PHD THESIS

DIRECT ANALYSIS OF DRIED BLOOD SPOT SAMPLES

By

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ABSTRACT

DIRECT ANALYSIS OF DRIED BLOOD SPOT SAMPLES

The aim of the research reported herein was to identify and develop a dried blood spot (DBS) direct analysis technique that could support high sample throughput quantitative bioanalysis in a regulated drug development environment.

An initial literature review, coupled with proof of concept testing of the most prominent direct analysis techniques coupled to mass spectrometers (MS), resulted in direct elution (direct extraction of DBS via a confined solvent, producing a liquid extract) being selected as the most suitable technique to develop for this application. Direct elution technology was then developed into fully automated techniques with sufficient functionality to enable compatibility with high sample throughput quantitative bioanalysis. Proof of concept robustness data demonstrated that direct elution, despite the lack of sample clean up, was a reliable technique which had no detrimental effects on detector or chromatographic performance compared to conventional wet plasma extraction and analysis. A proof of concept investigation also demonstrated that a method of improving internal standard (IS) performance by spraying IS solution onto DBS samples prior to extraction, allowed the analyte of interest and IS to be co-extracted, while retaining adequate analytical performance.

The foregoing proof of concept data was then combined to produce a fully automated DBS direct elution instrument designed to introduce sample extracts into a LC-MS/MS system. This instrument incorporated a 500 DBS card capacity, an intelligent visual recognition system, a dynamic IS applicator module, and a highly effective wash system that virtually eliminates carryover. Ultimately, this work led to the production of a fully automated DBS direct elution system that is now commercially available. Subsequent research focused on optimising the system, and using this technology to address some of the issues that are currently inhibiting the development of DBS usage in drug development applications, namely haematocrit (HCT) based assay bias, and the decreased sensitivity on offer from DBS sampling.

It was demonstrated that using the IS sprayer enabled the IS to integrate sufficiently with the DBS sample prior to extraction to nullify HCT based recovery bias. The direct elution mechanism was also optimised with a view to maximising assay sensitivity while retaining acceptable analytical and chromatographic (LC-MS/MS) performance. Generic optimised direct elution conditions were developed which demonstrated that increases in assay sensitivity of up to 30 fold (compared to conventional manual extraction methods) were possible using a set of representative small molecule compounds.

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1. Paul Abu-Rabie, Neil Spooner, Babur Z Chowdhry, Frank S Pullen
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Bioanalysis, 2015, in press

2. Paul Abu-Rabie, Neil Spooner, Babur Z Chowdhry, Frank S Pullen
Dried blood spot direct elution: determining generic conditions for optimising performance in high throughput quantitative LC-MS/MS analysis
Bioanalysis, 2015, in press (accepted for publication)

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2. Paul Abu-Rabie
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Conference Presentations

1. **Dried Blood Spots and Microsampling: A Pharmaceutical Industry Perspective and Beyond**
Toronto University Medical School, September 2013, Toronto, Canada

2. **Developing automated dried blood spot direct analysis techniques for high sample throughput quantitative bioanalysis**
BMSS (British Society of Mass Spectrometry) British Mass Spectrometry Society Annual Meeting, March 2012, Alderley Park, UK

3. **Dried blood spot direct analysis**
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ABBREVIATIONS

Abbreviation	Description
AC	Analytical column
ASMS	American Society for Mass Spectrometry
BMSS	British Mass Spectrometry Society
BST	Bioanalytical Science and Toxicokinetics (Dept in GSK)
CI	Chemical Ionization
CV	Coefficient of variation
DART	Direct analysis in real time
DBS	Dried blood spot
DESI	Desorption electrospray ionization
DMDG	Drug Metabolism Discussion Group
DMPD	Dried matrix in paper disc
DMF	Digital microfluidics
DMF	Dimethylformamide
DMPK	Drug Metabolism and Pharmacokinetics
DMS	Dried matrix spot
DPS	Dried plasma spot
DSA	Direct sample analysis
EBF	European Bioanalysis Forum
ESI	Electrospray
FDA	Food and Drug Administration
FT-ICR	Fourier-transform-ion cyclotron resonance
GC	Gas Chromatography

GC/MS	Gas Chromatography-Mass spectrometry
GSK	GlaxoSmithKline
HCT	Haematocrit
HIV	Human Immunodeficiency Virus
HLQ	Higher limit of quantitation
HPLC	High Performance Liquid Chromatography
HPLC-MS/MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry
IR-LADESI	Infrared Laser Assisted Desorption Electrospray Ionization
IS	Internal Standard
IR	Infrared
ISR	Incurred Sample Reanalysis
LAESI	Laser Ablation Electrospray Ionization
LC/MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LESA	Liquid Extraction Surface Analysis
LIMS	Laboratory Information Management System
LLE	Liquid-Liquid Extraction
LLQ	Lower Limit of Quantitation
LMJ-SSP	Liquid Microjunction-Surface Sampling Probe
LTP	Low Temperature Probe
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry/Mass Spectrometer
MS/MS	Tandem Mass Spectrometry
MW	Molecular Weight
PCDBS	Pre-Cut Dried Blood Spot

PDBS	Perforated Dried Blood Spots
PI	Photo-Ionization
PK	Pharmacokinetics
PS	Paper Spray
PSMS	Paper Spray-Mass Spectrometry
QC	Quality Control
QQQ	Triple Quadrupole
QTOF	Quadruple-Time of Flight
RF	Radio Frequency
SCAP	Sample Card and Prep
SIL	Stable Isotopically Labelled
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SSSP	Sealed Surface Sampling Probe
TC	Trapping Column
TD	Thermal Desorption
TK	Toxicokinetic
TLC-MS	Thin Layer Chromatography-Mass Spectrometry
TTP	The Technology Partnership
UV	Ultraviolet
UPLC	Ultra Performance Liquid Chromatography
UHPLC	Ultra High Performance Liquid Chromatography

KEY TERMS

Acetaminophen: Also called paracetamol. Drug used to relieve mild headache or muscle and joint pain and to reduce fever.

Direct analysis: A general term used to describe analytical techniques that eliminate manual extraction steps required for sample preparation prior to sample analysis.

Dried blood spots (DBS): Blood microsampling technique where small volumes of blood are spotted onto cellulose (or similar) matrix cards and stored at room temperature.

Direct elution: Direct liquid solvent extraction of DBS samples using a device coupled directly to the LC-MS/MS system, eliminating the manual extraction steps involved with conventional manual DBS extraction; can be automated for high sample throughput compatibility.

Dried matrix spot (DMS): A collective term for the technique for the collection, transport and storage of wet liquid matrix as a dried sample on a paper type substrate.

Dried plasma spot (DPS): A technique for the collection, transport and storage of plasma as a dried sample on a paper type substrate.

Haematocrit (HCT): Percentage of blood cells in whole blood by volume.

High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS): Analytical methodology routinely used for the detection and quantification of circulating drug concentrations.

Internal standard: A reference substance (ideally stable isotope-labelled) added to samples to correct for matrix effects and loss of analyte during sample preparation; conventionally added via the extraction solvent.

Manual DBS extraction: Conventional liquid solvent extraction of DBS samples carried out by hand in the laboratory (sometimes with partial liquid handling automation) resulting in an extract containing the analyte of interest that is analysed using LC-MS/MS.

Peak area ratio: The ratio of HPLC-MS/MS analyte peak response to internal standard peak area response.

Sitamaquine: Drug used for the treatment of visceral leishmaniasis.

Sub-punch extraction: A partial, fixed area disc (typically) from the centre of the DBS is used for extraction and analysis; also known as sub-sample analysis when using direct elution.

Whole spot extraction: Where the entire DBS is used for extraction and analysis. Eliminates the HCT based area bias associated with DBS sub-punch extraction.

CHAPTER 1: INTRODUCTION

This thesis details a project aimed at developing a dried blood spot (DBS) direct analysis technique, primarily focusing on its application to regulated high sample throughput quantitative bioanalysis of small molecules. This chapter introduces the fundamental concepts of both dried blood spots and direct analysis, and describes the scope of the challenge, and potential applications. This chapter includes both my own experiences of using various direct analysis techniques, and the results of a literature review, which was initiated at the start of the project, and updated throughout its duration. The literature review aimed to identify all published accounts of direct analysis techniques used to test (DBS) samples, plus evaluate other direct analysis techniques that have the potential to be DBS compatible.

The literature review was coupled to my own practical research of direct analysis techniques (e.g. Direct Elution, Digital Micro Fluidics (DMF), Paper spray (PS), Direct Analysis in Real Time (DART)) with the aim of identifying the most suitable technique that could fulfil the aims of this project. In the interests of conciseness, only direct elution techniques (section 1.9), which are directly relevant to the project, have been summarised in detail in this document. The conclusion to this chapter (section 1.10) describe how direct elution was chosen as the most suitable technique to fulfil the aims of this project, and how it was developed to meet the requirements of high throughput quantitative bioanalysis.

1.1 A brief history of dried blood spot sampling

Dried blood spot (DBS) sampling describes a simple process whereby whole blood is obtained via a simple skin prick (often heel or finger; or tail prick in rodents) or other means (venous cannula) from a human or study animal, and then collected and stored on a substrate (typically cellulose or polymer based paper). Once dry, this sample (substrate + blood) can be analysed by a variety of methods. Historically, the technique was introduced as it significantly simplified the collection, handling, and storage of blood samples compared to alternative techniques. The earliest report of using filter paper to collect blood samples and then apply an analytical method (here to determine glucose concentrations) is attributed to Bang, 1913[1]. Subsequent early developments in DBS methodology and application centred on syphilis testing, using serologic methods (diagnostic identification of antibodies). Chapman, in 1924, reported a DBS analytical method for syphilis testing, highlighting a number of advantages to the technique, namely the reduction in volume of blood required; the inexpensive and easy to perform methodology; reduced risk of sample spoilage; and, enhanced stability of the sample[2]. The origins of DBS methodology were further defined by Zimmerman in 1939, again testing for syphilis, who found DBS samples to be 'dry' after 2 hr of air drying at ambient temperature, and used a sharp hollow pipe to 'punch' out 15 mm diameter discs for sampling[3]. In 1950, Hogan suggested that a simple DBS sampling procedure (undertaken by microscopically analysing cut squares of DBS from filter paper strips) could improve congenital syphilis control programs[4]. Of particular note was Hogan's observation that the DBS methodology was particularly advantageous for mass testing programs for infants and home collections, where the use of finger prick blood collection overcame parents' reluctance to allow jugular puncture. This first report of using inked rings on substrate paper (a format which is still used today) to target the location of DBS was published by Anderson et al in 1961[5].

In 1961, Guthrie published an article describing the collection of a few drops of blood from a newborn heel prick onto filter paper, followed by a DBS bacterial inhibition assay for measuring phenylalanine to detect the metabolic disorder phenylketonuria (PKU)[6,7]. Despite the various DBS based research that was published prior to this work, this report is often regarded to be the first use of DBSs. Using this simple method, babies could be screened at the time of discharge after birth. This work had a huge impact, resulting in DBSs being used in a widely used critical prevention strategy in public health practices. By 1973, newborn DBS screening was extended to also test for congenital hypothyroidism, which if detected in the first 2-3 weeks after birth allows for treatment to be implemented that can prevent mental retardation[8]. Extracting DNA from DBSs (genetic testing) was demonstrated to be possible by McCabe in 1987[9]. Guthrie's original bacterial inhibition assay, has gradually been replaced by newer techniques such as tandem mass spectrometry (MS/MS), that can detect a wider variety of congenital diseases (such as maple syrup urine disease, cystic fibrosis, medium-chain acyl-CoA dehydrogenase deficiency (MCADD), and sickle cell disease)[10,11]. The use of MS/MS detection was instrumental in introducing a new-born recommended uniform screening panel (RUSP) using DBSs in the USA which currently includes over 50 conditions (using testing methods including high performance liquid chromatography (HPLC), MS/MS, immunoassays, and enzyme assays)[12]. DBS methods have been demonstrated to be

particularly effective in developing countries, where polymerase chain reaction (PCR) methods have been used to diagnose neonatal HIV-1 infection[13]. The simplified sample collection, storage, transport, and minimal biohazard risk, are of particular importance here. DBSs have been used in a wide range of applications and used to measure a large number of biomarkers and analytes, including hepatitis B virus, glucose, lipoproteins, trace elements, vitamin A, specific antibodies for multiple viruses and microorganisms, among many others[14].

DBSs have been successful in applications where they offer advantages over conventional liquid whole blood, plasma, or serum sample collection. The paper substrate is cheap, readily available, easily stored and handled. DBS samples can be reliably obtained with minimal training, are generally considered to be non-hazardous, and are easily transported from remote areas to sites of analysis[15]. DBS has been demonstrated to be a particularly cheap, and reliable method of large scale testing in remote populations[16]. The less invasive sampling methods that can be employed by DBSs (e.g. heel, finger, or ear lobe prick), rather than venous cannula needle insertion, aids in recruitment. Sample storage and transportation is easier and cheaper as the dry specimen is relatively robust, and generally does not need to be frozen or shipped in dry ice. The collection process reduces risk of infection, and crucially reduces the blood volume required compared to liquid blood collection methods[17,18].

Numerous disadvantages to DBS sampling have been established, generally centred around concerns over sample volume, haematocrit, sample homogeneity, effects of temperature and humidity during transportation and storage, analyte recovery, filter paper characteristics, and anticoagulants[19,20,21,22]. Another key issue is the relationship between quantitatively measured analyte in dry (DBS) and wet (liquid whole blood or plasma) samples[23].

1.2 Recent developments, and why the pharmaceutical industry is interested in dried blood spot sampling?

Until recently there had been relatively little interest in using DBSs to support the quantitative assessment of circulating drug concentrations in clinical and non-clinical samples derived from pharmaceutical discovery and development studies, despite reports of the potential benefits in discovery stage animal pharmacokinetic studies[24]. In 2008 and 2009 Spooner and co-workers, at GlaxoSmithKline, published two articles on the use of dried blood spots (DBS) as a collection technique to support toxicokinetic and pharmacokinetic studies in pharmaceutical drug development that sparked a surge of interest in DBS in the pharmaceutical arena and beyond, which has continued to the present day (Figure 1.1)[25,26].

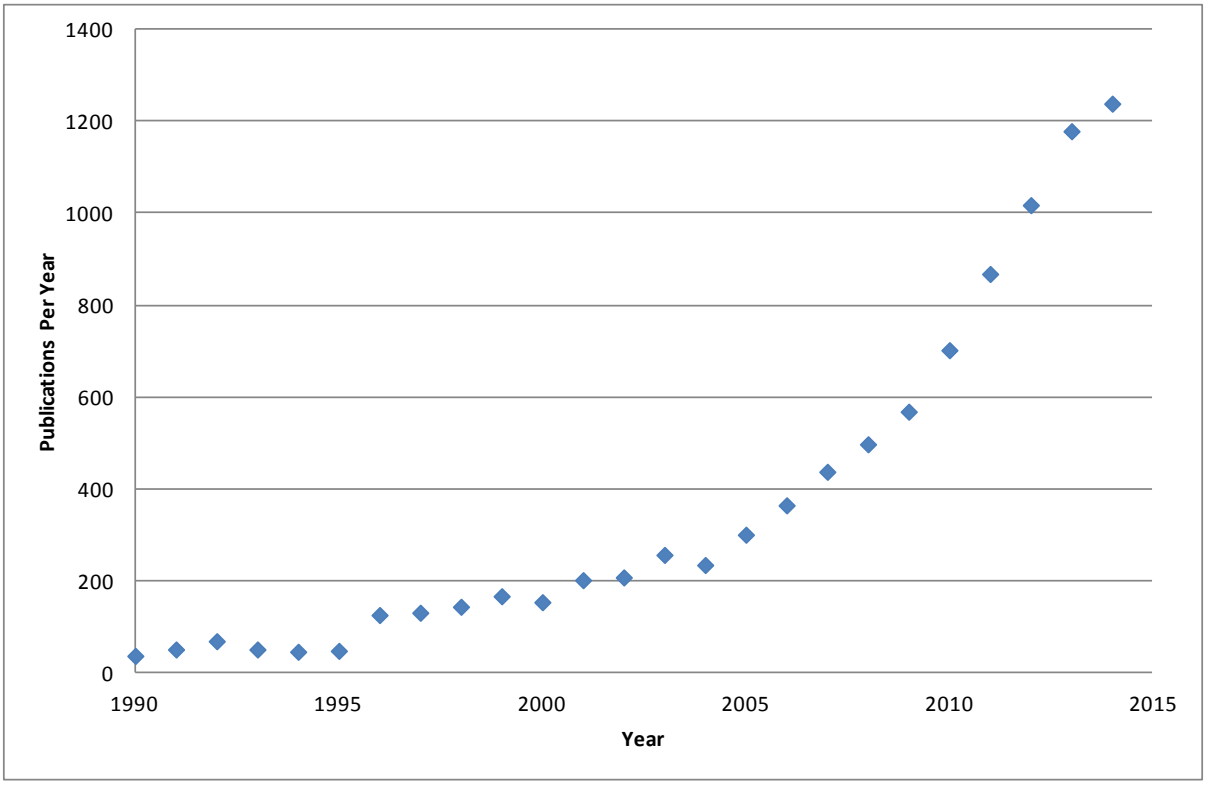


Figure 1.1: Number of publications per year for the last 25 years that contain the term 'dried blood spots'. Data collected using the Scopus search facility (Elsevier).

There are a number of reasons why, after being established as a concept for a century, DBS sampling suddenly became an interesting candidate for supporting quantitative drug bioanalysis[23]:

- Pressure from the public, governments, and regulators to achieve higher standards of welfare for animals in drug development. These expectations led to the implementation of the 3R's in animal welfare: replacement, reduction, and refinement, which push for the development of techniques which reduce the number of rodents used in drug development studies, and utilise procedures that are less stressful for the animals [27].
- Regulatory agencies required pharmaceutical companies to develop medicines for children as a first intent (European Union, 2006). This necessitated a process that could handle small blood volumes.
- There was a need to identify a biosampling procedure that could facilitate clinical drug trials in remote locations where critical equipment may not readily available (i.e. centrifuges to produce plasma from whole blood, facilities for shipping samples to an analytical laboratory in the frozen state).
- Therapeutic drug monitoring (TDM). There is a trend to expand the generation of drug exposure data into areas that were not previously accessible, such as home monitoring.

- Mass spectrometers have become increasingly sensitive, and readily available, which allows bioanalytical assays to be developed that are compatible with the small blood volumes typically used with DBS sampling.

Over the last ~five to seven years there has been much interest in applying the advantages on offer from DBS, when used as a microsampling technique, to new applications. The work in this project will focus on using DBS as an alternative to traditionally used wet plasma sampling in a pharmaceutical drug development environment. Wet plasma sampling is conventionally by far the most common technique to be used in quantitative and qualitative bioanalysis to support drug-development studies. This is due to the ease of handling plasma, shipping and storage compared to liquid whole blood. This conventional approach typically involves collecting whole blood in the clinic (or animal facility) from a volunteer (or animal) via venous cannula, which is then centrifuged under refrigeration to separate it into plasma and red blood cell fractions. Typically, the red blood cell fraction (which is typically around half of the original whole blood volume collected, depending on the haematocrit) will be discarded (along with the leukocytes and thrombocytes), and the plasma portion is transferred into a tube, placed on ice and maintained frozen during transportation to, and during storage at, the site of analysis. In order to derive the appropriate volume of plasma required for quantitative bioanalysis, typically >500 µL of blood needs to be collected[26].

The conventional wet sampling technique described above has been used successfully in this field for years. So, what are the advantages on offer from DBS that have generated such a high level of recent interest? These advantages can chiefly be categorised as ethical, financial or organisational, and are elaborated on below.

Ethical Considerations

DBS is often and readily employed as a micro-sampling technique. In a pharmacokinetic (PK) or toxicokinetic (TK) drug development study, typically a finger prick (or tail prick for rodents, for example) will be used to draw blood from a volunteer/animal at each sample time point, and typically three spots of blood (of approximately 15 µL each) will be added to a DBS sample card (using a pipette or glass capillary). The first spot is for primary analysis, with spots 2 and 3 being used for reanalysis or incurred sample reanalysis (ISR) when required. The sample cards used are typically composed of a cellulose based substrate (which may or may not contain a chemical treatment) which is sandwiched between two layers of thin cardboard (Figure 1.2).

This volume reduction (compared to conventional plasma analysis) is the basis of the ethical advantages of DBS. In practice DBS sampling (using three 15 µL spots), including wastage will typically require ~50-100 µL of blood, compared to ~500 µL for conventional plasma sampling. Plasma micro-sampling can also be employed, but using whole blood rather than plasma is always going to offer a volume advantage as a large proportion of the sample is not being separated and discarded.

In terms of animal testing, sample volume is important due to the physiological and ethical limitations of obtaining multiple serial plasma samples from individual animals, especially juvenile rodents, which means composite sampling is often required. This results in both more animals having to be used, and may result in lower quality TK data[25]. The smaller volumes (<100 µL) of blood required for DBS samples enables serial bleeds to be taken from the same central study animal. This often eliminates the need for satellite animals, reducing the overall number of animals needed to perform toxicology studies, and also increases data quality (as serial, rather than composite data is being used).

The reductions in the blood volume required for DBS sampling allows for significant benefits in the 3Rs (reduction, refinement, and replacement) for animal use in drug development. The 3Rs are a widely accepted ethical framework for conducting scientific experiments using animals humanely, that was first introduced in Russell and Burch's 1959 book 'The principles of humane experimental technique'[27]. For rodent studies the lower volumes required also mean that animal warming can be eliminated or reduced, further enhancing the ethical advantages of the approach. Pharmaceutical companies are under increasing pressure to demonstrate a progressive approach to animal use, and using DBS as a micro-sampling approach is an important step forward.

Financial and Logistic Considerations

The financial and organisational advantages on offer from DBS are largely intertwined, and can be categorised as either patent life, or logistical issues.

The patent life advantage on offer from micro-sampling stems from the ability to perform paediatric and juvenile toxicology studies that would otherwise be difficult or impossible with conventional large volume sampling techniques. Patent life is currently an area of increasing financial importance for pharmaceutical companies. There are currently significant extensions in patent life on offer for drugs that have been demonstrated to work effectively on juvenile populations[28].

Further financial advantages are on offer from a logistical perspective. In terms of sampling, transportation and storage, DBS has some clear advantages over conventional plasma sampling. For example, as mentioned above, DBS offers the advantage of less invasive sampling (finger or heel prick, rather than venous cannula in human studies) which may aid recruitment of subjects for clinical studies (a perpetual problem for drug development projects). DBS sampling also reduces costs in the form of shipping, storage, reduced animal numbers, simplified procedures, and reduction in the amount of test substance required. Wet plasma samples must be shipped and stored frozen (typically at -20°C). Test samples may be collected from hundreds of different sites all over the world, before being transported to a central laboratory for testing. The advantage of DBS samples is that they can be shipped and stored

at ambient temperature, thus eliminating the need for refrigeration/freezing, resulting in significant savings in shipping and storage costs[29].

The fact that DBS sampling eliminates the need for centrifugation and freezing also makes the process ideal for Phase II/III drug development in developing countries, where the need for specialist equipment and electricity may otherwise preclude studies being carried out in these locations. Similarly, the simplified blood sampling procedure makes DBS an ideal process for many drug screening processes where the collection of wet samples may not be feasible. DBS sampling has also been recognised as having great potential in therapeutic drug monitoring applications[30]. Finally, there are also advantages for programs involving small children and critically ill patients, where it may be important to minimise the volume of blood being taken [31,32].

The interest in DBSs has also spread beyond quantitative bioanalysis, into other areas of pharmaceutical development. For example, successful implementation into drug discovery studies have been demonstrated [24,33,34]. Application of DBS to large molecules has also been reported [35,36,37,38,39,40]. Small volume, dried sampling has also been applied to multiple other matrices including plasma, tears, synovial fluid, and cerebrospinal fluid [41,42].

1.3 Recent investigations into fundamentals principles associated with implementing DBS

In order to implement DBSs into pharmaceutical drug development, an area that must adhere to a range of criteria from regulatory authorities, many investigations into the fundamental principles of DBS have been carried out in the last ~5 years with the aim of understanding and underpinning the science behind the technique. It is beyond the scope of this document to discuss all these investigations in depth, but the following highlights some of the important work that has recently been performed to understand some of the limitations, and potential performance on offer from DBSs:

- Storage of control blood when used in the preparation of DBS quantitative calibration standards and quality control samples (up to 14 days was demonstrated)[43].
- Drying of DBS samples under standard laboratory conditions before analysis or shipment (2 hrs recommended)[44].
- Enhanced analyte stability of analytes when using DBS rather than conventional liquid storage [45,46,47,48].
- Alternative approaches to internal standard addition[20].
- Result of using sub-punch sampling methodology when using non-accurate volume DBSs on the accuracy and precision of quantitative data[26].
- DBS environmental conditions during ambient shipping and storage across international borders[49].
- DBS sample homogeneity [50,51,52,21].
- Analytical performance of DBS quantitation compared to liquid-based methods.

- Quality of incurred sample reproducibility (ISR) data when using DBSs [41,53].

A critical hurdle, that currently hinders the implementation of DBSs as a widely used technique to support pharmaceutical development studies, is acceptance from regulatory authorities. Specifically, regulators have raised concerns over the impact of haematocrit (HCT) on spot size and recovery, sample homogeneity, and best practices for IS addition [54,55,56]. Concerns have also been raised about making a change between determining systemic exposure in plasma to blood, particularly in the same regulatory submission package[57]. The result of these concerns is that regulators at this point do not appear to be ready to accept DBS-only exposure data to support clinical studies[29,58]. Recently, regulators have requested that prior to supporting clinical studies with DBSs alone, initial clinical exposure DBS data should be run in parallel with wet samples. This requirement currently puts the use of DBSs in pharmaceutical development at a significant financial disadvantage due to the additional costs associated with having to collect and analyse two sets of samples (wet and dry). Consequently, project managers are currently reluctant to select DBS as a sampling method (except in niche applications where a microsampling approach is the only option) due to resource constraints, and this means the important advantages on offer from using DBS are not being realised. Unfortunately this has created something of a vicious circle[59]. It appears that regulatory authorities want to review a (currently unspecified) number of study submissions that have generated wet and dry sample data in parallel before the requirement for parallel data will diminish, or hopefully, ultimately be eliminated. However, the additional costs associated with running wet and dry data mean that fewer DBS studies are currently being used in pharmaceutical development studies. It is hoped that pharmaceutical companies will realise the long term ethical and financial advantages on offer from DBSs, and make the additional resources available in the short term to allow DBSs to reach their potential.

1.4 Alternatives to DBS

As mentioned above, the recent interest in implementing DBSs in pharmaceutical development is as an alternative to conventional wet plasma sampling. It must be noted that other alternative sampling techniques are also available. Table 1.1 summarises these techniques and lists their primary pros and cons. In line with the aims of this project, I have only included those that can be, or have the potential to be applied to drug development workflows, though many of them could be applied to other applications. Note that not all of the techniques listed are commercially available, or have been formally introduced in the peer reviewed literature.

Table 1.1: Comparison of microsampling techniques.

Microsampling Technique	Description	Pros	Cons
Liquid Plasma Microsampling	As per conventional (large volume) wet plasma collection/analysis, but smaller volumes (~15µL) are collected	Mirrors established process, so no regulatory barriers to acceptance	Centrifugation and facilities to freeze samples (shipment and storage) required
			Significant sample wastage (plasma portion only used)
			Difficult to handle/automate small volume analysis
Liquid Blood Microsampling	As per conventional (large volume) wet plasma collection/analysis, but smaller volumes (~15 µL) are collected, and whole blood is not separated into components	Mirrors established process, so no regulatory barriers to acceptance	Facilities to freeze samples (shipment and storage) required
		No sample wastage	Whole blood thawing process can cause handling issues (e.g. rat blood crystallises on thawing)
			Difficult to handle/automate small volume analysis
Liquid Blood/Water microsampling	As per liquid blood microsampling, but a fixed accurate volume of water is added to the blood prior to shipment/storage	Mirrors established process, so no regulatory barriers to acceptance	Facilities to freeze samples (shipment and storage) required
		Easier to handle than liquid whole blood	Difficult to handle/automate small volume analysis
		Eliminate risk of crystallisation during thaw	An accurate fixed volume of water needs to be added to a fixed blood volume (requires skill and resource)

Liquid Blood/Water Capillary microsampling	Accurate blood volume collected using capillary, placed in tube with known volume of water and frozen	Mirrors established process, so no regulatory barriers to acceptance	Facilities to freeze samples (shipment and storage) required
		Easier to handle than liquid whole blood	Difficult to handle/automate small volume analysis
		Eliminate risk of crystallisation during thaw	An accurate fixed volume of water needs to be added (requires skill and resource)
Plasma Capillary Microsampling (AZ method) [60]	Accurate whole blood volume collected using capillary. Centrifuged to separate plasma component. Capillary is cut and blood 'half' is disposed of. Accurate plasma volume is collected in a second capillary, which is placed in tube and frozen prior to analysing the whole sample	Mirrors established process, so no regulatory barriers to acceptance	Facilities to freeze samples (shipment and storage) required
			Difficult to handle/automate small volume analysis
			Added complexity at sampling point
			Significant sample wastage (plasma portion only used)
Plasma capillary Microsampling (Drummond/GSK method) [61]	Blood collected into a modified capillary housed in a collection tube, and centrifuged to separate plasma. Wiretrol used to expel plasma portion from capillary into micro sample tube, and frozen. Sample is thawed and an accurate volume aliquotted for analysis.	Mirrors established process, so no regulatory barriers to acceptance	Facilities to freeze samples (shipment and storage) required
			Difficult to handle/automate small volume analysis
			Added complexity at sampling point
			Significant sample wastage (plasma portion only used)

Dried Blood Spot (DBS)	Blood is collected via finger prick, and applied to paper substrate via pipette or capillary	No sample wastage (if whole spot elution is used)	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Easy to handle	Haematocrit effect may necessitate accurate volume sampling
		Readily compatible with direct analysis	Method development/Validation/ analysis more complex than for liquid sample extraction
DPS (Dried Plasma Spot)	Blood is collected via finger prick, placed into a tube and centrifuged to separate plasma component. Plasma then applied to paper substrate via pipette or capillary	As DBS	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Haematocrit effect eliminated	Endogenous levels of plasma components (lipids, gylcoproteins) may have a similar effect as varying haematocrit
			Pale substrate difficult to recognise on non-specialist material (available at extra cost)
			Significant sample wastage (plasma portion only used)
			Method development/Validation/ analysis more complex than for liquid sample extraction
Solid Phase Micro Extraction (SPME) [62]	In-vivo sample preparation technique that involves the use of a fibre coated with an extracting phase inserted into the body. The quantity of analyte extracted by the fibre is proportional to its concentration in the sample as long as equilibrium is reached	No sample wastage	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		No blood withdrawal	Development at an early stage for many applications - use in practice untested
		Potential for integrated direct analysis	Invasive - Possibly unsuitable for Human sampling
		Cheap/easy sampling & shipment (no centrifugation/ freezing required)	Assay sensitivity uncertain

VAMS/MITRA (Phenomenex) [63]	Next generation blood collection technique. Substrate on a tip is dipped into liquid blood - it collects a fixed volume and is allowed to dry. Tip containing dried blood can then be extracted for analysis.	No sample wastage	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Relatively cheap/easy sampling & shipment (no centrifugation/ freezing required)	Method development/Validation/analysis more complex than for liquid sample extraction
		Accurate volume collection built into technology	Technology and testing at a very early stage
		Eliminate haematocrit based area bias	Not as easy to ship/store as DBS
			Specific automation required for analysis
			Currently no direct analysis options
Blood filtration devices (e.g. Yorktest plasma separation card, Noviplex plasma separation card) [64,65]	Whole blood (25-50µL) is applied to a membrane based filter card which removes the red blood cell component and dispenses an accurate volume plasma spot. This is allowed to dry before shipment/storage/analysis	Dispenses an accurate volume spot	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Cheap/easy sampling & shipment (no centrifugation/freezing required)	Endogenous levels of plasma components (lipids, glycoprotein) may have a similar effect as varying haematocrit
		Use of plasma spots avoids haematocrit based bias issues	Significant sample wastage (plasma portion only used)
			Method development/Validation/ analysis more complex than for liquid sample extraction
Digital Microfluidics (DMF) [66]	Whole blood can be directly applied to an integrated chip which is used as both a collection device and means of analysis	Relatively cheap/easy sampling & shipment (no centrifugation/ freezing required)	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Integrated direct analysis	Development at an early stage for many applications - Further product development required and use in practice untested
			Potentially expensive
			Accurate volume required

Paper Spray (PS) [67]	Whole blood can be directly applied to an integrated paper mounted device which is used as both a collection device and means of analysis	Relatively cheap/easy sampling & shipment (no centrifugation/ freezing required)	Unestablished technique for regulatory applications (thus faces barriers to acceptance)
		Integrated direct analysis	Development at an early stage for many applications - Further product development required and use in practice untested
			Potentially expensive
			Accurate volume required

1.5 Challenges associated with using DBSs?

As with any technique, there are also some disadvantages associated with using DBS. From an analytical perspective these are largely limited to increased complexity of sample extraction, and decreased assay sensitivity compared to conventional plasma sampling techniques. A further 'disadvantage' is that, like any new technique applied in the highly regulated field of pharmaceutical drug development, DBS also faces intense scrutiny from regulatory authorities (such as the U.S. Food and Drug Administration (FDA))[29,58]. The role of these bodies is to ensure there is maximum confidence in the data produced in support of new drugs, and ultimately that the drugs are safe for public consumption. This means that considerable research is required to prove that any new technique produces reliable data, and to do this requires significant time and money. It should be noted that the FDA recognise whole blood as a suitable matrix for pharmaceutical drug development. It should also be noted that many applications outside of regulated quantitative bioanalysis in drug development, such as qualitative, discovery, TDM, and screening applications do not face the same level of scrutiny, and thus it is far faster and easier for new techniques, such as DBSs, to become accepted.

As detailed above, In terms of sampling, ethics, cost, transportation and logistics, DBSs have some clear advantages over conventional wet plasma analysis. Once at the site of analysis however we begin to encounter some of the disadvantages. To understand the significance of the disadvantages of using DBS over conventional wet plasma sampling, one must consider both analytical procedures. The following section compares wet plasma, and DBS sample analysis, from the perspective of high throughput regulated quantitative bioanalysis of small molecules in pharmaceutical drug development.

Conventional Wet Plasma Bioanalysis

At the site of analysis a wet plasma sample is thawed and a volume (typically 20-150 µL) is accurately sub-aliquotted by pipette and taken through the manual extraction procedure. Sub-aliquotting has traditionally been carried out in the analytical laboratory as an accurate sample

volume is essential in generating good quality data, and this step has historically been deemed as difficult to manage when dealing with untrained personal (in analytical methodology) across multiple study centres. Extraction or clean up techniques prior to analysis range from very quick and simple protein precipitation methods, to more complex liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods. Concentration steps can also be utilised where low detection limits are required.

Elaborate laboratory automation is available that is compatible with all the above techniques and is capable of supporting high sample throughput (hundreds of samples per analyst per day). Resulting extract volumes are typically in the region of 50-200 µL. From here, separation and detection of the compounds of interest is typically undertaken using liquid chromatographic separation coupled to triple quadrupole mass spectrometers (HPLC-MS/MS). Typically 2-20 µL of extract will be introduced per injection. This technique offers proven selectivity and sensitivity, and often LC-MS/MS cycle run times short enough to be compatible with high sample throughput requirements (~1.5-3 min per sample).

This wet plasma sampling and analysis technique, when validated and performed to internationally accepted guideline criteria, is acknowledged as being suitable for measuring drug exposures by regulatory authorities[68]. Concentration of analyte in test samples is calculated by plotting the LC-MS/MS chromatographic peak area ratio (analyte/internal standard) response against a calibration line prepared at the site of analysis. Quality control (QC) samples, also prepared at the site of analysis, are used to ensure confidence in the data generated. As a guideline, calibration standard and QC samples need to be within ≤ ±15% of their nominal value for study sample data to be deemed acceptable.

DBS Bioanalysis

Current DBS manual extraction methods follow much the same procedure as above, but an additional step of punching out the DBS from the surrounding substrate is required prior to extraction. Two main categories of analysis exist: 'sub-punch', and 'whole-spot' analysis.

The initial procedure that was recommended for DBS sampling for use in pharmaceutical development involved collecting a non-accurate volume of blood (for example by taking blood from a finger prick into a glass capillary, then applying it to the DBS substrate), and then, at the site of analysis, punching a fixed diameter disc (or sub-punch) from the centre of the DBS using a sharp cutting tool (Figure 1.2)[25,26]. Typically a ~15 µL blood spot on cellulose substrate has a diameter of approximately 7-8 mm, and a 3 or 4 mm diameter disk is routinely punched out from the centre.

The original intent of this sub-punch methodology was to ensure a fixed volume is sampled in each analysis (remember, only an approximate volume is spotted in the clinic) and is the equivalent of taking an accurate volume of wet plasma using a pipette. Note that, to account for any inaccuracy in the volume of blood applied to the card in the clinic, it is proved during

method validation that blood volume variance (of 15 µL ±5 µL, for example) does not produce a significant assay bias[26]. Unfortunately, this methodology is only appropriate when there is control over the HCT ranges and homogeneity of the DBS samples being analysed. For example, blood HCT levels at extremities outside the normal range can cause a significant bias to the area of the DBS for a given volume, and to the recovery of the analyte during extraction[69]. This can result in a significant overall assay bias, and thus a lack of confidence in the analytical technique to accurately measure drug concentrations. To overcome some aspects of this issue, whole-spot DBS extraction can be utilised. As the name suggests, this involves punching out and extracting the entire DBS sample, rather than a sub-punch. This eliminates any spot area or homogeneity bias issues (though on its own does not solve issues around HCT based recovery bias). The drawback to using whole-spot analysis is that to analyse an accurate amount, an accurate volume of blood has to be applied to the substrate. This presents its own challenges, as the reason for using sub-punch analysis in the first place was to avoid having to collect an accurate volume!

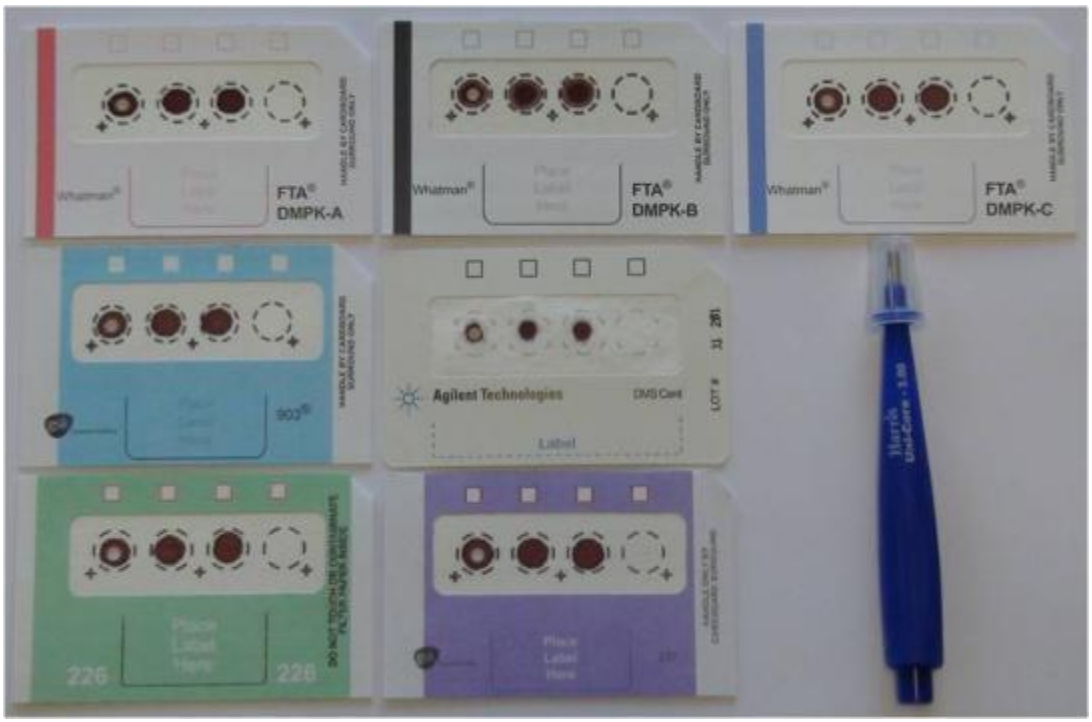


Figure 1.2: Dried blood spot 85 x 53mm 4-spot cards typically used in sampling for pharmaceutical drug development studies. Three 15µL spots of blood have been applied to each card. The first spot has had a 3mm diameter disk punched out using Harris Uni-core punching device (also shown). Top row left to right: Whatman FTA-DMPK-A, Whatman FTA-DMPK-B, Whatman FTA-DMPK-C; Middle row left to right: Whatman 903, Agilent Bond Elute DMS card; Bottom row: Ahlstrom 226, Ahlstrom 237.

To extract the drug or analyte of interest from the DBS sample, the punched out disk (sub-punch or whole spot) is added to a sampling tube (typically 96 well format) and extracted using the same variety of techniques used for wet plasma analysis. DBS sample analysis is typically carried out by solvent extraction, which transfers analytes from the substrate paper into an injectable solution that is compatible with liquid chromatography (LC). The simplest form of extraction involves adding 100 µL of highly organic solvent (typically 70:30 (v:v) methanol:water), containing a suitable internal standard (IS), to the tube containing the DBS disk, and extracting the analyte of interest by agitating the sample for ~2 hrs on an automated bench shaker. The sample is then centrifuged to move the disk to the bottom of the tube, and the supernatant is transferred to a fresh tube. The supernatant can then be analysed using HPLC-MS/MS in the same way as a wet plasma extract. LLE and SPE extraction can also be used, usually in cases where a cleaner extract and/or lower limits of quantitation (LLQ) are required.

In addition to extra complexity of extraction, current DBS manual extraction methods also exhibit less sensitivity then corresponding wet plasma extraction methods. This can partially be due to extra ion suppression caused by competitive ionisation of compounds present in chemically treated cards[46], but by far the biggest factor is the reduced quantity of sample being analysed. A 3mm diameter punch taken from the centre of a 15 µL DBS sample corresponds to only around 2.5 µL of blood. Compare this to the 20-150 µL that is typically sub-sampled during wet plasma analysis, and we have at least a 10 fold decrease in the amount of material being extracted and eventually injected into the detection system. Thus, using manual extraction techniques it is a challenge to meet low LLQ requirements using DBS sampling techniques. To date DBS sampling has not generally been compatible with the development of respiratory drugs that typically need LLQ's in the low pg/mL region. The result of this is that not all drugs can be supported with DBS sampling and thus the important ethical, financial and operational advantages on offer from the technique cannot be maximised throughout the pharmaceutical industry and beyond.

1.6 Why do we need a DBS direct analysis technique?

In addition to the general aspiration to make analytical measurements as quick, simple and cost effective as possible, the interest in using direct analysis techniques to analyse DBS samples has also stemmed from the desire to counter some of the analytical disadvantages. Swapping wet plasma for DBS samples results in a more complex extraction procedure, and increased difficulty in meeting target assay LLQ's due to the smaller sample volume. An additional problem, in areas such as pharmaceutical drug development, is the regulatory requirements for any new technique. The scrutiny (and associated costs) any new technique is subjected to in this field is so intense that, to be worth the effort, the advantages have to be significant.

A reader unfamiliar with high sample throughput bioanalysis will be forgiven for thinking that the need to punch out a disc from a DBS sample seems like a very minor increase in extraction complexity, compared to conventional wet plasma analysis. In isolation, this is certainly true. Indeed, it barely involves any more effort than the equivalent step in conventional wet plasma analysis (aliquotting an accurate volume into a sample tube via a pipette). To put the problem in context, it should be considered from a high sample throughput perspective, where an analyst may analyse five hundred or more samples per day. Aliquotting an accurate volume of wet plasma from this many samples using a hand held pipette is as equally time consuming as punching the same number of DBS samples. To eliminate this problem automated liquid handling robots are used in the laboratory, which reduces this part of the process from hours to minutes.

To eliminate any reluctance to using DBS sampling on the basis of bioanalytical efficiency, the manual handling limiting factor also needs to be addressed for DBS extraction. Indeed, some resistance to accepting DBS as a technique has occurred for this reason alone, by those who fail to appreciate 'the bigger picture' (i.e. the advantages that DBS can bring to an organisation as a whole)[59,70]. For many bioanalysts to even consider DBS to be a suitable alternative, the effort involved with sample extraction needs to be on a par with conventional wet plasma analysis. However the manual extraction burden is not the only barrier to be overcome; the regulatory requirements involved in implementing a new technique mean that there is considerable reluctance to move away from an established technique that is readily accepted and 'works', even if the overall advantages to the alternative are significant. Coupling these issues mean that any new analytical process needs to not only match the simplicity on offer from the current standard approach; ideally it needs to be demonstrated that the process is significantly simpler.

In summary, any reluctance to accepting DBS is a barrier to maximising the important ethical, financial and operational advantages that DBS sampling undoubtedly offers. A potential solution is the use of direct analysis techniques that could eliminate manual extraction steps completely. The term 'direct analysis' is used to describe techniques that eliminate the laborious manual extraction procedures traditionally used to analyse complex mixtures of samples (Figure 1.3). If such a technique could also offer additional advantages over manual extraction, such as higher sensitivity, enhanced IS performance, and online sample dilution, it would undoubtedly help reduce any reluctance to accepting the technique.

The ways in which direct analysis can benefit DBS sampling depends on the application. For simplicity, the applications and how they differentiate can be broadly categorised as follows.

1) Regulated quantitative bioanalysis (e.g. drug development)

Drug concentration measurements must have the highest level of confidence (in both the identity of the compound, and the quantity measured) and be determined according to internationally recognised guideline criteria. Throughput will often be an important factor. DBS samples will be collected by trained personnel and shipped internationally from multiple

study centres to a facility containing specialist preparation and instrument laboratories operated by trained personnel. This project will largely focus on this application.

2) Drug discovery and screening applications

This includes some drug discovery, and qualitative and quantitative screening applications that do not require the same levels of acceptance criteria required for regulated quantitative bioanalysis. Potentially this opens up applicability to direct analysis techniques that do not provide the level of selectivity (or the level of selectivity is unproven) required for regulated quantitative bioanalysis. Throughput will often be an important factor. As above, DBS samples will have been collected by trained personnel and analysed in specialist facilities containing specialist preparation and instrument laboratories operated by trained personnel.

3) *In situ* bioanalysis for therapeutic drug monitoring

'In situ' in this instance refers to analysis occurring at the site of sampling. DBS samples may be collected by trained (in sampling) personnel in medical clinics (which may be mobile), or possibly by patients themselves at home. In most cases high throughput will not be an important factor. Analysis will be carried out locally in the clinic by untrained (in MS) personnel, or theoretically at the home of the patient with a suitable portable device.

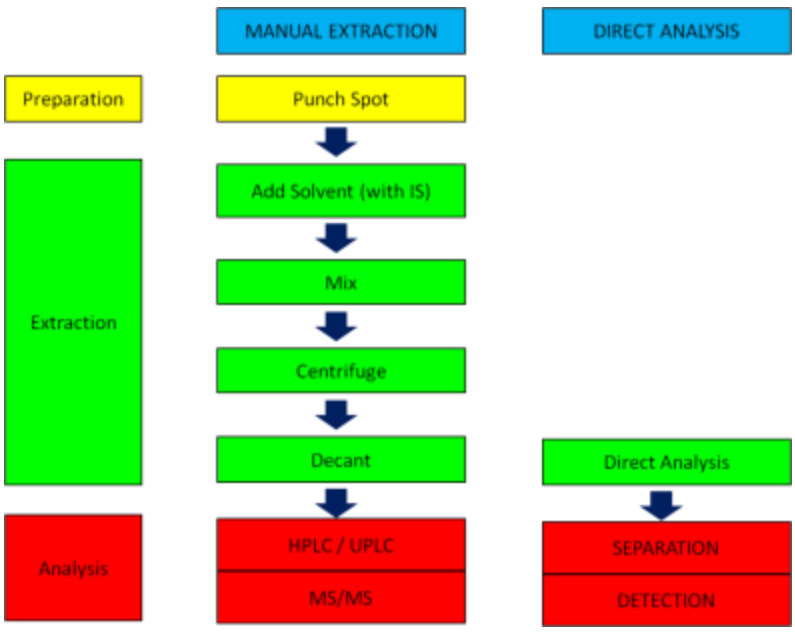


Figure 1.3: DBS manual extraction and direct analysis schematics.

Direct analysis techniques eliminate the laborious manual extraction procedures traditionally used to analyse complex mixtures of samples. Utilizing direct analysis could not only compensate for the extra analytical complexity involved in DBS analysis, but also significantly simplify the entire bioanalytical procedure. Clearly, direct analysis would also potentially be of a similar benefit to many other types of sample analysis, including conventional wet plasma analysis, wherever a cheaper, faster, and simpler alternative is desired. It so happens that dry

samples, such as DBS, are particularly amenable to many existing direct analysis techniques (see chapter 1.8), and the emergence of DBSs as a 'new' sampling technique has created an impetus to investigate different (hopefully improved) ways of performing bioanalysis.

Direct analysis is particularly desirable for DBS because, as discussed above, the manual extraction of these samples is more complex than for traditionally used wet plasma. Ideally, what is required is a technique where DBS samples, once shipped to the site of analysis are loaded directly onto an automated direct analysis device. From this stage no further manual intervention is required. Conventional sample preparation, carried out in the bioanalytical 'wet' laboratory, where extraction is carried out, is bypassed completely (except for the in-house preparation of associated calibration standards and QC's, where required). The direct analysis device extracts the analyte of interest, and is coupled to a suitable separation and detection technique (Figure 1.3). The direct analysis concept is fundamentally simple; however developing a technique in practice also requires a range of additional functionality depending on the application being supported (see section 1.7).

Direct analysis is desirable for any DBS application where a quicker, simpler and possibly cheaper analysis alternative is advantageous. The majority of bioanalytical measurements require the use of complex HPLC-MS/MS methodology, and consequently the use of both specialist sample preparation and instrumentation laboratories, and trained personnel to perform the analysis. DBS have an obvious application in the area of therapeutic drug monitoring and other applications where it is desirable to run MS applications outside traditional laboratory settings, such as doctor's surgeries for example. For in situ chemical analysis applications such as point-of-care diagnostics, the monitoring of drugs in whole blood is critical, as it is in therapeutic drug development, clinical disease treatment and forensic applications. In this situation, cheap and possibly easily portable devices are required that are simple enough for non-MS specialists to use, and which would allow them to obtain immediate results. There are three areas that need to be developed to make this a reality:

- A sampling technique that is simple enough for non-clinicians to perform with accuracy, and produces samples that can be safely shipped, preferably without precautions such as freezing or refrigeration and is free of contamination hazards.
- A method of direct sample extraction and ionization
- MS instrument miniaturization

It has been established that DBS has the potential to be a suitable sampling technique for this purpose. In drug development and discovery environments MS miniaturization will usually not be relevant, as the space taken by equipment will usually not be a critical consideration. However for some *in situ* bioanalysis applications it could be vital. The concept of portable detectors is certainly not a new one and some interesting progress has recently been made in this area demonstrating that the practical application of this process is realistic[71,72].

1.7 What do we need from a DBS direct analysis instrument?

No matter how well an analysis technique fundamentally performs, it is of limited use if it cannot be made to complement the requirements of the potential application. There is much more involved in creating a useable direct analysis technique than just getting analyte ions into gas phase for MS detection. This section details the additional functionality and other factors that must be taken into consideration in making the routine use of DBS direct analysis become a reality. Figure 1.4, which complements each part of this section, demonstrates a theoretical DBS direct analysis work flow for use in regulated drug development high sample throughput bioanalysis. Each of the following sub sections discusses part of this procedure. For most parameters discussed in this section, regulated drug development constitutes the most stringent workflow. For other applications some of these parameters may not be applicable.

A number of terms are used in this section that are not fully introduced until section 1.8. To aid understanding of the following section, brief descriptions are given below:

Direct analysis: used here to describe any analysis technique that eliminates conventional manual extraction steps undertaken in analytical preparation laboratories (encompasses both direct elution and direct desorption techniques).

Direct elution: (aka direct extraction): describes a number of similar techniques where the analyte of interest is extracted from a DBS sample through interaction with a suitable liquid solvent. Although the mechanism of extraction is the same as conventional DBS extraction (liquid extraction), direct elution is differentiated by the use (typically) of a sealed sampling area or confined liquid stream, low extraction solvent volumes, and direct introduction of the extract to the detector.

Direct desorption: For the purpose of this work the term direct desorption describes the host of direct analysis techniques that use a non-liquid elution basis for analyte extraction, and do not produce a liquid extract. This term encompasses techniques that have also been referred to as atmospheric pressure surface sampling/ionisation MS, ambient ionization MS, ambient MS, and ambient desorption ionization MS techniques, among others.

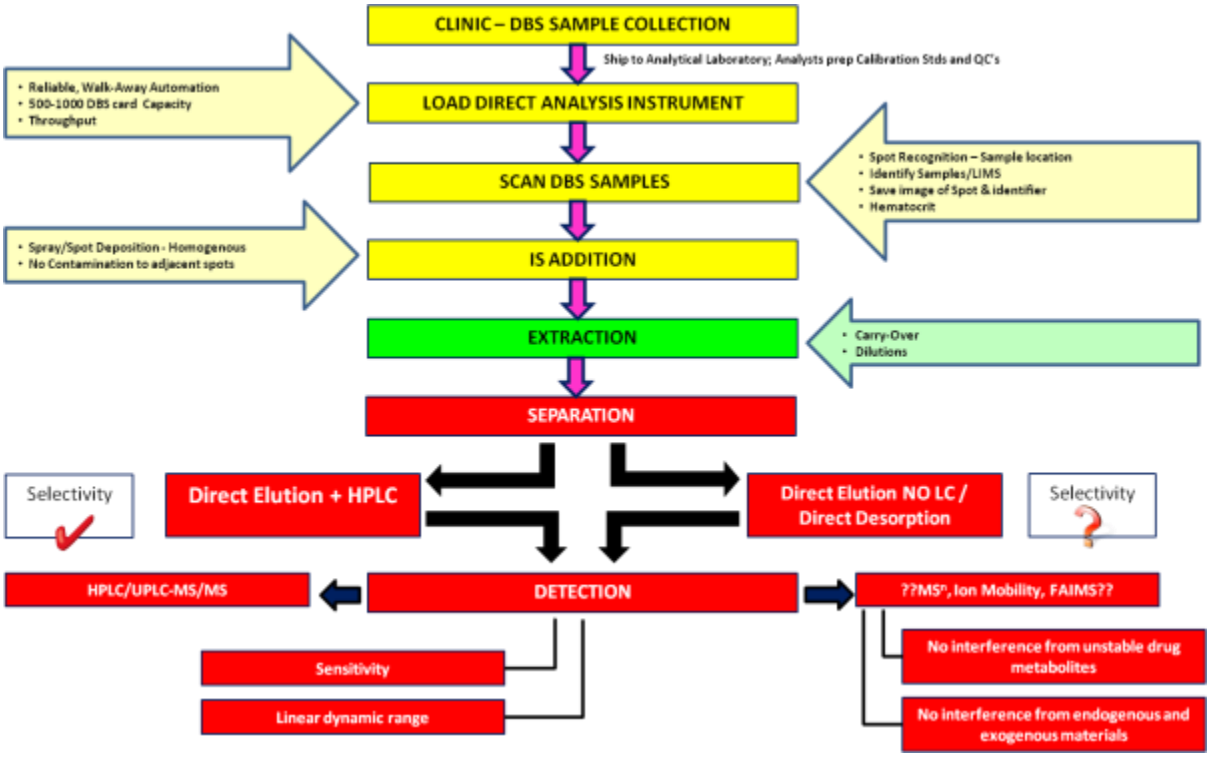


Figure 1.4: DBS direct analysis theoretical workflow.

1.7.1 Automation and throughput

The level of automation required for DBS direct analysis depends on the application. In its simplest form, a direct analysis setup consists of the extraction/ionization device coupled to a MS inlet with no additional functionality. In this arrangement each DBS sample would have to be manually loaded or inserted into the device for analysis. Many direct desorption techniques (section 1.8) can be used in this manner, as can manually operated direct elution techniques (section 1.8 and 1.9) such as the CAMAG TLC-MS interface (Figure 1.5). For low sample throughput requirements, such a simple setup may be entirely adequate. However, for other applications, such as routine drug development, discovery and some screening applications, there may be a requirement to analyse hundreds of DBS samples per day, ideally without the need for any human interaction once the 'run' or 'batch' of multiple samples has been started. In these situations reliable 'walk-away' automation, large sample capacities and run times short enough to suit high throughput applications are essential.

(Figure 1.5a)  (Figure 1.5b) 

***Figure 1.5: Examples of non-automated direct analysis**: (a) Direct analysis in real time (DART) being used to analyse a tablet by manually inserting it between the heated gas stream of the DART probe and the MS inlet (photo from JEOL USA, Inc. Used with permission); (b) CAMAG TLC-MS interface being used to directly elute a DBS sample. The DBS card is manually placed on extraction platform, and the centre targeted using a laser crosshair. A switch is pressed which lowers the extraction head onto the centre of the spot using a pneumatic system. Extraction is then carried out by manually switching the flow of solvent 'through' the DBS sample which then is directed into the HPLC mobile phase flow and into the MS source.*

To offer a realistic alternative to conventional wet plasma sampling, DBS direct analysis needs to offer similar levels of throughput. Direct analysis sample cycle times need to be a close as possible to existing wet plasma analysis timescales, although there is some scope for compromise here due to the efficiency gains brought by removing manual extraction (which will take several hours to perform for a typical batch containing several hundred samples). Sample capacity needs to be able to at least accommodate continuous overnight running (i.e. ~15 hrs with no human interaction) with run times per sample as low as 1.5 min. This equates to a minimum sample capacity of ~600 samples. Typically, cards will be stacked in racks and mounted vertically or horizontally on an instrument deck or platform. Automated sample handling is then required to move DBS sample cards from these racks on the instrument platform to the area of extraction/ionization. For the widely used 85 x 53 mm 4 spot substrate cards currently favoured by clinicians and bioanalysts (Figure 1.2) this will most likely be achieved by some sort of robotic arm that can pick out a sample card from the storage racks, transfer it around the deck so any additional functions can be performed (e.g. visual recognition, IS addition), and then move it into position so the sample can be extracted, before being returned.

Robust automation of this procedure is a challenge, due to the flexible nature of the 4 spot card format, where the paper substrate is sandwiched between two layers of thin card. The level of robustness desired is hard to quantify, but as an analogy, automation errors should not exceed the occurrence of automation errors or 'bad injections' seen in conventional sample injection systems in HPLC instruments, if direct analysis techniques are to be deemed a suitable

alternative. A number of commercially available DBS direct analysis systems (see section 1.9) have struggled to cope with this challenge, and not offered the automation reliability required from anything other than perfectly flat DBS cards (and they are often not perfectly flat, even straight from fresh packaging, before they have been shipped around the world). However, significant progress has been made in automation and the latest instruments utilise highly efficient/robust card handling with suitable error handling and generous card capacity.

Further challenges include making systems compatible with the variety of DBS substrate cards on the market, as they have different thicknesses, and different substrate material strengths (glass fibre based substrates tend to be much easier to punch than cellulose, for example). This latter parameter is an issue for automated punches and direct elution techniques, where a sealed sampling area is created on the DBS sample (section 1.9). A further challenge is to integrate compatibility with formats other than the 4 spot cards that has been widely accepted in drug development, as used in some screening applications. There has also been some demand for a multi-purpose integrated sample rack that combines the following functions:

- Drying, immediately after whole blood has been applied to the substrate card.
- Shipping to the site of analysis.
- Storage at the site of analysis.
- Analysis of samples (i.e. rack could be loaded directly onto direct analysis instrument).

Unfortunately, a solution that is cheap enough to produce (it would need to be sent out to multiple study centres), and compact enough not to be detrimental to shipping costs has yet to emerge. The majority of systems currently rely on the analyst to transfer sample cards to custom system storage racks.

A number of existing automation platforms have been modified with DBS punch and visual recognition modules to enable the use of automated DBS extraction. This approach could have cost advantages over purchasing new and elaborate DBS direct analysis instrumentation (section 1.8).

1.7.2 Visual recognition and sample identification

Many automated direct analysis systems will require a visual recognition system to accurately locate the position of each spot on the sample card. Sample cards contain guide marks for applying blood (Figure 1.2), and experienced bioanalysts apply perfect blood spots within these guidelines with ease. However, clinical personnel who are new to the technique, will often apply blood slightly outside these areas, and very occasionally produce spoiled samples that are unsuitable for analysis (i.e. where the volume applied is obviously much smaller or larger than what it should be, where the blood is smeared or spackled, the surface of the substrate is damaged, or a mistake is made) (Figure 1.6). In addition, there is some variation in the printing of substrate cards which means the position of the guide marks varies between manufacturers, and also from one batch of the same card to another. Hence an integrated visual recognition system is absolutely vital for accurate sampling.

