% and 23% ABV than at 40% ABV. It is hypothesised that as whisky is diluted from 40% ABV to 23% ABV for nosing purposes, the CMC of ethyl ester agglomerate formation is also reduced and hence that under these conditions the aroma balance is more sensitive to long-chain ethyl ester concentrations in the spirit. Distillates high in such esters might thus be expected to have a nosed aroma which (relatively speaking) emphasizes the aroma characters of the polar hydrophilic volatiles present.

Aqueous concentrations of ethanol (5-40% ABV) and ethyl hexadecanoate (0-500 μg/mL) significantly impacted on the partitioning behaviour of a test-set of 14 whisky aroma volatiles in aqueous model systems. These effects can be interpreted in terms of the physical chemistry of ethanolic solutions and the potential for agglomerate formation by long chain fatty acid ethyl esters in spirit samples, which selectively diminishes headspace concentrations of the more hydrophobic aroma compounds. When spirit is diluted to 23% ABV for nosing, spirits with ethyl esters present at higher concentrations are more likely to form agglomerates. These structures can incorporate small hydrophobic flavour compounds, thus lowering their headspace concentrations and changing the balance of the nosed aroma towards more polar, hydrophilic compounds. In whisky it is anticipated that micelle formation would incorporate long-chain ethyl esters of varying chain-lengths (as well as surface active alcohols and

aldehydes) and thus that the total concentration of these species, as influenced by the distilling process, would be a significant factor determining aroma balance.

6 THE IMPACT OF INCREASED WASH FATTY ACID LEVELS ON THE NUTTY/CEREAL/OILY AROMA VOLATILE COMPOSITION OF NEW MAKE MALT SPIRIT

6.1 AIM

To investigate the impacts of wash fatty acid content and of wash still distillation temperature on the chemical composition and sensory characteristics of new make spirits, with particular focus on the nutty/cereal flavour characters thought to be influenced by Maillard and lipid oxidation chemistry.

6.2 INTRODUCTION

Unlike in the brewing industry where a cloudy wort is not considered to be desirable, in a whisky distillery a cloudy wash may be a common sight. It has been said that a turbid wash serves as an indicator of what the character of the resulting spirit is likely to be; that a cloudy or turbid wash imparts a "nutty or oily character" to the distillate (Lindsay, 2009). The circumstances in which a cloudy, lipid-rich wash may arise are numerous. For example if the mashing temperature is high or the spent grain is compressed during filtration (Lea and Piggott, 2003). Similarly, in brewing, if the mashing process is aided by brisk and thorough raking this can also produce a wort high in fatty acid content (Yonezawa and Stewart, 2004). Lipids are necessary to promote yeast growth, increasing chances of yeast survival during fermentation. However increasing the level of lipids in the wash has been shown to adversely affect the production of ethyl esters during brewery and distillery fermentations (Saerens *et al.*, 2008). During distillation a range of thermally induced chemical reactions take place which impact upon the volatile composition of the distillate. These include Maillard reaction chemistry, lipid oxidation and the myriad of interactions which occur between the precursors and products of both (Farmer and Mottram, 1994; Hidalgo and Zamora, 2004). Due to the

flavour taints associated with excessive fatty acid content in beer, much of the research to date has concerned the causes and effects of cloudy wort in breweries. In doing these experiments, one aim was to address the dearth of knowledge of this readily accepted phenomena in distilling. Therefore, the research put forward in this chapter endeavoured to prove or disprove whether the turbidity, and thus the lipid content of the wash, could significantly affect the spirit quality with regard to nutty, cereal or oily character. In order to fulfil this objective, unsaturated fatty acids were extracted from industry-sourced fermented wash samples. Meanwhile, control fermented wash samples and fermented wash with a high level of oleic and linoleic fatty acids spiked into it were distilled in lab-scale copper stills. The aroma of the ensuing spirits were compared by sensory analysis. Once sensory analysis had proven the differences in aroma and the levels of lipids naturally occurring in the wash were known, samples of wash were spiked with various amounts of fatty acids relative to the lipid concentration already measured in the wash. The composition of the aroma volatiles of the control distillate and the spirit produced from the fermented wash with the highest lipid spike were further compared using Gas Chromatography-Olfactometry with mass spectrometric detection.

6.3 MATERIALS AND METHODS

6.3.1 Analysis of the fatty acid profile of fermented wash

Fermented wash (10 mL) sourced from a Scotch whisky distillery was centrifuged for 5 mins (6000 rpm). The liquid layer was transferred to a separating funnel where it underwent liquid-liquid extraction (LLE) with 2 aliquots of 9mL DCM/methanol (2:1). The solid pellet was also extracted with DCM/methanol (2:1; 9 mL) and shaking the mixture for 20 mins at 500 rpm. Once thoroughly mixed, separation was achieved by centrifugation (6000 rpm) for 5 mins. Both the solid and liquid solvent extracts were dried with anhydrous sodium sulphate before

being transferred to separate TurboVap cylinders (Biotage, Uppsala, Sweden). The cylinders were placed in the TurboVap II manifold where they were heated in a water bath (35 °C) whilst being concentrated under a stream of nitrogen gas to 2 mL. Internal standards (methyl tridecanoate and glyceryl triheptadecanoate) were added (100 µL; 83 µg/mL in sample) and the extracts were subsequently methylated with 100 µL Trimethylsulphonium hydroxide (TMSH) reagent (0.25 M in methanol) and quantified by GC-MS of their derivative Fatty Acid Methyl Esters (FAMEs). A purchased standard of 37 FAME compounds (Sigma Aldrich, Poole, Dorset, UK) was run alongside the sample extracts and used for compound identification. A set of calibration standards of oleic and linoleic acids (20, 40, 60, 80, 100 µg/mL) were made up in DCM, also methylated with TMSH and were also included in the run sequence and used for quantification.

6.3.1.1 GC Analysis of the fatty acid methyl esters

Solvent extracts (1 µL) were injected into a Finnigan Trace GC-MS (ThermoQuest Ltd, Hemel Hempstead, UK) fitted with a DB-WAX ETR column (J & W Scientific; 60 m x 0.32 mm, film thickness 0.5 µm). The oven was held at 120°C for 1 min then raised at 5 °C/min to 250°C, where it was held for 2 minutes. The injector temperature was 250°C and samples were injected splitless for 0.50 mins with a split flow of 35 mL/min thereafter. The carrier gas was helium at a constant flow of 1.4 mL/min with vacuum compensation. The transfer line was held at 250 °C throughout the run and the mass spectrometer scan range was set at 35-400 m/z at 2.0 scans per sec.

6.3.1.2 Lipid-spiked fermented wash recovery experiment

Samples of fermented wash (9 mL) had either 1 mL of 8% ABV solution with no lipid added (control) or 1 mL containing 100 µg/mL oleic and linoleic acids for the spiked sample (final concentration of 10 µg/mL in the 10mL sample). The spiked samples were made up in triplicate. All wash solutions were shaken for 20 mins at 500 rpm, followed by centrifugation at

6000 rpm for 5 mins. The solid and liquid portions were then solvent extracted and analysed by GC-MS as described in the above paragraph.

6.3.2 Experimental design to investigate the flavour impacts of changing wash fatty acid levels and wash distillation temperature

To determine the gross impacts of higher wash lipid levels on the sensory character of new make spirit, a comparison was made between distillates prepared from the control wash (as sourced) and wash which had been spiked with an additional 100 µg/mL of both oleic and linoleic acids. Subsequently, using the concentration of lipids seen in the fermented wash samples, an experiment was designed which would not only test the impacts of more subtle variations in wash lipid composition but also look at what effects a higher distillation temperature could have on the volatile flavour profiles of the resulting spirit samples. As each distillation requires a full day, we needed to get the most robust model from the minimum number of distillations, so we opted to use a response surface D-Optimal design (Design Expert v 8.0, Stat-Ease, Mn, USA). This required 18 distillations to be conducted, varying in respect of spiked linoleic and oleic acids and distillation temperature as indicated in Table 6.1.

6.3.3 Laboratory scale distillations

Fermented wash samples from a local distillery (1.8 L; 55hrs) were spiked with varying amounts of oleic and linoleic acids in ethanolic solution (2 mL) and hand shaken to distribute. The majority of spiked samples (1.65 L) was then poured into the wash still, where 500 µL antifoam Y-30 emulsion and a small amount of PTFE boiling stones (Sigma Aldrich, Poole, Dorset, UK) were added. The wash still was then positioned in a large heating mantle (Fisher Scientific) and the copper condensing apparatus was connected to the top of the still as shown in Figure 6.1 and the mantle was switched on. Each wash sample was double distilled using miniature copper wash and spirit stills (2 L and 1 L capacity respectively) with copper shell and

tube condensers, cooled with glycol to 5 °C. A standard procedure of distillation by volume was followed. Once the low wines (500 mL) were collected, the mantle was switched off and the wash still was allowed to cool before being cleaned thoroughly with a wire brush and rinsed with ethanol and water.

Table 6.1 A response surface D-Optimal design was used to give 18 distillations. Variables include a multi-level lipid spiking regime (0-x8 spike) and altering the mantle temperature settings from control (1) to high (2).

		Concentration of spike in wash (µg/mL)		Approx. final concentration in wash (µg/mL)		
Sample	Spiking level	OLEIC	LINOLEIC	OLEIC	LINOLEIC	Mantle temp. setting
1	0	0	0	5	7	2
2	0	0	0	5	7	2
3	0	0	0	5	7	1
4	x2	5	7	10	14	1
5	x2	5	7	10	14	2
6	x4	15	21	20	28	1
7	x4	15	21	20	28	1
8	x4	15	21	20	28	2
9	x4	15	21	20	28	1
10	x4	15	21	20	28	2
11	x4	15	21	20	28	2
12	x4	15	21	20	28	1
13	x4	15	21	20	28	1
14	x4	15	21	20	28	2
15	x6	25	35	30	42	1
16	x8	35	49	40	56	2
17	x8	35	49	40	56	2
18	x8	35	49	40	56	1

The low wines (500 mL) were transferred to the smaller spirit still, a few Teflon boiling stones were added, the heating mantle was switched on and the final distillation began. Foreshots (25 mL), spirit (100 mL) and feints (160 mL) were collected from the spirit still.

The temperature of the wash still isomantle (Fisher Scientific) was set at two levels: the control (normal SOP level of '8' on the mantle dial) and a higher setting (the maximum setting; '10' on

the dial). To record this difference in heat input, temperature was measured throughout each distillation at the outer side of the still using a thermocouple sensor (4 Channel Datalogging Thermometer Model 800024; SPER Scientific, Scottsdale, Arizona, USA). Average time and temperature data for distillations conducted under these two conditions were recorded, from which the rate of distillation for the low wines could be calculated.

Figure 6.1 Photograph of distillation apparatus in use. Both stills have a splash head with an upward sloping lyne arm. The blue liquid is the 5°C cooled glycol which is pumped into the bottom of the copper shell and tube condensers to facilitate condensation.

6.3.4 Sensory evaluation of distillates

Sensory evaluation was by the Scotch Whisky Research Institute (SWRI) trained Sensory Panel (n=16). Samples were rated using Quantitative Descriptive Analysis (Stone *et al.*, 1974) to score a minimum of 13 key new make spirit aroma characteristics; sulphury, feinty, cereal, green/grassy, floral, fresh fruit, solventy, soapy, sweet, oily, nutty, buttery, sour and in later tests, stale. Samples were blind coded and diluted to 20% ABV prior to assessment. In total, 4 sensory tests were carried out on 2 spirits at a time; in all cases one spirit resulted from a lipid

spiked wash and the other was a control. The first 2 sensory tests were formative, as the results informed the subsequent lipid-spiking experiment. Initially the control spirit was directly compared with the distillate of the extreme 100 µg/mL lipid spiked wash. When significant differences between scores were apparent at this level, another spirit with a much lower spike was sensory profiled, again alongside the control spirit. When this low level spike was also shown to be significant, this was set as the lower limit for lipid spiking in the designed experiment. Of the 18 distillates generated and using the experimental design shown in Table 6.1, 4 spirits were submitted for sensory analysis which represented the 4 'corners' of the design space; control and high 'x8' spike at the control temperature setting; and the control and high 'x8' spike at the higher temperature setting.

6.3.5 Extraction and analysis of distillate aroma volatiles

Solid Phase Extraction (SPE) cartridges (LiChrolut EN; Merck) were used to absorb and fractionate volatile compounds from the distillate samples (see Section 2.2.2 for a full description of this method). The fraction eluted from LiChrolut EN cartridges using DCM was concentrated by evaporation under a stream of nitrogen to 1 mL and analysed by GC-O-MS. Panellists sniffing at the GC odour port (n=7) generated descriptors for any odours perceived, whilst noting the chromatographic run-time and rating each odour intensity on a scale from 1 to 3 (1 = weak, 2 = clear but not intense, 3 = intense). Subsequently GC-O peak assignments were made on the basis of a combination of mass spectral library matching (NIST), comparison of Kovat's linear retention indices (LRI) with prior published data, the odour character reported by the panel in a particular chromatogram region, and wherever possible by chromatographic similarity with authentic standards. The GC-MS method used is a stated in section 2.2.5.

6.4 RESULTS & DISCUSSION

6.4.1 Lipid-spiked fermented wash recovery experiment

The predominant fatty acids in the fermented wash samples were found to be oleic (18:2) and linoleic acids (18:3). Fermented wash samples (10 mL) were spiked with 10 µg/mL of oleic and linoleic acids prior to solvent extraction to investigate the extraction efficiency of the proposed procedure. In the control sample of fermented wash, 11 µg/mL linoleic and 9 µg/mL oleic acid were extracted from the liquid and solid component parts combined (Table 6.2.), with the majority of the lipids being extracted from the solids (Figure 6.2).

Table 6.2 The level of oleic and linoleic acids extracted from control and 10 µg/mL lipid-spiked fermented wash samples (10 mL), and the separate concentrations in liquid and solid portions of the wash.

		Liquid portion extract conc^{n} (µg/mL)	Solid portion extract conc^{n} (µg/mL)	Total lipid extracted (µg/mL)	Recovery of 10 µg/mL spike (%)
OLEIC	*10 µg/mL Spiked Sample 1*	2.13	12.00	14.13	45.1
	10 µg/mL Spiked Sample 2	2.08	11.63	13.71	40.9
	10 µg/mL Spiked Sample 3	2.27	10.17*	12.44	28.2
	CONTROL	1.99	7.63	9.62	-
LINOLEIC	*10 µg/mL Spiked Sample 1*	3.13	22.07	25.20	139.7
	10 µg/mL Spiked Sample 2	2.75	21.67	24.42	131.9
	10 µg/mL Spiked Sample 3	3.45	11.42*	14.87	36.4
	CONTROL	2.85	8.38	11.23	-

*sample left in the fridge for a prolonged period of time before extraction

This experiment also brought to my attention the instability of the fatty acids in the solid portion of the wash. After centrifugation, the 10 mL spiked sample 3 was stored in a transparent disposable polypropylene test tube (50 mL capacity) in the refrigerator for a prolonged period (much longer than samples 1 and 2) before being solvent extracted. As can be seen in Table 6.2, there is a marked reduction in the amount of lipid retrieved when

compared with samples 1 and 2. One possible reason for this could be related to the fact that unsaturated fatty acids in organic matter have been shown to be particularly susceptible to photo-oxidation when exposed to light and chilled (Wise and Naylor, 1987). The third double bond present and therefore its heightened instability could have contributed to linoleic acid (18:3) degrading to a greater extent than that compared to oleic acid (18:2) under these unfavourable conditions. As a result of this discovery, I made sure that fermented samples were always extracted within 24 hrs of thawing. Figure 6.2 is a representation of the percentage distribution of the two fatty acids in the liquid and solid portions. Here we can clearly see that the solid portion contained the majority, averaging an 82% share of the fatty acids being extracted from the particulates.

