CHAPTER 3

Sample preparation

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3.1 *Introduction*

The quantitative determination of drugs and their metabolites in biological matrices (bioanalysis) includes a number of steps from sample collection to the final report of the results. The intermediate steps typically include sample storage, sample preparation, separation, identification and quantification of analyte(s). Sample preparation prior to the chromatographic separation has three principal objectives: the dissolution of the analyte in a suitable solvent, removal of as many interfering compounds as possible and pre-concentration of the analyte. A number of techniques such as protein precipitation, liquid–liquid extraction and solid phase extraction (SPE) are routinely used.

3.2 *Sample preparation techniques*

3.2.1 Protein precipitation

In protein precipitation, acids or water-miscible organic solvents are used to remove the protein by denaturation and precipitation. Acids, such as trichloroacetic acid

(TCA) and perchloric acid, are very efficient at precipitating proteins. The proteins, which are in their cationic form at low pH, form insoluble salts with the acids. A 5–20 per cent solution of these acids is generally sufficient and the best results can be achieved using cold reagents. Organic solvents, such as methanol, acetonitrile, acetone and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with high-performance liquid chromatography (HPLC) mobile phases (Blanchard, 1981). These organic solvents which lower the solubility of proteins and precipitate them from solutions have an effectiveness which is inversely related to their polarity.

3.2.2 Liquid–liquid extraction

Liquid–liquid extraction (LLE) is the direct extraction of the biological material with a water-immiscible solvent. The analyte is isolated by partitioning between the organic phase and the aqueous phase. The distribution ratio is affected by a number of factors: choice of extracting solvent, pH of aqueous phase and ratio of the volumes of the organic to aqueous phase. The analyte should be preferentially distributed in the organic phase under the chosen conditions. Although a number of factors influence the choice of solvent, the most important factor is the relative lipophilicity or hydrophobicity of the analyte. The analyte must be soluble in the extracting solvent. The solvent should also have a low boiling point to facilitate removal at the end of the extraction and a low viscosity to facilitate mixing with the sample matrix. Generally, selectivity is improved by choosing the least polar solvent in which the analyte is soluble.

The extraction process can be controlled using pH; ionised compounds are less efficiently partitioned in the organic phase. Changing the pH enables the process to be reversed; the charged analyte is re-extracted into the aqueous phase for further purification. The use of pH control allows the fractionation of the sample into acid, neutral and basic components. A large surface area is important to ensure rapid equilibrium. This is achieved by thoroughly mixing using either mechanical or manual shaking or vortexing.

There are several disadvantages of LLE; the technique is not applicable to all compounds. Highly polar molecules can be very difficult, although the use of an ion pairing reagent can extend LLE to molecules of this type. Another major problem is the formation of emulsions. These can be difficult to break even using centrifugation or ultrasonification and can cause loss of analyte by occlusion within the emulsion. The use of less rigorous mixing or larger volumes of extracting solvent can help reduce the problem with emulsions. LLE is also not very readily automatable.

Disposable columns containing diatomaceous earth as an adsorbent have been used to overcome many of the limitations of LLE. The diluted sample is poured onto the column and held on the support as a very thin film. The extracting solvent is then passed through the column. The high surface area of the sample allows a very efficient extraction.

3.2.3 Solid phase extraction

The principal mechanisms of separation and isolation utilised in solid phase separation (SPE), reversed-phase, normal phase and ion exchange are the same as those used in HPLC. Although the mechanisms of separation for the two techniques are the same, the dynamics of each technique is very different. In HPLC the compounds are separated in a continuously flowing system of mobile phase, while SPE is a series of discrete steps. In SPE the analyte is retained on the solid phase while the sample passes through, followed by elution of the analyte with an appropriate solvent. SPE can be considered as a simple on/off type of chromatography (Wells and Michael, 1987).