Emerging automated instruments use a camera or scanner for this purpose, and need to be coupled to intelligent software that can identify a number of variations that would otherwise be identified by eye. These include the absence of DBS samples, 'bad samples' (those that fall outside pre-determined size and shape parameters), and whether a sample has already been sampled. Figure 1.6 highlights a number of examples of unacceptable blood spots that would be rejected for analysis. A visual recognition system also provides the means for a number of other potential secondary functions. These include the ability to identify each sample, perhaps through a barcode reader system (using a Radio Frequency (RF) emitter chip is also an option). This facility could be linked to laboratory information management systems (LIMS), ensuring that the analysis and detector sequence lists are reconciled. An image of the DBS (before and after extraction) and identification may wish to be saved for regulatory reasons. Estimating the haematocrit level of a DBS sample has also been attempted by measuring the intensity of colour in the sample via the visual recognition system (see section 1.7.9).

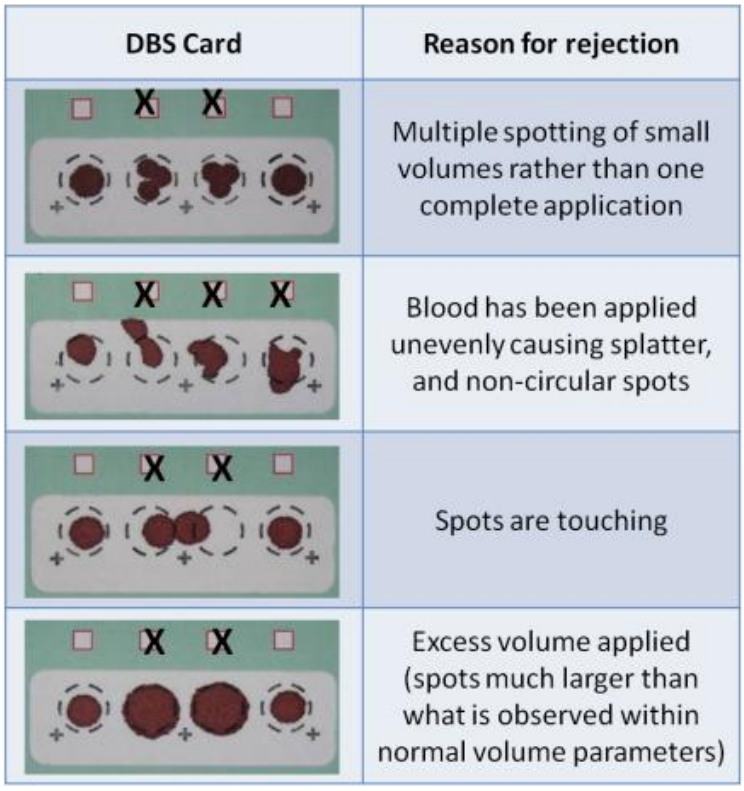


Figure 1.6: Examples of unacceptable DBS samples. 'X' in check box above DBS denotes DBS samples that would typically be deemed to be unacceptable for analysis.

1.7.3 Internal Standard addition

For many quantitative analysis applications, such as drug development, internal standard (IS) is added to correct for variable MS performance (ionization and detection), for losses and variability during preparation, and possibly variability in sample injection volume. The ideal

IS is a stable isotopically labelled (SIL) analogue of the drug of interest. A peak area ratio (the area under the analyte chromatographic peak divided by the IS area) is used for quantitation. The following summarises the options available for DBS IS addition:

1. IS incorporated into extraction/elution solvent

This method is widely used for manual extraction and some direct analysis techniques (e.g. direct elution, paper spray etc), but is obviously not an option for direct desorption techniques where a suitable extraction or elution medium (i.e. a solvent or liquid interaction) is not used. This method of adding IS is simple, widely used in drug development applications, and historically has been accepted by regulatory authorities. However, when applied in this way, the IS is not fully incorporated into the matrix components and sample paper prior to extraction and is therefore not being co-extracted with the analyte as part of the sample. Thus, we are only offered limited information on assay performance by this approach.

2. Substrate pre-treated with IS

Incorporating blank substrate (such as the cellulose or glass fibre cards currently in use) with internal standard prior to applying the wet matrix could ensure the IS is integrated and extracted with the analyte. This is an ideal technique for large scale applications such as certain therapeutic drug monitoring and screening applications, where a large number of samples will be analysed for a small finite number of compounds of interest. For other applications, such as drug development, this approach is unlikely to be logistically feasible when dealing with large numbers of studies, study centres and compounds, if the cost and procedural simplifications on offer from DBSs are to be kept intact.

3. **IS added to matrix before spotting onto paper at clinic**

Adding IS to liquid matrix prior to spotting onto paper substrate should ensure the IS is fully associated with matrix components, along with the compound of interest. This may be possible in practice for a small number of applications. However, in many cases this approach is even less logistically feasible than pre-treating the substrate with IS (option 2, above), as it involves accurate volumes being dispensed in a clinical environment. This would over-complicate the procedure in the clinic and move away from the simple process DBS sampling offers.

4. **IS applied to DBS prior to extraction**

A technique that applies IS to DBS samples prior to manual or direct analysis would improve the integration of IS to the sample compared to currently used manual extraction techniques. However, this is only holds if the IS is given sufficient time prior to extraction for it to bind to matrix and substrate components, and does not adversely affect the distribution of the analyte. Such an IS application would preferably be carried out once samples have been shipped to the site of analysis (e.g. analytical laboratory), but before undertaking the extraction procedure. This procedure is potentially easily compatible with direct analysis techniques and could be easily configured into a fully automated procedure.

In drug development applications the only feasible methods for IS addition are options 1 or 4 above. As detailed above, adding IS at the time of extraction means that IS is not fully integrated into the matrix components and substrate of the sample prior to the extraction process and therefore does not correct for any variability during the extraction process. It should be noted that, despite its limitations, when used in wet plasma analysis this methodology is widely accepted, and routinely used to support drug development studies submitted to regulatory authorities. However, this is not a reason not to try to improve bioanalytical techniques wherever possible, to ensure the best possible quality data is produced. Also as previously mentioned, for many direct desorption techniques no suitable medium to add IS via an extraction solvent exists, and thus applying IS prior to analysis becomes the only (logistically feasible) option.

Clearly, IS performance is an area in which the current manual extraction method can be improved on, and developing new DBS direct analysis techniques provides an opportunity to do this. Alternatives have been investigated with the aim of finding a technique that could better integrate IS with DBS samples prior to extraction, while still offering acceptable reproducibility; maintain the simplicity on offer from DBS sampling; and be compatible with manual extraction or direct analysis techniques. To date, research has focused on spray type IS applications. The ideal technique would involve the homogenous application of IS solution across the width and depth of the DBS to ensure its full interaction with matrix and paper substrate components of the sample, in a manner similar to the analyte. The application of the IS would also not significantly compromise the integrity of the sample in a way which irreproducibly alters the measured concentration of analyte.

After IS addition, the samples would then be left to dry for a suitable period of time before being analysed using a direct analysis. Chapter 3 details the initial work undertaken as part of this project to evaluate the potential of pre-analysis spray based IS application. In practice, it is likely in an automated system that a full batch of DBS samples will have IS applied and be allowed to dry, prior to any sample extraction. A fully automated instrument will then automatically move on to the direct analysis phase. Another consideration of note is that contamination of IS to adjacent samples on a single card, or from one card to another, must be avoided.

1.7.4 Sensitivity

Overall bioanalytical assay sensitivity is a combination of:

• The quantity of sample available.

• The quantity of compound recovered from the sample after extraction.

• The ability to concentrate the extract.

• The sensitivity of the detector.

For many applications, the therapeutic ranges, or compound concentrations likely to be encountered are unlikely to offer challenging levels of sensitivity for the extraction methods

and detectors used. However in drug development, the low levels of quantitation, especially in the analysis of respiratory drugs where low pg/mL levels of detection are often required, push the limits of sensitivity on offer from even the latest, highly sensitive triple quadrupole mass spectrometers when analysing wet plasma extracts. For this reason, DBS analysis when using typical microsampling volumes (using manual extraction) is not currently compatible with respiratory compounds. A typical DBS punched disk may contain around an order of magnitude less drug than in typical wet plasma samples. While the benefits of DBS sampling for this application have allowed the technique to progress despite this disadvantage, there is a desire to maximise the advantages of DBS by making it compatible with as many compounds as possible. Therefore direct analysis techniques must at least match the sensitivity of manual DBS extraction to be a suitable alternative. Any extra sensitivity beyond this would be a considerable bonus. Potentially, increased assay sensitivity could arise from:

• Analysing more sample than the small central sub-punch portion currently taken in manual extraction (e.g. 3 or 4 mm diameter disk punched from a 15 µL DBS).

• Maximising assay recovery (thus producing a greater detector response for the same sample input).

• Concentrating the extract.

• More sensitive detectors.

Mass spectrometry (MS) instrumentation is continuously being refined, and more sensitive instrumentation is launched year on year. Typically, triple quadrupole (QQQ) MS is the most sensitive platform when used in SRM (MRM) mode. However, even the latest QQQ MS do not have the sensitivity required to enable support for many respiratory based compounds in drug development using manual DBS extraction. Other types of MS, including accurate mass and high resolution instruments, and those utilizing other separation techniques (e.g. ion-mobility) may be required for non-HPLC compatible direct analysis techniques (see selectivity, section 1.7.5). Huge improvements in sensitivity have been made in hybrid platforms (e.g. quadrupole-time of flight (QTOF) MS) in recent years and while they still trail the latest triple quads in most cases, the gap is closing (Abu-Rabie, unpublished).

The other routes for improving sensitivity are through sampling as much of the DBS as possible, and maximising recovery. A major limiting factor in manual extraction is the small sub aliquot (3 or 4 mm punch) taken from a DBS sample (typically 15 µL). This equates to only around 2.5 µL of blood, which is why sensitivity suffers compared to plasma analysis. The ability to sample more of or the entire sample (see haematocrit section 1.7.9, for another reason why this is desirable) are obvious routes to improving sensitivity. Another limiting factor of current LC-MS/MS manual extraction techniques is that often only a small portion of the total extract can be injected onto a HPLC column if acceptable chromatography is to be maintained. For example, a 3mm diameter DBS punch may be extracted in 100 µL methanol. Typically, only 2-20 µL (i.e. 2-20% of the compound extracted), of this extract can be injected on column. Trapping column approaches can be used to increase the amount of analyte that

can be introduced to the detector, but this adds to cycle times and creates more complex methodology, which is highly undesirable in many high sample throughput applications.

1.7.5 Selectivity, separation and type of detectors

The selectivity required from DBS analytical techniques largely depends on the application being supported, and consequently how much confidence is required in the identity of the compound being measured. In regulated pharmaceutical drug analysis the upmost confidence in this factor is vital. HPLC coupled to highly sensitive triple quadrupole mass spectrometers is the current separation and detection method of choice in this field and is almost exclusively used. Liquid chromatographic separation is used to separate analytes of interest from co-existing components, and in conjunction with tandem MS monitoring (detecting the parent ion of a molecule, and a specific product fragment ion following passage through a collision cell) provides the selectivity required.

Any extraction technique that produces a liquid extract (e.g. manual/automated DBS extraction, or direct elution (section 1.8)) is potentially compatible with HPLC-MS/MS. Many direct desorption techniques do not produce a liquid extract and/or are not compatible with HPLC-MS/MS. In this case the extraction product is transferred directly to the MS without further separation (unless inherent in the extraction process). This results in a situation where a question mark is raised over the level of selectivity, and thus the level of confidence in analytical data, where direct desorption is used without LC (see Figure 1.4). In practice removing LC can result in poor sensitivity due to ion suppression and reduced selectivity, and risks assay interference via metabolite decomposition (e.g. *N*-oxides and glucuronides) into parent compounds during MS ionization. Potentially, circulating drug concentrations can be overestimated if fragile metabolites or pro-drugs, such as acyl glucuronides and *N*-oxide metabolites and esters convert back to the parent drug. To overcome this issue, further understanding is required into the likelihood of these 'risks' involved in analysis without liquid chromatographic separation.

Eliminating LC would offer highly desirable and significant simplifications to the bioanalytical workflow, but cannot be at the expense of adequate selectivity. It is here that perhaps different types of detectors and separation techniques than those commonly found in drug development bioanalytical laboratories (where QQQ MS is prevalent) could be of assistance. It is possible that high mass accuracy and high resolution MS, together with separation techniques such as ion mobility or high-field asymmetric waveform ion mobility MS (providing that fragmentation does not occur during the ionization process), or perhaps some other novel approach, could fill the selectivity gap vacated by LC and provide unambiguous assignment of the data obtained[73,70].

For applications outside of drug development (such as screening applications), HPLC compatibility, and the regulatory consequences of this, may not be such a major issue. Is it in these areas that direct analysis without LC has less barriers to overcome, so the benefits on offer can hopefully be reaped in a much shorter timescale.

1.7.6 Cross contamination/Carry over

Contamination between samples must be controlled to ensure confidence in analytical results. As a target guideline, in quantitative pharmaceutical drug development, ideally the level of contamination in blank samples following samples containing analyte should be ≤ 20% of the analyte response at the lower limit of quantitation (LLQ). In conventional wet plasma analysis, cross contamination can be avoided by careful sample preparation. During subsequent LC-MS/MS detection, contamination is controlled through the use of integrated wash systems that clean the injection syringe between samples. A similar system is required for DBS direct analysis. Very large levels of carryover is often observed in direct elution (section 1.9) systems, due to extract collecting in sealed sampling areas and the capillaries leading from them to the detection system. It has been demonstrated that suitable wash systems that rinse the sampling and extraction apparatus can reduce carry over levels to well within internationally accepted guideline criteria, and under what is observed in conventional wet plasma analysis[74,75]. The integration of such systems is thus vital if DBS direct elution techniques are used in a regulated development environment.

Carry over in direct desorption systems has not yet been identified as a major issue, and will obviously depend on the specific technique. Understanding of this will increase as direct desorption becomes more widely used in applications where guideline carry over level criteria must be adhered to.

1.7.7 Robustness and reproducibility

If direct analysis techniques are to be viewed as suitable candidates to replace existing manual extraction techniques, they must offer similar levels of robustness and reproducibility. Experience with LC-MS/MS analysis of manually extracted DBS has demonstrated that typically many thousands of samples can be analysed before mass spectrometer performance is affected by build-up of matrix components (at which point the MS interface will require cleaning to remove the build-up of co-extracted matrix components which eventually cause a decrease in sensitivity) (Abu-Rabie, unpublished, and see Chapter 2). Similarly, HPLC columns (and pre-column filters) will typically last many thousands of injections of manual DBS extracts, before build-up of co-extracted components causes performance to decrease below acceptable limits.

One of the potential problems with direct analysis is that conventional clean up steps are bypassed, often resulting in a ‘dirtier’ extract or product being introduced into the MS. Clearly this has the potential to compromise the robustness of the analytical procedure. Even where IS is added to correct for variation, there is a limit to how far the MS response can vary before it adversely effects the sensitivity and linear dynamic range of the assay. Large scale robustness studies are required to identify the significance of this issue in high throughput environments.

For example, direct elution systems lacking trapping columns demonstrably produce a relatively dirty extract. Experimental chapter 1 addresses the robustness of a DBS direct elution technique[75].

Reproducibility can be judged by the ability to meet guideline acceptance criteria (where it exists). In the case of regulated drug development, the acceptance criteria applied to the accuracy and precision of multiple quality control samples is a very direct way of identifying if a technique is suitable for the application and how well it compares to conventional manual DBS extraction[68]. A number of DBS direct elution and direct desorption techniques have been shown to produce assay validation data that meets internationally recognised guideline criteria for regulated drug development[67,74,76]. The acceptance criteria for this application states that the accuracy and precision of QC sample data should be ±15% (n=6) across a range of concentrations covering the range of the assay.

1.7.8 Dilution and linear dynamic range

For many applications, a direct analysis solution also needs to be able to accommodate sample dilution requirements. Typically, in drug development applications, current LC-MS/MS methods supporting DBS manual extraction will have an assay range limited to three orders of magnitude (e.g. 1-1000 ng/mL) in line with the 3-4 orders of magnitude linear dynamic range typically on offer from triple quadrupole MS. If a DBS sample is found to contain a concentration beyond the higher limit of quantitation (HLQ), a repeat analysis will be performed where the DBS extract is diluted by a known factor to bring it within range. This is typically carried out by diluting a DBS extract with matrix matched diluent (blank control DBS extract containing IS)[26,77]. An alternative 'doughnut punch' method has also been reported where a small diameter punch (e.g. 1.1 mm diameter) is taken from both the sample to be diluted and a blank blood sample. The sample punch is added to the extraction tube, and the blank punch is discarded. A second 4 mm diameter punch is then taken from this blank sample, centred over the existing 1.1 mm diameter punch (hence the term 'doughnut punch'). This is then added to the extraction tube containing the 1.1 mm diameter sample punch, resulting in an effective dilution of 11.1 in this example[77].

How can DBS direct analysis techniques accommodate sample dilution? The ideal solution would be to use detectors that have a much larger linear dynamic range (a minimum of 5-6 orders of magnitude) than what we currently use (typically 3-4 orders of magnitude), so that sample dilution would no longer be necessary. Analytical methods could be developed with larger ranges that would encompass the higher concentration samples sometimes encountered in early stage drug development studies. Clearly this is more of a work-around that a solution and unfortunately such detectors are not currently available, and are not likely to be in the short term future. A reasonably simple alternative is available for direct elution whereby the liquid eluate following extraction could be diverted and diluted with matrix matched solvent before being redirected to the HPLC column and/or detector. This could be reasonably easily integrated into an automated direct elution system. It is hoped that as the sampling mechanisms

occurring in DBS direct desorption become better understood, a reproducible way of sub sampling (by a known factor) to bring detector responses within assay ranges can be incorporated into the techniques.

1.7.9 Haematocrit

The relationship between DBS sample haematocrit and assay bias is likely to be one of the most important issues that must be overcome if DBS sampling is to be used to its full potential. A common view is that that this issue needs to be addressed before practical application of DBS analysis (in regulated quantitative bioanalysis) can progress to the next level, and any direct analysis technique needs to be compatible with this solution. This critical issue already has had a major influence on the progression of DBS direct analysis, and therefore the following section summarises the main issues.

It has been demonstrated that haematocrit values that deviate extensively from the 'normal' expected range can significantly affect assay bias when using sub-punch manual extraction of DBS samples for the quantitative bioanalysis of drugs[69,78]. The overall assay bias caused by changes in haematocrit is thought to be made up of three main components: area bias, recovery bias, and ion suppression bias (Figure 1.7)[79]. Analyte recovery appears to vary with haematocrit level, with high haematocrit levels resulting in lower recoveries. There is evidence to suggest that the effect of haematocrit based recovery bias becomes increasingly significant as overall assay analyte recovery decreases, resulting in a wider recovery bias range[80]. Therefore, maximising analyte recovery should reduce the range of the recovery bias as haematocrit changes.

An improved method of IS addition (see section 1.7.3) that integrates IS with the sample prior to extraction, theoretically could nullify HCT based recovery bias (Chapter 5). In conventional manual extraction techniques (where the IS is not fully incorporated into the matrix components and sample paper prior to extraction) recovery bias occurs because the IS is not co-extracted with the analyte. If both the analyte and IS were extracted together, the effect of haematocrit based recovery bias would be nullified. It should be noted that co-spiking does not eliminate the recovery bias, it just nullifies its effect, as both analyte and IS (provided it is a SIL) suffer the same extraction efficiencies.

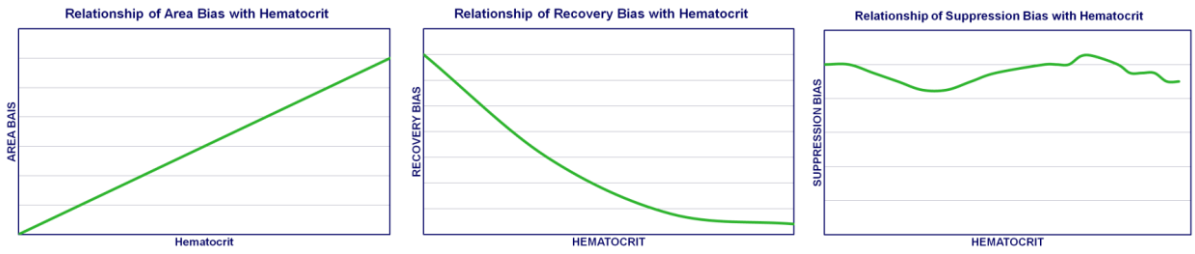


Figure 1.7: Components of haematocrit based assay bias, and how the bias varies with haematocrit level.

Where elevated levels of suppression, or a correlation with haematocrit level is observed, the effects can be minimised by utilizing additional sample clean up to remove interfering species, and/or by modifying chromatography to move the analyte away from areas of suppression.

This leaves the area bias which can be visualized as being influenced by the viscosity of the whole blood sample, which is directly related to its haematocrit. For a fixed volume of blood on cellulose substrate, high haematocrit gives us a relatively small spot area, and low haematocrit a relatively large area, compared to the control HCT level. This means that the analyte density of each spot will be different i.e. a fixed volume of blood will spread over different areas depending on the haematocrit value. To understand this issue consider this exaggerated example: Two patients both have the same concentration of a drug circulating in their bodies. Patient A has a very high haematocrit, patient B has a very low haematocrit. Both patients have ~15 µL of blood taken via finger prick. Both these samples contain the same amount of drug. However patient A's DBS eventually covers 20 mm^2, while patient B's covers 40 mm^2. When a fixed diameter (say, 3 mm) sub punch is taken from both DBS samples, a much larger proportion of the sample is taken from patient A. Thus when the samples are subsequently analysed, (assuming no variability in recovery) a much higher concentration of drug is found in patient A (as the blood spot and hence the drug have spread over a much smaller area) despite the drug concentrations circulating in the body actually being identical. Currently during method validation, the effect of haematocrit on assay bias is assessed over a range of normal haematocrit values for the matrix of interest. This ensures bias is within acceptable limits (typically $<\pm15\%$). The problem is that haematocrit values of study samples may be unknown and certain disease states, or exposure to certain drugs will take a patient's haematocrit level beyond normal ranges. A key factor in how HCT will affect overall assay bias is the extent to which recovery bias and area bias counteract each other[81], and an important consequence of this is that overall assay bias can actually appear to be worsened if only one of the these two factors is eliminated or nullified (see Chapter 5).

An ideal solution for controlling the area bias would be the use of a novel substrate that behaves independently of haematocrit, which would enable the current workflow to remain unchanged. This could be via a novel substrate material, substrate configuration or through the addition of a suitable modifying agent (that perhaps eliminated differences in drying rates, for example) to the current format. Likewise, a modifier could potentially be added to the whole blood sample prior to spotting. A number of novel substrates have been developed that claim to work independently to HCT, but most only aim to control the area bias, rather than the HCT based recovery bias contribution. When launched, Agilent's glass fibre based Bond Elut Dried Matrix Spotting (DMS) cards were informally promised to work independently of HCT. However, our group found HCT based bias was of a similar magnitude to that observed on cellulose based substrate (Denniff et al, GSK, unpublished). Agilent's specification on this product currently reads: 'Spot size, homogeneity and recovery are highly reproducible

independent of haematocrit levels: allowing confidence in assay development', which makes no claim of eliminating HCT based bias[82]. Other substrate developments that claim to reduce the HCT effect have been introduced, but to date have not been backed up with data to demonstrate their effectiveness[83].

An alternative method of eliminating HCT based area bias is to extract the whole-spot rather than a sub-punch. Unfortunately, this workflow would also require an accurate volume to be dispensed at the clinic. Of course, the reason for taking a sub punch from the DBS in the first place is to avoid this! Additionally, it was also previously considered not possible for accurate volumes to be reliably dispensed in the clinic! To work around this problem, devices that could enable accurate volume dispensing of blood to be feasible in the clinic are being explored (see chapter 5). This is no easy task as such a device would need to be cheap, disposable and easy enough to use accurately by non-analysts in a clinical environment.

A number of groups have reported DBS analysis techniques related to the whole spot extraction concept. Li et al reported the use of perforated dried blood spots (PDBS) which is essentially a simplified version of whole spot extraction[84]. This approach partially pre-cuts (or perforates) a small diameter circular perimeter in paper substrate. An accurate amount of blood is deposited that largely fills the substrate area within the perforations and is allowed to dry. The entire DBS can then be easily pushed into an extraction tube using a single use pipette tip. The technique is claimed to offer decreased wastage through complete sample utilization, no requirement for punching, ease of recovery assessments, and elimination of sampling influence due to haematocrit level (though crucially, this last advantage is no different to any other whole spot extraction technique). This technique could potentially be easily compatible with existing automation platforms with only minor modifications. Youhnovski et al published a similar concept named pre-cut dried blood spot (PCDBS) and reported good analytical performance and haematocrit independency[85]. Likewise Fan and Lee reported a similar commercially available approach named dried matrix in paper disc (DMPD)[86].

Another option could be to apply a correction factor that modifies the analytical result based on the haematocrit value. In some workflows, this would rely on an accurate volume of blood being spotted, and the haematocrit value of the samples and calibration standards being known. Such a correction factor could be derived from knowledge of the difference between the haematocrit of standards and samples[87]. Basic proof of concept work has demonstrated that visual recognition systems can measure both spot area and haematocrit level (through sample colour) with good accuracy, but calibration is required, and there are concerns over sample colour changing as the DBS ages (Spooner et al, GSK, unpublished data)[69]. Alternatively, if sufficient sample volume is available, automated analysers are available that can measure haematocrit (from wet blood samples) in isolation (typically using electrical impedance). Similarly, point-of-care testing systems are available that can measure the haemoglobin in blood, which enables the calculation of HCT[88]. Knowing the HCT value of a sample means it can be checked against the accepted HCT range, defined during method validation. A further option is to estimate DBS sample HCT by measuring an endogenous compound (such as potassium) that correlates with HCT[89]. Capiau et al measured potassium concentrations from

DBS extracts, and found this was a good HCT predictor. Further work demonstrated that an algorithm, using the predicted HCT, could be used to correct for the bias in DBS quantitation[90]. Using a correction factor, or measuring the HCT level of samples, is certainly worthy of consideration, as it could allow the current workflow to be maintained, albeit with some additional steps. The downside is the level of complexity this could add to the overall process, and the additional time it could take to prove and implement such a technique in a regulated environment. Also, the additional measurement will have its own errors, so increasing the overall errors of the method.

The use of dried plasma spots (DPS) has been highlighted as having potential to avoid the HCT issue[91]. The downside to this solution is that some of the important advantages to DBS sampling are compromised. For example, a centrifuge is required to produce plasma from whole blood, and more importantly in terms of microsampling, around half the sample taken from the patient, volunteer or animal, is wasted. A solution to the need for centrifugation could come from membrane filtration devices which produce a DPS following addition of whole blood[65,64]. It has also been demonstrated that natural variance in albumin concentrations can vary the diameter of plasma spots in much the same way to what is observed with blood and HCT (Spooner et al, GSK, unpublished).

The haematocrit issue certainly complicates the ultra-simple workflow on offer from DBS sampling, but the introduction of an elegant sampling device in the clinic should enable the benefits of DBS to be reaped without a significant compromise. What is the impact on direct analysis? For automated DBS analysis and direct elution the same techniques could be used with minor modifications (such as larger punches or sealed sampling areas to fully encompass the entire DBS, rather than a sub section). It was thought that a potential problem with whole spot direct elution could be that the extraction solvent would take the path of least resistance through the surrounding blank substrate rather than interact with the DBS. However it has already been shown that this is not the case and it has been demonstrated that direct elution is compatible with whole spot elution without loss of sensitivity or chromatographic performance[92]. Another advantage of whole spot extraction/elution is that potentially, sample volumes could be further reduced without assay sensitivity, as the same amount of blood could be sampled in total as is currently sampled in the sub punch (typically 3-4 mm diameter), eliminating the wastage that is currently encountered with manual extraction. Alternatively, current typical DBS volumes could be retained and many of the sensitivity issues with DBS would be eliminated.

It is not currently clear how haematocrit level effects direct desorption techniques, but it can be assumed that the area bias will be relevant in any analysis technique that samples a localised area rather than the whole sample. Further investigation is required into mechanisms of direct desorption sampling and how haematocrit variation will bias analytical results.

For many applications the haematocrit issue will not be relevant or significant, especially in situations where there is confidence that haematocrit levels are within ‘normal’ levels. It has been shown that in the vast majority of cases significant assay variation is only observed in

extreme haematocrit levels only observed in critically ill patients[69]. In this situation, where HCT values are all skewed in one direction, another alternative is to change the HCT of the calibration and quality control samples to match the study samples.

1.7.10 Advances in DBS substrate material

The last few years have seen numerous innovations in DBS substrate material and card format from various manufacturers such Agilent, Ahlstrom and Whatman. Advancements to the substrate material and card format could assist many of the functions and considerations discussed in this section, and influence the progression of DBS direct analysis. For example the use of more robust, rigid cards, which would sandwich the substrate in a tough plastic material (or similar) rather than cardboard, would have numerous advantages. Firstly, the cards would be more robust, and offer more physical resistance to tearing and deformation during shipping and storage. Some form of flip-over or sliding cover could also offer further physical protection and some additional confidence against contamination during drying. Secondly, a rigid card would greatly assist ease of automation. The currently used cards bend and twist easily. The less variability there is in the size and shape of the sample format, the easier it is to reliably automate. A number of prototype plastic card variants have been produced by various card and substrate manufacturers, and it is possible that these superior performing cards may one day replace the current cardboard versions. However, currently the additional cost of these new formats and the strong take up of the existing format is inhibiting the development of these alternatives. Additionally, it has been proven that robust, reliable automation of the cardboard format cards is possible, and a new format could mean existing automation has to be modified. It would also be desirable not to significantly increase the depth of the cards so the ease of shipping and storage advantages can be maintained.

As previously discussed, minor modifications to the existing format have also been made in an attempt to assist with efficient sampling and the haematocrit issue such as perforated dried blood spots (PDBS), pre-cut dried blood spots (PCDBS) and dried matrix in paper disc (PMPD) (section 1.7.9).

As discussed in section 1.7.9 the most important substrate development would be the introduction of a substrate that behaves independently of haematocrit. It is hoped that this can be achieved using the same paper type substrate currently used. For some applications the haematocrit issue could be a deal breaker for the future of DBS usage. If a paper type substrate that behaves independently of haematocrit, or a suitable method of applying accurate volumes of blood in the clinic (prior to whole spot extraction) does not emerge, other microsampling variants may emerge to take the place of the current format. The most obvious idealised approach would be to use a substrate that can only physically absorb a fixed volume of blood, regardless of haematocrit, thus eliminating the issue of variable blood volumes being applied in the clinic and the effect of haematocrit on assay bias. If this could be achieved without resorting to anything that would compromise the cost advantages and simplicity of DBS it would be a very neat solution! It is likely that such a substrate, unlike the current flat paper

format, will be pre-cut or moulded, or will incorporate some kind of physical or chemical barriers to limit the volume of blood that can be absorbed. Such a technique could also potentially significantly reduce wastage of both sample and substrate. To continue the goal of a simplified workflow, direct analysis instrumentation would need to modified and optimised to suit this new substrate, chiefly in the areas of automation and sample handling.

Another substrate innovation is the introduction of 'indicating' cards that enable colourless fluids (such as plasma, urine or cerebrospinal fluid) to be easily viewed (on 'normal' substrate these fluids can be almost invisible). These substrates contain a dye that is displaced by the additional of a sample, leaving a lighter-coloured area for easy identification[41]. For full flexibility/versatility direct analysis visual recognition systems should ideally be compatible with this type of substrate.

While the 85 x 53 mm 4 spot substrate card is particularly suitable for drug development applications, it is probable that alternative formats will be optimal for other applications. Ideally, for maximum flexibility, a direct analysis instrument should be compatible with these variants. Unfortunately this seems unlikely given the difficultly in automating the current cards. A more feasible solution is likely to be a modular instrument approach where different attachments can be fitted according to the format being used.

1.7.11 Communication, compatibility with other systems and error handling

In this proposed workflow, the automated direct analysis system takes the place of an autosampler in a conventional HPLC-MS/MS system. Thus it must be able to communicate and sync with the detector and HPLC system in the same way. It is also desirable for the visual recognition system to reconcile samples against a sample sequence list that may be submitted directly from a LIMS system and in turn reconcile this against the detector sequence. Intelligent error handling is essential in any automated high throughput 'walk away' system, and becomes increasingly important in regulated environments where sample tracking is required. It is vital that any automation, visual recognition or communication errors that occur are either immediately corrected, or if a sample cannot be analysed, this is reported at the end of the run.

1.7.12 Instrument specification document with potential instrument manufacturers

During the early stages of this project, in order to engage instrument manufacturers and other DBS users, GSK initiated the creation of a consortium of pharmaceutical companies and contract research organisations that were early adopters to using DBS (for pharmaceutical support). One aim of this consortium was to produce a document that could be shared with instrument manufacturers that detailed the requirements and specification of an ideal generic DBS direct analysis instrument capable of supporting high sample throughput quantitative bioanalysis. This document is reproduced in full in Appendix A.

1.7.13 Analytical acceptance criteria in regulated quantitative bioanalysis

Quantitative bioanalytical methods used to support drug development studies are validated against internationally recognised guideline acceptance criteria, to assess their suitability and reliability to produce good quality data[68]. Since application to pharmaceutical drug development is the main focus of this project, it follows that these analytical criteria, and statistics associated with this criteria, were used in this project to test the performance of new instruments and techniques. For DBS methods, the following criteria will typically need to be adhered to:

- A suitable validation procedure consists of a minimum of three separate and acceptable consecutive validation runs consisting of calibration standards and validation samples.
- The concentration of IS used should give an appropriate instrument response. Typically this is equivalent to the response observed at 30-70% of the assay HLQ. Any observed analyte response caused by the IS should be no greater than 20% of the response at the lower limit of quantitation (LLQ).
- A minimum of six calibration standards (plus a blank sample containing no analyte) should be prepared, with three standards within each order of magnitude of the concentration range. The lowest concentration standard must be at the lower limit of quantitation LLQ of the method.
- For calibration of a given analyte, regression analysis of the instrument response (using analyte: IS peak area ratio) versus the actual concentration of the calibration standard is used.
- A calibration standard must be omitted from the regression if the back-calculated concentration deviates from actual by more than 15% (20% at the LLQ). No more than 25% of the calibration standards can be rejected from the calibration line, and at least six calibration standards at different concentrations must be included.
- The LLQ is defined as the lowest concentration samples that produces a response at least five times the response observed in total blank (matrix with no analyte or IS added) and blank (matrix with no analyte added, which include the IS) standards.
- Replicate analysis of validation samples (aka quality control samples) are used to determine the bias and precision of methods at five concentration levels ((1) the LLQ; (2) three times the LLQ; (3) at the approximate geometric mean of the HLQ and LLQ); (4)75-85% of the HLQ; and, at (5) 100% of the calibration range (HLQ)). Six replicates at each concentration are tested in a minimum of 3 separate runs.
- Acceptance criteria for validation samples:

1. For each run, within run precision (CV) at all concentrations should be ≤15% (≤20% at the LLQ).

2. Between-run precision (CV) at all concentrations should be ≤15% (≤20% at the LLQ).

3. Within run bias (%) at all concentrations should be within ≤15% (±≤20% at the LLQ).

- Selectivity should be demonstrated by analysing a minimum of six independent sources of drug free blood. Any observed interference should be no greater than 20% of the analyte response at the LLQ.
- The extent of recovery of an analyte at each concentration level (2, 3 and 4) should be precise (within 15%).
- The extent of matrix effects between separate sources of matrix should be precise (within 15%) at each concentration level (2, 3 and 4).
- Impact of HCT on resulting measured quantitative concentrations should be assessed by preparing test validation samples (in replicates of 6) at two concentrations (levels 2 and 4) at HCT levels bracketing the anticipated HCT range (for normal subjects, this typically corresponds to 25% and 65%)[93,69,21]. These samples are analysed using a calibration curve and QC samples with a HCT level of 45%. Accuracy of HCT test samples should be within 15% of that for the QC samples at the standard HCT level.

The guideline analytical criteria and statistics listed above form the basis for the testing criteria used throughout this project. This creates a very clear (and widely accepted) set of performance targets for the testing of new techniques and instrument in this project. They are only suitable for use in regulated quantitative bioanalysis of pharmaceutical drugs if they can meet this set of acceptance criteria.

1.8 What are the options for DBS direct analysis?

A number of emerging options exist that can potentially aid the efficiency of DBS analysis. These can be summarised into the three categories: automated DBS analysis, direct elution and direct desorption.

Automated DBS Analysis

Automated direct elution instruments replicate the currently used DBS manual extraction technique described above, but automate one of more of the manually performed steps to relieve some of the manual burden of DBS analysis.

Direct Elution

DBS direct elution describes a number of similar techniques where the analyte of interest is extracted from a DBS sample through interaction with a suitable liquid solvent. Although the mechanism of extraction is the same as conventional DBS extraction (liquid extraction), direct elution is differentiated by the use (typically) of a sealed sampling area or confined liquid stream, low extraction solvent volumes, and direct introduction of the extract to the detector.

Direct Desorption

For the purpose of this work the term direct desorption describes the host of direct analysis techniques that use a non-liquid elution basis for analyte extraction, and do not produce a liquid

extract. This term encompasses techniques that have also been referred to as atmospheric pressure surface sampling/ionisation MS, ambient ionization MS, ambient MS, and ambient desorption ionization MS techniques, among others.

The initial work undertaken in this project involved performing a literature review on the above techniques, and testing as many of them in practice as possible. Ultimately, it was decided that within the timeframe of this study, direct elution would be the most suitable technique to develop (see conclusions, section 1.10). For this reason, only the background research on DBS direct elution is included in this document. The research and practical experiences on automated DBS analysis and direct desorption have been published separately[70].

1.9 History of DBS Direct Elution

DBS direct elution techniques can broadly be categorized as follows: on-line DBS; sealing surface sampling probe (SSSP); liquid-microjunction surface-sampling probe (LMJ-SSP), and a simplified LMJ-SSP variant, namely liquid extraction surface analysis (LESA); and, DBS digital microfluidics (DMF). One of the attractions of direct elution is that a liquid eluate is produced which can be separated and detected using LC-MS/MS – a technique that is readily available in bioanalytical laboratories and is familiar to, and accepted by, bioanalysts and where applicable, regulatory authorities. For some applications it therefore currently possesses a higher level of accessibility than direct desorption techniques that cannot be coupled to HPLC, or alternative separation techniques. Over the last few years commercially available and prototype technology has emerged from CAMAG (TLC-MS)[94], Spark Holland[95], Prolab (SCAP)[96], and Advion (LESA and LMJ-SSP utilizing the Triversa Nanomate)[97] and others that encompass a variety of approaches and varying levels of automation and additional functionality. This chapter attempts to summarise the various technology that has emerged so far.

1.9.1 On-line DBS

In 2009 Déglon et al introduced a method of on-line DBS extraction into a LC-MS system without sample pre-treatment. The procedure involved manually punching a disk from the centre of the DBS on a filter paper sample (as performed for manual extraction) and placing the punch in a desorption (or inox) cell that was compatible with LC capillaries[98] (Figure 1.8). HPLC users will recognise this device as being rather like an in-line HPLC pre-column filter cartridge, though the DBS punch is placed in the cell rather than a frit. An extraction solvent of choice is flowed thought the cell using a LC pump which extracts (or desorbs) the compound of interest from the DBS and allows it to flow to a HPLC column for separation and then the MS for detection. In this arrangement, a trapping column set up was used to ensure the purification and separation of compounds before detection (Figure 1.9). Pump 1 used a high organic concentration solvent to desorb and elute the analytes from the DBS samples and transfer them towards the trapping column. Pump 2 used a high aqueous concentration solvent to adjust the chemical properties of the desorption mobile phase.

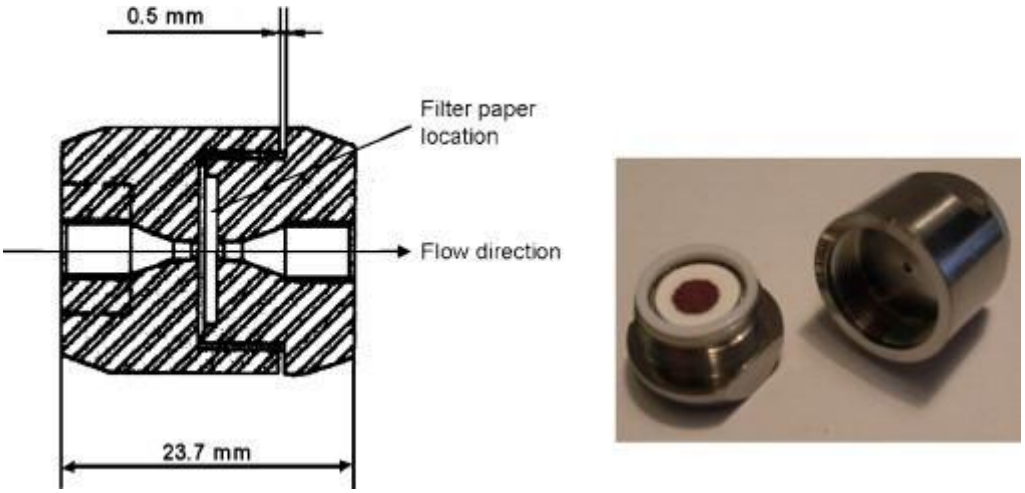


Figure 1.8: Desorption (Inox) cell used in Déglon et al's original on line DBS direct elution system.[98]

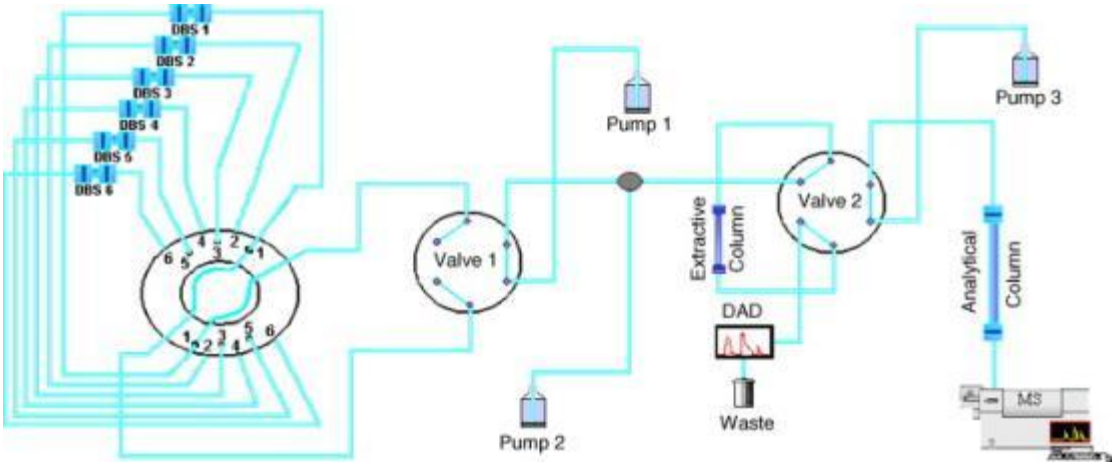


Figure 1.9: Global view of the on-line DBS procedure coupled to a column switching LC/MS system. [98]

Déglon et al reported that while acetonitrile was optimum for solvent extraction, it did not effectively trap analytes in a reverse phase trapping column. Thus, pump 2 was used to add water to reduce the organic content (to around 10% organic) to enable effective trapping. The extraction time was 5.5 min (which at a flow rate of 0.25 ml/min gave complete desorption), after which the trapping and analytical columns were connected. Trapped analytes were then transferred to the analytical column for 2 min, while the pre-column was regenerated, and finally a generic gradient was used by pump 3 to deliver the analytical mobile phase with a constant flow rate. Total run time was (a non-high throughput friendly) 18 min. There are advantages and disadvantages of using a trapping column coupled to an analytical column, over an analytical column only approach. One potential advantage is that more analyte may

be stored in a trapping column and subsequently transferred to the analytical column than may be possible with an analytical column only system. Thus sensitivity may be improved. Disadvantages include higher costs, complexity and substantially increased run times.

In Déglon et al's initial work it was demonstrated that the extraction step and analysis step could be integrated into a relatively simple process without sample pre-treatment. Using three small molecule compounds, this initial work demonstrated that this technique could offer reasonably good sensitivity (within therapeutic range of the drugs tested) and sufficient accuracy and precision to be suitable for regulated drug development DBS applications. A comparison of on-line DBS versus a validated LC-MS/MS procedure on patient samples was also undertaken and showed a close correlation. Interestingly, carry over observed was quoted as being (just) adequate for regulated development applications (carry over in a blank solution following an injection of double the HLQ was lower than 0.01%) despite the lack of a dedicated wash system.

In its initial form, on-line DBS extraction is of limited use in a high throughput environment as not only do the DBS samples still need to be punched by hand, the punches must then be manually inserted into the inox cells between extractions (using a 12 port valve it was shown that up to 6 DBS samples could be extracted per run). Additionally, very long run times were used. Clearly this approach would benefit from automation, and the concept has been subsequently developed into commercially available and prototype on-line DBS instrumentation by various groups. These include the DBS-MS/MS (and DBS SPE-MS/MS) from Spark Holland, the commercially available SCAP DBS from Prolab Instruments, and prototype instrumentation reported by Miller et al[(99)] and Déglon et al[(100)]. These devices all work around the same principle. Instead of having to punch the DBS samples and fit the disk into an on-line cartridge for analysis, the samples are instead left intact and are clamped from either side (Figure 1.10) using automation. These clamps are essentially two halves of the desorption cell and house integrated capillaries that allow the extraction solvent to flow through the DBS sample and onto the HPLC column and detector.

The Prolab SCAP (sample card and prep) system is based around existing CTC Pal automation technology, and has been designed to automate the 85 x 53 mm 4 spot substrate cards (Figure 1.11). This system is intended to work around a trapping column arrangement but can also be programmed to work with an analytical column only for shorter run times. The first version of this system had a small capacity (28 cards) and lacked both a wash system (other than rinsing the clamps) and a visual recognition system (relying on consistent printing alignment on the cards and accurate spotting). The latest versions have additional functionality, adding a camera for accurate spot location; larger trays (160 cards) for increased card capacity, more sophisticated error handling, and a new wash procedure that the manufacturer claims to significantly decrease carry over. The only provision for adding IS is via the extraction solvent, but its favour, the SCAP system has a cost advantage over some of the commercially available alternatives. My own experience with the first version of this device was that the automation on offer worked well with perfectly straight DBS cards but struggled with bent or over/undersized cards (Abu-Rabie et al, GSK, unpublished). The simplistic error handling

could not to cope with such errors, which were catastrophic to the sample runs. However, there are a number of reports in the literature where the SCAP system has been evaluated and the same problems have not been highlighted, suggesting that it can be used reliably, perhaps as a result of the modifications made to later versions. Heinig et al, and Ganz et al have both reported reliable automation performance and the ability to generate quantitative validation data within guideline acceptance criteria[101,102].

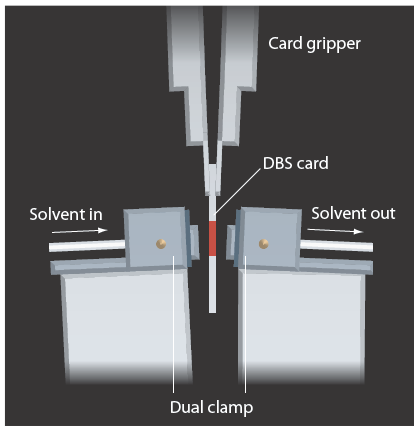


Figure 1.10: Automated on-line DBS desorption (Prolab SCAP schematic). The Spark Holland DBS SPE-MS/MS and automated prototypes from Déglon et al use the same concept.[101]

Figure 1.11: PROLAB SCAP (Sample card and prep) system, commercially available on-line DBS instrument. Photo from Prolab, Inc. Used with permission).

The DBS SPE-MS/MS from Spark Holland has been in development for a number of years and was first formally reported on in the literature in 2011[103]. The concept is the same as that used in the SCAP system, the DBS sample is clamped from both sides and solvent is allowed to flow through to desorb the analyte. Similar levels of assay sensitivity, accuracy and precision have been demonstrated using this prototype device. Recent developments with this system have focused on automation using either a CTC platform or a more elaborate robotic arm[95]. Déglon et al developed a similar automated clamp, on-line DBS prototype based on their on-line DBS concept[100] (Figure 1.12). The design is based on a rotating plate with multiple wells where the DBS punches are manually placed. While this system is likely to be perfect for some applications, the necessity to manually punch discs from DBS samples and transfer them to the device limits its use in high throughput applications.

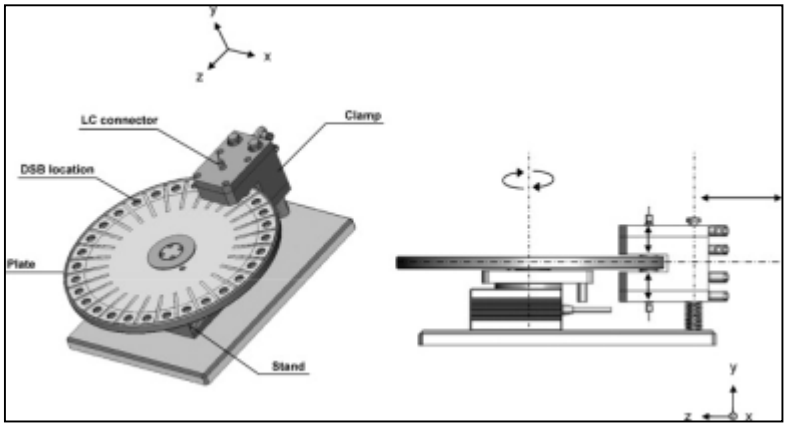


Figure 1.12: Automated on-line DBS from Déglon et al. [100]

1.9.2 Liquid microjunction-surface sampling probe (LMJ-SSP) and Liquid Extraction Surface Analysis (LESA)

The liquid microjunction-surface sampling probe (LMJ-SSP) approach uses an unconfined liquid microjunction in contact with a sample surface to extract analytes of interest. A probe is positioned an appropriate distance away from the surface and is configured so that an extraction liquid is both brought to the surface and is then carried on to the ionization source through a probe acting as a liquid conduit (Figure 1.13). This is achieved by reducing the self-aspiration flow rate of the probe to less than the flow rate volume pumped into the probe. The self-aspiration rate of the probe is then increased (controlled by altering the nebulising gas flow rate), allowing desorbed analytes on the sample surface to be aspirated back into the probe with the liquid that created the liquid microjunction and transferred to the MS. The technique has been reported as being fast enough to be compatible with high throughput sample analysis[104].

Unfortunately this wall-less microjunction and dynamic flow approach is not suited to sampling porous surfaces, such as DBS on paper substrate, where the liquid microjunction cannot be maintained. However it was recognised that the technique could be used without the

continuous flow provided by the LMJ-SSP, potentially being compatible with any probe device capable of both dispensing and retrieving a solvent from the sample surface. Thus, in an attempt to overcome the LMJ-SSP issue with porous DBS samples, the technique has been coupled to the Advion Triversa Nanomate chip-based infusion nanoESI system, which is also capable of automating the process (Figure 1.14a). The Nanomate system comprises a pipette based liquid handling robotic system coupled to chip-based electrospray ionization technology. For DBS analysis the Nanomate is used in LESA (liquid extraction surface analysis) mode and utilises a static microjunction. Automated liquid handling is enabled by the use of a robotic arm that positions pipette tips around the Nanomate instrument deck.

In LESA mode a pipette tip picks up a volume of extraction solvent, moves above a DBS sample, and is lowered to an appropriate distance above the sample. A portion of extraction solvent is then dispensed onto the sample surface forming a static liquid microjunction, which is the mechanism of analyte desorption. The solution containing the extracted analyte is then aspirated back into the tip and transferred to a nanospray nozzle for MS analysis (Figure 1.14b). Kertesz et al demonstrated performance of the technique using DBS samples containing sitamaquine (prepared by Abu-Rabie et al, as a suitable small molecule on DBS test sample) using 4 mm diameter punches mounted onto plates using double sided tape[105].

Acceptable accuracy and precision was reported down to 100ng/mL (liquid chromatographic separation was not used) using small volumes of highly organic extraction solvent (2 µL of MeCN / MeOH / H2O / formic acid (58/34/8/0.1 v/v/v/v)). Advantages of this approach include quick run times, the incorporation of an automation platform, and no carry over issues as pipette tips and nanospray nozzles are disposed of between samples. Disadvantages include relatively poor sensitivity compared to SSSP (see section 1.9.3), and the requirement to manually punch disks from DBS samples. Fixed area punched disks must be used as otherwise the extraction solvent will not form the microjunction and simply wicks out to the surrounding areas on the porous substrate. However the formation of the microjunction, even in this static incarnation on a punched disk, is tricky to perform reliably. A potential solution, reported by Henion reported the development of DBS substrate containing a non-porous silicon ring around the DBS area which would both aid the formation of the microjunction and enable analysis without the need to pre-punch the sample[106].

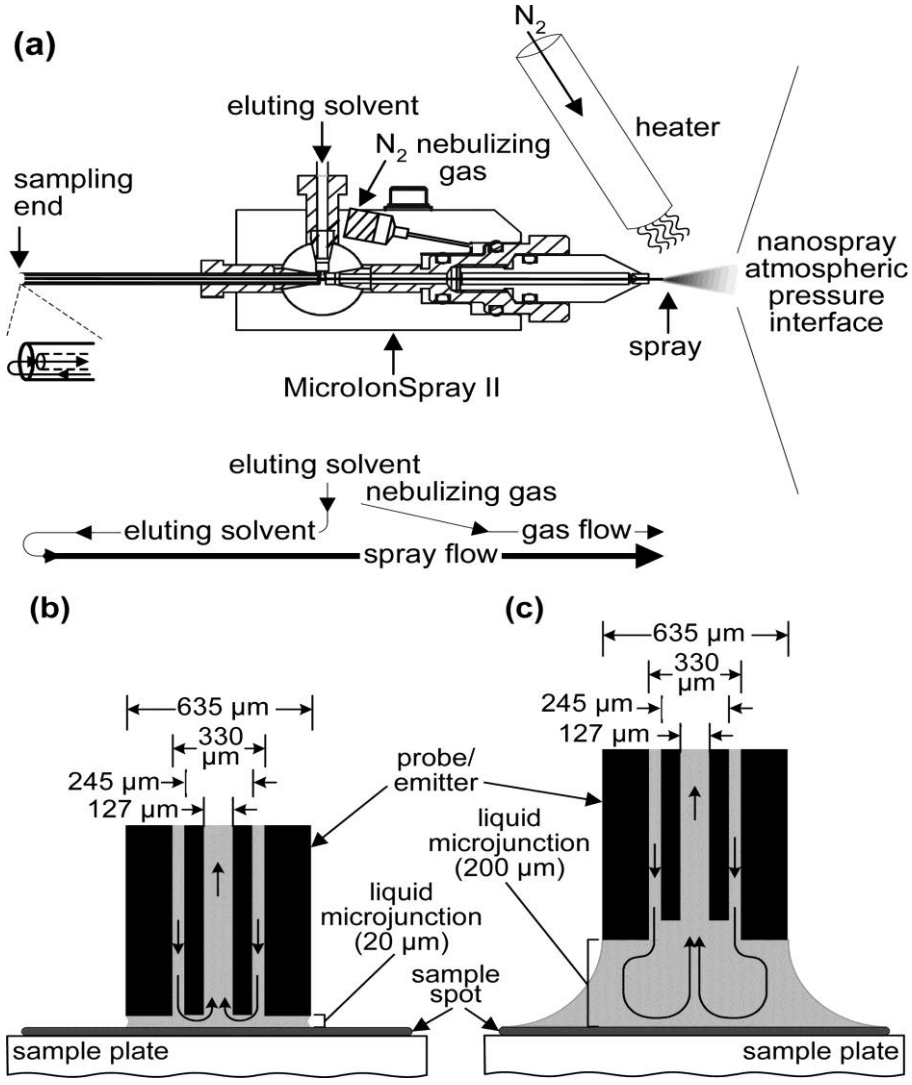


***Figure 1.13: LMJ-SSP**: (a) Schematic illustration of the liquid microjunction surface sampling probe/ESI-MS experimental setup with details of the surface sampling probe/emitter. (b) Schematic illustration showing the close probe-to-surface spacing and narrow liquid microjunction used for spot sampling. (c) Schematic showing larger probe-to-surface spacing for spot sampling and the resulting liquid microjunction developed in an updated system. Note that the probe-to-surface liquid microjunction was actually formed in the horizontal position and is only shown in parts b and c in the vertical position for ease of viewing.* [(104)]

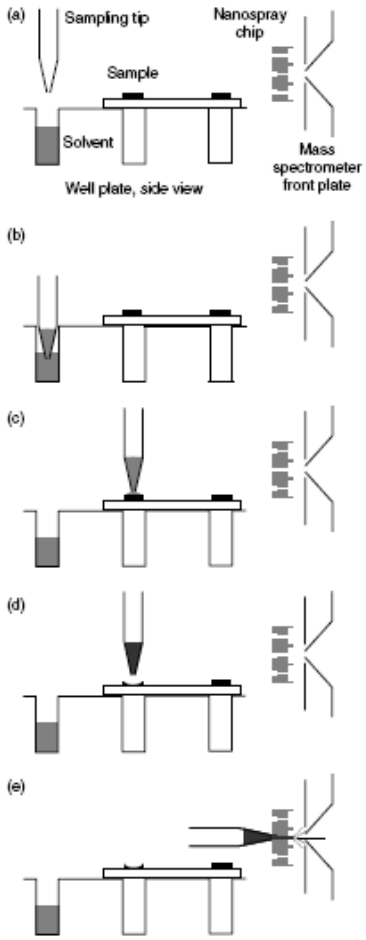


Figure 1.14a: Advion Triversa Nanomate chip-based infusion nanoESI system (left). Photo from Advion, used with permission.

Figure 1.14b: For DBS analysis the Nanomate is used in LESA (liquid extraction surface analysis) mode and utilises a static microjunction (right). Schematic shows the individual steps of the surface sampling process ***(105)***

1.9.3 Sealing Surface Sampling Probe (SSSP)

The Sealing Surface Sampling Probe (SSSP) concept is based on a device originally reported by Luftmann in 2004 which coupled thin layer chromatography (TLC) to MS detection[107]. This device was later commercialized by CAMAG into the TLC-MS interface, a simple and relatively cheap, pneumatically driven device that is operated manually and used to directly elute samples from TLC plates and transfer the extract to mass spectrometer interfaces. A number of groups have recognised the potential of extending the use of this device to directly elute DBS samples (Figure 1.15a). In a simple arrangement the TLC-MS can be positioned between the HPLC pump and the HPLC column and MS, essentially replacing the autosampler in a typical LC-MS set up (Figure 1.15b). Prior to extraction, mobile phase from the HPLC pump bypasses the TLC-MS and flows directly to the HPLC column (if used) and MS. DBS

extraction is carried out by lowering the plunger (or extraction head) onto the centre of a DBS sample forming (typically) a 4 mm diameter sealed sampling area. The exact site of extraction is located using a Laser crosshair (Figure 1.15a). Solvent is then allowed to flow down the inlet capillary of the plunger, filling the sealed sampling area on the DBS. The continuous flow of the mobile phase forces the extract to flow up the outlet capillary and towards the HPLC column and MS. It is this action of solvent flow through the DBS that transfers the compound of interest from the DBS sample into the solvent flow.

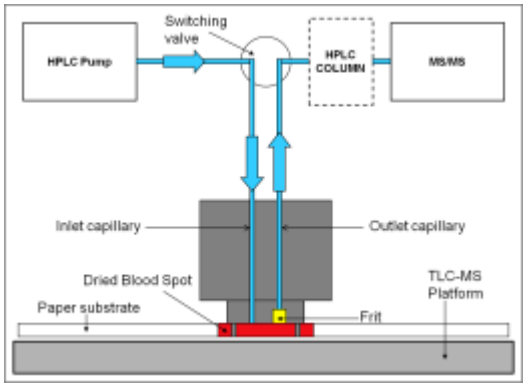
A)  B) 

Figure 1.15a: CAMAG TLC-MS instrument which utilizes the SSSP concept. Photo shows the instrument being used to directly elute a DBS sample. Figure 1.15b: Schematic diagram of dried blood spot direct elution assembly using the CAMAG TLC-MS interface. Reprinted with permission. [74]

In 2009 Abu-Rabie and Spooner, and Van Berkel and Kertesz, independently demonstrated that the TLC-MS interface was suitable for DBS analysis[74,108]. The work performed by Abu-Rabie and Spooner demonstrated that direct elution of DBS samples using the TLC-MS compared favourably with existing DBS manual extraction methods used for quantitative bioanalysis. It was clear from the initial evaluation that this direct elution technique warranted further investigation as not only was chromatographic performance maintained without method re-optimization, but assay sensitivity was considerably increased. Over a range of representative small molecule test compounds an average 10-fold increase in assay sensitivity was observed. This really sparked the interest in this technique as it appeared to offer multiple advantages; direct analysis with no sample pre-treatment, compatibility with existing LC conditions, and significant increases in assay sensitivity[74]. This last advantage is of course particularly of interest due to the sensitivity challenges faced when using DBS manual extraction.