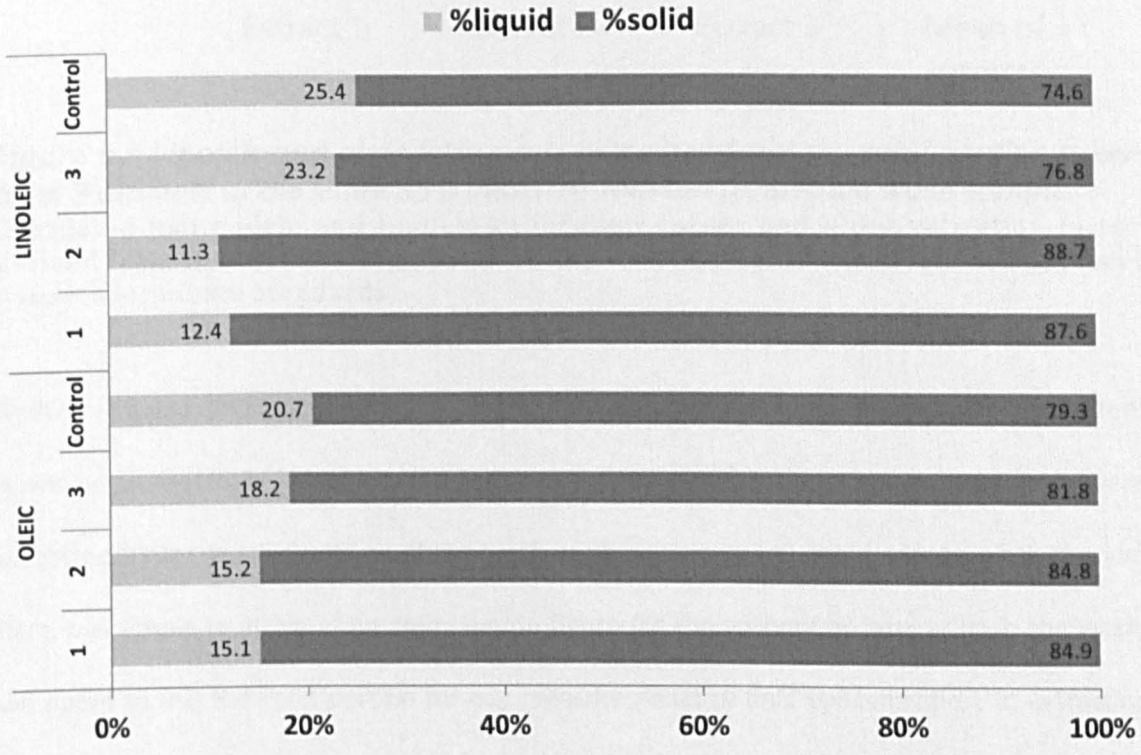


Figure 6.2 The percentage distribution of linoleic and oleic acids recovered from the solid and liquid portions of the fermented wash samples (data is the same as shown in Table 6.2).

6.4.2 Fatty acid content of the fermented wash samples

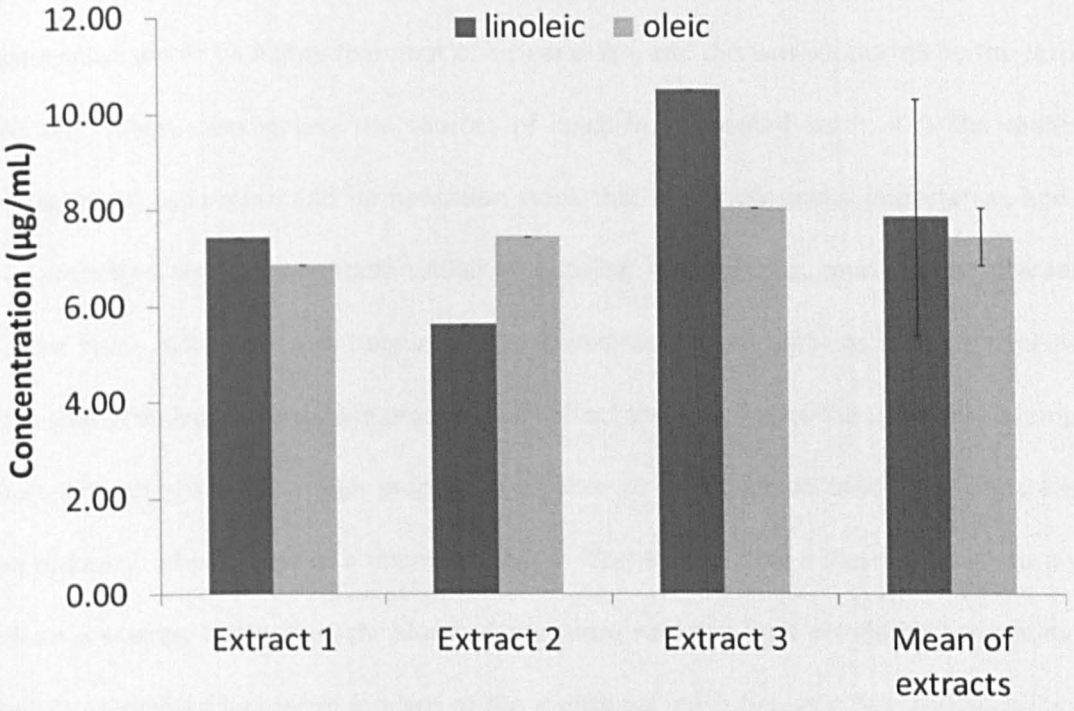


Figure 6.3 Linoleic and oleic fatty acids extracted from the solid portion taken from 3 aliquots of the same 55 h industry-sourced fermented wash sample. Calculated using oleic and linoleic calibration curves and a derivatisation factor derived from the methylating power of TMSH on methyl tridecanoate and glyceryl triheptadecanoate standards.

Both the liquid and solid portions of the fermented wash were exhaustively extracted, however as shown in Table 6.2 and Figure 6.2 significantly higher level of fatty acids were extracted from the solid portion of the wash as compared to the liquid. Hence, since the aim here was simply to arrive at an approximate figure for the amount of fatty acids in the wash, we opted to use the solid portion for our measurements of lipid concentration. In extracting the solid fraction alone, the levels of lipids recovered were between 5 and 11 µg/mL linoleic and between 6 and 8 µg/mL of oleic acid (Figure 6.3). It was decided to set the minimum level of spiking for the distillation experiment at 5 µg/mL oleic and 7 µg/mL linoleic acids, as these were the average contents of the 3 samples. Saerens *et al.* (2008) in brewing wort saw a much lower concentration lipids; 0.34 µg/mL oleic acid and 1.82 µg/mL linoleic acid. These figures are consistent with what would be considered normal in the brewing industry and the low

levels indicate that this may be a fairly clear wort. As our fermented wash was turbid, with a high proportion of particulate matter suspended in the solution, it was assumed that the lipid concentration would be higher than that of a clear wash, and this was supported by the results presented. When investigating the sources of lipids in fermented wash, it is the malting, mashing, mash separation and fermentation steps that are of particular importance, and as these processes are fundamentally similar in distilling and brewing, much of the literature concerns lipids extracted from beer as this is a well-researched area. As is to be expected, differences in milling and mashing practices will affect the turbidity of the mash. For example, a finely ground grist with a high proportion of flour or 'fines' would tend to give increased wash turbidity, whereas use of a membrane mash filter (rather than a mash or lauter tun) will produce a clearer, brighter wash. Many of the more recently built distilleries are opting to install an automated lauter tun in place of the traditional mash tun, as it has the capability for the wash to be pumped with negative pressure into the false bottom, which whilst affording a quicker wash run off, is more likely to take through more lipids in the particulate matter.

Anness and Reed (1985) in their research into lipids in the brewery, found that more than 80% of the lipids arriving in the fermenter had been derived from the malt. This figure was calculated on material balance, where total lipids were extracted from brewery samples that were collected across the brewing process to discern where the lipids were being generated and lost. It was noted that a relatively high proportion of the original 273.3g fatty acids in the grist (39.2g; 14.3%) were lost from the process altogether; unaccounted for in either the spent grain or the wort. Lipid oxidation during mashing is cited as the probable cause, especially as it was the recoveries of the unstable and oxidation-prone unsaturated fatty acids (oleic, linoleic, linolenic) that were more affected.

6.4.3 Sensory evaluation of distillates – preliminary tests

In order to ascertain the impacts of the investigated variables on the perceived sensory characteristics of new make spirit, Quantitative Descriptive Analysis (QDA) was employed.

Intensity scores (0-3) generated by the panellists for each selected attribute were averaged and ANOVA carried out to test for significant differences between sample treatments. The extreme effect of increasing wash fatty acid concentration by 100 µg/mL can be seen by comparing spider plots of the mean QDA data between the control and maximum added lipid concentration (Figure 6.4).

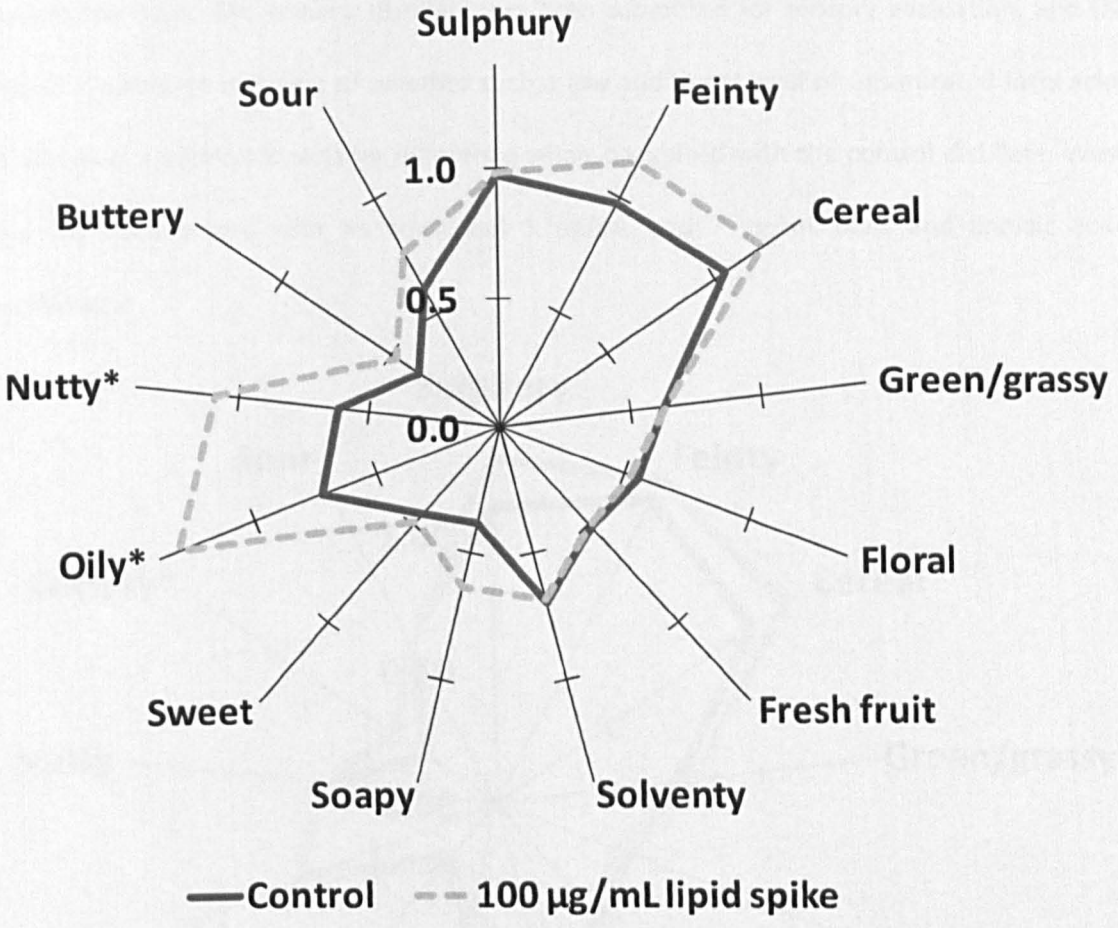


Figure 6.4 Sensory spider plot comparing the mean perceived flavour profile of the control distillate as compared to the distillate resulting when wash was spiked with 100 µg/mL of both oleic and linoleic acids.
*denotes a significant difference in mean sensory scores ($p < 0.05$). Data published in WDSC Proceedings (2012)

The lipid spiked spirit was scored as significantly higher than the control for both nutty ($p = 0.0203$) and oily ($p = 0.0034$) aroma characters (Figure 6.4). This supports the hypothesis that a higher level of lipid in the wash produces spirit richer in these characteristics. Whilst perception of some aroma characters (solventy, floral, green/grassy, sweet, sulphury) was clearly unaffected by the addition of 100 µg/mL linoleic and oleic acids to the wash, there were

(non-significant) trends towards increasing soapy, feinty, cereal, sour and buttery notes with this high level of added unsaturated fatty acids.

Once we had determined fatty acid concentrations in the distillery fermented wash sample, it was decided to spike up and distil another wash sample with a much lower spike, so that the final concentration in the wash would be equivalent to approximately double the lipid level seen in the wash. The ensuing distillate was then submitted for sensory evaluation, and the results taken as an indicator of whether such a low additional level of unsaturated fatty acids could cause a significant sensory difference when compared with the control distillate. Wash was therefore spiked with an additional 5 µg/mL and 7 µg/mL oleic and linoleic acids respectively.

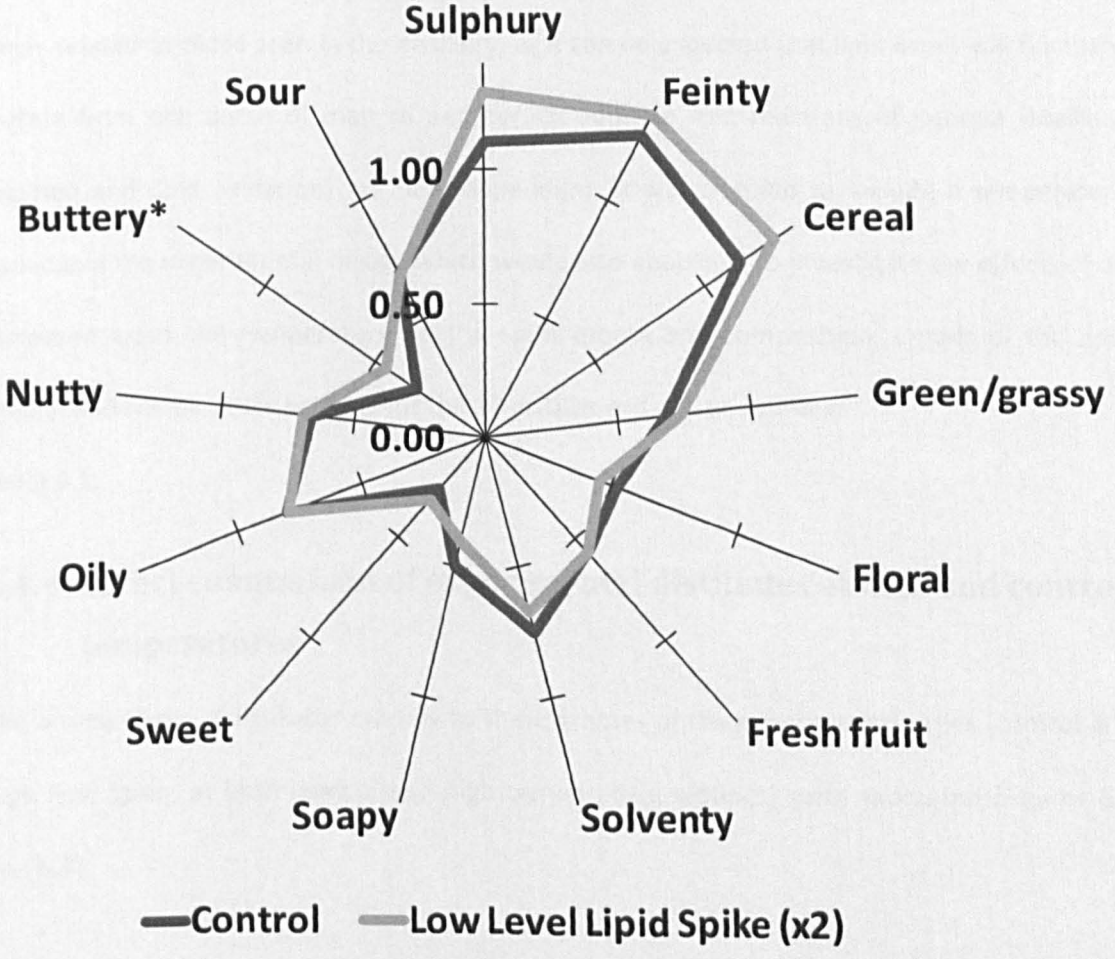


Figure 6.5 Sensory spider plot comparing the mean perceived flavour profile of the control distillate as compared to the distillate resulting when wash was spiked with 5 µg/mL of oleic and 7 µg/mL linoleic acids.
*denotes a significant difference in mean sensory scores ($p < 0.05$).

