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Review

Before the injection—modern methods of sample preparation for separation techniques

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Abstract

The importance of sample preparation methods as the first stage in an analytical procedure is emphasised and examined. Examples are given of the extraction and concentration of analytes from solid, liquid and gas phase matrices, including solvent phase extractions, such as supercritical fluids and superheated water extraction, solid-phase extraction and solid-phase microextraction, headspace analysis and vapour trapping. The potential role of selective extraction methods, including molecular imprinted phases and affinity columns, are considered. For problem samples alternative approaches, such as derivatisation are discussed, and potential new approaches minimising sample preparation are noted.
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Keywords: Reviews; Sample preparation; Solvent extraction; Supercritical fluid extraction; Solid-phase extraction; Solid-phase microextraction; Molecular imprinting

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1. Introduction

These days, when separation methods can provide high resolution of complex mixtures of almost every matrix, from gases to biological macromolecules, and detection limits down to femtograms or below, the whole advanced analytical process still can be wasted if an unsuitable sample preparation method has been employed before the sample reaches the chromatograph. Rather like the proverbial computer rule, garbage-in garbage-out (GIGO), poor sample treatment or a badly prepared extract will invalidate the whole assay and even the most powerful separation method will not give a valid result.

Yet sample preparation is often a neglected area, which over the years has received much less attention and research than the chromatographic separation or detection stages. However, getting the sample preparation stages correct can be economically valuable as well as analytically important. An inefficient or incomplete technique can represent a considerable constraint on the throughput of any method and involve a significant additional workload for staff. A survey in 1991 claimed that sample preparation can account for around two thirds (61%) of the effort of the typical analytical chemist and 92% of the respondents regarded sample preparation as moderately or very important [1]. However, a more recent comment was that ‘‘ . . . in analytical chemistry laboratories, sample preparation is not recognised as an important step in the whole analytical scheme and is often given to the less trained chemist’’ [2]. Although individual steps or sample preparation methods have been reviewed in detail, there are few general monographs or reviews on the subject [3–6], probably also emphasising the broad nature of the topic and the wide range of approaches that can be used.

The basic concept of a sample preparation method is to convert a real matrix into a sample in a format that is suitable for analysis by a separation or other analytical technique. This can be achieved by em-

ploying a wide range of techniques, many of which have changed little over the last 100 years. They have a common list of aims:

- The removal of potential interferents (for either the separation or detection stages) from the sample, thereby increasing the selectivity of the method.
- To increase the concentration of the analyte and hence the sensitivity of the assay.
- If needed, to convert the analyte into a more suitable form for detection or separation.
- To provide a robust and reproducible method that is independent of variations in the sample matrix.

With increasing demands on the analytical chemist to provide accurate and valid analytical measurements for regulatory requirements, poor manual reproducibility during the sample preparation stage can be a major cause of assay variability [7], hence a need for automation and reduced manual sample handling. However, robots and the automation of the laboratory bring their own problem of longer method development times and new skill requirements.

Many of these ideas could apply to any analytical process but we will concentrate here on preparations leading to assays by separation methods. In some ways, this simplifies the requirements of the sample preparation process, as the final assay step often already incorporates a powerful separation and discrimination technique.

Although many traditional sample preparation methods are still in use the trends in recent years have been towards:

- The ability to use smaller initial sample sizes even for trace analyses.
- Greater specificity or greater selectivity in extraction.
- Increased potential for automation or for on-line methods reducing manual operations.
- A more environmentally friendly approach (green chemistry) with less waste and the use of small volumes or no organic solvents.

These goals are being achieved in a number of different ways and are still the subject of active research and this has been recognised in the recent addition of a new topic heading in the *Journal of Chromatography A* on Sample Preparation. This review surveys the wide range of sample preparation methods and combinations of methods for low molecular mass compounds that have been developed over the century since chromatography was first reported, with selected recent samples. Within the scope of a review, the coverage is necessarily representative rather than comprehensive, as effectively almost every assay of a real sample requires some sample preparation and the potential examples are endless. Frequently references are therefore given to more specialised reviews or monographs.

2. The first theoretical plate?

There are close analogies between many sample preparation methods and analytical separations and frequently the sample preparation step can be considered to be the first theoretical plate in the separation process. However, it is one with often relatively low discrimination but high capacity. It is still based, as are chromatographic separations, on a phase distribution, charge interaction and/or size fractionation. Frequently an inherent increase in the concentration of the analyte can also be achieved through a chromatographic focusing effect. The skill of the analytical chemist has been in devising sample preparation methods to achieve the desired distribution by manipulating the polarity or ionic state of the analyte, or by the appropriate selection of the phases.

3. Problems with the old methods

In looking at current sample preparation methods, it is interesting to compare them with the methods used in the early days of chromatography and from the early volumes of the *Journal of Chromatography*.

3.1. Sample preparation 100 years ago

In many ways, the extraction of natural products has changed little. In his original work Tswett [8] utilised a number of alternative solvent extractions with alcohol–light petroleum, benzene, carbon tetrachloride or carbon disulfide to obtain the chlorophyll pigments from plant material, after neutralisation of the leaves with MgO and $CaCO_3$. The need to obtain a sample solution free of alcohol and water was

recognised, as the presence of these solvents in the extract gave indistinct chromatograms. Thus from the earliest days of chromatography, the influence of the sample preparation methods on the quality of the resulting chromatogram was identified, as was the potential of poor practice to destroy the advantages of the analytical technique.

3.2. Sample preparation in early volumes of the Journal of Chromatography

The coverage of volume 1 was very different from that found today and sample preparation in 1958 had advanced little from the methods utilised by Tswett. In the first few volumes of the journal, there were almost no papers on gas or column liquid chromatography, the principal techniques being ion-exchange separations, electrophoresis, and paper chromatography, with the most frequently examined analytes being radiochemicals and inorganic samples.

By volume 500, in 1990, still relatively few papers referred specifically to sample preparation but it was noticeable that gas chromatography was now the dominant technique. However, a review of carbohydrate analysis discussed recent derivatisation advances [9] and another paper considered derivatisation for electron-capture detection with electrophoric derivatives [10]. A fully automated method for nitrofuran in biological samples, using on-line dialysis and column switching, showed a more modern trend [11], although in a recent survey one third of the respondents suggested that automation was unnecessary in their laboratory mainly because of a low sample load [6]. Interestingly the preface worried that advance in electronics would not permit the journal to reach volume 1000.

In more recent years the importance of sample preparation has been reflected by special issues reporting related symposia and topics. These include solid-phase extraction (Vol. 885), preconcentration and sample enrichment techniques (Vol. 902), Ex-Tech 2001 (Vol. 963), and sample handling (Vol. 975). Similar influences are reflected in other separation science journals.

4. Filtration

Simple filtration can be an important part of sample preparation, particularly in liquid chromatography (LC), where any insoluble material will block the column or frits. The efficiency of filtration is determined by the porosity of the filter and would be typically 2 μm or less for LC. Different types of filters can be used include paper, glass fibre, and membrane filters [12,13]. In a recent development, filters have been built into standard sized sample vials so that sample handling and solution transfer is minimised, which can be important to avoid contamination of the sample and reduce biohazards to the operator [14].

For some samples, such as environmental solutions, the removal of relatively large solid material may be required as this may physically interfere with extractions or later stages and an initial simple filtration will suffice. However, care must be taken that there are no sample losses because of adsorption of analytes onto the solid material that is removed. Alternatively centrifugation can be used to remove insoluble material from solutions.

5. Extraction methods

The oldest and most basic sample preparation method is extraction, in which the analyst aims to separate the analyte of interest from a sample matrix using a solvent, with an optimum yield and selectivity, so that as few potential interfering species as possible are carried through to the analytical separation stage. Different extraction methods are used, including solvent extraction from solids and liquid–liquid extraction from solutions [15]. The solvents may be organic liquids, supercritical fluids and superheated liquids or the extraction liquid may be bonded to a support material, as in solid-phase extractions (SPEs). Selectivity can be obtained by altering the extraction temperature and pressure, by the choice of extraction solvent or liquid, and the use of pH and additives, such as ion-pair reagents.

5.1. Unification

All extraction methods make use of the same basic set of concepts to concentrate the analyte selectively in one phase. Any analyte will be distributed between two phases according to the distribution constant, temperature, and the relative volumes of

the phases. However, the extraction rates are based on the migration kinetics and hence are governed by temperature and the diffusion rates in the two phases. These parameters are essentially those that are manipulated in chromatographic separations, and one can therefore consider the extractions as a form of pre-assay chromatography.

In many of these methods, a balance must often be obtained between the complete extraction of all the soluble organic components and the selective extraction of only the compounds of interest. This conflict has been a constant theme throughout sample preparation methods in analytical chemistry. Exhaustive extraction techniques, such as Soxhlet extractions, are usually designed to give complete extractions irrespective of the matrix. This is an essential feature of a method that can be applied to a range of samples, such a different soil types, but limits selectivity.

