HEADSPACE ANALYSIS OF NATURAL YOGHURT USING HEADSPACE SOLID PHASE MICROEXTRACTION

A THESIS

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ERRATA

Page xxi, lines 23 & 24	"PA polyacrylate PA polyacrylate" should read "PA polyacrylate"
Page 10, line 2	"quantified" should read "were quantified"
Page 25, line 5	"or" should read "as"
Page 51, line 20	"was" should read "were"
Page 69, line 31	The sentence beginning "Variations to the sampling....." is missing some text and should read "Variations to the sampling procedures are detailed in the Materials and Methods sections in each chapter."
Page 70, line 1	The first sentence is missing some text and should begin with "Before headspace sampling, each sample ("
Page 82, Fig. 4.1 (A-F)	When regression lines are forced through the origin in Fig. 4.1, R^2 values do not change a great deal, with the majority changing by less than 1%. All trends in R^2 values are retained, thus discussions based on these values are unaffected.
Page 104, line 24	"were determined" should be deleted
Page 105, line 1	"were determined" should be deleted
Page 109, line 27	"very to 1" should read "very close to 1"
Page 143, line 25	"were" should be deleted
Page 147, line 3	"unfavorable" should read "unacceptable"
Page 177, line 10	"that" should read "those"

This thesis is dedicated to my darling daughter,

Sophia Jeevaranee Campbell Reid.

ABSTRACT

The Solid Phase Microextraction (SPME) method was originally developed to extract volatile and semivolatile compounds from wastewater samples but has since been applied to flavour compounds in foods and beverages. Research using the HS-SPME in related areas such as cheese and skim milk powder has been carried out but, to date, no work has been done on yoghurt flavours. The main objective of this study was to devise a methodology for the Headspace Solid Phase Microextraction (HS-SPME) technique to investigate and quantify six flavour analytes in natural, set yoghurts made from recombined milk.

The relevant literature was reviewed and from it, a research proposal for this work on yoghurts was drawn. The first step in analysing and quantifying the yoghurt volatiles was to set up a working methodology for the HS-SPME method. The 100 μm polydimethylsiloxane (PDMS) fibre was chosen along with 20 minutes being the optimum fibre adsorption time. General equipment, materials and methods used throughout this thesis are also detailed. The external standard (ES) method was used to calibrate the GC and quantify the analyte concentrations in this study. The internal standard (IS) method was not used as a quantitative tool in this study.

Once the HS-SPME methodology had been set up for the analysis of yoghurts, the classical Static Headspace (SH) method was compared with the HS-SPME method for extraction efficiency. The results suggested that the two methods were complementary in that the SH method extracted the more volatile compounds (acetaldehyde, acetone and 2-butanone) whereas, the HS-SPME method extracted the semi- to non-volatile compounds (ethanol, diacetyl and acetoin) more readily. However, the HS-SPME was found to be the more sensitive and effective method of the two techniques tested.

The next step in the thesis was to investigate the presence of the six analytes in milk and cultured yoghurt. The effects of the sample matrix, fat levels and incubation on the volatile concentrations were also examined. The results suggested that the six analytes were inherently present in milks but at low concentrations. No conclusive effects were found for the sample matrix, fat levels and incubation. However, it was evident that

fermentation of the milks using bacterial starter cultures resulted in a large increase in some of the volatiles being investigated.

Following this, the effects of fat levels, storage time and storage temperature on the six volatiles in yoghurts were examined. The results indicated that significant fat level effects were only seen for diacetyl and acetoin, while temperature effects were only observed for ethanol. In both trials, only general trends for the analytes concentrations were drawn because the data varied from day to day. The results suggested that most of the compounds decreased with time except for diacetyl, which seemed to increase.

The final part of this study looked at applying the devised HS-SPME methodology to a series of commercial yoghurts as a preliminary trial, with a view to investigating a potential application for the HS-SPME method. Fourteen commercial yoghurts were analysed and the six analytes quantified. The data obtained was analysed using Principle Component Analysis (PCA), which divided the yoghurts into groups based on their analyte concentrations. From these groupings, eight yoghurts were selected and fresh samples were analysed using HS-SPME and PCA. This was carried out parallel with an untrained consumer panel, which had to distinguish differences between the yoghurts in a series of triangle tests by smelling the headspace on opening the yoghurt containers. The conclusions drawn were that, unlike the HS-SPME method with PCA, the average consumer could not differentiate the yoghurts based on smell alone. PCA also showed that the HS-SPME results obtained were fairly reproducible.

In conclusion, the HS-SPME method was shown to be a useful analytical technique, which can be used to analyse and quantify flavour compounds in natural, set yoghurts. This area of investigation has a lot of scope, with the results from this study providing a basis or starting point for further investigations in this area. Future studies may lead to potential applications for the HS-SPME method, one of which may be quality control where correlation of sensory data with HS-SPME analytical data is required.

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and lastly, my darling daughter, Sophia.
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ABBREVIATIONS

A	peak area ratio
AnalaR	analytical reagent
ANOVA	analysis of variance
B	Biofarm
BA	Biofarm *Aciophilus*
BP	boiling point
BTEX	benzene, toluene, ethyl benzene and xylene isomers
CO_2	carbon dioxide
conc.	concentration
CW/DVB	carbowax/divinylbenzene
DA	descriptive analysis
DDI	distilled, deionised water
DH	dynamic headspace (Purge and Trap)
DH-GC	dynamic headspace - gas chromatography
DNA	deoxyribonucleic acid
ES	external Standard
FA	factor analysis
FID	flame ionisation detector
FN	Fresh 'n' Natural
g	gramme
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography-olfactory
HF	high fat
HPLC	high performance liquid chromatography
hr	hour
HS	headspace
HS-GC	headspace-gas chromatography
HS-SPME	headspace solid phase microextraction
IFNHH	Institute of Food Nutrition and Human Health
IR	infra-red

IS	internal Standard
k	partition coefficient
kg	kilogram
L	litre
L/hr	litre per hour
LDA	linear discriminant analysis
LF	low fat
min	minute
MITC	methyl isothiocyanate
mL	millilitre
MS	mass spectrometry or mass spectroscopy
N	Naturalea
N_2O	nitrogen oxide
NA	Naturalea *Acidophilus*
Na_2SO_4	sodium sulphate
NaCl	sodium chloride
NMR	nuclear magnetic resonance
NS	not significant
NZDRI	New Zealand Dairy Research Institute
O_2	oxygen
°C	degree Celsius
OFN	oxygen-free nitrogen
PA	polyacrylate
~~PA~~	~~polyacrylate~~
PCA	principal component analysis
PDMS	polydimethylsiloxane
PDMS/DVB	polydimethylsiloxane/divinylbenzene
ppb	parts per billion
ppm	parts per million
psi	pounds per square inch
QDA	qualitative descriptive analysis
R	regression coefficient
rep.	replicate

R_T	retention time
SAM	standard addition method
SC	Slimmers' Choice
SDA	stepwise discriminant analysis
secs	seconds
SFE	Supercritical fluid extraction
SH	static headspace (direct injection)
SH-GC	static headspace - Gas chromatography
SMP	skim milk powder
SPE	Solid phase extraction
SPME	Solid Phase Microextraction
Std.	standard
TSNF	total solids non-fat
UHT	ultra high temperature
units ^{2}	peak area units
VA	Verona *Acidophilus*
VB	Verona Bulgarian
WMP	whole milk powder
WW	Weight Watchers
YAB	Yoplus *Acidophilus Bifido bacterium*
YABC	Yotrim *Acidophilus Bifido bacterium Caseii*
YHF	Yoplait high fat
YLF	Yoplait low fat
µL	microlitre

CHAPTER ONE

INTRODUCTION

Volatile organic compounds (VOC) in food have significant impacts on the flavour quality. In order to identify which compounds significantly influence the flavour of a product, flavour analysis has to be undertaken. This involves separating and isolating the volatile flavour compounds, quantifying them and lastly, identifying them.

In flavour analysis, gas chromatography (GC) coupled with mass spectrometry (MS) have been the most important flavour-separating and identifying tools, to date. Various isolation and extraction methods have been developed to extract the flavour compounds from the sample matrix. The traditional methods include the classical (direct injection) static headspace (SH) method, the dynamic (Purge and Trap) technique (DH), supercritical fluid extraction (SFE), solid phase extraction (SPE) and the membrane extraction (ME) methods. The area of flavour analysis research has grown recently with the development of new and improved analytical tools.

One such analytical tool is the Solid Phase Microextraction (SPME) method, which was originally developed to extract volatile and semi-volatile compounds from wastewater samples (Arthur and Pawliszyn, 1990; and Arthur *et al.*, 1992b). It has since been applied to flavour compounds in foods and beverages (Yang and Peppard, 1994; Elmore *et al.*, 1997; and Chin *et al.*, 1996).

The SPME technique is a simple analytical tool. It is relatively cheap to run and easily automated with convenient on-site sampling capabilities. It is a solventless extraction method with advantages such as high precision and efficiency with increased sensitivity. The method can be used to extract analytes from the aqueous sample directly (SPME) or from the headspace above the sample matrix (headspace SPME [HS-SPME]). The SPME fibre is small and cylindrical with an analyte-specific coating on it. The fibre is easily incorporated into a syringe-like device, which makes it more durable and less fragile.

The main objective of this study was to devise a methodology for the HS-SPME technique to investigate six flavour volatiles in natural, set yoghurts. Yoghurt is produced when bacterial starter cultures are added to milk and incubated. The subsequent fermentation results in acidification of the milk and development of favourable flavours and organoleptic properties in the product.

Very little flavour analysis work has been done in the dairy area using HS-SPME (Chin *et al.* 1996; and Stevenson and Chen, 1997). To date, no work has been carried out on yoghurts and their flavour volatiles. The work in this study is a pilot study in the area and the results obtained provide a good starting point for further investigations in HS-SPME.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to Yoghurts

One of the oldest methods for preserving milk is fermentation. This process results in the acidification of the milk and the development of various, usually favourable, organoleptic qualities. There are many methods for carrying out this fermentation, which give rise to a range of products such as kefir, yoghurt, kumiss, and acidophilus milk. Yoghurt has become increasingly popular over the last fifteen to twenty years (McGregor and White, 1987) and besides its sour nature, the characteristic aroma is highly appreciated by consumers around the world.

There is still some controversy regarding the exact definition of yoghurt in terms of its chemical composition and types of starter organisms used. For the purpose of this review, yoghurt is defined as a product resulting from milk fermented using a mixed starter culture consisting mainly of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These cultures can be obtained from commercial starter manufacturers or starter banks. The temperature is controlled during fermentation and the yoghurt is cooled quickly once the desired acidity level is reached.

2.2 Classification of Yoghurts

Yoghurts have been classified into five groups depending on their chemical composition, production methods, flavour, nature of post-incubation processing and the types of starter cultures added.

2.2.1 Chemical Composition of Yoghurt

The standards for yoghurt chemical compositions depend on the fat content namely, full fat (~3% fat), medium fat (~1.5% fat), and low fat (~0.3%). Milkfat in fermented dairy products has several characteristics, which are believed to contribute to the overall flavour of the product (Schultz *et al.,* 1967).

2.2.2 Production Methods

There are two main types of yoghurt: set and stirred. These classifications depend on the method of production and the physical structure of the coagulum. Set yoghurt is the product formed when fermentation of milk is carried out in the retail container and the yoghurt produced is a continuous semi-solid mass. Stirred yoghurt, on the other hand, results when the coagulum is produced in bulk and the gel structure is broken prior to cooling and packaging

2.2.3 Yoghurt Flavourings

Flavouring of yoghurt is another method often used to differentiate between the various types of yoghurts. Flavoured yoghurts can be divided into three categories: plain or natural yoghurts, fruit yoghurts, and flavoured yoghurts. Plain or natural, unsweetened or sweetened yoghurt is the traditional yoghurt with its typical sharp "nutty" flavour. The addition of sugar to natural yoghurt can mask the acidic taste of the yoghurt. Fruit yoghurts are often made by adding fruit preserves, purees, or jams. Flavoured yoghurts are prepared by adding sugar or common sweetening agents as well as synthetic flavourings and colourings to plain or natural, unsweetened yoghurts. The fruit, flavourings and colourings can be incorporated and homogenously distributed in stirred yoghurts but in set yoghurts, the fruit settles to the bottom and has to be mixed in by the customer before eating (e.g. Swiss-type yoghurt).

2.2.4 Post-Incubation Processing of Yoghurts

The post-incubation processing of yoghurts can also give different types of yoghurts. For example, products such as pasteurised/UHT yoghurt, concentrated yoghurt, frozen yoghurt, and dried yoghurt which may be spray-dried, sun-dried, or freeze-dried, are results of the post-fermentation processing. These products vary in chemical composition, physical characteristics, and organoleptic qualities. With the pasteurised/UHT yoghurts for example, the yoghurt is heat-treated after the incubation/fermentation step. This leads to the denaturation of the yoghurt starter bacteria which, in turn, results in a reduction in the levels of the volatile flavour compounds produced during refrigerated storage. Refrigerated storage also slows down the metabolic activity of any viable starter culture present in the yoghurts and reduces the volatility of the analytes and concentrations.

2.2.5 Types of Starter Cultures Used to Make the Yoghurts

The microorganisms found in the starter cultures added to ferment the milk fall into three categories:

- Lactic acid-producing cultures - *Streptococcus lactic, Streptococcus thermophilus, Streptococcus cremoris,* and *Lactobacillus delbrueckii* subsp. *bulgaricus;*
- Associative citrate-fermenting aroma bacteria - *Leuconostoc citrovorum* and *Leuconostoc dextranicum* which produce quantities of diacetyl when grown in a milk medium; and
- Dual purpose lactic acid- and aroma-producing strains - *Streptococcus diacetilactis.*

Mixed strain cultures manufactured by starter banks contain combinations of the above-mentioned species. The type of fermented product to be manufactured is determined by the species of microorganisms added to the milk before fermentation.

2.3 Flavour Volatiles in Yoghurts and Milks and Their Origins

The two main roles of the starter culture during the manufacture of yoghurt are:

(a) production of lactic acid and

(b) the development of flavour in the product.

Although more than one hundred individual chemical compounds have been isolated from yoghurts (Badings and Neeter, 1980), the carbonyl compounds such as acetaldehyde, acetone, acetoin, diacetyl, and 2-butanone, have been found to have a major impact on the desired yoghurt flavour (Keenan and Bills, 1968; Sandine and Elliker, 1970; Sandine *et al.*, 1972; and Dwivedi, 1973, cited in Tamime and Deeth, 1980). Viani and Horman (1976, cited in Tamime and Deeth, 1980) identified various flavour compounds associated with plain or natural yoghurt aroma (Table 2.1 page 6), and their possible origins.

Table 2.1: Flavour Compounds in Natural Yoghurt and Their Origins (from Tamime and Deeth, 1980).

Flavour Component	**Origin and Precursor**
Acetaldehyde, acetoin and diacetyl	Microbial fermentation of lactose-citrate cycle
Acetylpropionate, 2-hydroxy-3-pentanone, 3-hydroxy-2-pentanone	Threonine cycle or thermal degradation of fat
Acetone, butanone, 3-penten-2-one, 2-hexanone	Thermal degradation of fat from ketoacids
2-heptanone, 2-nonanone, 2-undecanone	-
γ-Valerolactone, δ-caprolactone, δ-caprilactone, δ-tridecalactone	Thermal degradation of fat from hydroxyacids
Pentane, methylcyclopentane	-
Furfural, furfurylalcohol, 5-methylfurfural, furylmethylketone, 2,5-dimethylfurane, 2-furyl-3-propional, furylethylketone, 2-pentylfurane	Thermal degradation of lactose
Isobutyraldehyde	Thermal degradation of protein from valine
Benzaldehyde, benzyl alcohol, methyl benzoate	Thermal degradation of fat and/ or lactose
Dimethylsulphide, dimethylsulphone	Thermal degradation of protein from methionine
Phenylacetaldehyde	Thermal degradation of protein from phenylalanine

There are several metabolic pathways for the production of acetaldehyde, the dominant "nutty" flavour compound in plain, natural yoghurt (Tamime and Deeth, 1980). These pathways may operate simultaneously during fermentation. A small amount of acetaldehyde is necessary to impart a balanced yoghurt flavour to cultured products but in high concentrations, it causes a flavour defect described as "green", which is undesirable in yoghurt products (Keenan and Bills, 1968, cited in Tamime and Deeth, 1980). According to Keenan and Bills (1968, cited in Tamime and Deeth, 1980), acetaldehyde in yoghurt is

produced from lactose, valine, and acetyl phosphate (due to decarboxylation of pyruvate). Cleavage of threonine to form glycine and acetaldehyde has also been reported as a possible source (Sandine and Elliker, 1970, cited in Tamime and Deeth, 1980). Another potential source of acetaldehyde is the metabolism of glucose by lactic starters to form acetaldehyde and ethanol via the activity of aldehyde dehydrogenases [Lees and Jago (1976a; 1976b; and 1977, cited in Tamime and Deeth, 1980)]. These reduce acetyl-CoA to acetaldehyde, and acetaldehyde to ethanol. Another possible metabolic pathway for the production of acetaldehyde is via the activity of deoxyriboaldose, which degrades deoxyribonucleic acid (DNA) and breaks down thymidine to acetaldehyde. This enzyme has only been found in certain lactic acid bacteria, however.

Aroma production in cultured dairy products only occurs when the pH of the medium is below 6.0. The lactic acid produced by the starter cultures during fermentation reduces the pH of the milk medium and contributes to the yoghurt flavour. It imparts an acidic, refreshing taste, while the carbonyl compounds such as 2-butanone, ethanol, acetaldehyde, acetone and acetoin, are associated with the aroma and flavour of yoghurt. Pure lactic acid is odourless and does not contribute to the aroma while other acids, such as acetic acid, produced by culture organisms are volatile and contribute to the mildly acidic aroma. Formic, propionic, butyric, and valeric acids have also been found in small quantities in yoghurts and these, combined, impact on the overall flavour.

Laye *et al.* (1993) used high performance liquid chromatography (HPLC) to confirm that lactic acid was the major organic acid present in yoghurts. They found that the lactic acid concentration (mg/g quantities) increased substantially during storage. Pyruvic, uric, acetic and propionic acids were the four other organic acids found in very small amounts (μg/g quantities). It was believed that these acids contributed to the overall acidic flavour as well.

Certain other compounds are associated with flavour enhancement in yoghurts including volatile fatty acids (Turcic *et al.*, 1969, cited in Tamime and Deeth, 1980) and amino acids (Groux, 1976, cited in Tamime and Deeth, 1980). Yoghurt proteolysis by starter bacteria leads to changes in the physical structure of the product and contributes to the production of various flavour compounds. Fat metabolism in full fat yoghurt only occurs to a small

degree but it is thought to have a significant effect on the yoghurt flavour. However, according to Tamime and Deeth (1980), most of the volatile acid content of yoghurt is derived from non-fat components and not from the hydrolysed milkfat by-products.

The characteristic flavour of cultured dairy products is mainly due to the metabolism of the lactic acid bacteria. Its flavour elements are an acid taste and smell, superimposed by a typical cultured flavour, which depends on the type of mixed strain culture used. Badings and Neeter (1980) commented that the important compounds from lactic acid starter cultures were diacetyl, acetaldehyde, dimethyl sulphide, acetic acid, and lactic acid. It is the balance between these aroma compounds, the carbon dioxide produced by the starter cultures, and the aroma compounds originally present in the milk, which determines the overall yoghurt flavour.

According to Tamime and Deeth (1980), most of the acetaldehyde, diacetyl and acetoin formed in yoghurt is due to microbial fermentation in the lactose-citrate cycle, while acetone and 2-butanone were present due mostly to the thermal degradation of fat from ketoacids (Table 2.1, page 6). Lees and Jago (1976a, 1976b and 1977, cited in Tamime and Deeth, 1980) established that glucose was metabolized to acetaldehyde and ethanol by the yoghurt starter bacteria via the activity of acetaldehyde dehydrogenase.

Even though the cultured flavour of yoghurts and other fermented dairy products depends on microbial action and metabolism, some of the flavour compounds present in fermented dairy products are naturally present in fresh milk or are produced from the milk constituents during the manufacturing process (Lindsay, 1967).

Milk of good flavour quality has a bland but characteristic flavour. This is mainly due to low concentrations, in the parts per billion ranges, of various flavour compounds. Hundreds of volatile compounds have been identified in milk, many of which contribute to the overall flavour (Grosch, 1987, cited in Badings, 1991). Because of its nature and because of the absence of a strong flavour of its own, milk is very susceptible to tainting.

The flavour compounds of milk are thought to originate from various sources. Freshly-collected milk may have flavours present from forage the cow has eaten and also volatiles

transferred to the milk from the rumen and respiratory tract. Many flavours and off-flavours are also derived from milkfat, proteins, lactose, and other constituents in milk. Some flavour compounds are transferred from the feed to the milk during synthesis in the mammary glands of cows, and also during the manufacturing, packaging and distribution stages of milk. Cleaning agents and disinfectants may cause "aseptic" or "disinfected" odours. Odours from the environment and surroundings may also be a problem. When milk is heated, changes in the flavour occur as well. The kind and intensity of the flavour changes depend on the time and temperature of the heat treatment. Differently heated milks therefore, have a range of "heated" milk flavours. Some of the terms used to describe these flavours include "cooked flavour", "UHT" flavour and "sterilized" or "caramelised" flavours. The types of heated milks can be classified into five groups depending on the type of heating administered to the milks: low-pasteurised (LP) milk, medium-pasteurised (MP) milk, high-pasteurised (HP) milk, ultra-high temperature, short time (UHT) heated milk and sterilised milk.

"Sterilised" or "pasteurised" milk - this involves batchwise heating of milk to 115°C for 20 minutes. In such heating processes, the end product has undergone severe chemical changes, which are reflected in the strong UHT or ketone-like and caramelised or sterilised flavours. The colour of the milk is also often a little brownish from Maillard reactions and caramelization. The 2-alkanones (C_5, C_7, C_9, C_{11}) mostly contribute to this flavour along with other compounds such as furans and pyrazines. The recombined milks in the present study were pasteurised by heating to 91°C and holding for 5 minutes in the Alfa Laval UHT apparatus before inoculation with the mother culture was carried out.

Jaddou *et al.* (1978) found that the concentrations of all the compounds being analysed were higher in a 90 second heat treatment than a 3 second heat treatment administered to milk. This suggested that a stronger "cooked" flavour was present in the milk that was heated for longer. They also found that acetaldehyde, propanal, 2-hexanone, 1-butanol, 2-heptanone and cyclohexanol were not present in raw milk but were present in the heated milks suggesting that thermal degradation of certain compounds had occurred.

According to Vallejo-Cordoba and Nakai (1993), good- and poor-quality pasteurised milks have several aromatic compounds, which give them their characteristic heat-treated milk

flavour. Of these compounds, the volatiles namely, acetone, ethanol (in very small amounts), 2-propanol, 2-butanone, and diacetyl (only in poor quality milk) were quantified in the parts per billion (ppb) concentration range. Acetoin and acetaldehyde were not identified in the heated milks and therefore, were assumed to be absent.

2.4 Flavour Analysis of Volatile Organic Compounds

Volatile Organic Compounds (VOC) present in foods have significant impacts on the flavour quality. A flavour is the "sensation produced by a material when ingested and perceived by the senses of taste and smell in combination with the overall temperature and tactile sensations". To a flavour chemist, flavours are simply chemical compounds, which produce these sensations. The foods containing these flavours are complex systems. They are made up of an array of chemical substances ranging from simple inorganic molecules to complex biomolecules. The flavour analyst must pinpoint which chemical compounds are responsible for the perceived sensation by devising an analytical method to separate, identify, and quantify these volatile substances.

Such an analytical process would consist of three main steps:

- **Separation and isolation** of the analyte of interest from a sample matrix;
- **Qualitative identification** of the compound; and
- **Quantification** of the analyte concentration.

Each step is critical for accurate determination of the flavour compounds in the selected food. Gas chromatography (GC) has been the most important tool, to date, for the analysis of VOCs or flavours in foods (Shaath and Griffin, 1988).

GC is used to analyse either an aliquot of a solution of the analyte or an aliquot of vapour from above the solution (headspace analysis). The vapour sample is obtained either by DH sampling (Purge and Trap) or SH sampling (Mulligan and McCauley, 1995), and then separated using GC according to the specific boiling points of the flavour compounds. Flavour chemists have developed several new techniques in conjunction with GC for the isolation, separation, and identification of volatile flavour compounds in foods and beverages (Maarse, 1991).

The types and levels of VOCs present in materials such as foods, health care products, perfumes, and medicines determine, in part, the perceived aroma and flavour of the products. Due to the volatile nature of these compounds present in these flavour-full and aromatic formulations, headspace gas chromatography (HS-GC) has been successfully applied to the qualitative and quantitative analysis of the components (Coleman *et al.,* 1994). Headspace methods minimise the wear and tear on the chromatographic system as only the gaseous portion of the sample is subject to analysis. This also reduces the possibility of artefact formation during separation and isolation of the VOCs and relaxes some of the constraints on sample preparation.

It should be noted that some compounds observed as peaks in a gas chromatogram may contribute, little if anything, to the overall aroma of a product. Some highly potent aroma compounds that are not detected by GC due to their low concentrations may contribute significantly to the aroma of the product (Braggins *et al.*, 1999). However, the GC method does give a chromatographically detectable "fingerprint" of the headspace composition, while the aroma smelt, gives a physiologically detectable characterization of the headspace. Even though it is incorrect to state that the headspace composition completely describes the aroma and vice versa, there is much analogy between the two.

2.4.1 Procedures for Headspace Analysis of Volatile Compounds

The term "headspace" gas sampling or analysis is frequently misunderstood. It implies that the vapours above the sample in a closed container are in equilibrium with the sample material being analysed. In most cases where headspace gas analysis is reported, this is not so. The concentrations of volatile compounds in the headspace area are usually low. Thus, it is necessary to use a very large volume of sample vapour to produce a response in the GC analysis. Even then, trace components may not be detected or the large volume of vapour may be incompatible with GC analysis, resulting in broad, poorly resolved peaks. Hence, some form of sample-concentrating procedure is usually required prior to analysis.

The original chromatographic analyses of flavours were done by drawing vapours directly from above the food material (Buttery and Teranishi, 1961; Mackay *et al.,* 1961; and Teranishi *et al.,* 1967). Due to the large volume of gas sample required, packed columns were used. This resulted in poor resolution because only the volatile compounds with

relatively high concentrations were recorded. Other researchers tried to increase the sensitivity of GC analysis by concentrating the volatile compounds (Nawar and Ferguson, 1960) or by salting-out techniques, which enriched the vapours in the headspace (Bassette *et al.*, 1963; Kepmer *et al.*, 1964). Steam distillation (Azzouz *et al.*, 1976), cryogenic traps (Kaiser, 1973), use of solid adsorbent traps (Turk and d'Angio, 1962) and on-column trappings (Grob, 1973) were some of the other concentrating procedures used before volatile headspace analysis could be carried out.

The main limitations to these concentrating procedures are:

- the headspace profile does not accurately reflect the true quantitative distribution of volatile materials in the headspace. Instead, there is a distortion of the distribution of volatile compounds present in the sample due to the concentrating procedures (Bassette, 1984);
- the concentration of the volatile materials increases the sensitivity of HS-GC method but the concentration procedures are laborious and time-consuming; and
- artefacts from various sources during the concentration procedures may interfere with the analysis of VOCs. Therefore, chromatograms obtained from concentration procedures above should be analysed with caution.

2.4.2 Qualitative Analysis of VOCs Using GC

Many of the earliest reports on headspace analysis were of a qualitative nature. The gas chromatogram provides a single piece of qualitative information about each species present in the sample, that is, its retention time or its position on the stationary phase after a certain elution period. It is used for determining the presence or absence of specific components in the injected sample volume.

2.4.3 Quantitative Analysis of VOCs Using GC

As instruments, detectors, columns, and isolation techniques for HS-GC analyses have improved, quantitative analyses of trace levels of VOCs within defined matrices have been reported (Ozeris and Bassette, 1963). Quantitative analysis is based on the comparison of either the peak height or peak area of the analyte with that of one or more standard peaks.

2.4.4 Calibration Methods for Quantitative Analysis

(A) External Standard (ES) Method

This type of calibration involves preparing a progressive series of standard solutions containing the analytes of interest and obtaining the analyte peak heights and areas using HS-GC. These are then plotted as a function of the standard solution concentrations. A plot of the data obtained should yield a straight line passing through the origin. In quantitative headspace analysis, it is important that the standards used have similar matrix effects on the VOCs in the headspace to those of the samples investigated (Ulberth, 1991; De Haast *et al.,* 1978). This will ensure consistent calibrations throughout the investigation.

(B) Internal Standard (IS) Method

The internal standard (IS) method of calibration involves spiking a measured amount of a standard of known concentration, into each sample being analysed. The ratio of analyte to IS peak areas serves as an analytical parameter. It is desirable that the IS peak is well separated and distinct from the peaks of the other sample components. By using the IS method, external errors such as variable sample injection volumes are avoided.

For both calibration methods, it is important to consider the type of equilibrium in question. Considerations include:

- Whether the sample system is a static, closed equilibrium or a dynamic equilibrium;
- Whether the concentration in the condensed phase will change during sampling;
- The time needed for equilibrium vapour pressure to be established;
- Solvent effects and solute interactions with the sample constituents such as lipids, proteins, carbohydrates;
- Temperature of the sample;
- Possible degradation or alteration of the materials of interest; and
- Production of volatile materials due to decomposition or alteration of sample constituents.

When such factors are considered and the analytical methods are designed to give specific information on the analyte concentrations (for both the ES and IS methods), HS-GC can be

a very useful technique for a wide range of applications.

2.4.5 Identification of Volatile Organic Compounds

Mass Spectrometry (MS) may be used alongside a GC to identify the flavour compounds present in a sample mixture after they have been concentrated and separated. The mass spectrum does not automatically provide an identification of a compound. It can however, provide information about the molecular weight and the structure of the volatile compound from the way it fragmented after ionisation.

Infrared spectroscopy (IR) can also be particularly useful as a supplementary tool to MS. It provides information about the nature of the functional groups in the molecule along with a "fingerprint" that can be used for absolute identification when a reference compound is available. Nuclear Magnetic Resonance (NMR) spectroscopy can be used to determine the environment of the functional groups. Unfortunately, neither of these techniques has a degree of sensitivity comparable with that of MS. This leaves MS as the most favoured universal technique used for identifying a specific compound.

In order to detect important compounds present in concentrations too low to be detected by the GC-MS set-up, the human nose can be used as a substitute. This technique is known as Gas Chromatography-Olfactory (GC-O) and is essential for detecting taints and off-flavours. It involves smelling the components as they elute from the end of the GC column. This can be done by splitting the effluent gas, so one part goes to the GC detector while the other part is diverted to a smelling port. At the smelling port, odour descriptions can be recorded as they are eluted and assessed as being a potential contributor to the overall flavour of the product. GC-O is mainly used as a sensory tool to identify flavour compounds present in foods. The method focuses on individual compounds and not on their effect on the overall flavour of the food being investigated.

2.5 Solvent-Free Headspace Sampling Techniques

Despite the advances in instrumentation and technology, many sample preparations and extractions still use toxic organic solvents and multi-step procedures, which can result in the loss of analytes. Awareness of pollution and the hazards posed by organic solvents (including ozone depletion and carcinogenic effects) has resulted in initiatives to reduce the

use of organic solvents in sample preparations.

Solvent-free headspace sampling techniques include:

- Classical (direct injection) static headspace (SH) sampling;
- Dynamic (Purge and Trap) headspace (DH) analysis;
- Membrane extraction (ME);
- Solid Phase Extraction (SPE);
- Supercritical fluid extraction (SFE); and
- Solid Phase Microextraction (SPME).

Voice and Kolb (1994) compared the European and American techniques for the analysis of VOCs in environmental matrices such as soil and drinking water. An informal survey conducted in both continents indicated a clear trend away from using solvents to extract VOCs from aqueous samples whenever possible. The preferred technique in most of Europe was found to be SH analysis (direct injection) whereas DH analysis (Purge and Trap) was found to be common in the United States of America.

An ideal sample preparation technique should be solvent-free, simple, inexpensive, efficient, selective, and compatible with a range of separation methods and applications. The ideal separation procedure should simultaneously separate and concentrate the components and be portable.

Voice and Kolb (1994) compared the precision of routine SH and DH analysis. Ten replicate, 5 mL ground water samples, were analysed using both techniques. The results indicated that SH analysis was consistently more precise than the DH method. More peak tailing occurred on all DH chromatograms which was hypothesised as being due to the thermal desorption process.

In a similar study done by Wylie (1988), these two techniques were compared for the analysis of drinking water. It was reported that SH analysis could offer sensitivities comparable with those of Purge and Trap provided the sample to be analysed was placed in a 15 mL sample vial as opposed to the 5 mL vial that was used in the DH method. Salt was added before equilibration to enrich the vapours. The SH method was found to have

superior precision in such cases because of increased volatilisation of analytes from the sample matrix.

Voice and Kolb (1994) also compared four techniques for the analysis of soil samples: DH analysis of soil; methanol extraction of the soil followed by headspace analysis of the extract in water; direct headspace analysis of the soil at 95°C; and direct headspace analysis with stirring. It was found that direct headspace analysis of a complex matrix such as a soil sample with equilibration at 95°C was superior to the Purge and Trap method. Soils with very high organic matter however, produced variable results such as low recoveries of analytes in some soil samples and high recoveries in others. The methanol extraction technique was found to have poor analyte extracting abilities.

From Voice and Kolb's (1994) comparative report, it was concluded that the SH (direct injection) technique was the more precise and efficient method for analysis of volatile compounds under specific conditions. The technique is simple and inexpensive. It requires little sample preparation and analysis time is minimal.

2.5.1 The Classical Static Headspace (Direct Injection) Extraction Method

The classical SH (direct injection) method is the simplest form of solvent-free sample preparation and has been used for several decades to analyse VOCs (Charalambous, 1978 cited in Zhang *et al.,* 1994). The technique is based on the partitioning of volatile compounds between the sample matrix and the vapour phase above the sample at a rate dependent on various factors. These factors include the volatility of the analytes and their solubility in the sample matrix, the temperature of the sample and the homogeneity of the sample.

SH sampling is carried out by extracting an aliquot from the headspace above the sample, once equilibrium is established between the two. This is usually done using an airtight syringe, or as in the case of Miller and Stuart (1999), a sample loop was used. The aliquot is injected into the GC injector port where the headspace volume is eluted through the column and analysed for the volatiles of interest. One of the main advantages of SH sampling is its ability to analyse a sample for low molecular weight volatiles without interference from solvent peaks. This is especially important as some samples being

analysed by SH are being assayed for residual solvents in the finished product at the end of a process line, for example, in the pharmaceutical industry. SH sampling can also be fully automated which provides increased reproducibility compared to manual sampling. Other advantages include the relatively low cost per analysis, the speed and the simplicity of sampling.

There are some disadvantages associated with the SH technique. Due to the low headspace concentration of the analytes of interest only a fraction of the analytes are extracted and injected into the GC. These analytes are in equilibrium with those still in the sample matrix and therefore, only a small amount of the volatiles may be present in the headspace. Hence, the technique may lack sensitivity when identifying and quantifying specific analytes in the sample. Also, the analytes with high boiling points are hard to extract using the SH method, as these analytes are non-volatile and therefore, not in a gaseous state ready for extraction and analysis. The reproducibility of the technique depends on analyzing a sample after equilibrium has been reached within the sealed vial. The time for this may be longer especially when analyzing low volatility compounds. One way to overcome this problem may be to heat the sample, but for high boiling compounds, a lot of heat would be required before the compounds volatilize and this may lead to degradation of the sample, resulting in non-representative results.

SH analysis is based on the assumption that equilibrium partitioning controls the distribution of the analytes between the sample matrix and the headspace. Hence, the concentration of the analytes in the headspace volume is related to the concentration of analyte in the sample matrix volume by a partition coefficient. Aqueous partition coefficients depend on the chemical characteristics of the sample matrix so analytical errors may result if partitioning is not the same in both the standards and the samples analysed. By adding salt to the sample or heating the sample matrix, the partition coefficient is increased, thereby increasing the concentration of the analyte in the vapour phase above the sample. This topic is discussed further in Section 2.6.

Headspace analysis can be conducted by matching the standards prepared to the sample matrix being investigated. This can only be done if the matrix characteristics are known. Due to the lack of any concentrating effects, the SH technique suffers from low sensitivity

and an exhaustive extraction cannot be achieved. Careful calibration of the method is also required, which enables quantification of the analytes .

2.5.2 The Dynamic (Purge and Trap) Headspace Method

DH analysis involves moving the analytes away from the headspace above the sample matrix. Instead of allowing the sample analytes to reach equilibrium with the headspace, the atmosphere around the sample matrix is constantly swept away (purged) taking any analytes with it. The volatiles move out of the container and through to a short column containing a porous polymer adsorbent. The adsorbent on the column selectively retains the analytes while allowing water vapour and the purge gas to flow through. Once purging of the sample is completed, the column is heated to release the analyte compounds (desorption). These pass through the GC column and elute at different rates, finally reaching the detector where they are analysed. By continually stripping the sample of its organic components into the vapour phase, the partition coefficient is increased. This, in turn, increases the rate at which the analytes partition from the sample matrix, thus, enhancing the recovery of these compounds from the headspace. Often, quantitative removal of the analytes of interest from the sample matrix occurs and this can be especially useful when analyzing trace compounds.

According to Mulligan and McCauley (1995), the DH method is inherently more sensitive than equilibrium-dependent headspace methods and may be most appropriate for the analysis of solids (and yet, is generally used to analyse aqueous solutions). Other advantages of DH sampling include:

- easy sample preparation;
- no organic solvents are used and therefore, the analysis of volatiles without extraneous artefacts, such as solvent peaks, is possible; and
- increased sensitivity, which allows the determination of volatiles in the parts per billion (ppb) and parts per trillion (ppt) ranges;
- the type of sorbent used can be made more selective towards certain analytes and not others.

A disadvantage of the DH technique is that it is more complex than the SH method because the instrumentation used requires constant monitoring at several stages. For example, heating the column, opening and closing of the valves during purging, foaming of the sample during purging, and cross contamination. The DH system is harder to automate due to the number of steps involved, which results in more opportunity for malfunction. The DH technique has a slightly longer sample processing time than the SH method and usually requires a larger sample size.

Westendorf (1985) reported a fully automated technique for DH sampling. Samples were heated and purged with an inert gas, which swept any volatile compounds out of the sample, and onto the trap which was then thermally desorbed onto the GC column. Detection limits as low as parts per billion (ppb) were obtained using this technique with good reproducibility. This automated method has been successfully applied in the food industry in recent years and it has become useful in many laboratories for routine analysis of VOCs.

Although headspace methods are limited to VOC analysis, analysis of solid samples and less volatile compounds can be achieved by heating the sample. Volatile analytes can be thermally desorbed from solid samples so that the less volatile species in the aqueous or solid samples can be better partitioned into the gas phase. However, thermally labile analytes and the high moisture content of the desorbed gaseous mixture often prevent the use of thermal desorption, necessitating a more complicated form of analysis.

2.5.3 The Supercritical Fluid Extraction Method

SFE, analogous to liquid solvent extraction, is an attractive solvent-free sample preparation technique (Hawthorne, 1990, cited in Zhang *et al.*, 1994). Supercritical fluids are sometimes considered to be "super solvents". SFE is a quick extraction process and the solvent strength can be controlled as the concentration depends on the pressure and temperature used during the extraction process. Supercritical fluids such as carbon dioxide (CO_2) and dinitrogen oxide (N_2O) also have the added advantages of being pure, non-toxic, inert, and relatively inexpensive. However, SFE requires an expensive, high-pressure delivery system and a large quantity of high purity gas. Due to the heavy equipment needed for SFE, on-site field analysis is quite difficult. In the case of liquid solvent

extraction, the extracts need to be concentrated before the trace organic analytes can be analysed. As the "solvents" in SFE are mostly gases at ambient temperatures, the concentration steps after SFE are greatly simplified and it can be coupled to GC analysis directly.

2.5.4 The Membrane Extraction Method (ME)

In the ME method, a low pressure stripping gas is typically used for VOC analysis. The technique involves two simultaneous processes (Pratt and Pawliszyn, 1992, cited in Zhang *et al.,* 1994):

- polymeric extraction from the sample matrix - this is where VOC analytes adsorb onto the polymeric membrane and
- gas desorption of VOCs from the polymeric phase.

Disadvantages of ME techniques include slow response of the membrane to changes in concentration of the analytes and a limited ability to analyse polar compounds due to a lack of suitable commercially available polar hollow fibre membranes.

2.5.5 The Solid Phase Extraction Method (SPE)

Sorbent materials are used to extract organic compounds from matrices like water, air, and soil (Poole and Schuette, 1983, cited in Zhang *et al.,* 1994). The important feature of this technique is the concentration of analytes by the sorbent material. SPE is commonly used and it involves analytes being extracted together with the interfering compounds. This is done by passing an aqueous sample matrix through a plastic cartridge containing dispersed sorbent material on a particulate support. The analytes of interest are adsorbed onto the sorbent material while any interfering compounds are washed away using appropriate solvents. Once the interfering compounds are removed, the chosen analytes are removed by washing the sorbent with other analyte-specific solvents. This method is quite simple, inexpensive, easily automated and uses relatively little solvent. It can also be used on-site with relative ease.

SPE does however, have some limitations such as low recovery of analytes, which results from the interaction between the sample matrix and analytes. Plugging of the cartridge or

blocking of the pores in the sorbent by solid and oily components is quite common, resulting in less analytes being adsorbed on the sorbent material. SPE is limited however, to the analysis of semi-volatile compounds with boiling points above those of the solvents.

One solution to the limitations of SPE, is to improve the geometry of the sorbent by coating it on a fine rod such as fused silica fibres or wires. The cylindrical geometry of the recently-developed solid phase microextraction (SPME) system allows rapid transfer during extraction and desorption, it prevents plugging and facilitates handling and sample introduction directly into the GC instrument.

2.5.6 The Solid Phase Microextraction Method (SPME)

SPME consists of two processes:

- **partitioning of analytes between the fibre coating and the sample** - a coated fibre is exposed to the sample and the analytes are extracted from the sample matrix and adsorbed onto the coating, and

- **desorption of concentrated analytes into the analytical instrument** - the fibre with the concentrated analytes is transferred to the GC injection port where desorption of the analytes occurs.

SPME is a simple analytical technique, relatively low in cost, cheap to run and easily automated with convenient on-site sampling capabilities. It is a solventless extraction technique without the hassles of plugging found with SPE. SPME allows placement of the sorbent (fibre with analyte-specific coating) into a sampling matrix (aqueous or gaseous) or in the headspace above the sample to extract analytes. The small size and cylindrical geometry of the fibre allows it to be incorporated into a syringe-like device, which is easily accommodated in a GC injector port. The fused silica fibre is connected to stainless steel tubing, which is used to increase the mechanical strength of the fibre assembly for repeated sampling. The stainless steel tubing is contained in a specially designed syringe as seen in Figure 2.1 (page 22).

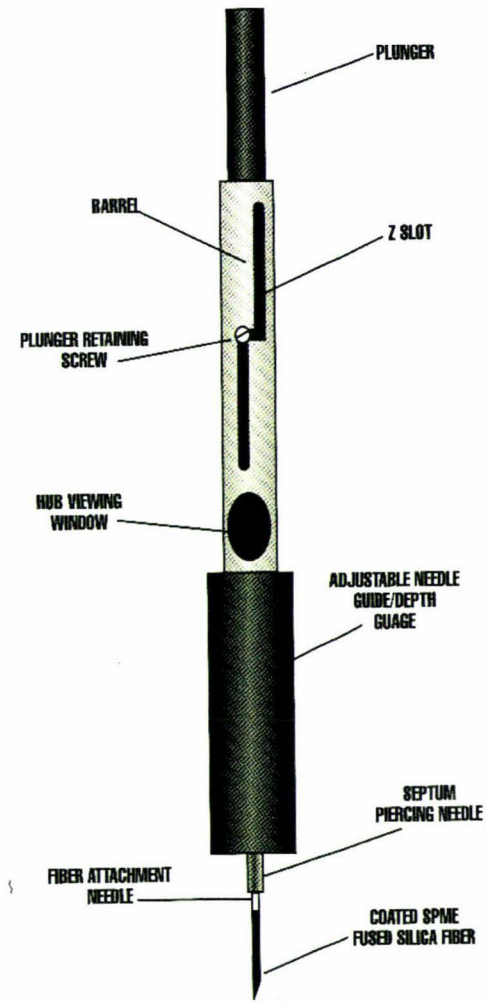


Figure 2.1: The SPME Device Produced by Supelco (Stevenson and Chen, 1997).

During SPME, the fibre is first withdrawn into the syringe needle. The syringe needle is typically lowered into a vial through a pre-pierced septum. The fibre is then exposed to the headspace or the liquid when the plunger is pressed down. The fibre is exposed for a certain length of time to extract analytes from the matrix or VOCs from the headspace above the matrix. The fibre coatings used in SPME have specific affinities for different organic compounds and therefore, SPME can have a very high concentrating effect, which leads to good sensitivity. Once sampling is completed, the fibre is withdrawn into the syringe, which is then transferred to the heated GC injector port. Inside the port, the fibre

is exposed and the analytes thermally desorbed. They are eluted through the column and analysed by the detector at the other end of the GC column.

SPME is a static, non-exhaustive extraction process. SPME reduces analysis time by combining sampling, extraction, concentration, and injection into a single uninterrupted process.

Hawthorne *et al.* (1992) stated that SPME followed by GC analysis, provided a simple and potentially useful quantitative analysis, which hardly required any sample preparation time or solvents. As SPME is an equilibrium technique, calibration is necessary for quantitation. Both IS and ES calibration techniques work well. For relatively clean aqueous samples such as drinking water (<1% organic), external calibration works well. This is usually done by spiking a known amount of target analytes into a clean matrix and then performing SPME. Analyte concentration in unknown samples can then be determined from the calibration curve.

For complex soil samples, the ES calibration method may not work well due to matrix effects. Therefore, the standard addition method (SAM) or IS method is used. The partition coefficients of the IS must be similar to those of the target analytes. Usually, isotopically labelled analogues of target analytes are the best internal standards for SPME as their chemical and physical properties are similar to those of their unlabelled counterparts. As spiked analytes may not interact with the matrix as strongly as the native ones, experimental conditions must be carefully adjusted to ensure effective release of native analytes into the extracting media.

For SPME quantitative analysis, Louch *et al.* (1992) stated that the coating/water distribution constant of the analyte should be determined, which in turn determined the slope of the calibration curve for a given coating thickness. Temperature variations, changes in polarity indices and the composition of the aqueous sample matrix affect the partition coefficient. For example, if sodium chloride had been added to the sample matrix, the ionic strength of the matrix would change and the sensitivity would improve. However, at salt concentrations below 1%, and only small temperature changes, the effects on the partition coefficients are minimal. Therefore, quantitative analysis of VOCs is viable

because partitioning of the analytes between the sample matrix and the headspace is unaffected.

SPME, like the SH method, is an equilibrium sampling method and through proper calibration, can be used to determine the concentration of target analytes in a sample matrix. The two sampling techniques are both solventless procedures, which involve non-exhaustive extractions. The two methods are similar except in their sensitivity and concentrating effects of VOCs. Only small samples are required for the two sampling techniques. Neither technique requires any specialized equipment except for the SPME syringe and fibre for HS-SPME and a gas-tight syringe for SH sampling. Neither method requires extensive sample preparation. Hence, the SH sampling technique was chosen for the present study because it was considered quite similar, in theory and practice, to the HS-SPME method. The HS-SPME method does however, share some common features with the DH technique. For example, they both have some form of an adsorbent trap within the system: the fused silica fibre in the case of HS-SPME and the adsorbent column (trap) in the case of DH. The volatile analytes are extracted and concentrated onto the coating or trap and then desorbed.

SPME was originally designed to extract and analyse water effluents (Arthur and Pawliszyn, 1990; and Arthur *et al.*, 1992). It was later discovered that the technique could be used for various samples ranging from pharmaceuticals to food. The HS-SPME method acts as an intermediate method, halfway between the two traditional headspace techniques (SH and DH). It has the best features of both headspace techniques. It does however, seem to have more in common with the SH method, from a theoretical and practical point of view, than with the DH technique and therefore, the SH method was chosen to be compared with the HS-SPME method in the present study. Other reasons for choosing SH over DH were that the instrumentation is relatively cheap and compact. A disadvantage of using DH sampling for milk and milk products is that a lot of water vapour is generated when the milks are heated, causing foaming during the purging phase (Vallejo-Cordoba and Nakai, 1993).

Like SH sampling, HS-SPME can be used for rapid quantitative determination of a sample with the use of an IS, similar to the analytes, but with a different R_T. ES calibration can

also be used as a means for quantitative analysis. The HS-SPME method can also be used to extract analytes from sample mixtures for qualitative work. This technique can also be fully automated which ensures consistent analyses aiding in quality control applications and quantitative aspects of the analysis. It is portable, making it easy for on-site sampling as the fibre, in its case, can be carried back to the laboratory for analysis.

2.6 Solid Phase Microextraction (SPME)

2.6.1 Advantages and Disadvantages of SPME

Louch *et al.* (1992) reported that SPME could be used to determine traces of organic contaminants at sub-parts per billion (ppb) levels. It is the ideal method for analysis of low concentration volatiles as the total amount of extracted material is transferred onto the column resulting in higher sensitivity. The detection limit of the method depends on the sensitivity, which is in turn, dependent on the coating/water partition coefficient.

Arthur *et al.* (1992) found that in the analysis of ground water for organic contaminants, the relatively inexpensive SPME method reduced sample preparation time by 3-7 times compared with that of the DH method. Another advantage of SPME is that it is solvent-free, which improves the chromatographic separation efficiency. In addition, such solvent-free sample injections allow the use of columns with thinner stationary phase layers and smaller internal diameter. This gives higher precision and sensitivity.

Precision in SPME is generally high as it is a single-step method and therefore, random sources of error associated with transfer of analytes are reduced. Thermal desorption of analytes from the SPME fibre coating in the GC injection port is effective. As the temperature increases, the coating/gas partition coefficients decrease and the ability of the coating to retain analytes diminishes quickly. The constant flow rate of carrier gas within the GC also aids the removal of analytes from the coating. Most volatile and semi-volatile analytes can be desorbed from the coating in the injector port at a temperature between 150°C and 250°C in a few seconds. This results in a narrow band of analytes in the column, which improves separation, precision and efficiency.

HS-SPME can be used to sample target VOCs in the headspace above sample matrices such as oily, aqueous or human blood. In such areas, direct SPME sampling would lead to

the fibre coating being covered with oil or large protein molecules, but with HS-SPME sampling, such problems are avoided.

Much of the work done in the area of SPME to date has been to study environmental pollutants, wastewater and ground water contaminants, and soil samples. MacGillivray *et al.* (1994) compared HS-SPME with the DH sampling method for the analysis of benzene, toluene, ethylbenzene, and the xylenes (BTEX), all common water contaminants. The DH method used for the analysis of VOCs in water was widely used in the United States for drinking water analysis but the technique has limitations. These include the equipment being expensive and prone to leakage. Sample carryover and problems associated with managing the water flow emerged as well. The DH system did however, have some advantages which included good reproducibility and accuracy with low detection limits and high sensitivity for VOCs. The advantages of using HS-SPME were simplicity, portability, low cost, and high precision. These advantages were considered more useful than the advantages of the DH technique and therefore, HS-SPME was the preferred method for headspace analysis of contaminated drinking water.

SPME has also been used to extract target analytes from food and drug samples. Accurate determination of the caffeine content in coffee and tea has been carried out using this technique (Hawthorne *et al.,* 1992). Isotopically labelled (trimethyl ^{13}C) caffeine was added to the samples as an IS so the caffeine concentration in tea and coffee could be quantified. Two significant problems were encountered. First, the authors found that when the analyst touched the syringe needle to help guide the needle into the sample vial or GC injection port, significant contamination occurred. Touching the syringe needle caused artefacts in the GC-MS chromatograms, resulting from biological acids present on the skin. The second experimental problem experienced by the authors was spoilage of the fused silica fibres due to improper treatment. They worked with an uncoated fibre, which was fairly brittle and therefore, had to be physically protected within the syringe needle. It was found that with reasonable care, the fibres had lifetimes of up to fifty or more injections.

2.6.2 Factors that Affect SPME Sampling Time

HS-SPME is an equilibration analytical method. When a sample is placed in a sealed vial with a headspace volume above it, a chemical equilibrium is eventually established between the sample and the headspace. For liquid and solid samples, three phases are generally involved:

- **the gas phase** (the headspace);
- **the condensed phase** (the liquid or solid sample); and
- **the adsorption phase** (the fibre coating).

During the HS-SPME sampling procedure, the polymer-coated fibre is inserted into the sample or placed in the headspace above the sample. The fibre is left for a set time to adsorb the volatiles present. The exposure time is determined by the time it takes for the analyte concentration to reach a state of equilibrium in all three phases within the system (Zhang and Pawliszyn, 1993). A series of transport processes occur from the sample phase to the gas phase and finally, to the polymer coating phase until equilibrium is finally established between all three phases. SPME eliminates any pre-concentration steps by directly extracting the analytes of interest onto the fibre coating (adsorption). The fibre is then transferred to the injector port of the GC where desorption and analysis take place (Zhang and Pawliszyn, 1993). The kinetics of the mass transport of the analytes from the aqueous phase to the headspace and then to the fibre coating are important as this is what determines the sampling (extraction) time for the HS-SPME method.

Louch *et al.* (1992) suggested a model for the SPME extraction process. They assumed that the extraction process was a diffusion-limited process, that is, that the slowest mass transport mechanism occurring in the system was by diffusion. This suggestion was only valid for liquid polymer fibre coatings, such as the polydimethylsiloxane (PDMS) coating, where transfer of the analytes between the sample and the coating did not require any activation energy to move through the different phases in the system.

The amount adsorbed by the stationary phase on the fibre is primarily determined by:

- the distribution coefficient (k) of the analytes partitioning between the aqueous sample matrix and the fibre coating;
- the volume of the stationary phase (how thick the fibre coating is and how long and wide the fibre is);
- the coating characteristics (polar or non-polar);
- cooling the fibre coating.
- the concentration of the analyte in the aqueous sample matrix;
- the sample matrix characteristics (solid, liquid [aqueous vs nonaqueous] or gas);
- the derivatization of target analytes;
- agitation of the sample matrix; and
- temperature of the sample matrix;

Yang and Peppard (1994) used the 100 μm non-polar PDMS fibre to investigate the flavours present in a fruit juice beverage, ground coffee and a vegetable oil. They mainly looked at possible applications for the HS-SPME method. It was apparent that the results obtained using SPME depended on experimental conditions and the sample matrix. Any changes in these conditions affected the adsorption of analytes by the fibre and this was reflected in the sensitivity and reproducibility of the analytical method used.

The amount of analyte extracted is directly proportional to the volume of the fibre coating. Harmon (1997) confirmed Potter and Pawliszyn's (1992) findings, which suggested that fibres coated with thicker films required a longer time to achieve equilibrium. As a result they provide greater sensitivity due to the higher analyte mass that can be adsorbed. Harmon, (1997) stated that at equilibrium, a linear relationship existed between moles of analyte adsorbed onto the fibre and the analyte concentration in the aqueous phase.

This linear relationship can be described by the equation shown below:

$$n_s = k V_s C_{aq}$$

where

n_s is the moles of analyte adsorbed onto the stationary phase (film coating),

k is the distribution constant of the analyte partitioning between the aqueous and stationary phases,

V_s is the volume of the stationary phase and

C_{aq} is the concentration of the analyte in the aqueous phase.

Potter and Pawliszyn (1994) put forward a similar linear relationship:

$$n = \frac{C_o V_1 V_2 k}{(kV_1 + V_2)}$$

where

n is the moles of analyte adsorbed onto the stationary phase ,

k is the distribution constant of the analyte partitioning between the aqueous and stationary phases,

V_1 and V_2 are the volumes of the stationary phase and aqueous phase, respectively and

C_o is the initial concentration of the analyte in the aqueous phase.

They stated that there is a linear relationship between the amount of analyte adsorbed by the fibre (**n**) and the initial analyte concentration in the sample solution (C_o). The linear range and sensitivity depended on two factors:

- the volume of the stationary phase (V_1); and
- the distribution or partition coefficient (**k**).

If **k** for a particular analyte is large, as is the case with non-polar semi-volatile compounds,

or the aqueous phase volume (V_2) is small, then, at equilibrium, the analyte concentration may be significantly depleted.

However, SPME is a sampling process where quantitative extraction of analytes is almost always impossible. This is because both the amount of analyte adsorbed onto the fibre and the sensitivity of the method are determined by adsorption kinetics and by the distribution coefficient of the analyte compounds. SPME is very sensitive to experimental conditions and if these are varied, the distribution coefficient and adsorption rate will also be affected. This, in turn, will affect the amount of analyte adsorbed onto the fibre and the corresponding reproducibility. Other techniques such as DH and SPE, which have similar theoretical principles are not as sensitive to experimental conditions as SPME and usually recover analytes of interest quantitatively.

Yang and Peppard (1994) found similar results to Potter and Pawliszyn (1994), that is, that the amount adsorbed on the SPME fibre is dependent on the initial sample concentration and the sample volume. In general, the adsorption rate is higher when the concentrations of the analyte are higher. Therefore, if an analyte exists mainly in the liquid phase, a liquid SPME sampling method would be most appropriate, as it would be more sensitive than the HS-SPME sampling method and vice versa. The liquid SPME and HS-SPME techniques were considered to be complementary as either method could be used to analyse analytes in any sample matrix depending on their volatility. Yang and Peppard (1994) went on to use the headspace SPME sampling technique to analyse flavour components (VOCs) in ground coffee, fruit juice, and vegetable oil. As mentioned earlier, the SPME sampling technique was found to be a rapid, sensitive and efficient technique for analysing food samples.

Kinsella (1989) reported that when a sample has a 50-90% water content, interactions between volatiles and food components do not occur. This is because, at this point, the solution of volatiles (the liquid phase of food) has similar characteristics to an ideal, infinitely dilute solution. In addition, Dalla Rosa *et al.* (1994) found that headspace volatile concentrations decreased rapidly when the water became totally free (infinitely dilute) and the water vapour pressure was close to that of pure water.

Fibre exposure time also has a dramatic effect on how much analyte is adsorbed onto the fibre coating and therefore, the signal intensity obtained for flavour components. Clark and Bunch (1997) found that signal intensity versus fibre exposure time was analyte-dependent. Certain compounds such as menthol reached maximum signal intensity (equilibrium) after 15 minutes exposure time, while tetramethylpyrazine needed only 5 minutes, after which, the signal intensity slowly decreased. They also found that longer sampling times, that is, more than 1 hour, gave no increase (fully saturated fibre coating) in signal intensity for analytes in a tobacco-flavour mixture. Arthur and Pawliszyn (1990) studied adsorption time profiles by monitoring the peak area counts as a function of exposure time. They found for the first 2 minutes, rapid adsorption occurred, after which, the rate slowed down. Therefore, in order to improve the precision of the method, the exposure time must be long enough for the equilibrium to be reached or for the rate of adsorption to have slowed down significantly.

The time required to reach equilibrium is the optimum sample adsorption/extraction time. Hence, the equilibrium time for each analyte must first be determined as part of setting up the methodology. This can be done by plotting an adsorption/extraction time profile curve for each analyte versus extraction time. Zhang and Pawliszyn (1993) observed a turning point on time profiles where the curves started to flatten off after initially rapidly rising when maximum mass transfer between the phases occurred. They found that for highly volatile compounds where the concentration of the analytes in the headspace was high, up to 90% of the defined equilibrium had been reached at the turning point on the time profile. The turning point is due to the rapid diffusion process of analytes in the headspace and very slow diffusion in the aqueous phase. At this turning point, the headspace layer around the fibre is depleted of analytes due to adsorption onto the fibre, diffusion within the headspace becomes the limiting factor and the rate of mass transport decreases and the extraction is slowed down. Figure 2.2 (page 32) shows an example of an adsorption time profile graph (Louch *et al.*, 1992). It shows the mass of analyte adsorbed by the fibre over a period of time.

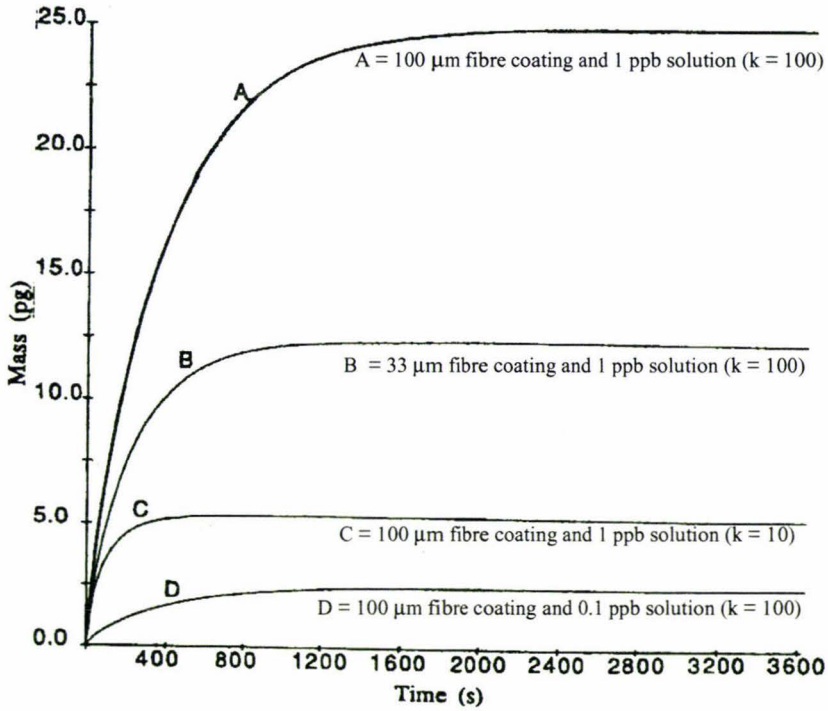


Figure 2.2: Mass Adsorbed by 1-cm-long, 33 µm and 100 µm PDMS Fibres [A - D are variables which alter the adsorption time profiles] (Louch *et al.* 1992).

