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REVIEW

SAMPLE PREPARATION FOR BIOMEDICAL ANALYSIS

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LIST OF ABBREVIATIONS

AASP	Advanced (Analytichem) Automated Sample Processor
ASPEC	Automated Sample Preparation with Extraction Columns
ASTED	Automated Sequential Trace Enrichment of Dialysates
BHT	Butylated hydroxytoluene
DMSO	Dimethyl sulphoxide
GAG	Glycosaminoglycan
GC	Gas chromatography
Gly	Glycine
HPLC	High-performance liquid chromatography
I S	Internal standard
ISRP	Internal-surface reversed-phase
LSE	Liquid–solid extraction
LLE	Liquid–liquid extraction
MLC	Micellar liquid chromatography

PBA	Phenyl boronic acid
Phe	Phenylalanine
SFE	Supercritical fluid extraction
SPE	Solid-phase extraction
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
TMA-OH	Tetramethylammonium hydroxide

1 INTRODUCTION

The analytical process is the means by which chemical information is obtained from a sample, shown in Fig. 1, it can be depicted as six stages. After a sample has been received, it must be prepared for analysis; the sample preparation stage is intended to improve the specificity of the assay by removing the majority of the matrix whilst concentrating the analyte. The specificity of any assay is derived partly from the analysis but also from the initial clean-up process After the analysis, the response is quantified and the analyte concentration or amount is calculated This result, together with any analytical observations, is incorporated in a final report for the submitter of the sample.

Over the past decades, technological advances have meant that analytical techniques can measure lower quantities of analyte; computer control of instruments has enabled the data produced to be mananged efficiently Until recently, these advances were not matched by improved sample preparation procedures; this meant that sample clean-up could become the rate-limiting step for a laboratory It is the aim of this paper to critically review the techniques available for sample preparation for biomedical analysis and to indicate the future directions in which progress might occur It is intended to limit this review to smaller molecular mass compounds as a recent paper has discussed sample preparation of proteins [1] Moreover, this review will concentrate on the main techniques of sample preparation such as protein precipitation, liquid–liquid extraction (LLE), liquid–solid extraction (LSE) and high-performance liquid chromatography (HPLC) The use of other techniques such as soxhlet extraction, distillation or headspace analysis will not be covered and the reader is referred to the books by Poole and Schuette [2] and Beyermann [3] for further details.

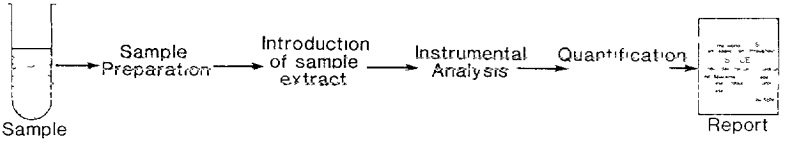


Fig 1 Stages of an analysis

1 1 Objectives of sample preparation

The isolation and measurement of organic compounds present, especially at low concentration, in a biological matrix presents a significant analytical challenge Therefore, a sample preparation scheme can have several objectives These include

1 Removal of unwanted protein or non-protein material that would interfere with analyte determination
2 Removal of material if the resolving power of the chromatographic column is insufficient to separate all the components in the sample completely or in a reasonably practical time
3 Removal of material that would affect chromatographic resolution or reproducibility
4 Solubilization of compounds to enable injection under the initial chromatographic conditions
5 Concentration of the analyte within the detection limits of the analytical instrument
6 Dilution to reduce solvent strength or avoid solvent incompatibility
7 Removal of material that could block the chromatograph tubing, valve, column or frits
8 Stabilization of the analyte to avoid hydrolytic or enzymatic degradation

The objectives of the assay method will indicate how much effort will be necessary to put into a sample preparation scheme, some of the factors to consider are the concentration of the analyte, the matrix involved and the assay specificity required A balance should be struck between the specificity that is obtained by the sample preparation scheme with that from the instrumental assay insufficient sample clean-up may result in interference with the analysis or too great a sample preparation effort may result in the chromatograph being under-utilized [4]

Assays tend to be developed for an individual application, as such, the clean-up should be tailored to the requirements of the method

1 2 Unit operations of sample preparation

Sample preparation can be considered as a number of unit operations, each of which are capable of a specific task [5] These techniques are the fundamental building blocks for any clean-up scheme as they can be taken and matched to the analytical challenge at hand It is important to realize that a particular clean-up technique should not exist in isolation, but should be used in combination with other techniques as required by the analytical method

A list of some typical unit operations that can be utilized for sample preparation is given in Table 1, these operations can be classified into four groups

1 Stabilization and release of analyte from the matrix

TABLE 1

CLASSIFICATION OF SAMPLE PREPARATION UNIT OPERATIONS BY FUNCTION

Group 1 Release of the analyte from the biological matrix		
Hydrolysis	Acid	
	Base	
	Enzyme	
		Proteases
		Lipases
		β-Glucuronidase
		Arylsulphatase
Sonication		
Group 2 Removal of endogenous compounds		
Liquid–liquid extraction		
Liquid–solid extraction (solid-phase extraction)		
High-performance liquid chromatography		
Precipitation	Organic solvents	
	Inorganic acids and salts	
	Ammonium sulphate	
Ultrafiltration		
Dialysis		
Immunoextraction		
Micellar liquid chromatography		
Supercritical fluid extraction		
Saponification		
Lyophilization		
Group 3 Procedures for liquid handling		
Aspiration		
Centrifugation		
Dilution		
Evaporation		
Filtering		
Freezing		
Mixing		
Pipetting		
Salting-out		
Separation		
Group 4 Enhancement of selectivity and sensitivity		
Pre-column derivatization	GC	
	HPLC	
Post-column derivatization	Enzyme reactors	
	Solid-phase reactors	
	Ion pair as a detector	
	Photochemical derivatization	
	Segmented flow reactors	
	Packed bed reactors	
Selective detection modes	Diode array detection	
	Electrochemical detection	
	Fluorescence detection	
	Sensors and biosensors	

2 Removal of endogenous compounds
3 Procedures for the addition, mixing, separation or removal of liquids
4 Enhancement of assay selectivity or sensitivity

This classification is an extension of the original scheme [5] which did not consider Group 4 methods to be part of sample preparation per se However, in light of the integrated approach to sample preparation and detection presented by Frei and Zech [6] it is now appropriate to include these methods and each group will now be discussed in more detail

1 2 1 Release of analyte from the matrix

The unit operations in Group 1 are either to cleave a molecule into a more convenient form to assay, to release an analyte by breakdown of the biological matrix or to stabilize the analyte to avoid artefact formation by undesirable reactions or enzymatic degredation

Molecular cleavage A common pathway of metabolism, for either endogenous or exogenous compounds, is conjugation, where a polar moiety, e g glucuronic acid, glutathione or sulphate, is covalently bonded to the compound [7] The main aim of conjugation is to make the compound more polar to aid excretion from the body Therefore, the aim of the techniques in this group are cleave the conjugate and release the original compound for assay

Specific cleavage of the bond to release the analyte can be accomplished by the use of enzymes, such as β-glucuronidase (EC 3 2 1 31) or aryl sulphatase (EC 3 1 6 1) However, there are a number of disadvantages that must be remembered the enzyme can be inhibited by substances in the sample, e g salt content of urine, therefore positive control experiments must be undertaken to observe the enzyme activity As the species from which the hydrolyzing enzyme is isolated can result in different rates of hydrolysis [8], optimization of the hydrolysis conditions should include testing the enzyme from different sources The enzymatic hydrolysis can be performed off-line in the traditional manner [9,10] but the introduction of a large amount of enzyme may interfere with the subsequent HPLC analysis Increasingly, hydrolysis can be accomplished on-line with a post-column reactor containing either immobilised enzyme [11,12] or acid [13] The on-line approach means a Group 4 method is now used with potential time-saving and convenience

The use of non-specific methods such as acidic or basic hydrolysis can also be employed to hydrolyse the sample The time to completion is usually shorter than that for the enzymatic hydrolysis, however, the conditions used are generally harsher (extremes of both pH and temperature can be encountered), therefore precautions should be taken to ensure that the resulting analyte(s) are stable under the conditions used

An alternative to cleavage of the molecule that should be considered is to determine the intact conjugate by HPLC [14–16], this should overcome the problems of hydrolysis by either specific or non-specific methods

The breakdown of macromolecules to fragments that are easier to analyze is another function of the techniques in this group, often macromolecules that are difficult to analyze per se due to heterogeneity can be split into component parts Glycosaminoglycans (GAGs) are long-chain polysaccharides composed of repeating disaccharides of either glucosamine or galactosamine, prior to separation and HPLC determination they are cleaved to their component disaccharides by enzymatic hydrolysis Chondroitinase ABC (EC 4 2 2 4) and chondroitinase AC (EC 4 2 2 5) can be used separately or in combination for the determination of chondroitin sulphate and hyaluronidase (EC 3 2 1 35) can be used to cleave hyaluronic acid into its substituent groups [17]

Breakdown of the biological matrix Where the analyte is bound to a component of the matrix, enzymes can be used to break down the components of the matrix and release, e g , protein-bound compounds Proteases, such as pepsin [18] and subtilysin [19], can be used to provide a relatively controlled breakdown of plasma and blood A problem with this approach can be the release of low molecular mass molecules that are normally bound to the macromolecules which can be co-extracted and interfere with the subsequent instrumental analysis [20], therefore further clean-up or more chromatographic resolution may be required with this approach

1 2 2 Removal of endogenous material

A biological matrix may be solid or particulate, e g muscle, tissue, milk, faeces or blood, or a mixed composition of organic compounds in an aqueous solution, e g urine or plasma A biological matrix consists of many components macromolecules such as proteins, carbohydrates and lipids which can be components of cellular structures, used as energy sources or involved with the transport of other molecules In addition smaller molecules, with differing polarities, are also present in the matrix The unit operations falling into Group 2 are considered by most analysts as 'sample preparation', these techniques are responsible for removing the majority of the biological material from the sample matrix prior to analysis

The techniques in this group comprise a variety of physico-chemical procedures such as adsorption or partition that aim to selectively isolate the analyte in preference to components of the sample matrix, e g LLE, LSE or HPLC Other techniques use ultrafiltration or precipitation to remove proteins and other macromolecules, this area will be discussed extensively in Section 2

1 2 3 Liquid handling procedures

Group 3 methods are mainly involved with the addition, mixing, removal or transfer of liquids, whilst they appear to be mundane and routine they provide the links between the techniques in the other groups For example, in an LLE the sample has to be pipetted into the extraction vessel, the internal standard added to the solution, the pH value of the aqueous phase may need to be ad-

TABLE 2

COMPARISON OF THE UNIT OPERATIONS REQUIRED FOR SOME SAMPLE PREPARATION SCHEMES

Table derived from Huber and Zech [102]

Liquid–liquid extraction [52]	Off-line liquid–solid extraction [14]	AASP [14]	Gilson-AASP [139]	On-line HPLC [102]
Aliquot sample	Centrifuge sample	Centrifuge sample	Centrifuge sample	Centrifuge sample
Add I S	Aliquot sample	Aliquot sample	Aliquot sample	Aliquot sample
Mix	Add I S	Add I S		
Add octanol	Mix	Mix		
Mix	Activate phase 1	Activate phase 1		
Centrifuge	Activate phase 2	Activate phase 2		
Transfer organic	Apply sample	Apply sample		
Add acid	Wash cartridge	Wash cartridge		
Mix	Elute analytes	HPLC analysis		
Centrifuge	Transfer to vials			
Aspirate organic	HPLC analysis			
Transfer aqueous				
Add acetonitrile				
Mix			Reload AASP cassettes	Repack pre-column
Add solid Na_2CO_3				
Mix				
Centrifuge				
Aspirate acetonitrile				
Transfer to vials				
HPLC analysis				

justed, and finally the extracting solvent is added, all of these stages are accomplished by liquid handling procedures

Liquid handling procedures can often be the rate-limiting steps in a sample preparation scheme as too many of them will result in a tedious and labour-intensive assay

1 2 4 Enhancement of selectivity and sensitivity

The unit operations that comprise Group 4 are mainly concerned with derivatization of an analyte to enhance the assay sensitivity and specificity such as pre-column derivatization reactions [21–23] and post column derivatization and reaction detectors [24–28] The use of specific detectors such as electrochemical detectors [29] and diode array detectors with associated computer interpretation [30] are also part of this group These techniques will not be discussed further here as they have been extensively reviewed

1 2 5 Use of unit operations

Unit operations provide the analyst with a means of evaluating any sample preparation scheme, simply listing all the individual operations within the extraction provides a means of critically evaluating a method If there are many individual operations in an extraction scheme, this means that the method will probably be slow and tedious to use and have a low throughput, whereas an assay with a few unit operations should be simpler to use and generally be capable of a higher throughput with respect to the sample preparation phase of the method

To illustrate this point, Table 2 lists the unit operations for a number of different sample preparation methods such as LLE, LSE and on-line HPLC As can be seen, the simpler or the more automated the method becomes, the fewer the number of unit operations are required and the quicker the method should be to perform However, it is important to realize that a critique of any sample preparation method should be viewed with the objectives of the individual analytical procedure in mind (limits of quantification, volume of sample, analyte properties, etc) and the resources available to put it into operation.