In contrast, when supercritical fluid extraction (SFE) was first introduced, it was claimed to be highly selective compared to Soxhlet extraction but in reality the carbon dioxide solvent was simply a weaker eluent and hence more selective extraction medium. With standards and model matrices, there were few problems but when the method was applied to real samples, yields were found to depend on the age of the sample [16] and type of soil being extracted [17]. The method might work for a simple matrix, such as sand, but real soil matrices with differing interactions, moisture content and organic components often caused difficulties and incomplete extractions. Interestingly, because compounds can be more tightly bound as a matrix ages, it has been suggested [18] that the mild SFE extraction conditions might give a closer indication of the bioavailability of the pollutant and thus be more environmentally significant than more comprehensive extraction methods.

One further example is the problems that can arise if methods are not fully tested. In SFE there were frequent reports that an extraction was complete if a repeat extraction under the same conditions yielded no further analyte (for example, Ref. [19]), it was subsequently found that the only reliable guide was the extraction of a standard sample of known composition. It was often observed that more powerful extraction conditions (modifier additive, higher temperatures or pressures) would result in additional analyte being released from a matrix [17]. The initial mild conditions, while selective, were simply not sufficiently strong to release the analyte from all the active matrix sites and give a quantitative yield.

These problems emphasise the need for extraction methods to be tested with a range of real samples of different types, not just with model systems (and in particular not just with spiked samples). Realistic robustness studies should be undertaken before the extraction is used in an analytical method. If possible alternative independent extraction methods should be used as a guide or the methods should be applied to samples of known composition, such as certified standard reference materials.

6. Analytes in solid samples

If the whole of a solid sample is readily soluble, dissolution in a suitable solvent or water followed by liquid partitioning is usually the easiest method (see Section 7). However, most solid samples, such as soils, environmental solids, plant material, and polymers, are largely insoluble and usually cannot be examined directly. In some cases, it is appropriate to digest the sample in strong acid but in most cases this would destroy the analytes and is principally of interest for the determination of inorganic elements or ions.

For most samples, it is necessary to extract the analyte of interest out of a residual matrix with 100% efficiency but with also achieving as much specificity and selectivity as possible to simplify the subsequent separation steps. Typical methods use exhaustive extraction in a Soxhlet system in which the solvent is continuously recycled through the sample for some hours. However, the analyte must be stable in the refluxing boiling solvent. Less efficient methods included stirring the sample in hot or cold solvents for prolonged periods. All these processes were often quite slow and required the use of significant amounts of sample and large volumes of organic solvents to ensure complete extraction. The subsequent work-up employed solvent evaporation and concentration of the sample was slow and manually laborious. There was the added disadvantage that any impurities in the extraction solvent were also concentrated.

The aims of most recent methods for the ex-

traction of solids have been to reduce the amount of solvent and sample, reduce the time required, and enhanced the selectivity of extraction. The first two aims have frequently been achieved but the last is harder as in any extraction process there has to be a balancing of selective and complete extraction. In most cases, smaller samples are now used but this does impose a restriction that the sample homogeneity may limit reproducibility. There have been two principal approaches, the use of conventional solvents in more efficient ways or the employment of alternative solvents, such as supercritical fluids.

6.1. Enhanced solvent extraction methods

The extraction process can be speeded up by heating or agitating the sample (in pressurised liquid extraction and microwave assisted extraction) or by using an alternative solvent, which has a higher diffusion rate (as in supercritical fluid extraction and superheated water extractions).

6.1.1. Pressurised liquid extraction

By employing a closed flow-though system, it is possible to use conventional organic solvents at elevated temperatures above their atmospheric boiling points. This method, known as pressurised liquid extraction (PLE) [20,21], has been commercialised in an automated or manual version as accelerated solvent extraction (ASE). A restriction or backpressure valve ensures that the solvent remains as a liquid but has enhanced solvation power and lower viscosities and hence a higher diffusion rates. Both changes increase the extraction rate. Both static and flow-through designs can be used. In the latter, fresh solvent is continuously introduced to the sample improving the extraction but diluting the extract.

As a consequence, extraction procedures, which would have taken many hours of Soxhlet refluxing, can be carried out in minutes on a smaller sample, considerably speeding up the sample pre-treatment and requiring a small fraction of the original solvent volume. An essential feature of the success of the system is the ability to carry out multiple extractions and hence move towards automation. The extracts are generally much more concentrated than from conventional extractions. They could often be analysed directly or the solvent could be cooled, and the analyte trapped on glass beads or a cartridge, and subsequently extracted into a smaller solvent volume.

The method has been applied to a number of matrices, including marine particulate materials [22], pesticides in soils [23,24], medicinal plants [25,26]. The many applications for soil [27] and environmental samples [28] have been reviewed. Frequently the studies have compared PLE with conventional alternative methods, such as SFE [29,30], including a comparison of methods for the extraction of environmental matrix standards [21]. In situ derivatisation of the sample can be used to enhance extractability [31]. Once the technique had been introduced, the US Environmental Protection Agency (EPA) rapidly adopted it for the analysis of pesticides in soils [32], as effectively it used the same solvent systems as conventional liquid extraction. Many other EPA methods using PLE have since been published. In contrast it has taken many years for the SFE method (Section 6.1.3) to be accepted.

The initial extraction can be often combined with a second sample preparation method, such as solid-phase extraction or stir-bar extraction (see later), to concentrate the analytes before analysis

6.1.2. Microwave and sonic wave assisted extraction

For a number of years microwaves have been employed to assist the digestion of solid samples by focusing energy into the sample, resulting both in heating and increased agitation [33]. This method can also be used to enhance solvent extraction methods but the main disadvantage is that it uses a single extraction vessel and the sample vessel has to been cooled, before the extract can be obtained. Multiple samples can be extracted simultaneously but it is difficult to employ the technique as a flow system and thus hard to automate.

The method has been used to extract pesticides and herbicides from soil [34,35], fungal metabolites [36] and essential oils from plant materials [37], and polycyclic aromatic hydrocarbons (PAHs) in sediments [38]. Comparisons have been made with other extraction techniques, such as supercritical fluid extraction [39,40] or Soxhlet extraction [41,42] and the application to solid matrices have been reviewed

[43]. Microwave extraction has also been combined with PLE [44] for the extraction of polymers.

Alternatively sonication can be used to enhance extraction [45] and this has been applied for the extraction of organophosphorous pesticides.

6.1.3. Supercritical fluid extraction

One area that stimulated an interest in enhanced fluid extractions was SFE. This is a long established method, which has been used industrially for many years. However, it was not until an interest was shown in supercritical fluids as a chromatographic medium that it started to be seriously studied as an extraction technique on an analytical scale. It has since been the subject of numerous books and reviews (for example, Refs. [46–50]).

Almost all practical work has employed carbon dioxide as the supercritical fluid as potential alternative solvents, such as nitrous oxide proved dangerous because of their oxidising power [51] and more exotic solvents like xenon were ruled out by their cost. In many ways carbon dioxide is an ideal solvent as it combines low viscosity and a high diffusion rate with a high volatility. The solvation strength can be increased by increasing the pressure and extractions can be carried out at relatively low temperatures. The high volatility means that the sample is readily concentrated by simply reducing the pressure and allowing the supercritical fluid to evaporate.

The principal problem is the relatively low polarity of the carbon dioxide, ideal for PAHs and halogenated pesticides, or lipids and fats, but unsuitable for most pharmaceuticals and drug samples. It has been quite a popular method for solid matrices, including powdered plant materials, herbal medicines, some foods, and polymers [52] but there are problems with liquids, such a biological fluids, which need immobilising on a solid support material. Although one advantage was claimed to be the mild extraction conditions, which would enable the extraction of thermally unstable compounds, there are few examples, such as the extraction of fire retardants from plastic foams [53]. Often the extractions were compared with alternative methods of sample preparation (Fig. 1) [54]. The addition of modifiers, such as methanol, to the carbon dioxide enables more polar analytes to be extracted and

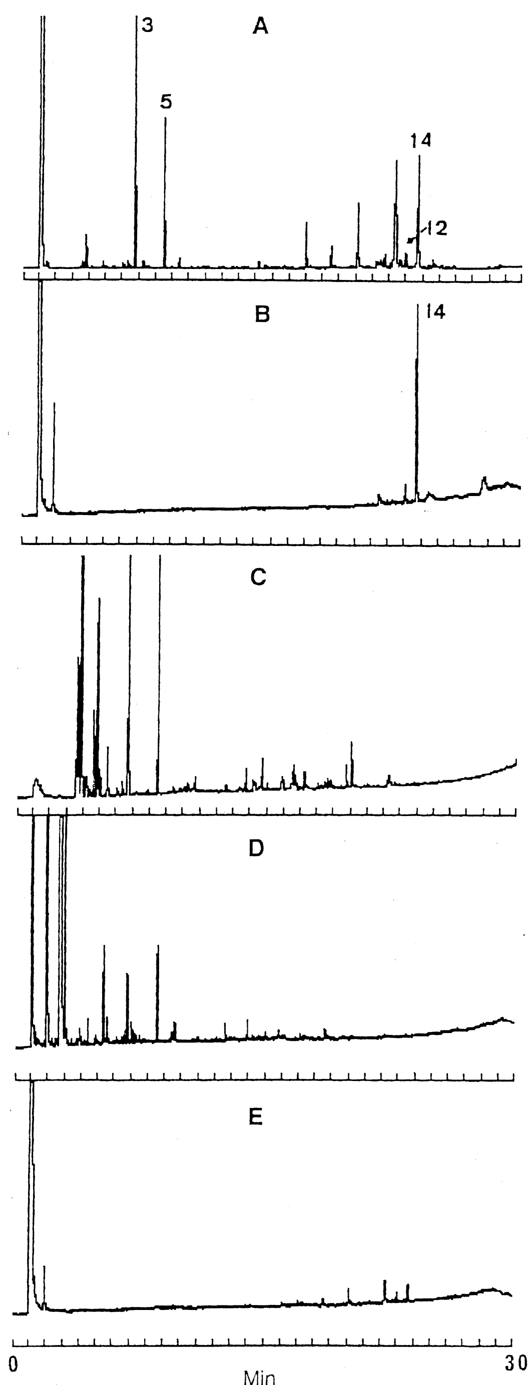

Fig. 1. Comparison of the gas chromatograms of extracts of feverfew obtained by different extraction methods. (A) SFE; (B) parthenolide standard; (C) steam distillation; (D) headspace analysis; (E) solvent extraction. Peaks: 3, camphor; 5 chrysanthenyl acetate; 12, dihydroparthenolide; 14, parthenolide [54].

increases the scope of the method [55,56]. The high pressures required have caused some problems in developing automated systems but commercial systems are now available.