With semi-volatile compounds however, it takes longer to reach equilibrium and therefore, longer to reach the turning point on the adsorption time profile. It is general practice to use an extraction time from the adsorption time profile at any time after the turning point of the curve. This is where maximum adsorption on the fibre has occurred and is therefore, the optimum extraction time for that particular analyte.

For volatile compounds, the release of analytes into the headspace is easier. The mass transfer of volatile compounds from the headspace to the fibre is fast because of their large diffusion coefficients in the gas phase. Hence, volatile compounds transfer more efficiently from aqueous sample phase to headspace to fibre coating than from water directly to fibre coating. With an efficient fibre coating however, HS-SPME can be used to extract both volatile and semi-volatile compounds from various sample matrices. The analytes tend to vaporize when they separate from the sample matrix, thereby increasing the distribution coefficient and also shortening the extraction times. For semi-volatile compounds, the lower volatility and relatively large molecular size reduces vaporization into the headspace to an extent and therefore, the mass transfer from the sample matrix to the headspace is slowed. This may result in longer extraction times as the distribution

coefficients are lowered. In the case of semi- to non-volatile compounds, the sample matrix adsorbs the analytes more strongly than the extracting medium (the fibre coating) and this results in long extraction times.

Louch *et al.* (1992) suggested that if the optimum extraction time was too long, a shorter adsorption time (that is, before the turning point on the profile) could be used as long as the extraction conditions were kept constant throughout the experiment. To ensure repeatability, they also recommended that there be no carry-over of the analytes from one GC run to the next. This can easily be done by desorbing the fibre for a second time and running the GC again, checking for any residual analytes in the gas chromatogram.

Ng *et al.* (1996) found that 2 minutes was sufficient to completely desorb the fibre of any analytes. It was necessary to cool the fibre back down to room temperature before the next extraction was carried out because a hot fibre is less efficient at extracting analytes (Zhang *et al.*, 1994).

The extraction time can be shortened by modifying the sample matrix, which can be carried out in several ways. The sample matrix can be stirred. This speeds up the mass transfer of analytes from the matrix to the headspace (Ng *et al.*, 1996). Stirring the sample matrix has been shown to enhance the convection in the headspace above the sample and the aqueous phase of the sample. This increases the adsorption of analytes onto the fibre coating as the partitioning of the analytes between the phases is greatly increased. In a stirred sample, the mass transfer of volatile and semi-volatile compounds from the headspace to the fibre coating is fast compared to static samples. According to Arthur *et al.* (1992), in a static aqueous sample matrix, the equilibrium between the phases is eventually reached, it just takes longer than stirred samples. For example, it took 10 minutes for benzene to equilibrate in a static solution but only 2 minutes when stirred. Hence, they confirmed that any change in experimental conditions that affects the distribution constants and adsorption rates also influences the amount of analyte adsorbed onto the fibre.

Agitation efficiency was found to be especially important for analytes with high coating/ water distribution constants (Louch *et al.*, 1992). If the agitation was perfect, then the thin, unstirred layer of water around the fibre would be negligible, resulting in short extraction

times even for compounds with higher molecular weights. Analytes with high coating/water distribution constants did not need thick fibre coatings to achieve good sensitivities when efficient mixing of the sample occurred. A thin coating could be used to decrease extraction times even further while retaining the required sensitivity.

Other methods used to improve agitation were to spray the aqueous solution into a fine mist before sampling. The drops of mist improved the mass transfer rate of analyte from the sample matrix to the fibre coating because of increased surface area to volume ratio and convection in the vapour phase. Alternatively, a smaller headspace volume above the sample would be appropriate, thus decreasing equilibration and extraction times but this would mean changing the dimensions of the SPME syringe and fibre.

Motlagh and Pawliszyn (1993) found extraction times using SPME from static solutions were unacceptably high. They investigated three agitation techniques to reduce the extraction times; magnetic stirring, intrusive mixing, and sonication. They found magnetic stirring to be the least expensive, cleanest and most convenient method to use. At maximum stirring speed, there were no problems with sample heating but the sample matrix structure was destroyed. With intrusive mixing, they found that at high mixing speeds, there was significant sample heating, which resulted in the fibre losing its ability to adsorb volatiles. Sonication on the other hand, was found to be a most effective mixing technique but it also resulted in temperature increases. A cooling jacket overcame this problem.

Heating the sample sped up sample extraction times, by releasing more analytes into the headspace from the matrix (Field *et al.*, 1996). Louch *et al.* (1992) found that heating the sample increased the vapour pressure, thereby releasing the less volatile organic compounds into the headspace above the sample matrix. However, at higher sample temperatures, the fibre coating starts to lose its ability to adsorb analytes. This slows down the mass transfer of compounds from the sample matrix to the fibre coating and extends the extraction period. According to Zhang and Pawliszyn (1995, cited in Vergnais *et al.*, 1998), heating provides some of the sample molecules with enough energy to overcome the interactions that tie them to the matrix. As the adsorption of analytes to the fibre coating is an exothermic process, heating the sample matrix can have an adverse effect on the

adsorption properties of the fibre because the partition coefficient decreases. This extends the extraction period and may result in non-representative results. There is usually an optimum extraction temperature for the SPME process where maximum analyte mass transfer from sample matrix to fibre occurs. The SPME device can be modified to maintain a low coating temperature while the sample is heated up to 250°C, for example, by using liquid carbon dioxide (CO_2) as a coolant (Zhang *et al.*, 1994). In such cases, the sensitivities improve and quantitative extraction may follow.

Another method for increasing the extraction rate is to add salts ('salting out' effect) such as sodium chloride (NaCl) or sodium sulphate (Na_2SO_4) to the sample matrix (Steffen and Pawliszyn, 1996). The ionic strength of the matrix increases, which increases the partitioning of the polar organic compounds (but not the ions) onto the fibre coating (Zhang *et al.*, 1994). As the analytes in their neutral form are more efficiently extracted by non-ionic polymeric coatings, the pH of the aqueous sample must also be adjusted to prevent ionisation of the analyte. As stated earlier, when the sample matrix adsorbs analytes more strongly than the extracting medium, extraction of analytes by SPME is inefficient and results in poor sensitivity. Hence, the sample matrix properties can have a large effect on the extraction of analytes by the SPME fibre.

Derivatization can also be used to reduce the polarity of compounds such as phenols and carboxylic acids, thereby increasing their coating/water partition coefficients and improving the extraction time and peak separation. In SPME, polar analytes can be derivatized in their aqueous matrix, for example, phenols can be converted to their acetate derivatives. Alternatively, the fibre coating can be doped with an appropriate derivatizing agent so that during extraction, the analytes are derivatized into compounds that are more easily adsorbed by the fibre. This results in shorter extraction times, and potentially increases the sensitivity of the method.

Field *et al.* (1996) found that the fibre thickness contributed significantly to the time required to reach equilibrium. When comparing a 30 μm with a 100 μm PDMS fibre, they found the extraction profiles were very similar except that the 30 μm fibre took a lot less time to reach equilibrium. However, the peak areas were 10-20 times higher for the 100 μm fibre when compared with that obtained for the 30 μm fibre. This was because the

thicker fibre coating adsorbed more of the analytes onto it. Hence, the authors used the 100 μm PDMS-coated fibre for more effective analyte extraction, together with greater sensitivity.

Because both the sample matrix and fibre coating are competing for the analytes, the affinity of the fibre coating for the target analytes is critical in SPME sampling. The stationary phase on the SPME fibre plays a key role in determining the signal intensity. Non-polar compounds are likely to be extracted more efficiently using non-polar film coatings, while polar compounds will be more easily extracted using a polar coating. However, for a polar coating to extract polar compounds from water, it must have a much stronger affinity for the analyte than the water does. For example, PDMS, a non-polar coating, extracts non-polar compounds such as benzene, toluene, and other non-polar hydrocarbons easily. It cannot however extract polar organic compounds such as phenols. The polar PA coating on the other hand, extracts phenols and other polar compounds efficiently but it cannot be used to extract non-polar compounds. Zhang and Pawliszyn (1993) commented that the selective adsorption of analytes by the fibre coating prevents oxygen and moisture from getting into the GC column, which is an advantage in the upkeep of the equipment and the column. The single step SPME method therefore, provides major advantages over previous solvent-less sampling techniques.

Clark and Bunch (1997) compared various fibre coatings with a range of polarities while investigating the flavour additives and natural flavours in tobacco. Fibre exposure times providing the highest signal-to-noise ratio were determined for an 85 μm polar PA fibre, a 65 μm carbowax/divinylbenzene, CW/DVB, (intermediate polar fibre), 65 μm PDMS/DVB fibre (intermediate non-polar fibre) and a 100 μm PDMS non-polar fibre. The polar fibres were more appropriate in this study because most of the tobacco headspace volatiles were polar. The PDMS fibre had a strong affinity for non-polar analytes, which made up the natural components present in tobacco. The authors found the 65 μm CW/DVB fibre to be most useful in their research as the flavour additives in tobacco were easily identified and there was less interference from the tobacco matrix components when using this fibre coating.

In the study of Chin *et al.* (1996), samples of cheese were analysed for their flavour volatiles using HS-SPME GC. Both the 100 μm non-polar PDMS and 85 μm polar PA fibres were used in this investigation. Based on the peak areas, the authors found that the polar PA fibre adsorbed the polar analytes (mostly fatty acids) almost 3 to 10 times more than the non-polar PDMS fibre. However, the PA fibre was not more effective than the PDMS fibre in extracting acetic acid. In some cases the PDMS fibre showed a higher extent of acetic acid adsorption compared with the PA fibre. Similar results were found in the study by Yang and Peppard (1994) where acetic acid was efficiently extracted from an aqueous solution using the PDMS fibre.

Song *et al.* (1997) used the 100 μm PDMS fibre and HS-SPME GC to look at apple flavour volatiles. They found that the PDMS fibre favoured the adsorption of larger, more lipophilic compounds such as acetone, esters and ketones. The authors also added that the type of fibre used could be tailored to fit the needs of a particular research thrust, that is, polar volatiles could be better extracted using a polar film coating and vice versa with non-polar volatiles. Thick film fibre coatings were found to be best for extracting semi-volatile compounds (low partition coefficients), while thin film fibre coatings were adequate for extracting very volatile compounds (high partition coefficients). They also observed that various volatiles took different times to reach equilibrium. Hence, they concluded that the kinetic behaviour of the adsorption process, which differs according to the type of analyte, is also a factor that must be determined before any work is carried out in this area.

Elmore *et al.* (1997) compared two headspace techniques - the DH method and HS-SPME method. They used the 85 μm PA and 100 μm PDMS fibres to investigate the volatile components in cola-flavoured drinks. They found that the PDMS fibre was more efficient at extracting a combination of medium to non-polar compounds whereas the PA fibre did not extract as many compounds, but did extract the very polar benzoic acid. The number of artefact peaks was greater in both SPME extracts when compared with the DH extract, but this may have been due to insufficient conditioning of the fibres. Overall, the PDMS fibre was found to give satisfactory results. The fibres did extract the more semi- to non-volatile compounds whereas the DH method extracted the more volatile compounds only.

Stevenson and Chen (1997) used the 100 μm PDMS fibre to look at wet and dry skim milk powders by HS-SPME, while Chin *et al.* (1996) used both the PA and PDMS fibres to study cheese volatiles.

HS-SPME has been used to investigate the flavours in foods and beverages. Page and Lacroix (1993) used HS-SPME to analyse 33 halogenated volatile contaminants in model aqueous solutions and in foods. The authors found HS-SPME to show a greater response for the less volatile analytes than those of greater volatility. The SH technique, in comparison, was found to be more sensitive toward the more volatile components. Hence, SH (direct injection) gas sampling and HS-SPME may be considered complementary headspace sampling procedures.

Page and Lacroix (1993) also found that when HS-SPME was used to determine volatiles in food, increased quantities of lipid material markedly reduced the sensitivity of the method, which was especially apparent for the least volatile analytes. The authors used the standard addition method (SAM) to quantify the halogenated volatiles in foods. They suggested that the HS-SPME GC technique would also be applicable to volatile non-halogenated analytes in complex matrices like food. They found the HS-SPME method simple, inexpensive and robust to use routinely.

2.7 Headspace Gas Chromatography of Yoghurts and Other Dairy Products

HS-GC has been used for decades in the dairy industry to determine the aroma and flavour of milk and fermented milk products such as buttermilk, yoghurts, and cheese (Imhof and Bosset, 1994; Marsili, 1981; McGregor and White, 1987; Ulberth, 1991; and Horwood, 1989). Such VOCs or flavours are often used as indicators of the quality of the milk products.

Jennings and Filsoof (1977) compared six headspace sampling methods to analyse a mixture of ten compounds in milk. These compounds varied in volatility and functional groups. The methods compared were:

- direct injection of the liquid;
- SH analysis of the liquid;
- headspace over a 100 ppm aqueous solution;

- SH analysis of a 100 ppm sodium chloride solution ("salting-out");
- DH analysis using a Tenax and Porapak Q trap; and
- a distillation-extraction procedure.

Their results indicated that no single sampling procedure was totally satisfactory. They concluded that the choice of sampling method to extract the volatile and non-volatile compounds depended on the sample composition and the analytes of interest. It was suggested that the best results could be obtained by using multiple sampling methods to quantitate and evaluate the sample composition.

De Haast *et al.* (1978) found that HS-GC and the use of a glass capillary column for separation was the best method for accurate quantification of aroma components in fermented dairy products such as yoghurt. Sample preparation time in HS-GC was also shortened to an absolute minimum and the results of such an analysis reflected the "true" equilibrium concentrations of the aromatic volatiles present in the headspace. Palo and Ilkova (1970) found that packed columns, as opposed to capillary columns, were prone to artefacts suffered from residue formation inside the column packing arising from non-volatile sample components.

De Haast *et al.* (1978) used the SH method to compare recoveries of VOCs from aqueous solutions, non-coagulated milk, whey, and coagulated milk (curd). By taking a sample directly from the headspace, it was found that the recoveries, expressed as peak areas, were of a similar order of magnitude for both milk and coagulated milk. From this result, it was suggested that both milk and coagulated milk had similar matrices and that these matrices had similar effects on the VOCs present in the headspace. The authors looked at nine volatiles (acetaldehyde, acetone, ethanol, dimethylsulphide, isopropanol, diacetyl, acetic acid and *n*-butanol) usually present in yoghurt and cultured milks. Headspace vapours of cultured milks were sampled at 60°C, 80°C and 95°C. They found that the samples at 95°C yielded the largest peak areas when analysed by gas chromatography. This was expected as the increase in temperature should increase the volatility of the compounds, increasing the partition coefficients of the analytes to give greater volatile concentrations in the headspace.

Ulberth (1991) compared the relative sensitivities of a 1 μL aqueous sample injection and headspace injection. Water- and milk-based samples containing the primary volatile compounds found in yoghurts at a 5 ppm level were analysed using these two techniques. The headspace method was found to have a 2- 6 fold increase in sensitivity for acetaldehyde, acetone, diacetyl, and 2-butanone relative to the liquid sampling. However, ethanol and propanol (headspace analysis) had lower sensitivities compared to the liquid sampling technique. Acetoin, which is produced from citric acid fermentation (Tamime and Deeth, 1980), was not detected in the headspace gas. This was probably because it is not as volatile (BP ~ 146°C) as the other chemical compounds found in yoghurt and it has a very small partition coefficient. Similar results were found by Marsili (1981). Ulberth's report concluded that SH (direct injection) analysis was an accurate and sensitive procedure for routine determination of the lower boiling yoghurt volatiles.

Ulberth (1991) used the SH (direct injection) method to estimate the amount of acetaldehyde, ethanol, acetone, diacetyl, and 2-butanone in yoghurts with varying amounts of milkfat. Milk samples were spiked with the compounds being analysed and then fermented. These samples were used as reference samples. Yoghurt samples were made for VOC analysis and compared with the reference samples. Using linear regression, the VOCs present in yoghurts were quantified. The author showed that fat content did not significantly affect the amount of VOCs present in milk and yoghurts. Both De Haast *et al.*'s (1978) and Ulberth's (1991) findings suggested that eventual differences in matrices between milk and fermented milks, do not affect the volatiles and headspace analysis, as they have similar matrices.

Marsili (1981) used the SH (direct injection) method to measure changes in acetaldehyde, acetone, ethanol, and diacetyl concentrations in the headspace above a sample of cultured buttermilk. The aroma and flavour of good quality cultured buttermilk was attributed to a balance of organic acids and VOCs produced as metabolites during fermentation. In Marsili's study, headspace samples were taken at the beginning, middle and at the end of fermentation. Acetaldehyde, ethanol, and diacetyl were identified using the standard addition method (SAM) and their concentrations were found to increase during the fermentation process. Acetoin was also found to increase during fermentation but this could not be analysed directly by HS-GC due to the high boiling point of acetoin. Instead,

acetoin had to be re-oxidised to diacetyl before analysis. Marsili (1981) found that little or no sample preparation was required for the SH method and that it was straightforward. The chromatographic method was also sensitive, specific and versatile.

Laye *et al.* (1993) found 23 volatile components present in yoghurt headspace samples. All the yoghurts contained seven major flavour volatiles: acetaldehyde, heptane, acetone, diacetyl, benzothiole, acetoin, and 2-butanone. A ratio of 1:1 acetaldehyde:diacetyl was reported as providing a typical yoghurt flavour (Bottazzi and Dellaglio, 1967) whereas a ratio of 2.8:1 of acetaldehyde:acetone resulted in a "full" yoghurt flavour. Changes in concentrations of these and other flavour-related compounds during storage were considered to be due to reactions that resulted in their formation or conversion to other compounds by bacterial metabolic enzymes. Their losses were thought to be due to volatilisation. The volatile compounds, acetaldehyde and heptane decreased in concentration during refrigerated storage while the other compounds did not.

Robbiani *et al.* (1985, cited in Badings, 1991) reported the analysis of an off-flavour in yoghurt described as "metallic", "disinfectant-like", and "lead-like". In their storage trial, the yoghurt samples showed a decrease in oxygen (O_2) and an increase in carbon dioxide (CO_2) concentration, which correlated with the perceived concentration of the off-flavour. From this, it was evident that organisms such as yeasts and moulds were growing in the samples. The off-flavour compound was identified as 1,3-pentadiene, which is known to be a degradation product of sorbic acid (Tamime and Deeth, 1980).

Jenq *et al.* (1988) used headspace gas analysis to determine the composition of volatile aldehydes in pasteurised milk, which had been exposed to copper ions, sunlight, and fluorescent light (oxidative degradation processes). The increase in aldehydes such as *n*-hexanal, acetaldehyde and *n*-pentanal in the milk, was an indication of off-flavours being present. Standard curves were used to quantify the amount of volatiles present in the milk. An aliquot was extracted from each sample headspace after the VOCs were concentrated by steam-distillation and placed in 5 mL vials. This sampling technique was found to produce quick and accurate results.

Imhof and Bosset (1994) used the DH method to identify and quantify the VOCs in pasteurised milk and fermented milk products using the SAM. Selected VOCs were added at various concentrations to pasteurised milk as references (spiking) and analysed by GC-MS. Using linear regression, the VOC concentrations in milk were quantified. The SAM was found to be a useful but time-consuming tool to quantify flavour volatiles of milk and related dairy products. Although lengthy, the DH method provided accurate results.

Mulligan and McCauley (1995) investigated the performance of a DH sampler coupled to a narrow-core capillary GC-MS. The authors found that the automated technique was accurate in identifying and quantitating VOCs in pharmaceuticals in the ppb range. The liquid sample analysed was confined in a 9 mL vial and equilibrated at a fixed temperature. The sample volatiles were purged using helium and trapped on a pre-column. The pre-column was heated in order to re-volatilise the analytes adsorbed, and chromatography was initiated. This effective technique was automated. Such a technique should be applicable to the analysis of flavours and fragrances.

In a study done by Lloyd *et al.* (1980), the DH method was used to assess the effect of adding a yoghurt culture to cheese during cheddar cheese manufacture. The gas chromatograms that resulted suggested that the total amount of volatiles such as diacetyl, acetoin, acetic acid, propan-1-ol and 3-methylbutanol was greater in the cheddar cheese inoculated with the yoghurt culture. This suggested that the yoghurt culture had fermented the cheddar cheese further by producing the above-mentioned chemical compounds and causing the cheeses to mature early. These inoculated cheeses were found to have a modified cheddar cheese flavour.

Horwood (1989) used the DH method to analyse the headspace volatiles present above a sample of finely grated cheddar cheese. The compounds were separated and identified using GC-MS. The method was used to determine the quality of the cheese and ensure that excessive butyric acid was not present in the headspace, as this signified an off-flavour. The flavour in cheddar cheese was also determined by DH analysis. The headspace method proved to be useful in assessing the quality of yoghurts by identifying and quantifying certain off-flavours.

These examples illustrate the use of different headspace methods (DH, SH and HS-SPME) in the analysis of volatiles present in a variety of cultured dairy products including yoghurt and cheese. Headspace sampling (SH and DH) has been used to analyse the VOCs present in other foods and beverages too. Both sampling techniques have been found to be versatile and efficient in numerous studies. For example, Paik and Venables (1991) used a simple HS-GC method to determine the change in concentration of VOCs present in orange juice during storage. Jones *et al.* (1986) developed a method for rapid analysis of total acetaldehyde in wine. The method involved the chemical conversion of acetaldehyde acetals and bisulphite addition products to free aldehydes. The acetaldehyde was then quantified using the SH method with high precision in the ppm range.

2.8 Effects of Storage Conditions on Volatile Concentrations in Yoghurts

Yoghurt is a perishable fermented dairy product, which has a shelf life of up to 3 weeks under refrigeration depending on the hygiene standards observed during manufacturing and packaging. Refrigeration usually occurs directly after the product has reached the required acidity (pH ~4.6). The main reason for the cooling is to reduce the metabolic activity of the starter culture, which controls the acidity of the yoghurt product (Tamime and Deeth, 1980). The starter cultures can be limited in their activity by reducing the temperature to between 10°C and 15°C directly after the fermentation step. In industrial situations, the yoghurts are cooled down to below 20°C and then packaged, before finally being cooled to 5°C or less.

Imhof and Bosset (1994) used the DH method to analyse various flavours in yoghurts two weeks after preparation. The authors commented that flavour generation occurred mainly during the fermentation step in yoghurt manufacture and that the flavours do not change significantly during the period of refrigeration at 4°C.

An important consideration when seeking specific information about the changes in volatile concentrations in yoghurts over a period of time is to investigate the changes that occur in the population of the starter cultures over that time period. Davis *et al.* (1970, cited in Hamann and Marth, 1984) found few changes in the viable counts of *S. thermophilus* and *L. bulgaricus* in yoghurt stored at 5°C over a 28-day period. When stored at 15°C however, the viable counts of starter cultures dropped dramatically after 14 days. This was probably

due to higher metabolic rates in the bacterial culture stored at 15°C, which resulted in further lactic acid production and lower pH.

From McGregor and White's (1987) results, it was evident that storage time and storage temperatures both have varying effects on the analyte concentrations present in the headspace above yoghurts. The authors carried out a study to determine the effect of sweeteners on the acceptability of low fat, plain and Swiss-style yoghurts. One of their product evaluations involved the analysis and quantification of three volatile flavour compounds (acetaldehyde, acetone and diacetyl) using SH sampling. The yoghurts were stored at 4°C and 10°C for a 24-day period. McGregor and White looked at the storage time and storage temperature variables independently of each other. No significant differences were found for the diacetyl concentrations between the two storage temperatures. Acetone and acetaldehyde concentrations were lower at 10°C than at 4°C. There was no significant difference in total bacteria or lactobacilli counts between the two temperatures but, yeast and mould counts were significantly higher at the 10°C temperature towards the end of the 24 days. For the storage time trial at 4°C, there was no significant difference in acetone levels. Diacetyl levels increased while acetaldehyde levels decreased over the storage period. The yeast and mould counts increased significantly over the time period. There was a significant decrease in the total viable bacteria and lactobacilli count as well.

2.9 HS-SPME as a Potential Quality Control Tool for Yoghurts

The flavour qualities of yoghurts and related products are highly appreciated by consumers around the world. As mentioned earlier, the main volatile compounds in yoghurts known to have an impact on the overall flavour are acetaldehyde, acetone, 2-butanone, ethanol, diacetyl and acetoin (Kneifel *et al.*, 1992). Sensory testing by an expert panel is usually used to evaluate the flavour and texture properties of cultured dairy products. Because of the time and effort required to operate a trained panel, researchers are constantly trying to find chemical and physical methods that can replace sensory testing. To achieve this, the data obtained using the chemical or physical methods have to be correlated with sensory data.

There are various benefits for such correlations being made. At present, most quality control work in the yoghurt industry utilizes a trained sensory panel to evaluate the various products prior to sale. This process can be very expensive and tedious. Through correct correlation of analytical and sensory data, the analytical method may be able to replace or complement sensory analysis. The headspace technique is a more objective analytical tool than the subjective sensory method where there is a greater chance of human error. The analytical method may be quicker, which could mean that the product reaches the market earlier. It should be noted that the correlational data cannot be applied to other situations unless the products in question undergo the same processing with similar environmental and storage conditions to those applied during the original correlational study.

As described in previous sections, HS-GC is an analytical method that has been used extensively in yoghurt flavour analysis (Ulberth, 1991; and Viani and Horman, 1973). Yoghurt volatiles have been quantified using the SH and DH methods (Ulberth, 1991; and De Haast *et al.*, 1978). HS-SPME has been used to analyse volatiles present in milk and fermented products such as cheese (Chin *et al.*, 1994; and Stevenson and Chen, 1997) but no work has been done, to date, on yoghurts using this HS-SPME technique.

Little work has been done in the area of correlating sensory with HS-GC data for yoghurts and other dairy products. However, complete sensory evaluation of yoghurt has been carried out with respect to flavour and texture defects (McGill, 1983; Richter, 1979; and Tamime *et al.*, 1987, cited in Rohm *et al.*, 1994). Consumer and descriptive panels have been used to assess commercial, flavoured and plain yoghurts (Barnes *et al.*, 1991 and Harper *et al.*, 1991, cited in Rohm *et al.*, 1994). Muir and Harper (1992, cited in Rohm *et al.*, 1994) developed a vocabulary for descriptive analysis (DA) of commercial fermented milks and then related sensory properties to overall acceptability. Stone and Sidel (1985, cited in Rohm *et al.*, 1994) developed Quantitative Descriptive Analysis (QDA) where sensory characteristics of food materials were described in mathematical terms.

2.9.1 Correlation Studies of Sensory with Analytical or Instrumental Data

Quality control in the past was largely achieved through sensory evaluations, supported by microbiological analysis. The determination of certain chemical-analytical indices, which were indicative of certain forms of deterioration, such as oxidation and lipolysis, have also

been achieved. These methods are useful but a more direct, wholly analytical approach is needed to determine flavour quality by identifying desirable and undesirable flavours in foods. To do this, the analyst has to devise an analytical method, which pinpoints the chemical compounds responsible for a perceived sensation. Such an analytical process consists of several steps, which include separation and isolation of the analyte of interest from a sample matrix, quantification of the analyte concentration, and data analysis. Each of these steps is critical for obtaining accurate results.

While analytical data can provide useful information about these flavour compounds, sensory data can provide information on the importance of these analytical measurements in flavour research. What the flavour scientist wants to know from the correlation between analytical and sensory data, is the cause-effect relationship between a group of components and the sensory properties of a product. The chemical compounds that are most related to sensory evaluation are then detected and identified using pattern recognition (a sensory analysis technique) and GC-MS methods, respectively.

Relating sensory data with instrumental data usually involves modelling large and complex data matrices, which are difficult to handle and interpret. Multivariate statistical analyses are useful in this regard. Traditionally, these correlations considered only one variable at a time, or the combined effects of a limited number of variables on a subjective response. Early flavour research concentrated on how the instrumental measurements co-varied with overall liking but did not attempt to predict every subjective response (Gaafar, 1992).

According to O'Mahony (1986), Y may represent a block of sensory variables, and X, a block of instrumental variables measured for objects in statistical analyses. In order to relate these two blocks of variables, statistical methods are needed which search for a few "between-block" factors or tendencies of variation, which are common to both blocks.

An ideal model should be able to:

- handle many X and Y variables simultaneously;
- handle small sample sets;
- should allow for some missing values and random noise in X and Y;
- should give warnings of outliers and give estimated parameters that are easy to interpret; and

- it should have predictive ability (X-Y) when required.

This list is based on the assumption that the experimental design for the data collection is optimized.

Regression analyses and equations were developed by Kramer (1976) to relate the objective physical properties of food products with consumer-perceived product quality. Currently, the scientific practice is to calculate either regression equations or correlations between two sets of measures, for example, physical measures and perceived texture. In some cases the objective may be to develop an equation that estimates the product's subjective sensory characteristics from instrumental measurements, while in other cases, the objective may be to find variables that correlate together. The equation developed may be a simple polynomial equation with only statistical explanatory powers or, on the other hand, it could be a 'model' of what is occurring at the interface of the physical stimulus and the sense organ. In either case, the equation conveys more information and is more useful to researchers than the calculation of simple and multiple correlations between sensory measurements and a set of instrumental measurements. It may also be possible to estimate the profile of sensory responses given the corresponding profile of instrumental measurements for a certain food product. There are two potential applications for such correlations:

- **Quality Control**

  Evaluating a product's quality and the consumer's response based on instrumental data. Ideally, instruments will eventually replace sensory evaluation. Given a set of instrumental measures, it may become possible to estimate consumer reactions at the time of food production.

- **Relating Expert Panels to Consumer Panels**

  For example, given a profile of expert panellists' ratings, then what ratings would be assigned by consumers? Ideally, instrumental data can be related to the expert panel data, which, in turn can be used as a guide to what the "consumer" data would be.

Analytical-sensory correlations are important for the advancement of flavour research and quality control methods. Analytical data can provide information on the concentration of any flavour component present in a food product but without sensory data, the importance of the analytical measurements cannot be established. Not only are the senses, especially the sense of smell, more sensitive than most instruments, but sensory analysis is also the only measure of the results obtained from them. It should therefore, be the overriding factor when deciding on the quality of a food product. However, by combining sensory and analytical data, many problems important to product development and quality control can be attacked. For example, what the acceptable variation in a given flavour compound is, or the types of compounds important in eliciting a given sensory response. Eventually, it is hoped that instruments will, in routine situations, make the evaluation of flavours and odours independent of human sensory assessment.

2.9.2 Statistical Analyses

Multivariate statistics refers to an assortment of descriptive and inferential techniques that have been developed to handle situations where sets of variables are involved either as predictors or as measures of performance. Researchers in all of the sciences, behavioural, biological, and physical, have abandoned their sole reliance on the classic, univariate statistical design as it has become clear that a given experimental manipulation will affect many different, but partially correlated aspects of the research. There are a whole host of multivariate techniques including multi-dimensional scaling, one-way multivariate analysis of variance, Pearson's correlation and bivariate regression, multiple correlation and regression, canonical correlation and discriminant analysis. The three statistical techniques used in the present study are described below:

- **Analysis of Variance (ANOVA)**

  This technique compares the means from several samples and tests whether they are all from the same population, or whether one or more of them is significantly different. When there are more than two levels of the independent variable, and therefore, more than two groups of subjects involved, the null hypothesis is tested by comparing a direct measure of the variance of the many sample means. An indirect estimate of how much these sample means would be expected to vary can then be made. ANOVA is used to investigate the effect of certain factors on some

response variables. ANOVA is the most common type of analysis performed on analytical data. This technique, while analysing one peak at a time, provides information about the variables and is often used as a preliminary step prior to other statistical techniques being carried out.

- **Binomial Test**

  This is a simple statistical test, which is generally used to determine whether more cases fall into one of two categories than into the other. In sensory analysis, the binomial test is used for the analysis of difference tests such as triangle tests or paired comparison tests.

- **Principal Component Analysis (PCA) and Factor Analysis (FA)**

  In PCA and FA, relationships within a single set of variables are investigated. Both these techniques can be used to reduce the dimensionality of the set of variables, that is, to describe the subjects in terms of their scores on a much smaller number of variables with minimal loss of information. If successful, the new variables (components and factors), can be considered as providing a description of the 'structure' of the original set of variables. FA and PCA have been used to reduce large numbers of sensory or instrumental variables to a smaller number of factors by looking at underlying common patterns between the variables. This type of correlation procedure is the first step in identifying which analytical variables are important in imparting a given sensory characteristic.

2.9.3 Correlation of Sensory With Analytical Data - Dairy Products

A lot of work in the area of correlating analytical with sensory data has been carried out with various food types such as nuts, rice, fruit and vegetables, but only a few studies have looked at dairy products. This review will focus only on correlation work carried out on dairy products, in particular, cultured dairy products.

2.9.3.1 Milk

Some researchers have tried to predict sensory qualities of a food product based on a single chemical measurement. For example, Greig and Manning (1983, cited in Vallejo-Cordoba and Nakai, 1994) found a good correlation between acetaldehyde concentration and

consumer acceptability in pasteurised milk. However, attempts to correlate a single chemical measurement to a sensory response have not always been successful. This is because flavour differences often relate to the balance between volatiles in a product rather than to a major change in one or two compounds. Therefore, complex correlation methods, usually a multivariate approach, are often used to extract information from complex GC peak profiles in relation to sensory data.

Vallejo-Cordoba and Nakai (1994) used Linear Discriminant Analysis (LDA) to classify the volatile compounds in pasteurised milk as determined by the DH method. LDA classified the milk into good, marginal, and poor quality milk and into fruity, rancid, and normal flavour groups according to sensory terms. The chemical compounds in the milk samples causing these off-flavours and reduction in shelf life were identified. Such a system has the potential for early detection of spoilage and for tracing defects in the milk. It also makes quality control easier and ensures that a standard milk product is produced every time.

Vallejo-Cordoba and Nakai's (1994) research led to the development of an analytical system for shelf-life prediction of pasteurised milk. Traditionally, the keeping quality of milk was assessed by bacterial counts and sensory evaluation, which do not always measure spoilage compounds known to cause off-flavours towards the end of the shelf life. They found that sensory evaluation remained the most useful means of assessing milk quality with the disadvantage of being a time-consuming and an expensive exercise. However, the authors went ahead and derived a system, which associated instrumental data obtained using the DH method for milk volatiles with the sensory responses obtained from flavour scores. PCA was used to correlate the sensory data with the DH data, and a model was derived. This model had the potential for application in the dairy industry where milk quality could be assessed using volatile detection. A good correlation between flavour quality and sensory evaluation was obtained and the technique could predict milk shelf life with an accuracy of $\pm$ 2 days. Also, the model enabled the objective evaluation (using the DH method) of unknown milk samples without the use of a sensory panel.

Jaddou *et al.* (1978) used spray distillation to collect heat-treated milk volatiles at room temperature and then DH sampling in conjunction with GC-MS to elute and identify various volatile compounds. A sensory panel was selected to smell and taste the milk

samples and to rate the overall acceptability of the milks. A good correlation was observed between total sulphur volatiles and the intensity of cabbagey off-flavours. However, it may well have been that the cabbagey note was the result of the interaction between sulphur-bearing compounds, especially hydrogen sulphide and methanethiol, and one or more of the carbonyls present in milk. Such a technique can only be used for quality checks on long-term storage of heated milks, where off-flavours develop. HS-GC may be used to distinguish these off-flavours instead of a sensory panel. Such an objective technique may be advantageous, especially since these staleness notes are well below consumer panellists' thresholds.

Christensen and Reineccius (1992) investigated the feasibility of using the SH method as an objective tool to measure milk flavour quality. They chose to look at heated milk off-flavours, which were caused by an increase in the concentrations of sulphur compounds such as hydrogen sulphide and dimethyl sulphide. In order to increase the sensitivity of the headspace method, several strategies were used such as "salting-out" and cryo-focussing during injections. The authors used a sulphur-specific detector to detect the sulphur volatiles in pasteurised skim milk. Milks that were heated to varying degrees were analysed and the GC results were related to the heated flavour as determined by a sensory panel. The sensory panel rated the milks according to the amount of cooked flavour that had developed. For skim milk, correlations for hydrogen sulphide and dimethyl sulphide were moderately strong (0.75 and 0.60, respectively). Correlations for whole milk ~~was~~ were weak (between 0 and 0.5) suggesting that the milk fat in the whole milk was a competing solvent for the flavour volatiles, thereby lowering their vapour pressure below detectable limits of the analytical system. Sulphur volatiles were found to be maximal after moderate heat treatment and then decrease or remain the same when a more severe heat treatment was applied. Hence, this may limit the method's usefulness in a "Quality Control" setting because a heated milk flavour is not a severe defect easily picked by consumers. However, much has been learnt from this study about the sulphur volatiles, which give rise to "cooked" and other off-flavours.

Leland *et al.* (1987) tried to determine whether a DH method could be used to distinguish different intensities of oxidized off-flavours in milk. A trained sensory panel also evaluated the samples. The authors used Stepwise Discriminant Analysis (SDA) to

separate the oxidized milk samples into different pre-defined intensities on the basis of headspace volatile profiles. Reliable classification (90%) of milk samples into four oxidized flavour intensity groups was achieved on the basis of combined information provided from only seven volatile components. A significant correlation (0.81) was found between the average oxidized flavour intensity and the sensory data. However, this aspect was not studied in detail, as the major purpose was to determine the feasibility of the instrumental approach using HS-GC with SDA. Even though the sensory data and instrumental data were reliable in themselves, fundamental differences existed between the two types of data. The headspace sampling closely approximated what the nose encountered. Also, GC is a separation process whereas olfaction is an integrative process. When relating GC profiles with sensory data using SDA, these differences must be bridged before useful correlation models are produced. This method however, is a good quality control technique as it identifies the objectionable flavours. It is a good research tool because it assesses the effects of processing or formulating on product flavour.

In a study done by Wellnitz-Ruen *et al.* (1982), ethyl butyrate and ethyl hexanoate were found to be primarily responsible for the fruity off-flavours in milk. The DH technique was used to optimize the recovery of these esters in whole milk where concentrations as low as 1 ppb could be identified, detected, and quantified. The headspace analytical threshold was compared with human sensitivity to fruitiness in milk by correlating the data obtained. The authors found the headspace technique capable of detecting and quantifying the very low ester concentrations whereas the human sensory system could only detect down to 5 ppb. Hence, this methodology is comparable to the sensitivity of the human sensory system. The authors concluded that this instrumental system was a feasible alternative to sensory detection of fruity off-flavours in milk.

From the research done to date, it is evident that correlating sensory data with instrumental GC data has many applications in the dairy industry but the most useful application has been one form or another of quality control.

2.9.3.2 Cheese

A variety of aromatic compounds contribute to the flavour and aroma of cheeses. It is generally considered that these flavour compounds reside mainly in the fat phase of the

cheese but work has been done where cheese has been distilled for flavours in both the aqueous and fat phases (Manning and Robinson, 1973). Little work has been done on finding any relationships that may exist between analytical and sensory data of cheeses.

Manning and Robinson used a reduced pressure distillation procedure to extract the volatile fractions from cheddar cheese. GC-MS was used to analyse and identify the various volatile compounds present in the distillate. A sniff-port adjacent to the GC detector was used by the panelists to detect and evaluate the odours that were being eluted simultaneously. The panelists agreed that the different fractions had significantly different odours and certain fractions had definite cheese flavours. Hydrogen sulphide, methanethiol, dimethyl sulphide, and diacetyl were among these cheese aromas. 2-butanone and 2-pentanone were not detected by the GC-MS, which suggested that the two compounds did not contribute significantly to the aroma of the cheese distillate. However, the two compounds were smelt in the effluent by the panelists suggesting they were in fact important to the overall aroma of cheese. This reinforced the fact that a sensory panel is important to instrumental analysis when picking odours and flavours of food products. The advantage of using instrumental analysis is that the individual chemical compounds making up the overall flavour are identified. With the sensory panel however, the overall aroma is detected and graded or quantified. By knowing the individual components that go in to make a specific flavour, the flavour scientist can combine the separate components in their different proportions and make up what is called the artificial nature-identical or synthetic flavours.

Nijssen *et al.* (1987) used the DH method with GC-MS to determine the cause of an off-flavour in blocks of packaged cheese. A sniffing panel was also used to evaluate the odours, which were described as onion-like and almond-like. The compound was identified as 2-methyl-2-pentanal, which was believed to originate from propanol. Again, the study shows how beneficial a sensory panel coordinated with chemical analysis can be.

Bosset and Gauch (1993) compared the concentration of volatile flavour compounds in six types of cheese using the DH method. They reached the conclusion that all of the cheeses contained similar volatiles but at different concentrations. Therefore, the flavour of these cheeses were found not to depend on any particular key component but rather, on a 'critical

balance' or a 'weighted concentration ratio' of all the components present. Considered individually, the compounds had very different smells and tastes, than when present together in the cheeses.

Urbach (1993) concluded that the flavour of cheddar cheese was highly elusive. The changes in volatile concentrations which occur during maturation reflect biochemical changes rather than being a direct reflection of flavour. It was suggested that without the sulphur compounds, there was no cheddar flavour. However, from the studies done in this area, no sulphur compound concentrations change with flavour. Therefore, the volatiles in the headspace are either due to the breakdown of various products or to some physiological interaction between the sulphur compounds and other chemical compounds, which produce the sensation of the odour and cheddar flavour.

2.9.3.3 Yoghurts

Acetaldehyde, present at 13-16 ppm contributes to good flavour quality in yoghurts (Badings and Neeter, 1980). GC techniques have been used for quality control in the dairy and yoghurt industry. Product spoilage was determined by measuring minor biochemical and flavour changes during storage. Gaafar (1992) looked at relating changes in volatile flavour compounds in Egyptian yoghurt to changes in the acceptability as judged by a taste panel. The SH (direct injection) sampling technique was used to collect the yoghurt volatiles. Calibration curves were set up so that acetaldehyde, diacetyl, acetoin, acetone, butanone, and acetic acid could be quantified. An untrained taste panel was used to assess the yoghurts by tasting and smelling the samples for acceptability and to describe any off-flavours. According to the GC results, all the volatile compounds, except for acetic acid, decreased in concentration during storage at 8°C. The acceptability of the yoghurt samples was found to rapidly decrease by Day 12. Off-flavours were described as sour and unclean by Day 10. Quantitatively, acetaldehyde was found in the headspace as the major volatile, contributing to the yoghurt flavour. This was originally suggested by Pette and Lolkema (1950, cited in Gaafar, 1992) and confirmed by Bottazzi and Vescovo (1969).

Gaafar (1992) also found that the decline in yoghurt acceptability was related to a decrease in acetaldehyde and diacetyl, and an increase in acetic acid concentrations. The author suggested that GC analysis of commercial natural yoghurts could easily be adapted to

quality control and shelf life studies. Gaafar's (1992) method offers rapid and reproducible results using a single HS-GC injection. Despite the advantages of correlating instrumental HS-GC sampling and sensory data, only a few reports have been found in the literature on fermented dairy products to date.

Different starter cultures influence the flavour and the texture of the final product (Ott *et al.*, 1999). As a result, a variety of starter microorganisms with specific properties, are available from the manufacturers. Kneifel *et al.* (1992) screened various commercially available yoghurt starter cultures for their flavour profiles and flavour intensities using sensory methods and HS-GC, respectively. Kneifel *et al.* found lower acetaldehyde concentrations (20.7 ppm) for a typical yoghurt than those claimed by Bottazzi and Vescovo (1969) [23.0-41.0 ppm for optimum yoghurt flavour]. Kneifel *et al.* argued that although acetaldehyde was the main yoghurt volatile, yoghurt and related products could not be classified according to this single component. It was then suggested that concentration ratios of distinct aroma compounds be used as an aid when differentiating between low- and strong-flavoured yoghurts. For example, an acetaldehyde:acetone ratio of 2.8:1 for a single strain yoghurt equates to a strong yoghurt flavour. The overall quality and therefore, the sensory properties of yoghurt, are also influenced by physical and chemical properties such as texture, acidity and proteolysis.

Ulberth and Kneifel (1992) then went on to classify the different starter cultures according to the flavour and sensory properties produced using a sophisticated computational procedure (cluster analysis). The calculations were based on data presented in their first study (Kneifel *et al.*, 1992). The concentrations of individual aroma compounds were regressed against flavour intensity scores from the sensory trials. The most pronounced relationship between these two variables was observed with acetaldehyde ($R = 0.435$ for yoghurts and 0.635 for yoghurt-related products). For both groups of products, significant correlation coefficients between acetaldehyde content and acidity were observed. As sensory impressions are known to be the result of a diverse array of stimuli, a multivariate method was chosen to classify the cultures according to their aroma properties. Five clusters were obtained from the classification of yoghurt cultures and four clusters from the yoghurt-related cultures. Each cluster had similar organoleptic properties. This grouping was recommended as a valuable tool for selecting starters for specific sensory.

The DH method was used by Laye *et al.* (1993) to separate and identify twenty-three plain yoghurt volatiles over a 12-day refrigerated storage period. An untrained sensory panel also evaluated the yoghurts on Day 6 for appearance, texture, flavour, aroma, and overall acceptability on a 9-point hedonic scale. The peak areas from the gas chromatogram gave an indication of the relative concentrations of the many volatile compounds present in the yoghurt headspace. Just as Gaafar (1992) had discovered, the authors also found a gradual decrease in certain volatile compounds and increases in acetic acid and lactic acid concentrations over time. Relatively small differences in concentrations of volatile organic compounds and organic acids picked up by the GC resulted in substantial differences in flavour, aroma and overall acceptability picked up by the sensory panel.

Rohm *et al.* (1994) used Quantitative Descriptive Analysis (QDA) to evaluate yoghurts produced by different starters in order to describe sensory properties and to sensorially characterise these starters. A score sheet was developed by a trained 10-member sensory panel, which included eight categories and a hedonic scale. This was used to evaluate the panelists' acceptability impressions of the yoghurts. Statistical analysis showed good performance by the panelists. Significant differences between the yoghurt products were found in each category for taste, mouthfeel, ropiness, flavour, acidity, total aroma, surface, viscosity, and overall acceptability. Several interrelationships between sensory categories, for example, 'flavour and aroma', and 'surface tension and mouthfeel' were found. By using multiple regression analysis, hedonic scores of overall acceptability of the yoghurts were found to be mainly determined by flavour (preferred) and ropiness (not preferred). From this study, it was obvious again, that sensory evaluation is an important tool for determining preferred or ideal flavour characteristics of a product.

According to Ott *et al.* (1997) who used the DH method, *L. bulgaricus* was identified as being mainly responsible for the production of aromatic compounds and the sharp acidity of the final yoghurt product. A fine balance between all the key aromatic compounds is what gives yoghurt its overall unique aroma. As the vapour phase odour is first perceived on opening a yoghurt pottle, its quality largely influences the panellists' preferences. This is because what is perceived as the response to an odour is mainly dependent on the volatile concentrations that are released from the food and how much reaches the olfactory sensors.

Ott *et al.* (2000) looked at the sensory properties of traditional acidic and mild, less acidic yoghurts. They used a trained panel and descriptive analysis to evaluate the yoghurts. The trained panelists had to first, evaluate the smell attributes of the yoghurts and then the appearance and texture with a spoon. Lastly, they had to sample the yoghurts in their mouths and rate them for flavour and texture attributes. The authors concluded that the intensity of yoghurt flavour perception was highly dependent on the acidity of the product. That is, the acidic aroma overrides the overall yoghurt aroma. The panel demonstrated an extreme sensitivity to the acidic smell of the yoghurts and therefore, their perception of acidity seemed to condition the perception of all the other attributes (texture and taste). They found important flavour differences between the two yoghurt types but these were mainly due to differences in the amount of acidity they perceived in the overall yoghurt aroma and not to the three impact volatiles (acetaldehyde, diacetyl and 2,3-pentanedione) being investigated. From this study, it was evident that acidity plays an important role in the flavour perception of yoghurt aroma and that it has a large impact on the consumers' perception of the yoghurt quality and their decision with regard to product acceptability.

In the area of correlation between sensory and instrumental responses, two things must be kept in mind. Firstly, when comparing reports of the correlation between sensory and GC data, many authors use a correlation coefficient (R). To be of good quality, R should be greater than 0.87 and nearing 1.00 (R^2>0.75). Secondly, the correlation equations generated from any correlation studies are only valid for particular samples with the particular panel used at the time.

The progress of research has brought us from the purely subjective impression of select individuals to a time when some have combined the statistically-oriented, multipanellist sensory panels with GC-MS evaluations to provide an in depth profile of flavour characteristics and flavour stability. None of this will change the basic problems the researcher faces in the labile nature of the chemical constituents to be measured.

2.10 Conclusion

In conclusion, it is apparent that the HS-SPME method in conjunction with GC, would be appropriate to use when identifying and quantifying yoghurt volatiles. The only HS-SPME work done to date in a related area (cheese) was very successful (Chin *et al.*,

1996) and therefore, the method should work well for yoghurts. Another potential application for the HS-SPME technique is in the area of quality control where the method would be to assess yoghurts based on the flavour analytes and to determine whether the yoghurts will be acceptable to the consumer. The advantage of such a technique would be that the HS-SPME method would replace time-consuming and expensive to run sensory analysis. This would result in a cheaper method for assessing the quality of yoghurts. However, in order to use such an objective analytical tool to assess the quality of yoghurts, there are many steps to take to ensure that the analytical method is measuring exactly what the consumer perceives. Hence, it is important that the analytical and sensory responses are correlated before the analytical tool can be used alone. This study investigates the steps taken to identify and quantify six yoghurt analytes in laboratory- and commercially-made yoghurts.

2.11 A Brief Outline of the Research Proposal for This Study

The present study was divided into three sections:

1. A sampling methodology for HS-SPME had to be developed. This involved determining the retention times (R_T) for each of the six analytes of interest. Next, two SPME fibres, namely, the 80 μm, polar PA fibre and the 100 μm, non-polar PDMS fibre, were compared for their extraction ability. Finally, the optimum volatile extraction (adsorption) time of the PDMS fibre was determined. This extraction time is the minimum exposure time required for the fibre coating to reach saturation point.

2. The second objective of the present study involved comparing the classical SH (direct injection) technique with the HS-SPME method. The present study looked at the concentrations of six aroma impact compounds namely, acetaldehyde, acetone, 2-butanone, ethanol, diacetyl and acetoin in natural, low fat (0.3%) and high fat (3.5%) yoghurts. The presence of the six analytes in plain and acidified (incubated/chilled) recombined low and high fat milks was investigated to determine whether these analytes were inherently present in milk. Natural, unsweetened yoghurts (low and high fat) were also made in the laboratory to compare the analyte concentrations in the milks versus the yoghurts. A storage trial followed where the yoghurts (low and high fat) were made using a standard method and stored at 2°C for a period of 24 days.

A similar storage trial was also run at 2°C and 10°C. The HS-SPME method was used to analyse and quantify the six analytes present in the yoghurts directly after the fermentation process and during both trials.

3. For the third and final part of this study, the HS-SPME method was used to determine the concentration levels of the six analytes in fourteen different commercial yoghurts. These included natural (sweetened and unsweetened) yoghurts with varying fat levels. PCA, a multivariate statistical method, was used to classify the yoghurts into groups depending on the analyte peak areas and to describe their relationships. For the second part of this objective, eight commercial yoghurts were selected from the initial fourteen yoghurts, which were visually different on the previous PCA plots. The yoghurts were presented to an untrained panel in the form of seven triangle tests carried over two days. The sensory study was carried out so as to determine whether the average consumer could pick the same differences in yoghurt aroma that the HS-SPME could pick up. This work provides a starting point for characterising the differences between yoghurts based on flavour volatiles, using a simple and efficient headspace technique and multivariate statistics.

CHAPTER THREE

MATERIALS AND METHODS.

3.1 General Materials and Equipment

- Anchor whole milk and skim (non-fat) milk powders were obtained from Foodtown Supermarket, Palmerston North branch.

- Freeze-dried Wiesby Joghurt 709 mother culture was purchased from Biolab Scientific Limited, Palmerston North.

- 4 mL glass vials with open-hole screw top lids were obtained from Alltech and butyl rubber resealable septa purchased from Chromspec Distributors (NZ) Ltd.

- 35 mL Lily portion cups with lids and 120 mL white plastic containers with lids were obtained from Carter Holt Harvey Distributors (Palmerston North) Ltd.

- Distilled, deionised water (DDI) was obtained from a MilliQ filter system in the laboratory. Henceforth, the term "water" refers to DDI.

- AnalaR grade acetaldehyde, diacetyl, acetoin and 2-butanone were obtained from BDH, Palmerston North. AnalaR grade concentrated lactic acid, ethanol, propan-2-ol and acetone were all obtained from Sigma. All chemicals were stored at 5°C. Once the bottles were opened they were stored under nitrogen and returned to the refrigerator.

- A Supelcowax 10 glass capillary column was purchased from Chromspec Distributors (NZ) Ltd. and used in the Gas Chromatograph GC 6000 Vega Series 2. The GC software package used was Data Acquisition, Plotting and Analysis (DAPA Chromatography System Version 1.43).

- The manual SPME syringe and coated fibres, 100 μm PDMS and 85 μm PA fibres, were purchased from Chromspec Distributors (NZ) Ltd.

- A 10 μL Hamilton gas-tight glass syringe was used to make the SH extractions and injections.

- A variety of equipment in the Institute of Food, Nutrition and Human Health (IFNHH) pilot plant were used extensively to make the natural, set yoghurts throughout this project (more details are given in Section 3.2).

- The holding tube attached to the Alfa Laval UHT system, described and used in Section 3.2, was borrowed from the New Zealand Dairy Research Institute (NZDRI), Palmerston North, during the experimental period.

- The nitrogen (oxygen-free, OFN), hydrogen and dry air gas cylinders, hooked up to the GC were supplied by BOC Gases (Palmerston North) Ltd.

- All equipment and containers were sterilised with Sanicol, purchased from Ecolab.

3.2 Yoghurt Preparation Method

The method for yoghurt preparation was adapted from the method used by Rover and van Veldhuizen (1995, cited in H. V. Bennett's Food Technology 4th year project, 1995). It was a batch process only suitable for small quantity samples. Rovers and van Veldhuizen's study on the shelf-life of recombined yoghurt found that adequate heat treatment of the milk could be achieved by heating to 91°C using UHT equipment and holding for 5 minutes in an insulated tube. Heat treatment of the milk in this study was carried out on an Alfa Laval UHT in the IFNHH pilot plant with an extended holding tube (Figure 3.1, page 62). The holding tube was constructed using 12 mm stainless steel (Grade 3.16) and was housed in a stainless steel box insulated with fiberglass. To achieve a holding time of 5 minutes, the UHT plant was run at a flow rate of 46 L/hr.

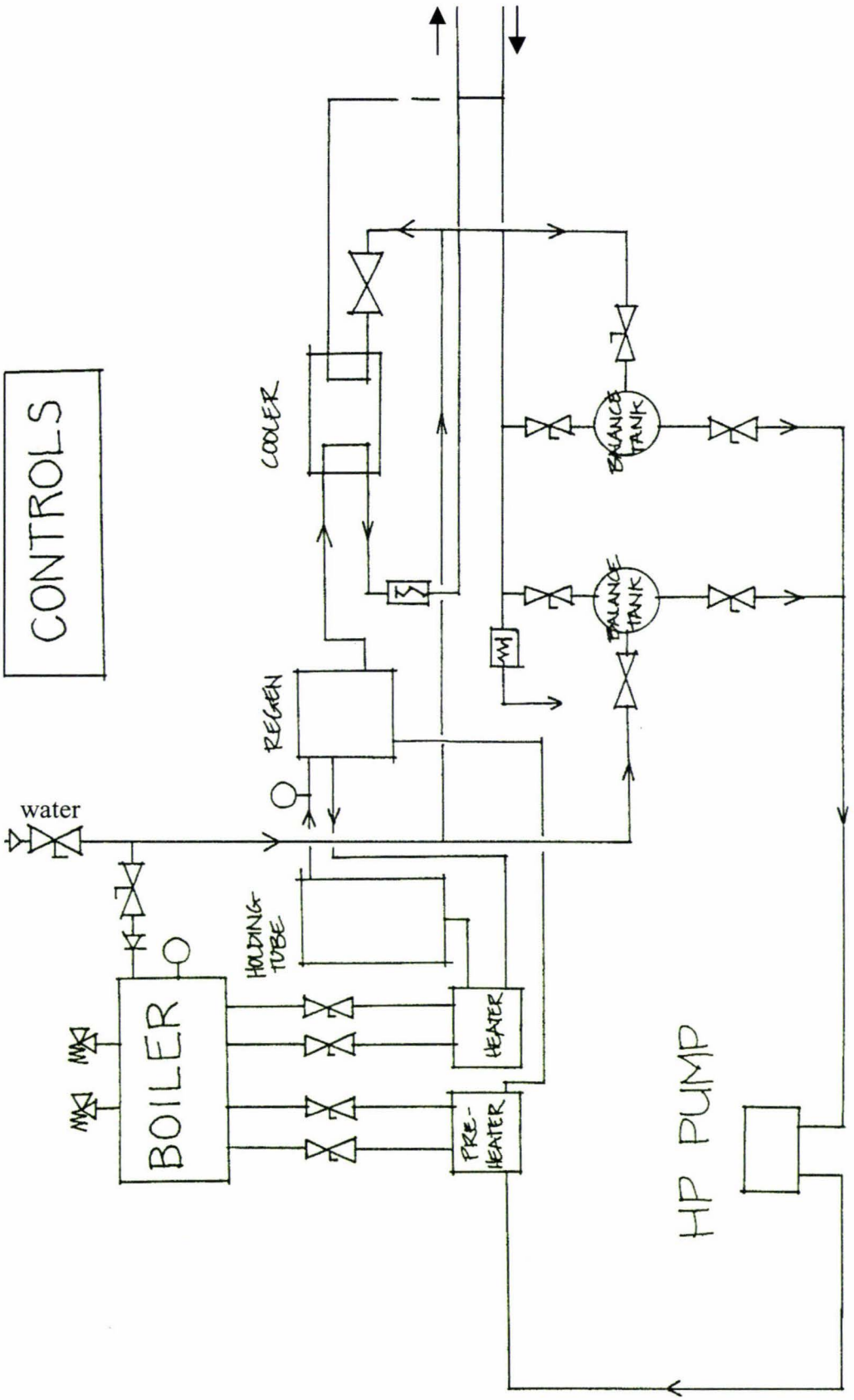


Figure 3.1: The Alfa Laval UHT Apparatus and Holding Tube.

The preparation of the yoghurt batches was carefully standardised. The yoghurt was formulated to a protein level of 4.5% and fat levels of 0.3% for low fat yoghurts and 3.5% for high fat yoghurts. A total solids non-fat level of ~12% was targeted. Simultaneous equations were used to calculate these formulations for the recombined yoghurts (Appendix I, page 214). A minimum 2 kg batch was made at any one time to account for any losses that occurred at the homogenisation and UHT-heating stages.

1. Mother culture preparation: The mother culture was prepared in 10% (w/w) recombined skim milk, which had been autoclaved at 15 psi for 12 minutes in medical flat bottles. Freeze-dried Wiesby mother culture was added aseptically to the sterile milk at 37°C at a rate of 250 mg per 60 mL (0.42%) milk. The inoculated milk was incubated at 37°C for 16 hours. At the end of this time, the inoculated milk was transferred to a refrigerator and held at 5°C. The mixture was discarded if not used within 4 hours.

2. Recombination and rehydration: Preweighed WMP and SMP were dispersed in accurately measured water and mixed thoroughly at high speed for 10 minutes using a Kenwood bowl mixer in the IFNHH pilot plant. The rehydrated milks were covered and allowed to stand for 1 hour at 5°C.

3. Removal of foam: The recombined milk was then placed in a rectangular container in a vacuum chamber (W. G. G. Curddon Ltd.), while the pressure was decreased to 90 kPa and held for a minimum of 5 minutes. The time under vacuum depended on the amount of foam that had formed during the mixing process.

4. Homogenization: The milk was then heated to 55°C in a waterbath set at 75°C and then homogenized using a two-stage Rannie homogenizer (200/30 psi).

5. Pasteurisation: Following this, the milk was then heated to 91°C in a Spiraflo UHT plant (Alfa Laval), held at 91°C for 5 minutes in an insulated holding tube and then rapidly cooled in the UHT plant to 20-25°C. The cooled milk exiting the UHT plant was collected in a sterile plastic keg.

6. Inoculation: The mother culture was added to the pasteurized milk in the sterile keg at a rate of 2% v/w of milk. The keg was then shaken to ensure thorough mixing and even distribution of the culture.

7. Sampling: Aliquots (2 mL) of each inoculated milk was pipetted into 4 mL glass vials as required and sealed. 15 mL of the same milk was also pipetted into the 35 mL Lily portion cups and sealed. These were used for monitoring the pH of the set yoghurts.

8. Incubation: The samples were then incubated at 40°C for about 5 1/2 hours or until the pHs of the yoghurts in the Lily portion cups dropped to ~4.6.

9. Refrigeration: At this point the yoghurt samples and Lily portion cups were transferred to the refrigerator where they were chilled at 5°C until required for HS-SPME analysis or pH measuring.

