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Use of Headspace Solid-Phase Microextraction for the Analysis and Characterisation of Volatile Compounds in Rumen Contents

A thesis presented in partial fulfillment of the requirements for the degree of

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Abstract

Volatile fatty acids (VFAs), alkyl phenols and indolic compounds are produced by rumen microbes during the fermentation of forages in ruminants. In this study, ruminal fluid obtained from sheep was examined by headspace solid-phase microextraction (SPME) sampling followed by GC-MS analysis. This technique provides a non-invasive, clean and selective method to characterize the volatiles in ruminal fluid from an *in vitro* fermentation system.

The factors which can influence the extraction efficiency were studied and include the SPME fibre, sample volume, pH of sample matrix (rumen fluid) and extraction time by the fibre in the headspace. The optimum experimental conditions for the analytes in question included: polyacrylate fibre to perform the headspace SPME above 20 mL of rumen fluid in a 68 mL vial for 5 min, followed by immediate GC-MS analysis. The pH of the rumen fluid sample greatly influenced VFA extraction efficiency.

Quantitative analysis of p-cresol, m-cresol, indole and skatole with SPME were compared with steam distillation simultaneous extraction. This comparison showed that the HS-SPME method was semi-quantitative.

The optimum *in vitro* system (16 mL of rumen fluid and 4 mL of artificial saliva in a 68 mL vial incubated at 39 ^{0}C) was utilised to study production of indole, skatole and p-cresol from the anaerobic fermentation of tryptophan and tyrosine.

Spirulina is an abundant source of dietary protein. Therefore, ^{13}C labelled spirulina was used to study the metabolism of protein and formation of analytes derived from ruminal metabolism of protein. A series of labelled end products, including toluene, acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, p-cresol, indole, skatole, dimethyldisulfide and dimethyltrisulfide were detected by GC-MS. This result indicates that these compounds are the products of ruminal metabolism of spirulina.

When applied to the *in vitro* rumen system the headspace SPME technique provides a fast approach to study metabolism of target compounds and allows the researcher to follow proposed pathways with labelled substrate.

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Abbreviations

BSTFA	bis(trimethylsilyl)trifluo-roacetamide
BTEX	benzene, toluene, ethyl-benzene and xylene
CAR	Carboxen
CE	capillary electrophoresis
CW	Carbowax
CZE	capillary zone electrophoresis
DI-SPME	direct immersion SPME
DMA	dimethylarsinic acid
DORM	dogfish muscle certified reference material
DVB	divinylbenzene
GC	gas chromatography
GC-AAS	gas chromatography atomic absorption spectrometry
GC-ECD	gas chromatography electron capture detection
GC-FID	gas chromatography flame ionization detection
GC-FPD	gas chromatography flame photometric detection
GC-MS	gas chromatography mass spectrometry
HPLC	high performance liquid chromatography
HS-SPME	headspace SPME
IT-MS	ion trap mass spectrometry
MEKC	micellar electrokinetic chromatography
MMA	monomethylarsonic acid
NPD	nitrogen-phosporous detection
O	olfactometry
PA	polyacrylate
PDECD	pulsed-discharge electron capture detector
PDES	ethoxypolydimethylsiloxane
PDMS	polydimethylsiloxane
ppb	part per billion
ppt	part per trillion
PPY	polypyrrole

PTV	Programmable Temperature Vaporizing
PVC	polyvinylchloride
RF	response factor
RT	room temperature
SBSE	stir bar sorptive extraction
SDE	steam distillation simultaneous extraction
SIM	selected ion monitoring
SPME	solid phase microextraction
TBT	tributyltin
TEA	thermal energy analysis
TOF-MS	time-of -flight mass spectrometry
TPR	templated resin
TPT	triphenyltin
VFA	volatile fatty acid
ZB	zebron

Chapter 1
Introduction and literature review

1.1 SPME

Solid phase microextraction (SPME) is a relatively new sample extraction technique, developed for the analysis of volatile organic contaminants in water samples by Pawliszyn and his co-workers in the early 1990s (Arthur and Pawliszyn, 1990). SPME can be combined with separation instruments such as GC and HPLC. There are two steps for analyses using SPME coupled with these instruments. Firstly, a fused-silica fibre coated with polymer is exposed to a sample or its headspace where the analytes partition between the sample or headspace and the fibre coating. Secondly the fibre is transferred to an instrument to desorb the analytes and perform the analysis of the target compounds.

In comparison with traditional sample preparation methods (**Table 1.1**), SPME integrates the sample extraction and concentration process into one single step and it requires no solvents, which allows SPME to be an on-site sampling technique. In addition, one step SPME not only provides a simple and fast sample preparation approach but also eliminates the loss of analytes during multi-step extraction techniques.

Although originally developed for sampling from a liquid, SPME has been shown to be more effective for sampling from the gas phase i.e. the headspace above a liquid (Zhang and Pawliszyn, 1993 and Ai, 1997). Headspace SPME can selectively extract the volatile or semi-volatile compounds from very complex matrices such as sludge (Zhang *et al.*, 1994).

Table 1.1 Procedures of three sample preparation techniques

Liquid-liquid extraction	Solid-phase extraction	Solid-phase microextraction
Addition of organic solvents to the sample	Condition cartridge or membrane	Exposure SPME fibre to the sample
Agitation in a separatory funnel	Sample elution	Desorption of the analytes in the analytical instrument
Separation of aqueous and organic phases	Solvent elution to remove interferences and analyte desorption	
Removal of organic phase		
Concentration of the organic phase	Concentration of the organic phase	
Injection into the analytical instrument	Injection into the analytical instrument	

1.2 SPME device

A commercial SPME device was first marketed by Supelco in 1993. The fibre SPME device includes a fibre holder and a fibre. The fibre is built in a needle, which can be assembled in a fibre holder, as described in **Figure 1.1**. The fibre holder consists of a spring-loaded plunger, a stainless-steel barrel and an adjustable depth gauge with needle. It is used with a reusable and replaceable fibre. The fibre is glued to a small stainless steel tube, which is built inside a syringe needle. The fibre is withdrawn into the needle when it pierces into the septum of the sample container. Then the fibre is extended into the sample or the headspace for a specific time to adsorb or absorb the analytes. The fibre is drawn back into the protective needle and transferred to the injection port of GC or HPLC.

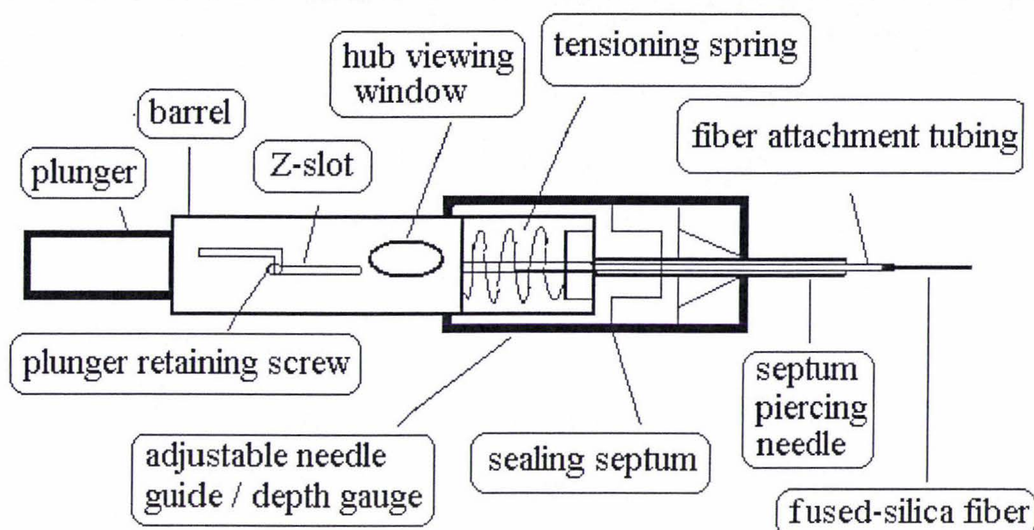


Figure 1.1 SPME fibre holder and fibre (Zhang *et al.*, 1994)

The fused-silica fibre is coated with a very thin film of polymeric stationary phase and the thickness of coating ranges from 7 to 100 μm. The fibre coating can concentrate organic compounds. ***Supelco*** (Bellefonte, PA, USA) can provide several kinds of fibres, as listed in the **Table 1.2**.

Non-bonded phases are stable with some water-miscible organic solvents, but slight swelling may occur. Non-bonded phases cannot be used or rinsed with non-polar organic solvents. Bonded phases are stable with all organic solvents. Slight swelling may occur when used with some non-polar solvents. Partially crosslinked phases are stable in most water-miscible organic solvents. They may be stable in some non-polar solvents, but slight swelling may occur. High-crosslinked phases are equivalent to partially crosslinked phases, except that some bonding to the core has occurred.

StableFlex ™ SPME fibres are coated on a flexible fused silica core. The coating partially bonds to the flexible core which results in a more stable coating and a less breakable fibre. The coating is more durable than a standard fused silica core. The selectivity may be slightly different from the standard fibre.

Table 1.2 SPME fibres currently available commercially from Supelco

Fibre coating	Thickness (μm)	Polarity	Coating Stability	Max Temp (^{0}C)	Application
PDMS	100	non-polar	non-bonded	280	GC/HPLC
	30	non-polar	non-bonded	280	GC/HPLC
	7	non-polar	bonded	340	GC/HPLC
PDMS/DVB	65	bi-polar	partially crosslinked	270	GC
	60 ☼☼	bi-polar	partially crosslinked	270	GC/HPLC
StableFlex ™	65	bi-polar	highly crosslinked	270	GC
PA	85	polar	partially crosslinked	320	GC/HPLC
CAR/PDMS	75	bi-polar	partially crosslinked	320	GC
StableFlex ™	85	bi-polar	highly crosslinked	320	GC
CW/DVB	65	polar	partially crosslinked	265	GC
StableFlex ™	70	polar	highly crosslinked	265	GC
CW/TPR	50 ☼☼	polar	partially crosslinked	240	GC/HPLC
StableFlex ™	50/30	bi-polar	highly crosslinked	270	GC
DVB/CAR/PDMS	50/30 ☼	bi-polar	highly crosslinked	270	GC

☼ The fibre is specially 2 cm length

☼☼ The fibre is more durable than others and it contains no epoxy.

Both PDMS and PA phases extract via absorption with analytes dissolving and diffusing into the bulk of the coating. The other types of coating, such as PDMS/DVB, CW/DVB, CW/TPR and DVB/CAR/PDMS, are mixed coatings and extract via adsorption with analytes staying on the surface of the fibre, while CAR/PDMS extract via both physiochemical procedures (Mills and Walker, 2000).

As shown in **Figure 1.2**, PDMS fibres have the lowest polarity while CW/TPR is the most polar coating among the seven kinds of coatings.

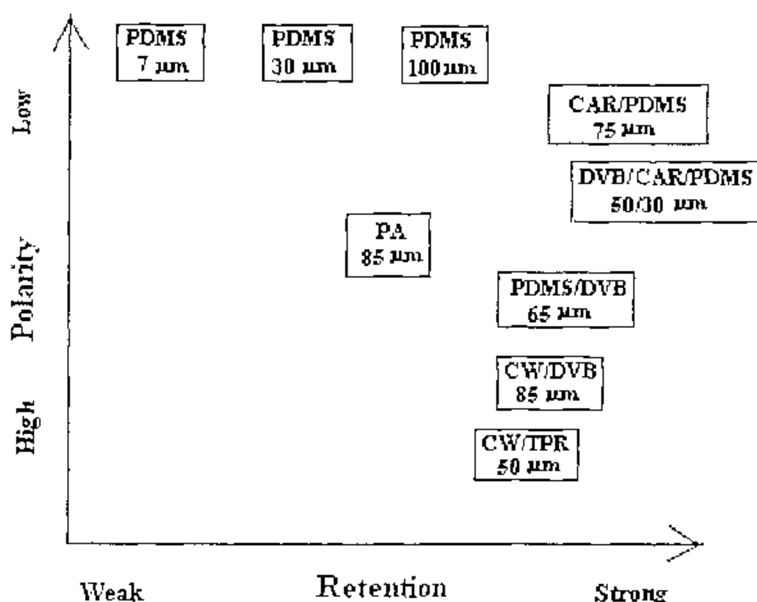


Figure 1.2 Properties of SPME fibres (Kataoka *et al.*, 2000)

1.3 Extraction and Desorption of SPME

There are four SPME modes, which require either fused-silica fibres or GC columns. Headspace (HS) and direct insertion (DI) SPME are fibre-extraction modes, GC column extraction is in-tube SPME (Kataoka *et al.*, 2000) and stir bar sorptive extraction is direct immersion SPME using a polymer-coated magnetic stir bar (Juan-García *et al.*, 2004)

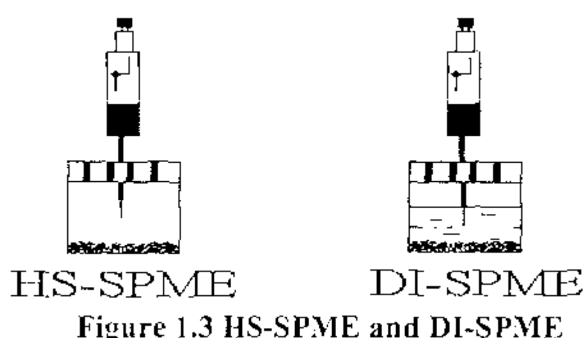


Figure 1.3 HS-SPME and DI-SPME

Direct insertion SPME involves inserting the fibre into the sample matrix and it is more sensitive than headspace SPME. Therefore DI-SPME is suitable for the extraction of semi- or less-volatile compounds.

Headspace SPME concerns inserting the fibre into the gas phase above the sample matrix, which provides a fast extraction. HS-SPME can protect the fibre and it can be

used to analyze the complex samples, such as biological samples, sludges and solid samples that release volatiles. The background in HS-SPME is low and it is suitable for volatile compounds.

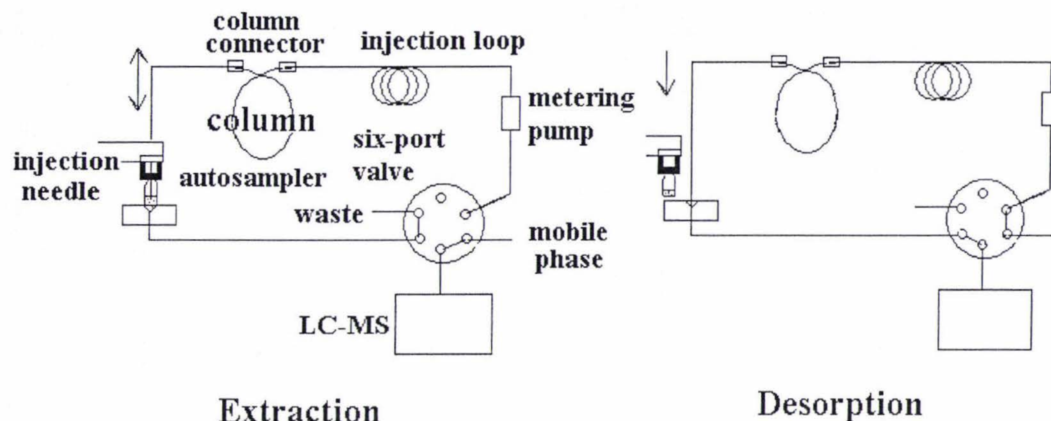


Figure 1.4 In-tube SPME (Kataoka *et al.*, 2000)

In-tube SPME (**Figure 1.4**) employs an open tubular capillary column as the SPME device. It is suitable for automation. Organic analytes are extracted from sample into the column, and then desorbed by a mobile phase. It is necessary to filter the particles in the sample before extraction to prevent plugging the column.

SPME samples are usually desorbed in a GC or HPLC. Thermal desorption in a GC is suitable for thermally reliable analytes. In solvent desorption, there are two methods, i.e. dynamic and static desorption. In the former, the analytes are removed by a moving steam of mobile phase while in the latter the fibre is soaked in a mobile phase or other strong solvents for a specified time by static desorption before injection into the HPLC.

A new development in SPME, stir bar sorptive extraction (SBSE), using a magnetic stir bar coated with PDMS to extract analytes, is much more sensitive compared with fibre and in-tube SPME. The volume of PDMS is approximately 300 μL while the SPME fibre or in-tube contains less than 0.5 μL, greatly improving the extraction ability of SPME (Vas and Vékey, 2004).

1.4 Theory of SPME

The geometry of headspace SPME is illustrated in the **Figure 1.5**.

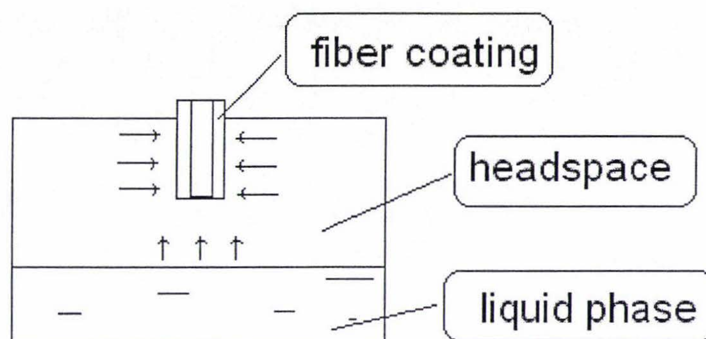


Figure 1.5 Process of headspace SPME

After a liquid sample is transferred to a closed container, equilibrium is established between the liquid phase and the headspace. Then a fused silica fibre coated with a thin layer of polymer is inserted into the headspace of the container. The fibre's coating absorbs the organic analytes from the headspace and then the organic compounds transfer from the liquid phase to the headspace. The whole process of the headspace SPME is from the liquid phase to the headspace, then from the headspace to the coating, until finally reaching equilibrium.

During the sample preparation using the HS-SPME, the analytes partition between the three phases, which are sample matrix, gas phase above the sample and the polymer coating. If reaching the equilibrium, the volatile compounds initially present in the sample will be distributed in the three phases, which can be described by the **Equation 1.1** (Pawliszyn, 1993 and Holt, 2001).

$$C_0V_s = C_fV_f + C_gV_g + C_sV_s \quad \textbf{(1.1)}$$

Where C_0 is the initial concentration of the analytes in the sample; V_f, V_g and V_s are the volumes of the fibre coating, the gas phase above the sample (i.e. headspace) and the sample. C_f, C_g and C_s are the concentrations of the analytes in the three phases at equilibrium.

The ratio of the concentrations of the analytes in the three phases at equilibrium can be described as the equilibrium constants.

$$K_1 = C_g/C_s$$

$$K_2 = C_f/C_g \qquad (1.2)$$

Where K_1 and K_2 are the equilibrium constants between the headspace and sample and between the fibre coating and the headspace, respectively.

Therefore, the mass of the analytes absorbed by the fibre coating, $n = C_fV_f$, can be expressed as:

$$n = \frac{K_1K_2V_fV_sC_0}{K_1K_2V_f + K_1V_g + V_s} \qquad (1.3)$$

Also, n is proportional to C_0 before equilibrium (Ai, 1997). The dynamic process of the HS-SPME can be described as:

$$n = [1 - exp(-2Am_2\frac{kK_1K_2V_f + kK_1V_g + kV_s}{2m_2K_2V_fV_s + kK_1V_fV_g + kK_fV_s}t)] \cdot \frac{K_1K_2V_fV_s}{K_1K_2V_f + K_1V_g + V_s}C_0 \qquad (1.4)$$

Where A is the surface area of the fibre coating; m_2 is the mass transfer coefficient of the analytes in the fibre coating and equal to D_2 / δ_2 (D_2 is the diffusion coefficient of the analytes in the fibre coating and δ_2 is the thickness of the fibre coating); k is the evaporation rate constant; t is the extraction time.

Equation 1.4 can also be written as:

$$n = [1 - exp(-at)]\ n_0 \qquad (1.5)$$

When the diffusion in the fibre coating is the rate-determining step, parameter "a" of **Equation 1.5** is independent of the evaporation rate constant k, i.e.

$$a = 2Am_2\frac{K_1K_2V_f + K_1V_g + V_s}{K_1V_fV_g + V_fV_s} \qquad (1.6)$$

When the evaporation from the sample phase is the rate-determining step, parameter "a" of **Equation 1.5** is independent of the mass transfer coefficient of the analytes in the fibre coating, i.e.

$$a = Ak\frac{K_1K_2V_f + K_1V_g + V_s}{K_2V_fV_s} \qquad \textbf{(1.7)}$$

In conclusion, whether equilibrium is reached or not, there is always a linear relationship between the amount of the analytes absorbed by the fibre coating and the initial concentration of these analytes in the sample.

1.5 Optimisation of SPME

Several factors can influence efficiency of SPME. These variables are discussed below.

1.5.1 Fibre coating

As listed in **Table 1.2**, there are several types of coating and thickness commercially available. The principles of selection of a suitable fibre are:

1. like dissolves like; for example, PDMS fibre, which is non-polar, can extract non-polar compounds such as BTEX (benzene, toluene, ethyl-benzene and xylene) from water very efficiently but cannot extract polar compounds such as phenol and its derivatives very well, while a polar PA fibre extracted phenol quite well but did not have good sensitivity of BTEX compounds (Zhang *et al.*, 1994).
2. the coating should be resistant to the chemical (pH, salts, additives) and physical (temperature) conditions;
3. a thick coating has higher sensitivity and longer equilibrium time than a thin one. (Krutz *et al.*, 2003 and Ulrich, 2000)

1.5.2 Extraction mode

HS-SPME is suitable for complex samples containing solids or large molecules such as proteins and lipids, i.e. biological specimens or sediments. Samples, such as body fluids, fruit juice, milk and so on, should use HS-SPME. HS-SPME prevents larger molecules or particles plugging the fiber coating and makes the lifetime of fibre

longer. On the other hand, DI-SPME can be applied in the analysis of clean water samples. It can extract both volatile and non-volatile analytes compared to HS-SPME.

1.5.3 Agitation

The equilibrium time is determined by the effectiveness of the sample agitation. The agitation methods include magnetic stirring, vortex mixing (moving vial), fibre movement and flow through agitation and sonication. An effective agitation is needed for highly viscous samples with low diffusion coefficients. Magnetic stirring can be used in both DI-SPME and HS-SPME. Fibre movement and moving vial methods can only be used with DI-SPME (Ulrich, 2000).

1.5.4 Extraction time

The amount of analytes removed by the SPME fibre is proportional to the concentration of the compounds in the sample when the extraction time is constant, no matter whether the equilibrium is reached or not. As described in **Figure 1.5**, the amount of analytes extracted increases with time, until the equilibrium is reached. Before the equilibrium is reached, a small change in extraction time can result in large change in extracted amounts, whereas the extraction time has little influence on the extracted amount after the equilibrium is reached. In practice, if the equilibrium time is too long or sample matrices vary too much with time, the extraction time should be chosen in the pre-equilibrium zone.

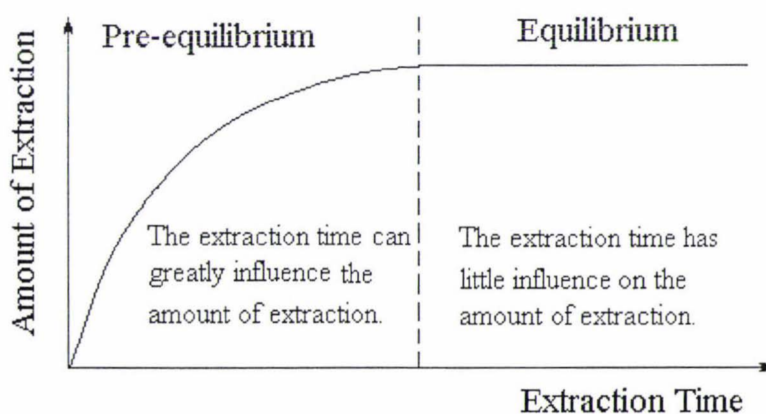


Figure 1.6 Effect of time on SPME extraction (Supelco Bulletin 929, Appendix 2)

As seen in **Figure 1.6**, the sensitivity of SPME is highest when the equilibrium between sample matrix and SPME fibre is reached. However, if the equilibrium time is too long, it is important to set a point where the sensitivity and precision are

acceptable. A wide range of extraction times, from 1 min to several hours, were reported in the literature (Krutz *et al.*, 2003).

1.5.5 pH

Sample matrix pH is very important for the SPME of the acidic and basic analytes and neutrals where pH influences the sample matrix. The extraction efficiency will fluctuate when the pH of the sample matrix changes. Acidic and basic compounds are in equilibrium with their conjugate base and acid. For example, the basic drugs such as the antidepressants, imipramine and desipramine, were analyzed in aqueous alkali (Ulrich *et al.*, 1999) and acid compounds such as chlorophenols showed better SPME performance at low pH (Lee *et al.*, 1998a).

1.5.6 Salts and other additives

Analyte solubility in the sample decreases as the concentration of salt increases, which improves the sensitivity by promoting analyte partitioning into the fibre coating or the headspace. This "salting-out" effect is compound-specific. For example, extraction efficiency decreases as ionic strength increases for phenoxy acid (Lee *et al.*, 1998b), while it increases for amides (Takino *et al.*, 2001). In addition, high salt concentration in the sample matrix facilitates salt deposition on the fibre, which decreases the extraction efficiency (Jinno *et al.*, 1996). The use of organic additives was recommended for matrices with polymer components, such as plasma. It is believed that the binding of the analytes to the proteins can be decreased, which can improve the sensitivity (Ulrich, 2000).