Whilst significant differences between spirits were not noted for either nutty or cereal characters in this experiment, the panellists' perception of buttery aroma in the distillate of the low level spike was found to be significantly greater ($p = 0.0194$) than when compared with the unspiked control (Figure 6.5) This was interesting – that even with a much lower level of lipid added there could still be a significant sensory effect, and added to that the fact that buttery aroma could be regarded as a 'fatty' attribute and could therefore potentially be related to lipid oxidation products such as aldehydes or lactones which are characteristic of these aromas. Following on from this, it was decided that the spiking range for the experimental design be nearer that of the concentration originally seen in the wash samples, in order that any compositional or aroma differences which came to light may then be more easily related to those seen in the distillery, as it can be expected that lipid levels will fluctuate slightly from one batch of malt to another. In addition, the reactions of interest (Maillard reaction and lipid oxidation) are heat dependent, it was decided to include a temperature variable in the experimental design which would also enable us to investigate the effects of an increased wash still temperature on the spirit aroma and composition. Details of the lipid spiking and temperature settings for the 18 distillations can be found in

Table 6.1.

6.4.4 Direct comparison of experimental distillates at high and control temperatures

The aroma of the 4 distillates relating to the extremes of the experimental series (control and high lipid spike, at both control and high temperature settings) were evaluated (Figures 6.6 and 6.7).

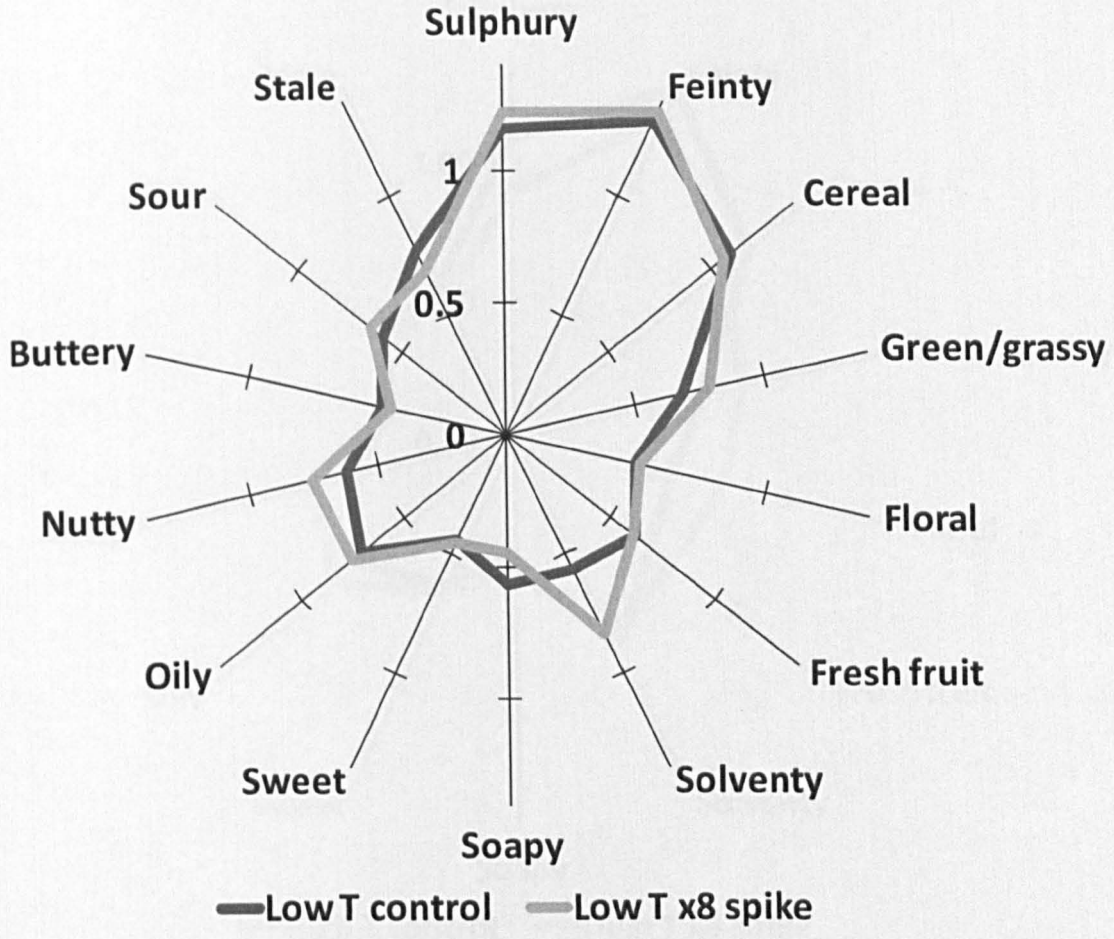


Figure 6.6 Sensory spider plot comparing the mean perceived flavour profile of the low temperature distillates: the control is compared to the distillate resulting when wash was spiked with 35 µg/mL of oleic and 49 µg/mL linoleic acids.

Similar trends were observed in the spider plots as illustrated in Figure 6.5, however the increase in mean sensory scores for nutty/oily aromas did not reach significance ($p > 0.05$) which is probably due to the lower amounts of oleic and linoleic acids added in these experiments.

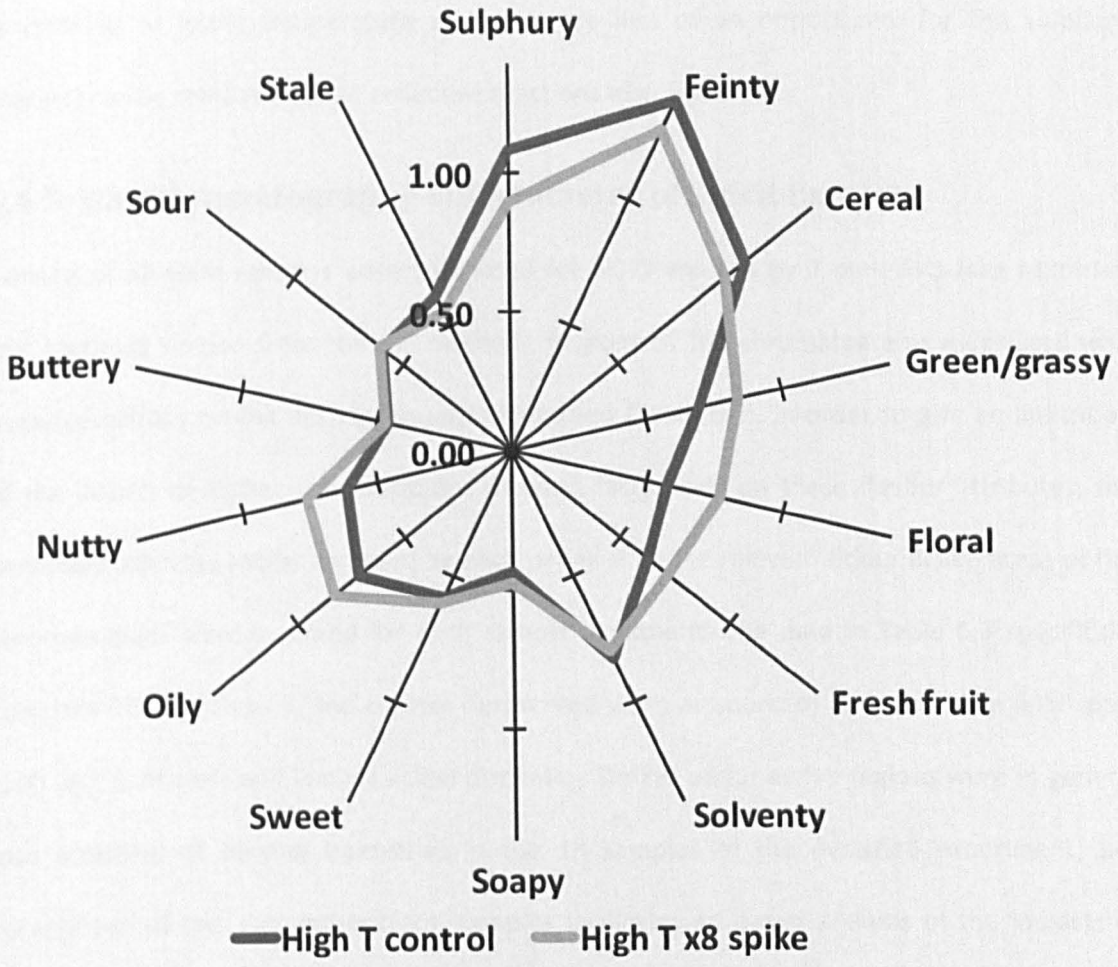


Figure 6.7 Sensory spider plot comparing the mean perceived flavour profile of the high temperature distillates: the control is compared to the distillate resulting when wash was spiked with 35 µg/mL of oleic and 49 µg/mL linoleic acids.

At the higher temperature setting both sulphur and feinty scores are reduced on lipid addition (Figure 6.7), whereas they have been shown to increase on the other sensory plots. Whilst this is not in line with scores seen for the other lower temperature distilled samples, overall the shape of the spider plot does not look dramatically different as there is still a bias towards the heavy aroma characters common to all spirits evaluated. As you would expected from a high temperature wash distillation, feinty has been scored relatively highly for both spirits. This is because the higher temperature means a quicker rate of distillation, which is therefore more like to allow heavier molecular weight compounds associated with fusel and burnt-type aromas to be distilled over into the low wines (Jack *et al.*, 2008). In addition, the sulphur compounds in the low wines would not be afforded as much interaction with the still surface

as distilling at lower temperature would, hence less of an opportunity for the sulphury character to be removed by the reductive reactions of copper.

6.4.5 Gas Chromatography-Olfactometry of Spirit Extracts

Extracts of all spirit samples were submitted for GC-O analysis by 7 panellists (see Materials and Methods section 0 for the full method). Regions of the chromatograms associated with nutty/cereal/oily aroma descriptors were identified (Table 6.3). In order to give an indication of the impact of higher concentrations of wash fatty acids on these flavour attributes, the perceived intensity scores awarded by each panellist to the relevant odour active areas of the chromatogram were summed for each sample treatment. The data in Table 6.3 specifically compare GC-O analysis of the control (fermented wash as sourced) and maximum lipid spike (100 µg/mL of oleic and linoleic acids) distillates. Similar odour active regions were in general also observed at varying intensities in the 18 samples of the designed experiment, but comparison of the 'top and bottom' samples facilitates an easier analysis of the impacts of increased wash fatty acid concentrations. From the data in Table 6.3 it is apparent that the odour-active areas with nutty, cereal or oily descriptors were in general scored as more intensely perceived in the lipid spiked sample as compared to the unspiked control. This is consistent with the sensory data for nosing of the distillates (Figure 6.4).

Table 6.3 Odour-active areas of the chromatogram which may contribute to nutty/oily aroma character.

	LRI	GC-O Descriptors	Compound Identity	Sum of Intensity Scores*		ID Confirmed by
				Control	Spiked	
1	1000	butter, caramel	2,3-butanedione	11	14	LRI, odour
2	1325	mushroom, earthy, raw vegetable, cereal	1-octen-3-one	9	12	LRI, odour
3	1370	nutty, malty, musty, nuts/popcorn/ savoury	2-acetylpyrroline	9	9	LRI, odour
4	1421	musty, nut, caramel, meaty sulphur	1-octen-3-ol	6	7	LRI, odour
5	1462	peaty, walnut, compost, shoes, savoury, vegetables, plastic, musty earthy, green	(E,E)-2,4-heptadienal	18.5	19	LRI, odour
6	1496	malty, savoury, baked, oaty, roast potatoes, oily	furfural	15	18	LRI, odour, NIST
7	1536	raw nut, snickers, green vegetable, earthy, nutty	not identified	4	6	n/a
8	1625	green, caramel, meaty	5-methylfurfural	np	5	LRI, odour, NIST
9	1710	beefy, musty, bovril, yeast extract, meaty, savoury, nutty, earthy, old socks	methionol	17	17	LRI, odour, NIST
10	1748	seafood, caramel, malty, oily, aldehyde	not identified	np	11	n/a
11	1762	chlorine, chips, cooked	not identified	np	6	n/a
12	1817	musty, leather, nutty, cooked, crispy, pyrazine	(E,E)-2,4-decadien-1-al	6	9	LRI, odour

Areas of the chromatogram were considered odour-active when 3 or more panellists (out of 7) detected an odour.

*The sum of intensity scores given by panellists describing corresponding attributes in the same region, within 0.05 seconds of one another.

np = the odour was not perceived by panellists in the corresponding region of the chromatogram.

As unsaturated acyl lipids, oleic and linoleic acids both contain allyl groups (R-CH=CH-CH2-R) which are readily oxidised to unstable hydroperoxides, which are known to give rise to many other compounds via their subsequent degradation (Belitz *et al.*, 2004). Peroxidation of lipids produces numerous volatiles (and non-volatiles), some of which are highly odourous, therefore the odour effects of even just a small amount of unsaturated acyl lipid will be apparent. The carbonyl compounds (E,E)-2,4-heptadienal, (E,E)-2,4-decadien-1-al and 1-octen-3-one are all known to arise from such hydroperoxides, in particular the oxidation of linoleic acid. The amount of 1-octen-3-one in the final spirit showed a significant positive correlation with the addition of fatty acids to the wash (Table 6.4) which gives further weight to this hypothesis. A compound which has been noted for its 'nutty/popcorn' aroma is 2-acetylpyrroline (Schieberle, 1991). All three panellists that were able to perceive this aroma awarded it a maximum intensity score, which is indicative of its low aroma threshold, which is reported to be as low as 0.01 ppb in cooked rice (Buttery, Turnbaugh and Ling, 1988). It is perhaps surprising that certain panellists did not record the odour at all, although with GC-O work this can relate to the ability of the panellist to note and record changing aromas individually in busy regions of a chromatogram.

6.4.6 Quantitative analysis of extracted compounds in experimental distillates

Chromatogram peak areas were integrated and expressed relative to that of the internal standard for all distillate samples. Concentrations thus calculated were modelled against the factors 'wash fatty acid concentration' and 'distillation temperature'. Table 6.4 lists compounds for which there was a significant effect of either or both factors, as well as indicating the direction of the effects.

Table 6.4 Compounds whose concentrations in new make spirit were significantly impacted upon by the factors: wash fatty acid concentration and wash still temperature.

Compound Name	Significant Factor(s)	p-value	Optimum condition for formation	Identity Confirmed by	LRI
ethyl hexanoate	Wash fatty acids & distillation temperature*	0.0041	high fatty acids, low temperature	LRI, NIST, odour	1260
1-octen-3-one	Wash fatty acids	0.0318	high fatty acids	LRI, NIST, odour	1325
furfural	Distillation temperature	<0.0001	low temperature	LRI, NIST, odour	1496
ethyl decanoate	Wash fatty acids & distillation temperature*	0.0449	high fatty acids, low temperature	NIST	n/a
diethyl butane-dioate	Wash fatty acids & distillation temperature*	0.0051	high fatty acids, low temperature	NIST	n/a

Quantification was by comparison with an internal standard (3-heptanone).
A p-value of < 0.05 indicates that the specified factor had a significant impact on concentrations of this compound in distillates across the 18 sample design space.
*the significant factor in each case was the interaction term between wash fatty acid level and distillation temperature.

