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Review

Microextraction of drugs

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Abstract

This review will attempt to provide an overview as well as a theoretical and practical understanding of the use of microextraction technologies for drug analysis. The majority of the published reports to date focus on the use of fibre solid-phase microextraction and so the review is significantly focused on this technology. Other areas of microextraction such as single drop and solvent film microextraction are also described. Where there are insufficient examples in the literature to illustrate important concepts, examples of non-drug analyses are presented. The review is intended for readers new to the field of microextraction or its use in drug extraction, but also provides an overview of the most recent advances in the field which may be of interest to more experienced users. Particular emphasis is placed on the effect various sample matrices have on extraction characteristics.

Keywords: Reviews; Preconcentration; Drugs

Contents

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1. Introduction

Microextraction is defined as an extraction technique where the volume of the extracting phase is very small in relation to the volume of the sample, and extraction of analytes is not exhaustive. In many cases only a small fraction of the initial analyte is extracted for analysis. The extraction efficiency is

determined by the partitioning of analyte between the sample matrix and the extraction phase. The higher the affinity the analyte has for the extraction phase relative to the sample matrix, the greater the amount of analyte extracted. Partitioning is controlled by the physicochemical properties of the analyte, the sample matrix and the extraction phase. Where sample matrix and extraction phase composition are constant, the degree of partitioning and hence the percentage of analyte extracted will be constant also. Because partitioning is not dependent on analyte concentration, quantification of sample concentration may be determined from absolute amount extracted.

Once sufficient extraction time has elapsed for the equilibrium to be established, further increases in extraction time do not affect the amount of analyte extracted. When extraction time does not impact the results, the extraction technique is simplified and precision is improved.

In many cases, the absolute amount extracted is insignificant relative to the initial amount present (<1%). Thus, there is no significant change in sample concentration during extraction. In this case, the amount extracted is independent of sample volume, allowing a further simplification of the technique. On-line or on-site analysis, either by means of a flow-through extraction cell or directly from the total matrix without need for sampling are facilitated.

Microextraction of drugs has to date, found its greatest application with the technique of solid-phase microextraction (SPME) and in particular fibre SPME. SPME is a relatively new sample preparation method, which has the potential to significantly simplify sample preparation, and integrate it with sample analysis. Introduced in 1990 by Arthur and Pawliszyn [1], it initially gained wide acceptance for the analysis of environmental samples. More recently it has been shown to be useful for many drug analysis applications, coupled to analysis by standard chromatography instruments, (GC, GC–MS, LC, LC–MS, CE). Sensitivity and precision are generally as good or better than standard methods, the methods themselves are simpler, and solvent use is eliminated.

In SPME, analytes move from a flowing liquid sample phase to an immobilized or supported liquid or solid-phase, and includes several embodiments. Fig. 1 illustrates several implementations of SPME that have been considered. They include mainly open

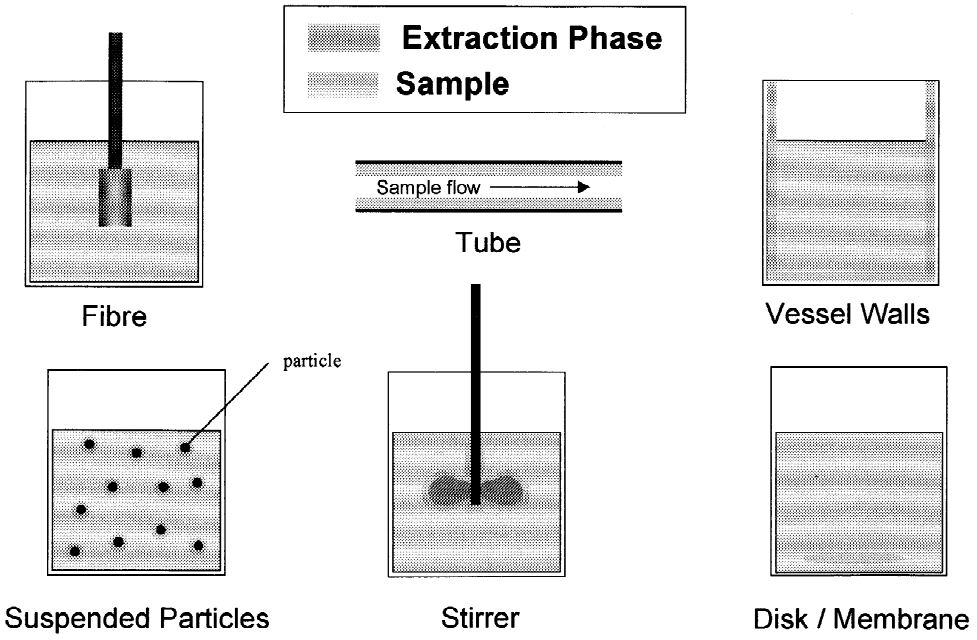


Fig. 1. Configurations of solid-phase microextraction.

bed extraction concepts such as coated fibres, vessels, agitation mechanism disks, but in-tube approaches are also considered. Some better address issues associated with agitation and others, ease of implementing sample introduction to the analytical instrument. It should be noted that solid-phase microextraction was originally named after the first experiment using an SPME device which involved extraction on solid fused-silica fibres, and later, as a reference to the appearance of the extracting phase, relative to a liquid or gaseous donor phase, even though it is recognized that the extraction phase is not always technically a solid. In the case of fibre solid-phase microextraction (SPME), analytes from a sample are extracted by a polymer film coated on a fine 1-cm long fused-silica rod. The rod with the polymer film and extracted analytes is then transferred to a hot injector port of a GC or GC–MS, where extracted analytes are thermally desorbed and transported in the carrier gas for standard separation and analysis. For LC applications, analytes are desorbed from the fibre into mobile phase or another desorption solvent, in a small volume desorption interface. In the technique of in-tube SPME, analytes are desorbed from a polymeric coating inside a capillary, also into mobile phase or a separate desorption phase. In the desorption process, the fibre and/or polymeric extraction phase are cleaned, and rendered ready for another extraction. Methods are typically developed so that extraction and analysis times are similar. In this way and with the use of an SPME autosampler, analysis is continuous, with the analysis occurring concurrently with the subsequent extraction.

This review provides an overview of microextraction theory and techniques for drug analysis, with the emphasis on SPME techniques. Newer microextraction techniques are also appearing, typically using a solvent film or micro-drop for extraction. Theoretical and practical aspects of this work, including single drop extraction, micro-LLE and LLLE are also reviewed here. Finally consideration is given to the practical aspects of microextraction analysis from typical biological and pharmaceutical samples. A review of published reports of SPME analysis in these samples is provided. While not all of the work reviewed deals specifically with drug extraction, experiences in extraction of toxicants and other non-drug compounds are immediately applicable to drug analysis, and serve to highlight considerations for these matrices.

Some users consider SPME to be a subset or miniaturization of the broader SPE technology, and certainly many of the considerations important for SPE method development also apply in SPME method development. The most significant difference between the two lies in the fact that whereas SPE is an exhaustive extraction, i.e. the goal is to extract as near as possible to 100% of the analytes from a sample, SPME is an equilibrium extraction. Thus while the goal of microextraction is to extract based on equilibrium partitioning, the goal of miniaturized extraction techniques such as microSPE is to exhaustively extract analyte from a small sample volume, using a small volume of extraction phase. The two techniques should be considered quite distinct and a more detailed comparison discussion is provided (Section 3.6.1).

Two very significant advantages of SPME and LLLE (liquid back-extraction) are firstly that solvents are completely eliminated from the injection, so the solvent peak does not obscure early eluting peaks. Secondly, even though less than 100% of analyte is extracted from the sample, everything that is extracted is injected, with the net result that sensitivities are often better. The most significant problem encountered with microextraction analysis is that any factors that alter the partition coefficient of an analyte in an extraction, or compete with the extraction phase for absorption or adsorption of the analyte, will change the proportion of analyte extracted. So long as these factors remain constant, analyses are quite reproducible. If however these factors vary from one sample to the next, or between samples and calibrators, this must be compensated by using proper calibration techniques or precision will suffer.

Of the drug analysis applications published to date, much emphasis has been placed on developing methods for the analysis of the common drugs of abuse, which are typically present at fairly high concentration. More recently, methods have appeared for analysis of legitimate pharmaceuticals at therapeutic concentrations.

2. Theoretical aspects

2.1. Partitioning

In the partitioning process upon which microextraction is based, analyte in a sample matrix distributes between the distinct phases present in the system. The analyte concentration in each phase at equilibrium is dependent on the affinity of the compound for each phase. When a discontinuous phase is introduced to an otherwise homogeneous sample, analyte moves into the discontinuous phase until concentration equilibrium is reached. By the law of conservation of mass, we know that the initial amount of analyte present in the sample will be equal to the sum of the individual amounts of analyte present in all discontinuous phases. This is expressed mathematically as:

$$n_0 = n_e + n_s + n_1 + n_2 + \cdots \quad (1)$$

Where n_0 is the mass of analyte initially present in the sample, n_e is the mass of analyte present in the extraction phase, n_s is the amount present in the homogeneous liquid phase, and n_1, $n_2 \ldots$ are the amounts present in discontinuous phases. It should be noted that a discontinuous phase is any compartment of a sample with a different affinity for the analyte of interest. This may include both distinct phases such as immiscible liquids, solids or a headspace, or dissolved phases such as proteins in colloidal solution. At equilibrium, or in fact any time before equilibrium, n_0 is equal to the sum of the amounts of analyte present in the sample (n_s) and the extraction phase (n_e) and any other phases present.

A mathematical consideration of the partitioning process provides both a demonstration of the linear dependence of amount extracted on initial sample concentration, and an understanding of the impact of other sample variables. The amount extracted by the extraction phase may be derived from Eq. (1). To simplify the discussion, a system consisting of just sample and extraction phase is considered initially.

$$n_0 = n_s + n_e \quad (2)$$

Because the partitioning process depends on a concentration equilibrium, concentrations in each of the phases must be introduced into Eq. (2).

$$C_0 V_s = C_e^\infty V_e + C_s^\infty V_s \quad (3)$$

C_0 is the initial concentration in the sample. V_s is the sample volume. C_e^∞ is the concentration in the extraction phase at equilibrium and V_e is the volume of the extraction phase. C_s^∞ is the sample concentration at equilibrium. The extraction phase/sample distribution constant is defined as shown:

$$K_{es} = \frac{C_e^\infty}{C_s^\infty} \quad (4)$$

The amount of analyte extracted by the fibre can be calculated by substituting $K_{es}C_s^\infty$ for C_e^∞ in Eq. (3), multiplying both sides by $K_{es}V_e$ and re-arranging as shown:

$$C_0 V_s = K_{es} C_s^\infty V_e + C_s^\infty V_s$$

$$K_{es} V_e C_0 V_s = K_{es} V_e K_{es} C_s^\infty V_e + K_{es} V_e C_s^\infty V_s$$

$$K_{es} V_e C_0 V_s = K_{es} V_e \frac{C_e^\infty}{C_s^\infty} C_s^\infty V_e + \frac{C_e^\infty}{C_s^\infty} V_e C_s^\infty V_s$$

$$K_{es} V_e C_0 V_s = K_{es} n_e^\infty V_e + n_e^\infty V_s$$

$$K_{es} V_e C_0 V_s = n_e^\infty (K_{es} V_e + V_s)$$

At equilibrium therefore the amount extracted is described as [2]:

$$n_e^\infty = \frac{K_{es} V_e C_0 V_s}{K_{es} V_e + V_s} \quad (5)$$

In Eq. (5), the equilibrium amount extracted is not dependent on extraction time. In practice, this means that once equilibrium is reached, the extracted amount is constant within the limits of experimental error and is independent of further increase of extraction time. In addition, if $V_s \gg K_{es}V_e$, then V_s cancels out and the amount extracted is no longer dependent on sample volume. Eq. (5) then becomes:

$$n_e^\infty = K_{es} V_e C_0 \quad (6)$$

Samples are typically more complicated than the two-phase system described above. Considerations

for common multi-phase extractions are discussed next.

2.2. Headspace vs. direct immersion extraction

Commonly, there is a headspace present in the sampling system, during microextraction. This headspace has the potential to act as a separate discontinuous phase. If the headspace has a low affinity for the analyte, its presence presents no complications for the extraction. This is true for analysis of many relatively low volatility drugs. Compounds with high volatility however, may have a significant concentration of compound present in the headspace. The repercussions of this effect are discussed.

When a headspace is considered as a separate phase with affinity for the analyte, the mass balance equation at equilibrium can be written as follows:

$$C_0 V_s = C_e^{\infty} V_e + C_s^{\infty} V_s + C_h^{\infty} V_h \quad (7)$$

C_h^{∞} is the equilibrium headspace concentration and V_h is the headspace volume. Similar to the discussion presented above, when Eq. (4) is rearranged to substitute $K_{es} C_s^{\infty}$ for C_e^{∞} the amount extracted by the extraction phase can be determined as follows:

$$n_e^{\infty} = \frac{K_{eh} K_{hs} V_e C_0 V_s}{K_{eh} K_{hs} V_e + K_{hs} V_h + V_s} \quad (8)$$

K_{eh} is the extraction phase/headspace partition coefficient, K_{hs} is the Henry's constant for the analyte, and $K_{eh} K_{hs} = K_{es}$. Therefore the amount extracted is proportional to the initial sample concentration, the volume of the extraction phase and the partition coefficient between the extraction phase and the sample. This is true whether the extraction phase is located in the sample or in the headspace. It is also true that if $V_s \gg K_{eh} K_{hs} V_e + K_{hs} V_h$, then extraction is independent of both sample and headspace volumes. This occurs when the sample volume is relatively large, or when the Henry's constant headspace volume and the overall partitioning coefficient extraction phase volume are small. If V_s is not $\gg K_{eh} K_{hs} V_e + K_{hs} V_h$, then the amount extracted also varies with both the sample volume and the headspace volume. Pawliszyn provides a thorough treatment of this subject [3]. The effect of headspace volume on extraction is shown in Fig. 2. Two features of this figure are noteworthy. Firstly, it can be seen that a large overall amount is extracted as the headspace volume is reduced, and this effect is exaggerated as Henry's constant increases. This is due to the fact that a larger Henry's constant, and a large headspace volume, will allow the headspace to hold a larger amount of analyte at equilibrium than in the case where Henry's constant and/or headspace volume are smaller. If more analyte is located in the headspace, less will be available for the extracting phase to extract.

Secondly, as Henry's constant increases, the slope of the relationship between amount extracted and headspace volume becomes steeper. Thus small variations in headspace volume have a larger impact on overall method precision with larger Henry's constants. Extraction sensitivity for a defined headspace extraction time is commonly improved by increasing extraction temperature. The effect here is to increase the headspace concentration of an analyte, to reduce the requirement of mass transfer from the sample, and speed up the overall extraction rate.

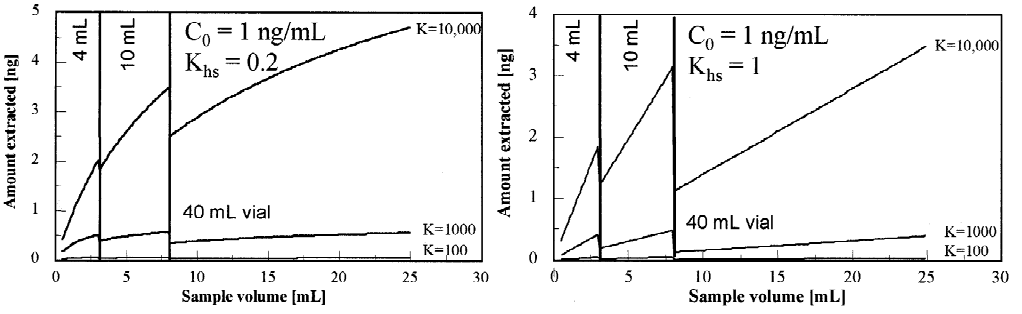


Fig. 2. The effect of headspace volume and Henry's constant on extraction.

In effect, the temperature increase, increases Henry's constant dramatically, and so variability in headspace volume has a greater impact on precision as extraction temperature increases.

2.3. Effect of additional phases in the sample

When additional phases are present in a sample, analyte can partition into those phases in addition to the sample matrix and the extraction phase. Any discontinuous phase in the sample having a significant affinity for the analyte of interest, will compete with the extraction phase for analyte, and therefore cause a reduction in the amount extracted. If the amount of such a competing phase varies from sample to sample, there will be a significant impact on method precision. Where competing phases are present, Eqs. (2) and (4) can be re-written to include the impact of each competing phase.

Mass balance equation:

$$C_0V_s = C_e^{\infty}V_e + C_1^{\infty}V_1 + C_2^{\infty}V_2 + C_3^{\infty}V_3 + \cdots + C_n^{\infty}V_n \quad (9)$$

Amount extracted:

$$n_e^{\infty} = \frac{K_{es}V_eC_0V_s}{K_{es}V_e + K_{1s}V_1 + K_{2s}V_2 + K_{3s}V_3 + \cdots + K_{ns}V_n} \quad (10)$$

A detailed discussion of these equations has been published [3]. In drug extractions, such phases could include tissue fragments, cells or cell fragments, or even receptors or antibodies to the analyte of interest. If the sample is a pharmaceutical compound, several competing phases may be present, originating as excipients in the drug formulation. While the presence of such phases could be problematic in an analysis of total concentration of an analyte, the nature of equilibrium extraction also provides an important advantage in assessing binding affinities in biological systems. A discussion of this important advantage has been published recently [4]. To briefly summarize, a simple microextraction of a multicomponent system will provide information on the concentration of free analyte present in the system, provided the amount extracted is insignificant relative to the total. By adding a standard addition spike and allowing the system to re-equilibrate, one can then assess the degree of binding to system components. By performing this analysis in systems containing the sample phase and one discontinuous phase at a time, one can assess the binding affinities of all system components individually.

2.4. Effects of extraction temperature, ionic strength, pH

2.4.1. Temperature

A change in extraction temperature has several effects on microextractions. With a temperature increase, diffusion coefficients and Henry's constants are increased, and partition coefficients to the extraction phase are decreased. Because Henry' s constants are increased, headspace concentrations will also increase. Because diffusion coefficients are increased, the time required to reach equilibrium is decreased. Finally, due to the lowered partition coefficients to the extraction phase, the equilibrium amount extracted is decreased. These effects can be observed in Fig. 3. Graph A shows the effect of increased extraction temperature on equilibrium profiles. The shortening equilibration times and lowered total amounts extracted with increased extraction temperature are clearly observed. When the data are re-plotted with extraction temperature on the *x*-axis, (graph B) the maximum extraction sensitivity given these competing forces, is easily observed for each extraction time.

2.4.2. Ionic strength

The addition of a salt can often improve recovery when conventional extraction methods are used. Sodium chloride (NaCl) is commonly used for this purpose [5]. Occasionally, one sees an initial increase in the extraction yield with an increase in salt concentration, with a maximum being reached, followed by a decrease in amount extracted with further increase in salt concentration [6–8].