1.5.7 Temperature

The sensitivity decreases as the temperature increases while the equilibrium time drops. The rule of choosing a suitable extraction temperature achieves acceptable sensitivity in a relatively short period. In the literature, the optimum DI-SPME extraction temperature, for example, is from 55 to 60 ^{0}C for triazines, thiocarbamate herbicides (Aguilar *et al.*, 1998). Moreover, the concentration of the analytes in the headspace depends on the temperature, so the optimum temperature for HS-SPME is higher. For example, the temperature ranges from 90 to 100 ^{0}C for acetamide in blood (Guan *et al.*, 1998).

1.5.8 Volume of sample and headspace

The amount of the analytes extracted increases with the volume of sample. On the other hand, the volume of the headspace should be as small as possible. However, capacity of the headspace should exceed that of the fibre by about 20 times to provide rapid extraction (Ulrich, 2000).

1.5.9 Desorption and separation in GC

The desorption-time should be as short as possible to prevent the carryover effect (Ulrich, 2000). The temperature selected must be below the highest temperatures the fibres can afford, which are listed in the **Table 1.2**. The reported range of desorption temperature and time in GC is from 200 to 300 ^{0}C and from 2 to 15 min (Krutz *et al.*, 2003).

Usually, the GC oven temperature program starts at a temperature lower than the desorption temperature, to focus compounds on the top of the column typically for 1 – 3 min. Then the temperature of the column is raised progressively to elute and resolve compounds of interest.

1.6 Applications of SPME

SPME has revealed itself as a convenient extraction and concentration tool in various fields. The main applications were encountered in environmental chemistry in the early development period. Since then, there have been constantly increasing numbers of publications in other areas such as food and biological analysis (**Figure 1.7**). It has been recognized that this technique is a promising alternative to the traditional extraction methods, affording a number of advantages such as simple sample preparation, increased selectivity and sensitivity, absence of solvent and facilitation of simple on-site sampling.

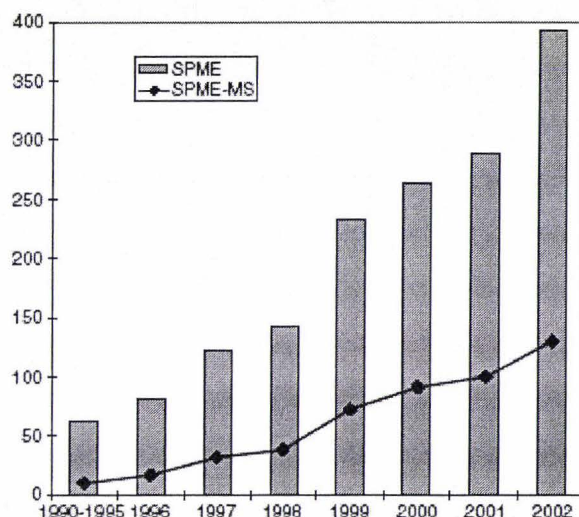


Figure 1.7 Number of published articles in recent years related to SPME and SPME/MS applications (Vas and Vékey, 2004)

1.6.1 Environmental applications

Although exhaustive removal of target analytes from the sample matrix is not usually obtained, the high concentration ability and selectivity of SPME allow highly sensitive analysis and parts per trillion (ppt) detection limits to be achieved (Magdic and Pawliszyn, 1996). Mostly organic compounds, such as alkyl sulfides (Abalos *et al.*, 2002) and nonylphenols (Diaz *et al.*, 2002), pesticides, herbicides in aqueous samples have been studied. Meanwhile, volatiles or semi-volatiles in solid samples have been analysed using HS-SPME, for example, BTEX compounds in sand and clay soil (Zhang and Pawliszyn, 1995), chloro-benzenes and nitroanilines in soils (Fromberg *et al.*, 1996) and organometallic compounds in sediments (Moens *et al.*, 1997). SPME techniques can also be applied for the determination of different components in air samples (Tuduri *et al.*, 2002 and Bartelt and Zilkowski, 1999).

Pesticide, fungicide and herbicide residue analysis in environmental samples has received increasing attention in the last decade. As the SPME technique has shown its advantages in terms of fast, simple, solvent free and on-site extraction, there have been an increasing number of its applications in this field. Summaries of some applications of SPME to the analysis of herbicides, fungicides and pesticides in different matrices such as aqueous and soil samples are presented in **Table 1, Appendix 1**.

The determination of arsenic compounds and organometallic pollution in the environment is critical because of their toxicities and bioaccumulative properties. Mester and Pawliszyn developed a method to determine two methylated arsenic species in human urine samples by SPME-GC–MS (Mester and Pawliszyn, 2000). They reported that direct extraction with SPME after thioglycol methylate derivatisation was suitable for the determination of trace levels of dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) in urine samples. Szostek and Aldstadt also described a method for the analysis of organoarsenic compounds, dimethylarsinic acid and phenylarsonic acid, which combined dithiol derivatisation with SPME-GC-MS (Szostek and Aldstadt, 1998). The method was applied to a series of water samples and soil/sediment extracts, as well as to aged soil samples that had been contaminated with Lewisite, the primary decomposition product of the chemical warfare agent. In 2000, Wu and co-workers used a polypyrrole (PPY) coated capillary for in-tube SPME coupled with LC-ESI-MS to analyse the organoarsenic compounds in aqueous samples. Organoarsenic compounds in water samples and arsenobetaine in a certified reference material (DORM-2) were analysed using this method. Cai and Bayona reported a SPME method for analysis of Hg^{2+} in fish and river water matrices (Cai and Bayona, 1995). The analytical procedure involved aqueous phase derivatisation of ionic mercury species with sodium tetraethylborate and subsequent extraction with a PDMS fibre. Yang *et al.* developed a HS-SPME-GC–MS method for separation and detection of methylmercury in fish tissue (Yang *et al.*, 2003). They used isotope dilution calibration to quantify the methylmercury. The limit of their method was 0.037 μg/g. Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used as insecticides, fungicides, bactericides, wood preservatives, plastic stabilisers and biocides in antifouling paints for ships (Yamada *et al.*, 1997). Like mercury, tin can also accumulate in the food chain, which can cause toxic effects on animals and humans. In 2002, Bancon-Montigny *et al.* developed a new approach to improve the precision of quantification of TBT in sediments by SPME-GC-MS using isotope dilution (Bancon-Montigny *et al.*, 2002). The isotope dilution technique eliminated the problem of poor reproducibility, which was the main disadvantage of SPME.

1.6.2 Applications in food and beverages

Food analysis is important for the evaluation of the nutritional value and quality of fresh and processed products, and for monitoring food additives and other toxic contaminants (Kataoka *et al.*, 2000). For example, flavour is a very important factor, which can greatly influence the consumption of food. In addition, monitoring of the toxic compounds is vital for human health. Therefore, various techniques have been developed for food analysis, such as steam distillation, headspace sampling and purge-and-trap methods. However, these methods involve some drawbacks. They are time consuming, labour intensive and the solvents required are hazardous. Therefore, SPME techniques have attracted increasing attention in the food analysis area due to their sensitive, selective, solvent free, simple characteristics.

Aroma and flavour are two of the most important quality criteria of fresh and processed foods and most aroma and flavour compounds are volatile. SPME can take advantage of this and provide a fast and clean approach to identify and characterise those compounds. The HS-SPME methods combined with GC-FID or GC-MS using 100 μm PDMS fibres have been widely used for the analysis of various volatile compounds in various foods like fruit, beverages and diary products. Summaries of some applications of SPME to the analysis of flavour compounds in food samples are listed in **Table 2**, **Appendix 1**.

Analysis of off-flavour compounds and contaminants such as pesticides and herbicides residues in food has also received a great deal of attention because they cause health risks and therefore impact on the daily life of people everywhere in the world. SPME methods have also been applied to this field. SPME methods for the analysis of off-flavour compounds and contaminants in food samples are listed in **Table 3**, **Appendix 1**.

1.6.3 Analysis of biological samples

Urine, blood, hair, breath and saliva are often sampled in clinical, forensic and toxicological analysis. The analytes can be drugs and their metabolites, alcohols, organometals, pesticides and so on. SPME has been one of the most popular sample preparation methods in the bio-analysis area since the 1990s. HS-SPME is ideal for the analysis of biological specimens as interference from large molecules (e.g.

proteins) in the matrix is reduced (Mills and Walker, 2000). In addition, successful coupling of SPME with LC and capillary electrophoresis (CE) enables the analysis of proteins, polar alkaloids, pharmaceuticals and surfactants which cannot be analysed by GC (Theodoridis *et al.*, 2000).

Jinno and co-workers coupled SPME with capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) successfully, which made it possible to study trace levels of drugs in bio-fluids (Jinno *et al.*, 1998). Studies of metal species in bio-samples can show the accumulation of Hg in the food chain. Dunemann and co-workers described a SPME GC/MS-MS method to determine Hg (II) and alkylated Hg, Pb, and Sn species in human urine (Dunemann *et al.*, 1999). Separation and identification of metal species were performed by capillary gas chromatography coupled with an ion-trap mass spectrometer with electron impact ionization in the tandem-MS mode. In 1998, He and co-workers combined SPME with GC-AAS to determine the methylmercury in bio-samples and sediments (He *et al.*, 1998).

Asakawa and co-workers (Asakawa *et al.*, 1999) studied BTEX in urine using SPME-GC-FID and Schimming's group analysed BTEX in blood using HS-SPME (Schimming *et al.*, 1999).

Chlorophenols were studied by Guidotti and co-workers (Guidotti and Vitali, 1998 and Guidotti *et al.*, 1999) using SPME-GC-MS methods, which were highly selective and sensitive for the determination of urinary chlorophenols at μg/L levels. Lord and Pawliszyn evaluated several factors affecting analyte recovery of amphetamines and methamphetamine in urine (Lord and Pawliszyn, 1997). Their method was useful for the analysis of narcotic analgesics.

SPME in conjunction with quadrupole ion trap GC–MS was applied to the determination of a series of barbiturates by Hall and Brodbelt (Hall and Brodbelt, 1997). Luo and co-workers developed a method using direct SPME coupled with GC-MS for the determination of five benzodiazepines in aqueous solution, urine, and serum (Luo *et al.*, 1998).

Li and Weber's SPME device was based on polyvinylchloride (PVC) as an extraction solvent coated on a primed steel rod, shown in **Figure 1.8**. A Teflon tube, with an inside diameter just larger than the PVC-coated extraction rod, was terminated at one end by a syringe. A few microliters of back-extraction solution were placed into the open end of the tube. After exposure to sample, the PVC-coated extraction rod containing the analytes was re-transferred into the back-extraction solution-containing tube. The device had been used in a CE-based determination of barbiturates. Extraction, back-extraction, and separation of 10 barbiturates took less than 30 min (Li and Weber, 1997).

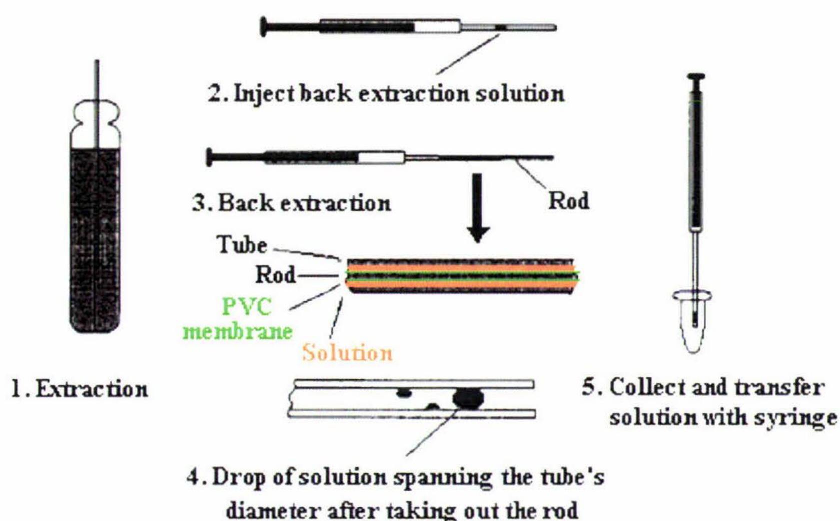


Figure 1.8 Device and operation of Li and Weber's SPME (1997)

Hall and co-workers established a SPME method to analyse cannabidiols in pure water and human saliva (Hall *et al.*, 1998). In comparison with the traditional liquid-liquid extraction, the SPME method was precise.

Okeyo and Snow described a SPME-GC-MS method with bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) headspace derivatisation on the SPME fibre for the analysis of steroids from aqueous and biological solutions (Okeyo and Snow, 1998). This method was a promising technique for the analysis of non-volatile compounds.

Myung and co-workers reported a SPME-GC-NPD technique for analysis of pethidine (meperidine) and methadone in human urine (Myung *et al.*, 1999).

Warfare agents are also the targets of SPME developers. Lakso and Ng (1997) described a novel analytical technique for detection of nerve agents in natural water samples at ppb and sub-ppb (v/v) levels with GC-NPD. This investigation showed that the SPME method was comparable to liquid-liquid extraction and had considerable potential for on-site inspections under the Chemical Weapons Convention. Sng and Ng's SPME-GC-MS method involved in-situ derivatisation prior to analysis (Sng and Ng, 1999). They analysed the degradation products of chemical warfare agents from water. This method was demonstrated during the 4th International Interlaboratory Proficiency Test organised by the Organisation for the Prohibition of Chemical Weapons to be comparable to existing recommended operating procedures for verification of degradation products of chemical warfare agents.

In addition to urinary and blood analysis, Röhrig and Meisch (Röhrig and Meisch, 2000) reported a method for monitoring organochlorine compounds in breast milk using the SPME-GC-ECD. The reproducibility of the results is very good down to the lower µg/L region. Grote and Pawliszyn applied SPME to the quantitative determination of ethanol, acetone, and isoprene in human breath (Grote and Pawliszyn, 1997). The method could detect concentrations of acetone and isoprene reported for healthy subjects. DeBruin's group (DeBruin *et al.*, 1998) used SPME-GC-MS to study monocyclic aromatic amines from milk as well as urine and blood. Gentili and co-workers developed a method to detect amphetamine-like drugs in hair (Gentili *et al.*, 2002). This method was suitable for routine clinical, epidemiological and forensic purposes and can be used for the preliminary screening of many other substances (amphetamine, methamphetamine, ketamine, ephedrine, nicotine, phencyclidine, and methadone) in hair and other biological matrices such as saliva, urine and blood.

1.7 Rumen study

Ruminant animals, such as cattle and sheep, are major sources of meat, milk, wool and leather. They can utilize cellulose and non-protein nitrogen, which are abundant in nature, such as grass and straw, while humans and other animals cannot digest these diets (Wu and Papas, 1997).

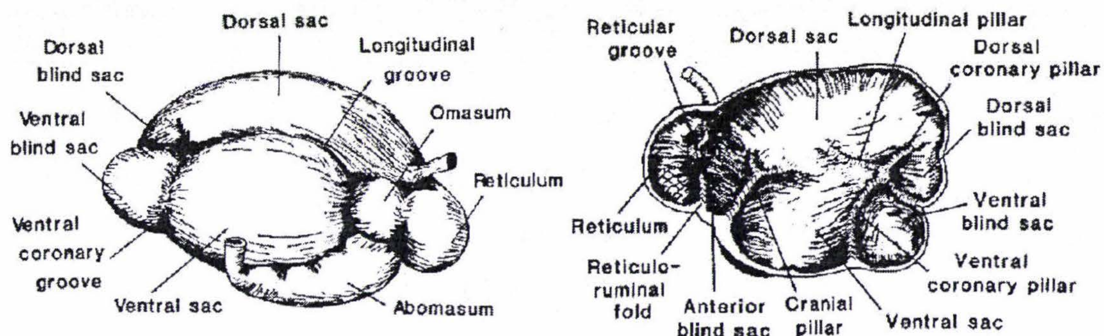


Figure 1.9 Diagrammatic view of the out side of the rumen (the right side) and the inner structure of the reticulo-rumen (based on left view)

As shown in **Figure 1.9,** the stomach complex of the ruminant animal consists of three functionally different parts, the reticulo-rumen, the omasum and the abomasum, and that the last mentioned corresponds to the stomach of the simple-stomach animals. The food enters through the oesophagus into a pear-shape sac, the reticulum, which communicates with the rumen proper, but is separated from it by a fold. Close to the reticulum is the omasum, which contains a large number of internal folds, directed towards the abomasum. The function of rumen is to degrade fibrous feed before it reaches the true stomach. The rumen proper, when viewed from the outside, consists of the large dorsal sac separated from the ventral sac by a horizontal fold. In addition, the caudal parts of the rumen have the dorsal and ventral blind sacs. There is another blind sac close to the reticulum and separated from it by the reticulo-rumen fold: this is the cranial blind sac. The position of the sacs is indicated by grooves, viewed from outside, and by pillars, viewed from inside the rumen. The pillars are composed of thick muscular bundles of tissue that project into the rumen. The dorsal and ventral sacs are separated by the longitudinal pillar and the dorsal and ventral blind sacs are formed by the dorsal and ventral coronary pillars. The cranial blind sac is separated from the ventral sac by the cranial pillar. The contents of the rumen are very heterogeneous and include a large proportion of semisolid digesta, particularly in the region of the longitudinal pillar, where it forms a "raft" of solid material. The contents above and below the raft are more fluid, but contain varying quantities of particles. There is usually some gas in the upper regions of the rumen (the gas cap). During the rumination, the solid digesta is regurgitated and the bolus is mixed with saliva and chewed by the animals. A part of the squeezed liquid is swallowed to the rumen firstly and then the squeezed bolus is also carried into the rumen. As a result,

the grass or other feeds freshly fed, squeezed liquid and solid produced via the rumination and a large population of rumen microbes, which can be divided into three groups: the bacteria, the protozoa and the fungi, constitute the rumen contents (Czerkawski, 1986).

The rumen is an enlarged forestomach in which the diet undergoes anaerobic fermentation. Various components are converted into volatile fatty acids, methane, carbon dioxide, ammonia and other compounds under the anaerobic conditions in rumen (Hobson and Stewart, 1997).

Traditional methods characterising these products involve sample collection, preservation, processing and use of reagents. For example, volatile fatty acids can be used to study the energetic efficiency of microbial fermentation in the rumen (Spinhirne *et al.*, 2003).

Several techniques have been used to determine the volatile fatty acids in rumen: (1) closed *in vitro* fermentation systems (Hungate, 1966), (2) *in vitro* continuous culture fermentation systems (Hoover *et al.*, 1976), (3) a suction pump with tubing for obtaining ruminal fluid through the esophagus, (4) rumenocentesis, and (5) gnotobiotic systems (Hungate, 1966). The disadvantages of these methods include invasive sampling and intensive sample preparation.

In 2002, Spinhirne and co-workers published their novel method, the application of solid phase microextraction to rumen study. Headspace SPME on a closed *in vitro* ruminal fluid fermentation system provided a non-invasive and selective approach to characterise the compounds released from the rumen fluid. This method also facilitated a rapid and immediate analysis coupled with GC-MS (Spinhirne *et al.*, 2003). They found toluene, dimethyl disulfide, pentadecane and volatile fatty acid, such as acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid and n-hexanoic acid from the headspace above the rumen fluid. However, quantification of the headspace gases was not performed in their studies.

1.8 Aims of the thesis

Firstly, develop a SPME method to study the volatiles in the headspace of the rumen contents, including:

1. Identifying the compounds in the headspace of the rumen contents using SPME-MS techniques.
2. Testing several factors which can influence the SPME extraction, such as SPME fibre type, pH, volume of headspace and rumen fluid and extraction time.
3. Quantifying the target compounds (VFAs, p-cresol, m-cresol, indole and skatole).

Then apply the technique to *in vitro* fermentation to study the conversion of VFAs, p-cresol, m-cresol, indole and skatole from proteins during the incubation of the material mentioned below in the rumen fluid:

(a) from tryptophan to indole and skatole.

(b) from tyrosine to p-cresol.

(c) from hydrolysed spirulina (unlabelled and ^{13}C labelled) to VFAs, p-cresol, m-cresol, indole and skatole.

(d) from spirulina (unlabelled and ^{13}C labelled) to VFAs, p-cresol, m-cresol, indole and skatole.

Chapter 2
Materials and methods

2.1 Rumen fluid

The rumen fluid for headspace SPME analysis was obtained from 2 – 4 sheep fitted with rumen canula maintained at AgResearch, Grasslands field facilities, Palmerston North, NZ. The sheep grazed pasture dominantly perennial ryegrass (Lolium perenne) containing a small quantity of white clover (Tifolium repens). The whole process of dealing with the rumen fluid is described in **Figure 2.1**. The sampling system included a 250 mL flask, a plastic funnel and two layers of cheesecloth. The rumen contents were squeezed through two layers of cheesecloth to remove the large particular matter. A 39 ^{0}C water bath was used to keep the whole system at the temperature of rumen. A PHM210 Standard pH Meter (Radiometer, Copenhagen) was used to measure the pH value of the rumen fluid. If the pH value was less than 5.5, the experiment was repeated on another day.

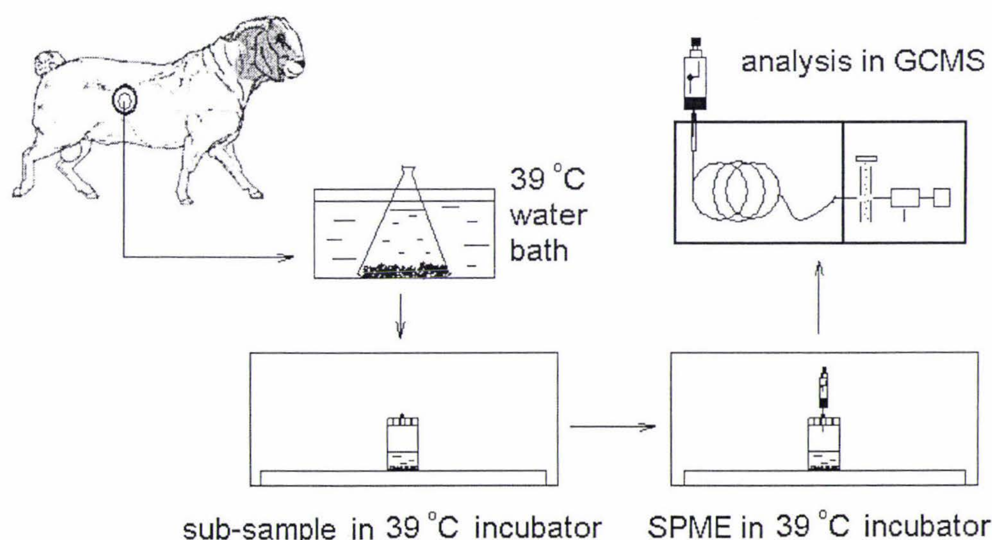


Figure 2.1 Sampling procedures

Typically a 20 mL of rumen fluid sub-sample was placed into a 68 mL glass bottle (Schott Duran) with a screw cap fitted with a septum. Additives, such as pH buffer solution and standard solution were added to the bottle prior to the addition of rumen fluid. Carbon dioxide was used to purge the headspace of the rumen fluid during setup to maintain anaerobic conditions. Subsequently, the bottles were moved into an

incubator, which was set at 39 ^{0}C and provided gentle agitation, 100 revolutions per minute.

An animal trial was carried out for the purposes of studying CH_4, VFAs and NH_3 production and bacteriology associated with the rumen for sheep received differing diets from 23-Feb to 20-Mar, 2004. White clover was fed to four sheep, No. 4859, 4862, 4863 and 4885, during the first ten days and then the sheep were given *Lotus pedunculatus* (Greater bird's foot trefoil) during the following ten days. Finally the feed was switched back to white clover for seven days. The rumen contents were sampled daily for the study of phenol and indole associated flavour compounds. This trial provided a chance to compare the headspace SPME with the traditional steam distillation simultaneous extraction method. The rumen fluid sampled from the four sheep were obtained and analysed individually. Whole rumen contents were squeezed through two layers of cheese cloth and a 5 mL sub-sample was immediately frozen in liquid nitrogen to arrest microbial activity and stored frozen – 25 ^{0}C until analysis. The remaining amount of rumen fluid was used to carry out the headspace SPME analysis immediately.

2.2 Chemicals and preparation of solutions

Acetic acid, $NaHCO_3$, Na_2HPO_4, Na_2HPO_4, NaCl, KCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$ and L-tryptophan were obtained from BDH Laboratory Supplies, Poole, Dorset, England. p-Cresol, m-cresol, indole, skatole, 2-isopropyl-phenol and 2-ethyl-phenol were obtained from Aldrich, Sheboygan Falls, Wisconsin, USA. D^3-skatole was made by D. Rowan, HortResearch, New Zealand. Propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid and n-hexanoic acid were from Sigma Chemical Co., St. Louis, USA. Heptanol was obtained from Riedel-de Haën, Germany. Tert-butylmethyl ether was obtained from Merck KGaA, Germany. L-tyrosine was obtained from S. M. A. Corporation, Chagrin Falls, Ohio, USA. Spirulina was purchased from Lifestream Research, Kenthurst, NSW, Australia. Spirulina universally labelled (^{13}C, + 98%) was obtained from Isotec Inc., Columbia, USA.

Artificial saliva, 500 mL, was prepared by dissolving 4.91 g $NaHCO_3$, 1.79 g Na_2HPO_4, 0.196 g NaCl, 0.296 g KCl, 0.297 g $MgCl_2{\cdot}6H_2O$ and 0.0145 g

$CaCl_2 \cdot 2H_2O$ in 500 mL Milli-Q water in accordance to procedure of McDouall (1948).

For standard addition analysis of p-cresol, m-cresol, indole and skatole, pH buffer solution was prepared by dissolving 1.214 g NaH_2PO_4 and 0.322 g Na_2HPO_4 in 100 mL Milli-Q water, resulting in a buffer strength of 100 mM and a pH of 6.1. The standard solutions included the internal standards, 2-isopropyl-phenol and 2-ethyl-phenol and the four target compounds prepared as follows. A 40 μg/mL 2-isopropyl-phenol and 20 μg/mL 2-ethyl-phenol solution was prepared by dissolving 2.0 mg 2-isopropyl-phenol and 1.0 mg 2-ethyl-phenol in 50 mL Milli-Q water. A 200 μg/mL p-cresol, 40 μg/mL m-cresol, 40 μg/mL indole and 40 μg/mL skatole solution was prepared by dissolving 10.0 mg p-cresol, 2.0 mg m-cresol, 2.0 mg indole and 2.0 mg skatole in 50 mL Milli-Q water.