A typical SPE sorbent consists of a 40–60 μm silica particle to which has been bonded a hydrocarbon phase. This bonding is achieved by the reaction of a chlorosilane with the hydroxyl groups of the silica gel to form a silicon–oxygen–silicon link (Gilpin and Burke, 1973). This monofunctional bonded phase was originally the most popular reversed-phase sorbent with 4, 8 or 18 carbon atoms attached (C4, C8 and C18). Other reversed-phase sorbents such as C2, cyclohexyl and phenyl have subsequently been developed. A three-carbon spacer chain is generally used to link both cyclohexyl and phenyl groups. Normal-phase sorbents and ion exchange sorbents also have a three-carbon linking chain. The degree of carbon loading varies from 5 to 19 per cent by weight depending on the length of the hydrocarbon chain. The greater the carbon loading the greater the capacity; hence C18 sorbents have the greatest capacity expressed in milligrams of analyte sorbed per gram of sorbent. The capacity of a sorbent is also dependent on the pore size of the silca. The pore size affects both the coverage density of alkly-bonded phase and the migration of analytes in and out of pores during sorption. Much of the variation encountered between bonded phases of different manufacturers and even between different batches from the same manufacturer can be attributed to variation in the surface composition of the silica (Nawrocki and Dabrowska, 2000). An important characteristic of a sorbent is the number of unreacted or free silanols. These silanols are capable of hydrogen bonding or weak cation exchange with any solute bound to the bonded phase of the sorbent, particularly weak basic compounds. These secondary interactions need to be carefully considered during method development. As in the development of chromatographic stationary phases the manufacturers have tried to minimise the effect of these silonal groups. The use of trifunctional derivatives, trichloroalkylsilanes, gives not only greater stability under acid conditions, because the hydrocarbon chain is attached at multiple sites, but reduces the number of free silanols. The number of free silanols is generally further reduced by endcapping, where the derivatised silica gel is reacted with a trimethylchlorosilane reagent.

The disposable syringe barrel format of SPE contributed significantly to its success as a sample preparation technique. The syringes are available in 1–25 ml and packing weights from 25 mg to 10 g. The syringe barrel is typically polypropylene with a male Luer tip fitting. The sorbent material is packed between two 20 μm polypropylene frits. A vacuum manifold is used to draw the sample and eluting solvents through the syringe barrel under negative pressure by applying

a vacuum to the manifold. Other types of sample processing that may be used include centrifugation and positive pressure. The SPE is typically carried out using a five-step process: condition, equilibrate, load, wash and elute. The solid phase sorbent is conditioned by passing a solvent, usually methanol, through the sorbent to wet the packing material and solvate the functional groups of the sorbent. The sorbent is then equilibrated with water or an aqueous buffer. Care must be taken to prevent the phase from drying out before the sample is loaded, otherwise variable recoveries can be obtained. Samples are diluted 1:1 with aqueous prior to loading to reduce viscosity and prevent the sorbent bed from becoming blocked. Aqueous and/or organic washes are used to remove interferences.

3.3 *Instrumentation*

A great deal of sample preparation in bioanalytical laboratories is still carried out manually. Indeed, automation of sample preparation in bioanlytical laboratories has progressed at a much slower rate than in other areas of the pharmaceutical industry. Specifically, the combinatorial chemistry, high-throughput screening and compound storage/distribution laboratories in drug discovery, as well as the analytical quality control laboratories in manufacturing plants, have been revolutionised by automation over the last 5–10 years. Until recently, the hesitation to automate bioanalytical sample preparation has been quite understandable. The diversity of sample preparation techniques and the uniqueness of each individual assay's methodology have made implementing the required functionality and flexibility into an automation system too difficult and expensive. The wide variety of extraction consumables (e.g. test tubes, vials, SPE cartridges, etc.) used for sample preparation has meant that automation system is often limited to only certain manufacturers' sample preparation products. Most bioanalytical methods are only used for a limited time and the long-term need for a particular assay is often uncertain. Therefore devoting significant resources to the automation of a method can be viewed as a risky venture.

Recent advances in both laboratory automation equipment and sample preparation products have made automating bioanalytical assays more appealing and practical than ever before. The establishment of LC–MS–MS, with its combination of high sensitivity, high specificity and potential for high sample throughput, as the technique of choice in bioanalysis has greatly accelerated the development of automated sample preparation systems. Trends towards parallel processing of samples in 96-well plates have made automated sample preparation procedures quicker and more cost-effective. Likewise, novel SPE products that yield high recoveries for a wide range of analytes, using a single generic extraction procedure, greatly reduce the amount of time spent reconfiguring robotic systems each time a new assay is needed.