This behaviour can be explained by considering two simultaneously occurring processes. Initially analyte recovery is enhanced due to 'salting out', whereby water molecules form hydration spheres around the ionic salt molecules. These hydration spheres reduce the concentration of water available to dissolve analyte molecules; thus it is expected this

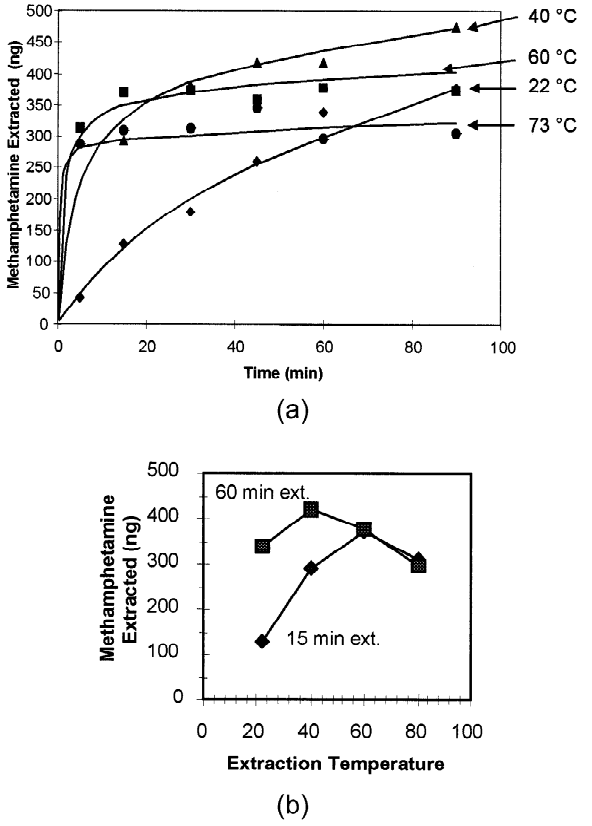


Fig. 3. (a) Effect of temperature on equilibrium profile; (b) effect of temperature on amount extracted by SPME.

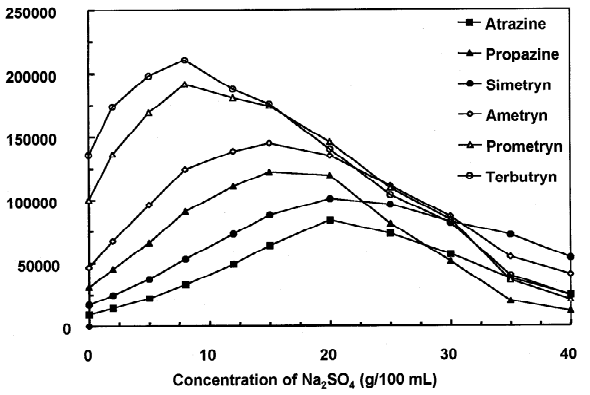


Fig. 4. Effect of partition coefficient and concentration of Na_2SO_4 on amount extracted by SPME.

will drive additional analytes into the extraction phase [9]. In competition with this process however, is the fact that polar molecules may participate in electrostatic interactions with the salt ions in solution [10], thereby reducing their ability to move into the extraction phase. Initially, it would be the interaction of the salt molecules with water that is the predominant process. As salt concentration increases further, salt molecules will begin to interact with analyte molecules. Thus it is reasonable that there should be an initial increase in analyte extracted with increasing salt concentration, followed by a decrease as the salt interaction with the analytes in solution predominates. Of further interest, it appears that the salt concentration producing maximum extraction is correlated to partition coefficient. In Fig. 4, the compound with the highest 'zero salt' level of extraction also has the highest partition coefficient, as all extractions were carried out at equilibrium with all analytes in equal concentration. In the figure legend, the partition coefficients increase as one reads down the list of compounds. Thus we see that the salt concentration producing maximum effect increases as analyte partition coefficient decreases. We expect that for compounds with lower partition coefficients, saturated salt conditions will be reached before the reduction in salt effect occurs.

2.4.3. Extraction pH

The pH of the extraction mixture is important for drugs possessing a pH-dependent dissociable group. It is only the undissociated form of the drug that will be extracted by an absorptive-type of fibre coating (PDMS or PA). This is important for extraction as drug that has partitioned into the fibre coating does not participate in the Henderson–Hasselbalch equilibrium between acid and base forms of a drug in an aqueous extraction mixture. In the case where the extraction mixture pH is controlled with a buffer, as the undissociated form of the drug is extracted by the fibre, more dissociated drug reassociates and is therefore available for extraction. Thus, in a buffered extraction mixture, more drug can be extracted by an absorptive fibre coating than in an extraction mixture where the pH is not buffered. In an unbuffered extraction mixture, the ratio of undissociated to dissociated forms of the drug can vary. Therefore, one does not achieve the continual transfer of drug from the dissociated to the undissociated form, and then to the fibre coating. This effect is seen in Fig. 5,

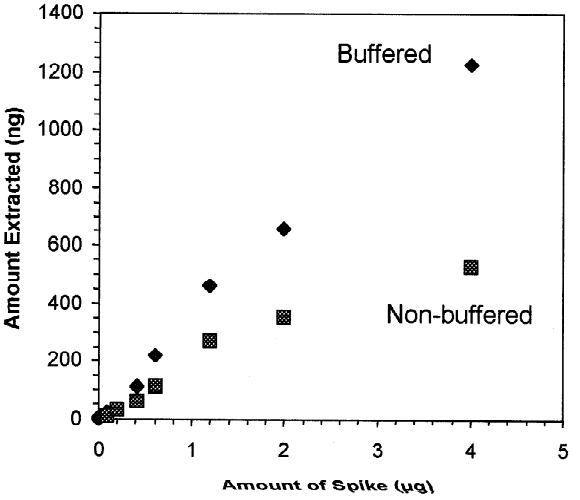


Fig. 5. Effect of buffering on sample extraction linearity.

which compares the amount of methamphetamine extracted from a solution where the pH is base adjusted with KOH, versus one where the pH is controlled at 12 with phosphate buffer [5]. Note there is a significant deviation from linearity in amount extracted as drug concentration increases in the non-buffered system. The effect is also used in the LLLE technique discussed below, where a donor, extraction and acceptor phase are all combined in one system.

Many drug formulations designed to be taken orally utilize the salt of the active ingredient, to aid dissolution in the stomach. Control of extraction pH is important in such cases, to ensure the undissociated form of the drug is present for extraction. Additionally, the formulation matrix of a pharmaceutical preparation may itself act to buffer the extraction matrix, and final extraction pH should be checked before extraction.

2.5. Absorptive extraction phases

The overall process of absorption depends on several discrete steps that must occur in sequence. Initially analyte moves towards the extraction phase through the bulk of the sample. This occurs by a combination of convection and diffusion, and the process can be enhanced by increasing either of these. Convection is increased by any of a number of agitation methods, the most common of which is magnetic stirring. As mentioned above, diffusion can be increased by increasing the extraction temperature. As analyte approaches an extraction phase, it encounters a region where sample matrix has reduced mobility. At the extraction phase/sample interface, the sample matrix is static. As one moves out from the interface, mobility of the sample increases, until it is equivalent to that in the bulk of the sample matrix. Degree of diffusion control of overall mass transfer varies from the level in the bulk of the sample matrix, to 100% at the boundary layer/extraction phase interface. A more detailed description of the nature of the boundary layer is available [11]. In practice it is defined as a region of specific thickness, in which analyte mass transfer is controlled by diffusion alone. Once analyte has diffused through the boundary layer, it must partition to the extraction phase, and then diffuse throughout the bulk of the extraction phase. In this system, overall extraction dynamic is controlled by diffusion through the boundary layer as it is the slowest process. For the theoretical discussion of extraction to be strictly valid, several assumptions are made. It is assumed that there is no displacement between different analytes on the extraction phase. The physical properties of the extraction phase cannot change during extraction in relation to the initial concentration of analyte in solution. Diffusion must be the only mass transport mechanism determining the migration of analyte molecules in the system. These assumptions are true for liquid extraction phases or in the case where a polymeric extraction phase is a normal liquid (amorphous rubbery compound). If the polymer were glassy, i.e. below its glass transition temperature during extraction, or if the polymer is a solid below its crystallization temperature, this assumption may not be valid. There must also be no activation energy involved in the transfer of analyte between solution and coating.

Of the commercially available SPME extraction phases, only poly(dimethylsiloxane) and polyacrylate meet these criteria. They meet the criteria of a fluid, in that the substance is amorphous and the molecules are disordered and mobile and are not rigidly fixed in position. Both exist at ambient temperatures as rubbery compounds. PDMS has a glass transition temperature (T_g) of -123°C. The polymer used for

PA fibres is much less well defined as it is a proprietary material. It is believed to be an acrylate co-polymer with a moderate degree of cross-linking, existing as an amorphous rubbery compound (i.e. above T_g) at ambient temperatures. In the presence of oxygen it darkens and starts to decompose at 150–170°C. Under inert atmosphere, the darkening occurs at 280–300°C. Incidentally, the polymer must undergo this partial decomposition during conditioning, or extraction rates will not be consistent. If the fibre becomes very dark or black, the decomposition is extensive and extraction efficiency deteriorates. Commercial extraction phases incorporating divinylbenzene are true crystalline solids. In this case, the molecules are ordered in a regular lattice. Extractions using these sorbents are discussed in more detail in the following section.

2.6. Solid sorbents

These sorbents possess a regular crystalline lattice in their structure. The kinetics of partitioning are the same as for the liquid sorbents discussed above, except that in the last stage, diffusion through the bulk of the polymer is extremely slow. Typically the amount of analyte migrating into the bulk of the polymer, even at very long extraction times, is negligible. For practical purposes extracted analyte remains on the surface. Because it is a crystalline solid, there may be activation energies involved in the transfer of analyte between the solution and the coating. Partitioning generally follows a Langmuir isotherm, with the following assumptions. (1) Molecules adsorb into an immobile state; (2) all sorption sites are equal; (3) each site can hold only one molecule and (4) there are no interactions between adsorbed molecules on adjacent sites [12].

The equilibrium amount of analyte on the fibre is:

$$n_f^\infty = C_f^\infty V_f = \frac{KC_s^0 V_s V_f (C_{f\,max} - C_f^\infty)}{V_s + KV_f(C_{f\,max} - C_f^\infty)} \quad (11)$$

$C_{f\,max}$ is the maximum concentration of active sites on the coating and C_f^∞ is the equilibrium concentration of analyte on the fibre. K is the analyte's adsorption equilibrium constant, and C_s^0 is the initial concentration of analyte in the sample. The form of Eq. (11) is quite similar to that for Eq. (5) where the coating extracts by absorption rather than adsorption. The main difference is the presence of the fibre concentration term $(C_{f\,max} - C_f)$ in both the numerator and denominator of Eq. (11). It should also be noted that in Eq. (11), K is very different from K_{es} in Eq. (5). $K_{es,}$ is the partition coefficient whereas K is the adsorption equilibrium constant. For very low analyte concentrations on the fibre, i.e. where the amount of analyte on the fibre is negligible compared to the total number of active sites, $C_{f\,max} \gg C_f$ and the dependence on sample concentration will be linear.

As available sites become saturated however, C_f is no longer insignificant and a non-linear dependance on amount extracted relative to sample concentration results.

In practice, it is very rare that only one analyte or compound with affinity for the fibre is present in a sample, and since adsorption is a competitive process, the presence of another compound (B) will affect the amount of analyte (A) extracted by the fibre. The equilibrium concentration of analyte A on the fibre in the presence of a competing compound B is given by the following equation:

$$C_{fA}^\infty = \frac{C_{f\,max} K_A C_{sA}^\infty}{1 + K_A C_{sA}^\infty + K_B C_{sB}^\infty} \quad (12)$$

K_A and K_B are the adsorption equilibrium constants for compounds A and B respectively, and C_{sA}^∞ and C_{sB}^∞ are the equilibrium concentrations of A and B in the sample. This can be re-arranged to give the amount extracted by the extraction phase (fibre) at equilibrium:

$$n_{fA}^\infty = C_{fA}^\infty V_f = \frac{C_A^0 V_s V_f K_A (C_{f\,max} - C_{fA}^\infty)}{(1 + K_B C_{sB}^\infty) V_s + K_A V_f (C_{f\,max} - C_{fA}^\infty)} \quad (13)$$

Thus the amount of analyte A extracted at equilibrium in the presence of a competing compound B, must be lower than for the situation where no competing compound exists as the additional term in the denominator of Eq. (13) relative to Eq. (11) can only be greater than one. If however the interfering compound is either present at a very low concentration or has low affinity to the coating, this first term may be insignificant, and there will be little

difference in the amount of analyte A extracted between samples with and without the presence of interfering compound B.

The presence of the competing compound B can also further impact the linear range of the calibration curve and can be understood as follows. When B adsorbs on the surface of the coating, it reduces the number of adsorption sites available for A. This means that $C_{f\,max}$ is effectively lowered for A, and hence the non-linearity for A becomes significant at lower concentrations of A, compared to the case when the sample contains no interfering compound. This is shown in Fig. 6.

In addition, when multiple analytes are present in the sample, the low affinity coefficient compounds adsorb quickly, and then are replaced as higher affinity coefficient compounds are adsorbed later. This is seen in Fig. 7.

The practical application of the foregoing for drug analysis, is that samples are almost always complex mixtures where multiple compounds could compete for binding sites on a solid sorbent. Where the analyte of interest has a low affinity for the sorbent, precision can suffer due to either displacement effects or non-linearity of response. Care must be taken in selecting a suitable internal standard. Ideally it would be an isotopically labelled compound or a close chemical analog that has a very similar affinity to the sorbent. Where headspace extraction can be employed, interferences from non-volatile compounds can be avoided.

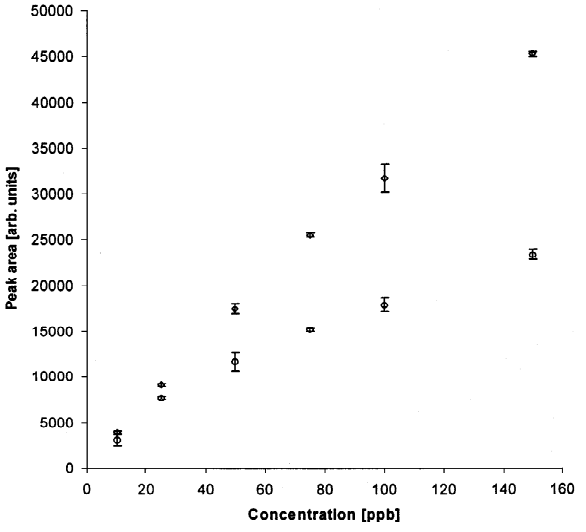


Fig. 6. Calibration curves for i-propanol in the presence of methyl-isobutyl ketone (MIBK). PDMS/DVB fibre, headspace sampling. Squares: MIBK concentration 10× lower than i-propanol concentration; circles: MIBK concentration equal to i-propanol concentration.

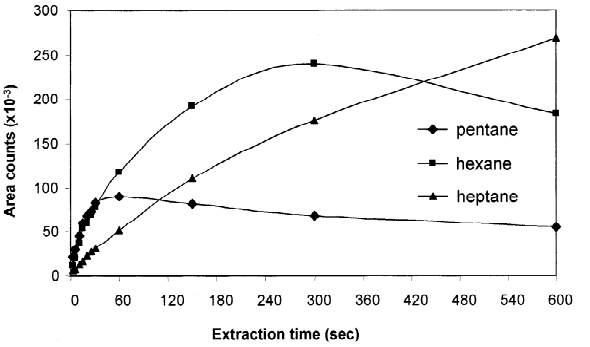


Fig. 7. Displacement effect on solid sorbents. Compounds with higher affinity gradually replace those with lower affinity.

2.7. Derivatization with SPME analysis

A significant challenge in organic analysis is for the determination of polar compounds. These can be difficult to extract from biological matrices and difficult to separate on a chromatographic column. Derivatization approaches are frequently used to address this challenge. Fig. 8 shows a summary of the various derivatization techniques that can be implemented in combination with SPME or other microextractions [13]. Some of the techniques, such as direct derivatization in the sample matrix, are

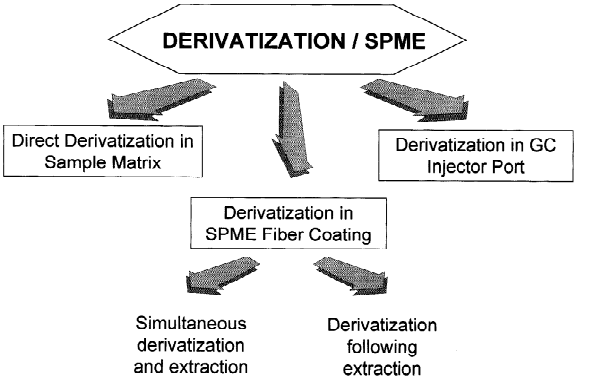


Fig. 8. Summary of derivatization techniques.

analogous to well-established approaches used in solvent extraction.

2.7.1. Direct derivatization in sample matrix

In the direct technique, the derivatizing agent is first added to the vial containing the sample and derivatives form there. The derivatives are then extracted by SPME and introduced into the analytical instrument. This approach has been applied to extract and separate phenols from aqueous samples by first converting the target analytes to their acetate derivatives [14]. More recently it has been used in the analysis of amphetamines in urine, by converting the amines to carbamates in solution, with chloroformates [15]. Briefly, a urine sample (1.2 ml) was buffered to pH 10.8 and NaCl was added to 5.5 *M*. 8 μl of chloroformate was added and a 16-min room temperature direct immersion extraction was performed with 100 μm PDMS fibre. Analysis was by GC–NPD. Throughput with automated extraction and analysis was 85 samples per day. Limits of detection were 5 ng/ml for methamphetamine, DMDA and MDEA and 15 ng/ml for amphetamine and MIDA. For all compounds and a range of concentrations, inter-assay precision averaged 4.3% (0.8–11.1%) and accuracy, measured as % bias averaged 7.5% (2–13%).

Hexylchloroformate derivatization has been used similarly for the analysis of benzylecgonine in urine [17]. One ml of urine was sonicated for 3 min with 12 μl of the chloroformate. A volume of 250 μl of the sample was then extracted with a 100-μm PDMS fibre and analysis of the resulting benzoylecgonine hexyl ester was performed by GC–MS. With quantification by deuterated internal standard, a detection limit of 0.03 μg/ml was realized, with inter-day precision of $\leq$3.3%.

2.7.2. On fibre derivatization after extraction

Because of the availability of polar coatings, extraction efficiency for polar underivatized compounds is frequently sufficient to reach the sensitivity required. Occasionally, however, there are problems associated with the separation of these analytes. Good chromatographic performance and detection can be facilitated by in-coating derivatization following extraction. This has been shown with high molecular mass carboxylic acids [13]. After exposing an SPME coating containing extracted analytes to diazomethane, the resulting ester derivatives can be separated as narrow bands on a GC column (see Fig. 9). In addition, selective derivatization to analogues containing high detector response groups will result in enhancement in sensitivity and selectivity of detection. This has been demonstrated for the analysis of amphetamines and their metabolites [18]. After extraction with a PDMS fibre, it was exposed to headspace over trifluoroacetic anhydride for 20 min at 60°C. The derivatives were subsequently desorbed in a GC injection port. LOQ were 10 ng/ml for amphetamine and methamphetamine and 20 ng/ml for MDA and MDMA.