For standard addition analysis of VFAs, pH buffer solution was prepared by dissolving 6.125 g NaH_2PO_4 and 1.622 g Na_2HPO_4 in 100 mL Milli-Q water, resulting in a buffer strength of 500 mM and a pH of 6.1. The standard solutions included the internal standard, heptanol, and the VFAs. A 0.81 mg/mL heptanol solution was prepared by dissolving 8.10 mg heptanol in 10 mL Milli-Q water. A 2 M acetic acid, 1 M propanoic acid, 0.2 M iso-butyric acid, 1 M n-butyric acid, 0.2 M iso-valeric acid, 0.2 M n-valeric acid and 0.04 M n-hexanoic acid solution was prepared by dissolving 1.2096 g acetic acid, 0.7339 g propanoic acid, 0.1831 g iso-butyric acid, 0.8601 g n-butyric acid, 0.2233 g iso-valeric acid, 0.2123 g n-valeric acid and 0.0463 g n-hexanoic acid in 100 mL Milli-Q water.

For the steam distillation extraction, phenol, indole and skatole standard solutions were prepared. A 2 mg/mL 2-isopropyl-phenol solution was prepared by dissolving 10 mg 2-isopropyl-phenol in 5 mL tert-butylmethyl ether. A 1 mg/mL 2-ethyl-phenol and 0.2 mg/mL D^3-skatole solution was prepared by dissolving 5 mg 2-ethyl-phenol and 1 mg D^3-skatole in 5 mL tert-butylmethyl ether. A 0.4 mg/mL phenol, 2 mg/mL p-cresol and 2 mg/mL m-cresol solution was prepared by dissolving 2 mg phenol, 10 mg p-cresol and 10 mg m-cresol in 5 mL tert-butylmethyl ether. A 1 mg/mL indole and 1 mg/mL skatole solution was prepared by dissolving 5 mg indole and 5 mg

skatole in 5 mL tert-butylmethyl ether. NaCl saturated solution was prepared by dissolving 300 g NaCl in 1 L Milli-Q water.

2.3 SPME

2.3.1 SPME fibres

SPME fibres were obtained from Supelco, Bellefonte, PA, USA. An 85 μm PA fibre and a 100 μm PDMS fibre were used in the preliminary investigation for the polar and non-polar compounds, respectively.

The behaviour of five types of fibres for the analysis of polar compounds, including 85 μm PA fibre, 65 μm PDMS/DVB fibre, 75 μm CAR/PDMS fibre, 65 μm CW/DVB fibre and 50/30 μm DVB/CAR/PDMS fibre and four types of fibres for the analysis of neutral compounds, including 100 μm PDMS, 65 μm PDMS/DVB fibre, 75 μm CAR/PDMS fibre and 50/30 μm DVB/CAR/PDMS fibre were tested. The polyacrylate fibre was chosen for the experiments described in this study.

Before initial use, all SPME fibres were conditioned in the GC injection port. The 85 μm polyacrylate fibre was conditioned at 270 ^{0}C for 120 min. The 65 μm PDMS/DVB fibre was conditioned at 260 ^{0}C for 30 min. The 75 μm CAR/PDMS fibre was conditioned at 280 ^{0}C for 30 min. The 65 μm CW/DVB fibre was conditioned at 200 ^{0}C for 30 min. The 50/30 μm DVB/CAR/PDMS fibre was conditioned at 270 ^{0}C for 60 min. The 100 μm PDMS fibre was conditioned at 250 ^{0}C for 60 min in accordance to manufacturer's recommendations.

2.3.2 HS-SPME

The headspace SPME was carried out after the rumen fluid was incubated for 15 min. The SPME syringe was pierced through the septum and the fibre was exposed in the headspace above the rumen fluid for a set period of time, 5 min for GC-MS analysis and 10 min for GC-FID analysis. The fibre was withdrawn and the needle was sealed by piercing it into a septum to prevent the loss of the analytes. Finally, the fibre was exposed into the injection port of the GC-MS or GC-FID system for 5 min to thermally desorb the analytes from the fibre onto the GC column.

2.4 Steam distillation simultaneous extraction

A Likens-Nickerson apparatus was used to extract the volatile analytes from the rumen contents. The apparatus is illustrated in the **Figure 2.2**. Saturated NaCl solution, 50 mL, rumen fluid, 5 mL, 50 μg of 2-ethyl-phenol and 10 μg of D^3-skatole were added into the left flask and 20 mL ter-butyl-methyl ether was added into the right flask. The two flasks were heated to reflux for 90 min. After they cooled down, 100 μg of 2-isopropyl-phenol was spiked into the 20 mL t-butyl-methyl ether. An aliquot 1 mL of the t-butyl-methyl ether extract was transferred into a GC vial.

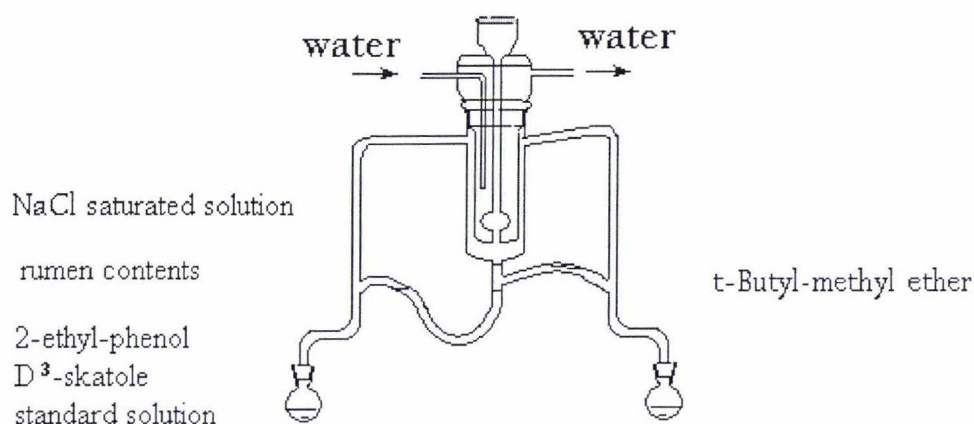


Figure 2.2 Steam distillation simultaneous extraction (Likens-Nickerson apparatus)

2.5 GC-MS and GC-FID

2.5.1 GC-MS conditions

The GC-MS analyses were performed on a Shimadzu GC-MS QP5050 gas chromatograph mass spectrometer. The GC injection port was fitted with a narrow-bore injection liner (Shimadzu). The GC was equipped with a 30m×0.25mm×0.25μm ZB-WAX (polyethylene glycol) capillary column (Phenomenex Ltd, NZ) for polar compound analysis as it was equipped with a ZB-5 (5% Phenyl Polysiloxane) 30m × 0.25mm × 0.25μm column (Phenomenex Ltd, NZ) for non-polar compounds analysis.

The GC-MS parameters are described below. A splitless injection was performed with the injection port was set at 250 ^{0}C. The spit vent was turned on after 1 min. The column inlet pressure was 69.0 kPa. Helium gas was used as carrier gas at a flow of 1.2 mL/min. The detector was turned on at 2.5 min. The mass spectrometry was

operated using scan mode scanning 40 – 400 m/z in range. Electron impact 70 keV was used to create positive ions.

When the polar ZB-WAX column was used, the initial column temperature was 50 ^{0}C and held for 1 min, followed by a ramp of 4 ^{0}C/min to 220 ^{0}C and held at final temperature for 6.5 min (the total program time was 50 min). The detector voltage was 1.6 kV. When non-polar ZB-5 column was used, the initial column temperature was 30 ^{0}C and held for 1 min, followed by a ramp of 4 ^{0}C/min to 260 ^{0}C and held at final temperature for 1.5 min (the total program time was 60 min). The detector voltage was 1.8 kV.

In the pre-investigation, the mass spectra were acquired in SCAN mode. Then in the following experiments, including the study of the effect of several factors on headspace SPME and the *in vitro* incubation of ^{13}C labelled spirulina, which only concerned polar compound analysis, the mass spectra were acquired in the selected ion monitoring (SIM) mode. The ions used for identification and quantitation of acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, hexanoic acid, p-cresol, m-cresol, indole and skatole were listed in **Table 2.1**. The GC-MS parameters in the study of the fibre sensitivities for neutral compounds were the same as those in the pre-investigation for neutral compounds.

Two representative ions were selected for the characterisation of each compound. The more abundant one was used to quantify the compound while the less abundant one served as a reference ion.

Table 2.1 Selected ions table for 11 polar compounds

Analyte	Ion (m/z)	Use
acetic acid	43 60	Quantitation Reference
propanoic acid	45 74	Quantitation Reference
iso-butyric acid	43 73	Quantitation Reference
n-butyric acid	60 73	Quantitation Reference
iso-valeric acid	60 74	Quantitation Reference
n-valeric acid	60 73	Quantitation Reference
hexanoic acid	60 73	Quantitation Reference
p-cresol	107 108	Quantitation Reference
m-cresol	108 107	Quantitation Reference
indole	117 90	Quantitation Reference
skatole	131 130	Quantitation Reference

GC-MS parameters employed in the analysis of the steam distillation extraction samples are listed below. An automated GC-MS system was utilised a SHIMADZU QP2010 gas chromatograph mass spectrometer was interfaced to a SHIMADZU AOC-20i auto injector equipped with a 10 μL SGE (Australia) syringe, coupled to a SHIMADZU AOC-20s auto sampler. The injection mode was splitless and the injection temperature was 240 ^{0}C. The column inlet pressure was 69.0 kPa. Helium carrier gas was utilised at a flow of 1.2 mL/min. The initial column temperature was 50 ^{0}C, held for 1min, followed by 4 ^{0}C/min to 220 ^{0}C and held at final temperature for 6.5 min (the total program time was 50 min). The ion source temperature was 200 ^{0}C and the interface temperature between GC and MS was 240 ^{0}C. The solvent cut time was 29.5 min. The detector voltage was 1.1 kV. Selected ion monitoring was performed. The ions used for quantitation and identification of 2-ethyl-phenol, p-cresol, m-cresol, 2-isopropyl-phenol, indole, D^{3}-skatole and skatole are given in **Table 2.2**.

Table 2.2 Selected ions for 2-ethyl-phenol, p-cresol, m-cresol, 2-isopropyl-phenol, indole, D^3-skatole and skatole

Analyte	Ion (m/z)	Use
2-ethyl-phenol	107 122	Quantitation Reference
p-cresol	107 108	Quantitation Reference
m-cresol	108 107	Quantitation Reference
2-isopropyl-phenol	121 136	Quantitation Reference
indole	117 90	Quantitation Reference
D^3-skatole	134 132	Quantitation Reference
skatole	131 130	Quantitation Reference

2.5.2 GC-FID conditions

The GC-FID analysis, including the quantitation of the 11 analytes and the some pre-investigation study for the *in vitro* incubation of ^{13}C labelled spirulina, was performed on a Shimadzu GC-17A gas chromatograph. The GC PTV injection port was fitted a narrow bore liner and the GC was equipped a ZB-WAX capillary column (30m×0.25mm×0.25μm).

The GC-FID parameters were the same as those of GC-MS except that the column inlet pressure was 119.0 kPa and the column flow rate was 1.74 mL/min. The temperature of FID was set to 250 ^0C.

2.5.3 Data analysis

Data was processed using GCMSsolution version 2.0 software for the GC-MS or ClassGC10 version 1.3 software for the GC-FID.

2.6 Experiment design

2.6.1 Preliminary investigation

Rumen fluid, 20 mL, was sub-sampled into a 68 mL vial and then incubated for 30 min. A PA fibre was inserted into the headspace for 10 min and then transferred to

the GC-MS for the analysis of polar compounds. Four vials of rumen fluid samples were prepared and headspace SPME was performed in each vial.

The headspace analysis using a PDMS fibre to identify the non-polar compounds was carried out on a separate day using identical conditions.

The whole rumen contents were sampled. The rumen fluid was obtained by squeezing the whole rumen contents. The rumen fluid was centrifuged to obtain the supernatant. The headspace SPME was carried out above the whole rumen contents, rumen fluid and the supernatant of rumen fluid to compare the polar headspace components of the three matrices using two PA fibres.

2.6.2 Optimisation of pre-incubation time

Rumen fluid was sub-sampled (20 mL) into six vials numbered 1 to 8 at various times Vial 1 and 2 were sub-sampled and incubated for 5 and 20 min, respectively. Two PA fibres were inserted into the headspace of the vials 1 and 2 for 5 min. After the two PA fibres were thermally desorbed for 5 min in the GC-MS injection port, they were inserted into the headspace for 5 min of vials 3 and 4, which were sub-sampled and incubated for 10 and 20 min. Pre-incubation time 20 min and 30 min were compared in the same way for vial 5 and 6.

2.6.3 Comparison of sensitivity of 5 kinds of fibres for polar compounds and 4 kinds of fibres for neutral compounds

Eight vials of rumen fluid were prepared and each one was filled with 20 mL. After 30 min incubation, a PA fibre was inserted into the headspace in vial 1 while a CW/DVB fibre was inserted into the headspace in vial 2 for 5 min. After 2.5 hours incubation, the headspace SPME of those 2 fibres was repeated in vial 3 and 4. After 4.5 hours incubation, a PA fibre was inserted into the headspace in vial 5 while a CAR/PDMS fibre was inserted into the headspace in vial 6 for 5 min. After 6.5 hours incubation, the headspace SPME of those 2 fibres was repeated in vial 7 and 8.

The comparison between PA and DVB/CAR/PDMS and between PA and PDMS/DVB was carried out in the same way on another day. GC-MS was used to

analyse the target compounds. The sensitivities of PDMS, PDMS/DVB, CAR/DVB and CAR/DVB/PDMS for neutral compounds were tested in the same way.

2.6.4 Effect of fibre age on the headspace SPME

Rumen fluid, 5 mL, and buffer solution (pH 6.1 and 100 mM buffer strength), 15 mL, were sub-sampled into vial 1 and 2. Heptanol solution was spiked into the rumen fluid as the internal standard. After 15 min incubation, a brand new PA fibre and an old PA fibre, which had been used for more than 80 times, were inserted into the headspace of vial 1 and 2 for 10 min. After two hours, the process was repeated in vial 3 and 4. In this case, GC-FID analysis was employed. The same experiments were carried out in the headspace of 10 mL rumen fluid and 10 mL buffer solution and 20 mL rumen fluid.

2.6.5 Effect of extraction time on headspace SPME

Firstly, extraction time within 10 min was compared. Six vials of sample were prepared and each one was filled with 20 mL rumen fluid. After 15 min incubation, two PA fibres were inserted into the headspace in vial 1 and 2 for 2.5 min and 10 min. Then two PA fibres were inserted into the headspace in vial 3 and 4 for 5 min and 10 min. Finally two PA fibres were inserted into the headspace in vial 5 and 6 for 7.5 min and 10 min. On another day, the process was repeated.

The extraction time within 20 min was then compared in a different way. On the first day, eight vials of sample were prepared and each one was also filled with 20 mL rumen fluid. After 15 min incubation, two 85 μm polyacrylate fibres were inserted into the headspace in vial 1 and 2 for 5 min and 20 min. Four replicates were carried out in the 8 vials. On the second day, the comparison of 10 min and 20 min extraction was carried out in the same way. GC-MS was used in this case for the comparison.

2.6.6 Effect of pH on headspace SPME

Rumen fluid, 10 mL, and pH buffer solution, 10 mL, were sub-sampled into each 68 mL vial. Rumen fluid at pH 5.5 and 6 was compared. After 15 min incubation, two 85 μm polyacrylate fibres were inserted into the headspace of vial 1 and 2 for 5 min. Another 3 replicates were carried out in the rest 6 vials. Then the comparisons

between pH 5.8 and 6, between pH 6 and 6.3 and between pH 6 and 6.7 were carried out in the same way using GC-MS.

2.6.7 Effect of sample and headspace volume on headspace SPME

Eight vials of rumen fluid samples were prepared. Rumen fluid, 20 mL, was added into vial 1, 3, 5, 7, and 40 mL, was added into vial 2, 4, 6, 8, respectively. After 15 min incubation, headspace SPME was carried out for 5 min using two PA fibres in vial 1 and 2. Then GC-MS was performed. Another three replicates of comparison of sample volume were carried out in remaining vials.

Four 68 mL vials were used in the study of headspace volume. Some glass beads (20 mL of volume) were put into vial 2 and 4, respectively. Then 20 mL of rumen fluid were sub-sampled into vial 1 and 2. After 15 min incubation, two 85 μm polyacrylate fibres were inserted into the headspace in vial 1 and 2 for 10 min. The experiment was repeated twice over two days.

2.6.8 Reproducibility

Eight vials of rumen fluid, 20 mL, were prepared and incubated at 39 ^{0}C. SPME was carried out after 15 min, 135 min, 255min and 375 min of incubation, respectively, using two PA fibres. For each analyte, the peak area extracted by fibre 1 was compared with that of fibre 2 at the same time point. The ratios of the peak areas between the two fibres were used to test the reproducibility of the method for the target compounds. The experiment was repeated four times on four days. During this period and the following standard addition experiments (**2.6.10**) for VFAs in May, 2004, heptanol was used as the internal standard for the VFAs. The response factor for heptanol, the peak areas *vs.* the amounts spiked (81 μg each vial), was also used to study the reproducibility of the technique.

During the animal trial mentioned in **2.1**, rumen fluid was sampled on 8, 9, 10, 12, 15, 18 and 19, March, 2004. 2-Ethyl-phenol and 2-isopropyl-phenol were used as the internal standard for p-cresol, m-cresol, indole and skatole. The response factors for the two internal standards, the peak areas *vs.* the amounts spiked (40 μg of 2-isopropyl-phenol and 20 μg of 2-ethyl-phenol each vial), were used to study the reproducibility of the technique.

2.6.9 Standard addition of p-cresol, m-cresol, indole and skatole

pH buffer solution, 15 mL, and rumen fluid, 5 mL, were added into a 68-mL vial. Then 40 μg of 2-isopropyl-phenol and 20 μg of 2-ethyl-phenol were spiked into the rumen fluid solution as the internal standards. Eight samples were prepared. Sample 1, 3, 5, 7 were used to measure background while 0.5, 1, 1.5, 2 mL of 200 μg/mL p-cresol, 40 μg/mL m-cresol, 40 μg/mL indole and 40 μg/mL skatole standard solution were spiked into sample 2, 4, 6 and 8, respectively. The standard addition SPME was carried out at four standard levels. After 15 min incubation, two 85 μm polyacrylate fibres were inserted into the headspace in vial 1 and 2, 3 and 4, 5 and 6, 7 and 8 for 10 min, respectively. Then the two fibres were thermally desorbed in the GC-FID injection port. The experiment was repeated twice over two days.

The background responses of the four compounds were subtracted from those extracted from the headspace above the spiked rumen fluid. The calibration curves for the four compounds were obtained by plotting the peak areas *vs*. the spiked amounts of those compounds. The slopes of the four curves were the response factors of GC-FID for the four analytes and they would be used in the calculation of the concentrations of those compounds in the SPME experiments parallel to the steam distillation extraction. Therefore, the conditions used in the SPME experiments parallel to the steam distillation extraction were the same as those in the standard addition experiments of p-cresol, m-cresol, indole and skatole. The applicability of the internal standards was evaluated by comparing the trend of the response factors of the target compounds and standard compounds on different days.

2.6.10 Standard addition of VFAs

pH buffer solution, 15 mL, and rumen fluid, 5 mL, were added into a 68-mL vial. Then 81 μg of heptanol was spiked into the rumen fluid solution as the internal standard. Eight samples were prepared. Sample 1, 3, 5, 7 were used to detect the background while 0.1, 0.2, 0.3 and 0.4 mL of 2 M acetic acid, 1 M propanoic acid, 0.2 M iso-butyric acid, 1 M n-butyric acid, 0.2 M iso-valeric acid, 0.2 M n-valeric acid and 0.04 M n-hexanoic acid standard solution were spiked into sample 2, 4, 6 and 8, respectively. The standard addition SPME for VFAs was also carried out at four standard levels. After 15 min incubation, two 85 μm polyacrylate fibres were inserted into the headspace in vial 1 and 2, 3 and 4, 5 and 6, 7 and 8 for 10 min,

respectively. Then the two fibres were thermal desorbed in the GC-FID injection port. Another replicate was performed on another day. The volume of VFAs standard solution spiked into the rumen fluid increased to 0.5, 0.75, 1 and 1.5 mL.

2.6.11 Pre-investigation of *in vitro* incubation

The study was performed on the GC-FID. Artificial saliva, 4 mL, and rumen fluid, 16 mL were mixed to prepare 80% rumen fluid, which would be used in the *in vitro* incubation.

Two amino acids, L-tryptophan and L-tyrosine, were used to test the *in vitro* design. Ten vials of samples were prepared for incubation with L-tryptophan or L-tyrosine. Vial 1, 3, 5, 7 and 9 were added 80% rumen fluid while vial 2, 4, 6, 8 and 10 were added L-tryptophan or L-tyrosine, about 3 mg. Then the 10 vials of samples were incubated at 39 ^{0}C. Two 85 μm polyacrylate fibres were inserted into vial 1 and 2, vial 3 and 4, vial 5 and 6, vial 7 and 8, vial 9 and 10 for 10 min, respectively, after 0, 2, 4, 6 and 8 hours incubation.

Acid hydrolysed spirulina, defatted via six hours Soxhlet extraction in pet ether, was then used to test the *in vitro* design. The acid hydrolysis conditions were spirulina defatted, about 300 mg, cooking in 10 mL of 6 M HCl, vacuum condition, at 110 ^{0}C for 20 hours. The rumen fluid samples were prepared in 8 vials and ca. 90 mg acid hydrolysed spirulina were added into vial 2, 4, 6, 8. The incubation of acid hydrolysate was performed for 6 hours and headspace SPME was carried out after 0, 2, 4 and 6 hours using two 85 μm polyacrylate fibres.

2.6.12 *In vitro* incubation of ^{13}C labelled spirulina

The acid hydrolysed spirulina (**^{13}C labelled**) and whole spirulina (**^{13}C labelled**) were incubated and headspace SPME was performed in the way described in **2.6.11**. The incubation lasted 24 hours and headspace SPME was carried out after 0, 2, 4, 6 and 24 hours incubation. Then GC-MS was use to identify the ^{13}C labelled compounds. The ions, which were selected to calculate the abundance of ^{13}C labelled compounds, are listed in **Table 2.3**. Both PA and PDMS fibres were used.

Table 2.3 Selected ions table for calculation of abundance of ^{13}C labelled compounds

	Ions (m/z)	
	labelled	unlabelled
acetic acid	62	60
propanoic acid	77	74
iso-butyric acid	76	73
n-butyric acid	76	73
iso-valeric acid	77	74
n-valeric acid	76	73
hexanoic acid	76	73
p-cresol	114	107
m-cresol	115	108
indole	125	117
skatole	140	131
dimethyldisulfide	96	94
dimethyltrisulfide	138	136

Chapter 3
Preliminary investigation

3.1 Introduction

The flavour-producing substances from the rumen are absorbed by the blood stream and transmitted to the milk or meat. In addition, feeds release or produce volatile flavours after partial digestion in the rumen. These volatiles are eructated or belched from the rumen, inhaled into the lungs and transferred to the milk via the blood stream. The major process in the rumen involves anaerobic microbes converting the components of the forages to volatile fatty acids which are absorbed into the blood stream for energy production (Keen, 1998). Therefore, it is important to study the rumen gas. Spinhirne and co-workers were the first group to apply the SPME technique to the study of rumen fluid fermentation (Spinhirne *et al.*, 2003). However, only a limited number of compounds were identified using HS-SPME-GC-MS method. A wider range of analytes were identified in this section using a different SPME method as outlined in this chapter.

Within an animal rumen the ingested material segregates into a raft of plant materials which tend to float at the top and a larger volume of fluid and fine solids which sink to the bottom in spite of the system being reasonably mixed by rumen muscular contractions. To sample the whole rumen contents representatively requires us to empty the entire rumen out into a bucket and mix. The bulk of rumen contents will be returned back to the animal after sub-sampling. It is a very intensive process. Therefore, the rumen fluid is obtained instead for the studies. The goal in using the rumen fluid is to obtain a collection of rumen microbes suitable for fermenting with plant fractions. The volatiles extracted from the headspace above the whole rumen contents, rumen fluid and the supernatant of rumen fluid were compared in this chapter. The representability of rumen fluid was observed and the influence of solids was ascertained.

When the rumen fluid was sub-sampled to the vial, the compounds started to volatilize from the liquid phase to the headspace. After a period of time, the

equilibrium between the rumen fluid and the headspace was reached. This pre-incubation time was optimised in this section.

3.2 Results and discussion

3.2.1 Analysis of polar compounds

A typical chromatogram of the polar compounds sampled from the headspace of the rumen fluid by the 85 μm polyacrylate fibre at natural rumen pH (5.6 – 6.8) is shown in **Figure 3.1**. As expected toluene and seven kinds of volatile fatty acids were found in the headspace of the rumen fluid. In addition, p-cresol, m-cresol, indole and skatole were identified. The chromatogram also included some peaks other than those of interest.