The number of commercially available automated sample preparation systems is still somewhat limited. Most of these are designed to support solid phase extraction. Protein precipitation and liquid–liquid extraction are often restricted to either semi-automated approaches, in which only the sample transfer and reagent addition steps are automated using a liquid handling workstation, or the more complex fully automated robotic systems which tend to be highly customised.

Instrumentation currently available can be grouped by their degree of automation. The five representative categories are:

1 SPE application-specific workstations or modules
2 On-line sample prep instruments
3 XYZ liquid handlers
4 Robotic workstations
5 Fully automated robotics systems.

This classification is not absolute since the diversity of these systems really represents a continuum. Some systems may overlap into two or more of the categories. On-line instruments include autosamplers with preparative, liquid addition, transfer capabilities, switching valves and other on-line and direct injection techniques.

3.4 *Bioanalytical automation strategy*

The switch to LC–MS–MS as the method of choice for bioanalysis made the sample preparation step the bottleneck. The development of SPE in the 96-well format and the development of systems using parallel processing have overcome this bottleneck and once again made the analysis time the rate-determining step. The original semi-automated systems used a customised vacuum manifold located on the deck of a Packard MultiPROBE™ RSP (Allanson *et al.*, 1996). The MultiPROBE performed all the liquid handling steps and controlled the vacuum. This allowed automation of the condition, load and wash steps, but the operator was required to manually place the collecting plate in the vacuum manifold prior to the elution step. Similar approaches to sample preparation in the 96-well format have been subsequently reported by a number of authors. In order to maximise the advantages of this approach and overcome some of the limitations a fully automated system was developed.

3.4.1 Fully automated sample preparation system

The initial system consisted of a Zymate XP robot, cooled storage carousel, a custom-built SPE station and an RSP (see Figure 3.1). The system running Easylab software (version 2.5) with a Visual Basic™ (version 4) user interface. The software

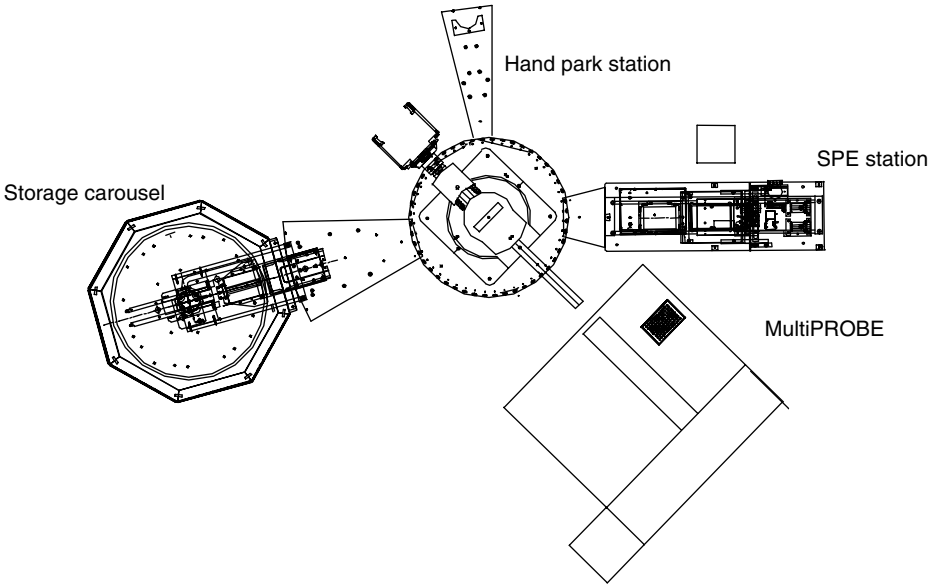

FIGURE 3.1 *Schematic representation of Zymark sample preparation system.*

was very quick and easy to use providing the facility to set up extraction procedures, change reagents and purge lines, and run procedures.