In Abu-Rabie and Spooner's initial evaluation of this technique, DBS bioanalytical method validations using the TLC-MS were performed using test compounds sitamaquine and paracetamol (aka acetaminophen). Linearity, accuracy and precision data well within guideline acceptance criteria was generated. Carry over was found to be large enough to significantly bias subsequent samples. In order to generate acceptable data, carry over was controlled using a time consuming procedure that involved back flushing the outlet capillary column using a secondary pump. A rudimentary recovery evaluation was also carried out that suggested that sensitivity could potentially be further increased with optimised extraction and chromatographic conditions. DBS direct elution was also reported using this arrangement where the HPLC column was removed, and the elute was transferred directly to the MS. It was found that using the same mobile phase conditions excellent accuracy and precision was achieved, but sensitivity was poor. Switching the extraction solvent to 70:30 (v/v) methanol water retained the accuracy and precision performance and greatly increased sensitivity, to the extent that it was comparable to what was achieved with chromatographic separation. Ultimately, the findings of this work proved to be key in determining which direction to follow in selecting a direct analysis technique to develop to meet the goals of this project (see section 1.9)[70]. The results can be summarised as follows:

- DBS sample extraction is extremely quick and easy to perform (typically well under 60 s per sample, compared to several hours for manual extraction)
- Assay sensitivity increased on average by an order of magnitude compared to using conventional manual extraction
- DBS direct elution coupled to HPLC-MS/MS produced linearity, and accuracy and precision data well within globally accepted guideline acceptance criteria.
- Recovery (of the entire sampling area, using extraction times of ~5 s) appeared to be low, suggesting that extraction optimisation could further improve assay sensitivity
- Existing HPLC-MS/MS assay methods and equipment could be utilised
- The direct elution assay technique is similar enough to widely used and accepted bioanalytical techniques, that it is unlikely that it would face a major regulatory barrier.
- Some preliminary work without HPLC (MS/MS only) demonstrated that, once optimised, similar sensitivity could be achieved as when using HPLC-MS/MS.

Around the same time Van Berkel et al also reported good accuracy and precision using the TLC-MS for the quantitation of sitamaquine and acetaminophen in DBS samples. In this study chromatographic separation was not used, and lower flow rates (0.2 mL/min) and longer extraction times (60 s) were utilized, with methanol (for sitamaquine) or methanol:formic acid 100/0.1 (v/v) (for acetaminophen) as the extraction solvent. A 60 second extraction of a blank sample (e.g. blank paper substrate) was used to wash the extraction head which reduced carry over to acceptable levels.

Following these initial reports, a number of other groups have published accounts of using the TLC-MS for direct elution of DBS samples in bioanalytical quantitation. For example, Heinig

et al integrated the TLC-MS into a column switching LC-MS/MS system that incorporated online SPE (a trapping column) for additional analyte collection and clean up[109]. Excellent sensitivity, linearity, accuracy and precision data were reported. An extraction time of 45 s onto a trapping column was used before the trapping column (TC) and analytical column (AC) were connected.

1.9.4 Digital Microfluidics (DMF)

In 2011 Jebrail et al reported proof of concept for a fast and efficient digital microfluidic (DMF) method for dried blood spot analysis[110]. DMF is a fluid handling technique where discrete droplets of samples and regents are manipulated on an open surface by applying a series of electrical potentials to an array of electrodes. Droplet actuation is driven by electromechanical forces generated on free charges in the droplet meniscus (for conductive liquids) or on dipoles inside of the droplet (for dielectric liquids). Jebrail et al demonstrated that sample analysis could be undertaken by spotting blood directly onto a 'chip' device and allowing it to dry, or by positioning a punched disk from a DBS (on paper substrate) onto the chip (Figure 1.16).

Extraction solvent is dispensed onto the chip and driven onto the dried blood spot, and actuated back-and-forth multiple times to facilitate extraction (Figure 1.17). Samples processed by this method can either be collected and then analysed off-line, or to meet the automation requirements of high throughput bioanalysis, the DMF platform can be directly coupled to a nanoelectrospray emitter for in-line MS analysis. To initiate analysis by mass spectrometry, a droplet was driven to the entrance of a pulled-glass emitter on the chip, and after filling by capillary action, a voltage was applied to the top plate of the DMF device to generate a nanoelectrospray into a mass spectrometer. Devices were translated horizontally in front of the mass spectrometer to switch between emitters. Potentially such emitters could be built into DMF DBS sampling devices.

The mechanism for DBS DMF analyte extraction is automated solvent extraction, however the execution is different enough from traditional large scale wet lab extraction that it is worth recognising it as a distinct technique in the context of this chapter.

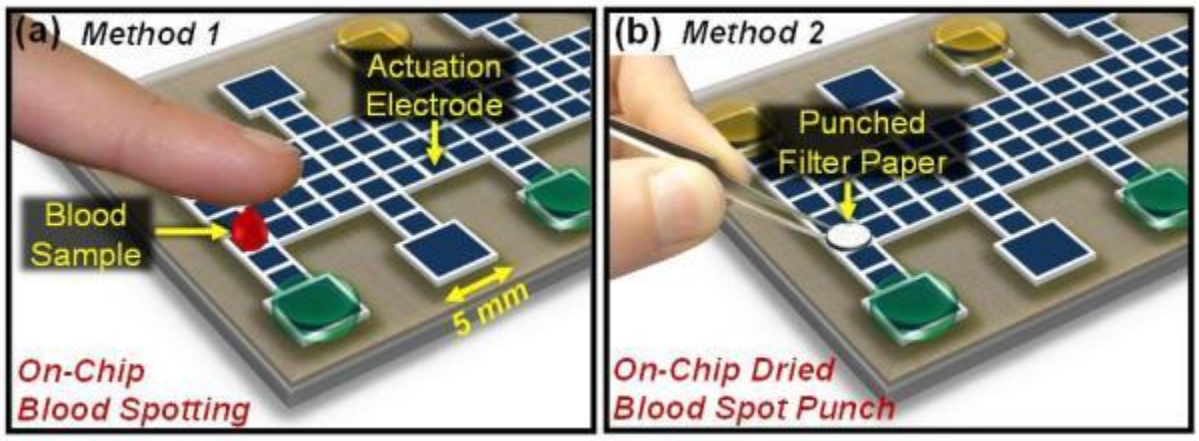


Figure 1.16: Two digital microfluidic methods designed to analyse dried blood spot samples. In method 1 (a), a 5 µL droplet of blood is spotted directly onto the device surface and allowed to dry. In method 2 (b), a 3.2-mm diameter punch from filter paper bearing dried blood is positioned on the device surface. [110]

In the initial publication Jebrail et al demonstrated that there was no significant difference in data generated using DMF or conventional DBS extraction techniques to quantify analytes that are commonly measured as biomarkers for amino acid metabolism disorders in new-born patients. A collaboration between Abu-Rabie and Spooner (GSK), and the Institute for Biomaterials and Biomedical Engineering at the University of Toronto was established to further investigate this technique[66]. Initial work using sitamaquine as a test compound showed that a linear MS response over 3 orders of magnitude, with a limit of detection of 3ng/mL (on a linear ion trap MS) could be achieved using a 3mm DBS punch. Initial testing also demonstrated that suitable assay sensitivity, and accuracy and precision data within guideline acceptance criteria for regulated pharmaceutical development could be achieved for sitamaquine QC samples. In line with other techniques where HPLC is not utilised, other compounds were significantly less sensitive than what can be achieved using conventional extraction techniques. Compatibility with HPLC methodology has yet to be reported, but potentially such technology could be integrated into a chip format for a seamless approach. Further testing is required to fully evaluate the performance on offer from this technique but DMF certainly offers considerable potential in terms of automation and miniaturization for DBS direct analysis. Further refinements to the technique, including simplified chip fabrication and improved liquid actuation, have recently been reported that are specifically aligned with optimising DBS direct analysis. This work compared newborn screening of succinylacetone (a marker for hepatorenal tyrosinemia) in DBS samples using a conventional manual extraction technique and DMF and demonstrated no significant difference in the two sets of data[111].

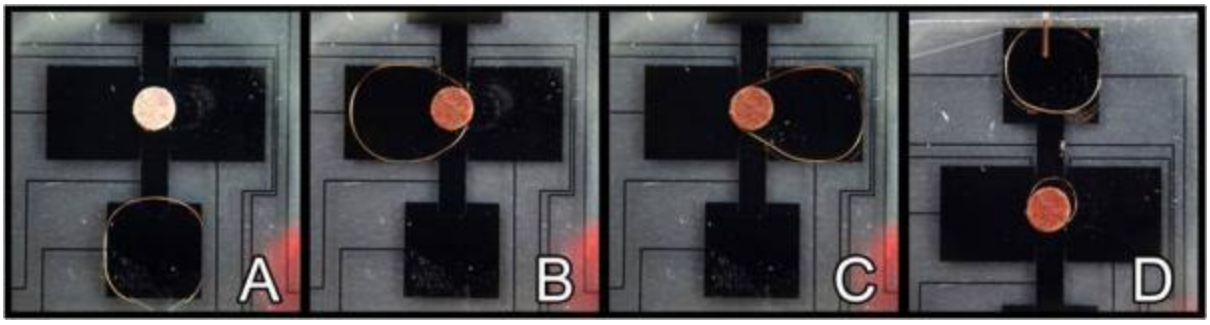


Figure 1.17: Frames from a video (left-to-right) illustrating a digital microfluidics (DMF) dried blood spot (DBS) extraction. (A) A reservoir is filled with extraction solvent (MeOH containing internal standard). (B - C) The solvent is then driven onto the dried blood spot, and actuated back-and-forth 10x before being allowed to incubate for 5 min. (D) After incubation, the droplet is moved to the final electrode, where it fills a pulled glass emitter by capillary action. Photo courtesy of Aaron Wheeler, University of Toronto, Department of Chemistry, used with permission.

1.10 Direct analysis comparison, current trends, and short term and long term prospects

The literature review and initial experimental work on automated DBS extraction; direct elution; and, direct desorption (summarised briefly in this chapter, and published in full elsewhere), enabled conclusions to be formed on the long and short term prospects for DBS direct analysis[73]. These conclusions, which shaped the aims and objectives of this project, are detailed below.

The recent surge in interest in DBSs has come from areas new to the technique, such as pharmaceutical discovery and development, and therapeutic drug monitoring. The requirements for the technique depend on the application being supported, and this reflects the levels of performance, compatibility, selectivity, sensitivity and additional functionality we need from a DBS direct analysis technique. Regardless of the application, it can be asserted with confidence that the ideal DBS direct analysis technique for any application will be fast, simple to perform, robust, reliable, and in many cases will need to offer a high level of selectivity and sensitivity. However, depending on the application, other parameters and additional functionality may also be important, such as full automation, sample capacity, the ability to add internal standard prior to sampling/desorption, the ability to perform sample dilution, and a method of controlling sample carry-over. The large number of different criteria to consider, and the way in which the relative importance of these varies for different DBS applications, makes comparing DBS direct analysis techniques a difficult task. In addition the multitude of direct analysis techniques reported are at a wide range of stages of development. Some have been tested using a wide range of representative compounds in DBS, others have had very limited DBS testing, while many direct analysis techniques that are potentially

applicable to DBS analysis have yet to be tested at all. Nonetheless, an attempt has been made to summarise the performance and compatibility of the most prominent direct analysis techniques that have been applied to DBS analysis, and how they compare to automated manual extraction (Table 1.2).

It should be noted that Table 1.2 only includes DBS direct analysis techniques where results and evaluations have either been adequately published to date, or were directly tested as part of this project. Summarising these techniques is not a straight forward task. In addition to some subjective criteria, the relative importance of many parameters will depend on the application being considered and of course the reader's point of view. Even where absolute values are being compared, such as limits of quantitation, numerous variables need to be considered, such as the sensitivity of the detector coupled to the direct analysis technique, the quantity sampled, whether an additional separation technique is being utilized, and the criteria used to determine these values. For example in terms of sensitivity, the inherent variability in MS performance from occasion to occasion, lab to lab, and between different models of detector, makes published limits of detection data difficult to reconcile. Also certain techniques have demonstrated excellent sensitivity with some test compounds, and poor sensitivity with others (sensitivity has been measured against what can be achieved with conventional manual extraction), which means that rating a technique as 'good or 'bad' in this respect does not tell the whole story. In these cases, a compromise has been used. For example, Desorption electrospray ionization (DESI) demonstrates sensitivity similar to what is achieved with conventional manual DBS extraction for some compounds, but is up to two orders of magnitude less sensitive for others[76]. For this reason the sensitivity ratings in the Table are weighted primarily towards my own experiences with these techniques (where assessments have been made using my own set of test compounds; a range of representative pharmaceutical small molecules), with a secondary weighting towards techniques that have been widely tested by a range of representative molecules. In a similar fashion, a technique that has demonstrated quantitative performance over a range of compounds, and on multiple occasions, will rate higher than a technique that has shown the same performance level but for few applications. To put sensitivity comparisons in context, it should be noted that in most cases a typical conventional manual extraction DBS assay exhibits roughly an order of magnitude less sensitivity than a corresponding conventional plasma based assay, due to much smaller amount being sampled (i.e. a 25 µL plasma sample versus a 3 mm diameter DBS punch). The remainder of this section summarises the current performance and future prospects of the three principle methods of improving the efficiency of DBS analysis.

Table 1.2: Summary and comparison of the current methods available to analyse DBS samples. The table refers to maximum current performance, hence manual extraction is being considered as a technique coupled to instrumentation that provides the best level of automation and sample throughput available.

Parameter	Manual Extraction	Direct Elution				Direct Desorption		
	Manual Extraction	Online DBS	SSSP	LMJ-SSP (LESA)	Digital Micro fluidics	DESI	DART	PAPER SPRAY
Automation/ Throughput	***	***	****	**	**	*	**	***
Sensitivity	***	****	****	**	***	**	*	***
Quantitative Performance	****	****	****	**	***	**	**	****
HPLC Compatibility[1]	Yes	Yes	Yes	No	No	No	No	No
Commercial Availability	Yes	Yes	Yes	Yes	No	Yes (not DBS specific)	Yes (not DBS specific)	Yes (Proposed)
Cost range	* Up to ****	** Up to ***	* Up to ****	***	tbd	**	**	tbd
Robustness	****	****	****	tbd	tbd	tbd	tbd	tbd

- [1]Where the direct analysis technique is being used as the sampling mechanism
- [tbd]To be determined
- Parameter performance: Low(*), medium(**), high(***), very high(****)
- Abbreviations: DBS Dried Blood Spot; SSSP Sealing Surface Sampling Probe; LMJ-SSP LESA Liquid MicroJunction-Surface Sampling Probe and Liquid Extraction Surface Analysis; DESI Desorption Electrospray; DART Direct Analysis in Real Time.

1.10.1 Automated DBS extraction

For many applications manual DBS extraction offers a reasonably quick, simple, cheap and reliable method of analysis. Automating the punching and liquid transfer steps can be used to relieve some of the manual burden, and this technique has been used in high throughput environments and demonstrated to work effectively. It also has the advantage of not significantly deviating from current methods of sample bioanalysis. However, the additional complexity of DBS bioanalysis over conventional plasma extraction techniques, particularly in high throughput applications, and the inherent sensitivity decrease encountered with microsampling, means that this technique has sometimes proved unpopular with laboratory analysts, and this has proved to be a barrier to DBS acceptance. Without investing prohibitive amounts of money to produce very complex integrated instruments, automated DBS extraction cannot provide the seamless, simplified workflows on offer from direct analysis techniques. For this reason, in high sample throughput environments, direct analysis will provide a more efficient method of analysis in applications where justified by sample throughput requirements.

Thus the direct analysis techniques that are emerging offer significant additional advantages that warrant the additional resource involved in their development.

1.10.2 Direct Elution

DBS direct elution describes a number of similar automated or semi-automated techniques where the analyte of interest is extracted through contact with a suitable extraction solvent. One of the advantages of DBS direct elution that differentiates it from direct desorption, is that a liquid eluate is produced which means that the technique can easily be made compatible with LC-MS/MS separation and detection[74]. This is a key feature, as LC-MS/MS is the predominantly used analysis technique in many high sample throughput quantitative bioanalysis applications, and this is an area that has generated considerable momentum in the recent interest in DBS sampling. LC-MS/MS is a technique that is readily available in many bioanalytical laboratories and is familiar to, and accepted by, bioanalysts and regulatory authorities. Thus, because of its compatibility with the existing bioanalytical LC-MS/MS workflow, using DBS direct elution in practice is a realistic short term goal, even for regulated applications where any changes to existing workflows can be extremely difficult, and slow, to implement. In the longer term it is possible that DBS direct elution could be coupled to different types of detector, or an alternative separation technique that replaces the selectivity lost if HPLC is not used. This would leave an even more seamless bioanalytical workflow, akin to what is offered by direct desorption techniques. Initial work has also shown that there is some promise in utilizing direct elution without HPLC[74]. This highlights that direct elution is a flexible technique that should be compatible with both currently used detectors and those in the future that may make other direct desorption techniques a realistic option.

Essentially, one of the main advantages of direct elution is that the concept is not significantly different to currently used DBS (or wet plasma or blood) liquid manual extraction LC-MS/MS techniques, and yet it offers considerable advantages over automated DBS extraction. HPLC coupled to highly sensitive triple quadrupole mass spectrometers is the current separation and detection method of choice in many quantitative bioanalytical applications and is very widely used. Triple quadrupole mass spectrometers are utilized as they offer very high levels of sensitivity, and are relatively cheap (compared to accurate mass MS). Liquid chromatographic separation is used to separate analytes of interest from co-existing components, and in conjunction with tandem MS monitoring (detecting the parent ion of a molecule, and a specific product fragment ion following passage through a collision cell) provides the selectivity required. MS instrumentation is the most expensive component in the bioanalytical workflow, so compatibility with pre-existing resource is a major advantage in the introduction of any new technique. DBS direct elution's compatibility with HPLC-MS/MS means that triple quadrupole mass spectrometers, which are prevalent in high sample throughput quantitative bioanalysis facilities, can be used and the level of selectivity required for regulated applications is retained. For some applications DBS direct elution thus has a financial and practical advantage over direct desorption techniques.

Another important benefit on offer from DBS direct elution is the increase in assay sensitivity that can be readily achieved. In a proof of concept study, an order magnitude increase in assay sensitivity compared to conventional manual DBS extraction, is regularly observed using existing HPLC-MS/MS methodology[74]. The importance of this feature cannot be overstated, as it breeches the sensitivity gap faced in switching from conventional plasma sample volumes (20-50 µL) to DBS micro-sampling (a 3mm diameter punch contains ~2 µL blood). This increase in sensitivity, coupled with the most sensitive detectors, could make DBS sampling applicable to a section of compounds (respiratory based pharmaceutical compounds) currently not compatible with the technique. Clearly the benefit here is that the wider the range of compounds that can be supported, the further the ethical and financial benefits on offer can be maximised.

1.10.3 Direct Desorption

Direct desorption techniques in general are still at a relatively very early stage of development, but a number of these techniques show massive potential for gains in efficiency over a range of applications. If the goal is to find the simplest and quickest DBS analysis technique, direct desorption techniques are theoretically the most favourable option. The potential advantages on offer from direct desorption, such as even simpler bioanalytical workflows, are significant, and the elimination of LC in particular would transform the way bioanalysis is performed, and the environments where analytical instrumentation could be used for non regulated applications (Doctors surgeries, for example). However the challenges described above clearly show that a series of barriers need to be overcome before direct desorption can be used to support some applications, such as regulated drug development studies. Thus it currently can only be considered a longer term goal. For other applications, such as drug screening, drug discovery and therapeutic drug monitoring, where the same level of confidence in the accuracy of the analytical data may not be required and the same regulatory restrictions do not exist, there are far fewer barriers to the use of direct desorption techniques.

As detailed in section 1.7.5, one of the potential advantages of direct desorption is the elimination of liquid chromatographic (LC) separation, and this is also the one of the challenges that must be overcome if it is to be introduced to a regulated bioanalytical environment. Eliminating LC would be a welcome and significant simplification of the bioanalytical workflow. However in practice removing liquid chromatography can result in poor sensitivity due to ion suppression, reduced selectivity, and risks assay interference via metabolite decomposition (e.g. *N*-oxides, glucuronides) into parent compounds during MS ionisation. Perhaps more selective detectors and different types of separation techniques than those commonly found in the bioanalytical laboratory could assist in this regard.

It is clear that utilizing DBS direct elution will be a significant change to current bioanalytical workflows, and this means a significant input of resource (financial and time) is required to develop these techniques and underpin the underlying science behind them to ensure that they

are acceptable for regulatory approval. However, this is not the only barrier to the practical application of direct desorption. Extensive investigation has yet to identify a direct desorption technique that offers adequate sensitivity across a range of representative pharmaceutical (small molecule) compounds. DBS analysis using direct analysis in real time (DART) on the widely used paper substrate format has demonstrated sensitivity considerably lower than that currently achievable from manual DBS extraction (Abu-Rabie et al, GSK, unpublished). Desorption electrospray ionization (DESI), and in particular Paper Spray, have demonstrated sensitivity close to, and in some cases exceeding that for manual DBS extraction for some compounds, but has also been equally disappointing for others[76,76]. No direct desorption technique to date has matched the sensitivity on offer from direct elution. For some applications this will not be an issue as the techniques will still offer enough sensitivity to put the technique within therapeutic range. However, for other techniques, this will be a limiting factor that could exclude the use of the techniques all together.

1.11 Project Rationale and Objectives

A range of industries and applications are interested in utilizing DBS due to the numerous ethical, financial, and logistic advantages the technique potentially offers (section 1.2). This project focuses on implementing DBS in a regulated quantitative bioanalytical high throughput environment. Here, a direct analysis technique to support DBS analysis is required to counter some of the disadvantages and challenges caused by using DBS, namely increased complexity of sample extraction, decreased assay sensitivity, bioanalytical acceptance of the technique, and intense scrutiny from regulatory authorities (section 1.5). A solution to these issues would reduce resistance to using DBS, and thus help the important ethical and financial advantages to be maximised throughout the industry. The aim of this project was therefore to identify a direct analysis technique, and subsequently develop a system around the technique, that is capable of supporting regulated quantitative high sample throughput bioanalysis, within the timeframe of this project. The introduction details the initial part of this project that identified that direct elution techniques were best suited for this task. The objectives for the rest of the project were thus as follows.

- Develop an automated direct elution system/instrument compatible with high throughput regulated bioanalytical requirements.
- Develop a means of adding internal standard that improves bioanalytical performance by enabling analyte and internal standard co-extraction.
- Develop a means of controlling the high level of cross contamination/carry over observed when using DBS direct elution. Aim to at least match what is currently achieved with conventional wet plasma analysis.
- Evaluate the robustness and reproducibility of DBS direct elution, with the aim of developing a system that at least matches the performance on offer from conventional bioanalysis techniques.
- Optimise the technique with an aim to produce guidance on best conditions for maximum sensitivity and reproducibility/robustness.
- Investigate methods of controlling DBS haematocrit based quantitation bias.

CHAPTER 2: DRIED MATRIX SPOT DIRECT ANALYSIS – EVALUATING THE ROBUSTNESS OF A DIRECT ELUTION TECHNIQUE FOR USE IN QUANTITATIVE BIOANALYSIS

An initial literature review on MS-based direct analysis techniques, and initial testing of various DBS compatible direct analysis techniques demonstrated that direct elution was the most promising technique to develop within the timeframe of this project[70,73]. In particular, the proof of concept study using the CAMAG TLC-MS interface to directly elute DBS samples produced such promising results that it cemented direct elution as the technique to focus on and develop further in this project[74]. The next stage was to further test and develop the technique to make it compatible with regulated high throughout quantitative bioanalysis, based on the parameters detailed in **section 1.7**, namely:

- Automation and throughput
- Visual Recognition and Sample Identification
- Internal Standard addition
- Sensitivity
- Selectivity, separation and type of detector
- Cross contamination/carry over
- Robustness and reproducibility
- Dilution and linear dynamic range
- Haematocrit
- Advances in substrate
- Communication, compatibility with other systems and error handling.

Part of the development process involved forming collaborations with a number of manufacturers with the aim of developing fully automated DBS direct elution instruments (or constituent components) made to meet our specification (see section **1.7.12,** and Appendix A: Instrument specification document with potential instrument manufacturers).

One of the first steps in developing DBS direct elution was to investigate automation options. Not only is automation ultimately essential for the intended application (high sample throughput), it also enabled further testing of the technique to be accelerated. The introduction of the DBS-MS16 prototype (Figure 2.1) was the first major step in developing the TLC-MS into an automated instrument which could meet our high sample throughput specification. This device automated the extraction of up to 16 DBS samples (over 4 cards). The DBS-MS16 lacks a true visual recognition system, instead relying on a laser crosshair to correctly position the cards on a moving platform which moves the spots in turn to under the extraction head for extraction. Therefore accurate spotting within the marked region of the cards was essential if the centre of the DBS was to be sampled. The DBS-MS16 is best viewed as a stop-gap prototype to aid in the development of the fully automated instrument. Its primary function

was to enable further investigation of the fundamental robustness of DBS direct elution by providing some limited automation and card handling (it greatly increased the speed at which multiple samples could be analysed).

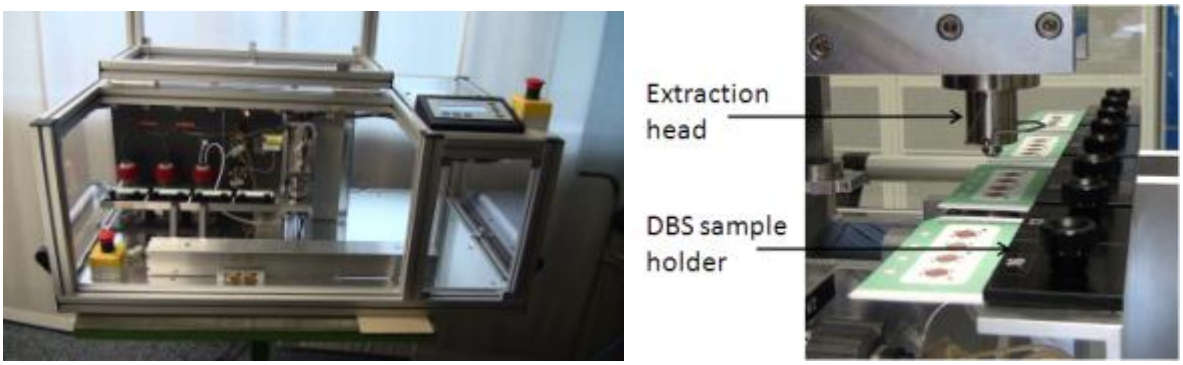


Figure 2.1: The CAMAG DBS-MS16 prototype automated direct elution instrument.

2.1 Introduction

Dried blood spots (DBS) and dried matrix spots (DMS) are now widely used for the collection of samples for the quantitative determination of circulating exposures of pharmaceuticals in animal toxicokinetic (TK), clinical pharmacokinetic (PK) and therapeutic drug monitoring studies at physiologically relevant concentrations[25,26,41,112,30]. The interest in switching from traditionally used wet plasma to DMS sampling is due to the significant ethical, financial, and practical advantages on offer from the technique[25,30]. Any disadvantages are largely limited to the bioanalytical issues associated with the manual extraction technique typically used in DMS analysis[25,26]. The established generic manual extraction technique for analysing DMS samples involves punching a disk from the centre of the DMS, or the entire spot; transferring the disk to a tube and adding an extraction solvent containing internal standard; shaking the sample for ~2 hrs; centrifuging the sample; transferring the supernatant to a fresh tube; then analysing the sample using LC-MS/MS[25]. This method of sample extraction is more complex than the typical protein precipitation method used for wet plasma analysis. Additionally, sensitivity is likely to be lower due to the smaller sample sizes, and ion suppression is generally higher due to extracted matrix components and chemicals added to the paper substrate, than for wet plasma analysis.

The benefits on offer from DMS sampling, and a desire to counter the associated bioanalytical disadvantages, has accelerated the development of numerous direct elution[71,74,105,113] and direct surface desorption[76,114] techniques, which offer the possibility to simplify analytical assay procedures. A DMS direct elution concept utilising the CAMAG thin-layer chromatography TLC-MS interface[94] coupled to a high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) system was previously reported[74]. The DMS sample was placed on a horizontal platform directly under the TLC-MS interface extraction

head, which was lowered onto the sample to create a 4 mm diameter seal on the centre of the spot. The compound of interest was then extracted by directing flow of extraction solvent through the DMS sample, from the solvent pump to the HPLC column and ultimately the mass spectrometer **(**Figure 2.2**)**. Chromatographic performance from validated (manual extraction) bioanalytical HPLC-MS/MS assays was maintained; validation data within internationally accepted guideline criteria[68] was produced; and significant increases in assay sensitivity over a range of representative test compounds was demonstrated[74].

The TLC-MS interface previously reported was a relatively simple, manually operated device[74]. To make this direct elution technique (and numerous similar direct elution and direct desorption techniques) compatible with high sample throughput quantitative bioanalysis, fully automated versions of the instrumentation need to be developed[70,115]. Our group first reported on an automated prototype, the CAMAG DBS-MS-16, in 2010[116,117].

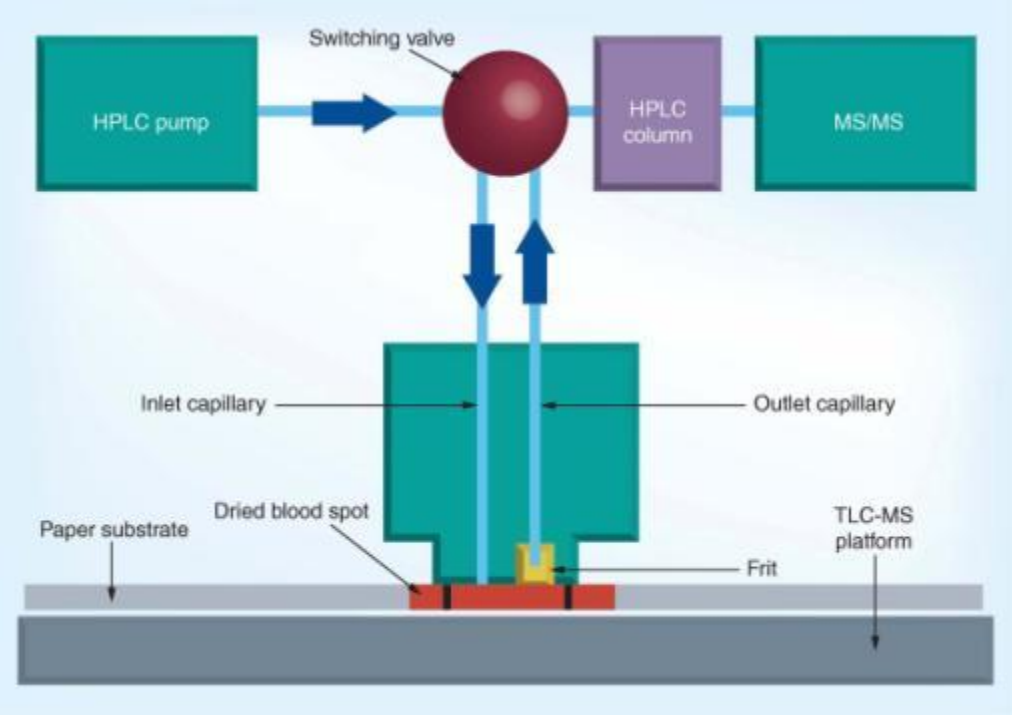


Figure 2.2: Dried blood spot direct elution assembly using the CAMAG TLC–MS interface.

This instrument works using the same principle as the TLC-MS but allows four 85 x 53 mm DMS substrate cards (so, 16 DMS in total) to be analysed without manual interference (Figure 2.3). The most important aspect of this instrument however is the incorporation of an automated wash system. One of the problems of using the TLC-MS for DBS direct elution was that the extraction head capillaries (Figure 2.2), and in particular the frit, appeared to be susceptible to contamination, and at high analyte sample concentrations this resulted in unacceptably high carry over between samples[74]. This was solved by manually reconfiguring the system and back-flushing the outlet capillary, but this was a time consuming and labour intensive process. The DBS-MS 16 utilises an automated 'dual capillary' wash system that

effectively minimises carry over well within the sample cycle time-frame required for high throughput bioanalysis. This wash system has also been demonstrated to be much more effective at eliminating carry over than the manual single capillary flush (Harlan Switzerland, unpublished data).

In addition, the DBS-MS 16 also provides more options for extraction optimisation. The only extraction parameter that could be easily adjusted on the TLC-MS was the 'extraction time' – the duration the extraction solvent was allowed to pass through the DMS sample. It was complex in practice to configure the TLC-MS so that anything other than the mobile phase was used as the extraction solvent (which in most cases is unlikely to offer optimised extraction performance). It was found that assay sensitivity could be increased by extending the extraction time, but a limit was reached where chromatographic peak shape became unacceptably broad[74]. The DBS-MS 16 incorporates additional pumps that allow an optimised extraction solvent to be used. The flow rate and volume of the extraction solvent used can also be optimised independently of the mobile phase conditions.

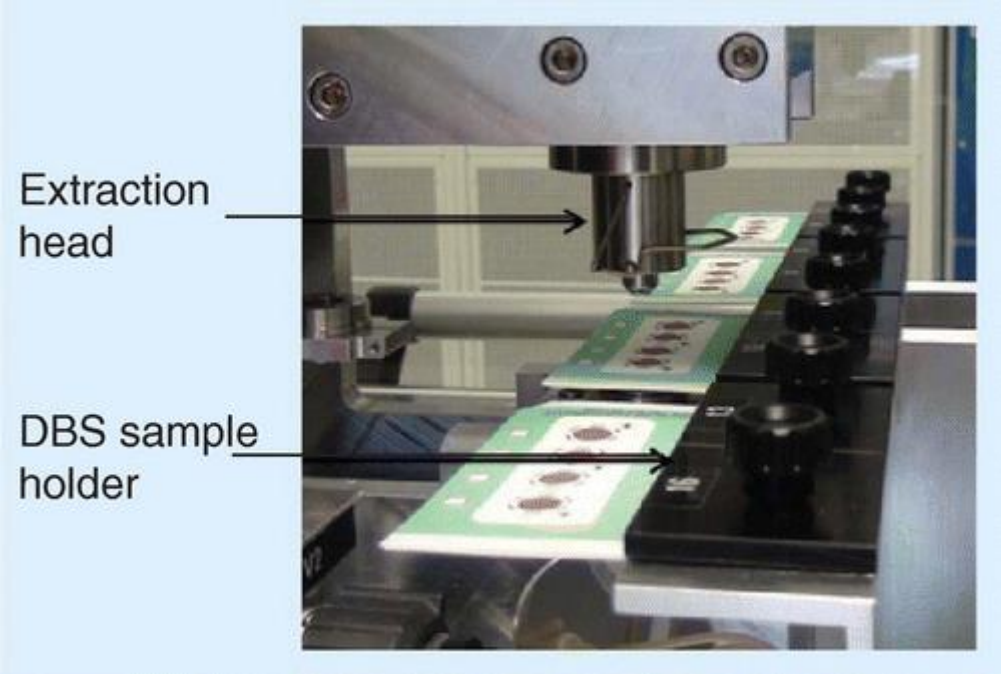


Figure 2.3: The CAMAG DBS–MS 16 direct elution prototype.

If such a direct elution technique is to be used in practice in pharmaceutical support, the robustness and reproducibility on offer is of paramount importance. This simple DMS direct elution technique does not include an elaborate sample or extract clean up step, resulting in relatively dirty extracts being introduced into the HPLC column and mass spectrometer. Additionally, it was previously reported that the technique produced significant increases in assay sensitivity compared to manual extraction procedures[74]. This sensitivity increase appears to be caused by the introduction of increased quantities of analyte into the separation

and detection systems (note: the novel aspect of the technique is that more analyte can be introduced to the HPLC column without causing unacceptably broad chromatography, unlike injecting larger volumes of manual extract). If a larger amount of analyte is being introduced, there is a very strong possibility that this is being accompanied by a larger amount of substrate and endogenous material (cellulose, blood, plasma, urine etc). Clearly there is a concern that the introduction of relatively dirty extracts will negatively impact on the robustness of the technique. This article presents a study where large test batches of both DBS and DPS samples were run to assess detector and chromatographic robustness, with the aim of providing an understanding of how DMS direct elution will perform in practice to support high sample throughput bioanalysis.

2.2 Experimental

Chemicals, materials and instrumentation

Methanol, acetonitrile and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd (Loughborough, UK). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, UK). Control human blood and plasma was supplied by GlaxoSmithKline (GSK) volunteers in accordance with current GSK polices on informed consent and ethical approval. In all cases blood was collected into containers lined with EDTA to prevent coagulation.

Acetaminophen, [$^{2}H_{4}$]-acetaminophen, sitamaquine and [$^{2}H_{10}$]-sitamaquine were obtained from GSK (Stevenage, UK). Ahlstrom 226 DMS cards were obtained from ID Biological Systems (Abbots Langley, UK). Sample tubes were obtained from Micronics (Sanford, USA). Resealable 100mmx140mm polyethylene bags were obtained from VWR International Limited (Poole, UK). 1g silica gel desiccant sachets were obtained from Süd-Chemie (Munich, Germany). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat were supplied by Ted Pella (Redding, USA). Benchtop sample shaker (model HS 501 D) was supplied by Janke and Kunkel, IKA Labortechnik (Staufen, Germany). Benchtop roller mixer (model STR1) was supplied by Stuart Scientific (Staffordshire, UK). Pipettes used were Microman M25, M50 and M250 from Gilson (Nottingham, England), and Micropette plus from Eppendorf (Hamburg, Germany).

The HPLC-MS/MS system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven. MS detection was by a Sciex API-3000 (Applied Biosystems/MDS Sciex, Canada) equipped with Turbo IonSpray™ source. HPLC-MS/MS data were acquired and processed (integrated) using Analyst software (v1.4.2 Applied Biosystems/MDS Sciex, Canada).

The CAMAG DBS-MS 16 prototype instrument was obtained from CAMAG (Basel, Switzerland).

Preparation of test 'standard' samples

Two validated bioanalytical DBS test methods (acetaminophen and sitamaquine (GSK, unpublished data)) were used as the basis of the test samples used in this evaluation **(**Supporting information Figure S2.1**)**. These assays both used stable isotopically labelled analogues as internal standards (IS; [$^{2}H_{4}$]-acetaminophen and [$^{2}H_{10}$]-sitamaquine, respectively). Primary stock solutions for each test compound and IS were prepared in dimethylformamide (DMF, 10 mg/mL for acetaminophen, 1 mg/mL for all other compounds). For each assay, working standards at suitable concentrations were made up in methanol:water (1:1, v/v). Analytical samples were prepared by diluting the appropriate working solutions with blank control whole blood or plasma. Control whole blood was stored at 4°C for up to two weeks[43]. Control plasma was stored frozen at -20°C and thawed before use. The spiking volume into blood was less than 5% non-matrix solvent.

Test 'standard' samples were prepared at concentration levels relevant for the physiological exposure of these drugs. For acetaminophen, test samples were made up at 10000 ng/mL, with the corresponding internal standard being added at 2000 ng/mL. For sitamaquine, test samples were made up at 500 ng/mL, with the corresponding internal standard being added at 100 ng/mL. To suit the aims of this experiment both analyte and internal standard were added to control blood/plasma prior to spotting. This is a deviation from our generic methods, where internal standard is adding via the extraction solvent. This deviation was deemed necessary in order to treat the control manual extraction samples and test direct elution samples as similarly as possible. It has been demonstrated that adding internal standard to blood prior to spotting slightly decreases the variability in internal standard LC-MS/MS peak response compared to adding via the extraction solvent (GSK, unpublished data). DBS/DPS samples were prepared by spotting a fixed volume (15 µL) of blood onto the paper substrate (Ahlstrom 226) using a Gilson Microman M25 pipette and drying for at least 2 hr at room temperature.

Control Samples: DMS manual extraction

Currently used manual DBS extraction methods (acetaminophen and sitamaquine (GSK, unpublished data) were used as a control for this experiment. The data obtained for this experiment provided a control against which the robustness of the direct elution technique could be measured under identical conditions.

For both manual analytical methods, a 3 mm diameter disc was punched from the centre of the DBS or DPS sample (or blank paper substrate for 'blank' samples) and transferred into a clean tube. This was then extracted by the addition of 100 µL methanol. The tube was shaken using a bench top shaker for 2 hr, centrifuged for 10 min at 3000xg to push the disc to the bottom of the tube, and the supernatant was then transferred to a clean sample tube.

Test samples: DMS direct elution

The extraction of the direct elution test samples was carried out using the automated CAMAG DBS-MS 16 direct elution prototype. 20µL of extraction solvent (70:30 methanol:water (v/v)) was applied at 50 µL/minute to extract the DMS samples. The DBS-MS 16 utilises an integrated three stage wash system to clean the extraction head. The first wash, 350 µL of 5:95 methanol:water (v/v) was used to clear away matrix components. The second wash constituted 350 µL of 4:3:3:0.1 acetonitrile:propan-2-ol:water:0.1% formic acid (v/v), which was used to clear away compound contamination. The final, third, wash flushed the system with 500 µL of extraction solvent (70:30 methanol:water (v/v)) in readiness for the next sample. This wash configuration was completed without compromising the MS cycle time or chromatographic performance.

HPLC-MS/MS analysis

In order the make the conditions in which the control samples (manual extraction of acetaminophen and sitamaquine from both DBS and DPS samples) and direct elution test samples (direct elution of sitamaquine and acetaminophen from both DBS and DPS samples) were run as similar as possible, a thorough mass spectrometer (curtain plate and Q0) clean was performed at the start of the experiment and then between runs to remove any contamination caused by matrix components. Photos were taken of the curtain plate before and after the sample batches were run so that a comparison of visible contamination could be made. In addition, all mobile phases, reagents (including wash solutions and extraction solutions) and consumables (including the HPLC column and precolumn filter frit) were swapped over for freshly prepared/unused replacements prior to starting new runs. This provided the best possible starting conditions for a fair comparison of each data set. Although no visible contamination was observed, the extraction head of the DBS-MS 16 was also thoroughly cleaned between runs using a tissue soaked in methanol.

For the manual extraction samples a CTC PAL-HTS (Zwingen, Switzerland) autosampler equipped with fast-wash station was used to inject extracts onto the HPLC column. The wash solvent used was 4:3:3:1 acetonitrile:propan-2-ol:water:0.1% formic acid (v/v), which was found to reduce carry over sufficiently to produce results within international accepted guideline criteria, during the original method validation (GSK, unpublished data). The maximum number of wash cycles were run that would fit into the MS cycle time (2.5 min for acetaminophen, 1.8 min for sitamaquine).

For the acetaminophen manual extraction control samples, a 2 µL aliquot of the extract was injected into a 50 x 4.0 mm i.d. YMC-Pack ODS-AQ 3 µm HPLC column (Dinslaken, Germany) at a column temperature of 40°C. Gradient chromatography was performed employing the mobile phases ammonium acetate (1 mM, native pH) (A) and methanol (B) at a flow rate of 800 µL/min. Following sample injection, the mobile phase was held at 100% A for 0.08 min. A ballistic gradient to 0% A at 1.08 min was followed by an isocratic period at

0% A to 1.25 min. The mobile phase was then returned to 100% A by 1.26 min and was held as this composition until 2.5 min, before the injection of the next sample.

For the sitamaquine manual extraction control samples, a 5 µL aliquot of the extract was injected onto a 50 x 2.0 mm i.d. Varian Polaris C18 5 µm HPLC column (Palo Alto, CA, USA) at a column temperature of 40°C. Isocratic chromatography was performed using 10 mM methyl ammonium acetate (pH 4.2) : acetonitrile (62:38; v/v) at a flow rate of 500 µL/min and a run time of 1.8 min.

In all experiments an inline precolumn filter containing a 2µm PEEK encapsulated stainless steel frit (Hichrom, Berkshire, UK) was used to protect the column from particulates.

The HPLC conditions for the direct elution tests were identical to the above, except for the volume injected into the column, which is described in the 'Test samples: DMS direct elution' section above.

For all the above methods, the HPLC eluent was introduced into the MS interface using a 1 in 4 split ratio. MS data was acquired in selected reaction monitoring mode (the transitions monitored are detailed in Supporting information Figure S2.1**)**. For both assays, the mass spectrometer was used in positive ion mode. Analyte and internal standard peak areas, peak area ratio (area ratios of analyte to its respective internal standard), analyte peak asymmetry, analyte retention time, peak width at half height and HPLC system pressure was monitored and recorded using Analyst software.

2.3 Results and Discussion

Data sets

In total eight series of samples or 'batches' each consisting of 576 consecutive DBS or DPS samples were run: For each analyte (acetaminophen and sitamaquine) manual extraction control batches were run for both DBS and DPS samples, followed by test direct elution batches, again for both DBS and DPS samples. Batches of 576 consecutive samples were chosen as this is the maximum number of samples that can be loaded onto the CTC autosamplers available (6 x 96 well blocks), and it is likely to be the close to the typical maximum number of samples in an overnight run, thus offering a reasonable duration in which to test robustness. Sample numbers were also somewhat limited by the low sample capacity of the prototype DBS-MS 16 instrument. Only 4 MS cards (16 DMS samples) could be analysed at once which made the process highly time consuming.

For all batches the 576 samples consisted entirely of the test samples except every 16th sample which was a paper blank (no matrix, analyte or internal standard present). Paper blanks were run in order to evaluate carry over performance over the duration of the runs.

Analyte, internal standard and peak area ratio response

Analyte peak area, internal standard peak area and peak area ratio response for each data series was recorded for acetaminophen (Figure 2.4a-c) and sitamaquine (Figure 2.5a-c). It is demonstrated that there is no significant difference in response over the duration of these runs using either manual extraction or direct elution. The only data series that displays a significant trend over the duration of the batch is the manual extraction of DBS samples containing sitamaquine, where a gradual decrease in analyte and internal standard response over time is observed (Figures 2.5a and 2.5b). The isotopically labelled sitamaquine internal standard appears to perform its role well here and mimics the behaviour of the analyte, as is demonstrated by the flat peak area ratio response (Figure 2.5c). The fact that this was the only data series to behave in this way suggests that this trend may have been caused by mass spectrometer performance variation unrelated to the sample type or method of extraction.

Coefficients of variation (CV) over 576 samples (excluding the paper blanks) for all eight data series was calculated **(Table 2.1)**. Peak area ratio CVs range from 3.1-4.7 and 2.8-4.1% for manual extraction and direct elution respectively. Within the context of the overall assay precision values applied to bioanalytical validations (±15%) by internationally accepted guideline criteria[68] these values are respectably low, and there is no notable difference in peak area response using manual extraction or direct elution.

Analyte peak asymmetry, number of theoretical plates, and chromatographic performance

Chromatographic performance over each data series was evaluated by calculating analyte peak asymmetry and the number of theoretical plates, and by visual inspection of chromatographic peak shape.

Analyte peak asymmetry (a measure of peak tailing) was calculated as follows:

Peak asymmetry = (peak end time) – (retention time) / (retention time) – (peak start time)

It is demonstrated that the analyte peak asymmetry for each data series for both acetaminophen and sitamaquine was not notably different for manual extraction or direct elution, and any variation over the duration of the individual runs was minimal (Figure 2.4d and 2.5d). The slightly lower analyte peak asymmetry values for acetaminophen direct elution compared to manual extraction are most likely due to the differences in extraction conditions and consequent minor changes to chromatographic peak shape.

Table 2.1: Analyte peak area, internal standard peak area, peak area ratio, analyte peak asymmetry and number of theoretical plates, coefficients of variation (CV) over 576 consecutive DBS and DPS samples for sitamaquine and acetaminophen using both manual extraction and direct elution.

	Analyte	Coefficient of variation (CV) over 576 consecutive samples (%)			
		Manual Extraction		Direct Elution	
		DBS	DPS	DBS	DPS
Analyte Peak Area	Sitamaquine	23.9	8.1	7.0	6.7
	Acetaminophen	8.8	8.0	4.8	5.2
Internal Standard Peak Area	Sitamaquine	22.2	8.5	8.2	6.8
	Acetaminophen	9.3	8.4	6.0	6.7
Peak Area Ratio	Sitamaquine	4.6	3.1	2.8	2.3
	Acetaminophen	4.7	3.1	4.1	3.8
Analyte Peak Asymmetry	Sitamaquine	14.6	14.6	13.4	11.0
	Acetaminophen	7.6	7.3	8.5	7.8
Number of Theoretical Plates, N	Sitamaquine	13.4	7.3	7.8	7.8
	Acetaminophen	8.5	16.5	15.7	13.7

The number of theoretical plates, N, was calculated using the following equation where t_R is the retention time and $W_{1/2}$ is the peak width at half height:

$$N = 5.55 \cdot \left(\frac{t_R}{W_{1/2}} \right)^2$$

All data series (bar sitamaquine DPS direct elution where the response is consistent throughout the duration of the run) show a gradual decrease in N towards the last quarter of the samples and a similar level of variation (Figure 2.4e and 2.5e). There is no indication that there is any significant difference in performance using manual extraction or direct elution.

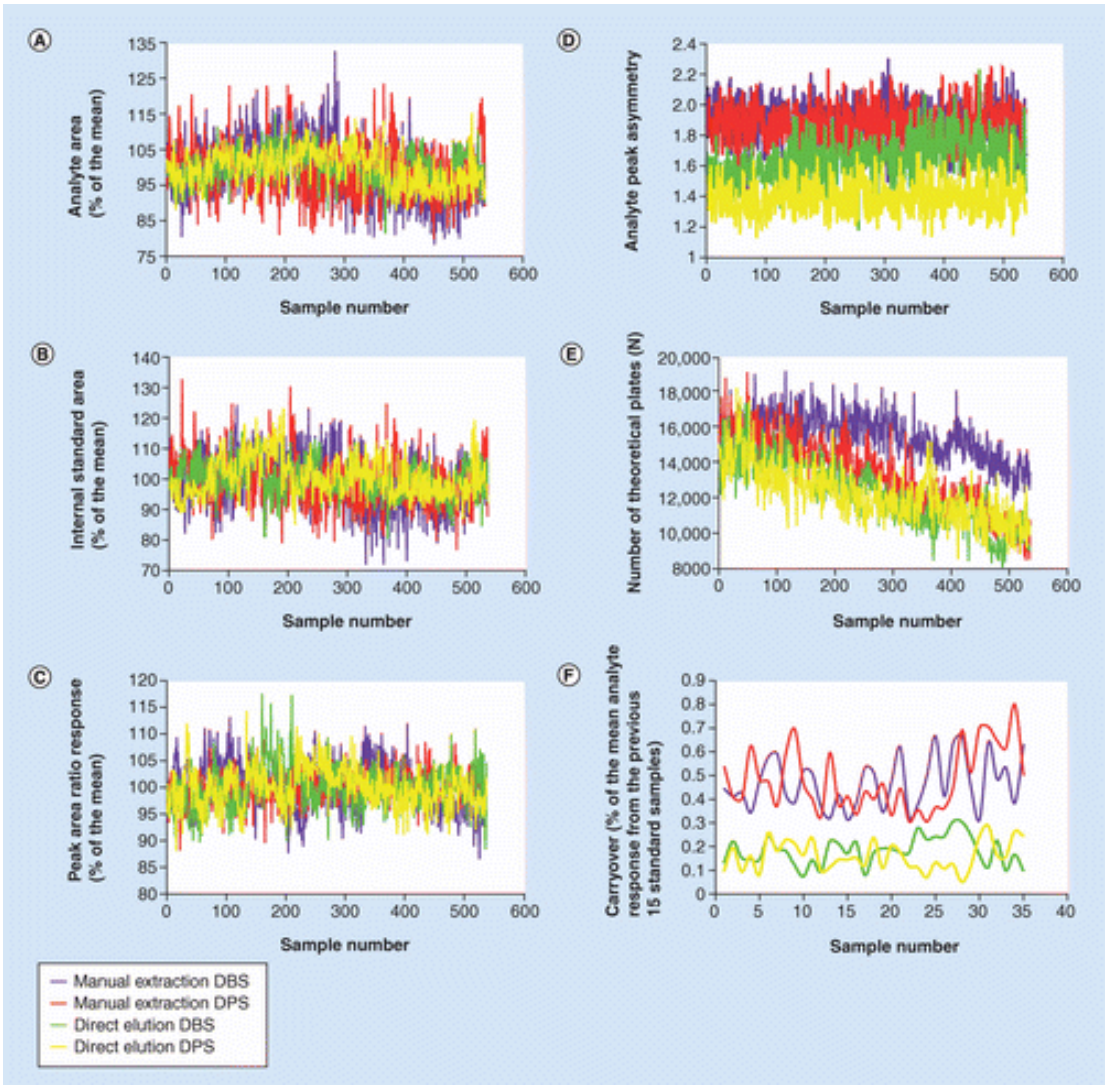


Figure 2.4: Acetaminophen HPLC–MS/MS data over 576 consecutive dried blood plasma spot samples (acetaminophen 10,000 ng/ml, [$^2H_4$]-acetaminophen 2000 ng/ml) using manual extraction and direct elution via the CAMAG DBS–MS 16. (A) Analyte peak area response. (B) Internal standard peak area response. (C) Peak area ratio. (D) Analyte peak asymmetry. (E) Number of theoretical plates, N. (F) Carryover. Charts (A–C) were produced by calculating the mean value for each data series, then expressing individual data points as a percentage of that mean. Charts (D–E) use absolute values. Carryover (F) was calculated using the analyte peak area response in each blank sample expressed as a percentage of the mean response analyte peak area from the preceding 15 standard samples. DBS: Dried blood spot; DPS: Dried plasma spot.

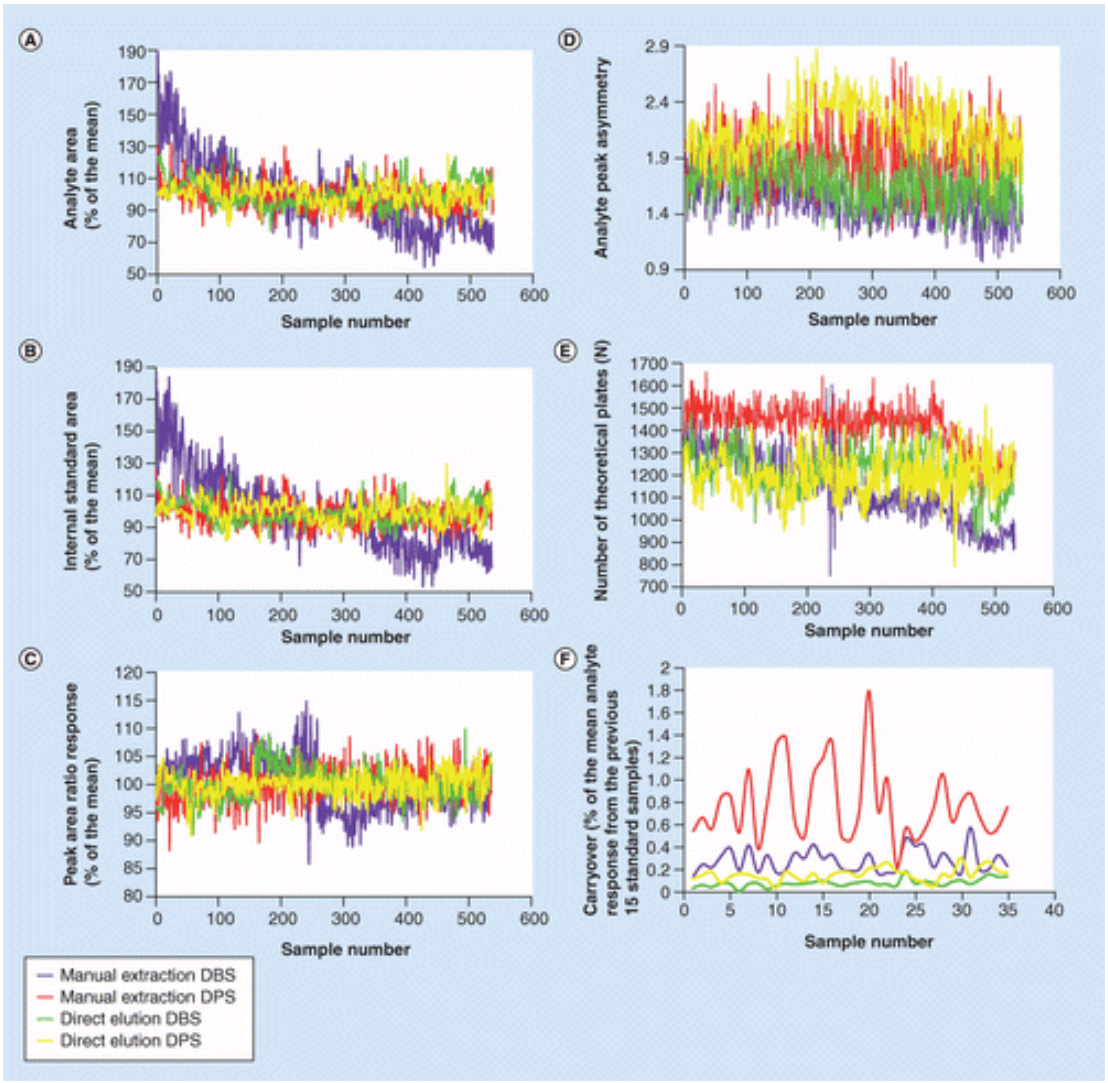


Figure 2.5: Sitamaquine HPLC–MS/MS data over 576 consecutive dried blood and plasma spot samples (sitamaquine 500 ng/ml, [$^2H_{10}$]-sitamaquine 100 ng/ml) using manual extraction and direct elution via the CAMAG DBS–MS 16. (A) Analyte peak area response. (B) Internal standard peak area response. (C) Peak area ratio. (D) Analyte peak asymmetry. (E) Number of theoretical plates, N. (F) Carryover. Charts (A–C) were produced by calculating the mean value for each data series, then expressing individual data points as a percentage of that mean. Charts (D–E) use absolute values. Carryover (F) was calculated using the analyte peak area response in each blank sample expressed as a percentage of the mean response analyte peak area from the preceding 15 standard samples. DBS: Dried blood spot; DPS: Dried plasma spot.

The consistency of chromatographic performance over all eight data series was also demonstrated by visually comparing chromatographic performance over the duration of each series (Figures 2.6 and 2.7). Again, no notable difference in chromatographic performance using manual extraction or direct elution was observed. For all eight data series, 576 consecutive DBS or DPS samples were run. Every sample was a 'standard' containing an identical concentration of analyte (either acetaminophen or sitamaquine), except every 16th sample which was a paper blank (no matrix, analyte or internal standard present).

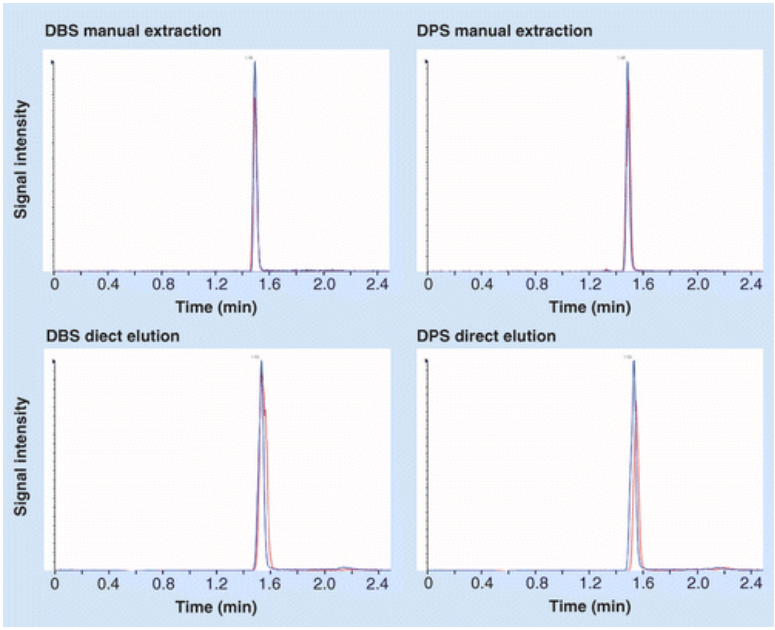


Figure 2.6: Robustness and reproducibility of LC–MS/MS chromatographic performance over each run (consisting of 576 consecutive dried blood spot or dried plasma spot samples) was evaluated by comparing chromatographic performance over the duration of each series. The Figure above overlays chromatography from acetaminophen 10,000 ng/ml samples at the start (sample#1 in blue) and end (sample #575 in red) of each series. DBS: Dried blood spot; DPS: Dried plasma spot.