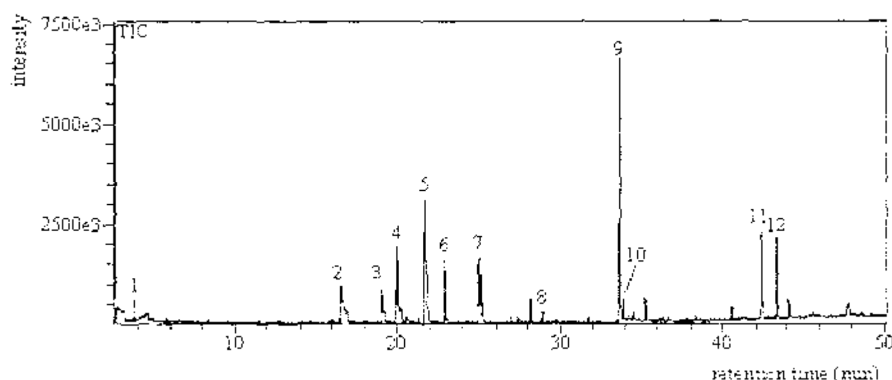


Figure 3.1 GC-MS chromatogram of polar compounds from the headspace of the rumen fluid by a 85 μm polyacrylate fibre: (1) toluene, (2) acetic acid, (3) propanoic acid, (4) iso-butyric acid, (5) n-butyric acid, (6) iso-valeric acid, (7) n-valeric acid, (8) hexanoic acid, (9) p-cresol, (10) m-cresol, (11) indole, (12) skatole.

The retention times of the target compounds extracted using PA fibre are listed in **Table 3.1**.Compounds were initially identified by spectral comparison to spectra compiled in the Wiley 139 library. Subsequently, spectra and retention time of authentic compounds were compared with the analytes initially assigned.

Table 3.1 Retention time of polar analytes

Compounds	Retention time (min)
toluene	3.78
acetic acid	16.97
propanoic acid	19.34
iso-butyric acid	20.11
n-butyric acid	21.97
iso-valeric acid	23.1
n-valeric acid	25.25
hexanoic acid	28.33
p-cresol	33.8
m-cresol	34.01
indole	42.47
skatole	43.5

Twelve compounds extracted from the headspace of the rumen fluid using PA fibre were identified. **Figure 3.2** gives their mass spectra.

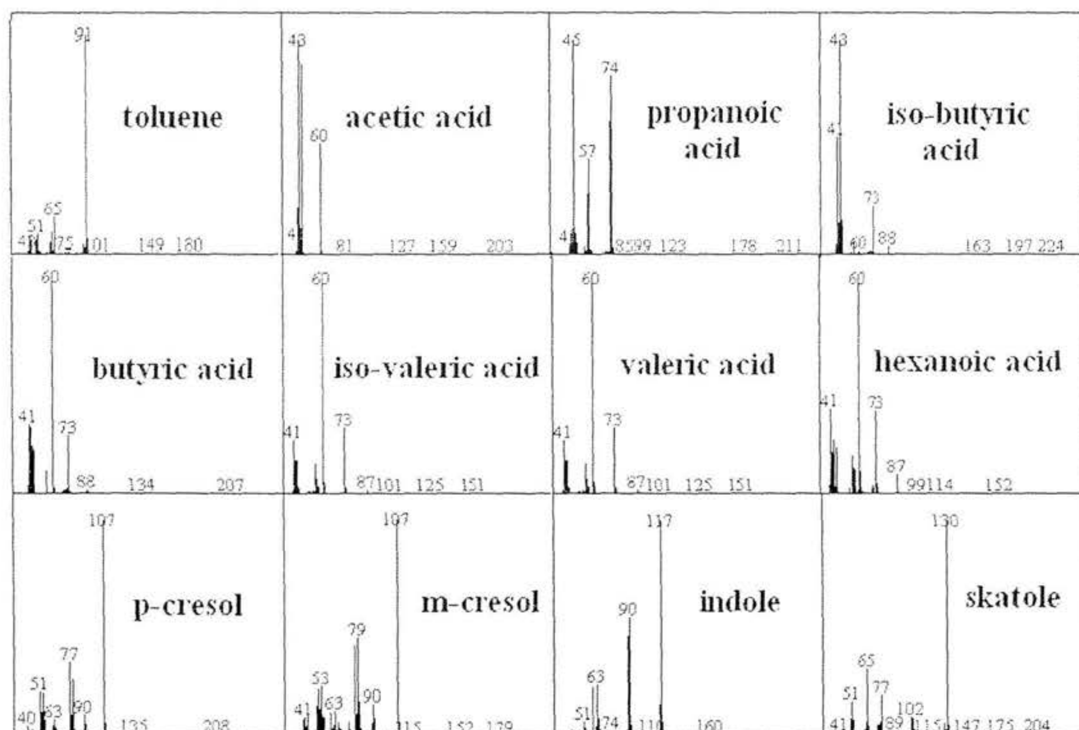


Figure 3.2 Mass spectra of twelve target compounds extracted using PA fibre from the headspace of rumen fluid, including toluene, acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, hexanoic acid, p-cresol, m-cresol, indole and skatole.

The mass spectra of most aromatic hydrocarbons show very intense molecular ion peaks (Pavia *et al.*, 2000). In the mass spectrum of toluene, loss of hydrogen from the molecular ion gives a strong peak at $m/e = 91$, which is a tropylium ion.

Aliphatic carboxylic acids generally show a weak molecular ion. With short chain acids, the loss of OH and COOH through α-cleavage on either side of the C=O group may be observed. With acids containing γ hydrogens, the principal pathway for fragmentation is the McLafferty rearrangement. In the case of carboxylic acids, this rearrangement produces a prominent peak at $m/e = 60$ (Pavia *et al.*, 2000).

In the spectrum of acetic acid, the molecular ion ($m/e = 60$) and $COOH^+$ ($m/e = 45$) are observed. Loss of OH gives rise to a peak at $m/e = 43$.

In the spectrum of propanoic acid, the molecular ion ($m/e = 74$) and $COOH^+$ ($m/e = 45$) are observed. Loss of OH gives rise to a peak at $m/e = 57$.

In the spectrum of iso-butyric acid, the molecular ion ($m/e = 88$) is observed. Loss of methyl group gives rise to a peak at $m/e = 73$. Loss of COOH group gives rise to a peak at $m/e = 43$.

In the spectrum of n-butyric acid, the molecular ion ($m/e = 88$) is observed. Loss of methyl group gives rise to a peak at $m/e = 73$. As the presence of γ hydrogens, the $m/e = 60$ appears.

In the spectra of iso-valeric acid and n-valeric acid, loss of hydrogen from the molecular ion gives a weak peak at $m/e = 101$. Loss of ethyl group gives rise to a peak at $m/e = 73$. As the presence of γ hydrogens, the $m/e = 60$ appears.

In the spectrum of iso-valeric acid and n-valeric acid, loss of two hydrogens from the molecular ion gives a weak peak at $m/e = 114$. Loss of ethyl group gives rise to a peak at $m/e = 87$. Because of γ hydrogens, the $m/e = 60$ also appears.

Phenols typically lose the elements of carbon monoxide to give strong M – 28 peaks. Phenols also lose the elements of the formyl radical (HCO·) to give strong M – 29 peaks. However, the M – 28 and M - 29 peaks of cresols are not very strong (Pavia *et al.*, 2000). In the spectra of p-cresol and m-cresol, loss of hydrogen from the molecular ion gives a strong peak at *m/e* = 107. The M – 28 peaks (*m/e* = 80) and M – 29 peaks (*m/e* = 79) appear.

Indole compounds yield a molecular ion, usually very intense (Jamieson and Hutzinger, 1970). In the spectra of indole and skatole, the molecular ions, indole at *m/e* = 117 and skatole at *m/e* = 130, respectively, are very strong. The loss of HCN (*m/e* = 27) gives a peak at M – 27 (Numan and Danielson, 2002). Peaks of M – 27, indole at *m/e* = 90 and skatole at *m/e* = 103, respectively, are also observed.

Volatile fatty acids are the products of the rumen fermentation from fibre, cellulose, proteins and lipids (Hobson and Stewart, 1997). Phenol and alkyl phenols are a group of flavour compounds that occur in milk or meat. For example, p-cresol is considered to be responsible for the formation of undesirable barny flavour in NZ cheese. Ruminal degradation of tyrosine and lignins is a likely source of phenolic compounds. Indole and skatole are two flavourful amines in milk and dairy products, leading to a faecal taint. Ruminal deamination of dietary tryptophan is a likely source of milk indoles (Keen, 1998).

3.2.2 Analysis of non-polar compounds

Figure 3.3 shows a typical chromatogram of the non-polar compounds sampled from the headspace of the rumen fluid by the 100 μm PDMS fibre at pH = 12.5. After eliminating the interference of volatile fatty acids with base, several compounds, including dimethyldisulfide, toluene, octane isomers, n-hexanol, α-pinene, dimethyltrisulfide, β-pinene, limonene, p-cresol, linalool, indole and skatole, were identified.

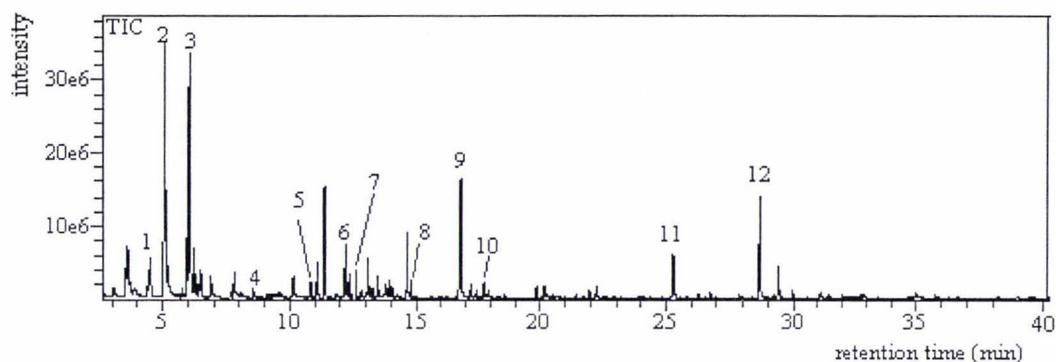


Figure 3.3 GC-MS chromatogram of neutral compounds from the headspace of rumen fluid by 100 μm PDMS fibre: (1) dimethyldisulfide, (2) toluene, (3) octane isomers, (4) n-hexanol, (5) α-pinene, (6) dimethyltrisulfide, (7) β-pinene, (8) limonene, (9) p-cresol, (10) linalool, (11) indole, (12) skatole.

The retention times of the target compounds extracted using PDMS fibre are listed in **Table 3.2**.

Table 3.2 Retention time of neutral analytes

Compounds	Retention time (min)
dimethyldisulfide	4.497
toluene	5.1
octene isomers	5.957, 6.08, 6.267, 6.503
n-hexanol	8.533
α-pinene	10.827
dimethyltrisulfide	12.183
β-pinene	12.55
limonene	14.74
p-cresol	16.8
linalool	17.743
indole	25.27
skatole	28.687

Figure 3.4 shows the mass spectra of three monoterpenes and one terpene alcohol, two sulfides, one alkene and alcohol, extracted from the headspace of the rumen fluid using PDMS fibre.

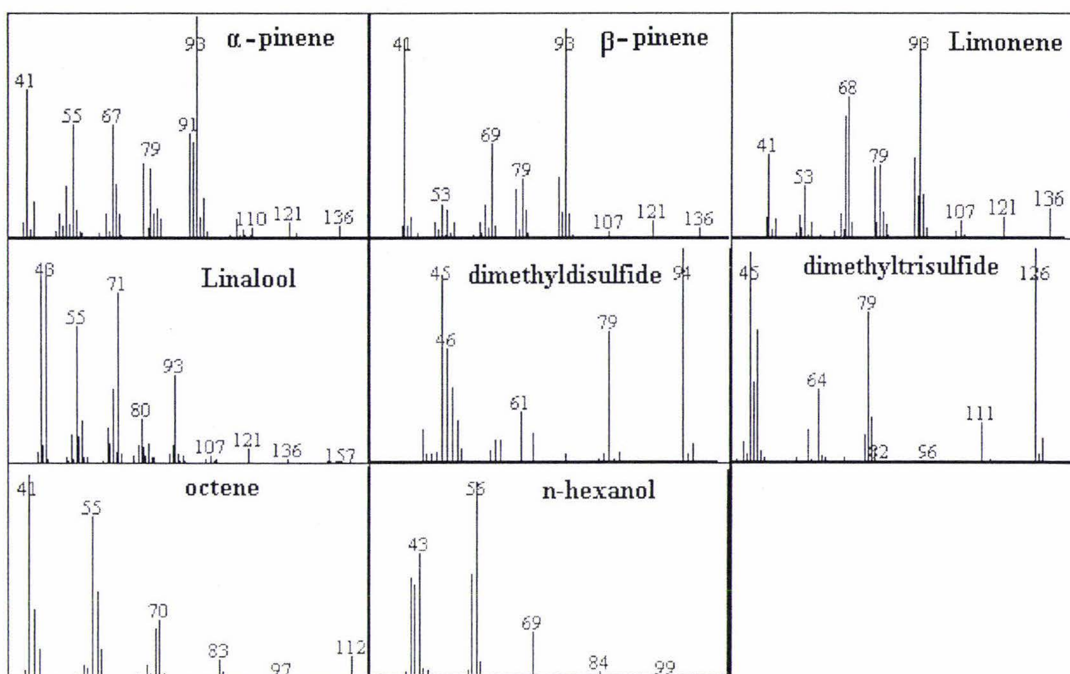


Figure 3.4 Mass spectra of eight target compounds extracted using PDMS fibre from the headspace of rumen fluid, including α-pinene, β-pinene, limonene, linalool, dimethyldisulfide, dimethyltrisulfide, octane and n-hexanol.

The spectra of terpenes or terpene derivatives contain fragment ions at *m/e* = 79, 93, 107 and 121 (Eriksson and Levin, 1996). In the spectra of α-pinene, β-pinene and limonene, the molecular ions at *m/e* = 136 are observed. The loss of CH_3, C_2H_5, C_3H_7 or C_4H_9 from the molecular ion gives ion *m/e* = 121, ion *m/e* = 107, ion *m/e* = 93 or *m/e* = 79. In the spectrum of linalool, the loss of H_2O from the molecular ion gives a peak at *m/e* = 136.

Thioethers tend to exhibit weak molecular ion peaks. Principal modes of fragmentation include α-cleavage and loss of alkylthio group (Pavia *et al.*, 2000). In the spectra of dimethyldisulfide and dimethyltrisulfide, the molecular ions at *m/e* = 94 and 136 are very strong. The loss of a CH_3 group from the molecular ions gives peaks at *m/e* = 111 and 79. In the spectrum of dimethyltrisulfide, the loss of an S from ion *m/e* = 111 gives rise to a peak at *m/e* = 79 and then the loss of another methyl group give the ion S-S at *m/e* = 64, which is also observed in the spectrum of dimethyldisulfide.

The intensity of the molecular ion peak in the mass spectrum of a primary and secondary alcohol is usually rather low. Fragmentation of alcohols involves the loss of alkyl group or the loss of a molecule of water (Pavia *et al.*, 2000). In the mass spectrum of n-hexanol, the loss of a molecule of water from the molecular ion gives ion m/e = 84, then the loss of a CH_3 group from ion m/e = 84 give rise to peak m/e = 69. The loss of a CH_3 and a CH_2OH group from the molecular ion gives a peak at m/e = 56.

The mass spectra of most alkenes show distinct molecular ion peaks. Apparently, electron bombardment removes one of the electrons in the π bond, leaving the carbon skeleton relatively undisturbed. Fragmentation to form an allyl cation (m/e = 41) is favoured (Pavia *et al.*, 2000). In the spectra of octene isomers, the molecular ion m/e = 112 is observed. The allyl cation m/e = 41 is very strong. The loss of a series of alkyl group gives peaks M – 15 = 97, M – 29 = 83, M – 43 = 69 and M – 57 = 55, respectively.

Some of the neutral compounds derive from the plant material. A number of compounds, such as toluene, octene, hexanol, linalool and p-cresol, were identified from the flavour components of Lucerne hay, Italian ryegrass and rice straw (Aii *et al.*, 1980). The monoterpenes, α-pinene, β-pinene and limonene have a contribution to dairy flavour (Keen, 1998).

Certain fermentation processes carried out in the rumen are able to degrade sulfur-containing amino acids in the diet (i.e. methionine, cysteine) to low molecular weight volatile sulphur compounds, such as dimethyldisulfide, which can lead to cowy or unclean flavour (Keen, 1998).

3.2.3 Comparison of rumen fluid, supernatant of rumen fluid and whole rumen contents

Table 3.3 shows the difference between the amounts of the analytes extracted from the rumen fluid and the supernatant using PA fibres. In comparison with rumen fluid, 50% of the toluene, 60% to 85% of the VFAs and approximately 90% of p-cresol, m-cresol, indole and skatole were extracted from the headspace of the supernatant of rumen fluid.

Higher amounts of analytes could be extracted from the headspace of the rumen fluid than the supernatant. Although there was a small quantity of solids present, approximately 5% (dry matter weight) for the rumen fluid, the results could reveal a considerable quantity of target compounds reside with these solids, e.g. ca 10 – 20 % of rumen fluid analytes reside with the small quantity of solids present. The relative standard deviations were very large, due to the variance distribution of the analytes in the rumen fluid because of the presence of solid. In addition, compounds such as VFAs could be lost more easily than cresols, indole and skatole during the process of centrifuging because they are more volatile. This might lead to the greater relative standard deviations for the VFAs. This may be due to the origin of these compounds, i.e. β-pinene is derived from the plant therefore more likely to reside in plant solids.

Table 3.3 Comparison of rumen fluid and supernatant of rumen fluid (The mean values are the ratios of peak areas of the analytes extracted from the headspace of supernatant of rumen fluid relative to rumen fluid.)

Compounds	mean value (%) n = 4	RSD (%)
toluene	49	48
acetic acid	83	71
propanoic acid	85	72
iso-butyric acid	80	69
n-butyric acid	74	56
iso-valeric acid	67	43
n-valeric acid	60	35
hexanoic acid	61	21
p-cresol	88	18
m-cresol	92	19
indole	87	11
skatole	85	11

The difference between the amounts extracted from the rumen fluid and the whole rumen contents using PA fibres is listed in the **Table 3.4**. Although the same amount of the whole rumen contents contains more compounds than the rumen fluid, the transfer rate in the whole rumen contents is smaller than the one in the rumen fluid. This might be the reason that the amounts of analytes extracted from the headspace of the whole rumen contents were very close to those from the headspace of rumen fluid

Consequently, the headspace analysis of the whole rumen contents introduces much more variables. However, more analytes were found in the headspace of the whole rumen contents than rumen fluid for most target compounds except n-butyric acid, iso-valeric acid and n-valeric acid.

Table 3.4 Comparison of rumen fluid and whole rumen contents (The mean values are the ratios of peak areas of the analytes extracted from the headspace of whole rumen contents *vs.* rumen fluid.)

Compounds	Mean value (%) n = 4	RSD (%)
toluene	133	31
acetic acid	124	76
propanoic acid	108	68
iso-butyric acid	104	54
n-butyric acid	94	42
iso-valeric acid	95	19
n-valeric acid	94	25
hexanoic acid	102	17
p-cresol	108	10
m-cresol	113	13
indole	111	6
skatole	114	6

3.2.4 The study of pre-incubation time

Four pre-incubation times, 5, 10, 20 and 30 min, were studied. The pre-incubation time profile is described in **Figure 3.5**. The results showed the release process of the analytes from the rumen fluid to the headspace as well as the production of those compounds in the rumen fluid.

Within 30 min pre-incubation time, the amounts of VFAs extracted from the headspace of rumen fluid rose very slowly in the range from 93.8 to 120 (relative peak area) except n-hexanoic acid, slightly fluctuating within the range from 100 to 105.9. The amount of toluene extracted from the headspace of rumen fluid increased steadily from 86.4 to 100 by increasing the pre-incubation time from 5 min to 20 min and then rose sharply to 148.9 when 30 min pre-incubation time was used. The pre-

incubation time profile of p-cresol, m-cresol, indole and skatole had the same trend as the toluene.

There was less than 25% increase for all the analytes released from the rumen fluid to the headspace when pre-incubation time rose from 5 to 20 min. On the other hand, a great increase was due to the production of several analytes, such as p-cresol, m-cresol, indole and skatole. Nevertheless, all the analytes reached a relative plateau from 10 to 20 min. Therefore, 15 min pre-incubation time was chosen to use in following experiments.

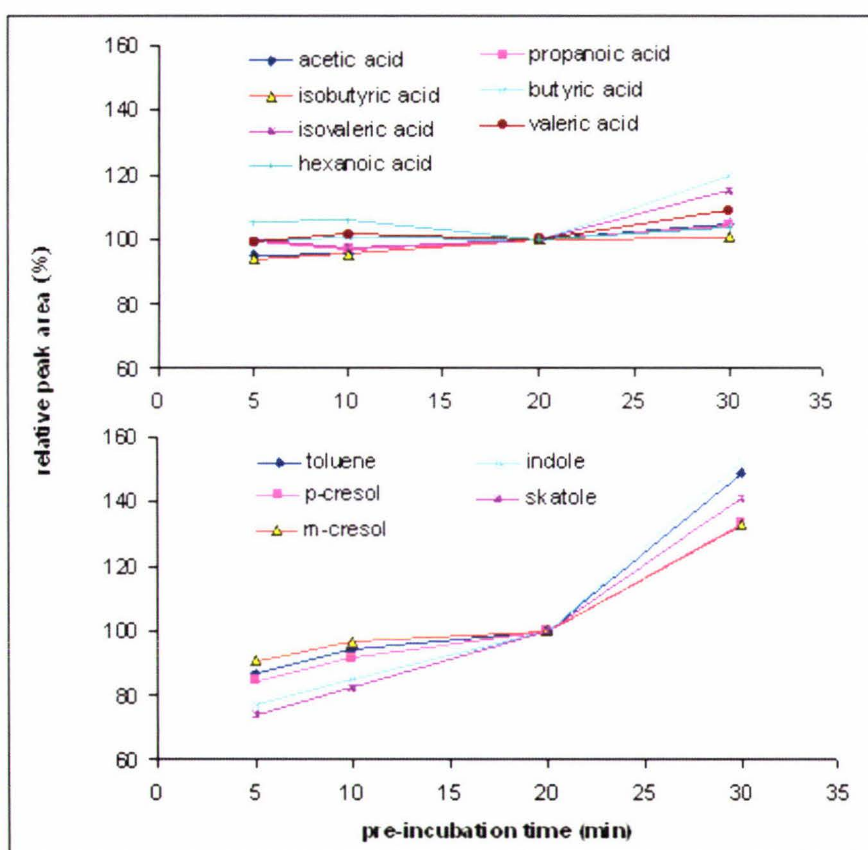


Figure 3.5 Influence of pre-incubation time on the extraction of the target compounds (The values of relative peak area of 5, 10 and 30 min pre-incubation were relative to 20 min pre-incubation, respectively.)

3.3 Conclusions

Application of headspace SPME technique to a closed *in vitro* rumen fluid incubation system provides a non-invasive approach to study the volatile compounds released from the rumen fluid. This method is selective and solvent free. Several classes of

compounds such as VFAs, flavour compounds (e.g. p-cresol etc.) and terpene compounds can be extracted using SPME fibres. This technique is fast, requires no preservation and gives immediate results. The polar compounds are the major interests in the following study.

All identified compounds could be extracted from the whole rumen contents, the rumen fluid and the supernatant. The logistics of rumen fluid is easier than the whole rumen contents while it still contains large population of rumen microbes relative to the supernatant, which determines that the rumen fluid is suitable for the rumen study using the headspace SPME technique.

One of the factors which can influence the headspace SPME, the pre-incubation time, was tested. Considering that the rumen fluid can produce target compounds, 15 min incubation would be carried out before SPME in the following experiments.

Chapter 4

Effect of several factors on SPME

4.1 Introduction

Several factors, such as fibre coating, temperature, pH, agitation, extraction mode, extraction time, salts and other additives and volume of sample and headspace can influence efficiency of headspace SPME (Krutz *et al.*, 2003).

In the study of volatile compounds in rumen contents, some factors can be optimized while the others cannot. Firstly, the rumen contents constitute a complex sample matrix, including solids and high molecular weight organics. This limits to use headspace SPME. Secondly, 39 ^{0}C, which is the rumen temperature, was chosen in all experiments to keep the rumen contents viable. The addition of salts and other additives were not suitable, upsetting the microbes. Although the incubator can be set in a shaking mode, the shake, a gentle agitation, is not designed to mix the contents thoroughly. While SPME extraction was carried out, the incubator was stationary. The rest of the factors, fibre coating, pH, extraction time and volumes of sample and headspace were readily changed.

Non-polar analytes are most effectively extracted with a non-polar fibre coating while polar analytes are most effectively extracted with a polar coating. The addition of an adsorbent material to the coating can improve the sensitivity of SPME. For example, the big surface area of Carbowax® and the small pores in Carboxen™ can improve the sensitivity for small polar molecules in SPME. The equilibrium time and sensitivity increase as the thickness of the fibre coating increases. Therefore the thick fibre is used to retain volatile compounds while the thin fibre is suitable for high boiling point compounds, which can ensure complete rapid thermal desorption of the analytes extracted in the SPME fibre **(Supelco Bulletin 923, Appendix 3)**.

The extraction yield increases by increasing the extraction time and reaches a plateau after the multi-phase equilibrium of SPME is reached. Although the equilibrium extraction has the advantages of high sensitivity and reproducibility, extraction time

are rarely set at equilibrium but rather at a point where sensitivity and precision are maximised over an acceptable experiment time (Krutz *et al.*, 2003).

Changing the pH of the sample can greatly influence the extraction. Acidic and basic compounds are effectively extracted at acidic and basic pH, respectively. For example, at low pH, the acid-base equilibrium of acidic compound is shifted toward the neutral form and the analyte partitioning into the fibre coating is enhanced.

