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

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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.

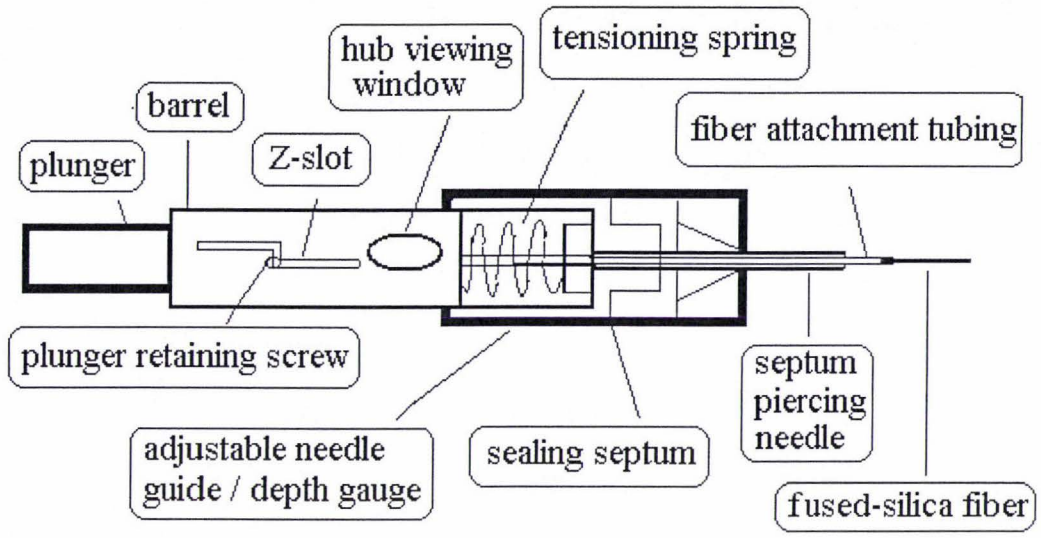

Figure 1.1 SPME fibre holder and fibre (Zhang *et al.*, 1994)

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 ^{TM} 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
StableFlex ™	60 ☼☼	bi-polar	partially crosslinked	270	GC/HPLC
	65	bi-polar	highly crosslinked	270	GC
PA	85	polar	partially crosslinked	320	GC/HPLC
CAR/PDMS StableFlex ™	75	bi-polar	partially crosslinked	320	GC
	85	bi-polar	highly crosslinked	320	GC
CW/DVB StableFlex ™	65	polar	partially crosslinked	265	GC
	70	polar	highly crosslinked	265	GC
CW/TPR	50 ☼☼	polar	partially crosslinked	240	GC/HPLC
StableFlex ™ DVB/CAR/PDMS	50/30	bi-polar	highly crosslinked	270	GC
	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.

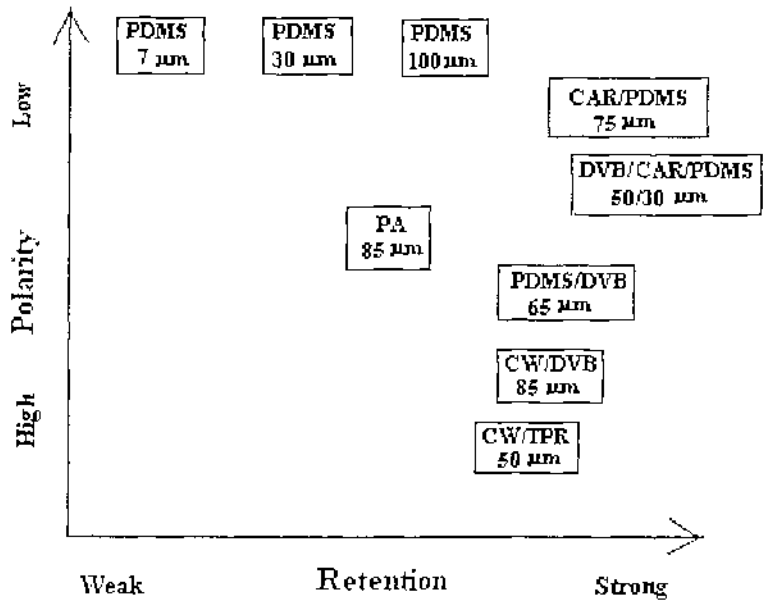

Figure 1.2 Properties of SPME fibres (Kataoka *et al.*, 2000)

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)

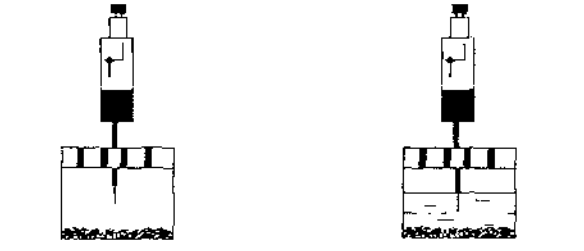

Figure 1.3 HS-SPME and DI-SPME

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.