6.1.4. Superheated water extraction

Because the polarity of water decreases markedly as the temperature is increased, superheated water (sometimes termed subcritical or pressurised hot water) at 100–200 °C, under a relatively low pressure, can act as a medium to non-polar solvent and is an efficient extraction solvent for many analytes [57]. Typical applications of superheated water extraction (SHWE) have included PAHs and polychlorinated biphenyls (PCBs) [58] or pesticides [59] from soils, and natural products [60] from plant material.

So far the equipment has usually been laboratory-made but PLE systems can also be employed at a higher temperature than normal extractions [61]. The conditions are usually lower than the critical point of water at 374 °C and 218 bar, because under those conditions the high temperature causes sample decomposition. At lower temperatures, the pressure has little effect on the density of water and is not a critical operating parameter unlike in SFE. As with other liquid extraction methods, superheated water extractions are most suitable for powdered samples. A number of linked methods have also been described, including SHWE–gas–liquid chromatography (GLC) [62], SHWE–LC–gas chromatography (GC) (Fig. 2) [63,64] and SHWE–superheated water chromatography [65].

6.2. Problems with solid matrices

6.2.1. Biological matrices and matrix solid-phase dispersion

Most of the previous methods cannot be applied to biological samples, such as meat or fish tissues and undried plant material, because they rely on a non-polar solvent and this cannot penetrate the largely aqueous matrix. Sometimes more polar water miscible solvents can be employed for plant material but this approach cannot be used with fatty tissues. One successful approach for pesticide analysis has been to disperse the solid tissue, such as liver or kidneys, by macerating with a dispersion matrix—typically thin-layer chromatography (TLC) grade octadecylsilyl (ODS)-bonded phase silica. This matrix solid-phase dispersion provides a porous structure and enables the solvent to penetrate and extract the analytes. It also appears to partially carry out the initial extraction from the aqueous sample phase. Sequential eluent then enables the analytes of interest to be released. The ODS phase has the advantage of retaining lipids so they do not interfere with the subsequent assays.

However, the method is fairly labour intensive requiring the tissue to be ground up with the matrix and packed into an SFE type tube for extraction. Its application in food analysis has been reviewed [66,67], including drugs in fish [68], sulfonamides in bovine and porcine muscle [69], and clenbuternol from bovine liver [70]. Other dispersion and desiccant agents can also be used including sodium sulfate and hydromatrix (particularly for SFE) [71].

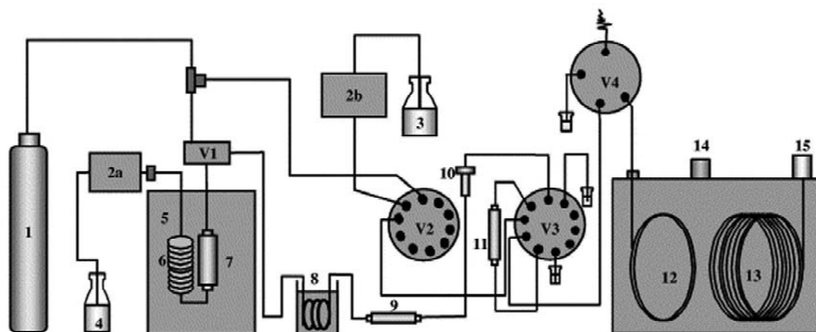

Fig. 2. PHWE–LC–GC apparatus. 1=N_2; 2a,2b=pumps; 3=elution and LC solvent; 4=water; 5=oven; 6=preheating coil; 7=extraction vessel; 8=cooling coil; 9=trapping column; 10=restrictor; 11=LC column; 12=precolumns; 13=analytical column; 14=SVE; 15=detector; V1=extraction valve; V2–V4=multiport valves [63].

6.2.2. Insoluble solid matrices—pyrolysis

The pyrolysis of samples to form characteristic fragments, which can be separated and analysed by GC [72,73], has been used for many years for the analysis of insoluble matrices, such as polymers [74,75] plastics, automotive paints [76] and some drugs. Some recent examples have examined Egyptian mummies (Fig. 3) [77] and have combined pyrolysis with in situ silylation to give trimethylsilyl (TMS) derivatives of resin acids from Manila copal [78]. A novel application was the use of a thermal probe on a scanning probe microscope to select and pyrolyse a small area on the surface of a polymer or plant material followed by GC–mass spectrometry (MS) [79].

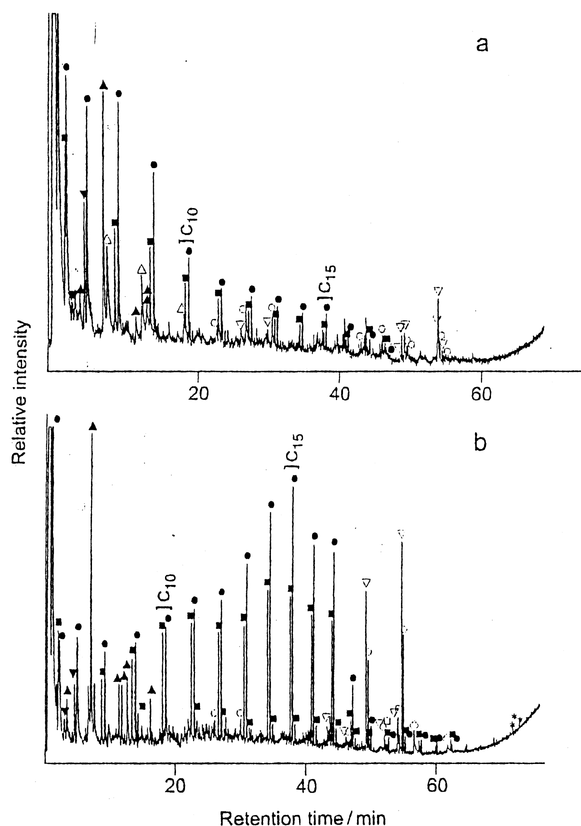

Fig. 3. Total ion current (TIC) of the pyrolysis profile of (a) Horemkensi, resin-like material) and (b) Khnum Nakht, bandage/resin/tissue after thermal desorption. Note: ■=alkenes; ●=alkanes, ▼=alicyclic hydrocarbons; ▲=aromatic hydrocarbons; ○=2-alkanones; □=3-alkanones; △=cyclic ketones; ▽=nitriles; ◊=amides; *=steroids [77].

6.2.3. Thermal desorption from solids

Volatile analytes in solid matrices can be released for analysis by thermal desorption, for example the analysis of chlorinated components in soils [80], or volatile constituents of oak wood [81].

7. Analytes in solution

The traditional method to obtain analytes from liquid samples has been either by partitioning into an immiscible solvent, trapping the analyte onto a column or solid-phase matrix of some sort, or as a last resort evaporation of the sample to dryness and selective solvation of the analytes. The most common method for an aqueous matrix was to use a separating funnel and extract any organic compounds into a non-polar solvent. The method would typically use large volumes of organic solvent (100–250 ml) from a similar volume of sample and the extraction would have to be repeated 2–3 times to achieve a high recovery. After drying, the solvent would be concentrated by evaporation. The resulting sample would frequently require a further clean-up stage. With some samples, the initial solvent extraction step results in the formation of an emulsion and the extraction process could become prolonged.

Overall the process was slow, required considerable manpower and was hence costly. It generated a large volume of organic waste, which was environmentally unfriendly, and its disposal is becoming increasing difficult (and costly). The repetitive manual operations often lead to errors and could be a boring task for the operator, although crucial to obtaining reliable results. There has also been a recognition that the use of large volumes of solvent poses hazards to the health of the laboratory worker and can have a direct impact on the environment. The final blow to the method came with the Montreal protocol, which limited the widely used chlorinated solvents because of their effect on the ozone layer. There has hence been a considerable interest in the reduction of solvent usage and/or alternatives to chlorinated solvents, and in methods capable of automation.

Two groups of methods have been developed, those which trap the sample out of solution onto a small volume of an immobilised phase, such as SPE

and solid-phase microextraction (SPME) and related methods, and those which transfer the analytes to a smaller volume of a second solvent, such as membrane extractions. Both methods are compatible with automation. In addition to the direct extraction, these methods can also be used to concentrate the analytes from extraction solutions of solid samples (see previous sections). Often the methods are directly integrated with the separation stages to further reduce sample handling.