For headspace SPME, the volume of headspace may be minimised to improve the sensitivity. Another way to increase the sensitivity is to increase the sample volume, an approach that is widely used in direct immersion SPME (Krutz *et al.*, 2003).

4.2 Results

4.2.1 Fibre

4.2.1.1 Effect of fibre types

4.2.1.1.1 Polar compounds

Five types of polar or mixed coating fibres, polyacrylate, CW/DVB, CAR/PDMS, DVB/CAR/PDMS and PDMS/DVB, were tested to determine the sensitivity towards polar compounds such as the VFAs, p-cresol, m-cresol, indole and skatole.

Figure 4.1 shows the values of relative peak areas. The peak areas of all the four mixed coating fibres were relative to those of polyacrylate fibre.

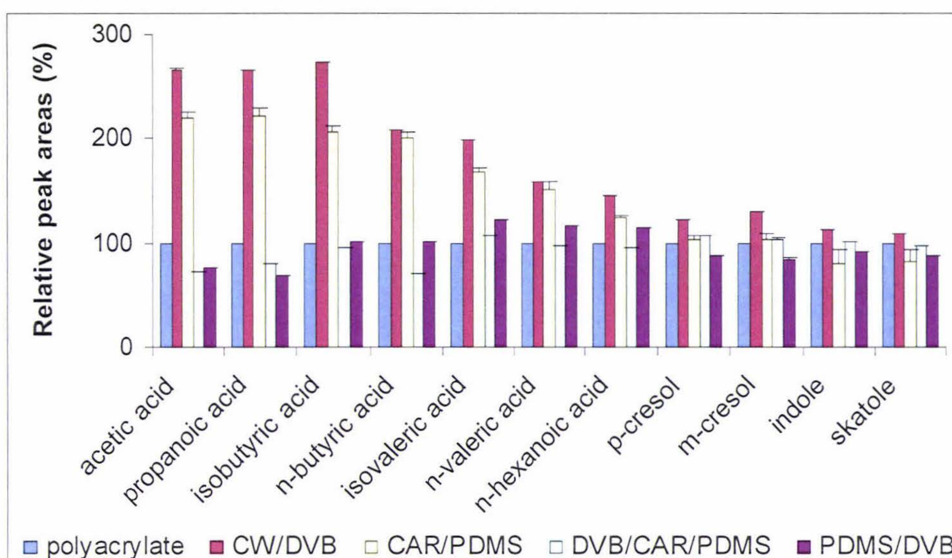


Figure 4.1 Comparison of the sensitivities of five types of fibres for polar compounds (the values are the mean of two replicates + standard deviation)

CW/DVB fibre is the most sensitive fibre towards all of the 11 compounds. It extracted 250% acetic acid, propanoic acid and iso-butyric acid, ca. 200% n-butyric acid and iso-valeric acid and ca. 150% n-valeric acid and n-hexanoic acids relative to the polyacrylate fibre(s). The ratios (CW/DVB relative to polyacrylate) for p-cresol, m-cresol, indole and skatole fell from 130% to 110%.

The sensitivities of the CAR/PDMS fibre towards the 11 compounds are slightly lower than those of CW/DVB fibre. In comparison with polyacrylate fibre, CAR/PDMS fibre extracted ca. 220% acetic acid and propanoic acid, ca. 200% iso-butyric and n-butyric acids, about 150% iso-valeric and n-valeric acids and 125% n-hexanoic acid. The amounts of p-cresol and m-cresol extracted using CAR/PDMS fibre were very close to those extracted using polyacrylate fibre while the CAR/PDMS fibre extracted 80% indole and skatole relative to polyacrylate fibre.

In general, the performance of DVB/CAR/PDMS fibre and PDMS/DVB fibre was poorer than polyacrylate fibre for the 11 compounds. The ratios, DVB/CAR/PDMS fibre relative to polyacrylate fibre, fluctuated between 70% and 107% while those, PDMS/DVB fibre relative to polyacrylate fibre, fluctuated between 68% and 123%.

4.2.1.1.2 Non-polar compounds

Figure 4.2 shows the comparison of PDMS, CAR/PDMS, DVB/PDMS and DVB/CAR/PDMS fibre extraction for neutral compounds. The toluene, p-cresol, indole and skatole are the major peaks in the chromatograms of PDMS in all three figures while the four peaks are much stronger and more peaks are clearly present in the chromatograms of CAR/PDMS, DVB/PDMS and DVB/CAR/PDMS.

In **Figure 4.2 a**, the chromatogram of CAR/PDMS shows the dimethyldisulfide peak just before the toluene peak at 4.5 min (retention time), (**Section 3.2.2**) and the octene isomers peaks at around 6 min (behind toluene, **Section 3.2.2**). There are some weak peaks, such as α-pinene and dimethyltrisulfide, between 10 min and 15 min.

In **Figure 4.2 b**, the dimethyldisulfide, octene isomers and the peaks between 10 and 15 min are stronger. Linalool, behind the p-cresol peak at 17.7 min, (**Section 3.2.2**) is detected. Some unknown peaks are present after 20 min.

In **Figure 4.2 c**, the chromatogram of DVB/CAR/PDMS is very close to the one of DVB/PDMS.

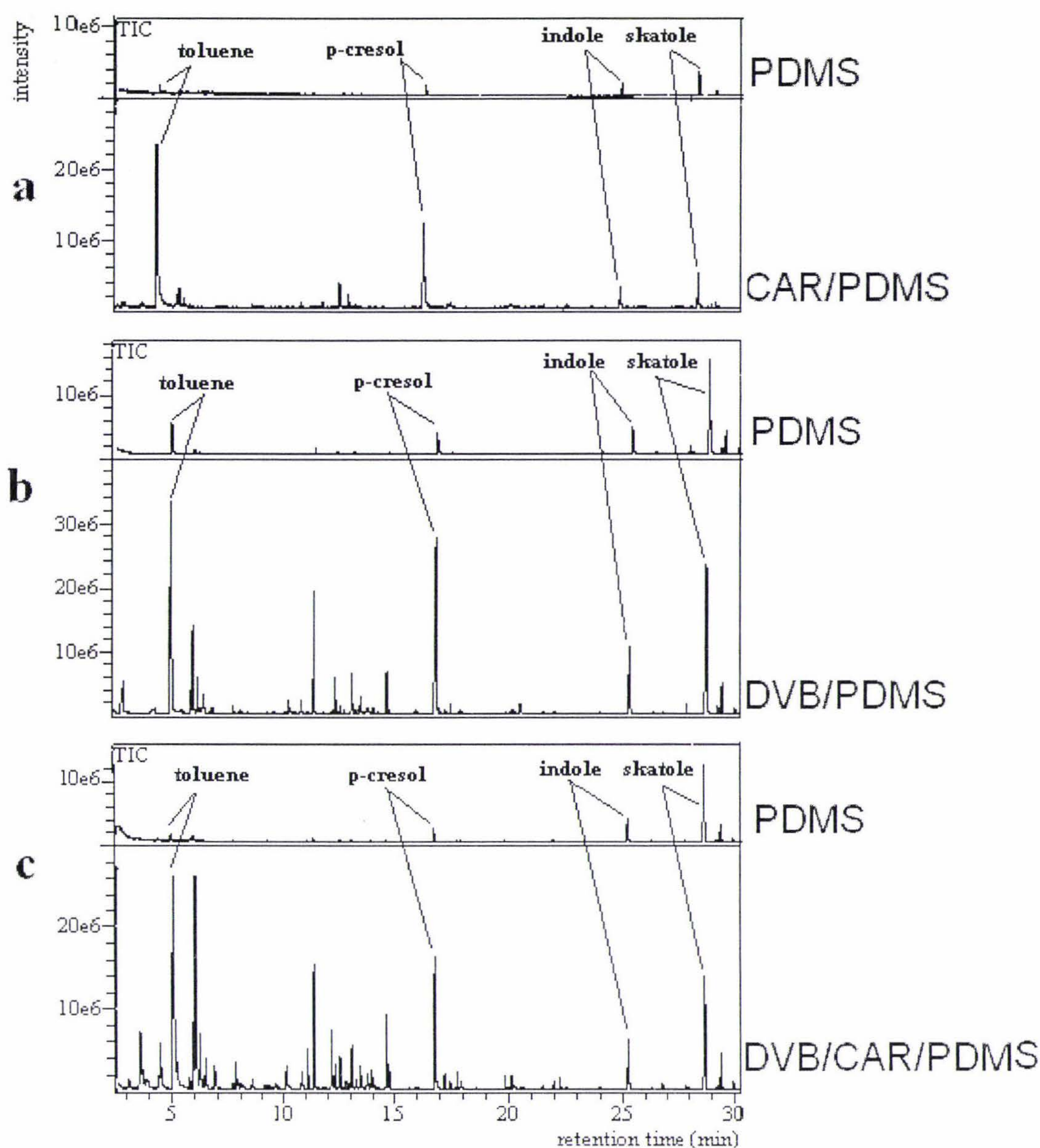


Figure 4.2 Comparison of the sensitivities of four types of fibres for neutral compounds: (a) PDMS *vs.* CAR/PDMS, (b) PDMS *vs.* DVB/PDMS and (c) PDMS *vs.* DVB/CAR/PDMS

4.2.1.2 Effect of fibre age

Table 4.1 reveals the life time of the polyacrylate fibre for the headspace extraction. The extraction efficiencies of a used fibre (> 80 injections) and a brand new one were tested for the polar compounds. The new fibre had a better extraction efficiency than the old one. In comparison with new fibre, the old one could extract less than 70% of acetic, propanoic and n-butyric acids, 70 to 77% of iso-butyric, iso-valeric and n-

valeric acids, 83% of n-hexanoic acid, approximately 90% of p-cresol, m-cresol, indole and skatole.

Table 4.1 Comparison of SPME efficiency between a brand new polyacrylate fibre and an old polyacrylate fibre over 80 times of injection to the GC for the 11 target compounds

	Ratios of peak areas (used fibre *vs*. new fibre, %) n = 5
acetic acid	60
propanoic acid	66
iso-butyric acid	70
n-butyric acid	65
iso-valeric acid	72
n-valeric acid	77
n-hexanoic acid	83
p-cresol	91
m-cresol	89
indole	93
skatole	92

4.2.2 Effect of extraction time

Figure 4.3 shows the extraction time profiles of the polyacrylate fibre for 11 compounds. The extraction time was chosen within 20 min. All the values are the means of two replicates.

Firstly, the extraction time 2.5, 5, 7.5 and 10 min were compared and the results are presented in **Figure 4.3 a** and **b**. The relative peak areas of 2.5, 5 and 7.5 min extraction were relative to 10 min extraction. For acetic acid, propanoic acid and iso-butyric acid, the relative peak areas were stable above 60% from 2.5 to 5 min and rose rapidly to 100% by increasing the extraction time to 7.5 min, followed by another plateau until 10 min. For n-butyric acid, iso-valeric acid, n-valeric acid and n-hexanoic acid, the relative peak areas increased by approximately 10% when extraction time increased from 2.5 to 5 min, then they jumped to above 95% at 7.5 min and increased slightly to 100% at 10 min. For p-cresol and m-cresol, the relative peak areas rose steadily from under 40% at 2.5 min to over 50% at 5 min, and increased significantly to above 97% at 7.5 min and then to 100% at 10 min. For

indole and skatole, the relative peak areas were constant under 45% from 2.5 to 5 min and then rose dramatically to 100% at 7.5 min.

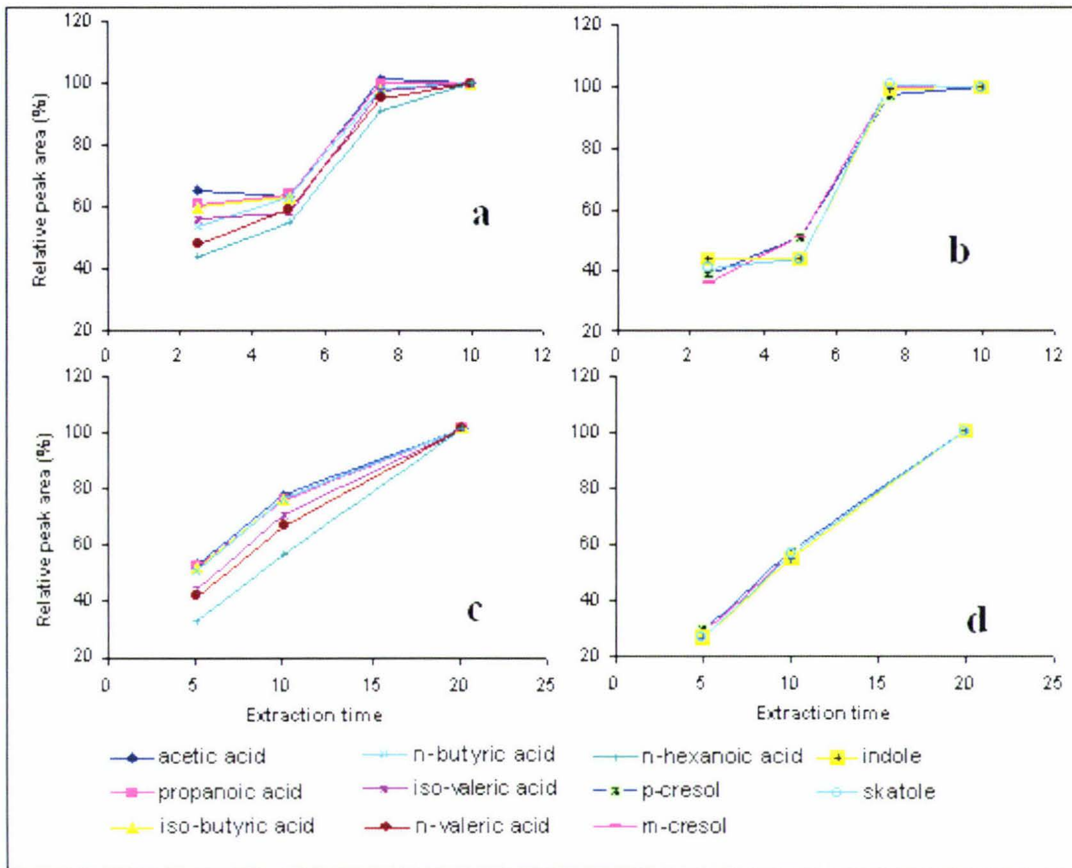


Figure 4.3 The time profiles of SPME in the headspace of rumen fluid using polyacrylate fibre: (a) for VFAs within 10 min, (b) for p-cresol, m-cresol, indole and skatole within 10 min, (c) for VFAs within 20 min and (d) for p-cresol, m-cresol, indole and skatole within 20 min

The comparisons of the extraction times 5, 10 and 20 min are presented in **Figure 4.3 c** and **d**. For acetic acid, propanoic acid, iso-butyric acid and n-butyric acid, the relative peak areas increased gradually from nearly 50% at 5 min to about 75% at 10 min and then to 100% at 20 min. For iso-valeric and n-valeric acids, the relative peak areas rose from above 40% at 5 min to over 65% at 10 min and finally to the top at 20 min. For n-hexanoic acid p-cresol and m-cresol, the relative peak areas increased from about 30% at 5 min to over 55% at 10 min and then jumped to 100% at 20 min. For indole and skatole, the relative peak areas increased gradually from 26% at 5 min to about 55% at 10 min and reached the top at 20 min.

The standard deviations of the two sets of time profiles are listed in **Table 4.2**. Generally, the variables ranges from 10% to 20% while some of them are under 8%.

Table 4.2 Relative standard deviations of the time profiles for 11 compounds

	Figure 4.3 a and **b**			**Figure 4.3** c and **d**	
	2.5 min	5 min	7.5 min	5 min	10 min
acetic acid	17.1	13.6	3.4	14.3	10.6
propanoic acid	18.3	9.4	3.6	13.9	9.9
isobutyric acid	18.7	1.1	6.0	12.8	9.6
butyric acid	17.7	5.8	8.4	15.6	11.3
isovaleric acid	13.6	0.0	10.2	5.6	8.6
valeric acid	14.1	5.0	12.1	10.9	7.8
hexanoic acid	14.5	6.7	17.5	11.5	7.5
p-cresol	17.6	15.4	16.3	13.6	11.3
m-cresol	13.0	18.3	12.1	10.1	11.0
indole	14.1	10.3	14.3	14.0	13.4
skatole	15.3	14.1	10.4	13.2	12.6

4.2.3 Effect of pH of the rumen fluid sample matrix

The **Figure 4.4** shows that the sample pH can influence the SPME for the 11 compounds. The peak areas at pH 5.5, 5.8, 6.3and 6.7 were relative to those at pH 6.0.

In comparison with pH 6.0, nearly 16% of VFAs were extracted at pH 6.7; approximately 50% of VFAs were extracted at pH 6.3; 170% of acetic acid, 1.5 times of propanoic acid and 130% of the rest of VFAs were extracted at pH 5.8. Ca. 200% of VFAs were extracted at pH 5.5. On the other hand, the effect of pH on SPME for p-cresol, m-cresol, indole and skatole was not as great. There was a range from 10% to 30% of increase when pH dropped from 6.0 down to 5.5 or rose from 6.0 to 6.7.

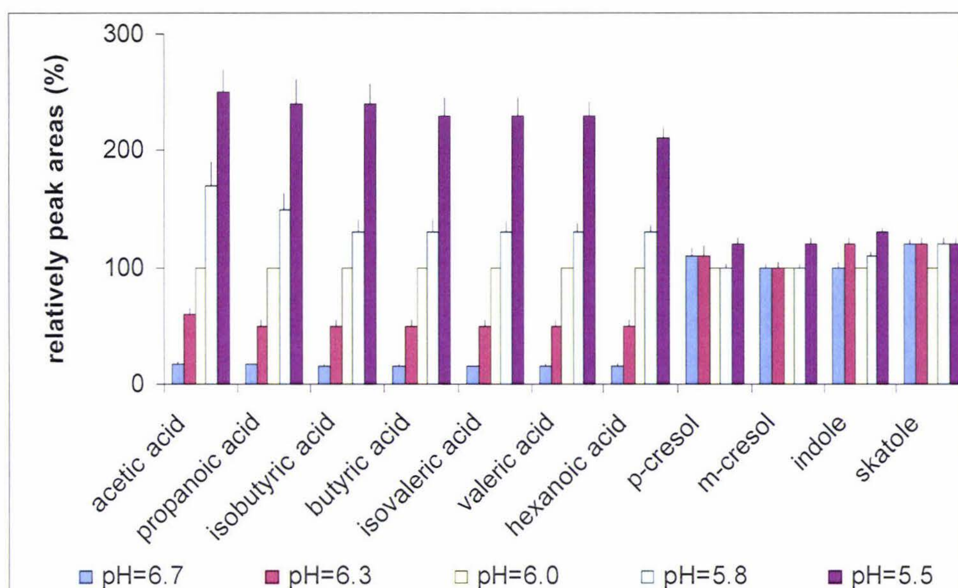


Figure 4.4 Effect of pH on SPME (the values are the means of two replicates + standard deviation)

4.2.4 Effect of sample and headspace volume

The amount of analytes absorbed into the polyacrylate coating increased as the sample volume increased (**Figure 4.5**). By increasing the sample volume from 20 mL to 40 mL, the extraction of acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid and n-valeric acid improved by over 30%. In addition, 27% of n-hexanoic acid increased. The extent of increase of the SPME efficiency for p-cresol, m-cresol, indole and skatole was 0 - 20%.

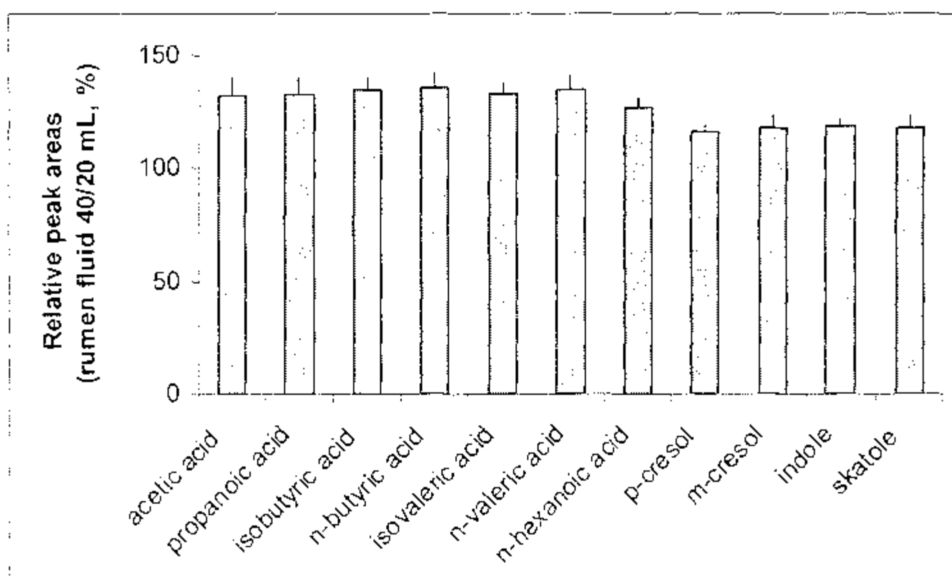


Figure 4.5 Effect of sample volume on SPME (the values are the means of four replicates + standard deviation)

The effect of headspace volume on the SPME is described in **Figure 4.6**. The amounts of VFAs increased by decreasing the headspace from 48 mL to 28 mL when adding 20 mL of glass beads into the vials. The amounts of acetic acid and propanoic acid rose by nearly 40%. Those of iso-butyric and n-butyric acids increased by over 25% and the other three acids increased by less than 10%. On the contrary, less p-cresol, m-cresol, indole and skatole were extracted when the headspace fell from 48 mL to 28 mL. These decreased ca. 13%, 12%, 5% and 9% for p-cresol and m-cresol, indole and skatole, respectively.

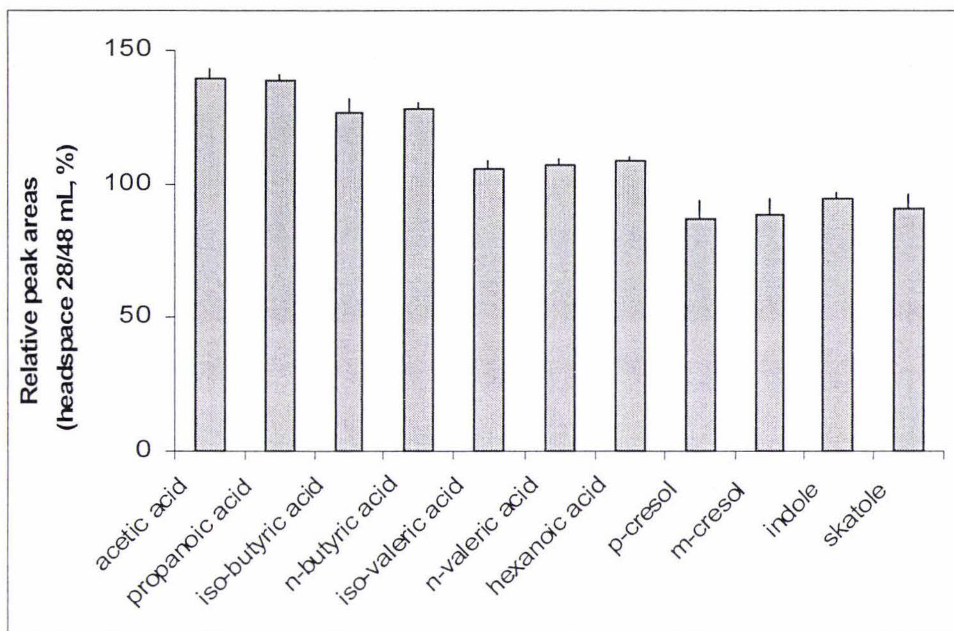


Figure 4.6 Effect of headspace volume on SPME (the values are the means of four replicates + standard deviation)

4.3 Discussion

4.3.1 Fibre types

Diffusion coefficients of organic compounds in PDMS are close to those in organic solvents and those of organic molecules in polyacrylate are lower by about one order of magnitude than in PDMS. These diffusion coefficients are large enough for absorption to be the primary extraction mechanism. On the other hand, diffusion coefficients of organic compounds in divinylbenzene (DVB) and Carboxen are so small that the diffusion times in these matrices can be days or weeks, which are out of the time frame of SPME (Górecki *et al.*, 1999). Therefore, the mixed coatings, such as PDMS/DVB and Carbowax/DVB, extract analytes via adsorption.

The coatings with a phase of DVB consist of porous particles of DVB which are held together by either PDMS or Carbowax as glue (Ulrich, 2000). Carboxen/PDMS coating is a mixed carbon (Carboxen 1006 adsorbent, surface area approximately 1000 m^2/g) phase and both absorption and adsorption mechanisms operate in this coating (Górecki *et al.*, 1999). DVB/Carboxen/PDMS is made up of two layers of coating, consisting of a layer of DVB/PDMS and a layer of Carboxen/PDMS.

The Carbowax/DVB is the most polar (**Figure 1.2**) fibre coating and the Carboxen/PDMS is designed to retain highly volatile analytes (Mills and Walker, 2000). Therefore, both of them showed larger extraction efficiency for the polar analytes except indole and skatole. On the other hand, their sensitivities decreased as the volatility and polarity of the compounds dropped in comparison with polyacrylate fibre. For p-cresol, m-cresol, indole and skatole, these two fibres have little advantage over the other three fibres, polyacrylate, DVB/Carboxen/PDMS and PDMS/DVB.

Although PDMS is non-polar, it can extract both polar and non-polar compounds (Krutz, *et al.*, 2003). Meanwhile the mixed coating fibres are bi-polar. Consequently, polar compounds such as volatile fatty acids can be extracted using these fibres. To avoid interference from these acids, the pH of rumen fluid was adjusted to above 12.