To run an existing procedure the user selected the appropriate procedure or procedures and entered the number of samples in each batch, up to four consecutive batches of samples could be processed using either the same or different procedures. When prompted, the user then loaded the required labware, SPE blocks and collection plates, into the storage carousel. The eight racks of the storage carousel are pre-assigned in the software to a particular type of labware. The cooled storage carousel, which could be maintained at 4–50 °C, not only acted as a warehouse for all the labware but also as a repository for the collection plates containing extracts.

To create a new procedure the user selected the type of SPE block (e.g. Micro-lute™ 2 or Empore™) and type of collection plate (e.g. deep well or square well), and specified the volume and reagent used for up to three condition steps, two wash steps and one elute step. The vacuum time and the bleed factor were also entered for each vacuum step. The power of the vacuum applied to the SPE block could be moderated using an extra valve that was open to the air. The setting on this valve, the bleed factor, was used to control the flow rates and ensure that the phase did not dry out before the sample load step. Conventional packed bed-reversed phase sorbents are prone to drying out. This can lead to poor or variable recoveries. It is also important to ensure that the flow rate for the sample load and elution step were optimised, otherwise recoveries could again be adversely affected.

The SPE station is a modified reagent addition station (RAS) which incorporates a two-stage vacuum manifold (see Figure 3.2). Reagents can be rapidly added row at

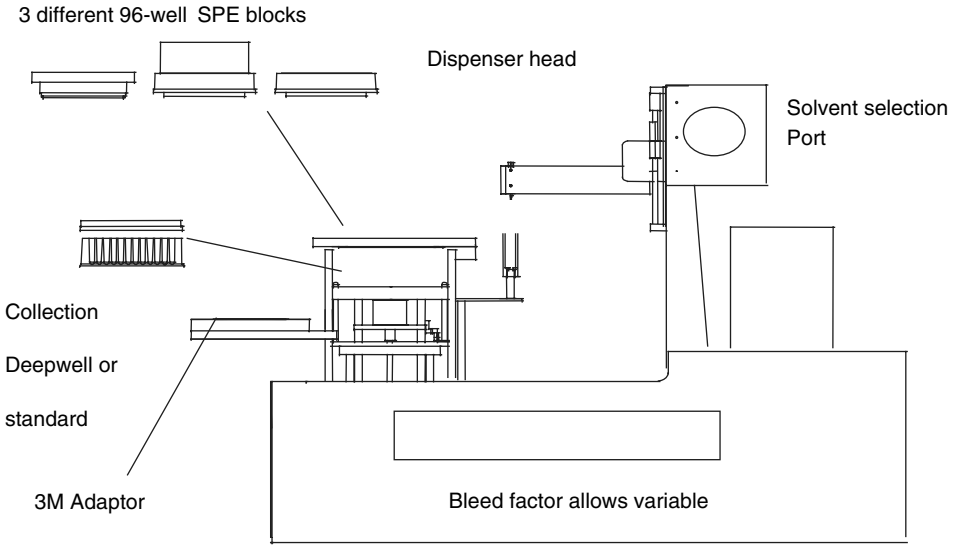

FIGURE 3.2 *Schematic representation of solid phase extraction station.*

a time and a switching valve allows up to nine different reagents to be used on the system enabling different procedures to be sequentially run. The variable height control on the dispenser head allows different height SPE blocks to be processed. The station can also cope with both Microlute™ and Empore™ type plates with their different skirt heights and length of flow directors, the Zymate places an adapter on the SPE station prior to running Empore™ type blocks (see Figure 3.2). The semi-automated system requires a different manifold for each type of SPE block.

This variable height control on the dispenser head also offers another advantage, in that it can be used to push down on top of the SPE blocks to ensure that the vacuum is consistently formed, inconsistent formation of the vacuum could be a problem with the semi-automated system. The variable height setting on the platform allows both deep well and standard micro titre collection plates to be used. Although the SPE station performs all the vacuum, conditioning, washing and elution steps the system utilises a MultiPROBE™ RSP to dilute samples, add internal standard and transfer samples from tubes to the SPE blocks.