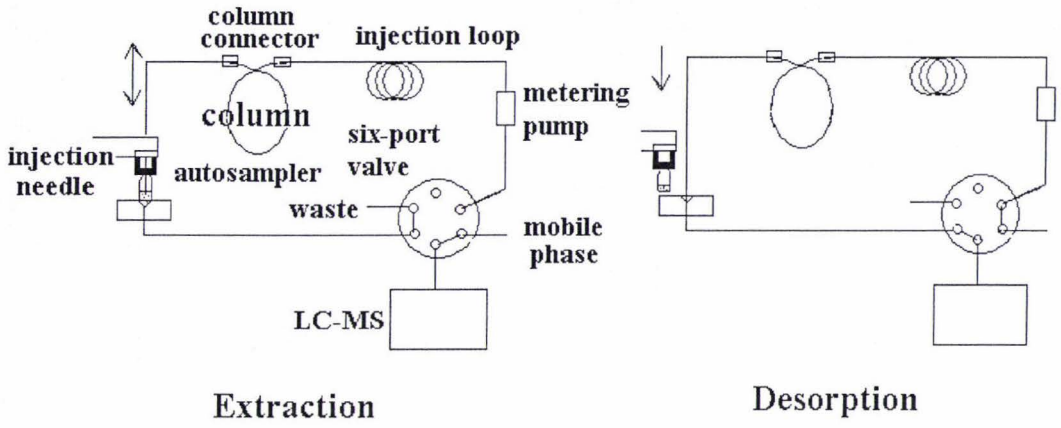

Figure 1.4 In-tube SPME (Kataoka *et al.*, 2000)

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**.

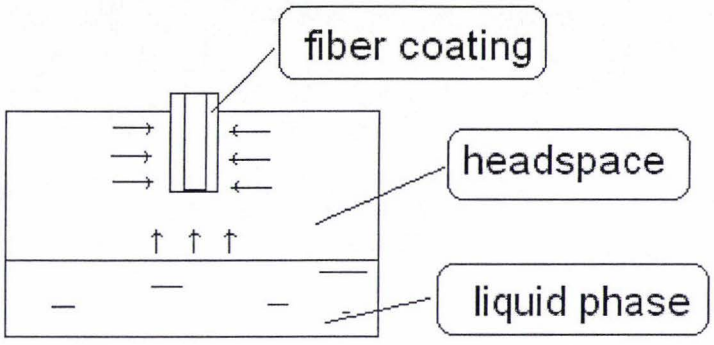

Figure 1.5 Process of headspace SPME

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).

$$C_0V_s = C_fV_f + C_gV_g + C_sV_s \qquad (1.1)$$

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, $n = C_f V_f$, can be expressed as:

$$n = \frac{K_1 K_2 V_f V_s C_0}{K_1 K_2 V_f + K_1 V_g + V_s} \qquad (1.3)$$

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.

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

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.

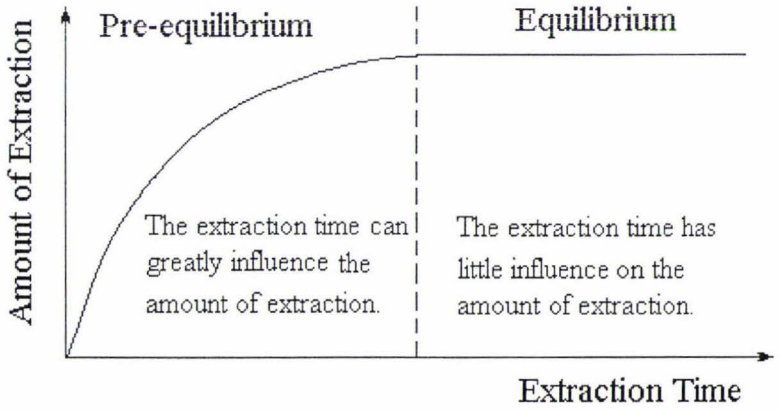

Figure 1.6 Effect of time on SPME extraction (Supelco Bulletin 929, Appendix 2)

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.