3.3 Gas Chromatography

A polar phase glass capillary column (Supelcowax 10 - 30 m x 0.32 mm, 0.25 µm bonded stationary phase) was used in the present study. The carrier gas used was oxygen-free nitrogen (OFN), while hydrogen and dry air were mixed at optimum proportions for the use of the flame ionisation detector (FID 40). The flow rates of the carrier and fuel gases were as follows:

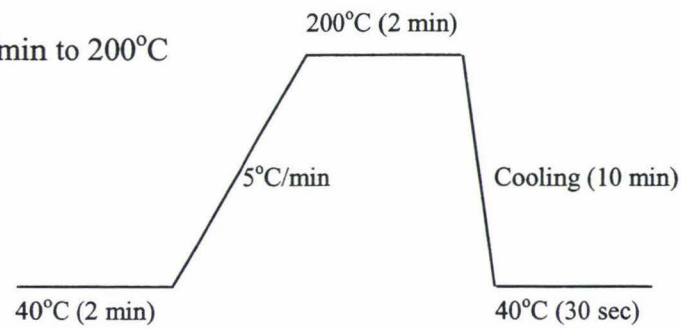
- Nitrogen (carrier gas) : carrier and make-up gases = 30 mL/min.
- Hydrogen (fuel) : 20 mL/min
- Dry Air : 300 mL/min
- Injector port temperature : 240°C
- Detector temperature : 250°C
- Maximum column temperature : 260°C

The splitless mode was used, as the concentrations of the volatiles being analysed were very low (0 - 100 ppm). This mode would allow the maximum volume of analyte to reach the detector from the injector port via the column. The column was conditioned overnight

before commencing a batch of GC experiments. This was done by heating the column slowly (5°C/ min ramp) to 255°C and holding it there for 1 hour and then cooling the column back down to 40°C. This cycle was carried out repeatedly throughout the night before the day of use, particularly if the GC had not been used for more than 2 weeks at a time.

The temperature programme used for GC analysis of yoghurt volatiles was:

- Temperature ramp at 5°C/min to 200°C
- 200°C for 2 minutes
- Cool back down to 40°C
- 40°C for 30 seconds
- Ready for sampling



3.4 Static Headspace (Direct Injection) Technique

A 10 µL Hamilton gas-tight glass syringe was used to extract 2.5 µL of the headspace above yoghurt, simulated yoghurt and the standard aqueous and milk solutions. The samples were left at room temperature for 10 minutes before sampling was carried out. It was necessary for the syringe needle plunger be in the 'closed' position when being pushed through the pre-pierced septum. This prevented any contamination of the headspace with the outside air in the sealed vial. The plunger was then 'pumped' three or four times again without introducing any outside air, before 2.5 µL from the headspace volume was taken and injected through the pre-pierced septum in the injector port.

3.5 Headspace Solid Phase Microextraction

For the main part of the study, the 100 µm, non-bonded PDMS fibre was used but in Chapter 4, the 100 µm PDMS fibre was compared with the 85 µm, partially cross-linked PA fibre for their extracting abilities. The SPME syringe was designed for manual injections only.

All new fibres were conditioned before use so as to prolong their extracting capabilities. Up to 50 or more injections were possible with each fibre after the conditioning treatment was applied. Conditioning for both types of fibres was carried out by heating the exposed fibre in the injector port for 1 hour at 250°C. The fibre was then retracted into the SPME syringe shell and the syringe removed from the GC. The fibres were left to cool down to room temperature on the laboratory bench before headspace extraction of yoghurt samples was carried out. The GC was left running for a further 30 minutes at 250°C in order to get the column "cleaned". This procedure got rid of any residual impurities in the column and had the added effect of conditioning the column simultaneously.

The following headspace extraction procedure was followed when analysing the yoghurt samples, standard simulated yoghurt samples and standard aqueous solutions for their headspace volatiles using the HS-SPME technique.

1. Just prior to headspace (SH and HS-SPME) sampling of the yoghurts, the septum of each glass vial was pierced using a drawing pin needle. This was done so as to avoid damaging the thin Hamilton syringe needle and the coated fibre housed inside the SPME syringe needle when they were pushed through the septum. The butyl rubber septum was specially bought for its resealing properties, which ensured the volatiles did not escape from the vials through the pre-pierced septum holes. Both syringe needles could then be pushed through the resealable septum, with reasonable ease, to a point just inside the septum, in the headspace above the sample.

2. At this point the SPME plunger was pushed down and the coated fibre exposed to the headspace. It was ensured that the exposed fibre was only in contact with the headspace and not touching the set yoghurt in any way. The same sampling requirements were used for the SH (direct injection) method.

3. According to the methodology in Chapter 5, the SPME fibre was left exposed to the headspace above the cultured or simulated yoghurts for 20 minutes, during which time, adsorption of volatiles onto the fibre took place.

4. When 20 minutes had elapsed and adsorption was completed, the fibre was retracted into the SPME syringe needle and removed from the glass vial.

5. The SPME syringe needle was then rapidly transferred to the injector port and the needle was pushed through the pre-pierced injector port septum. The SPME plunger was pushed down so as to re-expose the fibre. At this point, the fibre was left to desorb at 250°C for 5 minutes. As soon as desorption commenced, the GC run was started and the analyte peaks were eluted through the column, separated, detected and the data collected.

6. After 5 minutes of desorption, the fibre was retracted into the SPME syringe needle again and removed from the injector port and left to cool down.

After 20 minutes, the HS-SPME adsorption-desorption process (steps 1-6 above) was recommenced on the next sample to be analysed. This process was continued until all the yoghurt samples had been analysed.

3.6 Standard Solutions

3.6.1 1000 ppm Stock Solution

A 1000 ppm stock solution was made by measuring 100 mg of each analyte (i.e. acetaldehyde, acetone, 2-butanone, diacetyl, ethanol, and acetoin) into a 2 mL volumetric flask using the capillary technique (Bassette, 1984) and making it up with AnalaR grade methanol. Dissolving the analytes in this way resulted in a 50 000 ppm solution. This methanol solution was then emptied into a 100 mL volumetric flask and when made up to volume with water resulted in a 1000 ppm stock solution. The stock solution was stored at 5°C and was used as required throughout the project. The solution was good for up to 2 months at this temperature according to Ulberth (1991).

3.6.2 The External Standard Method

For the ES calibration method used throughout this thesis, a standard curve was set up at the start of every day that headspace sampling was carried out on the unknown yoghurt samples.

This was done for two reasons:

- to routinely calibrate and
- to determine the reproducibility and reliability of the GC from day to day.

The 1000 ppm stock solution, which contained all six analytes, was diluted down further to make standard aqueous solutions of varying concentrations ranging from 0 ppm to 500 ppm. These standard aqueous solutions were used for headspace analysis. For the standard simulated yoghurts the following preparations were carried out. In order to have a representative linear calibration curve, it was important to match the sample matrix of the standard solution with that of the set yoghurt samples. Pre-calculated quantities of SMP and WMP were measured to make low fat (0.3%) and high fat (3.5%) milks solutions. These were weighed into 50 mL volumetric flasks. A little water was added and mixed thoroughly to prevent the milk powders clumping. Then the required amounts of 1000 ppm stock solution were added to make standard solutions of the six analytes ranging from 0 to 500 ppm. These were then made up to 50 mL with water. The resulting standard milk solutions were formulated so that they contained the same protein, fat and total solids non-fat contents as those of the laboratory-prepared yoghurts.

2 mL aliquots of these standard milk solutions (usually 0, 12.5 and 25 ppm) were then transferred to 4 mL glass vials, which contained 60 µL concentrated lactic acid and 200 µL of 200 ppm propan-2-ol IS solution. The glass vials were sealed tight with the butyl rubber resealable septa in the open-hole screw-top lids. The concentrated lactic acid was added to the standard milk solutions to lower the pH to ~4.6 which caused the milk to curdle and set. Acidification of the standard milk solutions was done to simulate the cultured yoghurt sample matrix. Both low fat and high fat simulated yoghurts were made so as to mimic the low fat and high fat cultured yoghurts being investigated.

Initially, a standard curve was drawn using all the standard simulated yoghurts of varying concentrations (0, 5, 12.5, 25, 50, 100 and 500 ppm). However, as it was apparent from this initial standard curve that a straight regression line was almost always certain ($R^2 = 0.999$), only three standard simulated yoghurts of known concentration (0, 12.5 and 25 ppm) were used thereafter for the ES method.

The whole concentration range (0 - 500 ppm) of standard simulated yoghurts could not be analysed daily due to time restrictions, as each GC run took around 40 minutes. Also, both low and high fat simulated yoghurts had to be analysed, in duplicate, which extended the total analysis time. Calibration of the GC using the three standard simulated yoghurt concentrations in duplicate took about 4 hours every day, which then only allowed enough time for sampling the experimental yoghurts (in duplicate or triplicate). The standard concentrations (0, 12.5 and 25 ppm) were chosen because it was cited in Nijssen *et al.* (1996) that the six analytes of interest in yoghurts were mostly within the 0 - 25 ppm concentration range.

3.6.3 Internal Standard Method

The IS method was carried out by spiking each yoghurt sample to be analysed via headspace analysis with 200 µL of a 200 ppm propan-2-ol aqueous solution. The 200 ppm IS solution was made by dissolving 200 µL propan-2-ol in water and making it up to 1000 mL with water. This 200 ppm solution was then used as the IS throughout this study. The milk samples, commercial yoghurt samples, standard aqueous solutions, simulated yoghurt samples and standard simulated yoghurt samples were all "spiked" with this IS solution.

3.7 General Sampling Procedures for the Inoculated Milks

When the inoculated milk from Section 3.2 (part 6) was mixed thoroughly, 15 mL and 2 mL aliquots were transferred to Lily portion cups and glass vials, respectively, and sealed tight with butyl rubber septa inserted into open-hole, screw top lids. These vials all contained the IS (200 µL of 200 ppm IS solution). Enough vials were filled, as required, for each experiment in the present study and sampling was either carried out in duplicate or triplicate. The inoculated milks were then incubated at 37°C for about 5 ½ hours, where fermentation took place and the yoghurts set. The pH of the yoghurts was constantly checked (using an Orion pH meter) during the 5 ½ hours of incubation by determining the pH of the 15 mL aliquots of inoculated milks placed in the Lily portion cups. Once the pH in the portion cups had reached a pH ~ 4.6, it was assumed that the inoculated milks in the glass vials had also reached this same endpoint. The glass vials and Lily portion cups were then transferred from the incubator to the refrigerator and immediately chilled and stored at 5°C. The yoghurts were mostly stored at this temperature overnight or for around a week before headspace analysis was carried out on each sample. Variations to the sampling *

* See Errata information

simulated yoghurt samples, standard simulated yoghurt samples, laboratory-prepared yoghurt samples or commercial yoghurt samples) was extracted from the refrigerator and left at room temperature (20°C ± 2°C) for 10 minutes. From this point onwards, headspace sampling using the SH or HS-SPME methods were followed according to Sections 3.4 and 3.5, respectively.

For this thesis, only set yoghurts were studied. As explained earlier, both the IS and ES methods were used to quantify the analyte concentrations in the yoghurts throughout the present study. Table 3.1 (pages 71-74) gives a quick reference guide to the sampling details used in the six experiments carried out in this thesis. Which calibration method was used and how many replicates were made for each experiment are also detailed in this table.

3.8 Determination of Analyte Retention Times

Six 50 ppm aqueous solutions were made. Each solution contained one of the six analytes or propan-2-ol (IS) at this concentration. The solutions were made by taking 5 mg or 5 µL of each analyte and separately dissolving them in water (making up to 100 mL). Aliquots (2 mL) of each analyte solution were pipetted into 4 mL glass vials and sealed. The solutions were then individually analysed using the HS-SPME method as outlined in Section 3.5. The following retention times were observed for the six analytes and propan-2-ol (IS):

Analyte	Retention Time (R_T)
Acetaldehyde	~3.50 mins
Acetone	~5.50 mins
2-Butanone	~7.30 mins
Ethanol	~7.95 mins
Diacetyl	~9.20 mins
Acetoin	~19.30 mins
Propan-2-ol	~10.90 mins

Table 3.1: A Quick Reference Guide to Experimental and Sampling Details Used in the Present Study.

Chapter	Calibration method used	Number of replicates	Storage Time and Temperature	Sampling and pH Monitoring
- 4 - Comparison of the Performance of PA and PDMS Fibre Coatings in HS-SPME.	ES and IS methods	Duplicate	A. Overnight storage at 5°C before analysis.	A. Standard simulated yoghurts analysed. LF (~0.3%) and HF (~3.5%) standard milk solutions made (0, 12.5, 25 and 50 ppm). 2 mL of each milk + 60 µL conc. lactic acid + 200 µL of 200 ppm IS solution in glass vials.
		Duplicate	B. 1 week storage at 5°C before analysis.	B. Commercial yoghurts analysed. 15 mL of each yoghurt transferred to Lily portion cups for pH monitoring and 2 mL in glass vials + 200 µL of 200 ppm IS solution.
- 5 - Adsorption Time Profiles for Extraction of Analytes from Simulated Yoghurts Using the 100 µm PDMS Fibre.	-	Triplicate	Overnight storage at 5°C before analysis.	Standard (10 ppm and 100 ppm) aqueous solutions and LF standard simulated yoghurts analysed. Enough 1000 ppm stock solution added to milk powder mixtures + water to make 10 ppm and 100 ppm. Simulated yoghurts made from these milk solutions. 2 mL of the standard milk solutions were transferred to 4 mL glass vials containing 60 µL conc. lactic acid + 200 µL of 200 ppm IS solution.

Table 3.1ctd.: A Quick Reference Guide to Experimental and Sampling Details Used in the Present Study.

Chapter	Calibration method used	Number of replicates	Storage Time and Temperature	Sampling and pH monitoring
- 6 - Comparison of the Classical SH Method with the HS-SPME Technique.	ES and IS	Triplicate	A. Overnight storage at 5°C before analysis.	A. Aqueous standard solutions analysed. 1000 ppm stock solution diluted down to 0, 5, 12.5, 25, 50, 100 and 500 ppm. 2 mL aliquots of each standard solution transferred to 4 mL glass vials + 60 µL conc. lactic acid + 200 µL of 200 ppm IS solution.
		Triplicate	B. Overnight storage at 5°C before analysis.	B. Standard simulated yoghurt samples analysed. Standard low fat, milk solutions made (0, 5, 12.5, 25, 50, 100 and 500 ppm) using a combination of SMP and WMP and enough 1000 ppm stock solution and water. 2 mL aliquots of each milk solution were transferred to 4 mL glass vials containing 60 µL conc. lactic acid and 200 µL of 200 ppm IS solution.
- 7 - An Investigation into the Concentrations of the Six Yoghurt Analytes in Plain Milk, Acidified Milk and Cultured Yoghurts.	ES and IS	Duplicate	Overnight storage at 5°C before analysis.	Standard milks analysed. LF and HF milks made by recombining WMP and SMP with water and divided into 3 classes - plain, acidified and cultured yoghurts. 2 mL aliquots of each were transferred to 4 mL glass vials. For the plain version, the samples were left as they were, while for the acidified version, 60 µL conc. lactic acid + 200 µL of 200 ppm IS solution were added to each vial. Incubation or chilling variables were also investigated. Standards for the ES method were also made to quantify analytes.

Table 3.1ctd.: A Quick Reference Guide to Experimental and Sampling Details Used in the Present Study.

Chapter	Calibration method used	Number of replicates	Storage Time and Temperature	Sampling and pH monitoring
- 7 ctd. -				Cultured yoghurts analysed. LF and HF yoghurts made according to Section 3.2. 2 mL aliquots of inoculated milks transferred to 4 mL glass vials containing 200 µL of IS. 15 mL of inoculated milk also in Lily portion cups for pH monitoring. Incubated and chilled before analysis.
- 8 - An Investigation into the Effects of Storage Time and Storage Temperature on the Six Analytes in Yoghurt Flavours over 24-day Periods	ES and IS	Triplicate	2°C and 10°C storage over 24 days depending on storage trial. Initial headspace sampling taken on "Day 0" after one hour at respective storage temperature.	LF and HF yoghurts analysed in Two Storage Trials. 2 kg batches of LF and HF yoghurts were made according to Section 3.2. 2 mL of each milk transferred into 4 mL glass vials containing 200 µL IS. 15 mL of the inoculated milks was placed in Lily portion cups for pH monitoring. Milks were then incubated and chilled before analysis. HS-SPME sampling carried out on the yoghurts over a 24-day period. 0, 12.5 and 25 ppm standard simulated yoghurts were also made for GC calibration using ES method. 2 mL of each standard transferred to glass vials containing 200 µL IS and 60 µL conc. lactic acid. Enough standards and yoghurt samples made to cover two 24-day storage trials.

Table 3.1ctd.: A Quick Reference Guide to Experimental and Sampling Details Used in the Present Study.

Chapter	Calibration method used	Number of replicates	Storage Time and Temperature	Sampling and pH monitoring
- 9 - An Investigation of Commercial Yoghurts Using HS-SPME and Sensory Analysis	ES and IS	A. Duplicate	A. 2°C storage for one week before analysis done.	(A) Fourteen commercial yoghurts analysed. 2 mL of each yoghurt transferred to 4 mL glass vial containing 200 µL of 200 ppm IS solution for headspace analysis. 15 mL of each yoghurt placed in Lily portion cups for pH monitoring. 0, 12.5 and 25 ppm standard simulated yoghurt samples made to calibrate GC using ES method. PCA carried out on peak area results obtained.
		B. Triplicate for Day 1 and four replicates for Day 2	B. 2°C storage for one week before analysis done.	(B) Eight commercial yoghurts selected and analysed from A. above. 2 mL of each commercial yoghurt transferred to 4 mL glass vials containing 200 µL IS for headspace analysis. 15 mL of each yoghurt placed in Lily portion cups for pH monitoring. 0, 12.5 and 25 ppm standard simulated yoghurt samples made to calibrate GC using ES method. PCA carried out on peak area results obtained. Seven triangle tests administered to consumer panellists.

CHAPTER FOUR

COMPARISON OF THE PERFORMANCE OF POLYACRYLATE AND POLYDIMETHYLSILOXANE FIBRE COATINGS IN HEADSPACE SOLID PHASE MICROEXTRACTION.

4.1 Introduction

The first step in setting up a working methodology for the HS-SPME method was to select a fibre with a surface coating that was most appropriate for extracting the six aroma impact compounds (acetaldehyde, acetone, 2-butanone, ethanol, diacetyl and acetoin) present in natural, unsweetened yoghurts. When the work was carried out there was a limited number of fibre coatings manufactured by Supelco for the commercial market. These fibre coatings are listed below with their specific extracting properties:

- **100 µm Polydimethylsiloxane (PDMS)** coating for volatiles and semi-volatiles;
- **30 µm PDMS** coating for nonpolar semi-volatiles;
- **7 µm PDMS** coating for mid- to nonpolar semi-volatiles;
- **65 µm Carbowax/Divinylbenzene (CW/DVB)** coating for polar analytes; and
- **85 µm Polyacrylate (PA)** coating for polar semi-volatiles.

The 30 and 7 µm PDMS fibre coatings and the 65 µm CW/DVB coating were not considered for this study. The two PDMS fibre coatings were only suitable for extracting nonpolar semi-volatiles, while the 65 µm CW/DVB fibre coating was only suitable for polar analytes. As a mixture of polar and nonpolar flavour compounds (volatile and semi-volatile) were to be extracted from the yoghurts, these fibres were considered unsuitable for this study.

In addition, the 30, 7 and 65 µm coating layers were considered too thin compared with the 100 and 85 µm coatings of the PDMS and PA fibres, respectively. For HS-SPME the amount of analyte extracted is proportional to the volume of the fibre coating (Potter and Pawliszyn, 1992). According to Harmon, (1997), thicker coatings provide greater sensitivity due to the higher analyte mass that can be adsorbed by the fibre. The only

drawback is that the extraction process may be lengthened due to the longer time required to saturate the fibre coating and to achieve a state of an equilibrium within the sample system. In this study, time was less important than extracting as much analyte as possible. Thus, the thicker fibre coatings should enhance adsorption of a greater amount of each flavour compound, thereby, increasing the accuracy, efficiency and sensitivity of the method. Hence, the performance of the 100 μm PDMS fibre and 85μm PA fibre were compared in this study.

Notably, the PDMS and PA fibres were at opposite ends of the polarity scale with the nonpolar PDMS fibre having a preference for nonpolar compounds and the polar PA fibre having a preference for polar compounds. These two fibre coatings have been used in previous studies of volatiles in dairy products. Stevenson and Chen (1997) used HS-SPME and the 100 μm PDMS fibre to determine the flavour compounds present in wet and dry skim milk powders, while Chin *et al.* (1996) used both the PA and PDMS fibres to study cheese volatiles.

The PDMS fibre coating was quoted in the Supelco catalogue, as being able to extract "volatiles and semi-volatiles", while the PA fibre was quoted as being most suitable for "polar semi-volatiles". Even though the PA fibre did not theoretically adsorb nonpolar semi-volatiles, it was still tested for its extracting power, mainly because of the thickness of its coating.

The aim of this work was to determine which fibre coating (PA or PDMS) would be most efficient for extracting and analysing the six flavour compounds present in yoghurts.

4.2 Materials and Methods

The first part of this work consisted of making two 50 g batches of simulated yoghurt samples containing 0.3% fat (low fat) and 3.5% fat (high fat) as described in Section 3.6.2. The proportions used to make the simulated yoghurts are given in Table 4.1 below. Enough 1000 ppm stock solution was added to the milk powders and water resulting in milks with known analyte concentrations of 0, 12.5 and 25 ppm.

Table 4.1: Quantities of WMP, SMP and 1000 ppm Stock Solution Used to Make Standard Simulated Yoghurt Samples.

	Simulated Low Fat Yoghurt (0.3% fat)				**Simulated High Fat Yoghurt (3.5% fat)**			
Analyte Conc. Required (ppm)	**0**	**12.5**	**25**	**50**	**0**	**12.5**	**25**	**50**
1000 ppm Stock Solution (mL)	0	0.625	1.25	2.5	0	0.625	1.25	2.5
WMP	0.70g				12.75g			
SMP	10.35g				3.00g			

The 1000 ppm stock solution, which contained the six main yoghurt analytes was made according to the method in Section 3.6.1. 0.7 g of WMP and 10.35 g SMP were weighed into a 50 mL volumetric flask to make the simulated low fat yoghurts. Enough water was added to the mixture to prevent it from clumping. The required amounts of 1000 ppm stock solution (see Table 4.1) were then added to give the respective concentrations (0, 12.5, 25 and 50 ppm). The mixtures were then made up to 50 mL with water. For the simulated high fat yoghurts, 12.75 g of WMP and 3.00 g of SMP were weighed out and treated as described for the low fat version. 2 mL of each standard milk solution was then placed in 4 mL glass vials as described in Section 3.6.2 and sealed tight. In order to achieve the simulated yoghurt effect, each vial also contained 60 µL of conc. lactic acid. The simulated yoghurts were also spiked with the IS solution as described in Section 3.6.3. All the sample vials were sealed and stored at 5°C, overnight, before HS-SPME GC analysis.

For this section of the work, the IS was added at a concentration level of 200 ppm so as to determine the IS peak area using each fibre type. For the second part of this work however, the IS was added as a reference to each commercial yoghurt sample as a means of calculating the concentration of the six analytes in the yoghurt samples (IS method). The guide on page 71 of the Materials and Methods section (Chapter 3) gives details of the sampling procedures.

The second part of this work involved using the two fibres to analyse various commercial yoghurts for the six yoghurt volatiles. The same yoghurt brands were also used in Chapter 9, as part of a separate investigation where a possible application for the HS-SPME technique was investigated. The commercial yoghurts used in this chapter are listed in Table 4.2 (page 79). Table 4.2 outlines details of the commercial yoghurts presented on the labels. The details included what types of starter cultures were added to make the yoghurts, the fat content, the protein content and the fat class (that is, either low fat or high fat). The pH of each yoghurt sample was also measured just before HS-SPME sampling was carried out.

Each yoghurt (2 mL) was added to a glass vial spiked with the IS, as described in Section 3.6.3, and sealed tight. In addition, 15 mL of each yoghurt was transferred to a Lily portion cup for pH monitoring as described in Section 3.7. The yoghurts were sampled one week after the date of purchase, stored overnight at 5°C, and analysed using HS-SPME on Day 8. The yoghurts all had similar expiry dates (within 2 days of each other) and were chosen so they had at least a fortnight before their expiry date. From this, it was assumed that the yoghurts were all manufactured on or around the same date and therefore, were of similar ages.

Both fibres were tested for their linear responses (regression) and their sensitivity towards the six yoghurt analytes by comparing the peak areas obtained. The HS-SPME sampling procedure set out in Section 3.5 was followed for both parts of this work using the two fibre coatings. The IS and ES methods were used to quantify the analyte concentrations in the simulated and commercial yoghurts. The two fibre coatings (PA and PDMS) were compared for their extracting abilities.

Table 4.2: List of the Commercial Yoghurts Analysed Using the 100 µm PDMS and 85 µm PA Fibres.

Yoghurt Sample With Abbreviations	Yoghurt Culture	Fat Content (%)	Protein Content (%)	Fat Class	Yoghurt pH
Naturalea Acidophilus (NA)	*Acidophilus* and *B. bacterium*	3.8	3.2	HF	3.55
Naturalea (N)	Unspecified yoghurt cultures	3.3	4.2	HF	3.64
Slimmer's Choice (SC)	*Acidophilus, B. bacterium* and other unspecified yoghurt cultures (unsweetened)	0.2	4.6	LF	3.76
Verona Bulgarian (VB)	*L bulgaricus* culture and other unspecified yoghurt cultures	3.25	5	HF	3.71
Metchnikoff (M)	*Acidophilus* and *B. bacterium*	0.2	4.8	LF	3.66
Yoplait High Fat (Y)	*L. bulgaricus* and *S. thermophilus*, (sweetened)	2.9	5.3	HF	4.04
Fresh 'n' Natural (F&N)	Unspecified yoghurt cultures, (sweetened)	1.2	5	LF	3.91
Verona Acidophilus (VA)	Unspecified yoghurt cultures	2	5	HF	3.88
Biofarm Acidophilus (BA)	*Acidophilus* and *B. bacterium*	~3	4.5	HF	3.62
Biofarm (B)	*Acidophilus* and *B. bacterium*	~3	4.5	HF	3.68
Weight Watchers (WW)	Unspecified yoghurt cultures	0.1	5.2	LF	3.98

4.3 Results and Discussion

The peak areas for each analyte were measured and used to draw standard curves for each fibre type. This was done at both fat levels of the standard simulated yoghurt samples. The results obtained are shown in Table 4.3 (page 81). Regression lines were drawn in order to establish the precision of the SPME method for absorbing specific analytes at varying concentrations (Figure 4.1, pages 82, 83 and 84). From the results, it was apparent that both fibres produced good standard curves. Regression coefficients (R^2) were calculated and these were greater than 83% and often, close to 100%.

The two fibres being compared showed a high sensitivity towards the six analytes and even toward the IS. This was seen by the large peak areas obtained, e.g. IS peak of ~800 units^2 for the PA fibre and ~500 units^2 for the PDMS fibre. As accurate standard curves with high regression coefficients could be drawn, it was apparent that good correlations between analyte peak areas and their concentrations in the yoghurts existed. Therefore, the ES method was clearly a good calibration technique to use because the standard curves could be used to provide useful information about unknown analyte concentrations in yoghurts.

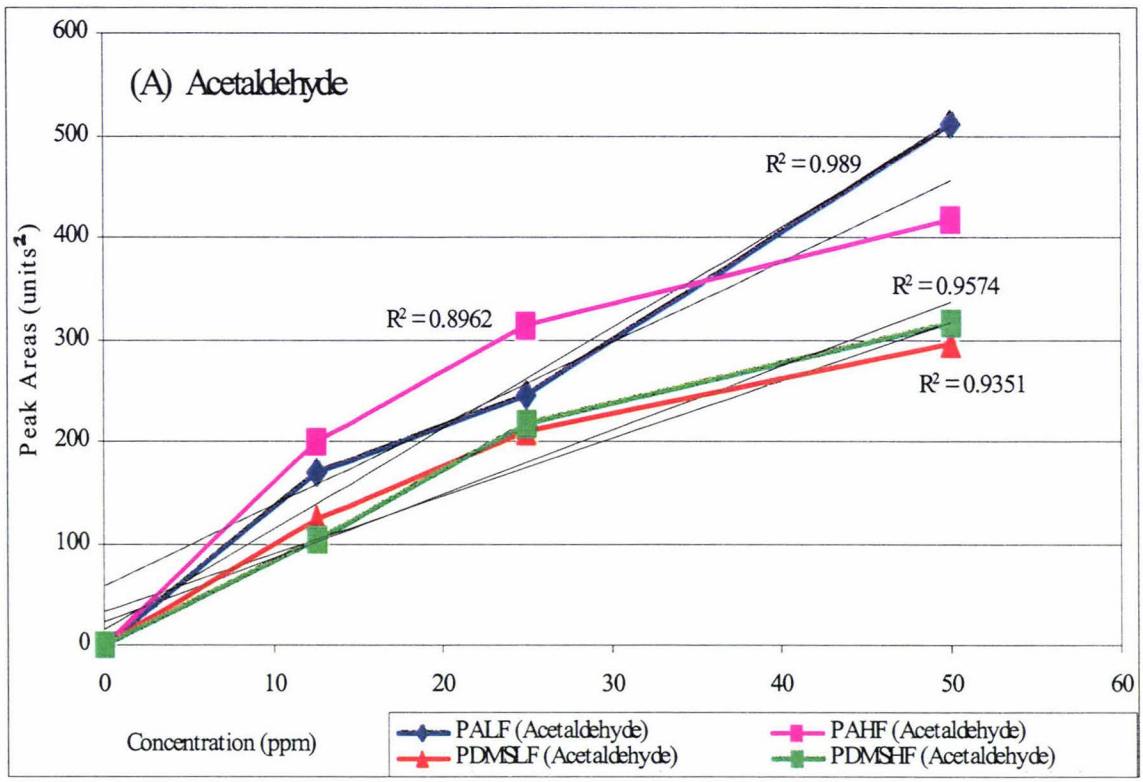
From the results in Table 4.3 (page 81), the PA fibre had higher peak area values for the polar compounds such as ethanol (208 units^2 vs. 77.5 units^2 for PDMS fibre) and propan-2-ol (873 units^2 vs. 544 units^2 for PDMS fibre) suggesting that it had a greater affinity towards these compounds. The PA fibre also extracted acetaldehyde marginally better than the PDMS fibre. For the more nonpolar analytes such as acetone, 2-butanone and acetoin, the PDMS fibre had greater peak areas e.g. for acetone, 506.5 units^2 vs. 320 units^2 for PA fibre). Both fibres adsorbed diacetyl to a similar extent producing peak areas that were similar, e.g. 217 units^2 for the PA fibre vs. 230 units^2 for the PDMS fibre. It should be noted that both fibres adsorbed all six analytes including the IS. However, the PA fibre did adsorb the more polar compounds, such as alcohols to a greater extent while the PDMS fibre mainly adsorbed the nonpolar volatile and semi-volatile organic compounds better.

Table 4.3: Regression Coefficients from Standard Curves Obtained for the Standard Simulated Yoghurt Samples (A) the PA Fibre and (B) the PDMS Fibre.

(A)	Peak Area (units ^{2}) Obtained for Analytes Using the Polyacrylate (PA) Fibre						
Conc. (ppm)	**Acetaldehyde**	**Acetone**	**2-Butanone**	**Ethanol**	**Diacetyl**	**Acetoin**	**Propan-2-ol 200 ppm**
	Low Fat						
0	0	0	0	0	0	0	873
12.5	170	320	1201	208	151	99	844
25	245.5	563.5	1816	251.5	133.5	170	998
50	510.5	1510	5419	578.5	335.5	600.5	893.5
Regress. Coeff. (R^2) (%)	**98.62**	**97.57**	**96.06**	**96.84**	**90.05**	**92.88**	
	High Fat						
0	0	0	0	0	0	0	845
12.5	199	326.5	1083	167.5	74.5	165	882.5
25	315	655.5	2203	365	217	231	801
50	417	1226	4366	574.5	394.5	363	759
Regress. Coeff. (R^2) (%)	**83.53**	**99.79**	**99.99**	**97.25**	**98.93**	**90.44**	

(B)	Peak Area (units ^{2}) Obtained for Analytes Using the Polydimethylsiloxane (PDMS) Fibre						
Conc. (ppm)	**Acetaldehyde**	**Acetone**	**2-Butanone**	**Ethanol**	**Diacetyl**	**Acetoin**	**Propan-2-ol 200 ppm**
	Low Fat						
0	0	0	0	0	0	0	544
12.5	123	506.5	2030.5	77.5	93	114	529.5
25	209	1014	3920	177	171	203	436
50	295	1651.5	7539	289	263	412	459.5
Regress. Coeff. (R^2) (%)	**89.92**	**97.75**	**99.89**	**98.19**	**95.30**	**99.85**	
	High Fat						
0	0	0	0	0	0	0	579
12.5	103	509	1854	99.5	134.5	96.5	543
25	218	961	3859	142	230	253	525
50	316	1859	7437	265	322	402	542.5
Regress. Coeff. (R^2) (%)	**94.36**	**99.87**	**99.95**	**97.05**	**89.58**	**97.61**	

See Errata in Front Cover



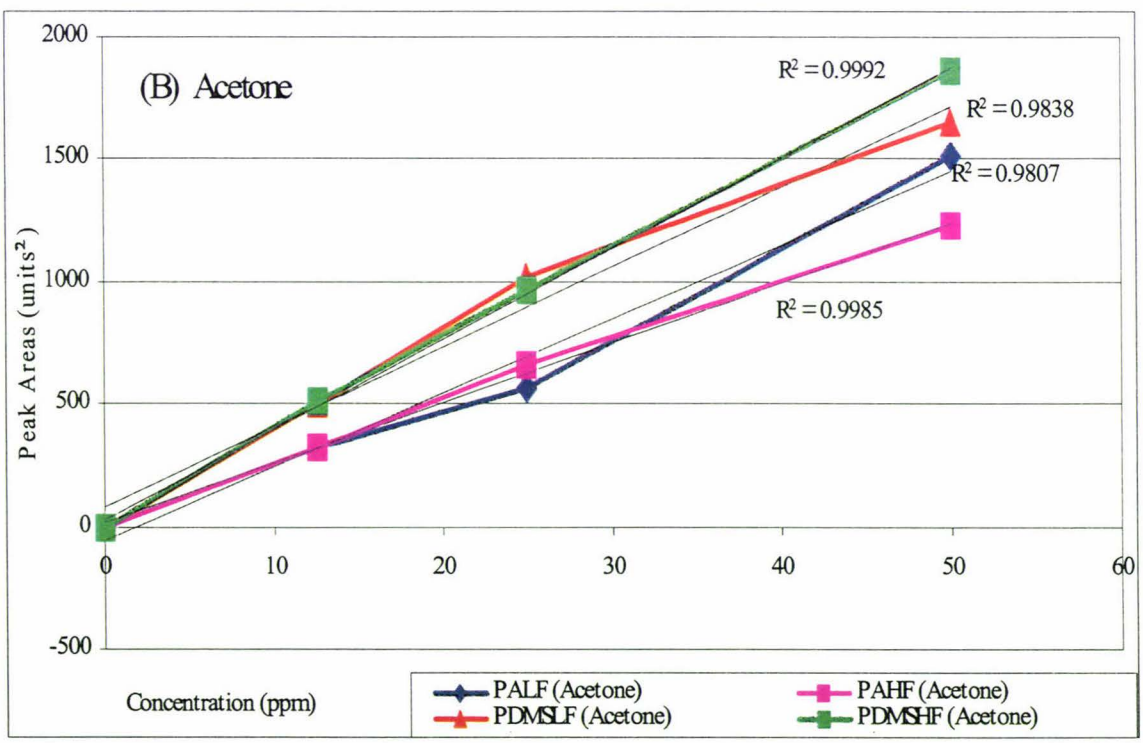
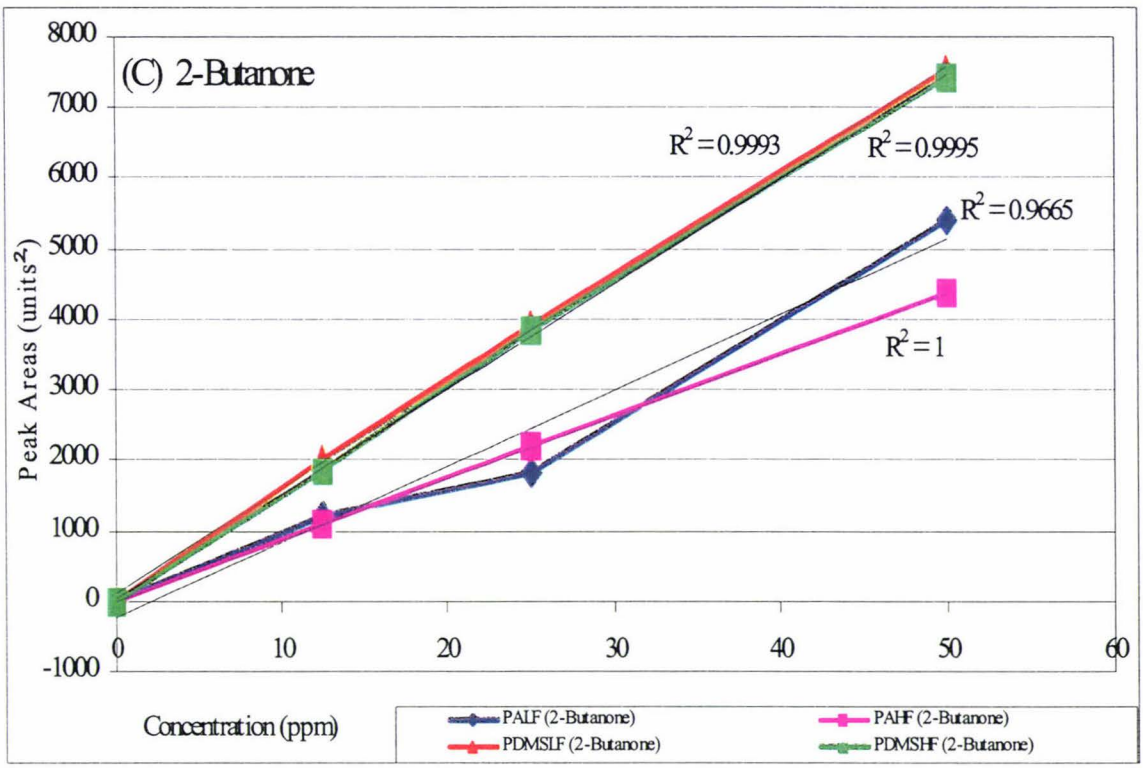


Figure 4.1: Standard Curves for Flavour Analytes in Low and High Fat Standard Simulated Yoghurts Using the 100 µm PDMS and 85 µm PA Fibres

N.B. PALF = Polyacrylate, low fat simulated yoghurt;
PAHF = Polyacrylate, high fat simulated yoghurt;
PDMSLF = Polydimethylsiloxane, low fat simulated yoghurt;
PDMSHF = Polydimethylsiloxane, high fat simulated yoghurt.



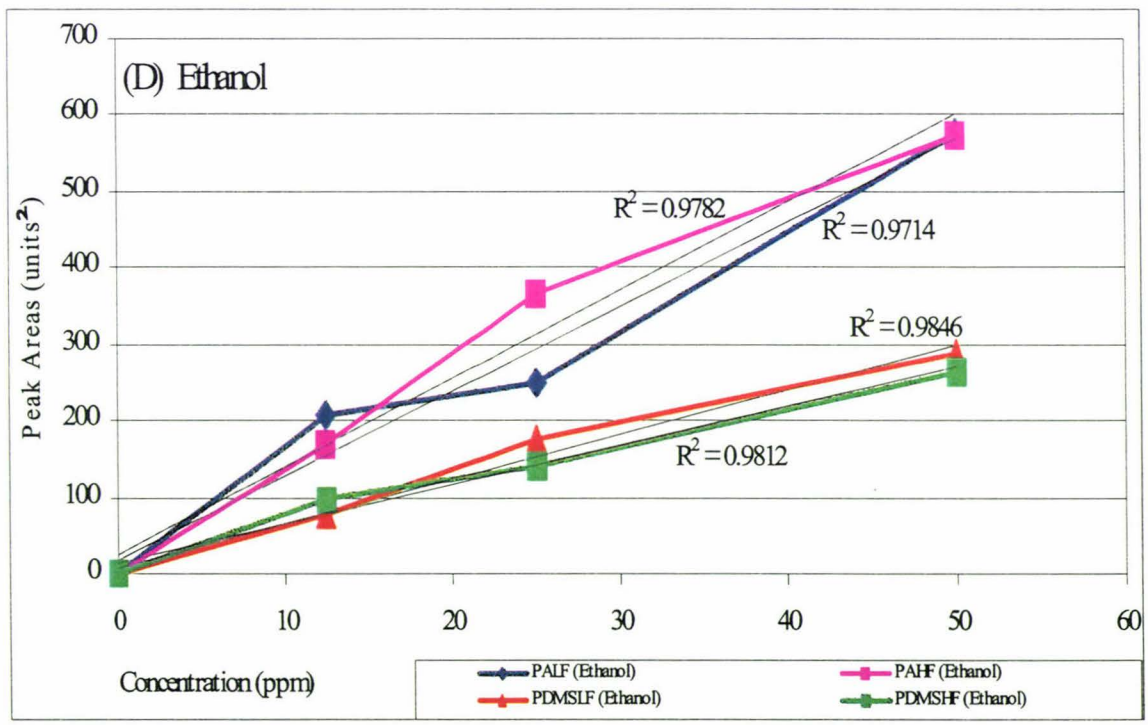
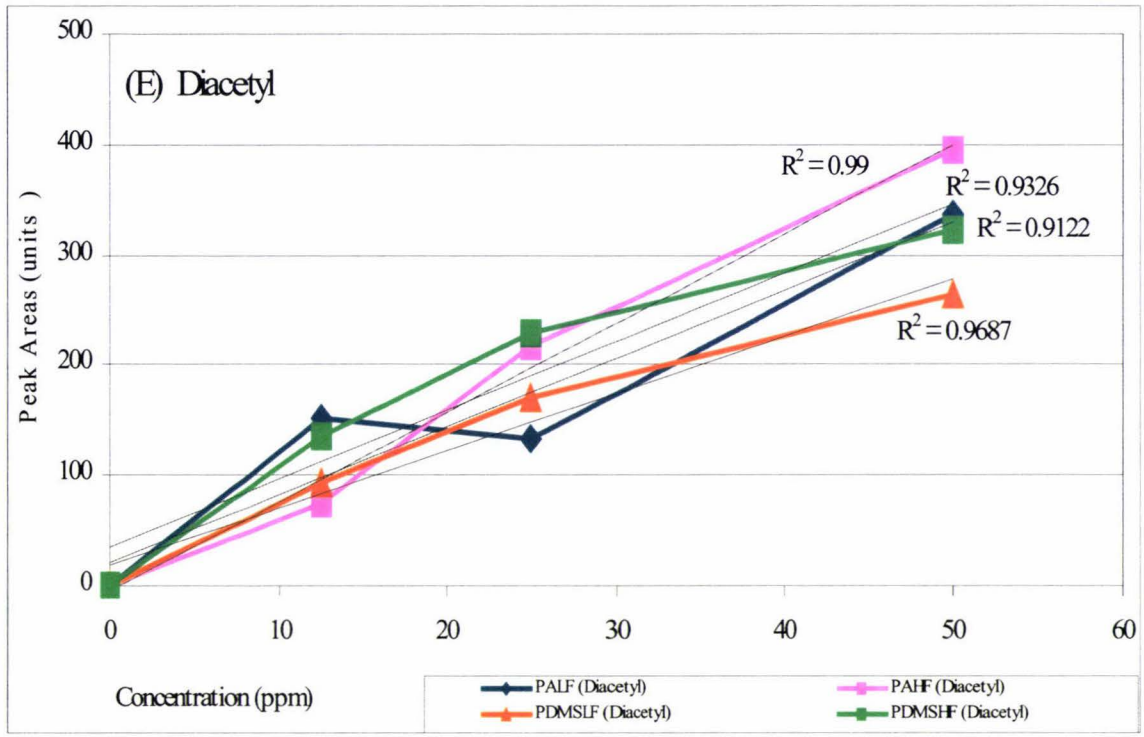


Figure 4.1 ctd.: Standard Curves for Flavour Analytes in Low and High Fat Standard Simulated Yoghurts Using the 100 µm PDMS and 85 µm PA Fibres

N.B. PALF = Polyacrylate, low fat simulated yoghurt;
PAHF = Polyacrylate, high fat simulated yoghurt;
PDMSLF = Polydimethylsiloxane, low fat simulated yoghurt;
PDMSHF = Polydimethylsiloxane, high fat simulated yoghurt



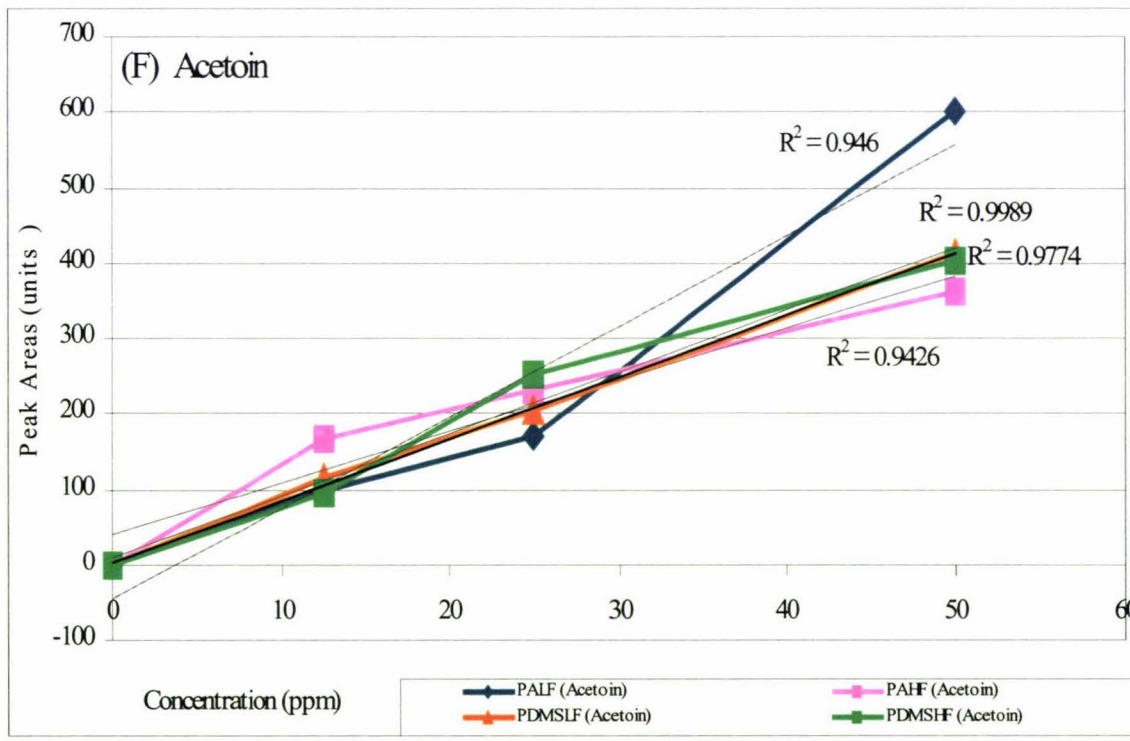


Figure 4.1 ctd.: Standard Curves for Flavour Analytes in Low and High Fat Standard Simulated Yoghurts Using the 100 µm PDMS and 85 µm PA Fibres

N.B. PALF = Polyacrylate, low fat simulated yoghurt;
PAHF = Polyacrylate, high fat simulated yoghurt;
PDMSLF = Polydimethylsiloxane, low fat simulated yoghurt;
PDMSHF = Polydimethylsiloxane, high fat simulated yoghurt

Comparing the peak areas obtained for each fat level using both fibres, it seemed that the fat level (0.3% vs. 3.5%) in the simulated yoghurts did not have a large effect on the adsorbing power of the fibre. Figure 4.2 (below) shows an example of the peak area results obtained at the 25 ppm concentration level for both fat levels using the PA and PDMS fibres. No consistent trend was seen for the peak areas obtained for each fibre between the low and high fat simulated yoghurts. Similar inconclusive results were also seen for the other analyte concentrations (12.5 and 50 ppm). From this, it was concluded that there were no fat effects at the 0.3% and 3.5% fat levels. That is, the fat present in the simulated yoghurt samples, at these levels, did not affect the analyte headspace composition or the HS-SPME extraction method.

If there had been fat effects present in the simulated yoghurt matrix, the high fat analyte peak areas would have been proportionally lower than those obtained for the low fat simulated yoghurts. This would be because the fat globules in the high fat sample compete with the fibre for analyte adsorption, resulting in the fibre being a poor adsorber of the flavour compounds. This, in turn, would result in lower peak area values and lower sensitivity of the HS-SPME method as was reported in a study carried out by Page and Lacroix (1993).

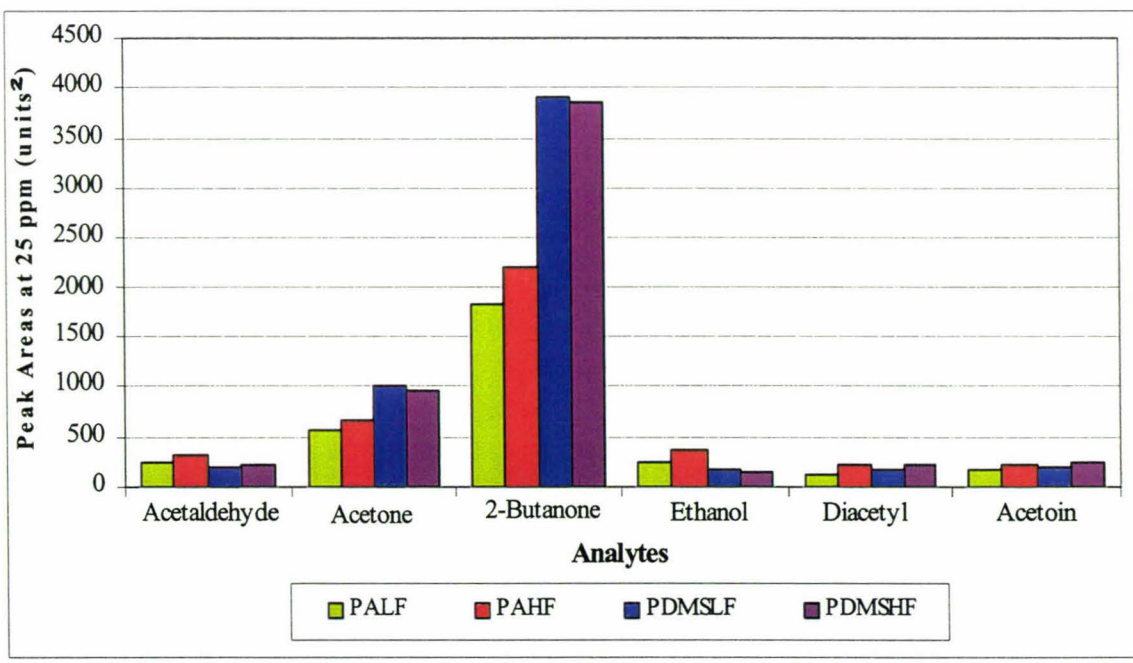


Figure 4.2: Comparison of the Peak Areas Obtained for Low and High Fat Simulated Yoghurts at 25 ppm Using the PDMS and PA Fibres.

N.B. PALF = Polyacrylate, low fat yoghurt; PAHF = Polyacrylate, high fat yoghurt; PDMSLF = Polydimethylsiloxane, low fat yoghurt; PDMSHF = Polydimethylsiloxane, high fat yoghurt.

The analyte concentrations in the commercial yoghurts were calculated using the IS and ES methods. The concentration results obtained using the IS method were unusually high compared to the values quoted in Nijssen *et al.* (1996). Therefore, it was concluded that the IS method, with the known concentration (200 ppm) peak area, could not be used to quantify the six yoghurt volatiles in the commercial yoghurts using the HS-SPME technique. This was because each fibre responds differently to each compound and therefore, the IS response factor for the PA fibre is very different to that of the PDMS fibre. Hence, the analyte peak areas could only be normalised against the IS peak areas. This gave semi-quantitative data, which was only suitable for analyte peak area comparisons between the simulated yoghurts using a specific fibre. Therefore, the IS method could not be used as a quantifying tool in this thesis.

Ulberth's (1991) study suggested a 50 ppm propan-2-ol solution be used for the IS. The author used the SH method where a volume containing a known IS concentration is injected directly into the GC. This method does not suffer from the fibre coating responses and therefore, quantitative analysis is possible. It should be noted however, that the propan-2-ol peak areas were consistent throughout this investigation (~800 units ^{2} for the PA fibre and ~ 500 units ^{2} for the PDMS fibre).

The ES method on the other hand, could be used to identify and quantify the analytes in the commercial yoghurts. This calibration technique did also overcome the limitation of the IS method. The only disadvantage of the ES method was that it was time-consuming, especially since the calibration technique had to be performed daily before the experimental yoghurts could be analysed.

The analyte concentrations (except acetoin) obtained for the commercial yoghurts using the ES method were well within the ranges quoted in Nijssen *et al.* (1996). The values quoted for acetoin from previous studies were between 13.5 and 28 ppm, while the findings in this work (using the ES method) suggested that the acetoin levels were higher (40 - 60 ppm). This could have been due to the HS-SPME technique being more efficient at extracting non-volatile compounds, such as acetoin, than various analyte extraction techniques used in the past. The studies quoted in the Nijssen *et al.* (1996) compilations used techniques such as steam distillation and acetoin derivatization parallel with GC to indirectly quantify the acetoin levels in yoghurts. Therefore, the

values quoted in the literature may not have been truly representative. More HS-SPME GC work, preferably with MS for analyte identification, could be carried out in order to confirm the presence of acetoin and its concentration in these yoghurts.

Table 4.4 (below) shows the differences in the extracting capacities of the two fibres (PA and PDMS) for extracting the six analytes from the standard simulated yoghurts.

Table 4.4: Peak Area Results Obtained Using the PA and PDMS Fibres for Analysis of Standard Simulated Yoghurt Samples (**PA** : PDMS).

N.B. 1. Peak areas are in units^2;
2. Table replicated from Miller and Stuart's (1999) study.

Sample	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin	Propan-2-ol
Low Fat							
12.5 LF	**170**:123	**320**:506.5	**1201**:2030.5	**208**:77.5	**151**:93	**99**:114	**844**:529.5
25 LF	**245.5**:209	**563.5**:1014	**1816**:3920	**251.5**:177	**133.5**:171	**170**:203	**998**:436
50 LF	**510.5**:295	**1510**:1651.5	**5419**:7539	**578.5**:289	**335.5**:263	**600.5**:412	**893.5**:459.5
High Fat							
12.5 HF	**199**:103	**326.5**:509	**1083**:1854	**167**:99.5	**74.5**:134.5	**165**:96.5	**882.5**:543
25 HF	**315**:218	**655.5**:961	**2203**:3859	**365**:142	**217**:230	**231**:253	**801**:525
50 HF	**417**:316	**1226**:1859	**4366**:7437	**574.5**:265	**394.5**:322	**363**:402	**759**:542.5

Table 4.5 (page 88) shows the scaling of the PA peak areas to that of the PDMS peak areas obtained for the six analytes in the standard simulated yoghurts. The "+" sign indicates the better absorbing capability of the PA fibre compared with that of the PDMS fibre, while the '-' sign indicates the opposite. Values equalling "1" show that both fibres have similar adsorbing capabilities.

Table 4.5: Comparison of 100 µm PDMS with 85 µm PA Fibres Using Standard Simulated Yoghurts (all recoveries were scaled to the 100 µm PDMS, which was assigned "1" for comparison purposes).

N.B. "+" values show that the PA fibre has better adsorbing properties than the PDMS fibre coating and "-" values shows that the PA fibre has worse adsorbing properties than the PDMS fibre coating.

Sample	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin	Propan-2-ol
Low Fat							
12.5 LF	+1.382	-0.632	-0.591	+2.684	+1.624	-0.868	+1.594
25 LF	+1.175	-0556	-0.463	+1.421	-0.781	-0.837	+2.289
50 LF	+1.731	-0.914	-0.719	+2.002	+1.276	+1.458	+1.945
High Fat							
12.5 HF	+1.932	-0.641	-0.584	+1.678	-0.554	+1.71	+1.625
25 HF	+1.445	-0.682	-0.571	+2.57	-0.943	-0.913	+1.526
50 HF	+1.32	-0.659	-0.587	+2.168	+1.225	-0.903	+1.399

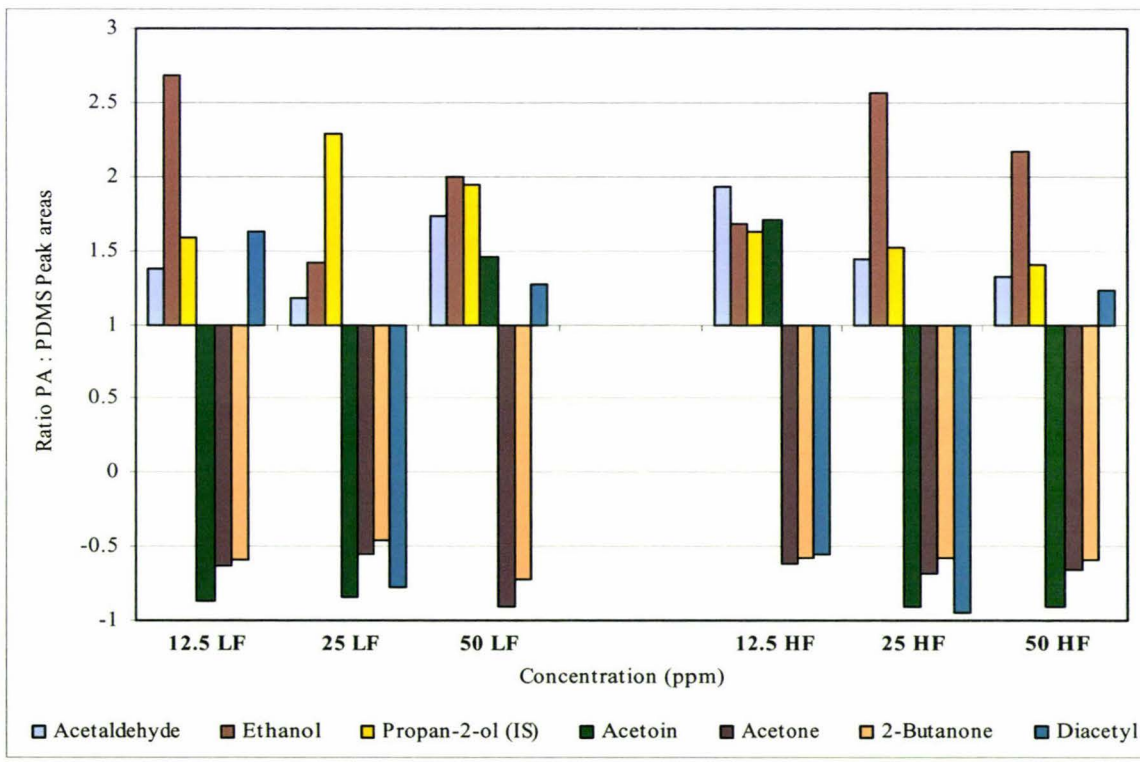


Figure 4.3: Comparison of 100 µm PDMS with 85 µm PA Fibres Using Standard Simulated Yoghurts (all recoveries were scaled to the 100 µm PDMS fibre, which was assigned "1" for comparison purposes).

N.B. LF = Low fat and HF = High fat

Figure 4.3 (page 88) visually depicts the results obtained in Table 4.5 (page 88) for the standard simulated yoghurts. As expected, acetone and 2-butanone were better extracted by the PDMS fibre, while ethanol, propan-2-ol and acetaldehyde were better extracted by the PA fibre. It was difficult to determine which fibre coating adsorbed diacetyl and acetoin better because both fibres seemed to have similar adsorption abilities. This was seen by the varying "+" and "-" signs obtained for these analytes in the simulated yoghurts analysed.

Similar fibre coating preferences were observed for the six analytes in the commercial yoghurts: the 100 μm PDMS fibre was found to adsorb acetone, 2-butanone (the nonpolar analytes) better than the 85 μm PA fibre, which was better at adsorbing the more polar compounds such as acetaldehyde, ethanol, and propan-2-ol. This essentially confirmed the results found for the extracting abilities of the two fibre coatings. Again, it was inconclusive as to which fibre adsorbed diacetyl and acetoin better. The peak area results are shown as a ratio of PA: PDMS in Table 4.6 (below).

Table 4.6: Peak Area Results Obtained Using the PA and PDMS Fibres for Analysis of Commercial Yoghurt Samples (**PA:**PDMS).

N.B. 1. Peak areas are in units^2;
2. Table replicated from Miller and Stuart's (1999) study.

Sample	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin	Propan-2-ol (IS)
NA	**165:**120	**121:**110.5	**45:**35.5	**55:**27.5	**50:**59.5	**626:**613	**861:**484.5
N	**133:**100.5	**120:**145	**27:**38.5	**158:**30	**45:**90.5	**574:**584	**865:**515.5
SC	**262:**125	**108:**163.5	**15:**38	**13:**19.5	**21:**62	**731:**393.5	**927:**559
VB	**164:** 86	**25:**119	**0:**31.5	**64:**43.5	**32:**50	**640:**572.5	**1093:**595.5
M	**173:**112.5	**119:**120	**37:**35	**19:**106	**10:**54.5	**432:**566.5	**845:**546
YHF	**293:**192.5	**39:**93.5	**29:**44	**20:**28	**82:**52	**604:**585	**941:**495
FN	**146:** 95.5	**75:**160.5	**15:**40	**39:**16	**53:**61.5	**777:**846.5	**735:**361.5
WW	**174:**156.5	**136:**150	**27:**59	**68:**20.5	**64:**108.5	**907:**846.5	**532:**405
VA	**106:**74	**128:**167	**15:**48.5	**166:**45	**53:**94	**658:**572.5	**1046:**637
BA	**122:**92.5	**81 :**136	**15:**31.5	**47:**30.5	**49:**75	**406:**488	**620:**5115
B	**131:**109	**105:**108	**0:**24	**48:**14	**79:**110	**168:**516.5	**1054:**536

The same scaling of the PA peak areas to that of the PDMS peak areas was carried out for the commercial yoghurts' analyte concentrations. Again, the "+" sign indicates the better absorbing capability of the PA fibre compared with that of the PDMS fibre, while the '-' sign indicates the opposite. Values equalling "1" show that both fibres have similar adsorbing capabilities. Table 4.7 (below) shows the comparison of extracting abilities of the two fibres for commercial yoghurts (PA peak areas were scaled to that of the PDMS fibre).