7.1. Trapping the analytes

These methods extract the analyte by trapping it onto an immobilised phase, the analyte is then washed off with a minimal small volume of solvent or eluted thermally. They are usually considerably faster and use significantly smaller volumes of solvent and sample than traditional extraction methods

7.1.1. Solid-phase extraction

The introduction of the disposable pre-packaged SPE cartridge had a major effect on methods for the examination of analytes in solution [82–86]. Although the concept of using a short column for sample clean-up has been employed for many years, usually hand-packed normal-phase materials were used, such as silica or Fluorisil. Their principal role was the retention of unwanted components from the sample, such as tars and polar or involatile compounds, in the clean-up of pesticide residues and environmental samples. The SPE cartridge introduced two important features, standardisation and hence greater reproducibility, and a much wider range of phases, importantly including reversed-phase and ion-exchange materials enabling aqueous solutions to be treated and additional trapping mechanisms to be utilised.

A wide range of phases means that either polarity, hydrophobicity or ionisation can be used as trapping mechanisms and the sample matrix may now be non-polar or aqueous. Once trapped, the analyte can be released into a small volume of an extraction solvent by altering the polarity or pH. In some examples, impurities are trapped and the analyte of interest passed through the cartridge, but it is usually then concentrated on a second cartridge. After some early problems, the retention properties of the cartridges can now be expected to be consistent between batches and the flow-rates and trapping efficiency will be reproducible. However, as with high-performance liquid chromatography (HPLC) columns, nominally equivalent (for example, ODS phases) from different manufacturers may have different bonding chemistries and carbon loadings and so can behave differently. It took some time for SPE to be widely adopted and for robust methods to be developed. For example, there was a need to understand the requirements of preconditioning and the importance of consistent flow control.

Although the cartridges are single-use and disposable and thus represent a significant consumable cost, this has been claimed to be much lower then the cost of chemicals and manpower needed for the corresponding traditional solvent extraction methods. Other formats have also been developed for solid-phase extraction, including flat disks with the stationary phase particles supported on a mesh, enabling very large volumes to be rapidly extracted [87]. Recent use of high flow-rates through extraction cartridges has been claimed to give improved extraction [88] but such ‘‘turbulent flow extractions’’ seem little different to conventional extractions.

The scope of SPE is considerable, with a wide range of reported permutations of cartridge material and eluents/sample matrices. Numerous methods have been developed and reported and libraries of applications are available on manufacturers’ websites and in the literature.

One of the principal applications of SPE has been in the extraction of drugs and their metabolites from body fluids. The disposable cartridges reduce the handling of body fluids, such as urine and blood, and hence the biohazard to the operator is minimised. When large numbers of related assays are required as in toxicology studies the process can be further automated using a robot [89,90] or an intelligent autosampler [91,92] almost completely eliminating sample handling. Extraction onto sample disks has been developed as a method for the determination of organochlorine pollutants in body fluids [93].

The second widespread application of SFE has been for environmental samples, such as river waters and sewage outflow, where large volumes of very dilute solutions have to be extracted [94]. With

conventional solvent extraction, large volumes of sample solution had to be manipulated to obtain sufficient analytes for assay. With SPE cartridges, the sample is simply pumped through the SPE bed and the analytes are then eluted with a small volume of organic solvent. Typical examples are the assays of trace levels of PAHs from river water or non-polar pesticides. A limit of the degree of concentration is imposed by the breakthrough volume of the cartridge (when even the weak aqueous eluent effectively starts to elute the sample) or the overloading of the cartridge by other sample components. The large sample volumes required are aided by the use of the disk format, such as the extraction of estrogen from sewage and river waters [95].

The extraction of the concentrated analytes from the cartridge can either use a solvent or the elution can be accelerated by heating, effectively combining SPE and PLE. The eluted sample can be linked directly to GC (Fig. 4) [94,96] or to an LC separation [97,98]. In recent work, the cartridge can also be eluted with superheated water [62,99] for off-line analysis by HPLC or to on-line gas chromatography [63]. A further method has been described in which the solution from a superheated water extraction is trapped on a short cartridge, then eluted thermally directly onto a superheated water chromatographic separation [65].

7.1.2. Solid-phase microextraction

In solid-phase extraction, it is still necessary to extract the sample from the column, usually with an organic solvent, before it can be injected into a separation method. This last step and the need for an organic solvent were eliminated in the ingenious SPME method, which was invented by Pawliszyn and co-workers [100–102]. They used a fibre coated with a stationary phase as the extraction medium. After carrying out an extraction from a sample solution, the fibre could be placed in the injection port of a gas chromatograph so that the analytes were thermally desorbed directly into the carrier gas stream. The method has been automated and commercial systems are available that will both extract, agitate the sample and inject into a GC system. Assay by HPLC can also be employed but the sample is extracted directly into the eluent stream rather than thermally desorbed (Fig. 5) [102]. A number of different fibre coatings are available, which offer a range of analyte solubilities and

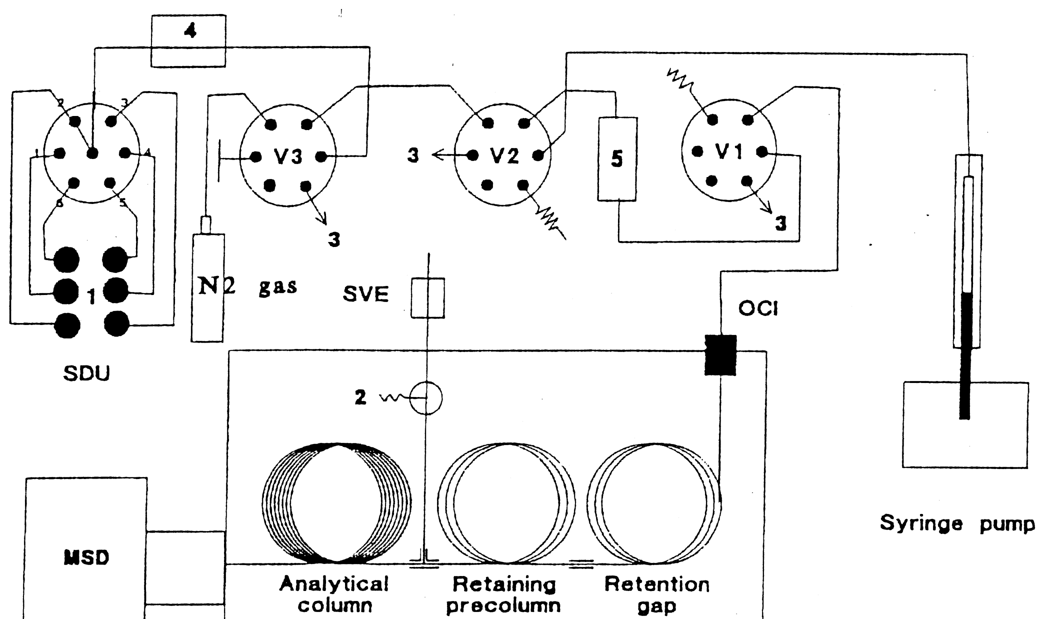

Fig. 4. Scheme of an on-line SPE–GC system consisting of three switching valves, two pumps and a GC system equipped with an SVE, and a mass-selective detector [96].

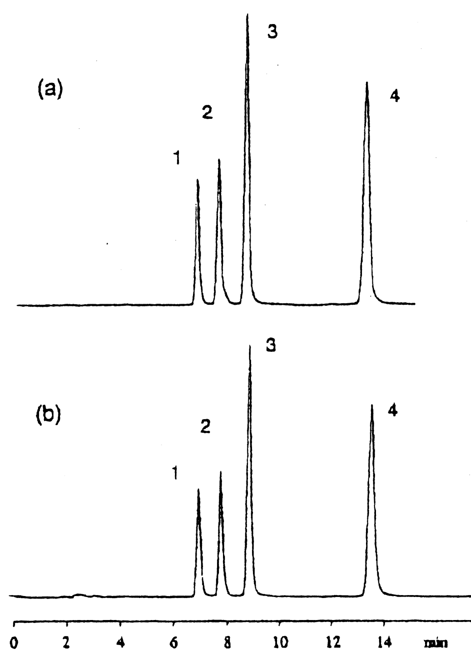

Fig. 5. Isocratic separation of a four-PAH mixture by (a) 1 μl loop injection and (b) fibre injection, 7 μm PDMS extraction for 30 min from 100 ppb of each compound spiked into water. Peaks: (1) fluoranthrene, (2) pyrene, (3) benz[*a*]anthracene and (4) benzo[*a*]pyrene [102].

porosities, including the non-polar polydimethyl siloxane (PDMS), semi-polar PDMS–divinylbenzene and polar polyacrylate, and Carbowax–divinylbenzene liquid like phases and the coated porous particle phase PDMS–Carboxen, They are available in increasing thicknesses from 7 to 100 μm, which increases the partitioning ratio and hence improves sensitivity but increases equilibration times.