The on-line methods in Table 2 are easier to operate but require a good technical understanding of the analytical technique used, whilst Group 4 methods may also require a detailed knowledge of chemistry employed

2 TECHNIQUES FOR SAMPLE PREPARATION IN BIOMEDICAL ANALYSIS

The main methods for the removal of endogenous material include dilution, precipitation, ultrafiltration, LLE, LSE [or solid-phase extraction (SPE)] and HPLC

However, these techniques require that the sample be liquid, therefore the first task of a method may be to liquidize a sample in order that the above-

mentioned techniques may be utilized Homogenization methods, generally known as destructive disruption, have been reviewed by Maickel [31], here the procedures by which solids or semi-solids can be made liquid and the advantages and disadvantages of the media used to liquidize them are discussed

2 1 Dilution

Where an analyte is present in a sufficiently high concentration or there is a specific detection system, then dilution is a very simple and effective means of sample preparation

A diluting fluid, such as water or a buffer, is added to the sample, which is mixed, centrifuged if necessary to remove particulate matter, and then assayed The diluting fluid can have several functions, it can reduce the signal due to endogenous material in the matrix, reduce the viscosity or ionic strength of the sample, ensure the compatibility of the sample with the mobile phase if HPLC is the method of analysis, or disrupt weak bonding between the analyte and plasma proteins

An example of dilution is the analysis of triamterene in human urine, this involves a 1 10 dilution of the sample with water before quantification by fluorimetry [32] As this is a non-specific procedure, metabolites as well as the parent drug are measured, and care should be taken in the interpretation of the results

2 2 Precipitation and deproteinization methods

Removal of proteins and precipitation of other endogenous material are simple and effective methods of sample preparation that can be used alone or in conjunction with other methods of sample clean-up

2 2 1 Protein precipitation

Removal of protein by denaturation or precipitation is an effective method of sample preparation that is often used on plasma and whole blood samples prior to analysis The main reason is to remove proteins that can precipitate when in contact with the mobile phase and thereby block tubing, cause increases in back pressure or deterioration of HPLC column performance

The advantages of this technique are the speed at which the sample can be prepared and its simplicity The main disadvantages are that there may be loss of the analyte by occlusion in the precipitate and when using strong acids the analyte must be stable at the low pH values encountered in the supernatant If the supernatant is to be injected directly into an HPLC system it should be compatible with the mobile phase, the latter may need a suitable buffering capacity if the pH value of the sample extract differs greatly from it The agents commonly used to precipitate proteins are either acids or organic solvents or

inorganic salts or metallic ions; each type of agent will now be presented with an assessment of their relative efficiencies

Acid precipitation methods The commonly used acids for the precipitation of proteins are trichloroacetic acid (TCA), perchloric acid and tungstic acid, some other acids that have been used as precipitating agents are molybdic, sulphosalicylic, phosphotungstic, metaphosphoric and picric acids These acids act by forming insoluble salts with the cationic form of the proteins at low pH values These agents are very efficient when used as a 5–20% solution, for best results the solutions should be cold [31]

Organic solvents Organic solvents that are miscible with water, such as acetone, methanol, ethanol and acetonitrile, are used to lower the solubility of proteins and precipitate them from solution [33] Occasionally the use of two solvents may be necessary for quantitative recovery of analytes as shown by Li et al [34] to extract porphyrins from liver, methanol was used with dimethyl sulphoxide (DMSO), the former solvent to precipitate proteins and the latter to release the bound porphyrins from the protein Care should be exercised with the use of acetone as it has a relatively high UV cutoff (330 nm) which could result in large peaks being seen on HPLC profiles as excess solvent in the sample elutes from the column

A combination of an acid and an organic solvent such as acetonitrile, methanol or DMSO may be useful as the organic solvent helps to solubilize and extract the analytes from the proteins [33] Care may need to be taken as methanol in acid may lead to esterification of some carboxylic acids [33]

Metallic salts in alkaline solution Proteins can also be removed from a sample by the use of cupric or zinc salts in alkaline solution [35,36], they act by forming insoluble salts with the proteins in solution It may be necessary to adjust the pH value of the solution before HPLC analysis and the method is unsuitable for analytes with a tendency to form metal complexes [33]

Ammonium sulphate Ammonium sulphate has been used as a protein precipitant for many years, a saturated solution is added to the sample, mixed and centrifuged to remove the precipitated proteins Ammonium sulphate is a relatively inefficient protein precipitant as 1 5 ml of a saturated solution will remove $<75\%$ of the protein in a 0 2-ml plasma sample [37], however, it is still possible to inject the supernatant directly into a liquid chromatograph [38] Furthermore, it should be noted that protein denaturation with ammonium sulphate is reversible, in contrast to the use of strong acids, organic solvents and metallic salts

Efficiency of protein precipitation methods Blanchard [37] has evaluated the relative efficiency of several protein precipitation methods, detailing the volumes of precipitation agent added to 0 5 ml of plasma and then determining the amount of protein remaining in the supernatant Table 3 lists the agents used, the pH value of the final supernatant and the volume of reagent required

TABLE 3

RELATIVE EFFICIENCIES OF SOME COMMON PROTEIN PRECIPITATION METHODS [37]

Precipitant	Supernatant pH	Volume of agent (ml) to precipitate > 95% protein in 0 5 ml plasma	Comments
Acids			
Trichloroacetic acid (10%, w/v)	1 4-2 0	0 2	Use at low temperatures [31]
Perchloric acid (6%, w/v)	<1 5	0 4	Use at low temperatures [31]
Tungstic acid	2 2-3 9	0 6	Prepare reagent fresh
Metaphosphoric acid (5%, w/v)	1 6-2 7	0 4	
Inorganic salts			
Copper sulphate-sodium tungstate	5 7-7 3	1 0	Unsuitable for analytes that form metal complexes
Zinc hydroxide	6 5-7 5	1 5	
Ammonium sulphate (saturated)	7 0-7 7	2 0	Relatively inefficient agent
Organic solvents			
Acetonitrile	8 5-9 5	1 0	Ultraviolet cut-off at 330 nm
Acetone	9 -10	1 0	
Ethanol	9 -10	1 5	
Methanol	8 5-9 5	1 5	

to precipitate >95% of the plasma protein, whilst Fig 2 shows the relative efficiencies of these reagents in a diagrammatic form.

From these results it can be seen that TCA and perchloric acid are very efficient at precipitating proteins; however, the pH value of the supernatant is very low and the analyte must be stable for these methods to work. The four organic solvents tested in this work are less effective than the acids above; however, they are commonly employed prior to an HPLC analysis and their relative effectiveness (acetonitrile > acetone > ethanol > methanol) is inversely related to their polarity [37]

Dilution occurs during protein precipitation and if no further sample prep-

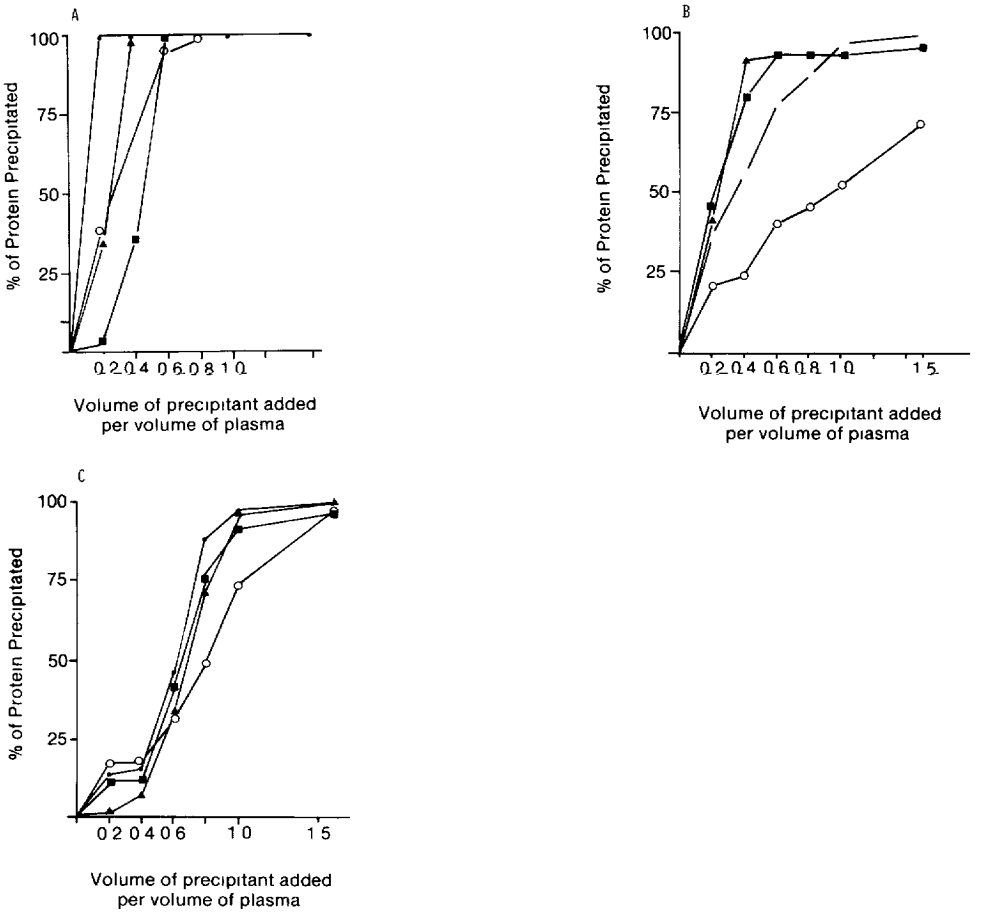


Fig 2 Relative efficiency of twelve protein-precipitating agents (A) Inorganic acids (●) 10% (w/v) TCA, (▲) 6% (w/v) perchloric acid, (■) tungstate-sulphuric acid, (○) 5% (w/v) phosphoric acid (B) Cationic agents and inorganic salts, (●) $CuSO_4$-Na_2WO_4, (▲) $ZnSO_4$-NaOH, (■) $ZnSO_4$-$Ba(OH)_2$, (○) saturated $(NH_4)_2So_4$ (C) Organic solvents (●) acetonitrile, (▲) acetone, (■) ethanol, (○) methanol

aration is undertaken it may result in a lower sensitivity of the assay method If HPLC is the analytical stage, this may be overcome by diluting the sample below the strength of the mobile phase and concentrating the analyte on the head of the column Alternatively, a protein precipitation method could be linked with an LLE [39] or LSE to provide further clean-up and concentration

A protein precipitation method should be matched to the analytical problem for creatinine determination it was found that TCA was very effective at removing interfering compounds but that sulphosalicylic, perchloric and tungstate acids were poor choices [40,41]

2 2 2 Precipitation of urine pigments and bile salts

Urinary pigments and bile salts can often produce high backgrounds that can prevent quantification of analytes, however, the removal of these compounds, even by extensive extraction schemes, can often be relatively difficult or time-consuming Therefore their removal by precipitation as insoluble lead salts may be preferable before further purification and analysis [39,42]

2 2 3 Ultrafiltration

Removal of proteins and other large macromolecules without precipitation can be achieved using ultrafiltration This can be used to measure the concentrations of 'free' or non-protein bound analyte in plasma or serum but is also a sample clean-up method in its own right The procedure uses cone-shaped membranes that fit into centrifuge tubes, aliquots of the sample are placed in the tops of the cones and centrifuged gently (ca 200 *g*) The membranes allow passage of molecules smaller than the exclusion limit (e g 25 000 or 50 000 molecular mass) that are not bound to macromolecules

Virtually all the protein in the plasma can be removed [37], but a potential disadvantage of the technique is the binding of the analyte to the membrane and control experiments should be undertaken to determine the extent of this One of the major advantages of ultrafiltration over dialysis is its speed, especially when hollow fibre membranes are used Another advantage of this technique over protein precipitation is its applicability to small sample volumes as no dilution occurs during filtration [43] Degradation of labile compounds can be avoided as there are no extremes of pH Also if an analyte is metabolized by a plasma enzyme the removal of this protein by ultrafiltration should stabilize the analyte by removing the source of degradation

Demonstration of ultrafiltration as a sample preparation technique has been shown by Duncan et al [44] for the determination of carboplatin, an antitumor agent which was virtually insoluble in organic solvents SPE and protein precipitation methods were also tried but did not provide sufficiently clean extracts As carboplatin was less than 3% bound to plasma proteins but a major metabolite was highly protein-bound, ultrafiltration provided a means of removing this compound and improving the assay specificity After centrifuga-

tion, the filtrate was evaporated, reconstituted in methanol–water (9·1, v/v) and assayed by HPLC Disturbance of the binding equilibrium was avoided by processing the samples without freezing and thawing and the internal standard for the assay was not added until after the centrifugation step

2 2 4 Dialysis

Dialysis can separate an analyte from the matrix by diffusion through a semi-permeable membrane rather than centrifugal force as with ultrafiltration The membrane restricts the size of molecule that can pass through it, holding back the macromolecules and allowing the lower-molecular-mass compounds to pass through into the dialysis medium. Diffusion is a slow process and is driven by a concentration gradient which continues to function until an equilibrium is established, therefore the maximum recovery of analytes in equilibrium is 50%, assuming that the sample and dialysis medium volumes are the same However, dialysis can only be applied to compounds that are relatively weakly protein-bound, those analytes that are strongly protein-bound require the binding to be disrupted The use of dialysis for sample preparation would normally be discouraged because it can involve a lot of tedious work and a large dilution of the sample

However, coupling dialysis with HPLC provides the ability to concentrate the analyte prior to analysis, ASTED (Automated Sequential Trace Enrichment of Dialysates) [45] is now a commercially available instrument The addition of a dialysis unit with a column-switching HPLC system (see Section 2 5 1) can be an alternative approach to ASTED [46] On-line dialysis needs to be fully evaluated to establish its potential.