Table 4.7: Comparison of 100 µm PDMS with 85 µm PA Fibres Using Commercial Yoghurts (all recoveries were scaled to the 100 µm PDMS, which was assigned "1" for comparison purposes).

N.B. "+" values show that the PA fibre has better adsorbing properties than the PDMS fibre coating and "-" values shows that the PA fiber has worse adsorbing properties than the PDMS fiber coating.

Sample	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin	Propan-2-ol (IS)
NA	+1.375	+1.095	+1.268	+2	-0.84	+1.021	+1.777
N	+1.323	-0.828	-0.701	+5.267	-0.497	-0.983	+1.678
SC	+2.096	-0.0661	-0.395	-0.667	-0.339	+1.858	+1.658
VB	+1.907	-0.21	-	+1.471	-0.64	+1.118	+1.835
M	+1.538	-0.992	+1.057	-0.179	-0.183	-0.763	+1.548
YHF	+1.522	-0.417	-0.659	-0.714	+1.577	+1.032	+1.901
FN	+1.529	-0.467	-0.375	+2.438	-0.862	-0.918	+2.033
WW	+1.112	-0.907	-0.458	+3.317	-0.059	+0.071	+1.314
VA	+1.432	-0.766	-0.309	+3.689	-0.564	+1.149	+1.642
BA	+1.319	-0.596	-0.476	+1.541	-0.653	-0.832	+0.121
B	+1.202	-0.972	-	+3.429	-0.718	-0.325	+1.966

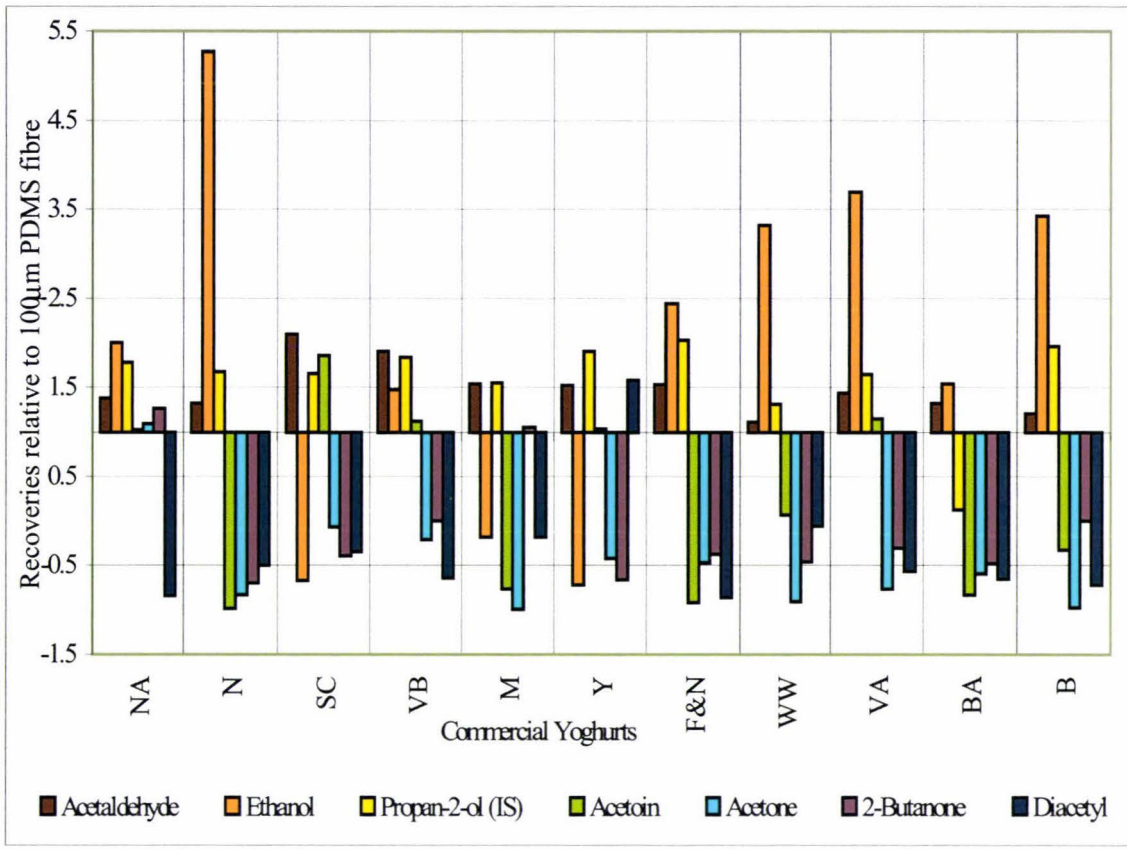


Figure 4.4: Comparison of 100 µm PDMS and 85 µm PA Fibres Using Commercial Yoghurts (all recoveries were scaled to the 100 µm PDMS, which was assigned "1" for comparison purposes).

Figure 4.4 (above) graphically displays the results obtained in Table 4.7. Similar results to those shown in Figure 4.3 (page 88) were obtained.

The 100 µm PDMS fibre was chosen as a result of the work undertaken in this chapter. This choice in fibre coating was also supported by the following citations:

- According to Harmon (1997), the nonpolar 100 µm PDMS fibre can be used for general purposes and provides high sensitivity with most compounds.

- Steffen and Pawliszyn (1996) investigated the presence of various flavour compounds present in orange juice using HS-SPME. They also compared the 85 µm PA with 100 µm PDMS fibre coatings. They commented that the PA coating is made of a crystalline polymer and therefore, analytes would diffuse through it at a

slower rate. This would result in a slightly longer equilibration time and also a longer extraction time.

- Miller and Stuart (1999) compared five different fibre coatings, of which, the 85 μm PA and 100 μm PDMS were two, for extracting volatiles from juice samples. They concluded that a coating combination of a nonpolar and polar coating such as 65 μm PDMS/DVB was most effective in extracting the orange juice analytes. This fibre coating was closely followed by the 100 μm PDMS fibre in extracting power. The work in this study was carried out before Miller and Stuart's results were published. However, it was interesting to note that the 100 μm PDMS was considered second-most effective out of a range of fibre coatings for extracting powers.

Steffen and Pawliszyn (1996) commented that the extraction efficiency could be increased using the "salting out" effect for both fibre coatings (see Section 2.6.2 for more details on this technique). In this study, salt was not added to the yoghurt samples, as it was preferred that the yoghurt samples remained as authentic as possible. Future work in this area could be carried out where the "salting out" effect is investigated. The addition of different salts to the recombined milks or yoghurts and their effects on extraction abilities of different fibre coatings can also be determined. However, it should be noted that excess salt might hinder the bacterial starter cultures during the storage and fermentation processes.

4.4 Conclusion

From this set of experiments, it was evident that both the PA and PDMS fibres adsorbed the six yoghurt volatiles. However, the 100 μm PDMS fibre was selected for the rest of the work carried out in this thesis. It was chosen because it performed satisfactorily in the headspace analyte extractions and because it adsorbed the nonpolar volatiles such as acetone and 2-butanone to a greater extent than the 85 μm PA fibre. It adsorbed the more polar volatiles such as acetaldehyde, ethanol and propan-2-ol to a lesser extent compared to the PA fibre but at a level that was still detectable by GC.

The ES method was proven to be a good analyte quantifying tool, whereas the IS method could only be used as a semi-quantitative tool due to the different response factors of each fibre coating. Hence, the IS method could only be used to normalise the analyte peak areas, which was done throughout the study ensuring no vast variations brought about by the GC and other equipment used.

Having decided that the 100 μm PDMS fibre was to be used for this study, the next step in setting up a working methodology was to determine the minimum adsorption or extraction time for the 100 μm PDMS fibre. This is described in Chapter 5.

CHAPTER FIVE

ADSORPTION TIME PROFILES FOR EXTRACTION OF ANALYTES FROM SIMULATED YOGHURTS USING THE 100 μm PDMS FIBRE.

5.1 Introduction

The second step in setting up the HS-SPME methodology was to determine the minimum time the fibre had to be exposed to the headspace to achieve maximum analyte adsorption. As stated in Section 2.6.2, equilibration is pivotal to the HS-SPME technique. As part of the extraction method, the fibre must be left exposed to the sample headspace to adsorb the volatiles for a set time. This is the time required for the analytes to equilibrate in the three phases (the gas phase or headspace, the condensed phase - liquid or solid sample, and the adsorption phase - the fibre coating) within the sample system.

The time required to reach equilibrium is the optimum volatile adsorption/extraction time. It is therefore, necessary to determine the equilibration time for each analyte of interest. This can be done by plotting an adsorption versus exposure time profile. The time at which the curve levels off to a plateau is the point at which equilibrium within the system has been reached. This turning point is due to the rapid diffusion of analytes in the vapour phase and the slow diffusion of analytes in the aqueous phase (Potter and Pawliszyn, 1992). The extraction time is determined by the diffusion of analytes from the sample to the vapour phase. Therefore, sampling time is short for VOCs due to the rapid diffusion, but relatively long for semi-volatile analytes and longer still, for non-volatile compounds. Hence, equilibration times depend on the volatility of each analyte, which results in a range of turning points on adsorption time plots.

The aim of this section of the investigation was to establish the adsorption time profiles for the six yoghurt analytes: acetaldehyde, acetone, 2-butanone, diacetyl, ethanol, acetoin, and IS (propan-2-ol). This was done to determine the optimum time required

for the 100 μm PDMS fibre to remain in the headspace before reaching maximum adsorption or saturation point.

Similar steps to those of Arthur *et al.* (1992a) were taken to determine the adsorption-time profiles for the volatiles of interest. The fibre was exposed to the headspace above the simulated yoghurt samples for times ranging from 1 to 120 minutes and then desorbed in the GC injector port for 5 minutes at 250°C. The peak areas for each volatile peak at a specific exposure time were then collated and plotted against the exposure times. This was done to determine the turning point on the adsorption time profile, manifested as a plateau on the adsorption curve. The plateau was an indication that the fibre was saturated with analytes from the headspace and equilibrium within the system has been reached. The optimum extraction time is then selected from the adsorption-time profile at any time after the turning point on the curve. It was desirable that the optimum adsorption time was relatively short in relation to the total GC run, but precise and with high sensitivity ensuring an efficient headspace technique.

Various steps such as heating the sample, using a thicker fibre coating, "salting out" and stirring the sample, can be taken to shorten the optimum adsorption time. This investigation did not explore these alternatives. The priority for this study was to set up a methodology for the HS-SPME technique, and then to investigate the analytes present in unadulterated yoghurts.

5.2 Materials and Methods

The 1000 ppm stock solution containing all six analytes and propan-2-ol was made according to the method described in Section 3.6.1. Propan-2-ol was added in order to determine its equilibrium time within the yoghurt system. Hence, propan-2-ol was used here as an analyte instead of as an IS. Two standard milk solutions were made. As outlined in Section 3.7, the required amounts of stock solution were added to low fat milk powder mixtures and then made up to 50 mL with water. The resulting solutions were two low fat (0.3%) milk solutions with analyte concentrations of 10 and 100 ppm. The amounts of each ingredient are shown in Table 5.1 below:

Table 5.1: Quantities of WMP, SMP and 1000 ppm Stock Solution Used to Make the Standard Simulated Yoghurt Samples (10 and 100 ppm) for Plotting the Analyte Adsorption Time Profiles.

Concentration (ppm)	Amount of WMP (g)	Amount of SMP (g)	Amount of 1000 ppm stock solution (mL)
10	0.7	10.38	0.5
100	0.7	10.38	5

2 mL aliquots of each milk were poured into 4 mL glass vials containing 60 µL of concentrated lactic acid as described in Section 3.6.2. Thus, standard simulated yoghurt samples of known analyte concentrations (10 and 100 ppm) were ready for analysis. The sample vials were sealed and left to equilibrate overnight at 5°C. Duplicate samples were prepared. HS-SPME was carried out on the simulated yoghurt samples as described in Section 3.5. The only difference in the sampling methodology was that the fibre was exposed to the headspace above the sample for times ranging from 0 to 120 minutes. At the chosen exposure time, the HS-SPME syringe was transferred to the injector port and desorbed at 250°C for 5 minutes as described in Section 3.5.

The two concentrations (10 and 100 ppm) were specifically chosen because the six analyte concentrations in yoghurts were encompassed within or were slightly below this concentration range (as quoted by various authors in Nijssen *et al.* (1996)). Also, according to Louch *et al.* (1992), the amount of analyte extracted is linearly proportional to the concentration of the analyte in the sample but the extraction time is independent of the analyte concentration. This simplifies the use of HS-SPME, as it does not require adsorption time profiles to be obtained for a range of analyte concentrations in order to carry out quantitative analysis. Therefore, only two concentrations (10 ppm and 100 ppm) were chosen in this study and their adsorption time profiles plotted.

5.3 Results and Discussion

The peak areas obtained for each analyte after the fibre was left in the simulated yoghurt headspaces for the set exposure times are shown in Tables 5.2 (a) and 5.2 (b) below:

Tables 5.2: Peak Areas Obtained for the Analytes after the Set Exposure Times for Standard Simulated Yoghurts (a) 10 ppm and (b) 100 ppm.

(a) 10 ppm Simulated Yoghurt.

Time (min)	Acetaldehyde	Acetone	2-Butanone	Diacetyl	Ethanol	Propan-2-ol	Acetoin
1	141	621	2221	98	107	233	112
2	275	620	2337	103	115	287	190
5	224	686	5432	185	124	344	225
10	195	536	5241	171	142	293	213
20	208	598	5362	207	163	278	191
30	148	544	5669	289	120	257	339
40	121	622	5429	252	158	250	166
50	113	638	5285	207	105	549	245
60	237	820	5433	304	198	269	115
120	273	722	5447	232	138	370	138

(b) 100 ppm Simulated Yoghurt.

Time (min)	Acetaldehyde	Acetone	2-Butanone	Diacetyl	Ethanol	Propan-2-ol	Acetoin
5	1732	6831	29 298	722	737	488	874
10	1897	6643	36 340	605	921	600	947
20	2180	7352	45 398	741	845	928	1210
30	2188	7513	45 637	600	941	824	2154
40	2235	7635	57 428	569	1029	606	1519
50	2335	7954	38 848	1158	1159	581	1402
60	2460	8524	43 878	487	1234	897	1644
120	2288	7060	43662	454	1044	1301	1280

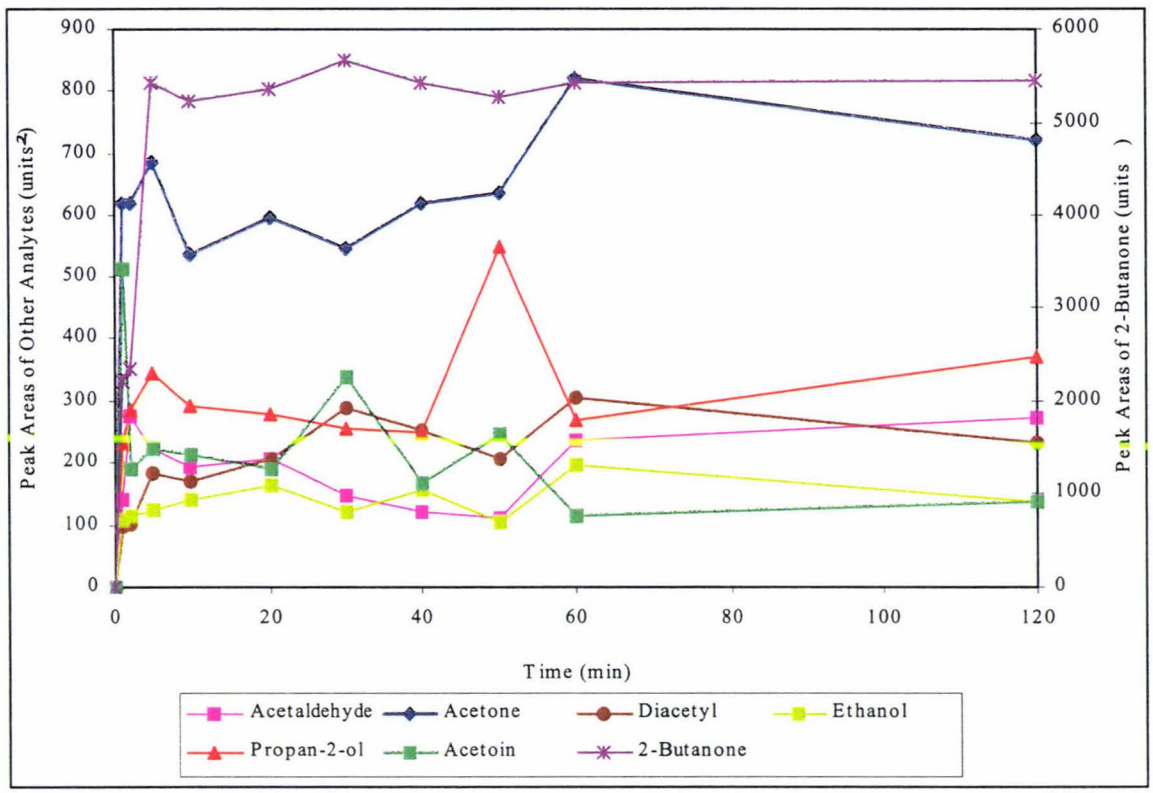


Figure 5.1(a): Adsorption Time Profiles for the 10 ppm Analyte Concentration in Standard Simulated Yoghurts.

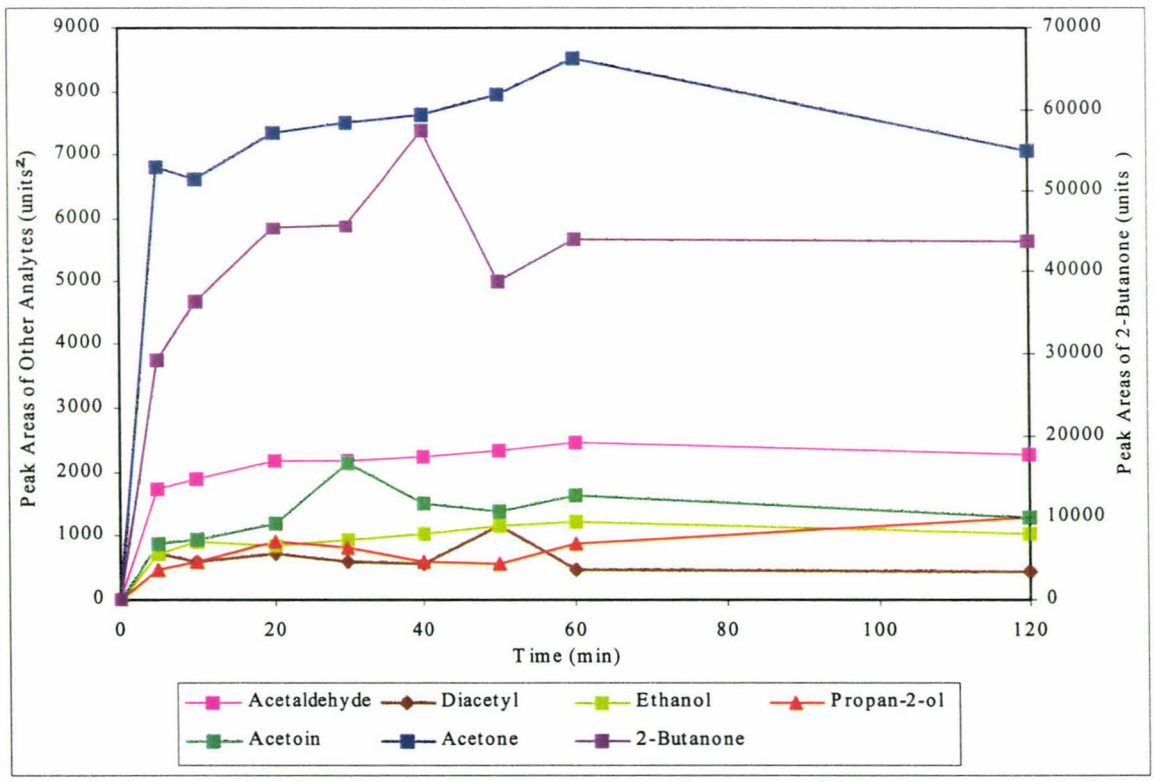


Figure 5.1(b): Adsorption Time Profiles for the 100 ppm Analyte Concentration in Standard Simulated Yoghurts.

From the results (Figures 5.1(a) and (b) - page 99), it was apparent that at an adsorption time of 20 minutes, the great majority of the six analytes and propan-2-ol had reached a state of equilibrium within the sealed sample vial. This was seen as a levelling off of each analyte curve on the adsorption profiles. In fact, it was evident that by 10 to 15 minutes, almost all of the analytes' adsorption curves had reached a plateau. It was decided however, that 20 minutes would be used as the standard adsorption time in the headspace method as it was assumed that the extra time would result in a more complete extraction.

This approach is supported by the work of Gandini and Riguzzi (1997) who used HS-SPME to investigate the levels of methyl isothiocyanate (MITC) in wine. They used a fibre coated with 65 μm partially cross-linked CW/DVB to perform their headspace extractions. The authors chose an extraction time that was a compromise between the times required to perform the analysis and the reproducibility of each analysis. They investigated two extraction times – 5 minutes and 30 minutes. The latter gave the higher detector response and a higher reproducibility and was therefore, chosen as the experimental sampling time. The 20 minute extraction time was chosen in this investigation, as opposed to the 10 minutes, for the same reason.

Potter and Pawliszyn (1994) commented that by choosing a fibre exposure time of between 20 and 30 minutes, the compounds with partition coefficient values (k) less than 10^4 should reach a state of equilibrium within that time. Those compounds with k values above this, won't be at equilibrium but would have ample sensitivity if they were extracted within this period of time. For semi-volatile compounds, a GC run takes longer than 30 minutes and therefore, for convenience and optimum sensitivity, the exposure time can be about the same as the time it takes for the GC to complete a chromatographic run. This was the case with the present study.

The 20 minute extraction time was convenient as it fitted in well with the total time required for each GC run. The GC run in this study took 50 minutes in total, that is, from pressing the start button to commence the temperature programme to the end, where the machine returned to its "ready state", ready for the next sample injection. During the GC run of the first sample, the second sample to be analysed had already been taken out of the refrigerator 10 minutes earlier to equilibrate to room temperature.

Following this, the SPME fibre, which had cooled down from its first desorption in the injector port, was inserted into the second sample's headspace. 20 minutes later, the GC was ready for the second sample injection. At this point, the SPME syringe was transferred to the GC injector port and desorbed of any analytes. The GC run took about 20 minutes thereafter to return to its ready state for the next sample. This sampling process made for a more efficient and effective HS-SPME extraction process, which could be easily automated if required.

Song *et al.* (1997) investigated the flavour-contributing volatile compounds present in apples using HS-SPME. They found that most of the compounds required 6-8 minutes to reach equilibrium, while some compounds such as hexyl-2-methylbutanoate took more than 24 minutes to reach equilibrium. They concluded that the adsorption process was related to the nature and kinetic behaviour of each analyte. Therefore, this must be allowed for in the experimental procedures. They also noticed that a competition between the analytes existed for the limited adsorption capacity onto the fibre. They recommended that HS-SPME could be used as a quantitative tool provided the adsorption time is precisely maintained. With qualitative analysis via HS-SPME, a volatile profile is only required and therefore, adsorption times do not need to be very precise. However, to ensure repeatability and accuracy, they advised that a closely monitored experimental procedure be undertaken. Hence, in order to ensure reproducibility and accurate results throughout this investigation, the experimental conditions were all kept constant and measured accurately. These experimental conditions included leaving samples at room temperature for 10 minutes, adsorption time of 20 minutes, GC run times, desorption time of 5 minutes, injector port (250°C) and detector (260°C) temperatures.

Various methods for reducing this equilibration time within the sample system thereby, reducing the optimum extraction time can be investigated in future work. The methods include using a mixing technique, such as low power sonication or magnetic stirring, to evaluate their effects on the fibre adsorption time. Other recommendations for further work in this area include heating the yoghurt samples and using the "salting-out" method in an effort to lower the fibre adsorption times.

5.4 Conclusion

Simulated yoghurts with the added analytes at known concentrations (10 ppm and 100 ppm) were made and analysed using the HS-SPME technique. The fibre exposure times were varied in order to plot adsorption time profiles for the six analytes and propan-2-ol. The adsorption time plots showed that the maximum adsorption time was around 10 to 15 minutes. However, 20 minutes was chosen as the standard adsorption time for the HS-SPME methodology in this investigation. According to the adsorption time profiles, longer adsorption times were unnecessary, as all the analytes had reached a state of equilibrium within the sealed sample vial.

From Chapter 4 and this chapter, a working methodology for the HS-SPME method was devised. The next step in this thesis was to compare the HS-SPME method with the classical SH (direct injection) technique.

CHAPTER SIX

COMPARISON OF THE CLASSICAL STATIC HEADSPACE METHOD WITH THE HEADSPACE SOLID PHASE MICROEXTRACTION METHOD.

6.1 Introduction

Chapters 4 and 5 of this thesis described the steps taken to set up the HS-SPME methodology for analysing the six yoghurt analytes. The HS-SPME method using the 100 μm PDMS fibre can be summarised as follows:

- Samples were left at room temperature for 10 minutes before headspace sampling commenced.
- The optimum extraction time selected for the 100 μm PDMS fibre was 20 minutes.
- The standard desorption time and temperature for the fibre were 5 minutes at 250°C in the injector port.
- The ES method was used to calibrate the GC method daily and quantify the analytes. The IS method was used to normalise the peak area data obtained.
- The GC temperature programme is set out in Section 3.3 and the analytes were eluted, detected and quantified accordingly.
- The SPME syringe was removed after desorption and left to cool to room temperature before the next sampling.

This chapter involves comparing and evaluating two headspace methods; the classical SH (direct injection) method with the HS-SPME technique. Various headspace techniques were reviewed in Section 2.5. From this, the SH method was chosen for comparison with the HS-SPME method because it has the most in common. They are both equilibrium-dependent, solventless extraction procedures, which only require small sample sizes and not much sample preparation. They both undertake non-exhaustive extractions and are fairly cheap to run compared with other methods such as the DH method, which requires specialised gear.

In order to determine which headspace technique was more sensitive and accurate for analysing six yoghurt volatiles, part of the work carried out by Ulberth (1991) was replicated. The method of Ulberth involved analysing and quantifying five volatiles (acetaldehyde, acetone, 2-butanone, ethanol and diacetyl) in water- and milk-based samples, and cultured yoghurt, using the SH method. Acetoin was not included in this list. In many SH studies done to date, acetoin concentrations have not been directly determined because of its low volatility (BP = 146°C). This chapter looks at using the SH and HS-SPME methods to extract these five analytes plus acetoin, from standard water- and milk-based simulated yoghurts.

Objective 1:

(a) To determine the linear responses (R^2) of each headspace method from the peak area data obtained using a range of known analyte concentrations.

This was done by plotting standard curves of the analyte peak areas for a range of concentration levels (0-500 ppm) using both headspace techniques. Both aqueous solutions and simulated yoghurt samples were analysed. Regression coefficients were calculated from these results and the linear responses of each method were observed.

(b) To determine the sample matrix effects on the linear responses of each headspace technique.

This was done by calculating peak area (A = peak area) ratios [A (simulated yoghurt)]/[A (aqueous solution)] as Ulberth (1991) did. Again, only the peak area results from three sample concentrations (12.5, 50, and 100 ppm were compared in this work.

Objective 2:

(a) To determine the relative sensitivities of the two headspace methods ~~were determined~~ by comparing the peak areas obtained for the 2.5 µL SH sample injection with those of the HS-SPME technique for both water- and milk-based sample. (The 2.5 µL sample injection volume was chosen from Ulberth's (1991) study).

(b) To determine the relative sensitivities of each headspace method (SH or HS-SPME) in relation to the respective peak areas obtained for the water- and milk-based samples ~~were determined~~.

The relative sensitivities of the two headspace techniques were compared at three concentrations (12.5, 50, and 100 ppm). Both techniques were also evaluated for their ease of handling and convenience.

6.2 Materials and Methods

6.2.1 Standard Aqueous Solutions

The 1000 ppm stock solution was prepared as outlined in Section 3.6.1. The stock solution was then diluted down with water to make standard aqueous solutions of known concentrations (0, 5, 12.5, 25, 50, 100, and 500 ppm). Aliquots (2 mL) of each standard aqueous solution were transferred to 4 mL glass vials spiked with 200 µL of 200 ppm IS solution and 60 µL of concentrated lactic acid. The vials were sealed with the resealable butyl rubber septa and open-hole lids. Sampling was carried out in triplicate for each headspace method. Hence, there were six standard aqueous samples (three for SH method and three for HS-SPME method) made for every concentration level (42 samples in all).

After preparation, all the samples were held overnight at 5°C. On the day of analysis, samples were taken out of the refrigerator and left at room temperature for 10 minutes prior to headspace sampling. The headspace techniques for SH and HS-SPME sampling are described in Section 3.4 and 3.5, respectively.

The headspace analyses of the aqueous samples were carried out over a week. As each headspace method had to analyse triplicate samples for each concentration, the two headspace methods were performed alternately as shown below.

Headspace Sampling Procedure:

The order of sampling commenced with the 0 ppm (rep. #1) SH method followed by the 0 ppm (rep.#1) HS-SPME extraction. The 0 ppm (rep. #2) SH method was next followed by the 0 ppm (rep. #2) HS-SPME sampling method. The third replicates were also sampled in this order, SH first and then HS-SPME. This sampling order was followed until all the replicates at each analyte concentration had been analysed. A similar sampling order was used for Section 6.2.2 (the simulated yoghurt samples). The triplicate peak area results were averaged and standard curves of the peak areas versus concentration (ppm) for each analyte were plotted. Regression coefficients were calculated and these gave an indication of the linear responses from the peak area data obtained for each headspace technique

Sampling Order	Analyte Concentration, Replicate Number and Headspace Method Used.
1	0 ppm (rep. #1) SH
2	0 ppm (rep. #1) HS-SPME
3	0 ppm (rep. #2) SH
4	0 ppm (rep. #2) HS-SPME
5	0 ppm (rep. #3) SH
6	0 ppm (rep. #3) HS-SPME
7 etc....	5 ppm (rep. #1) SH etc.....

6.2.2 Standard Simulated Yoghurt Samples

Only low fat (0.3%) simulated yoghurts were investigated in this chapter. Standard milk solutions (50 mL) were made by reconstituting a low fat milk powder mixture (1.4% WMP and 20.75% SMP, derived from simultaneous equations calculated as shown in Appendix I, page 214) with enough 1000 ppm stock solution and water. The resulting simulated yoghurts were standardised to 0.3% fat, 4.5% protein and 12% TSNF. Varying amounts of the 1000 ppm stock solution and water were added to the milk powder mixture to make a series of standard milk solutions with the same analyte concentrations (0, 5, 12.5, 25, 50, 100, and 500 ppm) as the aqueous standards (Table 6.1, page 108). These were then made up to 50 mL with water.

Aliquots (2 mL) of each standard low fat milk solution were added to 4 mL vials spiked with 200 µL of IS solution and 60 µL concentrated lactic acid. Sampling was carried out in triplicate for each headspace method as for Section 6.2.1. The samples were stored, overnight, in the refrigerator at 5°C. The vials were taken out of the refrigerator as required and left to stand at room temperature for 10 minutes before headspace extraction commenced. Both headspace sampling procedures were followed as described in Sections 3.4 and 3.5. The procedure for the order of headspace analysis was carried out according to that outlined in Section 6.2.1.

Table 6.1: Quantities of 1000 ppm Stock Solution and Low Fat Milk Powder Mixture Used to Make Standard Low Fat Milk Solutions.

Concentration (ppm)	Amount of Milk Powder Mixture (1.4% WMP and 20.7% SMP) Added (g)	Amount 1000 ppm stock solution added (mL)
0	11.08	0.0
5	11.08	0.25
12.5	11.08	0.63
25	11.08	1.25
50	11.08	2.5
100	11.08	5.0
500	11.08	25.0

All samples were made up to 50 mL with water

N.B. All peak areas shown in Figures 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8 are in units^2.

6.3 Results and Discussion

The work in this chapter was divided into two sections according to the two objectives:

Objective 1:

6.3.1 Standard Aqueous Solutions

Tables 6.2 and 6.3 (page 110) summarise the results obtained for the standard aqueous solutions using the SH and HS-SPME methods, respectively. Figures 6.1 and 6.2 (pages 111 and 112) show the standard curves obtained for each analyte using the two methods.

Regression coefficients were calculated from the standard curves for the analyte concentrations between 0 and 500 ppm. It was evident from the results obtained that the SH method struggled to detect ethanol and diacetyl (seen by peak area values of 0 units^2) in the 5 to 25 ppm range. Despite this, linear curve fits were carried out for all the yoghurt analytes.

There was some concern that the 100 and 500 ppm peak area results were causing an illusion of linearity where linearity was poor, especially within the 0-50 ppm range. Therefore, two different regression coefficients were calculated. The first incorporated all concentrations (0-500 ppm) for the six analytes, while the second (seen as bold, red numbers in Tables 6.2 and 6.3) used only the 0-50 ppm concentrations. Both regression calculations were carried out to determine whether linearity between the peak areas and concentration was good at the low analyte concentrations (< 50 ppm) and also, whether the regression coefficients were representative of the data obtained.

For the HS-SPME method, the regression coefficients were calculated using 0-500 ppm concentrations and the R^2 values ranged from 0.982 for acetoin to 0.999 for acetone. The values did not change significantly when the 0-50 ppm concentrations were used to calculate R^2 values, which ranged from 0.960 for acetoin and acetaldehyde to 0.999 for acetone. In both regression calculations, as the regression coefficients were very close to 1, excellent linearity for the HS-SPME method was indicated. The HS-SPME method also showed a high correlation between analyte peak areas and analyte concentrations. According to the regression results obtained, the method has good

linear responses for all six analytes including acetoin when extracting from the aqueous solutions.

Table 6.2: Peak Area Results Obtained Using the SH Method to Extract Analytes From Standard Aqueous Solutions.

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	Propan-2-ol (IS) (units ^{2})
0	0	0	0	0	0	No acetoin peak seen.	490
5	390	273	1080	0	0		470
12.5	1250	956	1570	0	0		360
25	1990	1840	2880	0	247		400
50	3820	3070	5 550	273	275		380
100	7350	6310	11220	1260	1320		450
500	38150	30120	54220	6820	6720		340
R^2	**0.999** ***cf*** **0.993**	**0.999** ***cf*** **0.984**	**0.999** ***cf*** **0.983**	**0.996** ***cf*** **no R^2**	**0.997** ***cf*** **no R^2**		**Average= 411.4**

- Regression coefficients obtained using the 0-50 ppm concentration range are shown as bold, red numbers in the R^2 row of table.

Table 6.3: Peak Areas Results Obtained Using the HS-SPME Method to Extract Analytes From Standard Aqueous Solutions.

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	Propan-2-ol (IS) (units ^{2})
0	0	0	0	0	0	0	696
5	230	425	3960	0	209	0	503
12.5	280	1160	5550	155	246	343	369
25	620	2240	10410	195	473	435	346
50	1020	4520	19730	505	890	901	566
100	2040	7610	36420	934	1 940	1 330	271*
500	8 150	37320	151 290	5 630	7 630	4 390	1 000*
R^2	**0.998** ***cf*** **0.960**	**0.999** ***cf*** **0.999**	**0.998** ***cf*** **0.981**	**0.998** ***cf*** **0.964**	**0.997** ***cf*** **0.967**	**0.982** ***cf*** **0.960**	**Average = 536.0**

- '*' denotes a possible error in the peak area integrations.
- Regression coefficients obtained using the 0-50 ppm concentration range are shown as bold, red numbers in the R^2 row of table.

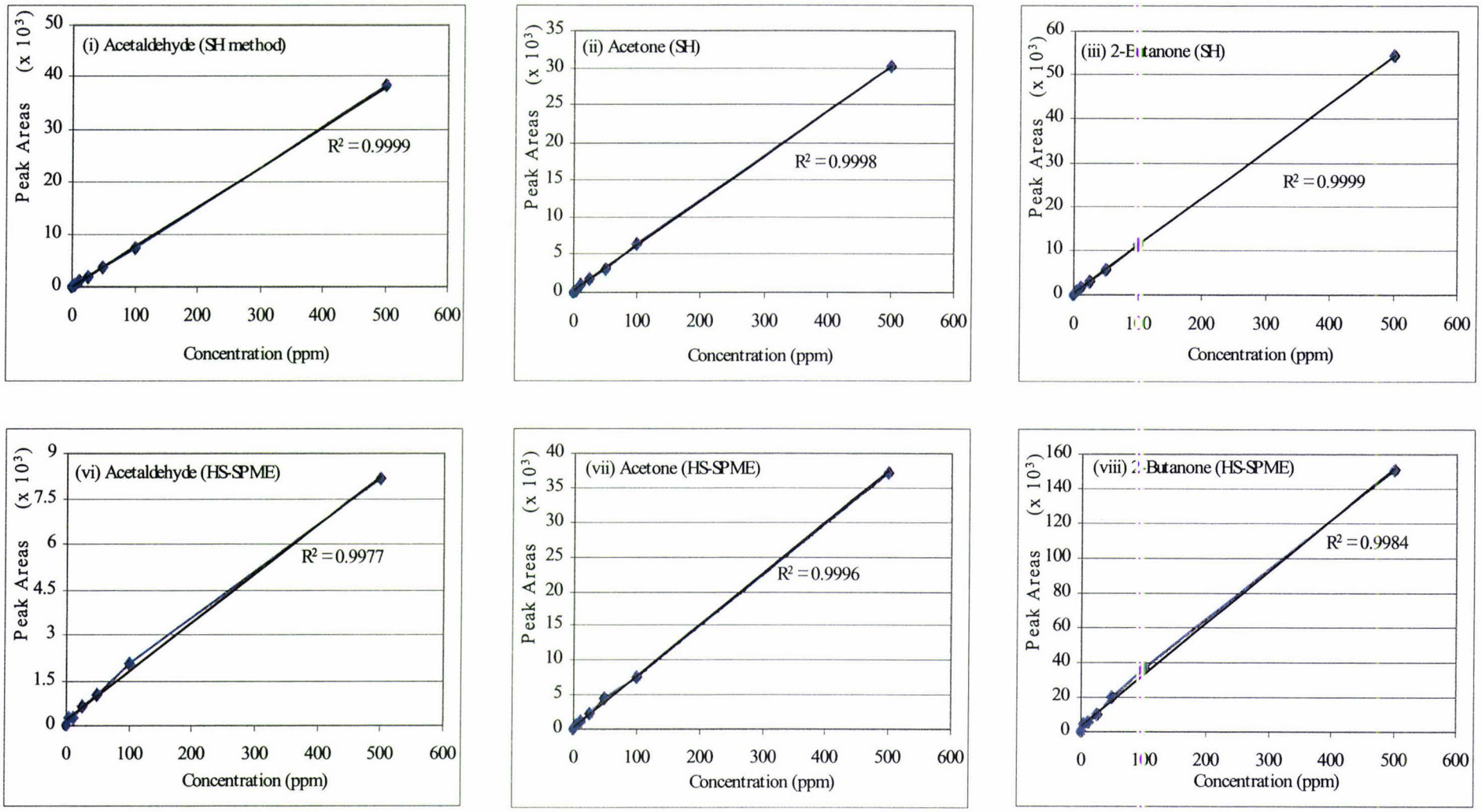
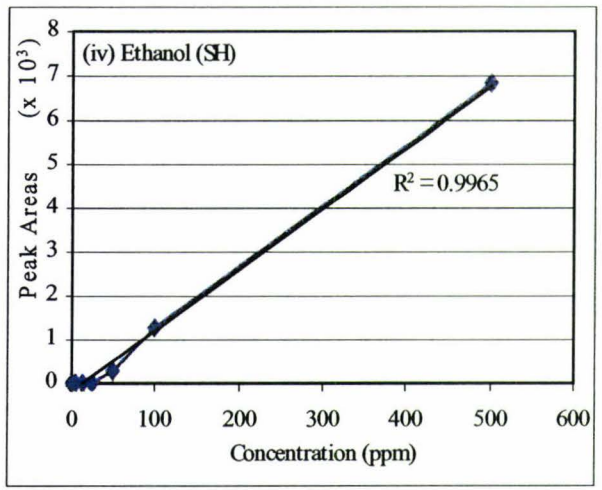
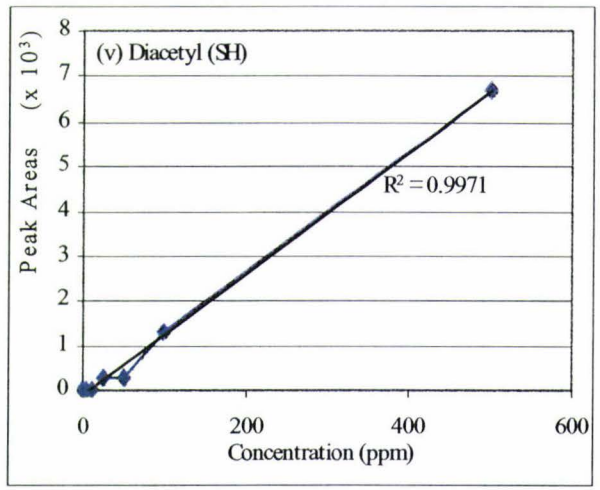
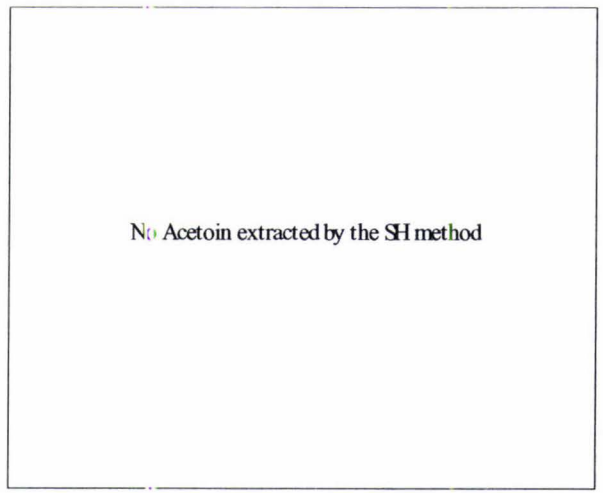
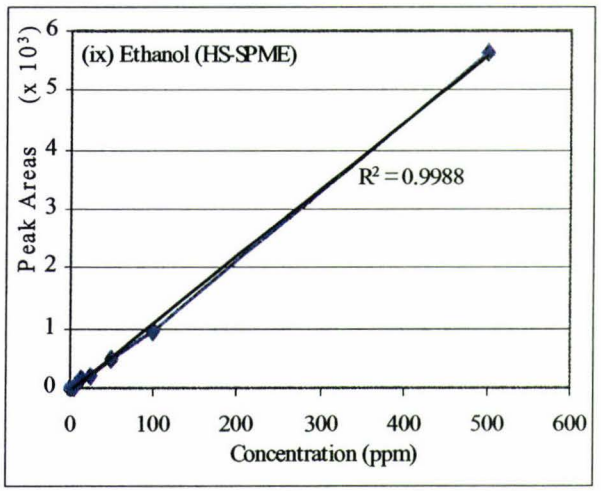


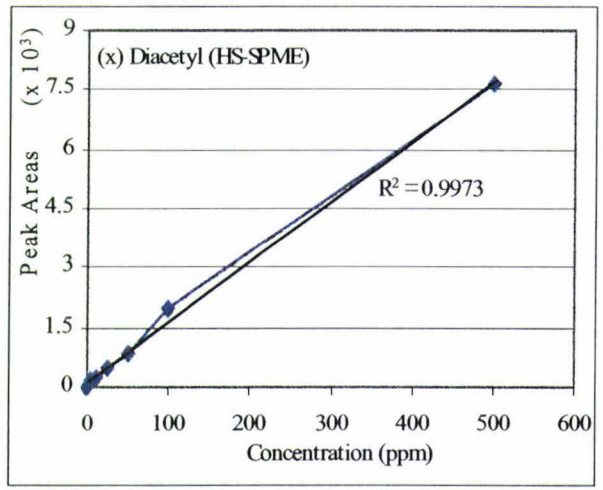
Figure 6.1: Standard Curves for the SH and HS-SPME Methods in Standard Aqueous Solutions.











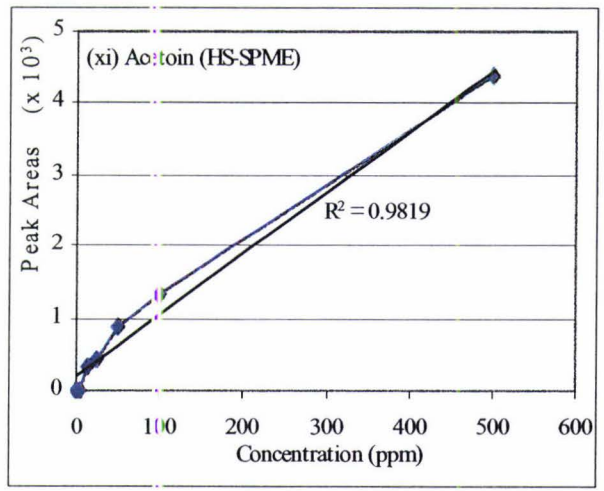


Figure 6.2: Standard Curves for the SH and HS-SPME Methods in Standard Aqueous Solutions.

The calculated R^2 values (0-500 ppm aqueous solutions) for the analyte peak areas using the SH method, ranged from 0.996 for ethanol to 0.999 for 2-butanone, acetone and acetaldehyde. These values were also close to unity suggesting very good linearity for the SH method. This was probably because of their volatility and high partitioning coefficients. No acetoin peaks were observed on the gas chromatogram using the SH method. This was due to acetoin's non-volatile nature (BP = 146°C), which resulted in the SH method being unable to extract it from the headspace owing to very little acetoin being present. As the SH method struggled to detect any ethanol and diacetyl at concentrations below 25 ppm, no regression coefficients were calculated for these analytes in the 0-50 ppm concentration range. The R^2 values calculated using 0-50 ppm concentrations for the other three analytes (acetaldehyde, acetone and 2-butanone) were still close to 1, and ranged from 0.983 for 2-butanone to 0.993 for acetaldehyde. These high R^2 values indicated that the 100 and 500 ppm analyte concentrations did not strongly skew the standard curves.

The IS peaks were ~400 units ^{2} for the SH method and ~500 units^2 for the HS-SPME method. The SH method may have been unable to detect ethanol and diacetyl because of increased solubility in the aqueous phase. This would have accounted for the SH method being unable to extract them from the headspace due to lower analyte concentrations being present. The propan-2-ol (IS) may have also been difficult to extract for this reason but, because of its high concentration (200 ppm), partitioning into the headspace may have been greater, thus enabling the SH method to extract the IS from the headspace.

6.3.2 Standard Simulated Yoghurt Samples

Tables 6.4 and 6.5 (page 114) show the peak area results and the regression coefficients obtained for the standard simulated yoghurt samples using the SH and HS-SPME methods, respectively. Figures 6.3 and 6.4 (pages 115 and 116) show the standard curves obtained for the simulated yoghurts using both headspace techniques with the R^2 values calculated for each plot. From the SH plots, it was again evident that the standard curves for ethanol and diacetyl could not be regressed due to peak area values of 0 units^2 obtained for the 5 to 25 ppm concentrations. Hence, these two analytes were again excluded from the SH 0-50 ppm regression calculations. R^2 values were still calculated for these analytes over the 0-500 ppm concentration range.

Table 6.4: Peak Areas Obtained Using the SH Method to Extract Six Yoghurt Volatiles Present in Standard Simulated Yoghurts.

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	Propan-2-ol (IS) (units ^{2})
0	0	0	0	0	0	No acetoin peak seen.	94
5	170	119	157	0	0		94
12.5	290	300	420	0	0		113
25	400	440	660	0	0		107
50	1070	1160	1690	86	102		86
100	2580	2680	4110	230	200		97
500	12870	12730	19150	840	936		82
R^2	**0.999** ***cf*** 0.973	**0.999** ***cf*** 0.980	**0.999** ***cf*** 0.985	**0.989** ***cf*** no R^2	**0.996** ***cf*** no R^2		**Average = 96.1**

- Regression coefficients obtained using the 50 ppm and lower concentration standard solutions are shown as bold, red numbers in the R^2 row of table.

Table 6.5: Peak Areas Obtained Using the HS-SPME Method to Extract Six Yoghurt Volatiles Present in Standard Simulated Yoghurts.

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	Propan-2-ol (IS) (units ^{2})
0	0	0	0	0	0	0	586
5	0	259	859	0	0	0	650
12.5	85	575	2330	93	96	89	562
25	148	964	39	111	155	134	577
50	360	1990	8070	219	280	272	649
100	841	4180	17600	416	578	443	621
500	3910	20900	88700	2290	2970	1915	862*
R^2	**0.999** ***cf*** 0.986	**0.999** ***cf*** 0.997	**0.961** ***cf*** 0.997	**0.999** ***cf*** 0.942	**0.999** ***cf*** 0.971	**0.997** ***cf*** 0.977	**Average = 643.57**

- Regression coefficients obtained using the 50 ppm and lower concentration standard solutions are shown as bold, red numbers in the R^2 row of table.
- '*' denotes a possible error in the peak area integrations.

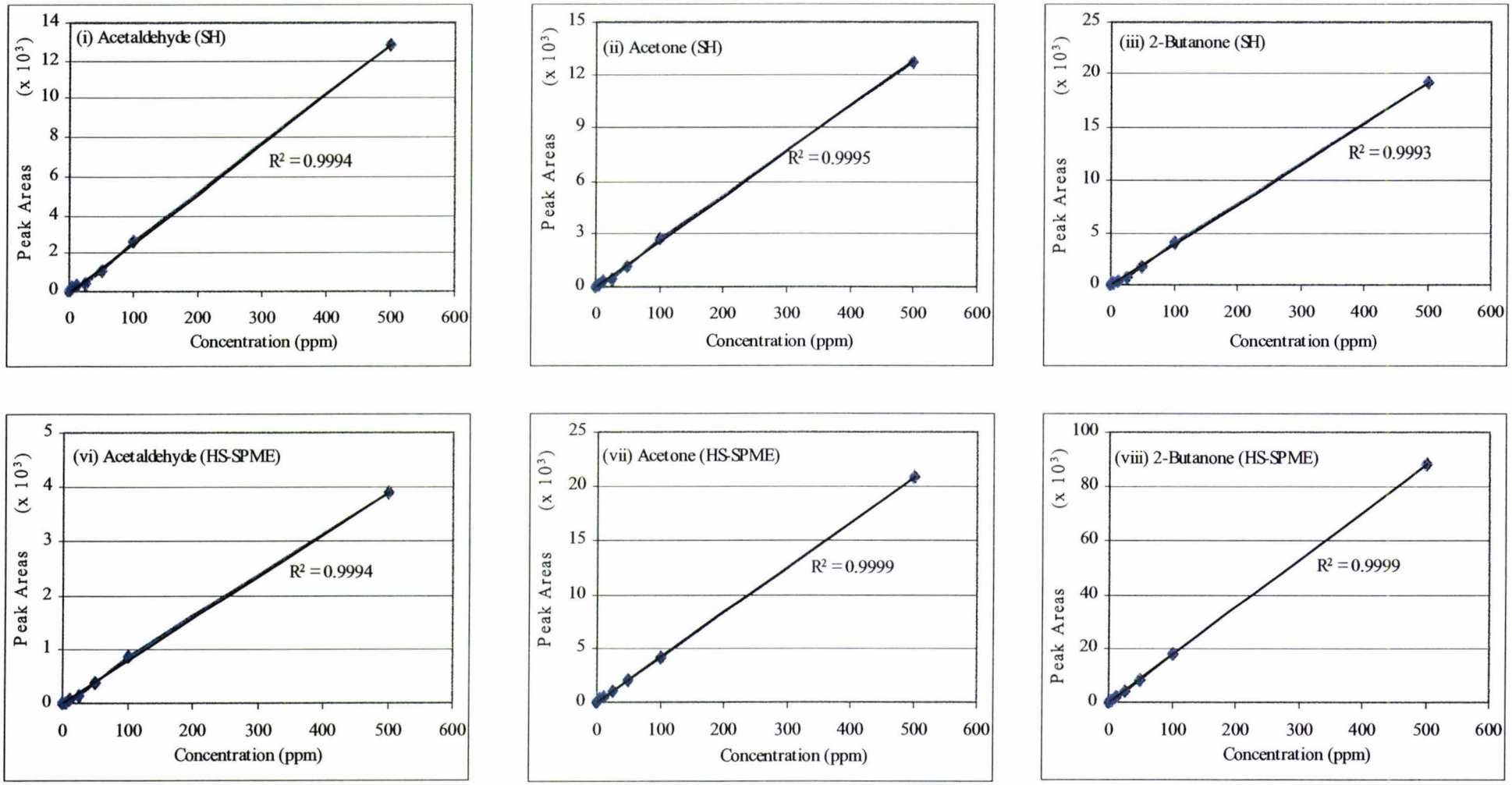
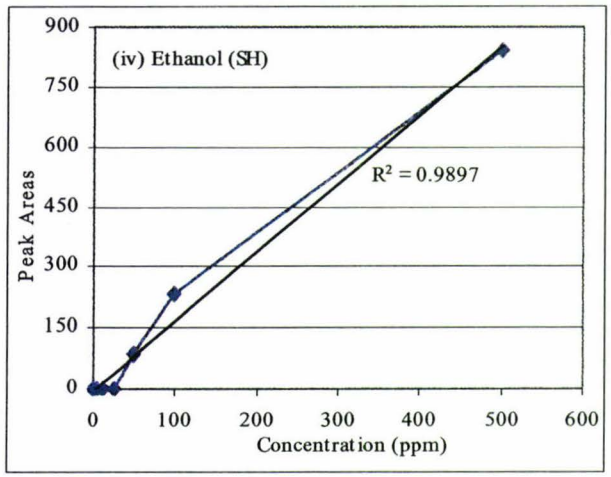
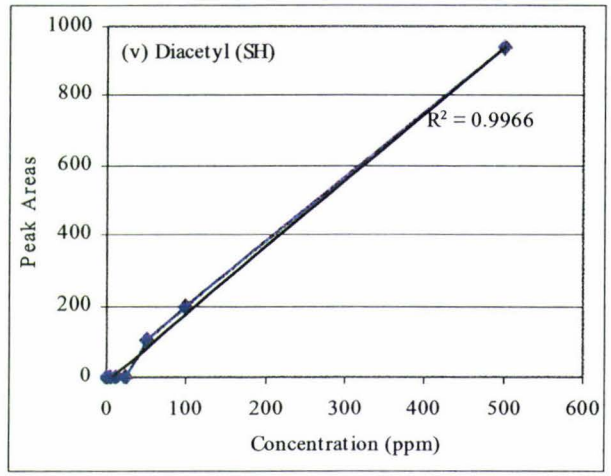
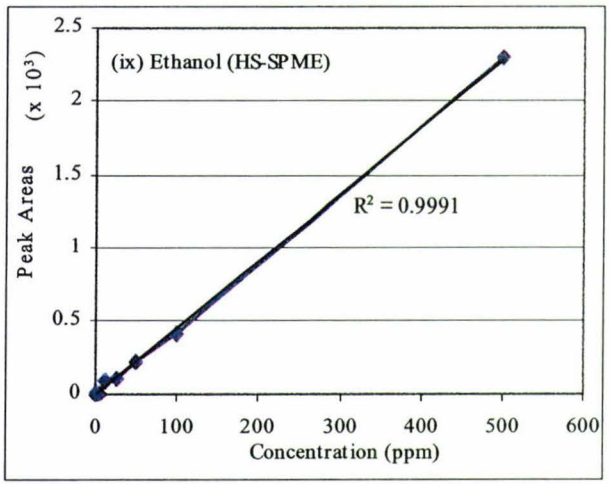


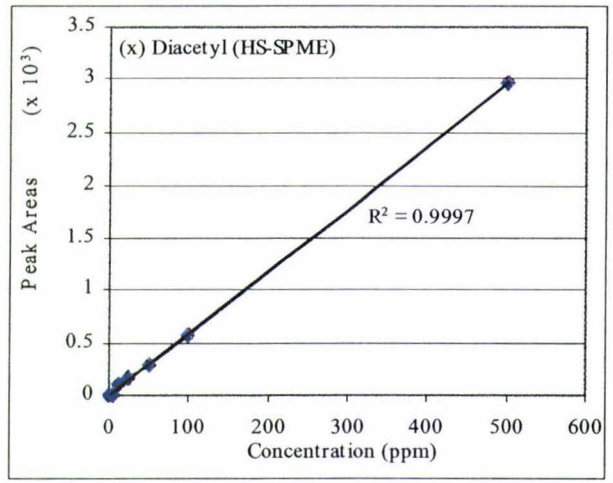
Figure 6.3: Standard Curves for the SH and HS-SPME Methods in Standard Simulated Yoghurts.





No Acetoin peak extracted by the SH method





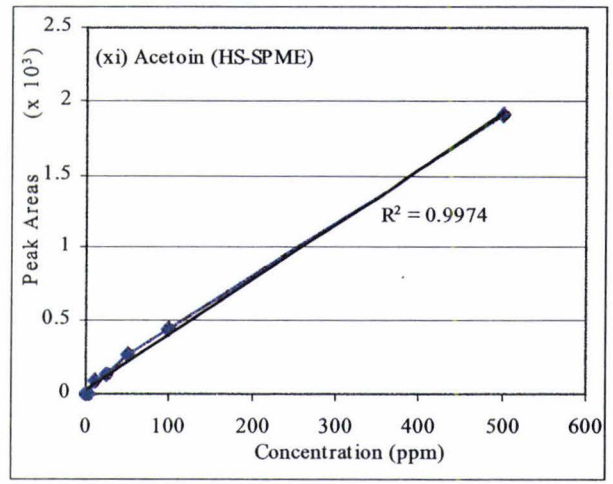


Figure 6.4: Standard Curves for the SH and HS-SPME Methods in Standard Simulated Yoghurts.

It was evident from the results obtained that the simulated yoghurt matrix had had a negative effect on the extraction methods and the analyte concentrations in the headspace above the sample. This was seen by the lower peak areas obtained for the simulated yoghurt analytes using both headspace methods. The peak areas obtained were approximately half those obtained for the aqueous standard solutions. This suggested that the interactions between the flavour volatiles and the simulated yoghurt matrix were stronger than those in the aqueous matrix. The strong matrix effects resulted in less partitioning of analytes (and therefore, smaller partition coefficients), which in turn, resulted in lower analyte concentrations in the headspace. Hence, the lower peak areas were obtained.

As in the case of the standard aqueous solutions (Tables 6.2 and 6.3, page 110), two regression coefficients were calculated. First, regression coefficients were calculated for all seven concentrations (0-500 ppm). These values are shown as bold numbers in the R^2 row of values in Tables 6.4 and 6.5 (page 114). Then, regression coefficients were calculated for the 0-50 ppm concentration range. These R^2 are shown in Tables 6.4 and 6.5 (pages 114) as bold, red numbers in the R^2 row of values. All regression coefficients were comparable to the aqueous standard solutions.

The R^2 values calculated using the 0-500 ppm concentrations for the HS-SPME method were very close to 1. The regression coefficients ranged from 0.961 for 2-butanone to 0.999 for acetaldehyde, acetone, ethanol and diacetyl. Not much difference was seen in R^2 values when the 0-50 ppm concentrations were used (ranges from 0.942 for ethanol to 0.997 for acetone and 2-butanone). Thus, it was decided that for the HS-SPME method, the R^2 values would be calculated using all seven concentrations. Again, acetoin was readily extracted using the HS-SPME method with excellent linearity.

The regression coefficients obtained for the SH method over the 0-500 ppm concentrations were 0.999 for acetaldehyde, acetone and 2-butanone indicating good correlation between peak areas and concentrations for these analytes (high linearity). The SH method did not have any trouble extracting these analytes. The high peak areas obtained at very low concentrations e.g 5 ppm for these analytes were indicative of this. When 0-50 ppm concentrations were only taken into account, the regression coefficients

were lower for these three analytes, but still close to unity, indicating good linearity. The R^2 values ranged from 0.973 for acetaldehyde to 0.985 for 2-butanone. Hence, the regression coefficients for acetaldehyde, acetone, and 2-butanone were calculated using all seven concentrations. Again, the SH method could not detect ethanol and diacetyl in the 5-25 ppm concentration range (seen by peak area values of 0 units^2). Therefore, the 0-50 ppm regression calculations were not used to calculate linear responses for these two analytes. However, linear curve fits were still carried out for these two analytes using all seven concentrations. The R^2 values for ethanol (0.987) and diacetyl (0.997) were also calculated over the 0-500 ppm concentration range. As the R^2 values were close to 1, it was evident that these analytes showed good linearity over this concentration range. Again, acetoin could not be analysed because, due to its non-volatile nature, the SH sampling technique could not extract it.

Ulberth (1991) used the SH method to look at five common yoghurt volatiles (acetaldehyde, acetone, 2-butanone, ethanol and diacetyl). The relative sensitivities of a 1 µL liquid sample injection was compared with a 2.5 µL headspace sample injection of water- and milk-based samples containing analytes at 5 ppm concentration. The author found that the headspace sampling method showed a two- to six-fold increase in sensitivity relative to the liquid sampling for acetaldehyde, acetone and 2-butanone. The headspace method only had a small increase in sensitivity to the liquid sampling for diacetyl. The liquid sampling was superior in sensitivity to the headspace sampling method for ethanol and propan-2-ol. This indicated that these two analytes (ethanol and propan-2-ol) were mostly present in the liquid, more polar phase (semi-volatile). The other analytes being investigated were more volatile, nonpolar and mostly present in the headspace. Ulberth also found that acetoin could not be extracted by the SH method due to its low volatility. This was the case in the present SH investigation.