Steroids have also been derivatized on the fibre subsequent to extraction [19]. In this case, a polyacrylate fibre (85 μm) with extracted steroids was exposed to the headspace of 5 μl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) in a 1-ml autosampler vial insert and incubated at 60°C for 1 h. After a 5-min desorption in a GC injector port at 280°C, GC–MS analysis was performed. The derivatization reaction was shown to quantitatively consume the parent compound and no evidence was seen of multiple derivatization products from a single steroid. While direct contact with the liquid BSTFA destroyed the polyacrylate coating, there was no damage observed with the headspace method. Derivatization significantly improved chromatographic resolution of estrone and estradiol compared to analysis of the parent compounds.

2.7.3. Derivatization in GC injector port

Derivatization in the GC injector is an analogous approach, but it is performed at high injection port temperatures. For example, long chain carboxylic acids can be extracted onto the coating as ion pairs when tetramethylammonium hydrogen sulfite is added to the sample. During volatilization, analytes are converted to methyl esters [13].

Injection port derivatization of amphetamines extracted by SPME has also been reported [20,21]. Amphetamines were extracted from the headspace above a whole blood samples with a 100-μm PDMS fibre. The fibre with extracted analytes was introduced to the injection port of a GC, after heptafluorobutyric anhydride had been injected. The compounds were desorbed and derivatized simultaneous-

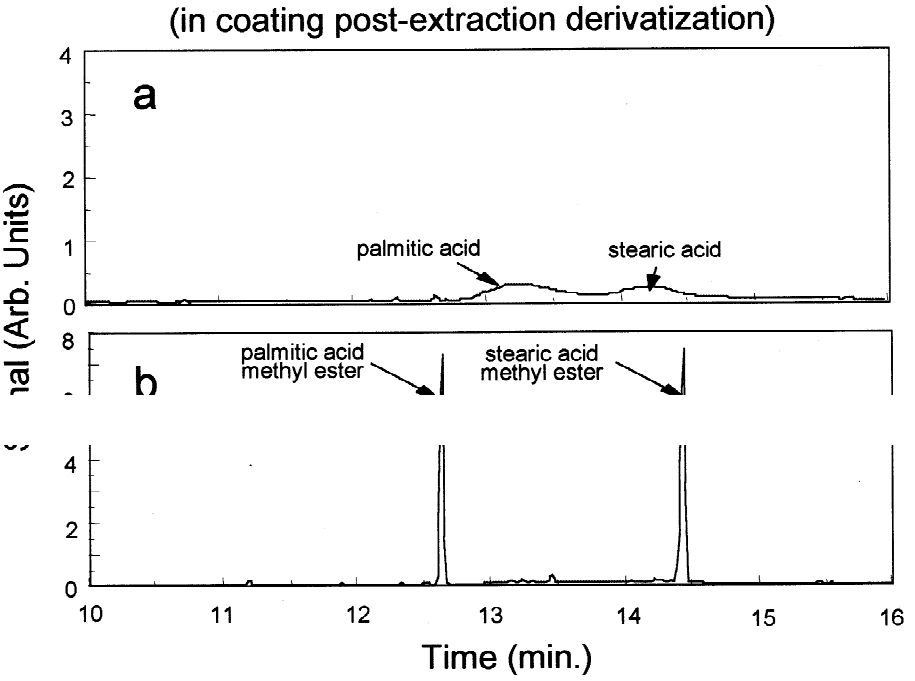


Fig. 9. Derivatization of carboxylic acids improves peak shape.

ly. For fenfluramine, amphetamine and methamphetamine, limits of detection were 5–10 ng/g. Intra-day and inter-day coefficients of variation for a range of concentrations were 1.0–4.8% and 1.6–9.2% respectively.

2.7.4. On fibre derivatization simultaneous with extraction

An interesting and potentially very useful technique is simultaneous derivatization and extraction, performed directly in the coating. This approach allows high efficiencies and can be used in remote field applications. The simplest way to execute the process is to dope the fibre with a derivatization reagent and subsequently expose it to the sample. Then the analytes are extracted and simultaneously converted to analogues having high affinity for the coating. This is no longer an equilibrium process as derivatized analytes are collected in the coating as long as extraction continues.

This approach, which is used for low molecular mass carboxylic acids, results in exhaustive extraction of gaseous samples [22]. When 1-pyrenyldiazomethane is used as the derivatization reagent, it is introduced into the coating by first dissolving the reagent in a volatile solvent. The fibre is then immersed in the solution. The fibre coating swells and is doped with the reagent. After evaporation of the solvent, the fibre is ready to perform extraction. The reagent, having low vapor pressure and high affinity toward the coating, remains on the fibre during its exposure to the sample. Volatilities of the pyrenylmethyl esters formed during the reaction are also low, resulting in the accumulation of the product onto the fibre until analyte or reagent is exhausted or decomposed. At a high injector temperature the derivatized analytes are removed from the coating and the fibre can be reused.

A similar approach is used for the analysis of formaldehyde from gaseous samples [23]. This approach is not an equilibrium extraction, but it is based on the reaction kinetics of the derivatization process. The derivatizing agent, *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine is first doped onto the fibre by room temperature headspace extraction from an aqueous solution. Formaldehyde is subsequently

extracted from an unknown sample, and converted to the oxime derivative in the fibre. In this case, kinetics of the derivatizing reaction are fast, and uptake is controlled by mass transport. If reaction kinetics are slow, uptake may be controlled by both the distribution constant and the reaction kinetics [3]. With fast derivatization reaction kinetics, the process is similar to the mass transport controlled extraction described in the Conclusion section, for VOCs analysis from air, where uptake rates for short sampling times are proportional to diffusion coefficients.

The simple but powerful procedure described for this analysis is limited to low volatility derivatization reagents. The approach can be made more general by chemically attaching the reagent directly to the coating. The chemically bound product can than be released from the coating either by high temperature in the injector, light illumination or change of an applied potential etc. The feasibility of this approach was demonstrated by synthesizing standards bonded to silica gel, which were released during heating [24].

2.8. Quantification

By definition, the amount extracted in a microextraction should be linearly correlated with sample concentration, with the exception described above for adsorptive phases used to extract complicated or high concentration samples. In a microextraction the extraction phase will always extract the same proportion of analyte from a sample. For this reason it is import to calibrate the amount extracted relative to the sample concentration. This is distinct from the separate calibration of detector response that must be completed for any analysis. There are a number of options available for calibrating a microextraction test.

2.8.1. External calibration

This is probably the most widely used calibration method. Several samples are prepared at different analyte concentrations typically in triplicate. Microextractions are performed and linear calibrations are calculated for each analyte. It is important that calibration samples have the same composition as test samples. If the matrix in test samples differs from that of calibration samples, partitioning will not be the same and the calibrations will not be valid. In addition, where amount extracted is dependent on sample volume, this must also be closely controlled. If the sample headspace has a significant affinity for the analyte, the headspace volume must also be constant, regardless of whether headspace or direct sampling is performed.

For extractions using an absorptive phase such as poly(dimethylsiloxane), the linear range can extend beyond that of the detector, particularly for high partition coefficient analytes. When assessing linearity of the method, it is important to consider the linearities of both the detector and the extraction.

External calibration is useful when there is little variance in test sample composition, or where test samples are all normalized prior to extraction. For instance, for urine analysis of drugs of abuse, particularly for overdose samples where analyte concentration is very high, a small amount of sample (ca. 50 μl) may be diluted in several ml of buffer. In this case, overall sample composition is very constant and external calibration will give satisfactory results. On the other hand, for analysis of trace amounts of drugs in urine, it is more likely to be whole urine that is analysed. Because of the large variability in ionic strength in urine, this will either have to be normalized by performing all analyses at high or saturated salt concentration, or another quantification means will have to be employed.

For complex matrices such as tissue, external calibration is impractical. The variability in sample matrix from one sample to the next will cause unacceptable imprecision in analyses.

2.8.2. Standard addition

For variable sample matrices, standard addition quantification generally gives very good results. The method is described in detail elsewhere [25]. Briefly, after the sample is analysed, a known spike of the analyte of interest is added to the sample, and the sample is re-analysed after it has re-equilibrated. The amount of the unknown in the original sample is calculated based on the amount of the known spike extracted, relative to the amount added. The same sample can be re-analysed only if an insignificant amount of the analyte is extracted in the first extraction ($<1\%$). Otherwise a separate positive

sample vial must be prepared, spiked with the standard and analysed. This method generally gives precision on the order of 5–10%. If better precision is required, several standard addition samples must be prepared. It is recommended to analyse three standard addition samples in triplicate, for best precision (2–5%). Obviously the major drawback to this method is the large number of additional samples that must be analysed for each unknown. The method becomes overly cumbersome if more than a handful of unknowns must be tested.

2.8.3. Internal standard

The use of internal standards for quantification is routine for many methods, and can give satisfactory results for microextraction as well. It is preferable that the internal standard selected is closely related to the analyte(s) of interest, particularly in terms of partition coefficient for the extraction phase. If the internal standard is extracted to a significantly different extent than the analyte, error in the analysis will be either under- or over-stated. Where the internal standard is poorly extracted relative to the analyte, error in the analysis of the analyte of interest may be overstated. On the other hand, if the internal standard is extracted to a much greater extent than the analyte of interest, analysis error may be understated. Also, extraction time profiles must be determined for both compounds. If the analyte of interest is extracted at equilibrium, and therefore extraction time is not closely controlled, large errors will result if the internal standard is not extracted at equilibrium. This can be the case where the internal standard has a higher partition coefficient than the analyte of interest. If the sample characteristics are optimized for the analyte of interest, poor extraction conditions can result for a poorly matched internal standard. For instance, if the pK of the analyte of interest is very different from that of the internal standard, and sample pH is adjusted to favour the analyte of interest but not the internal standard, results will be poor.

A limitation to the use of an internal standard exists where there is a significant and variable competing phase present in the sample. The internal standard may have a very different affinity for the competing phase, than the analyte of interest does. Where the composition of the competing phase is consistent, quantification by internal standard may still be possible, as the absolute amounts of binding of both compounds to the competing phase would be linearly correlated with the amount of the phase present. Where the competing phase is variable in composition however, the absolute amounts of internal standard and analyte of interest bound may not be linearly correlated, and peak ratio analysis would not give an adequate quantification.

Where the amount of the competing phase is small, the absolute amount bound to the phase would be insignificant, and could be ignored. This could be the case where an insoluble coating with affinity to the analyte of interest is used on a tablet, but where the absolute amount bound is insignificant relative to the total amount of analyte/internal standard present.

Where a competing phase is present in excess, such that only a small proportion of the surface active sites are occupied, and where the consistency of the competing phase is constant, internal standard calibration can be used reliably. A significant complication arises where the degree of saturation of surface active sites in a competing phase is high. In this situation, binding to the competing phase is non-linear, and if the absolute amount of binding is significant, internal standard calibration cannot be used reliably. The identification of which situation exists for a given sample type can be determined experimentally by performing a calibration curve in the range of analyte and competing phase concentrations expected to be encountered in the samples.

2.8.4. Isotopic label

By far the most accurate and simplest method of quantification is the use of an isotopically labeled standard. Unfortunately isotopically labeled standards are commercially available for only a small number of compounds, are expensive, and can be unstable. In many cases though, it is not complicated to prepare deuterated standards in-house. The method of course necessitates mass spectral detection. The only caution for using isotopically labeled standards, is that the standard should be added at roughly the same concentration (within one–two orders of magnitude) as the unknowns. The tendency is to add very little isotopically labeled standard, due to cost considerations. However, in this way, error in analysis of the standard is much larger than for the

unknown, and this will be reflected in a larger than normal error for unknown quantification.

The methods of standard addition and internal standard with deuterated pentobarbital were compared for the quantification of barbiturates in spiked urine [8]. For the two methods recoveries were determined to be 93% and 104% and precisions were 5.9% and 4.0% respectively.

3. Applications

3.1. Study of binding affinity

As mentioned above, because microextraction techniques typically extract an insignificant amount of the total analyte, depletion can be negligible and the extraction will not disturb the equilibrium in a system. The method can therefore be used relatively easily, to assess the affinities of various components of a complex system. This technique has been described recently for analysing protein and membrane binding, and determination of the free amount of a drug or other chemical in a system. Microextraction only measures the free concentration of an analyte. It is the free concentration that is most toxicologically and pharmacologically relevant, as opposed to the nominal or total concentration most commonly reported. The method is faster and more convenient than alternatives, which include equilibrium dialysis or headspace equilibration analysis. The method is particularly well suited to situations where the discontinuous phase of interest is dissolved (such as proteins and antibodies) or otherwise difficult to separate from the liquid phase. The theory and application of the method for fibre SPME analysis of several polar compounds in biological matrices has been reported [26,27]. The authors report criteria which must be met to successfully implement the method:

- The extraction itself must not influence the equilibrium between the aqueous phase of the sample and the discontinuous phase.
- The binding phase must not interfere with the extraction by binding to the extraction phase.
- The absorption profile of the compound should be the same in a calibration sample as in the sample containing the discontinuous phase.

The method was validated by comparing the amount of one of the compounds extracted by microextraction, both inside and outside of the membrane in an equilibrium dialysis experiment. There was no difference in the free concentrations determined in the samples from inside and outside of the dialysis tube. BSA concentrations were varied from 2.0 to 200 μ*M*. They therefore concluded that the protein itself is not adsorbed to the fibre, and that the protein does not influence the amount of chemical absorbed by the fibre in any other way.

In practice the free fraction (f_X) of analyte X in solution is calculated as follows:

$$f_X = [X]_a/[X]_t = 1/(1 + K_M[M]) \quad (14)$$

where $[X]_a$ is the concentration of the freely dissolved chemical, $[X]_t$ is the total concentration (free and bound to M), K_M is the protein–water partition coefficient of X and [M] is the concentration of protein M in the aqueous phase.

Experimentally, f_X is determined by calculating the slope of the linear plot of $[X]_a/[X]_t$. Compound X is first added to an aqueous solution without protein, and the total concentration determined. Protein is added to the system, and the system is allowed to re-equilibrate. Once equilibrated, the freely dissolved concentration is determined by microextraction. By varying $[X]_t$ the required linear correlation can be determined. The analyte/protein partition coefficient (K_M) can then be calculated. Eq. (12) can be re-arranged to give:

$$K_M = \frac{([X]_0 - [X]_a) \times \frac{V_a}{V_M}}{[X]_a} \quad (15)$$

where $[X]_0$ is the total (nominal) concentration, $[X]_a$ is the free concentration in the solution, V_a and V_M are the volumes of the aqueous and protein phases respectively.

It is well established that a compound's octanol/water partition coefficient correlates well with therapeutic potency of many drug compounds, and is used as a general parameter to describe effective concentrations and kinetic behaviour in biological systems. However the molecular structures and overall natures of octanol versus membrane lipids are very different. Perhaps most importantly, octanol is a bulk

solvent, whereas membrane lipids exhibit a bilayer structure. A simple means to correlate with the membrane partition coefficient would be much more valuable. Currently, most biological, toxicological and pharmacological effects are expressed based on nominal concentrations, even though it is only freely available concentrations that are responsible for biological effect. Efforts have been made to correlate K_{ow} to actual free concentration in cell cultures. Vaes et al. demonstrated that K_{ow} is well correlated with membrane partition coefficients, and not surprisingly, that as partition coefficient increases, the free fraction is reduced. Partitioning was studied in hepatocyte primary culture, S9 post-mitochondrial supernatant, as well as microsomal cell fractions.

A similar strategy has been employed for the determination of the equilibrium constant of protein binding between diazepam and human serum albumin (HSA) [4]. In this work, a Scatchard plot was used to evaluate the binding data. An equilibrium constant and an indication of one independent binding site were determined, and was consistent with literature results obtained by gel filtration analysis. For diazepam analysis, direct extraction with a 100-μm PDMS fibre was performed from a 0.5 μg/ml solution of diazepam in 1 mg/ml HSA dissolved in 0.066 *M* phosphate buffer pH 7.4. Trace amounts of methanol were observed to effect the precision of analysis. Because methanol could not be eliminated entirely due to its use as a solvent for diazepam standards, methanol content was kept consistent for all analyses.

By the Scatchard plot relationship:

$$r/[D] = -rK + NK \quad (16)$$

where r is the moles of drug bound per mole of protein and [D] is the free concentration of diazepam. K is the equilibrium constant of drug binding to protein and N is the number of identical independent sites. In these experiments HSA (molecular mass 67 000 D) at 1 mg/ml corresponded to a solution protein concentration of 1.45 E-5 *M*. Free drug concentration is determined by SPME extraction and bound drug is determined by subtracting free drug from the total spiked into the sample. When $r/[D]$ is plotted on the y-axis and r is plotted on the x-axis, the resulting slope of the linear regression analysis of the data is K and the x-intercept is N.

3.2. Study of drug metabolism and chemical reaction kinetics

For the same reasons that microextraction is well suited to monitor biological binding affinities, it is also very useful for monitoring biological reaction kinetics. The use of SPME to investigate drug metabolism in keratinocytes has been demonstrated [28]. The authors reported that relative to liquid–liquid extraction and SPE, SPME produced the best recovery rate and precision, in addition to the advantages of reduced solvent use, a saving in time, the re-usability of the fibre and the potential to automate the entire process.

The technique is quite straight-forward. Once an extraction procedure has been identified, one need only measure the appearance of a product (or disappearance of a substrate etc.) over time. The resulting substrate and/or product concentrations are typically plotted versus incubation time. The data are then easily converted to biochemical measures of enzyme kinetics (Michaelis–Menten constant) or expressions of reaction kinetics.

The monitoring of chemical reaction kinetics can also be beneficial. In the report on alkylchloroformate derivatization of amphetamines [15], it is shown that the derivatization agent undergoes hydrolysis simultaneously with the derivatization reaction, and the reaction conditions must be optimized to minimize the hydrolysis and maximize the product formation. A detailed study of the kinetics of these two competing reactions has been reported [29]. In this report, the carbamate formed was extracted into 5 ml of methyl isobutylketone (MIBK) containing an internal standard and excess amine was back extracted into 1 *M* phosphoric acid. An aliquot of the organic phase was injected into a GC with flame ionization detection and the peak height ratio between the carbamate and an internal standard was calculated.

More recently, it has been demonstrated that SPME analysis is much more efficient for this study. Values obtained for the pseudo-first order reaction rate constant of the hydrolysis and the second order rate constants for the reactions between the amine and chloroformates, agreed very well with the previously reported values [30].

For the hydrolysis determination, a chloroformate

solution (3 m*M*) was prepared in 5 ml of buffer. At the required time, the hydrolysis reaction was stopped by the addition of excess (100 μl) *N*-methylbutylamine (*N*-mba). Unhydrolysed chloroformate was thereby converted to the corresponding carbamate and the degree of hydrolysis was determined by the difference between moles of chloroformate added and moles of carbamate formed. The carbamate was extracted using PDMS/DVB fibre. Extraction was performed directly from the aqueous solution for 30 min and the fibre was injected into a GC for analysis. Desorption was 1 min at 250°C. For the carbamate formation experiments, an aqueous solution of *N*-mba was prepared (3.7 E-6 *M*) in 5 ml of buffer. Isobutylchloroformate and propylchloroformate were added at initial concentrations of 0.75 to 6.0 E-3 *M* and the solutions were stirred for 60 min to ensure complete reaction. The carbamates formed were extracted and analysed as above.