The robot sequence begins with SPE block being ejected from the storage carousel. All the SPE blocks are stored upside down in the storage carousel so they can easily slide. The block is transferred to the SPE station where it is conditioned. The conditioned block is transferred to the MultiPROBE™ for the sample transfer step. The Zymark controller and the MultiPROBE™ controller are networked so that the transfer method can be initiated from the Zymark controller. When the MultiPROBE™ flags that the transfer is complete the Zymate collects the loaded block and returns it to the SPE station. The SPE station applies a load vacuum, carries out the wash step(s) and the elution step. The plates containing the extracts

are returned to the cooled storage carousel until manually transferred to an autosampler. This system effectively halves the time to extract 96 samples compared with the semi-automated system, a batch takes about 30–40 minutes depending on the number of steps and the reagent volumes. This system was originally designed to perform a minimum of four consecutive runs from sample tubes (384 samples) and to be capable of handling all the currently available labware (Microlute™ and Empore™ blocks).

This system has been used to support clinical studies for over three years and has proven to be very reliable, with minimal unscheduled down time. The performance of the Zymark 96-well system will be highlighted by looking at the data from one of the first studies supported by the system.

3.4.2 Application

The study was repeat dose Phase I study for a compound being developed for rheumatoid arthritis. There were three dosing occasions each generating 288 samples (8 patients × 36 samples) a total of 864 samples. A rapid turn round of the data was required to enable the pharmacokinetic (PK) analysis to be carried out prior to the next dosing occasion.

The SPE method although straightforward and requiring no concentration step does highlight the fine vacuum controlled that can be achieved on the SPE station allowing SPE blocks containing only 15 mg of sorbent per well to be routinely processed. The Microlute™ block, containing 15 mg of varian C2 sorbent, was conditioned with 0.1 ml of acetonitrile followed by 0.1 ml of water. The samples were diluted 1:1 with the internal standard solution and loaded onto the block; the RSP aspirates internal standard followed by sample and dispenses these sequentially onto the block. The block was washed with 0.1 ml of water and the analyte was then eluted into a deep well micro titre plate with 0.1 ml of mobile phase, acetonitrile:0.1 per cent formic acid (50:50). The plate was then covered with foil to prevent evaporation and placed on the autosampler. The LC–MS–MS conditions are tabulated in Table 3.1.

Table 3.1 *LC–MS–MS conditions*

Column	**Columbus C18 5 µ 150×4.6 mm**
Mobile phase	Acetonitrile:0.1% aq HCOOH (1:1)
Flow rate	1.0 ml/min with 1/10 post column split
Temperature	40 °C
Injection	50 µl
Typical retention time	1.9 minutes
Detection	PE Sciex API-300 with turbo ionspray

The following transitions were monitored: m/z 395 ⇑ 86 for the parent and m/z 399 ⇑ 86 for the internal standard. The data was processed using MacQuan™ Software (PE Sciex). Peak area ratios of analyte to internal standard were used to construct a calibration curve for interpolation of sample concentrations. The regression model was linear with $1/x^2$ weighting. With a 0.1 ml sample volume the validated range for the assay was 1–2,000 ng/ml. The serum (SRM) chromatographs for the 1 ng/ml standard are shown in Figure 3.3. The assay is both sensitive and specific for the analyte. The precision and accuracy obtained for the back interpolated standard values and the quality control samples are tabulated in Tables 3.2 and 3.3, respectively. Both the %CV and %bias for both sets of data are less than 5 per cent and well within our acceptance criteria of 15 per cent.

The data in Tables 3.2 and 3.3 shows that the assay performance was very good. The sample preparation time is 28 minutes a block and with an analysis time of 2.5 minutes/sample each block took four hours to run. We were able to give next day turn round for the data on all three dosing occasions.

3.4.3 Enhanced system

A number of new modules have been added to the system to enhance its capability (see Figure 3.4). A plate rotator station is used to switch plates between portrait and landscape. A heated dry down station is used to evaporate the SPE extracts to