The theory and practice of the method has been examined in considerable detail in recent years [103] and numerous applications has been reported and reviewed [104,105]. The basic theory is that of a phase distribution and the amount extracted depends on the partition coefficient between the sample solution and the fibre. However, the fibre volume is small so that the target analyte is often not completely extracted. However, a representative sample is obtained that can be compared with the extraction of a standard solution. The yield can be susceptible to matrix effects, if these alter the distribution constant, such as changes in the ethanol content between different alcoholic drinks [106]. For some routine applications, non-equilibrium conditions can be used as long as the extraction conditions are reproducible.

The main advantages of the system are that no solvent is required to elute the sample from the fibre and there is a direct transfer from the sample solution to the separation method. Unless the matrix is very complex or involatile, the fibre can be reused numerous times as the thermal elution step also cleans the fibre. The disadvantages are that the fibre is fragile even though it is shielded when out of the sample and it can be damaged by a build-up of involatile materials from the samples. The extraction process can be relatively slow because it relies on sufficient stirring or diffusion to bring the analytes into the location of the fibre and good reproducibility requires that an equilibrium is established. The fibre can be also used to assay the headspace above the sample (see Section 8.2) and this method is preferred for volatile analytes as the fibre avoids contact with the matrix solution.

The scope of SPME–GLC can be expanded for some involatile analytes by on-fibre derivatisation to enhance either separation [107] or detection, for example the reaction of chlorophenol with pentafluorobenzoyl chloride to give increased response from the electron-capture detector [108].

Although conventional SPME uses a coated fibre, which is immersed in the sample solution, an interesting variant employs an internally coated capillary through which the sample flows or into which the sample is sucked up repeatedly [109,110]. The extraction components are then eluent by a solvent. In recent developments, a restricted access coated tube using an alkyl diol-coated silica material (Fig. 6) has been used to selectively trap drugs from serum without suffering protein fouling of the surface [111,112].

7.1.3. Stir-bar extractions

Because the SPME fibre has a relatively small volume of bound stationary phase, the extraction is frequently incomplete. Even with a favourable distribution constant, the phase ratio between the fibre and sample solution are often unfavourable, so that the partitioning can still leave a significant amount of the analyte in the sample phase. This problem prompted the development of the stir-bar extraction

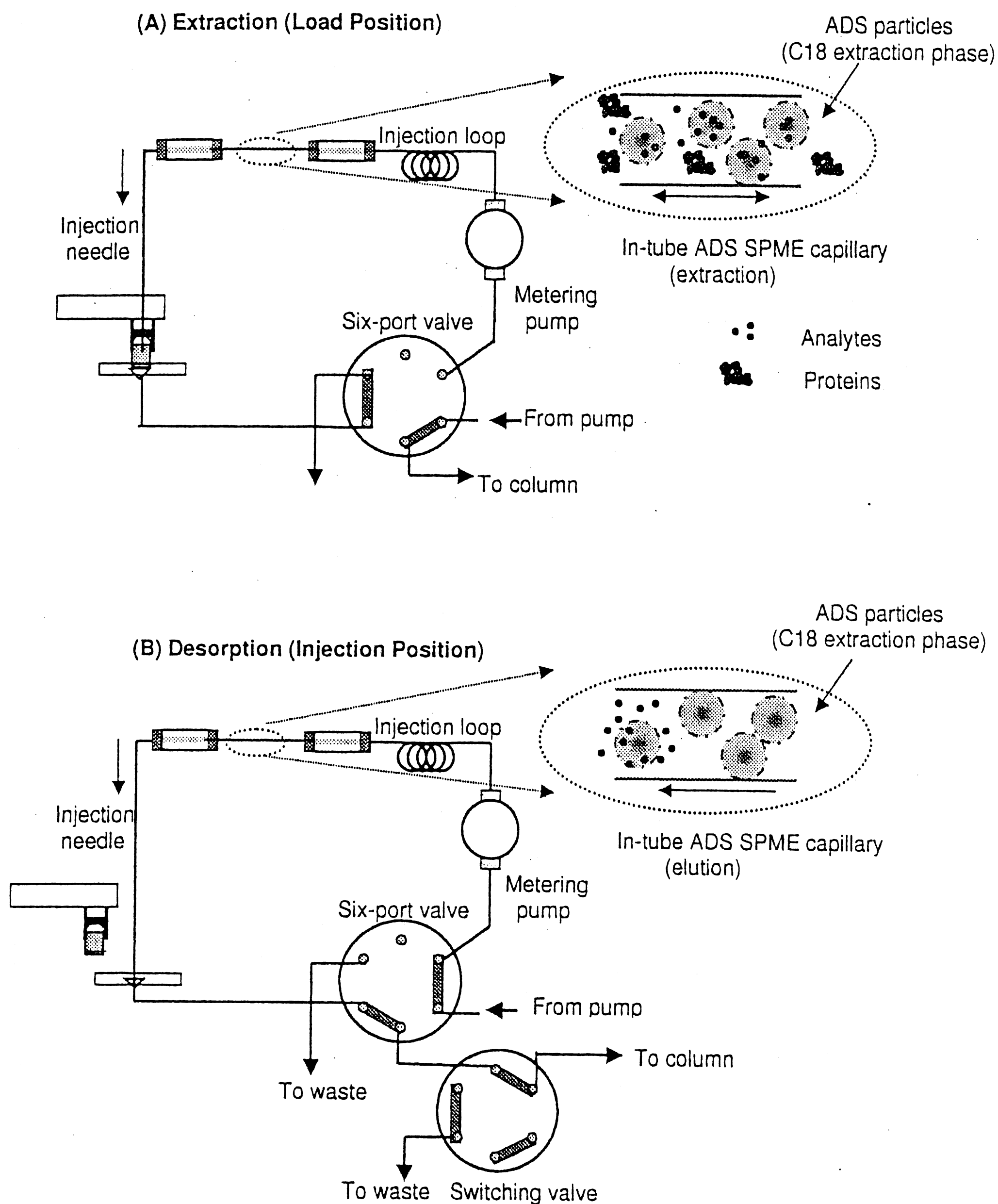

Fig. 6. In-tube alkyl diol silica restricted access SPME system in (A) load position for extraction from serum and (B) injection position (elution onto analytical column) [111].

system (marketed commercially as the Twister), which uses a magnetic stirrer bar or flea coated with a bonded adsorbent layer (such as a polymethyl dimethyl siloxane) [113]. Alternatively a magnetic stirrer can be inserted into a short length of PDMS tubing. The surface area of the stirrer bar is higher than a fibre and the volume of the adsorbent layer is much larger so that there is a higher phase ratio than in SPME and hence a higher extraction yield.

The stir-bar is simply rotated in the sample,

removed and extracted thermally for gas chromatography [113] (using a thermal desorption unit) or into a solvent for liquid chromatography [114,115]. It has proved very good for complex and semi-solid matrices, such as yoghurt or beer, and pesticides in wine [116]. More unusual applications, included the assay of PCBs in human sperm (Fig. 7) [117]. The main difficulty is that it is hard to automate the removal of the stir-bar from the sample matrix, rinse it, and extract.

As with a number of related methods, it can also be used to concentrate the analytes in an extract from an alternative extraction process, for example it has be used to concentrate the analytes from a PLE solution to determine the pesticides in strawberries [118].

7.2. *Extraction of the analytes into a liquid phase*

Rather than distribute the sample between a pair of immiscible (usually polar and non-polar) solvents in a traditional separating funnel, three alternative liquid–liquid extraction methods have been reported, which give a more concentrated extract ready for direct chromatographic examination. However, true liquid–liquid counter-current methods, in which two immiscible liquids flow through a tube in opposite directions are now fairly rarely used, largely because of the time taken to set up and the difficulty of obtaining two truly immiscible liquids.

7.2.1. *Membrane extraction*

A membrane can act as a selective filter, either

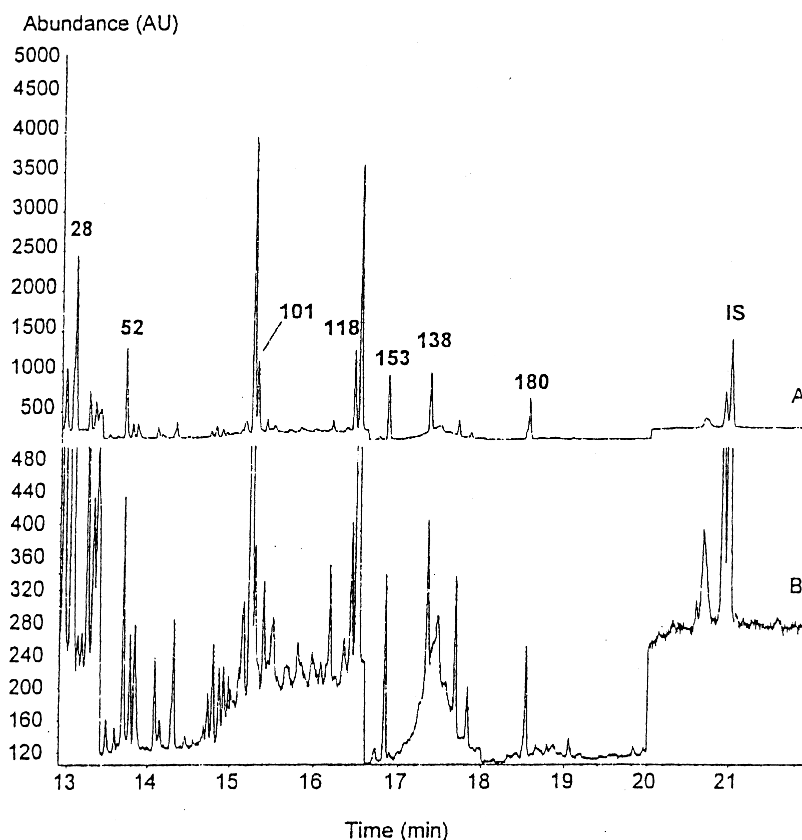

Fig. 7. GC in the selected ion monitoring (SIM) mode of seven PCBs extracted using a stir-bar from human sperm at 10 ppt (A) and 1 ppt (B) [117].