In agreement with the GC-O results, 1-octen-3-one, which was associated with mushroom, earthy, raw vegetable/cereal characters (Table 6.3) was shown to increase significantly in concentration with the addition of oleic/linoleic acids to the wash. Furfural levels were reduced as the wash still temperature increased ($p < 0.0001$, Table 6.4). Since the mean temperature difference between the two wash still temperature settings was only 3 °C (Table 6.5), it is unlikely that the difference in distillate furfural concentration would relate to a direct impact of temperature on furfural formation pathways. More likely it relates to changes in the rate and cut-points of distillation at the different temperature control settings.

Table 6.5 Average temperatures and timings of low wine collections during distillation for the control and high mantle temperature settings.

		Control Temperature			Higher Temperature		
Timings (mins)	Time at which low wines collection started (t_1)	41.2	±	0.7	34.6	±	2.3
	Time at which low wines collection ended (t_2)	119.8	±	2.5	97.0	±	1.8
	Total time of collection for low wines (t_2-t_1)	78.6	±	2.3	62.4	±	1.3
Temperatures (°C)	Temperature of still before mantle switched on (T_0)	23.8	±	0.9	24.3	±	0.7
	Temperature at which low wines collection started (T_1)	102.2	±	1.9	105.1	±	2.9
	Temperature at which low wines collection ended (T_2)	109.7	±	1.7	112.9	±	3.7
	Maximum temperature reached during distillation (T^{max})	110.1	±	2.1	113.4	±	4.3
Distillation rate (mL/min)		7.0	±	0.2	8.8	±	0.2

On average, the rate of distillation of the low wines distilled at the higher mantle setting increased by 1.8 mL/min as compared with the lower 'control' setting (Table 6.5). Thus, at the higher still temperature setting, distillation process times to collect the specified volume of low wines were shorter, allowing less time for reactions catalysed by copper still surfaces. This would be anticipated to impact upon the volatile composition of the spirit as is evident from the significant effect of still temperature setting on the concentrations of several volatile compounds listed in Table 6.4.

2-acetylpyrroline was a compound which was clearly associated with a nutty/popcorn-like character in GC-O studies. However, whilst detectable by the human nose due to its low odour threshold, as already noted, the SPE extraction coupled with Total Ion Chromatogram (TIC) GC-MS analysis were not sensitive enough to quantify 2-acetylpyrroline. Hence correlations with lipid concentrations or still temperature could not be sought for this compound.

6.5 CONCLUSIONS

Spiking oleic and linoleic acids into the wash at concentrations of 100 µg/mL each was shown to significantly increase the perceived nutty/oily aroma characteristics of new make malt spirit. These findings are consistent with the hypothesis that increasing levels of lipid in the fermented wash results in a spirit which is perceived to be significantly richer in nutty and oily character. Furthermore, GC-O was used to identify the congeners which potentially contribute to these characters. Some of these congeners (e.g. 1-octen-3-one) were found to increase in concentration as a result of adding oleic/ linoleic acids to the wash. The nutty/cereal flavor character of whisky appears to be of complex origins, which is perhaps not surprising, bearing in mind its likely origins in Maillard chemistry and the evident interactions with fatty acid concentrations. As the mashing process is becoming increasingly automated (an example of this is the switch to automated Lauter tuns with pressurised pumps) increased knowledge of the origins of spirit characters such as nutty/cereal/oily may enable the distiller to alter parameters to suit whatever spirit character is required.

7 THE NUTTY/CEREAL AROMA CHARACTER OF NEW MAKE SPIRITS: A STATISTICAL APPROACH

7.1 AIMS

To examine the correlations between concentrations of odour active congeners and the sensory scores of 35 new make spirits. This was done by using univariate (ANOVA) and multivariate (PCA) statistical analyses to determine trends within the data. To identify further congeners which could be related to nutty/cereal aroma by comparing nutty spirit B and control spirit E with a distillate made using roasted malt.

7.2 INTRODUCTION

Whilst univariate statistics are useful tools to assess the significance of individual variables on measured outputs, multivariate statistical applications are required to pick out trends in large sets of data where multiple underlying factors are at play. Such multivariate techniques have been used in the investigation of the odour active aromas compounds in various fermented beverages (Lee *et al.*, 2001; Campo *et al.*, 2005; Câmara *et al.*, 2006; Vilanova *et al.*, 2010). In particular, Principal Component Analysis (PCA) can be used to show statistical trends in the data by way of scatter plots, and serves to highlight the similarities and differences between variables. In this chapter we used PCA to gain a better understanding of the relationships between the spirit sample sensory scores and the concentrations of odour active compounds in the 35 samples surveyed. In addition to these 35 spirits, a different new make spirit was also donated to us by an industrial source. A portion of the usual low temperature kilned malt (the distiller's usual choice due to the preservation of enzymes required for saccharification of the mash) used in manufacture of this spirit had been substituted with an unknown amount of highly roasted chocolate malt. This spirit was thought to have a particularly nutty character and was put forward by a company for analysis during this research project. Although not

included in the multivariate analysis of the 35 commercial new make spirit samples, odour port work on this spirit was used to try and identify compounds contributing to nutty character – on the assumption that the use of chocolate malt was likely to have increased the content of several Maillard-derived compounds with nutty/ cereal characters.

7.3 MATERIALS AND METHODS

7.3.1 Analysis of the new make spirit samples

35 industry-sourced new make spirits were extracted by LiChrolut SPE in triplicate and in a randomised order (for the full LiChrolut EN SPE method see section 2.2.2). Included in these samples were the subset of 5 samples (A-E) which had been used in previous solvent and solid phase extractions (see Chapters 3 and 4). These samples were included in the randomised order of solid phase extractions, concentrated and subsequently analysed in the same GC-MS sequence as the other 35 samples for direct comparison (see section 2.2.6 for the full GC parameters used). At the time of writing, the sensory ('nosing') profile of the chocolate malt spirit is not known, however we were informed by the manufacturer that it was noted for a considerably nutty aroma quality. All other spirits were scored by a trained panel using Quantitative Descriptive Analysis (QDA) (Stone *et al.*, 1974).

7.3.2 Compound quantification and data analysis

The internal standard used was 3-heptanone, which was added to 30mL of diluted sample at a concentration of 0.1 µg/mL prior to extraction (See section 2.2.2). Compound concentrations were calculated by normalisation to the internal standard, mass balance calculations, and where possible, application of the individual relative response factors calculated from external standard of a known concentration (10 µg/mL) for that compound.

7.3.2.1 Statistical analysis

Analyses of variance (ANOVA) was carried out to assess which compounds showed a significant relationship with the 4 spirit sensory QDA attributes under investigation (Design-Expert software, MN v.7). 'Responses' consisted of the 4 sensory scores and the variables investigated were the concentrations (µg/mL) of the analytes. Principal Component Analysis (PCA) was carried out using Unscrambler software (v9.0 CAMO A/S, Oslo, Norway). Average compound concentrations for each spirit were used. In order to give each variable an equal weighting in the PCA, data were normalised by division of their standard deviation.

7.4 RESULTS AND DISCUSSION

7.4.1 Odour active compounds identified and quantified in 35 new make samples

Until this point in the research, only univariate statistical analyses have been used. This is because it suited our purpose to take each compound in turn, looking to see whether there were any significant differences in the concentration of that compound between the 5 samples; the 4 nutty new make spirits and the 1 control 'non-nutty' spirit. In obtaining the compositional and sensory data for the 35 new make spirits, we now had a much fuller range of sensory scores and concentration data to interpret. Hence, multivariate analysis such as PCA was used to show any sensory differences and similarities between spirits as influenced by their congener concentrations. This can show trends within the data which help to interpret which groups of compounds may be of importance to the nutty/cereal aroma of the spirits. Prior to the Principal Component Analysis of data, (as a check on the integrity of subsequent PCA models), ANOVAs were conducted for each of the odour active compounds of interest (the concentrations and some odour thresholds of these are shown in Table 7.1).

Table 7.1 LRI, concentration and odour threshold data for 25 volatile compounds identified in odour active areas of the chromatogram where GC-O panellists described odours which could contribute to nutty/cereal character (mean of 35 samples, all with triplicate measurements).

Nutty/ cereal OAA	RT	LRI	Lit LRI	Compound	Conc[n] in Spirit (µg/mL)		Odour threshold in water (µg/mL)
					Mean	SD	
2	5.53	993	978	2-pentanone	6.33	± 0.81	
3	5.83	1012	792	**2,3-pentanedione**	0.02	± 0.02	
4/5	6.39	1047	1045	**propan-1-ol**	0.84	± 0.72	
4	6.52	1055	1057	ethyl butyrate[n]	2.82	± 2.89	0.001
5	7.27	1105	1108	**2-methylpropan-1-ol**	19.75	± 13.75	0.065
-	7.96	1138	1132	isoamyl acetate[c,o,f]	12.11	± 6.11	
6	9.40	1217	1206-1230	fusel alcohols	124.26	± 26.19	
6	10.23	1263	1244	pentan-1-ol[n,c,o,f]	6.96	± 1.76	4
6	10.53	1286	1270	**thiazole[o]**	0.36	± 0.09	
7	10.84	1297	1272	**2-methyl pyrazine[n]**	0.08	± 0.03	60
8	11.83	1353	1331	**2,5-dimethyl pyrazine[n]**	0.04	± 0.03	1.7
9	12.87	1412	1386	**2-nonanone[o]**	1.14	± 0.50	
10	13.70	1461	1461	**1-octen-3-ol**	0.84	± 0.21	0.0013
11	14.34	1498	1485	**furfural[n,o]**	12.41	± 4.40	14.1
12	15.05	1542	1499	**2-acetylfuran**	1.40	± 0.67	80
13	15.49	1575	1515	**benzaldehyde[o]**	0.33	± 0.11	3.5
14	16.18	1612	1560	**5-methylfurfural**	0.80	± 0.28	20
	16.97	1663	1630	ethyl decanoate[f]	116.99	± 231.26	0.2
15	17.26	1682	1686	**2-furanmethanol[n,c]**	0.69	± 0.25	2.4
17	17.65	1708	nf	ethyl benzoate[n,o,f]	2.47	± 1.15	0.06
18	18.21	1746	1745	methionol[n,c,o,f]	0.53	± 0.28	1
n/a	19.95	1869	1822	ethyl dodecanoate[c,o]	75.68	± 132.80	2 (beer)
20	21.08	1961	nf	2-phenethyl alcohol[n,c,o,f]	680.46	± 191.70	14
21	22.82	2088	1991	**gamma-nonalactone[c,o]**	1.35	± 0.60	0.030
22	25.00	2198	nf	ethyl hexadecanoate[f,o]	6.30	± 19.57	

Compounds with aromas which could contribute to nutty/cereal character are highlighted in bold text. Where a compound was found to be significant it is shown with an superscript n = nutty, c = cereal, f = feinty, o = oily. For full statistical significances ($p < 0.05$) see Table 8.2.
Odour active areas refer to the overall Odour activity identification Table 8.1.

References for LRI (WAX GC columns) and odour threshold data: Fors, 1988; Grosch and Schieberle, 1991; Takeoka *et al.*, 2008; Ferreira *et al.*, 2001; Mottram, 1987; Umano *et al.*, 2002; Vilanova *et al.*, 2010; Valim *et al.*, 2003; Cullere *et al.*, 2004; Sanz *et al.*, 2001; Ferreira *et al.*, 2000.

Where ANOVAs showed significant correlation with any of the sensory scores, even if compounds had not previously been characterised as nutty/cereal in the literature, they were included in the PCA. Also included in the multivariate dataset were compounds with known nutty/cereal attributes that had been detected in odour active LRI ranges where nutty/cereal descriptors had been recorded by the panellists during GC-O studies. These were 1-octen-3-ol, 2-acetylfuran and 5-methylfurfural. In total, 20 compounds were entered into the PCA.

7.4.2 Principal component analysis of congeners which showed significance for nutty/cereal/oily/feinty scores

Nutty/cereal odour active compounds that had been identified in the three sets of GC-O analysis and which were shown to significantly correlate with any of the 4 sensory attributes were taken forward to the multivariate statistical analysis to see if any patterns in the data could be visualised. Table 7.2 details these 17 aroma volatiles, their associated aroma quality and any significance (p value < 0.05) they were shown to have in the ANOVA for the sensory attributes of interest.

Table 7.2 Compounds which were identified within odour active regions of the chromatogram (as measured by GC-O; see Table 7.1) and have been found to have a significant correlation for at least one odour attribute as scored by the sensory panel

LRI	Compound	Odour	*Compound had +/- significance (p value < 0.001)*			
			Nutty	**Cereal**	**Oily**	**Feinty**
1055	ethyl butyrate	fruity	0.0005 -	-	-	-
1138	isoamyl acetate	pear drops	-	0.0038 +	0.0026 -	0.0018 -
1263	pentan-1-ol	fermented	< 0.0001 +	0.0097 -	0.0361 -	0.0264 +
1286	thiazole	rubber	-	-	0.0410 +	-
1297	2-methylpyrazine	nutty	0.0013 +	-	-	-
1353	2,5-dimethylpyrazine	nutty	0.0004 +	-	-	-
1412	2-nonanone	fatty	-	-	0.0295 -	-
1498	furfural	bready	0.0431 -	-	0.0257 +	-
1575	benzaldehyde	almond	-	-	0.0418 +	-
1663	ethyl decanoate	grape	-	-	-	< 0.0001 -
1682	2-furanmethanol	burnt	< 0.0001 +	0.0417 -	-	-
1708	ethyl benzoate	musty	< 0.0001 +	-	0.0314 -	0.0121 +
1746	methionol	meaty	0.0004 +	0.0018 -	< 0.0001 -	0.0010 +
1869	ethyl dodecanoate	leaf	-	0.0068 -	0.0004 -	-
1961	2-phenethyl alcohol	floral	0.0147 +	0.0355 -	0.0013 -	0.0351 +
2088	gamma-nonalactone	coconut	-	0.0102 -	0.0055 -	-
2198	ethyl hexadecanoate	waxy	-	-	0.0281 +	0.0039 +

Biplots of one PCA are shown in Figures 7.1 and 7.2. Biplots consist of loadings (the mean concentration of compounds) overlaid on the 'scores' for the PCA, which were the spirits (letter coded).

In total, the 4 PCs generated explained 71% of the variance within the dataset. The PCA in Figure 7.1 is representative of 44% of the variation with 26% being explained by PC1, and 18% by PC2. The PCA in figure 7.2 is representative of 27% of the variation with 15% being explained by PC3, and 12% by PC4.

Only 3 of the compounds in Table 7.2 were shown to be significant for all sensory attributes. These were all alcohols: pentan-1-ol, methionol and 2-phenethyl alcohol. Furthermore, they all shared the same pattern of significance for the attributes, in that they were positively

correlated with nutty and feinty, and showed a negative correlation with cereal and oily. Higher alcohols such as these are formed via the Ehrlich pathway during fermentation. In this way methionol and 2-phenethyl alcohol are derived from the amino acids methionine and phenylalanine respectively. The mechanisms involved are the transamination of the amino acid, which is then decarboxylated to its aldehyde, which in turn is reduced to obtain the higher alcohols (Etschmann *et al.*, 2008). Methionol has been shown to contribute pungency, negatively correlating with pleasant descriptors used to describe the odour of red wine (Aznar *et al.*, 2003). In the PCA shown in Figure 7.2, these compounds are shown near each other which further illustrates this co-correlation (Grouping 2). Also correlated to the compounds are other volatiles which could be termed as pungent in odour: meaty thiazole, fatty 2-nonanone and musty ethyl benzoate, as well as 2-furanmethanol, known for its burnt type aroma.

In both PCAs, The ethyl esters ethyl decanoate (C10) and (C12) are close to isoamyl acetate, with fruity ester ethyl butyrate (C4) also nearby. In Figure 7.1 these volatile esters, which are not known for having nutty/cereal character, are grouped together in the opposing quadrant to the 4 nutty/cereal sensory descriptors, showing that they are not allied to these attributes and on the whole have been shown to display negative correlations with them.