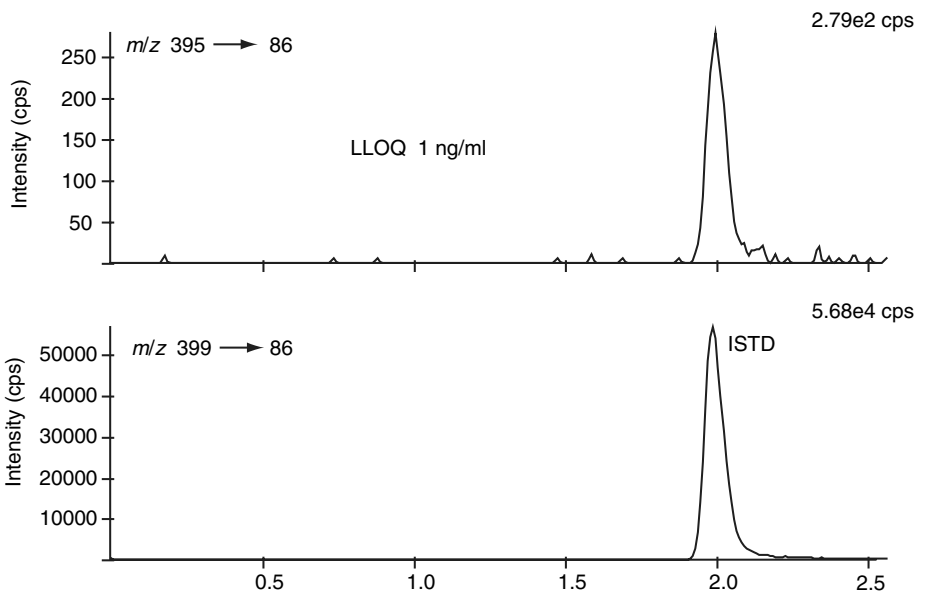

Figure 3.3 *SRM chromatographs for the 1 ng/ml standard.*

TABLE 3.2 *Back calculated calibration standard concentrations*

Nominal	1.08	3.23	10.75	32.25	107.50	322.50	1075.00	2150.00
Mean	1.09	3.13	10.29	31.86	108.49	328.71	1100.70	2182.59
SD	0.05	0.14	0.28	0.89	2.63	11.09	28.76	51.08
%CV	4.67	4.44	2.76	2.79	2.42	3.37	2.61	2.34
%Bias	1.34	–2.88	–4.24	–1.20	0.92	1.92	2.39	1.52

TABLE 3.3 *Quality control data*

Nominal	3.17	846.40	1692.80
Mean	3.27	862.57	1701.41
SD	0.13	36.75	40.65
%CV	4.12	4.26	2.39
%Bias	2.95	1.91	0.51
N	25.00	26.00	25.00

dryness and allow a concentration step. The software controls the gas temperature and the dry down time; the gas flow is set manually. The dry down head is fixed but the plate can be moved up with time by setting the start height and end height; this can improve the dry down rate for larger volumes. To reduce evaporation rates from SPE extracts prior to analysis an automated plate sealer (Advanced Biotechnologies,