2 3 Liquid–liquid extraction

A method of sample preparation for many years has been LLE, this commonly entails the direct extraction of the biological material with a water-immiscible solvent. The isolation of the analyte is achieved by partitioning it between the organic phase and an aqueous medium, an equilibrium distribution is established between the two phases which follows the Nernst Distribution law [2,47] The distribution ratio between the two phases will be influenced by the choice of the extracting solvent, pH value of the aqueous phase and the ratio of the volumes of the organic to aqueous phases The initial conditions of the extraction should be such that the analyte is preferentially distributed into the organic solvent

If there is a low recovery of the analyte this can be enhanced by successive extractions of the sample to produce acceptable recoveries [2,48], but in practice it is often the case that a large excess of extracting solvent can be used in order to save time and achieve the same result

2 3 1 Choice of extracting solvent

The relative lipophilicity or hydrophobicity of the analyte will determine the choice of the solvent as the analyte must be soluble in it for the extraction to function Solvents suitable for extraction should have a low boiling point in order to remove them efficiently at the end of the extraction and a low viscosity to aid mixing with the sample matrix The solvents for LLE can be arranged in order of polarity into an elutropic series (see Table 4) A factor for consideration is the amount of water that can dissolve in the solvent, this can influence the amount of co-extracted interference in the final extract In general for extraction of biological samples, the less polar the solvent, the more selective it is, therefore the solvent of choice is usually the least polar one in which the analyte is still soluble

A major advantage of LLE is selectivity, depending on the choice of solvent, the drug of interest can be extracted from most of the endogenous components Moreover, this can be used to greater advantage where a non-specific spectrophotometric assay is employed For example, if a drug is extensively metabolised, and the metabolites have the same chromophore as the parent compound, they then have the potential to interfere with the assay, but the drug may be selectively removed, by using a lipophilic solvent to extract the sample, leaving the metabolites in the biological fluid Alternatively, if chromatography is utilized to give the separation, a hydrophilic solvent may extract the drug as well as the metabolites to allow concomitant determination of each analyte

When considering the use of LLE, any pH can be utilized, subject to the stability of the compound to be isolated and the extracting solvent (e g ethyl acetate)

Solvent-stabilizing agents and additives A problem with LLE that is often overlooked is the addition of compounds by manufacturers to prevent oxidation or decomposition of their product

For example, to prevent phosgene formation in chloroform the solvent is

TABLE 4

PHYSICAL PROPERTIES OF SOME SOLVENTS FOR LIQUID-LIQUID EXTRACTION

Solvent	Boiling point (°C)	Solvent polarity (p')	Solubility in water (%)
n-Hexane	69	0	<0 001
Carbon tetrachloride	77	1 7	0 08
Cyclohexane	81	0	0 01
Chloroform	61	4 4	0 82
Dichloromethane	40	3 4	1 30
1,1-Dichloroethane	57		5 03
Diethyl ether	35		6 04
Ethyl acetate	77	4 3	8 08

often stabilised with 2% ethanol In itself, this is no problem but when a method is to be established from the literature it may not be immediately obvious to the reader if the solvent contains ethanol (this assumes that the author knew the composition of the extracting solvent) The presence of ethanol can change the polarity of the solvent and affect the specificity and recovery of a method

Diethyl ether can have any one of several chemicals added to it to prevent peroxide formation These include 2% ethanol (Merck, Darmstadt, F R G), 2–3 ppm pyrogallol (May and Baker, Dagenham, U K) and <0 1 ppm butylated hydroxytoluene (BHT) (Aldrich, Gillingham, U K), sometimes the addition of the compound is not mentioned in the specification of the solvent These compounds can affect the outcome of an assay Ethanol, as mentioned above, can affect the polarity of the solvent and hence its selectivity, BHT could be concentrated upon evaporation and form a discrete peak on a chromatogram, whilst pyrogallol is electrochemically active and in the author's experience can interfere with an HPLC analysis when using an electrochemical detector

Solvent impurities It can be the case that the extracting solvent can introduce impurities into an analysis, the term 'impurity' is a function of the detection system used in the analytical scheme [thin-layer chromatography (TLC) spray reagents or HPLC detection, UV, fluorescence or electrochemical] and the limits of detection required [49] It may be necessary to re-distill or purify the solvent prior to use, e g ethyl acetate [50]

2 3 2 Liquid–liquid extraction in practice

Control of extraction by pH For an extraction to be successful, the analyte should be un-ionised to facilitate partition into the organic phase, therefore, the pH value of the aqueous phase may need adjustment to ensure that the molecule is uncharged

This has been demonstrated with the extraction of a molecule with a carboxylic acid moiety, when the pH is below 3 the compound can be extracted into an organic solvent whilst above pH 5 it remains in the aqueous phase [48] After an initial extraction and isolation of the extracting solvent, pH adjustment can be used to reverse the process and re-extract the charged analyte into an aqueous phase for further purification In this way it is possible to build up an extraction scheme to further purify an analyte Moreover, the use of pH adjustment allows the fractionation of a sample into acid, neutral and basic fractions, each of which can be processed further [2,51]

Inherent in the technique are drug losses because of an inability to transfer 100% of the extracting solvent, for example, when transfer losses are taken into account, the extraction procedure for oxmetidine has a maximum theoretical recovery of 75% [10]

Mixing and emulsion formation To promote a rapid equilibrium of the extraction, a large surface area is an advantage and the organic solvent is usually

present in excess The method of mixing can be manual or mechanical shaking (rolling, reciprocating, tumbling or wrist action) or by vortex, this should ensure good mixing of the two phases for an efficient extraction

A major problem, regardless of the mode of mixing, is the formation of emulsions which are extremely difficult to break and often cannot be separated by either centrifugation or ultrasonication Emulsion formation causes loss of analyte by occlusion within the emulsion, and lower recoveries ensue, it can be minimized by less rigorous mixing or by the use of larger volumes of extracting solvent If the latter is used, then it is usually removed from the extraction process at some later stage

Removal of solvent The removal of solvent (e g evaporation, freezing in liquid nitrogen, pipetting, etc) is frequently a rate-limiting step and can present several problems

1 There is the safety hazard involved in handling the solvent, as nearly all those used in LLE are toxic or inflammable, so the vapours generated need to be vented efficiently to the atmosphere
2 The conditions used to evaporate the solvent may also cause low recovery of the compound due to degradation by heat, volatilization or adsorption to glass
3 The costs of removing waste solvent from a site are expensive and time-consuming.

Salting out As indicated above, the evaporation of solvent can be a time-consuming task, therefore a technique known as salting out can be applied to separate a mixture of water and water-soluble solvent such as acetonitrile or ethanol This has been used successfully in the analysis of the histamine H_2 receptor antagonists cimetidine [52], oxmetidine [10], SK&F 93479 [53] and SK&F 93319 [54] Following extraction into an organic solvent and re-extraction into an acid phase, acetonitrile (200 μl) is added to the acid followed by circa 5 g of solid potassium carbonate The addition of the carbonate increases the ionic strength and polarity of the aqueous phase which forces the acetonitrile containing the analytes to form a discrete layer on the surface This layer is recovered and injected directly into the chromatograph

2 3 3 Extraction of polar molecules

LLE is not suitable for all compounds, for example, highly polar molecules are not usually extracted by this method However, the use of a suitable ion-pairing reagent can extend LLE to molecules of this type [48]

It is also possible to form complexes to extract polar molecules so that the polarity of the newly formed molecule is amenable to extraction One such example is the formation of phenyl boronic esters with molecules containing vicinal hydroxyl groups, this reaction has been applied to catecholamines [55,56] and nucleosides [57] This concept has also formed the basis of a phenyl

boronic phase (PBA) solid-phase packing which has been reviewed previously by McDowall et al [58]

2 3 4 Direct derivatization

A discussion of some examples of direct derivatization of drugs in untreated biological samples prior to gas chromatographic (GC) analysis has been presented by Vessman et al. [59] This paper discusses derivatization in the sample matrix followed by extraction of the analyte as well as two-phase reactions where derivatization takes place in the organic phase whilst extraction of the analyte is continuing from the aqueous sample It would be advantageous to take the principles described above and adapt them to HPLC for greater applicability

2 3 5 Liquid–liquid extraction on a solid support

An attempt to overcome some of the limitations of LLE is the use of disposable Chem Elut or Tox Elut (Analytichem International, Harbor City, CA, U S A) columns, which contain diatomaceous earth as an adsorbent. The diluted sample to be extracted is poured through the cartridge and held on the support as a very thin film, the extracting solvent is then passed through the column, and the high surface area of the film allows very efficient extraction of the analytes of interest This approach avoids emulsion formation but not the evaporation of the solvent used in the extraction. If TLC is the analytical method used then Tox Elut columns are preferred, but Chem Elut columns are better suited for HPLC and GC analysis as higher-purity silica is used in the cartridges

2 3 6 Summary of liquid–liquid extraction

LLE is a major technique for sample preparation, it is very versatile and well documented, but due to the drawbacks of the technique it is beginning to be replaced by LSE and HPLC for routine applications with high throughput LLE can be used to assay efficiently large numbers of samples and can be adapted to batch-mode operation, however, the transfer steps involved make the process labour-intensive and tedious

2 4 Liquid–solid (solid-phase) extraction

An alternative method of isolating an analyte from a biological matrix is LSE, which consists of mixing the biological fluid with an absorbent, separating the solid phase and eluting the analyte with an appropriate solvent The success of this approach depends on the relative affinities of the analyte between the biological matrix and the adsorbent and the relative ease of eluting the compound for subsequent analysis LSE should be seen as an important addition to sample preparation techniques and not as a replacement [60]

LSE is very easy to use as it is considerably easier to separate a liquid and a solid than two immiscible liquids The most common approach utilized today involves the solid adsorbent packed in a small cartridge or column, the flow through this packed bed provides the surface area necessary to ensure a high extraction and clean-up This approach means that most LSE schemes can have a higher throughput than a comparable LLE because of the ease of handling the solid phase Also the potential for automation is easier compared with other methods of sample preparation

2 4 1 Classical adsorbents

Adsorbents such as carbon, celite and alumina have been used for many years, the advantage is that they avoid the formation of emulsions that is a disadvantage of LLE, but often the analytes were eluted from the solid phase by organic solvents which still had to be evaporated [61,62] Alumina is still frequently used for the isolation of catecholamines prior to separation and detection by HPLC although it was originally used by Anton and Sayer in 1962 [63]

Ion-exchange resin-loaded papers were introduced by Dole et al [64] for early urine screening programmes for detecting misuse of drugs, however, their performance was unreliable and there were variable recoveries of different classes of misused drugs Recently, more reliable products with various types of ion-exchange resins incorporated into a PTFE membrane have been introduced [65], these are packaged in a disposable filter through which the sample is passed and the analytes extracted prior to analysis or components of the matrix removed, e g metallic cations to prevent decomposition of analytes in the remaining sample

Amberlite XAD-2, a non-ionic resin, first used by Fujimoto and Wang [66], and subsequently refined [67], has been used successfully for screening drugs in urine and extended to drug metabolite isolation XAD-2 is a styrene divinylbenzene copolymer with a macroreticular structure which provides a high surface area-to-volume ratio, it binds relatively lipophilic but water-soluble organic molecules by a mixture of hydrophobic forces [68] This approach could be used for the simultaneous extraction of acidic, neutral and basic drugs in one step and saved time and effort

2 4 2 Use of bonded phase silica for sample preparation

The recent trend has been to use chemically modified silicas for sample preparation, these are available commercially from an increasing number of vendors (Analytichem International, Bond Elut, Waters Assoc , Sep-Pak, Baker, BakerBond, Supelco, Superclean, Alltech, Extract-Clean, etc) The majority of solid-phase cartridges are packed in a Luer tipped polypropylene reservoir with 100–500 mg sorbent dry packed into the bottom between two fritted discs

For use the extraction cartridges are mounted on a vacuum manifold, which can hold eight to thirty extraction cartridges for batch extraction [58]