In Figure 7.2, there is more grouping together of the majority spirit scores in the centre of the PCA suggesting that a large number of the spirits are similar.

As the scores show the distribution of the spirit samples, it is telling that Spirit BO is an obvious outlier in both PCA plots as it is ranked highly for all sensory characters of interest: 1st for oily, 4th for feinty, 9th for nutty, and 6th for cereal character. Out of 35 spirits, this tells us that not many of these spirits scored as highly for all these attributes (See Appendix 8 for spirit rankings of sensory score data).

The pyrazines 2,5-dimethylpyrazine (2,5-dmp) and 2-methylpyrazine showed positive correlations with nutty sensory scores in both Table 7.2 and in the PCA biplots. As known

contributors to nutty odours in other food systems (Bonvehi, 2005; Aceña *et al.*, 2010), this is not overly surprising. In Figure 7.2 the pyrazines are also co-localised with the furan compounds (5-methylfurfural, furfural and 2-acetylfuran), pentan-1-ol and gamma-nonalactone, showing that these compounds arise in similar relative levels in the spirits (have a tendency to co-vary).

In both PCAs, the nutty, feinty, cereal and oily scores are near each other and can be grouped together, showing that they are co-correlated. A scatter graph which illustrates this co-correlation can be seen in Figure 7.3.

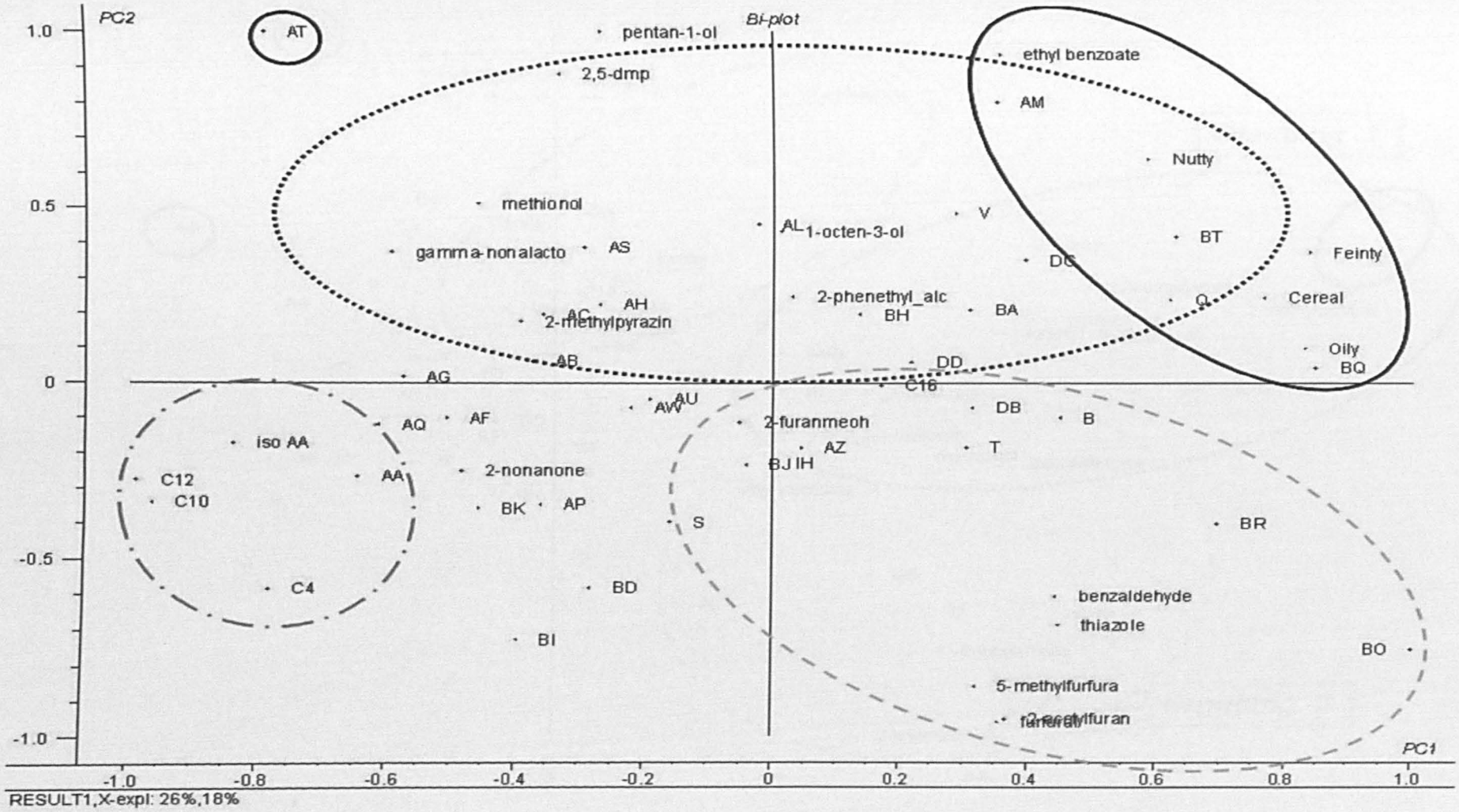


Figure 7.1 Biplot (PC1 vs PC2) of principal component analysis of 4 sensory attributes and 20 odour active compounds selected for their nutty/cereal aromas and/or statistical significances for nutty/cereal/oily/feinty attributes

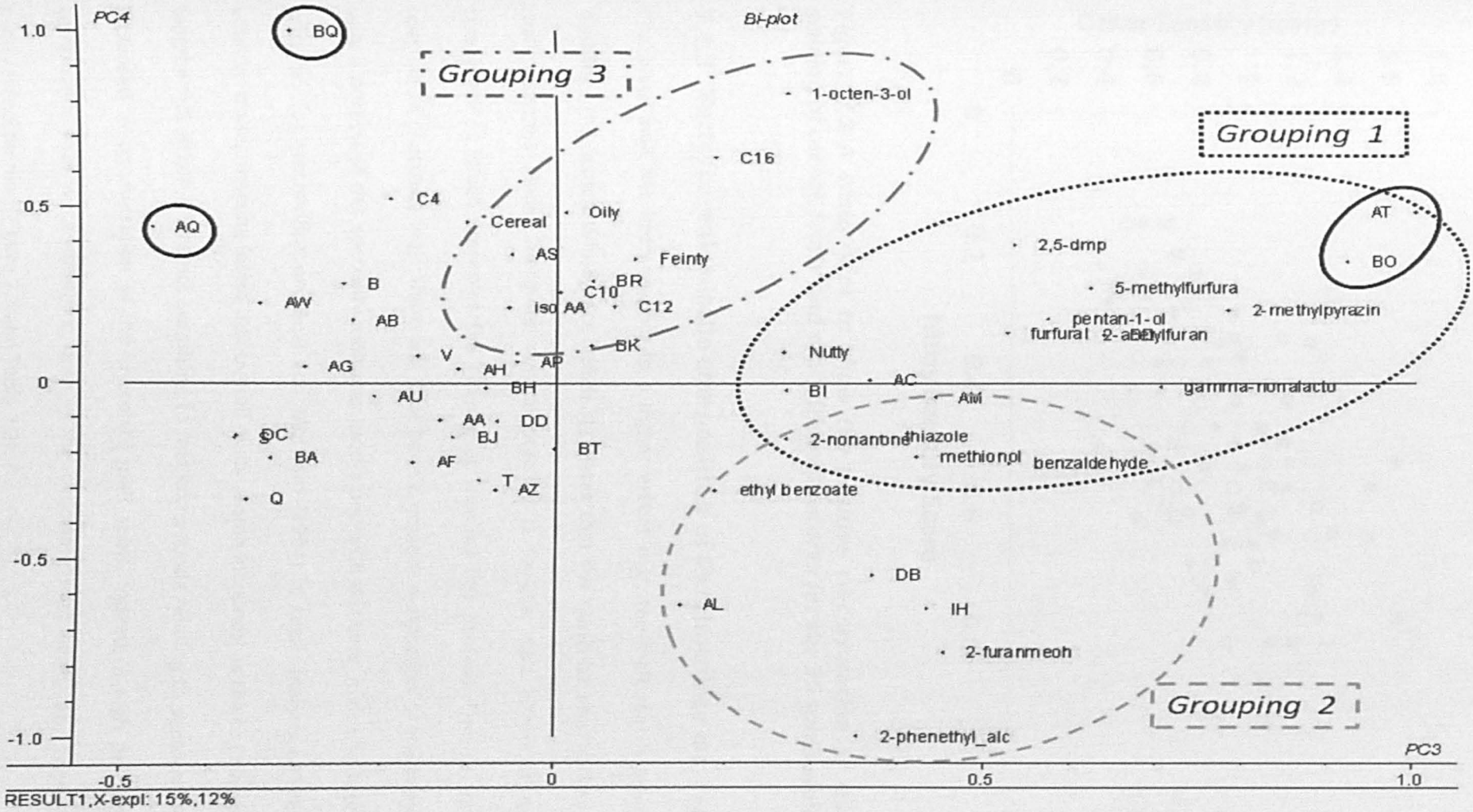


Figure 7.2 Biplot (PC3 vs PC4) of principal component analysis of 4 sensory attributes and 20 odour active compounds selected for their nutty/cereal aromas and/or statistical significances for nutty/cereal/oily/feinty attributes

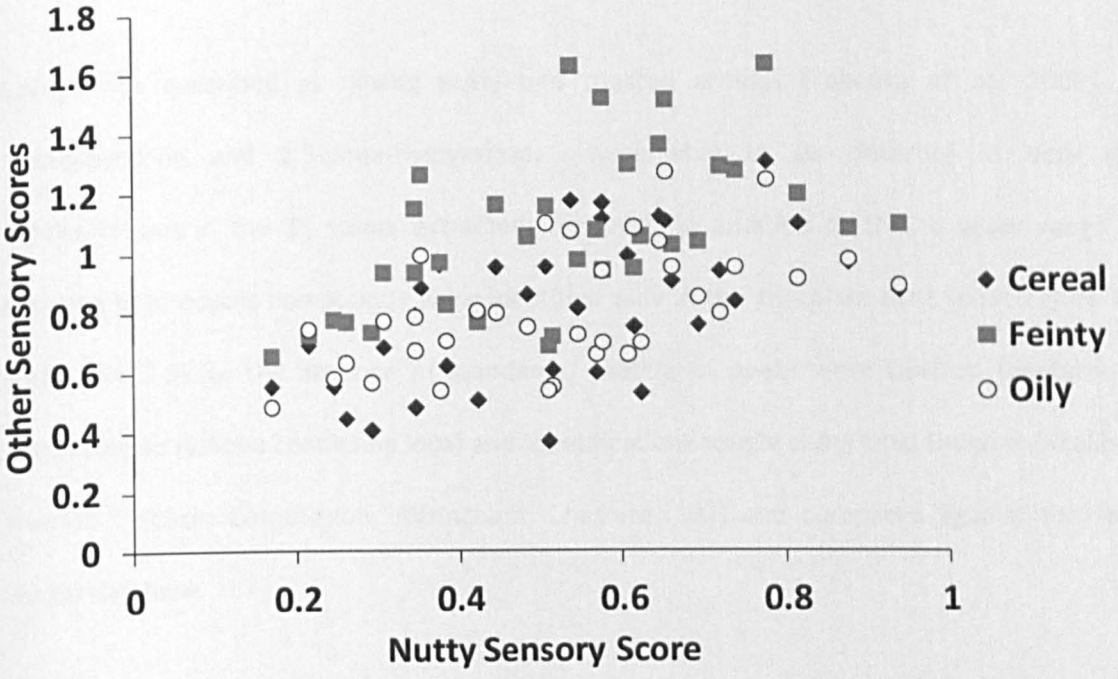


Figure 7.3 A scatterplot to show the positive co-correlation of the sensory scoring of cereal, feinty and oily with nutty scores for the 35 spirit samples.

7.4.3 Nutty/cereal volatile composition of the chocolate malt spirit

Chocolate malt has been roasted to a higher extent than the malt that is usually used in distilling. The Scotch Whisky Act (1988) stipulates that the mash be saccharified by natural malt enzymes alone, therefore normal practice is to use malt kilned at much lower temperatures which preserves the enzyme activity for this process. Due to the extent of roasting of chocolate malt, there will have been complete destruction of the enzymes Hence only a portion of this speciality malt was used along with distillers' malt which provided the enzymes for saccharification. Beal and Mottram (1994) in their analysis of malted barley volatiles during roasting found that overall malty aroma increased as the roasting process was lengthened, hence it was not surprising to find compounds relating to such characters to be increased in concentration in the chocolate malt spirit. Indeed, a high proportion of the compounds that were identified only in the chocolate malt spirit, are known to exhibit nutty/cereal aromas (Figure 7.3 and Table 7.2).

Compounds described as having nutty and roasted aromas (Takeoka *et al.*, 2008), 2-methylpyrazine and 2,5-dimethylpyrazine, were able to be detected at very low concentrations in the 35 spirits extracted. However, in addition to this, a wider range of nitrogen heterocyclic compounds were identified only in the chocolate malt spirit (Figure 7.4 and Table 7.3). In the absence of standards, spectra of peaks were isolated (background subtracted to remove conflicting ions) and identifications sought using Qual Browser (Xcalibur; Thermo Electron Corporation, Altrincham, Cheshire, UK)) and compared against the NIST library database.

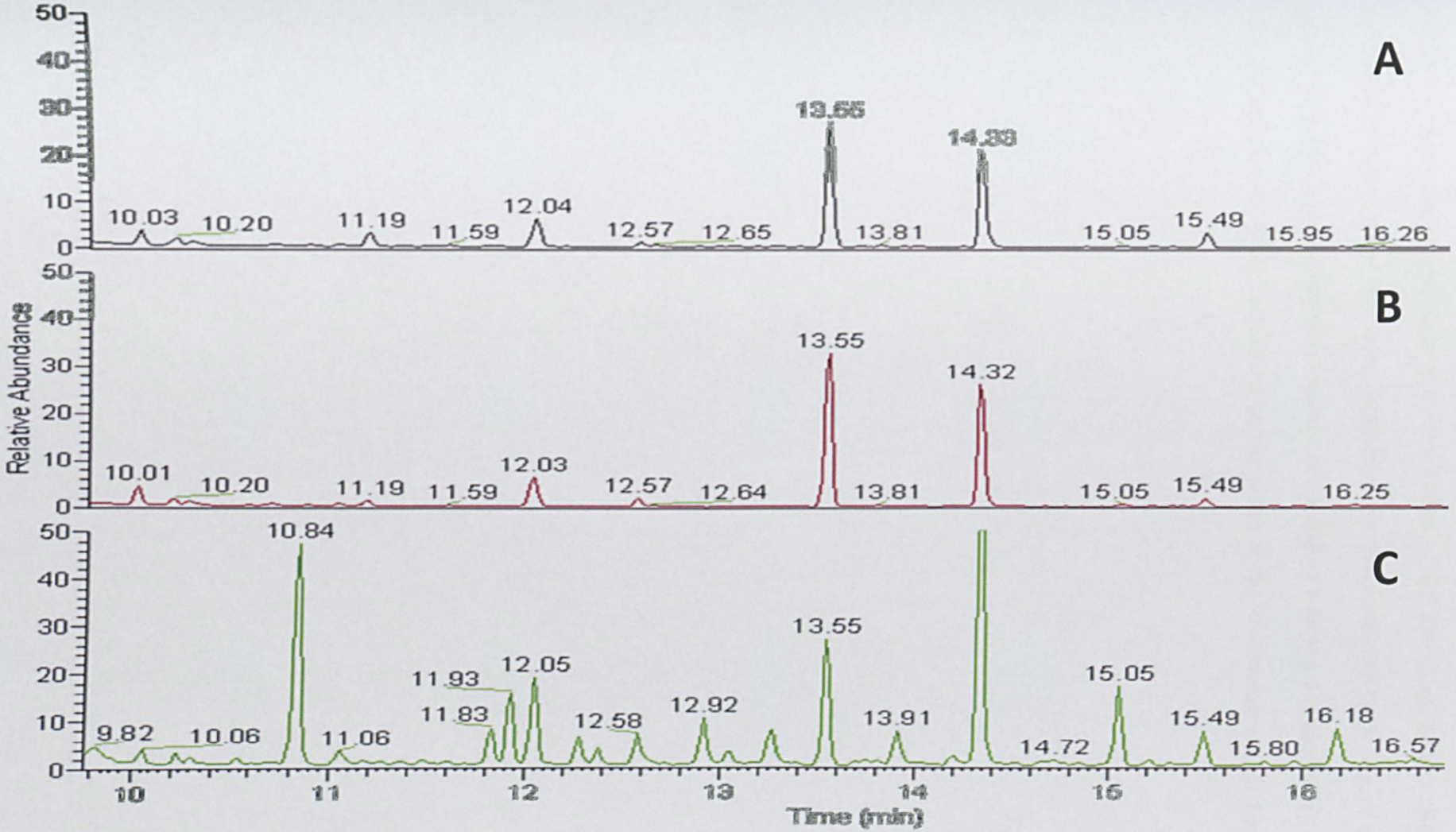


Figure 7.4 Total Ion Chromatograms of new make spirit E (A), new make spirit B (B) and the chocolate malt spirit (C) showing the additional peaks relating to compounds in Table 7.3.