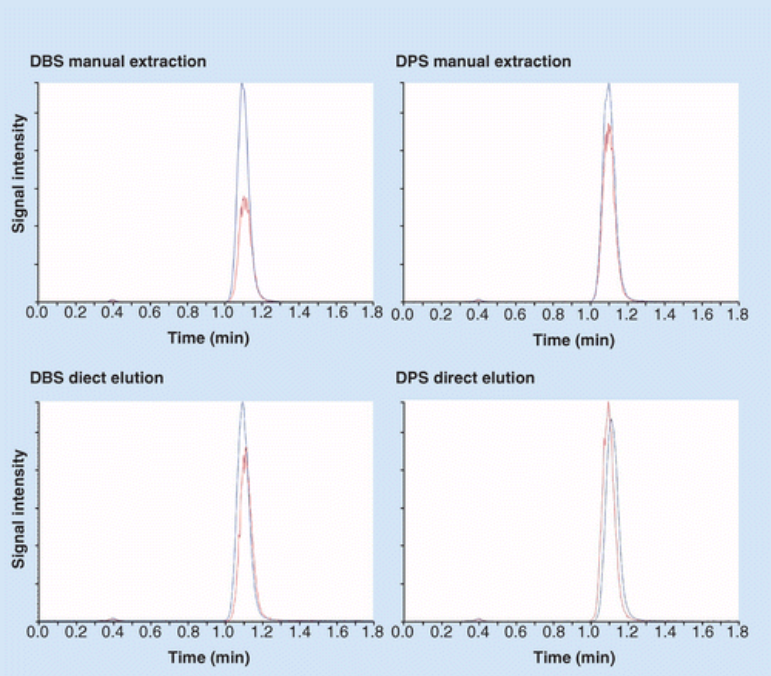


Figure 2.7: Robustness and reproducibility of LC–MS/MS chromatographic performance over each run (consisting of 576 consecutive dried blood or plasma samples) was evaluated by comparing chromatographic performance over the duration of each series. Overlaid chromatography from 500 ng/ml sitamaquine samples at the start (sample #1 in blue) and end (sample #575 in red) of each series. DBS: Dried blood spot; DPS: Dried plasma spot.

Carry Over

The purpose of this sample was to measure carry over performance over the duration of the run. The carryover was assessed by calculating by the analyte response in the blank sample as a percentage of the mean response from the preceding 15 standard samples (Figures 2.4f and 2.5f). The carry over data obtained in this study suggest that the direct elution technique used would have no problems in meeting the criteria required to support regulated quantitative analysis. For both acetaminophen and sitamaquine, percentage carry over is significantly lower than that calculated for manual extraction. For all data series carry over levels do not vary notably over the duration of the runs.

No notable variation in HPLC system pressure over the data set was observed for the manual extraction assays for acetaminophen. A sharp increase in system pressure (~+5%) occurred right at the beginning of the data series for the acetaminophen direct elution samples, after which there was no further significant increase in column pressure (Figure 2.8a). The sitamaquine assay behaved in broadly a similar way, but there was a gradual increase in system pressure for the manual extraction assays. The sitamaquine direct elution samples behave in a similar way to the manual extraction samples up to ~sample#200, where a sharp increase (~+10%) in system pressure occurred, followed by a further gradual increase (Figure 2.8b).

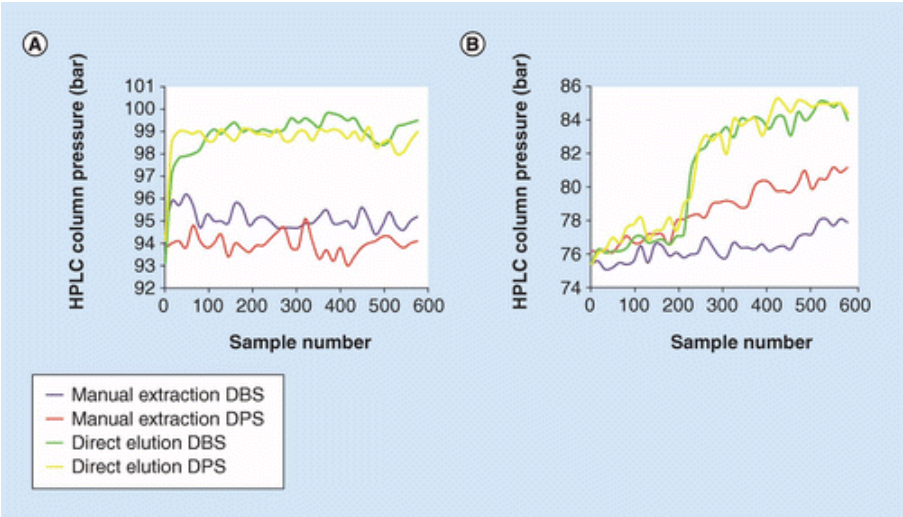


Figure 2.8: (A) Acetaminophen and (B) sitamaquine assay HPLC system pressure over 576 consecutive dried blood spot and dried plasma spot samples. DBS: Dried blood spot; DPS: Dried plasma spot.

It is possible that these differences were due to the gradient and isocratic chromatography used for the acetaminophen and sitamaquine methods respectively, but this was not explored further in this evaluation. The majority of the increased system pressure for the direct elution assays appeared to be due to build up in the precolumn filters rather than the HPLC columns (Table 2.2). When these were replaced with new frits, after the 576 samples had been run, the system pressures dropped to levels not notably different to the manual extraction assays. Additionally, it should be noted that the increase in column pressure observed for direct elution did not appear to have any impact on the analyte peak area response, internal standard peak area response, or chromatographic performance (as detailed above).

Therefore within the scope of this evaluation, the increased system pressure observed with direct elution had no notable negative impact on assay performance. The point at which the build up of material on the precolumn filter/column could produce a significant difference to assay performance was not reached during this study. The use of fully automated direct elution devices with larger samples capacities will make extending studies of this type a more viable proposition. Within the scope of this experiment it would appear that system pressure increase could be controlled by changing frits between overnight runs (a cheap option compared to HPLC column cost), but no data has been generated on how many consecutive occasions this could be performed before a decrease in assay performance was observed.

Table 2.2: HPLC system pressure measured at the start of each data series and at the end after 576 consecutive DBS or DPS samples run either using manual extraction or direct elution. System pressure was also measured at the end of run after removing the inline precolumn filter, and when replaced with an unused frit.

Time of measurement	HPLC system pressure/Bar							
	Acetaminophen				Sitamaquine			
	Manual Extraction		Direct Elution		Manual Extraction		Direct Elution	
	DBS	DPS	DBS	DPS	DBS	DPS	DBS	DPS
@ Sample #1	75.4	76.2	75.5	75.4	94.7	93.8	93.1	94.1
@ Sample #576	77.9	81.2	84.0	84.4	95.2	94.1	99.5	99.0
@ Sample#576 without precolumn filter	75.6	77.2	77.6	78.1	94.1	95.1	94.2	95.1
@ Sample#576 with new pre-column filter frit	75.6	77.6	77.9	78.6	94.9	95.6	94.0	95.4

Mass spectrometer interface visual inspection

As an additional assessment of system robustness the mass spectrometer curtain plate was visually inspected after each data series to investigate whether the introduction of direct elution extracts produced more contamination of endogenous material than what is observed with manual extraction (Supporting information Figure S2.2 and S2.3**)**. It is routinely observed that a sufficient build-up of endogenous material on the curtain plate will eventually have a negative effect on assay performance in terms of magnitude of response and quality of peak shape. The direct elution of DBS samples appeared to produce more contamination (that could be observed visually) than the manual extraction, and DPS sample direct elution batches. However there is no indication in the analyte and internal standard response, and chromatographic performance data generated (above) that the additional contamination has any significant negative impact on assay performance. A longer term study would be needed to get a better idea of how build-up of endogenous material on the mass spectrometer interface would affect assay robustness.

2.4 Conclusions

The advantages on offer from DMS sampling to pharmaceutical companies and drug development organisations are considerable. However, one part of the drug development process that does not directly benefit is bioanalysis. Compared to traditionally used wet plasma analysis, DMS sample analysis adds complexity to the bioanalytical process. Unfortunately, some resistance to accepting the technique has been observed for this reason alone, which fails to acknowledge the overall benefits on offer. A suitable direct analysis technique could help solve this issue by eliminating manual extraction steps completely. DMS analysis would then be as attractive to the bioanalyst as it is to the rest of the drug-development organisation, which

would counter any resistance to accepting the technique, and allow the important ethical, financial and data quality benefits on offer to be maximised throughout the industry.

The fundamental robustness and reproducibility of the technique needs to be evaluated if direct analysis is to be used in practice to support pharmaceutical analysis. The direct elution technique used in this article utilises an impressively simple and quick sample extraction method (cycle times of 1.8 and 2.5 min for sitamaquine and acetaminophen, respectively), but lacks an elaborate clean up step when used in its most simple form (i.e. extracts are introduced directly to HPLC columns and mass spectrometers). Thus, relatively 'dirty' extracts are introduced which potentially could adversely affect the robustness and reproducibility of the technique. The use of similar direct elution techniques have been reported where extract clean-up has been introduced by using a trapping column in conjunction with an analytical HPLC column[98]. However, even when employed in a fully automated direct elution system this approach would still add complexity to the technique, and can dramatically increase sample cycle time (in the region of 6-8 min), both of which are highly undesirable in high sample throughput applications. While this may aid robustness, and may be required for some applications, for high sample throughput use the ideal direct elution technique would be as simple as possible. Therefore the robustness on offer from the simplest method of direct elution should be fully assessed before more complex procedures utilising clean up steps are used.

The data obtained in this study suggest that the direct elution technique employed is suitable for high sample throughput quantitative bioanalysis, without additional sample or extract clean up. Over run lengths that are likely to be approximately the maximum number of samples analysed in a single batch, the direct elution of DBS and DPS samples produced data that was at least as robust as that generated using manual extraction methods. This was demonstrated both by the LC-MS/MS peak area ratio data generated, and through evaluation of chromatographic performance (peak shape and column efficiency). Differences in HPLC system pressure using manual extraction and direct elution were observed, but this did not appear to affect assay performance in any way. While further studies are required to gain more understanding of the reproducibility and robustness of the technique, the data generated in this study suggests that the relatively dirty extracts produced by direct elution do not negatively impact on assay performance. This suggest that this simple direct elution technique is suitable to support bioanalytical studies and offers enough potential benefits to warrant the development of the technique into a fully automated instrument incorporating the additional functionality required to fulfil that role[70,115].

Following the research detailed in this article, a further advancement of this direct elution technique has been developed which incorporates some of the additional functionality required to enable compatibility with high throughput quantitative bioanalysis. This new instrumentation (the CAMAG DBS-MS 500) uses the same direct elution technique but also incorporates robotic card handling for card automation (with a capacity of 500 DMS cards); a visual recognition system to accurately locate the position of DBS on the sample cards; and a method of adding internal standard to samples prior to the direct elution extraction process[92,118]. Such large sample capacity instruments will make it easier to extend the work

described in this manuscript and further the understanding of how reliable and robust this DMS direct elution technique will be in practice.

The use of direct elution without HPLC (where extracts are introduced directly to the mass spectrometer) was not assessed as part of this study as in the authors opinion this approach does not offer the selectivity required to be compatible with regulated quantitative bioanalysis when coupled to triple quadrupole mass spectrometers[70]. It is possible in the future that the use of techniques not commonly employed in high throughput bioanalysis, such as ion mobility, high-field asymmetry waveform ion mobility, or some novel approach, could replace the selectivity currently provided by using HPLC. The robustness of the technique would then need to be evaluated where extracts were being introduced directly into the detection system.

Note: Supplementary information can be found in Appendix B.

CHAPTER 3: METHOD OF APPLYING INTERNAL STANDARD TO DRIED MATRIX SPOT SAMPLES FOR USE IN QUANTITATIVE BIOANLAYSIS

3.1 Introduction

A novel technique is presented that addresses the issue of how to apply internal standard (IS) to dried matrix spot (DMS) samples that allows the IS to integrate with the sample prior to extraction. The TouchSpray, a piezo electric spray system, from The Technology Partnership (TTP), was used to apply methanol containing IS to dried blood spot (DBS) samples. It is demonstrated that this method of IS application has the potential to work in practice, for use in quantitative determination of circulating exposures of pharmaceuticals in toxicokinetic and pharmacokinetic studies. Three different methods of IS application were compared: addition of IS to control blood prior to DBS sample preparation (control 1), incorporation into extraction solvent (control 2), and the novel use of TouchSpray technology (test). It is demonstrated that there was no significant difference in accuracy and precision data using these three techniques obtained using both manual extraction and direct elution.

Dried blood spots (DBS) and dried matrix spots (DMS) are now widely used for the collection of samples for the quantitative determination of circulating exposures of pharmaceuticals in animal toxicokinetic (TK), clinical pharmacokinetic (PK), and therapeutic drug monitoring studies at physiologically relevant concentrations[25,26,41,119,120]. The ethical, financial, and practical advantages[25,26,30] made possible when switching from traditionally used wet plasma to DMS sampling have also accelerated the development of numerous direct elution[74,99,100,101,121] and direct surface desorption[71,76,113,114] techniques, which offer the possibility of simplification of processes in the analytical laboratory. These techniques are collectively termed direct analysis in this manuscript.

The widely used manual extraction method for analyzing DMS samples involves punching a disk from the sample and extracting the analyte with a solvent (typically methanol) containing an appropriate concentration of internal standard[25]. A number of direct analysis techniques apply the internal standard (IS) in a similar way, namely, the incorporation of IS into the elution solvent[99,100,101,114,121]. This approach provides reproducible results. However, we argue that these techniques are not entirely satisfactory, as the IS is not integrated into the sample prior to the extraction process and therefore does not correct for any variability during the extraction process. Thus, we have been investigating the use of an alternative technique that could better integrate IS with DMS samples prior to extraction, while still offering acceptable reproducibility, maintain the simplicity on offer from DMS sampling, and be compatible with manual extraction or direct analysis techniques. The following summarizes the IS application options available.

(1) IS incorporated into extraction/elution solvent: This method is widely used for manual extraction and direct analysis techniques (e.g., direct elution, paper spray, etc). Using this

technique is simple in practice and has been shown to work reproducibly. However, the IS is not fully incorporated into the matrix components and sample paper and is therefore not being coextracted with the analyte as part of the sample. Thus, we are only offered limited information on assay performance by this approach.

(2) Paper pre-treated with IS: Incorporating blank paper substrate with internal standard prior to applying the matrix spot would ensure the IS is integrated and extracted with the analyte. However, this technique is logistically not feasible when dealing with multiple studies, study centres, and compounds if the cost and procedural simplifications on offer from DMS are to be kept intact.

(3) IS added to matrix before spotting onto paper at clinic: Adding IS to liquid matrix prior to spotting onto paper substrate would ensure the IS is fully associated with matrix components along with the analyte. However, as above, this method is logistically not feasible, as it would over complicate the procedure in the clinic and move away from the simple process DMS sampling offers.

(4) IS applied to DMS prior to extraction: A technique that applies IS to DMS samples prior to manual or direct analysis would improve the integration of IS to the sample compared to currently used manual extraction techniques, as long as the IS is given sufficient time prior to extraction for it to bind to matrix components and paper substrate and does not adversely affect the distribution of the analyte. Such an IS application would preferably be carried out once study samples have been shipped to the analytical laboratory but before undertaking the extraction procedure. This procedure is potentially easily compatible with direct analysis techniques and could be easily configured into a fully automated procedure.

It is our opinion that the final option is the only solution that offers the potential to improve IS assay performance, while retaining the procedural simplification offered by DMS technology. The ideal technique would involve the homogeneous application of IS solution across the width and depth of the DMS to ensure its full interaction with matrix and paper substrate components of the sample, in a manner similar to the analyte. The application of the IS would also not cause any significant chromatography effect that could significantly affect the concentration or distribution of analyte within the sampling area. To explore such a solution, we have investigated the use of a piezo electric spray technology, the TouchSpray (Figure 3.1) from The Technology Partnership (TTP) as a method of adding IS solution to DMS samples.

Figure 3.1: TouchSpray piezo electric spray device utilized in this study. Note that the photo demonstrates a different spray geometry to that used in this evaluation.

This manuscript describes an initial evaluation and partial optimization of the TouchSpray technology as a technique to apply IS to DMS samples. All the work was performed with DBS samples, as blood is the most commonly used matrix for DMS in our laboratories, but the technique has the potential to be used for any type of DMS sample. Experiments were performed with the aim of evaluating the robustness and performance of this technique using analytical methods that had been previously validated with the IS incorporated into the extraction solvent. These methods were validated to internationally recognized guideline criteria[68]. DBS samples were allowed to dry (for at least 2 hrs) before IS was added using TouchSpray technology. Drying time after IS application was not evaluated in this manuscript, but samples were touch dry within seconds (and samples were analyzed at least 1 day after IS application). Performance and reproducibility was then evaluated by analyzing the samples by both manual extraction and direct elution with a CAMAG TLC-MS interface.

The CAMAG TLC-MS interface is a relatively simple device that is being used here as a DMS direct elution tool. This direct elution technique works by flowing a suitable extraction solvent over the DMS sample (an action which extracts the analyte of interest) and introduces the extract into the separation/detection system. We have previously published an article demonstrating the suitability of this technique to support quantitative bioanalysis of DBS samples[122]. Both manual extraction and direct elution approaches employed LC-MS/MS detection. Quality control (QC) accuracy and precision data were compared for sample sets where the IS was added either via the TouchSpray (test samples), where IS was added to control blood prior to sample preparation (control 1 samples), or where IS was added via the manual extraction solvent (control 2 samples). IS contamination to adjacent samples using the TouchSpray was also evaluated.

3.2 Experimental Section

Chemicals, Reagents, and Equipment

Methanol, acetonitrile, and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd. (Loughborough, UK). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, UK). Control rat blood containing EDTA was obtained from Harlan (Hull, UK). Acetaminophen, [$^2H_4$]-acetaminophen, sitamaquine, and [$^2H_{10}$]-sitamaquine were obtained from GlaxoSmithKline (Stevenage, UK).Whatman FTA-DMPK-A (previously Whatman FTA) and FTA-DMPK-B (previously Whatman FTA Elute) cards were obtained from Whatman (Kent, UK). Ahlstrom grade 226 paper for blood spots was supplied by ID Biological Systems (Abbots Langley, UK). Sample tubes were obtained from Micronics (Sanford, USA). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). The Harris punch and cutting mat were supplied by Ted Pella (Redding, USA). The benchtop sample shaker (model HS 501 D) was supplied by Janke and Kunkel, IKA Labortechnik (Staufen, Germany)The CAMAG TLC-MS interface was obtained from Omicron Research Ltd. (Wiltshire, UK). The HPLC-MS/MS system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven. MS detection was by a Sciex API-3000 (Applied Biosystems/MDS Sciex, Canada) equipped with Turbo IonSpray source. HPLC-MS/MS and MS/MS data were acquired and processed (integrated) using Analyst software v1.4.2 (Applied Biosystems/MDS Sciex, Canada).

Preparation of Test Samples

Two existing manual extraction bioanalytical DBS methods, validated to internationally accepted guidelines[68], were adapted to evaluate performance. The test methods, for sitamaquine and acetaminophen (Supporting Information, Table S3.1 and Figure S3.1), both used stable isotopically labelled analogues as internal standards ([$^2H_{10}$]-sitamaquine and [$^2H_4$]-acetaminophen). Primary stock solutions for each test compound and IS were prepared in dimethylformamide (DMF, 1 mg/mL for sitamaquine, 10 mg/mL for acetaminophen). For each assay, working standards at suitable concentrations were made up in methanol/water (1:1, v/v). Three types of analytical DBS spiked samples were used in this evaluation. Control 1 samples had IS prespiked into control blood prior to standard preparation. Control 2 samples were prepared in blank (no IS) control blood and had the IS added via the extraction solvent (during manual extraction). Test samples had IS added via the TouchSpray prior to extraction. Calibration and QC standards were prepared over a concentration range relevant for the physiological exposure of the drugs by diluting the appropriate working solutions with control blood. In all cases, the spiking volume into blood was less than 5% nonmatrix solvent. For sitamaquine, the concentrations of calibrants were 5, 10, 20, 50, 100, 200, 500, 800, and 1000 ng/mL and QC's were 5, 20, 100, 800, and 1000 ng/mL. For acetaminophen, the concentrations of calibrants were 50, 100, 200, 500, 2000, 5000, 10 000, 20 000, 40 000, and 50 000 ng/mL and QC's were 50, 200, 2500, 40 000, and 50 000 ng/mL. In this evaluation, control rat blood used for sample preparation was used within 1 week of collection[43]. DBS samples were less

than 1 week old when the IS was applied using the TouchSpray. DBS samples were prepared by spotting a fixed volume (15 μL) of blood onto the paper and drying for at least 2 h at room temperature. If required, samples were stored at ambient laboratory conditions (21 ± 2 °C) in a sealed plastic bag containing desiccant.

Internal Standard Application

The TouchSpray was set up with a simple platform and targeting system (Figure 3.2) that ensured that the spray head was positioned at a set distance (20 mm) from, and directly above, the centre of each DBS sample. The TouchSpray device was controlled by a bespoke lab drive system comprising a waveform generator and amplifier unit. Once optimized, the TouchSpray produced a light mist dispensed as a cylindrical arc that covered a diameter a few mm larger than a typical 15 μL DBS (approximately 10 mm) (Figure 3.3). The combined internal standard solution was made up in methanol at a concentration of 125 ng/mL [$^{2}H_{10}$]-sitamaquine and 2500 ng/mL [$^{2}H_{4}$]-acetaminophen. A one second spray duration was used for each application that dispensed 8 μL of internal standard solution per sample. This relatively low volume was selected as it ensured no obvious visual chromatographic effect occurred when the dry blood spot was rewetted with IS solution. Drying times were not investigated in this initial evaluation. Visual observation suggested the IS solution only partially penetrated the depth of the paper (Figure 3.3).

Initial investigations with different spray head geometry produced a much narrower diameter application arc (around 3 mm at the DBS sample when dispensed from a distance of 20 mm) and then relied on larger volume applications and the spread of solution via a wicking effect to visibly cover the diameter of the spot. With this smaller diameter application arc, a 3 s application (approximately 24 μL) was the minimum volume required to reproducibly cover the entire spot diameter across all the paper types used, but in some cases, this created a clear chromatographic effect and thus a significant risk of changing the distribution of drug within the sampling area (Supporting Information, Figure S3.1).

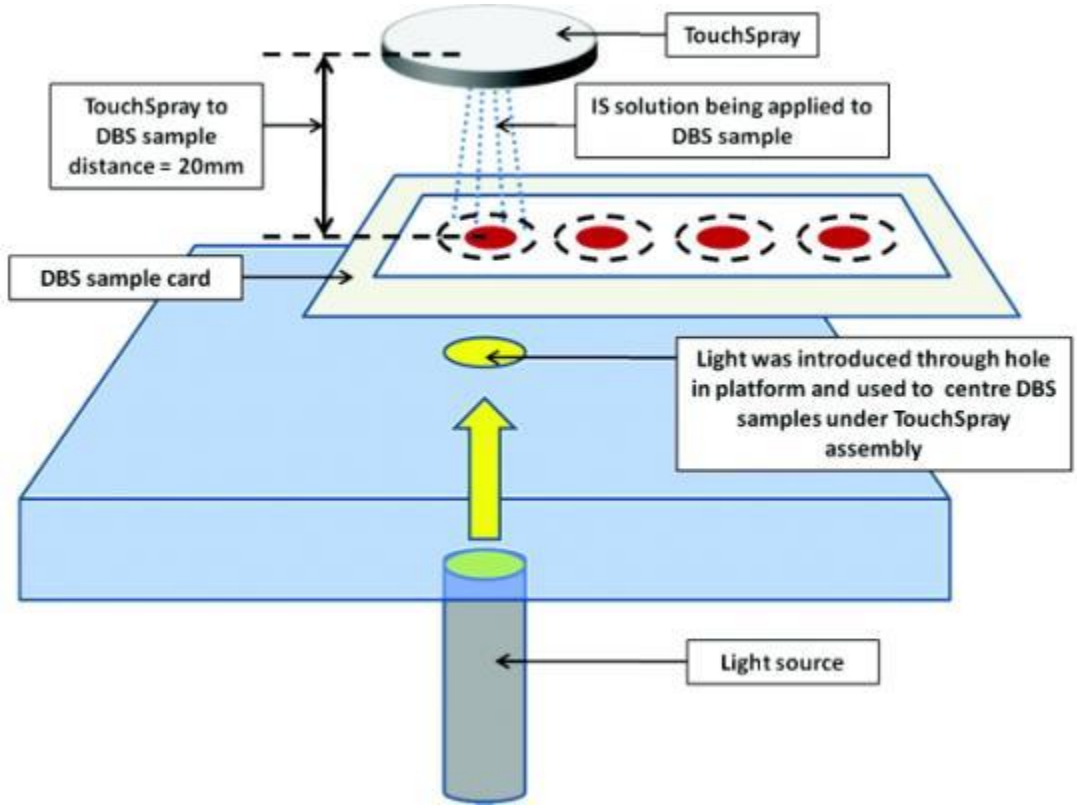


Figure 3.2: TouchSpray experimental setup. The TouchSpray was set up with a simple platform and targeting system that ensured that the spray head was positioned at a set distance (20 mm) directly above the centre of each DBS sample. The TouchSpray was secured in place relative to the platform, and a light was shone through a hole in the centre of the platform that could be viewed through the DBS sample. This enabled the IS spray to be applied to the centre of the DBS sample on each occasion.

Using these conditions, it was shown that test sitamaquine validation data was highly variable, while the acetaminophen assay produced accuracy and precision data well within normal acceptance criteria[68] (Supporting Information, Table S3.2). Possible explanations for the variation in accuracy and precision data shown for sitamaquine and acetaminophen are the differences in blood binding characteristic for these compounds (sitamaquine demonstrates high blood binding, and acetaminophen demonstrates low blood binding[69]) and the bearing this has on analyte distribution if a chromatographic effect occurs. This was not explored further; as it was deemed essential that a reliable IS application technique would have to eliminate any possibility of significant chromatographic effect on the DMS sample.

Thus, a more suitable spray geometry that applied a wider arc just over the diameter of a 15 µL DBS sample (approximately 8 mm) was used with lower volumes of solution. This was found to be less likely to cause a chromatographic effect. The disadvantage of the use of a lower volume was that we risked not covering the entire depth of the DBS paper substrate and thus have less confidence that the sample contains a homogeneous application of IS. It could be argued that, as long as the IS application is reproducible, having a homogeneous application across the depth of the sampling area is not necessarily essential and still offers an advantage over current IS usage, where IS is applied in the extraction solvent.

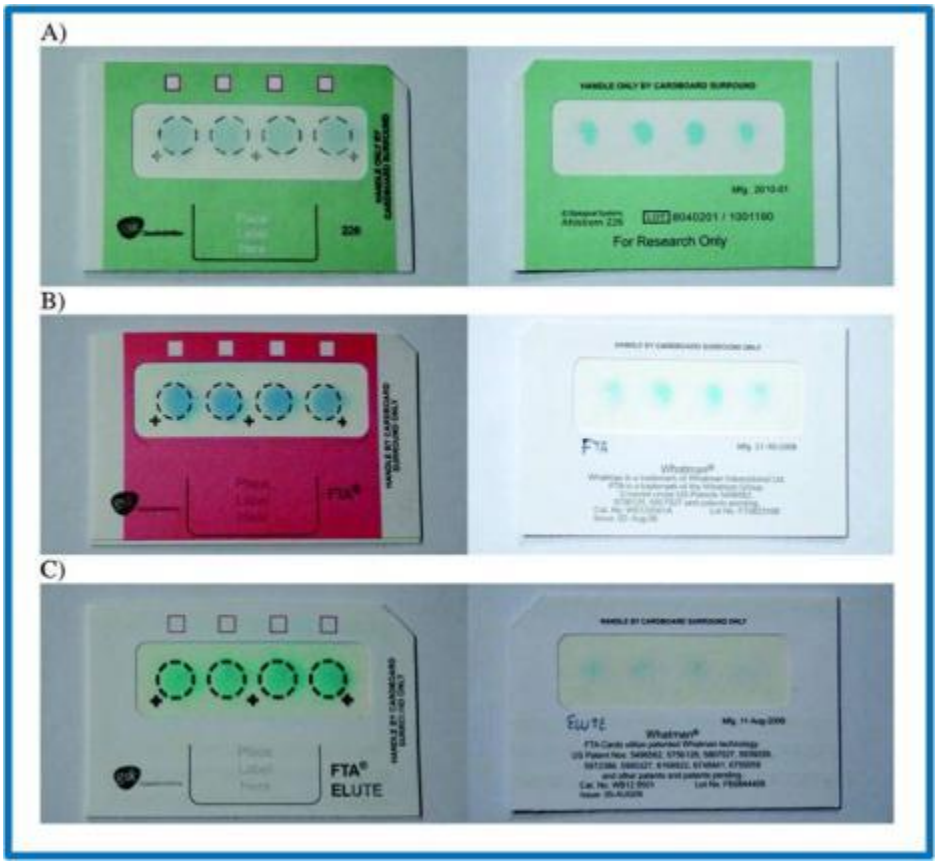


Figure 3.3: TouchSpray was used to spray cards with a methylene blue dye solution to demonstrate spray pattern. Methylene blue in methanol solution (0.05 g/L) was dispensed onto Ahlstrom 226 (front and reverse of card) (A), Whatman FTA-DMPK-A (front and reverse of card) (B), and Whatman FTA-DMPK-B (front and reverse of card) (C) sample cards using an optimized Touchspray device to demonstrate the area being covered by IS solution. A 1 s spray application was used that dispensed 8 µL of solution. Visual inspection of the reverse of the cards suggest that depth penetration is only partial using this volume.

As this approach involves the spraying of the IS onto the spot surface, the risk of contamination to other adjacent spots is potentially high (but could be readily avoided in practice). In this initial evaluation, no attempt was made to control carry over between adjacent dried blood spots on the same card. Thus, for the validation samples only, one DBS sample was spotted per card.

Manual Extraction Procedure

A 3 mm diameter disk was punched from the centre of the DBS sample and dispensed into a clean tube. This was then extracted by the addition of 100 µL of methanol. For control 1 (IS prespiked into control blood) and test (TouchSpray) samples, the extraction solvent was methanol only. For control 2 (IS in extraction solvent) samples, the methanol contained an appropriate concentration of IS (10 ng/mL $[^2H_{10}]$-sitamaquine, 200 ng/mL $[^2H_4]$-acetaminophen). For all samples, the tube was then shaken using a benchtop shaker for 2 hrs and centrifuged for 10 min at 3000 g to push the disk to the bottom of the tube, and the

supernatant was then transferred to a clean sample tube. An aliquot (2 µL for sitamaquine, 5 µL for acetaminophen) of this extract was then injected onto the HPLC-MS/MS system.

Direct Elution Using TLC-MS Interface

A direct elution technique, utilizing the CAMAG TLC-MS interface, was used to analyze control 1 samples (IS prespiked into control blood) and test samples (TouchSpray). The TLC-MS interface was located between the HPLC pump delivering solvent and a HPLC column coupled to the MS system. This direct elution technique works by forming a sealed area at the centre of the DBS sample and flowing an extraction solvent across this area[74]. The appropriate mobile phase for each analyte was used as the extraction solvent, and an extraction time of 5 s was used.

HPLC-MS/MS Analysis

For manual extraction and direct elution experiments, the following HPLC conditions were used. The sitamaquine assay used a 50 × 2.0 mm i.d. Varian Polaris C18 5 µm HPLC column (Palo Alto, CA, USA), a flow rate of 500 µL/min, column temperature of 40 °C, run time of 1.5 min, and isocratic chromatography; 62:38 (v/v) 10 mM methyl ammonium acetate (pH 4.2 with acetic acid)/acetonitrile. The acetaminophen assay used a 50 × 4.0 mm i.d. YMC-Pack ODS-AQ 3 µm HPLC column (Dinslaken, Germany), a flow rate of 800 µL/min, column temperature of 40 °C, run time of 2.5 min, and gradient chromatography employing the mobile phases ammonium acetate (1 mM, native pH) (A) and methanol (B). Following sample injection, the mobile phase was held at 100% A for 0.08 min. A ballistic gradient to 0% A at 1.08 min was followed by an isocratic period at 0% A to 1.25 min. The mobile phase was then returned to 100% A by 1.26 min and was held as this composition until 2.5 min, before the injection of the next sample.

For all methods, the HPLC eluent was introduced into the MS interface using a 1 in 4 split ratio. MS data was acquired in selected reaction monitoring mode. The transitions monitored are detailed in Supporting Information, Table S3.1. MS source conditions were optimized to give the maximum response for a given analyte/assay. Concentrations of test compounds were determined from the peak area ratios of analyte to internal standard using Analyst software.

3.3 Results and Discussion

General Observations

The TouchSpray technology is a novel piezo-electrically activated droplet generation system, which can be used for deposition or spraying. It operates by physically vibrating a nozzle plate which contains hundreds of nozzles at around 80 000 vibrations per second, producing

approximately one droplet per nozzle per cycle. The ability to deliver precise quantities of liquid arises from activating the device for a digitally controlled number of vibration cycles. A resistance to nozzle blockage arises from the ultrasonic vibration of the nozzles and inherent dislodging of particles. By arranging the pattern of nozzles in the device to coincide with the physical target area, the TouchSpray can dispense evenly over a broad target area, due to the activity of multiple nozzles simultaneously. By dispensing a known quantity of IS over the target area, it was hoped that a homogeneous coverage on the DBS sample could be achieved. Droplet size is generally and readily controlled by the nozzle diameter with a narrow droplet size population easily maintained and thus can be used to reduce the propensity for chromatographic effects caused by rewetting DMS samples. This technology has been applied to applications including drug delivery and spray drying, but this represents the first report of its use in DMS analysis.

Using the optimized spray conditions, there were no visual changes in the appearance of DBS samples once IS was added using the TouchSpray. Samples were faintly wet to touch immediately after IS addition but were touch dry within seconds. No notable differences in LC-MS/MS chromatographic peak shape (of the analyte or IS) was observed using the three methods of adding IS described in this manuscript (Supporting Information, Figures S3.3 and S3.4). The IS solution concentration used was sufficient to produce a LC-MS/MS response of suitable magnitude for the volume of IS solution (8 μL) dispensed from the TouchSpray.

Visual observation of the reverse side of blank paper substrates which had been sprayed with methanol solution containing methylene blue dye suggested only a portion of the internal standard solution penetrated the depth of the paper (Figure 3.3). It is likely that the balance point between increasing the volume of IS solution sprayed to ensure full DBS depth penetration and minimizing the volume to avoid chromatographic effects could be refined to further improve performance. However, further optimization to obtain greater depth penetration was not performed in this study.

Validation Sets

Running test validation assays is the best method we have of evaluating if a technique will work in practice for bioanalytical study support, as it examines the precision, accuracy, linearity, and sensitivity of the approach. Test validation runs on three paper types (Ahlstrom 226, Whatman FTA DMPK-A, and Whatman FTA-DMPK-B) were performed for control 1, control 2, and test samples. These experiments were based on existing validated methods for the analytes sitamaquine and acetaminophen which use manual extraction of a 3 mm diameter DMS punch, with the IS present in the extraction solvent, and meet internationally accepted validation criteria[68]. Accuracy and precision QC data for control 1 (IS prespiked into blood) and control 2 (IS incorporated into extraction solvent) samples, using TLC-MS direct elution and manual extraction, respectively, was within guideline acceptance criteria (±15% precision and accuracy) (Supporting Information, Tables S3.3 and S3.4). For the analysis of test (TouchSpray) samples, IS was added to DBS samples using the TouchSpray and allowed to

dry. Replicate samples were extracted using both manual extraction and TLC-MS direct elution. Note, a TLC-MS extraction head that was compatible with Whatman FTA-DMPK-B substrate was not available during testing so direct elution data was not collected for this substrate. For test samples, the validation QC accuracy and precision data was also well within internationally accepted guideline acceptance criteria (Table 3.1a–d). For test samples, the calibration plot correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9969 (across all substrate types and both analytes). This data demonstrates that this novel technique of adding IS is capable of producing results as good as that obtained for both control methods: the original (manual extraction) bioanalytical method validation where IS was added via the extraction solvent and where IS was spiked into control blood prior to sample preparation.

Table 3.1: Bioanalytical validation assay accuracy and precision test sample data for two compounds on three paper types where IS was applied using the TouchSpray[a].

Table 3.1a. Sitamaquine assay, Touchspray IS addition, manual extraction.

		Manual Extraction					
Sitamaquine QC Level		226		FTA-DMPK-A		FTA-DMPK-B	
	N	%CV	Accuracy	%CV	Accuracy	%CV	Accuracy
5ng/mL	6 of 6	6.8	104.3	5.5	101.4	5.2	101.3
20ng/mL	6 of 6	4.9	99.4	4.3	95.1	4.9	101.7
100ng/mL	6 of 6	7.1	105.8	5.6	102.1	4.0	101.1
800ng/mL	6 of 6	6.0	102.2	2.9	106.1	4.5	99.9
1000ng/mL	6 of 6	4.6	105.2	3.3	102.6	6.3	98.3

Table 3.1b. Sitamaquine assay, Touchspray IS addition, TLC-MS direct elution.

		Direct Elution TLC-MS					
Sitamaquine QC Level		226		FTA-DMPK-A		FTA-DMPK-B	
	N	%CV	Accuracy	%CV	Accuracy	%CV	Accuracy
5ng/mL	6 of 6	4.9	103.2	7.2	97.4	N/A	N/A
20ng/mL	6 of 6	7.2	102.8	4.0	97.1	N/A	N/A
100ng/mL	6 of 6	8.1	99.5	6.8	97.4	N/A	N/A
800ng/mL	6 of 6	4.3	100.1	8.6	103.4	N/A	N/A
1000ng/mL	6 of 6	8.6	104.0	6.8	100.4	N/A	N/A

Table 3.1c. Acetaminophen assay, Touchspray IS addition, manual extraction.

		Manual Extraction					
Paracetamol QC Level		226		FTA-DMPK-A		FTA-DMPK-B	
	N	%CV	Accuracy	%CV	Accuracy	%CV	Accuracy
50ng/mL	6 of 6	5.0	102.1	4.1	98.0	4.8	101.0
200ng/mL	6 of 6	4.7	96.5	4.9	97.7	5.4	101.9
2500ng/mL	6 of 6	6.5	102.8	8.6	100.6	8.7	97.7
40000ng/mL	6 of 6	9.8	102.3	7.2	99.6	5.2	92.3
50000ng/mL	6 of 6	5.5	99.7	6.9	98.6	5.9	93.7

Table 3.1d. Acetaminophen assay, Touchspray IS addition, TLC-MS direct elution.

		Direct Elution TLC-MS					
Paracetamol QC Level		226		FTA-DMPK-A		FTA-DMPK-B	
	N	%CV	Accuracy	%CV	Accuracy	%CV	Accuracy
50ng/mL	6 of 6	5.7	102.8	7.1	102.4	N/A	N/A
200ng/mL	6 of 6	3.0	106.1	6.4	96.7	N/A	N/A
2500ng/mL	6 of 6	5.7	99.6	9.0	100.6	N/A	N/A
40000ng/mL	6 of 6	7.1	101.6	8.5	99.7	N/A	N/A
50000ng/mL	6 of 6	7.0	99.1	4.7	104.5	N/A	N/A

[a]Internal standard was added to DBS samples using the TTP Touchspray technology (8 µL of IS solution containing 125 ng/mL [$^2H_{10}$]-sitamaquine and 2500 ng/mL [$^2H_4$]-acetaminophen dispensed in one second) prior to either manual extraction (a and c) or direct elution using the CAMAG TLC-MS (b and d). Note:

A TLC-MS extraction head compatible with FTA-DMPK-B paper was not available during testing.

Carry-Over/Contamination

Using the TouchSpray to apply IS, the theoretical risk of contamination to adjacent spots was high. In this initial evaluation, no attempt was made to shield the adjacent spots from the IS spray. To estimate the potential level of contamination to neighbouring DBS samples, four control blank DBS samples were spotted per card and IS (10 ng/mL [$^2H_{10}$]-sitamaquine, 200 ng/mL [$^2H_4$]-acetaminophen in methanol) was applied to spot 2 only via the TouchSpray using the same conditions as for the validation samples (Figure 3.4 and Table 3.2). All four DBS samples per card were analyzed (using manual extraction with methanol), and the IS response was calculated. Note that control total blank samples on separate cards gave no IS response when analyzed as part of this test. Figure 4 shows that mean contamination to adjacent spots on the same card varies between 0.4 and 2.8% of the IS response measured on the IS-applied spot (across 8 replicate cards). Potentially, this is a significant amount for the intended application and would have to be addressed if this technique progressed into routine use. The values for the nonadjacent spot (spot 4) were typically 2–4 times less than those measured on the adjacent spots (1 and 3), as we might expect due to the extra distance between this spot position and the spray head.

Potential solutions to this issue include altering the TouchSpray geometry or blanking off the rest of the DBS card with a physical screen ensuring the IS spray was only applied to the single intended DBS sample. Applying internal standard solution as discrete micro droplets (rather than a mist spray) is an alternative method that could be explored that could offer more control over the application and, thus, reduce the risk of carry over. This however may be at the expense of speed and simplicity.

Another potential solution could be to spray the entire card with IS. This would apply IS to all DBS on the card in a single step, eliminating any issue with contaminating adjacent spots. This may be suitable for some applications and could simplify automation, but it could also potentially introduce other issues if used for regulated quantitative bioanalysis. This is because multiple DBS on a single card tend to be replicate aliquots of the same sample. Usually, only the first sample would be analyzed, with the second and third only analyzed in a subsequent analytical run if repeat analysis was required.

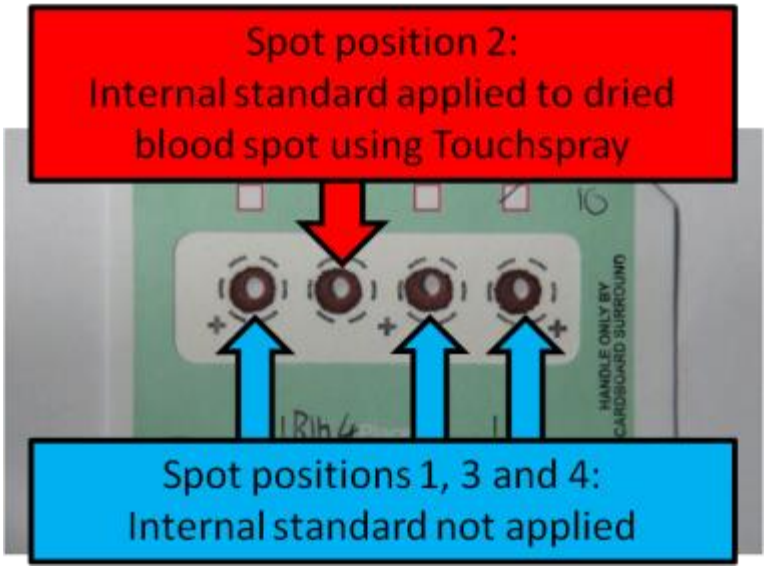


Figure 3.4: Dried blood spot (DBS) sample card demonstrating how internal standard (IS) contamination was estimated in this study. A 15µL control blank DBS sample was applied to spot positions 1-4. IS was applied to spot position 2 only, using the TouchSpray. In this evaluation no attempt was made to control IS contamination between adjacent dried blood spots on the same card. Risk of IS contamination to spot 4 was less likely than for spots 1 and 3 which were directly adjacent. All 4 DBS per card were analysed to evaluate IS contamination to adjacent areas. Figure shows DBS sample card post manual extraction where all 4 spot positions have had a 3mm diameter punch taken from the spot centre.

Table 3.2: Estimated internal standard (IS) contamination to adjacent dried blood spot (DBS) samples caused by Touchspray application of IS solution. Four control blank DBS samples were spotted per card (see Figure 4). IS was applied to DBS in position 2 only using the Touchspray. All DBS samples were analysed using manual extraction and the internal standard LC-MS/MS peak area response was measured for each sample. Mean IS contamination to adjacent samples was estimated by calculating the measured IS response as a percentage of the IS response for spot 2 (8 replicate DBS cards were analysed).

Analyte	Sitamaquine						Paracetamol					
Card Location	directly adjacent spots			Not directly adjacent spots			directly adjacent spots			Not directly adjacent spots		
	Spot 1 and 3			Spot 4			Spot 1 and 3			Spot 4		
Paper Substrate	226	FTA DMPK-A	FTA DMPK-B	226	FTA DMPK-A	FTA DMPK-B	226	FTA DMPK-A	FTA DMPK-B	226	FTA DMPK-A	FTA DMPK-B
Reps	16	16	8	16	16	8	16	16	8	16	16	8
Mean internal standard concentration to adjacent DBS samples on same card (as a % of IS response to applied spot)	2.75	2.14	1.83	0.87	0.56	0.51	2.24	2.14	2.24	0.44	0.76	0.65

The reason for repeat analysis could be to clarify an unexpected result or for routine incurred sample reanalysis (ISR). The problem is that, especially in the case of ISR of large clinical studies, the period between the initial analysis and subsequent reanalysis could be many months. This raises two issues. First, IS stability over this period would need to be proven. Second, ideally, the same batch of IS solution should be added to the study samples, calibration standards, and QC samples in a single analytical run, to eliminate any potential issues with batch to batch variation in IS concentration and the consequence this has on calculated concentrations of test samples. This is a logistical issue which, while not insurmountable, would add complexity to the procedure in practice and would be preferable to avoid.

3.4 Conclusions

This manuscript investigates the use of a novel technique for applying IS to DMS samples that offers a potential improvement in the utility of the IS for monitoring analyte extraction, which is not offered by the current approach of adding the IS to the extraction solvent. The assay performance obtained by this spray technique was at least as good as that offered by the current approach.

Given sufficient time between IS addition and DMS extraction, the addition of IS solution to DMS samples prior to extraction allows the IS to become better incorporated with the sample, offering a potential advantage over widely used manual extraction techniques, with no significant difference observed in calculated accuracy and precision. The TouchSpray technology used to apply the IS proved to be well suited to the task and offered good reproducibility, as demonstrated by the validation accuracy and precision data, which were well within internationally accepted guideline criteria[68]. It was also shown that there is no significant difference in validation accuracy and precision data between using the TouchSpray and other methods of IS addition (IS incorporated into manual extraction solvent, and IS added

to control blood prior to standard preparation). This technique of IS application could be used prior to manual extraction or a compatible direct analysis technique. We are currently exploring the use of fully automated high throughput DMS direct analysis instruments[70]. We envisage an automated card handling instrument that incorporates a visual recognition system used to locate the position of DMS on sample cards. This would enable IS to be accurately applied to the DMS sample to be analyzed. This sample would then be left to dry for a suitable period of time before it was analyzed using a direct analysis technique. Minimum drying times need to be established through further testing.

The most important parameter in need of further optimization is the volume of IS solution applied. This initial evaluation has erred on the side of caution and ensured that the addition of IS solution did not cause a significant chromatographic effect in the DMS sample. However, this has probably compromised the full depth penetration of IS into the sample. In order to maximize the potential performance increases on offer, it is vital that we find a balance between full depth penetration and avoiding a chromatographic effect. It is likely that this could be achieved by further optimizing the volume applied and other parameters such as flow rates, dispensed particle size, solvent, and application distance.

Note: Supplementary information can be found in Appendix C.

CHAPTER 4: OPTIMISATION OF AN AUTOMATED INTERNAL STANDARD SPRAY ADDITION SYSTEM FOR USE IN HIGH THROUGHPUT QUANTITATIVE LC-MS/MS ANALYSIS OF DRIED BLOOD SPOT SAMPLES

At this stage in the overall project, the following conclusions had been made.

- Direct elution was the most promising and accessible technique (within the timeframe of this project) for DBS directs analysis in a regulated high sample throughput bioanalytical environment (see introduction).
- Direct elution provides improved DBS assay sensitivity across a range of representative small molecule compounds[74].
- DBS Direct elution was demonstrated to be capable of generating validation data (linearity, sensitivity, accuracy and precision) well within guideline acceptance criteria[74].
- DBS direct elution robustness data, utilising HPLC-MS/MS, demonstrated that the technique is compatible with high sample throughput bioanalysis. This proof of concept data indicates that DBS direct elution offers a level of robustness and reproducibility that is not significantly different to conventional manual extraction techniques.
- The semi-automated DBS-MS16 direct elution prototype incorporated a wash system that was demonstrated to reduce carry over to levels below that observed for manual DBS extraction using a high performance conventional autosampler.
- A 'spray-type' application that aimed to improve the integration of IS to DBS samples prior to extraction was demonstrated to be capable of producing bioanalytical validation data well within globally accepted guideline acceptance criteria. This proof of concept work suggested that this method of IS addition will be a suitable alternative to conventional methods of adding IS.

The proof of concept work proved that direct elution was a viable option, and that it was a suitable basis to develop a fully automated instrument that could meet all the criteria required to be compatible with high sample throughput regulated bioanalysis (section 1.7.1). The success of the semi-automated DBS-MS16 prototype led to the creation of the fully automated CAMAG DBS-MS500 (Figure 4.1), which was designed to meet the specifications set out in section 1.7. This instrument retains the SSSP extraction mechanism, automated wash system concept, and enhanced extraction options of the DBSMS16 and adds the following integrated functionality:

- large sample capacity (500 cards)
- Fully automated card handling (via a robotic arm)
- Intelligent visual recognition system
- Enhanced pre-extraction 'dynamic' IS addition module
- Intelligent error handling

Figure 4.1: CAMAG DBS-MS500, DBS direct elution system. Photo from CAMAG, used with permission.

When coupled to a HPLC-MS/MS rig, the DBS-MS500 creates a system that can follow the DBS direct analysis theoretical workflow proposed in this project (Figure 1.4). This direct analysis instrument now incorporates all the primary additional functionality, outlined in section 1.7, bar the ability to handle DBS dilutions. The DBS-MS500 enables the following integrated workflow:

1) Load up to 500 DBS sample cards onto the instrument deck (from this stage the process is fully automated using a robotic arm to transfer cards around the deck).
2) Samples are scanned using a camera based visual recognition system to determine the exact position of the DBS on the card (barcode information can also be read at this stage).
3) IS solution is sprayed onto DBS using a 'dynamic' spray system, and allowed to dry.
4) DBS sample is extracted (DBS-MS500 is coupled to HPLC-MS/MS instrument and communicates with data acquisition software).
5) The wash system clears contamination between sample extractions.
6) The visual recognition system records image of sample post extraction.
7) Samples returned to storage racks on deck.

The downside to this robustness and functionality is the expense, which compared to the TLC-MS is now quite considerable, although still in the same range as existing liquid handling robotics. Of course this outlay could quite quickly be made back in resource savings given enough sufficient sample throughput. CAMAG have now made this instrument commercially available, with further refinements and capabilities made based on the results of the collaboration.

Card handling automation on the DBS-MS500 has proved to be extremely reliable, with no errors observed after many thousand extractions. The DBS-MS500 visual recognition system, which scans the cards prior to IS addition and extraction, works by measuring a number of (adjustable) parameters for each sample: card type; spot size; spot roundness; and also detects if the 'reject spot' check box has been marked (see section 1.7.2). Like the DBS-MS16, the DBS-MS500 provides a number of options for sample extraction, the most important being that, unlike the TLC-MS, an optimised solvent can be utilised for sample extraction in place of the mobile phase. The DBS-MS500 aids practicality by having 4 separate solvent lines. Volume and flow rate of extraction solvent can be varied enabling extraction optimisation. Options for wash parameters are similar to the DBS-MS16. Three wash lines are available and the volume and flow rate from each can be varied to enable sample carry-over to be minimised.

4.1 Introduction

When employed as a microsampling technique, dried blood spot (DBS) analysis (a technique that has been used in areas such as new born screening for many decades) provides well documented ethical and financial benefits compared to conventionally used wet plasma analysis [25,26]. In the last few years there had been considerable interest in implementing DBS analysis into new areas and applications. Unfortunately the momentum behind introducing DBS analysis as a widely used technique into highly regulated applications (such as pharmaceutical drug development) has recently been slowed due to a number of barriers. These include uncertainty over regulatory acceptance, scientific uncertainty over assay bias caused by varying haematocrit (HCT), and also concerns relating to the increased complexity of extraction, and lower assay sensitivity associated with DBS analysis [123,70]. To try and counter some of these issues our group has recently carried out research on nullifying haematocrit (HCT) based assay bias, and maximising assay sensitivity via the development of direct elution techniques in dried blood spot (DBS) quantitative bioanalysis [81,124]. Both of these studies involved the use of an optimised technique that applied internal standard (IS) to DBS samples prior to analysis, using a commercially available spray technology. This article describes the optimisation of this IS spray system, a crucial process both in the success of the aforementioned research, and for any workflow where the spray system is to be utilised.

In conventional DBS manual extraction techniques the IS (ideally a stable isotopically labelled, or SIL-IS) is routinely added via the extraction solvent. While this simple approach is widely accepted, analytical performance is not optimal as the IS does not fully integrate with the bioanalytical sample and hence the analyte and IS are not coextracted [125,73]. Other methods

of IS addition are available that enable coextraction, such as spiking IS into whole blood prior to spot deposition, or imbuing the DBS substrate with IS prior to spot deposition, but neither of these will usually be practical options in pharmaceutical development applications [125,73]. For such applications it was previously defined that the only practical alternative was to find a reliable method of adding IS to DBS samples prior to extraction [125,73]. Ideally such a technique would deliver a highly reproducible volume of IS solution that was homogenously incorporated into the DBS sampling region, without spoiling the integrity of the sample (i.e. altering the sample in such a way that the measured analyte concentration using LC-MS/MS methodology is significantly changed).

Initial proof of concept research into spray IS applications was undertaken using a prototype device (the TouchSpray from The Technology Partnership) [125]. This work demonstrated that it was possible to generate data using this technique that met internationally recognised guideline acceptance criteria[68]. However it also demonstrated that without optimising the technique, there was a high risk of spoiling the sample integrity by flooding a localised section of the sample, which caused a chromatographic effect - a flow of sample constituents (certainly red blood cell components, and possibly analyte) outside the original DBS sample periphery [125]. The extent of this chromatographic or mass-flow effect, was proportional to the volume of IS solution added [125]. In this initial work a compromise of only adding small volumes of IS (delivered as a fine mist) to DBS samples was used. This prevented any large scale (i.e. observable by the naked eye) chromatographic effects, but it was speculated that this type of application was unlikely to penetrate the full depth of the DBS, which may limit the full integration of IS within the sample.

The work then progressed to utilising a modified dynamic spray system developed by CAMAG (and later integrated into the DBS-MS500 automated direct elution system) that applies IS in a 10x10 mm grid of IS centred over DBS samples. This IS spray system allows greater volumes of IS solution to be applied (thus, more likely penetrating the full depth of the DBS sample, and potentially enabling greater integration of IS within the sample) before the limit at which flooding occurs is reached [81,73,92,126]. In a proof of concept study, Zimmer et al demonstrated that this dynamic spray system produced a homogenous IS application within the DBS sampling area [126]. Using methanol based IS solutions, It was reported that the IS spray produced a central 8-9mm diameter homogenous application within the 10x10 mm grid, with a slight enrichment of IS at the grid perimeter. Good analytical precision (<3%) was demonstrated using spray volumes between 5 and 20 µL (measured using LC-MS/MS), which rose significantly (~6%) if 40 µL was used. Contamination of the IS between adjacent DBS was also demonstrated to be negligible.

In the current report we significantly expand on the previous proof of concept studies by testing a much larger range of variables (solvent choice, volume, and speed of application) in order to optimise the commercially available dynamic spray technique for use in regulated quantitative bioanalysis of small molecules. The aim was to identify a generic set of conditions that could be used for most small molecule analyses, which would give the best possible precision/reproducibility and, crucially, also avoid spoiling the integrity of the DBS sample.

The optimised parameters were then used in the validation of four representative small molecule LC-MS/MS bioanalytical methods to assess if the technique could produce acceptable analytical performance. Validations were carried out using sub-punch, whole spot DBS sampling, as well as both manual extraction and automated direct elution, in order to maximise the scope of the study. To our knowledge this is the first time such a study has been reported in the literature.

4.2 Experimental

Chemicals, Reagents, and other Equipment

Human volunteer control blood was collected via the GlaxoSmithKline blood donation unit (Stevenage, UK) in accordance with current GSK policies on informed consent and ethical approval.

Methanol, acetonitrile, and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd (Loughborough, UK). All other chemicals were of analytical grade and supplied by Fisher Scientific Ltd (Loughborough, UK). Paracetamol (*aka* acetaminophen), [$^{2}H_{3}$]-paracetamol, sitamaquine, [$^{2}H_{10}$]-sitamaquine, and [$^{2}H_{3}{}^{13}C_{3}$]-midazolam were obtained from GlaxoSmithKline (Stevenage, UK). Midazolam was obtained from Tocris Bioscience (Bristol, UK). Naproxen and [$^{2}H_{3}$]-naproxen were obtained from Sigma Aldrich (Pool, UK). Ahlstrom grade 226 paper for blood spots was supplied by Perkin Elmer (Buckinghamshire, UK). Sample tubes were obtained from Micronics (Sanford, USA). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat were supplied by Ted Pella (Redding, USA). Benchtop sample shaker (model HS 501 D) was supplied by Janke and Kunkel, IKA Labortechnik (Staufen, Germany). The HPLC-MS/MS system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven. MS detection was undertaken by using a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada) system equipped with a Turbo IonSpray source. HPLC-MS/MS data were acquired and processed (integrated) using Analyst software v1.6.1 (Applied Biosystems/MDS Sciex, Canada).

DBS Preparation

All control blood was shipped and stored at 4°C, used within 30 hr of collection and was artificially altered to create pooled batches with HCT 0.45, as described previously [69]. Standard preparation for the sitamaquine, paracetamol, midazolam, and naproxen assays have been described previously [81,125,74,63]. These compounds were chosen as they encompass a wide range of characteristics, particularly DBS assay recovery (SI, Table S4.1) [81]. For each assay at least 9 calibration standards were used, covering the ranges of 5-1000, 50-50000, 5-5000, and 100-10000 ng/mL for sitamaquine, paracetamol, midazolam, and naproxen, respectively. For each assay validation run, 6 replicate quality control samples were analysed

at 5 concentrations (QC levels 1-5: the assay lower limit of quantification (LLQ); 3x the LLQ; at the approximate geometric mean of the assay range; ~75% of the higher limit of quantification (HLQ); and, at the assay HLQ, respectively).

DBS samples were prepared specifically for sub-punch/sub-sample, and whole spot analysis. For sub-punch/sub-sample analysis a fixed volume (15 µL) of blood was added by pipette onto DBS cards and allowed to dry overnight at room temperature. For whole spot analysis, 2 µL of blood was accurately spotted onto DBS cards. This lower volume was used to ensure the 4 mm diameter sampling area on the DBS-MS500 could comfortably fully encompass the entire sample. If required, samples were stored under ambient laboratory conditions (21 ± 2°C) in a sealed plastic bag containing desiccant.

Manual Extraction

The generic manual extraction method involved punching a 4 mm diameter disc from a 15 µL DBS for sub-punch analysis; or punching the entire spot from a 2 µL DBS for whole spot analysis. For the control data, where IS was added via the extraction solvent, the disc was then placed in a tube and 200 µL of 70:30 (v:v) methanol:water containing the stable isotopically labelled (SIL) IS at a suitable concentration was added (10, 500, 25, and 250 ng/mL for the sitamaquine, paracetamol, midazolam, and naproxen assays, respectively) (SI, Table S4.2) [81]. The tube containing the disc was agitated on a laboratory shaker for 2 hr, centrifuged, and the supernatant transferred to a fresh tube. Analysis was then carried out by injecting the maximum volume of supernatant into the LC-MS/MS system that produced the maximum assay response with acceptable chromatography (5 µL for sitamaquine, midazolam, and naproxen, 2 µL for paracetamol).