In the analysis of neutral compounds, the mixed coating fibres had much greater extraction efficiency than the PDMS fibre. The sensitivities of the two layers of coating fibre (DVB/Carboxen/PDMS) and the DVB/PDMS fibre were very close while the Carboxen/PDMS was less sensitive, which suggests that the Carboxen/PDMS coating is not so sensitive as the DVB/PDMS coating.

4.3.2 Fibre age

Thermal desorption in the GC injection port which can steadily damage the polyacrylate phase might be one of the main reasons of loss of sensitivity of the fibre. Moreover, the absorption of analytes could not be desorbed completely each time. The accumulation of these memories may be converted to active carbon residues material in the polyacrylate phase when it is heated at 250 ^{0}C in the GC injection port, a temperature which can also change the properties of the extraction phase.

The fibre age can affect the SPME efficiency for the polar analytes as shown in the comparison between an older fibre (> 80 times injection to the GC injection port) and a brand new one. However, the extent of influence for different compounds varies in a range from 60% to 93% (experimental data). One possible reason is dependent on the amounts of the analytes in the rumen fluid. If the amount of an analyte is larger than the extraction capability of used fibre, a different extraction efficiency can be revealed. Otherwise, when the amount of an analyte is smaller than the extraction

capability of used fibre, of course less than that of new fibre too, the difference between the two fibres would not be apparent.

In **Table 4.3**, the concentrations of volatile fatty acids in rumen contents match the ratios, extraction efficiency of used fibre *vs*. new fibre, very well: high concentrations leading to low ratios and low concentrations leading to high ratios. The concentration levels of p-cresol, m-cresol, indole and skatole are nearly several μg/mL, which is much smaller than those of VFAs. Therefore, the effect of the fibre age (> 80 times injection to the GC injection port) is less than 10% of extraction capability.

Table 4.3 The approximate concentrations of VFAs in rumen fluid (measured by D. Li from AgResearch Grassland, Palmerston North) and the influence extent of fibre age

	mM	Ratios of peak areas (used fibre *vs*. new fibre, %)
acetic acid	50	60
propanoic acid	20	66
iso-butyric acid	5	70
n-butyric acid	20	65
iso-valeric acid	5	72
n-valeric acid	5	77
hexanoic acid	< 1	83

4.3.3 Extraction time

The equilibrium times of the target compounds in the headspace SPME analysis from the rumen fluid are not known. In addition, the analytes can be produced by microbial fermentation and released to the headspace with increasing time. Therefore, the amounts of analytes extracted were examined by increasing the extraction time up to 20 min.

The time profile within 10 min shows that there was little improvement of extraction by increasing the extraction time from 2.5 to 5 min and from 7.5 to 10 min while the extraction increased dramatically in comparison 5 min with 7.5 min extraction (**Figure 4.3 a** and **b**). The different extraction capability between the two PA fibres

could lead to this problem. As the poor fibre was exposed into the headspace for a long time and the better fibre was inserted into the headspace for a short time, the relationship between the extracted amounts of compounds and the extraction time would not be present properly. However, if the difference of the extracted amounts of compounds is larger as the extraction time increases, the trend in another time profile, from 5 min to 20 min extraction, was that the extraction increased steadily as the extraction time increased but no equilibrium was reached (**Figure 4.3 c** and **d**).

The ratios of peak area (5 min *vs.*10 min extraction) in the time profile (from 5 min to 20 min extraction) were calculated and listed in **Table 4.4** and compared with those in the time profile (from 2.5 min to 10 min). The results are very close, so the reliability of the two time profiles is high and they can be used to determine the extraction time used in the following experiments.

Table 4.4 The relative peak areas (5 min *vs.*10 min extraction, %)

	Time profile within 20 min	Time profile within 10 min
acetic acid	67	63
propanoic acid	68	64
isobutyric acid	67	63
butyric acid	65	63
isovaleric acid	61	58
valeric acid	62	59
hexanoic acid	56	55
p-cresol	51	51
m-cresol	50	51
indole	48	44
skatole	46	44

4.3.4 pH

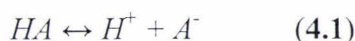
The rumen pH ranges from 5.7 to above 7.0 (Hungate, 1966). The influence of pH on SPME varies depending on the nature of compound. The pKa values of volatile acids are around 4.8 and those of p-cresol and m-cresol are above 10 (**Table 4.5**).

Table 4.5 pKa values of the analytes

compounds	p Ka
acetic acid	4.76
propanoic acid	4.88
isobutyric acid	4.84
butyric acid	4.82
isovaleric acid	4.77
valeric acid	4.84
hexanoic acid	4.88
p-cresol	10.3
m-cresol	10.1
indole	11.6 *
skatole	10.6 *

* p K_b value

In the rumen fluid there is an equilibrium between the HA molecule, H^+ and A^- ions, described in **Equation 4.1**.



$$HA \leftrightarrow H^+ + A^- \qquad \textbf{(4.1)}$$

$$Ka = \frac{a_{H^+} \cdot a_{A^-}}{a_{HA}} \qquad \textbf{(4.2)}$$

The equilibrium concentration of HA in the rumen fluid, a_{HA}, is shown in **Equation 4.3**,

$$\frac{a_{HA}}{C_0} = \frac{a_{H^+}}{K_a + a_{H^+}} \qquad \textbf{(4.3)}$$

where C_0 is the original concentration of HA in the rumen fluid and a_{H^+} is the equilibrium concentration of H^+ ion.

When Ka = 4.8 in **Equation 4.3**, a_{HA}/C_0 can be calculated at pH 5.5, 5.8, 6.0, 6.3 and 6.7. Relative to pH 6.0, approximately 21%, 50%, 150% and 280% of HA are present in the rumen fluid at pH 6.7, 6.3, 5.8 and 5.5, respectively. These data calculated match the trend of volatile fatty acids at decreasing pH in **Table 4.6** and some of them are slightly higher than the practical results.

Table 4.6 The proportions (%) of VFAs (a_{HA}/C_0) at different pH, both practical and calculated values are relative to those at pH 6

	pH							
	6.7		6.3		5.8		5.5	
	Prac.	Calc.	Prac.	Calc.	Prac.	Calc.	Prac.	Calc.
acetic acid	17	21	60	50	170	150	250	280
propanoic acid	17	21	50	50	150	150	240	270
isobutyric acid	16	21	50	50	130	150	240	280
butyric acid	16	21	50	50	130	150	230	280
isovaleric acid	15	21	50	50	130	150	230	280
valeric acid	16	21	50	50	130	150	230	280
hexanoic acid	16	21	50	50	130	150	210	270

When $K_a > 10$ in **Equation 4.3**, a_{HA}/C_0 are also calculated at those pH values. In comparison with pH 6.0, under 0.05% of variable are found for pH 6.7, 6.3, 5.8 and 5.5, which also matches the practical data.

Using K_b and a_{OH^-} to replace the K_a and a_{H^+} in **Equation 4.3**, the equilibrium concentration of a basic compound can be calculated. The basic compounds, indole and skatole, have large pK_b values, which determine that pH range studied cannot affect their SPME results just like p-cresol and m-cresol.

4.3.5 Volume of sample and headspace

Figure 4.5 shows the combination of the effect of increasing the sample volume from 20 mL to 40 mL and decreasing the headspace volume from 48 mL to 28 mL. Although this method improved the recovery of the SPME efficiency by from 20% to 30% for the analytes, respectively, it introduced a problem of foaming, which could contaminate the fibre during the SPME procedure.

Another way to increase the sensitivity of headspace SPME is to decrease the headspace. Glass beads, 20 mL of volume, were used to increase the height of the surface of the rumen fluid in the vials. The headspace volume was thus decreased from 48 mL to 28 mL without changing the surface area of the rumen fluid.

Theoretically, the effect of the combination of the effect of increasing the sample volume and decreasing the headspace volume is greater than that of decreasing the headspace volume only. However, the practical results of acetic acid and propanoic acid did not show very large difference while those of the other compounds matched the theoretical calculation. One possible reason is that a large amount of acetic acid and propanoic acid saturated the headspace, from both 28 mL and 48 mL of rumen fluid. Consequently, the effect of 40 mL rumen fluid and 28 mL headspace on SPME was very close to that of 20 mL rumen fluid and 28 mL headspace.

For p-cresol, m-cresol, indole and skatole at low concentrations, the addition of glass beads into the rumen fluid might decrease the transfer rate of those compounds in the rumen fluid. As a result, less of them were released into the headspace during the 10 min extraction time and then about 10% sensitivities of the four compounds were lost comparing the condition of 20 mL rumen fluid, 20 mL glass beads and 28 mL headspace with that of 20 mL rumen fluid and 48 mL headspace.

4.4 Conclusions

The polyacrylate, Carbowax/DVB and Carboxen/PDMS fibres were suitable for the analysis of polar compounds. The Carbowax/DVB and Carboxen/PDMS fibres were better than the polyacrylate fibre but not substantially. As four polyacrylate fibres were available and the order of new SPME fibre would take at least six weeks, polyacrylate fibres were chosen in the following analyses due to their availability.

The PDMS, DVB/PDMS and DVB/Carboxen/PDMS fibres can extract the neutral compounds effectively. DVB/PDMS and DVB/Carboxen/PDMS fibres show great advantages over the PDMS fibre.

As all the analytes were still approaching the equilibrium plateau even after 20 min extraction, 5 min extraction would be used in the future study, which could provide enough analytes for GC-MS analysis.

The influence of pH is crucial for the SPME of volatile fatty acids while it hardly affects the SPME of p-cresol, m-cresol, indole and skatole. Therefore it is very

important to keep the pH constant for SPME when the pK_a or pK_b values of analytes are small.

Although the volume of sample and headspace can influence the SPME, both decreasing the headspace using glass beads and increasing the sample volume brought additional variables. Consequently, 20 mL rumen fluid with 48 mL headspace was chosen in the next steps.

Chapter 5
Quantitative analysis

5.1 Introduction

During the development of SPME, the technique was initially qualitative. In the theory of SPME, the amount of analyte extracted by the fibre is proportional to the concentration of the compound in the sample. The research has been widely carried out for quantitative SPME analysis. For example, Boyd-Boland and Pawliszyn (1995) studied pesticides in aqueous sample using direct immersion SPME; Zhang and Pawliszyn (1995) reported quantitative extraction was obtained for the BTEX compounds from the headspace of a spiked sand matrix and a spiked clay matrix using an internally cooled SPME device.

Analytical methods need to be:

a. reproducible,
b. accurate or representative,
c. possessing defined scope of application and limitations.

Reproducibility can be determined by making repeated measurements of a set of identical samples. The accuracy of the method can be assessed by comparing the quantity of analyte that is extracted with how much was there initially, commonly referred to as analyte recovery, or comparing the method's quantitative results with alternative methods analysing the same samples with established recoveries.

Unless SPME completely extracts the analyte from the headspace and the sample matrix, the extracted analyte quantity will represent a proportion of the sample analyte concentration. Rumen fluid contains water, large molecules (e.g. protein), a portion of solid such as plant particles and even live rumen microbes, which is a complex matrix. Headspace SPME will be incomplete and the proportion of the analytes extracted is likely to vary with subtle method variations (pH, fibre age, extraction time, stirring variability, temperature) and matrix differences.

Quantitation of analytes can be achieved by performing analysis of the sample prior to and after addition of a known quantity of the target analyte, a technique referred to as "standard addition". Alternatively, quantitation of analytes can be performed using addition of a known quantity of a compound that mimics the behaviour of the target analyte, e.g. use of stable isotopically enriched analyte referred to as "internal standardisation" and "isotope dilution" respectively. These techniques require the added analyte to behave and partition identically to the endogenous analyte.

MS detection is an optimal quantitation technique as it allows isotopically labelled (^{2}H or ^{13}C) analogues to be spiked into the sample, mimicking the behaviour of target compounds (Vas and Vékey, 2004). Isotopically enriched analytes are not easy to resolve on a GC column therefore cannot be use in association with GC-FID.

Therefore, it is suitable to use standard additions to quantify the target compounds. In the following experiments the headspace SPME method was applied to rumen contents collected from sheep receiving differing diets. The reproducibility of the technique was determined.

Standard additions of the major rumen fluid analytes were performed to characterise behaviour of the added analytes to rumen samples collected from sheep receiving similar differing diets. Standard addition establishes a set of response factors for the GC-FID which can be used to compare the day to day extraction efficiencies for the similar and differing rumen samples. From the standard additions one can calculate the concentrations of the analytes present and contrast these results with traditional steam distillation extraction analysis of the same samples.

5.2 Results

5.2.1 Reproducibility of the HS-SPME technique

The SPME behaviour of two polyacrylate fibres, which would be used in the quantitative analysis of the volatile fatty acids, p-cresol, m-cresol, indole and skatole, are presented in **Table 5.1**. The mean ratios of the peak areas extracted by fibre 1 relative to fibre 2 are very close to one, which suggests that the variability between the two fibres or between replicate exposure to the same sample or between replicate experiments is very small. The relative standard deviations of the volatile fatty acids

range from 16.5% to 27.4% and those of p-cresol, m-cresol, indole and skatole are around 6.5% and 11%.

Table 5.1 Comparison of two polyacrylate fibres

	Mean (%) (n = 16)	RSD %
acetic acid	109	27.4
propanoic acid	108	22.3
iso-butyric acid	103	23.4
n-butyric acid	106	19.0
iso-valeric acid	102	18.2
n-valeric acid	105	18.7
hexanoic acid	104	16.5
p-cresol	101	6.4
m-cresol	101	11.2
indole	101	6.7
skatole	102	11.1

The retention time of heptanol is just before acetic acid and was used as an internal standard for volatile fatty acids in the quantitative experiments. **Figure 5.1** gives the response factor of GC-FID for heptanol. During the period of eight trial days, the results fluctuated between 7300 and 8600 (peak area/μg). The relative standard deviation was 5.67% during the whole period.

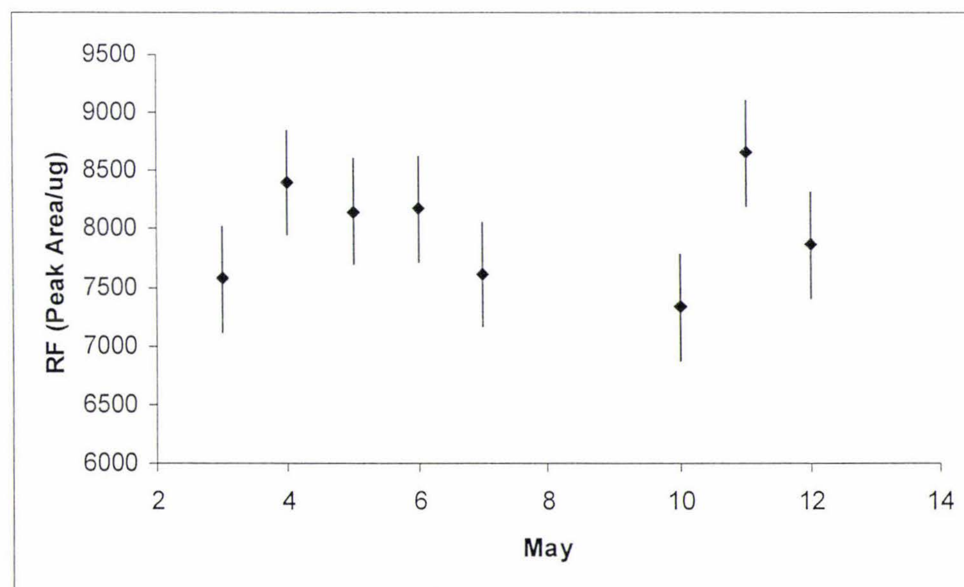


Figure 5.1 Response factor of heptanol (± daily standard deviation)

The internal standard for p-cresol, m-cresol, indole and skatole was 2-ethyl-phenol and 2-isopropyl-phenol. The response factors of GC-FID for those two compounds are shown in **Figure 5.2**.

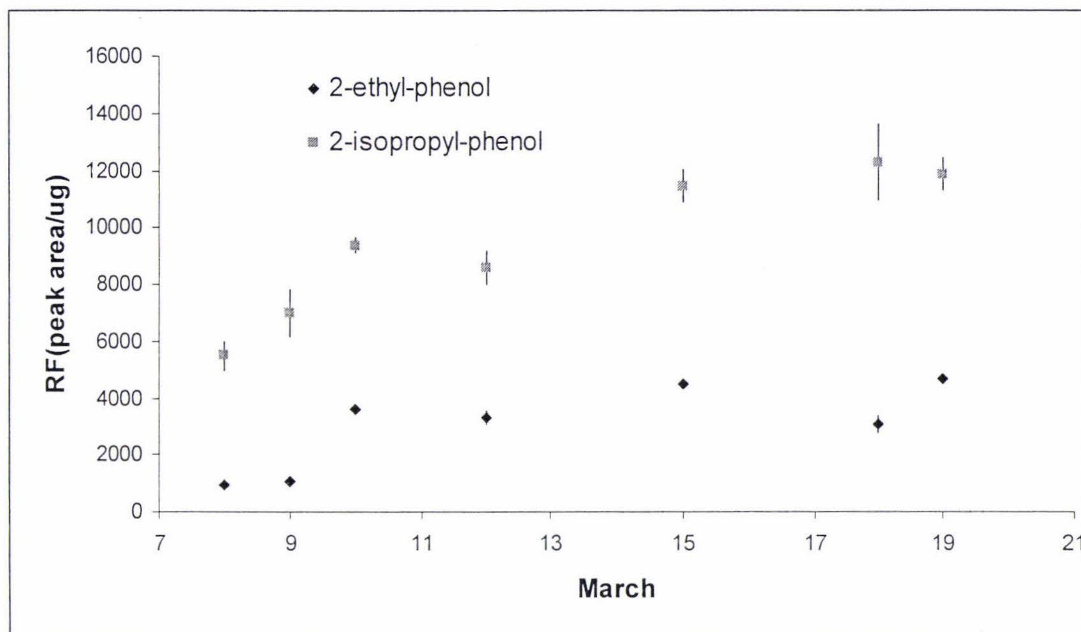


Figure 5.2 Response factors of 2-ethyl-phenol and 2-isopropyl-phenol (± daily standard deviation, n = 4 on 8, 12 and 19 March, n = 8 on 9, 10 and 18 March)

The response factor of GC-FID for 2-ethyl-phenol was close to 1000 on 8 and 9 March, then jumped to over 3300 on 10 and 12 March, and finally increased to above 4500 after 15 March with a drop to about 3000 on 18 March. On the other hand, the response factor rose from 5500 on 8 March to 9400 on 10 March, then fell slightly to 8600 on 12 March, and at last jumped to over 11000 after 15 March. The daily standard deviation for 2-ethyl-phenol and 2-isopropyl-phenol ranged from 3% to 12%.

5.2.2 Pre-investigation of quantitation

The rumen fluid was diluted to 25% and 50% and then headspace SPME was carried out, respectively. The amounts extracted from the headspace of 25% and 50% rumen fluid were compared. Generally, the extraction efficiency was nearly half for the headspace of the 25% rumen fluid compared to the 50% rumen fluid. The ratios (25% *vs*. 50% rumen fluid) dropped from 56% to 49% as the number of carbon in the acid

molecules increases. Those of p-cresol, m-cresol, indole and skatole were approximately 46%, 51%, 48% and 51%, respectively.

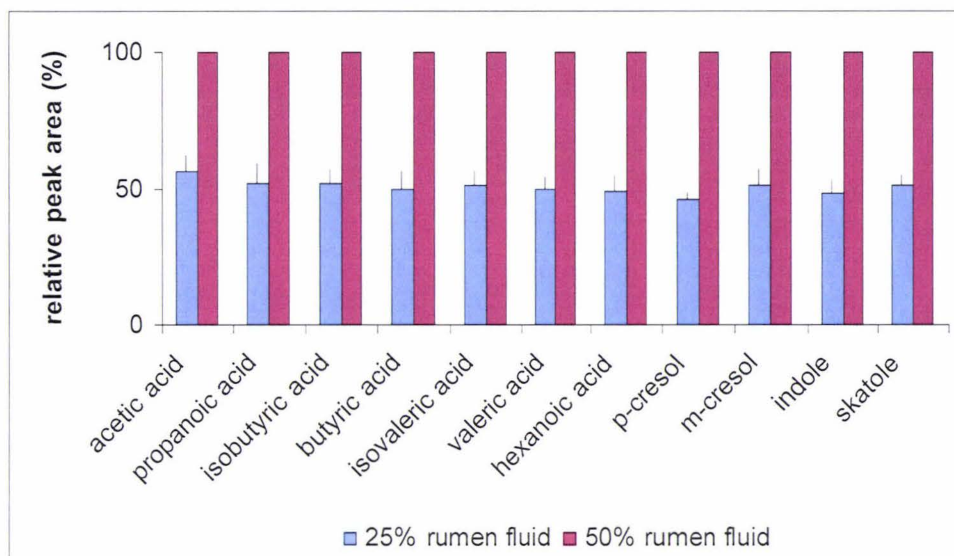


Figure 5.3 Pre-investigation of quantitation (the mean value + standard deviation, n = 6; the peak areas are relative to those from 50% rumen fluid)

5.2.3 Quantitation of p-cresol, m-cresol, indole and skatole

5.2.3.1 Standard addition

The quantitative calibration curves were generated using two rumen fluid matrices, sampled from the sheep fed with *Lotus* pedunculatus and white clover, respectively. The linearity ranged from 5 to 20 μg/mL for p-cresol, from 1 to 4 μg/mL for m-cresol, indole and skatole, which are the possible concentration ranges for the four analytes in the rumen fluid. The standard addition was performed twice on two days for the *Lotus pedunculatus* diet and once for white clover diet using GC-FID.

The response factors for the four target compounds, p-cresol, m-cresol, indole and skatole, were compared with those for the internal standard, 2-ethyl-phenol and 2-isopropyl-phenol in **Figure 5.4**. As the sheep ate differing diets on 9, 10 and 18 Feb, when the standard addition experiments were carried out, the response factors for the four analytes showed a similar trend while those for the internal standard did not match that. This suggested that the two compounds, 2-ethyl-phenol and 2-isopropyl-phenol, were not suitable to be the internal standard for the four target compounds. Therefore, the response factor values, listed in **Table 5.2**, were calculated without using the internal standard.

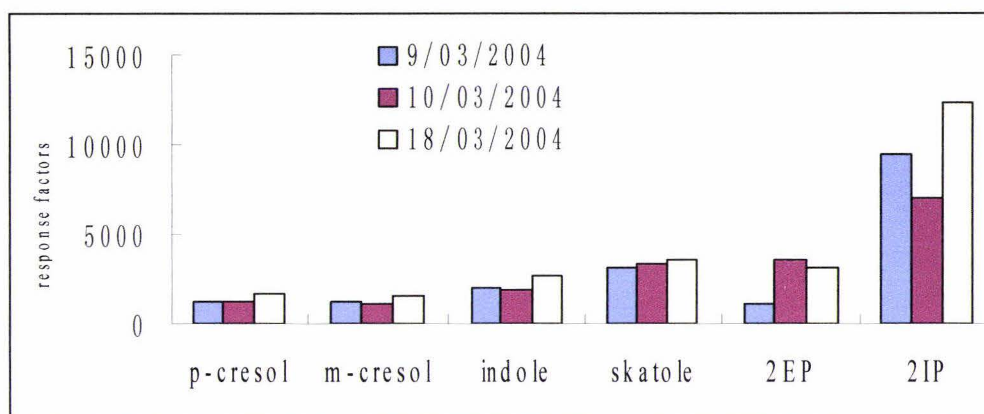


Figure 5.4 Comparison of the response factors between the analytes (p-cresol, m-cresol, indole and skatole) and the internal standard (2EP and 2IP)

2EP : 2-ethyl-phenol, 2IP : 2-isopropyl-phenol

Table 5.2 gives the response factors of GC-FID for the four analytes. The linear correlation coefficients, R^2, are over the order of 0.98, except that 0.95 of R^2 were obtained for the indole and skatole in the rumen fluid sampled from the sheep fed with white clover.

Table 5.2 The response factors of GC-FID (peak area/µg) for p-cresol, m-cresol, indole and skatole (rumen fluid were sampled from sheep fed with two diets, *Lotus pedunculatus* and white clove)

Diet	*Lotus pedunculatus*				White clover	
	9-Mar		10-Mar		18-Mar	
	RF	R^2	RF	R^2	RF	R^2
p-cresol	1186	0.9983	1176	0.9876	1654	0.9524
m-cresol	1210	0.9924	1068	0.9879	1576	0.9584
indole	2030	0.9979	1925	0.9839	2631	0.9474
skatole	3066	0.9966	3333	0.9792	3520	0.9267

5.2.3.2 Comparison between SPME and steam distillation extraction

The rumen fluid was sampled during the animal trial from 23-Feb to 20-Mar, 2004. The rumen fluid was divided into two parts: one was frozen and preserved in the freezer and the other was used to carry out headspace SPME immediately. The response factors of GC-FID for p-cresol, m-cresol, indole and skatole were used to calculate the concentrations of the four compounds in the rumen fluid. Meanwhile,

the steam distillation simultaneous extraction (SDE) was performed and results were compared with those obtained from headspace SPME technique (**Table 5.3**).