Figure 7.4 shows the chromatograms for the RT 9.8 – 16.6 (LRI range 1240 - 1640) which contained additional peaks that were detected in the chocolate malt spirit (Figure 7.4C), the majority of which were undetected in spirits E and B (Figure 7.4A and B respectively). Table 7.3 lists the compounds which correspond to the peaks in the chromatograms of Figure 7.4C.

Table 7.3 Peak identification data for chromatogram C seen in Figure 7.4.

RT†	Compound Name	m/z values	LRI	LRI in Lit.	Aroma descriptors	Nutty OAA*
9.81	pyrazine	80	1240	1194	nutty	6
10.05	ethyl hexanoate	88, 99, 115	1254	1231	apple	
(10.53)	thiazole	85, 58	1286	1270	rubber	
10.83	2-methylpyrazine	94, 67	1297	1272	nutty	
11.05	1-octen-3-one	70, 55, 88	1309	1317	mushroom	7
11.83	2,5-dimethylpyrazine	108, 42	1353	1331	nutty	
11.93	2,6-dimethylpyrazine	108, 42	1358	1324	nutty	7/8
12.04	ethyl lactate	45, 75	1363	1330	fruit	n/a
12.05	ethyl pyrazine	107, 56, 108, 80	1365	1354	nutty	8
12.27	2,3-dimethylpyrazine	108, 67	1378	1315	nutty	
(12.87)	2-nonanone	58, 59, 71, 142	1412	1401	fatty	9
12.91	2-ethyl,6-methylpyrazine	121, 122	1415	1411	roast potato	
13.04	2-ethyl,5-methylpyrazine	121, 122	1422	1419	coffee, nutty	
13.26	2-ethyl-3-methylpyrazine	121, 122, 67	1435	1422	baked, nutty	
13.54	ethyl octanoate	88, 127, 172	1451	1446	fatty	n/a
(13.70)	1-octen-3-ol	57, 72	1461	1463	mushroom	10
13.75	2,6-diethylpyrazine	135, 136	1464	1463	hazelnut	10
13.90	3-ethyl-2,5-dimethylpyrazine	135, 136	1473	1474	potato, roast	10
(14.05)	2,4-dimethyl-3-pentylpyrazine	94, 121, 135	1481	nf	raw nutty	11
(14.09)	2,3-diethylpyrazine	121, 136, 135	1484	1458	nutty	
14.33	furfural	96, 97	1498	1485	bready	
15.00	octan-1-ol	41, 56, 55	1539	1530	waxy	12?
15.01	2,3,5-trimethyl-6-ethylpyrazine	149, 150	1539	nf	nutty	12
15.04	2-acetylfuran	95, 110	1541	1500	caramel	12
15.48	benzaldehyde	*105, 106, 77*	1568	1515	almond	*13*
16.17	5-methylfurfural	110, 109, 53	1610	1560	caramel	14
16.92	2-isoamyl-6-methylpyrazine	108, 121	1656	nf	nutty	15
16.97	ethyl decanoate	88, 101, 200	1660	1630	grape	15
17.04	1-methylpyrrole-2-carboxaldehyde	109, 108	1664	nf	baked nuts	15
17.26	2-furanmethanol	98, 81	1677	1686	caramel	16

†RTs in brackets were not visible on the chromatogram in Figure 6.1.
**For corresponding nutty OA Areas, see final Odour Activity Table 8.1*
References for LRI and odour threshold data: Mottram, 1987; Fors, 1988; Grosch and Schieberle, 1991; Ferreira et al., 2001; Umano et al., 2002; Valim et al., 2003; Takeoka et al., 2008; Vilanova et al., 2010.

Of particular note is that the previously undetected pyrazines which arose in the chocolate malt spirit are of large molecular weight with more branched and alkyl groups substituted to the aromatic ring. An explanation for this increase in pyrazine molecular complexity may because of the prolonged heating of the chocolate malt, the Maillard pathways which produced them were further developed, with more sets of reactions taking place to form these di- and tri-substituted alkylpyrazines. Further evidence of the increased rate of Maillard reactions taking place can be seen in the spirit concentrations of furan derivative compounds (Figure 7.5).

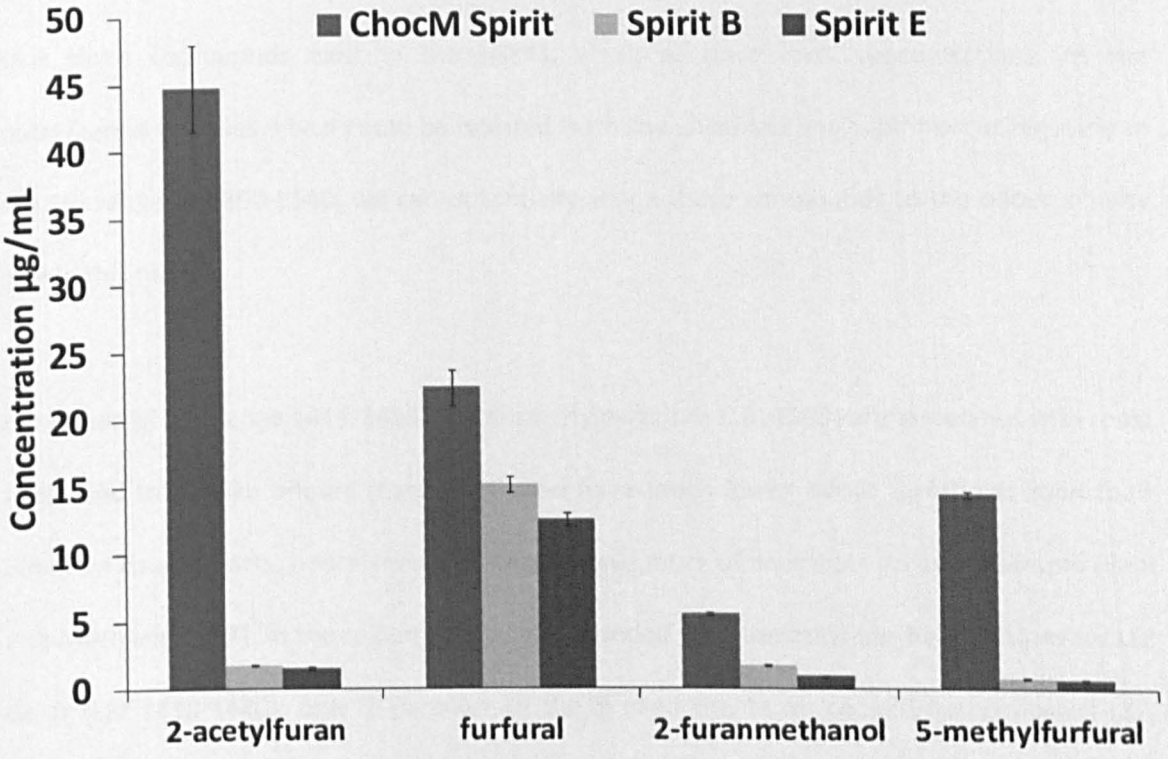


Figure 7.5 The concentrations (µg/mL) of furan derived compounds SPE extracted from the chocolate malt, B and E spirits

Significantly higher concentrations of furan compounds were extracted from the chocolate malt spirit than from samples B and E. As seen in previous chapters, the differences between nutty sample B and non nutty E were also significant; in both the LLE and SPE extracts 2-furanmethanol showed positive correlation with nutty ($p < 0.0001$ in both cases) and in the SPE furfural correlated positively with nutty and 2-acetyl furan showed significance for cereal

scores, again both at the highest level ($p < 0.0001$). These compounds have caramellic, burnt odours and are present above their odour thresholds. They therefore appear to be contributing to the perception of nutty/cereal character in the spirit.

As many of the new compounds detected have extremely low odour thresholds (in the ppb range), therefore if they were present in the other spirit samples analysed by GC-O yet their concentration was below the detection limits of the MS, then they would have been perceived at the odour port. Thus we can deduce that if the LRI of any of these revealed nutty/cereal compounds fall within the areas where nutty/cereal aromas were perceived, then it is possible that these compounds exist in the spirits, albeit at trace level concentrations. As the nutty/cereal volatiles which could be isolated from the chocolate malt spirit occur regularly in the LRI range of 1350-1540, we can potentially assign these compounds to the odour activity within this region.

Ethyl-methyl (LRI range 1415-1435) and trimethylpyrazines (LRI 1539) are associated with roast, nutty and toffee-like odours (Fors, 1988) and have much lower odour thresholds than their dimethyl counterparts, hence they are likely to have more of an impact on overall aroma (Beal and Mottram, 1994). In the region which corresponded with the ethyl-methyl-pyrazines for LLE GC-O (LRI 1410-1440), only 1 panellist of the 9 used the term 'cooked/nutty/roasted (2)', whilst all the others describe sulphur-like aromas. Although not detected in the samples, the compound responsible for this may be dimethyl trisulphide. With an LRI of 1442 (Qian and Reineccius, 2003) it has an extremely low odour threshold of 3 ppt, which accounts for the fact that the majority of GC-O panellists were able to detect it - in the LLE extract of spirit E all 9 of the panellists described sensing a sulphury odour. Originally, the LRI range 1460-1470 was found in LLE and SPE to contain a peak for 1-octen-3-ol, which has a characteristic mushroom odour. Whilst one panellist out of the 9 recorded 'mushroom (3)' in the GC-O of the LLE extract for spirit E, the more common descriptors that were detected in all 3 GC-O analyses in the

region of LRI 1460-1470 were those relating to a nutty, roasted, earthy and green, 'fresh peapod' type aroma, with one panellist using the term 'popcorn, but greener' for the LLE extract. In fact, in the SPE extracts 100% of the GC-O panellists detected these kinds of aromas. In the chocolate malt extract at LRIs 1464 and 1473, nutty pyrazines 2,6-diethylpyrazine (hazelnut) and 3-ethyl-2,5-dimethylpyrazine (roasty aroma) were identified and their LRIs confirmed with the literature (Sanz *et al.,* 2001). It is therefore possible that these are responsible for the odours noted in the SPE extracts. Cross-referencing the trimethylpyrazine peak identified at LRI 1539 for the chocolate malt spirit with the lipid-spiked and control lab distillates (Chapter 6), potentially significant GC-O descriptors 'snickers/nutty , raw nut, earthy' were given between LRI 1520-1540 which could suggest the presence of this compound. In both the control and lipid-spiked samples, 3 panellists out of the 7 detected this odour, and average intensities recorded were 2, suggesting that those who did smell this aroma were able to describe it with some degree of certainty.

7.5 CONCLUSION

Combining the results of the 3 GC-O analyses in chapters 3, 4 and 6, 25 volatile compounds were identified and their concentration quantified in nutty/cereal odour active areas of the chromatogram.

Of these 25, 20 volatiles which had shown significance for sensory scores were taken forward for PCA visualisation of patterns. The PCA illustrated a grouping between heavy fusel-type aroma compounds which were positively correlated with nutty sensory scores. Knowing that nutty character is likely to be a result of synergistic interactions between various compounds, these findings could suggest that pungent compounds have a role to play in nutty aroma, in that they may provide the basis for Maillard products commonly known for their nutty and cereal character, furans and pyrazines were grouped together which suggested they were present in relatively similar amounts in the spirits. The chocolate malt spirit served as a

possible marker for identification of nitrogen heterocylics known to contribute nutty and cereal characters. In total 13 pyrazines were identified which had not previously been seen in the other extractions. These findings suggest that the malting regime, and in particular the extent of roasting, is a key factor in the contribution of nutty/cereal compounds in new make spirit.

8 OVERALL DISCUSSION, CONCLUSIONS & RECOMMENDATIONS FOR FUTURE WORK

The findings of this research will now be discussed in the light of current knowledge, with particular emphasis on odour active compounds whose concentrations in new make spirit have shown significant correlations with the sensory QDA scores of nutty, cereal, oily and feinty aromas (Table 8.1) and the nutty/cereal odour active regions which have been identified in Table 8.2 - both Tables are located at the end of this chapter. Comparisons will also be made between the different extraction methodologies employed. Potential stages during the whisky manufacturing process where these compounds could be formed are also discussed, with a view to developing knowledge as to the process control of these characters.

As this research was led primarily by the sensory scores and the detection frequency, intensity and descriptors generated from GC-O, it is important to take into account what limitations this may have involved. The opinion of what constitutes nutty or cereal aromas may differ greatly between panellists, as they (the GC-O panel) were not specifically trained to detect these aromas, nor had they been given specific terms to use. My purpose here was to identify any areas of the chromatograms which could have the potential to relate to the general aroma of nutty and cereal, hence it was not in my interest to limit the output of descriptors by assigning the panellists a strict lexicon. A recently published paper by Miller (2013) sought to define specific terms for the description of nutty aroma in different food systems. They developed a lexicon by reference to standards which included nut mixtures, flours and cereals. In terms of defining what the term nutty means for whisky, these references would not be suitable because the aroma complexity of new make spirits makes the perception of specific odours less clear cut. Therefore, the trained sensory panellists (at the Scotch Whisky Research Institute) who evaluated the new make spirits used in this research had been trained to measure nutty aroma by reference to other known nutty spirit samples.

In my quest to identify which compounds may be contributing to these aromas, regions of the chromatogram were further investigated if the related GC-O descriptors were in a broad sense related to nutty/cereal characters and were sufficiently odour active (detection frequency was 60% or more). As can be seen from the odour activity tables within chapters 3, 4 and 6, these included a wide range of terms, with panellists making reference to oily, fatty, musty, earthy, green or vegetable-type odours, as well as malty, caramel, popcorn, burnt aromas and those relating to specific nuts such as walnut, peanuts or coconut. It was due to the varied nature of the descriptors used here that prompted me to include oily and feinty spirit scores when correlations were sought between sensory QDA scores for spirit samples and concentrations of these congeners in the spirit.

Liquid-liquid extraction and solid phase extraction techniques were compared for their ability to extract compounds of a nutty/cereal character. Although the compounds which were extracted and analysed in the solvent extract were of a higher concentration than those extracted by SPE, on comparison of the two techniques it was clear that losses had potentially occured during the exhaustive concentration procedure. Hence, with a high surface area for increased retention, and more specific retention interactions between the analyte and the sorbent, LiChrolut SPE was shown to be better suited to the isolation of low level polar compounds, and was able to successfully extract 2,5-dimethylpyrazine as a result of its specificity. This was borne out by the GC-O analysis where a total of 26 odour active areas were recorded by panellists, 19 of which could be regarded as having qualities which could relate to nutty/cereal aromas. This technique was then used for the extraction of the 35 spirits in the new make spirit survey in Chapter 7. Within these areas, compounds which were shown to have significantly positive correlations with nutty scores included 2-methyl-pyrazine, 2,5-dimethylpyrazine, 2-furanmethanol, methionol, ethyl benzoate and 2-phenylethyl alcohol.