just limiting diffusion between two solutions or as an active membrane in which the chemical structure of the membrane determines the selectivity of sample transfer [119–122]. In most cases, the driving force for the movement of the analyte across the membrane is a concentration gradient. This can be enhanced by effectively removing the analyte from the receiving phase by either ionisation using buffers, complexation, or derivatisation, so that the free solution concentration of the analyte species is reduced. By altering the flow-rate of the solutions passing either side of the membrane, a low concentration in a large volume can be converted into a higher concentration in a smaller volume (Fig. 8). The extraction can be also carried out to transfer a volatile analyte from a liquid to a gas phase by using hollow fibre membranes, linked directly to a GC system (Fig. 9) [123]. Recently a microporous membrane has been incorporated into a superheated water extraction to concentrate a sample of PAHs from soil before GC analysis [124].

Dialysis methods and microdialysis [125] are closely related to membrane separation, with a controlled pore structure providing a separation diffusion process based on molecular size. In vivo microdialysis with the end of the microdialysis probe placed in living tissue enables real time measurements of body chemicals in test animals [126]. The membrane or dialysis method can be directly connected to the sample loop of a HPLC injection port so that the dialysate can be directly injected [127].

7.2.2. Single drop extraction

In a recently developed microscale method, rather than using an immobilised phase, a single liquid drop is utilised as the collection phase [128,129]. Although elegant the method appears to require high manual dexterity. It requires a collection phase with a sufficiently high surface-tension to form a distinct drop, which can be exposed to the analyte solution (Fig. 10). It has been used for pollutants and can readily be linked to GC.

7.2.3. Purge and trap

Purge and trap systems in which a volatile analyte is expelled from a solution by flushing it out with a gas [130] and then trapping the components of interest in a cryogenic trap, solvent or solid-phase

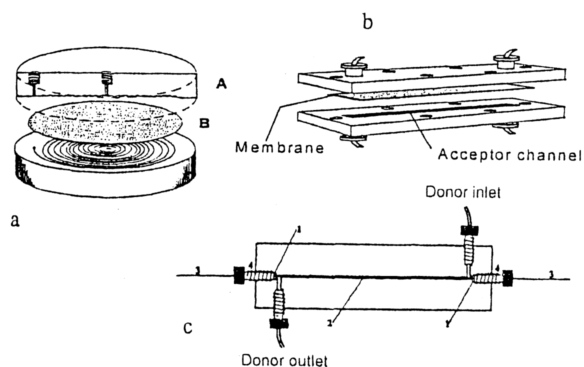

Fig. 8. Different membrane modules for flow systems. (a) Flat membrane module with spiral channel; (b) flat membrane module with 10 μl channel volume; (c) hollow fibre module with 1.3 μl acceptor channel [122].

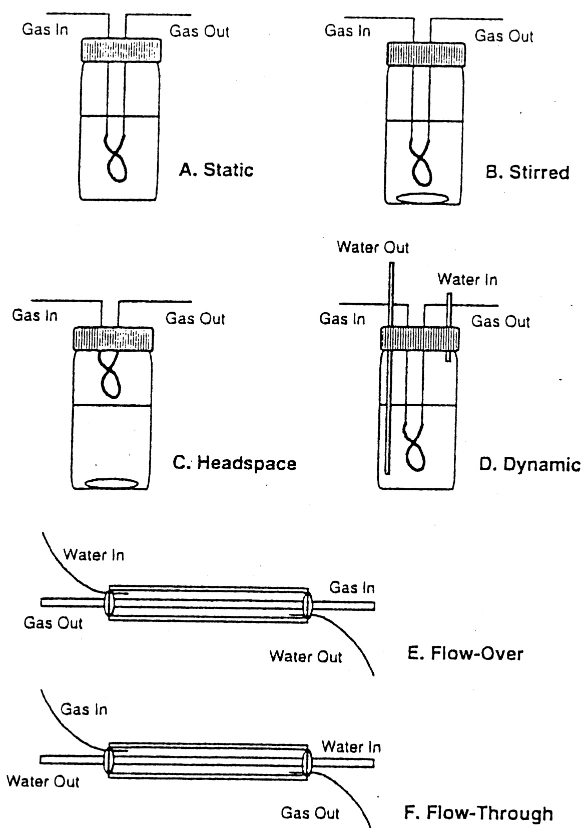

Fig. 9. Different configurations of hollow fibre membrane extraction modules for volatile organic compounds [123].

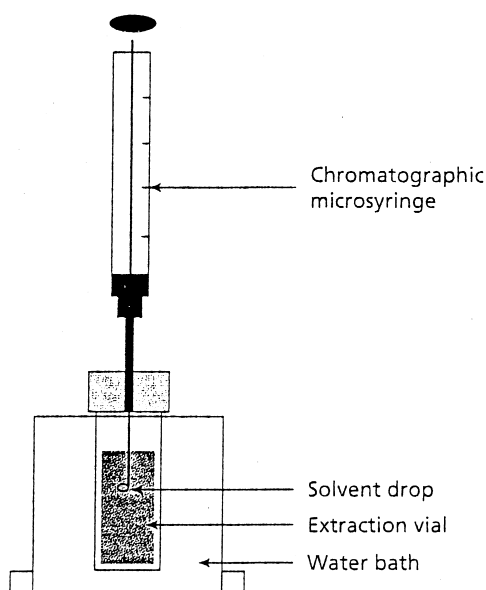

Fig. 10. Schematic of a single drop microextraction apparatus [129].

trap (see also the next section) have been useful for low levels of analytes in environmental solutions. For example it can be used to examine sulfur-containing analytes in beer, coffee and water [131].

8. Analytes in the gas phase

It might seem that little sample preparation of gases should be needed as they can be analysed directly by gas chromatography. The whole sample is volatile and thus will leave no residues. However, the analytes of interest are often at low concentration near the limit of detection and the high diffusion rates in gases mean that the integrity of the sample is hard to maintain from the collection point to the analyser. There has therefore been considerable interest in concentrating, focusing, or trapping out the analytes of interest to increase sensitivity and transportability.

Early methods tried to trap out the analytes using a cold trap or solvent trap from a flowing stream. However, misting rather than condensation can occur or the flowing gas bubbling through a trap can partially desolvate volatile components, causing low yields and under-estimating real concentrations. In recent years alternative trapping methods have been used and these are still developing.

Gaseous samples are of interest directly as a measurement of the environment, for example in workplace exposure to solvents, or as the products of a chemical process or combustion. The vapour above a sample is also of analytical interest as the concentration of volatile analytes in the vapour phase can be directly related to their concentration in the matrix.

8.1. Trapping analytes from vapour samples

A number of methods have been used to trap and concentrate components from gases. Some of the more efficient methods have effectively passed the gas over a cold adsorption tube packed with a form of GC stationary phase, including adsorptive materials, such as porous carbon, or sorptive polymers, such as Tenax, polystyrene–divinyl benzene or PDMS [132]. The gas may be pumped for a specific time or can be allowed to diffuse into the trap in long-term workplace exposure studies. The trapped components are then usually desorbed thermally and passed directly into a gas chromatograph for separation and quantification. A typical recent example is the indoor air monitoring of monoterpenes [133]. Alternatively, the adsorption tube can be eluted using a volatile solvent. Typically carbon disulfide is used because of its high volatility and lack of response in a flame ionisation detector. However, it is a hazardous chemical and this method is difficult to automate, whereas automated thermal desorption (ATD) systems are commercially available, although large sample numbers are needed to justify the investment.

8.2. Headspace analysis

If the components of interest in a solid or involatile matrix are volatile, a well established method [134–136] is to assay them by examining their concentration in the headspace gas above the matrix, either by taking a direct gaseous sample or trapping the volatile material on an SPME fibre (see below). The sample is usually heated to increase the vapour phase concentration and both manual and automated systems are available, the latter giving higher reproducibility.

Either a sample can be taken directly from the headspace (static headspace analysis) or the gas above the matrix can be flushed from the sample vessel and trapped as in the previous section (dynamic headspace analysis). The latter effectively flushes the full headspace gas and concentrates the sample and thus is inherently more sensitive. The time of extraction and the degree of sample agitation are important, as these will influence the rate of release of the analyte from the matrix. The dynamic method is very similar to purge and trap except that the incoming gas flow is not passed over not through a liquid matrix.

Typical analytes and matrices are solvents in body fluids (in particular ethanol in blood as a test for drunken drivers), solvents in matrices, such as polymers or paints, and plastic monomers in food packaging plastics. There are also numerous applications to food samples, such as tomatoes [137], the sulfur components of beer [106], fatty acid esters in rum [138] and spice samples [139], such as coriander [140].