LSE separates the different compounds in a sample by utilizing the principles of modern liquid chromatography In this process, the sample passes over the stationary phase, the analytes being separated according to the degree of which each component is partitioned or adsorbed by the stationary phase The objective of an extraction scheme is to achieve 'digital chromatography' [69], this is different from the normal aims of a chromatographer who requires good peak shape and relatively short retention times Extraction schemes based on chromatographic principles should either retain the analyte on the phase allowing isolation and clean-up or elute it rapidly in the smallest possible volume prior to analysis In other words, the capacity factor, k', which in elution chromatographic analysis should be in the range 1–10, should be > 1000 for retention and $< 0\,001$ for elution when using LSE to isolate molecules [70]

Choice of phase The types of silica bonded phase available for LSE are presented in Table 5 and are grouped according to functionality, e.g non-polar, polar, etc Also listed is information on four manufacturers' phases such as the average percentage carbon loading, whether or not the phase is endcapped and the method of bonded silica synthesis It should be noted that most non-polar phases are endcapped but the polar or ion-exchange phases are not, the degree of endcapping can be important as the residual silanols can often play an important role in retention and elution of compounds (see respective paragraphs in Section 2 4 2) In addition to the packings listed in Table 5, florisil and alumina (acid, neutral and basic washed) are also available in Bond Elut and Sep-Pak cartridges Although not mentioned in the table, the porosity and particle size distribution of the packing material may also be factors influencing selectivity of a separation

The same packing material from different manufacturers can have an effect on the selectivity of an assay, Ruane and Wilson [71] tested the ability of two manufacturers' C_{18} cartridges to extract four β-blocking drugs With two of the drugs tested there were large differences in recovery between the two makes, this may provide a valuable source of selectivity when developing assays This selectivity may depend, in part, on the original silica particle, the extent and coverage of the bonded silica phase, the production methods and the number of silanols left after endcapping

Method development The following guidelines have proved useful to the author

1 Search the literature for chromatographic assay methods published for similar structures If the analyte is a novel compound, it may be possible to deduce chromatographic conditions from similarities in structure to existing chemical entities

TABLE 5

CHOICE OF PHASES FOR LIQUID–SOLID EXTRACTION

The phases manufactured by Waters Associates are monomeric, all the remaining phases are all polymeric N A data not available

Type of phase	Manufacturer of phase							
	Analytichem International		Applied Separations[a]		J T Baker		Waters Assoc	
	% Carbon	Endcapped	% Carbon	Endcapped	% Carbon	Endcapped	% Carbon	Endcapped
Non-polar phases								
C_1	4 4	Yes	6–8	No				
C_2	5 8	Yes	7–9	No	4 8	Yes		
C_4	8 3	Yes			5 9	Yes		
C_6	10 4	Yes						
C_8	12 1	Yes	10–14	Yes	14	Yes		
Cyclohexyl	9 7	Yes			12	Yes		
Phenyl	10 3	Yes			10 6	Yes		
C_{18}	19 1	Yes	17–22	Yes	17 2	Yes	10	Yes
C_{18}-OH	N A	No						
Polar and weak ion-exchange phases								
Silica	0	No	0	No	0	No	0	No
Cyanopropyl	7 4	No[b]	5–8	No	10 5	Yes	N A	Yes
Diol	6 4	No			8 6	Yes	N A	No
Aminopropyl	5 0	No	4–6	No	6 4	Yes	N A	No
Primary/secondary amine	N A	No			N A	Yes		
Propylcarboxylic acid	6 9	No	4 5–6	Cleaved	N A	Yes		
Diethylamino	N A	No						
Strong ion-exchange phases								
Propylsulphonic acid	1 8	No	4 5–6	No	N A	Yes		
Benzenesulphonic acid	8 4	No			N A	No		
Quaternary amino	6 7	No	6–8	No	N A	No		

[a]The range of % carbon loading given by Applied Separations is the range possible, synthesis is normally tightly controlled within 0 5% but custom packings within the ranges in the table are possible

[b]Endcapped CN is also available from Analytichem International

2 Consider the physico-chemical properties of analyte, e g solubility and ionisation, this knowledge should give the conditions for a potential retention mechanism such as ion exchange or hydrophobic on a particular type of phase
3 The physico-chemical properties of the sample matrix should be considered polar or non-polar This combined with the analyte conditions should help in further developing the extraction scheme
4 Consider the properties of the HPLC mobile phase as this will give information about the elution conditions for the compound from the solid-phase cartridge
5 Evaluate the retention of the analyte on various bonded phases if the HPLC separation is reversed phase It is important to remember that pH can have a profound effect on retention of a compound (see 2 above) Generally a stationary phase of similar polarity to the compound of interest is used with the sample dissolved (and applied to the column) in a solvent of opposite polarity The analyte is eluted by again switching solvent polarity Thus a relatively lipophilic organic molecule would be applied to a C_{18}, C_8 or C_2 cartridge in a polar solvent and eluted with a relatively non-polar solvent
6 Compare the recoveries from aqueous and biological matrices Differences in the behaviour of some drugs in these two matrices have been seen, for example oxmetidine has a recovery of $>90\%$ from water but this falls to 50% in plasma due to competition from endogenous compounds [20] In contrast, failure to elute all the analyte from the phase after extraction from water ($<30\%$ recovery) compared with $>95\%$ recovery from plasma has been observed by Doyle et al [72] The masking of active silanols by components in the plasma may be a possible explanation of this phenomenon
7 Elution of the analyte from the cartridge must occur efficiently so that the solution to be assayed is not too dilute
8 The sorbent size can be optimised if required although one group of workers prefer 100 mg size on the grounds of easier handling, smaller elution volumes and economy [60]

To evaluate any sample preparation scheme, a radiolabelled form of the drug can be used, the amount of radioactivity in the various fractions will quickly identify any problem areas of the potential extraction scheme Alternatively, if radioactively labelled analyte is not available, Whelpton and Hurst [60] pass a solution down the columns and examine the eluent for presence of analyte Those phases that retain the compound are then successively eluted with 1-ml portions of methanol, 0 1 *M* ammonia in methanol and 0 1 *M* acetic acid in methanol until elution occurs

Guidelines are available from the main vendors of these cartridges to develop methods, which also include troubleshooting sections to help the analyst with any problems that may be encountered [73–75] Care must be taken to think the extraction through carefully the retention mechanism can often include

secondary interactions which could affect the outcome of an extraction (see the respective paragraphs in Section 2 4 2)

After the use of an SPE clean-up step, there may still be in the eluate compounds from the sample matrix, which interfere with the final measurement One solution may be to vary the nature of the washing solvent to elute selectively the unwanted compounds If this approach is unsuccessful, it may be necessary to use a second extraction column to provide a more efficient clean-up technique known as Chromatographic Mode Sequencing (CMS) An example of CMS is the analysis of tricyclic antidepressants in human plasma [76] The drug is retained on a C_2 cartridge and interfering material is washed to waste with dilute sodium hydroxide Chloroform is used to elute the drug from the C_2 cartridge onto a CN cartridge, effecting a further purification, the analytes are then eluted with HPLC mobile phase and aliquots of this are taken for analysis

Alternatively, LLE can be combined with LSE [77], prilocaine can be extracted from plasma with toluene, the organic phase is passed through a normal-phase activated diol AASP [Advanced (Analytichem) Automated Sample Processor] cartridge, washed with acetonitrile and eluted off the phase by a reversed-phase solvent from HPLC analysis This approach couples LLE with the on-line elution into a chromatograph via LSE, thereby achieving high selectivity

Conditioning and use of solid-phase cartridges Before using LSE cartridges, there is a basic level of knowledge required in order to obtain the best results

1 Wet the column with an organic solvent, e g methanol This serves two purposes (i) to open up the hydrocarbon chains and hence increase the surface area available for interaction with the analyte, (ii) to remove residues from the packing material that might interfere with the analysis In the author's experience, failure to carry out this stage effectively will result in poor recoveries of analyte due to reduced retention on the column and interference peaks on the chromatogram which are unrelated to the original sample
2 Wash the sorbent bed with HPLC-grade water or a suitable buffer This will remove excess methanol and prepare the surface for the sample This conditioning step should be as similar as possible in polarity, ionic strength and pH value to the sample to be extracted It should not be necessary to use a large volume of solvent, three or four times the bed volume of the cartridge is usually sufficient
3 Apply the sample, allow it to flow through the sorbent bed and discard the waste Biological samples are generally viscous and wherever possible should be diluted to speed the passage through the sorbent bed and certainly centrifuged prior to extraction to remove particulate matter that could block the column Blood samples can be assayed by LSE if the erythrocytes are lysed and the sample is centrifuged to remove the membranes If a large

volume of sample is used, the column may no longer be 'wetted' and a reduction in recovery will be observed To overcome this problem, an organic solvent, e.g methanol (1–3%), should be added to a sample of large volume prior to processing This will help to maintain the equilibrium between the stationary and mobile phases. Flow can vary from cartridge to cartridge due to a number of factors such as particle size distribution or the packing characteristics of individual columns In some applications it may be necessary to stop the flow through individual cartridges with the use of commercially available stopcocks to prevent the sorbent bed drying out Flow-rates through the sorbent bed should be controlled, based on personal experience, a maximum flow generated by circa 30 kPa vacuum is recommended. If an analyte is strongly protein-bound then lower flow-rates may be necessary to achieve a good recovery of the analyte [60].

4 Wash the column with water or a suitable solvent to remove selectively endogenous compounds from the sample matrix which might interfere with the subsequent chromatography.

5 Elute sample with a suitable solvent and collect eluent for immediate analysis or further work-up The volume of the eluting solvent should be as small as possible to avoid dilution of the extract and lower limits of sensitivity. If the retention/elution mechanism is known it should be possible to elute the analyte in a very small volume, depending on the bed volume of the cartridge Two elutions of 100 μl are generally more efficient than a single application of 200 μl An interesting method of preparing the cartridge for elution of the analytes has been presented by Whelpton and Hurst [60]: after the final wash, the cartridge is centrifuged and 50 μl of the elution solvent are added to the cartridge and centrifuged to waste This appears to

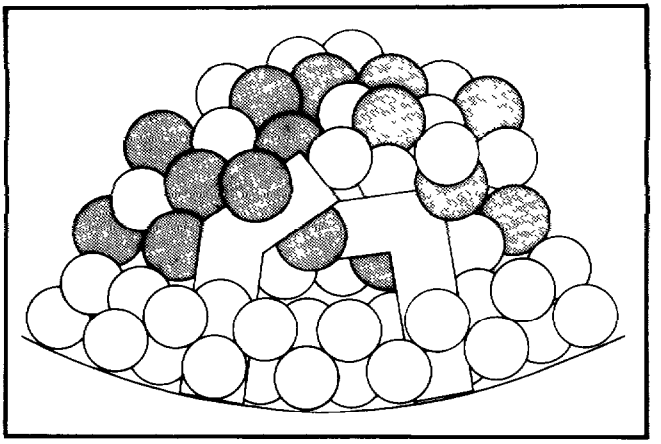
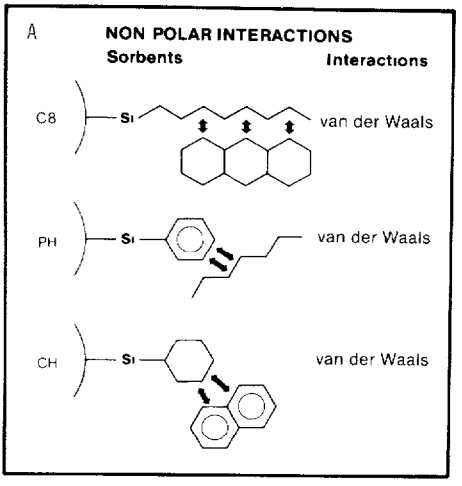


Fig 3 Solvated bonded silica surface (Reproduced with permission from Mike Burke)

displace water from the sorbent bed and no analyte to date has been detected in this volume [60]; elution then proceeds normally.