3.3. Solvent microextraction

Liquid–liquid extraction is a widely used and generally accepted sample preparation method for a large variety of applications. Although it suffers from several limitations, such as large volume of solvent use, labour intensity, tendency for emulsion formation, and poor potential for automation, almost half of respondents in one survey [31] reported they still use liquid–liquid extraction for sample preparation. Its advantages are general acceptance for standard methods, simplicity of method development, generally good reproducibility and high sample capacity. Initial efforts to address the problems of large solvent consumption and poor automation included the development of flow injection extraction [32,33] which had the advantages of high speed, low cost and reduced solvent/sample consumption. In this method extraction is quantitative and analyte determination is performed by measuring optical absorption in the organic phase. While the method is attractive, solvent consumption is still on the order of several hundred microlitres per analysis. More recently effort has been placed on miniaturising the extraction process. This has involved using a small amount of organic solvent (<200 μl) and a relatively larger aqueous sample. Several early reports appeared on the method [34–36] and an excellent overview of these efforts has appeared recently [37]. The main findings reported by these latter authors and others in the field, are reviewed here.

The primary goal of techniques to miniaturise LLE sample preparation, has been to greatly reduce the solvent: aqueous phase ratios. As phase ratios are reduced, the methods developed have made use of microextraction techniques as exhaustive extraction is abandoned for equilibrium extraction. Two general methods have evolved. These are single drop extraction, where the extraction phase is a discrete drop of immiscible solvent suspended in a sample, or extraction into a liquid film in contact with the sample.

Recent publications in this field have demonstrated applications for drug analysis. While much of the preliminary work in the field has been performed on non-drug compounds, a review of both drug and non-drug publications is presented to provide a more thorough understanding of the field.

3.4. Single drop extraction

In early work Liu and Dasgupta [38] reported extraction of SDS ion pairs into a stationary organic drop suspended inside a flowing aqueous drop. While the kinetics of the process are not described in detail, the importance of convective transport of analyte is described. Vibration in the aqueous drop and elimination of organic microdrop evaporation are discussed. Analysis is by optical absorbance and the authors propose drop in drop in drop extraction for further enhancement of extraction efficiency.

At the same time, Jeannot and Cantwell [39] reported on solvent microdrop extraction of 4-methylaceotphenone, with *n*-dodecane employed as an internal standard. The microdrop (*n*-octane) is supported on a teflon rod. After extraction the drop is injected directly into a gas chromatograph for analysis. The article provides a good treatment of the kinetics and diffusion of the process.

Jeannot and Cantwell [40] later reported on the extraction of progesterone, malathion, 4-methylacetophenone and 4-nitrotoluene into a 1 μl drop of *n*-octane suspended from tip of microsyringe needle. After extraction the drop was analysed directly by GC. Mass transfer characteristics of the system were evaluated, and it was found that mass transfer is

proportional to diffusion coefficients. The evidence provides support for the film theory of convective–diffusive mass transfer rather than penetration theory. A good description of the boundary layer effect is also provided.

Jeannot and Cantwell [41] later extended the method for use as a speciation tool to study progesterone binding to BSA with GC analysis. Progesterone was extracted into a 1 μl drop of n–octane suspended on a microsyringe needle tip. A very low phase ratio was used. Equilibrium binding constants were determined for both equilibrium and non-equilibrium extraction, and were shown to be equivalent. The values obtained were consistent with macro-scale solvent extraction and equilibrium dialysis.

He and Lee [42] reported on static and dynamic liquid phase microextraction of 1,2,3-trichlorobenzene and l,2,3,4,5-pentachlorobenzene into <2 μl organic solvent (toluene) in a microsyringe. The extraction was performed by drawing aqueous sample into the syringe. Extraction occurred both into the solvent plug located near the syringe plunger and into the solvent film that formed on the walls of the syringe barrel. Extraction into the film was more efficient than extraction into the plug. The method was compared with static extraction where the 1-μl microdrop was exposed directly to the sample, attached to the tip of the needle. Static extraction gave a $12\times$ enrichment, 15-min extraction time and 10% RSD. In the dynamic extraction 1 μl of toluene is first drawn into the needle. The needle tip was placed into the aqueous sample and 3 μl of sample was drawn and dispensed 20 times. This extraction scheme resulted in a $27\times$ enrichment, a 3-min extraction, and 13% RSD. Extraction occurred primarily into the renewable microfilm within the syringe. All analyses were by GC.

This work was extended [43] for the analysis of ten chlorobenzenes with GC analysis. The extraction efficiencies of several extraction solvents were investigated with enrichment factors reported as follows: toluene (6.6–12), chloroform (10–20), butylacetate (2.7–10), isooctane (8.8–23). The effects of sample volume drawn, plunger speed and dwell time were investigated. Salt in the sample was found to decrease extraction, possibly by an adverse effect on the extraction film.

Ma and Cantwell [44] further extended the technique by combining microextraction into a solvent film with back extraction into a microdrop. The final receiving phase was a 0.5–1 μl aqueous microdrop (pH 2), suspended in a 30-μl *n*-octane liquid membrane confined in a teflon ring. The n–octane membrane was exposed to an aqueous sample at pH 13. The method provided convenient preconcentration and clean-up of samples containing methamphetamine, mephentermine, 2-phenylethylamine and methoxyphenamine, for HPLC analysis. No solvent evaporation or desorption was required for analysis. The aqueous receiving phase (microdrop) was introduced directly to HPLC for analysis. Solute adsorption at the *n*-octane aqueous interface reduced mass transfer rate. Convection in the organic membrane phase, caused indirectly by magnetic stirring of the sample, was shown to accelerate the extraction rate.

3.5. LLE and LLLE

Liquid–liquid microextraction and liquid–liquid–liquid microextraction (back extraction) using immiscible films have been a somewhat more recent development in the field.

Ma and Cantwell [37] provided early work showing simultaneous forward and back-extraction across a microliter-size organic liquid membrane. The method was used for the analysis of the ionizable compounds mephentermine and 2-phenylethylamine, prior to LC analysis.

Pedersen-Bjergaard and Rasmussen [45] demonstrated a novel method for concentration of methamphetamine from samples prior to CE analysis. CE requires relatively high sample concentrations, but many biological samples of interest contain analyte at trace levels. CE injection volumes are typically small. Although attempts have been made to facilitate large sample volume injection for CE, results have been mixed to date. The method reported preconcentrates basic analytes, and separates them from large molecules (proteins, DNA). Neutral or acidic compounds would also be separated from ionized species. Briefly, polypropylene hollow fibre membrane (8 cm, I.D. 600 μm, wall thickness 200 μm, 0.2-μm pores) is first dipped into solvent (1-octanol), which fills the pores within the membrane. The

membrane is flushed with air to remove solvent from the lumen, and an aqueous acceptor solution (25 μl) is added. The outer surface of the membrane is exposed to sample (2.5 ml in a 4-ml vial). After extraction, the acceptor solution is transferred to a 200-μl vial insert by air pressure.

The authors provide a comprehensive explanation of the theoretical aspects of the extraction. For an analyte i, the extraction process may be illustrated with the equation:

$$i_{a1} \overset{K_1}{\leftrightarrow} i_o \overset{K_2}{\leftrightarrow} i_{a2} \tag{17}$$

where the subscript a1 represents the aqueous donor phase (sample solution), o the organic phase within the pores of the hollow fibre, and a2 the aqueous acceptor phase. At equilibrium the distribution ratios for the analyte i in the three-phase system are:

$$K_1 = \frac{C_{o,eq}}{C_{a1,eq}} \tag{18}$$

and

$$K_2 = \frac{C_{o,eq}}{C_{a2,eq}} \tag{19}$$

where $C_{o,eq}$ is the equilibrium concentration of i in the organic phase, $C_{a1,eq}$ is the equilibrium concentration of i in the donor phase and $C_{a2,eq}$ is the equilibrium concentration of i in the acceptor phase.

At equilibrium, the mass balance in this three component system can be expressed as:

$$C_{a1,initial} = \frac{K_2 C_{a2,eq}}{K_1} + \frac{K_2 C_{a2,eq}}{V_{a1}} + \frac{C_{a2,eq} V_{a2}}{V_{a1}} \tag{20}$$

where $C_{a1,initial}$ is the initial concentration of i in the donor phase (sample), V_{a1} is the volume of donor solution (sample) V_o, is the volume of organic solvent in the pores of the hollow fibre, and V_{a2} is the volume of acceptor solution inside the hollow fibre.

The enrichment factor (E_e) is define as the ratio $C_{a2,eq}/C_{a1}$, and is calculated by rearranging Eq. (20):

$$E_e = \frac{1}{\frac{K_2}{K_1} + \frac{K_2 V_o}{V_{a1}} + \frac{V_{a2}}{V_{a1}}} \tag{21}$$

Assuming V_0 is small, Eq. (21) simplifies to:

$$E_e = \frac{1}{\frac{1}{K} + \frac{V_{a2}}{V_{a1}}} \tag{22}$$

where:

$$K = \frac{C_{a2,eq}}{C_{a1,eq}} \tag{23}$$

It was determined that K values of 100 or more are required for the analytes of interest in order to obtain high enrichment factors (>50). Also, the donor: acceptor volume ratio should not be below 100 in order to obtain high enrichments and to ensure analyte preconcentrations are sufficient for practical work with biological samples.

1 *M* NaOH in the donor solution provided maximal enrichment factors, but 0.1 *M* was selected for further studies for handling reasons. The strength of the acceptor solution was more critical. At 1 *M* HCl, significant distortions were observed with the CE runs. At 0.1 *M* HCl, peak shape was significantly improved, although overall extraction efficiency was somewhat compromised. At lower concentrations, the enrichment factor was reduced significantly. After optimization, the method produced an enrichment factor of 75. The method had an overall extraction efficiency of 75%. Diphenylhydramine was used as an internal standard, as it had approximately the same enrichment factor, and was well separated from methamphetamine in the CE run. The method had a limit of detection for methamphetamine analysis of 5 ng/ml, and precision on the order of 6% (with use of I.S.), and was successfully applied for the analysis of urine and plasma.

Rasmussen et al. [46] extended the above work for application to GC and HPLC in addition to CE analysis. For HPLC and CE, the method used was as described above. For GC, the hollow fibre was filled with 15 μl *n*-octanol. Extraction time was 30 min, with vibration of the system at 1000 rpm. This is a departure from the previous stirring with stir bars and eliminated cross-contamination. HPLC/CE extractions employed 15 s of ultrasonication after fibre doping prior to extraction, to remove small droplets of organic solvent from the outside of the hollow

fibre. A volume of 25 μl of acceptor phase was added and extraction was for 45 min with vibration at 1000 rpm. Between 10 and 30 samples could be extracted in parallel. The hollow fibre selected (polypropylene) is compatible with both aqueous solutions and a broad range of organic solvents.

The GC model compounds used were diazepam and prazepam. Samples were adjusted to pH 5.5 with sodium acetate. With a 30-min extraction time, extraction efficiencies were diazepam: 69%, prazepam: 103%. LOD from plasma was 2 ppb. The organic acceptor selected should have low volatility and high solubility for analytes. *n*-Octanol met these requirements.

The model compounds employed for CE analysis were, methamphetamine (basic) from plasma and naproxen (acidic) from urine. A 75% extraction efficiency was observed and other parameters employed were as per the previous and next references.

For HPLC the model compounds were citalopram and its *N*-desmethyl metabolite. A 75% extraction efficiency (30× preconcentration) and LOD of 0.7 ppb were observed with fluorescence detection.

Pedersen-Bjergaard and Rasmussen [47] further extended the work to analysis of the acidic drugs ibuprofen, naproxen and ketoprofen. In this case the polypropylene hollow fibre membrane was impregnated with dihexyl ether. The donor solution was acidified with HCl (0. 1 *M* final), and the 25 μl aqueous acceptor solution was 10 m*M* NaOH, with or without 25% methanol. The extractor was vibrated at 400 rpm during extraction. Near 100% extraction efficiency was observed, so this is not a microextraction technique. Sample volume was 2.5 ml. With larger sample volume, microextraction conditions would be met. The hollow fibre was sonicated following impregnation. Extraction time was 45 min, and the acceptor phase was injected directly to CE. Precision was 2–5% with use of an internal standard and 8–13% without. A LOD of 1 ppm was found with UV detection. The extraction efficiency from urine was inferior to that from water, but the inclusion of methanol in the acceptor phase improved this extraction efficiency. Several other peaks appeared, as urine is known to contain several acidic compounds. In general, backgrounds are more complicated when extracting acidic compounds than when extracting basic compounds.

Several method requirements are described:

- Organic solvent in pores should be immiscible with water.
- Analytes should be more soluble in organic phase than donor phase.
- Analytes should be more soluble in acceptor phase than organic solvent.
- Organic solvent should be easily immobilized in the hollow fibre pores, and be non-volatile.

The acceptor phase must strike a compromise between high ionic strength (high preconcentration for the analyte) and compatability with CE analysis (narrow bands).

In related experiments, a polyacrylate SPME fibre was doped with *n*-octanol prior to extraction of a series of benzodiazepines from human urine and plasma [48]. Approximately a three-fold increase in peak area was observed for nordiazepam extraction, after 4 min of pre-doping in *n*-octanol. It was felt that this immobilization increased the hydrophobic nature of the fibre coating, thereby increasing the partition coefficient of the analytes for the fibre coating. Absorption of the solvent may also cause swelling of the fibre coating, with the inherent increased coating volume also contributing to enhanced extraction efficiency.

3.6. Micro-SPE

3.6.1. Comparison of SPME and SPE

As discussed previously, solid-phase microextraction is often considered as an extension of solid-phase extraction or as micro-SPE. There are significant differences between the methods however. Solid-phase extraction is essentially a three-step process. A sample is initially passed through the sorbent bed, and analytes present in the sample are exhaustively extracted from the sample matrix to the solid sorbent. In a second step, unwanted analytes are selectively desorbed from the solid sorbent by washing with a solution capable of desorbing unwanted analytes, but leaving desired analytes retained on the sorbent. In the final step, the wash solution is changed for one able to desorb analytes of interest. The resulting eluent may then be concentrated by evaporation to the desired volume. Solid-phase microextraction however takes advantage of

equilibrium extraction and selective sorption from the matrix onto the coating. In the first step, the fibre coating is exposed to the sample and analytes with a high affinity for the sorbent are selectively extracted. In the second step, everything extracted by the fibre is desorbed into the analytical instrument. No intermediate clean-up step is normally implemented. Micro-SPE is more related to SPE as it is a total extraction method, but utilizes a reduced sorbent volume. A comparison with SPME is therefore inappropriate.

A degree of selectivity is required for any sample preparation method. It is impractical to introduce all compounds present in a sample to an analytical instrument. The method developed must eliminate compounds incompatable with the instrument including matrix components. It is also desirable to remove as many of the unwanted analytes as possible, to make the resulting data interpretation clean and simple. Thus, with selective extraction, sample preparation is simplified and typically results in a significant savings in time and precision. The foregoing is also true for other microextraction techniques where the entire extraction phase is introduced to the analytical instrument.

Selectivity is therefore quite important when choosing an extraction phase. High capacity, even for a range of analytes, is more important for solid-phase extraction, where prevention of breakthrough is a significant concern. Because break-through is not an issue to be addressed in an equilibrium extraction method, more emphasis may be placed on sorbent selectivity.

SPME differs from SPE in another significant way, in that SPE sorbent, because of the large volume of sorbent required relative to SPME, has the potential to retain non-adsorbed components in the void volume. It is difficult to design a wash regime that removes unwanted compounds completely, without impacting retention of the analyte(s) of interest. In this way there is the potential that unwanted compounds may remain, either adsorbed, or present as non-adsorbed analytes in the bulk of the sorbent. Because of the geometery of the SPME device, and the modes of extraction used, unwanted analytes are not normally present in the sorbent at the time of desorption.

3.7. Fiber SPME

The majority of examples of the use of microextraction for drug analysis reported to date, employ fibre SPME with GC analysis. Publications now exist covering most of the major drug classes. Table 1 provides a partial summary of literature references.

The method was originally thought to be applicable only to forensic samples where concentrations of analytes are quite high. More recently however methods have appeared for the analysis of drugs at therapeutic concentrations. As techniques and coatings continue to improve, it is likely that more trace analysis at successively lower levels will be possible.

3.8. SPME coupled to liquid chromatography

The receiving phase from a microdrop extraction or a LLL microextraction, where the receiving phase is aqueous, may be directly introduced to liquid chromatographic instruments. Analytes extracted by SPME however must be desorbed into a suitable receiving solvent prior to analysis. There are two modes by which SPME may be coupled to LC, and there are some minor variations in the way interfaces are incorporated, depending on the LC manufacturer. Either conventional fibre coupling, or the newer in-tube SPME may be incorporated by placing the interface in the position of either the sample loop or a transfer line. Fibre extractions for LC applications are completely analogous to those for GC applications. With conventional fibre coupling, analysts are currently limited to performing manual extractions and desorptions. For automated extraction and analysis, in-tube SPME is relatively simple to implement.