Similar results to that of Ulberth (1991) were obtained in this study. In the present study, ethanol and diacetyl were hard to detect at concentrations lower than 25 ppm, using the SH method, while propan-2-ol was probably easily extracted from the water- and milk-based samples because it was present at a relatively higher concentration (200 ppm).

Excellent linear correlations between the analyte concentrations and the peak areas were obtained ($R^2 > 0.999$) in Ulberth's (1991) study. In the present investigation, high R^2 values were obtained for all five analytes ($R^2 > 0.999$) using the SH method over the 0-500 ppm range, while ethanol and diacetyl showed very poor linearity when regression coefficients were calculated using the peak areas between 0 and 50 ppm.

The peak areas obtained for the propan-2-ol (200 ppm) in the standard simulated yoghurts were very low (~96 units^2) compared with those obtained for the standard aqueous samples (~400 units^2). It was apparent that the yoghurt sample matrix had significantly reduced the propan-2-ol partitioning to the headspace (lower partition coefficient), which in turn, resulted in lower peak area values.

Ulberth (1991) found the peak areas obtained for the SH method of milk-based samples to be larger than the water-based equivalents. This suggested that there were no matrix effects on the extraction method. The opposite was observed in the present study, suggesting that there were strong matrix effects on the volatiles in the headspace. A possible explanation for the difference may have been due to the fact that Ulberth heated the samples to 60°C. Heating would have increased the vapour pressure, thereby releasing the less volatile organic compounds into the headspace Louch *et al.* (1992). The increased analyte concentration in the headspace would, in turn, increase the amount extracted by the SH method.

Jelen *et al.*, (1998) looked at using the HS-SPME method to analyse some higher alcohols and esters in beer such as isobutanol and ethyl acetate. They compared the technique with an automated SH method. For both methods, six- or seven-point standard curves were prepared encompassing the analyte concentrations found in the beers. Regression coefficients using the two methods were >0.9 suggesting that both techniques had good linear responses to the analytes of interest. The authors concluded that both methods were characterised by high linearity in the concentration ranges investigated. The present study also showed that both headspace methods had excellent linearity for most of the yoghurt analytes investigated. Good replication within the two sampling methods was also found, as was the case with Jelen *et al.*'s investigation. Jelen *et al.* also found the two methods to be highly correlated, which suggested that the HS-SPME method could be used as an alternative to the automated SH method when

analysing beer volatiles. In the present study, however, the results suggested that the SH method was more appropriate for volatile analytes such as acetaldehyde, 2-butanone and acetone, but not for the semi- to non-volatile compounds such as ethanol, diacetyl and acetoin. The HS-SPME method was found to have better linear responses towards the semi- to non-volatile compounds such as diacetyl, ethanol and acetoin. Unlike the SH method, the HS-SPME technique did in fact extract all six analytes readily, with good linearity, even at the concentrations below 25 ppm. From this, the two headspace techniques could be considered complementary to a limited extent. However, it was clear that the HS-SPME method was a superior headspace method, especially in its extracting ability of semi- to non-volatile compounds and its sensitivity.

Miller and Stuart (1999) compared the SH method with HS-SPME methods when analysing the volatile compounds present in various juice samples. The authors found the SH technique quite limited in its extraction ability of certain analytes at very low concentrations. This was the case for ethanol and diacetyl in the present study. Miller and Stuart (1999) found that the HS-SPME method on the other hand, had the ability to concentrate the analytes on the fibre and therefore increase the amount of analyte placed on the GC column. Dramatic increases in the recoveries of the volatile flavours were found when the HS-SPME was used.

Miller and Stuart (1999) concluded that the SH method lacked the sensitivity needed to perform well while HS-SPME was found to be almost three times more sensitive than SH sampling in this case. They commented that this value was large and that other authors noted lesser improvements in sensitivity when comparing the two methods probably because their work dealt with more volatile compounds in simpler matrices. This allowed the SH method to perform better where it almost matched the HS-SPME technique's extracting performance, such as Jelen *et al's.* (1998) findings, which suggested that the two headspace methods were fairly equal in sensitivity. Miller and Stuart also suggested that both headspace techniques could be considered complementary, that is, the HS-SPME method performs the headspace analyses for the semi- to non-volatile analytes, while SH is used for the more volatile compounds.

Page and Lacroix (1993) looked at the application of HS-SPME for the analysis of halogenated volatiles in selected foods. An interesting finding in their study of model

systems was that a 1 μL sample injection of headspace volume using the SH method was progressively more sensitive as the volatility of the analyte increased. That is, the sensitivity of the SH method decreased for the semi- to non-volatile compounds. With HS-SPME however, the method was found to be more sensitive towards the less volatile compounds and this was seen by the increase in analytes extracted with larger masses, lower polarity and lower volatility. They also concluded from their study that the HS-SPME method complemented the SH sampling method in relation to extracting the more volatile compounds, while HS-SPME was more sensitive to the semi- to non-volatile compounds. Similar conclusions were drawn in the present study as it was evident that the SH method had good linear responses towards the more volatile compounds (acetaldehyde, acetone and 2-butanone), while HS-SPME showed higher linearity towards the semi- to non-volatile compounds (ethanol, propan-2-ol, diacetyl and acetoin.

De Haast *et al.* (1978) also found the SH sampling technique most suitable for the more volatile compounds such as acetaldehyde. Similar results were obtained in this investigation where the more volatile compounds such as acetaldehyde (BP = 21°C), acetone (BP = 56°C), and 2-butanone (BP = 80°C) were easily detected using the SH method while ethanol (BP = 78°C) and diacetyl (BP = 88°C) were not. The SH method could not detect the non-volatile compound, acetoin (BP = 146°C), due to its low partitioning coefficient. A possible explanation for 2-butanone to be extracted with greater sensitivity by the SH method, even though it had a higher boiling point than ethanol, is that, 2-butanone, being nonpolar, did not adhere to the sample matrix components (aqueous- or milk-based) to the same extent that ethanol did. The ethanol has increased solubility in aqueous phases compared to 2-butanone.

The results from the present study also suggested that the two methods could be considered complementary. However, it was evident that the HS-SPME method is definitely superior in its extracting/detecting ability compared with the SH method (R^2 values were only obtained for three out of six analytes). Therefore, the HS-SPME was selected for use throughout the rest of this thesis.

Part two of the first objective involved investigating whether sample matrix effects affected the two headspace methods' extracting abilities. Ulberth (1991) claimed that if a peak area ratio [A(simulated yoghurt)/A(aqueous)] exceeded unity (>1), it meant that the detector response of a given analyte increased due to a higher partition coefficient of the analyte between the sample matrix and headspace regardless of the sample temperature. This increased analyte concentration in the headspace results in larger peak areas as more analyte was detected by the headspace method. A value less than 1 indicates that the peak areas observed are influenced by sample matrix effects. The calibration of the methods must then be carried out using standards with similar matrices to the samples being analysed. More of the theory behind matrix effects is discussed in Section 2.6.2.

Table 6.6 (below) shows the peak area ratios obtained for the three concentration levels (12.5, 50 and 100 ppm) using both headspace methods in this investigation. By calculating the peak area ratios, the size of the sample matrix effects for each headspace method could be estimated.

Table 6.6: Peak Area Ratios [A(simulated yoghurt)/A(aqueous)] Calculated for (A) SH and (B) HS-SPME Methods.

(A) SH Method

Conc. (ppm)	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin
12.5	0.228	0.316	0.267	0	0	0
50	0.280	0.379	0.305	0.315	0.371	0
100	0.351	0.425	0.366	0.183	0.152	0

(B) HS-SPME Method

Conc. (ppm)	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin
12.5	0.299	0.497	0.419	0.603	0.390	0.259
50	0.353	0.441	0.409	0.433	0.315	0.301
100	0.412	0.549	0.483	0.445	0.297	0.332

It was evident from the above results that the HS-SPME method was more sensitive than the SH method (seen by the higher peak area ratios obtained for the HS-SPME). Both methods showed a reduced ability to extract analytes from the simulated yoghurt samples compared with the aqueous solutions (peak area ratios <1), which suggested that there were significant matrix effects in the simulated yoghurt samples.

Objective 2

6.3.3 Sensitivities of the Two Headspace Methods

The first part of the second objective in this study was to determine and compare the sensitivities of the two headspace techniques. The results are tabulated as shown in Tables 6.7 and 6.8 (below and page 124, respectively) and graphically presented in Figures 6.5 (page 125) and 6.6 (page 126), respectively.

Table 6.7: Comparison of Peak Areas Obtained for the Standard Aqueous Solutions Using the Two Headspace Methods (A) SH and (B) HS-SPME Methods.

(A) SH Method

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	IS 200 ppm (units ^{2})
12.5	1250	956	1570	0	0	0	360
50	3820	3070	5550	273	275	0	380
100	7350	6310	11220	1260	1320	0	450

(B) HS-SPME Technique

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	IS 200 ppm (units ^{2})
12.5	280	1160	5550	155	246	343	369
50	1020	4520	19730	505	890	901	566
100	2040	7610	36420	934	1940	1330	271

Table 6.8: Comparison of Peak Areas Obtained for the Standard Simulated Yoghurts Using the Two Headspace Methods (A) SH and (B) HS-SPME Methods.

(A) SH Method

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	IS 200 ppm (units ^{2})
12.5	290	300	420	0	0	0	113
50	1070	1160	1690	86	102	0	86
100	2580	2680	4110	230	200	0	97

(B) HS-SPME Technique

Conc. (ppm)	Acetaldehyde (units ^{2})	Acetone (units ^{2})	2-Butanone (units ^{2})	Ethanol (units ^{2})	Diacetyl (units ^{2})	Acetoin (units ^{2})	IS 200 ppm (units ^{2})
12.5	85	575	2330	93	96	89	562
50	360	1990	8070	219	280	272	649
100	841	4180	17600	416	578	443	621

Figure 6.5 (plots i to iii, page 125) shows a comparison of the analyte peak areas obtained using both headspace methods for the standard aqueous solutions at three different concentrations. Figure 6.6 (plots i to iii, page 126) shows similar results obtained for the standard simulated yoghurt samples at the same concentrations.

It was evident that at 12.5 ppm (Figure 6.5 plot i, page 125), all six analytes including the IS were extracted using the HS-SPME method. The SH method, on the other hand, could only extract acetaldehyde, acetone, 2-butanone (most volatile compounds in the samples) and IS. Of these, acetaldehyde was better detected using the SH method compared with the HS-SPME method (peak areas = 1252 units^2 vs 284 units^2, respectively). This was probably due to acetaldehyde's high volatility (BP = 21 °C) and therefore, higher headspace concentration resulting in larger peak areas.

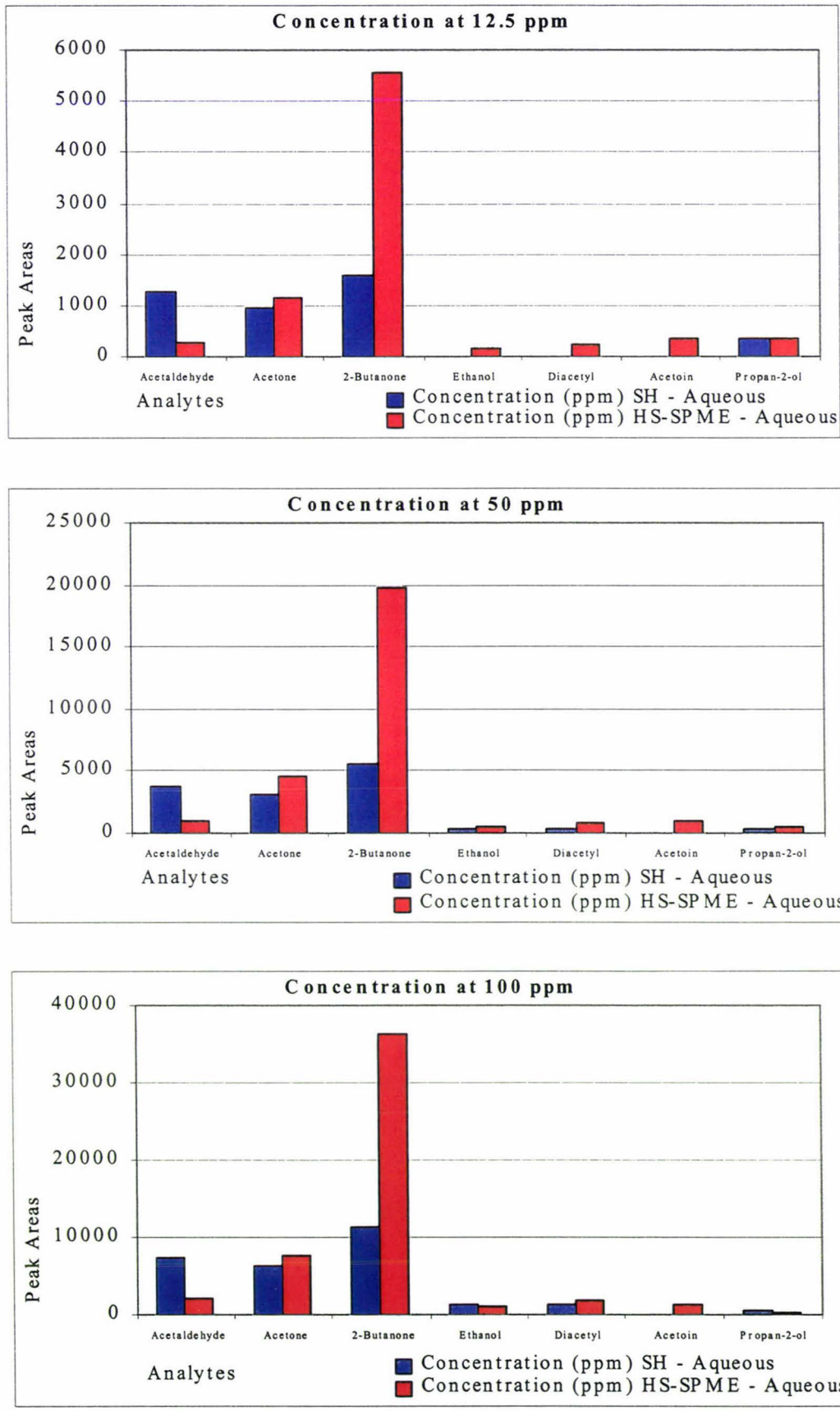
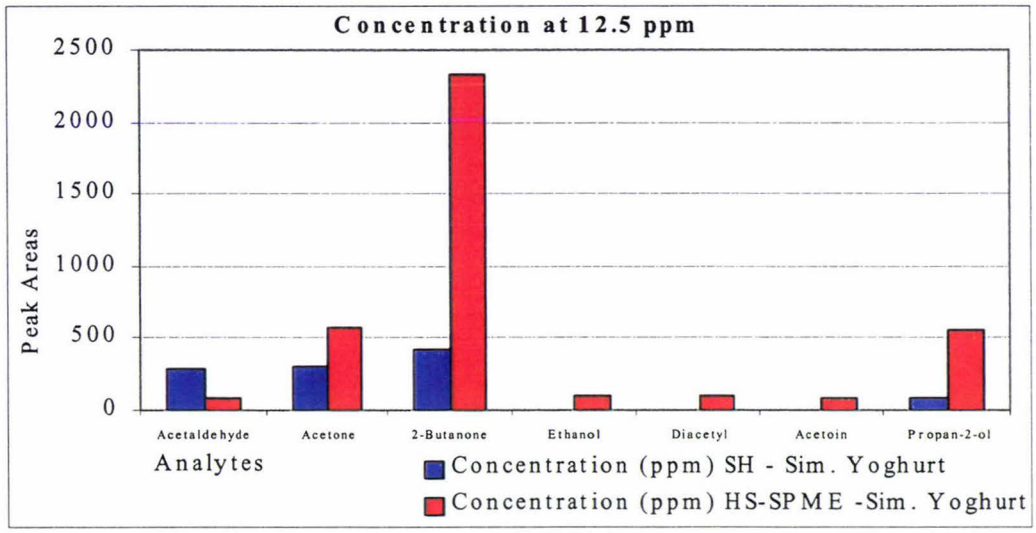
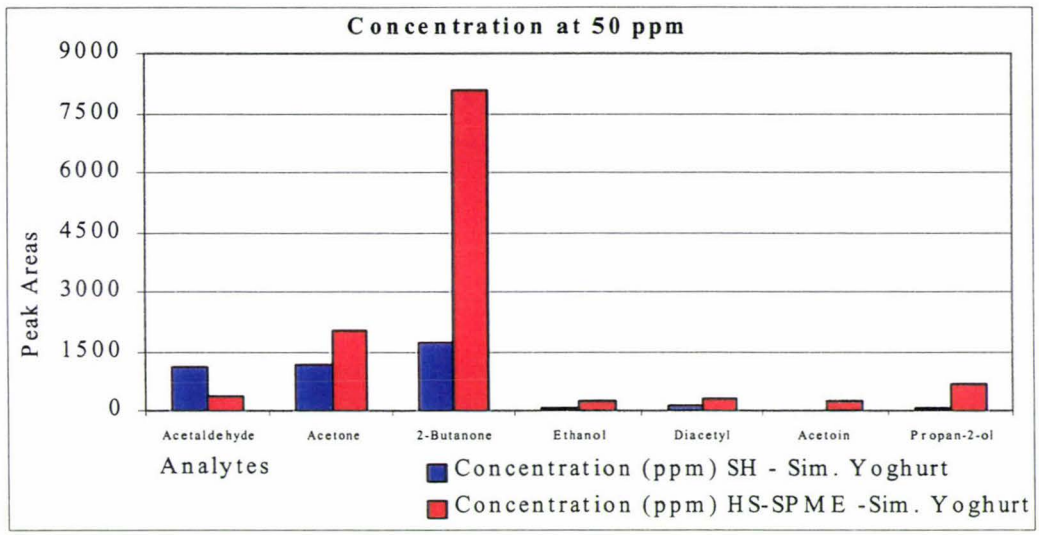


Figure 6.5: Plots Showing the Relative Sensitivities of (SH:HS-SPME) for the Standard Aqueous Solutions (i) 12.5 ppm, (ii) 50 ppm and (iii) 100 ppm.





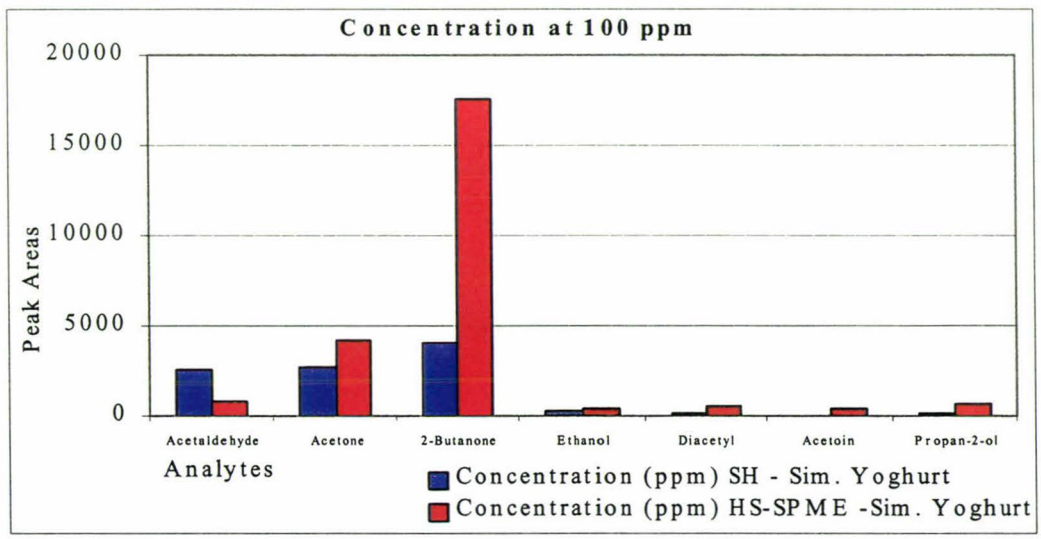


Figure 6.6: Plots Showing the Relative Sensitivities (SH:HS-SPME) for the Standard Simulated Yoghurt Samples (i) 12.5 ppm, (ii) 50 ppm and (iii) 100 ppm.

At the 50 ppm level (Figure 6.5, page 125, plot ii), similar results to that at the 12.5 ppm concentration (HS-SPME) were found. The only differences were for the SH method. In addition to acetaldehyde, acetone and 2-butanone, a little ethanol and diacetyl were also extracted from the sample headspace using this method. The amount extracted was less than the respective values obtained for the HS-SPME method (273 units^2 for SH method vs 505 units^2 for HS-SPME method). A larger amount of acetaldehyde (3822 units^2) was still extracted by the SH method compared with that of the HS-SPME (1019 units^2) technique at this concentration level. At the 100 ppm level in the standard aqueous solution (Figure 6.5, plot iii, page 125), the SH method extracted acetaldehyde and ethanol to a greater degree than the HS-SPME method.

Similar analyte trends to those obtained for the standard aqueous solutions were found for the simulated yoghurt samples (Figure 6.6, plots i to iii, page 126). The only difference was that the peak areas were approximately half the value of those obtained for the aqueous solutions. At the 12.5 ppm level (plot i, page 126), it was noted that the HS-SPME technique could adsorb all six analytes including acetoin and the IS. The SH method could only detect acetaldehyde, acetone, 2-butanone and IS at this concentration and no acetoin peak was seen. The SH method still extracted more acetaldehyde (286 units^2) than the HS-SPME technique (85 units^2). This was probably due to acetaldehyde's high partitioning coefficient as a direct result of its high volatility (BP = 21°C).

A similar trend was seen at the 50 ppm level for the standard simulated yoghurts (Figure 6.6, plot ii, page 126) to that seen for the aqueous samples. The HS-SPME technique extracted all the analytes including IS and acetoin. Once again, the SH method extracted more acetaldehyde (1072 units^2) than the HS-SPME method (360 units^2) and again, acetoin was not extracted by the SH method.

At the 100 ppm level (Figure 6.6, plot iii, page 126), the SH technique only extracted acetaldehyde (2582.5 units^2) to a greater extent than the HS-SPME method (840.5 units^2). This was probably because of its volatile nature and high partitioning coefficient which was not affected much by matrix effects. The other analytes were better extracted by the HS-SPME method (seen by peak areas almost double those obtained using the SH method).

These peak area results suggest that the milk-based samples had strong matrix effects on the release of the volatiles into the headspace compared with the aqueous samples. The reduced headspace analyte concentrations resulted in the headspace techniques extracting less from the headspace, which in turn, produced the lower peak areas. This was especially noticeable in the SH case. These matrix effects were also confirmed by the low peak area ratios calculated for both headspace methods in Section 6.3.2.

According to Yang and Peppard (1994), HS-SPME is an equilibrium extraction process, dependent on experimental conditions and the type of sample matrix used. Any changes in the experimental conditions or sample matrix that affect the partition coefficients will be reflected in the sensitivity and reproducibility of the method. The authors concluded that when using external calibration to quantitate analyte concentrations, it is vital that a sample matrix similar to that being tested is used. This is because the sample matrix has a large impact on what analytes the sample ingredients absorb and what analytes are released into the headspace.

In this investigation, the results suggest that matrix effects had an impact on the linear responses and sensitivities of the two headspace methods. Hence, plain, unsweetened simulated yoghurt samples spiked with known analyte concentrations were used to set up calibration curves as part of the ES method. The simulated yoghurt samples had physical and chemical characteristics similar to the natural, unsweetened yoghurts being investigated throughout this thesis. Voice and Kolb (1994) reiterated Yang and Peppard's (1994) findings that the analyte concentration in the headspace gas injected is directly related to the concentration in the sample by a partition coefficient. They also agreed that similar matrices should be used for the standards (especially for ES calibration) and samples. This was another reason for using a simulated yoghurt matrix that would closely mimic a yoghurt matrix for both external and internal calibration.

From the results obtained, it was evident that the HS-SPME method is a more sensitive headspace sampling method. It was shown to be less affected by sample matrix effects when compared with the SH method. Overall, the results suggest that the HS-SPME technique had no trouble detecting all six analytes from the headspace of both the water- and milk-based samples whereas, the SH method had trouble detecting the analytes from the milk-based samples, especially at low concentrations (<25 ppm).

An additional advantage was that acetoin could be extracted using the HS-SPME method, despite its low volatility. To date, no other method has been developed where acetoin can be extracted so easily.

For the second part of the sensitivity comparisons (Objective2), the peak areas obtained for the standard aqueous solutions (at each concentration level) were compared with the respective standard simulated yoghurt samples in relation to each headspace method. Again, this comparison was carried out to determine what effect the sample matrix has on the extracting power of each headspace method.

Figure 6.7 (plots i to iii, page 130) graphically depicts the peak area differences obtained using the SH method for the aqueous standard solutions and the standard simulated yoghurt samples. Figure 6.8 (plots i to iii, page 131) describes similar results but for the HS-SPME method. From these, it was evident that larger peak areas (often double or more) were obtained for the aqueous standard solutions compared to the simulated yoghurts. This was the case for both headspace methods at all three analyte concentrations.

At the 12.5 ppm concentration level (Figure 6.7, plot i, page 130), only acetaldehyde, acetone, 2-butanone and propan-2-ol were extracted from both types of samples using the SH method. The standard aqueous solution peak areas were a lot larger (three to four times larger) than those obtained for the simulated yoghurts.

Similar results were seen at the 50 ppm level using the SH method (Figure 6.7, plot ii, page 130) except that there were peaks seen for ethanol and diacetyl as well. Again, the peak areas obtained for the standard aqueous solutions were a lot larger (about two to three times larger) than those obtained for the simulated yoghurts. At the 100 ppm level (Figure 6.7, plot iii, page 130), all the analytes were extracted except acetoin and the peak areas were almost two to seven times larger for the aqueous standard solutions than for the simulated yoghurts.

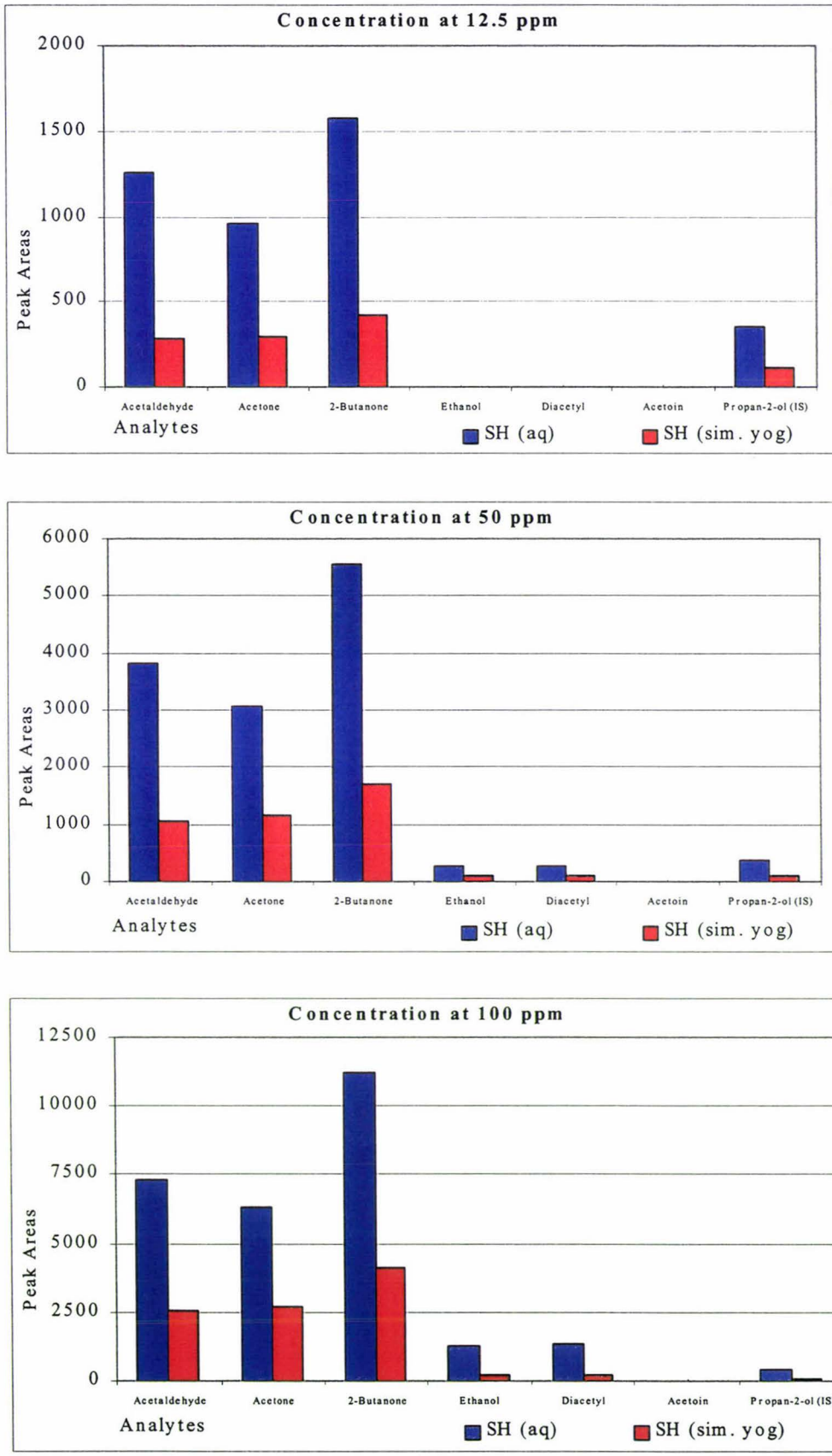


Figure 6.7: Plots Showing the Relative Sensitivities of Aqueous:Simulated Yoghurt Samples Using the SH Method at the (i) 12.5 ppm, (ii) 50 ppm and (iii) 100 ppm Levels.

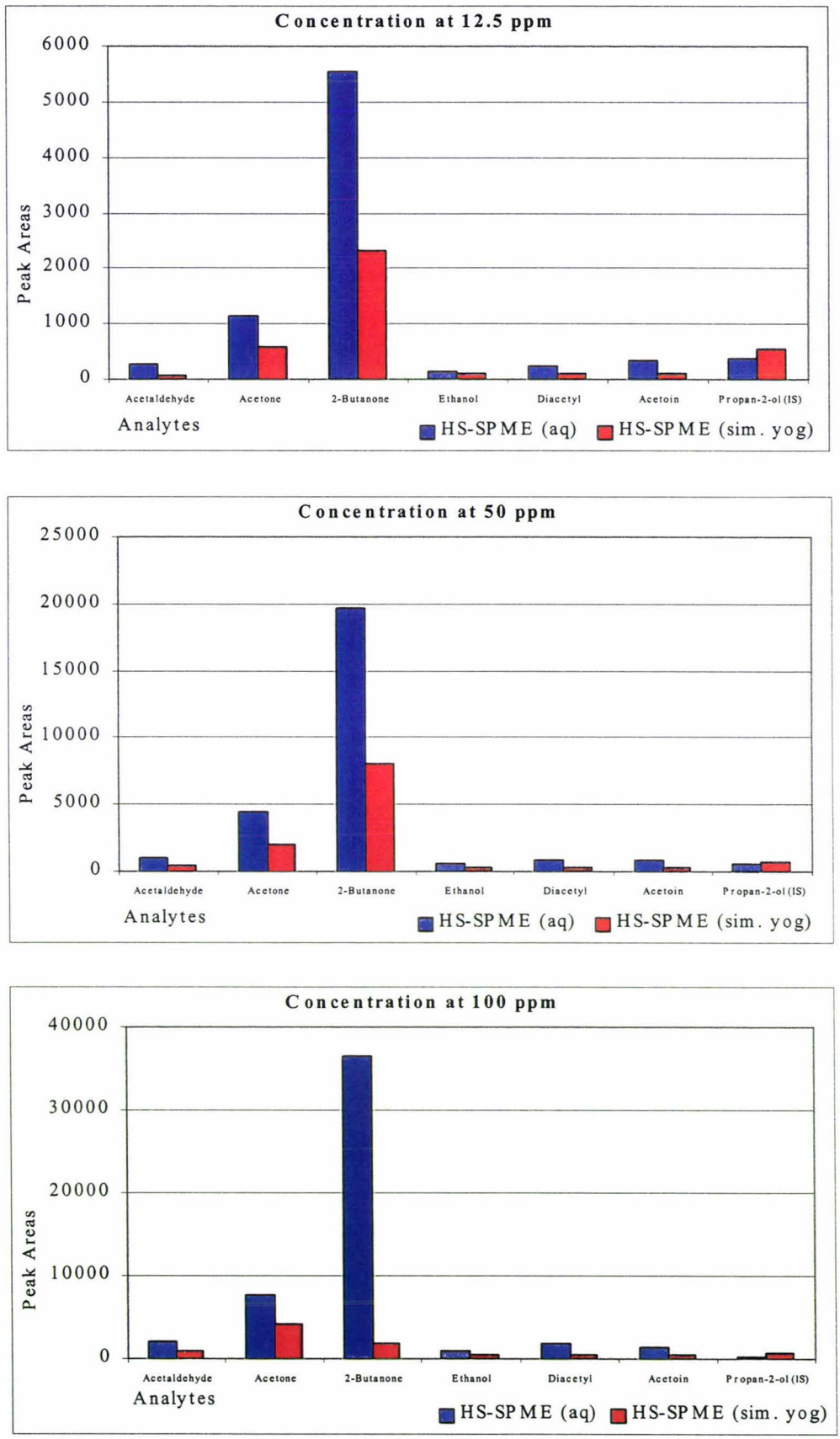


Figure 6.8: Plots Showing the Relative Sensitivities Aqueous:Simulated Yoghurt Samples Using the HS-SPME Method at the (i) 12.5 ppm, (ii) 50 ppm and (iii) 100 ppm Levels.

For the HS-SPME method (Figure 6.8 (i to iii), page 131), the peak areas obtained for all six analytes were larger (almost double) for the aqueous standard solutions compared with those for the simulated yoghurts at all three concentration levels. However, a larger propan-2-ol peak area was observed for the simulated yoghurt than for the aqueous solution. The opposite was expected due to matrix effects preventing volatilisation of the analytes into the headspace, which in turn, would have resulted in lower peak area values. The only plausible explanation for propan-2-ol's behaviour was that it was present in the samples at a very high concentration (200 ppm) in relation to the other analytes. It is possible that because of this high concentration, partitioning of propan-2-ol into the headspace above the aqueous sample was high, resulting in a greater headspace concentration. This may have saturated the fibre coating preventing it from adsorbing more IS, and therefore, less was extracted.

Matrix effects can be reduced by diluting the sample or adding salts to increase the ionic concentration. Heating the sample can result in increasing the partition coefficients of the analytes thereby, releasing more analytes into the headspace. This can reduce the matrix effects in the sample. None of these techniques were carried out in this study as it was thought unnecessary especially since, the ES method with the standard simulated yoghurts was used and successfully quantified the yoghurt analytes. In addition, altering the sample matrix would have altered the composition of the simulated yoghurt samples and therefore, the experimental conditions. This would have resulted in changes in the headspace techniques' extracting abilities and sensitivities.

Miller and Stuart (1999) found that the poor sensitivity of a headspace method could be overcome using similar modes of action as that described by Zhang *et al.* (1994). One suggestion was to heat the sample beyond all the analytes' boiling points forcing them to volatilise into the headspace. However, this can cause other problems especially since most flavour compounds have boiling points greater than 100°C and such heating cannot be used safely with aqueous samples. Another way to enhance the sensitivity of the headspace method is to cryogenically trap and concentrate the analytes in the GC inlet. However, there are problems associated with this as well. For example, when large amounts of water vapour are present in the sample and ice builds up in the inlet.

Page and Lacroix (1993) looked at matrix effects of a mixed vegetable oil and its effects on the HS-SPME extraction of analytes from the oil sample. The recoveries of the analytes obtained were hugely reduced due to the oil competing with the fibre coating for the nonpolar analytes. They found that the less volatile analytes were affected more compared with the volatile compounds. The authors then went on to look at halogenated volatiles in beverages and milks. One of their findings was that the percentage partitioning from the food-water matrix to the fibre coating decreased with the increase in food lipid content. From the results obtained in Chapter 4, it was evident that the two fat levels (0.3% and 3.5%) of the yoghurts investigated in this study, did not have any effect on the extracting ability of the HS-SPME method or presence of analytes in the headspace.

Clark and Bunch (1997) confirmed that sample matrix changes lead to significant differences in signal intensity of analytes with varying structures. They found quite a big difference in signal intensity, when analytes (standards) were added to tobacco instead of to water. Similar findings were seen in the present study where the milk-based solutions produced smaller signal intensities of analytes compared with those of the aqueous solutions.

From this section of work, it was shown that the yoghurt sample matrix had an effect on the analyte concentrations in the headspace above the samples. It may have been that components of the yoghurt matrix, such as proteins and carbohydrates, interact with the flavour analytes and reduce the partitioning coefficients. Thus, this reduces the release of the volatiles into the headspace. This in turn, results in lower peak areas being obtained. Rankin and Bodyfelt (1996) found this in their studies of the effects of compositional ingredients on flavour volatiles. The HS-SPME method proved to be the more sensitive technique, of the two investigated, despite these matrix effects.

6.4 Conclusion

From the results obtained in this chapter, the HS-SPME method was found to have better linear responses towards the analytes investigated compared to the SH method. The HS-SPME method could extract all six analytes even at low concentrations unlike the SH method, which could only detect acetaldehyde, acetone, 2-butanone and

propan-2-ol (IS) in the concentration range investigated. The SH method could not detect ethanol and diacetyl at concentrations below 25 ppm. Acetoin was not detected by the SH method at all due to its low volatility. Both headspace methods showed excellent linearity for the analytes they could detect.

The results suggest that the two headspace methods are fairly complementary and can be used together to ensure all analytes over a range of volatilities are analysed and quantified. However, the HS-SPME sampling method was found to be superior in extracting ability in relation to the SH method. Therefore, it would be the preferred headspace technique to use when analysing for the six common yoghurt volatiles. The HS-SPME technique was used throughout the rest of this investigation.

The HS-SPME method was also found to be the more sensitive technique of the two investigated and less affected by sample matrix effects. Both headspace methods were easy to handle and use, but the HS-SPME method has the additional advantage of being automated easily. Another advantage to the HS-SPME technique is its ability to extract acetoin.

The next chapter uses the HS-SPME method to analyse and quantify the six analytes in laboratory-prepared yoghurts. Milk samples, which had undergone similar treatments to the milks used to make the yoghurts, were also analysed to determine if the six analytes were inherently present or were produced by microbial fermentation.

CHAPTER SEVEN

AN INVESTIGATION INTO THE CONCENTRATIONS OF THE SIX YOGHURT VOLATILES IN PLAIN MILK, ACIDIFIED MILK AND CULTURED YOGHURT.

7.1 Introduction

The cultured flavour of yoghurts and other fermented dairy products depends on microbial action and metabolism (see Section 2.3). However, many of the flavour compounds present in the fermented dairy products are naturally present in fresh milk or are produced from the milk constituents during processing and manufacture (Lindsay, 1967). The work in this chapter investigated the concentrations of the six yoghurt volatiles in a series of recombined, UHT-treated (pasteurised) milks and yoghurts.

Good quality milk has a bland but characteristic flavour. This is due to low concentrations (in the parts per billion (ppb) range) of certain flavour compounds. Heat-treated milks have distinctive "cooked" flavours. The recombined milks used in this thesis were effectively "heat-treated" due to the spray drying of the milk powder ingredients. In addition, the milks were sterilised in UHT equipment (pasteurising at 91°C for 5 minutes). According to Badings (1991), such strong heating would have a large impact on the flavour of milk. Hence, it was assumed that a "cooked" flavour was present in the recombined milks used throughout this thesis, along with caramelised notes and other heat-induced flavour compounds such as dimethylsulphide, diacetyl, 2-pentanone, dimethyl disulphide and 2-hexanone (Calvo and de la Hoz, 1992).

The primary goal of the work reported in this chapter was to investigate the contribution of the milk itself to the six key flavour compounds present in yoghurts. This was to be achieved by detecting and quantifying these volatiles in a series of specially formulated milks and yoghurts. As pasteurised, recombined milks (with their inherent "cooked" flavours) were used to make the natural, set yoghurts throughout this thesis, this same class of milk was used as the basis for preparing the milks and yoghurts in this chapter.

The first factor to be investigated was the effect of the sample matrix on the volatile concentrations. Steffen and Pawliszyn (1996) used HS-SPME to determine the concentrations of specific analytes in orange juice. They found that the complex juice matrix "trapped" some of the target analytes resulting in a decrease in headspace volatiles. Thus, lower analyte concentrations were observed. Such matrix effects also affect the rate and efficiency of extraction by the HS-SPME method. Thus, to investigate these matrix effects, a series of plain and acidified milks were prepared and their headspace volatiles analysed. The plain milks were chosen to represent milk matrices, while the acidified milks were chosen to simulate yoghurt matrices.

The second factor investigated was the effect of fat levels on the volatile concentrations. Page and Lacroix (1993) used HS-SPME to determine flavour compounds present in certain dry foods and beverages such as biscuit mix, white flour, apple juice and orange juice. They found that increased lipid quantities markedly reduced the sensitivity of the headspace method. Thus, low fat (0.3%) and high fat (3.5%) milks were prepared and their headspaces analysed using the HS-SPME method. This was done to test Page and Lacroix's (1993) findings, and to confirm the results obtained in Chapter 4, which suggested that these fat levels (0.3% and 3.5%) did not affect the headspace extraction method.

The third part of the investigation was to see if the step of incubating the milks influenced the concentrations of the flavour volatiles. Thus, some milks were incubated for 5½ hours at 40°C prior to HS-SPME analysis, while control milks were left in the refrigerator for the same time period.

Yoghurt samples were also prepared from the low and high fat milks, to enable direct comparisons of the headspace volatiles in milks and yoghurts.

The standard HS-SPME method described in Section 3.5 was used in this investigation to extract the volatiles from the various milks and yoghurts (see Section 7.2). The analyte concentrations for each milk class were quantified and compared using the ES method and GC calibration as outlined in Section 3.6.

7.2 Materials and Methods

Three-kilogram batches of low fat (0.3%) and high fat (3.5%) milks were made using WMP (1.4%), SMP (20.75%) and water. The milks were standardised to contain ~4.5% protein and ~12% TSNF. The quantities used to make the milks are given in Table 7.1 below:

Table 7.1: Quantities of WMP, SMP and Water Used to Make Standardised Low Fat (0.3%) and High Fat (3.5%) Milks.

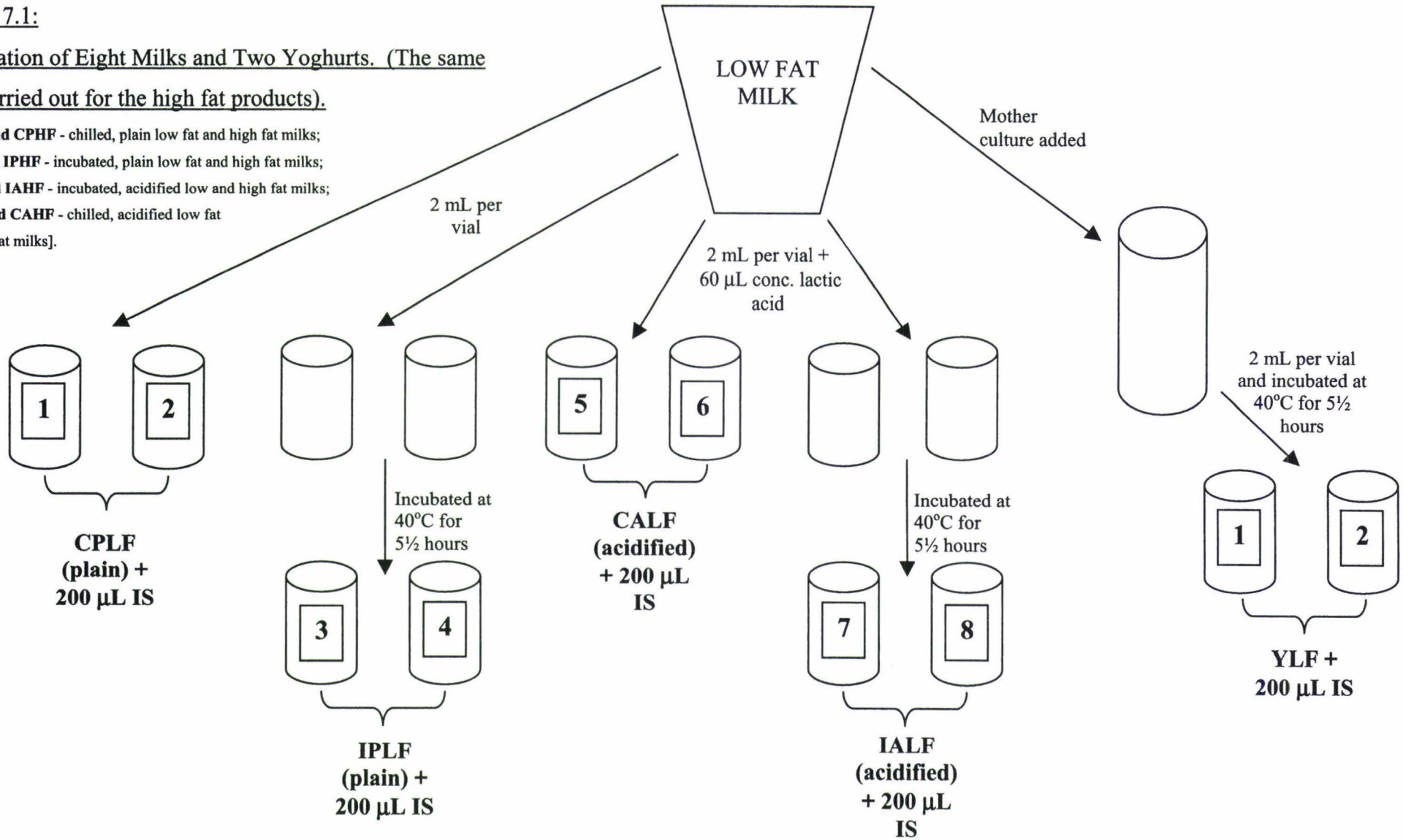
	Low Fat Milk	**High Fat Milk**
WMP (g)	42	766.2
SMP (g)	622.5	151.8
Water (g)	2335.5	2082

The method outlined in Section 3.2 (Parts 2-5) was used to prepare and sterilise the milks. After pasteurisation, the low fat and high fat milks were treated as outlined in Figure 7.1 (page 138) to make a series of eight milks and two yoghurts.

Figure 7.1:

Preparation of Eight Milks and Two Yoghurts. (The same was carried out for the high fat products).

[**CPLF and CPHF** - chilled, plain low fat and high fat milks;
IPLF and IPHF - incubated, plain low fat and high fat milks;
IALF and IAHF - incubated, acidified low and high fat milks;
CALF and CAHF - chilled, acidified low fat and high fat milks].



In the case of the low fat milks, 2 mL portions were placed in eight glass vials. Four vials were "plain", while the other four vials were each acidified with 60 µL of lactic acid. It had been previously ascertained that the addition of 60 µL of concentrated lactic acid to 2 mL of milk was sufficient to lower the pH to ~4.6, which is typical of natural, good quality yoghurts. Of the four plain samples, two were incubated for 5 ½ hours at 40°C while the other two were chilled at 2°C. The four acidified samples were treated similarly. All the sample vials were refrigerated overnight, at 2°C before headspace analysis. The high fat milks were prepared in exactly the same manner.

Yoghurt samples were generated by inoculating a one-kilogram portion of each milk with the mother culture (prepared according to the method outlined in Section 3.2. Inoculated (LF) milk (2 mL) was placed into two glass vials and sealed tight. 15 mL of each milk was also poured into 35 mL Lily portion cups. All four samples including the portion cups were incubated at 40°C for 5 ½ hours. The pHs of the inoculated milks in the portion cups were monitored during the 5 ½ hour incubation period using an Orion pH meter. Once the pH in the portion cups had reached pH 4.6, it was assumed that the inoculated milks in the glass vials had also reached this same endpoint. All four glass vials and portion cups were then refrigerated overnight at 2°C and HS-SPME GC was carried out the following day.

The ES method outlined in Section 3.6.2 was used to calibrate the GC and the standard curve was used to calculate the concentrations (ppm) of the six analytes in the various classes of milk/yoghurts. The results are summarised in Table 7.2 (page 141). As explained in Chapter 4 (Section 4.3), even though 200 µL of the IS solution was added to each sampling vial, the IS method was not used in this investigation. This was because the IS method could not be used to quantify the analyte concentrations. Instead, all the peak areas were normalised using the IS peak area in order to adjust for any variations in the GC method.

7.3 Results and Discussion

The results obtained using the HS-SPME-GC method are summarised in Table 7.2 (page 141). Unfortunately, the chilled, acidified, low fat milk samples (CALF) were accidentally destroyed and there are no results for this set. From Table 7.2 it was apparent that the six analytes of interest were present in both the milks and the yoghurts. However, in some cases, duplicates showed poor agreement, which casts doubt over the validity of some of the values obtained. This makes it difficult to assess the influences of matrices, fat levels and treatment conditions. Due to time and resource constraints, this section of the work was not repeated. It should be noted that repeatability was satisfactory for the yoghurt samples (YLF and YHF).

Some analyte peak areas were very small and the calculated concentrations therefore, very low - parts per billion or less. The GC software could not detect and integrate such small analyte peaks. As a result, some peaks were manually integrated after first, detecting the peak using the known retention times (R_T). This increased the potential for errors in determining the peak areas and thence, analyte concentrations. It may be that the concentration of a number of volatiles in the milks were in a region of borderline sensitivity for this machine and method.

Table 7.2: Concentrations (ppm) of the Six Yoghurt Volatiles Present in the Eight Milks and Two Yoghurts (CPLF, CPHF, CALF, CAHF, IPLF, IPHF, IALF, IAHF, YLF AND YHF).

Analytes	CPLF (plain, chilled)	CPHF (plain, chilled)	IPLF (plain, incubated)	IPHF (plain, incubated)	CALF (acidified, chilled)	CAHF (acidified, chilled)	IALF (acidified, incubated)	IAHF (acidified, incubated)	YLF (cultured yoghurt)	YHF (cultured yoghurt)
pH	6.64	6.74	6.65	6.46	4.55	4.56	4.66	4.64	4.78	4.65
Acetaldehyde (ppm)	3.28	1.38	2.29	0.41	NO RESULTS	1.89	4.04	1.75	6.71	6.33
Acetone (ppm)	0.21	0.18	0.03	2.15		0.31	0.34	0.27	1.12	0.76
2-Butanone (ppm)	0.08	0.12	0.04	0.20		0.09	0.11	0.11	0.12	0.05
Ethanol (ppm)	1.32	4.27	1.07	7.73		2.36	1.65	2.73	5.87	2.00
Diacetyl (ppm)	2.74	2.09	1.24	7.32		2.30	3.80	1.95	6.53	8.02
Acetoin (ppm)	2.18	2.97	1.32	6.53		1.43	1.69	1.43	64.96	88.41

Despite the questions over data validity, some aspects of the data obtained were noteworthy. From the results in Table 7.2, it seemed that there was an increase in acetaldehyde, diacetyl and acetoin concentrations in the yoghurts when compared with the milk samples. It was also evident that all six analytes were present in the various milk samples prior to the fermentation step. This is in keeping with the observations of Ott *et al.* (1997). They found that many of the volatiles in their studies, using the Purge and Trap method, were common to both milk and yoghurt. They also found that some impact flavourings, which were present in milks, were of similar or lower concentration in yoghurts, while other compounds such as acetaldehyde and diacetyl were much higher. Thus, the overall yoghurt flavour is due to milk odour contributors and some specific flavours, which result from starter culture metabolism. Ott *et al.* found that diacetyl, acetaldehyde and 2,3-pentadione were also some of the high impact flavour compounds present in yoghurt. Hence, the increases for acetaldehyde, diacetyl and acetoin observed in this study for the yoghurts were attributed to microbial action on the milk components.

The data in Table 7.2 (page 141), do not point to any matrix effects. This was evident from the similar analyte concentrations obtained for the plain versus acidified milks. Imhof and Bosset (1994) used the DH method and found that milk and fermented milk have very similar matrix effects and that matrix differences between them do not affect headspace analysis. According to their conclusion, variations in analyte concentrations between the milk and simulated yoghurts were not expected.

De Haast *et al.* (1978) made up standard solutions containing nine volatile compounds (acetaldehyde, acetone, ethanol, dimethylsulphide, isopropanol, diacetyl, acetic acid and n-butanol) in different media: (a) aqueous, (b) milk, (c) in the whey after coagulation and separation and (d) in the coagulated milk. They used the SH (direct injection) method to analyse the headspace composition. The results suggested that the recovery of acetaldehyde, ethanol, acetone, and isopropanol from the non-coagulated milk corresponded best with the aqueous standard solutions while the recovery of these volatiles from the whey, corresponded best with the coagulated milk. In contrast to this study, and that of Imhof and Bosset (1994), it was apparent that the type of sample matrix had a significant impact on volatile concentration.

No trends were observed in the data collected from low fat versus high fat milks and yoghurts. This confirmed the results found in Chapter 4 where the effects of fat levels (0.3% versus 3.5%) on volatile concentrations were investigated. According to Page and Lacroix (1993), high levels of fat affect the extracting ability of the HS-SPME method, which in turn, results in lower volatile concentrations. Hence, it was assumed in this case that the 3.5% fat level was not large enough to have an impact on the extracting method. The results in Table 7.2 (page 141) also suggested that incubating the milks at 40°C had no significant effect on the target analyte concentrations.

It must be noted that until the recent development of the HS-SPME method, acetoin could not be measured directly using the purge and trap method or the SH method due to its low volatility. The acetoin peaks for all of the yoghurts analysed in this thesis are large, equating to around 40 - 80 ppm. The acetoin levels quoted Njissen *et al.* (1996) were between 13 ppm and 28 ppm for unsweetened, natural yoghurts (low and high fat). The reason for this increased value is not known. However, the ability of the HS-SPME method to extract the acetoin from the headspace, may explain it in part.

Another possible explanation for the large increase in acetoin levels in the yoghurts is that there might be other compounds co-eluting at the same retention time as acetoin ($R_T = \sim 18.90$ minutes). If this was the case, it could be assumed that this compound(s) was a product of the fermentation process as such a high peak was not visible for the plain milk samples. The best way to investigate this possibility would be to analyse the yoghurts using the HS-SPME with a GC-MS. Unfortunately, GC-MS was not available for this study and therefore, it was assumed that the peak seen at R_T ~18.90 minutes for the yoghurts was solely acetoin.

7.4 Conclusion

This work set out to determine whether the six volatiles ~~were~~ inherently present in milk are a direct result of bacterial fermentation. The results suggested that the six analytes were present in UHT-pasteurised milks, but only at very low concentrations compared to the yoghurts where acetaldehyde, diacetyl and acetoin were found at increased levels.

Three factors - matrix effects, fat levels (low fat versus high fat) and conditions (incubated versus chilled) - were investigated to determine whether these had an effect

on the volatile concentrations. From these results, no distinctive trends were observed suggesting that these factors did not affect the volatile concentrations.

The level of sensitivity for the GC instrument was questionable, especially for analysing the very low analyte concentrations in the milk samples. Therefore, it is recommended that this work be repeated. Also, the use of HS-SPME in conjunction with GC-MS is preferred for accurate identification of the analytes eluting through the GC column.

This investigation of volatile concentrations present in UHT-pasteurised milks and yoghurts was also carried out to test the HS-SPME method in relation to other potential applications for the headspace technique. As this headspace technique is relatively new in this area, it was useful to investigate other avenues for its application.

The next chapter looks at another possible application for the HS-SPME technique. Storage trials were carried out where the yoghurts were stored at 2°C for 24 days and at 2°C and 10°C for the same time period. The six analyte concentrations were analysed using the HS-SPME method and quantified accordingly. Analyte profiles were plotted for the time and temperature variables.

CHAPTER EIGHT

AN INVESTIGATION INTO THE EFFECTS OF STORAGE TIME AND STORAGE TEMPERATURE ON THE SIX ANALYTES IN YOGHURTS.

8.1 Introduction

The aim of this section of work was to use HS-SPME to quantify the flavour volatiles in natural, laboratory-made yoghurts. Low and high fat set yoghurts were investigated in storage time and storage temperature trials. The study was thus divided into two parts:

- A storage time trial where the yoghurts were stored at 2°C for a period of 24 days and
- A storage temperature trial where the yoghurts were stored at 2°C and 10°C for 24 days.

In both trials, the effects of storage time and storage temperature on the six analytes concentrations were investigated. It was envisaged that the results from the storage time study would provide a flavour profile of the six analytes during extended refrigeration. The storage temperature trial would provide a similar profile of the six analytes during storage at 2°C and 10°C.

The term "flavour profile" is not totally correct because the peaks that are observed in a GC chromatogram do not all represent compounds that contribute to the aroma. On the other hand, compounds that are highly potent and contribute to the overall product aroma, may be undetectable by the GC. However, the GC method does give a chromatographically detectable "fingerprint" of the headspace composition, while the aroma gives a physiologically detectable characterisation of the headspace. Even though it is incorrect to state that the headspace composition completely describes the aroma and vice versa, there is much similarity between the two.

McGregor and White (1987) investigated the effect of sweeteners on the acceptability of two low fat yoghurts - plain and Swiss-style. One of their product evaluations

involved the analysis and quantification of three flavour compounds (acetaldehyde, acetone and diacetyl) in yoghurts using the SH-GC method. The yoghurts were stored at 4°C and 10°C over a 24-day period. The authors looked at the storage time and storage temperature variables, independently of each other. The work described in this chapter was similar to the headspace research carried out by McGregor and White. Some key differences are stated below:

- Six flavour compounds were investigated (acetaldehyde, acetone, 2-butanone, ethanol, diacetyl and acetoin), as opposed to three;

- The storage temperature trial was carried out at 2°C and 10°C as opposed to 4°C and 10°C (2°C was selected for this study firstly, because it was the temperature at which the particular refrigerator used was set and secondly, because all the laboratory-made and commercial yoghurts analysed throughout this thesis were chilled at this temperature);

- Based on the total bacterial counts obtained in McGregor and White's (1987) yoghurt study, it was presumed that similar bacterial counts would be obtained in this study. Thus, no bacterial counts of the experimental yoghurts were performed in this study.

  This decision was supported by the findings of Davis *et al.* (1970) and Glattli *et al.* (1974, cited in Hamann and Marth, 1984), who concluded that there were no significant changes in total bacterial counts in yoghurts stored between 0°C and 10°C for up to 40 days. Hamann and Marth found similar results including incubation temperatures and type of milk mixes having very little effect on the survival of the yoghurt cultures during subsequent refrigeration. As the length of the storage trials was well under forty days and the temperature was within 0-10°C in this chapter, it was presumed that the total bacterial count would slowly decrease over the 24-day storage period, but not to a point where bacterial viability was questionable; and

- The headspaces of low (0.3%) and high (3.5%) fat yoghurts were analysed for their volatile concentrations in the storage trials.

It was envisaged that if flavour profiles for the six analytes using HS-SPME could be quantified during storage and plotted, then the results might be used to indicate when yoghurts become ~~unfavourable~~ unacceptable. In order to achieve this however, the HS-SPME analytical data would have to be related to the appropriate sensory data, as observed by the consumer, to extract any useful information about the state of the yoghurts. It is only then, that the HS-SPME analytical data might be used as a guide to determine yoghurt acceptability.

The goal of this thesis was only to develop an HS-SPME methodology to analyse yoghurt volatiles with a view to it eventually being used as a tool in industry to evaluate the quality of the yoghurts based on these flavour volatiles. Therefore, it was important to determine if the HS-SPME method could detect small changes in the individual analytes during a storage period. It was not in the scope of the work in this thesis to relate the flavour profiles (analytical data) obtained to consumer flavour perception of yoghurts (sensory data).

Such an analytical tool would be advantageous, as it could be routinely used to check for "palatability" of yoghurts. If successful, the HS-SPME technique may be used to complement or supplement sensory panels, which are used regularly to screen commercial yoghurts prior to marketing, and savings in time, labour and money could be made.

8.2 Materials and Methods

Two kilogram batches of low (0.3%) and high (3.5%) fat yoghurts were made according to the method described in Section 3.2. The yoghurts were standardised at ~4.5% protein, 0.3 or 3.5% fat and ~12% TSNF. The two batches were made with the appropriate quantities of SMP and WMP as shown in Table 8.1 below.

Table 8.1: Quantities of Water, WMP and SMP Used to Make Two Batches of Standardised Low Fat (0.3%) and High Fat (3.5%) Yoghurts.

Ingredients	Low Fat (0.3%)	High Fat (3.5%)
WMP (g)	28	510.8
SMP (g)	415	101.2
Water (g)	1557	1388

The general sampling procedure outlined in 3.7 was followed for this section. Aliquots (2 mL) of each inoculated milk (low fat and high fat) were transferred to 4 mL glass vials and sealed. Sampling was carried out in triplicate for each fat class. Enough glass vials were filled with the inoculated milks and labelled "Day 0" through to "Day 24". This was done so that headspace analyses could be carried out daily for the 24-day periods of both storage trials.

15 mL of each inoculated milk was also poured into 35 mL Lily portion cups and used for pH determination of the yoghurts. This was done in duplicate and the portion cups were also labelled "Day 0" through to "Day 24". The glass vials and portion cups were then incubated at 40°C for 5½ hours. During incubation, the pH of the yoghurts in the portion cups labelled "Day 0" was monitored using a pH meter. When the pH of the yoghurts had dropped to ~4.6, it was assumed that the yoghurts in the glass vials had also reached this same endpoint. The experimental yoghurts in the glass vials and Lily portion cups were then transferred to the various storage temperatures (2°C or 10°C) for 1 hour before pH measurements and headspace analyses were carried out. The results obtained from this first headspace analysis (directly after incubation and cooling) were designated "Day 0" data. Thereafter, headspace analysis of the yoghurts was carried out on set days as required.