Table 5.3 The concentrations of p-cresol, m-cresol, indole and skatole
Trial conditions: rumen fluid were sampled from four sheep; the diets included white clover (from 23-Feb to 8-Mar and from 15-Mar to 20-Mar) and *Lotus pedunculatus* (from 9-Mar to 14-Mar)

# sheep	4859		4862		4863		4885	
	SPME	SDE	SPME	SDE	SPME	SDE	SPME	SDE
diet: white clover (8-Mar)								
p-cresol	26.2	33.9	21.1	24.0	35.7	24.2	25.0	26.9
m-cresol	1.1	3.0	0.9	0.5	2.8	0.4	1.2	0.8
indole	6.5	6.5	4.9	4.3	7.9	4.2	6.5	5.6
skatole	10.1	9.9	11.5	9.6	9.5	12.9	10.5	13.7
diet: white clover (19-Mar)								
p-cresol	22.6	20.6	29.8	22.7	45.5	18.4	32.0	19.2
m-cresol	1.1	0.8	1.4	1.0	1.5	1.0	0.9	0.8
indole	6.2	7.0	8.2	5.8	11.6	5.9	9.4	6.1
skatole	9.9	12.9	10.6	10.0	21.4	9.6	16.0	9.2
diet: *Lotus pedunculatus* (11-Mar)								
p-cresol	14.2	11.0	7.4	6.9	18.7	22.2	21.6	23.6
m-cresol	2.1	1.8	2.3	2.1	1.8	2.2	4.1	5.0
indole	0.3	0.4	0.2	0.2	0.7	1.1	1.0	1.4
skatole	2.1	1.7	1.5	1.5	2.6	3.4	3.3	4.0
diet: *Lotus pedunculatus* (12-Mar)								
p-cresol	17.0	10.2	36.4	19.8	34.6	17.4	26.8	15.6
m-cresol	1.9	1.4	5.6	3.6	4.6	2.6	6.3	3.4
indole	0.5	0.9	1.0	1.4	1.2	1.2	1.5	1.1
skatole	2.6	1.6	3.4	2.2	6.7	2.5	4.6	2.2

When white clover was fed to sheep, the concentration of p-cresol was above 20 μg/mL and that of m-cresol mainly fluctuated under 2 μg/mL. The concentration levels of indole and skatole were above 4 and 9 μg/mL, respectively.

In general, when *Lotus pedunculatus* was fed to the sheep, the concentration of p-cresol was less than 20 μg/mL and m-cresol reached 6 μg/mL. The indole and skatole levels were clearly low, less than 5 μg/mL.

5.2.4 Quantitation of VFAs

Standard addition also was performed for the quantitative analysis of volatile fatty acids. **Figure 5.5** gives the calibration curves for the seven target acids in rumen fluid. Phosphate buffer solution was used to keep the pH of rumen fluid at about 6.2 (the error < 0.1).

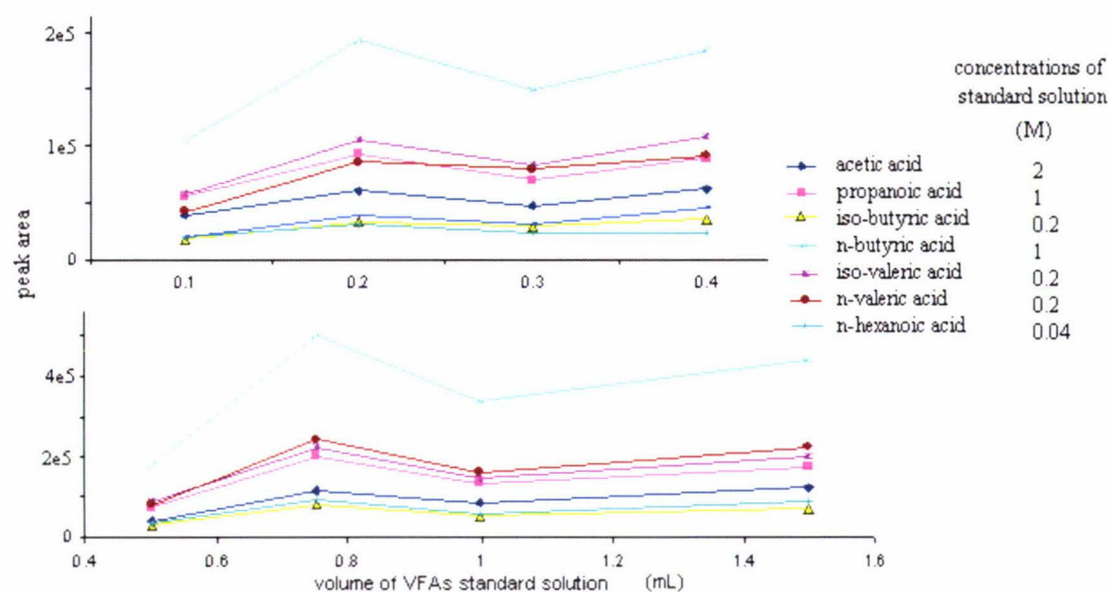


Figure 5.5 Calibration curves for VFAs

The VFAs standard solution, 0.1, 0.2, 0.3 and 0.4 mL, were spiked into the rumen fluid as well as the volume of 0.5, 0.75, 1.0 and 1.5 mL. The curves did not show the trend that the peak areas increased gradually as the volume of standard solution of VFAs spiked into the rumen fluid increased. In general, the peak areas increased from the first spike to the second one and then all the curves fluctuated at their specific levels, respectively.

The peak areas of the VFAs extracted from the rumen fluid without spiking standard solution were close to those from the rumen fluid with spiking 0.1 – 0.4 mL of standard solution.

5.3 Discussion

5.3.1 Reproducibility

The difference between the two polyacrylate fibres was small, less than 2%, for p-cresol, m-cresol, indole and skatole while it seemed larger, ranging from 2% to 9%, for the volatile fatty acids. In addition, the relative standard deviations for the acids were around 20% except that of acetic acid (27%) while those for p-cresol, m-cresol, indole and skatole were about 6% and 11% (**Table 5.1**). It is suggested that the variability of the two different fibres studied is small while the large amounts of VFAs and their high volatility might be disadvantages for the headspace SPME analysis and lead to the large variability for the SPME of VFAs. pH can greatly affect the SPME of VFAs, which is another disadvantage for the VFAs analysis. When the pH value increases from 6.0 to 6.1 or decreases from 6.0 to 5.9, the concentrations of VFAs change approximately 20%, which can influence the SPME effectively. In practice for the *in vitro* trials, it is impossible to keep the rumen fluid at exactly the same values. If pH values at some time points change dramatically, the results might be unreliable.

On the other hand, p-cresol, m-cresol, indole and skatole have no drawbacks which interfered with the VFA analysis. Although their concentrations are low but they are still far above the limitation of detection. pH has little effect on them and they are not so volatile that this might lead to large amounts of loss. Therefore, their reproducibility was good.

The change of the response factors of GC-FID for 2-ethyl-phenol and 2-isopropyl-phenol reveals that the sample matrix can influence the headspace SPME. The 9 March was the first day the sheep were fed *Lotus pedunculatus*. The response factor for 2-isopropyl-phenol was 7000, which is between 5500 (8 March) and 9400 (10 March). However, the response factors for 2-ethyl-phenol and 2-isopropyl-phenol did not fall back to the values in the first period when sheep were fed with white clover and even increased to a higher level when the diet was switched from *Lotus pedunculatus* back to white clover on 15 March.

5.3.2 Quantitation of p-cresol, m-cresol, indole and skatole

The pre-investigation of quantitative analysis suggested that the analyses of the 11 target compounds were quantitative using headspace SPME technique. The amounts of analytes extracted from the headspace of rumen fluid were proportional to the concentrations of the analytes. However, the matrix might change as the concentration of rumen fluid decreased from 50% to 25%. Consequently, more than 50% of analytes were extracted from the headspace of 25% rumen fluid in comparison with 50% rumen fluid. It is understood that the rumen fluid can trap more of these target compounds than water and therefore more analytes could be released to the headspace from the 25% rumen fluid.

The standard addition technique was performed for p-cresol, m-cresol, indole and skatole. ^{3}D labelled skatole, which was used as the internal standard of indole and skatole in the steam distillation extraction, was not used as the internal standard of indole and skatole in the headspace SPME, because the GC-MS was not available at that moment and the detector was FID.

Since the amount extracted is influenced strongly by the matrix, quantitative analysis of real samples on the basis of calibration in a model rumen fluid would not be feasible. As a result, the standard addition was carried out twice for the rumen fluid sampled from the sheep fed with *Lotus pedunculatus*. The standard addition was also performed twice for the rumen fluid sampled from the sheep fed with white clover. GC-MS was used for the first standard addition experiment for white clover on 5 March after which equipment failure did not allow further study until August. Because of the limitation of the rumen fluid sample, the standard addition using GC-FID was performed once for the rumen fluid sampled from the sheep fed with white clover.

The comparison between headspace SPME and steam distillation extraction indicates that the headspace SPME is semi-quantitative for p-cresol, m-cresol, indole and skatole as the steam distillation simultaneous extraction technique has been proven reliable at AgResearch, Grassland.

The rumen fluid for both steam distillation extraction and headspace SPME was sampled from the same four sheep. But the samples for steam distillation extraction were frozen immediately while the rumen fluid for headspace was incubated for 15 min (rumen fluid from sheep 4859 and 4862) and 2 hours (rumen fluid from sheep 4863 and 4885) after it was sub-sampled into the 68 mL vials, which might introduce some difference. Moreover, the response factors of GC-FID for the four compounds were obtained on the different days from those sampling days in **Table 5.3**. Therefore, the matrices of rumen fluid could change, which also could decrease the accuracy.

Although the headspace SPME technique did not provide accurate results in comparison with the steam distillation simultaneous extraction method, the concentrations of the analytes obtained via the two methods were in the same order of magnitude. In general, the results from the steam distillation simultaneous extraction changed in a tendency close to those from the headspace SPME. Thus it is helpful to conclude the analyte concentrations change when the diets differed. The concentrations of p-cresol, indole and skatole in the rumen fluid obtained when the diet was white clover, were higher than those obtained when the sheep received *Lotus pedunculatus*. However, the m-cresol level was opposite relative to those three compounds.

5.3.3 Quantitation of VFAs

The standard addition of VFAs was not successful. As shown in **Figure 5.4**, when the standard solution spiked into the rumen fluid increased, the peak areas of the VFAs did not increase but fluctuated. The larger volume (> 1.5 mL) of standard solution was spiked, but the pH of rumen fluid changed too much (> 0.5). The small volume was also tried while the amounts of VFAs extracted from the parallel rumen fluid were very close to those from the rumen fluid spiked the VFA standard solution.

There are several disadvantages for the standard addition of VFAs using the headspace SPME method. Firstly, it is very difficulty to keep the pH of rumen fluid constant. The H^+ in the VFA standard solution spiked into the rumen fluid ranged from 50 to 400 mM in the experiment while the buffer strength of phosphate buffer solution was 500 mM theoretically and in practice the phosphate buffer solution did

not work very well sometimes. Therefore the experiments had the risk of fluctuation of pH. The variables of the extraction for VFAs using PA fibres were not small, ranging from 16% to 27% (**Table 5.1**). Finally, the amounts of VFAs extracted from the headspace of rumen fluid were relative large in comparison with the phenols and indoles and the thermal desorption might not be complete. The memories of VFAs in PA fibres were considerable in the test. This also could lead to failure of the quantitation.

5.4 Conclusions

The reproducibility of the 11 analytes was reasonable, less than 22% except acetic acid 27% due to its larger concentration in the rumen fluid and high volatility. The variables of VFAs were larger than those of p-cresol, m-cresol, indole and skatole, which might influence the quantitation of VFAs.

The response factors of GC-FID for heptanol, 2-ethyl-phenol and 2-isopropyl-phenol changed with the rumen fluid matrices. It is suggested that the response factors should be obtained from the same sample matrix.

The pre-investigation of quantitation indicated that the headspace SPME has potential as a quantitative technique for the headspace SPME analysis above the rumen fluid. The response factors of GC-FID for p-cresol, m-cresol, indole and skatole were calculated from the calibration curves. The standard addition of VFAs was not successful. Then the response factors of GC-FID for p-cresol, m-cresol, indole and skatole were used to quantify the rumen fluid samples. In comparison with steam distillation extraction, the headspace SPME method is semi-quantitative. The accuracy can be improved if the difference of sample matrix is eliminated.

Chapter 6

The *in vitro* study of protein derived metabolites

6.1 Introduction

The analytes in this project play important roles in ruminant agriculture. It is well known that the paramount importance of volatile fatty acids, mostly acetic, propanoic and butyric acids, to herbivores is that they are absorbed and serve as the animal's major fuel for energy production (Hobson and Stewart, 1997). Ruminants, such as cattle, sheep and deer, fed pasture develop a characteristic meat or dairy flavour called 'pastoral' flavour (Young *et al.*, 2003). It is important to recognise that the major component of pastoral flavour is actually aroma. Most of the literature on pastoral flavour has addressed volatiles (Young and Baumeister, 1999). Skatole is a nauseating faecal-smelling compound known as a component of the boar-taint flavour in pork (Hansson *et al.*, 1980) and as the cause of the faecal taint in milk from cows fed *Lepidium* spp. (Park, 1969). Ha and Lindsay (1991) discussed the contribution of methylphenols and other alkylphenols to pastoral flavour in cooked meat aroma. Lane and Fraser (1999) reported studies of two classes of flavour compounds found in beef fat, phenols and indoles. In addition to pastoral flavour, sheepmeat has a characteristic species flavour due to the presence of branched-chain fatty acids in storage fats (Duncan and Garton, 1978).

Proteins are the important substrates for ruminal fermentation. They are digested by microbial proteases and peptidases to release peptides and amino acids. Then a significant amount of amino acids are deaminated and metabolised to acids. Some of these acids might be further converted to other compounds, such as phenols and indoles (Austgen *et al.*, 1996).

Spirulina is an abundant source of protein therefore it is an ideal substrate to observe protein derived metabolites. In this section, ^{13}C labelled spirulina was digested in the rumen fluid so that the abundance of the ^{13}C labelled end products via the ruminal fermentation could be studied and it was summarised that protein served as a source

of those target compounds in rumen. Before the *in vitro* fermentation, tryptophan, tyrosine and acid hydrolysed spirulina were incubated with the rumen fluid to test the feasibility of the *in vitro* design.

6.2 Results

6.2.1 Design of the *in vitro* fermentation

6.2.1.1 Incubation of tryptophan in rumen fluid

Tryptophan was one of the substrates used to test the feasibility of the *in vitro* design of eight hours incubation. **Figure 6.1** gives the production of indole and skatole in the rumen fluid in comparison with the addition of tryptophan. The mean of the response factors of GC-FID for p-cresol, m-cresol, indole and skatole in **5.2.3** were used to calculated the concentrations of those compounds in this section and the following experiments.

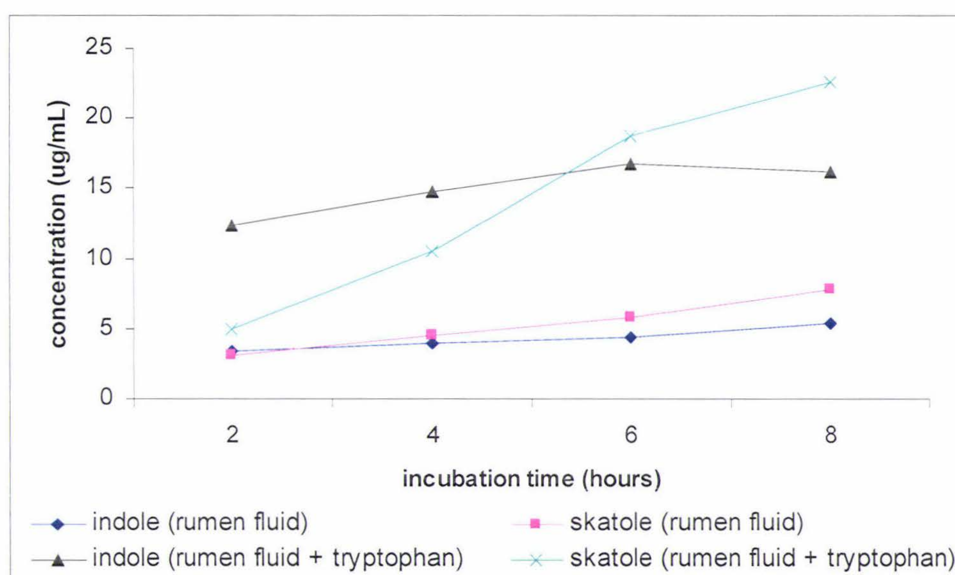


Figure 6.1 The production of indole and skatole in the rumen fluid from tryptophan (Figure 6.1 is one of three replications, which is representative)

As the incubation time increased from 2 to 8 hours, indole and skatole increased steadily from 3.4 to 5.4 μg/mL and from 3.2 to 7.8 μg/mL, respectively. On the other hand, the addition of tryptophan to the rumen fluid caused a dramatic change. After 2 hours incubation, the concentration of indole was above 12 μg/mL, much larger than that in the rumen fluid. However, the production of skatole was not as fast as indole, which was about 5 μg/mL. After 6 hours incubation, the level of indole reached a

peak at approximately 16.5 μg/mL. Nevertheless, the concentration of skatole increased greatly to 22.6 μg/mL. The concentration of skatole exceeded that of indole after nearly 5 hours incubation.

For other analytes, the addition of tryptophan did not lead to additional production.

6.2.1.2 Incubation of tyrosine in rumen fluid

Tyrosine was the second reagent. As seen in **Figure 6.2**, the concentration of p-cresol in the rumen fluid rose from nearly 18 μg/mL to the peak value, 58 μg/mL, at 6 hours, and then dropped slightly to 52 μg/mL at 8 hours. After the addition of tyrosine, the production of p-cresol was very close to that in the rumen fluid before 4 hours, showing an increase from about 18 to above 40 μg/mL. However, after 4 hours, the two curves started to split and the concentration jumped significantly to about 140 μg/mL at 8 hours.

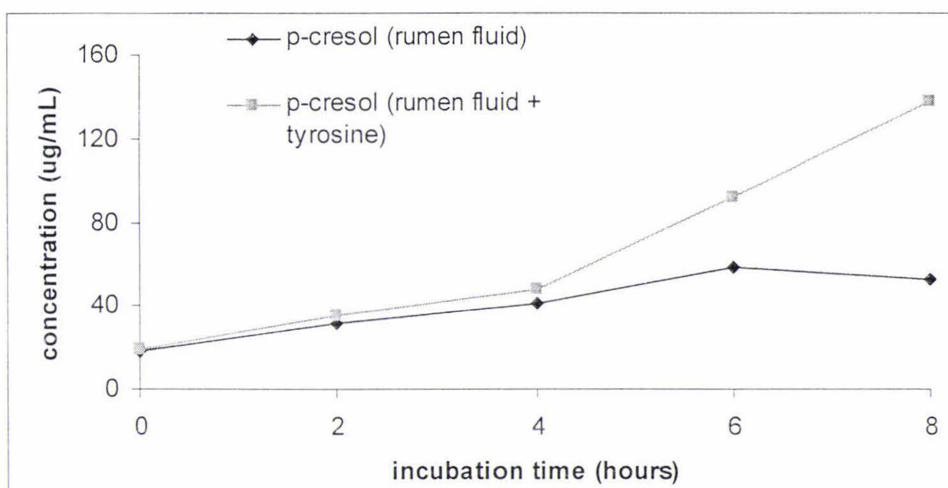


Figure 6.2 The production of p-cresol in the rumen fluid from tyrosine (Figure 6.1 is one of three replications, which is representative)

It is noted that, the addition of tyrosine did not cause additional production for other analytes.

6.2.1.3 Incubation of acid hydrolysed spirulina in rumen fluid

Finally spirulina, acid hydrolysed, was used in the *in vitro* experimental design. **Figure 6.3** shows that more p-cresol was produced as a consequence of the addition of acid hydrolysed spirulina. The concentration of p-cresol in the rumen fluid

increased from nearly 13 μg/mL to 36 μg/mL while the slope of the curve for the p-cresol production in the rumen fluid with addition of acid hydrolysed spirulina was greater, from 15 μg/mL to 55 μg/mL.

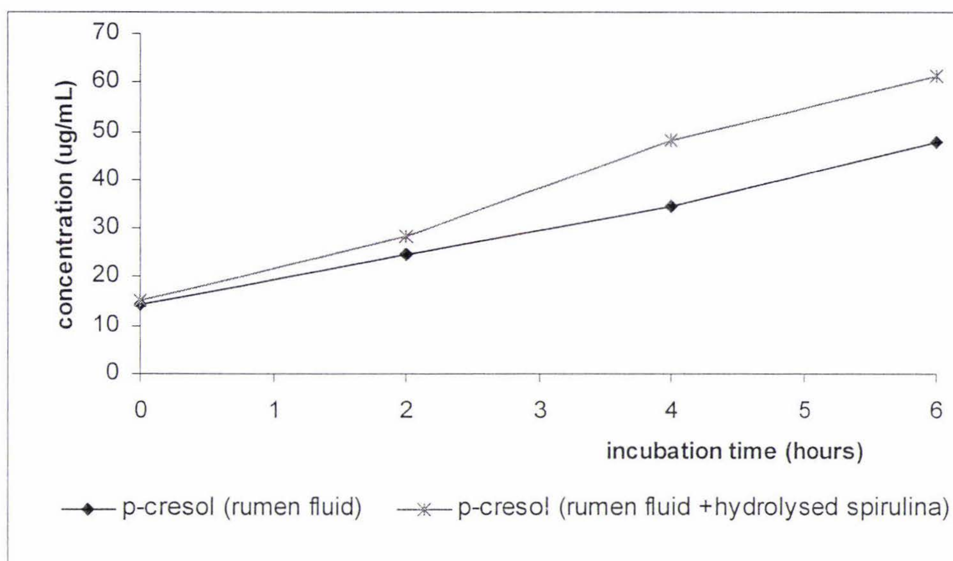


Figure 6.3 The production of p-cresol in the rumen fluid from acid hydrolysed spirulina (Figure 6.1 is one of three replications, which is representative)

No other compound changes were found during the incubation process. The concentrations of m-cresol, indole and skatole were relatively stable, which were around 2, 4 and 3 μg/mL, respectively.

6.2.2 The *in vitro* fermentation

6.2.2.1 Incubation of acid hydrolysed ^{13}C labelled spirulina

The acid hydrolysed ^{13}C labelled spirulina was incubated in the rumen fluid for 24 hours at 39 ^{0}C. The ^{13}C labelled compounds were produced during the incubation. **Figure 6.4** gives the trend of the abundance of those ^{13}C labelled compounds during the 24 hours of incubation. The abundance relative to ^{12}C labelled compounds was calculated by M : M+1 ion ratio. As the acid hydrolysis of protein destroys tryptophan (Fountoulakis and Lahm, 1998), no ^{13}C labelled indole and skatole were found in the experiment. Generally, the abundance of ^{13} labelled compounds was under 1% during the parallel incubation of the acid hydrolysed spirulina.

Figure 6.4 The profile of the abundance of ^{13}C labelled polar compounds during the incubation of acid hydrolysed spirulina (SPME carrying out by using PA fibres)

The abundance of ^{13}C labelled toluene increased gradually to 25% after 24 hours incubation. That of acetic acid reached the peak value 3.2% at 8 hours and then dropped slightly to 2.9%. The performance of propanoic acid was similar to acetic acid, reaching the peak value 2.6% at 4 hours and then decreasing to 1.8% at 24 hours. The abundance of ^{13}C labelled iso-butyric acid jumped to the peak value 5.6% at 4 hours and then dropped slowly to 3.0% at 24 hours. That of n-butyric acid reached the plateau above 6% after 4 hours with a fluctuant at 8 hours. The abundance of ^{13}C labelled iso-valeric acid rose sharply to 66% at 4 hours and then decreased to 29% at the end of the incubation. That of n-valeric acid increased quickly to 18% at 2 hours, fluctuating to 15% at 4 hours, and decreased to 14% at 24 hours. The abundance of n-

hexanoic acid was under 0.4% during the whole incubation. The abundance of p-cresol rose sharply to the peak value 40% at 2 hours and then dropped gradually to 14% at 24 hours while that of m-cresol was under 1% during the whole process.

The neutral compounds from the headspace of rumen fluid were also studied using PDMS fibres. Dimethyldisulfide and dimethyltrisulfide were found labelled. The profiles of the two sulfides were similar presented in **Figure 6.5**. At the beginning of the incubation, no sulfides were found in the parallel incubation of unlabelled acid hydrolysed spirulina in rumen fluid, so 100% of the two sulfides were labelled. The abundance of ^{13}C labelled dimethyldisulfide

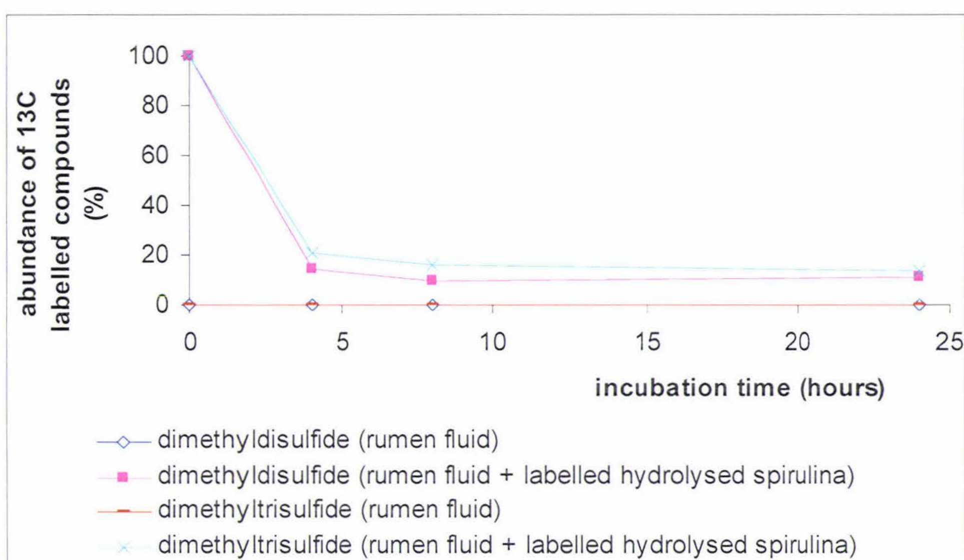


Figure 6.5 The profile of the abundance of ^{13}C labelled sulfides during the incubation of acid hydrolysed spirulina (SPME carrying out by using PDMS fibres)

6.2.2.2 Incubation of ^{13}C labelled spirulina

The whole ^{13}C labelled spirulina was also incubated in the rumen fluid for 24 hours. In this case, ^{13}C labelled indole and skatole were found during the experiment in addition to the other compounds, which were found labelled during the incubation of the ^{13}C labelled acid hydrolysed spirulina.