For conventional fibre coupling, the commercial desorption chamber (Supelco) is essentially a chromatographic tee, with two of the ports connected in the position of the sample loop. In the third position, a fitting seals the fibre and either static or dynamic desorption is used. In static desorption, a suitable desorption solvent is introduced to the tee before the fibre is introduced. After a predetermined desorption time, the valve is switched and mobile phase sweeps desorbed analytes to the column. Where desorption into the mobile phase is efficient, dynamic desorp-

Table 1
Summary of literature references for fibre SPME methods of drug analysis

Compound class	Compounds	Matrix	Fibre	Extraction	Desorption	Separation/ detection	Limit of detection	Linearity	References
Illicit drugs									
Amphetamines [5]	Amphetamine, Methamphetamine	Urine	PDMS 100 μm	1 ml Urine, pH 12 1 ml PO_4 buffer (0.25 *M* pH 12) sat'd. NaCl, 60°C Headspace, stirred 1500 rpm, 15 min	15 min, 250°C	GC–FID	1.5 ng/ml	0.005–2.0 μg/ml	Blood [20] Urine[a] [49–54] Urine, derivat. [15,16] Blood, derivat. [21] Hair [55] Illicit tablets [56]
Benzodiazepines [57]	Oxazolam, haloxazolam, Flunitrazepam, Nimetazepam, Clonazepam, diazepam, Bromazepam, Fludiazepam, nitrazepam	Urine	PDMS 100 μm	1 ml Urine Acid hydrolysis, Adjust to pH 9.4 with 0.4 ml borate Buffer, ambient Temperature, Immersion, 30 min	5 min, 270°C	GC–ECD	2–20 ng/ml	20–500 ng/l	Plasma [58,59] Serum [60] Urine[a] [59–62]
Cannabinoids [63]	Cannabidiol, cannabinol Δ^8-Tetrahydrocannabinol Δ^9-Tetrahydrocannabinol	Saliva	PDMS 100 μm	2 ml Saliva 1 ml Water 1 ml Acetic acid Centrifuge, Immersion, 30 min	12 min, 270°C	GC–MS	1.0 ng/ml	5–500 ng/ml	Saliva[a] Hair [64]
Cocaine [65]	Cocaine	Urine	PDMS 100 μm	0.5 ml Urine NaF, ambient, Immersion, 30 min	3 min, 240°C	GC–NPD	12 ng/ml	60–500 ng/ml	Urine[a] [17,52]
GHB [66]	Gamma-hydoxybutyric acid	Plasma, urine	Carbowax/DVB templated resin, 50 μm	0.5 ml Plasma/urine, acid hydrolysis at 80°C Neutralize (pH 6–7) with phosphate buffer, 70°C, Headspace, 15 min	n/r	GC–PICI–MS	Plasma 0.05 μg/ml Urine 0.1 μg/ml	Plasma 1–100 μg/ml Urine 5–150 μg/ml	Plasma, urine[a]
Opiates [67]	Methadone EDDP	Hair	PDMS 100 μm	50 mg Hair 12 h Deproteination DTT, Pronase E, Tris buffer pH 7.2 diluted 1:4 borax buffer, pH 9.2, NaCl, immersion, 30 min	5 min, 250°C	GC–MS	Methadone 2.48 ng/mg EDDP 0.15 ng/mg	1–50 ng/mg	Urine [5,68,69] Hair[a]
Other stimulant [70]	Carphedon	Urine	Carbowax/DVB 65 μm	3 ml Urine, Carbonate buffer to pH 9.6, NaCl, Ambient Immersion, 10 min	3 min, 250°C	GC–NPD	Water 0.01 μg/ml	Urine 0.1–10 μg/ml	Urine[a]
Phencyclidine [71]		Whole blood Urine	PDMS 100 μm	1 ml Urine NaOH, K_2CO_3 1 ml Blood Perchloric acid, Centrifuged, NaOH, K_2CO_3, 90°C, Headspace, 30 min	250°C, time not reported	GC–SID	Blood 0.25 ng/ml Urine 1.0 ng/ml	Blood 2.5–100 ng/ml Urine 0.5–100 ng/ml	Blood, urine[a]

Table 1. Continued

Compound class	Compounds	Matrix	Fibre	Extraction	Desorption	Separation/ detection	Limit of detection	Linearity	References
Therapeutic drugs									
Local anaesthetics [72]	Lidocaine, 2,6-Xylidine glycinexylidide monoethyl-glycinexylidide	Plasma, urine	Carbowax/DVB 65 μm	1 ml Plasma/urine NaOH, pH 9.5 NaCl 35°C, immersion, 40 min	5 min, 250	GC–NPD	4.0–21 ng/ml	2,6-Xylidine, lidocaine 0.035–7.7 μm Glycinexylidide, Monoethylglycinexylidide 0.1–3.5 μ*M*	Blood [73,74] Plasma[a] [75] Urine [76] Hair [77]
Anorectic compounds [78]	Norfenfluramine Fenfluramine Phenmetrazine Phendimetrazine	Urine	PDMS 30 μm	1 ml Urine, 1 ml sat'd $NaHCO_3$, Immersion, 20 min	1 min, 250°C	GC–MS	Phendimetrazine 40 ng/ml Fenfluramine 30 ng/ml	Phendimetrazine 80–800 ng/ml Fenfluramine 60–900 ng/ml	Blood [21] Urine[a] [79]
Antibiotics [80]	Tetracycline, minocycline, Oxytetracycline, Demeclocycline, Anhydrotetracycline, 4-Epianhydrotetracycline, Methacycline	Milk	Carbowax/DVB templated resin, 50 μm	3.5 ml Milk, 65°C, Immersion, 30 min	5 min, static, Acetonitrile:water (15:85), 40°C	LC–ionspray–MS–MS	Water 4–40 ng/ml	Water 30 ppb Milk 100 ppb	Erythromycin[a] [81] Tetracyclines[a]
Antidepressants [82]	Maprotiline Mianserin Setiptiline	Whole blood	PDMS 100 μm	0.5 g Blood 0.5 ml NaOH (1 N) 120°C Headspace, 45 min	5 min, 250°C	GC–MS	Maprotiline 25 ng/g Mianserin, Setiptiline 5 ng/g	Maprotiline 0.025–25 μg/g Mianserin, Setiptiline 0.005–5.0 μg/g	Blood[a] [83] Plasma [84] Urine [85,86]
Antiepileptic [87]	Valproic acid	Plasma	PDMS 100 μm	200 μl Plasma dialysate, Phosphoric acid to pH 2.5, immersion, 3 min	1 min, 210°C	GC–FID	1 μg/ml	2–20 μg/ml	Plasma[a]
Antihistamines [88]	Diphenhydramine, Doxylamine, orphenadrine Terodiline, carbinoxamine, Chlorpheniramine, Diphenylpyraline, Triprolidine, benactyzine, Homochlocyclizine, Cloperastine, clemastine, Piperilate	Urine, Whole blood	PDMS 100 μm	0.5 ml Whole blood, 0.5 ml Water, NaOH 98°C, headspace, 10 min, No water dilution for urine samples	5 min, 250°C	GC–FID	Blood 76–473 ng/ml Urine 13–186 ng/ml	Blood 0.18–1.4 to 5.6–5.7 μg/ml Urine 0.14–0.35 to 2.2–5.7 μg/ml	Blood, urine[a]
Barbiturates [8]	Barbital, butabarbital, Butalbital, amobarbital Pentobarbital, secobatbital Hexobarbital, Phenobarbital	Water Urine	Carbowax/DVB 65 μm	4 ml Water/urine 20 min, ambient, immersion	12 min, 250°C	GC–MS	1 ng/ml	10–1000 ng/l	Serum [89] Urine[a]
Neuroleptic [90]	Clozapine	Plasma	PDMS 100 μm	0.25 ml Plasma, 8× dilution with water, NaOH, NaCl, 30°C, immersion, 30 min	1 min, 260°C	GC–NPD	30 ng/ml	100–1000 ng/ml	Plasma[a] [91]

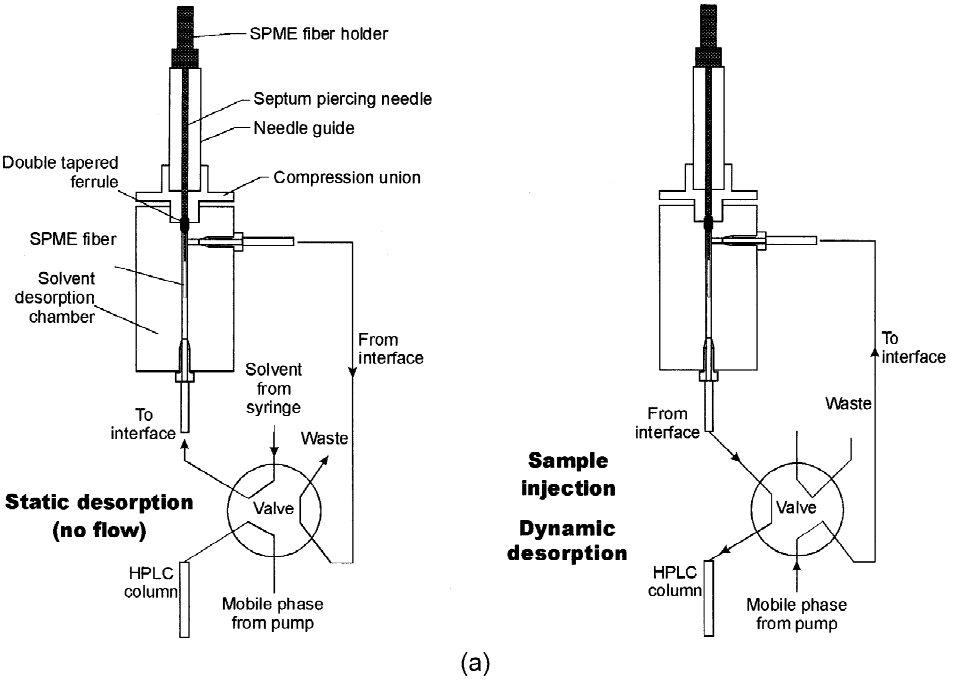
Table 1. Continued

Compound class	Compounds	Matrix	Fibre	Extraction	Desorption	Separation/ detection	Limit of detection	Linearity	References
Steroids [92]	Estriol-3-sulfate Triamcinolone, Prednisone, cortisone, Prednisolone, Hydrocortisone, Fludrocortisone, estrone-3-sulfate, flumethasone, 6-Methylprednisolone, Dexamethasone, Deoxycorticosterone, Budesonide epimer A, Budesonide epimer B	Urine	Carbowax/DVB templated resin, 50 μm	3 ml Urine, NaCl, Immersion, 15 min, Ambient	5 min, static, Methanol:water (1:1)	LC–ESI–MS	4–30 ng/ml	20–20 000 ng/ml	Serum, derivat. [93] Urine[a]
UV absorbent [94]	Benzophenone-3 2,4-dihydroxy Benzophenone 2,2′-dihydroxy-4-Methoxybenzophenone	Urine		4 ml, Immersion, 15 min	13 min, 250°C	GC–MS	5–10 ng/ml	50–1000 ng/ml	Urine[a]
Toxicants									
Cyanide [95]	cyanide	Whole blood	Carbowax/DVB 65 μm	0.5 ml Blood, Na_2SO_4/H_3PO_4 50°C, headspace, 45 min	30 s, 150°C	GC–NPD	0.02 μg/ml	0.04–4.0 μg/ml	Whole blood[a]
Ethanol [96]	Ethanol, acetone, isoprene	Breath	PDMS/DVB 65 μm	30 s Breath Stream through mouthpiece	15 s, 200°C	GC–MS	Ethanol 5.8 nmol/l Acetone 1.8 nmol/l Isoprene 0.3 nmol/ml	Ethanol 0–8 μmol/l Acetone 0–545 μmol/l Isoprene 0–100 μmol/l	Whole blood and urine [97] Breath[a] Hair [98]
Nicotine [99]	Nicotine, Cotine	Urine	PDMS 100 μm	1 ml Urine, K_2CO_3, 80°C, headspace, 5 min	3 min, 250°C	GC–MS	Nicotine 0.005 μg/ml Cotinine 0.3 μg/ml	Nicotine 0.01–0.2 μg/ml Cotinine 0.5–10 μg/ml	Urine[a]
VOCs [100]	Trichloroethylene Tetrachloroethylene	Tissues		1 g Tissue, Homogenized on ice, headspace, 60°C, 1 min	15 s, 250°C	GC–ECD	Trichloro 5 ng/g Tetrachloro 3 ng/g	Trichloro 10–750 ng/g Tetrachloro 10–150 ng/g	Blood [97,101,102] Urine[a] [97,103] Tissues[a] Gastric sample [103]

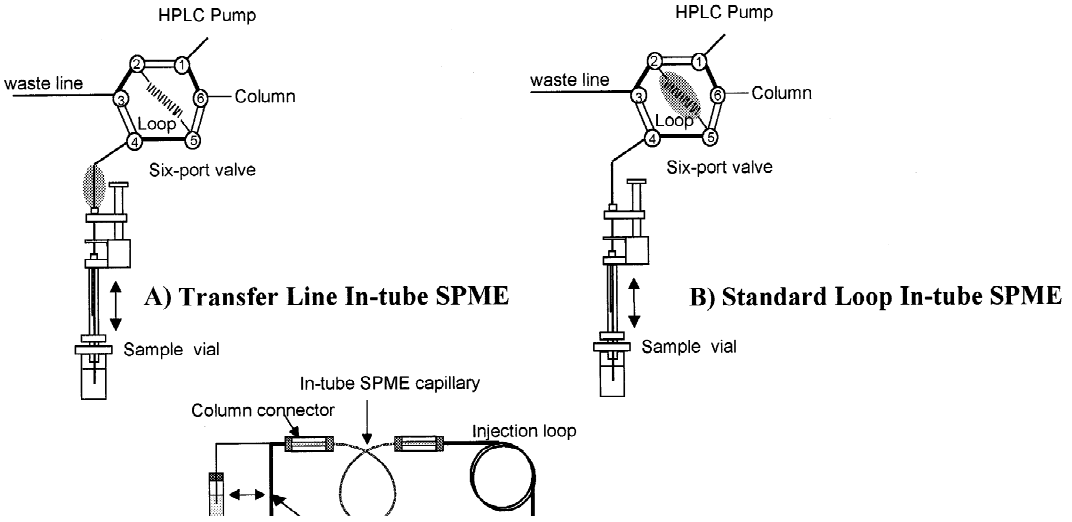
[a] Denotes the reference used as an illustrative example.

tion may be accomplished by introducing the fibre to the desorption chamber, and then immediately switching the valve to desorb analytes as mobile phase flows over the fibre. A schematic of this interface, is shown in Fig. 10a.

For in-tube SPME, a section of fused-silica GC capillary, coated on the inside with an appropriate extraction phase, is used in place of a section of tubing in a conventional autosampler. Schematics of the possible arrangements are shown in Fig. 10b. To date, most of the published reports have used sections of standard GC capillaries, coated primarily with poly(dimethylsiloxane) and poly(ethylene glycol) based phases. When implemented on the HP1100 autosampler or related models, the existing sample loop is normally left in place, with the capillary connected between it and the injection needle. This prevents contamination of the metering



(a)



(b)

Fig. 10. (a) Commercial fibre SPME/HPLC interface design (Supelco), static vs. dynamic desorption; (b) in-tube SPME approaches: (A) extraction capillary in place of transfer line; (B) extraction capillary in place of standard loop; (C) extraction capillary incorporated in flush loop injection.

pump during the extraction phase. The capillary is connected by adding a sleeve of appropriate ID PEEK tubing over the ends of the capillary, and then adding standard stainless steel fittings and ferrules.

During extraction, sample is aspirated from the sample to the capillary, and then dispensed back into the sample. This process is repeated until either an equilibrium extraction has been accomplished, or sufficient analyte is extracted to allow the desired method sensitivity. While this method is limited to relatively clean matrices, due to the potential for plugging the system if dirty samples are extracted, the method has a clear benefit in the automation of analysis.

3.8.1. Extraction time profile/aspirate–dispense step profile

Whether for fibre or in-tube SPME, it is beneficial to understand the extraction profile over time. If analyses are performed under equilibrium conditions, extraction conditions such as extraction time and agitation can be ignored as they no longer have an effect on the amount of analyte extracted. In the case of fibre SPME, an absorption–time profile is constructed by performing several extractions at different extraction times, exactly as is done for SPME–GC applications. Fig. 11 shows extraction time profiles for methamphetamine and amphetamine, for headspace fibre SPME with GC–FID detection. The amount of analyte extracted normally increases rapidly at first, and then slows until at equilibrium extraction, no further increase in amount extracted occurs. Compounds with a low partition coefficient are characterized by shorter equilibrium extraction times, and lower equilibrium amounts extracted. Compounds with a high partition coefficient by contrast take longer to reach equilibrium, and have higher equilibrium amounts extracted. In Fig. 11 methamphetamine has the higher partition coefficient. In this figure we see that methamphetamine does not reach equilibrium extraction within the 180 min tested. Amphetamine by contrast, has reached equilibrium extraction after about 1 h.

In the case of in-tube SPME, the number of aspirate/dispense steps is varied, instead of the extraction time, in order to construct an extraction profile. Fig. 12 shows an example of this for the in-tube SPME extraction of amphetamines with Supelco's Omegawax 250 capillary incorporated into the Hewlett-Packard 1100 autoinjector, and ESI–MS detection. The very small error values seen in this figure are representative of method precision for automated extraction and injection. Because of this, non-equilibrium extraction is quite practical to improve throughput, where the added sensitivity possible with equilibrium extraction is not important.

In some cases, it has proven difficult to establish equilibrium extraction for in-tube SPME. It is assumed that this is due to the fact that in every aspirate/dispense step, a plug of analyte free mobile phase follows the plug of sample during its travel

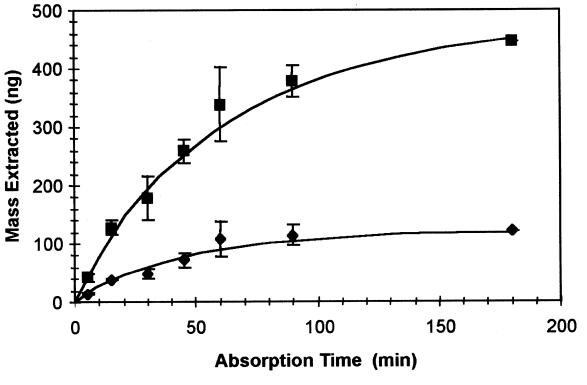


Fig. 11. Effect of partition coefficient on equilibrium time profile.

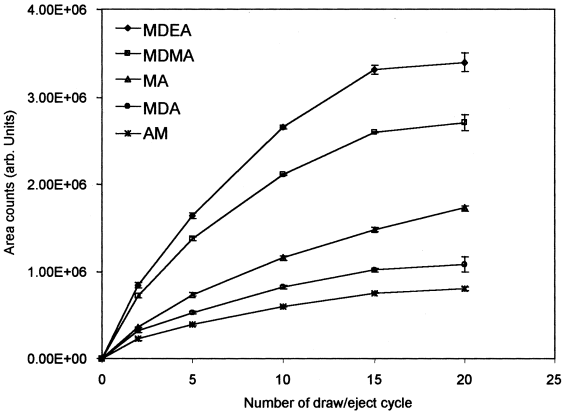


Fig. 12. Extraction profiles for in-tube SPME, extraction of amphetamine and related compounds.

through the capillary. Thus, during the dispense step, analyte previously extracted by the capillary coating may desorb into the mobile phase plug. While this can complicate the determination of equilibrium extraction levels, extraction at equilibrium levels is not necessary, as discussed above.

Several early papers on in-tube SPME studied the technique for the analysis of a range of pesticides [104–106]. In addition to the more recent results obtained for the analysis of amphetamines [107] by in-tube SPME, encouraging results have also been obtained for ranitidine [108] and a range of β-blockers [109].

3.8.2. Extraction coating selection-in-tube vs. fibre

With both fibre and in-tube SPME the decision of which extraction phase to select must be balanced between attaining equilibrium extraction conditions which provide good precision, versus maximal sensitivity and throughput. Fig. 13 shows the results of headspace fibre SPME extraction of amphetamines. While analysis here is by GC–FID, it is completely analogous to LC methods. In this case, the PDMS coating would appear to exhibit the lowest partition coefficient for these drugs, based on the fact that both drugs reach equilibrium extraction quickly (amphetamine, <5 min; methamphetamine, 15 min). It is expected that both the poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) and PA coatings would eventually reach equilibrium extraction at levels higher than those achieved by PDMS. If one wanted to maximize sensitivity with a shorter extraction time, and was able to accurately control extraction time and agitation conditions, the PDMS/DVB fibre would provide the highest levels of drug extracted in the times tested, and thus would produce the best limits of detection. If method precision was the overriding determinant, the PDMS coating would be preferable, as equilibrium extractions will normally provide the best precision. It is convenient to select an extraction time equal to or less than the separation/detection time, to maximize throughput.