Retention mechanisms in liquid–solid extraction To understand the retention mechanisms involved in an isolation it is necessary to discuss the nature of the bonded silica surface. Fig. 3 depicts the surface after conditioning. The siloxane structure of the silica binds a permanent layer of water molecules which is only removed by heating [78], protruding from the water layer are



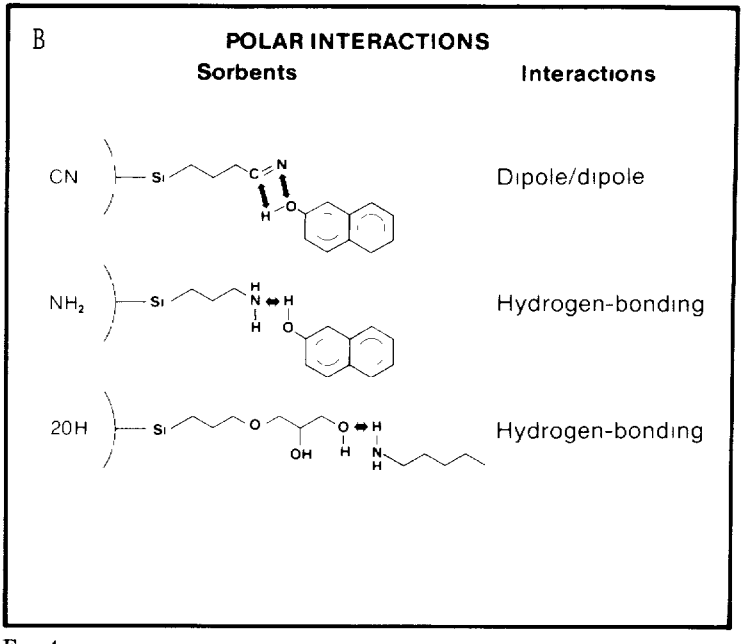
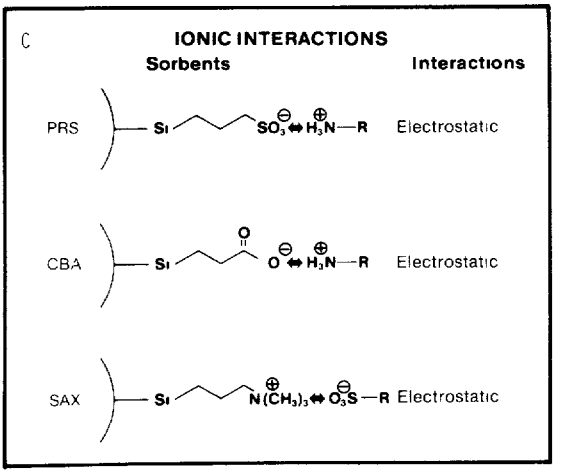


Fig 4



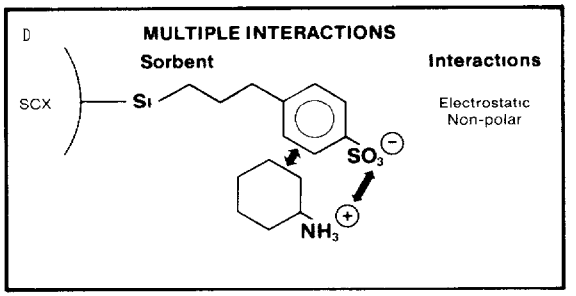


Fig 4 Interactions involved in analyte isolation using bonded silicas (A) Non-polar interactions, (B) polar interactions, (C) ionic interactions, (D) multiple interactions (Reproduced with permission from Analytichem International)

the bonded silica chains and interdispersed between them are molecules of water and the conditioning solvent. This configuration is controlled by solvation. the chains are more extended the higher the organic content [79]. This swelling and shrinking behaviour will occur during the application and elution stages of the extraction. Also present on this surface, but not shown in the diagram, are residual silanols which can play a significant role in the extraction scheme

The analyte can be bound to the solid phase by a number of different mechanisms which are the same as for HPLC, i e hydrogen bonding, dipole–dipole interactions, hydrophobic dispersion forces and electrostatic (ionic) interaction [80] Any or all of these forces will be involved during an extraction, it is the mastery of these forces that will determine the specificity of the method developed.

The use of small molecular probes by Schunk and Burke [81] show that non-polar molecules appear to favour interaction near the centre of solvated

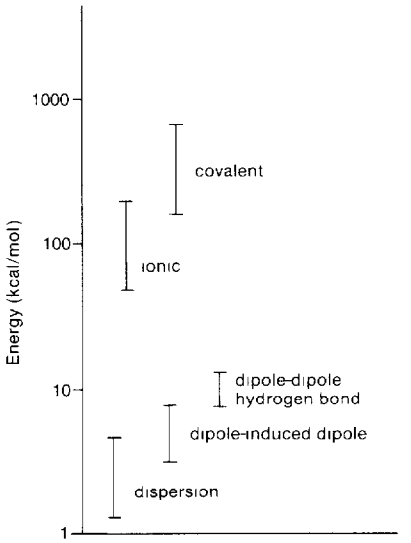


Fig 5 Bonding energies of the various interactions involved with a chromatographic isolation (Reproduced with permission from Mike Burke)

ligand chains in contrast to polar basic molecules that can interact with acidic sites on the silica surface.

Bonding energies and selectivity The energies involved in the various forces involved in bonding vary considerably Hydrophobic bonding energies (dipole–dipole, dipole–induced dipole and dispersive interactions) range from 1 to 10 kcal/mol Hydrogen bonding between suitable polar groups involves 5–10 kcal/mol but there are many opportunities for this type of interaction on the silica surface (Fig 4) Ionic or electrostatic interactions between oppositely charged species involve energies of 50–200 kcal/mol [82]. The relative energies of these bonding forces are shown in Fig 5

It has been suggested by Harris [69] that a more selective extraction may possibly be the result of utilizing higher-energy interactions that would be less likely to be formed. To a certain extent, this is borne out by the general observation that the selectivity of an extraction rises from C_{18} through C_2 to the more polar phases like diol, CN and amine, however, there is insufficient evidence to support this theory at present.

Silanol activity Under the most rigorous endcapping procedures it is only possible to endcap 70% of all silanol groups remaining after synthesis of the bonded phase [83], therefore, it is probable that silanol groups may be involved in an isolation of an analyte.

The pK_a of a silanol group is not easily determined as it is influenced by the surrounding environment; however, at pH values of 2 the silanol is uncharged [78] Above this value it becomes increasingly dissociated and able to influ-

ence an extraction by virtue of its negative charge From the binding energies outlined in the last section it can be seen that electrostatic interactions involve higher energy than hydrophobic interactions Therefore, if a mixed retention mechanism is present the analyst must know the steps that can be taken to reduce or enhance the influence of residual silanols depending on the extraction mechanism desired

Reduction of silanol activity To reduce the influence of silanol groups during an isolation, the analyst should chose a phase that is endcapped The residual silanols can be masked by the use of a competing base such as triethylamine which has been used in HPLC for many years [84–86] Ammonium acetate is another method of masking silanol groups [87] and is shown in the analysis of caffeine in serum and saliva [88]

Alternatively ion suppression can be employed by choosing a pH value where either the silanol (<pH 4 [89]) or the ionisable groups on the analyte molecule are uncharged If this latter approach is taken then the analyst should ensure that the analyte is stable at the pH value of the buffer used to suppress the ionisation

An option that is becoming increasingly possible is to change to the supplier of the column in order to reduce silanol activity It is possible to rank HPLC C_{18} columns on the basis of relative acidity [89] and in the future it might be possible to characterize extraction cartridges in the same way

If silanol activity cannot be reduced by the above suggestions then it may be possible to increase the ionic strength of the conditioning solvent, sample and wash solvent to compete with the silanols and prevent analyte bonding

To illustrate the elimination of silanol activity, Kupferschmidt and Schmidt [90] used a C_{18} non-specific extraction column to isolate relatively lipophilic drugs such as chlorpromazine The phase is activated first with methanol followed by water containing tetramethylammonium hydroxide (TMA-OH), the purpose of the amine is to neutralize the silanol groups and isolate the analytes by non-specific hydrophobic binding alone The sample and water wash are applied but both samples contain the amine to prevent loss during passage of solvent through the cartridge The cartridge is dried under vacuum and the analytes are eluted by chloroform (TMA-OH is insoluble in chloroform) which is then passed through a pre-conditioned AASP (see Section 3 2 2) silica cartridge to isolate the analytes by a specific retention mechanism The use of the AASP allows on-line elution of the analytes onto an analytical HPLC column

Enhancement of silanol interaction To use silanol groups in an extraction scheme, use a phase that is not endcapped and a conditioning buffer with a pH value of at least pH 4 or above should ensure that the residual silanol groups are ionised A buffer is recommended as the second conditioning solvent as water can have a variable pH value and has little buffering capacity

An example of this approach to extraction is the work of Bland [91] for the determination of salbutamol in plasma, the phase used was silica which was

activated by successive aliquots of methanol and water Plasma was passed through the cartridge and the positively charged salbutamol was extracted by ionic interaction with the silanol groups Washing of the phase was undertaken with water and then acetonitrile The use of the latter solvent is interesting as the retention mechanism is essentially ionic, acetonitrile can be used as a wash solvent to remove material bound by non-polar bonding as it does not have the capacity to interfere with the binding Elution of the analyte was effected by methanol containing 0 5% ammonium acetate

Reuse of sorbent cartridges Some publications [92,93] mention the reuse of solid-phase cartridges up to ten times, this practice, often for economic reasons, must be carefully evaluated so that the results generated are not compromised Reuse of bonded phase silica will inevitably affect its selectivity by the coating of the bonded phase with endogenous plasma constituents making it similar to the pre-column in column switching

2 4 3 Alternative sorbent phases

The main sorbents discussed in this section have been mainly bonded silicas, however, modified carbon black has been evaluated as an alternative [94,95] The phase is activated by passing successive volumes of chloroform, methanol and water in a fashion analogous to bonded silicas, then the sample is passed through the bed, followed by a water wash and hexane to remove the water. Elution of the analyte, with good recovery, was achieved with chloroform–methanol (1 1, v/v) [95] Evaluation of this sorbent is still being undertaken

2 4 4 Applications of liquid–solid extraction

The main advantages of LSE are selectivity due to the wide choice of bonded phases available, speed and time saving being able to process large numbers of samples efficiently [20] LSE can also cope with a wide range of analyte polarity, e g Doyle et al [72] extracted temalastine and eight of its metabolites including compounds with carboxylic acid and primary amine functions and Smith et al [96] isolated chlorpromazine and thirteen of its metabolites with a single extraction on a C_8 phase Isolation of quaternary amino compounds can also be accomplished using LSE [97] An interesting use for LSE is for clean-up prior to NMR analysis for metabolite identification in urine [98,99]

A disadvantage of the technique is the need to centrifuge all frozen and thawed plasma samples prior to analysis to remove fibrins and other particulate matter which might block the flow of solvents through the sorbent bed The operator should concentrate fully whilst extracting samples as it is possible to add the wrong solvent or forget to collect the eluate One disadvantage identified previously [58] was a lack of information about the technique, this is being remedied but some users still need to be educated

2 5 High-performance liquid chromatography

A liquid chromatograph is also capable of separation and clean-up, so that it can either enhance any preparation scheme already undertaken or perform the both extraction and quantification stages It can be argued that elaborate purification procedures make redundant the separating power of a chromatographic column, but insufficient purification can lead to deterioration of the column efficiency [4] Therefore, there is a balance to be struck between an external clean-up and that due to the chromatographic column, if there is a strong element of selectivity and trace enrichment in the sample preparation step, fewer demands will be placed on the quality of the chromatography On the other hand the choice of a selective detection mode may simplify the preparation stage to a point where it is relatively easy to automate for greater throughput [4]

2 5 1 On-line sample preparation by HPLC

Using standard equipment plus a switching valve and timed events a liquid chromatograph can be used to prepare and analyze biological samples. According to Uhlein [49] there are three main approaches that can be used

1 Solvent programming where the column and stationary phase are not changed
2 Column switching where the mobile phase is constant but different columns are used to effect the separation
3 Column and solvent switching where one chromatograph provides a crude separation of the sample and a fraction containing the analytes is transferred to another chromatograph for the analytical separation

The reader is also referred to the reviews by Nielen et al [100], Roth and Beschke [101] and Huber and Zech [102] for more information about this technique

Solvent programming and trace enrichment The simplest method of HPLC sample preparation is the use of trace enrichment where the sample, either diluted or neat, is pumped onto a pre-column of the chromatograph, as shown in Fig 6 Firstly, in flushing mode, water or a low-strength wash solvent is pumped through the pre-column such that the analyte is retained on the pre-column, but the more polar endogenous material is washed to waste When flushing is complete, the valve is switched and elution of the analyte onto the analytical column is effected by a gradient After the analysis is complete, the two columns will need to be re-equilibrated before the next run, slowing the throughput of the assay The arrangement described above is backflushing, less band broadening occurs compared to front flushing, the disadvantage is the elution of particulate matter retained from the sample by the pre-column but this can be overcome by a guard column placed prior to the analytical column

Column switching front and heart cutting In the previous section, all of the

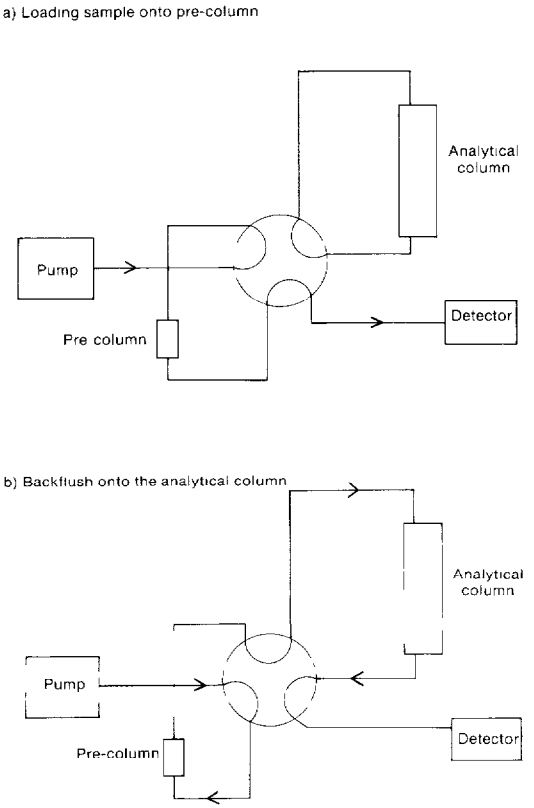


Fig 6 Column switching I trace enrichment (a) Loading sample into the pre-column, (b) backflush onto the analytical column

sample eluted from the pre-column by the gradient was loaded onto the analytical column This may be undesirable as unwanted material may be present on the column giving rise to more complex separations or longer run times due to late eluting peaks To overcome this problem, the first column can be used to give a limited separation of the sample extract, therefore the packing material must have a low retention of the components in the sample The eluent from this column is run into a switching valve and to waste until the analyte fraction is eluted from the first column, the valve is switched to divert the eluent onto the analytical column for the main separation and detection In the simplest variation of this technique the valve can be switched back to complete the elution of the sample off of the first column to waste whilst the analyte is held on the head of the analytical column. After all material has been eluted from the first column the sample flow is routed to the analytical column to complete the assay. In this form there is a delay whilst excess material is removed from the first column