The analyte concentrations were determined using the ES method. For the ES method, 0, 12.5 and 25 ppm standard simulated yoghurts were made as outlined in Section 3.6.2. Duplicate standard samples were analysed daily using HS-SPME to set up a standard curve and to calibrate the GC, before headspace analysis. 200 μL of propan-2-ol (IS) solution (Section 3.6.3) was also added to each sample vial so that it could be used to normalise the peak area data.

The HS-SPME method outlined in Section 3.5 was used to extract the six yoghurt volatiles (acetaldehyde, acetone, 2-butanone, ethanol, diacetyl and acetoin).

Data analysis was carried out using Analysis of Variance (ANOVA) and Tukeys comparison tests. These statistical comparison tests were used to determine significant changes of the analyte concentrations over the storage periods along with fat and temperature effects for the six analytes. Statistical tables for these two statistical tests can be found in Appendix II (page 215).

8.3 Results and Discussion

8.3.1 pH of Yoghurts Over a 24-Day Period

The pH of the low and high fat yoghurts stored in the Lily portion cups was monitored over the 24-day period. This was done in both storage trials to determine the change in pH over the 24 days. The "Day 0" pH reading in both trials was taken after the samples had been cooled at their respective storage temperatures (2°C and 10°C) for 1 hour directly after the 5 ½ hour incubation step. The pH readings were taken at the start of every day assigned for headspace analysis. Tables 8.2 (below) and 8.3 (page 152) show the pH measurements taken during the storage time (2°C) and storage temperature (2°C vs 10°C) trials, respectively, over the 24-day periods.

Table 8.2: pH of Low and High Fat Yoghurts Stored at 2°C Over a 24-Day Period.

Day	Low Fat Yoghurts (pH)	High Fat Yoghurts (pH)
0	4.63	4.61
1	4.58	4.64
2	4.48	4.5
3	4.61	4.63
4	4.57	4.55
6	4.52	4.66
7	4.41	4.32
8	4.44	4.33
10	4.41	4.46
12	4.38	4.38
15	4.40	4.35
17	4.38	4.31
21	4.25	4.25
24	4.18	4.12

Over the 24 days at 2°C, the pH of the low fat yoghurt dropped from 4.63 to 4.18. The high fat yoghurt pH dropped from 4.61 to 4.12.

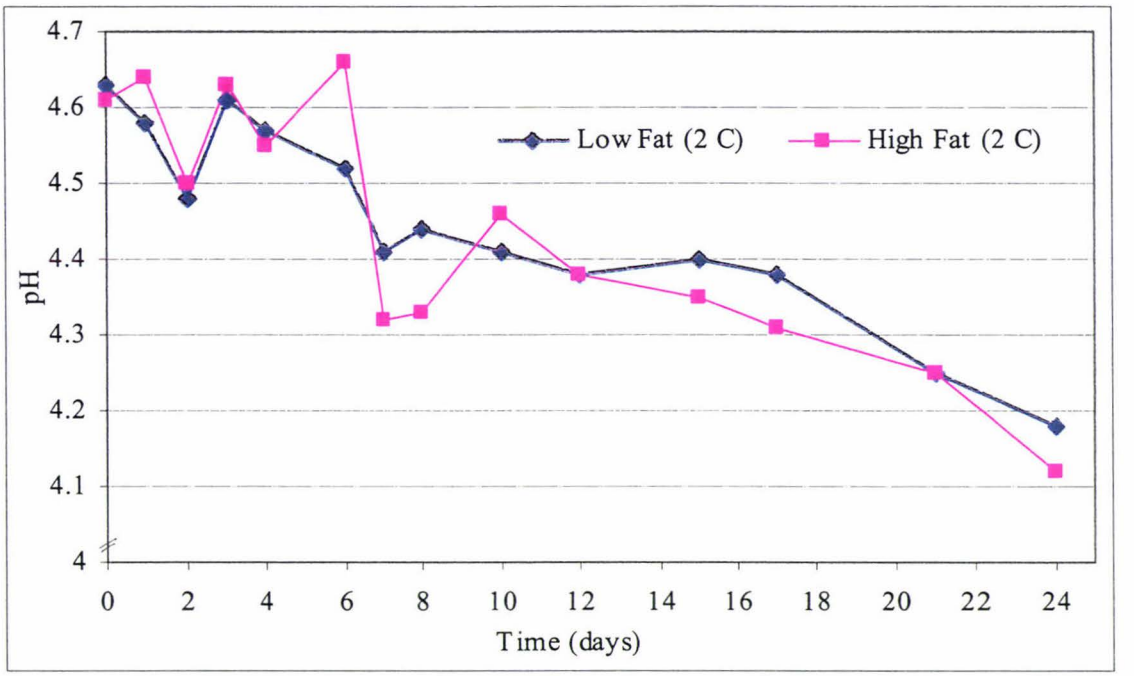


Figure 8.1: Change in pH for Low and High Fat Yoghurts Stored at 2°C Over a 24-Day Period.

From the data, it was evident that the pH of the low and high fat yoghurts was similar. They both showed an overall downward trend indicating that the acidity increased with time. However, the pH values did oscillate a little during the 24-day period. This may have been due to the fact that separate portion cups containing the yoghurt samples (in duplicate) were used to measure the pHs. It should be noted here that a single portion cup containing the yoghurt sample could not be used for pH determination throughout the storage period. This was because the yoghurt structure would have been destroyed (leading to increased syneresis) every time the pH probe was inserted for measuring the pH.

An alternative method of monitoring acidity in yoghurts would have been to measure the titratable acidity (TA) of each sample over the time period. However, due to the numerous yoghurt samples and yoghurt standards that had to be analysed daily using HS-SPME, TA measurements were not a practical option.

The pH measurements obtained for the low and high fat yoghurts stored at 2°C and 10°C over the 24 days are tabulated (Table 8.3, page 152). Figures 8.2 and 8.3 (page 152) depict the pH changes measured during the storage period.

Table 8.3: pH of Low and High Fat Yoghurts Stored at 2°C and 10°C Over a 24-Day Period.

Day	Low Fat Yoghurts (pH)		High Fat Yoghurts (pH)	
	2 °C	10 °C	2 °C	10 °C
0	4.61	4.32	4.56	4.25
1	4.46	4.16	4.41	4.21
2	4.46	4.17	4.52	4.18
3	4.39	4.07	4.50	4.09
4	4.45	4.15	4.43	4.14
5	4.42	4.14	4.50	4.06
7	4.44	4.17	4.42	4.06
9	4.37	4.09	4.35	4.08
11	4.39	4.05	4.43	4.06
15	4.35	4.07	4.33	4.07
17	4.32	4.10	4.29	4.05
23	4.26	4.08	4.25	4.00
24	4.21	4.05	4.19	3.99

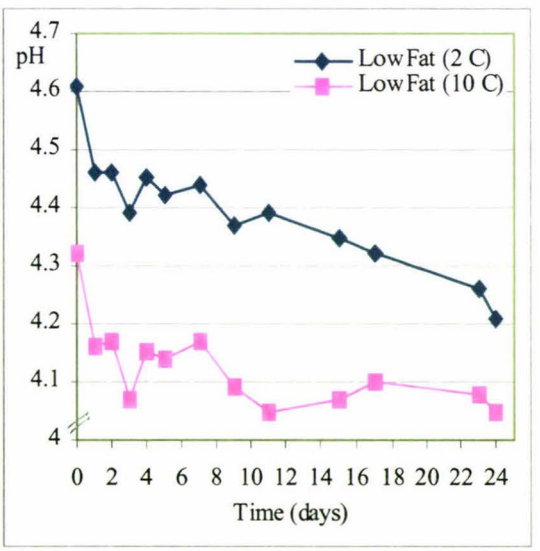


Figure 8.2: Change in pH for **Low Fat** Yoghurts Stored at 2°C and 10°C Over a 24-Day Period.

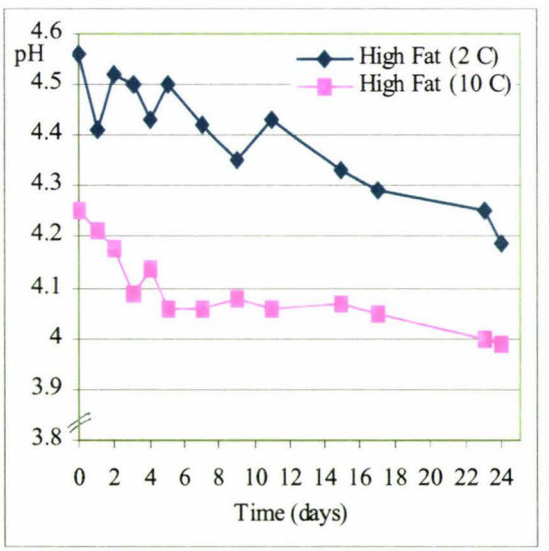


Figure 8.3: Change in pH for **High Fat** Yoghurts Stored at 2°C and 10°C Over a 24-Day Period.

The pH of the low fat yoghurt stored at 2°C dropped from 4.61 to 4.21 over the 24-day period. The high fat yoghurt pH dropped from 4.56 to 4.19. The low and high fat yoghurts stored at 10°C had lower pHs on "Day 0" (4.32 for the low fat yoghurts and

4.25 for the high fat yoghurts). This was probably due to the initial storage temperature (1 hour post incubation) being higher (10°C) resulting in the starter culture's metabolism not slowing down as fast. Hamann and Marth (1984) reported similar results in their study of starter activity in yoghurts. At the end of the storage temperature trial, the low fat yoghurt pH dropped to 4.05, while the high fat yoghurt pH dropped to 3.99.

The storage temperature pH plots were similar to those obtained for the storage time trial. The pH trend was the same for both types of yoghurts; decreasing with time.

8.3.2 The Storage Time Trial - Headspace Analysis of Yoghurts Stored at 2°C Over a 24-Day Period

As explained in Chapters 4 and 7, the IS method was not used in this work to quantify the analyte concentrations. Instead, it was used to normalise the analyte peaks obtained to account for any variations in the GC method during analyses. The ES method was used for quantification. Standard curves of peak areas versus concentrations were drawn for each analyte and from these, the unknown analyte concentrations were calculated. The values obtained for the low and high fat yoghurts are given in Tables 8.4 and 8.5 (page 154) and plotted in Figures 8.4 (page 155) and 8.5 (page 156), respectively.

Even though headspace analyses were carried out on consecutive or alternate days, the tabulated results and graphs shown are only for Days 0, 1, 2, 3, 4, 7, 15, 21 and 24. This was done so that the results from both storage trials could be directly compared on specific days.

(a) Low Fat Yoghurts

Table 8.4: Analyte Concentrations in the Low Fat Yoghurts Stored at 2°C for 24 Days.

Day	Acetaldehyde (ppm)	Acetone (ppm)	2-Butanone (ppm)	Ethanol (ppm)	Diacetyl (ppm)	Acetoin (ppm)
0	23.20	0.50	0.30	5.00	6.60	84.50
1	19.50	0.60	0.30	7.00	5.30	80.60
2	7.20	0.80	0.20	7.30	4.80	73.20
3	12.90	0.60	0.25	3.20	2.40	59.10
4	15.10	0.60	0.10	3.60	4.90	55.70
7	17.60	0.50	0.10	7.20	10.20	49.40
15	16.50	0.50	0.10	5.40	9.80	40.90
21	11.40	0.40	0.10	3.50	7.10	39.40
24	19.10	0.70	0.09	3.50	9.60	37.80

(b) High Fat Yoghurts

Table 8.5: Analyte Concentrations in High Fat Yoghurts Stored at 2°C for 24 Days.

Day	Acetaldehyde (ppm)	Acetone (ppm)	2-Butanone (ppm)	Ethanol (ppm)	Diacetyl (ppm)	Acetoin (ppm)
0	15.40	0.60	0.20	5.00	10.20	78.70
1	24.10	1.00	0.40	7.10	7.80	85.70
2	20.70	0.95	0.20	3.50	9.30	74.60
3	14.40	0.40	0.10	2.30	4.80	71.30
4	17.50	0.50	0.10	4.10	3.70	68.10
7	12.10	1.00	0.10	6.00	13.70	67.20
15	21.20	0.60	0.10	3.20	11.60	56.80
21	10.70	0.70	0.10	6.80	9.80	44.30
24	12.80	1.10	0.10	5.50	10.20	48.10

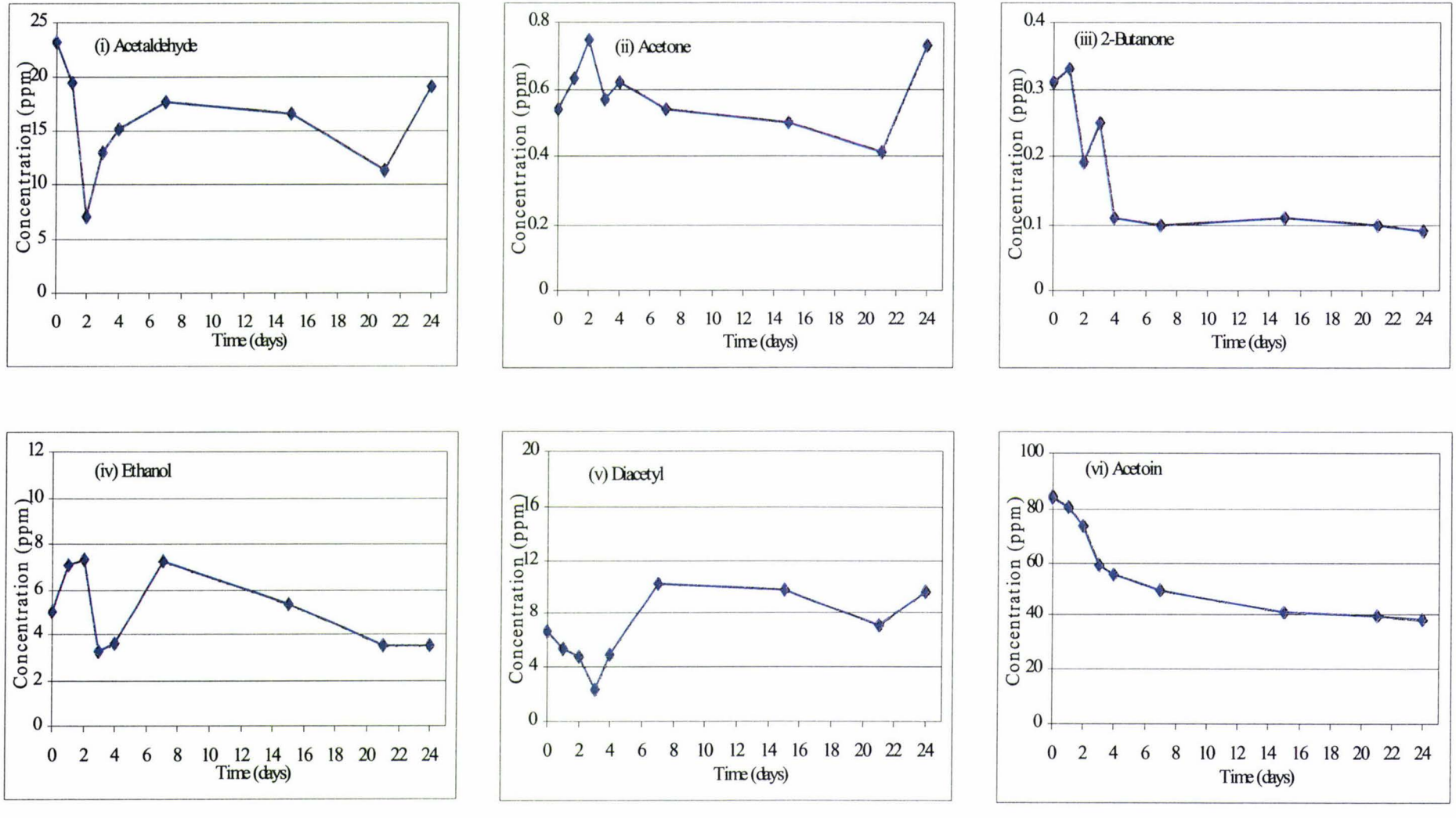


Figure 8.4: Analyte Concentrations in Low Fat Yoghurts Stored at 2°C for 24 Days.

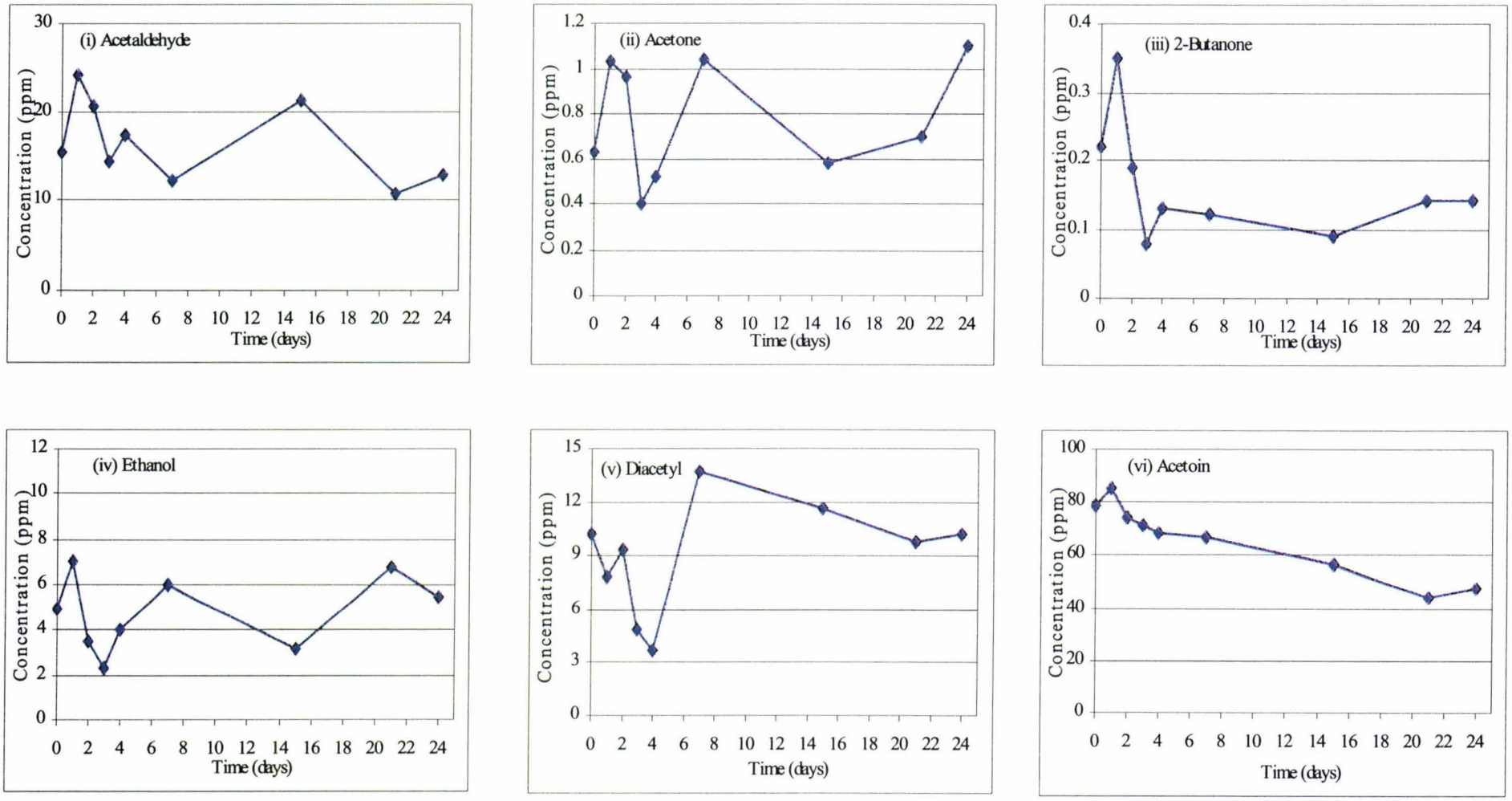


Figure 8.5: Analyte Concentrations in High Fat Yoghurts Stored at 2°C for 24 Days.

From Figures 8.4 and 8.5 (pages 155 and 157, respectively), it was apparent that the analyte concentrations oscillated a fair bit over the 24 days and therefore, no statistical analyses were carried out and only general trends could be drawn from the data plotted.

The general trends seen on the plots in Figure 8.4 (page 155) for the **low fat** yoghurts over the 24 days were:

- Acetaldehyde - no marked change (~15-18 ppm);
- Acetone - no marked change (~0.6 ppm);
- 2-Butanone - gradual decrease from ~0.3 to ~0.1 ppm;
- Ethanol - no marked change (~5 ppm);
- Diacetyl - gradual increase from ~5 ppm to ~9 ppm; and
- Acetoin - decrease in concentration from ~80 ppm to ~40 ppm.

Similar analyte concentration trends were seen for the **high fat** yoghurts stored at 2°C for 24 days (Figure 8.5, page 156).

Even though statistical tests were not carried out on the results obtained in this storage time trial, the general trends seen for acetone and diacetyl were fairly similar to those that McGregor and White (1987) observed. These authors found a significant ($P< 0.05$) difference in diacetyl, which increased between day 0 (1.0 ppm) and day 24 (1.4 ppm) but no change was seen for acetone. In the present study, no marked change was observed for acetaldehyde over the time period. McGregor and White on the other hand, found that acetaldehyde decreased significantly ($P< 0.001$) over the storage period from day 0 (29.9 ppm) to day 24 (22.2 ppm).

As the HS-SPME data obtained for the storage time trial were quite variable, ANOVA and Tukeys comparison tests were not carried out to statistically determine whether the analyte concentrations varied over the storage period. The statistical tests were however, used to determine the effects of fat level on the analyte concentrations during the storage period. Table 8.6 (page 158) shows the Tukeys test results for the low and high fat yoghurts over the 24-day period.

Table 8.6: Tukeys Test Results for Fat Levels in Yoghurts Over 24-Day Period.

Fat Level	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin
	Fat Tukeys Test ($\alpha = 0.05$)					
Low Fat	16.10 [a]	0.56 [a]	0.17 [a]	5.27 [a]	8.10 [b]	55.43 [b]
High Fat	17.90[a]	0.76 [a]	0.18 [a]	5.57 [a]	10.60 [a]	63.45 [a]

"a, b" = means within a column followed by the same superscript letter are not significantly different ($P < 0.05$).

For the fat effects on the analyte concentrations, significant differences were only seen for diacetyl (8.08 ppm for LF vs 10.56 ppm for HF) and acetoin (55.43 ppm for LF vs 63.45 ppm for HF). In both cases, the high fat yoghurts had higher analyte concentrations than the low fat yoghurts. Page and Lacroix (1993) found that the amount of fat in samples did affect the concentration of analyte present in the headspace and therefore, also the extracting ability of the HS-SPME method. As the high fat yoghurts in this study had the higher analyte concentrations, it was concluded that the 3.5% fat level in the experimental yoghurts did not significantly affect the diacetyl and acetoin concentrations in the headspace or the HS-SPME method's ability to extract them. These results confirm the findings in Chapters 4 and 7, where the 3.5% fat level in the yoghurts did not appear to have any effect on the HS-SPME extraction method.

8.3.3 The Storage Temperature Trial - Headspace Analysis of Yoghurts Stored at 2°C and 10°C Over a 24-Day Period.

Tables 8.7 and 8.8 (page 159) show the analyte concentrations (calculated using the ES method) for the laboratory-prepared yoghurts stored at 2°C and 10°C. Figures 8.6 (page 160) and 8.7 (page 161) show the analyte concentrations of the low and high fat yoghurts, respectively, over the 24 days.

(a) Low Fat Yoghurts

Table 8.7: Analyte Concentrations in the Low Fat Yoghurts Stored at 2°C and 10°C for 24 Days.

Day	Acetaldehyde (ppm)		Acetone (ppm)		2-Butanone (ppm)		Ethanol (ppm)		Diacetyl (ppm)		Acetoin (ppm)	
	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C
0	10.60	10.60	2.70	2.70	0.40	0.40	7.10	7.00	5.60	5.60	2.20	2.20
1	27.90	35.60	3.50	2.80	0.50	0.30	9.30	12.00	10.50	10.90	108.00	135.40
2	23.10	20.40	3.40	1.60	0.20	0.20	8.80	11.00	12.70	10.30	73.70	69.20
3	14.50	21.10	1.10	1.30	0.10	0.40	7.60	9.40	5.70	12.10	115.00	106.40
4	18.70	24.00	0.60	1.20	0.20	0.40	4.60	5.95	7.40	12.40	65.30	66.70
7	16.70	15.90	0.90	0.90	0.10	0.10	7.40	5.60	8.10	7.70	102.70	95.43
15	11.80	11.40	0.90	1.00	0.10	0.20	9.00	15.40	6.70	10.50	119.50	135.40
21	16.40	12.30	0.50	0.40	0.20	0.20	5.90	10.70	12.50	12.50	107.70	112.10
24	13.90	14.10	0.90	1.40	0.10	0.20	6.50	9.30	11.20	11.20	89.40	87.00

(b) High Fat Yoghurts

Table 8.8: Analyte Concentrations in the High Fat Yoghurts Stored at 2°C and 10°C for 24 Days.

Day	Acetaldehyde (ppm)		Acetone (ppm)		2-Butanone (ppm)		Ethanol (ppm)		Diacetyl (ppm)		Acetoin (ppm)	
	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C	2°C	10°C
0	19.00	19.00	2.60	2.60	0.10	0.10	4.00	4.00	1.30	1.30	14.80	14.80
1	48.80	46.50	4.10	4.10	0.50	0.30	8.90	8.30	11.80	8.50	120.40	98.70
2	19.60	38.60	3.30	2.20	0.30	0.20	9.70	14.40	8.40	8.60	96.30	75.10
3	13.40	17.40	1.50	1.00	0.10	0.20	6.10	5.40	10.90	8.70	124.80	101.00
4	13.80	17.80	0.60	1.10	0.20	0.30	5.70	5.70	9.50	8.90	87.70	90.50
7	33.70	25.50	0.60	1.50	0.10	0.10	9.60	9.40	9.50	11.70	43.60	43.30
15	8.90	13.40	1.00	1.30	0.20	0.10	3.70	10.60	8.00	9.80	141.40	150.50
21	9.00	11.30	1.00	0.70	0.10	0.30	4.30	6.10	5.60	6.40	106.90	104.70
24	11.90	8.60	1.10	1.50	0.20	0.10	6.40	5.70	7.80	10.10	93.00	88.50

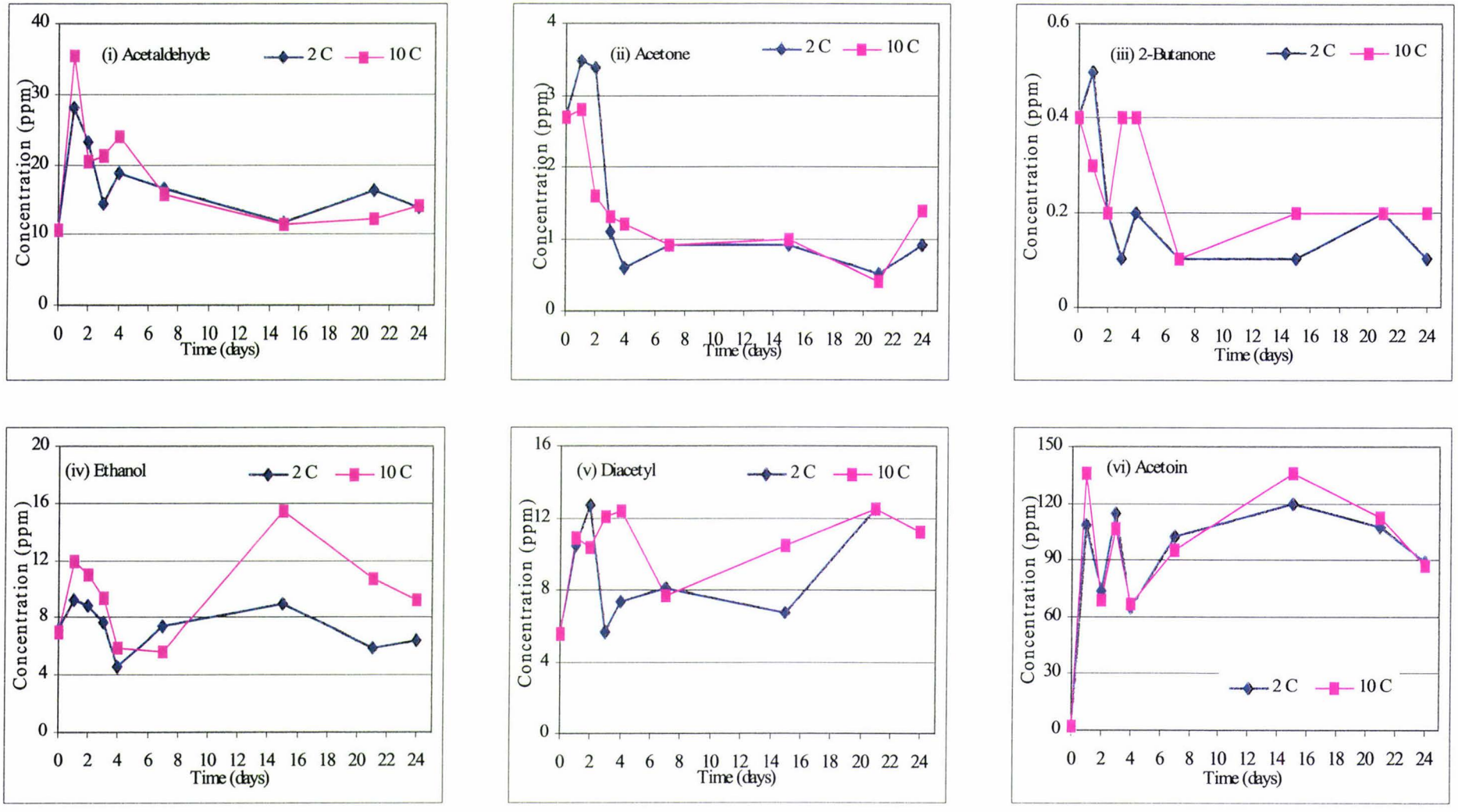


Figure 8.6: Analyte Concentrations in Low Fat Yoghurts Stored at 2°C and 10°C for 24 Days.

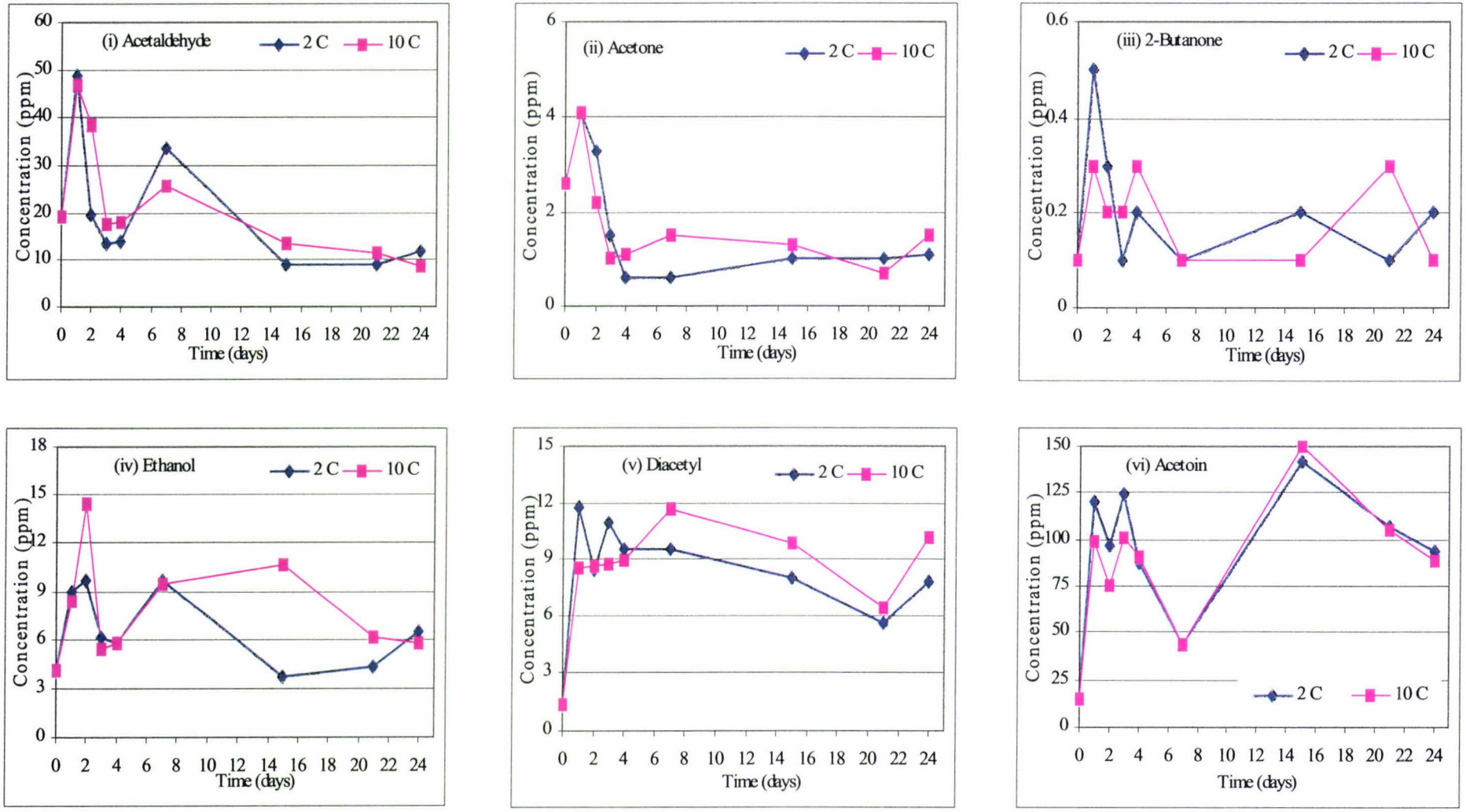


Figure 8.7: Analyte Concentrations in High Fat Yoghurts Stored at 2°C and 10°C for 24 Days.

For the low and high fat yoghurts stored at 2°C and 10°C, a large increase in the analyte concentrations was observed between the "Day 0" and "Day 1" headspace results. A small increase in analyte concentrations was also observed for the yoghurts in the storage time trial (Section 8.3.2). In the storage temperature trial, the most pronounced increase was seen for acetoin (from ~10 ppm to ~100 ppm) at both storage temperatures between "Day 0" and "Day 1". A possible explanation for this may have been due to the higher storage temperature (10°C), which may have resulted in prolonged metabolism of the starter culture and production of more flavour analytes. However, this does not explain the increase in analyte concentrations for the yoghurts stored at 2°C.

In Imhof and Bosset's (1994) study, yoghurts were analysed for various flavours at age 14 days using the SH method. The authors commented that flavour generation mainly occurred during the fermentation step (incubation) in yoghurt manufacture and that the flavours do not change significantly during refrigerated storage at 4°C. The present study used the HS-SPME method to investigate and quantify six analytes in yoghurts stored at 2°C and 10°C for a 24-day period. The results from this work partly confirm Imhof and Bosset's (1994) in that most flavour production seems to occur during the fermentation step. However, from the results obtained in this work, especially the storage temperature trial, it was evident that residual flavour production may also occur during the initial storage period, mostly between "Day 0" and "Day 1". This was seen by a large increase in analyte concentration between the "Day 0" and "Day 1" readings. Unfortunately, due to time constraints this work could not be repeated at the time. Repeating this experiment would be recommended in future work in order to confirm or deny this outcome.

Again, ANOVA and Tukeys comparison tests were not carried out on the analyte concentrations for the yoghurts stored at 2°C and 10°C because the results obtained were quite variable over the storage period.

Ignoring the increases in analyte concentrations between "Day 0" and "Day 1", the general trends seen on the time plots for the **low fat** yoghurts stored at 2°C and 10°C were:

- Acetaldehyde - gradual decrease from ~20 ppm to ~15 ppm;
- Acetone - gradual decrease from ~2 ppm to ~1 ppm;
- 2-Butanone - gradual decrease over the time period (~0.4 to ~0.2 ppm);
- Ethanol - no marked cahnge (~6 ppm);
- Diacetyl - gradual increase from ~8 ppm to ~10 ppm; and
- Acetoin - no marked change (~95 ppm).

Similar trends were observed for the **high fat** yoghurts in the storage temperature trial.

In Section 8.3.2 (storage time trial), only diacetyl and acetoin appeared to be affected by fat level. In the storage temperature trial however, only diacetyl was significantly affected ($P < 0.05$) by the fat level (9.35 ppm for LF vs 6.63 ppm for HF). According to Tamime and Deeth (1980), both diacetyl and acetoin are products of microbial fermentation. It was not understood why the fat levels in the yoghurts would affect them. Table 8.9 (below) shows the Tukeys test results for the fat effects in the yoghurts of the storage temperature trial.

Table 8.9: Tukeys Test Results for the Fat Effects on Yoghurts Stored at 2°C and 10°C for the 24-Day Period.

Fat Level	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin
	Temperature Tukeys Test ($\alpha = 0.05$)					
Low Fat	15.40 [a]	1.50 [a]	0.22 [a]	8.10 [a]	9.35 [a]	85.20 [a]
High Fat	15.60 [a]	1.64 [a]	0.19 [a]	6.56 [a]	6.63 [b]	72.20 [a]

"a, b" means within a column followed by the same superscript letter are not significantly different ($P < 0.05$).

From the Tukeys comparison test for the effect of storage temperature on analyte concentration, it was apparent that the only analyte affected by the storage temperature

was ethanol ($P<0.05$). The ethanol concentration was significantly higher at 10°C (8.25 ppm) than at 2°C (6.40 ppm). These results suggested that the storage temperature (2°C and 10°C) did not have a major effect on the amount of analyte present in the headspace and the amount extracted by the HS-SPME method. The fact that there was only one (ethanol) significant difference seen for the two temperatures supports this idea. Table 8.10 (below) shows the results obtained from the Tukeys tests carried out for the storage temperatures in the storage temperature trial.

Table 8.10: Tukeys Test Results for the Temperature Effects on Yoghurts Stored at 2°C and 10°C for the 24-Day Period.

Temperature	Acetaldehyde	Acetone	2-Butanone	Ethanol	Diacetyl	Acetoin
	Temperature Tukeys Test ($\alpha = 0.05$)					
2 °C	15.50 [a]	1.60 [a]	0.20 [a]	8.30 [a]	7.80 [a]	78.30 [a]
10 °C	15.50 [a]	1.50 [a]	0.20 [a]	6.40 [b]	8.20 [a]	79.10 [a]

"a, b" means within a column followed by the same superscript letter are not significantly different ($P < 0.05$).

The general trends observed for the analytes in the two storage trials were very similar except for acetoin. It was not clearly understood why the acetoin levels behaved differently in the storage temperature trial as opposed to the storage time trial. In the former case, the acetoin levels decreased marginally during the storage period, while, in the latter case, acetoin concentrations were seen to drop to almost half its original value over the 24-day period. Again, the acetoin levels were unusually high compared to the values reported to date in Njissen *et al's.* (1996) compilation, but this was attributed to the efficiency of the HS-SPME method being used.

McGregor and White (1987) found no significant differences for diacetyl concentrations between the 4°C and 10°C samples in the storage temperature trial. Acetone concentrations were significantly ($P<0.001$) lower at 10°C (1.8 ppm) than at 4°C (2.3 ppm). Acetaldehyde concentrations were also ($P<0.001$) lower at the higher storage temperature (25.1 ppm at 10°C vs 27 ppm at 4°C). McGregor and White also

found there were no significant differences in total bacteria or lactobacilli counts between the yoghurts stored at the two temperatures but yeast and mould counts were significantly higher at the 10°C temperature. A significant decrease in the total viable bacterial count was found for the yoghurts stored at 4°C however. The starter culture viability dropping may result in less lactic acid being produced, which in turn, might result in an alteration of the overall yoghurt flavours perceived. This brings us back to the question of the effects of pH on the yoghurt sample and on flavour concentrations present in the headspace of the yoghurts. Future work in this area could investigate these effects.

It is also recommended that the work in this chapter be repeated to confirm or deny the analyte concentration trends seen in this study. The use of HS-SPME in conjunction with GC-MS would also be beneficial because the analytes, especially acetoin, can be positively identified and quantified.

8.4 Conclusion

In conclusion, this work showed that the HS-SPME extraction method could be used to investigate the six analytes in yoghurts over a period of time. The ES method was successfully used to quantify the analyte concentrations in the laboratory-prepared yoghurts.

The pH of the yoghurts decreased over the 24 days in both storage trials indicating that the starter culture were producing more acid as a result of metabolic action. No major differences were seen between the two fat levels but the yoghurts stored at 10°C were generally more acidic (lower pH) than the yoghurts stored at 2°C. This was probably due to the warmer environment for the starter cultures, enabling them to continue their metabolic activity.

For both storage trials, the analyte concentrations varied from day to day over the storage periods and therefore, statistical analyses could not be carried out on this data. Only general trends for the analyte concentrations could be drawn for the two storage trials.

For the storage time trial 2-butanone and acetoin seemed to decrease gradually over the 24-day period, while diacetyl increased slightly. For the storage temperature trial, acetaldehyde, acetone and 2-butanone were seen to gradually decrease with time, while acetoin and ethanol showed no marked change over the 24 days. Again, diacetyl increased slightly during storage.

Acetoin decreased significantly in the storage time trial but showed very little change in the storage temperature trial over the 24-day periods. It was not certain why this occurred but it was suggested that the work be repeated, preferably with the use of HS-SPME coupled to GC-MS. The use of an MS would give a positive identification for the acetoin and with the ES method, it may be possible to quantify the acetoin levels in order to confirm or deny the findings from this study.

Fat effects were observed for diacetyl and acetoin in the storage time trial and in the storage temperature trial, only diacetyl was affected by fat levels. From this, it was concluded that there were no major fat effects in the yoghurts as only two/one out of six analytes were affected by the level of fat in the yoghurts. It was uncertain why only diacetyl and acetoin were affected by the fat levels.

Temperature effects were only seen for ethanol in the storage temperature trial. From this, it was concluded that the storage temperatures (2°C and 10°C) did not have any major temperature effects on the analyte concentrations in the yoghurt headspace. This was because only one compound out of the six analysed seemed to be affected by storage temperature.

From this study, it was apparent that the HS-SPME method could be used to extract, detect and quantify the six yoghurt flavour compounds. However, it was not possible to draw flavour profiles for the six analytes in the two storage trials as the analytes varied from day to day quite a lot. Only general trends for each analyte were observed. It was concluded that this work needs to be repeated in order to confirm or deny the results obtained in this study.

A potential application of setting up flavour profiles using HS-SPME may be to determine the quality of yoghurts. By relating the analytical data obtained, with

relevant sensory data, it may be possible to determine when yoghurt becomes unpalatable based on the headspace aroma and the analyte concentrations. With this eventual goal in mind, it was the intention of the work in this chapter to produce flavour profiles using the HS-SPME method for the six analytes in laboratory-prepared yoghurts. However, it is evident from the results obtained that more work in this area is required.

Having established that the HS-SPME method can be used to evaluate and quantify the six analytes in laboratory-prepared yoghurts, our attention was turned to commercial yoghurts (Chapter 9).

CHAPTER NINE

AN INVESTIGATION OF COMMERCIAL YOGHURTS USING HEADSPACE SOLID PHASE MICROEXTRACTION AND SENSORY ANALYSIS.

9.0 Introduction

The work in this chapter was a preliminary trial carried out to investigate whether the HS-SPME technique could be used to extract and quantify analytes in a range of commercial yoghurt products. The investigation then went on to determine whether the yoghurts could be differentiated based on the HS-SPME results of analyte concentrations (headspace aroma) together with Principal Component Analysis (PCA). An untrained consumer panel was used to determine whether the consumers could differentiate between the yoghurts based on the headspace aroma.

An untrained panel was selected for this study because the panellists were available at the time to participate in the sensory tests. An expert panel was not available, besides which, it was not vital for a preliminary study such as this.

It was envisaged that the results from this preliminary trial would provide useful information for further research in this area, the eventual goal being that the HS-SPME technique is used as a tool to evaluate yoghurt quality and consumer acceptability based on analyte concentrations. At present, trained panellists do most of the quality checking before commercial yoghurts are marketed. In order to determine consumer acceptability using analytical/instrumental techniques, the instrumental data must first be related to the respective sensory data. The sensory data is usually obtained using trained/expert panellists and methods such as Quantitative Descriptive Analysis (QDA), Descriptive Analysis (DA) and Gas Chromatography-Olfactory (GC-O). These processes are usually laborious, time-consuming and expensive. If an analytical technique such as HS-SPME, which is simple, accurate, and efficient to use routinely, was to replace or supplement the use of expert sensory panellists, then the quality checking step during the manufacturing process could be shortened significantly and costs reduced. This, in turn, would ensure that the product is marketed very soon after production.

The work in this chapter was divided into two parts:

(1) **HS-SPME Analysis of the Fourteen Commercial Yoghurts** - the first part was a general investigation into the analyte concentrations in a range of commercial yoghurts. The HS-SPME method was used to analyse and quantify the six analytes present in fourteen commercial yoghurts purchased from the supermarket. (A similar selection of commercial yoghurts was investigated in Chapter 4 where the extraction powers of two fibre coatings were compared). Once the HS-SPME data had been collated and Principal Component Analysis (PCA) carried out, the commercial yoghurts were classified into clusters according to their analyte concentrations. The eight yoghurts, which were visibly either very different or very similar on the PCA plots, were then chosen for the second part of the study.

Principal Component Analysis (PCA) is a multivariate statistical technique that is used to simplify and/or describe interrelationships among multiple dependent variables and among objects. It provides a graphical description of how various dependent variables are related to each other and this is sometimes more informative than a block of numerical data. PCA only works on data that are partly correlated, which can then be reduced to a lower dimension requiring only a few components (principal components) to explain the overall variation in the data.

(2) **HS-SPME Analysis and Sensory Evaluation of Eight Commercial Yoghurts** - the second part involved re-analysing fresh samples of the eight, newly-bought yoghurts using HS-SPME. The HS-SPME results were again classified into groups using PCA. The 8 yoghurts were also administered to untrained panellists in a series of triangle tests where the panellists had to pick the odd yoghurt sample out of three presented to them, based on smell alone.

According to Ott *et al.* (1997), the perception of headspace aroma in the vapour phase is first perceived on opening a yoghurt pottle. This greatly influences the panellists' preferences of the product. Hence, in the triangle tests carried out in this study, the consumer panellists were asked to smell the yoghurt by opening the yoghurt container and taking a few short sniffs. They had to then decide which of the three yoghurt samples presented to them was the odd sample.

9.2 Materials and Methods

9.2.1 HS-SPME Analysis of the Fourteen Commercial Yoghurts.

A list of the commercial yoghurts and their details is shown in Table 9.1 below. All fourteen yoghurts (six low fat and eight high fat yoghurts) were chosen from the supermarket based on their expiry date. The yoghurts all had similar expiry dates (within two days of each other), which were at least three weeks before their expiry date. From this, it was assumed that the yoghurts had been manufactured around the same time and were therefore, of similar age.

Table 9.1: The Fourteen Commercial Yoghurts Used in this Study With Specifications as Seen on the Product Labels.

Yoghurt Sample With Abbreviations	Yoghurt Culture	Fat Content (%)	Protein Content (%)	Fat Class
Naturalea Acidophilus (NA)	*Acidophilus* and *B. bacterium*	3.8	3.2	HF
Naturalea (N)	Yoghurt cultures unspecified	3.3	4.2	HF
Verona Bulgarian (VB)	*Bulgaricus* culture and other unspecified yoghurt cultures	3.25	~5	HF
Yoplait High Fat (YHF)	*L. bulgaricus* and *S. thermophilus* (sweetened)	2.9	5.3	HF
Verona Acidophilus (VA)	Yoghurt cultures unspecified	2	~5	HF
Biofarm Acidophilus (BA)	*Acidophilus* and *B. bacterium*	~3	~4.5	HF
Biofarm (B)	*Acidophilus* and *B. bacterium*	~3	~4.5	HF
Yoplus (YAB)	*Acidophilus* and *B. bacterium*	2.9	5.3	HF
Yoplait Low Fat (YLF)	*L. bulgaricus* and *S. thermophilus*	0.5	5.3	LF
Slimmer's Choice (SC)	*Acidophilus*, *B.bacterium* and other unspecified yoghurt cultures (unsweetened)	0.2	4.6	LF
Metchnikoff (M)	*Acidophilus* and *B. bacterium*	0.2	4.8	LF
Fresh 'n' Natural (FN)	Yoghurt cultures unspecified (sweetened)	1.2	5	LF
Weight Watchers (WW)	Yoghurt cultures unspecified	0.1	5.2	LF
Yotrim (YABC)	*Acidophilus*, *B. bacterium* and *L. caseii*	0.2	5.3	LF

The commercial yoghurt samples for the HS-SPME analysis were prepared in duplicate as outlined in Section 3.7. 15 mL of each commercial yoghurt was also transferred into 35 mL Lily portion cups to measure the pH prior to HS-SPME analysis. Both the headspace sample vials and pH portion cups were sealed and refrigerated for 1 week at 2°C before HS-SPME was carried out as outlined in Section 3.5.

It was decided that whenever the yoghurt cultures were unspecified, that is, whenever "Yoghurt Cultures" was stated on the labels, it was to be assumed that a mixed strain starter culture made of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* was used. Other starter culture varieties, if used, such as *Acidophilus* and *Bifidus bacterium*, were generally stated on the product labels. However, this was not always clear on the product labels.

The ES method was used to quantify the analytes in the commercial yoghurts. Standard low fat simulated yoghurts (0, 12.5 ppm and 25 ppm) containing the six analytes were made in duplicate according to Section 3.6.2. 200 µL of the 200 ppm propan-2-ol (IS) solution was added to each yoghurt sample and its peak area was used to normalise the analyte peak areas obtained before analyte concentrations were calculated. Enough standard simulated yoghurt samples were made so that daily GC calibration could be carried out ahead of the commercial yoghurts being analysed. Standard curves of analyte peak areas versus concentration were drawn, and from these, the analyte concentrations were calculated.

PCA was carried out on the HS-SPME data obtained using SAS (Version 6.12). The PCA plots derived from the statistical analyses are shown in Section 9.3.1. From these plots, eight of the fourteen commercial yoghurts were selected for the second part of this study.

9.2.2 HS-SPME Analysis and Sensory Evaluation of Eight Commercial Yoghurts.

Fresh samples of the eight commercial yoghurts chosen from the first part of this study were then analysed using HS-SPME. Consumer panellists were also employed to evaluate the yoghurts by participating in the sensory triangle tests. The selected yoghurts were Weight Watchers (WW), Fresh 'n' Natural (FN), Slimmers' Choice (SC),

Metchnikoff (M), Yoplait High Fat (YHF), Yoplus *Acidophilus* and *Bifido Bacterium* (YAB), Yotrim *Acidophilus, Bifido Bacterium* and *Caseii* (YABC), and Biofarm *Acidophilus* (BA). 35 mL of each yoghurt was transferred to 120 mL white plastic containers with lids and refrigerated for 1 week, at 2°C, before sensory evaluation was carried out. The plastic containers were labelled only with predetermined, random, three-digit codes specifically chosen for each commercial yoghurt in each triangle test. Enough yoghurt samples were made for the various triangle tests administered to the panellists.

The yoghurt samples for headspace analysis were prepared as outlined in Sections 3.7 and 9.2.1. The only difference was that sampling was carried out in triplicate for Day 1 of the sensory testing and four replicates of each yoghurt were analysed on Day 2. Fewer yoghurts were tested on Days 1 and 2 and therefore, it was possible to analyse these extra replicates. 15 mL of each yoghurt was also transferred into 35 mL Lily portion cups for pH measurements. All the samples were refrigerated for 1 week at 2°C. Samples were left at room temperature for 10 minutes prior to headspace sampling and pH measurement. Standard simulated low fat yoghurts (0, 12.5 and 25 ppm) were made as part of the ES method (as outlined in Sections 3.6.2 and 9.2.1), to calculate the analyte concentrations in the eight commercial yoghurt samples. Again, PCA was carried out on the analyte peak areas obtained for the eight yoghurts.

For the sensory evaluation, a total of 41 consumer panellists (26 females and 14 males) were chosen to undertake the triangle tests. All 41 panellists were first year Hospitality students at the Universal College of Learning (UCOL), Palmerston North, with ages ranging from 17 to 28 years. All the panellists had to be yoghurt eaters without having a great aversion to the sour, fermented smell of yoghurts. Any panellists, who were suffering from colds or similar health problems, such as hay fever, were removed from the sensory testing, as their sense of smell would have been affected.

The panellists were instructed on how to carry out the triangle tests administered to them. Each test consisted of three yoghurt samples, which were randomly coded with three-digit numbers. Two of the yoghurt samples had been taken from one batch of homogeneous commercial yoghurt and were identical, while the third sample had been taken from a different yoghurt. The instructions given to the panellists for the sensory

testing were to slowly lift the lid and take a few short sniffs of the yoghurt aroma. They were then asked to pick the odd sample out of the three presented to them based on what they smelt. The yoghurt samples used in these triangle tests were left to equilibrate to room temperature (around 21°C) for 2 hours before they were presented to the panellists.

The triangle test was chosen because the aim was to answer a simple question. That is, whether consumers (untrained panellists) could tell which sample was different out of the three presented to them at any one time. These sensory results would give immediate information about the panellists' ability to differentiate yoghurts based on smell. The chance probability for this test is 1/3, which, accounts for its claim of greater sensitivity above other discrimination tests such as duo-trio and paired comparison tests (Pal *et al.*, 1995). The triangle test is also considered a more difficult test than the duo-trio and the paired comparison tests, as the panellist has to recall the sensory characteristics of two products before evaluating the third, and then make a decision.

The six possible presentation arrangements for the triangle tests were AAB, ABA, BAA, BBA, BAB, and ABB. Each combination was presented an equal number of times. The panellists were asked to sniff each yoghurt sample presented to them and to choose the sample, which smelt different to the other two. A copy of the questionnaire form presented to the panellists for each triangle test can be found in Appendix III (page 223).

The triangle tests were done on two Tuesdays, a week apart. A timetable for all the triangle tests administered to the panellists is listed in Table 9.2 (page 174). Day 1 consisted of four triangle "sniff" tests. The panellists had to leave the room for a 10-minute break after the second test so as to prevent panellist fatigue. Day 2 consisted of three triangle tests divided by a 10-minute break at the end of the second test. Test 3 on Day 2 was a repeat of Test 4 on Day 1, except that the yoghurt samples were placed in sealable 120 mL glass bottles instead of the 120 mL plastic containers. This test was carried out to determine whether a plastic odour from the plastic containers tainted the yoghurt aroma smelt by the panellists, thereby altering their judgements. Biofarm *Acidophilus* (BA) and Yoplait *Acidophilus* and *Bifido Bacterium* (YAB) were chosen

for this test as they were found to be most different to each other according to the PCA plots drawn in Section 9.3.1.

Table 9.2: Timetable Showing the List of Triangle Tests Administered to the Consumer Panellists for (a) Day 1 and (b) Day 2.

(a) Day 1

Triangle Test No:	Test Type	Number of Panellists
Test 1	SC vs FN	40
Test 2	YAB vs YABC	40
10 MINUTE BREAK		
Test 3	M vs WW	40
Test 4	BA vs YAB (plastic containers with lids)	40

(b) Day 2

Triangle Test No:	Test Type	Number of Panellists
Test 1	YHF vs YAB	40
Test 2	SC vs YAB	40
10 MINUTE BREAK		
Test 3	BA vs YAB (sealable glass bottles)	39

9.3 Results and Discussion

9.3.1 HS-SPME Analysis of the Fourteen Commercial Yoghurts.

(A) Analyte Concentrations in the Commercial Yoghurts

Table 9.3 (below) lists the fourteen commercial yoghurts analysed with measured pHs and analyte concentrations calculated using the ES method.

Table 9.3: Calculated Analyte Concentrations for the Fourteen Commercial Yoghurts.

Yoghurt Sample With Abbreviations	pH	Acetaldehyde (ppm)	Acetone (ppm)	2-Butanone (ppm)	Ethanol (ppm)	Diacetyl (ppm)	Acetoin (ppm)
Naturalea Acidophilus (NA)	3.55	16.90	2.90	0.22	5.10	9.50	89.50
Naturalea (N)	3.64	14.20	3.80	0.20	5.60	14.50	85.30
Verona Bulgarian (VB)	3.71	12.10	3.10	0.20	8.10	8.00	83.60
Yoplait High Fat (YHF)	4.04	27.20	2.50	0.28	5.20	8.30	82.70
Verona Acidophilus (VA)	3.88	10.50	4.40	0.30	8.40	15.00	83.60
Biofarm Acidophilus (BA)	3.62	13.10	3.60	0.20	5.70	12.00	71.20
Biofarm (B)	3.68	15.40	2.80	0.15	2.60	17.60	75.40
Yoplus (YAB)	4.75	39.60	2.70	0.20	14.90	16.20	153.60
Yoplait Low Fat (YLF)	4.38	34.30	1.60	0.43	0	9.40	106.70
Slimmer's Choice (SC)	3.76	17.70	4.30	0.24	3.60	9.90	57.40
Metchnikoff (M)	3.66	15.90	3.20	0.22	19.70	8.70	52.00
Fresh 'n' Natural (FN)	3.91	13.50	4.20	0.25	2.98	9.80	85.40
Weight Watchers (WW)	3.98	22.10	3.90	0.37	3.80	17.30	123.60
Yotrim (YABC)	4.76	37.50	1.10	0.27	13.20	11.20	129.50

The pHs for the above yoghurts ranged from 3.55 for Naturalea Acidophilus to 4.76 for Yotrim ABC. Investigations as to why the commercial yoghurts were more acidic after about one week of refrigeration at 2°C were not undertaken, but, various explanations are possible.

For example, the definite age of the yoghurts was unknown as the date of manufacture was estimated based on the expiry dates. So the yoghurts could have been a lot older than estimated, resulting in the observed lower pHs. The yoghurts may have also contained high lactose concentrations, which would have resulted in more lactic acid being produced thereby, lowering the pH further. The types of starter culture used to ferment the yoghurts may have also played a part in lowering the pH of the commercial yoghurts. The processing conditions may have also been different to that of the laboratory-prepared yoghurts resulting in some effect on the pH. For instance, there may have been a delay before the yoghurts were transferred from fermentation to chilling stages or gradual cooling may have been used instead of instant chilling. Such processing conditions would have resulted in the gradual inactivation of the cultures enabling them to produce more lactic acid (= lower pHs) in the yoghurts during this transition time. This was found in Chapter 8 where the yoghurts stored at 10°C directly after incubation had a lower pH than those stored at 2°C.

The level of acidity in the yoghurts is important because, as the pH reaches the isoelectric point of milk (pH = 4.6), the milk coagulates and possibly traps a number of the flavour volatiles within. And when the pH is lowered further, the milk coagulum begins to redissolve, possibly releasing certain yoghurt flavour volatiles. The relationship between pH and analyte concentrations in yoghurts was not investigated in this study. However, there is much scope in this area of research, which could be pursued in the future.

From Table 9.3 (page 175), it was evident that acetaldehyde, acetone, 2-butanone, ethanol and diacetyl were within the concentration ranges stated in Nijssen *et al.* (1996). Acetaldehyde levels ranged from 10.50 ppm (Verona Acidophilus) to 39.60 ppm (Yoplus AB). Acetone concentrations ranged from 1.10 ppm (Yotrim ABC) to 4.40 ppm (Verona Acidophilus). The 2-butanone levels were low and ranged from 0.20 ppm (Biofarm) to 0.40 ppm (Yoplait LF). Ethanol and diacetyl levels were within the

reported ranges (2.60 ppm (Biofarm) to 19.70 ppm (Metchnikoff) for ethanol and 8.00 ppm (Verona B) to 17.60 ppm (Biofarm) for diacetyl).

The acetoin concentrations in this study were higher than what had been reported in Nijssen *et al.* (1996). The concentrations ranged from 52.00 ppm (Metchnikoff) to 153.60 ppm (Yoplus AB). These results were attributed to the increased extracting power of the HS-SPME method compared with other headspace methods such as steam distillation-GC and SH, quoted in previous studies (for further information on these headspace methods refer to the references quoted in Nijssen *et al.* (1996).

The acetaldehyde and acetoin levels for the four Yoplait yoghurts (Yoplait Low Fat, Yoplait High Fat, Yoplus AB and Yotrim ABC) were almost double ~~that~~ THOSE obtained for the other commercial yoghurts. The reason for the increase in acetaldehyde and acetoin concentrations was not ascertained but may have been due to the addition of certain starter cultures specifically to the Yoplait yoghurts. Abrahamsen (1978) determined the acetaldehyde content in yoghurts made with different starter cultures using GC and found that this was the case. According to Tamime and Deeth (1980), acetaldehyde, acetoin and diacetyl are products generated through microbial fermentation. Another cause for higher concentrations of these two compounds in the Yoplait yoghurts may have been due to the addition of various functional ingredients, such as stabilisers and thickeners. Rankin and Bodyfelt (1996) found that compositional ingredients had effects on the types and amounts of headspace volatiles present. The Yoplait ingredient specifications were confidential and therefore, not available in this study. The Weight Watchers yoghurt also had relatively high levels of acetaldehyde (22.10 ppm) and acetoin (123.60 ppm), which may have been due to similar reasons as those described.

(B) Principal Component Analysis (PCA) of the Analytical HS-SPME Data

The peak area values obtained for the six analytes in the fourteen commercial yoghurts were statistically analysed using PCA (SAS output for "14 yoghurts" results are shown in Appendix V, page 226). The results of the PCA are given in Figure 9.1 (a) and (b) (pages 178 and 179, respectively). The first four principal components accounted for ~ 91% of the variation in the data. The PCA results suggested there were some strong correlations between the analytes in these commercial yoghurts.