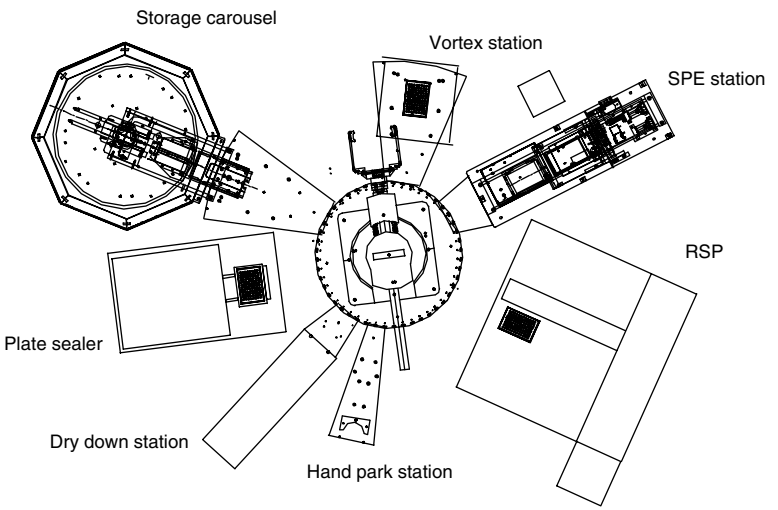

FIGURE 3.4 *Enhanced Zymark sample preparation system.*

Epsom, UK) is used to heat seal the plates with polypropylene-backed aluminium foil. The sealer can be adjusted to accommodate plates of different heights and sizes. The unsealed plate is placed on a shuttle that stands clear of the main body of the sealer, allowing robotic access. The shuttle is drawn into the unit and the plate is sealed in 10–15 seconds, depending on the plate material. The shuttle then slides out, presenting the plate in its original position. The temperature and the time for sealing the plate are manually set. The punch cuts the film to cover the entire plate surface without any overhang down the sides, which could affect the handling of the plate. This system also has the added advantage that each individual well of the collection plate is sealed. To ensure that the reconstituted extracts are adequately mixed prior to analysis a vortex station has also been added to the system. This station consists of a Bellco mini-orbital shaker mounted on a fixing plate with a pneumatically controlled plunger that is used to hold the plate in position. The speed control is manually set and the length of vortex time is software controlled.

The VB interface has also been upgraded (VB 6) and enhanced to improve its flexibility and to control the new modules; the number of condition, wash and elution steps is no longer limited, and procedures can be created by copying and editing an existing procedure. The software can schedule tasks, one plate will be dried down while the next plate is extracted; it is also dynamic, work can be added onto the system once the system is running. The MultiPROBE interface has also been improved; it now uses the MPTable™ software to allow the user to set the sample volumes, reagent volumes, source rack and destination rack positions. MPTable™ is a comma-separated values file that contains the sequence of pipetting operations and instrument optimisation parameters. This allows total control over the order in which samples are pipetted and the way in which all pipetting operations are performed. The VB interface uses Microsoft Excel™ to edit the MPTable™ file.

3.5 *Future development*

The development of more robust water wettable polymeric phases such as Oasis hydrophilic lipophilic-balanced (HLB) has led to the development of generic approaches to sample preparation. The two-stage design of the Oasis plate has enabled the use of a 5 mg sorbent mass without loss of recovery due to channelling. This enables conditioning and wash steps to be reduced to 0.1 ml and elution volumes to 0.2 ml. Coupling standard SPE procedures with standard fast gradient LC conditions has enabled method development times to be reduced. Standardisation also has a benefit in maximising the efficiency of these systems.

This approach has also highlighted many other areas that require improvements. The sample format (e.g. tubes) is not ideally suited to high-throughput bioanalysis. The choice of serum removes the necessity to centrifuge the samples prior to analysis, but the logistics of removing the caps, racking the samples and then

replacing the lids afterwards on 400 tubes still remains a laborious task for the analyst. A switch to microtitre plates or strips of tubes (8 or 12) which can be plugged into a microtitre plate format would reduce this potential bottleneck. Switching to a microtitre plate format for samples would enable samples to be stored in the carousel prior to extraction; this would also increase the capacity of the existing systems more than 20 unattended runs. The automation of the sample reception processes is currently under development.

Data processing particularly with multiple analytes or cassette-dosing studies would quickly become a bottleneck; with a single quantifiable analyte the data processing was not as tedious as expected. The current sample control software on the PE Sciex API-365 cannot run multiple batches. When running four consecutive assays one large batch (384 samples) has to be set up in sample control via the Excel import, and the data retrospectively separated into individual assays before processing. This problem should be overcome with the introduction of the new NT-based Analyst^{TM}. Processing large numbers of assays makes the requirement for a laboratory data management system (LIMS) with automated report templates more important.

There are many future challenges for automation, shorter LC–MS–MS run times, lower limits of detection, miniaturisation and demands for increased instrumentation utilisation. The development of multiplexing systems, where multiple LC pumping systems are used to increase the frequency of sample introduction into the mass spectrometer, will increase the requirements for sample throughput. The requirement to develop more sensitive methods is likely to increase the complexity of sample preparation procedures. The switch to the 96-well format is likely to be only the first step, already 384 is being looked at as the next step, particularly as the benefits of LC miniaturisation become a practical reality. The need to increase the efficiency of utilisation of mass spectrometers makes it inevitable that sample preparation and sample analysis will be on-line, making it possible to operate round the clock seven days a week.

None of these problems are insurmountable and should not detract from the key success of these robot systems, i.e. sample preparation is no longer a problem or rate limiting.

3.6 *References*

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