Direct Elution

The CAMAG DBS-MS500 automated direct elution platform (CAMAG, Basel, Switzerland) was used for all direct elution experiments, and was operated using Chronos software [81]. Optimised extraction conditions were used, as described previously [124]. For each assay a dual wash solution was used between extractions to prevent carry over using the DBS-MS500 integrated wash system. The sampling apparatus was rinsed with combinations of 70:30 methanol: water (v/v) to remove any remnants of analyte, and 5:95 methanol: water (v/v) to remove matrix components [81].

Chromatographic and MS Conditions

Chromatographic conditions and MS optimisation for the sitamaquine, paracetamol, midazolam, and naproxen assays have been described previously [81,74].

IS addition

In the initial control experiments, where IS was added via the extraction solvent, the concentration of SIL-IS in the extraction solvents was optimized so that the LC-MS/MS IS response was approximately 50% of the magnitude of the analyte LC-MS/MS response at the HLQ for each assay (SI, Table S4.2). For the alternative methods of IS addition (either by spiking IS into blood prior spot deposition, or via the optimised IS Sprayer) concentrations were adjusted so the IS MS/MS responses were as close to this control level as possible, to ensure that any conclusions made were not influenced by the magnitude of the IS response [81]. For the control data where IS was spiked into whole blood prior to deposition on DBS substrate, samples were prepared by spiking a suitable volume of IS working solution into a bulk volume of whole blood (and gently mixing for 15 min) and then using sub-aliquots of this bulk to prepare calibration standards or QC's as per the normal method in place of control blood. Note that the non-matrix volume in whole blood was, in all cases, <5%.

For the test method of IS addition, the integrated CAMAG DBS-MS500 IS spray module was utilized. The visual recognition system on this instrument is designed to locate the centre of DBS samples and use this point to spray a 10×10 mm grid of IS solution over the sample. Following application of IS via the spray technique, DBS samples were left to dry for at least 24 hr prior to extraction for the optimisation phases of this work, and for at least 4 hr prior to extraction using optimised conditions[124].

LC-MS/MS Performance

To ensure all the optimisation data collected was comparable, two system suitability test (SST) methods were used to ensure that LC-MS/MS performance (which can vary dramatically from day to day, depending on a number of factors) was consistent between tests. The performance of both the direct elution instrument and LC-MS/MS system was assessed by monitoring a reference DBS extraction (10 µL of 100% methanol @ 20 µL/min) of one of the test samples prepared for this study. Performance of the LC-MS/MS system in isolation was assessed using a reference mixture of test compounds (Abu-Rabie, unpublished). The manual extraction or direct elution response was required to be within ±15% of the reference response to be acceptable. When the SST fell outside this limit, action was taken to identify what was causing the variation in performance, and once rectified the SST was repeated.

Area Bias (ImageJ)

DBS area bias with varying HCT was assessed by measuring the area of DBS using ImageJ software, as reported previously [81,69]. During IS optimisation, the areas of the DBS were measured before and after IS spray addition.

4.3 Results and Discussion

The IS spray module parameters were optimised using three experimental phases. The first phase was an initial screen to eliminate any spray conditions that clearly spoilt the integrity of the DBS sample. The second phase determined the analytical reproducibility (precision) of the assays when the parameters that made it past the initial screen were used, as well as further investigating if the spray application had any effect on the integrity of the sample. In the third and final phase, the conditions that produced the best analytical results from the previous tests were used to perform validation studies (using methods that had previously been validated using conventional IS addition via the extraction solvent). This tested if the optimised IS spray application technique was capable of producing analytical data across a full validation study within internationally accepted guideline acceptance criteria[68], and how it compared to identical methods using conventional IS addition.

Phase 1: Sample integrity screen

The first phase of the optimisation was to eliminate solvent/volume combinations which clearly compromised the integrity of the DBS sample. Previous work had shown that large volumes of solvent sprayed onto DBS could flood localised areas of the sample and cause the constituents of the sample to flow outside of the original DBS perimeter [125]. In this project, IS was applied to DBS samples using the IS module integrated to the CAMAG DBS-MS500 DBS direct elution instrument. This module uses a dynamic IS spray system in place of the static spray apparatus reported previously [92]. Rather than applying the IS solution in a single spray arc covering the entire DBS, this system applies a much finer stream, covering only a very small section of the sample (spray arc diameter less than 2 mm), and uses automation to move the sample relative to the spraying device (a dynamic spray application) (Figure 4.1). The result is a 10×10 mm grid of IS solution applied centred around the DBS. This system allows greater volumes of IS solution to be applied (thus, more likely penetrating the full depth of the DBS sample, and potentially enabling greater integration of IS within the sample) before the limit at which flooding occurs is reached [73,81,126].

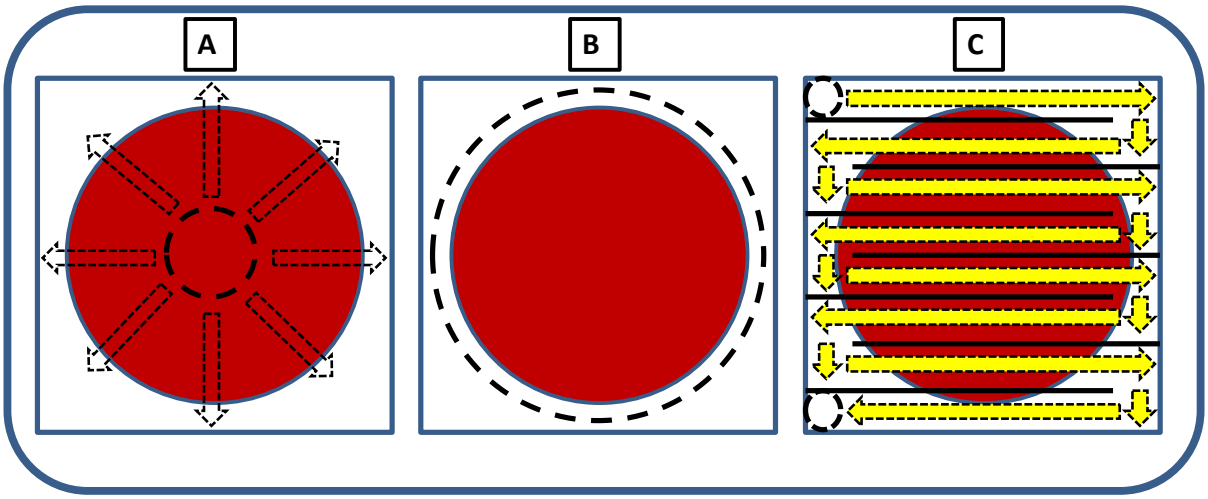


***Figure 4.2: Methods of IS addition via static and dynamic spray type technologies**. The dashed lines represents the solvent spray arc diameter that initially impacts DBS surface. (a) To cover an entire DBS using a static spray arc smaller than the DBS diameter, the DBS has to be flooded and rely on a wicking effect to reach the perimeter of the DBS. This causes a significant chromatographic effect of the sample components which can effect analyte concentration for some compounds. (b) A larger diameter static spray arc can be used to cover the entire DBS. Lower application volumes (~8 µL) avoid the undesirable chromatographic effect but can limit IS penetration into the full depth of the DBS. It may be possible to reach an effective compromise using this technique. (c) A Dynamic spray application, as used in this study, can be used to apply larger IS volumes (up to ~40 µL, thus more likely to penetrate the depth of the DBS) without causing a significant chromatographic effect.*

Nine different solvents were sprayed onto blank blood DBS samples using 5 different volumes (5 to 40 µL), using both the slow and fast application setting on the DBS-MS500. The area of each DBS was measured using imageJ before and after each IS application (after a 24 hr drying time). As described previously, a large volume addition of IS solution onto a small localised area of the DBS appears to 'flood' that region of the sample i.e. saturate that region of the DBS [125]. Any additional IS solution added to the saturated area then flows into the surrounding areas (Figure 4.2a). If sufficient IS volume is added, a chromatographic effect occurs whereby dried blood components in the sample can be visually observed to flow outside the original perimeter of the DBS, ultimately producing a sample spread over a greater area of substrate. Clearly any technique that spoils the integrity of the sample (and thus risks altering the measured quantity of analyte with the sampling area) is not suitable for use in quantitative bioanalysis. Measuring the area of the sample before and after IS addition was thus used to determine if sample integrity was spoilt. This test acted as an initial screen to identify which IS spray parameters could either be immediately rejected, or investigated further. Eight replicates were taken for each solvent/volume/application speed combination, and the mean percent increase in spot area after IS addition was calculated (Table 4.1). Differences in area of less than 5% were deemed to be within experimental error, and thus not significant, in line with previous experience of using ImageJ to measure DBS areas [69]. As an additional screen, the spots were also visually inspected to look for signs that the integrity if the sample had been visually affected (Table 4.2). It is noted that a visual inspection is a subjective measure, and

thus it has only been used in conjunction with other (non-subjective) data, and not used in isolation to make any critical judgements.

In practice, any distortion of the original sample periphery measured by area calculations appeared to also be easy to spot with the naked eye, and the results of the visual inspection closely matched the area measurements. It was noted that in almost all cases that a maximum limit of 7 µL of aqueous component of the IS spray could be applied before DBS sample integrity was compromised (Table 4.3). The only exception was 100% DMSO, where volumes of 30 µL and above compromised the integrity of the DBS sample, without any aqueous component. In all of the tests, there was no noticeable difference between using the fast and slow IS application (which took either ~10 s in fast, or ~20 s in slow, to apply the same volume of IS solution) so from that point forward, only the fast setting was used for all further investigations. While the area evaluation and visual experiments cannot guarantee whether measured analyte concentration using LC-MS/MS methodology will be significantly changed, it was used to eliminate parameters that clearly risked DBS sample integrity. Based on the results of the above tests, 27 solvent/volume combinations were rejected, and 18 were taken forward for further optimisation (those which showed an area increase of <5%) (Table 4.1, SI Table S4.3).

It is notable that previous research indicated that despite DBS appearing to be touch dry within ~30 min following IS spray application (using 20 µL of a methanol based solution), the sample may not be at equilibrium until after 150 min[124]. However this previous work also demonstrated that in practice, using only a 10 minute drying time after IS application (again spraying 20 µL of a methanol based solution) produced acceptable analytical performance, and the IS still integrated sufficiently with DBSs to allow HCT based recovery bias to be nullified[124]. As larger spray volumes were tested, and the effects of using other solvents were unknown in this study, a drying time of 24 hr was used in the phase 1 screening tests.

The physical mechanism of blood absorption onto DBS substrate, and microscopic analysis of how the dried sample then reacts to subsequent addition of IS solution, was not investigated as part of this study, though other groups have studied similar situations. Starov et al considered the process of liquid droplets spreading over a porous layer as consisting of two different and simultaneously occurring processes: blood spreading over substrate; and capillary motion inside the substrate [127,128]. Chao et al then investigated the application of this theory to the mechanism of blood spreading on cellulose substrate [129]. This work suggested that red blood cells blocked micro-pores within DBS substrate which influenced the further penetration of plasma into adjacent pores. There does not appear to be any literature on the redistribution mechanism of components that occurs when solvent is added to DBSs. In the absence of further information on these microscopic mechanisms, the conclusions in this study rely on area measurements and the end results of LC-MS/MS methodology, but further work in this area is desirable and would enhance our understanding of the processes that are occurring.

Table 4.1: Percent increase in blank blood DBS area after spray application. Areas were measured using imageJ software. Differences in area of less than 5% were deemed to be within experimental error, and thus not significant. The solvent/volume parameters coloured green in the table did not demonstrate a significant increase in area, and were taken through to the next stage of testing. Those coloured red showed a significant increase in area post IS spray application (indicating sample integrity had been spoilt), and were not considered further.

IS Spray Volume/ µL	Spray application speed	% Difference in spot area after IS addition (mean value n=8)								
		Water	Methanol	MeOH:H_2O 70:30	MeOH:H2O 50:50	MeOH:H2O 25:75	ACN	ACN:H2O 50:50	DMSO	DMSO:H2O 50:50
5	Fast	1.8	1.5	-0.3	-1.8	-0.5	-0.8	-1.4	-1.4	-1.6
	Slow	3.6	1.3	1.7	2.1	3.1	1.7	2.5	1.1	1.1
10	Fast	45.9	-2.4	-1.3	1.2	22.2	-1.8	-0.1	-0.1	-0.1
	Slow	33.3	0.4	3.5	3.5	16.6	1.1	3.9	0.5	1.6
20	Fast	161.8	-3.2	0.3	77.4	73.7	-2.0	36.1	0.7	16.1
	Slow	128.5	0.2	3.7	64.5	83.9	1.4	29.3	3.8	33.0
30	Fast	407.7	-3.0	13.9	80.5	167.5	-1.6	70.1	19.2	48.1
	Slow	290.6	-0.6	85.0	57.0	110.4	0.2	55.5	29.2	24.0
40	Fast	388.1	-0.7	51.5	107.6	224.2	0.1	70.2	52.4	116.4
	Slow	364.6	-0.7	52.7	168.6	229.0	0.7	98.1	95.0	155.6

No significant area increase measured post IS spray application (<5%)
Significant area increase measured post IS spray application (>5%)

Table 4.2: Visual inspection of changes to DBS following IS spray addition.

IS Spray Volume/ µL	Spray application speed	Visual description after IS addition								
		Water	Methanol	MeOH:H2O 70:30	MeOH:H2O 50:50	MeOH:H2O 25:75	ACN	ACN:H2O 50:50	DMSO	DMSO:H2O 50:50
5	Fast	2	1	1	1	1	1	1	1	1
	Slow	2	1	1	1	1	1	1	1	1
10	Fast	3	1	1	2	3	1	2	1	2
	Slow	3	1	1	2	2	1	2	1	2
20	Fast	4	1	2	3	4	1	3	2	3
	Slow	4	1	2	3	4	1	3	2	3
30	Fast	4	1	3	4	4	1	3	3	3
	Slow	4	1	3	4	4	1	3	3	3
40	Fast	4	1	3	4	4	1	3	4	4
	Slow	4	1	3	4	4	1	3	4	4

1 No visual effect
2 Minor distortion around spot pheriphery (less than 1mm distortion of pheriphery)
3 Noticable distortion of spot pheriphery (less than 3mm distortion from original spot periphery)
4 Major distortion of spot pheriphery (more than 3mm distortion from original spot periphery)

Table 4.3: Aqueous volume component of IS spray dispensed onto DBS. Green area overlaid on table indicate the solvent/volume combinations.

IS Spray Volume/ µL	Spray application speed	IS Spray Aqueous Volume Component Dispensed/µL								
		Water	Methanol	MeOH:H2O 70:30	MeOH:H2O 50:50	MeOH:H2O 25:75	ACN	ACN:H2O 50:50	DMSO	DMSO:H2O 50:50
5	Fast	5.00	0.00	1.50	2.50	3.75	0.00	2.50	0.00	2.50
	Slow	5.00	0.00	1.50	2.50	3.75	0.00	2.50	0.00	2.50
10	Fast	10.00	0.00	3.00	5.00	7.50	0.00	5.00	0.00	5.00
	Slow	10.00	0.00	3.00	5.00	7.50	0.00	5.00	0.00	5.00
20	Fast	20.00	0.00	6.00	10.00	15.00	0.00	10.00	0.00	10.00
	Slow	20.00	0.00	6.00	10.00	15.00	0.00	10.00	0.00	10.00
30	Fast	30.00	0.00	9.00	15.00	22.50	0.00	15.00	0.00	15.00
	Slow	30.00	0.00	9.00	15.00	22.50	0.00	15.00	0.00	15.00
40	Fast	40.00	0.00	12.00	20.00	30.00	0.00	20.00	0.00	20.00
	Slow	40.00	0.00	12.00	20.00	30.00	0.00	20.00	0.00	20.00

No significant area increase measured post IS spray application (<5%)
Significant area increase measured post IS spray application (>5%)

Phase 2: Analytical precision/reproducibility

The second phase of optimisation was carried out by investigating which solvent/volume combinations for IS application gave the best analytical performance, while further investigating if IS spray application affected the integrity of the DBS sample. To do this, batches of samples were prepared containing 3 DBS sample types: total blanks (blank DBS substrate containing no blood); blood blanks (control blank DBSs not spiked with any compound); and DBS QC samples (spiked at ~75% of the assay HLQ i.e. QC4). 18 separate batches (per analyte) were run, each one using one of the solvent/volume IS spray combinations carried forward from the initial screen, to apply IS to the above samples (SI Table 4.3). Also in each run, as controls, the above samples were prepared where no IS was added at all, and where IS was spiked into whole blood prior to spotting (12 replicates of each sample type).

These samples were taken through the manual, sub punch extraction method, and analysed using the LC-MS/MS methods described above. For each sample type, mean analyte response (where applicable), IS response, and peak area ratio (where applicable) were recorded, and the mean precisions of each value were calculated to evaluate reliability and reproducibility. To gain an understanding of how the presence of analyte, and the method of IS addition affect the magnitude of LC-MS/MS responses, the intra-run means (mean LC-MS/MS response of that sample type within an individual run) of these values were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches) [SI, Tables S4.4-4.11]. A summary of the data for the sitamaquine assay is shown in Supplementary Information Table S4.12, and Figure 4.3. The control data, where IS was spiked into whole blood before spotting the DBS, was used to provide a guide to the reproducibility, accuracy and precision expected when the IS is fully incorporated with the blood components [81,73,20]. The spray test data was then evaluated to see which IS spray parameters provided the performance closest to this ideal, and if IS spray application had any effect on the measured analyte concentration (i.e. potentially any effect on the integrity of the DBS sample).

For the sitamaquine data (Figure 4.3), the IS response of the control data provides a guide to the batch to batch response variance typically observed with LC-MS/MS analysis using conventional DBS extraction methods. The control method of IS addition gave an intra-run IS response CV range of between 4.2 and 20.0% (for blank blood), and 2.3 to 9.1% (for QC4). Control data between run CV's were 9.4% and 6.6% (for blank blood and QC4, respectively). Control data intra-run peak area ratio CV range was 1.0 to 8.9%, with a between run CV of 5.6% (QC4 (IS in blood).

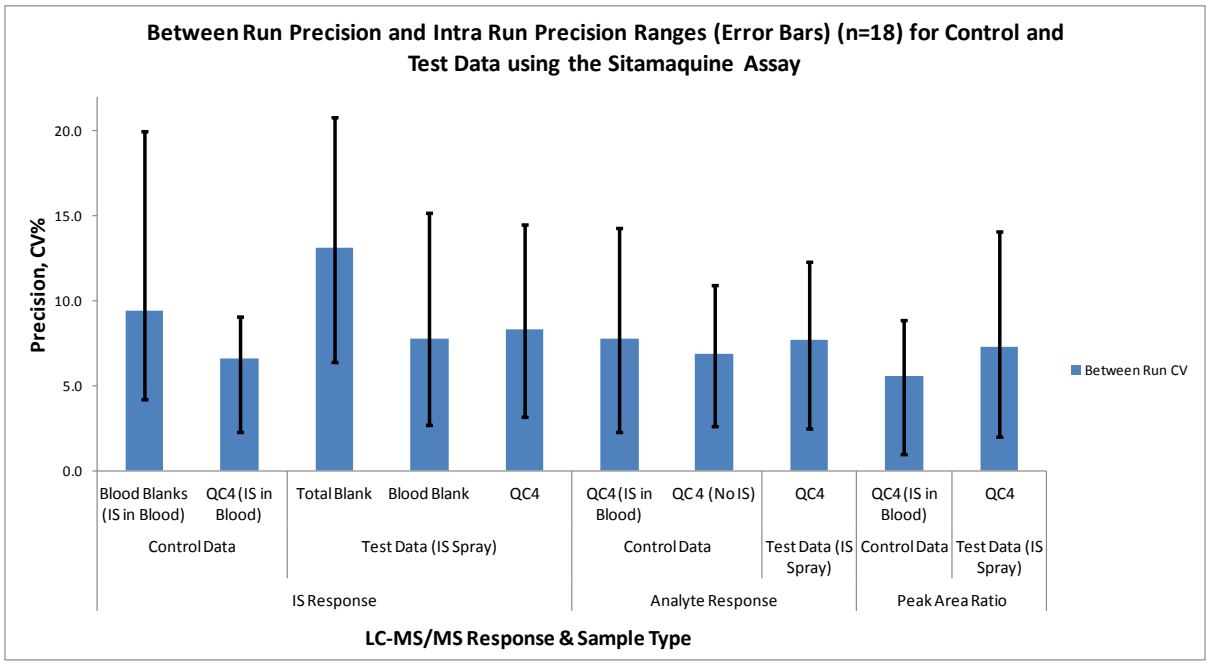


Figure 4.3: Summary of the control and test IS Spray optimisation precision data using the sitamaquine assay. A total of 18 batches were run using 18 different IS spray volume/solvent combinations (test data). In each batch, control data was also generated where IS was spiked into whole blood prior to spot deposition, or not added at all. In each run, 12 replicates of each sample type were included. Blocks (blue) show the between run precision (n=18); 'error bars' show the corresponding intra run precision range (the highest and lowest inter run mean response across the 18 runs, as a percentage of the between run mean response).

In comparison, the IS spray test data showed CV's very close to what was observed for the control data (intra-run CV range, and between run CV's were 6.4 to 20.8% and 13.1%; 2.7 to 15.2% and 7.8%; and 3.2 to 14.5% and 8.3%, for total blank, blood blank, and QC4 samples, respectively) (Figure 4.3). The control data and test data were not notably different, indicating that the reproducibility of using IS spray application, across nearly all the combinations tested, is likely to match that of an ideal IS application (IS in blood). The much wider intra-run mean IS response range observed for IS spray applications is due to the range of IS spray addition volumes applied which obviously had an effect on the total IS deposited on the samples.

The QC4 intra run range of analyte responses were 66.8-160.3%, 75.6-141.4%, and 64.9-154.9% (of between run mean responses) for IS in blood, no IS added, and IS added via spray, respectively. These mean measured analyte responses, and their corresponding mean CV values across these three IS variants (Figure 4.3) were remarkably close, indicating that the IS spray addition was not noticeably affecting the integrity of the DBS sample (in terms of altering the amount of analyte available for analysis). The range of analyte response intra-run and between run CV's for the three IS variants were also very similar (Figure 4.3), indicating that the IS spray technique did not inhibit the production of precise analytical data (the lowest intra-run analyte response CV's for no IS, IS in blood, and IS spray, were 2.6%, 2.3% and 2.5%, respectively).

Comparing the peak area ratio CV's for the control and test data demonstrated that the best performing spray parameters were able to achieve intra-run CV's (range 2.0-14.1%) very close to the range observed where IS was added to blood (1.0 to 8.9%) (Figure 4.3 and SI, Table S4.12). Overall, the test data shows that an optimised IS spraying technique is capable of producing analytical reliability and precision that is comparable to what can be achieved using the ideal method of IS addition (IS in whole blood prior to spotting).

The above IS optimisation experiment was repeated for the paracetamol, midazolam and naproxen assays, and generated very similar data, providing further confidence to the conclusions made above (Supplementary Information Tables S4.13-15). It was clear from the above results that a number of the solvent/volume spray combinations were capable of providing sufficiently good analytical performance to be a realistic alternative to conventional IS addition via the extraction solvent. To simplify the data and help select the best candidate, the data was ranked in order of best analytical precision (i.e. lowest intra-run CV) for each response and sample type (SI, Table S4.16). A number of solvent/volume combinations gave similar results, but overall using 20 µL of methanol containing SIL IS was the most consistent performer, so this was chosen as the optimal combination to use for the remainder of this project.

Phase 3: Test validations using optimised spray conditions

To gain further confidence in the optimisation, the final phase of testing involved taking 4 representative small molecule quantitative bioanalytical assays through a typical validation procedure using the optimised spray parameters to apply IS. The accuracy and precision of each assay was investigated over three consecutive runs. Each validation batch consisted of two calibration lines bracketing QC samples (5 concentration levels, 6 replicates). This procedure was then repeated using the typical conventional method of IS addition (via the extraction solvent), and an ideal but impractical method of IS addition (spiked into whole blood prior to spot deposition), to produce a direct comparison of these three methods of IS addition. To maximise the scope of this work, validations were carried out using manual extraction and direct elution techniques, and using both sub punch, and whole spot extraction methodology. For the phase 3 validation experiments using optimised conditions, a minimum drying time after IS spray application of 4 hr was used before extraction. Previous work has indicated that the DBS sample should be at equilibrium after this drying time[124].

All accuracy and precision values across the three different methods of IS addition were within internationally accepted guideline acceptance criteria (Tables 4.4 and 4.5) [68]. Note that these accuracy and precision values combine the variability of the IS addition methods with the additional variability of the extraction and LC–MS/MS analysis process. Overall there was little difference in analytical performance between the three methods of IS addition, demonstrating that the optimised IS spray technique was capable of producing analytical data to a suitable standard for use in regulated quantitative bioanalysis [68].

Subsequent work has been undertaken using this IS spray technique that also demonstrated that analytical data can be generated within internationally accepted guideline acceptance criteria [81,124]. Other groups have also demonstrated some success using spray technology to apply IS. For example, in a limited range of experiments Van Baar et al also showed that the IS spray technique could produce good analytical performance, similar to that obtained using conventional methods of IS addition [20].

Table 4.4: Mean accuracy and precision of four DBS assays using sub-punch analysis (n=18; 6 replicates at each concentration over 3 validation runs). Data was generated using 3 methods of Internal Standard (IS) addition ((i) The conventional method via the extraction solvent; (ii) IS spiked into control blood prior to deposition onto substrate; (iii) IS added via the optimised sprayer prior to extraction), and 2 methods of extraction (conventional manual extraction, and direct elution).

Method of IS addition		IS added via extraction solvent				IS spiked into control blood prior to spot deposition				IS added via optimised spray prior to extraction			
Extraction method		Manual extraction		Direct elution		Manual extraction		Direct elution		Manual extraction		Direct elution	
Compound	QC Level	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %
Sitamaquine	QC1	2.9	5.4	-1.3	6.7	4.0	6.0	9.4	6.6	6.3	8.8	3.4	10.0
	QC2	2.6	5.7	10.7	4.7	7.1	4.2	3.9	5.4	5.9	4.2	4.6	3.1
	QC3	-0.2	5.2	-0.5	3.7	3.0	5.6	5.1	5.0	2.4	3.9	3.9	5.2
	QC4	-7.0	3.9	-9.5	3.4	-4.3	5.1	-1.9	5.3	-0.6	3.6	1.1	6.1
	QC5	-4.4	5.7	-1.0	5.2	-4.6	2.8	-0.6	5.0	.2.1	4.8	-2.0	4.0
Paracetamol	QC1	9.5	4.0	-5.3	3.6	7.1	4.9	3.5	6.7	8.1	6.2	4.5	7.1
	QC2	-9.2	4.8	-9.3	7.7	4.5	7.0	4.1	4.7	6.0	5.6	10.1	5.4
	QC3	-13.1	5.5	-11.1	6.6	-2.1	3.9	1.2	5.4	-4.3	5.9	3.2	3.9
	QC4	1.5	6.5	-6.4	9.0	-4.3	4.7	-6.1	6.5	-6.7	5.1	2.4	4.7
	QC5	6.4	6.8	7.1	6.4	-5.7	4.9	-3.3	4.8	-6.3	4.8	-5.3	6.8
Midazolam	QC1	8.2	9.3	6.0	11.4	5.0	9.0	6.5	8.9	-3.1	10.5	3.0	8.8
	QC2	6.1	5.6	4.5	5.5	6.1	4.9	4.8	4.6	4.6	3.9	8.2	6.3
	QC3	4.3	5.0	9.0	7.0	5.5	5.0	4.5	1.5	7.8	4.6	5.6	5.6
	QC4	-0.5	6.1	-4.4	4.3	7.0	3.7	8.2	0.9	-6.2	5.7	-0.5	6.8
	QC5	-3.7	5.7	-7.1	3.7	4.1	3.2	9.1	3.8	-3.4	2.9	-5.6	3.9
Naproxen	QC1	-5.4	11.2	-4.2	9.5	-1.5	7.0	3.1	8.3	2.1	10.3	1.2	7.0
	QC2	-3.5	4.3	-1.4	6.7	3.2	6.0	4.3	5.1	-2.5	7.8	-4.2	6.1
	QC3	5.4	4.3	4.3	5.4	6.1	3.7	4.5	5.6	4.1	9.2	2.5	5.8
	QC4	-2.3	3.7	-5.5	5.3	1.2	4.4	-5.5	4.8	2.3	5.4	-4.4	5.6
	QC5	-6.1	5.2	-8.1	3.2	-4.3	5.9	-4.9	2.9	1.0	5.2	-10.2	6.0
Mean		-0.4	5.7	-1.7	6.0	1.9	5.1	2.5	5.1	0.9	5.9	1.1	5.9
Minimum value		-13.1	3.7	-11.1	3.2	-5.7	2.8	-6.1	0.9	-6.7	2.9	-10.2	3.1
Maximum value		9.5	11.2	10.7	11.4	7.1	9.0	9.4	8.9	8.1	10.5	10.1	10.0

Experiments to investigate carry-over to adjacent DBS samples (on the same card) were also performed as part of this study, repeating the work Zimmer et al carried out previously on a prototype version of the spray apparatus [126]. As per the rest of this study, Ahlstrom 226 cards were used containing four identical DBS samples (numbered 1-4, from left to right). On each card, samples 1 and 3 were sprayed with IS (using optimised conditions), while the two adjacent spots (2 and 4) had no IS added. This was carried out on 10 cards for each test assay. On every card tested no IS peak was detected when the samples in positions 2 and 4 were analysed, indicating that no significant carry over occurred using the IS spray technology with the assays tested in this study. This is a factor that should be investigated on an assay by assay basis, as it is possible that carry over could be detected in methods where higher concentrations of IS, and lower limits of quantitation are used.

Table 4.5: Mean accuracy and precision of four DBS assays using whole-spot analysis (n=18; 6 replicates at each concentration over 3 validation runs). Data was generated using 3 methods of Internal Standard (IS) addition ((i) The conventional method via the extraction solvent; (ii) IS spiked into control blood prior to deposition onto substrate; (iii) IS added via the optimised sprayer prior to extraction), and 2 methods of extraction (conventional manual extraction, and direct elution).

Method of IS addition		IS added via extraction solvent				IS spiked into control blood prior to spot deposition				IS added via optimised spray prior to extraction			
Extraction method		Manual extraction		Direct elution		Manual extraction		Direct elution		Manual extraction		Direct elution	
Compound	QC Level	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %	Bias %	Precision CV %
Sitamaquine	QC1	-1.2	7.0	5.7	8.9	-4.6	10.2	9.4	13.4	3.8	9.4	-4.1	8.9
	QC2	-1.7	4.3	8.6	4.7	-4.3	6.3	5.5	4.0	-6.1	4.5	2.7	2.2
	QC3	-0.3	5.6	8.3	3.9	2.5	2.9	-4.3	5.6	-2.4	5.0	0.8	2.9
	QC4	-8.3	3.1	4.2	4.0	-4.3	3.8	-6.6	5.9	-6.9	3.1	1.4	1.9
	QC5	-5.4	3.3	11.1	5.7	-7.0	4.1	-8.9	3.9	-10.8	5.9	-3.4	4.6
Paracetamol	QC1	10.0	11.2	9.2	8.9	7.7	8.9	-9.0	9.9	5.6	8.6	0.4	10.8
	QC2	-5.9	8.5	-4.9	5.1	7.9	3.4	-7.8	3.1	3.1	5.4	1.4	4.3
	QC3	-9.9	6.0	0.4	4.5	-5.4	3.9	5.4	4.8	-0.4	5.9	-9.4	4.9
	QC4	8.4	5.7	12.1	4.9	-8.2	4.8	-7.1	4.1	-1.2	4.6	-7.6	5.3
	QC5	10.4	4.5	9.7	5.2	-11.5	5.9	-10.5	5.6	-4.9	6.0	-3.6	4.0
Midazolam	QC1	-6.5	9.5	4.2	11.4	4.2	8.7	-5.1	9.7	11.1	11.2	-8.4	8.0
	QC2	1.2	3.0	1.2	5.0	-5.4	4.1	-5.6	3.9	6.9	3.9	-3.2	5.4
	QC3	0.9	3.1	2.5	5.4	-12.3	3.8	2.4	3.5	0.3	4.8	4.6	5.2
	QC4	-2.9	3.7	-5.6	4.5	-9.9	4.4	-10.5	3.5	-7.8	3.0	-0.9	5.6
	QC5	-4.8	4.5	-11.7	6.1	-5.3	5.7	-2.5	4.2	-1.4	6.1	1.4	4.0
Naproxen	QC1	4.9	10.9	-1.3	12.1	0.2	8.0	4.3	12.2	1.3	10.6	4.5	10.1
	QC2	6.2	7.7	-2.4	5.6	0.9	2.4	3.6	4.5	-3.9	5.4	7.4	5.3
	QC3	5.7	5.5	-5.6	6.6	-3.9	2.0	6.7	3.8	4.5	5.1	-5.5	4.9
	QC4	6.0	5.1	-8.1	3.6	5.6	3.9	0.3	4.3	-7.8	6.0	-3.1	4.9
	QC5	-2.3	4.9	-7.9	4.9	1.5	6.1	-2.9	4.9	-11.1	4.5	-0.9	6.3
Mean		0.2	5.9	1.5	6.1	-2.6	5.2	-2.2	5.7	-1.4	6.0	-1.3	5.5
Minimum value		-9.9	3.0	-11.7	3.6	-12.3	2.0	-10.5	3.1	-11.1	3.0	-9.4	1.9
Maximum value		10.4	11.2	12.1	12.1	7.9	10.2	9.4	13.4	11.1	11.2	7.4	10.8

4.4 Conclusions

The work reported in this study has identified optimised conditions for an automated dried blood spot internal standard spray addition system. It has been demonstrated that the optimised technique is capable of delivering a highly reproducible volume of IS solution, without compromising sample integrity. Subsequent research, that relied on this optimisation, demonstrated that when the IS is applied in this manner it integrates sufficiently with the DBS sample to enable analyte and IS coextraction[81,124]. It is well established that when using conventional DBS IS addition techniques (via the extraction solvent) significant assay bias with varying haematocrit (HCT) can occur[69]. This phenomenon, coupled with other issues such as regulatory approval, a reluctance to switch to DBS assays due to perceived increase in extraction complexity, and decreased assay sensitivity, has led to reluctance in using DBSs to support quantitative bioanalytical studies in regulated environments. Consequently, the potential ethical and financial advantages of DBSs are currently not being fulfilled. When coupled to automated whole spot direct elution, the optimised IS spray addition technique described here has been demonstrated to both effectively nullify HCT based assay bias (through analyte and IS coextractrion), and the manual extraction burden associated with DBS bioanalysis [81,124]. It is hoped that the optimisation parameters detailed in this article will offer DBS users a starting point for creating new DBS methodology that is able to counter the current issues with the technique. While the above issues in regulated quantitative bioanalysis are

being addressed, it is hoped in the meantime that the techniques described here can also be applied to other applications where there are less barriers to acceptance, and the advantages can be reaped in a shorter timescale.

Note: Supplementary information can be found in Appendix D.

CHAPTER 5: INVESTIGATION OF DIFFERENT APPROACHES TO INCORPORATING INTERNAL STANDARD IN DBS QUANTITATIVE BIOANALYTICAL WORKFLOWS AND THEIR EFFECT ON NULLIFYING HAEMATOCRIT BASED ASSAY BIAS

5.1 Introduction

Work carried out by Barfield *et al* and Spooner *et al* sparked considerable interest in implementing dried blood spot (DBS) analysis (a technique that has been used in areas such as new born screening for many decades) within the pharmaceutical industry for the development of new drugs[25,26]. DBS analysis as a microsampling technique provides well documented ethical and financial benefits compared to conventional wet plasma analysis[25,26]. However, recently the momentum behind introducing DBS analysis into highly regulated applications (such as pharmaceutical drug development) has hit a significant bottleneck due to a number of factors. The primary issues are uncertainty over regulatory acceptance, and scientific uncertainty over assay bias caused by varying haematocrit (HCT); while secondary issues include barriers to bioanalytical acceptance caused by the increased complexity of extraction, and lower assay sensitivity associated with DBS analysis[123,70,23].

The work detailed in this article demonstrates the effects of HCT based assay bias in DBS quantitative bioanalysis, and investigates if alterations to current workflows using existing technology can offer solutions to these analytical issues. To understand how varying HCT affects assays that measure analyte concentrations in DBS samples, one must be familiar with the workflow that was initially proposed for DBS analysis[25]. In brief, an approximate volume of whole blood (typically we used 15 µL ±5 µL) would be applied to a cellulose based paper substrate (a DBS 'card') in the clinic. The DBS sample would then be allowed to dry, and transported to the site of analysis. In this initial workflow, it was thought that an accurate volume of sample could then be analysed by taking a fixed diameter sub-sample (e.g. a 4 mm punched disc from a ~8 mm diameter 15 µL DBS) from the centre of the original DBS sample. This sub-punch would then be manually extracted, typically by adding ~200 µL of highly organic solvent (e.g. 70:30 methanol: water (v:v)) containing internal standard (IS) (the stable isotopically labelled (SIL) version of the target analyte); agitating the sample for approximately 1 hr; followed by centrifuging the sample; and aliquotting the supernatant into a fresh tube. A small volume (typically 2-10 µL) of the resulting extract would then be injected onto a liquid chromatography-tandem mass spectrometric (LC-MS/MS) system for analysis. Analyte concentrations are ultimately calculated using peak area ratio values (the area under the analyte chromatographic peak divided by the IS area). The problem with the aforementioned workflow is that blood HCT values at extreme levels can cause an assay bias which, if significant, can have a major effect on the confidence we have in the technique. The HCT value (also known as packed cell volume, or erythrocyte volume fraction) is the proportion of blood volume that is occupied by red blood cells, and is typically 0.41-0.51 for men and 0.37-0.47 for women[93].

HCT can vary beyond these typical levels in certain populations (e.g. neonates and children), and disease states.

In this article we test the theory that the chief components of HCT based overall assay bias are area bias, recovery bias and suppression bias. The area bias effects of varying HCT on DBS are well understood and well documented in the literature, but are described briefly here for those unfamiliar with this issue[69,21]. HCT is directly proportional to the viscosity of blood. Thus, a given volume of blood with a higher HCT (e.g. >0.45) tends to spread over cellulose-based DBS substrate more slowly, and ultimately produces a DBS with a smaller area (compared to the calibration standards made up with control blood, at a standard HCT e.g. 0.45). When a fixed diameter sub-punch is taken for analysis from this smaller diameter spot, a larger proportion of this sample is therefore taken, which creates a positive assay bias, and in this case the calculated concentration of circulating analyte (or drug/compound of interest) in the sample will be overestimated. To overcome this issue, whole-spot extraction methods have been introduced which eliminate area bias by punching out the entire DBS, rather than a sub-punch. To facilitate this workflow an accurate volume of blood must be spotted at the clinic. Note that it was initially envisioned that obtaining an accurate volume in the clinic by untrained (in bioanalysis) personal would be difficult to achieve with any confidence, and potentially expensive (accurate volume pipettes would have to be supplied to multiple clinical sites per study, calibrated, and the staff trained to use them). The requirement for accurate volume blood dispensing clearly compromises the simplicity of the original workflow that was envisioned for DBS, but the potential combined ethical, financial and logistic advantages on offer from DBS sampling still far outweigh the extra complexity and cost associated with an accurate volume DBS workflow, if practical accurate volume collection methods can be utilised[23,59]. In the last few years, new technology and whole blood dispensing techniques have been reported that potentially make accurate volume spotting, and whole-spot sample extraction options a realistic proposition[84,85,86,61,64,65,63]. New blood collection technologies such as the DBS-System (from Déglon et al), and Mitra or volumeric absorptive microsampling (VAMS) (from Phenomenex, albeit in a different format to card based DBS) demonstrate that practical, and cost effective solutions to accurate volume blood microsampling are available[63,130]. We acknowledge that recent issues with regulatory acceptance and uncertainty over HCT based bias have stalled the progress of DBS in many new applications, and thus the widespread use of these accurate volume workflows are largely untested in practice. An accurate volume DBS workflow is used throughout the work described in this article. It is hoped that this article will provide guidance to DBS users in the future where accurate volume DBS workflows are used, as the technology to support this workflow is now available. HCT based recovery bias is much less well recognised and understood than area bias, with few reports in the literature[131,20]. To our knowledge this is the first time an in depth investigation and potential solution to this phenomenon has been reported in the literature. We believe this is a major oversight as we demonstrate that recovery bias has the potential to cause significant assay bias, and anyone performing DBS analysis should be aware of the risk.

HCT based recovery bias in the conventional DBS extraction process (described above) occurs because the IS does not correct for variations in recovery (caused by variable HCT). In an ideal assay the IS would compensate for any variability or losses during extraction, and correct for variable LC-MS/MS performance, and variations in sample injection volume. However when the IS is added to the DBS sample via the extraction solvent the IS is not integrated into the matrix and substrate components of the sample prior to extraction, and is therefore not co-extracted with the analyte as part of the sample. This means that if varying HCT causes a recovery bias, the IS will not correct for this (the recovery bias affects the analyte only, not the IS), and the analyte concentration calculated using peak area ratio will be incorrect. Since the analyte/IS ratio is fixed at the time of the IS addition it only compensates for any variables after that point (e.g. variability in LC-MS/MS performance, and variation in sample injection volume).

Co-spiking IS into the whole blood (in the same way analyte is added to whole blood in the preparation of calibration and quality control samples) before it is spotted onto the paper substrate results in analyte and IS co-extraction, and allows the IS to correct for recovery bias. It should be noted that co-extraction would not eliminate HCT based recovery bias, but since both analyte and IS (provided it is a SIL) would suffer the same extraction efficiencies, the effect will be nullified.

In this research, the two methods of IS addition described above (conventional addition via the extraction solvent; and, co-spiking IS into whole blood prior to deposition onto DBS substrate) were used to generate control data. The conventional method of IS addition via the extraction solvent (where IS is not co-extracted with the analyte) provides data where HCT based recovery bias will occur. The other end of the spectrum is where IS is co-spiked (with analyte) into whole blood prior to deposition on DBS substrate. This ensures analyte and IS are fully and equally integrated into DBS samples prior to extraction, resulting in analyte and IS co-extraction, which nullifies the effects of HCT based recovery bias. Clearly, co-spiking blood with IS prior to applying it to the substrate will not be a practical solution for most DBS bioanalytical workflows. The use of a novel spray method for applying IS to DBS at the site of analysis, prior to extraction was previously reported[125]. For many high sample throughput drug development applications this type of IS addition will be the only practical alternative to conventional IS addition (via the extraction solvent). This IS spray technique was shown to work reliably, and it was theorized that the IS would be more likely to correct for losses as it was integrated (to some degree) with the sample prior to extraction[125,126]. The aim of the research in this new article was to investigate if the IS spray technique would allow IS to integrate sufficiently with DBS samples to enable analyte and IS co-extraction, and thus nullify HCT based recovery bias. This theory was tested by measuring the HCT based overall assay bias using this method of IS addition. This ‘test’ data was then compared to the two sets of control data (representing both ends of the IS integration spectrum, within the scope of this work), to provide a comparative measure of how well the IS and analyte is integrating with the sample prior to extraction using this technique.

In addition to the issues associated with HCT, another notable barrier to introducing DBS analysis to pharmaceutical drug development was persuading bioanalysts to accept a technique that was new and both more complex, and offered less assay sensitivity than the conventional wet plasma analysis technique it was potentially replacing[59]. To counter this, numerous groups have investigated DBS direct analysis techniques that eliminate manual preparation prior to analysis and (in some cases) enable increased assay sensitivity through injecting the entire extracted volume onto the analytical system[70,131,73]. We have found that the most accessible DBS direct analysis technique for use in regulated quantitative bioanalysis is currently direct elution, as it offers reliable performance, compatibility with HPLC, and increased assay sensitivity[70,73,74]. In order to maximise the scope of this work, for each method of IS addition, analysis was investigated using both conventional manual extraction (using both sub-punch, and whole-spot extraction), and direct elution (using both sub sampling, and whole-spot elution).

5.2 Experimental

Standard laboratory equipment and reagents used in this work are detailed in the supporting information.

DBS Preparation

Control human blood was pooled and artificially altered to create a range of HCT values as described previously by the addition and removal of plasma[69]. All blood was shipped and stored at 4°C and used within 30 hr of collection. Standard preparation for the sitamaquine, paracetamol, and midazolam assays have been described previously[63,125]. For the naproxen assay, primary stock solutions for test compound and IS ([$^2H_3$]-Naproxen) were prepared separately in dimethylformamide (DMF, 10 mg/mL and 1 mg/mL, respectively). Working standards at suitable concentrations were made up in methanol/water (1:1, v/v). Calibration standards and quality control (QC) samples were prepared over a concentration range relevant for the physiological exposure of the drug by diluting the appropriate working solutions with control blood. In all cases, the spiking volume into blood was less than 5% non-matrix solvent. For naproxen, the concentrations of calibrants were 100, 200, 400, 1000, 4000, 10000, 20 000, 40 000, and 50 000 ng/mL, and QC's (levels 1 to 5) were 100, 300, 4000, 40 000, and 50 000 ng/mL.

DBS samples for all analytes were prepared by spotting a fixed volume (15 or 2 µL) of blood using a calibrated pipette onto DBS cards and drying for at least 2 hr at room temperature. If required, samples were stored at ambient laboratory conditions (21 ± 2°C) in a sealed plastic bag containing desiccant. For the assay bias experiments, QC's at low and high concentration levels (i.e. QC levels 2 and 4; 3 times the LLQ and ~75% of the HLQ, respectively) were used. To test the bias with varying HCT levels, blood HCT values of 0.2, 0.35, 0.45, 0.55 and 0.70 were used at a high and low QC concentration level (with the control HCT level (used to prepared calibration standards) being 0.45).

Manual Extraction

The conventional manual extraction DBS method is described in the introduction. Note that for whole-spot extraction, smaller 5 µL volumes were used for the DBS samples, and the entire sample was punched out using a 6.35mm diameter punch.

Direct Elution

Direct elution was carried out using the CAMAG DBS-MS500 This instrument fully automates the DBS direct elution process first reported in 2009[73]. Specific details on direct elution conditions can be found in the supporting information.

Chromatographic Conditions

Identical chromatographic conditions were used for both manual extraction and direct elution assays. The conditions for the sitamaquine, paracetamol, and midazolam assays have been reported previously[63,125]. Conditions for the naproxen assay are detailed in the supporting information.

Area Bias (ImageJ)

DBS area bias with varying HCT was assessed by measuring the area of DBS using ImageJ software, as reported previously[69]. During IS optimisation, the areas of the DBS were measured before and after IS spray addition.

IS addition

For the control experiment, where IS was added via the extraction solvent, the concentration of SIL IS in the extraction solvent was optimised so that the LC-MS/MS IS response was approximately 50% of the magnitude of the analyte LC-MS/MS response at the higher limit of quantitation (HLQ) for each assay. For the alternative methods of IS addition, concentrations were adjusted so the IS MS/MS responses were as close to this control level as possible, to ensure that any conclusions made were not influenced by the magnitude of the IS response (Table S5.1). For the control data where IS was spiked into whole blood prior to deposition on DBS substrate, samples were prepared by spiking a suitable volume of IS working solution into a bulk volume of whole blood (and gently mixing for 15 min), and then using sub aliquots of this bulk to prepare calibration standards or QC's as per the normal method in place of control blood. For two test methods of IS addition, the integrated CAMAG DBS-MS500 IS spray module was utilised[126]. The visual recognition system on this instrument is designed to locate the centre of DBS samples and use this point to spray a 10x10mm grid of IS solution over the sample. To apply to blank substrate (DBS cards), the DBS-MS500 was set up to apply the IS grid centred on the pre-marked black circles of the blank DBS cards (usually used for guiding where the DBS are applied). This later made it easy to know that whole blood was being applied on top of the substrate containing IS. The substrate was left to dry for at least 24hrs before DBS addition.

5.3 Results/Discussion

In this research, HCT based overall assay bias, and the individual HCT based area, recovery and suppression components were calculated for four representative small molecule DBS assays used for DBS quantitative analysis of drug concentrations. Assays used to measure DBS drug concentrations for four test compounds (sitamaquine, paracetamol (aka acetaminophen), midazolam, and naproxen), were used to test each variable. These analytes were selected as they offer a suitably diverse mix of compound characteristics. In particular a wide range (~40-100%) of overall assay recoveries (Table 5.1). In this work, overall assay bias refers to the percentage deviation from the nominal analyte concentrations (accuracy) in quantitative DBS methods measuring drug concentrations. Overall bias data using variable HCT was initially generated using conventional DBS manual extraction techniques using a sub-punch (from an accurate volume (15µL) spot). IS was added using four techniques ((A) via the extraction solvent; (B) spiked into whole blood prior to deposition onto DBS substrate; (C) IS sprayed on DBS prior to extraction; and (D) IS sprayed onto substrate before whole blood is applied). In this article, data generated using conventional IS addition via the extraction solvent (option A), and co-spiking IS into whole blood (B) is termed control data, while data generated using alternative methods that could be applied in practice (options C and D) will be referred to as test data. This process was then repeated using whole-spot extraction to remove the area bias component. The entire process was then repeated again using automated direct elution in place of conventional manual DBS extraction.

Control Data

DBS assays using accurate volume sampling followed by both sub-punch and whole-spot extraction, for four test compounds (sitamaquine, paracetamol (aka acetaminophen), midazolam, and naproxen) were taken through a validation process using control human blood with a HCT of 0.45. Separate control data validations were performed where the IS was added via the extraction solvent (option A); and where IS was spiked into whole blood prior to deposition into DBS substrate (option B)). Each validation batch consisted of 2 calibration lines bracketing QC samples (5 concentration levels, 6 replicates), plus non-matrix spiked, and post extracted matrix spiked QC's (6 replicates at 2 concentration levels: 3 times the assay LLQ, and 75% of the HLQ; QC2 and QC4, respectively) to assess assay bias, recovery and suppression (Supporting information Appendix A). Accuracy and precision, area, recovery, and suppression data was calculated for each assay by taking the mean of 18 individual data points on three occasions. As part of the validation, test QC's at low and high concentrations (QC2 and QC4) were also prepared using blood with varying HCT levels (0.2, 0.35, 0.45, 0.55, and 0.70) and analysed to investigate how the HCT level affected assay bias (using 0.45 HCT QC's as the control) (Tables S5.3-S5.6). In all cases the assays were shown to offer reproducibility, linearity, and accuracy and precision performance within internationally recognised guideline acceptance criteria[68].

Control data overall assay bias with varying HCT, using sub-punch extraction, where IS was added via the extraction solvent is plotted in Figure 5.1A. Note that in all the validations run

in this study no significant difference in the trends between QC2 and QC4 for a given assay was observed (the mean %bias difference between QC2 and QC4 data at a given HCT for a given assay was 2.8%, with a minimum and maximum deviation of -6.8% and 7.1% respectively). Since no significant concentration dependency was observed, mean bias values (of QC2 and QC4) have been plotted throughout this work. The area bias, recovery bias, and suppression bias contributions to this overall assay bias were also calculated (Figure 5.2). As expected, based on previously published data, it was demonstrated that area bias increases with HCT consistently across all analytes, as this factor is related to the changes in the viscosity of the blood with HCT and is therefore unlikely to be affected by analyte structure, or concentration (Figure 5.2A)[69]. There is a dramatic bias in recovery with varying HCT for some analytes, and this appears to be closely related to the absolute recovery of the assay (Figure 5.2B and Table 5.1). Midazolam and naproxen have high absolute recoveries, and as a result the recovery bias does not display a noticeable trend with HCT. Paracetamol (medium recovery), and to a greater extent sitamaquine (low recovery), exhibit a dramatic decrease in recovery bias with increasing HCT. There were no notable trends in suppression bias with changes in HCT (Figure 5.2C).

Based on this data it appears that the overall assay bias, using conventional sub-punch manual extraction (Figure 5.1A), is a result of the competing effects of area bias and recovery bias, with suppression bias having no significant contribution (Figure 5.3). Midazolam and naproxen have flat recovery bias curves (Figure 5.2B), and it follows that their overall assay bias (Figure 5.1A) mimics the area bias curve (Figure 5.2A). Conversely, the assays with lower overall recovery (sitamaquine and paracetamol) display a significant trend in recovery bias (Figure 5.2B), and it follows that this recovery bias has a significant contribution to the overall assay bias (Figure 5.1A). The level of overall assay bias for all four assays at the HCT extremities, while clearly undesirable and clearly displaying a trend, is barely above the typical inherent analytical variance we would expect from these assays (~±5%). Thus, the assay bias for these analytes is unlikely to be a significant issue for most analytical applications where analyte concentrations in DBS are being quantified when the sub-punch DBS method is used.

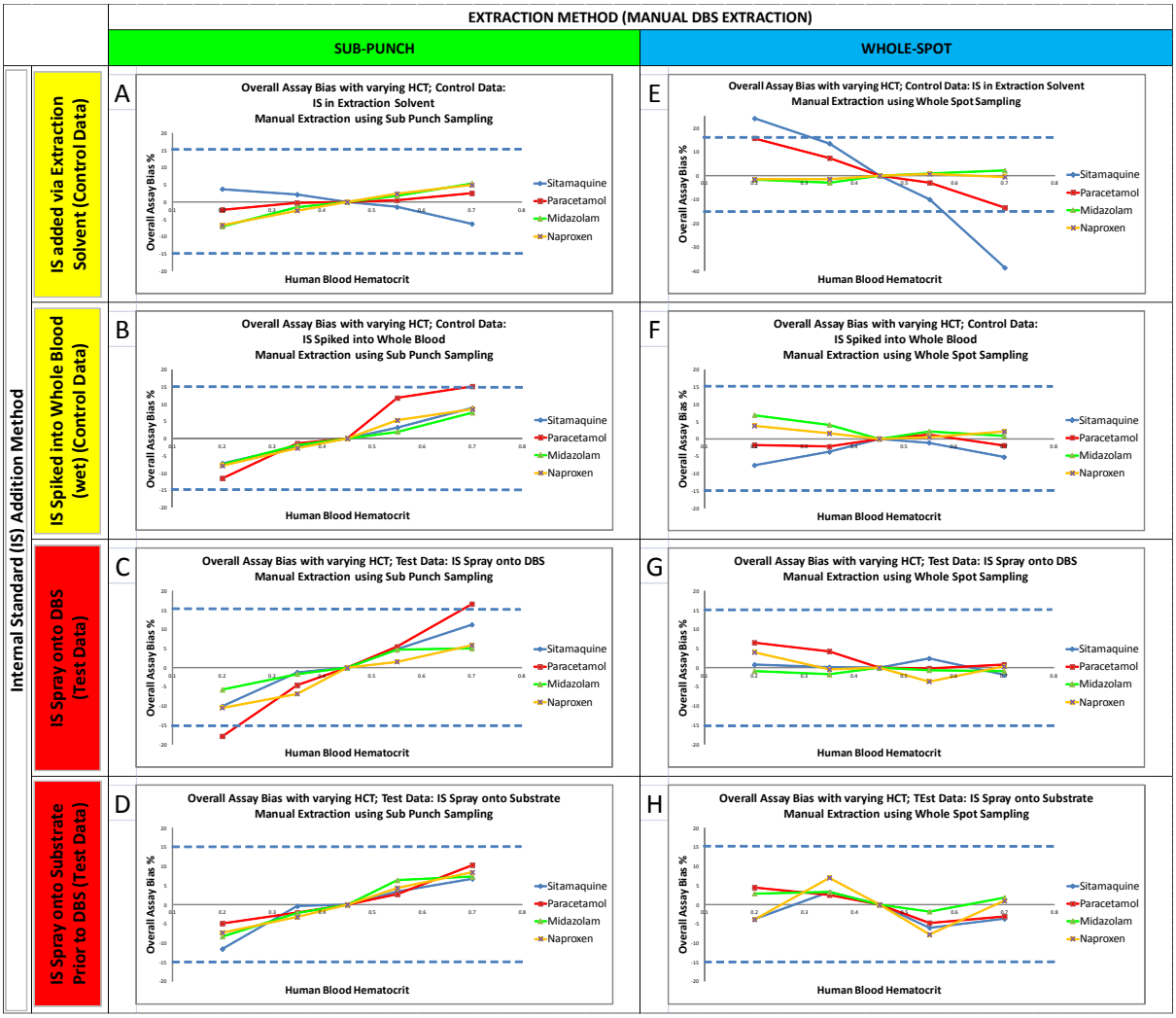


Figure 5.1: Overall assay bias with varying haematocrit (HCT) for DBS sub-punch and whole-spot manual extraction using four methods of internal standard (IS) addition (two control, and two test methods) for four quantitative bioanalytical assays measuring drug concentrations. Figures 1A-D show sub-punch extraction where the IS is added via the extraction solvent (A) (control data); by spiking IS into whole blood prior to blood deposition on substrate (B) (control data); by spraying IS onto the DBS prior to extraction (C) (test data); and, by spraying IS onto blank substrate prior to blood deposition (D) (test data). Figures 1E-H show the data in the same order, where whole-spot extraction is used. Each data point plotted is a mean bias value of the low and high concentration QC's (3x the assay LLQ, and 75% of the assay HLQ respectively), as no significant concentration dependency was observed. The blue dashed lines represent ±15% bias (the limit of total error allowable according to internationally accepted guideline acceptance criteria). To display the data concisely, bias results using variable HCT have been calculated using the result at the control HCT level (0.45) as the nominal value.

Overall assay bias with varying HCT for whole-spot manual extraction, where IS is added via the extraction solvent, is easy to reconcile for all compounds (Figure 5.1E). Here the effect of area bias is eliminated by taking the whole spot; again the suppression bias appears to have no significant effect; and therefore the overall assay bias closely mimics the recovery bias (Figure

5.2B). It is interesting that the whole-spot overall assay bias with varying HCT is significantly worse for paracetamol and sitamaquine where the area bias is eliminated (Figure 5.1E), than it is for sub-punch, where the area bias is also present (Figure 5.1A). This highlights how area bias and recovery bias can compete against each other to produce the overall assay bias (Figure 5.3), and how removing one component can have the effect of making the assay appear worse! The midazolam and naproxen assays demonstrate that this effect can be eliminated or minimised by ensuring an assay has very high recovery, but this is not always possible. Note that in all control experiments precision (%CV) was demonstrated to be within guideline acceptance criteria (±15%) (Tables S5.3-S5.6).

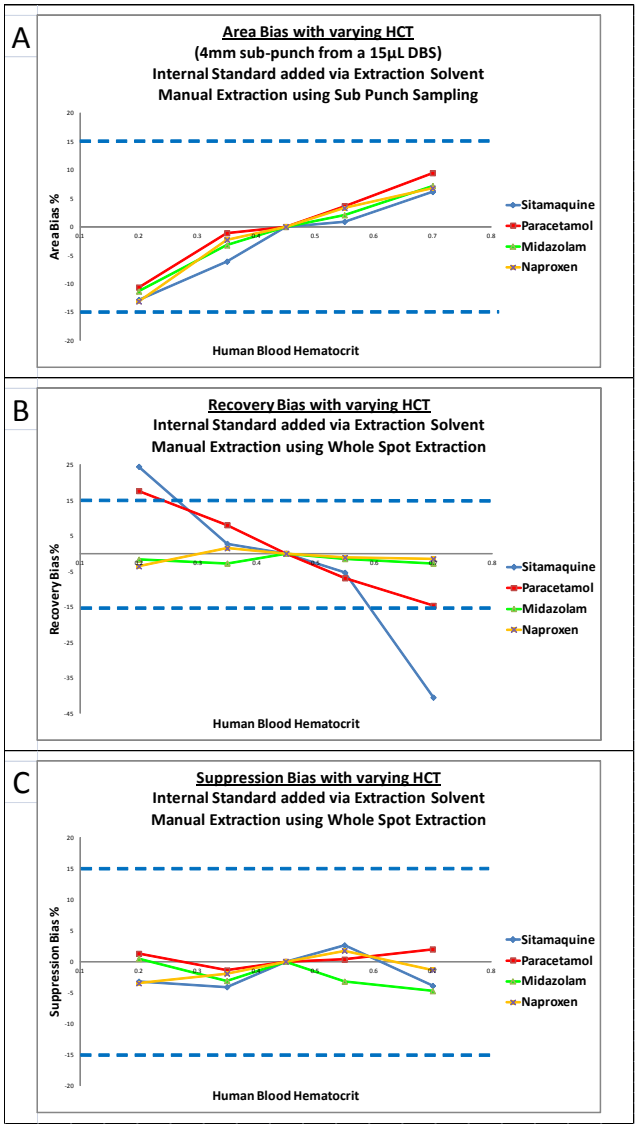


Figure 5.2: Area bias, recovery bias, and suppression bias (A, B and C, respectively) contributions to overall assay bias for DBS conventional manual extraction where internal standard is added via the extraction solvent. The blue dashed lines represent ±15% bias (the limit of total error allowable according to internationally accepted guideline acceptance criteria).