In particular, looking across all three sets of GC-O analyses, the Maillard reaction product 2-furanmethanol consistently occurred at higher concentrations as the sensory perception of nuttiness in the spirit increased. In GC-O, the odours in the region which contained 2-furanmethanol were recorded by all panellists in the SPE extracts, with more panellists observing these aromas in the nutty spirit B LLE extract. Descriptors such as popcorn, nutty, cereal, burnt toffee and musty were used. For methionol, the positive correlation for this sulphurous volatile seen in for the 35 new make spirits is in line with what was seen in the detection frequencies, with more assessors noting the odour in nutty spirit B than the non-nutty control E. Although not traditionally thought of as nutty character compounds, it is postulated that these heavier aromas could in some way be enhancing the perception of nutty in the spirit, by perhaps providing the base notes on top of which other lighter aromas are layered. As a spirit can contain over 80 compounds, we cannot view each concentration or odour in isolation, but must consider that there may be synergistic effects between them which results in the overall aroma.

Early on in the project, when it was evident that the compositional differences between the 2 spirits were subtle and that their gross volatile compositions were remarkably similar, it was decided to investigate any differences, whether they related to nutty aroma volatiles or not. One such difference was noted between long-chain ethyl ester levels, whereby more were seen in the extracts of nutty spirit B. This led us to investigate the possible partioning effect the esters might be having on the headspace aroma of the spirit. Ethanolic APCI-MS was used to measure the headspace concentrations of whisky volatiles with a wide range of physicochemical properties (specifically varying in volatility and polarity). The results showed that adding ethyl hexadecanoate (C16) at concentrations above its critical micelle concentration in ethanolic solution significantly reduced the headspace concentrations of other hydrophobic volatile compounds. The ethyl hexadecanoate was assumed to form agglomerates which incorporated relatively hydrophobic compounds, thus removing them

from the headspace and altering the perceived aroma. This effect was more pronounced at lower ethanol concentrations, where the solubilising effects of ethanol were reduced. Alternatively, polar compounds such as 2-methylpyrazine and furfural were also significantly affected by ethyl ester addition and showed a slight salting out affect at 5% ABV. In terms of the impact this could be having on the nutty and cereal aromas, one could argue that a higher level of ethyl esters in spirit B would mean that when the spirits are diluted for nosing at 23 % ABV, there is a greater likelihood that the hydrophobics in the headspace may be reduced, potentially allowing for the aroma of relatively polar compounds to become more accentuated in the headspace. In order to discern whether there were any differences in this respect, some informal sensory work was done on the compound mixtures containing low and high concentrations of added ethyl ester in aqueous ethanolic solutions at the nosing concentration. Overall, the aroma was regarded as stronger the less ethyl ester was added, suggesting that the aroma balance had been altered.

Another area of research that was explored in Chapter 6 was the widely accepted dogma in the whisky industry that a cloudy, lipid-rich wash produces a spirit high in nutty and oily character. In order to test this hypothesis, samples of fermented wash were spiked with a high level of fatty acids and distilled in the laboratory. There was a significant increase in the sensory scores of both the nutty and oily attributes for the lipid spiked spirit which supported the assumptions held in industry. When lower concentrations of fatty acids were added to the fermented wash, sensory work showed a significant positive correlation with buttery aroma and lipid levels. GC-O of the laboratory distilled samples revealed 3 additional regions of nutty/cereal odour activity. One of these areas which was characterised by a strong nutty/popcorn aroma – every panellist who perceived it gave it the highest intensity rating. This was tentatively identified by its odour and LRI as being 2-acetypyrroline, a nitrogen heterocyclic with an extremely low odour threshold (0.2 ng/L) known to arise from Maillard chemistry (Schieberle, 1991). Another Maillard product, furfural, was shown to be significantly

decreased when a higher temperature was applied to the wash still. As the rise in temperature itself was not deemed to be significant enough of a factor to affect the reaction rate, and as the distillations were done on a volume basis, this served to highlight the compositional differences which can be brought about by altering the spirit cut. There are many possible reasons why a cloudy wash may occur. One of these may be when a quicker mashing procedure is used, whereby instead of allowing the wort to slowly filter through the mash bed, the wort is pumped out of the mash tun, bringing with it the particulate matter.

This research has shown that the Maillard reaction and lipid oxidation are of great importance to the production of nutty and cereal compounds during the manufacture of new make spirit. In particular the key malting and distillery processes in which these reactions are most at work is during the high temperature processes of kilning and distilling. A wide range of compounds which are known to be produced by these reactions and have nutty/cereal character were identified in the spirits. Among these were thiazole, furan compounds, Strecker aldehydes, and a variety of pyrazines.

The influence of malt-derived compounds on the composition of Maillards was shown in Chapter 7, where a malt was used to produce a spirit with very high levels of nitrogen heterocyclics, with pyrazines being the greatest in number and variety. Pyrazine production has been shown to increase with temperature, which would explain why higher levels of pyrazines were seen in the spirit which had been produced with roasted chocolate malt than those that had used standard low temperature kilned distillers' malt.

To summarise, the compounds which have shown significantly positive correlations for the nutty scoring of the spirits are as follows: 2-furanmethanol (which showed significance in all 3 analyses), methionol, 2-phenethyl alcohol, 2-methylpyrazine and 2,5-dimethylpyrazine. Whilst positive correlations were seen with oily were thiazole and 1-octen-3-ol. Whilst the kilning of the malt and distillation are where nutty/cereal compounds are likely to be formed, other

distillery parameters will have a marked effect on the final spirit flavour. The mashing protocol, whether this is a lauter tun, or a traditional mash tun will affect the fatty acid composition of the wash. Similarly, the compounds formed in the fermentation decide what precursors are available for the reactions in the still, where the subsequent spirit cut will ultimately influence what aroma volatiles end up in the spirit.

8.1 SUGGESTIONS FOR FUTURE WORK

For a more complete characterisation of the chocolate malt spirit, sensory evaluation by QDA and Gas-Chromatography Olfactometry analyses would be able to confirm the nutty/cereal odour activity of the sample, which the GC-MS has shown are potentially in abundance.

An experiment to deduce whether the levels of certain ethyl esters can have an effect on the aroma by spiking into the spirit that have the lowest levels of ethyl esters, and adding in ethyl esters to the level of the highest concentration seen. Sensory evaluation would then be carried out on the unspiked and spiked spirits. This would give us information regarding the effects of additional hydrophobics on the aroma balance.

Finally, it would be beneficial to know the process parameters used in the different distilleries where the spirits were sourced from, in order to possibly trace what processes may be affecting the production of the compounds responsible for nutty/cereal character.

Table 8.1 Compounds identified in odour active areas as seen in LLE, SPE and lipid spiked distillation Gas Chromatography-Olfactometry work, showing their detection frequency (%), their descriptors, and their positive or negative significant correlations with the sensory attributes.

		LLE		SPE													
OAA	LRI range	B	E	B	E	control	lipid spiked	GC-O Descriptors	Nutty	Cereal	Oily	Feinty	OA Compound	LRI	Lit LRI	Lit. Descriptors	ID
1	930-945	78	67	71	60	n/a	n/a	malty, popcorn, nutty, bready					2-methyl butanal (96-17-3); 3-methyl butanal (590-86-3)	n/a	926; 936	green, almond, malty, cocoa	O, LRI
2	960-1000	100	100	100	100	n/a	n/a	bubblegum, solvent, nutty, malty					2-pentanone (107-87-9)	993	978	solventy/ fruity ketone, woody	O, LRI, NIST
3	1000-1040	89	89	86	60	86	86	butter, caramel, popcorn, toffee					2,3-butanedione (431-03-8)	n/a	983	buttery, nutty, cheese	O, LRI
													2,3-pentanedione (600-14-6)	1012	1041	buttery, caramel, cream, fruit, spirit	O, LRI, NIST, Std
4	1045-1080	89	67	86	100	n/a	n/a	malty, solvent, fruity, floral, nuts, chocolate, alcohol					propan-1-ol (71-23-8)	1047	1045	fruity, pungent, musty	O, LRI, NIST, Std
5	1100-1120	89	67	71	60	n/a	n/a	earthy, malty, chocolate, solvent, popcorn					2-methylpropanol (78-83-1)	1105	1085	solvent, bitter, glue, alcohol	O, LRI, NIST, Std

6	**1230-1280**	100	100	100	100	100	100	malty, alcoholic, solventy, nutty, sweaty, almond/marzipan cherry brandy, biscuit, chocolate, tutti frutti, plastic/phenolic, sweet/cheesy					fusel alcohols: A. 2-methyl-1-butanol (137-32-6); B. 3-methyl-1-butanol (123-51-3); C. 1-pentanol (71-41-0)	1230-1280 O/L	A. 1206 ; B. 1230 ; C. 1244	malty, pungent, balsamic, alcohol, fruity	O, LRI, NIST, Stds
											+		thiazole (288-47-1)	*1287*	1270	rubber	O, LRI
									+				2-methylpyrazine (109-08-0)	*1297*	1272	nutty, roasted	O, LRI
7	**1300-1330**	n/a	n/a	57	80	71	86	mushroom, metallic, earthy, raw vegetable, cereal					1-octen-3-one (4312-99-6)	1309	1317	mushroom, metallic	O, LRI, NIST, Std
8	**1350-1370**	n/a	n/a	n/a	n/a	57	43	roasted pyrazines, nutty popcorn, nutty baked pyrazine, cheesy feet, malty/must, nuts/popcorn/savoury					2-acetylpyrroline (99583-29-6)	n/a	1333	popcorn, nutty	O, LRI, (Std)
													(E,E)-2,4-Hexadienal (80466-34-8)	n/a	1337	green , vegetable	O, LRI
									+				2,5-dimethylpyrazine (123-32-0)	*1353*	1331	nutty, roasted	O, LRI, Std
													2,6-dimethylpyrazine (108-50-9)	*1358*	1324	nutty, roasted	O, LRI
9	**1410-1440**	89	100	n/a	n/a	57	57	meat/chicken, oily, faecal, sulphury, eggy, musty, cooked/nutty/roasted, rotten veg, metallic, musty, nut, caramel, meaty sulphur		+	- -	-	2-nonanone (821-55-6)	1412	1401	fatty green earthy	O, LRI, NIST
													dimethyl trisulphide (3658-80-8)	n/a	1442	garlic, sulphur	O, LRI
													2-ethyl,6-methylpyrazine (13925-03-6)	*1415*	1411	roast potato	O, LRI

														2-ethyl-5-methyl pyrazine (13360-64-0)	*1422*	1419	coffee bean, nutty	O, LRI
														2-ethyl-3-methylpyrazine (15707-23-0)	*1435*	1422	baked	O, LRI
10	**1460-1470**	100	100	100	100	100	100	roasted, fresh peapods, warm barley, metal, walnut, popcorn, green, nutty, mushrooms/oily, peaty,compost, shoes, savoury, vegetables, plastic, musty earthy, green/burnt, fusel/pungent	-	-	+		1-octen-3-ol (3391-86-4)	1461	1463	mushroom	O, LRI, Std
														(E,E)-2,4-heptadienal (4313-03-5)	n/a	1480	fatty, nutty	O, LRI
														2,6-diethylpyrazine	*1464*	1463	roast nuts	O, LRI
														3-ethyl-2,5-dimethylpyrazine	*1473*	1455	baked	O, LRI
11	**1480-1520**	100	89	86	60	100	100	earthy/must/ potato, malt/maltesers, wet grass, potatoes/vegetables cooking, malty, savoury, baked, oaty, roast potatoes, oily, peanuts, cooking, milky, fatty becomes roasted/burnt, nutty, green	+		+	-	furfural	1496	1485		O, LRI, Std
									-		-	-						
											+							
													2,4-dimethyl-3-pentylpyrazine	*1481*	nf	nf	O, LRI,
													2,3-diethylpyrazine (15707-24-1)	*1484*	1458	raw nutty	O, LRI,
													camphor	n/a	1510	burnt	O, LRI
12	**1520-1540**	n/a	n/a	n/a	n/a	43	43	raw nut, snickers, green vegetable, earthy, nutty					undecanal (112-44-7)	1536	1537	waxy soapy floral aldehydic citrus green fatty fresh laundry	O, LRI

														2,3,5-trimethyl-6-ethylpyrazine (17398-16-2)	*1539*		nf	O, LRI,
									-	+	-	-	2-acetylfuran (1192-62-7)	1542	1500	sweet balsam almond cocoa caramel coffee	O, LRI, Std
13	**1540-1580**	78	89	86	100	n/a	n/a	feints, aldehyde, green/grassy marzipan/almond, solvent, creamy, sweet, fresh peas, pine, mallow, 'new things', oily, leather, putty/plastic, minty, solventy, sweet		+	-	-	benzaldehyde	1575	1572	burnt almond, marzipan	O, LRI, NIST, Std
										+	+	-					
													trans-2-nonenal	n/a	1555	green, fatty	O, LRI
14	**1625-1645**	56	78	71	60	71	57	cucumber, green, mint, metal, floral, nutty, green leaves, onion, mossy/peaty, coconut, green, caramel, meaty	-	+		+	5-methylfurfural	1612	1560	caramel	O, LRI, NIST, Std
													(E,Z)-2,6-nonadienal	n/a	1605	cucumber, melon	O, LRI
15	**1650**	67	44	n/a	n/a	n/a	n/a	popcorn, socks/musty, earthy, feet					2-isoamyl-6-methylpyrazine	*1656*	1660		O, LRI
													1-methylpyrrole-2-carboxaldehyde	1668		baked nuts (Serra Bonvehi 2005)	O, LRI

16	**1680-1710**	89	78	100	100	100	100	corny, cereal, stale, nutty, musty, socks, burnt toffee, malt (brewery), popcorn, grass, baking cheesy, savoury, smoky/rubbery, popcorn, baked nutty, nutty/savoury, sweaty feet, floral, yeasty, sour/putrid, musty	+ + +	- -	- -	- -	2-furanmethanol (98-00-0)	1682	1686	burnt sugar, fermented, creamy, caramellic note	O, LRI, NIST, Std
17	**1710-1740**	100	100	71	80	29	86	beefy, musty, bovril, yeast extract, meaty, savoury, nutty, earthy, old socks, seafood, caramel, malty, oily, aldehyde	+		-	+	ethyl benzoate (93-89-0)	1708	nf	minty, musty, wintergreen	O, LRI, NIST
													1-(5-methylfuran-2-yl)propan-1-one	1720	nf	green, hazelnut	O, LRI, NIST
18	**1740-1780**	67	44	86	60	n/a	n/a	yeasty, baking, cheesy, solventy/green, plastic, metallic, malt/wheat, meaty, potato, clean, plants/ green/solventy, waxy, tea leaves,	+ +	+ -	-	- +	methionol	1746	1745	Meaty, sulphurous, vegetable	O, LRI, NIST

								balsam/cosmetics / chlorine, chips, cooked					propyl decanoate (30673-60-0)	1746	nf	Fatty, green, woody, oily	O, LRI, NIST
19	**1810-1820**	n/a	n/a	n/a	n/a	71	57	musty, nutty, crispy, pyrazine/roast nut, leather					(E,E)-2,4-decadien-1-al (25152-84-5)	n/a	1816	oily cucumber melon citrus pumpkin nut meat	LRI, O
20	**1900**	n/a	n/a	57	80	n/a	n/a	chestnuts, cake, almond, cinnamon	+	-	-	+	2-phenethyl alcohol	1913	nf		O, LRI, NIST, Std
21	**2040**	n/a	n/a	100	100	n/a	n/a	sweet/popcorn/ caramel, waxy/coconut		-	-		gamma-nonalactone	2088	1991	coconut, waxy	O, LRI, NIST, Std
22	**2160-2190**	n/a	n/a	100	100	n/a	n/a	musty/oily, sweets, old books, fruity, incense shop, liquorice					gamma-decalactone	2156	**2165**	fatty, sweet, peach-like	O, LRI, NIST
23	**2330-2350**	n/a	n/a	57	60	n/a	n/a	white chocolate, vanilla, creamy, baking, soapy, waxy					vanillin	n/a	2589	sweet, vanilla, creamy, choc	O, LRI
													gamma-undecalactone	2305	2270	fatty, fruity	O, LRI