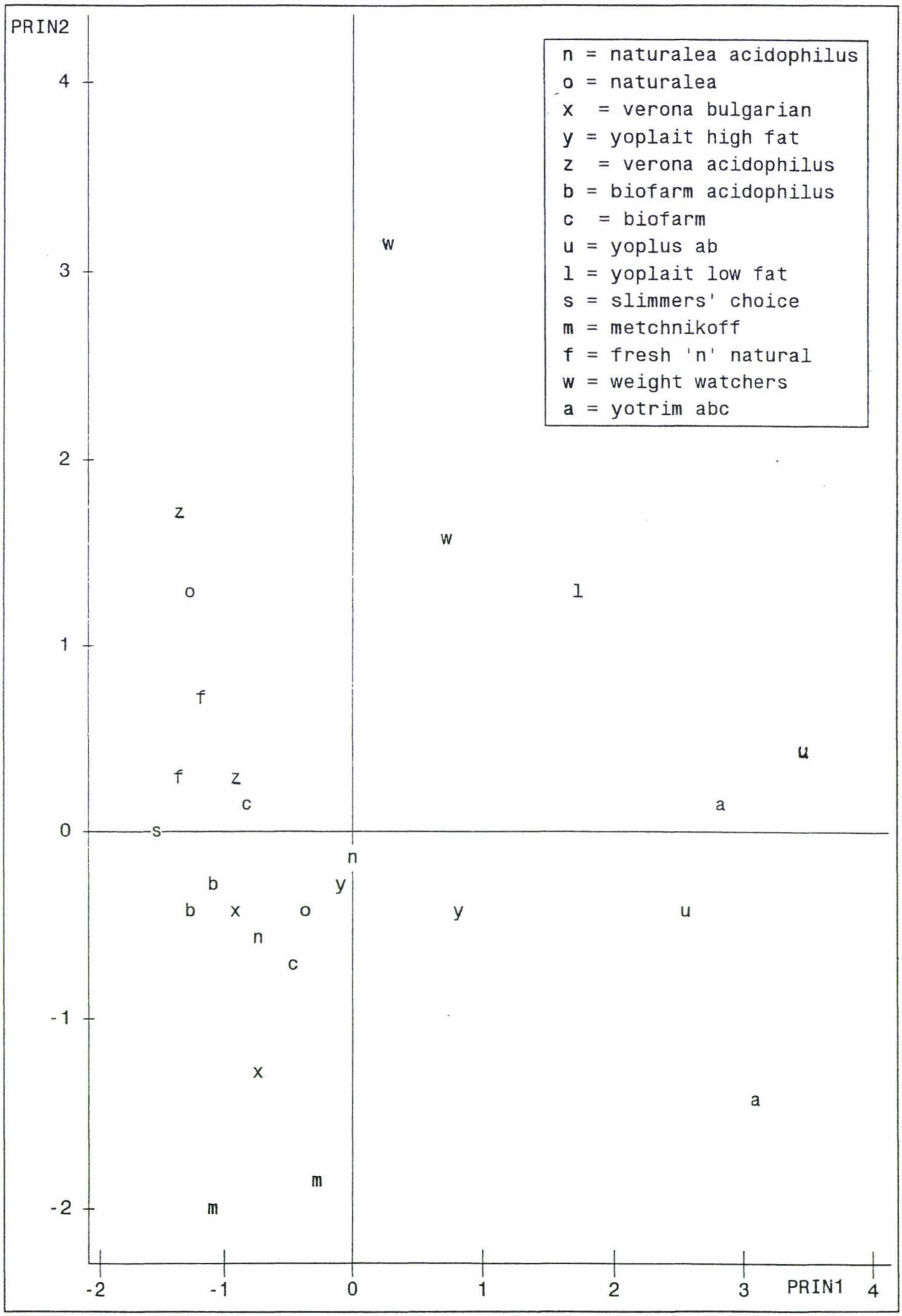


Figure 9.1 (a): PCA Plot Showing the Fourteen Commercial Yoghurts Along the P1 and P2 Axes.

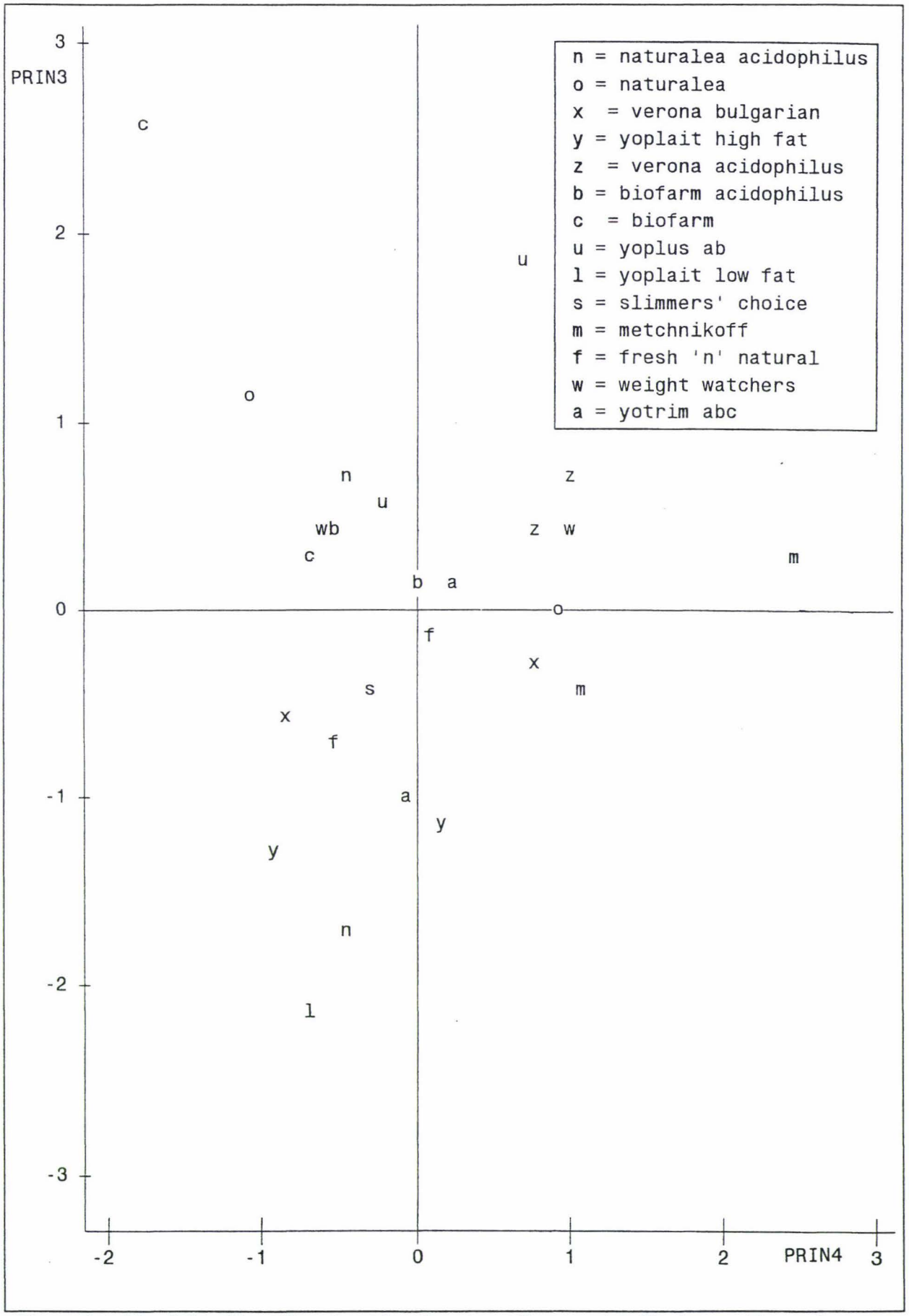


Figure 9.1 (b): PCA Plot Showing the Fourteen Commercial Yoghurts Along the P3 and P4 Axes.

Acetaldehyde and acetoin had a positive correlation of ~ 0.76 indicating that these two analytes did influence each other to a certain extent. As explained earlier, these two compounds and diacetyl are mainly produced during microbial fermentation (Tamime and Death, 1980) and therefore, their concentrations in the yoghurts may depend on the types of starter cultures used and length of fermentation time during the yoghurt-making process.

Acetaldehyde (0.62) and acetoin (0.55) loaded highly on the first principal component (P1), while 2-butanone (0.63) contributed to the second principal component (P2). P1 was highly correlated (Pearson correlation) with acetaldehyde (0.94) and acetoin (0.83), and negatively correlated with acetone (-0.72). P2 showed a high Pearson correlation with 2-butanone (0.72). In Figure 9.1 (a) (page 178), the yoghurt species were well separated along the P1 axis suggesting that most of the variation in the yoghurts was due to acetaldehyde and acetoin levels. Not as much variation was seen along the P2 axis, except for the Weight Watchers' and Metchnikoff yoghurts, suggesting that the rest of the yoghurts did not vary much in 2-butanone levels.

From Figure 9.1 (a), it was apparent that two Yoplait yoghurts, Yoplus AB and Yotrim ABC both had high levels of acetaldehyde and acetoin (~ +3 on the P1 axis). The other two Yoplait yoghurts, Yoplait Low Fat and Yoplait High Fat, and Weight Watchers, had lower amounts of acetoin and acetaldehyde (~ +1 on the P1 axis). The remaining yoghurts fell into a cluster around -1 (P1 axis) suggesting even lower levels of acetaldehyde and acetoin. Only Metchnikoff (~ -2, low 2-butanone levels) and Weight Watchers (~ +2 to +3, high 2-butanone levels) were classed as two distinct groups along the P2 axis. The other yoghurts were mostly placed along the -1 to +1 marks along the P2 axis suggesting that they all had similar 2-butanone levels.

Figure 9.1 (b) (page 179) shows the PCA plot for principal components P3 versus P4. Diacetyl (0.83) loaded high on P3, while ethanol (0.83) loaded high on P4. P3 and P4 were highly correlated (Pearson's correlation) with diacetyl (0.86) and ethanol (0.75), respectively. As seen on the plot, the Yoplait LF yoghurts were around -2 (P3 axis) and the Yoplait HF samples were around –1 (P3 axis), which suggested that they both had lower diacetyl levels than the rest of the yoghurts were centred around the 0 point (P3 axis). Not much variation was seen along the P4 axis except for Metchnikoff (~+1 to

+3, high ethanol) and Slimmers' Choice (~-2, low ethanol), which suggested that the majority of commercial yoghurts analysed had similar ethanol levels.

From the PCA plot (Figure 9.1 (a), page 178), it was decided that the following yoghurts would be used for the second part of this study (HS-SPME and sensory analysis). Yoghurts that were visibly different (e.g. Metchnikoff vs Weight Watchers) or very similar (e.g. Slimmers' Choice vs Fresh 'n' Natural) were selected for the second part (Section 9.3.2) of this work.

DAY 1

Triangle Test 1: **Slimmers' Choice (SC) vs Fresh'n'Natural (FN)**

- both yoghurts were rated very similar to each other as they were found around the same area on the PCA plot.

Triangle Test 2: **Yoplus AB (YAB) vs Yotrim ABC (YABC)**

- both these yoghurts were also found to be very similar to each other according to the PCA plot.

Triangle Test 3: **Metchnikoff (M) vs Weight Watchers (WW)**

- these two yoghurts were classed as very different to each other according to PCA (found on opposite ends along the P2 axis).

Triangle Test 4: **Biofarm Acidophilus (BA) vs Yoplus (YAB)–plastic containers**

- these two yoghurts were also rated very different to each other according to PCA (found on opposite ends along the P1 axis).

DAY 2

Triangle Test 1: **Yoplait High Fat (YHF) vs Yoplus (YAB)**

- both yoghurts were rated different to each other as seen on PCA plot.

Triangle Test 2: **Slimmers' Choice (SC) vs Yoplus (YAB)**

- both these yoghurts were found to be very different to each other as seen on PCA plot.

Triangle Test 3: **Biofarm Acidophilus (BA) vs Yoplus (YAB) - glass bottles**

- this triangle test was a replication of Test 4 (Biofarm Acidophilus (BA) vs Yoplus (YAB) - plastic containers) on Day 1.

Each letter on the PCA plot represents one yoghurt sample replicate analysed by HS-SPME. From the plot, it was evident that the headspace method was fairly reproducible in its extracting ability because duplicate yoghurt samples, which had similar analyte peak areas, were found clustered in specific regions on the PCA plot. The repeatability of the HS-SPME technique is reinforced later in the second part of this work where triplicate samples of each commercial yoghurt were analysed on Day 1 and four replicates of each yoghurt type were analysed on Day 2 (see Section 9.3.2 (B)).

9.3.2 HS-SPME Analysis and Sensory Evaluation of the Eight Commercial Yoghurts.

(A) Analyte Concentrations for the Eight Commercial Yoghurts

Fresh samples of the selected eight commercial yoghurts (newly-bought) were prepared for both HS-SPME and sensory analysis as outlined in Section 9.2.1 and 9.2.2, respectively. The selected yoghurts were Biofarm Acidophilus (BA), Yoplus AB (YAB), Slimmers' Choice (SC), Metchnikoff (M), Fresh 'n' Natural (FN), Weight Watchers (WW), Yotrim ABC (YABC), and Yoplait High Fat (YHF). Table 9.4 (page 183) lists the measured pHs and the calculated analyte concentrations for the yoghurts analysed on Days 1 and 2.

The acidity of these yoghurts from Days 1 and 2, ranged from pH 3.65 for Biofarm Acidophilus yoghurt to 4.83 for the Yoplus AB yoghurt. These values were similar to those obtained for the fourteen yoghurts in Section 9.3.1. Again, an assumption was made regarding the age of the yoghurts based on expiry dates stamped on the product labels.

Table 9.4: Calculated Concentrations for the Selected Eight Commercial Yoghurts (a) Day 1 and (b) Day 2.

(a) Day 1

Yoghurt Samples With Abbreviations	pH	Acetaldehyde (ppm)	Acetone (ppm)	2-Butanone (ppm)	Ethanol (ppm)	Diacetyl (ppm)	Acetoin (ppm)
Biofarm Acidophilus (BA)	3.65	7.20	10.00	0.70	23.70	21.30	78.10
Yoplus (YAB)	4.83	16.60	2.60	0.40	22.50	17.10	109.90
Slimmer's Choice (SC)	3.85	8.70	2.60	0.40	6.60	13.90	76.90
Metchnikoff (M)	3.84	12.20	5.40	0.50	12.12	1.30	86.40
Fresh 'n' Natural (FN)	3.95	14.20	3.60	0.60	0	0	143.50
Weight Watchers (WW)	3.99	23.90	4.10	0.50	0	19.50	164.00
Yotrim (YABC)	4.76	18.30	1.40	0.40	21.60	10.70	224.20

(b) Day 2

Yoghurt Sample With Abbreviations	pH	Acetaldehyde (ppm)	Acetone (ppm)	2-Butanone (ppm)	Ethanol (ppm)	Diacetyl (ppm)	Acetoin (ppm)
Yoplait High Fat (YHF)	4.12	24.20	2.10	0.20	6.80	6.30	111.90
Biofarm Acidophilus (BA)	3.65	14.60	13.40	2.10	8.60	7.80	99.70
Yoplus (YAB)	4.83	24.00	1.30	0.30	30.60	13.20	192.40
Slimmers' Choice (SC)	3.85	18.90	3.60	0.50	7.10	7.90	88.50

The calculated concentrations for all the analytes except acetoin for both days were within the ranges quoted in Nijssen *et al.* (1996). They were also quite similar to the results obtained in Section 9.3.1. The acetoin levels for the yoghurt samples were again

found to be very high for both days in relation to the values quoted. As mentioned earlier, this may have been due to the increased extraction capability of the HS-SPME method as opposed to other headspace techniques (DH and SH sampling), previously used to extract nonvolatile acetoin (Viani and Horman, 1973). Acetoin levels ranged from 76.90 ppm for the Slimmers' Choice yoghurt to 224.20 ppm for Yotrim ABC yoghurt. Again, as seen in Section 9.3.1, the highest acetoin levels were found for the Weight Watchers and Yoplait yoghurts. The acetaldehyde concentrations in these yoghurts were also proportionally higher compared to the other yoghurts analysed on both days. Reasons as to why these two analytes were higher in these yoghurts were not ascertained. However, as explained earlier, it may have been due to various functional ingredients and starter cultures being added during yoghurt manufacture. Nevertheless, the results suggested that these two analytes might have been related to and dependent on each other, reinforcing Tamime and Deeth's (1980) findings, which stated that acetaldehyde and acetoin were products of microbial fermentation in yoghurts.

(B) Principal Component Analysis and Sensory Evaluation Results

The peak area values obtained for the six analytes in the eight commercial yoghurts were then analysed statistically using PCA (SAS output for Day 1 results are shown in Appendix V, page 230).

- **PCA Carried Out on the HS-SPME Data Obtained on Day 1**

Figures 9.2 (a) and (b) (pages 185 and 186) are the PCA plots obtained for Day 1. The first four principal components shown accounted for about 87% of the variation in the data. Acetoin (0.56) loaded fairly high on the first principal component (P1), while 2-butanone (-0.52) had a negative loading on P1. For the second principal component (P2), acetone (0.70) loaded quite highly. P1 was negatively correlated (Pearson correlation) with 2-butanone (-0.76) and positively correlated with acetoin (0.81), while P2 was positively correlated with acetone (0.84).

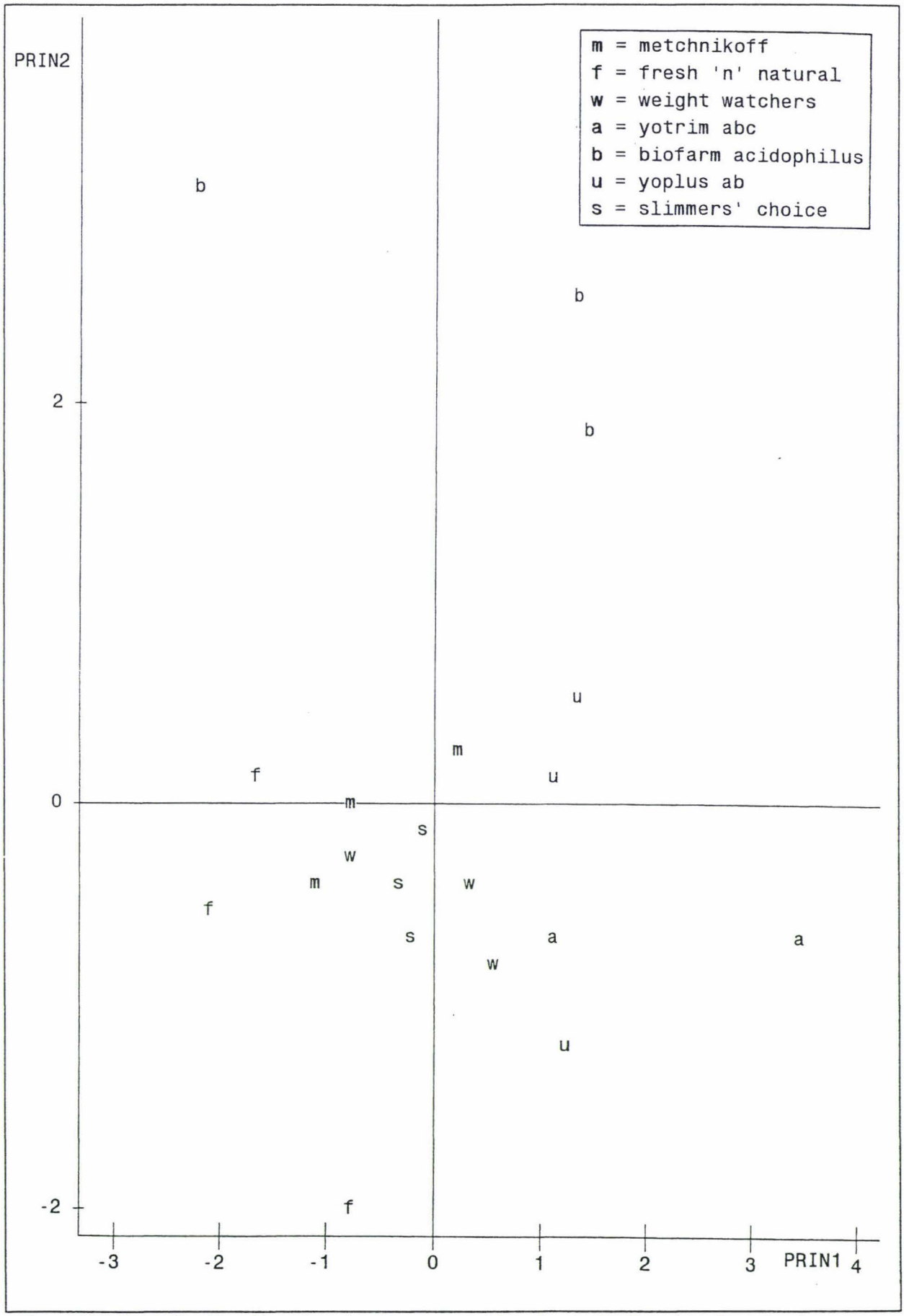


Figure 9.2 (a): PCA Plot Showing the Commercial Yoghurts Analysed on Day 1 Along the P1 and P2 Axes.

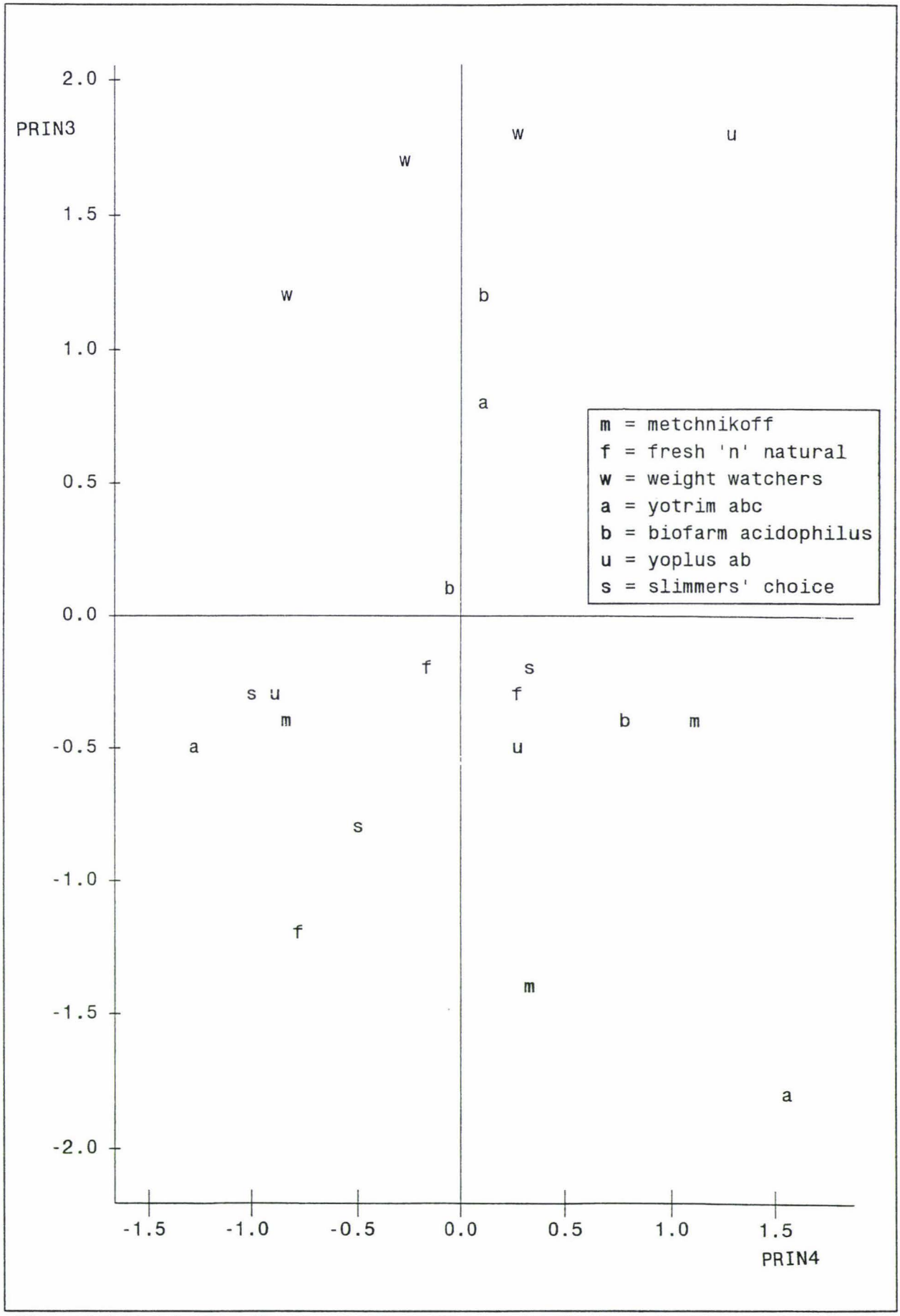


Figure 9.2 (b): PCA Plot Showing the Seven Commercial Yoghurts Analysed on Day 1 Along the P3 and P4.

According to Figure 9.2 (a) (page 185), it was apparent that the commercial yoghurts were well separated along the P1 axis but not as much along the P2 axis. Most of the yoghurts, except for Biofarm Acidophilus, were found along the 0 mark (P2 axis). The Biofarm Acidophilus yoghurt was placed between +2 and +3 (P2 axis), distinctly separate from the other yoghurts, suggesting that it had higher levels of acetone and acetoin but lower levels of 2-butanone.

The Fresh 'n' Natural yoghurt was placed around the -2 mark (P1 axis), while the Metchnikoff yoghurt was seen around the -1 mark (P1 axis). The Slimmers' Choice and Weight Watchers yoghurts were placed between the -0.5 and +0.5 region (P1 axis), while the Yoplus AB and Yotrim ABC yoghurts were seen around the +1 mark. The yoghurts, which were found on the positive end of the P1 axis, had higher levels of acetoin and lower 2-butanone levels, while the opposite was the case for the yoghurts on the negative side of the P1 axis.

It was evident from the PCA plot drawn for Day 1, that the yoghurt sample replicates were mostly clustered into distinct groups (similar to the PCA plot drawn for the 14 yoghurts), which reinforced the HS-SPME method's reproducibility. It also indicated that HS-SPME peak area results, in conjunction with PCA, could be used to divide various yoghurts into groups based on their analyte concentrations.

Acetaldehyde (0.70) and diacetyl (0.55) loaded highly on P3, while acetaldehyde (0.54) and ethanol (0.70) loaded highly on P4. P3 was correlated with acetaldehyde (0.74) and diacetyl (0.58), while P4 was correlated with acetaldehyde (0.42) and ethanol (0.56). Figure 9.2 (b) (page 186) shows the PCA plot for the commercial yoghurts along the P3 and P4 axes. It was apparent that the yoghurts were more randomly distributed across the plot and therefore, conclusions about the analyte levels in the yoghurts could not be made with confidence. Despite this, some interpretations can be made. For example, the Weight Watchers yoghurts were found around the +2 mark (P3 axis) suggesting that the yoghurt contained high levels of acetaldehyde and diacetyl.

According to Lindsay (1967), a desirable, "nutty-flavoured" yoghurt product requires a diacetyl:acetaldehyde ratio of between 3:1 to 5:1, while a "green" flavour in the yoghurts becomes apparent when this ratio falls to below 3:1. When the ratio exceeds

5:1, a harsh diacetyl flavour in the yoghurt is apparent. In this study, the diacetyl: acetaldehyde ratios for these yoghurts were very variable. Therefore, conclusions about the presence of desirable, "nutty" flavour or "green flavour" yoghurts, based on the diacetyl:acetaldehyde ratios, could not be made. The majority of the 14 yoghurts analysed in Section 9.3.1 however, had a diacetyl:acetaldehyde ratio of around 1:1 suggesting that the yoghurts had a strong "green" flavour at the time of HS-SPME analysis.

- **Results of Sensory Tests Carried Out on Day 1**

Table 9.5 (below) summarises the sensory evaluation results obtained from the tests carried out on Day 1.

Table 9.5: Results for Triangle Tests Carried Out on Day 1.

Triangle Test (DAY 1)	Total Number of Correct Answers for Each Triangle Test	Total Number of Panellists	Significance at $P < 0.05$ and $P < 0.01$
Test 1: SC vs FN	19	40	NS
Test 2: YAB vs YABC	14	40	NS
Test 3: M vs WW	12	40	NS
Test 4: BA vs YAB (plastic containers)	18	40	NS

"NS" = Not Significant

A one-tailed binomial test (statistical tables can be found in Appendix IV, page 224) was used to statistically analyse the triangle test results obtained. The null hypothesis (H_o) stated that the panellists could tell the difference between the various commercial yoghurts presented to them in the sensory testing. No significant results were obtained at the 95% or 99% confidence intervals for the triangular tests administered to the panellists on Day 1. This suggested that there was a 95% or 99% chance that the consumer panellists could not distinguish the difference between the commercial yoghurts based on smell alone.

- **PCA Carried Out on the HS-SPME Data Obtained on Day 2**

As there were only four commercial yoghurts (Yoplus AB, Slimmers' Choice, Yoplait High Fat, and Biofarm Acidophilus) being tested on Day 2, there was enough time to carry out HS-SPME on four replicates for each yoghurt. The analyte peak areas were then statistically analysed using PCA (SAS output for Day 2 results are shown in Appendix V, page 234). The "P1 vs P2" and "P3 vs P4" PCA plots obtained for the Day 2 results are shown in Figures 9.3 (a) and (b) (pages 190 and 191), respectively. The four principal components accounted for about 95% of the variation in the data.

From the PCA, a very strong correlation was observed between acetone and 2-butanone (0.95). This suggested that acetone and 2-butanone influenced each other to a significant extent. Both acetone and 2-butanone are volatile compounds known to originate from the breakdown of fat in cultured yoghurt products (Tamime and Deeth, 1980). A negative correlation was observed between acetone and acetaldehyde (-0.70) and between acetaldehyde and 2-butanone (-0.70). This suggested that yoghurts with high acetaldehyde concentrations had low levels of acetone and 2-butanone and vice versa. No other significant correlations were observed.

De Haast *et al.* (1979) commented that the "full yoghurt" flavour increased with increases in the acetaldehyde:acetone ratio. A strong yoghurt flavour is produced with an acetaldehyde:acetone ratio of about 2.8:1. In this study, the Yoplait High Fat, Slimmers' Choice (low fat) and Yoplus AB (high fat) yoghurts had a higher acetaldehyde:acetone ratio compared to the ratio quoted by De Haast *et al.*, suggesting that these three yoghurts had very strong yoghurt flavours. The Biofarm Acidophilus (high fat) yoghurt on the other hand, had a lower ratio than that quoted (~1:1) indicating that they had a weak yoghurt flavour.

Acetone (0.53) and 2-butanone (0.50) loaded highly on the first principal component (P1), while ethanol (0.55) and diacetyl (0.76) contributed greatly to the second principal component (P2). P1 was positively correlated with acetone (0.93), 2-butanone (0.89) and acetoin (0.62), and negatively correlated with acetaldehyde (-0.82) and ethanol (-0.58). These results are another indication of the fact that when the acetaldehyde concentrations are high, acetone and 2-butanone levels are relatively low. The second principal component was positively correlated with ethanol (0.62) and diacetyl (0.87).

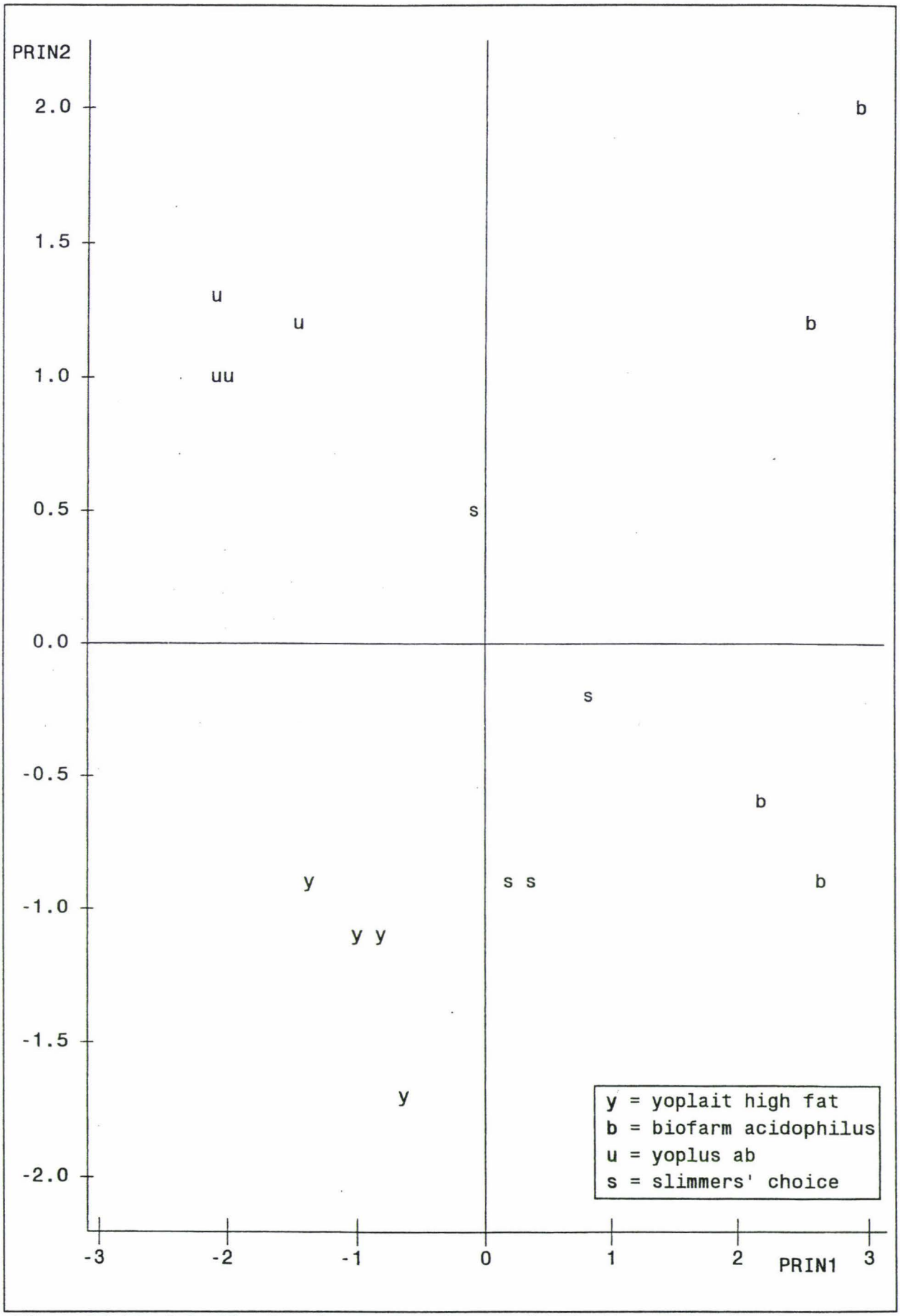


Figure 9.3 (a): PCA Plot Showing the Four Commercial Yoghurts Analysed on Day 2 Along the P1 and P2 Axes.

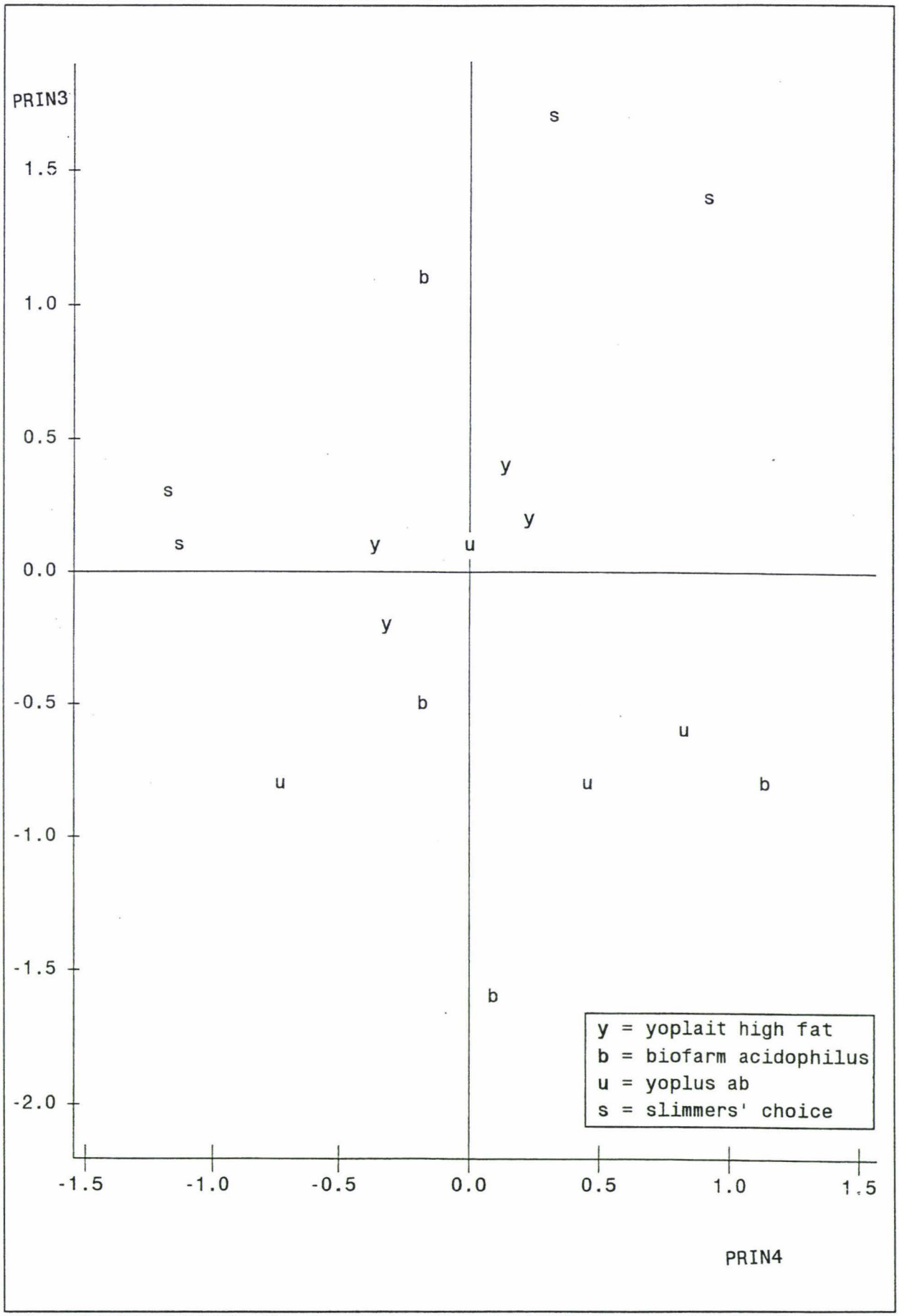


Figure 9.3 (b): PCA Plot Showing the Four Commercial Yoghurts Analysed on Day 2 Along the P1 and P2 Axes.

From the P1 vs P2 plot (Figure 9.3 (a) page 190), it was apparent that the HS-SPME method was fairly reproducible in its extracting procedure. This was seen on the PCA plot by the distinct clusters of similar analyte peak areas from replicate samples for each commercial yoghurt. The Yoplus AB yoghurts were found in a tight cluster around the -2 (P1 axis) and between the +1 to +1.5 (P2 axis). These results suggested that the Yoplus AB yoghurt had low levels of acetone and 2-butanone but higher ethanol and diacetyl concentrations. The Yoplait High Fat yoghurts were found in a cluster around -1 (P1 axis) and between -1 to -2 (P2 axis). This suggested that the Yoplait High Fat yoghurts had similar acetaldehyde, acetone and 2-butanone concentrations as the Yoplus AB yoghurts but varied in ethanol and diacetyl levels. The Biofarm Acidophilus yoghurts were placed around +2 to +3 (P1 axis) suggesting that the yoghurts had fairly high acetone and 2-butanone levels but low acetaldehyde concentrations. The replicates were positioned around -1 to +2 (P2 axis) suggesting that the replicates varied in ethanol and diacetyl concentrations.

The Slimmers' Choice yoghurts were found in a cluster around the intersection of the P1 and P2 axes suggesting that the yoghurt did not have outstanding amounts of acetone and 2-butanone levels compared with the other yoghurts. The replicates did vary along the P2 axis (-1 to ~+0.5), which indicated that the ethanol and diacetyl levels varied slightly between replicates, but not that much.

Figure 9.3 (b) (page 191) shows the "P3 vs P4" PCA plot for the replicates of the four yoghurts analysed on Day 2. Acetoin (0.78) loaded high on P3, while acetaldehyde (0.63) contributed fairly highly to P4. P3 had a fairly high correlation with acetoin (0.69), while P4 was correlated with acetaldehyde (0.42). It was evident from the PCA plot that the peak area values for the yoghurt replicates were randomly dispersed. Therefore, definite conclusions based on the P3 and P4 loadings could not be made. However, it was observed that Yoplait High Fat yoghurts were sited in a cluster around the intersection of the P3 and P4 axes. This suggested that the Yoplait High Fat yoghurts, unlike the other yoghurts, did not have outstanding acetoin and acetaldehyde levels.

- **Results of Sensory Tests Carried Out on Day 2**

The results from the triangle tests administered on Day 2 to the panellists are summarised in Table 9.6 (below).

Table 9.6: Results for Triangle Tests Carried Out on Day 2.

Triangle Test (DAY 2)	Total Number of Correct Answers for Each Triangle Test	Total Number of Panellists	Significance at $P < 0.05$ and $P < 0.01$
Test 1: YHF vs YAB	17	40	NS
Test 2: SC vs YAB	17	40	NS
Test 3: BA vs YAB (glass bottles)	8	40	NS

"NS" = Not Significant

Again, the null hypothesis (H_o) stated that the panellists would be able to differentiate between the yoghurts based on smell. A one-tailed binomial test was used to analyse the data and test this hypothesis. No significant results were found at the 95% and 99% confidence intervals (CI) for the sensory tests carried out on Day 2, concluding that the consumer panellists could not distinguish between the yoghurts based on smell alone.

Subsequent to this study, Ott *et al.*, 2000 found that the intensity of yoghurt flavour perception is highly dependent on the pH or acidity of the product. That is, the acidic aroma overrides the overall yoghurt aroma. At the time the present study was carried out, this confounding acidic aroma was not an issue in the headspace and sensory analyses.

Ott *et al.*, (2000) investigated the sensory properties of two types of yoghurts - traditional acidic yoghurt and mild, less acidic yoghurt. The two classes of yoghurts were characterised by a trained sensory panel using a descriptive approach. The yoghurts were evaluated first for smell attributes and then for appearance and texture, using a spoon. Finally the samples were tasted and rated for flavour. The authors found that the panel was very sensitive to acidic aroma variations between samples. Important differences were found between the two classes of yoghurts but these were mainly due

to the acidic aroma differences and not to the three analytes (acetaldehyde, diacetyl and 2,3-pentanedione) being investigated. Hence, the authors concluded that yoghurt flavour perception was pH dependent and that the yoghurt acidic aroma seemed to moderate or alter the perception of other attributes, such as overall liking and consumer acceptability. Thus, it was evident that in the present study, only smelling the yoghurt samples was an inadequate differentiating method for the untrained panellists. This was probably because the acidic aroma of the yoghurts, rather than the volatile flavour compounds, may have influenced their decisions. In this study therefore, an expert yoghurt panel trained to differentiate yoghurts based on smell, may have been of more use than the untrained consumer panel.

It was evident from the one-tailed binomial test results (95% and 99% CI) obtained for triangle Test 4 (Day 1) and Test 3 (Day 2), that the panellists could not differentiate the yoghurts stored in the glass or plastic containers. Therefore, it was concluded that the plastic containers did not taint the overall yoghurt aroma with a plastic odour.

Servili *et al.* (1995) attempted to relate the sensory and instrumental data obtained for olive oil samples using PCA and partial least squares regression analysis. They analysed thirty samples of virgin olive oil using both a trained sensory panel and an instrumental method (GC-MS). Using PCA, they observed that the instrumental data obtained for the oil samples formed groups or clusters on the PCA plots depending on the flavour analyte concentrations. This was very similar to what was found in the present study. The partial least squares regression method used in Servili *et al's.* study made good predictions of the quality of the oil samples from the headspace and sensory data obtained. Similar work to theirs could be carried out in future investigations where a trained sensory panel is used to relate sensory with HS-SPME data. Such an investigation may give some insight into the perception of the six analytes of interest and what their effect may be on yoghurt flavour preferences. It should be noted however, olive oil has a much simpler sample matrix than yoghurt, which is not affected as much, by variables such as pH and fat levels.

The untrained panellists were also selected for this preliminary trial based on Roberts and Vickers' (1994) findings. Roberts and Vickers (1994) carried out an investigation to compare the preferences and performance of three groups of judges (Descriptive

Analysis judges, American Dairy Science Association (ADSA) - trained dairy judges and untrained judges). All the judges were asked to rate their liking and the sensory attributes after tasting five Cheddar cheeses. They found that the trained panels rated a couple of cheeses lower in liking than did the untrained panel. The trained judges tended to find larger differences in liking among the cheeses than the untrained panel. They also found that training did not improve the agreement among the judges on the cheeses. The authors also showed that the type of training given to an expert panel is important. In their case, the dairy judges were mostly trained to focus on cheese defects and not on the desirable attributes of the cheeses. The Descriptive Analysis (DA) trained panel and the untrained judges however, paid attention to both the desirable and undesirable attributes. There were some indications that trained panellists were more sensitive to defects in general than consumers, who either did not detect the defect or did not consider it undesirable. Despite this, they found that all three panels generally agreed on the relative liking rating, which suggested that the panels had similar preferences.

It should be noted that Roberts and Vickers' study involved tasting the cheeses to rate them, while the present study required the panellists to smell the yoghurts. This was done mainly because headspace volatiles above the yoghurts (yoghurt aroma) were of particular interest instead of the taste.

It is recommended that these triangle tests be repeated to determine whether the sensory results are reproducible. It is also recommended that different consumer panels be used to determine whether the results were representative of untrained panellists. Future investigations could include correlating sensory with analytical data. In order to do this, a trained panel should be used to determine the effects of the flavour compounds on the overall aroma of the yoghurt product. The panel should be able to quantify and describe the analytes based on the aroma of the yoghurt product and from this, the data could possibly be correlated with the instrumental data. Other multivariate statistical methods could be investigated with a view to correlating the two data sets and obtaining meaningful information about the aromatic headspaces above yoghurts.

9.4 Conclusion

In conclusion, it was apparent that the HS-SPME method used to extract the six analytes from the commercial yoghurts was fairly reproducible. This was evident on the PCA plots where the replicate samples for each yoghurt were found grouped in specific regions on the PCA plots. Hence, it was concluded that the PCA method was a useful statistical tool to classify various commercial yoghurts based on the analyte peak areas obtained from the HS-SPME data.

It was evident from the sensory results that the untrained panellists were unable to differentiate yoghurts based on smell alone as the HS-SPME technique could. A sensory panel specifically trained to differentiate yoghurts in this manner may have been more useful.

CHAPTER TEN

SUMMARY AND FUTURE DIRECTIONS

To date there are no published studies where HS-SPME GC has been used to study yoghurt volatiles. Thus, this study is unique. The first step was to develop a prototype method for HS-SPME GC analysis of yoghurts. This was done by first selecting a suitable fibre coating. Five fibres were available of which, three were not considered on the basis of their specifications. The remaining two fibres (85 μm PA and 100 μm PDMS) were tested on simulated and commercial yoghurts. The results from this comparison, together with data from SPME studies in the literature, indicated that the 100 μm PDMS fibre would be most appropriate for examining yoghurt headspace.

In order to quantify the analytes in the chromatograms, two calibration techniques were investigated. The IS method involved spiking samples with a 200 ppm propan-2-ol solution. However, this method could not be used to quantify the analytes because the response factors for each fibre/analyte combination were not known. Hence, the IS peak was only used to normalise peak areas in each chromatogram. The ES method, although time-consuming, was used on a day-to-day basis throughout this study to quantify the six analytes in the yoghurt headspace. This method involved analysing standard water- or milk-based samples with known analyte concentrations prior to sample analysis. Calibration curves of peak area versus concentration were plotted and the sample analyte peak areas converted to concentrations.

Having selected the PDMS fibre, it was necessary to determine the optimum exposure time in the headspace. This was done by plotting analyte peak areas versus exposure times for each analyte. After evaluation of the six adsorption time profiles, 20 minutes was selected as the best compromise between a satisfactory equilibration time within the sample system and an excessive total analysis time.

The performance of the prototype HS-SPME technique was then compared with an established headspace procedure, the classical SH method. The two methods were used to extract and quantify analytes from aqueous samples and milk-based simulated

yoghurts. The HS-SPME GC method detected all six analytes over the 0-500 ppm range. The SH method could not detect acetoin and had trouble picking up ethanol and diacetyl at concentrations below 25 ppm. The technique did however, detect more acetaldehyde than the HS-SPME method at all concentrations. Hence, the two procedures were considered complimentary to a certain extent, but it was clear that the HS-SPME method was a more effective and sensitive method.

The influence of the sample matrix on the extraction ability of the HS-SPME method was investigated at several points in this study. Stronger detector responses (higher analyte peak areas) were observed for the aqueous samples than for the milk and yoghurt samples, indicating strong matrix effects in the latter. At the 0.3% and 3.5% fat levels, no significant differences were observed suggesting that changes of this magnitude did not affect the analyte concentrations or the HS-SPME method.

The new HS-SPME method was used to monitor the six analytes in low and high fat yoghurts during storage time and storage temperature trials. The storage trial results suggested that most of the analyte production occurred during the fermentation step. During the 2°C storage time trial, the concentration of 2-butanone and acetoin decreased with time, diacetyl increased while no marked changes were observed for the rest of the analytes. Storage at 10°C led to decreases in acetaldehyde, acetone and 2-butanone levels, a slight increase in diacetyl, while no marked changes were observed for ethanol or acetoin during the 24-day period. In the storage time trial, fat effects were only seen for diacetyl and acetoin, while in the storage temperature trial, fat effects were observed for diacetyl only. Ethanol was the only analyte to be affected by storage temperature. As only one or two analytes out of the six analysed were affected by the storage temperature (2°C or 10°C) or fat levels, it was concluded that these variables did not have major effects on the analyte concentrations.

The HS-SPME method was used to quantify the six analytes in fourteen commercial yoghurts. Analysis of the resulting data using PCA allowed a statistical picture to be built of the yoghurts. From the PCA analysis, eight of the yoghurts were selected and fresh samples reanalysed using HS-SPME and then PCA. These eight yoghurts were then passed on to an untrained consumer panel for sensory evaluation. On the basis of

smell, the panel was unable to distinguish between pairs of the yoghurts despite the samples being generally well separated on the PCA plots.

The strengths of this method, were: simplicity; it is a solvent-free technique with the possibility of being automated; and analytes with a range of volatilities (such as acetaldehyde [BP 21°C] and acetoin [BP 146°C]) could be extracted and quantified with good sensitivity. However, data variability was encountered throughout this study. This is partly inherent in relatively inhomogeneous biological systems such as yoghurt. Any variability stemming from the prototype HS-SPME method needs to be addressed before the method is used routinely, for yoghurt headspace analysis.

There are several areas that need further exploration:

- New fibres may become available that are more suitable for yoghurt headspace extraction/analysis;

- Using GC-MS may improve analyte detection and allow a wider range of analytes to be quantified; and

- Mixing (sonication, magnetic stirring) and/or heating of samples during analyte adsorption onto the fibre may make the extraction faster and more complete.

The pH of the yoghurt matrix may be a factor in determining volatile concentrations in the headspace. The commercial yoghurts investigated in this study had a pH range from3.5 to 4.8. This relatively wide range includes the isoelectric point of casein. Thus the solubility of the proteins in the yoghurt matrix may vary depending on how close the pH is to this isoelectric point. This may in turn, impact on the distribution of volatiles between the sample matrix and headspace. A study to determine the dependence of headspace volatile concentration on pH of the analyte matrix may be useful.

Once the HS-SPME-based method has been perfected, it will be ideal for routine analysis of a range of cultured dairy products such as yoghurts, kefir, buttermilk and sour cream. It could be used in a quality control role – checking flavour profiles and detecting off-flavours. In this way, it might supplement and hasten existing quality

control work, which might result in faster release to market. An accurate technique would also be useful in product development where it might give rapid feedback on whether the product's headspace flavour profile matches the target required.

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APPENDIX I Simultaneous Equations.

Anchor whole milk powder (WMP) [25 g of WMP in 100 mL milk].

3.4% milk fat

3.4% protein

4.6% carbohydrates

Anchor skim milk powder (SMP) [18.75g of SMP in 100 mL milk].

0.1% milk fat

3.8% protein

4.8% carbohydrates

% Fat WMP =>3.4/25g x 100% = 13.6%

% Protein WMP=>3.4/25g x 100% = 13.6%

%Fat SMP =>0.1/18.75g x 100% = 0.53%

%Protein SMP =>3.8/18.75g x 100% = 20.27%

Simultaneous Equations:

$13.6x + 0.53y = 0.3\%$ (Low Fat) {1}

$13.6x + 20.27y = 4.5\%$ {2}

$27.2x + 21.23y = 4.8$

$\left\{ y = \frac{3.5 - 13.6x}{0.53} \right\}$

Substituting in {2}

Low Fat

$\Rightarrow 13.6x + 20.27 \left\{ \frac{0.3 - 13.6x}{0.53} \right\} = 4.5$

$\Rightarrow 13.6x + 11.47 - 520.14x = 4.5$

$\Rightarrow 506.54x = 6.97$

$\Rightarrow x = 0.014$ (x 100%)

$\Rightarrow x = 1.4\%$ WMP (LF)

Substituting in {1}

$13.6(0.014) + 0.53y = 0.3$

$\Rightarrow 0.53y = 0.3 - 0.19$

$\Rightarrow y = 0.207$ (x 100%)

$\Rightarrow y = 20.7\%$ SMP (LF)

High Fat

$\mathbf{\Rightarrow 13.6x + 20.27 \left\{ \frac{3.5 - 13.6x}{0.53} \right\} = 4.5}$

$\Rightarrow 13.6x + 11.47 - 520.14x = 4.5$

$\Rightarrow 506.54x = 129.36$

$\Rightarrow x = 0.255$ (x 100%)

$\Rightarrow x = 25.5\%$ WMP (HF)

$13.6(0.255) + 0.53y = 3.5$

$\Rightarrow 0.53y = 3.5 - 3.47$

$\Rightarrow y = 0.060$ (x 100%)

$\Rightarrow y = 6.00\%$ SMP (HF)

APPENDIX II Statistical Tables for ANOVA Tests

(from O'Mahony, M. 1986).

Degrees of freedom for lesser mean square (denominator)	Degrees of freedom for greater mean square (numerator)																								
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
14	4.60	3.74	3.34	3.11	2.96	2.85	2.77	2.70	2.65	2.60	2.56	2.53	2.48	2.44	2.39	2.35	2.31	2.27	2.24	2.21	2.19	2.16	2.14	2.13	14
	8.86	6.51	5.56	5.03	4.69	4.46	4.28	4.14	4.03	3.94	3.86	3.80	3.70	3.62	3.51	3.43	3.34	3.26	3.21	3.14	3.11	3.06	3.02	3.00	
15	4.54	3.68	3.29	3.06	2.90	2.79	2.70	2.64	2.59	2.55	2.51	2.48	2.43	2.39	2.33	2.29	2.25	2.21	2.18	2.15	2.12	2.10	2.08	2.07	15
	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.89	3.80	3.73	3.67	3.56	3.48	3.36	3.29	3.20	3.12	3.07	3.00	2.97	2.92	2.89	2.87	
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49	2.45	2.42	2.37	2.33	2.28	2.24	2.20	2.16	2.13	2.09	2.07	2.04	2.02	2.01	16
	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.78	3.69	3.61	3.55	3.45	3.37	3.25	3.18	3.10	3.01	2.96	2.98	2.86	2.80	2.77	2.75	
17	4.45	3.59	3.20	2.96	2.81	2.70	2.62	2.55	2.50	2.45	2.41	2.38	2.33	2.29	2.23	2.19	2.15	2.11	2.08	2.04	2.02	1.99	1.97	1.96	17
	8.40	6.11	5.18	4.67	4.34	4.10	3.93	3.79	3.68	3.59	3.52	3.45	3.35	3.27	3.16	3.08	3.00	2.92	2.86	2.79	2.76	2.70	2.67	2.65	
18	4.41	3.55	3.16	2.93	2.77	3.66	2.58	2.51	2.46	2.41	2.37	2.34	2.29	2.25	2.19	2.15	2.11	2.07	2.04	2.00	1.98	1.95	1.93	1.92	18
	8.28	6.01	5.09	4.58	4.25	4.01	3.85	3.71	3.60	3.51	3.44	3.37	3.27	3.19	3.07	3.00	2.91	2.83	2.78	2.71	2.68	2.62	2.59	2.57	
19	4.38	3.52	3.13	2.90	2.74	2.63	2.55	2.48	2.43	2.38	2.34	2.31	2.26	2.21	2.15	2.11	2.07	2.02	2.00	1.96	1.94	1.91	1.90	1.88	19
	8.18	5.93	5.01	4.50	4.17	3.94	3.77	3.63	3.52	3.43	3.36	3.30	3.19	3.12	3.00	2.92	2.84	2.76	2.70	2.63	2.60	2.54	2.51	2.49	
20	4.35	3.49	3.10	2.87	2.71	2.60	2.52	2.45	2.40	2.35	2.31	2.28	2.23	2.18	2.12	2.08	2.04	1.99	1.96	1.92	1.90	1.87	1.85	1.84	20
	8.10	5.85	4.94	4.43	4.10	3.87	3.71	3.56	3.45	3.37	3.30	3.23	3.13	3.05	2.94	2.86	2.77	2.69	2.63	2.56	2.53	2.47	2.44	2.42	
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32	2.28	2.25	2.20	2.15	2.09	2.05	2.00	1.96	1.93	1.89	1.87	1.84	1.82	1.81	21
	8.02	5.78	4.87	4.37	4.04	3.81	3.65	3.51	3.40	3.31	3.24	3.17	3.07	2.99	2.88	2.80	2.72	2.63	2.58	2.51	2.47	2.42	2.38	2.36	
22	4.30	3.44	3.05	2.82	2.66	2.55	2.47	2.40	2.35	2.30	2.26	2.23	2.18	2.13	2.07	2.03	1.98	1.93	1.91	1.87	1.84	1.81	1.80	1.78	22
	7.94	5.72	4.82	4.31	3.99	3.76	3.59	3.45	3.35	3.26	3.18	3.12	3.02	2.94	2.83	2.75	2.67	2.58	2.53	2.46	2.42	2.37	2.33	2.31	
23	4.28	3.42	3.03	2.80	2.64	2.53	2.45	2.38	2.32	2.28	2.24	2.20	2.14	2.10	2.04	2.00	1.96	1.91	1.88	1.84	1.82	1.79	1.77	1.76	23
	7.88	5.66	4.76	4.26	3.94	3.71	3.54	3.41	3.30	3.21	3.14	3.07	2.97	2.89	2.78	2.70	2.62	2.53	2.48	2.41	2.37	2.32	2.28	2.26	
24	4.26	3.40	3.01	2.78	2.62	2.51	2.43	2.36	2.30	2.26	2.22	2.18	2.13	2.09	2.02	1.98	1.94	1.89	1.86	1.82	1.80	1.76	1.74	1.73	24
	7.82	5.61	4.72	4.22	3.90	3.67	3.50	3.36	3.25	3.17	3.09	3.03	2.93	2.85	2.74	2.66	2.58	2.49	2.44	2.36	2.33	2.27	2.23	2.21	
25	4.24	3.38	2.99	2.76	2.60	2.49	2.41	2.34	2.28	2.24	2.20	2.16	2.11	2.06	2.00	1.96	1.92	1.87	1.84	1.80	1.77	1.74	1.72	1.71	25
	7.77	5.57	4.68	4.18	3.86	3.63	3.46	3.32	3.21	3.13	3.05	2.99	2.89	2.81	2.70	2.62	2.54	2.45	2.40	2.32	2.29	2.23	2.19	2.17	
26	4.22	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22	2.18	2.15	2.10	2.05	1.99	1.95	1.90	1.85	1.82	1.78	1.76	1.72	1.70	1.69	26
	7.72	5.53	4.64	4.14	3.82	3.59	3.42	3.29	3.17	3.09	3.02	2.96	2.86	2.77	2.66	2.58	2.50	2.41	2.36	2.28	2.25	2.19	2.15	2.13	

APPENDIX II ctd. Statistical Tables for ANOVA Tests.