A more practical version of the technique requires an additional pump, a diagram of a system is shown in Fig 7 The second pump allows mobile phase to flow through both columns and after the analyte cut has been made the first column can be prepared whilst the analysis takes place simultaneously. When the analyte fraction has been eluted onto the analytical column the valve is switched to divert the pre-column eluent to waste whilst the main column continues its separation of the analyte fraction

The same principle can be used to cut other sections of the eluent from the first column

Column and solvent switching Initially developed by Roth et al [103] this approach consists essentially of two chromatographs with a short manually packed pre-column that has a low-strength mobile phase by which the analytes are retained without precipitation of protein, simultaneously another pump delivers the final mobile phase through the analytical column, and the two columns are connected by a switching valve Both systems are run parallel and the eluent containing the analyte fraction is switched onto the analytical column for the final separation A more complex arrangement, shown in Fig 8, can use a second pre-column so that one is re-equilibrating whilst the other is in use [103]

Boxcar chromatography A further refinement of column switching is 'boxcar' chromatography which involves the partial separation of a fraction containing the compounds of interest on a first column, with diversion of the resulting fraction to a second column [104] The concept of this approach is that the second column will be filled with several samples at the same time, but the

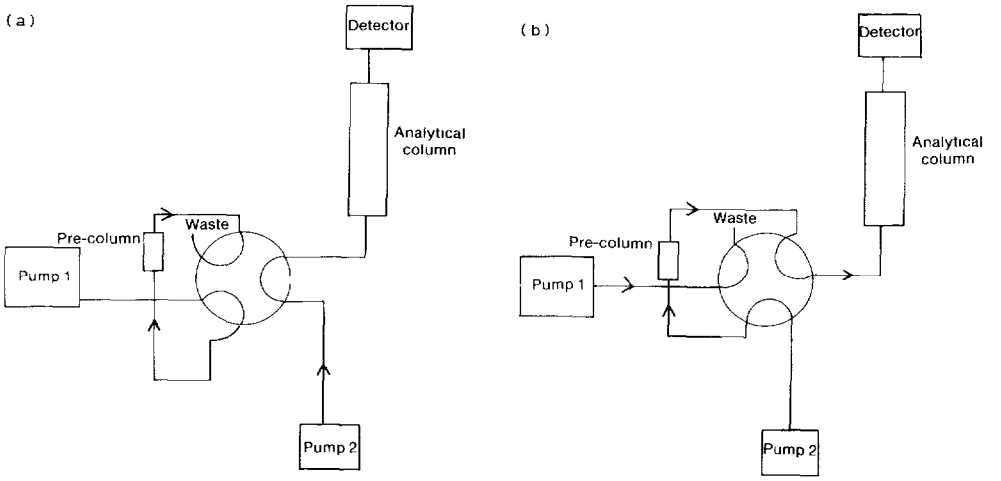
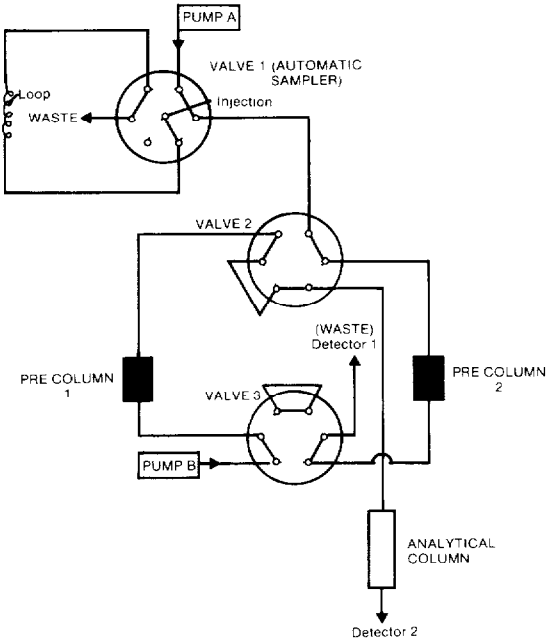


Fig 7 Column switching II heart cutting (a) Primary separation by pre-column, (b) valve setting for separation by analytical column



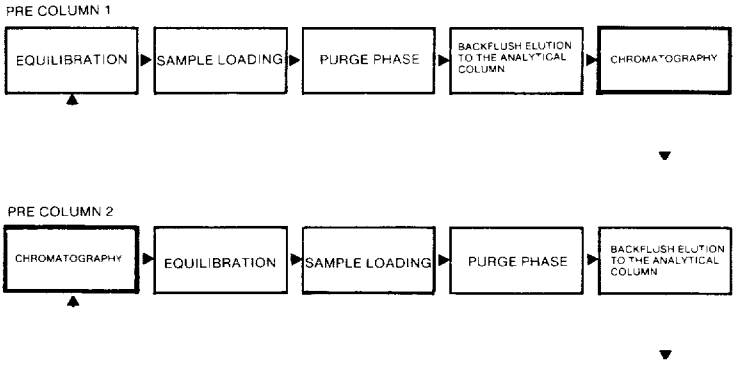


Fig 8 Column switching III column and solvent switching (automated two-pre-column system from Roth et al [103])

separation that is undertaken by the second column will be simpler than with a conventional extract

2 5 2 Practical considerations of HPLC sample preparation

The pre-column The length of the pre-column used for HPLC sample preparation can vary between 10 and 40 mm and the internal diameter between 1 and 4 6 mm. It is dry packed with 25–40 μm bonded silica particles to accommodate the relatively large volume of biological sample that will be injected into the chromatograph The most common packings for the pre-column are C_{18}, C_8 and C_2; similar to LSE, the longer the chain length the lower the selectivity of the phase C_2 is the preferred packing in our laboratories as the retention of an analyte is lower on this phase but has the advantage that it is easier to elute from, band broadening is minimized if a C_8 analytical column is used, as the analyte can be concentrated at the top of this column [105].

Fine mesh gauzes instead of sintered metal frits are used to retain the packing of both the column and pre-column and avoid clogging due to protein precipitation [102] The tubing through which the neat biological samples are pumped should have a relatively large bore, again to avoid blockage [106]

Mobile phase The first function of the mobile phase is to adjust the sample to allow retention of the analyte on the pre-column, the parameters to consider are the proportion of organic modifier and the pH value and buffering capacity of the final mixture These must be optimised to ensure good recovery of the analytes without precipitating proteins [102] The buffering capacity of the mobile phase should be tested with respect to the volume of the sample injected [107] to ensure that when the sample reaches the pre-column it is at the correct pH value for maximum recovery (Fig 9) To cope with changes in pH that occur with the injection of the sample, the mobile phase should be at least 150

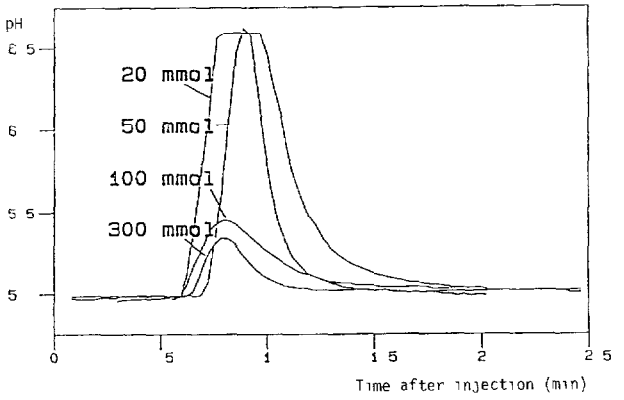


Fig 9 Plot of the pH of the mobile phase at the pre-column outlet after the injection of 200 μl of serum The pH of the mobile phase before entering the pre-column was 5 and the molarity was varied between 20 and 300 mmol (Reproduced from Huber et al [107])

m*M* [107] To allow the best extraction rate by the pre-column, the flow-rate of the mobile phase through the pre-column should be in the range 1–1 5 ml/min to overcome the effects of protein binding in the sample

Injection volume and pre-column life and performance When using HPLC to prepare and analyze biological samples it is best to keep the volume of the sample injected as small as practicable, bearing in mind the requirements of the assay. It has been estimated that the capacity of a 10 mm×4 6 mm I.D pre-column packed with C_2 phase is approximately 15 ml serum [102] This means either 150 100-μl injections to 15 1000-μl injections, there may be problems in scaling up the injection volume, i e. the autosampler may take a long time to load the sample or elution of endogenous material from the matrix may interfere with the assay.

The performance of the pre-column is crucial to the success of an assay, therefore it is essential to monitor the resultant chromatograms to ensure their quality. The experience gained in operating an assay will allow an analyst to judge when to repack the pre-column before unacceptable chromatograms are produced Many investigators have found that poor chromatography is obtained following the first two or three injections onto a fresh pre-column, therefore before an analytical run starts, the silica surface is primed by injecting blank plasma to obtain reproducible results [108]

Huber and Zech [102] offer practical guidelines for the development of analytical methods that start from an HPLC separation and describe the stages that should be undertaken to produce an automated HPLC assay

2 5 3 *Internal-surface reversed-phase HPLC columns*

The major problem when injecting samples containing proteins directly onto HPLC columns has been the problem of precipitation which leads to blockage of the chromatograph or deterioration of the column performance. As mentioned in the preceding sections, the use of HPLC for direct injection involves the use of a pre-column containing large-particle-size silica with the consequent use of valve switching, etc.

To overcome this problem Hagestam and Pinkerton [109] introduced an internal-surface reversed-phase (ISRP) silica packing, where the outer surface of the particle is hydrophilic and non-adsorptive to proteins whilst the internal surface is hydrophobic. The columns are prepared by bonding a hydrophobic di- or tripeptide (Gly-Phe or Gly-Phe-Phe) to a silica support. The support is then incubated with carboxypeptidase which cleaves the peptide from the outside of the silica leaving the surface coated with carboxylic acid moieties, because of size exclusion the enzyme cannot enter the pores which retain their hydrophobic character [109,110]

Keeping the pore size of the silica small ensures that large proteins are excluded from the internal phase and, as they are not attracted to the external

surface, are eluted at or near the void volume Only smaller-sized molecules can enter the pores and are retained by hydrophobic interaction

The only unit operation that is required prior to HPLC analysis with these columns is either filtration or centrifugation to remove particulate matter In the light of experience, the best applications for these columns are for relatively hydrophilic analytes in plasma that require only a small proportion (ca 2 5%) of organic solvent in the mobile phase [111,112] When the mobile phase contains a larger amount of organic solvent (ca 15–20%) small amounts of precipitated protein have been found on the top frit of the column resulting in a rise in back-pressure To prevent protein precipitation, it is recommended that a maximum of 25% organic solvent is used in the mobile phase and that the pH value be between 6 8 and 7 5 and the ionic strength 0 1 *M* or greater [110]

2 5 4 Applications of HPLC sample preparation

There are many applications of automated HPLC sample preparation in the literature confirming that the technique is capable of producing precise and accurate results [101,102] for a wide variety of compounds Applications include the use of specific detectors such as fluorescence [106] and electrochemical [113] detectors in the chromatographic system, whilst HPLC sample preparation can be applied to highly protein-bound compounds [114,115] and labile compounds [116,117] The coupling of Group 4 unit operations has been shown by Essers [118] and Werkhoven-Goewie et al [119] with pre- and post-column derivatization, respectively

The use of column-switching techniques has usually been limited to the analysis of either plasma or serum, recently, however, direct injection of whole blood has been investigated by Tamai et al [120–122] The choice of the packing material in the pre-column was crucial for the success of the assay as its function was to separate the analyte from the erythrocyte membranes, proteins and other endogenous material For hydrophobic drugs, hydrophilic compounds and hydrophobic drugs bound to red blood cell membranes the packings used in the pre-column were C_{18}, butyl Toyopearl 650-M and TSK Gel HW-65F (a polyvinyl resin), respectively Using this approach, it was also possible to perform on-line lysis and deproteinization of the blood samples, clogging of the chromatograph was overcome by surfactants (0 5% sodium dodecyl sulphate) to help solubilize the proteins in the samples (see Section 4 1 2)