ID: Identification was on the basis of O: Odour, LRI: Linear Retention Indices, NIST: NIST library database, Std: confirmed by a standard

Table 8.2 Combined significance of all compounds identified in nutty/cereal odour active areas and included in the Principal Components Analysis

OA *	Significant Compounds	Nutty			Cereal			Oily			Feinty		
		LLE	SPE	NMS	LLE	SPE	NMS	LLE	SPE	NMS	LLE	SPE	NMS
6	*pentan-1-ol*	-	-	< 0.0001 +	-	-	0.0097 -	-	-	0.0361 -	-	-	0.0264 +
6	*2-methyl pyrazine*	nf	-	0.0013 +	-	-	-	-	-	-	-	-	-
6	*thiazole*	-	-	-	-	-	-	-	-	0.0410 +	-	-	-
6	*3-methylbutanol*	-	-	-	-	-	-	-	-	0.0328 -	-	-	-
8	*2,5-dimethyl pyrazine*	nf	-	0.0004 +	-	-	-	-	-	-	-	-	-
9	*2-nonanone*	-	-	-	0.0408 +	-	-	0.0006 -	-	0.0295 -	0.0049 -	-	-
10	*1-octen-3-ol*	0.0004 -	-	-	0.0053 -	-	-	0.0001 +	-	-	-	-	-
11	*furfural*	-	< 0.0001 +	0.0431 -	-	-	-	0.0075 +	0.0141 -	0.0257 +	0.0078 -	< 0.0001 -	-
12	*2-acetylfuran*	-	0.0040 -	-	-	< 0.0001 +	-	-	0.0022 -	-	-	< 0.0001 -	-
13	*benzaldehyde*	-		-	0.0009 +	< 0.0001 +	-	-	0.0002 -	0.0418 +	0.0001 -	< 0.0001 -	-
14	*5-methylfurfural*	-	0.0403 -	-	-	< 0.0001 +	-	-	-	-	-	< 0.0001 +	-
16	*2-furanmethanol*	< 0.0001 +	< 0.0001 +	< 0.0001 +	-	-	0.0417 -	-	< 0.0001 -	-	< 0.0001 -	< 0.0001 -	-
17	*ethyl benzoate*	-	-	< 0.0001 +	-	-	-	-	-	0.0314 -	-	-	0.0121 +
18	*methionol*	0.0124 +	-	0.0004 +	0.0134 +	-	0.0018 -	-	-	< 0.0001 -	0.0177 -	-	0.0010 +
20	*2-phenethyl alcohol*	-	-	0.0147 +	-	-	0.0355 -	-	-	0.0013 -	-	-	0.0351 +

21	***gamma-nonalactone***	-	-	-	-	-	0.0102 -	-	-	0.0055 -	-	-	-
n/a	***(isoamyl acetate)***	-	-	-	-	-	0.0038 +	-	-	0.0026 -	-	-	0.0018 -

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Appendix 1 – Mean Sensory QDA scores for spirit samples A-E from the SWRI Panel (n=15)

Spirit Sample	*Pungent*	*Sulphury*	*Meaty*	*Solventy*	*Fruity/ estery*	*Green/ grassy*	*Floral*	*Cereal*	*Sweet*	*Soapy*	*Peaty*	*Feinty*	*Oily*	*Nutty*	*Sour*	*Stale*	*Clean*
A	1.3	1.2	1.0	1.1	1.0	0.9	0.8	1.1	0.9	0.6	0.3	1.2	0.9	0.8	0.7	0.6	1.4
B	1.4	0.8	0.7	1.2	1.1	1.1	0.9	0.9	0.7	0.9	0.2	1.1	0.9	0.9	0.8	0.7	1.4
C	1.3	1.2	1.0	0.8	0.6	1.0	0.5	0.9	0.6	0.6	0.3	1.3	0.8	0.7	0.7	0.8	1.1
D	1.2	1.0	0.7	0.8	1.0	0.6	0.7	0.8	0.8	0.7	0.3	1.0	0.9	0.7	0.4	0.8	1.4
E	0.9	0.5	0.3	1.2	1.4	1.0	1.2	0.4	1.2	0.7	0.0	0.7	0.5	0.5	0.4	0.3	1.9

Appendix 2 - Long chain ethyl ester addition: the effect on headspace concentrations of whisky aroma volatiles (Chapter 5)

The whisky volatiles used in the ethyl ester addition experiment: examples of where they have been found in the literature, which extraction techniques have been used in their detection in my spirit samples, and their known odour properties.

Compound	Reported in distilled beverages/whisky	Detected in my sample extracts	Odour descriptors (flavornet.org)
ethyl hexanoate	Bourbon Whisky (Poisson & Schieberle, 2008)	LLE, SPE (LiChrolut)	apple peel, fruit
ethyl octanoate	Bourbon Whisky (Poisson & Schieberle, 2008) Chinese Maotai liquor (Qian *et al.*, 2007)	LLE, SPE (LiChrolut)	fruit, fat
2-phenylethyl acetate	Bourbon Whisky (Poisson & Schieberle, 2008)	LLE, SPE (LiChrolut)	rose, honey, tobacco
β-damascenone	Bourbon Whisky (Poisson & Schieberle, 2008)	LLE, SPE (LiChrolut)	apple, rose, honey
benzaldehyde	Malt, blended & grain whisky (Postel & Adam, 1977) Chinese Maotai liquor (Qian *et al.*, 2007)	LLE, SPE (LiChrolut)	burnt sugar, almond
furfural	Scotch whisky (Paterson & Piggot 1989)	LLE, SPE (LiChrolut)	bread, almond, sweet
ethyl-(L)-lactate	Brandy (Bertrand, 1989) Cachaca, whisky, rum (Nascimento *et al.*, 2008)	LLE, SPE (LiChrolut)	fruit
2,5-dimethylpyrazine	Chinese Maotai liquor (Qian *et al.*, 2007)	SPE (LiChrolut)	cocoa, roasted nut, roast beef, medicine
isoamyl acetate	Brandy (Bertrand, 1989) Scotch whisky (Camara et al., 2006)	LLE, SPE (LiChrolut)	banana

Appendix 3 - Ethyl esters and their organoleptic and physical characteristics (Chapter 5)

Ethyl ester	MW	Carbon chain length	Organoleptics (flavornet.org)	Log P ($K_{o/w}$)	Vap P (mm. Hg 25°C)
Ethyl hexanoate	144	6	apple peel, fruit	2.83	1.8E+00
Ethyl octanoate	172	8	fruit, fat	3.81	2.1E-01
Ethyl decanoate	200	10	grape	4.96	3.1E-02
Ethyl dodecanoate	228	12	leaf	5.78	8.7E-03
Ethyl tetradecanoate	256	14	ether	6.76	2.5E-03
Ethyl hexadecanoate	284	16	wax	7.74	2.7E-04

Note how their odour properties change from fruity to waxy as the carbon chain length increases.

Appendix 4 – Glossary of distilling terms

Congeners – a term commonly used in distilling to describe the odour-active volatile compounds generated during the production of whisky

Feinty – aroma character relating to the heavier, fusel alcohol type aromas which are associated with the unpotable spirit that is collected nearer the end of the distillation known as the 'feints'.

Grist – malt that has been milled to a coarse flour consisting of (in decreasing size) husks, grits/middlings and flour.

Lautering – originally a brewing term; the process where spent grains and wort from the mash tun are separated

Wash – the liquor which results from mashing; in the text, the term 'wash' will either refer to the unfermented (directly after mash, before yeast is added) or fermented variety. Context will distinguish which term is meant.

Wort – brewing parlance for what distillers refer to as 'wash'; the term is associated primarily with the sugar-rich liquor which is the result of mashing

Appendix 5 – Stock solutions and spiking regime for multi-level lipid spiking experiment (Chapter 6)

Stock Solutions

Stock 1: 1 mL linoleic & 0.710 mL oleic in 10 mL EtOH = 100 mg/mL linoleic & 71 mg/mL oleic

Stock 2: 5 mL of Stock 1 in 50 mL = 10 mg/mL linoleic & 7.1 mg/mL oleic

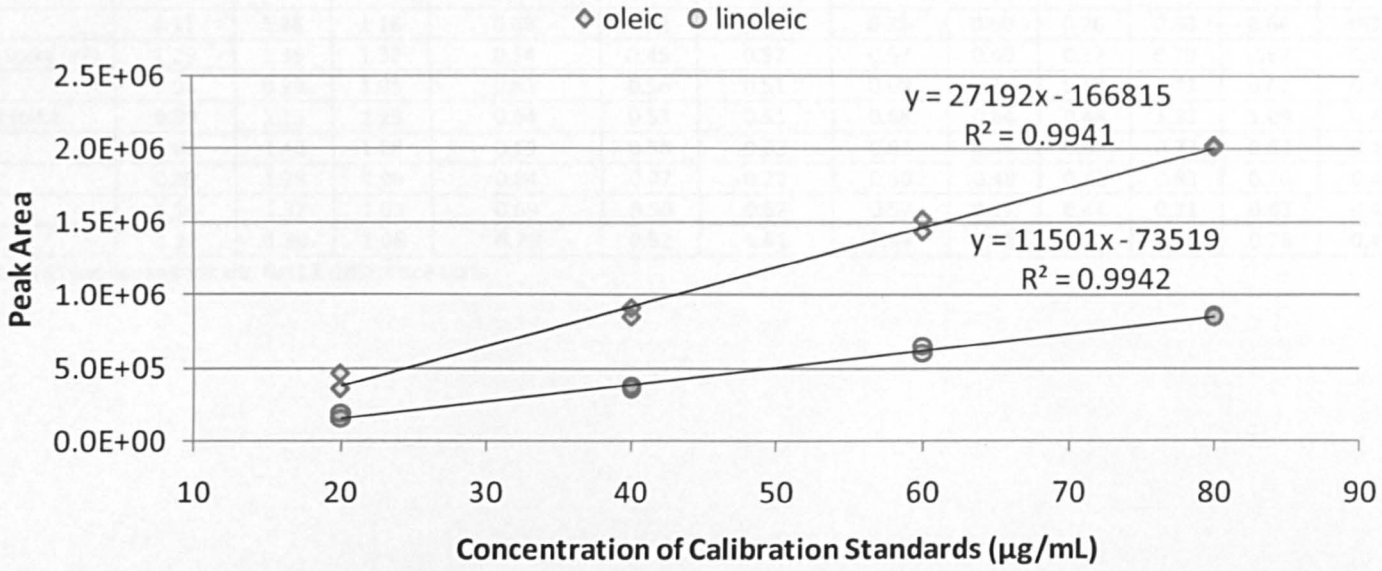
Stock 3: 4.4 mL of Stock 1 in 20 mL = 22 mg/mL linoleic & 15.62 mg/mL oleic

Spiking Regime

Each 1.8 L fermented wash sample will be spiked with 4 mL total ethanol. 1.65L of this will then be distilled.

Level Spiking	Stock Used	Vol. Spike (mL)	Vol. EtOH (mL)
Control	n/a	0	4
x2	2	1.3	2.7
x4	2	3.8	0.2
x6	3	2.9	1.1
x8	3	4	0

Appendix 6 – Calibration curves of fatty acid standards and fatty acid concentration calculation (Chapter 6)



The above values used for the calibration curves were adjusted using the derivatisation factor (C17/C13)/3) taken from the ratio of methyl tridecanoate to methyl heptadecanoate (derivatisation product of glyceryl triheptadecanoate).

Appendix 7 – Mean sensory scores for difference testing of lipid spiked and control distillates (Chapter 6)

Figs	Sample Description	Sulphury	Feinty	Cereal	Green/ grassy	Floral	Fresh fruit	Solventy	Soapy	Sweet	Oily	Nutty	Buttery	Sour	Stale*
6.5	Control (low T)	1.11	1.28	1.16	0.68	0.52	0.57	0.75	0.50	0.26	0.80	0.66	0.32	0.69	-
	Low Level Lipid Spike (x2)	1.29	1.36	1.32	0.74	0.45	0.57	0.67	0.40	0.32	0.79	0.69	0.45	0.68	-
6.4	Control (low T)	0.98	0.98	1.05	0.63	0.56	0.51	0.69	0.38	0.44	0.73	0.62	0.38	0.61	-
	100 µg/mL lipid spike	0.99	1.15	1.23	0.64	0.53	0.51	0.68	0.64	0.48	1.32	1.09	0.48	0.77	-
6.7	High T control	1.08	1.40	1.08	0.69	0.58	0.62	0.83	0.44	0.58	0.72	0.62	0.46	0.59	0.62
	High T x8 spike	0.89	1.28	0.99	0.84	0.77	0.72	0.80	0.48	0.61	0.83	0.76	0.47	0.61	0.55
6.6	Low T control	1.16	1.32	1.09	0.69	0.50	0.62	0.57	0.57	0.44	0.71	0.63	0.48	0.59	0.78
	Low T x8 spike	1.22	1.36	1.06	0.79	0.52	0.61	0.84	0.45	0.46	0.76	0.76	0.45	0.65	0.69

*Stale aroma character was not scored for the first 2 difference tests

Appendix 8 – Ranking of Sensory Scores of the 35 new make spirits surveyed by the SWRI Panel (Chapter 7)

Spirit Samples	Nutty Score	Nutty ranking	Cereal Score	Cereal Ranking	Oily score	Oily Ranking	Feinty Score	Feinty Ranking
AA	0.262	32	0.446	33	0.633	29	0.771	29
AB	0.377	26	0.962	10	0.540	34	0.971	22
AC	0.608	13	0.992	8	0.660	27	1.300	6
AF	0.515	19	0.608	26	0.553	32	0.721	33
AG	0.346	29	0.485	32	0.673	26	0.936	25
AH	0.446	23	0.954	11	0.800	17	1.164	11
AL	0.569	16	0.600	27	0.660	28	1.079	16
AM	0.738	5	0.838	18	0.953	9	1.279	8
AP	0.308	30	0.685	24	0.773	19	0.936	26
AQ	0.385	25	0.623	25	0.707	23	0.829	27
AS	0.577	15	1.115	5	0.940	11	0.943	24
AT	0.623	11	0.531	30	0.700	24	1.057	17
AU	0.423	24	0.508	31	0.807	15	0.771	30
AW	0.662	8	0.908	14	0.953	10	1.029	20
AZ	0.346	28	0.792	20	0.787	18	1.150	13
B	0.508	21	0.954	12	1.100	3	1.157	12
BA	0.354	27	0.885	15	0.993	7	1.264	9
BD	0.546	17	0.815	19	0.727	22	0.979	21
BH	0.485	22	0.862	17	0.753	20	1.057	18
BI	0.169	35	0.554	29	0.487	35	0.657	35
BJ	0.246	33	0.554	28	0.580	30	0.779	28
BK	0.292	31	0.408	34	0.567	31	0.736	31
BO	0.654	9	1.108	6	1.273	1	1.514	4
BQ	0.777	4	1.308	1	1.247	2	1.636	1
BR	0.538	18	1.177	2	1.073	5	1.629	2
BT	0.646	10	1.123	4	1.040	6	1.364	5
DA	0.815	3	1.100	7	0.915	12	1.200	10
DB	0.938	1	0.877	16	0.892	13	1.100	14
DC	0.720	6	0.940	13	0.800	16	1.293	7
DD	0.693	7	0.760	21	0.853	14	1.040	19
E	0.510	20	0.370	35	0.540	33	0.690	34
Q	0.577	14	1.169	3	0.700	25	1.521	3
S	0.215	34	0.692	23	0.747	21	0.729	32
T	0.615	12	0.754	22	1.087	4	0.950	23