Table 5.1: Absolute recoveries calculated for each test compound as part of the control data (whole-spot manual extraction with IS added in the extraction solvent).

HCT	Recovery (%)							
	Sitamaquine		Paracetamol		Midazolam		Naproxen	
	QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
0.2	45.9	52.5	71.0	74.6	94.5	84.0	97.0	94.9
0.35	38.6	41.6	65.5	67.8	90.9	87.1	98.3	104.0
0.45	38.0	41.1	60.9	62.1	92.8	90.8	99.8	101.1
0.55	37.7	37.4	56.5	58.5	91.1	87.1	96.0	103.1
0.7	24.1	23.2	49.0	55.0	91.0	86.9	95.7	101.9

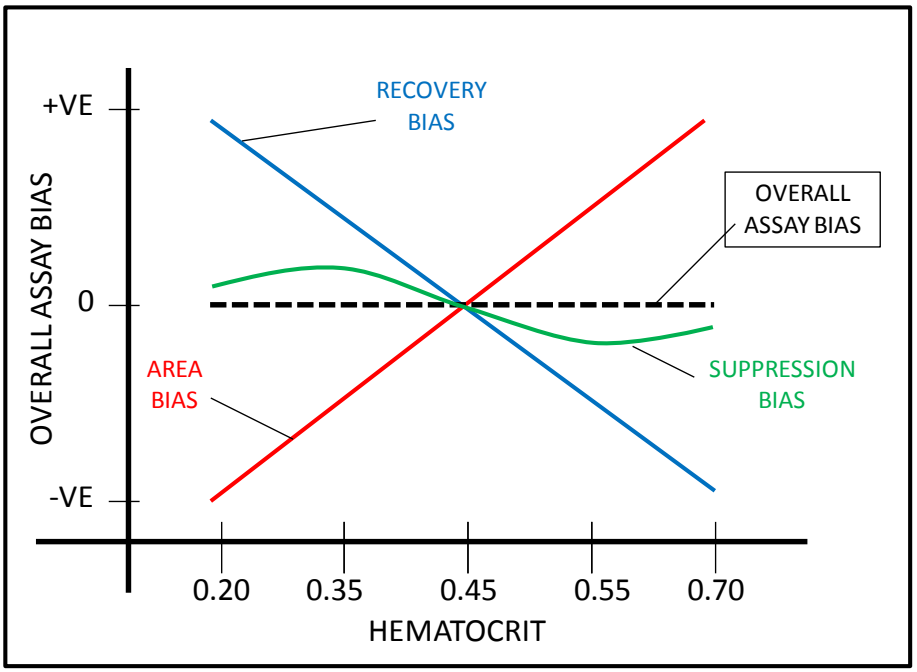


Figure 5.3: Schematic demonstrating the components of HCT based assay bias using conventional manual DBS extraction using an accurate blood volume dispense, followed by sub-punch sampling. In this simplified schematic the area bias (red line) and recovery bias (blue line) work in a competitive fashion with changing HCT and are of the same magnitude. Their effects therefore have the potential to cancel each other out, resulting in no overall assay bias with varying HCT being observed (dashed black line). The suppression bias (not to scale) shows no clear trend and does not contribute significantly to the overall assay bias. All bias values are normalised to those obtained for a typical HCT value of 0.45.

A further set of control data was collected where IS was spiked into whole blood prior to deposition into DBS substrate (option B). As described previously, this produces data where (because the analyte and IS are co-extracted) recovery bias should be nullified. This is demonstrated for sub-punch extraction using this method of IS addition (Figure 5.1B), where the overall assay bias for each analyte mirrors the area bias (Figure 5.2A), with no apparent contribution from recovery based bias (Figure 5.2B). If the area bias is then also eliminated by using whole-spot extraction (Figure 5.1F) it is demonstrated that no strong bias trend occurs

with HCT, with bias data at varying HCT well within acceptable limits. This demonstrates that eliminating HCT based area bias, and nullifying recovery bias will effectively eliminate HCT based overall assay bias. Unfortunately, this method of IS addition will not be a practical alternative for most applications, hence the test data using spray methods of IS addition were used to see if this effect could be replicated.

The above control data was also collected using direct elution. Direct elution was carried out using the CAMAG DBS-MS500, which is one of the few commercially available DBS direct elution instruments[73]. This instrument fully automates the DBS direct elution process first reported in 2009[74]. Further work has shown this technique to be suitably reliable and reproducible, and it is used here as an example of how to make the DBS workflow more efficient and reduce the additional manual burden associated with DBS[70]. Less emphasis is placed on this data in this article as the component HCT recovery bias data was not generated, meaning we have less understanding of the overall recovery levels involved. It is conceivable that a recovery experiment could be designed where the direct elution extraction product is collected and used to calculate recovery and suppression, but it was thought that this process would be difficult to perform accurately and optimise within the timeframes of the study. Also, while a better understanding of direct elution recovery is desirable, ultimately it's absence did not detract from the conclusions made in this study. Control data for overall assay bias with varying HCT using direct elution shows markedly different trends compared to the manual extraction data (Figures S5.1A, B, E, and F). Using sub-sample direct elution where the IS is added via the extraction solvent, midazolam and naproxen exhibit a very minor negative bias with increasing HCT, while sitamaquine and paracetamol both exhibit the same trend, but to a much more significant degree (Figure S5.1A). The area bias effect will be the same as for manual extraction (Figure 5.2A), so we theorise that the recovery bias (and absolute recovery) is significantly different for direct elution, and clearly the dominant factor in overall assay bias.

Control data for whole-spot direct elution where IS was added via the extraction solvent showed very high levels of overall assay bias with varying HCT (Figure S5.1E). We theorise that recovery bias for whole-spot direct elution is significantly different than for sub-sample direct elution, and the cause of the differences in overall assay bias. Likewise, this data suggests that some of the differences seen in the control data between manual extraction and direct elution (for both sub-punch and whole-spot analysis) (Figures 5.1A and E, and Figures S1A and E), are likely to be caused by differences in recovery, which in turn are likely caused by significantly different mechanisms of extraction. The direct elution mechanism involves extraction solvent being forced across the DBS sample under pressure for a matter of seconds, while for manual extraction the sample is agitated gently in extraction solvent for an hour. In addition, further variation in recovery may occur for whole-spot direct elution as the sampling probe covers the DBS sample, plus a surrounding area of blank substrate. It is possible that this ring of blank substrate creates a path of least resistance around the DBS sample that significantly affects how the extraction solvent interacts with the DBS, and subsequently the recovery. Further work is required to better understand the mechanisms of DBS direct elution recovery in these different circumstances.

The control direct elution data, where IS was spiked into whole blood prior to deposition on DBS substrate allows some conclusions to be made despite the lack of recovery data (Figures S5.1B and F), as it mirrors the effect observed for manual extraction. Where recovery bias is nullified (Figure S5.1B) the overall assay bias mirrors the trend for area bias only (Figure 5.2A). This trend contrasts strongly with the data where IS was added via the extraction solvent (Figure S5.1A) suggesting that the recovery bias for direct elution is significant, and adding the IS in this way (option B) is nullifying HCT based recovery bias. If both area bias and recovery bias are eliminated, no trend in overall assay bias with varying HCT is observed (Figure S5.1F).

Test Data

The control data demonstrated that integrating IS into the DBS sample, so that IS and analyte were co-extracted, allowed HCT based recovery bias to be nullified. Coupling this with whole-spot extraction to eliminate HCT based area bias allowed HCT based overall assay bias to be effectively eliminated. However, as mentioned above, this method of IS addition is not a practical workflow. The test data in this study, where IS was sprayed onto DBS samples prior to extraction (option C), or the substrate was sprayed with IS prior to whole blood deposition (option D) aimed to investigate if practical methods of IS addition could also nullify HCT based recovery bias (and thus overall assay bias, when also coupled to whole-spot extraction).

To generate the test data, the integrated CAMAG DBS-MS500 IS spray module was utilised. This is a more refined version of the IS spray technique we previously reported[125]. This IS module eliminates the 'flooding' issues we experienced during our previous report by replacing the static application used with the Touchspray device (The Technology Partnership, Hertfordshire, UK) with a dynamic spray application[126,73]. Rather than applying the IS solution in a single spray arc covering the entire DBS, this system applies a much finer stream, covering only a very small section of the sample (spray arc diameter less than 2mm), and uses automation to move the sample relative to the spraying device (a dynamic spray application). The result is a 10 x 10mm grid of IS solution applied centred around the DBS (guided by the integrated visual recognition system). This system allows greater volumes of IS solution to be applied (thus more likely penetrating the full depth of the DBS sample, and potentially enabling greater integration of IS within the sample) before the limit at which flooding occurs is reached. Prior to the IS spray experiments being performed, the technique was optimised. It was found that applying 20 µL of IS solution made up in 100% methanol using the 'fast' application setting, produced the best precision and reproducibility (Abu-Rabie et al, unpublished). Crucially, these optimised parameters also showed no evidence of flooding (or any physical mass flow of sample components), that is highly likely to spoil the integrity of the DBS sample[125]. The optimisation experiments also demonstrated that the mechanism of spraying IS onto DBS prior to extraction using the above conditions made no significant difference to analyte concentrations measured using the LC-MS/MS assays described in the article (Abu-Rabie et al, unpublished).

Overall assay bias with varying HCT for sub-punch and whole-spot manual extraction using the test methods of IS addition closely mirrored the control data where IS was spiked into

whole blood (option B) (compare Figure 5.1B and F to Fig 5.1C, D, G and H). This suggests that both test methods of IS addition enable the IS and analyte to be co-extracted, nullifying the recovery bias effect. Again, when coupled with whole-spot extraction this produces a method where HCT based overall assay bias is effectively eliminated. The test data for direct elution produce similar results (Figures S5.1C, D, G, and H). The data in this study does not allow us to make a strong statement about the spray application methods ability to fully integrate IS into DBS samples. However the data clearly demonstrates that these methods of IS addition allow IS and analyte to be co-extracted in a manner that is sufficient to effectively nullify HCT based recovery bias and that they perform better in that respect than the established workflow of including the IS in the extraction solvent.

Precision levels for all 4 test assays using the test methods of IS addition were all within internationally recognised acceptance criteria (Table S5.7). Of the four methods of IS addition, spiking the IS into blood prior to spotting (B) consistently produced the best analytical precision (lowest CV). Generally there was little difference in precision between adding IS via the extraction solvent (A), and using the IS sprayer (C), while applying IS to the substrate (D) tended to produce the worst precision of the four methods, by a small margin. These results generally agree with a previously published investigation by Van Baar et al[20]. This work also used IS spiked into blood prior to spotting as a reference value, and also found this method of IS addition produced the best precision of the application methods tested. The use of a IS sprayer was also found to produce good analytical performance. Unlike this work, spraying onto blank substrate before blood deposition was found to give slightly better precision than when IS was deposited onto DBS prior to extraction, albeit by a small margin. There was little difference in precision between manual extraction sub-punch, manual extraction whole-spot, and direct elution sub-punch techniques. Direct elution whole-spot analysis produced slightly worse analytical precision, albeit by a small margin. Possibly explanations for this reduced precision include higher variance associated with spotting the smaller volumes (2 µL) of blood required for this test, and our relative lack of experience in optimising direct elution whole-spot assays.

5.4 Conclusions

The data presented in this study has shown that HCT based recovery bias becomes increasingly important as the absolute recovery of DBS assays decreases. In this study, assays with recoveries of over 90% do not suffer significant HCT based recovery bias. However, HCT based recovery bias shows an increasingly significant contribution to the overall assay bias in assays with lower recovery (60% and 40% absolute recovery in this study). It has been demonstrated in this study that eliminating a single factor contributing to bias (such as using whole-spot extraction in place of sub-punch, to eliminate HCT based area bias) can actually cause overall assay bias to be more significant as the competitive effect between area and recovery bias is minimised. Therefore it is important that both DBS HCT based area and recovery bias is understood during assay development, and steps are taken to eliminate, or minimise them, particularly where the absolute assay recovery is low. A lack of understanding

of these competing effects during assay development and validation could result in an assay being used inappropriately and inaccurate study data being released.

Of the three alternative IS addition methods that nullify HCT based recovery bias described in this study, only the spray addition onto DBS technique prior to extraction (option C) is likely to be practical for drug development and discovery applications. Spiking IS into whole blood (option B), and spraying IS onto blank substrate prior to blood spotting (option D) will logistically not be feasible when dealing with multiple studies, study centres, and compounds, if the cost and procedural simplifications on offer from DBS are to be kept intact. However, pre-treating blank substrate with IS (option D) could be feasible for some therapeutic drug monitoring applications[125]. The IS spray application (option C) would most likely be carried out once study samples have been shipped to the analytical laboratory, and before undertaking the extraction procedure. We have demonstrated that this procedure is easily compatible with direct elution techniques and has already been integrated into at least one commercially available automated DBS direct elution instrument (the CAMAG DBS-MS500).

By combining the investigation of IS application techniques to nullify HCT based recovery bias, with whole-spot extraction, and direct elution, we present in the literature for the first time an accurate volume DBS analysis workflow that simultaneously eliminates HCT based area bias and recovery bias, and the burden of manual DBS extraction using commercially available automation technology. It is hoped that the information generated in this study will help improve confidence in using DBS analysis. To do this, workflows need to be introduced that combine the various techniques described in this article. Such a workflow would involve taking an accurate volume DBS sample; using one of the alternative IS addition methods to eliminate HCT based recovery bias; using whole-spot analysis to eliminate HCT based area bias; and using direct elution to eliminate the additional manual burden associated with DBS analysis.

Note: Supplementary information can be found in Appendix E.

CHAPTER 6: DRIED BLOOD SPOT DIRECT ELUTION: DETERMINING GENERIC CONDITIONS FOR OPTIMISING PERFORMANCE IN HIGH THROUGHPUT QUANTITATIVE LC-MS/MS ANALYSIS

6.1 Introduction

In the last few years there has been considerable interest in implementing dried blood spot (DBS) analysis (a technique that has been used in areas such as new born screening for many decades) into new areas and applications[25,26]. When employed as a microsampling technique, DBS analysis provides well documented ethical and financial benefits compared to conventionally used wet plasma analysis[25,26]. Unfortunately the momentum behind introducing DBS analysis as a widely used technique into highly regulated applications (such as pharmaceutical drug development) has been slowed due to a number of barriers. These include uncertainty over regulatory acceptance, scientific uncertainty over assay bias caused by varying haematocrit (HCT), and also concerns relating to the increased complexity of extraction, and lower assay sensitivity associated with DBS analysis[123,70].

We have previously demonstrated that direct elution techniques offer the potential to eliminate the manual extraction burden associated with DBS[74]. Our initial work used the commercially available CAMAG TLC-MS interface as a simple, manually operated direct elution device that enabled the direct on-line extraction of DBS samples by flowing mobile phase over a central sealed sampling area on the DBS. It was demonstrated that this direct elution technique produced acceptable chromatography, and quantitative performance within internationally recognised guideline acceptance criteria[68]. Also, compared to conventional manual extraction (where the amount of extract injected into the LC system is limited by the need for the resultant chromatography to be acceptable) an average ten-fold increase in assay sensitivity was observed (while retaining acceptable chromatographic performance) across seven representative small molecule compounds. It was observed in this initial investigation that the non-optimised direct elution actually had quite a poor recovery (~15% of the available analyte). As previously detailed, theoretical increases on offer from direct elution over manual extraction will depend on the maximum extract volume that can be injected, and recovery of the individual assay, but will often be over 50-fold (for a given DBS sample size), based on the assays we have examined[74]. We have chosen to develop direct elution, over other direct analysis techniques, as it is the only process that offers enhanced sensitivity and the potential to be easily compatible with regulated pharmaceutical bioanalysis (unlike techniques that do not utilise LC as an separation technique)[70,73,59].

Subsequent research has further demonstrated the advantages on offer from DBS direct elution, and it's suitability to support regulated quantitative bioanalysis, and other applications[104,109,100,101,132,102,103]. Prototype semi-automated, and fully automated direct elution instruments have been used to demonstrate that the technique offers comparable reliability to conventional manual extraction[75]. Further research has also shown that spraying

internal standard (IS) onto DBS samples prior to extraction offers improved analytical performance, and the ability to nullify haematocrit based recovery bias, by enabling co-extraction of analyte and IS[125,81]. The current article details the research carried out into identifying generic direct elution conditions that maximise assay sensitivity while retaining acceptable chromatography, and quantitative performance. The aim was to identify optimal starting conditions for high throughput DBS direct elution, and thus options such as additional on-line SPE, and trapping column LC apparatus, which add complexity and use longer cycle times were not considered.

The automated direct elution platform (CAMAG DBS-MS500) used in this study offers many advantages over its predecessor, the TLC-MS, that enables DBS extraction recovery, and thus assay sensitivity to be maximised. In our initial investigation using the TLC-MS, the mobile phase conditions and flow rate from the LC system were used as the extraction conditions, with the only variable being the extraction period (the time mobile phase was allowed to flow over the DBS). The DBS-MS500 allows the use of an independent extraction solvent, with variable flow rates and extraction volumes. Previously published articles (on manual extraction and various types of direct elution) have recommended a variety of extraction conditions, but have tended to focus on specific challenges, or have been proof of concept initial investigations, rather than attempting to identify generic conditions[73,132,47,98,131]. To the best of our knowledge, the work reported herein attempts to recommend, for the first time, generic direct elution conditions for the high throughput bioanalysis of typical small molecule pharmaceutical compounds. In this investigation a range of extraction conditions (including variations in solvents, pH and temperature) were tested using a large range of flow rates and extraction volumes. In addition the pre-wetting of DBS samples as a means of increasing assay sensitivity was also investigated using the IS spray module integrated into the DBS-MS500. LC-MS/MS responses and chromatographic performance were both assessed to identify which generic parameters produced the best results.

6.2 Experimental

Chemicals, Reagents, and other Equipment

Human volunteer control blood was collected via the GlaxoSmithKline blood donation unit (Stevenage, UK) in accordance with current GSK policies on informed consent and ethical approval. Methanol, acetonitrile, and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd (Loughborough, UK). All other chemicals were of analytical grade and supplied by Fisher Scientific Ltd (Loughborough, UK). Paracetamol (*aka* acetaminophen), [$^{2}H_3$]-paracetamol, sitamaquine, [$^{2}H_{10}$]-sitamaquine, and [$^{2}H_3{}^{13}C_3$]-midazolam were obtained from GlaxoSmithKline (Stevenage, UK). Midazolam was obtained from Tocris Bioscience (Bristol, UK). Naproxen and [$^{2}H_3$]-naproxen were obtained from Sigma Aldrich (Pool, UK). Ahlstrom grade 226 paper for blood spots was supplied by Perkin Elmer (Buckinghamshire,

UK). Sample tubes were obtained from Micronics (Sanford, USA). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat were supplied by Ted Pella (Redding, USA). Benchtop sample shaker (model HS 501 D) was supplied by Janke and Kunkel, IKA Labortechnik (Staufen, Germany). The HPLC-MS/MS system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven. MS detection was undertaken by using a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada) system equipped with a Turbo IonSpray source. HPLC-MS/MS data were acquired and processed (integrated) using Analyst software v1.6.1 (Applied Biosystems/MDS Sciex, Canada).

DBS Preparation

All blood was shipped and stored at 4°C and used within 30 hr of collection. Blood was pooled and artificially altered to create a range of HCT values as described previously[69]. Standard preparation for the sitamaquine, paracetamol, midazolam, and naproxen assays have been described previously[125,81,63]. DBS samples were prepared specifically for sub-punch/sub-sample, and whole spot analysis. For sub-punch/sub-sample analysis a fixed volume (15 µL) of blood was added by pipette onto DBS cards and allowed to dry overnight at room temperature. For whole spot analysis, 2µL of blood was accurately spotted onto DBS cards. This low volume was used to ensure the 4mm diameter sampling area on the DBS-MS500 could comfortably fully encompass the entire sample. If required, samples were stored at ambient laboratory conditions (21 ± 2°C) in a sealed plastic bag containing desiccant. For the experiments comparing performance of direct elution parameters each analyte was spiked at a single concentration (250 ng/mL for sitamaquine and midazolam; 2500 ng/mL for naproxen and paracetamol). To test the bias with varying HCT levels, blood HCT values of 0.2, 0.35, 0.45, 0.55 and 0.70 were used at a high and low QC concentration level (i.e. QC levels 2 and 4; 3 times the LLQ and ~75% of the HLQ, respectively) (with the control HCT level being 0.45).

Manual Extraction

The generic manual extraction method involved punching a 4 mm diameter disc from a 15 µL DBS for sub-punch analysis; or punching the entire spot (using a 6.35 mm diameter punch) from a 2 µL DBS for whole spot analysis. The disc was then placed in a tube and 200 µL of 70:30 (v:v) methanol:water containing the stable isotopically labelled (SIL) IS at a suitable concentration was added (250, 5000, 1000, and 5000 ng/mL for the sitamaquine, paracetamol, midazolam, and naproxen assays, respectively)[81]. The tube containing the disc was agitated on a laboratory shaker for 2 hr, centrifuged, and the supernatant transferred to a fresh tube. From experience of running scores of small molecule DBS assays, we know that these generic conditions give us the best chance of maximising recovery. Analysis was then carried out by injecting the maximum volume of supernatant into the LC-MS/MS system that produced the maximum assay response with acceptable chromatography (5 µL for sitamaquine, midazolam, and naproxen, 2 µL for paracetamol).

Direct Elution

The CAMAG DBS-MS500 automated direct elution platform (CAMAG, Basel, Switzerland) was used for all direct elution experiments, and was operated using Chronos software[73].

For the direct elution optimisation, 10 different extraction solvents were tested (100% methanol; aqueous methanol mixtures containing 70, 50 and 25% methanol; 100% acetonitrile; aqueous acetonitrile mixtures containing 70, 50 and 25% acetonitrile; 100% water; and, a mobile phase (MP) matched extraction solvent). The MP matched solvents used the proportions of MP for each assay at the retention time (RT) of the analyte. For each extraction solvent tested, total extraction volumes of between 1 and 100 µL, and extraction solvent flow rates (or delivery speeds) of between 2 and 500 µL/min were assessed. For each set of optimisation conditions 6 replicate samples were tested and the mean response was calculated.

Acidified versions of acetonitrile and methanol (0.1% formic acid added to both) were also tested. Further, heated extraction solvents (70% methanol and 70% acetonitrile) were also tested, using a very basic experimental procedure where extraction solvent was simply heated and maintained at 70°C on a hot plate situated on the DBS-MS500.

For each assay a dual wash solution was used between extractions to prevent carry over using the DBS-MS500 integrated wash system. The sampling apparatus was rinsed with combinations of 70:30 methanol: water (v/v) to remove any remnants of analyte, and 5:95 methanol: water (v/v) to remove matrix components[75].

Assay Rangefinder Tests

In order to compare the sensitivity that could be achieved from both conventional DBS manual extraction, and direct elution, range finder tests were carried out for each assay. This involved spiking calibration standards for each compound that would far exceed both the expected lower limit of quantitation (LLOQ) and higher limit of quantitation (HLQ). These samples were then extracted and analysed using the generic optimised conditions to determine the analytical ranges. The LLOQ was based on the lowest concentration sample that produced a LC-MS/MS peak area response value with a signal to noise ratio of better than 5:1 (the noise value being measured from a blank sample), and acceptable accuracy and precision, as per internationally recognised guideline criteria[68]. The HLQ was based on the maximum concentration calibration standard that demonstrated accuracy and precision within guideline criteria, which is generally limited by the linear dynamic range of the MS instrument.

Chromatographic and MS Conditions

Chromatographic conditions and MS optimisation for the sitamaquine, paracetamol, midazolam, and naproxen assays have been described previously[74,125,81,63].

IS addition

For the control manual extraction assays, IS was added via the extraction solvent, as described previously[125,81,63]. Part of the direct elution optimisation experiments involved pre-wetting the DBS prior to extraction. This was carried out using the IS addition module integrated with the DBS-MS500, which sprays a fine jet of IS solution in a 10x10 mm grid centred on the DBS[73,126]. The optimisation of the IS sprayer and the conditions used in for these assays have also been described previously[81].

LC-MS/MS Performance

To ensure all the optimisation data collected was comparable, two system suitability test (SST) methods were used to ensure that LC-MS/MS performance (which can vary dramatically from day to day, depending on a number of factors) was consistent between direct elution optimisation tests. The performance of both the direct elution instrument and LC-MS/MS system was assessed by monitoring a reference DBS extraction (10 µL of 100% methanol at 20 µL/min) of one of the test samples prepared for this study. Performance of the LC-MS/MS system in isolation was assessed using a reference mixture of test compounds (Abu-Rabie, unpublished). The direct elution response was required to be within ±15% of the reference response to be acceptable. When the SST fell outside this limit, action was taken to identify what was causing the variation in performance, and once rectified the SST was repeated.

6.3 Results/Discussion

The aim of this investigation was to determine optimised DBS direct elution conditions that produced the maximum LC-MS/MS response, while retaining acceptable chromatographic performance (comparable to the original manual extraction assays). To achieve this LC-MS/MS peak area response optimisation data was collated and then compared with data measuring the quality of chromatography.

DBS Direct elution optimisation was performed by extracting samples spiked at a single fixed concentration using 14 different extraction solvents, 8 different extraction volumes (1-100 µL), and 9 different extraction solvent delivery speeds (2-500 µL/min). This was undertaken for 4 different LC-MS/MS bioanalytical methods (sitamaquine, midazolam, naproxen, and paracetamol), using both sub-sample, and whole spot direct elution. Note that not every combination of extraction volume and delivery speed was possible to test (e.g. attempting extreme combinations such as 100 µL at 2 µL/min exceeded the maximum permitted 'wait' period of the DBS-MS500 software, and caused the system to 'time-out'). For all 4 compounds, the greatest LC-MS/MS responses were obtained when eluting with 70% methanol (Table 6.1). In order to display a very large data set concisely, the data in Table 6.1 has been

summarised to only display the maximum LC-MS/MS peak area response achieved from the 57 different combinations of extraction volumes and delivery speeds for each extraction solvent. Each value is expressed as a percentage of the maximum response recorded for each compound/sampling method (e.g. sitamaquine/sub-sample). At this stage the aim was to identify which extraction solvent produced the largest peak area responses, so presenting the data in this way demonstrates how the optimal extraction solvent was selected.

Table 6.1: Summary of maximum LC-MS/MS responses (normalised) measured during DBS direct elution optimisation. Each value represents the maximum LC-MS/MS peak area response recorded from the 57 different combinations of extraction volumes and delivery speeds tested for each extraction solvent. Values are expressed as a % of the maximum response of each compound/sampling method (e.g. sitamaquine/sub-sample). For each set of optimisation conditions 6 replicate samples were tested and the mean response was calculated. Note that 'MP Matched' refers to mobile phase (MP) matched solvents where the proportions of MP for each assay at the retention time (RT) of the analyte is used as the extraction solvent.

		Maximum LC-MS/MS peak area responses (expressed as a % of the maximum response of each compound/sampling method series)						
Compound	Sampling Method	Extraction Solvent						
			Methanol					
		MP Matched	100%	70%	70% + Heat	50%	25%	100% Acidified
Sitamaquine	Sub-Sample	84.8	67.7	100.0	93.4	55.8	24.3	50.3
	Whole-Spot	73.9	66.3	100.0	94.0	66.3	19.8	54.4
Midazolam	Sub-Sample	59.4	80.4	100.0	98.9	75.3	40.5	71.2
	Whole-Spot	55.1	85.6	92.7	100.0	69.7	43.2	77.9
Naproxen	Sub-Sample	36.5	90.2	96.8	100.0	55.3	30.8	79.4
	Whole-Spot	47.8	88.1	100.0	90.3	50.9	38.7	78.0
Paracetamol	Sub-Sample	49.8	76.4	100.0	80.4	54.4	36.4	90.4
	Whole-Spot	45.1	100.0	85.5	88.1	40.0	33.5	91.7
Compound	Sampling Method	Extraction Solvent						
			Acetonitrile					
		Water	100%	70%	70% + Heat	50%	25%	100% Acidified
Sitamaquine	Sub-Sample	22.6	4.5	20.1	21.3	28.9	24.5	3.7
	Whole-Spot	20.1	3.6	24.9	26.7	23.3	24.1	3.5
Midazolam	Sub-Sample	16.4	11.8	18.7	17.4	28.9	20.1	9.6
	Whole-Spot	10.9	8.9	23.4	21.3	33.4	22.3	9.0
Naproxen	Sub-Sample	11.3	12.5	25.4	30.4	19.9	18.6	8.9
	Whole-Spot	8.8	16.6	19.7	22.8	23.1	20.5	11.0
Paracetamol	Sub-Sample	26.7	6.9	22.2	25.1	28.8	28.7	6.7
	Whole-Spot	28.1	4.5	24.0	29.8	31.1	26.9	6.1

Note that increasing the temperature of the extraction solvent did not appear to have a notable effect on the magnitude of the response. We acknowledge there are possible limitations to this experiment, due to the likelihood of heat being lost between the solvent bottle and DBS-MS500

extraction head. Prior to extraction the extraction tubing was purged several times, to try and 'heat up' the direct elution apparatus, but relatively small heated volumes are being delivered to extraction capillaries housed in a large metal block, so it is difficult to guess what temperature the solvent was when it interacted with the DBS sample. In theory, more elaborate apparatus could be designed where extraction solvent temperature could be increased and monitored at the extraction head, but this was not possible as part of this study. In most cases acidifying the extraction solvent had a minor detrimental effect on the magnitude of the response. Overall, there was some variation in responses according to the compound used, but generally the trends are the same across the 4 compounds.

The next step was to determine the generic optimal extraction volume and delivery speed, using the optimised extraction solvent (70% methanol). The results demonstrated that the total volume of extraction solvent used had the primary influence on the magnitude of the LC-MS/MS response; with the delivery speed being the secondary factor (Supplementary Information Figures S6.1 and S6.2). Extraction volumes of 5, 10 and 20 µL produced the largest responses for all 4 compounds. At these optimal extraction volumes, the effects of delivery speed showed slightly different trends according to compound and sampling method (Figures 6.1 and 6.2).

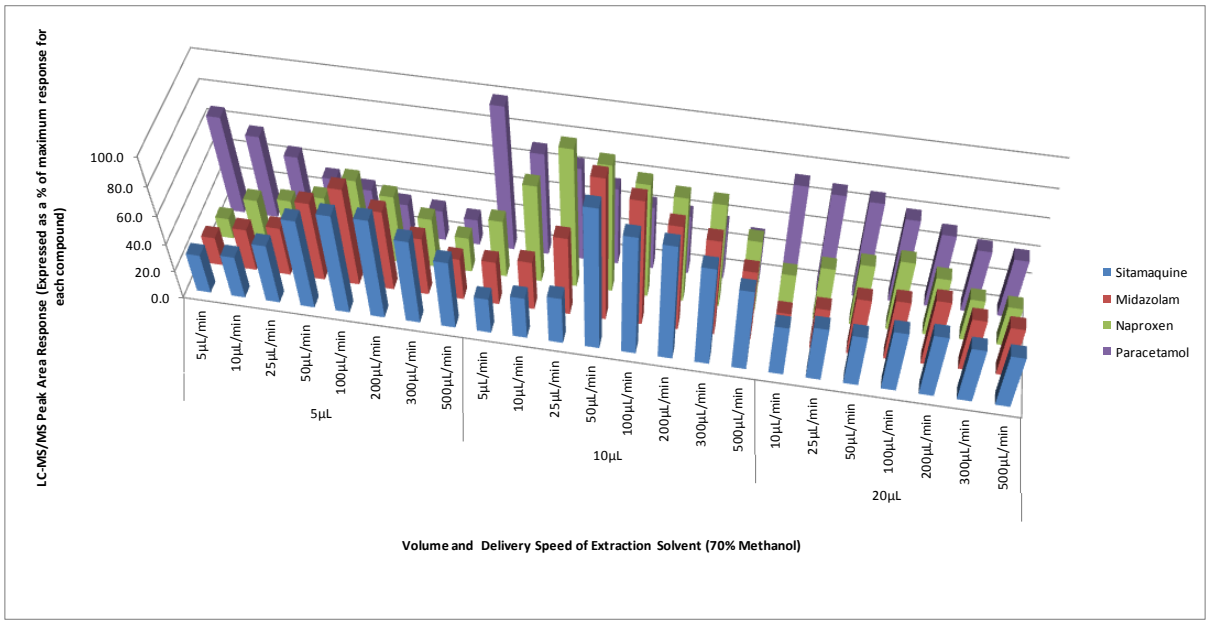


Figure 6.1: Sub-sample direct elution: Variation in LC-MS/MS peak area response with volume and delivery speed of optimised extraction solvent (70% methanol). Only the 3 highest performing extraction volumes are shown. Each value is a mean of n=6.

For sub-sample extraction, the effect of delivery speed for sitamaquine, midazolam, and naproxen showed a similar trend; as delivery speed increased peak area response increased (up to a maximum at 50 or 100 µL/min), then gradually dropped off as the speed increased further (Figure 6.1). The paracetamol extraction behaved slightly differently, with the greatest

responses observed at the lowest delivery speeds, with a gradual decrease in response as speed increases (Figure 6.1). Based on the greatest observed sub-sample responses across the 4 compounds, generic extraction conditions of 10 µL at 50 µL/min using 70% methanol would offer maximum performance for sub-sample direct elution. For whole spot direct elution the greatest responses were observed at the lowest delivery speeds, with a gradual decrease in response as speed increases (Figure 6.2). Based on the peak area response data, extraction conditions of 20µL at 10 µL/min using 70% methanol would offer the best generic extraction conditions. Differences in trends between sub-sample and whole spot direct elution are likely to be caused by the physical differences in extraction mechanisms (e.g. whole spot direct elution includes flowing extraction solvent through a band of blank substrate which may enable a path area of least resistance, and thus effect recovery)[81].

It is vital that the optimised extraction parameters, as well as producing the greatest LC-MS/MS response, also offer suitable chromatography. Other than the retention factor (k'), suitability of chromatography (for methods supporting regulated quantitative bioanalysis of pharmaceutical drugs) is often determined through somewhat subjective means (i.e. visual inspection). Traits of unacceptable chromatography include broad peaks, significant fronting or tailing, and non-symmetrical peak shape. To try and compare the control manual DBS extraction data with the direct elution optimisation experiments in a less subjective manor, the number of theoretical plates, N, which is a measure of chromatographic column efficiency (the higher the number, the better the column performance) was calculated for each response (Supplementary Information appendix F part A). This enabled both the change in N with different direct elution conditions to be monitored, as well as a comparison with the control data. The visual inspection and calculated N data for each set of optimisation parameters was then overlaid (Tables 6.2 and 6.3).

In all experiments, chromatographic peak shape and N was influenced by the total volume of extraction solvent used, with delivery speed at a given volume having no notable effect (data not shown). Therefore to summarise the data concisely, a mean N value for each extraction volume used was calculated (including data from all the delivery speeds used at that volume). Precision (CV) values for N across all compounds/sampling methods for a given extraction volume (including all delivery speeds) ranged from 0.9 to 7.4, demonstrating that delivery speed had no significant effect on N for a given extraction volume.

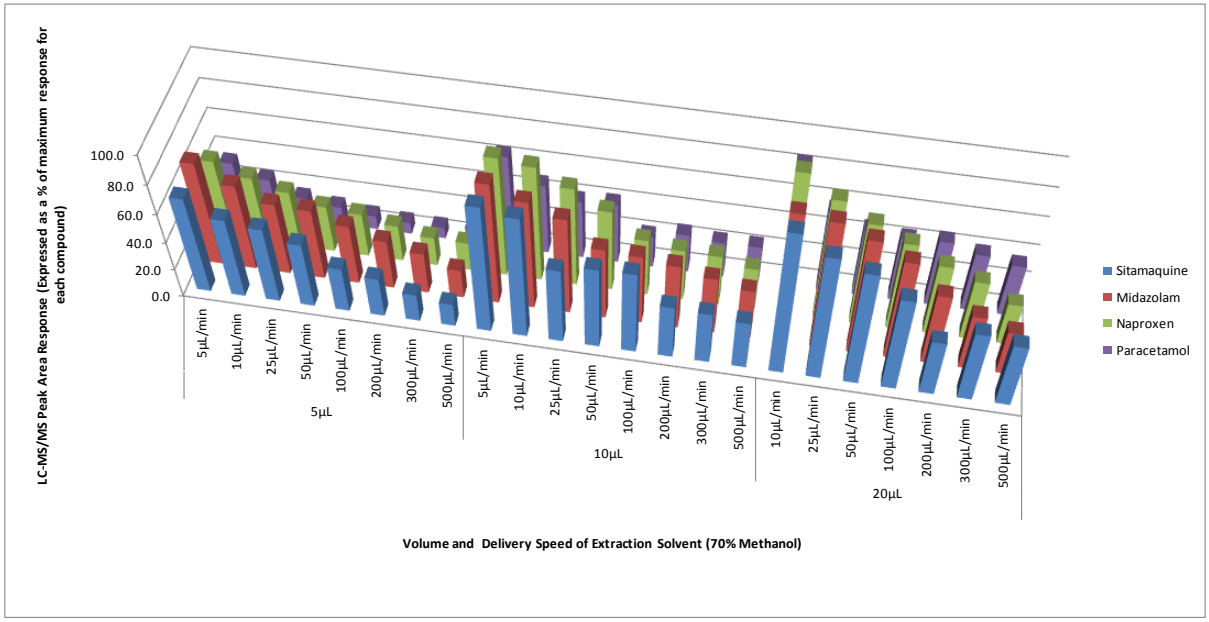


Figure 6.2: Whole-spot direct elution: Variation in LC-MS/MS peak area response with volume and delivery speed of optimised extraction solvent (70% methanol). Only the 3 highest performing extraction volumes are shown. Each value is a mean of n=6.

In most cases the subjective point at which chromatography became unacceptable was matched by the point at which N dropped below 500-1000. However the data demonstrates numerous exceptions, which excludes the possibility of forming a hard and fast rule. The N values for the control manual extraction data are shown in Table 6.4. In determining optimisation parameters both visual inspection and the calculated N value was considered to ensure that the chromatography was not significantly different to what was achieved for the control methods. Note that column packing and dimensions have a large effect on the N value, and thus values were only compared between manual and direct elution methods for each compound, but not between compounds/methods where different HPLC columns were used.

The extraction parameters that produced maximum LC-MS/MS responses for sub-sample direct elution (10 µL of 70% methanol at 50 µL/min) produced acceptable chromatography for all 4 compounds. The extraction parameters that produced maximum LC-MS/MS responses for whole spot direct elution (20 µL of 70% methanol at 10 µL/min) produced acceptable chromatography for all compounds except paracetamol. For paracetamol, the transition between 10 µL and 20 µL of extraction solvent proved to be the tipping point at which chromatographic peak shape became unacceptable (Figure S6.3). For this reason the generic optimised extraction conditions for whole spot direct elution were adjusted to 10 µL at 5 µL/min using 70% methanol, to ensure acceptable chromatography was obtained for each compound.

Table 6.2: Number of theoretical plates, N, and subjective visual inspection of chromatography for Sub-Sample Direct Elution Optimisation. Chromatography was deemed to be unacceptable if peak shape was very broad or displayed major peak asymmetry compared to control DBS manual

extraction chromatography. Delivery speed had no obvious effect on N, so to demonstrate the data concisely a mean N value from each delivery volume is shown.

		Number of Theoretical Plates and Visual Interpretation of Chromatographic Peak Shape													
		Extraction Solvent													
Compound	Direct elution extraction volume µL	MP Matched	Methanol						Acetonitrile						Water
			100%	70%	70% + Heat	50%	25%	100% Acidified	100%	70%	70% + Heat	50%	25%	100% Acidified	
Sitamaquine	1	2422	2424	1932	1904	2916	2246	2878	2492	2101	2001	2314	1876	560	767
	2	1942	2276	1936	1967	2944	2430	2828	2891	2070	2230	1943	1655	435	2290
	5	2284	1980	2008	1923	2766	1916	2658	2909	2360	2512	2120	1550	390	2783
	10	1290	1450	1876	1701	3115	1715	1734	2109	2341	2425	2801	1490	416	2980
	20	802	860	1070	1024	3257	1872	1322	907	1563	2012	1246	1301	89	2867
	40	456	652	644	711	3024	1654	908	244	1019	1324	653	875	36	3267
	60	222	361	475	375	2994	1599	233	201	720	992	480	330	28	2591
	100	108	248	316	298	3307	1801	119	98	213	311	290	182	19	2160
Midazolam	1	21900	23939	25878	24902	22014	22067	22780	16923	18010	19134	20265	20139	14000	19606
	2	19540	21057	23711	22777	19965	19888	12001	12199	16989	17043	17130	18008	11282	20321
	5	18644	18476	20321	21403	18544	17004	15690	5340	14391	15861	16980	1634	7949	22343
	10	15143	16352	19489	19983	16089	13722	10029	1551	6231	7424	14545	1286	2302	23363
	20	6623	4023	5279	4902	1002	4680	3710	409	2098	3021	10021	9830	1388	19480
	40	1532	1521	1920	1298	798	1620	1420	267	1002	1285	3471	1997	700	11192
	60	902	887	890	502	501	788	631	131	686	702	992	660	423	3988
	100	701	595	625	189	268	156	189	54	432	420	360	404	225	601
Naproxen	1	28329	25643	23309	22102	25401	24402	23081	18340	20281	21622	22310	22309	19372	14993
	2	26403	23334	21900	20203	2113	21454	19083	17230	18060	19582	20098	20141	15342	20100
	5	22381	20128	19872	19080	19232	18354	17355	12401	15243	16260	17265	18202	12109	23412
	10	20479	18966	18976	17098	17230	14062	16003	8973	13609	14352	15551	14432	8290	19882
	20	12320	11987	13892	13923	16759	12977	12930	6203	7021	8121	10928	9909	1510	17644
	40	1599	1307	6003	7080	9680	7004	1411	897	1750	1832	2392	1698	788	13042
	60	841	731	1087	1403	997	890	980	231	398	502	955	704	194	4701
	100	178	297	620	660	233	340	450	89	201	340	301	187	73	309
Paracetamol	1	3420	2843	2708	2450	2011	1844	2664	880	1880	2001	1506	1220	230	1154
	2	3098	3220	2433	2390	2544	1620	2871	540	1792	1821	1504	1267	165	1621
	5	3145	2034	2134	2231	1892	1300	1630	479	1683	1801	721	1183	102	3021
	10	2391	1946	1926	1822	1001	1249	440	230	1721	1699	410	1289	301	2672
	20	1023	145	184	199	567	980	300	501	1878	1872	499	1190	418	2241
	40	560	80	105	203	348	762	410	520	1230	1539	480	571	509	1541
	60	340	43	82	156	298	652	460	611	981	1301	701	230	260	388
	100	354	40	45	67	69	59	420	67	480	412	678	119	18	209

Visually acceptable chromatography

Visually unacceptable chromatography

As detailed in the experimental section, rangefinder tests were performed to benchmark the sensitivity that could be achieved from conventional DBS manual extraction for each assay.

The rangefinder test was then repeated for direct elution using the above generic direct elution optimised parameters (Table 6.4). Large increases in assay sensitivity (assay LLOQ's reduced by ~25 fold) between the manual extraction methods and optimised generic direct elution methods were observed. Note that acceptable chromatography was retained for each test compound.

Table 6.3: Number of theoretical plates, N, and subjective visual inspection of chromatography for Whole-Spot Direct Elution Optimisation. Chromatography was deemed to be unacceptable if peak shape was very broad or displayed major peak asymetry compared to control DBS manual extraction chromatography. Delivery speed had no obvious effect on N, so to demonstrate the data concisely a mean N value from each delivery volume is shown.

		Number of Theoretical Plates and Visual Interpretation of Chromatographic Peak Shape													
		Extraction Solvent													
			Methanol						Acetonitrile						
Compound	Direct elution extraction volume µL	MP Matched	100%	70%	70% + Heat	50%	25%	100% Acidified	100%	70%	70% + Heat	50%	25%	100% Acidified	Water
Sitamaquine	1	3046	2876	1899	1934	1799	1159	2699	3006	2971	2830	3098	2018	2843	120
	2	3144	2649	1921	1922	1604	983	2504	3124	2830	2765	3234	1902	2730	201
	5	2467	2750	2045	1899	1397	774	2402	1840	2341	2398	2854	1760	1654	424
	10	1450	2401	1862	1723	1214	710	1987	774	1432	1651	2410	1763	551	732
	20	968	1935	963	1562	1238	401	882	238	1120	1231	1880	1509	98	2001
	40	224	780	701	1011	481	380	465	56	865	1003	1654	920	40	2834
	60	231	602	645	655	317	287	376	50	314	447	620	570	24	2932
	100	210	540	499	442	304	192	279	34	91	139	202	215	18	2230
Midazolam	1	23212	23099	24003	25099	22450	20490	23763	15930	20930	21302	20048	20160	14023	22003
	2	22433	20033	21309	22845	18943	18091	21767	11941	20333	19482	19843	20204	10022	21330
	5	21566	17653	21398	21402	17480	1588	18503	8914	14444	15542	18279	17828	9920	18736
	10	20152	16450	20603	19301	14430	10100	14980	5434	10503	9380	14232	15223	2098	16832
	20	6329	4290	5472	6870	4380	2391	5648	1402	1765	2710	12308	11829	973	10944
	40	921	987	2304	3671	1001	1244	1322	622	821	920	1702	1751	690	1654
	60	491	612	1212	1093	899	780	902	389	308	591	802	990	602	1321
	100	282	400	980	820	507	510	487	215	187	220	333	445	40	473
Naproxen	1	3054	2763	2250	2348	2602	2590	2540	1909	2331	2400	2713	2801	2110	18672
	2	2766	2590	2046	2133	2099	2133	2219	1401	2080	2137	2312	2312	1670	23641
	5	2132	2190	1777	1879	1690	1702	2032	1002	1321	1543	2015	1972	923	24687
	10	2059	1753	20601	892	1480	1388	1432	730	970	1102	1342	1433	691	20350
	20	1423	876	632	700	920	1090	762	584	609	720	1122	1098	499	17832
	40	456	390	421	510	621	677	301	388	423	504	566	695	198	13241
	60	299	203	236	241	420	391	165	154	201	256	199	342	102	5871
	100	153	102	97	150	355	289	84	64	150	111	79	123	23	622
Paracetamol	1	3122	2412	2816	2709	2531	1980	501	977	1530	1464	1562	1221	244	1240
	2	2911	2650	2488	2314	2240	1833	431	801	1621	1621	1232	1099	166	1408
	5	2655	1890	2003	2193	1841	1672	435	722	1670	1762	877	921	110	2871
	10	2310	541	1666	1700	1222	1022	489	204	1340	1524	433	876	398	2652
	20	1870	438	290	301	303	722	366	172	1289	1354	366	560	424	2621
	40	770	411	145	220	287	320	376	132	1187	1233	211	422	512	2899
	60	288	487	132	210	161	111	308	88	489	578	89	129	230	621
	100	127	579	100	87	106	95	330	66	309	222	56	99	12	433

Visually acceptable chromatography

Visually unacceptable chromatography

Table 6.4: Summary of DBS assay sensitivity and chromatographic performance using generic manual extraction, and the optimised generic direct elution conditions identified in this study.
[1] Assay LLOQ (lower limit of quantification) represents the lowest spiked calibration standard that produced a LC-MS/MS peak area response with a signal to noise ration greater than 5:1, and produced acceptable accuracy and precision.
[2] The number of theoretical plates, N was calculated using the mean of N for every calibration standard that fitted into the linear dynamic range of the assay.
[3] The sub-punch (manual extraction) and sub-sample (direct elution) involved taking a 4 mm diameter punch (manual extraction) or utilizing a 4 mm diameter sealed sampling area (direct elution) from the centre of the 15 µL DBS.
[4] Whole spot extraction involved sampling an entire 2 µL DBS either through manual extraction (punching the entire sample) or direct elution (encompassing the entire sample within the sealed sampling area).
[5] Assay HLQ (higher limit of quantification) represents the highest spiked calibration standard that demonstrated accuracy and precision within guideline acceptance criteria.

Compound	Sampling Method	Assay LLOQ ng/mL[1]		Assay HLQ ng/mL[5]		Number of Theoretical Plates, N [2]	
		Manual Extraction	Direct Elution	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution
Sitamaquine	Sub-Punch/Sub-Sample[3]	2	0.1	10000	500	2156	1886
	Whole -Spot[4]	10	0.3	30000	600	2298	1862
Midazolam	Sub-Punch/Sub-Sample[3]	2	0.1	6000	300	20570	19461
	Whole -Spot[4]	10	0.5	30000	2000	20381	20603
Naproxen	Sub-Punch/Sub-Sample[3]	30	1	60000	3000	18679	19039
	Whole -Spot[4]	60	2	120000	6000	20476	20508
Paracetamol	Sub-Punch/Sub-Sample[3]	30	2	30000	2000	2200	1924
	Whole -Spot[4]	100	3	100000	3000	2063	1866

As part of this investigation into direct elution optimisation, the effect of pre-wetting DBS samples prior to extraction, with respect to drying time was also investigated. In previous investigations it had been noticed that extracting samples that were still 'wet' following IS spray application appeared to cause an increase in LC-MS/MS response, though this was not investigated further or reported until now. To ensure this did not cause a bias in previous studies, following IS spray addition, DBS were left to dry overnight prior to extraction. In this study, IS solution was sprayed onto test DBS samples (spiked at a single concentration) using previously optimised conditions[(81)], left for drying times varying from immediately after IS addition (~1 min) to 24 hr, and then extracted using optimised direct elution (Figure 6.3). Note that after IS addition, DBS samples were left to dry in the DBS-MS500 storage racks on the enclosed instrument deck, where drying rate may be slower than on an open bench. LC-MS/MS responses for each analyte were increased by up to 2.5 fold immediately after IS spray application, and the response dropped gradually until a drying time of ~150 min was used, at which point the samples appear to reach an equilibrium and responses show no significant difference up to 1440 min (24 hr). This point (~150 min) could be interpreted as the point at which the DBS sample have fully dried following IS spray application. DBS samples appear to be touch-dry following IS application after ~30 min, but this data suggests the samples may

not be at full equilibrium until ~150 min. Note that there was no significant difference in analyte response between a sample with no IS added, and a sample that had IS applied and allowed to dry overnight before analysis. More work is required to optimise the IS addition in terms of maximising analyte recovery, and identifying how much assay sensitivity can be increased further using this method.

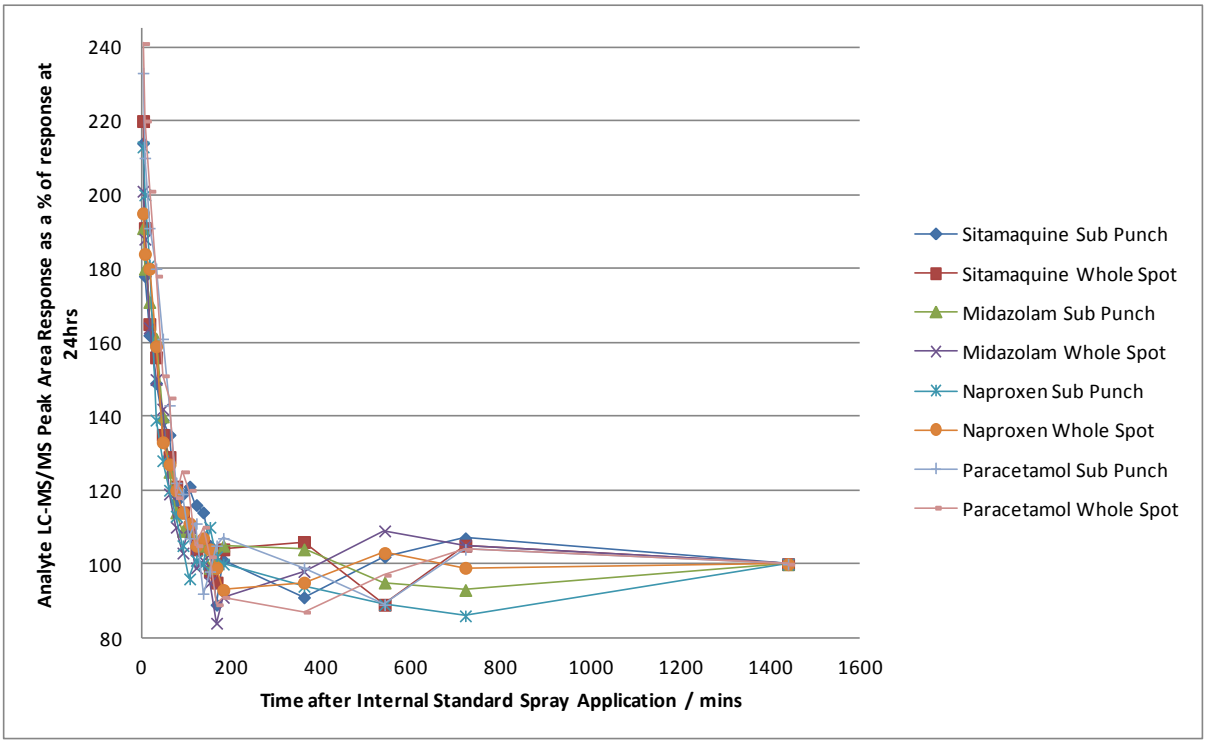


Figure 6.3: Effect of post internal standard spray application drying time on analyte LC-MS/MS response. DBS test samples were spiked at 250 ng/ml (sitamaquine and midazolam), and 2500 ng/mL (Naproxen and Paracetamol).

We theorize that this increase in assay sensitivity is due to the pre-wetted sample allowing more analyte (and IS) to be recovered for given extraction times i.e. it facilitates a more efficient extraction mechanism. The IS LC-MS/MS response following IS spray application followed a similar trend with drying time, although the increase in response immediately after application was slightly higher, and the equilibration point appeared to occur slightly earlier (~ 100 min) (Figure 6.4).

A final set of experiments were undertaken to investigate the feasibility of using a short drying time after IS spray addition to enhance assay sensitivity, while still producing acceptable quantitative performance, and retaining the ability to nullify HCT based recovery bias. In a previous study DBS samples were allowed to dry overnight after IS spray application, and we theorised that the IS drying on the sample may be critical as it enables the IS to become integrated with the sample, allowing the compound and IS to be co-extracted (which is vital to

nullifying HCT based recovery bias when using peak area ratios for quantitation)[81]. To do this whole spot direct elution was used (using the optimised conditions described above), as we have demonstrated previously that whole spot elution in conjunction with IS spray addition (followed by a 24 hr drying time) nullifies HCT based assay bias completely[81]. Test validation runs using quality control samples spiked with varying HCT (0.20 to 0.70), and analysed using a 10 min drying time post IS spray application, demonstrated that this technique could offer quantitative performance within internationally accepted guideline acceptance criteria, and retain the ability to nullify HCT based recovery bias (Table 6.5). Further work is required to test the minimum drying time that could be used that still allows HCT based recovery bias to be nullified. A 10 min drying time obviously hinders the efficiency of high throughput DBS analysis, so either shorter drying times, or the ability to accurately stagger IS addition and extraction on the same platform could help produce a more efficient process. Further work needs to be performed to determine how much assay sensitivity could be increased by extracting pre-wetted samples, but based on the data obtained, a useful two to three fold reduction to the assay LLOQ seems possible.

Table 6.5: Accuracy and precision of quality control samples spiked at varying HCT using optimized direct elution and IS spray drying times of 10 min and 24hr. Assay accuracy values are normalized to the values at the control HCT level (0.45). QC levels 2 and 4 represent 3 times the LLQ and ~75% of the HLQ, respectively.

DBS Drying time Post IS Spray application: 10 mins		Whole Spot Direct Elution							
		Assay Accuracy %							
	HCT	Sitamaquine		Midazolam		Naproxen		Paracetamol	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.20	109.8	100.6	96.4	101.2	108.3	102.5	108.4	102.1
	0.35	97.0	107.3	110.3	107.7	103.2	93.1	87.9	95.2
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	94.2	106.5	94.0	93.5	97.0	111.5	110.0	112.0
	0.70	98.0	101.3	101.2	94.9	102.9	89.6	107.4	97.8

Whole Spot Direct Elution							
Assay Precision CV %							
Sitamaquine		Midazolam		Naproxen		Paracetamol	
QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
3.4	9.8	9.0	5.6	6.7	4.9	10.6	7.9
6.8	5.0	6.0	8.9	3.9	4.0	5.4	9.0
5.1	6.0	5.4	6.1	4.5	6.2	7.4	8.8
9.1	6.7	11.6	10.1	5.5	5.0	5.9	7.3
8.4	5.3	8.1	6.0	7.4	4.3	6.1	5.9

DBS Drying time Post IS Spray application: 24hrs		Whole Spot Direct Elution							
		Assay Accuracy %							
	HCT	Sitamaquine		Midazolam		Naproxen		Paracetamol	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.20	105.1	101.6	106.4	103.2	97.8	96.4	105.8	104.3
	0.35	114.6	108.1	106.2	97.5	99.8	99.1	106.8	96.2
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	100.6	94.5	99.8	102.7	102.1	93.9	99.4	90.9
	0.70	103.7	100.5	102.3	96.3	95.6	104.5	107.2	98.9

Whole Spot Direct Elution							
Assay Precision CV %							
Sitamaquine		Midazolam		Naproxen		Paracetamol	
QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
6.0	8.5	6.7	4.9	5.1	7.1	9.3	6.9
7.4	7.7	5.8	3.8	5.3	5.0	8.0	5.3
3.6	5.5	7.1	5.4	4.8	4.9	6.2	9.9
6.8	4.5	4.8	5.9	3.9	5.8	5.1	8.0
4.6	5.4	4.7	8.1	6.1	4.8	5.4	4.8

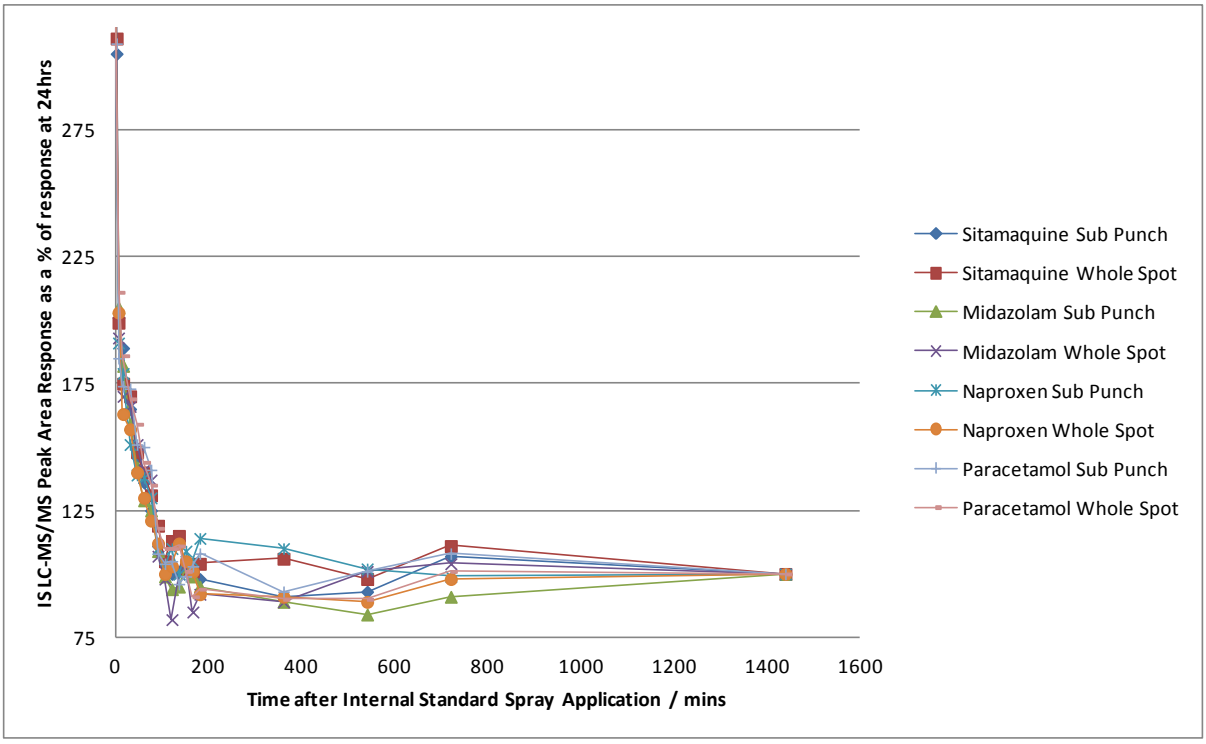


Figure 6.4: Effect of post internal standard spray application drying time on IS LC-MS/MS response.