Another consideration in fibre selection is the ability of the fibre coating to withstand the extraction medium. This is equally true for in-tube SPME. Poly (dimethylsiloxane) coating is not very tolerant of basic conditions. With mildly basic conditions, it shows visual degradation after repeated exposure to these conditions. With strongly basic conditions, the polymer is immediately stripped from the fused-silica core. The same holds true for the siloxane coatings used to date for in-tube SPME.

In the case of in-tube SPME, capillary selection may be determined by the shape of the extraction profile (profile of extraction versus number of aspirate/dispense steps) as seen in Fig. 12. If extraction time is determined by analysis time, capillary selection may simply involve testing sensitivities of several coatings in that time as seen in Fig. 14 for the analysis of a series of β-Blockers [110]. In addition to monitoring the extent to which various coatings will extract the analyte of interest, it is also important to monitor the coating durability under exposure to the necessary mobile phases, and any desorption solvents required. This is more difficult than monitoring a fibre coating, as one cannot directly visualize the coating on the inside of the

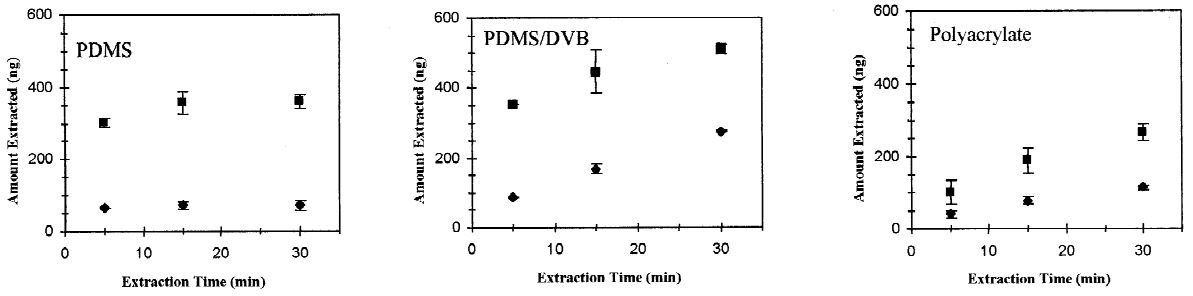


Fig. 13. Use of equilibrium profiles in extraction phase selection.

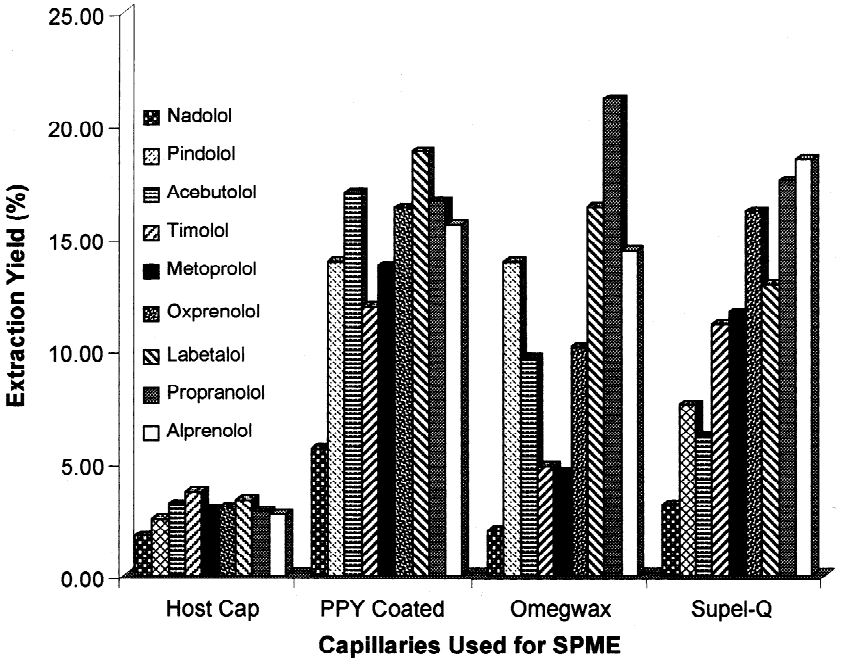


Fig. 14. Effect of polymer chemistry on extraction efficiency for in-tube SPME.

tube. Thus it is necessary to monitor for loss in response to known analytes.

3.8.3. Agitation during extraction

The degree of agitation in an extraction affects the dimensions of the boundary layer around the extraction phase. As the extraction phase removes analyte from the sample, the sample in the immediate vicinity of the extraction phase becomes depleted of analyte, and the size of the resulting boundary layer becomes limiting in terms of equilibrium extraction time. Efficient agitation reduces the size of the boundary layer and thus enhances the speed of extraction.

For fibre SPME, agitation is typically accomplished by vortexing of the vial, or magnetic stirring. For maximal agitation the fibre should be positioned half-way between the vial wall and the edge of the vortex. A vortex extending about three-quarters of the way down the sample generally indicates efficient agitation. For in-tube SPME, the rate of the aspirate/dispense fluid flow affects the speed of extraction. There is an upper limit to the speed at which the fluid can be drawn along the capillary. At some point, the draw rate is high enough that bubbles start to form inside the capillary, which causes a drop in extraction rate. At lower rates of fluid flow during aspirate/dispense steps, extraction time becomes very long. For 0.2–0.25 mm I.D. capillary, flow-rates of 60 to 100 μl/min are typically used during extraction.

We have investigated the possibility of a physical mixing of the sample with the mobile phase at their interface in the extraction capillary. While mixing does occur, if extraction efficiency is high enough, the amount of analyte present in the capillary due to mixing is not significant relative to the amount present due to extraction and pre-concentration. The flush-loop implementation is particularly prone to difficulties with mixing if extraction efficiency is low and there is no possibility of excluding the mixture of mobile phase and sample from the injection plug. The other implementations seen in Fig. 10b are more convenient for eliminating the mixture.

3.8.4. Applications of SPME–LC

Fibre SPME–LC methods have been described by Lock et al. for the analysis of tetracycline antibiotics [80]and Volmer and Hui for a series of corticosteroids [92]. For the tetracycline analysis, desorption from the fibre (carbowax/templated resin) was performed in the commercial desorption chamber in static mode, with acetonitrile:water (15:85) for 5 min at 40°C, followed by LC–MS analysis. The article by Lock et al. is discussed in more detail in the section on milk analysis. Volmer and Hui also used the carbowax/templated resin fibre for the analysis of corticosteroids in urine. After direct extraction from urine supplemented with 25% NaCl, the fibre was placed in the desorption chamber filled with methanol:water (50:50) for a 5-min static desorption, followed by separation and detection by LC–MS. Limits of detection ranged from 4 to 30 ng/ml, with the sensitivity limited by the mass spectrometric detection, not the absolute amount injected.

Lam et al. describe the use of fibre SPME–LC for the analysis of microcystins as a cost effective unequivocal correlation between density of cyanobacteria known to produce microcystins and the toxin level in water [111]. Given that these potent toxins and tumour promoters are effective at trace levels, and a general lack of effective water treatment processes, particularly in developing countries, a simple cost effective method for their analysis would allow the generation of a complete risk assessment to determine the likelihood of exposure to these toxins through various channels.

Saito et al. have recently described an interesting innovation for in-tube SPME, where a fine wire is incorporated in the lumen of the extraction capillary, to effectively increase the surface-to-volume ratio in the analysis, thought to limit the extraction efficiency [86]. In addition, the development of extraction phases better suited to extraction of relatively polar compounds from aqueous samples will enhance the sensitivity and overall utility of the method. Initial steps in this direction have been shown by Wu et al. with the development of pollypyrrole polymers [110]. Their superior performance for the analysis of β-Blockers, relative to the initial publication [109] hold promise for improved and broader application of the method in the future.

3.9. Considerations for biological matrices

3.9.1. Urine

Urine is one of the most commonly studied biological matrices for drug analysis, particularly because of its relative ease of collection, and because it is a nearly universal means of excretion of parent drug compounds, metabolites or both. As a matrix, it has moderate complexity, and typically contains both organic and inorganic constituents, as well as a relatively high salt content. In addition, urine suffers from high variability. Results are typically normalized by expressing drug concentration per gram creatinine, in order to circumvent the highly variable dilution that results from the body's attempts to maintain water balance [99,100].

Drug extraction efficiency can vary due to several reasons. Variations in urine pH effects both elimination of many drugs, as well as extraction efficiency when effects on the drug's acid/base balance are significant. Variations in ionic strength can also have a significant effect on drug extraction. The additional components of the sample may also provide for competitive sorption, further reducing the amount extracted. While this provides an opportunity for studying distribution of a drug between the various components of a urine sample, it is often total urine concentration which must be reported. This is accomplished by using an appropriate internal standard, or standard addition for quantification. If urine samples must be significantly diluted prior to extraction, because of very high drug concentrations, variability in the matrix becomes less of a significance for extractions.

Felix et al. studied the effect of urine matrix on the extraction of benzophenone-3 and its metabolites, relative to extraction from pure water [94]. They found a reduction in extraction efficiency of 31 to 58%. Drug concentrations were quantified using a three-point standard addition curve and precision at 50 ng/ml ranged from 5 to 8% RSD. The detection limit for the extraction ranged from 5 to 10 ng/ml for the various compounds.

Koster et al. [76] provide a helpful discussion of the SPME optimization for urine analysis. In particular they report on the impact of the turbidity that commonly results from increasing sample pH. They

observed significant turbidity in samples adjusted to pH 9.5 with 0.3 g/ml NaCl. The turbidity was eliminated from the sample in two ways. First samples were centrifuged after a 1:1 dilution with 0.2 *M* borate buffer pH 9.5 and addition of NaCl (0.3 g/ml). The clear supernatant was analysed and there was no difference in extraction yield of samples spiked before and after centrifugation. Second, samples were diluted at least five times with 0.1 *M* borate buffer pH 9.5 containing 0.3 g/ml NaCl. This method has the advantage that no additional steps (salt addition, centrifugation) are required, and the final sample still contains sufficient salt to enhance extraction (0.24 g/ml).

Koster et al. also provide a good discussion of the 'salting out' effect in terms of use of the Setschenow equation for predicting the impact of salt concentration on partition coefficients. Variation in ionic strength in urine samples is commonly normalized by the addition of salt. This is only feasible where the addition of salt does not decrease extraction efficiency, or where there is sufficient sensitivity such that a decrease in extraction efficiency is not detrimental. Commonly 15 to 30% salt (w/v) is employed. It has been reported that the maximum salt content in urine is about 65 mg/ml [112], which if 15% salt were employed for normalizing, would represent an approximately ±4% variation in ionic strength.

Abdel-Rehim et al. have described the use of an internal standard for the urine and plasma analysis of lidocaine and three of its metabolites [72]. The internal standard selected was structurally related to the other compounds. Absorption time profiles were given for all five compounds, demonstrating that the internal standard was intermediate in terms of both its equilibrium extraction time, and extraction efficiency. A standard curve with at least six standard concentrations and one zero concentration was prepared. The peak area ratios of the analytes relative to the internal standard were measured. The standard curve was constructed without the zero concentration. The calibration curve was described as:

$$y = Bx + A \quad (24)$$

where y is the peak-area ratio, x is the concentration and A and B are the y-intercept and slope respectively. The linear regression equation was weighted ($1/x$). Accuracy at just above the LOQ ranged from 88% to 103%. Precision for all compounds at concentrations from the LOQ to the upper end of the linearity range varied from 3.9% to 13.3% (intra-day) and 3.8% to 14.9% (inter-day). The internal standard was included at a concentration in the upper end of the linear range.

3.9.2. Hair

Hair is a popular target for drug analysis as it is relatively non-invasive to collect, and provides a historical record of exposure, even though it does suffer from variability in drug concentrations by hair type, due to drug affinity variations. An additional advantage of hair as a matrix for analysis, is that it is fairly nonpolar and so tends to absorb parent drug molecules, as they are typically less polar than metabolites. Because of this it is an ideal matrix for extraction of analytes to non-polar extraction phases, particularly where the parent drug is extensively metabolised and often non-detectable in other tissues.

Throughout most of its length the hair shaft consists of the keratinized remains of cells in three distinct layers [113]. The outermost layer (cuticle) is formed of overlapping squames (flattened cells) with their free edges pointing towards the tip of the hair. Internal to this is the cortex, which forms the greater part of the hair. It consists of tightly packed hard keratin which contains pigment and a few small air spaces. The medulla forms the core of the hair. It consists of soft keratin, and may be broken into discontinuous pieces. It only forms a significant part in coarse hairs. It contains larger air spaces fat globules and pigment.

Koide et al. have demonstrated the use of SPME for the analysis of amphetamines in human hair [55]. One mg of hair is hydrolysed in 0.2 ml of 5 *M* sodium hydroxide solution in a tightly sealed vial, by shaking at 75°C for 5 min. Because amphetamines are basic compounds, no further pH adjustment was necessary. A 100-μm PDMS fibre was exposed to the headspace in the vial and the sample was extracted at 50°C for 20 min. Desorption in a 220°C GC injector port required 30 s. Relative standard

deviations were below 10%, limits of detection were 0.1 and 0.4 ng/mg hair and the method was linear in the ranges of 0.4 to 15 and 4 to 160 ng/mg hair for amphetamine and methamphetamine respectively.

A recent review of the use of SPME for hair analysis surveys the application of HS–SPME to hair samples from several forensic and clinical cases [114]. The authors report that HS–SPME coupled with the hydrolysis of hair by 4% sodium hydroxide in the presence of excess sodium sulphate proved to be a convenient one-step method for the measurement of may lipophilic basic drugs. Detection limits were between 0.05 and 1.0 ng/mg. For cannabinoids, because of the phenolic hydroxyl group, it was necessary to change the sample pH after alkaline hair digestion, by adding excess orthophosphoric acid, although the detection limits were such that only cannabinol could be detected in the hair of a user. Clomethiazole, a compound hydrolyzed in alkali, was measured by HS–SPME after extraction with aqueous buffer, to a detection limit of 0.5 ng/mg.

Quintela et al. also reported on SPME hair analysis of cocaine, methadone, and cannabinoids [115]. In the method, 50 mg of hair was washed with petroleum ether to remove passive contamination. It was then hydrolyzed with NaOH, neutralized, and SPME sampling was by direct immersion. A deuterated internal standard was used for quantification with analysis by GC–MS. Limits of detection were 0.1 to 0.2 ng/mg for cannabinol, cannabidiol and $\triangle^9$-tetrahydrocannabinol. 11-nor-$\triangle^9$-tetrahydrocannabinol-carboxylic acid was not detected, and is known to have an extremely low rate of incorporation into hair. The effectiveness of different decontamination procedures was also studied on passively contaminated hair. Limits of detection for cocaine, cocaethylene and methadone were not reported. Because of the nature of the hair digests, a maximum of 20–30 samplings was possible from the same fibre. The authors note that the efficiency of the fibre is easily evaluated by monitoring the chromatographic abundance of the internal standard, which would suddenly fall in the case of a deterioration of the fibre surface.

Sporkert and Pragst have studied lidocaine in hair of drug fatalities, and shown that it had been consumed for a long period of time as an adulterant of cocaine and heroin preparations [77]. Pragst et al. have demonstrated an SPME method for the analysis of ethyl palmitate, ethyl stearate and ethyl oleate in hair, as a marker of ethanol consumption [98]. The method has a distinct advantages over the direct determination of ethanol in identifying chronic alcohol abuse, as ethanol itself is found in almost every hair sample, mostly due to absorption from the environment. The ethyl esters of the fatty acids studied however, are formed through the metabolism of ingested ethanol.

3.9.3. Saliva

Similarly for analysis in hair, saliva is attractive for analysis of drugs because as it is non- invasive to sample. Also, quantitative measurement may reflect the non-protein-bound (i.e. therapeutically relevant) concentration of the drug in plasma. While it is a complex viscous mixture, compared to other biological samples, levels of protein and lipids are quite low, making it amenable to microextraction. Hall et al. have demonstrated the use of SPME for analysis of several cannabinoids in saliva without derivatization [63]. Reduced extraction efficiency was found for direct extractions, due to a coating of the 100-μm PDMS fibre polymer with protein and cellular material. The effect was minimized by diluting the samples 1:1 with deionized water, after spiking with standards, but just prior to extraction.

An additional substantial loss in extraction efficiency was noted relative to extraction from pure water, unrelated to coating build-up on the polymer. Extraction efficiency from saliva varied from 5 to 9% relative to 100% for extractions from pure water. The authors stated that the effect was likely due to a combination of reduced mass transfer kinetics in the sample due to its viscous nature, and binding of the compounds to proteins present in the sample. It was found that by acidifying the samples with acetic acid, the samples were clarified and proteinaceous material and cellular debris was precipitated and removed prior to SPME. After acidification, extraction efficiencies improved to 20–50% relative to extraction from pure water, and precision was also improved. This may be also due in part to complete protonation of the hydroxyl groups on the molecules during

extractions. The method was found to be linear from 5 to 500 ng/ml and the limit of detection was below 1 ng/ml.

The effect of coffee and beer drinking and cigarette smoking were also evaluated for potential negative impact on extraction, but none was observed. The method was shown to be accurate relative to an established liquid–liquid extraction method. For quantification, the use of a deuterated internal standard was compared with external calibration and standard addition. The internal standard quantification gave the best precision in analysis.

3.9.4. Tissue

The use of microextraction techniques, and SPME in particular is relatively new to the field of tissue analysis. An interesting article available at the time of writing concerns the use of headspace SPME to monitor tissue distribution of the volatile dry-cleaning and industrial solvents tetrachloroethylene (CH_2Cl_4) and trichloroethylene (TCE), in a fatality due to exposure to these compounds [100]. Tissue homogenates were prepared from liver, myocardium, muscle, kidney cortex, cerebellum and brain stem. Blood and urine were also monitored. For tissue preparation, 1 g of tissue was homogenized on ice in a glass vial. Tissue standards were prepared from solvent-free tissue. Tissue homogenates were transferred to 2-ml glass vials with 25 μl internal standard solution (chloroform) or a methanolic solvent standard solution. After sealing, the vials were stirred and heated at 60° for 5 min. The headspace was extracted for 1 min with a 100-μm PDMS fibre. Trichloroacetic acid (TCAA) is a metabolite of both CH_2Cl_4 and TCE and was analysed after derivatization, by adding five volumes of 3-methyl-1-*p*-tolyltriazene (1 g/l) in diethyl oxide to one volume of homogenized tissue adjusted to pH 2.3 with phosphate buffer [116]. While metabolites were not detected in the tissue samples, both parent compounds were, with TCE found predominantly in the more liposoluble tissues.

3.9.5. Plasma

Both serum and plasma are routinely used for drug analysis. As for whole blood, the concentration of drug in this matrix gives a direct assessment of tissue exposure to the compound. Serum is the straw-coloured liquid that separates from the clot that forms in whole blood. Plasma is prepared from whole blood that has been treated with an anti-clotting substance such as heparin. It is the supernatant that results when the cellular components of blood are removed by centrifugation. As matrices for drug analysis, the significant difference between the two is that serum does not contain fibrinogen and some clotting factors (2.5–5% of proteins). A method developed for plasma can normally be applied without modification to serum.