The abundance of ^{13}C labelled toluene reached a plateau at 2 hours with a small fluctuation. That of acetic acid was 2.2% before 2 hours and the constant at 2.4% after4 hours. The performance of propanoic acid was similar to toluene, with a

plateau at 1.6%. The abundance of [13] labelled iso-butyric acid jumped to the peak value 2.8% at 4 hours and then dropped slowly to 1.8% at 24 hours. That of n-butyric acid reached the plateau above 4% after 4 hours with a fluctuant at 8 hours. The abundance of ^{13}C labelled iso-valeric acid rose sharply to 30% at 4 hours and then decreased to 14% at the end of the incubation. That of n-valeric acid increased quickly to 10% at 4 hours and decreased to 7.4% at 24 hours. The abundance of n-hexanoic acid was under 0.84% during the whole incubation. The abundance of p-cresol rose sharply to the peak value 38% at 4 hours and then dropped gradually to 17% at 24 hours while that of m-cresol was under 0.6% during the whole process except 1.5% at 2 hours. The abundance of indole reached the peak value 29% at 2 hours while the peak of skatole, 18%, appeared at 4 hours.

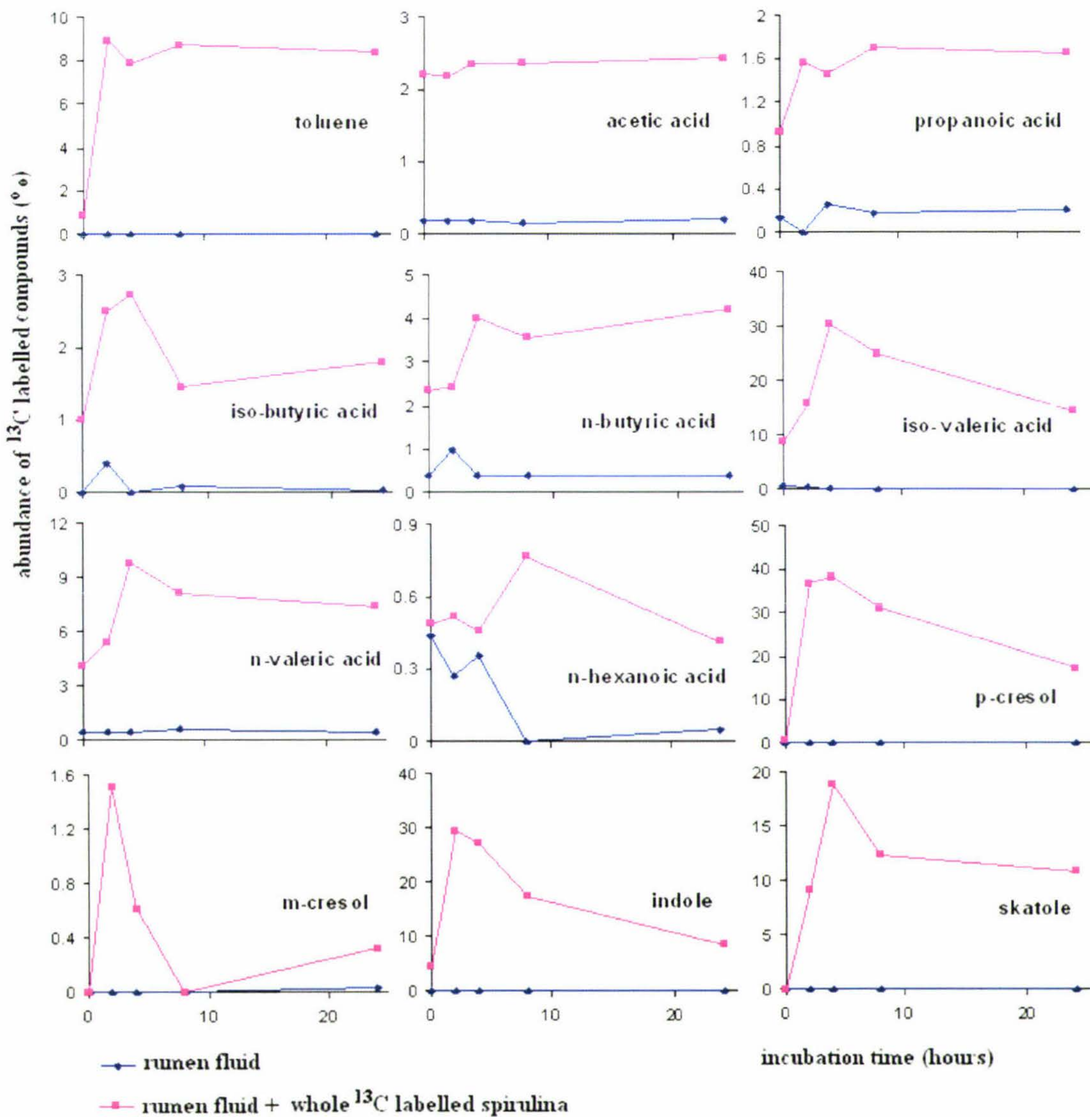


Figure 6.6 The profile of the abundance of ^{13}C labelled compounds during the incubation of whole spirulina (SPME carrying out by using PA fibres)

6.3 Discussion

6.3.1 Incubation of tryptophan, tyrosine and acid hydrolysed spirulina

In **Figure 6.1**, the amounts of indole and skatole in the rumen fluid with addition of tryptophan was significantly larger than that in the parallel rumen fluid, which indicates that tryptophan leads to the formation of indole and skatole in the rumen fluid. Deslandes *et al.* reviewed the microbiological and biochemical effects of skatole on animal production in 2001. Skatole is harmful for the beef and pork industries, It causes acute bovine pulmonary edema and emphysema (ABPE) in cattle and boar taint in pigs. **Figure 6.7** gives the biochemical pathway of the fermentation of tryptophan. L-tryptophan is degraded to indole or converted to indole-3-acetic acid and then metabolised to skatole.

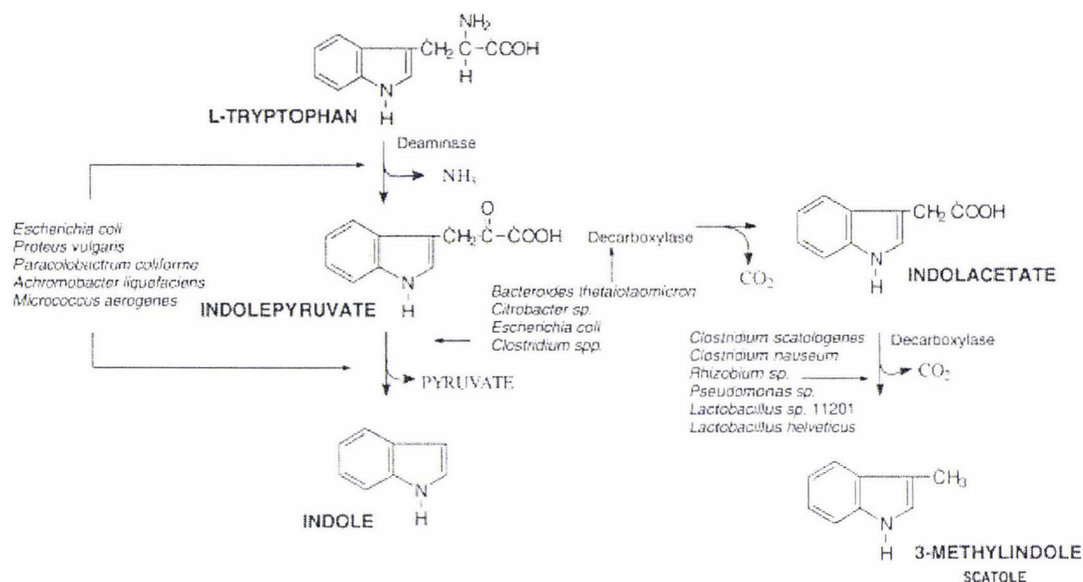


Figure 6.7 Tryptophan fermentation leading to the formation of indole and skatole (Deslandes *et al.*, 2001)

In **Figure 6.1**, the concentration of indole was larger than that of skatole before 5 hours of incubation and then the indole level kept constant while the skatole was still produced and exceeded the indole finally, which suggests that indole production was faster than the formation of skatole. The indole/skatole ratio might be determined by the diet (Deslandes *et al.*, 2001).

Tyrosine is converted to phenylpropionic acid and p-hydroxyphenyl-acetic acid (Scott *et al.*1964). Then the *Lactobacillus* strains produced *p*-cresol by the decarboxylation of *p*-hydroxyphenylacetic acid, but did not produce either *o*-cresol or *m*-cresol from

the corresponding hydroxyphenylacetic acid isomers (Yokoyama and Carlson, 1981). The formation of p-cresol could also be found in **Figure 6.2**. The concentration of p-cresol in the rumen fluid with addition of tyrosine increased dramatically after 4 hours of incubation and exceeded three times of that in the parallel rumen fluid at 8 hours. The concentration of p-cresol in the rumen fluid with addition of tyrosine was very close to that in the parallel rumen fluid from 0 to 4 hours, which points to the production of p-cresol in rumen fluid being slower than the formation of indole and skatole.

Table 6.1 gives the concentrations of the amino acids in the acid hydrolysed spirulina. The weight percentage of tyrosine was 5.4% and tryptophan was destroyed.

Table 6.1 The amino acids in the acid hydrolysed spirulina

	mg/g (DM)	% wt
Aspartate	78.5	10.2
Threonine	41.1	5.3
Serine	38.9	5.0
Glutamate	89.1	11.5
Proline	57.0	7.4
Glycine	39.7	5.1
Alanine	62.8	8.1
Valine	46.7	6.1
Isoleucine	47.5	6.2
Leucine	73.6	9.5
Norleucine	-	-
Tyrosine	41.4	5.4
Phenylalanine	40.1	5.2
Histidine	14.2	1.8
Lysine	33.8	4.4
Tryptophan	-	-
Arginine	67.2	8.7

The weight of tyrosine in the 90 mg of acid hydrolysed spirulina added into the rumen fluid was 4.86 mg. If the conversion rate from tyrosine to p-cresol is 100%, ca. 2.9 mg of p-cresol is produced. In **Figure 6.3**, the production of p-cresol from tyrosine at 6 hours is 0.4 mg, which indicates that only 14% tyrosine was converted to p-cresol. It is noted that the response factor of GC-FID for p-cresol might introduce

errors because it was calculated for p-cresol in the rumen fluid, when sheep were fed with white clover and *Lotus pedunculatus* and in this case the sheep grazed pasture.

6.3.2 Incubation of ^{13}C labelled acid hydrolysed and whole spirulina

With few exceptions, all dietary proteins can serve as substrates for microbial fermentation. They are digested by microbial proteases and peptidases and then peptides and amino acids are released. Then a large quantity of amino acids ingested by fermentative microbes are deaminated and enter some of the same pathways used for carbohydrate metabolism. The net result is that much dietary protein is metabolised to VFAs (Austgen *et al.*, 1996).

Elsden *et al.* (1956) found LC (a strain of *Peptostreptococcus elsdenii*) fermented L-serine to acetate, carbon dioxide and ammonia, and fermented L-threonine to propionate, small amounts of acetate and valerate, carbon dioxide and ammonia. Two strains of *Escherichia coli* together fermented lysine to acetic acid, butyric acid, carbon dioxide and ammonia (Dohner and Cardon, 1954). During the *in vitro* incubation of both acid hydrolysed and whole spirulina (^{13}C labelled), ^{13}C labelled acetic acid, propanoic acid, n-butyric acid and n-valeric acid were detected.

The presence of ^{13}C labelled toluene, iso-butyric acid and iso-valeric acid during the fermentation of both acid hydrolysed and whole spirulina (^{13}C labelled) suggests that protein can be a source of toluene and the two branch-chain fatty acids.

No literature reported that the hexanoic acid and m-cresol arise from protein. During the *in vitro* incubation, the abundances of the ^{13}C labelled hexanoic acid and m-cresol were quite low, which might be artefact or rearrangement of p-cresol to m-cresol.

p-Cresol, indole and skatole, which have been discussed in **6.3.1**, are from the degradation of tyrosine and tryptophan, which are amino acids composition of spirulina. Consequently, it is reasonable to find ^{13}C labelled p-cresol, indole and skatole present during the incubation.

Dimethyldisulfide and dimethyltrisulfide were found labelled. SPME was carried out in the headspace of the ^{13}C labelled acid hydrolysed spirulina before it was added into

the rumen fluid indicated no labelled dimethyldisulfide and dimethyltrisulfide were present, which indicates that the two labelled sulfides were produced in the rumen fluid original from the spirulina.

More ^{13}C labelled compounds were found when acid hydrolysed ^{13}C labelled spirulina was incubated in the rumen fluid than the whole ^{13}C labelled spirulina. This indicates that the proteolytic activity of the rumen fluid is not as effective as acid hydrolyses.

6.4 Conclusions

The 20 mL of 80% rumen fluid, artificial saliva, 4 mL, and rumen fluid, 16 mL, was suitable for the 8 hours of *in vitro* fermentation of feed. The single amino acids, such as tryptophan and tyrosine, the mixed amino acids, acid hydrolysed spirulina and the protein, spirulina, were incubated in the system and the formation of indole, skatole and p-cresol was found.

The ^{13}C labelled spirulina was fermented in the 80% rumen fluid. A series of labelled end products, including toluene, acetic acid, propanoic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, p-cresol, indole, skatole, dimethyldisulfide and dimethyltrisulfide were detected, which indicates that these compounds are the products of spirulina degradation.

Headspace SPME technique applied to an *in vitro* system demonstrated its usefulness in establishing compound metabolism and elucidating compound origin.

Chapter 7
Conclusions and future work

7.1 Conclusions

A non-invasive headspace SPME method was developed to characterise the volatile compounds from a closed *in vitro* rumen fluid incubation system. The advantages of the method include high selectivity, immediate results and solvent freedom.

Several factors which can affect the efficiency of the headspace SPME, such as pre-incubation time of the rumen fluid in the vial, SPME fibre coating, pH of the sample matrix, extraction time, volume of the sample matrix and the headspace, were studied.

The volatile compounds identified from the headspace above the rumen fluid include several classes of compounds such as VFAs, flavour compounds (e.g. p-cresol etc.) and terpene compounds.

The reproducibility of the 11 analytes was reasonable. The subtle change of experiment conditions, such as the sample matrix and the difference of SPME fibres, can influence the experiment error.

Standard addition was carried out for the analytes. The response factors of GC-FID for p-cresol, m-cresol, indole and skatole were calculated from the calibration curves. They were used to calculate the concentrations of the target compounds extracted from the samples on the other days. In comparison with steam distillation simultaneous extraction, the headspace SPME is semi-quantitative.

An *in vitro* fermentation method was developed, which can be used to incubate tryptophan, tyrosine, acid hydrolysed spirulina and spirulina. The metabolism of ^{13}C labelled compounds was observed.

Headspace SPME technique can serve as a simple and fast method for characterisation of ruminal fermentation end products of microbial digestion.

7.2 Future work

It is possible to seek the origin of m-cresol using the headspace SPME technique. One of the candidates is lignin. Lignin is a complex aromatic biopolymer formed of hydroxycinnamyl alcohols, which are connected to each other with various linkages, shown in **Figure 7.1**.

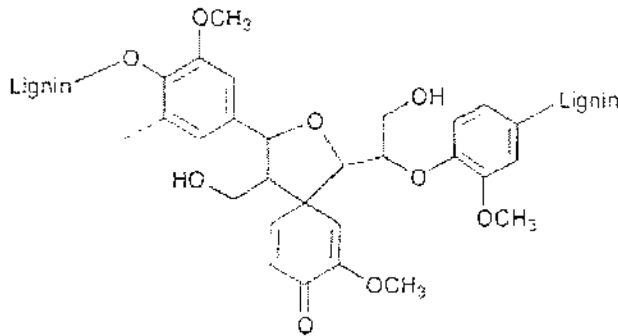


Figure 7.1 Structure of lignin

Another application of the headspace SPME method is to study effects of different diets or feed additives on the formation of end products. For example, efficiency of protein inhibitors in the rumen fermentation can be studied via testing the indole and skatole levels in the rumen contents. Use of the SPME method could give a fast answer to that question.

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Appendix 1

Table 1 Applications of SPME to the identification and characterisation of herbicides, fungicides and pesticides

Analyte	Sample matrix	SPME conditions					Detection	Ref.
		Fiber	Extraction mode	Temp. (°C)	Time (min)	Salt		
Organophosphorus pesticides	Groundwater	100 µm PDMS 85 µm PA	DI		60	NaCl	GC-NPD	Beltran *et al.*, 1998
Organophosphorus pesticides	River water	100 µm PDMS	DI		20		GC-AED	Eisert *et al.*, 1994
Organophosphorus pesticides	Tap water, sea water, wastewater	100 µm PDMS	DI	60	45		GC-NPD	Valor *et al.*, 1997
Organophosphorus pesticides	Surface water	85 µm PA	DI		45		GC-FID GC-NPD GC-MS	Magdic *et al.*, 1996
Organophosphorus pesticides	Ultrapure water	65 µm PDMS/DVB	DI		30		GC-FID	Sng *et al.*, 1997
Organophosphorus pesticides, thiocarbamate herbicides, fungicides	Surface water	85 µm PA	DI	60	180	NaCl	HPLC-UV	Jinno et al.,1996
Organochlorine pesticides	River water	100 µm PDMS	DI		2		GC-ECD	Jackson *et al.*, 1998
Organochlorine pesticides	Surface water	100 µm PDMS	DI		90		GC-FID GC-ECD GC-MS	Magdic and Pawliszyn, 1996
Organochlorine pesticides, thiocarbamate herbicides	Surface water, drinking water	85 µm PA	DI	55	45	NaCl	GC-MS	C. Aguilar *et al.*, 1998
Organochlorine pesticides, Triazine herbicides	Groundwater	100 µm PDMS	HS	87	45, 60	NaCl	GC-ECD GC-MS	Page *et al.*, 1997
Triazine herbicides	Surface water, sewage water	85 µm PA	DI		10		GC-FID GC-ECD	Eisert *et al.*, 1996
Triazine, thiocarbamate, substituted uracils herbicides	Ultrapure water	85 µm PA	DI		50		GC-FID GC-NPD GC-MS	Boyd-Boland, 1995
Triazine herbicides	Ultrapure water	100 µm PDMS	DI		10		GC-NPD	Barnabas *et al.*, 1995
Phenoxyacids herbicides	Ultrapure water	100 µm PDMS	DI		50	NaCl	GC-MS	Lee *et al.*, 1998b
Phenoxyacids herbicides	Ultrapure water	65 µm PDMS/DVB	DI		60		GC-MS	Nilsson *et al.*, 1998
Herbicides	Run-off water	100 µm PDMS	DI		15		GC-ECD	Graham *et al.*, 1996
Fungicides	River water, sea water	85 µm PA	DI	60	60	NaCl	GC-MS	Peñalver *et al.*, 1999
herbicides	Surface and groundwater, soil	65 µm CW/DVB	DI		30	NaCl	GC-MS	Hernandez *et al.*, 2000
Organophosphorus pesticides	Soil	65 µm CW/DVB	DI		30	NaCl	GC-MS	Ng *et al.*, 1999
Organophosphorus, Organochlorine pesticides, triazine, thiocarbamate herbicides	Drinking water, river water	65 µm CW/DVB	DI		30	NaCl	GC-NPD	Dugay *et al.*, 1998

Table 2 SPME methods for the analysis of flavour compounds in food samples

Analyte	Food sample	SPME conditions					Detection	Ref.
		Fiber	Extraction mode	Temp. (°C)	Time (min)	Salt		
Vegetables and fruits								
Sulphur aroma	Truffle	95 µm PDMS	HS	80	30		GC-IT-MS	Pelusio *et al.*, 1995
Volatiles	Apple	100 µm PDMS	HS		5 - 90		GC-FID	Matich *et al.*, 1996
Volatiles	Apple	100 µm PDMS	HS		2 - 30		GC-TOF-MS	Song *et al.*, 1997
Volatiles	Apple	100 µm PDMS	HS		20		GC-MS	Paliyath *et al.*, 1997
Volatiles	Fruits	65 µm PDMS-DVB	HS		12		GC-TOF-MS	Song *et al.*, 1998
Volatiles	Fruits	100 µm PDMS	HS		30		GC-FID	Ibanez *et al.*, 1998
Juices and other soft drinks								
Volatiles	Fruits juice	85 µm PA	HS		40 - 60	NaCl	GC-FID	Steffen *et al.*, 1996
Volatiles	Beverages	100 µm PDMS	DI, HS		2, 60		GC-MS	Yang *et al.*, 1994
Aroma volatiles	Cola	100 µm PDMS	HS	60	30		GC-MS	Elmore *et al.*, 1997
Volatiles	Coffee	7, 100 µm PDMS	DI, HS	40, 60	30, 120		GC-FID	Bicchi *et al.*, 1997
Orange flavour	Orange juice	100 µm PDMS	HS	40, 60	30, 20		GC-MS	Jia *et al.*, 1998
Volatiles	Beverages	100 µm PDMS	HS	49	30	NaCl	GC-IT-MS	Miller *et al.*, 1999
Alcohol beverages								
Alcohols, esters	Beer	85 µm PA	HS	50	60		GC-FID	Jelen *et al.*, 1998
Sulphur aroma	Wine	100 µm PDMS	HS	30	15		GC-FPD	Mestres *et al.*, 1998
Sulphur aroma	Wine	75 µm CAR-PDMS	HS	25	30		GC-FPID	Mestres *et al.*, 1999 a
Sulphur aroma	Wine	75 µm CAR-PDMS	HS	25	30		GC-FPD	Mestres *et al.*, 1999 b
Diacetyl	Wine	60 µm CW-DVB	HS	40	10	NaCl	GC-MS	Hayasaka *et al.*, 1999
Aroma volatiles	Wine	100 µm PDMS	HS	20	15	NaCl		Ong *et al.*, 1999
Others								
Volatiles	Whey protein	100 µm PDMS	HS	23	120		GC-MS	Stevenson *et al.*, 1996
Volatiles	Whey protein	100 µm PDMS	HS	40	30		GC-MS	Quach *et al.*, 1999
Menthol, menthone	Candy, tea, etc.	7 µm PDMS, 10µm PEMS	HS	30	15		GC-FID	Ligor *et al.*, 1999
Aroma	Spaghetti	65 µm CW-DVB	DI		10	NaCl	GC-FID	Roberts *et al.*, 1997
Volatiles	Meat	100 µm PDMS	HS	60	60		GC-MS	Ruiz *et al.*, 1998

Table 3 SPME methods for the analysis of off-flavour compounds and contaminants in food samples

Analyte	Food sample	SPME conditions					Detection	Ref.
		Fiber	Extraction mode	Temp. (°C)	Time (min)	Salt		
Off-flavour								
Oxidized products	Milk	75 µm CAR-PDMS	HS	45	12 - 15	NaCl	GC-MS	Marsili, 1999
Cork taint	Wine	100 µm PDMS	DI, HS	20	20	NaCl	GC-MS	Fischer *et al.*, 1997
Geosmin, etc.	Catfish	100 µm PDMS	DI		25	NaCl	GC-IT-MS	Zhu *et al.*, 1999
Methylisoborneol	Catfish	100 µm PDMS	HS	40	15	NaCl	GC-MS	Lloyd *et al.*, 1999
Contaminants								
Herbicides	Wine	65 µm PA	HS		50	NaCl	GC-MS GC-NPD	B-Boland *et al.*, 1995
Pesticides	Wine	30, 100 µm PDMS	DI		30		GC-MS	Gandini *et al.*, 1997
Methylisothio-cyanate	wine	65 µm CW-DVB	HS		30	NaCl	GC-FID	Urruty *et al.*, 1996
Pesticides	Honey	100 µm PDMS	DI	30	120	NaCl	GC-EDC	Jimdnez *et al.*, 1998
Pesticides	Fruits, Fruit juice	100 µm PDMS	DI	RT	20		GC-FPD	Simplicio *et al.*, 1999
Antibiotics	Milk	50 µm CW-TPR	DI	65	15	NaCl	LC -MS	Lock *et al.*, 1999
Nitrosamines	Smoked ham	85 µm PA	HS	80	60	NaCl	GC-TEA	Sen *et al.*, 1997

Corrections: MSc Thesis by Shengyi Lu

Use of Headspace Solid-Phase Microextraction for the Analysis and Characterisation of Volatile Compounds in Rumen Contents

p. xii BSTFA bis(trimethylsilyl)trifluoroacetamide
BTEX benzene, toluene, ethylbenzene, and xylene
Add FID – flame ionization detector/detection

Figure 1.9 Diagrammatic view if the outside of the rumen *(left)* and the inner structure of the reticulo-rumen *(right)*.

p. 26 line 4, *t*-butylmethyl ether, line 15, split vent

p. 39 para 6 and 7, change 'As the presence' to 'Due to the presence'

p. 48 para 2, line 3, change 'to use' to 'the use'

p. 74 para 1, line 11, 'effectively' is superfluous here

p. 81 para 3, line 9, change ^{13} to ^{13}C

p. 82 Figure 6.4 caption change ('SPME carrying out' to 'SPME carried out')

p. 104 Pelusio reference change 'Headspace Solid-phase' to 'Headspace solid-phase'