6.4 Conclusions

The work in this study has resulted in the identification of a generic starting point for small molecule direct elution. Generic optimised direct elution conditions for 4 representative small molecule compounds was demonstrated to lower assay LLOQs around 25 fold, compared to generic conventional manual DBS extraction. In each case LC-MS/MS chromatography was demonstrated to be of acceptable quality, and not significantly different to that obtained using manual extraction. These gains in sensitivity significantly expand the range of assays that DBS sampling is compatible with, and thus enable the important ethical, logistic, and financial advantages on offer from DBS to be maximised.

We acknowledge that there are numerous procedures that could improve the sensitivity of conventional manual extraction, such as solid phase extraction, liquid-liquid extraction, or trapping column/2D LC methods. However, this is equally true for direct elution, where on-line SPE, and 2D chromatography can also be utilised[109,103]. All these processes add considerable complexity, and the aim of this study was to investigate quick and simple methods of DBS analysis that are compatible with high throughput bioanalysis, which is often a critical factor in pharmaceutical discovery and development and other screening applications. We present an automated DBS direct elution methodology that simultaneously eliminates manual extraction, nullifies HCT based assay area and recovery bias, and offers high levels of sensitivity. The combination of these advantages should further enhance the appeal of DBS

analysis, and counter many of the barriers associated with DBS becoming a more widely used sampling technique.

After gathering great interest in recent times, the introduction of DBS sampling to new applications has suffered a drop in momentum due to a number of issues. The automated DBS direct analysis workflow described in this article demonstrates that the HCT, manual extraction burden, and assay sensitivity issues can be overcome relatively easily. The regulatory issue is more challenging. For DBS, or similar microsampling techniques to become widely used in regulated drug development, regulatory authorities require a certain number of these studies to be submitted, along with additional requirements to prove that these new techniques produce reliable, safe data. Unfortunately these additional requirements have a cost implication that currently makes DBS sampling unattractive (outside of niche applications), as well as a regulatory risk until fully approved. It is hoped that breaking down some of the barrier to acceptance will encourage the use of microsampling, so that the potential advantages on offer can be fulfilled. Only by allocating the resource now to support microsampling can the advantages be fully realised later. In the meantime it is hoped that the techniques described here can also be applied to other applications where there are less barriers to acceptance, and the advantages can be reaped in a shorter timescale.

Note: Supplementary information can be found in Appendix F.

CHAPTER 7: OVERALL CONCLUSIONS & FUTURE WORK

7.1 Overall Conclusions

The main aim of this project, to develop an automated DBS direct elution system that was compatible with high sample throughput quantitative bioanalysis in a drug development environment, has been achieved. The collaboration with CAMAG resulted in the DBS-MS500, which has been produced to meet our design and specification (see section 1.7), and is now commercially available. This system counters the manual extraction burden associated with DBS analysis, and has been demonstrated to significantly increase DBS assay sensitivity compared to conventional manual extraction[124].

Beyond the development of the direct elution system, the status of using DBSs within drug development studies changed during the course of this project, and this shaped the direction of subsequent research. The scope of this project changed as a result of the response/recommendations of regulatory authorities (in particular the FDA) concerning the use of DBS data to support drug development studies, and the impact this had on the attractiveness of using DBS[19]. At the start of the project, the momentum behind, and level of interest in implementing DBSs in drug development applications suggested that DBS sampling was a certainty to become a widely used technique in this environment within the timeframe of this project. However, as more research was undertaken to underpin the science behind DBSs, a number of uncertainties arose, such as the homogeneity of DBS samples, the optimum method of adding IS and performing dilutions, the stability of aged DBSs, and most notably the impact of HCT on assay bias[19]. The FDA currently recommend that, until instructed otherwise, DBS data to support drug development studies should be run in parallel with conventionally used wet plasma or whole blood sampling[123,29,23]. It is assumed that the FDA will only recommend DBS data to be used in isolation once they have received, monitored and approved enough drug development studies for them to have sufficient confidence that the technique produces reliable and accurate data[123,29,23]. Unfortunately the requirement to generate parallel wet and dry data has a significant cost implication[59], and consequently the interest in, and momentum behind using DBSs for drug development has dropped significantly as project managers in pharmaceutical development were not able to justify the extra costs (except in niche applications where other sampling alternatives were not possible). However, the use of DBS sampling for therapeutic drug monitoring, collection of samples in remote locations and paediatric studies has continued to gain interest.

The original plan for this project was to develop an automated direct elution technique that could be used to significantly improve the efficiency of high sample throughput quantitative DBS bioanalysis. This was achieved with the development and subsequent testing of the DBS-MS500 instrument, but it has not been possible to use it support drug development studies on a large scale as originally intended, due to the aforementioned issues in using DBSs. Instead, the remainder of this project has focused on using the technology developed to try and solve

some of the issues that are currently preventing DBSs from become a widely used technique, and thus maximising the benefits on offer. The main achievement has been the improved understanding of HCT based DBS assay bias, and it has been demonstrated that the addition of IS via a spray system prior to extraction nullifies recovery bias by allowing analyte and IS co-extraction. The work in this project has demonstrated a workflow that couples accurate volume DBS sampling, to an automated process where IS is integrated to the samples prior to whole-spot direct elution. This workflow eliminates the manual extraction burden associated with DBSs, significantly increases the assay sensitivity on offer from DBS, and effectively eliminates/nullifies the area and recovery bias caused by varying HCT[81,124]. The downside to this workflow is that it requires the use of accurate volume sampling, which complicates the simple workflow originally proposed for DBS sampling. While various technology has already been introduced that enables accurate volume DBS sampling[81,130], other microsampling technology, such as VAMS, may have a fundamental advantage over DBSs as it offers built-in accurate volume sampling[63]. This means that currently the future of DBS sampling, outside of niche applications, is rather uncertain in drug development, despite solutions now being available for all the main issues that have caused concerns in recent years.

The development of DBS usage in drug development is currently being constricted due to the desire to reduce costs in the short term. It is hoped that the drug development industry will take a long term view, and allocate sufficient resource to develop DBSs (or similar microsampling techniques) resource now, so that the ethical and financial advantages on offer can be fulfilled in the future.

7.2 Future Work

As described above, the role that was envisioned for DBS's in pharmaceutical drug development has not come to fruition during the course of this investigation, and it has not been possible to use the direct elution system developed in this project to support a drug development study in practice. Ideally, future work would involve following up the proof of concept work carried out in this investigation by using the technology and techniques described herein to support drug development studies in practice. Likewise, it would be of great interest to see the technology applied to alternative applications, such as in discovery or screening applications.

If the widespread use of DBSs in drug development applications is now unlikely to develop as previously expected in the short term, it is hoped that the advantages on offer from the direct elution and HCT nullification work detailed in this project can be applied to alternative microsampling techniques (such as VAMS). It has already been demonstrated that HCT based recovery bias affects VAMS tips in a similar fashion to DBSs (Denniff et al, GSK, unpublished), so it is clear that some of the same issues apply, and will need to be addressed in the same way.

Further development and testing of DBS direct analysis techniques that do not utilize HPLC would also be of great interest. In any application where sample throughput and efficiency is

important, eliminating the additional time and complexity of using HPLC would be of considerable value. Direct elution without HPLC was demonstrated to be capable of producing assay sensitivity similar to that when HPLC was being utilized for some compounds[74]. Numerous DBS direct desorption techniques (DESI, DART, PaperSpray) were tested in the early parts of this project and showed some promise, though notably none offered the robustness and sensitivity on offer from direct elution. Clearly more development on these techniques would be of value, and potentially offer important advantages in a number of applications. Part of this work would have to assess the impact on selectivity without HPLC, and how this may need to be compensated using other techniques (such as high mass accuracy/resolution MS, perhaps with ion mobility, for example).

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129. Chao TC, Trybala A, Starov V, Das DB: Influence of haematocrit level on the kinetics of blood spreading on thin porous medium during dried blood spot sampling. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 451, 38-47 (2014).

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131. Wagner M, Tonoli D, Varesio E, Hopfgartner G: The use of mass spectrometry to analyze dried blood spots. *Mass spectrometry reviews* (2014).

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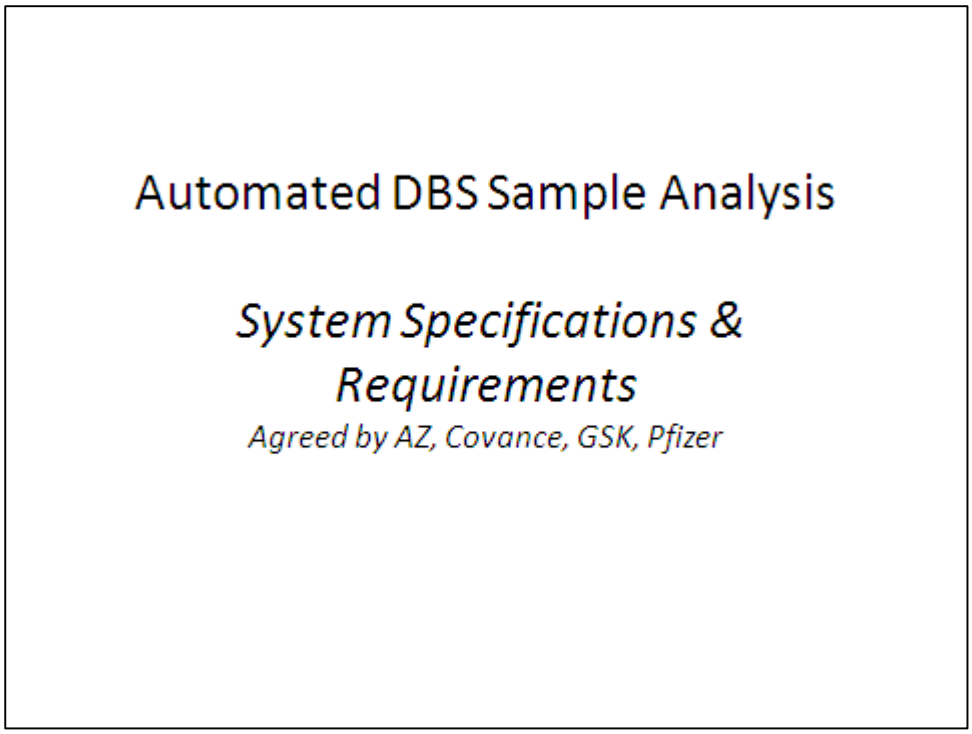
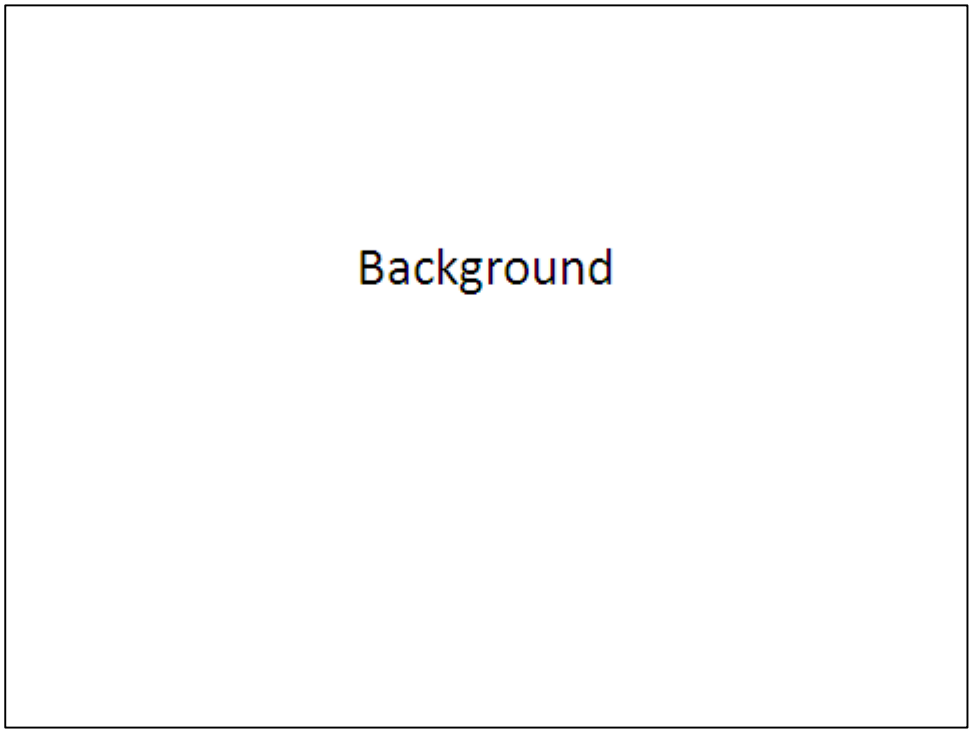
9. APPENDICES

Contents

APPENDIX A: Instrument specification (DBS Consortium)



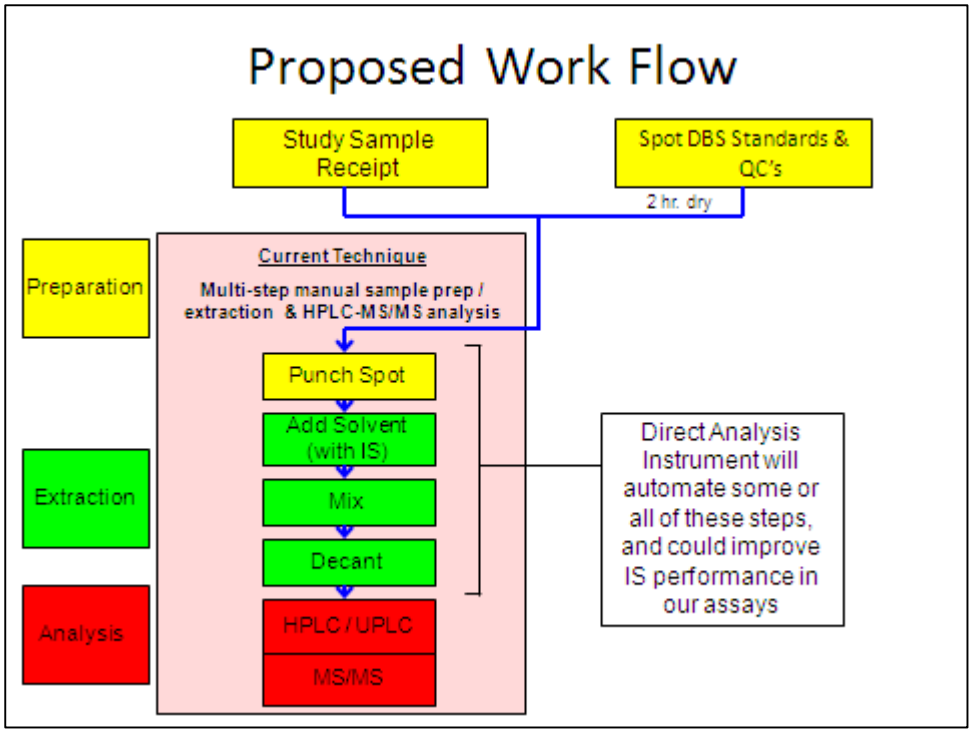


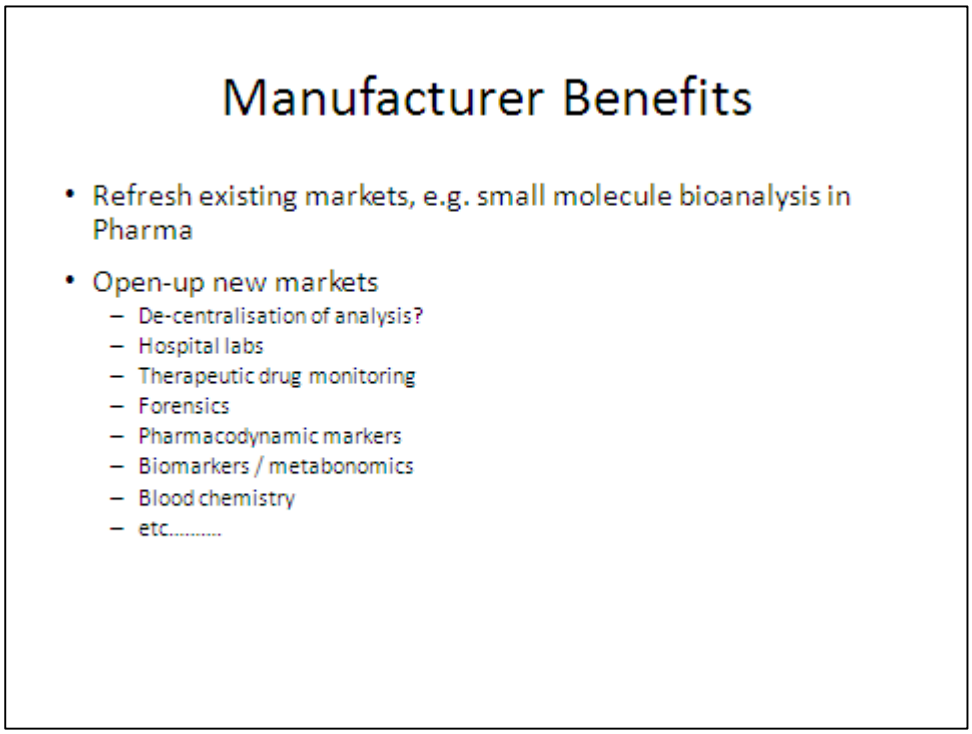
Existing Work Flow

- Study centre sampling
  - Phase II & III clinical studies can be multi centre – 200+ sites
- Ship samples to site of analysis
- Check shipment
- Prepare standards and QC samples
  - 2 hr dry
- Punch and elute samples, standards & QCs
  - 3 mm punch
  - Extract in 100 µL methanol containing IS
  - Shake 1 hr
  - Transfer supernatant
- HPLC-MS/MS analysis
- Data process samples
- Repeat sample analysis if required
- Retain analysed DBS samples

Reasons for Developing an Off-Line integrated System

- Sample traceability
  - Improved linking of data to sample / subject
- Improved compliance
  - Error log
  - Fully validated process
- True walk-away system
- Increased utilisation of instrument systems
  - Overall reduction in capital expenditure


Proposed Work Flow
Study Sample Receipt
Spot DBS Standards & QC's
2 hr. dry
Preparation
Extraction
Analysis
Current Technique
Multi-step manual sample prep / extraction & HPLC-MS/MS analysis
Punch Spot
Add Solvent (with IS)
Mix
Decant
HPLC / UPLC
MS/MS
Direct Analysis Instrument will automate some or all of these steps, and could improve IS performance in our assays


Manufacturer Benefits
• Refresh existing markets, e.g. small molecule bioanalysis in Pharma
• Open-up new markets
– De-centralisation of analysis?
– Hospital labs
– Therapeutic drug monitoring
– Forensics
– Pharmacodynamic markers
– Biomarkers / metabonomics
– Blood chemistry
– etc.....

Instrument Specifications / Requirements

Specifications 1 of 2

Specification	Priority
Compatibility with current (4x1) card format (for development use)	Essential
Capacity ≥ 600 cards (would accept 200 cards for initial models)	Essential
Throughput: No longer than current automated plasma analysis and needs to at least match manual preparation time	Essential, with some flexibility…
Compatibility with multiple punch sizes	Essential
Easy to change between 1, 3 and 6mm diameter punch size by user	Essential
Option to change to custom punch sizes	Essential*
Ability to select punch positions (default to centre with off-centre options)	Essential

*availability as optional extra acceptable

Specifications 2 of 2

Specification	Priority
Ability to select number of punches per spot	Essential
Ability to perform cleaning strikes	Essential
Compatibility with Dilution Techniques: i) Doughnut Technique, ii) punch size matched Technique	Essential
Reproducibility better than 10% for analytical result (response ratio of analyte to internal standard)	Essential
Instrument should be modular in nature for maximum flexibility A simple 'base-level' model should be available that can be upgraded with additional features/requirements A 'multi-purpose' platform that could also handle plasma analysis would be preferable if 'upgrading' from an existing platform.	Preferred, not essential
Compatibility with additional future format for discovery use	Preferred, not essential*

*availability as 'optional extra' acceptable

Requirements 1: General

Specification	Priority
Simple to use, robust, walk away system	Essential
Minimal, simple cleaning /user maintenance	Essential
Readily available global service provision	Essential
Readily available global user support (with good application support)	Essential
Compatibility with all current paper types (Whatman A, B & C / ID Biological 226) *(...and compatibility with new types...)*	Essential
Minimal carryover - No notable interference with subsequent sample(s)	Essential
Dried Matrix Spots - Compatibility with dried blood, plasma, urine and possibly other matrices.	Essential

*availability as 'optional extra' acceptable

Requirements 2: Sample input and Identification

Specification	Priority
Ability to interface with currently used LIMS systems (worklist generation and data output)	Essential
Able to accept samples in multiple formats i.e. 1 sample per time point per card (study samples); all spots on card sampled (standards); 2 Spots per card sampled (QCs)	Essential
Ability to run 'repeat' samples in single or duplicate	Essential
Able to run ≥4 assays in a single overnight batch	Essential
Intelligent spot recognition: Detection/rejection of spot; recognition of 'invalid spot' check box; define the centre of the spot for sampling; detection of previously sampled spot; allows for inaccuracy in spot position	Essential
Intelligent invalid spot recognition: Size (i.e. ±50% of mean area), homogeneity, joined spots, touching frame, splatter etc	Essential
Quick Card Input - Sample input 'hopper/stacker' rather than 'rack' preferred for speed. Automated sample ordering desirable (according to user specifiable preferences). Possibility of using this as a stand alone unit for sample management only, or first pass use for multi use systems.	Preferred, not essential*
Card input forces card to be entered correct side only	Preferred, not essential
Upfront sample quick-check (reconcile samples) before user departs	Preferred, not essential
'Disk in well' confirmation	Preferred, not essential
Image of spot & identifier, before & after sampling must NOT be saved	Essential?

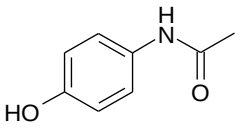
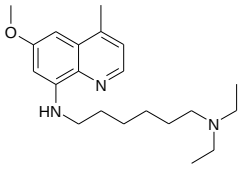
*availability as 'optional extra' acceptable

Requirements 3: Additional

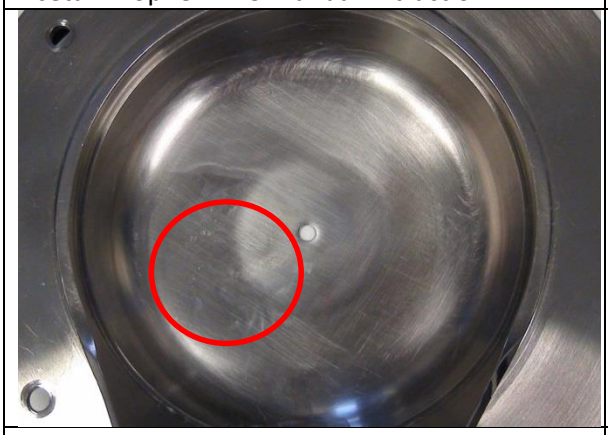
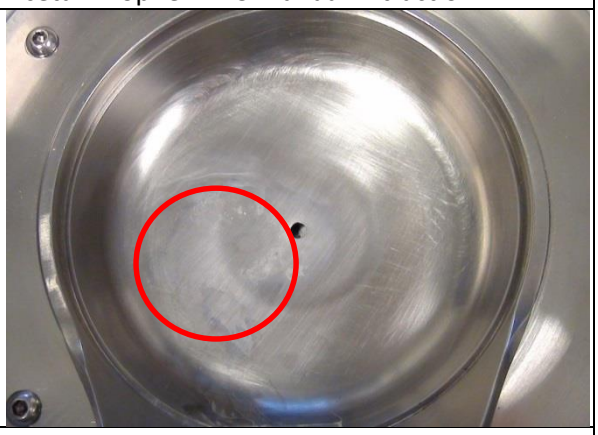
Specification	Priority
Homogenous application of internal standard to dry sample: Applied to spot to be analysed only – No contamination of adjacent spots & samples, or subsequent runs. (Compatible with ≥4 assays in a single overnight batch)	Preferred*
ISR (Incurred Sample Re-analysis) Automatically take 10% of study samples and re-analyse for ISR.	Preferred, not essential
Control from remote desktop	Preferred, not essential

*availability as 'optional extra' acceptable

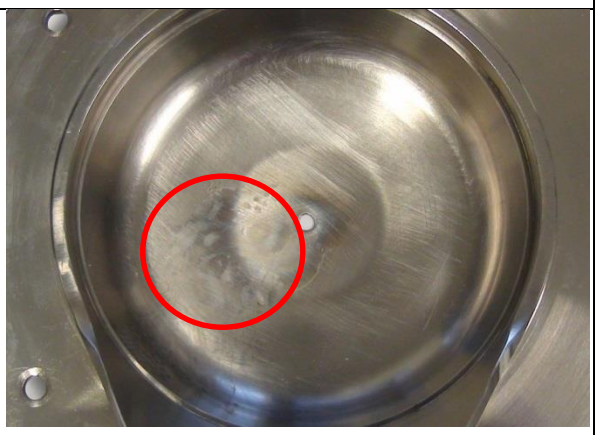
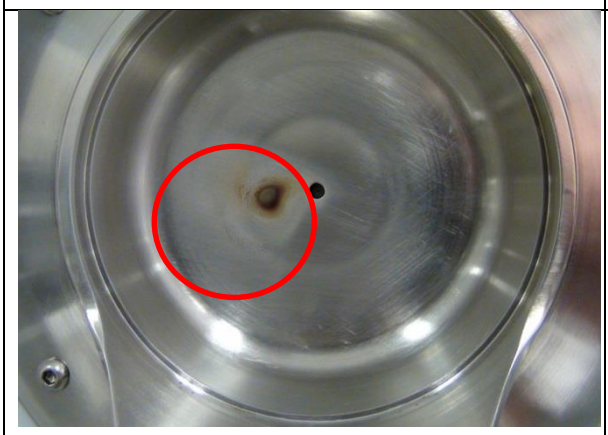
APPENDIX B: Supplementary Information for Chapter 2

Acetaminophen (Neutral)			**Sitamaquine (Base)**		
MW	151		MW	343	
pKa	9.4(OH)		pKa	2.7(NH) 10.3(NR3)	
Log P	0.49		Log P	5.59	
Precursor Ion (m/z)		152	Precursor Ion (m/z)		344

Supporting Information Figure S2.1: Acetaminophen and sitamaquine. Structure and properties with selected reaction-monitoring transitions are listed.

Acetaminophen DBS Manual Extraction	Acetaminophen DPS Manual Extraction
	
Acetaminophen DBS Direct Elution	**Acetaminophen DPS Direct Elution**
	

Supporting information Figure S2.2: Photos of Sciex API3000 Mass spectrometer curtain plate taken after 576 consecutive DBS or DPS samples containing 10000ng/mL acetaminophen using either manual extraction or direct elution. Curtain plate was thoroughly cleaned before start of each run so no sample residue was visible.

Sitamaquine DBS Manual Extraction	Sitamaquine DPS Manual Extraction
	
Sitamaquine DBS Direct Elution	Sitamaquine DPS Direct Elution
	

Supporting information Figure S2.3: Photos of Sciex API3000 Mass spectrometer curtain plate taken after 576 consecutive DBS or DPS samples containing 500ng/mL sitamaquine using either manual extraction or direct elution. Curtain plate was thoroughly cleaned before start of each run so no sample residue was visible.

Table S2.1. Sitamaquine accuracy and precision quality control (QC) data for the original (manual extraction) DBS method.

Sitamaquine QC Level	N	%CV	Accuracy
5ng/mL	6 of 6	10.9	95.9
20ng/mL	6 of 6	9.5	96.7
100ng/mL	6 of 6	6.4	93.6
800ng/mL	6 of 6	6.0	94.0
1000ng/mL	6 of 6	6.3	94.5

Table S2.2: Acetaminophen accuracy and precision quality control (QC) data for the original (manual extraction) DBS method.

Acetaminophen QC Level	N	%CV	Accuracy
50 ng/mL	6 of 6	9.9	108.5
200 ng/mL	6 of 6	7.9	109.2
2500 ng/mL	6 of 6	11.6	106.4
40000 ng/mL	6 of 6	6.4	98.9
50000 ng/mL	6 of 6	4.0	97.7

APPENDIX C: Supplementary Information for Chapter 3

Table S3.1: Summary of MS parameters and SRM transitions used for sitamaquine and acetaminophen.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Polarity	Dwell Time
Acetaminophen	152	110	Positive	100
[2H4] Acetaminophen	156	114	Positive	100
Sitamaquine	344	271	Positive	100
[2H10] Sitamaquine	354	271	Positive	100

Table S3.2: DBS validation set data using a non-optimized TouchSpray configuration to apply internal standard (IS) solution in methanol (125 ng/mL [$^2H_{10}$]-sitamaquine and 2500 ng/mL [$^2H_4$]-acetaminophen) to 15µL dried blood spot (DBS) samples. This configuration used a small diameter application arc that covered approximately a 3 mm diameter area within the centre of the DBS sample. The extent to which the IS solution then wicked out from this area depended on the volume applied. A 3 second application (approximately 24 µL) covered the entire spot diameter across all the paper types used. This created a clear chromatographic effect and thus a significant risk of changing the distribution of drug within the sampling area. Accuracy and precision data obtained for sitamaquine was outside internationally accepted guideline criteria, but was well within for acetaminophen.

A): Sitamaquine

		Whatman FTA DMPK-A		Whatman FTA DMPK-B		Ahlstrom 226	
QC	N	% Accuracy	% CV	% Accuracy	% CV	% Accuracy	% CV
QC5	6	No data	No data	142.3	9.5	111.5	4.4
QC20	6	117.2	13.8	120.7	17.4	103.4	11.2
QC100	6	111.0	5.6	121.6	17.4	110.2	17.6
QC800	6	158.3	18.7	132.2	29.3	119.0	3.2
QC1000	6	175.8	18.1	158.4	8.0	123.8	3.7

B): Acetaminophen

		Whatman FTA DMPK-A		Whatman FTA DMPK-B		Ahlstrom226	
QC	N	% Accuracy	% CV	% Accuracy	% CV	% Accuracy	% CV
QC50	6	106.2	5.7	98.5	4.0	98.1	5.9
QC200	6	106.5	8.8	105.2	5.2	98.6	6.7
QC2500	6	100.0	9.0	105.2	5.8	106.6	4.8
QC40000	6	100.8	13.8	101.7	7.5	101.2	7.0
QC50000	6	103.4	11.6	106.4	5.4	106.3	4.7

Table S3.3: Sitamaquine accuracy and precision QC validation run data. *Control 1* (where IS was spiked into control blood prior to sample preparation) samples were assayed using the CAMAG TLC-MS interface as a direct elution technique. *Control 2* (IS is added via the extraction solvent) samples were assayed using a generic manual extraction technique.

Sitamaquine QC Level	N	Direct Elution Control 1		Manual Extraction Control 2	
		226		FTA DMPK-A	
		%CV	Accuracy	%CV	Accuracy
5ng/mL	6 of 6	5.5	104.7	10.9	95.9
20ng/mL	6 of 6	10.2	104.4	9.5	96.7
100ng/mL	6 of 6	13.2	110.7	6.4	93.6
800ng/mL	6 of 6	3.0	110.5	6.0	94.0
1000ng/mL	6 of 6	1.1	110.2	6.3	94.5

Table S3.4: Acetaminophen accuracy and precision QC validation run data. *Control 1* (where IS was spiked into control blood prior to sample preparation) samples were assayed using the CAMAG TLC-MS interface as a direct elution technique. *Control 2* (IS is added via the extraction solvent) samples were assayed using a generic manual extraction technique.

Acetaminophen QC Level	N	Direct Elution Control 1		Manual Extraction Control 2	
		226		FTA DMPK-A	
		%CV	Accuracy	%CV	Accuracy
50ng/mL	6 of 6	6.4	103.3	9.9	108.5
200ng/mL	6 of 6	12.8	101.3	7.9	109.2
2500ng/mL	6 of 6	6.8	106.0	11.6	106.4
40000ng/mL	6 of 6	9.9	99.3	6.4	98.9
50000ng/mL	6 of 6	4.1	104.5	4.0	97.7

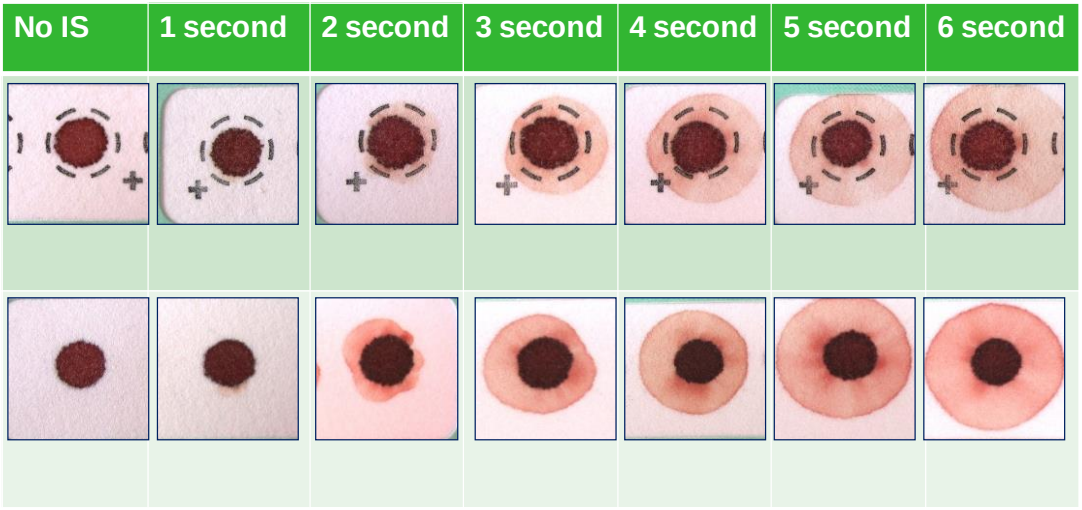


Figure S3.1: Initial attempts with a non-optimised TouchSpray to apply internal standard solution in methanol to 15 µL dried blood spot samples. A narrow distribution arc was used that relied on larger volumes and a wicking effect for the IS solution to visually cover the entire DBS. The touch spray dispensed 8 µL per second (8-48 µL). An undesirable chromatographic effect was visually observed at the larger volumes. Upper photo shows front side of sample, lower photos the reverse. Figure shows application on Ahlstrom 226 paper.

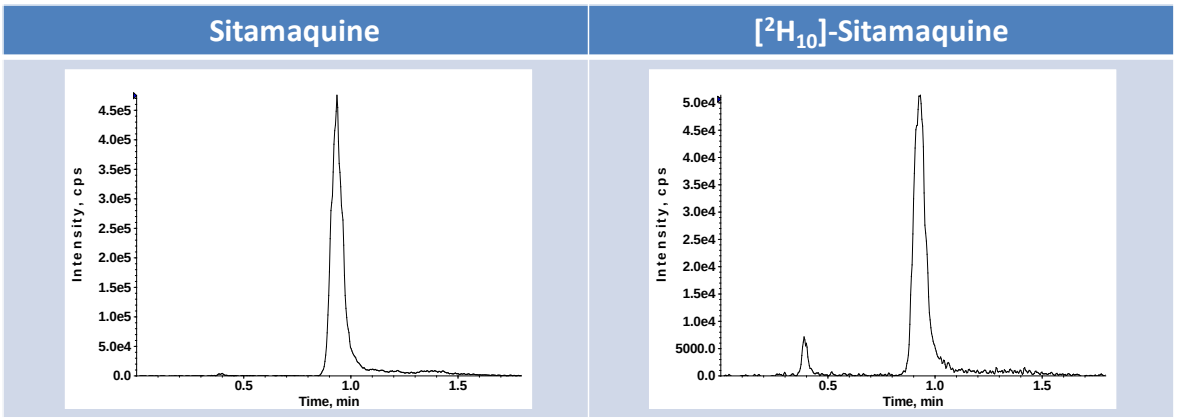


Figure S3.2a: IS added using the TouchSpray device (8 µL of 125 ng/mL [$^2H_{10}$]-sitamaquine)

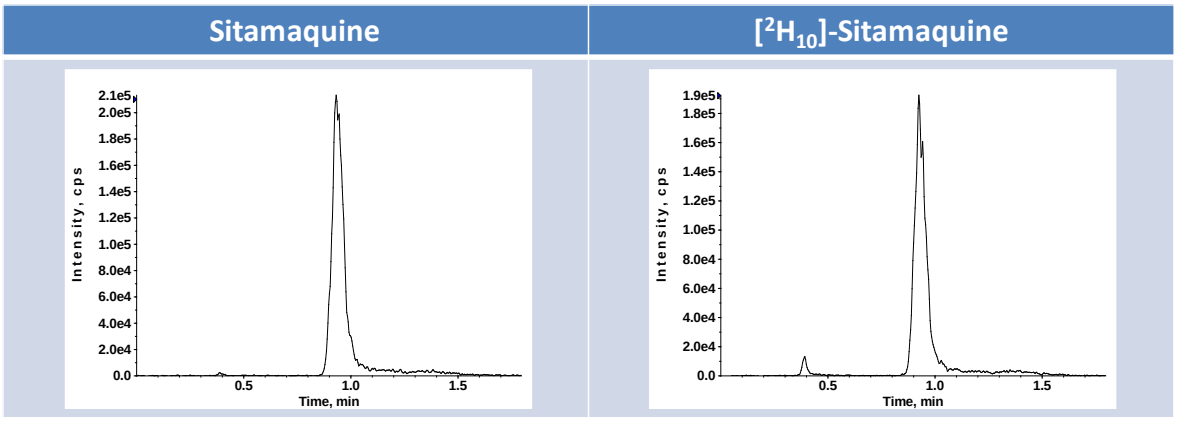


Figure S3.2b: IS spiked into control blood (200 ng/mL [$^2H_{10}$]-sitamaquine) prior to sample preparation.

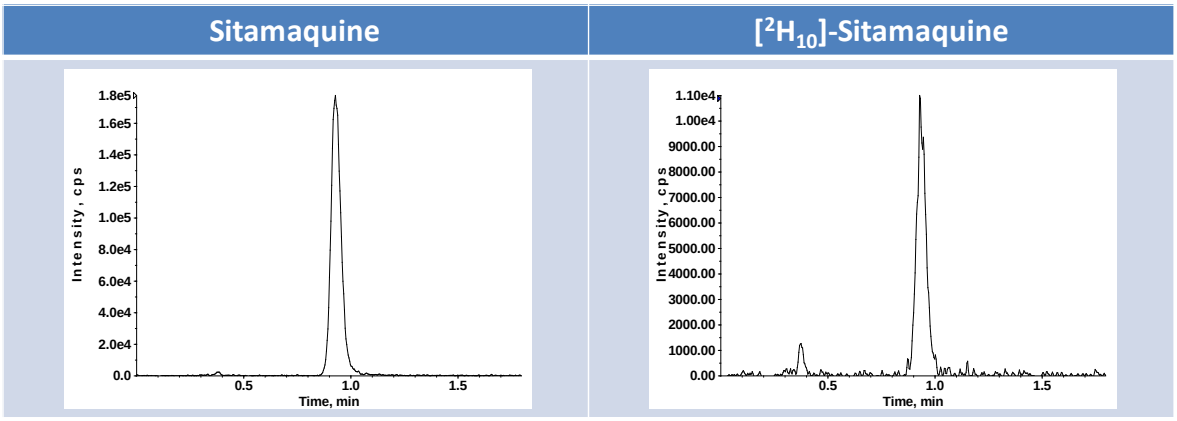


Figure S3.2c: IS incorporated into manual extraction solvent (10 ng/mL [$^2H_{10}$]-sitamaquine)

Figure S3.2: Representative chromatography obtained for sitamaquine and [$^2H_{10}$]-sitamaquine using manually extracted dried blood spot (DBS) samples where internal standard (IS) was applied using three different methods. In each case the DBS sample contained 200 ng/mL sitamaquine in control rat blood on Ahlstrom 226 paper.

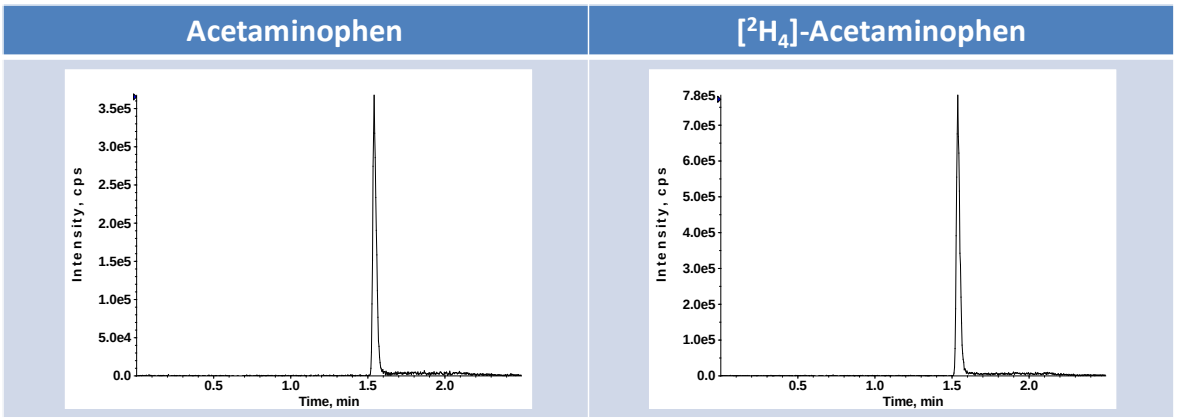


Figure S3.3a: IS added using the TouchSpray device (8 µL of 2500 ng/mL [$^2H_4$]-acetaminophen)

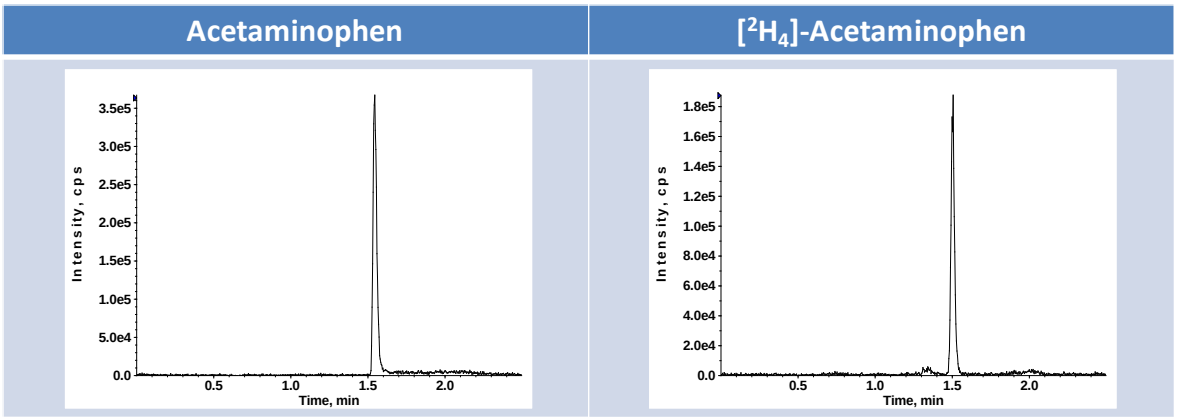


Figure S3.3b: IS spiked into control blood (2000 ng/mL [$^2H_4$]-acetaminophen) prior to sample preparation.

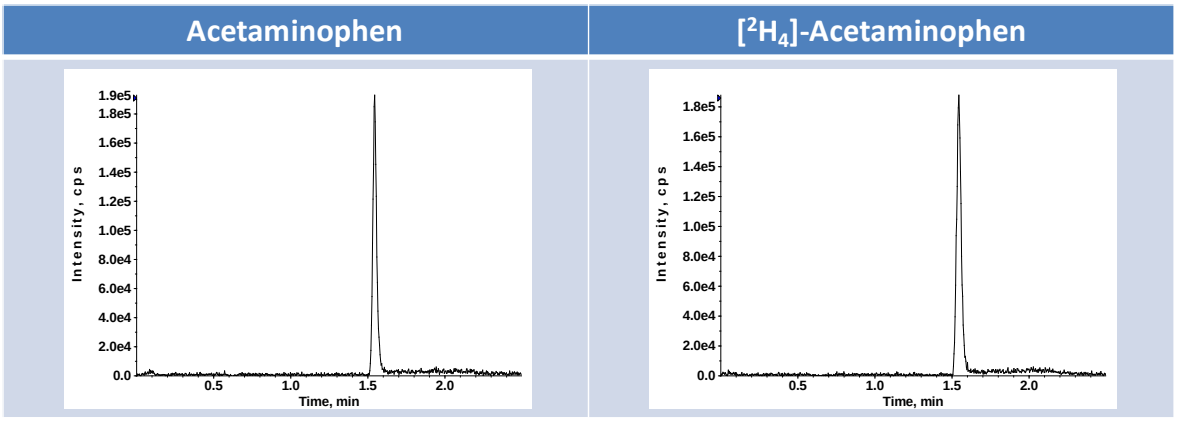


Figure S3.3c: IS incorporated into manual extraction solvent (200 ng/mL [$^2H_4$]-acetaminophen)

Figure S3.3. Representative chromatography obtained for acetaminophen and [$^2H_4$]-acetaminophen using manually extracted dried blood spot (DBS) samples where internal standard (IS) was applied using three different methods. In each case the DBS sample contained 5000 ng/mL acetaminophen in control rat blood on Ahlstrom 226 paper.

APPENDIX D: Supplementary Information for Chapter 4

Table S4.1: Characteristics of the representative compounds used in this study.

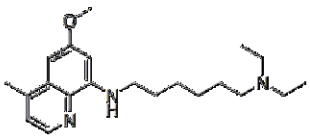
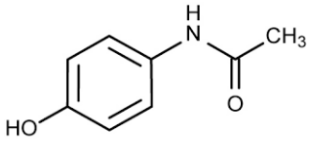
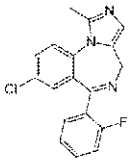
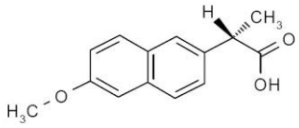
Compound	Mol Weight	Structure	DBS Assay Recovery	Acid/Base/ Neutral	Assay range ng/mL	pKa	SIL	MS/MS Parent/Product Transition Da
Sitamaquine	343.51		40	Base	5 to 1000	10.3	$^{2}H_{10}$	344-354
Paracetamol	151.16		60	Neutral	50 to 50000	9.4	$^{2}H_{3}$	152-110
Midazolam	327.78		90	Neutral	5 to 5000	5.9	$^{2}H_{3}{}^{13}C_{3}$	328-291
Naproxen	230.26		98	Acid	100 to 10000	4.4	$^{2}H_{3}$	231-185

Table S4.2: Concentrations of IS added for each IS application type.

Method of IS addition	Concentration of SIL IS used ng/mL							
	[$^{2}H_{10}$]-Sitamaquine		[$^{2}H_{3}$]-Paracetamol		[$^{2}H_{3}$,$^{13}C_{3}$]-Midazolam		[$^{2}H_{3}$]-Naproxen	
	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution
IS in Extraction Solvent	10	1	500	100	25	5	250	50
IS spiked into whole blood prior to spotting	100	25	2500	1000	250	100	2500	1000
IS spray onto DBS (optimised Conditions)	250	250	5000	5000	1000	1000	5000	5000

Table S4.3: IS Spray solvent/volume combinations taken forward for further optimisation (highlighted in green), and rejected following initial DBS sample integrity experiments (highlighted in red).

IS Spray Volume/ µL	Spray application speed	Solvent/Volume Combinations taken forward for further optimisation								
		Water	Methanol	MeOH:H2O 70:30	MeOH:H2O 50:50	MeOH:H2O 25:75	ACN	ACN:H2O 50:50	DMSO	DMSO:H2O 50:50
5	Fast	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
10	Fast	No	Yes	Yes	No	No	Yes	No	Yes	No
20	Fast	No	Yes	No	No	No	Yes	No	No	No
30	Fast	No	Yes	No	No	No	Yes	No	No	No
40	Fast	No	Yes	No	No	No	Yes	No	No	No

Table S4.4: IS Spray optimisation control data using sitamaquine: For control data, IS was either not added, or spiked into whole blood before DBS samples were prepared and spotted. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																			
sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
BLANK BLOOD (IS IN BLOOD 100 ng/mL)	IS response	Intra Run Mean	230436.3	245982.9	211438.3	275622.6	300121.1	255112.2	253323.4	209766.4	198554.4	200971.2	213421.1	199811.2	200987.9	278912.1	230988.4	280988.2	290877.3	233431.7		239485.9
		Intra run mean as a % of between run mean	96.2	102.7	88.3	115.1	125.3	106.5	105.8	87.6	82.9	83.9	89.1	83.4	83.9	116.5	96.5	117.3	121.5	97.5		100.0
		Intra run CV	11.4	4.5	4.2	7.0	7.6	8.8	9.7	8.1	5.2	8.9	7.1	12.6	18.0	20.0	7.5	13.1	6.8	9.0		9.4
QC4 (IS IN BLOOD 100 ng/mL)	IS response	Intra Run Mean	193440.8	344858.5	486636.7	323669.1	366511.8	297198.2	282882.6	259853.3	265824.6	198894.6	188112.6	189773.1	212212.9	214728.1	250042.3	192585.1	196953.5	285874.6		263891.8
		Intra run mean as a % of between run mean	73.3	130.7	184.4	122.7	138.9	112.6	107.2	98.5	100.7	75.4	71.3	71.9	80.4	81.4	94.8	73.0	74.6	108.3		100.0
		Intra run CV	7.6	7.2	9.1	7.9	9.0	3.6	8.9	7.2	2.4	2.3	6.7	5.0	9.0	4.0	7.7	6.7	8.5	5.8		6.6
	Analyte response	Intra Run Mean	239167.3	401667.3	512388.9	388767.9	412766.6	374440.1	310988.1	289766.4	315644.2	240991.2	213455.4	222965.5	250899.1	380973.2	311244.5	282344.2	261765.3	343049.5		319626.9
		Intra run mean as a % of between run mean	74.8	125.7	160.3	121.6	129.1	117.1	97.3	90.7	98.8	75.4	66.8	69.8	78.5	119.2	97.4	88.3	81.9	107.3		100.0
		Intra run CV	10.2	5.5	14.3	7.6	3.3	6.1	8.8	7.5	6.3	4.5	9.0	6.7	5.6	11.7	8.1	2.3	12.0	10.2		7.8
	Peak Area Ratio	Intra Run Mean	1.19	1.22	1.21	1.22	1.18	1.19	1.15	1.14	1.17	1.17	1.14	1.13	1.14	1.20	1.21	1.16	1.19	1.20		1.2
		Intra run mean as a % of between run mean	101.1	103.6	102.8	103.2	100.0	100.7	97.5	96.8	99.6	99.2	96.6	95.8	97.1	101.9	102.8	98.8	100.7	101.9		100.0
		Intra run CV	1.5	1.3	1.0	1.1	5.6	5.4	7.4	4.4	6.9	6.7	9.3	5.9	4.0	8.9	9.4	7.4	7.9	7.2		5.6
QC4 (NO IS)	Analyte response	Intra Run Mean	276161.4	307480.6	267647.4	307641.8	281594.9	303114.5	370256.7	299385.6	227767.4	259325.7	198061.5	271562.0	225586.2	230043.8	221443.0	228597.5	223510.2	214034.9		261845.3
		Intra run mean as a % of between run mean	105.5	117.4	102.2	117.5	107.5	115.8	141.4	114.3	87.0	99.0	75.6	103.7	86.2	87.9	84.6	87.3	85.4	81.7		100.0
		Intra run CV	7.4	8.8	5.8	6.6	6.1	6.7	10.9	7.4	10.8	6.1	3.9	6.2	4.2	4.2	9.6	8.6	2.6	7.9		6.9

Table S4.5: IS Spray optimisation test data using sitamaquine. IS was added via the CAMAG DBS-MS500 IS module. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																			
sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
TOTAL BLANK (IS Spray 250 ng/mL)	IS response	Intra Run Mean	145418.7	189643.0	218369.4	349184.1	412444.7	106799.0	154244.3	154391.8	90959.1	81824.6	140778.1	209181.2	274194.3	295491.5	99931.7	89370.5	108838.9	89459.8		178362.5
		Intra run mean as a % of between run mean	81.5	106.3	122.4	195.8	231.2	59.9	86.5	86.6	51.0	45.9	78.9	117.3	153.7	165.7	56.0	50.1	61.0	50.2		100.0
		Intra run CV	13.9	10.9	6.4	6.9	8.8	16.0	13.7	7.2	18.2	15.7	14.3	9.3	8.3	10.3	17.3	17.8	20.8	20.2		13.1
BLANK BLOOD (IS Spray 250 ng/mL)	IS response	Intra Run Mean	144121.0	170979.9	231768.6	305221.3	369100.4	103195.4	131354.7	99401.6	91966.8	62434.0	97559.3	121765.5	171826.0	202733.4	65305.4	62280.8	71661.7	61185.6		142436.7
		Intra run mean as a % of between run mean	101.2	120.0	162.7	214.3	259.1	72.5	92.2	69.8	64.6	43.8	68.5	85.5	120.6	142.3	45.8	43.7	50.3	43.0		100.0
		Intra run CV	11.7	9.1	2.7	3.0	4.9	8.7	10.1	6.7	7.3	13.2	7.9	4.6	4.7	8.9	15.2	4.9	9.7	6.1		7.8
QC4 (IS Spray 250 ng/mL)	IS response	Intra Run Mean	120241.9	180695.3	198517.8	269098.6	300059.7	88469.8	104674.7	77114.4	76576.2	90832.0	99574.1	140592.4	161990.3	189311.6	80695.9	72066.3	81800.7	80610.6		134051.2
		Intra run mean as a % of between run mean	89.7	134.8	148.1	200.7	223.8	66.0	78.1	57.5	57.1	67.8	74.3	104.9	120.8	141.2	60.2	53.8	61.0	60.1		100.0
		Intra run CV	10.3	4.2	3.2	4.1	4.7	9.9	5.4	10.8	11.0	12.1	10.1	7.2	7.4	6.8	11.1	14.5	6.9	10.3		8.3
	Analyte response	Intra Run Mean	264321.9	342259.9	382446.2	257314.6	280854.8	371376.4	340380.6	426750.8	379142.9	262898.6	178771.0	190097.3	191623.9	202227.1	202033.9	212601.1	250890.2	224072.3		275559.1
		Intra run mean as a % of between run mean	95.9	124.2	138.8	93.4	101.9	134.8	123.5	154.9	137.6	95.4	64.9	69.0	69.5	73.4	73.3	77.2	91.0	81.3		100.0
		Intra run CV	8.6	10.2	8.8	7.1	6.6	9.5	3.3	7.1	10.1	8.9	4.0	7.0	7.9	3.6	2.5	12.3	10.9	10.4		7.7
	PAR	Intra Run Mean	2.2	2.1	1.9	1.2	1.1	4.2	3.1	4.9	5.2	2.9	2.0	2.0	1.5	1.2	2.7	3.2	3.3	3.2		2.7
		Intra run mean as a % of between run mean	82.9	79.2	70.9	45.8	40.2	157.9	114.5	183.5	195.9	109.9	74.7	75.7	57.8	46.2	100.2	121.2	124.2	119.3		100.0
		Intra run CV	12.8	6.9	2.1	2.0	3.3	7.4	5.0	5.0	10.6	9.8	14.1	5.4	4.3	5.9	7.5	7.9	13.0	8.7		7.3

Table S4.6: IS Spray optimisation control data using paracetamol. For control data, IS was either not added, or spiked into whole blood before DBS samples were prepared and spotted. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
BLANK BLOOD (IS IN BLOOD 2500 ng/mL)	IS response	Intra Run Mean	1839843.2	1787544.2	1678999.1	1599243.2	1825499.3	1821354.7	1911323.4	2043272.5	1892344.1	1812445.6	1934562.3	1675499.3	1687392.2	1564322.7	1489755.5	1765988.1	1772349.4	1987633.4		1782742.9
		Intra run mean as a % of between run mean	103.2	100.3	94.2	89.7	102.4	102.2	107.2	114.6	106.1	101.7	108.5	94.0	94.7	87.7	83.6	99.1	99.4	111.5		100.0
		Intra run CV	6.7	8.4	8.9	11.1	11.4	8.0	6.1	6.9	5.6	7.0	8.9	12.3	12.2	9.9	10.9	7.8	9.4	8.9		8.9
QC4 (IS IN BLOOD 2500 ng/mL)	IS response	Intra Run Mean	1792446.4	1897662.3	2098256.4	1712345.1	2033244.5	1923422.7	2032485.7	2132975.7	2002344.9	1834222.1	1576444.6	1802877.3	1624343.2	1643231.7	1435023.1	1692374.4	2793422.5	2921759.8		1941604.6
		Intra run mean as a % of between run mean	92.3	97.7	108.1	88.2	104.7	99.1	104.7	109.9	103.1	94.5	81.2	92.9	83.7	84.6	73.9	87.2	143.9	150.5		100.0
		Intra run CV	6.5	4.5	9.2	8.0	10.4	11.3	5.6	6.1	3.3	5.0	6.8	11.1	7.4	12.3	5.4	7.2	8.0	3.3		7.3
	Analyte response	Intra Run Mean	6988644.3	7232145.6	8976344.2	7564122.2	9123422.9	8001243.3	8197304.1	8020333.1	8022987.8	7699342.6	6788902.0	8024904.3	7232566.1	6898344.4	6132433.5	7133977.1	12475604.3	12045278.7		8142105.6
		Intra run mean as a % of between run mean	85.8	88.8	110.2	92.9	112.1	98.3	100.7	98.5	98.5	94.6	83.4	98.6	88.8	84.7	75.3	87.6	153.2	147.9		100.0
		Intra run CV	14.1	9.9	10.2	7.1	6.7	5.5	10.9	8.8	4.4	3.9	9.0	4.3	5.4	11.1	5.9	3.9	9.3	7.0		7.6
	PAR	Intra Run Mean	3.63	3.71	4.09	4.14	4.19	4.02	3.98	3.89	3.80	3.85	3.87	4.06	3.97	3.81	3.88	3.78	4.11	3.97		3.9
		Intra run mean as a % of between run mean	92.4	94.4	104.1	105.3	106.6	102.3	101.3	99.0	96.7	98.0	98.5	103.3	101.0	96.9	98.7	96.2	104.6	101.0		100.0
		Intra run CV	4.0	2.5	5.5	3.2	3.8	1.9	2.3	4.5	6.4	5.5	6.3	4.5	3.8	4.2	5.0	3.2	4.0	3.6		4.1
QC4 (NO IS)	Analyte response	Intra Run Mean	7944356.3	7822534.2	7698703.2	7811203.2	8013325.0	7652034.3	7564409.1	7345334.3	7289992.0	7120034.3	8004354.2	8123343.3	7899020.2	8100231.2	7902032.5	7303411.2	6902355.6	6823412.2		7628893.7
		Intra run mean as a % of between run mean	104.1	102.5	100.9	102.4	105.0	100.3	99.2	96.3	95.6	93.3	104.9	106.5	103.5	106.2	103.6	95.7	90.5	89.4		100.0
		Intra run CV	4.9	3.3	6.4	3.9	3.8	6.6	6.1	5.5	7.9	8.0	4.2	5.2	4.1	4.0	8.7	6.7	3.2	3.3		5.3