Degrees of freedom for lesser mean square [denominator]

	Degrees of freedom for greater mean square [numerator]																							
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
27	4.21 7.68	3.35 5.49	2.96 4.60	2.73 4.11	2.57 3.79	2.46 3.56	2.37 3.39	2.30 3.26	2.25 3.14	2.20 3.06	2.16 2.98	2.13 2.93	2.08 2.83	2.03 2.74	1.97 2.63	1.93 2.55	1.88 2.47	1.84 2.38	1.80 2.33	1.76 2.25	1.74 2.21	1.71 2.16	1.68 2.12	1.67 2.10	27
28	4.20 7.64	3.34 5.45	2.95 4.57	2.71 4.07	2.56 3.76	2.44 3.53	2.36 3.36	2.29 3.23	2.24 3.11	2.19 3.03	2.15 2.95	2.12 2.90	2.06 2.80	2.02 2.71	1.96 2.60	1.91 2.52	1.87 2.44	1.81 2.35	1.78 2.30	1.75 2.22	1.72 2.18	1.69 2.13	1.67 2.09	1.65 2.06	28
29	4.18 7.60	3.33 5.42	2.93 4.54	2.70 4.04	2.54 3.73	2.43 3.50	2.35 3.33	2.28 3.20	2.22 3.08	2.18 3.00	2.14 2.92	2.10 2.87	2.05 2.77	2.00 2.68	1.94 2.57	1.90 2.49	1.85 2.41	1.80 2.32	1.77 2.27	1.73 2.19	1.71 2.15	1.68 2.10	1.65 2.06	1.64 2.03	29
30	4.17 7.56	3.32 5.39	2.92 4.51	2.69 4.02	2.53 3.70	2.42 3.47	2.34 3.30	2.27 3.17	2.21 3.06	2.16 2.98	2.12 2.90	2.09 2.84	2.04 2.74	1.99 2.66	1.93 2.55	1.89 2.47	1.84 2.38	1.79 2.29	1.76 2.24	1.72 2.16	1.69 2.13	1.66 2.07	1.64 2.03	1.62 2.01	30
32	4.15 7.50	3.30 5.34	2.90 4.46	2.67 3.97	2.51 3.66	2.40 3.42	2.32 3.25	2.25 3.12	2.19 3.01	2.14 2.94	2.10 2.86	2.07 2.80	2.02 2.70	1.97 2.62	1.91 2.51	1.86 2.42	1.82 2.34	1.76 2.25	1.74 2.20	1.69 2.12	1.67 2.08	1.64 2.02	1.61 1.98	1.59 1.96	32
34	4.13 7.44	3.28 5.29	2.88 4.42	2.65 3.93	2.49 3.61	2.38 3.38	2.30 3.21	2.23 3.08	2.17 2.97	2.12 2.89	2.08 2.82	2.05 2.76	2.00 2.66	1.95 2.58	1.89 2.47	1.84 2.38	1.80 2.30	1.74 2.21	1.71 2.15	1.67 2.08	1.64 2.04	1.61 1.98	1.59 1.94	1.57 1.91	34
36	4.11 7.39	3.26 5.25	2.86 4.38	2.63 3.89	2.48 3.58	2.36 3.35	2.28 3.18	2.21 3.04	2.15 2.94	2.10 2.86	2.06 2.78	2.03 2.72	1.98 2.62	1.93 2.54	1.87 2.43	1.82 2.35	1.78 2.26	1.72 2.17	1.69 2.12	1.65 2.04	1.62 2.00	1.59 1.94	1.56 1.90	1.55 1.87	36
38	4.10 7.35	3.25 5.21	2.85 4.34	2.62 3.86	2.46 3.54	2.35 3.32	2.26 3.15	2.19 3.02	2.14 2.91	2.09 2.82	2.05 2.75	2.02 2.69	1.96 2.59	1.92 2.51	1.85 2.40	1.80 2.32	1.76 2.22	1.71 2.14	1.67 2.08	1.63 2.00	1.60 1.97	1.57 1.90	1.54 1.86	1.53 1.84	38
40	4.08 7.31	3.23 5.18	2.84 4.31	2.61 3.83	2.45 3.51	2.34 3.29	2.25 3.12	2.18 2.99	2.12 2.88	2.07 2.80	2.04 2.73	2.00 2.66	1.95 2.56	1.90 2.49	1.84 2.37	1.79 2.29	1.74 2.20	1.69 2.11	1.66 2.05	1.61 1.97	1.59 1.94	1.55 1.88	1.53 1.84	1.51 1.81	40
42	4.07 7.27	3.22 5.15	2.83 4.29	2.59 3.80	2.44 3.49	2.32 3.26	2.24 3.10	2.17 2.96	2.11 2.86	2.06 2.77	2.02 2.70	1.99 2.64	1.94 2.54	1.89 2.46	1.82 2.35	1.78 2.26	1.73 2.17	1.68 2.08	1.64 2.02	1.60 1.94	1.57 1.91	1.54 1.85	1.51 1.80	1.49 1.78	42
44	4.06 7.24	3.21 5.12	2.82 4.26	2.58 3.78	2.43 3.46	2.31 3.24	2.23 3.07	2.16 2.94	2.10 2.84	2.05 2.75	2.01 2.68	1.98 2.62	1.92 2.52	1.88 2.44	1.81 2.32	1.76 2.24	1.72 2.15	1.66 2.06	1.63 2.00	1.58 1.92	1.56 1.88	1.52 1.82	1.50 1.78	1.48 1.75	44
46	4.05 7.21	3.20 5.10	2.81 4.24	2.57 3.76	2.42 3.44	2.30 3.22	2.22 3.05	2.14 2.92	2.09 2.82	2.04 2.73	2.00 2.66	1.97 2.60	1.91 2.50	1.87 2.42	1.80 2.30	1.75 2.22	1.71 2.13	1.65 2.04	1.62 1.98	1.57 1.90	1.54 1.86	1.51 1.80	1.48 1.76	1.46 1.72	46
48	4.04 7.19	3.19 5.08	2.80 4.22	2.56 3.74	2.41 3.42	2.30 3.20	2.21 3.04	2.14 2.90	2.08 2.80	2.03 2.71	1.99 2.64	1.96 2.58	1.90 2.48	1.86 2.40	1.79 2.28	1.74 2.20	1.70 2.11	1.64 2.02	1.61 1.96	1.56 1.88	1.53 1.84	1.50 1.78	1.47 1.73	1.45 1.70	48

APPENDIX II ctd. Statistical Tables for ANOVA Tests.

Degrees of freedom for greater mean square [numerator]

Degrees of freedom for lesser mean square [denominator]	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
50	4.03 7.17	3.18 5.06	2.79 4.20	2.56 3.72	2.40 3.41	2.29 3.18	2.20 3.02	2.13 2.88	2.07 2.78	2.02 2.70	1.98 2.62	1.95 2.56	1.90 2.46	1.85 2.39	1.78 2.26	1.74 2.18	1.69 2.10	1.63 2.00	1.60 1.94	1.55 1.86	1.52 1.82	1.48 1.76	1.46 1.71	1.44 1.68	50
55	4.02 7.12	3.17 5.01	2.78 4.16	2.54 3.68	2.38 3.37	2.27 3.15	2.18 2.98	2.11 2.85	2.05 2.75	2.00 2.66	1.97 2.59	1.93 2.53	1.88 2.43	1.83 2.35	1.76 2.23	1.72 2.15	1.67 2.06	1.61 1.96	1.58 1.90	1.52 1.82	1.50 1.78	1.46 1.71	1.43 1.66	1.41 1.64	55
60	4.00 7.08	3.15 4.98	2.76 4.13	2.52 3.65	2.37 3.34	2.25 3.12	2.17 2.95	2.10 2.82	2.04 2.72	1.99 2.63	1.95 2.56	1.92 2.50	1.86 2.40	1.81 2.32	1.75 2.20	1.70 2.12	1.65 2.03	1.59 1.93	1.56 1.87	1.50 1.79	1.48 1.74	1.44 1.68	1.41 1.63	1.39 1.60	60
65	3.99 7.04	3.14 4.95	2.75 4.10	2.51 3.62	2.36 3.31	2.24 3.09	2.15 2.93	2.08 2.79	2.02 2.70	1.98 2.61	1.94 2.54	1.90 2.47	1.85 2.37	1.80 2.30	1.73 2.18	1.68 2.09	1.63 2.00	1.57 1.90	1.54 1.84	1.49 1.76	1.46 1.71	1.42 1.64	1.39 1.60	1.37 1.56	65
70	3.98 7.01	3.13 4.92	2.74 4.08	2.50 3.60	2.35 3.29	2.23 3.07	2.14 2.91	2.07 2.77	2.01 2.67	1.97 2.59	1.93 2.51	1.89 2.45	1.84 2.35	1.79 2.28	1.72 2.15	1.67 2.07	1.62 1.98	1.56 1.88	1.53 1.82	1.47 1.74	1.45 1.69	1.40 1.62	1.37 1.56	1.35 1.53	70
80	3.96 6.96	3.11 4.88	2.72 4.04	2.48 3.56	2.33 3.25	2.21 3.04	2.12 2.87	2.05 2.74	1.99 2.64	1.95 2.55	1.91 2.48	1.88 2.41	1.82 2.32	1.77 2.24	1.70 2.11	1.65 2.03	1.60 1.94	1.54 1.84	1.51 1.78	1.45 1.70	1.42 1.65	1.38 1.57	1.35 1.52	1.32 1.49	80
100	3.94 6.90	3.09 4.82	2.70 3.98	2.46 3.51	2.30 3.20	2.19 2.99	2.10 2.82	2.03 2.69	1.97 2.59	1.92 2.51	1.88 2.43	1.85 2.36	1.79 2.26	1.75 2.19	1.68 2.06	1.63 1.98	1.57 1.89	1.51 1.79	1.48 1.73	1.42 1.64	1.39 1.59	1.34 1.51	1.30 1.46	1.28 1.43	100
125	3.92 6.84	3.07 4.78	2.68 3.94	2.44 3.47	2.29 3.17	2.17 2.95	2.08 2.79	2.01 2.65	1.95 2.56	1.90 2.47	1.86 2.40	1.83 2.33	1.77 2.23	1.72 2.15	1.65 2.03	1.60 1.94	1.55 1.85	1.49 1.75	1.45 1.68	1.39 1.59	1.36 1.54	1.31 1.46	1.27 1.40	1.25 1.37	125
150	3.91 6.81	3.06 4.75	2.67 3.91	2.43 3.44	2.27 3.14	2.16 2.92	2.07 2.76	2.00 2.62	1.94 2.53	1.89 2.44	1.85 2.37	1.82 2.30	1.76 2.20	1.71 2.12	1.64 2.00	1.59 1.91	1.54 1.83	1.47 1.72	1.44 1.66	1.37 1.56	1.34 1.51	1.29 1.43	1.25 1.37	1.22 1.33	150
200	3.89 6.76	3.04 4.71	2.65 3.88	2.41 3.41	2.26 3.11	2.14 2.90	2.05 2.73	1.98 2.60	1.92 2.50	1.87 2.41	1.83 2.34	1.80 2.28	1.74 2.17	1.69 2.09	1.62 1.97	1.57 1.88	1.52 1.79	1.45 1.69	1.42 1.62	1.35 1.53	1.32 1.48	1.26 1.39	1.22 1.33	1.19 1.28	200
400	3.86 6.70	3.02 4.66	2.62 3.83	2.39 3.36	2.23 3.06	2.12 2.85	2.03 2.69	1.96 2.55	1.90 2.46	1.85 2.37	1.81 2.29	1.78 2.23	1.72 2.12	1.67 2.04	1.60 1.92	1.54 1.84	1.49 1.74	1.42 1.64	1.38 1.57	1.32 1.47	1.28 1.42	1.22 1.32	1.16 1.24	1.13 1.19	400
1000	3.85 6.66	3.00 4.62	2.61 3.80	2.38 3.34	2.22 3.04	2.10 2.82	2.02 2.66	1.95 2.53	1.89 2.43	1.84 2.34	1.80 2.26	1.76 2.20	1.70 2.09	1.65 2.01	1.58 1.89	1.53 1.81	1.47 1.71	1.41 1.61	1.36 1.54	1.30 1.44	1.26 1.38	1.19 1.28	1.13 1.19	1.08 1.11	1000
∞	3.84 6.64	2.99 4.60	2.60 3.78	2.37 3.32	2.21 3.02	2.09 2.80	2.01 2.64	1.94 2.51	1.88 2.41	1.83 2.32	1.79 2.24	1.75 2.18	1.69 2.07	1.64 1.99	1.57 1.87	1.52 1.79	1.46 1.69	1.40 1.59	1.35 1.52	1.28 1.41	1.24 1.36	1.17 1.25	1.11 1.15	1.00 1.00	∞

Critical Values of F[a]

a. 0.05 level in roman type, 0.01 level in boldface

Degrees of freedom for lesser mean square [denominator]	Degrees of freedom for greater mean square [numerator]																								
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
1	161 **4,052**	200 **4,999**	216 **5,403**	225 **5,625**	230 **5,764**	234 **5,859**	237 **5,928**	239 **5,981**	241 **6,022**	242 **6,056**	243 **6,082**	244 **6,106**	245 **6,142**	246 **6,169**	248 **6,208**	249 **6,234**	250 **6,261**	251 **6,286**	252 **6,302**	253 **6,323**	253 **6,334**	254 **6,352**	254 **6,361**	254 **6,366**	1
2	18.51 **98.49**	19.00 **99.00**	19.16 **99.17**	19.25 **99.25**	19.30 **99.30**	19.33 **99.33**	19.36 **99.36**	19.37 **99.37**	19.38 **99.39**	19.39 **99.40**	19.40 **99.41**	19.41 **99.42**	19.42 **99.43**	19.43 **99.44**	19.44 **99.45**	19.45 **99.46**	19.46 **99.47**	19.47 **99.48**	19.47 **99.48**	19.48 **99.49**	19.49 **99.49**	19.49 **99.49**	19.50 **99.50**	19.50 **99.50**	2
3	10.13 **34.12**	9.55 **30.82**	9.28 **29.46**	9.12 **28.71**	9.01 **28.24**	8.94 **27.91**	8.88 **27.67**	8.84 **27.49**	8.81 **27.34**	8.78 **27.23**	8.76 **27.13**	8.74 **27.05**	8.71 **26.92**	8.69 **26.83**	8.66 **26.69**	8.64 **26.60**	8.62 **26.50**	8.60 **26.41**	8.58 **26.35**	8.57 **26.27**	8.56 **26.23**	8.54 **26.18**	8.54 **26.14**	8.53 **26.12**	3
4	7.71 **21.20**	6.94 **18.00**	6.59 **16.69**	6.39 **15.98**	6.26 **15.52**	6.16 **15.21**	6.09 **14.98**	6.04 **14.80**	6.00 **14.66**	5.96 **14.54**	5.93 **14.45**	5.91 **14.37**	5.87 **14.24**	5.84 **14.15**	5.80 **14.02**	5.77 **13.93**	5.74 **13.83**	5.71 **13.74**	5.70 **13.69**	5.68 **13.61**	5.66 **13.57**	5.65 **13.52**	5.64 **13.48**	5.63 **13.46**	4
5	6.61 **16.26**	5.79 **13.27**	5.41 **12.06**	5.19 **11.39**	5.05 **10.97**	4.95 **10.67**	4.88 **10.45**	4.82 **10.29**	4.78 **10.15**	4.74 **10.05**	4.70 **9.96**	4.68 **9.89**	4.64 **9.77**	4.60 **9.68**	4.56 **9.55**	4.53 **9.47**	4.50 **9.38**	4.46 **9.29**	4.44 **9.24**	4.42 **9.17**	4.40 **9.13**	4.38 **9.07**	4.37 **9.04**	4.36 **9.02**	5
6	5.99 **13.74**	5.14 **10.92**	4.76 **9.78**	4.53 **9.15**	4.39 **8.75**	4.28 **8.47**	4.21 **8.26**	4.15 **8.10**	4.10 **7.98**	4.06 **7.87**	4.03 **7.79**	4.00 **7.72**	3.96 **7.60**	3.92 **7.52**	3.87 **7.39**	3.84 **7.31**	3.81 **7.23**	3.77 **7.14**	3.75 **7.09**	3.72 **7.02**	3.71 **6.99**	3.69 **6.94**	3.68 **6.90**	3.67 **6.88**	6
7	5.59 **12.25**	4.74 **9.55**	4.35 **8.45**	4.12 **7.85**	3.97 **7.46**	3.87 **7.19**	3.79 **7.00**	3.73 **6.84**	3.68 **6.71**	3.63 **6.62**	3.60 **6.54**	3.57 **6.47**	3.52 **6.35**	3.49 **6.27**	3.44 **6.15**	3.41 **6.07**	3.38 **5.98**	3.34 **5.90**	3.32 **5.85**	3.29 **5.78**	3.28 **5.75**	3.25 **5.70**	3.24 **5.67**	3.23 **5.65**	7
8	5.32 **11.26**	4.46 **8.65**	4.07 **7.59**	3.84 **7.01**	3.69 **6.63**	3.58 **6.37**	3.50 **6.19**	3.44 **6.03**	3.39 **5.91**	3.34 **5.82**	3.31 **5.74**	3.28 **5.67**	3.23 **5.56**	3.20 **5.48**	3.15 **5.36**	3.12 **5.28**	3.08 **5.20**	3.05 **5.11**	3.03 **5.06**	3.00 **5.00**	2.98 **4.96**	2.96 **4.91**	2.94 **4.88**	2.93 **4.86**	8
9	5.12 **10.56**	4.26 **8.02**	3.86 **6.99**	3.63 **6.42**	3.48 **6.06**	3.37 **5.80**	3.29 **5.62**	3.23 **5.47**	3.18 **5.35**	3.13 **5.26**	3.10 **5.18**	3.07 **5.11**	3.02 **5.00**	2.98 **4.92**	2.93 **4.80**	2.90 **4.73**	2.86 **4.64**	2.82 **4.56**	2.80 **4.51**	2.77 **4.45**	2.76 **4.41**	2.73 **4.36**	2.72 **4.33**	2.71 **4.31**	9
10	4.96 **10.04**	4.10 **7.56**	3.71 **6.55**	3.48 **5.99**	3.33 **5.64**	3.22 **5.39**	3.14 **5.21**	3.07 **5.06**	3.02 **4.95**	2.97 **4.85**	2.94 **4.78**	2.91 **4.71**	2.86 **4.60**	2.82 **4.52**	2.77 **4.41**	2.74 **4.33**	2.70 **4.25**	2.67 **4.17**	2.64 **4.12**	2.61 **4.05**	2.59 **4.01**	2.56 **3.96**	2.55 **3.93**	2.54 **3.91**	10
11	4.84 **9.65**	3.98 **7.20**	3.59 **6.22**	3.36 **5.67**	3.20 **5.32**	3.09 **5.07**	3.01 **4.88**	2.95 **4.74**	2.90 **4.63**	2.86 **4.54**	2.82 **4.46**	2.79 **4.40**	2.74 **4.29**	2.70 **4.21**	2.65 **4.10**	2.61 **4.02**	2.57 **3.94**	2.53 **3.86**	2.50 **3.80**	2.47 **3.74**	2.45 **3.70**	2.42 **3.66**	2.41 **3.62**	2.40 **3.60**	11
12	4.75 **9.33**	3.88 **6.93**	3.49 **5.95**	3.26 **5.41**	3.11 **5.06**	3.00 **4.82**	2.92 **4.65**	2.85 **4.50**	2.80 **4.39**	2.76 **4.30**	2.72 **4.22**	2.69 **4.16**	2.64 **4.05**	2.60 **3.98**	2.54 **3.86**	2.50 **3.78**	2.46 **3.70**	2.42 **3.61**	2.40 **3.56**	2.36 **3.49**	2.35 **3.46**	2.32 **3.41**	2.31 **3.38**	2.30 **3.36**	12
13	4.67 **9.07**	3.80 **6.70**	3.41 **5.74**	3.18 **5.20**	3.02 **4.86**	2.92 **4.62**	2.84 **4.44**	2.77 **4.30**	2.72 **4.19**	2.67 **4.10**	2.63 **4.02**	2.60 **3.96**	2.55 **3.85**	2.51 **3.78**	2.46 **3.67**	2.42 **3.59**	2.38 **3.51**	2.34 **3.42**	2.32 **3.37**	2.28 **3.30**	2.26 **3.27**	2.24 **3.21**	2.22 **3.18**	2.21 **3.16**	13

[a]The values in the table are the critical values of F for the degrees of freedom listed over the columns (the degrees of freedom for the greater mean square or numerator of the F ratio) and the degrees of freedom listed for the rows (the degrees of freedom for the lesser mean square for the denominator of the F ratio): The critical value for the 0.05 level of significance is presented first (roman type) followed by the critical value at the 0.01 level (boldface). If the observed value is *greater than or equal to* the tabled value, reject H_0. The function, F = c with exponent 2z, is computed in part from Fisher's table VI (7). Additional entries are by interpolation, mostly graphical.

APPENDIX II ctd. Statistical Tables for ANOVA Tests.

0.001 level

Deg of freedom for denom.	Degrees of freedom for numerator									
	1	2	3	4	5	6	8	12	24	∞
1	405284	500000	540379	562500	576405	585937	598144	610667	623497	636619
2	998·5	999·0	999·2	999·2	999·3	999·3	999·4	999·4	999·5	999·5
3	167·0	148·5	141·1	137·1	134·6	132·8	130·6	128·3	125·9	123·5
4	74·14	61·25	56·18	53·44	51·71	50·53	49·00	47·41	45·77	44·05
5	47·18	37·12	33·20	31·09	29·75	28·84	27·64	26·42	25·14	23·78
6	35·51	27·00	23·70	21·92	20·81	20·03	19·03	17·99	16·89	15·75
7	29·25	21·69	18·77	17·19	16·21	15·52	14·63	13·71	12·73	11·69
8	25·42	18·49	15·83	14·39	13·49	12·86	12·04	11·19	10·30	9·34
9	22·86	16·39	13·90	12·56	11·71	11·13	10·37	9·57	8·72	7·81
10	21·04	14·91	12·55	11·28	10·48	9·92	9·20	8·45	7·64	6·76
11	19·69	13·81	11·56	10·35	9·58	9·05	8·35	7·63	6·85	6·00
12	18·64	12·97	10·80	9·63	8·89	8·38	7·71	7·00	6·25	5·42
13	17·81	12·31	10·21	9·07	8·35	7·86	7·21	6·52	5·78	4·97
14	17·14	11·78	9·73	8·62	7·92	7·43	6·80	6·13	5·41	4·60
15	16·59	11·34	9·34	8·25	7·57	7·09	6·47	5·81	5·10	4·31
16	16·12	10·97	9·00	7·94	7·27	6·81	6·19	5·55	4·85	4·06
17	15·72	10·66	8·73	7·68	7·02	6·56	5·96	5·32	4·63	3·85
18	15·38	10·39	8·49	7·46	6·81	6·35	5·76	5·13	4·45	3·67
19	15·08	10·16	8·28	7·26	6·62	6·18	5·59	4·97	4·29	3·52
20	14·82	9·95	8·10	7·10	6·46	6·02	5·44	4·82	4·15	3·38
21	14·59	9·77	7·94	6·95	6·32	5·88	5·31	4·70	4·03	3·26
22	14·38	9·61	7·80	6·81	6·19	5·76	5·19	4·58	3·92	3·15
23	14·19	9·47	7·67	6·69	6·08	5·65	5·09	4·48	3·82	3·05
24	14·03	9·34	7·55	6·59	5·98	5·55	4·99	4·39	3·74	2·97
25	13·88	9·22	7·45	6·49	5·88	5·46	4·91	4·31	3·66	2·89
26	13·74	9·12	7·36	6·41	5·80	5·38	4·83	4·24	3·59	2·82
27	13·61	9·02	7·27	6·33	5·73	5·31	4·76	4·17	3·52	2·75
28	13·50	8·93	7·19	6·25	5·66	5·24	4·69	4·11	3·46	2·70
29	13·39	8·85	7·12	6·19	5·59	5·18	4·64	4·05	3·41	2·64
30	13·29	8·77	7·05	6·12	5·53	5·12	4·58	4·00	3·36	2·59
40	12·61	8·25	6·60	5·70	5·13	4·73	4·21	3·64	3·01	2·23
60	11·97	7·76	6·17	5·31	4·76	4·37	3·87	3·31	2·69	1·90
120	11·38	7·32	5·79	4·95	4·42	4·04	3·55	3·02	2·40	1·54
∞	10·83	6·91	5·42	4·62	4·10	3·74	3·27	2·74	2·13	1·00

Source: Table V of Fisher and Yates, *Statistical Tables for Biological, Agricultural and Medical Research*, published by Longman Group Ltd., London (previously published by Oliver and Boyd Ltd., Edinburgh) and by permission of the authors and publishers.

APPENDIX II ctd. Statistical Tables for Tukeys Comparison Tests (from O'Mahony, M. 1986).

Significant Studentized Ranges for Newman-Keuls' and Tukey Multiple-Comparison Tests

Significant Studentized Ranges (two-tailed)

Error *df*	α	*r* = number of means or number of steps between ordered means									
		2	3	4	5	6	7	8	9	10	11
5	.05	3.64	4.60	5.22	5.67	6.03	6.33	6.58	6.80	6.99	7.17
	.01	5.70	6.98	7.80	8.42	8.91	9.32	9.67	9.97	10.24	10.48
6	.05	3.46	4.34	4.90	5.30	5.63	5.90	6.12	6.32	6.49	6.65
	.01	5.24	6.33	7.03	7.56	7.97	8.32	8.61	8.87	9.10	9.30
7	.05	3.34	4.16	4.68	5.06	5.36	5.61	5.82	6.00	6.16	6.30
	.01	4.95	5.92	6.54	7.01	7.37	7.68	7.94	8.17	8.37	8.55
8	.05	3.26	4.04	4.53	4.89	5.17	5.40	5.60	5.77	5.92	6.05
	.01	4.75	5.64	6.20	6.62	6.96	7.24	7.47	7.68	7.86	8.03
9	.05	3.20	3.95	4.41	4.76	5.02	5.24	5.43	5.59	5.74	5.87
	.01	4.60	5.43	5.96	6.35	6.66	6.91	7.13	7.33	7.49	7.65
10	.05	3.15	3.88	4.33	4.65	4.91	5.12	5.30	5.46	5.60	5.72
	.01	4.48	5.27	5.77	6.14	6.43	6.67	6.87	7.05	7.21	7.36
11	.05	3.11	3.82	4.26	4.57	4.82	5.03	5.20	5.35	5.49	5.61
	.01	4.39	5.15	5.62	5.97	6.25	6.48	6.67	6.84	6.99	7.13
12	.05	3.08	3.77	4.20	4.51	4.75	4.95	5.12	5.27	5.39	5.51
	.01	4.32	5.05	5.50	5.84	6.10	6.32	6.51	6.67	6.81	6.94
13	.05	3.06	3.73	4.15	4.45	4.69	4.88	5.05	5.19	5.32	5.43
	.01	4.26	4.96	5.40	5.73	5.98	6.19	6.37	6.53	6.67	6.79
14	.05	3.03	3.70	4.11	4.41	4.64	4.83	4.99	5.13	5.25	5.36
	.01	4.21	4.89	5.32	5.63	5.88	6.08	6.26	6.41	6.54	6.66
15	.05	3.01	3.67	4.08	4.37	4.59	4.78	4.94	5.08	5.20	5.31
	.01	4.17	4.84	5.25	5.56	5.80	5.99	6.16	6.31	6.44	6.55
16	.05	3.00	3.65	4.05	4.33	4.56	4.74	4.90	5.03	5.15	5.26
	.01	4.13	4.79	5.19	5.49	5.72	5.92	6.08	6.22	6.35	6.46
17	.05	2.98	3.63	4.02	4.30	4.52	4.70	4.86	4.99	5.11	5.21
	.01	4.10	4.74	5.14	5.43	5.66	5.85	6.01	6.15	6.27	6.38
18	.05	2.97	3.61	4.00	4.28	4.49	4.67	4.82	4.96	5.07	5.17
	.01	4.07	4.70	5.09	5.38	5.60	5.79	5.94	6.08	6.20	6.31
19	.05	2.96	3.59	3.98	4.25	4.47	4.65	4.79	4.92	5.04	5.14
	.01	4.05	4.67	5.05	5.33	5.55	5.73	5.89	6.02	6.14	6.25
20	.05	2.95	3.58	3.96	4.23	4.45	4.62	4.77	4.90	5.01	5.11
	.01	4.02	4.64	5.02	5.29	5.51	5.69	5.84	5.97	6.09	6.19
24	.05	2.92	3.53	3.90	4.17	4.37	4.54	4.68	4.81	4.92	5.01
	.01	3.96	4.55	4.91	5.17	5.37	5.54	5.69	5.81	5.92	6.02
30	.05	2.89	3.49	3.85	4.10	4.30	4.46	4.60	4.72	4.82	4.92
	.01	3.89	4.45	4.80	5.05	5.24	5.40	5.54	5.65	5.76	5.85
40	.05	2.86	3.44	3.79	4.04	4.23	4.39	4.52	4.63	4.73	4.82
	.01	3.82	4.37	4.70	4.93	5.11	5.26	5.39	5.50	5.60	5.69
60	.05	2.83	3.40	3.74	3.98	4.16	4.31	4.44	4.55	4.65	4.73
	.01	3.76	4.28	4.59	4.82	4.99	5.13	5.25	5.36	5.45	5.53
120	.05	2.80	3.36	3.68	3.92	4.10	4.24	4.36	4.47	4.56	4.64
	.01	3.70	4.20	4.50	4.71	4.87	5.01	5.12	5.21	5.30	5.37
∞	.05	2.77	3.31	3.63	3.86	4.03	4.17	4.29	4.39	4.47	4.55
	.01	3.64	4.12	4.40	4.60	4.76	4.88	4.99	5.08	5.16	5.23

[a]To use table, locate *df* and *r* values at the α level desired. The point of interaction (q) is the minimum significant value; this value multiplied by the standard error of the means is the minimum critical value. For the Tukey test, only the value for the largest range is needed.

APPENDIX II ctd. Statistical Tables for Tukeys Comparison Tests.

r = number of means or number of steps between ordered means									α	Error *df*
12	13	14	15	16	17	18	19	20		
7.32	7.47	7.60	7.72	7.83	7.93	8.03	8.12	8.21	.05	5
10.70	10.89	11.08	11.24	11.40	11.55	11.68	11.81	11.93	.01	
6.79	6.92	7.03	7.14	7.24	7.34	7.43	7.51	7.59	.05	6
9.48	9.65	9.81	9.95	10.08	10.21	10.32	10.43	10.54	.01	
6.43	6.55	6.66	6.76	6.85	6.94	7.02	7.10	7.17	.05	7
8.71	8.86	9.00	9.12	9.24	9.35	9.46	9.55	9.65	.01	
6.18	6.29	6.39	6.48	6.57	6.65	6.73	6.80	6.87	.05	8
8.18	8.31	8.44	8.55	8.66	8.76	8.85	8.94	9.03	.01	
5.98	6.09	6.19	6.28	6.36	6.44	6.51	6.58	6.64	.05	9
7.78	7.91	8.03	8.13	8.23	8.33	8.41	8.49	8.57	.01	
5.83	5.93	6.03	6.11	6.19	6.27	6.34	6.40	6.47	.05	10
7.49	7.60	7.71	7.81	7.91	7.99	8.08	8.15	8.23	.01	
5.71	5.81	5.90	5.98	6.06	6.13	6.20	6.27	6.33	.05	11
7.25	7.36	7.46	7.56	7.65	7.73	7.81	7.88	7.95	.01	
5.61	5.71	5.80	5.88	5.95	6.02	6.09	6.15	6.21	.05	12
7.06	7.17	7.26	7.36	7.44	7.52	7.59	7.66	7.73	.01	
5.53	5.63	5.71	5.79	5.86	5.93	5.99	6.05	6.11	.05	13
6.90	7.01	7.10	7.19	7.27	7.35	7.42	7.48	7.55	.01	
5.46	5.55	5.64	5.71	5.79	5.85	5.91	5.97	6.03	.05	14
6.77	6.87	6.96	7.05	7.13	7.20	7.27	7.33	7.39	.01	
5.40	5.49	5.57	5.65	5.72	5.78	5.85	5.90	5.96	.05	15
6.66	6.76	6.84	6.93	7.00	7.07	7.14	7.20	7.26	.01	
5.35	5.44	5.52	5.59	5.66	5.73	5.79	5.84	5.90	.05	16
6.56	6.66	6.74	6.82	6.90	6.97	7.03	7.09	7.15	.01	
5.31	5.39	5.47	5.54	5.61	5.67	5.73	5.79	5.84	.05	17
6.48	6.57	6.66	6.73	6.81	6.87	6.94	7.00	7.05	.01	
5.27	5.35	5.43	5.50	5.57	5.63	5.69	5.74	5.79	.05	18
6.41	6.50	6.58	6.65	6.73	6.79	6.85	6.91	6.97	.01	
5.23	5.31	5.39	5.46	5.53	5.59	5.65	5.70	5.75	.05	19
6.34	6.43	6.51	6.58	6.65	6.72	6.78	6.84	6.89	.01	
5.20	5.28	5.36	5.43	5.49	5.55	5.61	5.66	5.71	.05	20
6.28	6.32	6.45	6.52	6.59	6.65	6.71	6.77	6.82	.01	
5.10	5.18	5.25	5.32	5.38	5.44	5.49	5.55	5.59	.05	24
6.11	6.19	6.26	6.33	6.39	6.45	6.51	6.56	6.61	.01	
5.00	5.08	5.15	5.21	5.27	5.33	5.38	5.43	5.47	.05	30
5.93	6.01	6.08	6.14	6.20	6.26	6.31	6.36	6.41	.01	
4.90	4.98	5.04	5.11	5.16	5.22	5.27	5.31	5.36	.05	40
5.76	5.83	5.90	5.96	6.02	6.07	6.12	6.16	6.21	.01	
4.81	4.88	4.94	5.00	5.06	5.11	5.15	5.20	5.24	.05	60
5.60	5.67	5.73	5.78	5.84	5.89	5.93	5.97	6.01	.01	
4.71	4.78	4.84	4.90	4.95	5.00	5.04	5.09	5.13	.05	120
5.44	5.50	5.56	5.61	5.66	5.71	5.75	5.79	5.83	.01	
4.62	4.68	4.74	4.80	4.85	4.89	4.93	4.97	5.01	.05	∞
5.29	5.35	5.40	5.45	5.49	5.54	5.57	5.61	5.65	.01	

Source: Table 29 of E. S. Pearson and H. O. Hartley, *Biometrika Tables for Statisticians*, Vol. 1, 3rd ed., 1966. Used by permission of the Biometrika Trustees.

Distribution of the Studentized Range Statistic

df for $s_{\bar{X}}$	$1-\alpha$	r = number of steps between ordered means													
		2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	.95	18.0	27.0	32.8	37.1	40.4	43.1	45.4	47.4	49.1	50.6	52.0	53.2	54.3	55.4
	.99	90.0	135	164	186	202	216	227	237	246	253	260	266	272	277
2	.95	6.09	8.3	9.8	10.9	11.7	12.4	13.0	13.5	14.0	14.4	14.7	15.1	15.4	15.7
	.99	14.0	19.0	22.3	24.7	26.6	28.2	29.5	30.7	31.7	32.6	33.4	34.1	34.8	35.4
3	.95	4.50	5.91	6.82	7.50	8.04	8.48	8.85	9.18	9.46	9.72	9.95	10.2	10.4	10.5
	.99	8.26	10.6	12.2	13.3	14.2	15.0	15.6	16.2	16.7	17.1	17.5	17.9	18.2	18.5
4	.95	3.93	5.04	5.76	6.29	6.71	7.05	7.35	7.60	7.83	8.03	8.21	8.37	8.52	8.66
	.99	6.51	8.12	9.17	9.96	10.6	11.1	11.5	11.9	12.3	12.6	12.8	13.1	13.3	13.5
5	.95	3.64	4.60	5.22	5.67	6.03	6.33	6.58	6.80	6.99	7.17	7.32	7.47	7.60	7.72
	.99	5.70	6.97	7.80	8.42	8.91	9.32	9.67	9.97	10.2	10.5	10.7	10.9	11.1	11.2
6	.95	3.46	4.34	4.90	5.31	5.63	5.89	6.12	6.32	6.49	6.65	6.79	6.92	7.03	7.14
	.99	5.24	6.33	7.03	7.56	7.97	8.32	8.61	8.87	9.10	9.30	9.49	9.65	9.81	9.95
7	.95	3.34	4.16	4.69	5.06	5.36	5.61	5.82	6.00	6.16	6.30	6.43	6.55	6.66	6.76
	.99	4.95	5.92	6.54	7.01	7.37	7.68	7.94	8.17	8.37	8.55	8.71	8.86	9.00	9.12
8	.95	3.26	4.04	4.53	4.89	5.17	5.40	5.60	5.77	5.92	6.05	6.18	6.29	6.39	6.48
	.99	4.74	5.63	6.20	6.63	6.96	7.24	7.47	7.68	7.87	8.03	8.18	8.31	8.44	8.55
9	.95	3.20	3.95	4.42	4.76	5.02	5.24	5.43	5.60	5.74	5.87	5.98	6.09	6.19	6.28
	.99	4.60	5.43	5.96	6.35	6.66	6.91	7.13	7.32	7.49	7.65	7.78	7.91	8.03	8.13
10	.95	3.15	3.88	4.33	4.65	4.91	5.12	5.30	5.46	5.60	5.72	5.83	5.93	6.03	6.11
	.99	4.48	5.27	5.77	6.14	6.43	6.67	6.87	7.05	7.21	7.36	7.48	7.60	7.71	7.81
11	.95	3.11	3.82	4.26	4.57	4.82	5.03	5.20	5.35	5.49	5.61	5.71	5.81	5.90	5.99
	.99	4.39	5.14	5.62	5.97	6.25	6.48	6.67	6.84	6.99	7.13	7.26	7.36	7.46	7.56
12	.95	3.08	3.77	4.20	4.51	4.75	4.95	5.12	5.27	5.40	5.51	5.62	5.71	5.80	5.88
	.99	4.32	5.04	5.50	5.84	6.10	6.32	6.51	6.67	6.81	6.94	7.06	7.17	7.26	7.36

Source: From WADC Tech. Rep. 58-484, vol. 2, 1959, Wright Air Development Center.

APPENDIX III **Yoghurt Triangle Test Questionnaire.**

Day 1

YOGHURT TRIANGLE TEST

Panellist No: _____

This triangle test has been set up to look at differences in the overall yoghurt flavour (aroma) between samples. There are three yoghurt samples for you to evaluate. Two samples are the same and one is DIFFERENT.

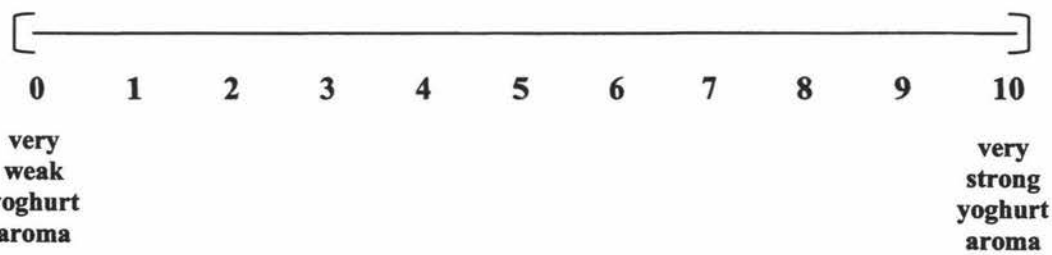
Instructions:

1. Sniff each sample in the order indicated below (from left to right). Do this by lifting the lid slightly and smell each yoghurt sample taking short, shallow sniffs.
2. Two samples are identical; determine which is the odd sample. Circle the number of the sample which is **DIFFERENT**.

REMEMBER, you are only evaluating the overall yoghurt aroma of each sample.

233 363 778

Once you have chosen the DIFFERENT yoghurt sample, place the sample on a scale of 0 to 10 (0 = very weak yoghurt aroma and 10 = very strong yoghurt aroma).



Remarks: If you wish to comment on the reasons for your choice or if you wish to comment on the yoghurt flavour, please do so below:

_____

_____

_____

APPENDIX IV Statistical Tables for Binomial Tests (from O'Mahony, M. 1986).

Probability of X or More Correct Judgments in n Trials (one-tailed, p = ½)[a]

n \ X	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	34	35	36
5	969	812	500	188	031																														
6	984	891	656	344	109	016																													
7	992	938	773	500	227	062	008																												
8	996	965	855	637	363	145	035	004																											
9	998	980	910	746	500	254	090	020	002																										
10	999	989	945	828	623	377	172	055	011	001																									
11		994	967	887	726	500	274	113	033	006																									
12		997	981	927	806	613	387	194	073	019	003																								
13		998	989	954	867	709	500	291	133	046	011	002																							
14		999	994	971	910	788	605	395	212	090	029	006	001																						
15			996	982	941	849	696	500	304	151	059	018	004																						
16			998	989	962	895	773	598	402	227	105	038	011	002																					
17			999	994	975	928	834	685	500	315	166	072	025	006	001																				
18			999	996	985	952	881	760	593	407	240	119	048	015	004																				
19				998	990	968	916	820	676	500	324	180	084	032	010	002																			
20				999	994	979	942	868	748	588	412	252	132	058	021	006	001																		
21				999	996	987	961	905	808	668	500	332	192	095	039	013	004	001																	
22					998	992	974	933	857	738	584	416	262	143	067	026	008	002																	
23					999	995	983	953	895	798	661	500	339	202	105	047	017	005	001																
24					999	997	989	968	924	846	729	581	419	271	154	076	032	011	003	001															
25						998	993	978	946	885	788	655	500	345	212	115	054	022	007	002															
26						999	995	986	962	916	837	721	577	423	279	163	084	038	014	005	001														
27						999	997	990	974	939	876	779	649	500	351	221	124	061	026	010	003	001													
28							998	994	982	956	908	828	714	575	425	286	172	092	044	018	006	002												
29							999	996	988	969	932	868	771	644	500	356	229	132	068	031	012	004	001											
30							999	997	992	979	951	900	819	708	572	428	292	181	100	049	021	008	003	001										
31								998	995	985	965	925	859	763	640	500	360	237	141	075	035	015	005	002										
32								999	997	990	975	945	892	811	702	570	430	298	189	108	055	025	010	004	001									
33								999	998	993	982	960	919	852	757	636	500	364	243	148	081	040	018	007	002									
34									999	995	988	971	939	885	804	696	568	432	304	196	115	061	029	012	005	002								
35									999	997	992	980	955	912	845	750	632	500	368	250	155	088	045	020	008	003	001							
36									999	998	994	986	967	934	879	797	691	566	434	309	203	121	066	033	014	006	002	001						
37										999	996	990	976	951	906	838	744	629	500	371	256	162	094	049	024	010	004	001						
38										999	997	993	983	964	928	872	791	686	564	436	314	209	128	072	036	017	007	003	001					
39										999	998	995	988	973	946	900	832	739	625	500	375	261	168	100	054	027	012	005	002	001				
40											999	997	992	981	960	923	866	785	682	563	437	318	215	134	077	040	019	008	003	001				
41											999	998	994	986	970	941	894	826	734	627	500	373	266	174	106	059	030	014	006	002	001			
42												999	996	990	978	956	918	860	780	678	561	439	322	220	140	082	044	022	010	004	001			
43												999	997	993	984	967	937	889	820	729	620	500	380	271	180	111	063	033	016	007	003	001		
44												999	998	995	989	976	952	913	854	774	674	560	440	326	226	146	087	048	024	011	005	002	001	
45													999	998	994	982	964	932	884	814	724	617	500	383	276	186	116	068	036	018	008	003	001	
46														999	998	994	987	973	948	908	849	769	671	558	442	329	231	151	092	057	027	013	006	002	001
47														999	998	996	991	980	961	928	879	809	720	615	500	385	280	191	121	072	039	020	009	004	002	001
48															999	997	993	985	970	944	903	844	765	667	557	443	333	235	156	097	056	030	015	007	003	001
49															999	998	995	989	978	957	924	874	804	716	612	500	388	284	196	126	076	043	022	011	005	002	001
50																999	997	992	984	968	941	899	839	760	664	556	444	336	240	161	101	059	032	016	008	003	001

[a] Initial decimal point has been omitted.

Source: E. B. Roessler et al., *Journal of Food Science*, 1978, *43*, 940-947. Copyright © by Institute of Food Technologists. Reprinted with permission of author and publisher.

APPENDIX IV ctd. Statistical Tables for Binomial Tests.

Probability of X or More Agreeing Judgments in n Trials (two-tailed, p = ½)[a]

n \ X	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
5	625	312	062																																
6		688	219	031																															
7			453	125	016																														
8			727	289	070	008																													
9				508	180	039	004																												
10				754	344	109	021	002																											
11					549	227	065	011	001																										
12					774	388	146	039	006																										
13						581	267	092	022	003																									
14						791	424	180	057	013	002																								
15							607	302	118	035	007	001																							
16							804	454	210	077	021	004	001																						
17								629	332	143	049	013	002																						
18								815	481	238	096	031	008	001																					
19									648	359	167	064	019	004	001																				
20									824	503	263	115	041	012	003																				
21										664	383	189	078	027	007	001																		
22										832	523	286	134	052	017	004	001																	
23											678	405	210	093	035	011	003																	
24											839	541	307	152	064	023	007	002																
25												690	424	230	108	043	015	004	001															
26												845	557	327	169	076	029	009	002	001														
27													701	442	248	122	052	019	006	002														
28													851	572	345	185	087	036	013	004	001													
29														711	458	265	136	061	024	008	002	001												
30														856	585	362	200	099	043	016	005	001												
31															720	473	281	150	071	030	011	003	001											
32															860	597	377	215	100	050	020	007	002	001										
33																728	487	296	163	080	035	014	005	001										
34																864	608	392	229	121	058	024	009	003	001									
35																	736	500	310	175	090	041	017	006	002									
36																	868	681	405	243	132	065	029	011	004	001								
37																		743	511	324	188	099	047	020	008	003	001							
38																		871	627	418	256	143	073	034	014	005	002							
39																			749	522	337	200	108	053	024	009	003	001						
40																			875	636	430	268	154	081	038	017	006	002	001					
41																				755	533	349	211	117	060	028	012	004	001					
42																				878	644	441	280	164	088	044	020	008	003	001				
43																					761	542	360	222	126	066	032	014	005	002	001			
44																					880	652	451	291	174	096	049	023	010	004	001			
45																						766	551	371	233	135	072	036	016	007	002	001		
46																						883	659	461	302	184	104	054	026	011	005	002	001	
47																							771	560	382	243	144	079	040	019	008	003	001	
48																							885	665	471	312	193	111	059	029	013	006	002	001
49																								775	568	392	253	152	085	044	021	009	004	001
50																								888	672	480	322	203	119	065	033	015	007	003	001

APPENDIX V PCA Results Obtained Using SAS (14 Commercial Yoghurts).

```
data yog;
infile "c:\My Documents\comyog.prn";
input sample$ rep acetal acetone but ethanol diacet acetoin;
proc princomp out=graph outstat=stats;
var acetal acetone but ethanol diacet acetoin;
run;

title "14 Yoghurts";
proc corr data=graph;
var prin1-prin4; with acetal acetone but ethanol diacet acetoin;
run;
proc print data=stats uniform;
run;

proc plot data=graph;
format sample;
plot prin2*prin1=sample/href=0 vref=0;
run;

proc plot data=graph;
format sample;
plot prin3*prin4=sample/href=0 vref=0;
run;
```

APPENDIX V ctd. PCA Results Obtained Using SAS.

14 Yoghurts 16:29 Tuesday, August 1, 2000 1

Principal Component Analysis

26 Observations
6 Variables

Simple Statistics

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
Mean	143.3846154	120.3461538	39.50000000	40.00000000	75.80769231	633.3076923
StD	71.6441634	40.0249345	13.46179780	31.33560275	26.22063192	188.7234525

Correlation Matrix

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
ACETAL	1.0000	-.6440	0.1585	0.2430	0.0431	0.7571
ACETONE	-.6440	1.0000	0.1475	-.2587	0.0844	-.3322
BUT	0.1585	0.1475	1.0000	-.1041	-.0313	0.2542
ETHANOL	0.2430	-.2587	-.1041	1.0000	0.0343	0.0966
DIACET	0.0431	0.0844	-.0313	0.0343	1.0000	0.3036
ACETOIN	0.7571	-.3322	0.2542	0.0966	0.3036	1.0000

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.29996	1.01223	0.383326	0.38333
PRIN2	1.28773	0.22387	0.214621	0.59795
PRIN3	1.06385	0.25036	0.177309	0.77526
PRIN4	0.81349	0.41604	0.135582	0.91084
PRIN5	0.39745	0.25993	0.066242	0.97708
PRIN6	0.13752	.	0.022920	1.00000

91% 4 pcs

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5	PRIN6
ACETAL	0.619398	0.008152	-.140875	-.102017	0.168248	0.746806
ACETONE	-.476408	0.410316	0.139766	0.281930	0.639229	0.311518
BUT	0.106385	0.632241	-.456767	0.455401	-.415058	-.025582
ETHANOL	0.246139	-.434319	0.219366	0.832401	0.077212	-.061711
DIACET	0.122006	0.358415	0.834676	-.024225	-.376146	0.133780
ACETOIN	0.550086	0.338750	0.084727	-.096124	0.493313	-.568224

APPENDIX V ctd. PCA Results Obtained Using SAS.

14 Yoghurts 16:29 Tuesday, August 1, 2000 3

OBS	_TYPE_	_NAME_	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
1	MEAN		143.385	120.346	39.5000	40.0000	75.8077	633.308
2	STD		71.644	40.025	13.4618	31.3356	26.2206	188.723
3	N		26.000	26.000	26.0000	26.0000	26.0000	26.000
4	CORR	ACETAL	1.000	-0.644	0.1585	0.2430	0.0431	0.757
5	CORR	ACETONE	-0.644	1.000	0.1475	-0.2587	0.0844	-0.332
6	CORR	BUT	0.158	0.147	1.0000	-0.1041	-0.0313	0.254
7	CORR	ETHANOL	0.243	-0.259	-0.1041	1.0000	0.0343	0.097
8	CORR	DIACET	0.043	0.084	-0.0313	0.0343	1.0000	0.304
9	CORR	ACETOIN	0.757	-0.332	0.2542	0.0966	0.3036	1.000
10	EIGENVAL		2.300	1.288	1.0639	0.8135	0.3975	0.138
11	SCORE	PRIN1	0.619	-0.476	0.1064	0.2461	0.1220	0.550
12	SCORE	PRIN2	0.008	0.410	0.6322	-0.4343	0.3584	0.339
13	SCORE	PRIN3	-0.141	0.140	-0.4568	0.2194	0.8347	0.085
14	SCORE	PRIN4	-0.102	0.282	0.4554	0.8324	-0.0242	-0.096
15	SCORE	PRIN5	0.168	0.639	-0.4151	0.0772	-0.3761	0.493
16	SCORE	PRIN6	0.747	0.312	-0.0256	-0.0617	0.1338	-0.568

APPENDIX V ctd. PCA Results Obtained Using SAS (Day 1).

```
data yog;
infile "h:\thesis\Chapter 9\8day1yogsas.prn";
input sample$ rep acetal acetone but ethanol diacet acetoin;
proc princomp out=graph outstat=stats;
var acetal acetone but ethanol diacet acetoin;
run;

title "8 Yoghurts Day 1";
proc corr data=graph;
var prin1-prin4; with acetal acetone but ethanol diacet acetoin;
run;
proc print data=stats uniform;
run;

proc plot data=graph;
format sample;
plot prin2*prin1=sample/href=0 vref=0;
run;

proc plot data=graph;
format sample;
plot prin3*prin4=sample/href=0 vref=0;
run;
```

APPENDIX V ctd. PCA Results Obtained Using SAS.

The SAS System 16:37 Wednesday, August 2, 2000 1

Principal Component Analysis

21 Observations
6 Variables

Simple Statistics

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
Mean	70.09523810	90.76190476	50.23809524	31.28571429	60.38095238	19.00000000
StD	47.40559541	63.02372947	29.59037810	34.81830389	45.99290836	23.58177262

Correlation Matrix

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
ACETAL	1.0000	-.1193	0.0041	-.3108	0.0339	-.0910
ACETONE	-.1193	1.0000	0.4950	0.0484	0.1394	-.0908
BUT	0.0041	0.4950	1.0000	-.3498	-.1321	-.4685
ETHANOL	-.3108	0.0484	-.3498	1.0000	0.2529	0.3162
DIACET	0.0339	0.1394	-.1321	0.2529	1.0000	0.4858
ACETOIN	-.0910	-.0908	-.4685	0.3162	0.4858	1.0000

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.08902	0.664788	0.348170	0.34817
PRIN2	1.42423	0.309956	0.237372	0.58554
PRIN3	1.11428	0.502056	0.185713	0.77126
PRIN4	0.61222	0.156321	0.102037	0.87329
PRIN5	0.45590	0.151554	0.075983	0.94928
PRIN6	0.30435		0.050724	1.00000

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5	PRIN6
ACETAL	-.143777	-.390295	0.697272	0.540849	0.169057	0.140402
ACETONE	-.198872	0.703220	0.165565	0.183634	0.505201	-.386743
BUT	-.523259	0.402497	0.126409	-.117869	-.234935	0.692192
ETHANOL	0.452263	0.282458	-.327876	0.702579	-.121125	0.316045
DIACET	0.387502	0.325778	0.553012	-.125841	-.609193	-.225689
ACETOIN	0.557923	0.072340	0.238948	-.387833	0.524605	0.448071

APPENDIX V ctd. PCA Results Obtained Using SAS.

8 Yoghurts Day 1 16:37 Wednesday, August 2, 2000 2

Correlation Analysis

6 'WITH' Variables: ACETAL ACETONE BUT ETHANOL DIACET ACETOIN
4 'VAR' Variables: PRIN1 PRIN2 PRIN3 PRIN4

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
ACETAL	21	70.095238	47.405595	1472.000000	16.000000	182.000000
ACETONE	21	90.761905	63.023729	1906.000000	0	260.000000
BUT	21	50.238095	29.590378	1055.000000	0	121.000000
ETHANOL	21	31.285714	34.818304	657.000000	0	107.000000
DIACET	21	60.380952	45.992908	1268.000000	0	158.000000
ACETOIN	21	19.000000	23.581773	399.000000	0	83.000000
PRIN1	21	0	1.445345	0	-2.190366	3.445011
PRIN2	21	0	1.193412	0	-2.045547	3.058487
PRIN3	21	0	1.055593	0	-1.849819	1.844918
PRIN4	21	0	0.782446	0	-1.298978	1.570484

Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N = 21

	PRIN1	PRIN2	PRIN3	PRIN4
ACETAL	-0.20781	-0.46578	0.73604	0.42319
	0.3660	0.0333	0.0001	0.0559
ACETONE	-0.28744	0.83923	0.17477	0.14368
	0.2064	0.0001	0.4486	0.5344
BUT	-0.75629	0.48034	0.13344	-0.09223
	0.0001	0.0275	0.5642	0.6909
ETHANOL	0.65368	0.33709	-0.34610	0.54973
	0.0013	0.1351	0.1243	0.0098
DIACET	0.56007	0.38879	0.58376	-0.09846
	0.0083	0.0815	0.0055	0.6711
ACETOIN	0.80639	0.08633	0.25223	-0.30346
	0.0001	0.7098	0.2700	0.1811

APPENDIX V ctd. PCA Results Obtained Using SAS.

8 Yoghurts Day 1 16:37 Wednesday, August 2, 2000 3

OBS	_TYPE_	_NAME_	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
1	MEAN		70.0952	90.7619	50.2381	31.2857	60.3810	19.0000
2	STD		47.4056	63.0237	29.5904	34.8183	45.9929	23.5818
3	N		21.0000	21.0000	21.0000	21.0000	21.0000	21.0000
4	CORR	ACETAL	1.0000	-0.1193	0.0041	-0.3108	0.0339	-0.0910
5	CORR	ACETONE	-0.1193	1.0000	0.4950	0.0484	0.1394	-0.0908
6	CORR	BUT	0.0041	0.4950	1.0000	-0.3498	-0.1321	-0.4685
7	CORR	ETHANOL	-0.3108	0.0484	-0.3498	1.0000	0.2529	0.3162
8	CORR	DIACET	0.0339	0.1394	-0.1321	0.2529	1.0000	0.4858
9	CORR	ACETOIN	-0.0910	-0.0908	-0.4685	0.3162	0.4858	1.0000
10	EIGENVAL		2.0890	1.4242	1.1143	0.6122	0.4559	0.3043
11	SCORE	PRIN1	-0.1438	-0.1989	-0.5233	0.4523	0.3875	0.5579
12	SCORE	PRIN2	-0.3903	0.7032	0.4025	0.2825	0.3258	0.0723
13	SCORE	PRIN3	0.6973	0.1656	0.1264	-0.3279	0.5530	0.2389
14	SCORE	PRIN4	0.5408	0.1836	-0.1179	0.7026	-0.1258	-0.3878
15	SCORE	PRIN5	0.1691	0.5052	-0.2349	-0.1211	-0.6092	0.5246
16	SCORE	PRIN6	0.1404	-0.3867	0.6922	0.3160	-0.2257	0.4481

APPENDIX V ctd. PCA Results Obtained Using SAS (Day 2).

```
data yog;
infile "h:\thesis\Chapter 9\8day2yogsas.prn";
input sample$ rep acetal acetone but ethanol diacet acetoin;
proc princomp out=graph outstat=stats;
var acetal acetone but ethanol diacet acetoin;
run;

title "8 Yoghurts";
proc corr data=graph;
var prin1-prin4; with acetal acetone but ethanol diacet acetoin;
run;
proc print data=stats uniform;
run;

proc plot data=graph;
format sample;
plot prin2*prin1=sample/href=0 vref=0;
run;

proc plot data=graph;
format sample;
plot prin3*prin4=sample/href=0 vref=0;
run;
```

APPENDIX V ctd. PCA Results Obtained Using SAS.

8 Yoghurts 16:34 Wednesday, August 2, 2000 6

Principal Component Analysis

16 Observations
6 Variables

Simple Statistics

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
Mean	95.56250000	104.9375000	77.56250000	33.62500000	48.12500000	53.43750000
StD	24.47030513	104.3203200	82.49441900	27.68362934	25.29789188	32.70671440

Correlation Matrix

	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
ACETAL	1.0000	-.6973	-.6965	0.3899	0.1318	-.3605
ACETONE	-.6970	1.0000	0.9508	-.3603	-.1064	0.5445
BUT	-.6965	0.9508	1.0000	-.2709	-.1464	0.4010
ETHANOL	0.3899	-.3603	-.2709	1.0000	0.4996	-.3329
DIACET	0.1318	-.1064	-.1464	0.4996	1.0000	0.0629
ACETOIN	-.3605	0.5445	0.4010	-.3329	0.0629	1.0000

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	3.14131	1.83798	0.523551	0.52355
PRIN2	1.30333	0.52230	0.217221	0.74077
PRIN3	0.78103	0.33435	0.130171	0.87094
PRIN4	0.44668	0.15027	0.074447	0.94539
PRIN5	0.29641	0.26517	0.049402	0.99479
PRIN6	0.03125	.	0.005208	1.00000

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5	PRIN6
ACETAL	-.463425	-.068476	0.243030	0.629071	0.570090	0.027426
ACETONE	0.526476	0.189215	-.146889	0.231695	0.292549	-.725381
BUT	0.502540	0.179153	-.356991	0.267585	0.254624	0.671923
ETHANOL	-.327536	0.546800	-.384476	0.428359	-.505298	-.084201
DIACET	-.155139	0.762785	0.197620	-.455053	0.380771	0.054572
ACETOIN	0.352372	0.215829	0.777844	0.297357	-.352514	0.107344

APPENDIX V ctd. PCA Results Obtained Using SAS.

8 Yoghurts 16:34 Wednesday, August 2, 2000 7

Correlation Analysis

6 'WITH' Variables: ACETAL ACETONE BUT ETHANOL DIACET ACETOIN
4 'VAR' Variables: PRIN1 PRIN2 PRIN3 PRIN4

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
ACETAL	16	95.562500	24.470305	1529.000000	55.000000	125.000000
ACETONE	16	104.937500	104.320320	1679.000000	17.000000	306.000000
BUT	16	77.562500	82.494419	1241.000000	10.000000	238.000000
ETHANOL	16	33.625000	27.683629	538.000000	7.000000	91.000000
DIACET	16	48.125000	25.297892	770.000000	0	87.000000
ACETOIN	16	53.437500	32.706714	855.000000	18.000000	124.000000
PRIN1	16	0	1.772373	0	-2.123279	2.941564
PRIN2	16	0	1.141634	0	-1.673598	2.007570
PRIN3	16	0	0.883758	0	-1.635563	1.693154
PRIN4	16	0	0.668342	0	-1.190737	1.154925

Pearson Correlation Coefficients / Prob > |R| under Ho: Rho=0 / N = 16

	PRIN1	PRIN2	PRIN3	PRIN4
ACETAL	-0.82130	-0.07817	0.21478	-0.42043
	0.0001	0.7735	0.4244	0.1049
ACETONE	0.93317	0.21601	-0.12981	0.15485
	0.0001	0.4217	0.6318	0.5669
BUT	0.89069	0.20453	-0.31549	0.17884
	0.0001	0.4474	0.2339	0.5075
ETHANOL	0.58052	0.62425	-0.33978	0.28629
	0.0184	0.0097	0.1979	0.2824
DIACET	-0.27496	0.87082	0.17465	-0.30413
	0.3027	0.0001	0.5177	0.2521
ACETOIN	0.62453	0.24640	0.68743	0.19874
	0.0097	0.3576	0.0033	0.4606

APPENDIX V ctd. PCA Results Obtained Using SAS.

8 Yoghurts 16:34 Wednesday, August 2, 2000 8

OBS	_TYPE_	_NAME_	ACETAL	ACETONE	BUT	ETHANOL	DIACET	ACETOIN
1	MEAN		95.5625	104.938	77.5625	33.6250	48.1250	53.4375
2	STD		24.4703	104.320	82.4944	27.6836	25.2979	32.7067
3	N		16.0000	16.000	16.0000	16.0000	16.0000	16.0000
4	CORR	ACETAL	1.0000	-0.697	-0.6965	0.3899	0.1318	-0.3605
5	CORR	ACETONE	-0.6973	1.000	0.9508	-0.3603	-0.1064	0.5445
6	CORR	BUT	-0.6965	0.951	1.0000	-0.2709	-0.1464	0.4010
7	CORR	ETHANOL	0.3899	-0.360	-0.2709	1.0000	0.4996	-0.3329
8	CORR	DIACET	0.1318	-0.106	-0.1464	0.4996	1.0000	0.0629
9	CORR	ACETOIN	-0.3605	0.545	0.4010	-0.3329	0.0629	1.0000
10	EIGENVAL		3.1413	1.303	0.7810	0.4467	0.2964	0.0312
11	SCORE	PRIN1	-0.4634	[illegible]526	-0.5026	-0.3275	-0.1551	0.3524
12	SCORE	PRIN2	-0.0685	0.189	0.1792	[illegible]5468	0.[illegible]7628	0.2158
13	SCORE	PRIN3	0.2430	-0.147	-0.3570	-0.3845	0.1976	0.[illegible]7778
14	SCORE	PRIN4	[illegible]6293	0.232	0.2676	0.4284	-0.4551	0.2974
15	SCORE	PRIN5	0.5701	0.293	0.2546	-0.5053	0.3808	-0.3525
16	SCORE	PRIN6	0.0274	-0.725	0.6719	-0.0842	0.0546	0.1073