The principal difficulty is accurate quantitation, although this is aided by automation, and standards need to be prepared by the method of standard additions or matrix spiking. Because the assay is based on the distribution of the analyte between the gaseous and matrix phases, the concentration in the vapour phase can be altered by the solubility of the analyte in the matrix phase. For example, with alcoholic beverages the concentration will vary with the ethanol content of the drinks [141,142]. Desirably a similarly volatile internal standard should be used. Quantitation can also be obtained by sequential extraction [143,144] and back-calculation.

Rather than extracting the vapour or flushing it from the analysis bottle, the headspace can be trapped on a SPME fibre [145]. However, the analyst needs to be aware that the distribution is between the fibre and matrix. Thus raising the temperature reduces the deposition onto the fibre (because it increases the vapour concentration above the fibre as well as above the sample), even though it increases the concentration in the headspace. Thus SPME sampling can give a very different selectivity to direct headspace analysis. The headspace sample will favour the volatile analytes but the fibre will favour the less volatile components. This approach has been applied to organic pollutants [146,147], arson samples [148], packaging materials (Fig. 11) [149]. Although a sealed system might seem necessary, open-capped vials in which there is a narrow restricted inlet have also been used and are easier to handle in automated systems [150]. Another recent innovation has been to use microwaves to assist the evaporation into the headspace coupled with SPME [151]. Gas-phase membrane extraction has also been used to trap analytes from the headspace of samples [152].

9. Direct combination of sample preparation and separation

To reduce the manual stages involved in sample preparation, analysts have spent considerable effort to link extraction or sample clean-up steps directly to the separation methods. These linkages can be relatively simple, like thermal desorption into gas chromatographs, to automated sample stations like AASP [153] and ASTED [91] in which a sample can be extracted onto a SPE cartridge after addition of an internal standard and the extract eluted and injected into a HPLC system. More complex sequences can be a carried out by robotic arms. However, these require more careful and extended setting-up and verification procedures and the time and effort spent at this stage must be balanced by a saving over an extended series of analyses [89,154].

Virtually every possible combination and multiple combinations have been explored; including supercritical fluid extraction to supercritical fluid chromatography [155], SFE to LC [156], PLE–SPE–HPLC [157]. As an excess of solvent is usually employed in an extraction frequently some type of focusing of the sample is usually required at the injection point of the separation method, such a low temperatures in GC.

9.1. Large volume injections in GC

The amount of liquid that can be injected directly into a gas chromatographic capillary column without causing band spreading can be very limited. A fairly recent development has been methods, which enable quite large samples to be injected. By the addition of

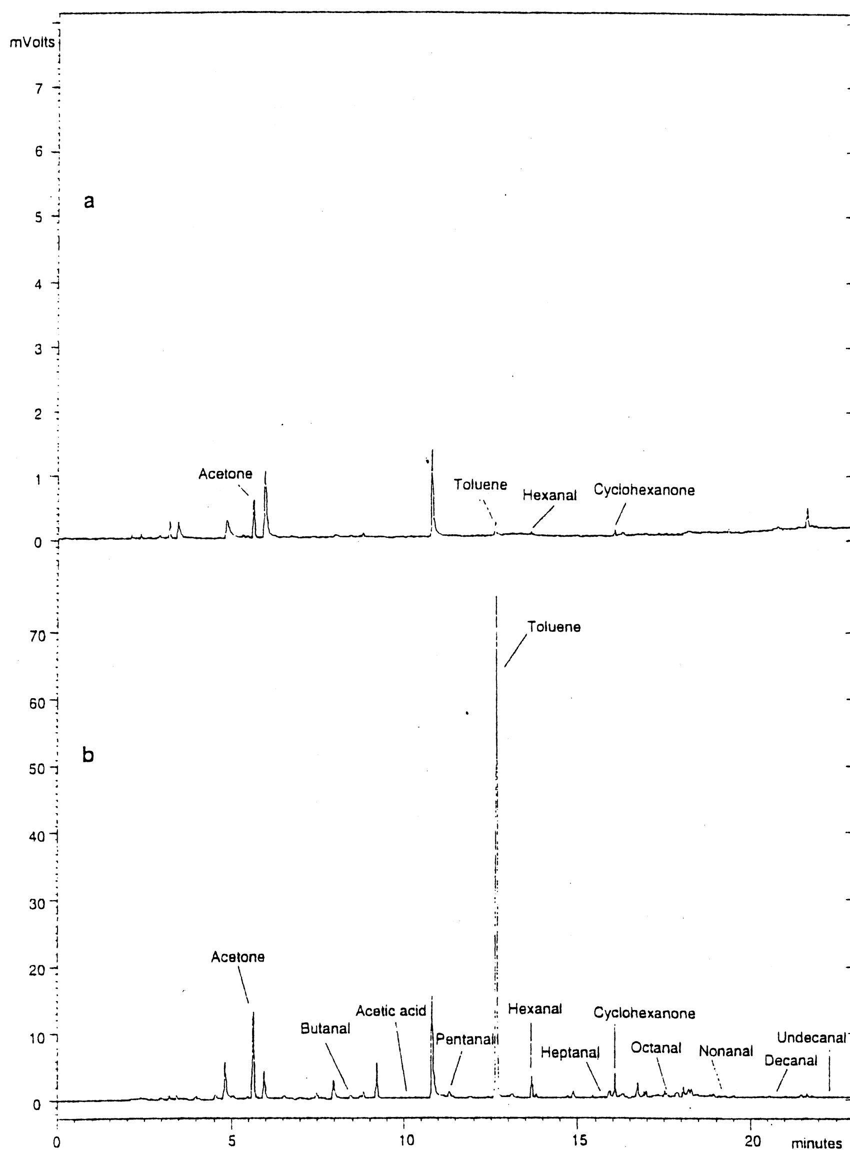

Fig. 11. GC–flame ionisation detection (FID) chromatograms from a packaging material with an unacceptable odour obtained by (a) headspace analysis (b) headspace SPME analysis. Reproduced from Ref. [149].

a vent after a pre-column, large amounts of solvent can be vaporised prior to the main analytical columns but leaving a film on the pre-column wall which solvates the analyte [158–160]. As the evaporation ends, the vent is closed and the residual sample is chromatographed. The technique has been used to inject 100–200 μl or up to 500 μl of aqueous environmental samples. Examples have used the concept to determine the hydrolysis products of sulfur mustards [161] and triazines after membrane extraction [162].

9.2. Coupled column systems LC–LC or GC–GC

Coupled-column separations or multidimensional chromatography can be considered as a form of

sample preparation, as one column is used to derive fractions for the second column. Most of the concepts have been well developed and reported as coupled or multidimensional chromatography [163]. Related methods include column-switching techniques, such as heart-cut, in which a fraction from one column is transferred to a second column for an additional separation and back-flushing, in which more highly retained materials are washed back from a column system through the inlet. These methods are more commonly used in GC than LC as in the latter case the reversal of the flow is harder and more likely to disturb the bed of the column. The complete combination is two-dimensional chromatography in which fractions from the first column are continuously passed to a second column to give a very high sample capacity. These can include GC×GC [164,165], which can generate very high resolution (Fig. 12).

9.3. Isotachophoresis in capillary electrophoresis

In capillary electrophoresis, dilute samples can be focused within the separation capillary by isotachophoresis, utilising differences in the migration rates of a pusher solution so that the analyte is focused to a single point before the electromigration technique occurs [167].

10. Selectivity enhancement

In most of the methods described so far, the discrimination between analytes has been based on differences in their physical properties, which is exploited as solubility, partitioning or volatility differences enabling discrimination. A further distinction is also possible in which discrimination can be obtained by a specific structural difference in interaction, either utilising or mimicking a biological difference.

10.1. Affinity methods

Affinity chromatography is a long employed technique that uses the very specific interactions that occur between analytes and biological systems to specifically retain or trap compounds because the

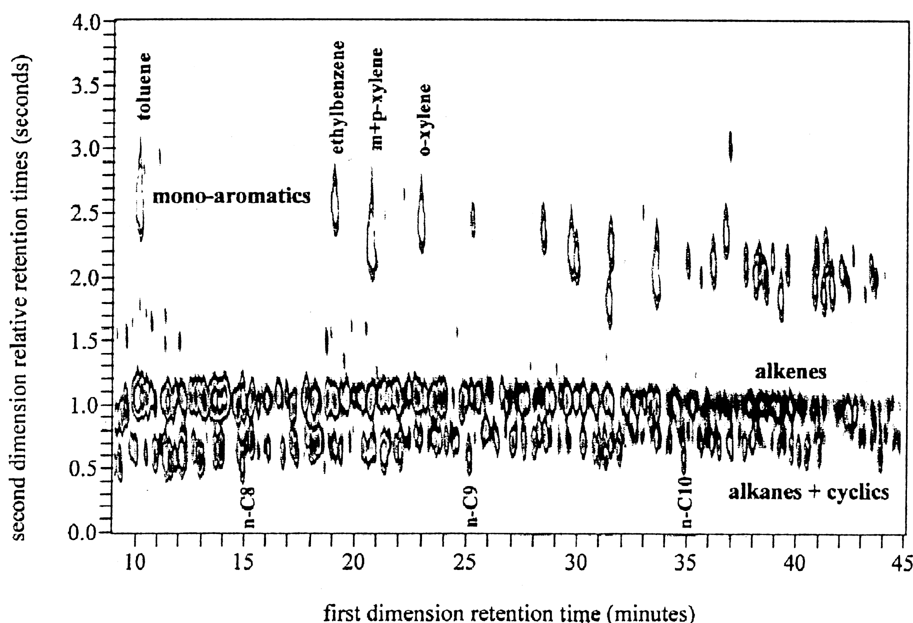

Fig. 12. GC×GC analysis of cracked gasoline using column 1, 10 m DB-1 and column 2, 0.5 m OV1701 with an oven temperature programme of 2 °C/min from 30 to 200 °C [166].

column coating recognises a particular structural shape or interaction [168–170]. The most specific form is immunoaffinity chromatography, which employs an antibody of the analyte to interact and specifically retain it from a solution. The interaction is then broken by solvent or pH changes. Apart from very closely related analytes, the method is highly specific. For example, a test for one barbiturate might trap other barbiturates to different extents [171]. However, the need for the antibody mean that few commercial columns are available and it is therefore difficult to obtain columns for specific assays. However, if the number of assays required can justify the method it can provide a very simple and efficient clean up.