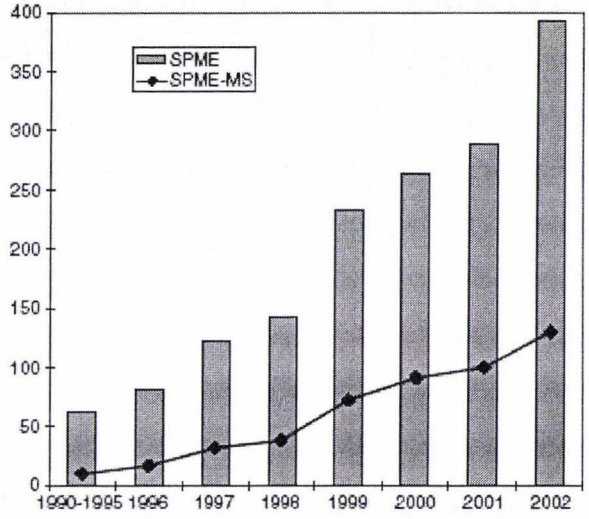

Figure 1.7 Number of published articles in recent years related to SPME and SPME/MS applications (Vas and Vékey, 2004)

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).

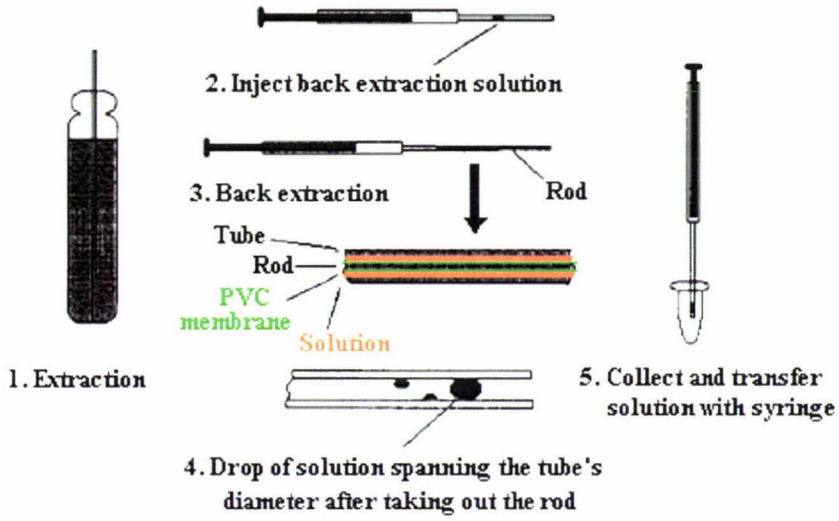

Figure 1.8 Device and operation of Li and Weber's SPME (1997)

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).

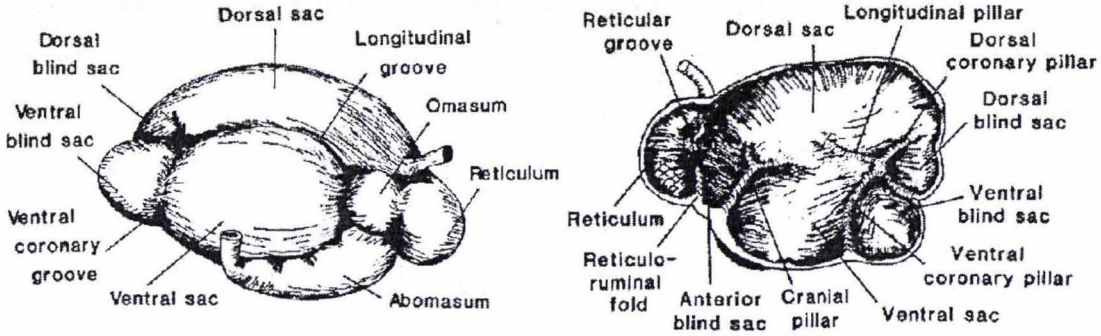

Figure 1.9 Diagrammatic view of the out side of the rumen (the right side) and the inner structure of the reticulo-rumen (based on left view)

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.