2 6 Miscellaneous techniques

2 6 1 Lyophilization

Lyophilization or freeze-drying is essentially the removal of water and other volatile compounds by vacuum sublimation, it is a technique that has been used widely in biochemistry for homogenates and protein-rich liquids It is

useful for concentrating analytes as once the water is removed, the residue is easier to manipulate This process can be also applied to semi-liquid matrices (e g plasma) and tissue homogenates

In the author's experience, lyophilization is an efficient process for reducing a large liquid or semi-liquid sample to a stable solid (ca 10% of its original volume) which can then be stored at ambient temperatures Further sample preparation can then be carried such as slurrying with methanol, the extracts thus obtained are very clean and can be injected into a chromatograph for analysis Lyophilization is preferred to evaporation for the removal of water from aqueous samples containing thermolabile analytes [49], however, there may be a problem with samples with a high salt content, such as urine, since it is a pre-requisite that the sample remains frozen during the whole process As a high salt content might lower the melting point when put into the freeze-dryer, dilution with water is a solution to this problem [49]

Commercial equipment is available, but the technique has not become popular because it is time-consuming and tedious for large numbers of samples

2 6 2 Saponification

Saponification is the hydrolysis of an ester with either sodium or potassium hydroxide, and fats form soaps by this process which can be removed easily As a method for eliminating the bulk of neutral lipids in biological samples, saponification is an efficient process, but the analytes must be stable in aqueous base, subsequently the unsaponified fraction can be extracted with an organic solvent [123] The technique is useful but can be inconvenient to use practically

3 AUTOMATION OF SAMPLE PREPARATION

The techniques for sample preparation that have been discussed in Section 2 are essentially manual, of these HPLC and LSE are the two major techniques that have the greatest potential for automation Other methods such as protein precipitation and ultrafiltration are difficult to automate or may not be easily justified on the basis of a cost/benefit analysis It is possible to automate LLE using robotics [124] or continuous flow methods [125] but these, in the author's view, will not be financially viable in the light of recent developments in automated sample preparation involving LSE and HPLC This section will discuss briefly approaches to the automation of these two techniques To justify automation fully, the laboratory must have a large throughput for the assay to be automated to automate an assay with few samples would not be cost-effective

3 1 Rationale for automation

There are many pressures on laboratories today Some of these are cost containment whilst maintaining the quality of output, efficient use of resources

and regulatory compliance An attempt to solve some of these problems is laboratory automation Automation in various forms has been available for many years (e g chromatographic autosamplers and integrators), however, until recently little has been undertaken with the automation of sample preparation. The situation has now changed and there are several approaches to take when considering automation robotics, dedicated automation systems and on-line HPLC

The automation of a process should produce

1 Greater increases in productivity (either in numbers of samples assayed per unit of time or speedier turnaround time)
2 Systems that should be capable of the same or better precision and accuracy as the existing manual methods
3 The freeing of trained laboratory staff to do more productive work than extracting samples
4 Reduced human contact with biohazards
5 Release of staff from undertaking tedious tasks thus improving morale

3 2 Laboratory automation

Liscouski [126] has divided laboratory automation into two areas laboratory management automation and instrument automation The former area is concerned with management of the data and information produced by the laboratory, typified by laboratory information management systems [127], whilst the latter area covers increased sample throughput with more efficient data capture and processing Instrument automation can be classified further into two main types flexible and dedicated [128]

Flexible automation This can be reprogrammed but also re-engineered to change the task that can be undertaken by it, this type of automation is typified by robotic arms A description of the robotic arms and operations that they can undertake is beyond the scope of this paper and the reader is referred to the reviews by Dessy [129,130] and Last [131] Moreover, general applications of robotics and specific sample preparation examples can be found in the series 'Advances in Laboratory Automation – Robotics' [132–135]

The acquisition of a robotic system can take the analyst into an area in which he has little experience often the task that has to be undertaken must be specified or designed in comparison to the purchase and use of equipment off-the-shelf Recognising this problem, the first robot system purchased in our laboratory was a commercially available system that was customised by the vendor to our specification [136] This allowed us to perform the immediate task at hand and also to develop our own expertise in robotics Moreover, the purchase was a joint project with out Research Engineering Department so that further re-engineering could be accomplished in-house [5]

The main advantage of robotic systems lies with their flexibility to be mod-

TABLE 6

OUTLINE OF SOME DEDICATED SAMPLE PREPARATION INSTRUMENTS [137]

AASP	Off-line manual sample preparation of a cassette of ten bonded phase cartridges Elution of the analytes is achieved on-line using the AASP HPLC module that takes the place of the autosampler in the liquid chromatograph, therefore, a method must be designed for analyte elution by the mobile phase A new cartridge is used for every sample [14,58]
Gilson-AASP	On-line sample preparation and analysis A Gilson autosampler–dilutor is coupled to the AASP valve to enable on-line activation of the cartridge, application of the sample and washing of the sorbent bed A fresh cartridge is used for each sample Must centrifuge samples to reduce blockages in system Uses the same purchased solid-phase cartridges as the AASP [139]
Auto SPEed	Off-line sample preparation unit that is capable of sequential preparation of purchased solid-phase cartridges Manual transfer of the sample extracts to the analytical instrument is necessary [137]
Millilab	Instrument capable of carrying out manipulative tasks, e g pick up and transfer cartridges via an inflatable tip of a probe Can be used for off-line or on-line sample preparation The samples can be cooled or heated and evaporation of solvents is also possible, alternatively the sample extract can be collected for further sample preparation [137]
ASPEC	Instrument based on a Gilson autosampler for the off-line and on-line preparation of solid-phase cartridges Racks hold the samples and extraction cartridges, the latter can be positioned over waste or collection trays A pipettor–dilutor with a moveable probe is used to condition the cartridges, transfer sample and elute the analyte fraction into collection tubes The probe can then be used to transfer the sample to an HPLC injector for on-line analysis [137]
Prospekt	Automated cartridge exchange module for use with on-line HPLC Uses a fresh disposable pre-column for every sample Instrument and cartridges specifically designed to minimize band broadening when using smaller-bore columns [161]

ified or re-engineered to undertake new tasks Obviously, a modification of an existing procedure is the easiest to achieve and this can be done relatively quickly depending on the degree of work to be done If the task is completely different, then it will be more involved, especially if the work requires the custom manufacture of specific peripheral components In the author's experience, input from engineers is very helpful and shows the multidisciplinary nature of robotics Once installed the application is relatively simple to operate

Dedicated automation These units are usually limited to a specific task, which may be varied by programming This can take the form of autosamplers that offer a cost-effective means of automating an instrument assay, the development of autosamplers with moveable probes, that move in the x,y,z planes, can also be used for sample preparation where the tasks involve the transfer or pumping of fluids

In an earlier review [58], it was predicted that sample preparation was an area for instrument manufacturers to exploit in the future This prediction has been born out in 1984 there were two automated systems commercially available, at present there are at least seven Descriptions of the main instruments available can be found in a recent article [137] and are summarized in Table 6

3 3 Comparison of flexible and dedicated automation for sample preparation

In 1986 [138] a comparison was made between the manual method of preparing AASP cassettes and two automated methods involving a robot to automate the off-line preparation stage [136] and an autosampler coupled to the AASP injection port for on-line sample preparation and HPLC analysis [139] In the light of experience, it is time to update that report and extend the comparison to include on-line HPLC

The manual and automated techniques were compared against the following criteria

1 Precision and accuracy of a drug assay in plasma
2 The number of samples that could be assayed per 24 h
3 Relative cost and ease of implementation
4 Human involvement in the assay scheme

The results are listed in Table 7

Precision and accuracy of the assay are shown in Table 7. As can be seen the precision, as measured by the coefficient of variation, of all methods is better

TABLE 7

SUMMARY OF PERFORMANCE OF A MANUAL AND THREE AUTOMATED METHODS OF ANALYSIS

Sample throughput is based on a 20-min chromatographic run plus a 10-min re-equilibration in the case of gradient elution

Performance criterium	Manual	Automated methods			
	Vac Elut	Gilson-AASP	Robot system	On-line HPLC	
				Gradient	Isocratic
Number of samples prepared per h	10	3	6	2	3
Maximum number of samples per 24 h	72	72	72	48	72
Reliability	Poor	Very good	Very good	Very good	Very good
Relative cost	1 00	1 35	2 75	1 35	1 00
Implementation time	10 min	Two days	Two weeks	Two days	Two days
Human involvement	Labour-intensive	Minimal	Minimal	Minimal	Minimal

than 5%, and accuracy, measured as bias, are acceptable Overall, the quality of the data from the automated method were comparable with the original manual method of sample preparation using the Vac Elut

Performance of the systems was evaluated· as the overall throughput of an assay is dependent on the chromatographic run time, it is obvious that a method with a relatively short run time (ca 10 min) will have a higher throughput than an assay with a 20-min run time This affects the way systems can be compared, therefore two figures are given in Table 7, the first is the number of samples able to be processed per hour and the second is the overall assay throughput with a chromatographic run time of 20 min

The off-line methods using the robot and manual extraction procedures have throughputs of six and ten, respectively The manual approach is marginally faster, however, it is limited by the time the analyst is able to extract samples When total assay performance is assessed over a 24-h period both systems are capable of a throughput of 72 but sample extracts in both cases must be transferred to the chromatograph for final analysis

The Gilson AASP and HPLC have similar throughputs, it is the operation that differs purchased and disposable versus hand-packed and reusable precolumns These on-line methods have similar throughputs compared to the off-line methods, but a difference is the ability to operate outside of normal hours Our previous comparison of the robot to the dedicated automated systems show that the dedicated example (autosampler) is capable of a higher throughput (30%) than the more flexible robotic system [138] However, this was due to a much shorter chromatographic run time (6 min) than the 20 min used for the calculations here It is important to realize that a shorter run will show automated systems in a better light than a longer separation

Reliability of automated systems is very important as unattended operation is highly desirable, if not essential The robot arm is very robust, it has worked with 100% reliability, it has been the peripheral devices such as the solenoid valves on the master laboratory station that have presented a few minor problems The Gilson AASP system has been used for a number of applications within our laboratories, it is reliable but can suffer from blockage in the AASP valve and leakage of the syringe used with the Gilson autosampler Therefore, to reduce these problems all samples must be centrifuged and the samples are diluted as much as practicable to reduce viscosity prior to loading onto the AASP cartridge The manual Vac Elut system for off-line preparation can be unreliable especially after long periods of use as the seals wear moreover, the cartridge and reservoir have to be positioned carefully A pneumatically operated version of this system is now available which has overcome the above problems

Relative capital cost is expressed by comparison to the HPLC equipment required to undertake the manual assay set as 1 0 The HPLC equipment required for automated analysis includes an autosampler in place of the AASP,

a switching valve and a pre-column for isocratic analysis, but another pump or gradient system is required for more sophisticated methods Total automation of the AASP requires the purchase of a Gilson 222, Model 401 syringe and a relay box [140] The robotic arm and peripherals are the requirements for the off-line automatic preparation of the AASP cassettes It can be seen that the manual system and on-line isocratic HPLC are the cheapest options, full automation with the gradient HPLC and the Gilson–AASP can be achieved relatively cost-effectively The cost of the robotic arm indicates that the task which it will undertake must be thoroughly investigated and justified before purchase Human involvement is at its greatest with the Vac Elut where a trained analyst is required in all sample preparation stages Less is needed with the robot where samples are laid out and the prepared cassettes are transferred manually to the chromatograph The Gilson–AASP and HPLC methods require the least human involvement as they can be set up and left to operate all samples are prepared and analyzed on-line

Speed of implementing automation also rises in a similar fashion the manual system can be shown and implemented within 10 min in contrast to the Gilson–AASP and HPLC which would take approximately two days to install and become operational The robot is relatively complex and would, in the opinion of the author, require about two weeks to become familiar with the programming language and how to use the instrument

3 3 1 Automation summary

The four approaches presented here can be used to analyze compounds in plasma, all are sufficiently precise and accurate However, the type, the degree of automation and the costs that a laboratory has to pay are varied Flexible robotic systems are expensive and the most complex to implement, but can be justified with a high throughput assay and can also undertake manipulative tasks that the dedicated systems cannot The dedicated automation (Gilson–AASP and HPLC), outlined here, is more cost-effective and quicker to implement, but have the disadvantage that they are limited to one task, however, they have the potential to provide the greatest throughput with the lowest cost for an automated system

One way of assessing a laboratory's needs for automation is to break the analytical method to be automated down into the unit operations Once this has been achieved, critically assess what is required, if liquids are moved or transferred from one location to another then a dedicated instrument may be the best approach If there is extensive manipulation of the sample, e g dissolution of a tablet or homogenization of a biological tissue, then the manipulative advantages of a robotic arm should be apparent.