Drugs will bind to plasma proteins to varying degrees depending on their individual physicochemical properties. In general, acid and neutral drugs bind primarily to albumin, and basic drugs primarily to α-acid glycoprotein. Only free drug is available for extravascular distribution and elimination, is able to cross cellular membranes and interact with drug receptors [117]. Although it is general practice to report total drug concentration (free plus protein-bound) in serum, SPME is ideally suited to measuring free drug (therapeutically relevant) concentration, as the protein binding equilibrium is not disturbed where an insignificant amount of free drug is removed in a microextraction

Ulrich and Martens provide an excellent overview of the use of SPME for drug analysis in plasma [84]. They describe a method for the analysis of antidepressant drugs and metabolites with the use of the structurally related chloramitriptyline as an internal standard.

The authors also report that other than water and electrolytes, human plasma consists of 7 to 8% plasma proteins. Of this, albumin is the main component (55%). Antidepressants also bind to α-acid glycoprotein (αAGP) which comprises about 1% of plasma proteins. With regard to the antidepressants studied, albumin binds with low effectivity but high capacity, and αAGP binds with high effectivity and low capacity. Because protein binding will influence the amount extracted in an SPME study, variations from normal protein content can influence the results and could introduce a significant bias in the analytical method.

While blood plasma normally has quite consistent composition, some disease states can drastically alter this. For instance, in hepatic and nephrotic diseases albumin can be decreased to about 50% of the normal level. In inflammatory diseases αAGP can increase by as much as a factor of six. In healthy individuals the αAGP concentration can vary by about a factor of three.

While the impact of this variation is largely eliminated by the use of an internal standard that is a chemical analog of the compounds studied, Ulrich and Martens also provide a thorough discussion of the impact on the calibration curve of differences in protein binding between analytes and an internal standard, caused by changes in the protein composition of the sample. They conclude that only minimal changes of calibration occur within normal variations of plasma proteins (between 7 and 8%). Pathological changes in αAGP concentration also do not pose a significant problem for calibration. However, pathological changes in normal total plasma protein concentration can negatively impact calibration by internal standard. For some compounds, a variance from a normal level of 7.5% to the pathological level of 3.75% produced a 50% difference in the peak area ratios of the drug to the internal standard. The dependence of peak area ratios on plasma protein concentration is however reasonably linear. If plasma protein concentration is determined, and composition is consistent, an accurate assay of the drug concentration is possible.

For some of the compounds studied, the protein binding was so significant that the sensitivity of the method was insufficient. However, absolute peak areas were increased at lower concentrations of plasma proteins. Thus the sensitivity of the method can be significantly improved by diluting the samples with water. The authors cite two reasons for this. Firstly, they cite that the resulting decrease in viscosity will increase diffusion, allowing for a higher total amount extracted for a given extraction time. The assumption here is that the extraction is performed under non-equilibrium conditions. The increased diffusion would allow the extraction to proceed closer to equilibrium levels for a given extraction time.

Secondly, they describe the ‘extraction of proteins with water’ as a significant contributing factor for the increased extraction levels. This can be explained by examining the equation for the equilibrium constant of association–dissociation reaction equilibrium, described by the authors. The chemical reaction equilibrium for the association of drug and protein is described as:

$$\mathrm{Drug_{aq}} + \mathrm{protein} \xrightarrow{K^{\mathrm{pr}}} \mathrm{drug_{protein}} \tag{25}$$

K^{pr} is the equilibrium constant of the association–dissociation reaction equilibrium and can be described as:

$$K^{\mathrm{pr}} = \frac{c_{\mathrm{b}}}{c_{\mathrm{fr}}(S_{\mathrm{v}}^{0} - c_{\mathrm{b}})} \tag{26}$$

where c_{b} is the concentration of drug bound to plasma proteins, c_{fr} is the concentration of unbound drug, (free concentration) and S_{v}^{0} is the maximal number of sites for drug binding per unit sample volume. As K^{pr} is a constant, the ratio on the right side of Eq. (26) must remain constant as the sample is diluted with water. Although c_{b}, c_{fr} and S_{v}^{0} all decrease with dilution, the decrease in c_{b} will occur at a faster rate than the decrease in c_{fr}. This is because while the numerator of the ratio in Eq. (26) contains only c_{b}, the denominator contains a multiplication of two concentration terms. The result is that the ratio of $c_{\mathrm{b}}/c_{\mathrm{fr}}$ decreases with dilution, and hence relative extraction efficiency of the analyte increases.

Given the foregoing discussion, the most suitable plasma analyses for SPME are those where either protein binding is minimal, or drug concentration is sufficiently high (e.g. toxicological samples) that sufficient sensitivity is attainable.

3.9.6. Blood

Much of the discussion for analysis of drugs from plasma also applies to analysis from whole blood. The difficulties in analysing low concentrations of highly bound drugs are similar, and the greatest application is in analysis of either compounds with minimal binding, or samples with high drug concentrations.

Whole blood is often extracted with no pre-treatment. The blood sample is added to a sample vial, and extraction is initiated. It is preferable to extract from blood collected with heparin or another anti-clotting agent. As heparin is a mucopolysaccharide and may interact with some analytes, EDTA or one of the other anticoagulants may be preferable depending on circumstances. EDTA of course should not be used where metallics or organometallics are to be analysed.

Protein degradation by acid treatment prior to extraction has been shown in some cases [71,88], but there has been no clear evidence that this has had any impact on rates of extraction.

Recoveries as high as 86% have been reported for the analysis of malathion from whole blood, after a ten× dilution [118]. Recoveries of 0.3 to 5% for a range of antihistaminics [88] and 10% for phencyclidine [71] are more typical.

Most reports of SPME analyses of whole blood employ headspace analysis. Direct immersion extraction has been reported for a range of local anaesthetics [74]. In this case though it was the clear supernatant resulting from deproteinization with perchloric acid that was analysed.

Internal standards are used for quantification in virtually all studies reported to date, and generally a chemical analog of the compound(s) of interest is employed.

3.9.7. Milk

For human milk the main constituents are water (88%), proteins (3%), lipids (ca. 3%) and carbohydrate in the form of lactose (6.8%). The lipids are in the form of fat droplets suspended in the watery matrix [119]. The collostrum that appears in early lactation differs significantly from true milk in that it contains less lactose and virtually no fat [120]. The composition of cow's milk has remained fairly constant over the years, although total production has increased significantly. It has a similar total water content (87.4%), 3.9% fat, 3.2% protein, 4.6% lactose and 0.9% other solids (minerals, vitamins etc.). The non-water constituents are present in different physical forms; dissolved (lactose), colloidally dispersed (protein) and emulsified in water (lipids or fats) [121]. The authors also provide an overview of milk composition by cattle breed.

Cow's milk can serve as a means of exposure of the population to a variety of compounds, and in most jurisdictions, is carefully monitored in this regard. Human milk can serve both as a means of exposure of a newborn to compounds the mother has been previously exposed to, and provide a relatively convenient means of biomonitoring for toxicant exposure. Human milk is quite constant in composition from the third week on, but the colostrum from early lactation, has significantly different and variable proportions of protein, fat and carbohydrate as noted above. Such variation could impact analyses if not taken into account.

The number of reports of drug analysis from milk is not high at the time of writing, but reports of analyses of non-drug compounds serve to highlight some considerations for SPME milk analysis that would be applicable for analysis of drugs also. Rohrig and Meisch reported on the analysis of organochlorines in breast milk, as a fast biomonitoring method to survey pesticide contamination [122]. A headspace method was selected, and proteins were denatured with perchloric acid prior to analysis. The authors reported that this was sufficient to disturb the protein/analyte complexes and protonize the phenolic compounds, thereby enhancing amount extracted, although the absolute binding of analytes to milk matrix components was not reported. Sample ionic strength was increased with sodium sulphate and samples were heated to 100°C for extraction.

DeBruin et al. reported a method for the headspace analysis of monocyclic aromatic amines from human milk, as a means of biomonitoring for the exposure to these potentially carcinogenic compounds [123]. The method involved treating the samples with strong base, salt and heat, to optimize sensitivity. This resulted in the saponification of the milk fat. The authors report that the resulting contamination of the samples with base-hydrolyzed lipid components significantly complicated the extraction. Lipids were subsequently removed from the samples by centrifugation prior to analysis. Because the analytes were not highly lipophilic, the loss to milk fat was not significant. The degree of loss however, correlated well with the components' K_{ow} values. It

was found that milk was intermediate between urine and blood, for degree of analyte binding to matrix components.

Lock et al. have reported on the analysis of a series of seven tetracycline analogues, with analysis by LC–MS [80]. Tetracycline and its analogues have seen broad use as additives for animal feed, and their presence has been noted in both meat and milk supplies. The method developed was proven rapid and sensitive and as it eliminated the need for lengthy sample clean-up, was extremely economic in comparison with wet chemistry procedures. During method optimization, the addition of saturated KCl to aqueous samples was shown to significantly enhance sensitivity. When milk samples were tested however, the KCl caused a precipitation of the protein component of the milk sample. The resulting mixture had a very high viscosity and efficient SPME extraction was precluded. While it would have been possible to filter the samples to remove the denatured protein, the authors decided to eliminate the addition of KCl instead, to maintain the simplicity of the method. The observed LOD of the method was ca. 100 ppb from 2% homogenized milk. The authors state that the sensitivity of the method may be improved by lengthening the extraction time, thus making the method in better accord with the U.S. Food and Drug Administration limit of 80 ppb.

3.9.8. Breath

Analysis of compounds in breath is a newer area of microextraction investigation, with reports appearing for analysis of ethanol, acetone and for isoprene as an indicator of metabolic state [96,124]. The authors used differing approaches to sampling in these two reports. Grote and Pawliszyn used a custom designed mouthpiece that was fitted over the end of the manual SPME sampler, allowing direct sampling of expired breath. Hyspler et al. collected expired breath in Tedlar bags, and the fibre was subsequently exposed directly to the contents of the bag.

Hyspler et al. noted that both temperature and humidity can have a large effect on extraction, and their values must be controlled. However conventional methods employing adsorbent cartridges or cryotrapping are also sensitive to variations in both carbon dioxide and moisture, and generally suffer from poor reproducibility. The impact of carbon dioxide on the SPME method was not reported, but a 35% decrease in amount extracted by carboxen/PDMS fibre, in a water vapour saturated atmosphere compared with extraction from dry air was observed. They employed 40°C sampling from the Tedlar bags to ensure complete evaporation of condensed water and isoprene in the bags.

Variation in ethanol and acetone, two common components of expired breath produced no variation in adsorption of isoprene. As it was not feasible to perform GC–MS at the patient's bedside, the effect of transportation and storage on the method was analysed. Samples were stable for 6 h in the Tedlar bags, but extended storage was not feasible. The authors speculate that the isoprene likely diffused through the wall of the bag, or slowly adsorbed to it. Samples stored in SUMMA cannisters were stable for at least two weeks. The method allowed for the determination of isoprene content in human breath in the range of 0.25 to 40 nmol/l. Isoprene content reported by other authors ranged from 1.6 to 48.5 nmol/l, indicating the reported method is generally suitable for monitoring of isoprene in breath.

Grote and Pawliszyn also studied the impact of temperature and humidity on the extraction. They noted an advantage of SPMF over conventional methods, in that it does not concentrate H_2O or CO_2 or allow a build-up of contaminants. They studied the relationship between $\log K$ and $1/T$, and its use in the calibration of the method for any given temperature, thus eliminating the impact of sampling temperature variation on accuracy.

The partition coefficient between the fibre coating and the gaseous matrix can be expressed as:

$$K = \frac{n_f^{\infty}}{c_s^{\infty} V_f} \tag{27}$$

where n_f^{∞} is the amount of analyte extracted from the gas sample by the fibre at equilibrium, V_f is the volume of the fibre coating and c_s^{∞} is the equilibrium concentration of the analyte in the sample. Where an insignificant amount of analyte is removed by the fibre, relative to the total amount present, c_s^{∞} can be replaced by c_s^0, the initial sample concentration. This

simplification is possible when the sample volume is at least two orders of magnitude larger than the product KV_f [125]. This assumption is true when sample volume is 1 l, considering a constant fibre volume of the 100-μm PDMS fibre of 6.91×10^{-4} cm^3, and K values less than 1000. Such K values are reasonable for the range of highly volatile compounds often found in breath. In this case, the determination of K values is possible by the following equation:

$$K = \frac{n_f^{\infty}}{c_s^0 V_f} \tag{28}$$

In practice various values of K are determined in a calibration step, at a series of known temperatures. Sampling is then performed on-site, and the sampling temperature noted. Because of the linear relationship between $\log K$ and $1/T$, K at the sampling temperature can be calculated. Thus the determination of n_f^{∞} for the unknown sample can be correlated to the sample concentration at any sampling temperature, so long as n_f^{∞} is small.

3.9.9. Illicit pharmaceuticals

Illicit preparations of drugs of abuse may be prepared by various synthetic routes, and for each of these, incomplete and side reactions may also occur. Extensive purification of the active ingredients is not normally performed following synthesis. As a result, several impurities are typically present in these preparations, and impurity profiling may be used to track the source of these products when they are confiscated. Liquid–liquid extraction or occasionally SPE have been used to prepare samples of confiscated materials for profiling by either GC or HPLC.

Recently, the use of SPME has been evaluated for impurity profiling in confiscated amphetamine and ecstasy tablets [126]. For ecstasy tablets, 10 mg of granulated tablet was mixed with 5 ml of 0. 1 *M* acetate buffer (pH 5.0), sonicated for 10 min and heated to 90°C. SPME headspace extraction was performed for 30 min. For amphetamine powders, 10 mg was mixed with 5 ml of 0. 1 *M* acetate buffer (pH 5.0) and the SPME fibre was immersed in the sample for 30 min at 20°C. Acidic extraction pH was employed to reduce the amount of the basic active ingredient extracted, in order that impurities would be more easily observed.

For ecstasy tablets, it was observed that the PDMS/DVB fibre extracted more impurities than the 100-μm PDMS fibre, substantially increasing the information content of the profile. Headspace extraction was preferred to avoid contamination of the fibre by tablet components. For amphetamine powders, direct immersion extraction was feasible, and the two fibres provided equivalent profiles. The repeatability of profiles was consistent with those attained from liquid–liquid extraction, indicating similar abilities in differentiating between closely related but different drug seizures. Interestingly, for ecstasy, isosafrole was identified as a precursor for synthesis. With liquid–liquid extraction, this compound was difficult to analyse, due to its high volatility. With SPME extraction however, it could be quantified to 2 ng/g tablet.

There has also been a report on the use of SPME for profiling of manufacturing by-products and impurities from an illicit drug seizure of 4-methoxyamphetamine [127]. The results indicated the synthetic route likely used in the preparation of the compound, and the method was found to be rapid, non-destructive and give results complementary to those from conventional liquid–liquid extraction.

3.10. Pharmaceutical analysis

In the design of pharmaceutical products, the nature of the active ingredient is only one of several important considerations. It is well known that pharmaceutical products from different manufacturers, containing the same active ingredient and dosage, can produce markedly different circulating concentrations of the active ingredient. Optimal drug formulation is a crucial step in the design of products that produce optimal therapeutic effect. In addition to meeting regulatory standards for active ingredient identity, purity and stability, products are typically optimized utility and bioavailability to assure the best competitive advantage. The nature of the formulation procedures and/or auxiliary (non-medical) components of a product can for example, make a product easier to handle, limit degradation, maintain

homogeneity, enhance bioavailability and targeting of the active ingredient to receptor sites, provide for additional routes of administration, and target the product for additional therapeutic indications for use [128]. These auxiliary in-active ingredients, known collectively as excipients, can also solubilize the active ingredient, suppress the growth of micro-organisms, provide bulk or a coating for a tablet or capsule, provide colour or mask an unpleasant taste or odour. A good working definition of excipients is ''agents in a medicinal preparation regardless of the nature, purpose or quantities employed, other than the components intended as the active ingredients'' [129].

Table 2
Summary of common excipients for pharmaceutical preparations

Bulk materials	**Controlled release binding agents**	**Flavourings**
Oral-solids	Stearate esters	Sugars
Starch	Cellulose derivatives	Artificial sweeteners
Lactose	Talc	Sodium/potassium chloride
Calcium salts	Aliphatic alcohols	Fruit flavours
Citric acid	Carboxylate polymers	Essential oils
Bicarbonate salts	Gel polymers	Imitation flavours
Dextrose	Copolymeric derivatives	
Cellulose	Resins	**Preservatives**
Oral-liquids	Phospholipids	Antimicrobials
Water	Waxes and wax esters	Antioxidants
Alcohol	Silicon dioxide	Chelating agents
Syrups		
Glycerin	**Capsules**	**Colouring**
Glycols	Gelatin	Tartrazine
Unabsorbed monosaccharides	+sucrose (hardening)	Carmine
Oils	+glycerin (softening)	Amaranth
Topical-solids	+sorbitol (softening)	Eosin
Mineral hydrocarbons	Microencapsulation	Erythrosine
Glycols	Plastics/proteins	Other D and C
Lanolin	animal and vegetable origin	
Silicates	Albumin	**Suspending, stiffening, emulsifying**
Beeswax	Phospholipids	Ionic agents
Topical-liquids	Cholesterol	Non-ionic agents
Water	Poly(methyl methancrylate)	Tragacanth and acacia
Alcohol		Mucilages and gums
Oils		Methylcellulose
Glycerin		Paraffin
Dimethylsulfoxide		Lubricants — talc
Injectables	**Coatings**	Lubricants — edible oils
Water (5% glucose, 0.9% saline)	Shellac	Fatty alcohols
Glycols	Silicones	
Glycerin	Mucilages/gums	**Propellants**
Nutrient fats	Gluten	Hydrocarbons
Fatty acid esters	Acidic polymers	Flurocarbons
Oils	e.g. carbomers	Other halocarbons
Suppositories	Basic polymers	Compresssed ambient gases
Fats with mp below 37°C, solidification >20°C	e.g. poly-L-lysine	
Mono-di-tri-glycerides	Cellacephate	**Buffers**
Hydrogenated, ethoxylated derivatives	Paraffins	Phosphate
Beeswax	Waxes	Borate
Polyethylenes	Beeswax	Acetate
Glycerin	Vegetable waxes	Citrate
Gelatin		Amino acid
		Tris

With so many tasks to perform, it is obvious therefore that the nature and numbers of excipients in various pharmaceutical products can vary widely. It is essential therefore, that for analysis of pharmaceutical products, the nature of these excipients is taken into account, for their potential impact on the process of analysis of the active ingredient. In microextraction, it is effects on the partitioning to an extraction phase that are of utmost concern. A good overview of the types of excipients used in different pharmaceutical preparations has been published previously [129], and is briefly summarized here.

The three most significant effects excipients can have on an extraction are:

- Providing a phase with competing affinity for the analyte of interest (e.g. polymers, proteins, immiscible liquids, amorphous or crystalline solids).
- Changing the pH of the sample (e.g. acids, bases, buffers).
- Changing the polarity of the sample (e.g. miscible liquids).

Because excipients can be present in concentrations far in excess of those of the active ingredient, use of solid sorbents for microextraction is often precluded for direct extractions. For other extraction phases, care should be taken to ensure that the interface between the sample and the extraction phase does not become fouled by an excipient. It is recommended that headspace extraction be used where possible.