10.2. Molecular imprinting polymers

Attempts have been investigated to mimic the selectivity of interaction in affinity separations by making a synthetic polymer, which contains imprinted cavities generated by a template molecule. These molecular imprinted polymers (MIPs) have been used for both separations and sample clean-up as SPE cartridges [172–174]. However, the degree of selectivity has been questioned and often they function as group-selective systems for compounds related to the original template. This may have advantages in areas, such as pesticide analysis, when only a group separation is required. Attempts have also been examined to provide chiral selectivity although the discrimination is relative rather than absolute. Because the specificity of the interaction is often dependent on a hydrogen-bonding interaction the MIPs are often restricted to use with normal-phase solvents as aqueous solutions preferentially bond and deactivate the interaction sites.

Recent examples of the use of MIPs have included phases to trap caffeine [175], which also show some selectivity toward theophylline and theobromine, salicylic acid [176], cholesterol [177] and quercetin (Fig. 13) [178].

10.3. Restricted-access media

One concept that was examined with some success, was developed originally by Hageston and Pinkerton [179], who to designed a HPLC column whose packing had a hydrophilic external surface and a hydrophobic internal surface, which acted as a reversed-phase material. These restricted-access phases could be effectively used as an on-column sample preparation media, which excluded biopolymers, which were rapidly eluted, but retained smaller analytes for separation [180,181]. More recently the same types of materials have been used in SPE cartridges and in-line traps designed for repeated use, in which the external biocompatible outer layer is based on a α_1-acid glycoprotein [111,182]. The

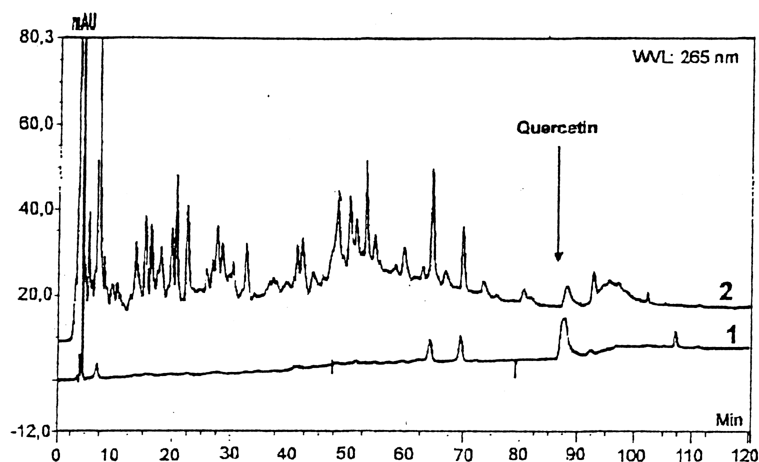

Fig. 13. HPLC separation of merlot (2) before MIPS extraction and (1) fraction eluted from MIPS cartridge with acetonitrile at 265 nm on a Kromasil C_{18} column [178].

phase materials can be polymeric [183] or based on silica.

11. When separation alone is not enough—derivatisation to see the sample

The above methods have generally tried to convert a sample into a form for direct analysis, however, because of analytical and detection limitations, many samples are incompatible with the separation methods. The derivatisation can either be as part of sample preparation (pre-column) or as an aid to detection (post-column) although often the two roles are combined and pre-column reagent are selected to also enhance detection. The original methods were driven by the inability of GLC to handle directly many of the involatile or polar analytes found in biochemistry, such as carbohydrates, lipids, fatty acids and sterols. Frequently these analytes were also aliphatic and although could eventually be examined by HPLC, they had detection problems as they contained only weak chromophores, such as many amino acids and sugars.

A very large number of reactions have been reported but in reality only a few have been used in routine analyses. Even though many textbooks and monographs have reported compilations of derivatisation techniques as part of sample preparation [184–186], this is an approach that most analytical chemists will avoid for a number of reasons. The problem is that derivatisation adds an additional step to the sample preparation procedure. As well as the extra costs involved, care must be taken to ensure that the reaction is working by introducing derivatisable standards. The additional manual or reagent addition stages introduce additional uncertainty into quantitation. Despite their limited role many research groups still study derivatisation reactions but often propose methods that in reality offer little advance on existing methods and frequently employ reagents that have to be specifically synthesised.

11.1. Derivation to enhance volatilisation and separation

The main application of derivatisation is to increase the volatility of analytes for GLC analysis, which still has the advantage of high efficiency and easy linkage to mass spectrometry needed for many studies, such as drug screening. The principal reactions are the formation of trimethylsilyl ethers from sugars, steroids and alkaloids, the methylation of fatty acids and transesterification of lipids, and the acylation of amines.

Some early methods for chiral separations used derivatisation to create diastereoisomeric mixtures enabling separation on achiral column but so many chiral separation columns are now available that this method has fallen into disuse. There also concern that the reaction could itself be stereoselective and hence the results would not reflect the original enantiomeric ratio

11.2. Derivatisation to enhance thermal stability

Although often mentioned in texts this concept is rarely applied in practice. It was principally a GC concern but most affected compounds can now be examined by LC.

11.3. Derivatisation to enhance detection

Particularly in HPLC, some analytes are more difficult to detect and pre-column reagents were selected, which introduced chromophores or fluorophores to enhance detectability and often also reduced interaction problems on the column by reducing the ability of the analysts to ionise. However, in more recent years the use of less active stationary phases, and the introduction of ion-pair separations (and even ion chromatography) and more universal detection, with the mass evaporative and the now increasing spread of mass spectroscopic detectors, has changed the situation considerably. Consequently, few routine methods would now use derivatisation unless the limits of detection were being examined. In many cases laboratories will examine almost any alternative to avoid derivatisation.

Derivatisation is still used for a few samples, such as amino acid separations, or in fields, such as capillary electrophoresis, capillary electrochromatography and microbore LC, where detection is a problem because of the limited cross-column path length for spectroscopic detection. It is also often

also applied in some lab-on-a-chip applications where sample mass is limiting.

12. Can sample preparation be avoided?

Because of the extra work in the inclusion of a sample preparation stage methods in an assay, there is considerable interest in simplifying method or in finding ways to combine the preparation and assay in a single stage. Many examples have already been indicated; the reduction of solvent extraction by using SPE and SPME, and reduced use of derivatisation. More specific extraction can help so there is a continuing interest in MIPs. However, some sample preparation is often still required to overcome the influences that differences in the sample matrix might have on the analytical step. A further problem can arise if residues of the matrix are left in the injection or separation system, as they can affect later separations. For example, a build-up of lipids on a reversed-phase column can change the separation characteristics.

An alternative approach has been to make the detection process more selective so that interfering species are simply not detected. Here single and multiple ion monitoring in GC–MS are a help and LC–MS–MS methods can greatly increase specificity. Effectively these methods filter the ions produced by the MS process to separate the characteristic ions for the analyte and to ignore those from the matrix or from other components, hence reducing the background signal.

However, some sample preparation still may needed, otherwise interferences and signal suppression for LC–MS can occur, even if when there is no obvious sample signal [187,188]. The sensitivity of MS can also be dependent on the composition of the mobile phase. LC–MS–MS is generally more sensitive for readily ionisable analytes. This has the advantage that smaller sample volumes of biological fluids, such a blood samples, are required. Although it was thought that very short separation runs could be used it has now been found that more conventional separations may be needed as these will separate analytes from endogenous interferents. High resolution LC–MS and other approaches such as matrix-assisted laser desorption ionisation time-of-flight may also increase discrimination by using improved mass discrimination as a form of resolution avoiding the need for clean up but expensive

However, the view was expressed at a recent meeting that one effect of the use of LC–MS had been the disappearance of a thorough knowledge of sample preparation [189] and it was felt that to get the full advantages of LC–MS, extensive work-up of the sample could still be needed.

13. Conclusions

As can be seen sample preparation is still evolving and may still be required as even highly discriminatory detector methods may suffer interferences. Generally extraction methods are becoming more selective and more readily combined directly with separation methods. Temperature, alternative solvents, and smaller sample sizes are reducing the use of organic solvents but care is still needed that with real samples that the amount taken for the assay is representative of the total sample.

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