4 FUTURE TECHNIQUES FOR SAMPLE PREPARATION

This section will discuss techniques that are emerging or have the potential for use in sample preparation for biomedical analysis

4.1 Supercritical fluid extraction

The use of supercritical fluid chromatography for separations of mixtures is becoming established [141,142], however, it is supercritical fluid extraction (SFE) that offers promise as a sample preparation technique for biomedical analysis SFE has been used for several years in the food industry for industrial-scale processing such as removal of caffeine from coffee beans [143]

Supercritical fluids are attractive extraction agents as the solvent power (density) can be manipulated over a wide range of temperatures and pressures Supercritical fluids have densities close to liquids and the viscosities and diffusivities intermediate between those of gases and liquids, the result is a highly effective extracting solvent that is capable of dissolving many compounds that would have been sparingly soluble in the same fluid under gaseous conditions

The solvating power of a supercritical fluid is varied by controlling the pressure or by adding modifiers such as methanol, a reduction in solvent density brought about by changes in temperature and/or pressure allows for the recovery of the analyte Solubility of an analyte depends on the density of the supercritical fluid which in turn is dependent on the temperature and pressure of the solvent Increases in selectivity of the extraction can be observed as the critical pressure of the solvent is approached [143]

There are three approaches to SFE depending whether the extraction is effected by pressure reduction causing a solubility decrease, a temperature change to change the analyte solubility or the adsorption of the analyte Solubility in supercritical fluids is dependent on both temperature and pressure In practice a combination of pressure, temperature and adsorption tends to be used for the separation of the analyte from the fluid [144]

Supercritical carbon dioxide has been the most used fluid for both chromatography and extraction as it has the advantages of being non-toxic, non-flammable, relatively inexpensive, leaves no solvent residue and has a low critical temperature (31 °C), thus thermal degradation of analytes is minimal SFE can be coupled to an on-line gas chromatograph without the need to modify commercially available instruments [141], coupling to mass spectrometers is also possible [141,145]

Only now is SFE being applied to analytical-scale problems and has yet to be fully evaluated for extraction of biological samples

4.2 Micelles

Surfactants have been used for many years as ion-pairing agents in reversed-phase HPLC. They are amphiphillic molecules with a hydrocarbon chain of at least eight carbon atoms with a polar group at the head. They can be classified as non-ionic, anionic, cationic or zwitterionic on the ionisation characteristics of the polar group. Their present use in HPLC has been at low concentrations but at higher concentrations they tend to aggregate together and above the critical micelle concentration they form micelles. Micelles are spherical aggregations of the surfactant molecules with the hydrocarbon chains pointing to the centre excluding water molecules whilst the polar heads are arranged on the surface of the sphere, this arrangement is thermodynamically stable [146].

Whilst a micellar solution appears homogeneous at the macroscopic level it provides a microenvironment which is different from the bulk solvent and which will enhance the solubility of hydrophobic compound in aqueous media. Micelles are beginning to be useful in extraction schemes, micellar liquid chromatography (MLC) [147,148] and a variety of analytical methods [149,150].

The main area of interest is MLC which may become a bioanalytical tool for the future as the technique offers a combination of advantages not offered by any other form of HPLC [147,149]. Essentially the surfactant replaces the conventional organic modifiers, such as methanol and acetonitrile, used in the mobile phase for reversed-phase HPLC [148]. The MLC mobile phase offers a dual hydrophobic and hydrophilic character so that molecules can be bound to the surface via ionic attraction and within the micelle hydrophobically. Retention of a hydrophobic analyte is inversely proportional to the micelle concentration, the higher the concentration the shorter the retention time. Selectivity can be controlled also by the concentration of the micelle but also by the surfactant used to form the micelle [147]. Gradients in MLC are very quick to run and as the surfactant does not enter the stationary phase the column does not need to be equilibrated before the next injection allowing a faster throughput of samples.

Direct injection of the sample is possible [151,152] without the need for any prior sample preparation, this, coupled with the possibilities of enhanced analyte response when using electrochemical or fluorescence detectors [147] makes it a very useful tool for the near future.

4.3 High-performance affinity chromatography

High-performance affinity chromatography is a technique for the purification of biologically active molecules that combines the specificity of affinity chromatography with the speed and resolution of HPLC. This subject has been the subject of recent reviews [153,154] and the reader is referred to these for

further information It is a technique that offers the possibility of 'designer' columns where the analyte can be selectively extracted and eluted on-line

4 4 Immunoextraction or antibody-mediated extraction

Antibody-mediated extraction or immunoextraction has been used for the past few years for the selective extraction of endogenous compounds, such as leukotrienes and prostaglandins, from biological samples [155–157] These compounds are present in low concentrations, and to improve the specificity of the assays to measure them, antibodies to the compound are bound to a Sepharose 4B support, packed in a column and the sample passed through it to extract the analyte In effect this approach is a variation of LSE where the 'bonded phase' is specific for the compound of interest Elution of the analyte usually requires a very strong solvent such as dioxane, acetonitrile or acetone to disrupt the strong binding forces between the analyte and the antibody, and the eluting solvent is evaporated prior to analysis The analytes that have been extracted by this technique are relatively lipophilic and are soluble in the eluting solvent Extraction of polar compounds which are not as soluble in these elution solvents may require more stringent methods, such as chaotropic agents, to disrupt the antigen–antibody binding forces These extraction columns can recover $>90\%$ of the analyte and can be reused up to fifty times [155]

As immunoextraction offers a more selective method of extracting compounds, analytical chemists are now using this technique to extract drugs and other molecules There are two general approaches. The first is off-line where the extract is evaporated before analysis and is outlined above The second approach is on-line preparation and analysis, where the antibody bound to Sepharose is packed into a pre-column and the column switching approaches outlined in Section 2 5 are used to enrich and separate the analyte Farjam et al [158] describe the analysis of β-19-nortestosterone, where the analyte was desorbed from the antibody–antigen complex by a solution containing an excess of a cross-reacting steroid hormone, norgestrel

This approach offers considerable advantages the specificity of the antibody could be 'designed' such that it is specific for only one molecule and or its metabolites Alternatively, if the compound is part of a series of homologues, e g barbiturates or opiate analgesics, the specificity of the antibody could be lowered so that all similarly structured compounds could cross-react and be extracted for the liquid chromatograph to separate and quantify

There are a few disadvantages that must be overcome before this technique becomes routine The antibody molecules are relatively large and offer the possibility for non-specific binding, this may be reduced by the use of enzymes such as papain (EC 3 4 22 2) to cleave the F_{ab} fragments (containing the active site of the antigen attachment) from the antibody and to bind these to the solid support The orientation of the antibody on the support should be such

that the binding sites are pointing away from the support and into the interstitial space to improve extraction efficiency This could be achieved by treating the antibody with pepsin (EC 3 4 23 1) producing a smaller, but still active, antibody combining site which could be bonded to a support via more selective chemical reactions, for example, through the thiol groups on the antibody fragment

The desorption of the analyte from the antibody for on-line analysis has used a cross-reacting compound [158] which required a relatively large volume of competing agent solution to elute the analyte. An alternative approach may be to isolate the low-affinity antibodies and bind these to the pre-column which should result in weaker analyte binding and hence easier elution Monoclonal antibodies usually have weaker binding compared to polyclonal antibodies and this area might be fruitful to investigate Finally, supports that can withstand high pressures need to be investigated for use in pre-columns of a liquid chromatograph

4 5 Microwave processing

Microwave processing can be used to accelerate diffusion processes, to speed up chemical reactions, to solubilize solid samples, to thaw frozen samples and to denature proteins Microwave irradiation, as a method of solubilization, is a technique in which energy (heat) is used for the extraction of compounds from solid materials The samples are mixed with a suitable solvent (e.g methanol) and irradiated for 30 s. After cooling and repeated irradiation, the samples are centrifuged and an aliquot of the resulting supernatant is injected into the HPLC system for analysis.

Microwave irradiation can also be used to denature the quaternary and ter-

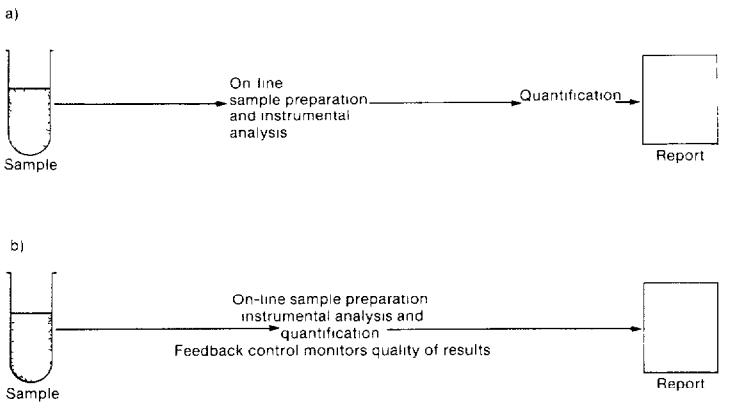


Fig 10 Stages of an analysis (a) Integration of on-line sample preparation and analysis, (b) integration of on-line sample preparation, analysis and quantification

TABLE 8

COMPARISON OF THE MAJOR SAMPLE PREPARATION METHODS

Sample preparation method	Advantages	Disadvantages
Precipitation agents	Rapid Very efficient for removal of protein	Possible occlusion of analyte in precipitate Analyte stability may be a problem Dilution of sample Possible incompatibility with HPLC mobile phase Relatively difficult to automate
Dialysis	Measures non-protein bound analyte Automation possible [40,41]	Short equilibration times Dilution of sample 50% recovery of analyte Difficult to use with avidly protein-bound analytes
Ultracentrifugation	Measures non-protein bound analyte No dilution of sample Removal of enzymes that degrade an analyte	Samples should not be frozen Binding to membranes a potential problem Relatively difficult to automate
Lyophilization	Produces extracts stable at ambient temperatures Clean extracts from subsequent extractions	Slow and tedious to use
Saponification	Efficient removal of neutral lipids	Tedious to use Analyte stability may be a problem
Liquid-liquid extraction	Well documented Easy to perform Unaffected by sample viscosity Unaffected by particulate matter Specificity	Emulsion formation Hazards of solvents used Removal of excess solvent Laborious, tedious, time-consuming Only extracts acids or bases under normal conditions Relatively difficult to automate [124,125]

Liquid–solid extraction (off-line)	Speed Wide range of analyte polarity extracted Ease of use Disposable cartridge	Particulate matter can block column Viscosity of sample affects flow Mental concentration required Documentation of methods lacking
Off-line preparation, on-line elution (AASP)	Speed Compact Disposable pre-column Wide range of analyte polarity extracted	Particulate matter can block column Viscosity of sample affects flow Mental concentration required Documentation of methods lacking
On-line liquid–solid extraction (Gilson–AASP)	On-line preparation and analysis Totally automated Disposable pre-column Labour-saving Standard curves prepared	Blockage by particulate matter Viscosity of sample Learning curve
HPLC column switching	On-line preparation and analysis Totally automated Ease of use Labour-saving	Carry-over from previous injections Renew pre-column Learning curve Blockage by particulate matter

tiary structures of protein As the structures denaturate within 1 s, it is a relatively fast and clean procedure for the removal of proteins from a sample [159] Furthermore, the technique is an excellent way of thawing frozen biological samples, e g doxorubicin and epirubicin are stable when stored at $-20°C$ and when thawed with microwave irradiation no significant degradation is observed [160]

5 INTEGRATION OF SAMPLE PREPARATION WITH THE ANALYTICAL PROCESS

It must be remembered that sample preparation was originally depicted as a separate stage in the analytical process (Fig 1), however, when viewing some sample preparation techniques presented here, it can be seen that there is a merging of the sample preparation stage with the instrumental analysis This is shown by HPLC column switching [102] and the use of the AASP in either semi [14] or fully automated mode [139] Therefore, the trend in sample preparation is clear the technique will be on-line and part of the instrumental analysis as shown in Fig 10a To use this approach, the sample must be liquid before the analysis can commence

A problem with merging preparation with analysis is that the instrumentation is not yet perfected to avoid loss of sample when the instrument malfunctions [137] Feedback control mechanisms will enable the quality of the analysis to be monitored and if required the instrument will be shut down pending an appraisal by the analyst This is shown in Fig 10b

6 CONCLUSION

There are many options available for sample preparation, the main ones discussed in this review are presented in Table 8 with their advantages and disadvantages The choice of which unit operations to use in an assay will be influenced by

1 Aims of the assay specificity, detection limit, etc
2 Physico-chemical properties of the analyte
3 The biological matrix
4 Required assay throughput
5 Time and resources to develop the assay and the experience of the analytical staff

In general, the method used should be the simplest one available that is consistent with the objectives of the assay

7 SUMMARY

A review of sample preparation techniques for biomedical analysis is presented Firstly, sample preparation techniques can be divided into unit operations which can be classified into four groups release of the analyte from the matrix, removal of endogenous material, liquid handling procedures and the enhancement of selectivity and sensitivity The concept of unit operations gives an analyst a tool with which to evaluate critically any method for preparing a sample for analysis Secondly, the major techniques of sample preparation (protein precipitation, liquid–liquid extraction, liquid–solid extraction and high-performance liquid chromatography, HPLC) are discussed and their advantages and disadvantages presented Thirdly, the rationale for the automation of sample preparation is reviewed, in general liquid–solid extraction and HPLC are the best techniques for automation The means by which this can be effected (either flexible or dedicated automation) is discussed Finally, techniques such as supercritical fluid extraction, micellar liquid chromatography, microwave energy and immunoextraction which may be applied to biomedical sample preparation are evaluated briefly

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