Excipients can be divided based on either formulation type or excipient action. In this discussion, formulation type and significant excipient actions for each are described, followed by a listing of types of excipients commonly used for different actions with impacts they may have on microextractions. Table 2 provides an overview of common excipients for various drug formulations. Material for this section has been largely derived from the two textbooks referenced above.

3.10.1. Excipients by formulation type

3.10.1.1. Oral solid formulations: e.g. tablets

In addition to fillers, excipients are commonly used to control disintegration, dissolution, release kinetics, pH, compressability and provide effervescence, encapsulation, coating, flavour and sweetness. Excipients may also be used to improve product flow during production although it is more common to carefully choose all excipients such that the flow of the end product is satisfactory. It is difficult to 'fix' a blend with poor flow characteristics by the addition of one or more excipients, and makes the product more expensive and difficult to manufacture.

3.10.1.2. Oral liquid formulations: e.g. elixirs

Elixirs are sweetened alcoholic solutions with an ethanol content that is either low (8–10%) or high (73–78%), with glycerol and amounts of water varying from none to over 90%. Common excipients include fillers, flavourings, preservatives and those to address solubility and stability problems. Controlled release is often achieved by entrapping or coupling the active ingredient in an unabsorbable complex. Encapsulating may also be used. Ethyl alcohol is often used to enhance solubility of the active ingredient.

3.10.1.3. Topicals

Excipients in topicals may be present to assist the active ingredient in penetrating the skin, preventing it from washing off, or providing for an occlusive dressing at one extreme, to a vanishing effect at the other. In terms of penetration, the formulation may be required to allow penetration to the skin only, to the underlying tissues, or to the circulatory system. Topicals are further subdivided into solid and liquid formulations.

[Sub category of 3.10.1.3] Solid formulations: e.g. creams/ointments/gels

It is often difficult to distinguish between solid and liquid topicals. While dusting powders are obviously solid, other formulations considered solid for this treatment include anything that does not run when poured. In addition to fillers and vehicles to control tissue penetration, excipients are used to solubilize the active ingredient, provide antibacterial action, enhance stability, emulsify and act as suspending agents. Mineral hydrocarbons are commonly used as the base for ointments and gels and creams typically have a more aqueous base.

[Sub category of 3.10.1.3] Liquid formulations: e.g. lotions/emulsions/suspensions

Water as a bulk filler is probably the most common excipient for liquid topicals. Excipients to control pH and suppress microbial growth are also common. Isopropyl and larger alcohols are commonly used to improve consistency, feel and solubilization. Oils are used to produce emulsions and suspensions, and other compounds provide humectant (water-retaining) and emollient (softening or soothing) properties. Topicals for opthalmologic applications require excipients to control tonicity, pH and sterility as well.

3.10.1.4. Injectables

Injectables are prepared for intramuscular (IM), intravenous (IV) and subcutaneous (SC) administration. Critical factors for these preparations are their sterility and tolerance of tissue at the site of injection. Water is the predominant solvent, although non-aqueous solvents can also be used to improve the solubility of the active ingredient. Glucose (5%) and saline (0.9%) are generally used, although some preparations may be hypertonic. Some preparations may also use so-called fixed oils, and nonglyceryl esters of fatty acids.

3.10.1.5. Suppositories

Excipients for these are generally fatty preparations with melting points below body temperature and solidification points above room temperature.

3.10.1.6. Inhalants

The most obvious excipient in these preparations is the propellant. These may be hydrocarbons, fluorocarbons, other halocarbons or compressed ambient gases such as nitrogen and carbon dioxide. Other major excipients in inhalants can also include solid or liquid components. For example, dry powder inhalers often use a filler such as lactose.

3.10.2. Excipients by activity

3.10.2.1. Bulk materials

These are generally considered as carriers of the active ingredient. Where the amount of active ingredient is too small for convenient handling, an additive is required to provide suitable bulk. In many cases, the amount of bulk material far surpasses the amount of active ingredient in a product. Additional uses for bulk materials are as controlled release agents, or agents to improve flow characteristics, stability or compressibility.

Matrix variation in a particular product, due to variation in a bulk material should be minimal. Due to increasingly stringent standards for pharmaceutical products, particularly in the United States, suppliers tend to have only one manufacturer for an excipient of a particular grade. Drug manufacturers will tend to use one supplier, but often have a back-up supplier to ensure continuity of supply in case of emergency. It is important to note though that ''fillers'' are often mixtures of several ingredients, often of biological origin, the nature of which can vary. Some matrix variation may therefore result between lots for either of these reasons.

The list of compounds that can potentially be used as fillers in pharmaceutical preparations is substantial (see Table 2). In many cases fillers will not dissolve in a sample and will therefore be present as a discontinuous phase that may compete with the extraction phase for the drug.

3.10.2.2. Capsules

These small single-dose containers are pre-manufactured and then filled with either a solid or liquid medication. They are manufactured primarily from gelatin. Common additives are sucrose, to increase hardness, and glycerin or sorbitol to increase softness.

As a protein, it would be expected that gelatin could bind to some drug compounds, and therefore compete with the microextraction phase. Gelatin content should not be permitted to vary significantly without the use of a suitable internal standard.

3.10.2.3. Coatings

Of the wide variety of tablet coatings used, the proteins and polymers would be expected to compete with the extraction phase for the analyte of interest, while the waxes and paraffins have the potential to change the sample polarity, and hence impact partition coefficients. If the total amount of coating present is low, the effect may not be significant.

3.10.2.4. Flavourings

Pharmaceuticals have for the most part, left the era where nasty-tasting medicine was considered strong, and therefore a positive characteristic. The public now expects oral pharmaceuticals to be palatable, particularly in pharmaceuticals for children. Flavour preference and consistency for pharmaceuticals are areas of significant concern.

Sucrose and glucose are commonly used sweeteners, and glycerin may be used as both a sweetener and a solvent. Other sweeteners may be present because of the use of natural flavourings. Sorbitol, xylitol and artificial sweeteners may be used where there is concern over disturbing the carbohydrate control of diabetics. In addition to its use to provide desired tonicity or solubility, sodium chloride may also be used to improve palatability. Many fruit flavours prepared as syrups from natural sources are used in flavourings. However, with the significant variability that exists in these preparations it is difficult to precisely predict their impact on extraction, where their concentrations are high. Synthetic flavourings by contrast, are more consistent and cost effective. There is however a common consensus, whether founded or not, that natural flavours taste better and are safer than synthetics, a fact that may limit the use of synthetics.

Where significant variation in flavouring composition exists between samples extraction efficiency may be variable and hence external calibration impractical. Standard addition or the use of an internal standard may be required.

Essential oils are also commonly used in flavouring, and like natural fruit flavours, can be a rather ill-defined mixture of many compounds. Because they consist mainly of compounds with low boiling points, they could interfere with headspace extraction of pharmaceuticals by solid sorbents.

3.10.2.5. Colourings

While colour in pharmaceuticals may be looked upon as a matter of aesthetics rather than of necessity, the adding of colour for psychological or aesthetic reasons has been shown to influence therapeutic response. Colouring also enhances the effect of flavouring. Most importantly, colour (and tablet shape) can also give a unique identity to a product, reducing confusion, errors and noncompliance.

Colourings used in pharmaceuticals can be divided into two classes. Dyes are soluble coloured materials that go into true solution, generally in water. Pigments on the other hand are coloured particulate material. A subclass of the pigments is the ‘lakes’, which are prepared by the tight adsorption of dyes to otherwise uncoloured insoluble particulate materials such as alumina. Particulate colouring agents, if present in significant amounts, may compete with the extraction phase for affinity for the active ingredient. Where amounts vary from one sample to another, some consideration will have to be given in analytical method development.

3.10.2.6. Controlled-release binding agents

By their very nature, most of these compounds are designed to compete strongly with the extraction phase, for the active ingredient. A very specific internal standard, or standard addition will be required to adequately quantify total concentration of the active ingredient. By contrast, microextraction techniques are ideal for studying or monitoring the therapeutically relevant dose delivered by these preparations.

3.10.2.7. Buffers

The pH of a pharmaceutical product typically must be controlled, either to optimize solubility, bioavailability or stability, or for compatibility with the site of administration. A salt of a drug compound is more water soluble than the free acid or free base form, and upon dissolution is less likely to result in a strongly acidic or basic solution. Non-ionic species however are more lipid soluble and better absorbed. It is therefore critical in many drug formulations that buffering components are included to achieve an appropriate balance between water and lipid solubility so that a compound reaches the required absorptive surface, with an adequate concentration of the lipid-soluble moiety to permit absorption. In the case of injectable medications, solutions for IM and SC injection must be close to the pH of the tissue into which they are injected. Modest deviation from physiologic pH is better tolerated for solutions for slow IV infusion, particularly where the molarity (buffering capacity) is low. Topicals for skin application are slightly acidic to avoid skin irritations, intravaginal preparations typically have a pH of 4–5

to maintain normal vaginal flora, and opthalmologic topicals require physiologic pH.

The pH appropriate for optimum bioavailability may not be appropriate for optimal extraction, and significant pH adjustment may be required to counter the buffering capacity of the excipients, and provide for adequate extraction efficiency.

3.10.2.8. Suspending, emulsifying and stiffening agents

These excipients are used in both liquid and solid dosage forms, typically in a small quantity. Many of these compounds are surfactants that act as suspending and emulsifying agents to aid in the development of solutions and topical formulations of materials that may otherwise be difficult to prepare in an acceptable form. They may also be used in aqueous solutions to be inhaled, to hydrate and liquify bronchiopulmonary secretions or to help clear debris. They also have cleansing and antimicrobial action and may be used as preservatives. The surfactants are either ionic or non-ionic. Other compounds that may be used include mucilages and gums, methylcellulose, paraffin, fatty alcohols and lubricants such as talc and edible oils.

All of these compounds could potentially change the polarity of a sample, and the partitioning of an analyte between a sample and an extraction phase. If only a very low concentration of one of these excipients is employed, the impact on extraction efficiency/precision would be low. Where the concentration is higher, the primary concern would be in ensuring consistency between samples, and between samples and standards. Poor precision would result if there was variation in the nature and/or concentration of these excipients.

3.10.2.9. Preservatives

There are three primary classes of preservatives, antimicrobials, antioxidants and chelating agents. The surfactants discussed previously may also be employed as preservatives. Antimicrobials include such things as phenol and thymol, thimerosal, parabens, chlorobutanol and benzyl alcohol. Concentrations range from about 0.1% to 10%. Compounds present at the high end of this range may interfere with extraction. Antioxidants include BHA and BHT at about 0.02% for non-aqueous formulations, and sulfites at the ppm level for aqueous formulations. At these levels, interference with extraction is unlikely. The primary chelating agent used is ethylenediamine tetraacetic acid (EDTA). It is typically the disodium salt that is employed. Unless the compound being extracted is a metal species, interference with extraction is unlikely.

4. Conclusions

4.1. Eliminating the fibre as a source of imprecision with fibre SPME

A variety of factors may influence the fibre response or absolute amount extracted with a given sample. These include coating of the fibre surface with sample matrix components, physical damage or deterioration of the fibre coating and lot to lot or fibre to fibre variation in coating character or length [125,130,131]. Even headspace analyses have been observed to result in a coating of the fibre with matrix components after many extractions. In order to produce adequate levels of precision, it is important to monitor fibre response on a regular basis. This is analogous to monitoring of detector response in a GC. This is most easily accomplished by performing a standard extraction once each day to verify that the absolute amount extracted does not vary. One should select as a standard, a compound that is related to those of interest in the work at hand. In most cases it is possible to use the same fibre extraction/injection to monitor both fibre and detector response. Only if the response factor changes significantly does the analyst need to perform a second injection to determine if the source of the variance is detector or fibre response.

Additionally, it is helpful to visually inspect the fibre coating under a 30× magnification top-lit stereomicroscope. This should be done prior to initial conditioning, after conditioning but before use, and daily during use. Any variance or irregularities should be noted and the fibre cleaned or discarded as required.

A typical cleaning may include soaking in an appropriate water or solvent (e.g. methanol) bath with stirring, depending on the nature of the contamination. Some authors employ heating during the cleaning step as well. Fibres may then be dried, typically for a few hours or overnight, and reconditioned at the recommended temperature for a short time before the next use. Where high salt concentrations are used, or where sample matrix is particularly prone to coating of the extraction polymer, a quick cleaning may be employed between extraction and injection, by directing a stream of purified water from a wash bottle down the length of the fibre for a few seconds. While this would impair precision for highly volatile compounds, it is effective for compounds with lower volatilities typical to most drugs.

With polyacrylate fibres, after the initial conditioning, the polymer should take on a transparent light caramel appearance. Any further darkening in the polymer is an indication of further degradation in the coating, and a change in fibre response can be expected.

If these precautions are observed, the fibre itself will be eliminated as a source of unacceptable method imprecision, leaving the analyst to be concerned solely with optimizing method development parameters.

4.2. Non-equilibrium extraction

While the limitations with extraction by solid sorbents that are observed for complex matrices may appear to limit their utility, they typically have much higher partition (affinity) coefficients than absorptive sorbents. Lower partition coefficients limit method sensitivity. This can be a particular difficulty with drug extractions where therapeutic drug concentrations can be very low. While solid sorbents typically have higher partition coefficients, they suffer from competitive displacement and limited linear range when concentrations of analytes or other sample constituents become too high. They are therefore most appropriate for monitoring of either low concentration samples or higher concentrations for shorter times.

It is expected that as solid sorbent affinities increase, they will find utility primarily for performing non-equilibrium extraction at a point very far from equilibrium. While the total amount extracted far from equilibrium is quite small, the very high affinity of these surfaces for analytes of interest can allow for sufficient or enhanced sensitivity. Affinity or immunoaffinity surfaces would be good examples of high affinity sorbents that exhibit high specificity and affinity at low analyte concentrations, but which must be employed in a sample range that does not significantly saturate active sites in order to achieve reasonable linearity of analysis.

At extraction times far from equilibrium uptake is determined by the rate of diffusion through the boundary layer and amount extracted is proportional only to the analyte's diffusion coefficient, rather than its partition coefficient. The differences in diffusion coefficients between compounds are small compared to the differences in distribution constants. This makes it easier to calibrate the system. Because of the large differences in distribution constants between analytes, chromatograms from equilibrium extractions are characterized by small peak areas for compounds with small distribution constants, and large areas for those with large constants. With uptake dependent on diffusion coefficients, all compounds in a chromatogram with similar molecular masses and sample concentrations will have similar peak areas, given similar detector responses. Also, it is relatively simple to calculate the diffusion coefficients for given analyte and therefore correct for the small differences in it. It must be understood that this system is only suitable for trace analysis. When sample concentrations become too high, saturation of the active sites is more likely, and uptake rates are no longer linear. Also, at higher concentrations, samples are easily extracted and analyzed with conventional fibres and SPME extraction methods. Results of extraction by the diffusion type of approach are shown in Fig. 15. Accumulation of volatile components (early eluting) on the solid coating in 10 s is much larger compared to the 10-min equilibrium extraction on PDMS. This approach to extraction is not limited to devices using the fibre geometry, but is generally applicable. In the early stages of the adsorption time profile, extraction

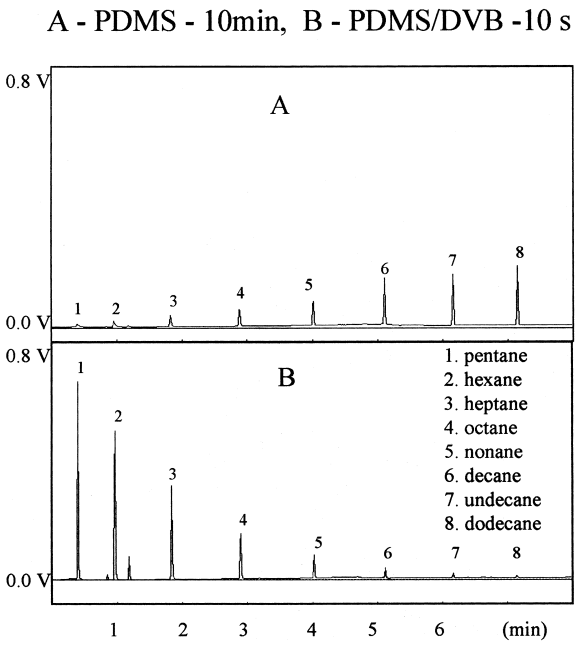


Fig. 15. Partition vs. diffusion-based extraction. (A) Partition-based extraction, PDMS fibre, 10-min extraction; (B) diffusion-based extraction, PDMS/DVB fibre, 10-s extraction.

is controlled primarily by diffusion, and uptake versus time is linear. This is observed in Fig. 16 for BTEX compounds. Therefore where the diffusion coefficient is known or can be estimated, sample concentration can be estimated with reasonable accuracy, based solely on amount extracted. This greatly simplifies analysis.

4.3. Miniaturization of analytical instrumentation

Previously, limitations in sample preparation limited on-site analysis. In addition to on-site as understood for environmental or production environments, this may also include such circumstances as physician office laboratories (POL) or point-of-care (POC). The significant advances in miniaturization of analytical instruments and 'lab-on-a-chip' technologies that are underway, will allow widely applicable on-site analysis, so long as simple, integrated sample preparation schemes are available. Advances in better integration of sampling/sample preparation and instrumental analysis will allow wider use of on-site analysis in the future, and eliminate much of the difficulties currently encountered by sampling, transport and storage requirements of current methods. It will also allow for faster, near-real time analyses that will aid in the more effective monitoring and control of patients both at home and in hospital.

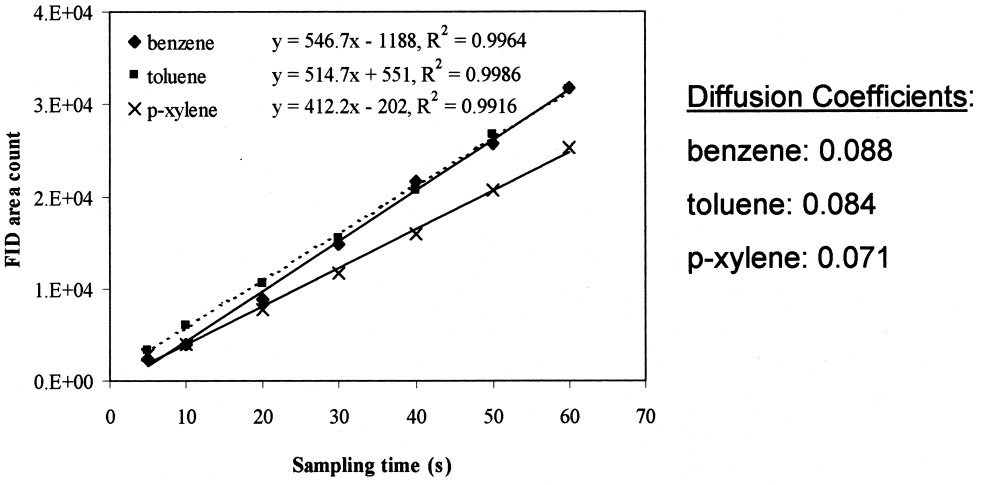


Fig. 16. Mass transport control of overall extraction rate, analogy to the non-equilibrium extraction of benzene, toluene and *p*-xylene using porous coatings.

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