Table S4.7: IS Spray optimisation test data using paracetamol. IS was added via the CAMAG DBS-MS500 IS module. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
Sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
TOTAL BLANK (IS Spray 5000ngmL)	IS response	Intra Run Mean	1434304.2	2200433.2	3082344.0	3800257.3	4199208.5	1737455.2	1997923.2	1620744.2	1592022.0	1392340.2	1752902.2	2923404.3	3450606.0	3939820.2	1599332.6	1334330.3	1899322.3	1599405.3		2308675.3
		Intra run mean as a % of between run mean	62.1	95.3	133.5	164.6	181.9	75.3	86.5	70.2	69.0	60.3	75.9	126.6	149.5	170.7	69.3	57.8	82.3	69.3		100.0
		Intra run CV	8.1	5.9	4.0	3.9	3.6	10.2	10.0	11.1	8.9	10.1	5.7	8.0	6.4	6.3	10.6	14.2	13.1	15.0		8.6
BLANK BLOOD (IS Spray 5000ngmL)	IS response	Intra Run Mean	1592022.0	2093765.6	3421355.4	4186344.9	4401243.3	1607763.2	1820345.5	1598230.4	1406743.3	1365201.3	1823004.3	2833457.3	3624063.2	4225382.0	1702632.4	1504432.9	1698903.4	1611851.8		2362041.2
		Intra run mean as a % of between run mean	67.4	88.6	144.8	177.2	186.3	68.1	77.1	67.7	59.6	57.8	77.2	120.0	153.4	178.9	72.1	63.7	71.9	68.2		100.0
		Intra run CV	6.0	6.2	2.8	3.4	3.3	7.9	8.9	10.2	12.1	11.5	7.2	5.0	5.0	6.9	14.3	9.9	9.1	6.1		7.5
QC4 (IS Spray 5000ngmL)	IS response	Intra Run Mean	1563452.0	2234755.6	2974899.0	3950476.3	4565887.9	1659703.7	2097676.0	1500757.2	1365743.8	1123980.7	1567587.3	2077630.4	2530231.8	3860524.8	1547556.6	1292304.7	1754778.9	1575334.7		2180182.3
		Intra run mean as a % of between run mean	71.7	102.5	136.5	181.2	209.4	76.1	96.2	68.8	62.6	51.6	71.9	95.3	116.1	177.1	71.0	59.3	80.5	72.3		100.0
		Intra run CV	8.0	5.5	2.5	4.1	3.8	6.9	7.7	8.8	10.0	9.1	6.4	5.0	6.8	7.1	9.6	11.4	11.2	13.2		7.6
	Analyte response	Intra Run Mean	7997901.6	8144683.3	9001743.2	8659487.1	8012376.8	7895342.1	7231998.2	6987612.3	6899472.3	6534672.3	6658911.2	6342998.2	6911253.2	7452988.3	8011293.4	8231982.2	8213198.2	8537743.2		7651425.4
		Intra run mean as a % of between run mean	104.5	106.4	117.6	113.2	104.7	103.2	94.5	91.3	90.2	85.4	87.0	82.9	90.3	97.4	104.7	107.6	107.3	111.6		100.0
		Intra run CV	6.3	6.0	8.1	6.1	5.9	5.5	3.4	7.1	11.1	6.2	7.6	7.0	8.9	4.3	5.8	6.1	4.3	7.0		6.5
	PAR	Intra Run Mean	4.99	3.83	3.23	2.35	1.69	4.67	3.51	4.51	4.93	5.70	4.29	3.01	2.96	2.06	4.93	6.04	4.45	5.36		4.0
		Intra run mean as a % of between run mean	123.9	95.1	80.2	58.3	42.0	115.9	87.1	112.0	122.4	141.5	106.5	74.7	73.5	51.1	122.4	149.9	110.5	133.1		100.0
		Intra run CV	8.8	6.0	2.4	2.5	3.3	7.4	7.6	6.4	10.6	9.8	5.2	4.9	4.3	4.0	7.5	12.4	13.0	8.7		6.9

Supplementary Information Table S4.8: IS Spray optimisation control data using midazolam. For control data, IS was either not added, or spiked into whole blood before DBS samples were prepared and spotted. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
BLANK BLOOD (IS IN BLOOD 250 ng/mL)	IS response	Intra Run Mean	2046448.0	1834985.7	1869458.4	2454687.2	2654189.3	3026478.5	2891115.1	2135699.9	2643990.4	2435162.8	2012341.4	2348112.3	1846811.5	2622473.2	2599811.2	1949536.2	1876489.0	2098212.1		2297000.1
		Intra run mean as a % of between run mean	89.1	79.9	81.4	106.9	115.6	131.8	125.9	93.0	115.1	106.0	87.6	102.2	80.4	114.2	113.2	84.9	81.7	91.3		100.0
		Intra run CV	6.0	8.5	6.0	4.2	6.7	10.4	7.9	11.2	12.1	9.2	8.9	5.9	9.0	7.9	8.1	11.5	5.1	13.1		8.4
QC4 (IS IN BLOOD 250 ng/mL)	IS response	Intra Run Mean	2162783.3	1966584.3	1963622.1	2388574.9	2664773.3	2863222.2	2817723.4	2347278.8	2499455.2	2399003.2	1893321.5	2511202.3	2100043.2	2490803.1	2435123.3	1892303.6	2004356.2	2183432.6		2310200.4
		Intra run mean as a % of between run mean	93.6	85.1	85.0	103.4	115.3	123.9	122.0	101.6	108.2	103.8	82.0	108.7	90.9	107.8	105.4	81.9	86.8	94.5		100.0
		Intra run CV	4.8	3.9	8.8	4.3	4.8	7.0	3.6	5.5	5.6	3.9	2.9	2.0	1.8	1.6	2.6	12.9	10.4	11.0		5.4
	Analyte response	Intra Run Mean	4016623.4	4290832.2	3299883.4	4964222.2	4390987.1	4491080.6	4415643.2	4092453.6	4189336.5	5014622.6	2967008.4	3548893.2	3657774.3	4061120.3	4239980.4	3410203.3	3659182.3	3541123.2		4013942.8
		Intra run mean as a % of between run mean	100.1	106.9	82.2	123.7	109.4	111.9	110.0	102.0	104.4	124.9	73.9	88.4	91.1	101.2	105.6	85.0	91.2	88.2		100.0
		Intra run CV	6.7	8.1	9.8	7.6	6.2	9.0	10.2	5.4	5.6	8.4	6.9	4.6	7.8	9.1	8.3	9.3	8.1	6.7		7.7
	PAR	Intra Run Mean	1.95	1.89	1.75	2.01	1.73	1.66	1.63	1.82	1.70	1.99	1.66	1.59	1.75	1.62	1.88	1.80	1.86	1.65		1.8
		Intra run mean as a % of between run mean	109.9	106.5	98.6	113.3	97.5	93.6	91.9	102.6	95.8	112.1	93.6	89.6	98.6	91.3	105.9	101.4	104.8	93.0		100.0
		Intra run CV	3.9	4.6	7.1	6.7	8.1	9.5	5.2	5.4	4.6	4.4	4.3	7.0	8.9	3.6	3.7	4.0	3.8	3.9		5.5
QC4 (NO IS)	Analyte response	Intra Run Mean	3869554.7	4023356.1	4185569.0	3789221.1	3768840.2	3699841.1	3567411.1	4009533.1	4687411.0	4587204.5	3897740.1	3968744.1	3784425.1	3691142.1	3364911.1	3224765.1	3517010.1	3800214.1		3857605.2
		Intra run mean as a % of between run mean	100.3	104.3	108.5	98.2	97.7	95.9	92.5	103.9	121.5	118.9	101.0	102.9	98.1	95.7	87.2	83.6	91.2	98.5		100.0
		Intra run CV	5.6	7.2	6.0	6.2	4.6	3.9	8.1	8.8	10.1	5.3	5.5	4.3	8.8	6.1	5.4	9.1	7.6	4.3		6.5

Table S4.9: IS Spray optimisation test data using midazolam. IS was added via the CAMAG DBS-MS500 IS module. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
Sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
TOTAL BLANK (IS Spray 1000 ng/mL)	IS response	Intra Run Mean	1531565.1	1834985.7	2503218.3	3066521.8	3781421.2	1437775.2	1895543.1	1134566.1	1166787.4	1452989.8	2156897.6	2713987.0	3653323.5	4138755.8	1002741.4	926654.7	987455.4	978412.4		2020200.1
		Intra run mean as a % of between run mean	75.8	90.8	123.9	151.8	187.2	71.2	93.8	56.2	57.8	71.9	106.8	134.3	180.8	204.9	49.6	45.9	48.9	48.4		100.0
		Intra run CV	6.0	5.1	4.5	4.1	5.0	8.3	9.0	9.8	10.7	7.0	4.6	3.9	4.0	6.2	7.2	14.3	12.1	11.5		7.4
BLANK BLOOD (IS Spray 1000 ng/mL)	IS response	Intra Run Mean	1640523.1	1795206.3	2399871.4	2895063.8	3589710.1	1649809.8	1922643.2	1081964.7	1200871.5	1617935.4	2164751.4	2846552.3	3473655.1	4019440.1	1123544.4	1026444.4	1100031.0	941145.0		2027175.7
		Intra run mean as a % of between run mean	80.9	88.6	118.4	142.8	177.1	81.4	94.8	53.4	59.2	79.8	106.8	140.4	171.4	198.3	55.4	50.6	54.3	46.4		100.0
		Intra run CV	6.8	4.3	4.0	4.1	4.6	8.1	8.9	4.5	10.4	5.1	5.0	5.1	3.8	6.1	11.5	14.4	13.6	15.9		7.6
QC4 (IS Spray 1000 ng/mL)	IS response	Intra Run Mean	1467807.8	1905468.1	2698544.1	3064977.8	3564199.7	1658213.4	2062562.9	1231445.4	1280631.5	1625889.1	1987778.4	2540804.7	3346608.8	3987556.2	1097874.0	958743.6	1066482.0	1060387.4		2033665.3
		Intra run mean as a % of between run mean	72.2	93.7	132.7	150.7	175.3	81.5	101.4	60.6	63.0	79.9	97.7	124.9	164.6	196.1	54.0	47.1	52.4	52.1		100.0
		Intra run CV	7.0	2.8	3.6	4.3	4.0	10.5	4.1	7.8	12.1	5.5	5.0	6.1	3.7	5.5	9.8	9.9	18.1	16.5		7.6
	Analyte response	Intra Run Mean	3856214.1	3625530.0	4035960.3	4123698.6	4199056.6	3744561.0	3856445.8	4156006.3	3784405.6	3611245.1	4311253.8	4477450.6	3328904.4	3126945.0	3211548.4	3514264.8	3641512.6	3745651.4		3797258.6
		Intra run mean as a % of between run mean	101.6	95.5	106.3	108.6	110.6	98.6	101.6	109.4	99.7	95.1	113.5	117.9	87.7	82.3	84.6	92.5	95.9	98.6		100.0
		Intra run CV	8.0	6.4	5.8	4.0	4.5	9.4	9.9	5.8	6.4	6.8	9.1	10.6	11.2	6.6	5.5	5.9	9.8	6.9		7.4
	PAR	Intra Run Mean	2.49	2.00	1.61	1.40	1.32	2.21	1.91	3.21	2.90	2.34	2.31	1.69	1.12	3.01	2.89	3.59	3.31	3.56		2.4
		Intra run mean as a % of between run mean	104.5	84.0	67.6	58.8	55.4	92.8	80.2	134.8	121.8	98.3	97.0	71.0	47.0	126.4	121.3	150.7	139.0	149.5		100.0
		Intra run CV	6.8	7.1	3.8	4.0	4.2	9.1	6.8	10.4	8.9	8.1	6.9	4.7	3.8	4.0	8.8	12.5	11.9	16.6		7.7

Table S4.10: IS Spray optimisation control data using naproxen. For control data, IS was either not added, or spiked into whole blood before DBS samples were prepared and spotted. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
BLANK BLOOD (IS IN BLOOD 2500 ng/mL)	IS response	Intra Run Mean	489665.0	521326.2	536887.0	504203.5	521600.9	465223.1	481202.1	469503.1	450603.8	500199.6	598663.5	600595.1	615396.9	529966.6	485159.2	459663.1	486991.6	473565.4		510578.7
		Intra run mean as a % of between run mean	95.9	102.1	105.2	98.8	102.2	91.1	94.2	92.0	88.3	98.0	117.3	117.6	120.5	103.8	95.0	90.0	95.4	92.8		100.0
		Intra run CV	5.6	7.9	3.9	8.4	8.9	9.1	4.6	4.8	5.0	8.6	8.4	7.5	7.6	5.0	5.5	9.1	9.8	5.5		7.0
QC4 (IS IN BLOOD 2500 ng/mL	IS response	Intra Run Mean	496141.7	517468.2	526988.0	512004.4	518467.3	479562.2	476532.1	480005.1	469813.9	491223.9	605191.0	584322.1	625314.1	532998.0	501211.6	481264.4	507449.2	492132.5		516560.5
		Intra run mean as a % of between run mean	96.0	100.2	102.0	99.1	100.4	92.8	92.3	92.9	91.0	95.1	117.2	113.1	121.1	103.2	97.0	93.2	98.2	95.3		100.0
		Intra run CV	7.9	6.6	6.5	5.0	5.1	10.1	8.1	8.5	4.6	4.5	3.9	4.5	5.8	6.4	6.8	5.8	6.0	6.1		6.2
	Analyte response	Intra Run Mean	1165484.1	1259632.5	1098536.4	1087741.1	1100589.0	986348.4	936541.4	905467.1	1145794.0	1294561.1	1308852.5	1187456.3	1198416.4	952649.8	998606.0	908746.3	1017529.4	115693.7		1037147.0
		Intra run mean as a % of between run mean	112.4	121.5	105.9	104.9	106.1	95.1	90.3	87.3	110.5	124.8	126.2	114.5	115.5	91.9	96.3	87.6	98.1	11.2		100.0
		Intra run CV	8.0	7.6	6.1	6.8	5.1	5.2	4.8	10.2	10.9	6.6	6.0	5.8	8.1	6.0	4.8	5.1	4.9	4.8		6.5
	PAR	Intra Run Mean	2.45	2.39	1.98	2.01	2.10	2.01	1.89	1.79	2.34	2.52	2.31	1.95	1.85	1.79	1.94	1.76	2.00	2.21		2.1
		Intra run mean as a % of between run mean	118.3	115.4	95.6	97.0	101.4	97.0	91.2	86.4	113.0	121.6	111.5	94.1	89.3	86.4	93.6	85.0	96.5	106.7		100.0
		Intra run CV	4.0	5.1	3.8	3.9	7.2	5.0	6.9	5.8	6.8	4.0	5.8	8.1	7.0	9.1	5.5	6.9	8.0	9.2		6.2
QC4 (NO IS)	Analyte response	Intra Run Mean	1096532.5	1141560.4	1012684.0	1125689.0	1256593.2	975268.1	956974.6	923005.8	1094261.5	1198562.5	1400156.0	1285866.5	1312554.1	1100996.2	1099598.1	958639.9	1035564.8	1105654.5		1115564.5
		Intra run mean as a % of between run mean	98.3	102.3	90.8	100.9	112.6	87.4	85.8	82.7	98.1	107.4	125.5	115.3	117.7	98.7	98.6	85.9	92.8	99.1		100.0
		Intra run CV	6.9	5.0	5.8	9.6	8.5	4.9	10.2	9.8	6.4	4.8	4.9	6.6	6.9	7.1	6.9	8.1	8.0	6.7		7.1

Table S4.11: IS Spray optimisation test data using naproxen. IS was added via the CAMAG DBS-MS500 IS module. Inter-run mean LC-MS/MS responses for each sample type (within an individual run) were expressed as a percentage of the mean between-run values (mean LC-MS/MS response of that sample type across the 18 batches).

			Mean LC-MS/MS Response (Peak Area cps) n=18																		
Sample Type	LC-MS/MS Response	IS Spray solvent and volume (where IS spray was used)	MeOH 5µL	MeOH 10µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH: Water 70:30 5µL	MeOH: Water 70:30 10µL	MeOH: Water 50:50 5µL	MeOH: Water 25:75 5µL	MeCN 5µL	MeCN 10µl	MeCN 20µL	MeCN 30µL	MeCN 40µL	MeCN: Water 50:50 5µL	DMSO 5µL	DMSO 10µL	DMSO: Water 50:50 5µL		Between Run Mean
TOTAL BLANK (IS Spray 5000 ng/mL)	IS response	Intra Run Mean	354695.5	435898.1	502988.3	568429.6	645899.0	295369.5	345963.3	305981.4	284565.5	378529.9	464440.1	550080.8	597780.4	689554.4	345121.1	285363.1	331861.6	264405.7		424829.3
		Intra run mean as a % of between run mean	83.5	102.6	118.4	133.8	152.0	69.5	81.4	72.0	67.0	89.1	109.3	129.5	140.7	162.3	81.2	67.2	78.1	62.2		100.0
		Intra run CV	6.1	3.8	4.2	5.0	4.5	8.1	9.0	10.5	11.8	6.0	6.8	4.0	5.8	6.0	12.1	14.5	15.0	16.1		8.3
BLANK BLOOD (IS Spray 5000 ng/mL)	IS response	Intra Run Mean	335646.4	425909.5	489600.3	654442.1	626315.0	315465.2	364559.0	290081.1	296336.6	392112.1	470008.1	569214.8	600017.8	701144.7	365098.8	305651.5	329964.1	283696.6		434181.3
		Intra run mean as a % of between run mean	77.3	98.1	112.8	150.7	144.3	72.7	84.0	66.8	68.3	90.3	108.3	131.1	138.2	161.5	84.1	70.4	76.0	65.3		100.0
		Intra run CV	8.0	6.5	3.0	3.4	2.9	10.1	10.8	3.7	7.9	6.9	5.5	4.0	4.9	5.1	9.6	11.7	8.9	16.2		7.2
QC4 (IS Spray 5000 ng/mL)	IS response	Intra Run Mean	330364.9	429008.8	481218.8	589296.9	645897.7	302006.8	367084.1	278986.5	314544.8	389066.5	478852.6	549998.4	615594.8	684854.7	378962.5	310069.8	335652.8	281145.4		431255.9
		Intra run mean as a % of between run mean	76.6	99.5	111.6	136.6	149.8	70.0	85.1	64.7	72.9	90.2	111.0	127.5	142.7	158.8	87.9	71.9	77.8	65.2		100.0
		Intra run CV	8.1	4.5	2.9	3.1	4.0	4.5	3.5	10.9	9.8	5.0	3.8	3.3	4.9	5.2	11.8	16.1	12.8	9.8		6.9
	Analyte response	Intra Run Mean	1252365.1	1159874.5	1098565.5	1056369.8	1100508.8	969535.2	995464.8	939658.0	991478.8	986488.7	974326.4	1010087.8	1115890.7	1299830.5	1194014.5	1305648.4	1285464.4	1298547.6		1113006.6
		Intra run mean as a % of between run mean	112.5	104.2	98.7	94.9	98.9	87.1	89.4	84.4	89.1	88.6	87.5	90.8	100.3	116.8	107.3	117.3	115.5	116.7		100.0
		Intra run CV	6.0	5.8	5.9	4.0	4.5	2.9	3.8	7.8	6.9	8.8	5.8	5.9	6.1	6.8	5.8	6.9	7.1	7.8		6.0
	PAR	Intra Run Mean	3.61	2.81	2.31	1.85	1.76	3.21	2.64	3.38	3.01	2.46	1.99	1.79	1.84	1.88	3.15	4.08	3.91	4.34		2.8
		Intra run mean as a % of between run mean	129.9	101.1	83.1	66.6	63.3	115.5	95.0	121.6	108.3	88.5	71.6	64.4	66.2	67.7	113.4	146.8	140.7	156.2		100.0
		Intra run CV	4.0	2.5	1.9	3.0	4.1	4.8	3.4	6.0	6.2	5.8	6.8	3.6	5.0	5.8	5.1	7.1	6.0	8.1		5.0

Table S4.12: Summary of the control and test IS Spray optimisation data using the sitamaquine assay (using 18 different IS spray volume/solvent combinations). The inter run ranges show the highest and lowest inter run mean response across the 18 runs, as a percentage of the between run mean response (n=18).

	Control Data												IS Spray optimisation Test Data											
n = 18 (x18 runs)	Blood Blanks (IS in Blood)				QC4 (IS in Blood)				QC4 (No IS)				Total Blanks (IS spray)				Blood Blanks (IS spray)				QC4 (IS spray)			
	Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV	
	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run
IS Response	82.9 to 121.5% of between run mean	239485.9 cps	4.2 to 20.0%	9.4%	71.3 to 184.4% of between run mean	263891.8 cps	2.3 to 9.1%	6.6%	N/A	N/A	N/A	N/A	50.1 to 231.2% of between run mean	178362.5 cps	6.4 to 20.8%	13.1%	43.0 to 259.1% of between run mean	142436.7 cps	2.7 to 15.2%	7.8%	53.8 to 223.8% of between run mean	134051.2	3.2 to 14.5%	8.3
Analyte Response	N/A	N/A	N/A	N/A	66.8 to 160.3% of between run mean	319626.9 cps	2.3 to 14.3%	7.8%	75.6 to 141.4% of between run mean	261845.3 cps	2.6 to 10.9%	6.9%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	64.9 to 154.9% of between run mean	275559.1 cps	2.5 to 12.3%	7.7%
PAR	N/A	N/A	N/A	N/A	95.8 to 103.6% of between run mean	1.2	1.0 to 8.9%	5.6%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	40.2 to 195.9% of between run mean	2.7	2.0 to 14.1	7.3%

Table S4.13: Summary of the control and test IS Spray optimisation data using the paracetamol assay. The inter run ranges show the highest and lowest inter run mean response across the 18 runs, as a percentage of the between run mean response (n=18).

	Control Data												IS Spray optimisation Test Data											
n = 18 (x18 runs)	Blood Blanks (IS in Blood)				QC4 (IS in Blood)				QC4 (No IS)				Total Blanks (IS spray)				Blood Blanks (IS spray)				QC4 (IS spray)			
	Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV	
	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run
IS Response	83.6 to 114.6% of between run mean	1782742.9 cps	5.6 to 12.3%	9.0%	73.9 to 150.5% of between run mean	1941604.6 cps	3.3 to 12.3%	7.3%	N/A	N/A	N/A	N/A	57.8 to 181.9% of between run mean	2308675.3 cps	3.6 to 15.0%	8.6%	63.7 to 186.3% of between run mean	2362041.2 cps	2.8 to 14.3%	7.5%	51.6 to 209.4% of between run mean	2180182.3 cps	2.5 to 13.2%	7.60%
Analyte Response	N/A	N/A	N/A	N/A	75.3 to 153.2% of between run mean	8142105.6 cps	3.9 to 14.1%	7.6%	89.4 to 106.5% of between run mean	7628893.7 cps	3.2 to 8.7%	5.3%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	82.9 to 117.6% of between run mean	7651425.4 cps	3.4 to 11.1%	6.5%
PAR	N/A	N/A	N/A	N/A	92.4 to 106.6% of between run mean	3.9	1.9 to 6.4%	4.1%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	42.0 to 149.9% of between run mean	4	2.4 to 13.0	6.9%

Table S4.14: Summary of the control and test IS Spray optimisation data using the midazolam assay. The inter run ranges show the highest and lowest inter run mean response across the 18 runs, as a percentage of the between run mean response (n=18).

	Control Data												IS Spray optimisation Test Data											
n = 18 (x18 runs)	Blood Blanks (IS in Blood)				QC4 (IS in Blood)				QC4 (No IS)				Total Blanks (IS spray)				Blood Blanks (IS spray)				QC4 (IS spray)			
	Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV	
	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run
IS Response	79.9 to 131.8% of between run mean	2297000.1 cps	4.2 to 13.1%	8.4%	81.9 to 123.9% of between run mean	2310200.4 cps	1.6 to 12.9%	5.4%	N/A	N/A	N/A	N/A	45.9 to 187.2% of between run mean	2020200.1 cps	3.9 to 14.3%	7.4%	46.4 to 177.1% of between run mean	2027175.7 cps	3.8 to 15.9%	7.6%	52.1 to 196.1% of between run mean	2033665.3 cps	2.8 to 18.1%	7.6%
Analyte Response	N/A	N/A	N/A	N/A	73.9 to 124.9% of between run mean	4013942.8 cps	4.6 to 10.2%	7.7%	83.6 to 118.9% of between run mean	3857605.2 cps	3.9 to 10.1%	6.5%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	82.3 to 117.9% of between run mean	3797258.6 cps	4.0 to 11.2%	7.4%
PAR	N/A	N/A	N/A	N/A	89.6 to 113.3% of between run mean	1.8	3.6 to 9.5%	5.5%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	55.4 to 150.7% of between run mean	2.4	3.8 to 16.6%	7.7%

Table S4.15: Summary of the control and test IS Spray optimisation data using the naproxen assay. The inter run ranges show the highest and lowest inter run mean response across the 18 runs, as a percentage of the between run mean response (n=18).

	Control Data												IS Spray optimisation Test Data											
n = 18 (x18 runs)	Blood Blanks (IS in Blood)				QC4 (IS in Blood)				QC4 (No IS)				Total Blanks (IS spray)				Blood Blanks (IS spray)				QC4 (IS spray)			
	Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV		Mean		CV	
	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run	Intra run range	Between Run
IS Response	88.3 to 117.6% of between run mean	510578.7 cps	3.9 to 9.8%	7.0%	91.0 to 117.2% of between run mean	516560.5 cps	3.9 to10.1%	6.2%	N/A	N/A	N/A	N/A	62.2 to 133.8% of between run mean	42482903 cps	3.8 to 16.1%	8.3%	66.8 to 161.5% of between run mean	434181.3 cps	2.9 to 16.2%	7.2%	64.7 to 158.8% of between run mean	431255.9 cps	2.9 to 16.1%	6.90%
Analyte Response	N/A	N/A	N/A	N/A	87.3 to 126.2% of between run mean	1037147.0 cps	4.8 to 10.9%	6.5%	82.7 to 125.5% of between run mean	115564.5 cps	4.8 to 10.2%	7.1%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	84.4 to 117.3% of between run mean	1113006.6 cps	2.9 to 8.8%	6.0%
PAR	N/A	N/A	N/A	N/A	85.0 to 121.6% of between run mean	2.1	3.8 to 9.2%	6.2%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	63.3to 156.2% of between run mean	2.8	1.9 to 8.1%	5.0%

Table S4.16: Top 5 ranking IS Spray solvent/volume combinations based on intra-run IS response and peak area ratio CVs.

Ranking of IS LC-MS/MS Response Intra-Run Calculated CV	IS Spray Solvent/Volumne Combination															
	Sitamaquine				Paracetamol				Midazolam				Naproxen			
	IS Response			Peak Area Ratio	IS Response			Peak Area Ratio	IS Response			Peak Area Ratio	IS Response			Peak Area Ratio
	Total Blank	Blood Blank	QC4	QC4	Total Blank	Blood Blank	QC4	QC4	Total Blank	Blood Blank	QC4	QC4	Total Blank	Blood Blank	QC4	QC4
Lowest CV	MeOH 20µL	MeOH 20µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH 20µL	MeOH 20µL	MeOH 20µL	MeCN 20µL	MeCN 30µL	MeOH 10µL	MeOH 20µL MeCN 30µL	MeOH 10µL	MeOH 40µL	MeOH 20µL	MeOH 20µL
2nd Lowest CV	MeOH 30µL	MeOH 30µL	MeOH 30µL	MeOH 20µL	MeOH 30µL	MeOH 40µL	MeOH 40µL	MeOH 30µL	MeCN 30µL	MeOH 20µL	MeOH 20µL	MeOH 30µL MeCN 40µL	MeCN 20µL	MeOH 20µL	MeOH 30µL	MeOH 10µL
3rd Lowest CV	50:50 MeOH:Water 5µL	MeCN 20µL	MeOH 10µL	MeOH 40µL	MeOH 20µL	MeOH 30µL	MeOH 30µL	MeOH 40µL	MeOH 30µL	MeOH 30µL	MeCN 30µL	MeOH 30µL	MeOH 20µL	MeOH 30µL	MeCN 20µL	MeOH 30µL
4th Lowest CV	MeCN 30µL	MeCN 30µL	MeOH 40µL	MeCN 30µL	MeCN 10µL	MeCN 20µL MeCN 30µL	MeCN 20µL	MeCN 30µL	MeOH 20µL	MeOH 10µL	MeOH 40µL	MeCN 20µL	MeOH 40µL	50:50 MeOH:Water 5µL	70:30 MeOH:Water	70:30 MeOH:Water
5th Lowest CV	MeOH 40µL	MeOH 40µL DMSO 5µL	70:30 MeOH:Water 10µL	70:30 MeOH:Water 10µL MeOH:Water 50:50 5µL	MeOH 10µL	MeOH 5µL	MeOH 10µL	MeCN 40µL	MeCN 10µL	50:50 MeOH:Water 5µL	70:30 MeOH:Water 10µL	MeOH 5µL MeOH:Water 50:50 5µL	MeOH 30µL	MeCN 20µL	MeCN 10µL	MeCN 20µL

APPENDIX E: Supplementary Information for Chapter 5

Content

Appendix E1: Supporting Information Experimental Details

Chemicals, Reagents, and other Equipment

Human volunteer control blood was collected via the GlaxoSmithKline blood donation unit (Stevenage, UK) in accordance with current GSK policies on informed consent and ethical approval.
Methanol, acetonitrile, and water were of HPLC gradient grade and were obtained from Fisher Scientific Ltd (Loughborough, UK). All other chemicals were of analytical grade, supplied by Fisher Scientific Ltd (Loughborough, UK). Paracetamol (aka acetaminophen), [$^2H_3$]-paracetamol, sitamaquine, [$^2H_{10}$]-sitamaquine, and [$^2H_3{}^{13}C_3$]-midazolam were obtained from GlaxoSmithKline (Stevenage, UK). Midazolam was obtained from Tocris Bioscience (Bristol, UK). Naproxen and [$^2H_3$]-Naproxen were obtained from Sigma Aldrich (Pool, UK). Ahlstrom grade 226 paper for blood spots was supplied by Perkin Elmer (Buckinghamshire, UK). Sample tubes were obtained from Micronics (Sanford, USA). The centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat were supplied by Ted Pella (Redding, USA). Benchtop sample shaker (model HS 501 D) was supplied by Janke and Kunkel, IKA Labortechnik (Staufen, Germany). The CAMAG DBS-MS500 was obtained from CAMAG (Basel, Switzerland) and was operated using Chronos software. The HPLC-MS/MS system consisted of an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven. MS detection was by a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada) equipped with Turbo IonSpray source. HPLC-MS/MS data were acquired and processed (integrated) using Analyst software v1.6.1 (Applied Biosystems/MDS Sciex, Canada).

Direct Elution

Direct elution was carried out using the CAMAG DBS-MS500, which is one of the few commercially available DBS direct elution instruments[73]. This instrument fully automates the DBS direct elution process we first reported in 2009[74]. Further work has shown this technique to be suitably reliable and reproducible, and it is used here as an example of how to make the DBS workflow more efficient and reduce the additional manual burden associated with DBS[75]. The DBS-MS500 essentially takes the place of the autosampler in a typical LC-MS/MS rig. In brief, the direct elution process works by flowing a suitable extraction solvent over the DBS sample, which removes the analyte of interest, and transferring the extract into the HPLC flow for analysis. The DBS-MS500 has a capacity of 500 DBS cards, an intelligent visual recognition system to accurately determine the position of spots on the DBS cards, and an integrated IS addition spray module, which means the vast bulk of the manual extraction burden of DBS extraction is removed. We were able to facilitate whole-spot extraction using direct elution, despite our DBS-MS500 not being specifically designed to do so. It was found that the visual recognition software could locate and centre DBS of less than the diameter of the sealed sampling area with 100% accuracy over the course of this study (Chronos software saves an image of each DBS post extraction allowing this to be verified). However, because our DBS-MS500 unit uses a 4mm diameter sealed sampling probe (the equivalent of the 4mm diameter punch, used for manual extraction), 2 µL DBS were used to ensure the entire spot was covered with a reasonable amount of

overlap. For the control experiments, IS was added to the extraction solvent, so it was not co-extracted with the analyte, similar to conventional manual extraction.

Note that, in accordance with our typical validation procedures, recovery and suppression (and their corresponding bias) were calculated using whole-spot extraction. As the extraction mechanisms for whole-spot and sub-punch manual extraction are identical, for the purpose of this discussion, it is assumed that there was no significant difference in recovery or suppression behaviour between the two techniques.

For all direct elution assays, 70:30 (v/v) methanol: water was used as the extraction solvent. The sitamaquine, midazolam, and naproxen assays delivered 10 µL of extraction solvent at 100 µL/min. The paracetamol assay delivered 4µL of extraction solvent at 25 µL/min. For each assay a dual wash solution was used between extractions to prevent carry over using the DBS-MS500 integrated wash system. The sampling apparatus was rinsed with combinations of 70:30 methanol: water (v/v) to remove any remnants of analyte, and 5:95 methanol: water (v/v) to remove matrix components.

Chromatographic Conditions

Identical chromatographic conditions were used for both manual extraction and direct elution assays. The conditions for the sitamaquine, paracetamol, and midazolam assays have been reported previously[63,125]. The naproxen assay used a Phenomenex Kinetex 50 × 2.0 mm i.d. C18 2.6 µm HPLC column (Cheshire, UK), a flow rate of 800 µL/min, column temperature of 60°C, run time of 3.0 min, and gradient chromatography employing the mobile phases water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Following sample injection a ballistic gradient from 95% to 5% A at 1.80 min was followed by an isocratic period at 5% A to 2.00 min. The mobile phase was then returned to 95% A by 2.01 min and was held as this composition until 3.0 min, before the injection of the next sample.

Methods and Equations used to calculate recovery, suppression, assay bias, recovery bias, suppression bias, and area bias.

$$\%Overall\ Assay\ Bias = \frac{DBS\ Peak\ Area\ Ratio\ Response}{DBS\ Peak\ Area\ Ratio\ Response\ @\ 0.45\ HCT} * 100\%$$

Overall DBS assay bias was determined by comparing the DBS sample LC-MS/MS peak area ratio responses at varying HCT levels with those prepared using the control HCT level (0.45).

$$\%Recovery = \frac{Response\ for\ Extracted\ Spiked\ Sample}{Response\ for\ Post\ Extracted\ Matrix\ Spike\ Sample} * 100\%$$

$$\%Recovery\ Bias = \frac{\%Recovery}{\%Recovery\ @\ 0.45\ HCT} * 100\%$$

Recovery of analyte from DBS cards was determined by comparing the LC-MS/MS analyte peak area responses for 'extracted spiked samples' and 'post extraction matrix spike samples' prepared at concentrations that represent 100% recovery. 'Extracted spiked samples' were prepared and extracted using the standard conditions detailed in the experimental section. 'Post extraction matrix spiked samples' were prepared by spiking an appropriate amount of analyte into the extract of the punched DBS disc of a blank matrix sample. For all recovery experiments whole spot extraction methods were used.

$$\%Suppression = \frac{(Non\ Matrix\ Spiked\ Sample) - (Post\ Extraction\ Matrix\ Spike\ Sample)}{(Non\ Matrix\ Spiked\ Sample)}$$

$$\%Suppression\ Bias = \frac{(100 - \%Suppression)}{(100 - \%Suppression\ @\ 0.45\ HCT)} * 100\%$$

DBS analyte suppression was determined by comparing the LC-MS/MS analyte peak area responses for 'post extraction matrix spike samples' and 'non matrix spike samples'. 'Post extraction matrix spiked samples' were prepared by spiking an appropriate amount of analyte into the extract of the punched DBS disc of a blank matrix sample. 'Non matrix spiked samples' were prepared by spiking the same amount of analyte into the extract of the punched DBS disc of a blank substrate sample. For all suppression experiments whole spot extraction methods were used.

Area bias was calculated by measuring spot areas using ImageJ software (mean of 8 replicates) and calculating the proportion of the fixed volume (15µL) of blood present in the 3mm diameter punched disc (this assumes a homogenous blood application on the substrate), as described previously[69]. These values were then normalised to the mean values obtained for 0.45 HCT control blood, and expressed as a percentage.

Appendix E2: Supporting Information Tables

Table S5.1: Concentrations of IS added for each IS application type.

Method of IS addition	Concentration of SIL IS used ng/mL							
	[$^2H_{10}$]-Sitamaquine		[$^2H_3$]-Paracetamol		[$^2H_3$,$^{13}C_3$]-Midazolam		[$^2H_3$]-Naproxen	
	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution	Manual Extraction	Direct Elution
IS in Extraction Solvent	10	1	500	100	25	5	250	50
IS spiked into whole blood prior to spotting	100	25	2500	1000	250	100	2500	1000
IS spayed onto blank substrate prior to DBS	250	250	5000	5000	1000	1000	5000	5000
IS spray onto DBS	250	250	5000	5000	1000	1000	5000	5000

Table S5.2: Characteristics of the representative compounds used in this study.

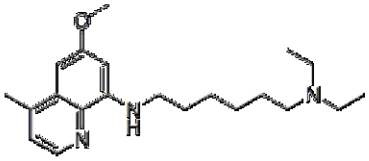
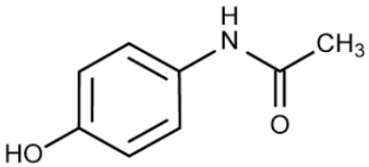
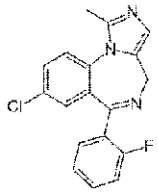
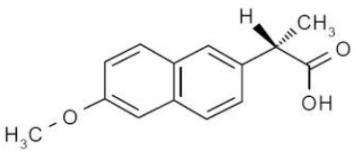
Compound	Mol Weight	Structure	Assay range ng/mL	pKa	SIL	MS/MS Parent/Product Transition Da
Sitamaquine	343.51		5 to 1000	10.3	$^2H_{10}$	344-271
Paracetamol	151.16		50 to 50000	9.4	$^2H_3$	152-110
Midazolam	327.78		5 to 5000	5.9	$^2H_3$ $^{13}C_3$	328-291
Naproxen	230.26		100 to 10000	4.4	$^2H_3$	231-185

Table S5.3: Mean assay bias and corresponding precision values for sitamaquine assay using sub-punch and whole-spot sampling for both manual extraction and direct elution, using four methods of IS addition.

		Mean Assay Bias n=18							
		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	77.0	79.1	125.1	122.9	110.1	114.6	140.9	135.0
	0.35	94.6	96.1	110.9	115.9	102.1	104.6	106.0	103.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	101.9	100.9	89.9	89.9	86.8	87.1	78.4	74.8
	0.7	80.6	79.5	62.8	59.8	62.6	60.1	61.2	66.2

		Corresponding CV n=18							
		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	4.7	4.3	2.2	2.7	3.1	2.9	4.0	3.6
	0.35	6.5	2.7	2.1	7.2	5.0	3.1	5.1	4.0
	0.45	4.7	3.4	3.0	3.6	3.2	3.4	1.5	7.1
	0.55	4.5	4.0	2.3	9.9	3.9	4.5	3.6	3.2
	0.7	4.8	3.1	6.7	3.1	4.9	0.8	5.9	4.0

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	93.5	92.1	93.1	91.6	89.8	92.5	92.1	92.3
	0.35	101.9	93.6	103.1	89.4	102.0	89.3	101.9	92.2
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	103.1	103.3	96.2	101.5	94.5	101.5	97.6	101.2
	0.7	107.9	109.8	95.1	94.3	99.9	104.0	97.5	96.9

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	3.5	1.2	3.3	1.1	3.1	2.0	2.4	1.4
	0.35	2.4	1.2	1.9	0.8	1.7	0.7	1.6	6.9
	0.45	2.3	1.1	1.3	1.2	1.4	1.6	2.7	1.7
	0.55	2.8	1.4	1.6	1.4	2.7	1.7	3.4	5.3
	0.7	2.7	0.8	2.7	2.0	1.9	3.0	3.0	1.1

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	87.2	92.7	101.3	100.4	84.6	89.7	105.1	101.6
	0.35	97.9	99.7	101.7	98.6	99.2	97.6	114.6	108.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	106.6	103.0	99.2	105.7	96.1	101.9	100.6	94.5
	0.7	108.4	114.0	97.4	99.0	103.2	106.0	103.7	100.5

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	6.3	4.5	1.9	4.3	4.9	4.9	6.0	8.5
	0.35	4.5	2.9	3.8	2.7	6.5	2.9	7.4	7.7
	0.45	5.7	5.9	3.2	2.3	5.3	5.0	3.6	5.5
	0.55	10.6	2.6	4.3	6.5	4.1	3.7	6.8	4.5
	0.7	5.9	5.1	2.0	5.3	3.2	3.7	4.6	5.4

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	89.7	87.3	99.5	92.9	86.8	91.3	100.1	103.6
	0.35	97.6	101.6	107.4	99.1	103.2	99.1	101.2	109.5
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	103.0	104.0	90.5	97.5	100.5	103.7	90.4	106.9
	0.7	107.1	106.4	98.9	93.8	110.7	105.6	107.3	92.3

		Sitamaquine							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	4.9	7.8	5.3	6.1	9.7	10.1	9.9	7.9
	0.35	7.0	6.1	8.0	5.7	9.6	3.2	10.4	10.5
	0.45	7.6	6.8	9.0	9.0	5.1	11.4	9.9	6.0
	0.55	3.6	7.0	5.2	7.5	8.0	4.5	10.6	11.7
	0.7	8.6	7.6	6.1	4.6	10.2	4.9	8.7	10.8

Table S5.4: Mean assay bias and corresponding precision values for paracetamol assay using sub-punch and whole-spot sampling for both manual extraction and direct elution, using four methods of IS addition.

		Mean Assay Bias n=18							
		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	91.9	90.8	117.3	114.0	112.3	108.9	315.6	302.1
	0.35	100.0	99.4	107.8	106.9	108.1	106.5	132.0	128.4
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	103.3	102.8	97.1	96.8	94.6	98.4	73.3	75.7
	0.7	104.9	106.1	84.5	88.4	73.3	77.3	51.0	54.1

		Corresponding CV							
		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	6.0	5.7	6.6	11.9	4.1	1.6	8.3	6.6
	0.35	3.7	6.4	8.5	5.4	1.8	3.2	6.9	6.2
	0.45	4.8	6.3	7.0	6.8	2.9	3.2	4.9	6.7
	0.55	6.1	6.6	9.6	8.5	2.9	4.0	8.8	7.9
	0.7	3.9	6.6	6.2	12.7	4.5	2.8	3.1	4.4

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	85.1	91.8	95.5	101.0	97.7	98.9	94.4	98.5
	0.35	94.3	102.9	93.9	101.6	95.9	101.2	91.7	100.9
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	113.8	109.8	93.1	109.4	107.2	108.1	101.6	102.8
	0.7	112.6	117.7	92.8	103.4	105.0	114.6	97.4	98.1

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	1.9	0.7	2.2	0.6	1.7	4.8	2.0	0.6
	0.35	1.5	0.7	2.7	1.4	1.5	0.5	0.4	0.9
	0.45	2.0	1.0	2.3	1.3	3.8	0.5	1.4	0.9
	0.55	2.4	1.1	2.1	1.1	4.5	1.6	1.9	0.8
	0.7	2.2	1.1	1.9	2.9	1.5	11.6	1.9	2.5

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	84.0	80.3	104.4	108.6	100.6	94.3	105.8	104.3
	0.35	93.5	97.3	106.6	101.9	102.6	95.5	106.8	96.2
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100
	0.55	103.1	107.9	100.8	98.8	99.6	100.7	99.4	90.9
	0.7	118.8	114.4	97.0	104.6	98.4	105.0	107.2	98.9

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	2.7	5.7	2.5	3.5	4.0	2.1	9.3	6.9
	0.35	4.1	2.4	2.6	2.5	7.5	2.7	8.0	5.3
	0.45	4.0	4.9	2.9	3.0	2.7	7.4	6.2	9.9
	0.55	4.5	2.2	2.3	4.0	5.4	2.3	5.1	8.0
	0.7	3.8	3.1	3.6	4.4	2.5	3.1	5.4	4.8

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	97.8	92.4	105.5	103.4	99.3	96.1	107.7	103.6
	0.35	101.2	94.6	102.1	103.0	100.5	99.9	102.5	108.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	102.2	103.3	93.9	96.5	101.2	109.9	94.0	102.4
	0.7	112.9	107.7	95.4	98.5	112.9	118.6	104.3	99.8

		Paracetamol							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	4.9	4.2	4.1	5.0	5.2	8.7	9.0	10.1
	0.35	4.5	3.0	2.4	1.4	4.3	2.3	10.9	9.5
	0.45	5.1	4.5	6.3	2.4	1.8	3.8	14.9	11.4
	0.55	4.9	3.7	6.1	3.2	2.9	1.8	13.0	12.2
	0.7	5.5	3.1	5.7	4.8	2.9	2.8	10.2	11.2

Table S5.5: Mean assay bias and corresponding precision values for midazolam assay using sub-punch and whole-spot sampling for both manual extraction and direct elution, using four methods of IS addition.

		Mean Assay Bias n=18							
		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	92.4	93.4	98.7	97.8	104.6	106.4	151.6	141.3
	0.35	97.1	99.9	97.4	96.6	98.9	103.6	110.9	118.4
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	101.5	102.0	100.3	101.6	94.8	98.7	81.6	82.4
	0.7	106.2	104.6	101.2	103.0	95.4	99.1	49.7	42.1

		Corresponding CV n=18							
		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	2.9	6.1	5.0	1.9	1.7	3.1	5.0	4.8
	0.35	2.8	3.8	5.4	5.1	3.4	3.9	4.7	6.1
	0.45	4.1	2.6	3.0	1.7	4.1	4.5	3.9	5.0
	0.55	3.8	4.1	2.9	1.1	3.8	4.0	3.1	3.8
	0.7	1.9	4.0	2.1	1.4	2.0	1.8	4.8	7.1

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	89.4	95.6	105.6	108.1	94.5	92.6	98.4	101.2
	0.35	100.0	96.1	102.5	105.6	98.9	99.8	97.5	107.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	101.1	102.8	99.8	104.5	99.8	105.5	96.4	104.2
	0.7	109.4	105.6	104.1	97.6	110.1	108.7	99.8	100.9

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	0.9	2.1	2.1	0.8	2.9	2.8	2.1	4.5
	0.35	1.2	2.1	2.8	2.7	4.1	3.0	2.6	3.0
	0.45	3.1	0.9	1.2	1.5	1.6	4.1	1.8	3.0
	0.55	2.8	3.0	1.9	0.9	2.5	0.9	1.8	5.3
	0.7	1.7	2.1	2.6	3.2	2.4	1.1	4.1	2.4

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	95.6	92.9	96.8	101.5	95.7	94.7	106.4	103.2
	0.35	95.8	100.9	100.9	95.6	99.7	102.4	106.2	97.5
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	100.9	108.4	94.6	104.1	102.1	98.4	99.8	102.7
	0.7	105.4	104.7	98.7	99.4	102.4	104.7	102.3	96.3

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	4.2	5.3	4.9	3.7	3.8	2.9	6.7	4.9
	0.35	3.9	6.4	5.3	3.5	2.9	1.9	5.8	3.8
	0.45	6.1	3.8	2.9	5.8	4.1	3.5	7.1	5.4
	0.55	5.0	3.2	4.7	6.1	3.6	4.0	4.8	5.9
	0.7	1.9	2.3	4.7	4.0	5.0	3.1	4.7	8.1

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	93.6	89.8	100.9	104.9	90.1	96.5	106.2	104.4
	0.35	98.6	97.0	105.8	100.8	95.1	96.7	107.1	95.8
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	105.6	107.1	96.6	99.8	101.3	105.0	105.5	103.9
	0.7	106.6	108.0	96.6	107.1	105.6	109.8	102.2	94.9

		Midazolam							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	6.1	3.9	5.2	7.1	3.9	5.5	6.9	5.0
	0.35	5.6	4.5	4.4	4.9	4.5	4.7	5.0	8.1
	0.45	5.5	4.1	5.0	3.5	7.1	5.3	6.1	4.9
	0.55	6.1	6.0	3.9	7.0	6.3	4.0	3.9	5.1
	0.7	4.2	5.8	6.1	5.1	3.0	4.9	5.9	5.6

Table S5.6: Mean assay bias and corresponding precision values for naproxen assay using sub-punch and whole-spot sampling for both manual extraction and direct elution, using four methods of IS addition.

		Mean Assay Bias n=18							
		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION A IS added via extraction solvent	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	93.9	92.6	99.8	97.0	103.2	102.8	189.6	171.4
	0.35	96.1	98.9	101.0	96.1	99.9	98.7	102.0	105.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	103.4	101.4	103.0	98.4	95.8	100.8	79.6	71.8
	0.7	103.1	106.7	102.1	96.7	93.0	99.8	45.8	42.0

		Corresponding CV n=18							
		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	3.5	7.1	4.9	9.5	4.1	3.9	8.1	4.9
	0.35	4.6	5.2	7.1	7.0	8.0	5.1	5.1	6.8
	0.45	4.6	5.9	5.1	7.1	7.6	4.5	6.0	9.1
	0.55	2.1	4.9	8.4	6.1	4.9	7.3	5.8	5.5
	0.7	2.8	3.8	4.8	5.8	6.0	6.8	4.8	4.2

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION B IS SPIKED INTO WHOLE BLOOD PRIOR TO DEPOSITION ON DBS SUBSTRATE	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	95.1	89.1	101.2	106.3	92.8	96.7	98.8	104.1
	0.35	99.8	94.6	98.9	104.2	96.6	99.9	99.8	101.2
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	108.7	102.0	99.8	101.1	100.9	101.0	96.3	104.5
	0.7	110.1	106.9	98.7	105.5	105.6	107.0	97.4	102.3

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	2.9	1.9	1.9	1.5	4.1	1.8	3.2	4.1
	0.35	3.1	0.8	3.6	0.9	2.3	2.0	2.0	3.9
	0.45	4.0	1.8	2.0	1.8	2.0	1.7	2.4	4.1
	0.55	3.9	3.2	2.5	3.0	1.9	3.1	2.5	2.6
	0.7	2.8	2.0	1.8	2.3	1.8	4.0	2.4	1.8

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION C IS SPRAY ADDITION TO DBS PRIOR TO EXTRACTION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	90.4	88.7	102.0	106.0	94.6	95.8	97.8	96.4
	0.35	94.6	91.8	94.6	104.5	99.8	96.2	99.8	99.1
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	101.1	102.0	93.2	99.6	103.9	104.5	102.1	93.9
	0.7	106.6	105.1	98.7	101.9	111.9	108.0	95.6	104.5

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	4.0	6.1	3.9	3.8	5.0	3.2	5.1	7.1
	0.35	3.1	4.5	1.9	6.0	3.9	4.6	5.3	5.0
	0.45	4.8	3.0	2.5	4.9	4.1	4.5	4.8	4.9
	0.55	5.2	3.2	3.6	2.7	4.1	6.1	3.9	5.8
	0.7	2.9	4.0	5.0	2.9	2.3	2.1	6.1	4.8

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
OPTION D IS SPRAY ONTO SUBSTRATE PRIOR TO WHOLE BLOOD DEPOSITION	HCT	Assay Bias		Assay Bias		Assay Bias		Assay Bias	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	92.5	93.0	90.9	101.4	90.4	97.1	95.8	103.8
	0.35	97.1	96.5	108.5	105.5	95.5	98.0	111.2	105.6
	0.45	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	0.55	103.4	105.4	87.9	96.6	105.0	104.0	106.4	110.1
	0.7	109.1	107.8	104.6	97.4	107.0	105.5	103.2	104.5

		Naproxen							
		Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
	HCT	CV		CV		CV		CV	
		QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
	0.2	7.3	7.1	8.1	6.5	5.1	8.1	8.6	6.9
	0.35	5.0	7.0	5.9	7.8	6.0	5.6	9.1	5.1
	0.45	4.2	6.5	7.1	6.4	5.1	5.6	5.4	9.5
	0.55	4.8	4.2	4.0	2.6	7.8	4.0	5.6	4.7
	0.7	5.3	4.0	4.8	3.4	8.1	7.8	6.2	4.5

Table S5.7: Summary data displaying range of precision values (CV, n=18) for each type of sampling method, extraction method, and method of IS addition carried out in this study.

Assay	Method of IS addition	Manual Extraction				Direct Elution			
		Sub Punch		Whole Spot		Sub Punch		Whole Spot	
		CV range % (n=18)		CV range % (n=18)		CV range % (n=18)		CV range % (n=18)	
		Min	Max	Min	Max	Min	Max	Min	Max
Sitamaquine	Via extraction solvent (control)	3.1	6.5	2.1	9.9	0.8	4.9	1.5	7.1
	IS spray	2.6	10.6	1.9	6.5	3.2	6.5	3.6	8.5
	IS in blood prior to spotting	0.8	3.5	0.8	3.3	0.7	3.1	1.1	6.9
	IS on substrate priot to spotting	3.6	8.6	4.6	9.0	3.2	11.4	6.0	11.7
Paracetamol	Via extraction solvent (control)	3.7	6.6	5.4	12.7	1.6	4.5	3.1	8.8
	IS spray	2.2	5.7	2.3	4.4	2.1	7.4	4.8	9.9
	IS in blood prior to spotting	0.7	2.4	1.1	2.9	0.5	11.6	0.4	2.5
	IS on substrate priot to spotting	3.0	5.5	1.4	6.3	1.8	8.7	9.5	14.9
Midazolam	Via extraction solvent (control)	1.9	6.1	1.1	5.4	1.7	4.1	3.1	7.1
	IS spray	1.9	6.4	2.9	6.1	1.9	5.0	3.8	8.1
	IS in blood prior to spotting	0.9	3.1	0.9	2.8	0.9	4.1	1.8	5.3
	IS on substrate priot to spotting	3.9	6.1	3.9	7.1	3.9	7.1	3.9	8.1
Naproxen	Via extraction solvent (control)	2.1	7.1	4.8	9.5	4.1	8.0	4.2	9.1
	IS spray	2.9	6.1	1.9	6.0	2.1	6.1	3.9	7.1
	IS in blood prior to spotting	0.8	4.0	0.9	3.6	1.7	4.1	1.8	4.1
	IS on substrate priot to spotting	4.2	7.1	2.6	8.1	4.0	8.1	4.5	9.5
	Mean	2.4	6.0	2.4	6.5	2.1	6.5	3.6	8.0

Appendix E3: Supporting Information Figures

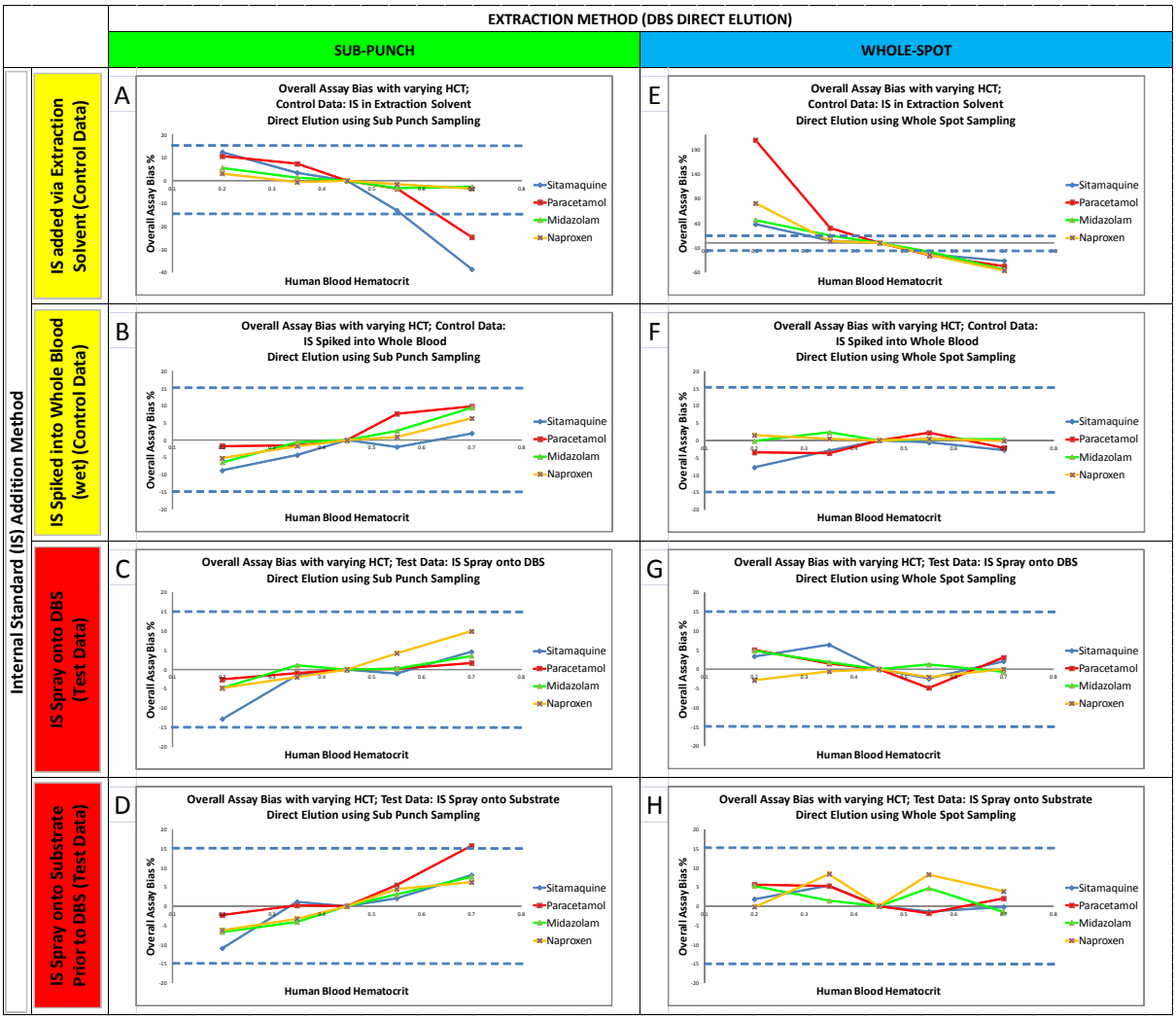


Figure S5.1: Overall assay bias with varying haematocrit (HCT) for DBS sub-punch and whole-spot direct elution using four methods of internal standard (IS) addition (two control, and two test methods) for four quantitative bioanalytical assays measuring drug concentrations. Figures 1A-D show sub-punch extraction where the IS is added via the extraction solvent (A) (control data); by spiking IS into whole blood prior to blood deposition on substrate (B) (control data); by spraying IS onto the DBS prior to extraction (C) (test data); and, by spraying IS onto blank substrate prior to blood deposition (D) (test data). Figures 1E-H show the data in the same order, where whole-spot extraction is used. Each data point plotted is a mean bias value of the low and high concentration QC's (3x the assay LLQ, and 75% of the assay HLQ, respectively), as no significant concentration dependency was observed. The blue dashed lines represent ±15% bias (the limit of total error allowable according to internationally accepted guideline acceptance criteria).
To display the data concisely, bias results using variable HCT have been calculated using the result at the control HCT level (0.45) as the nominal value.

APPENDIX F: Supplementary Information for Chapter 6

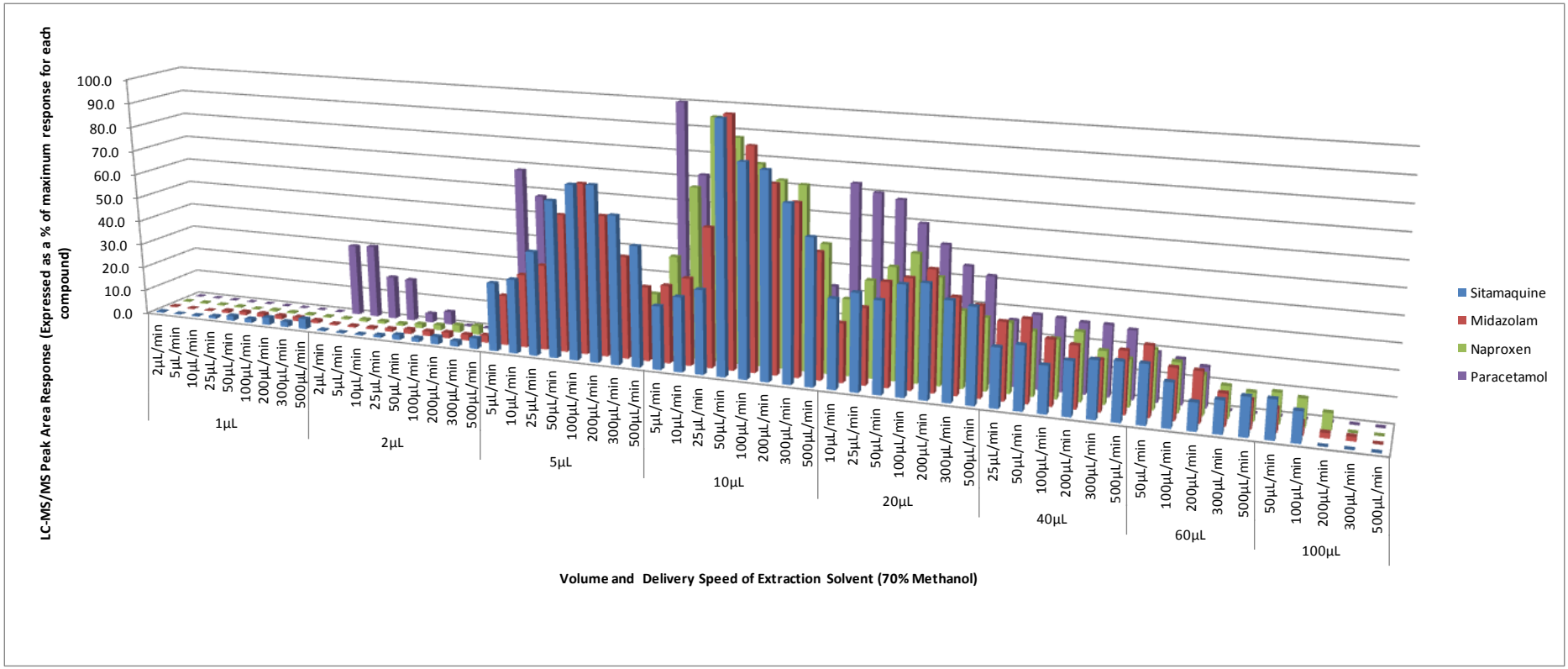


Figure S6.1: Sub-sample direct elution: Variation in LC-MS/MS peak area response with volume and delivery speed of optimised extraction solvent (70% methanol). Each value is a mean of n=6.

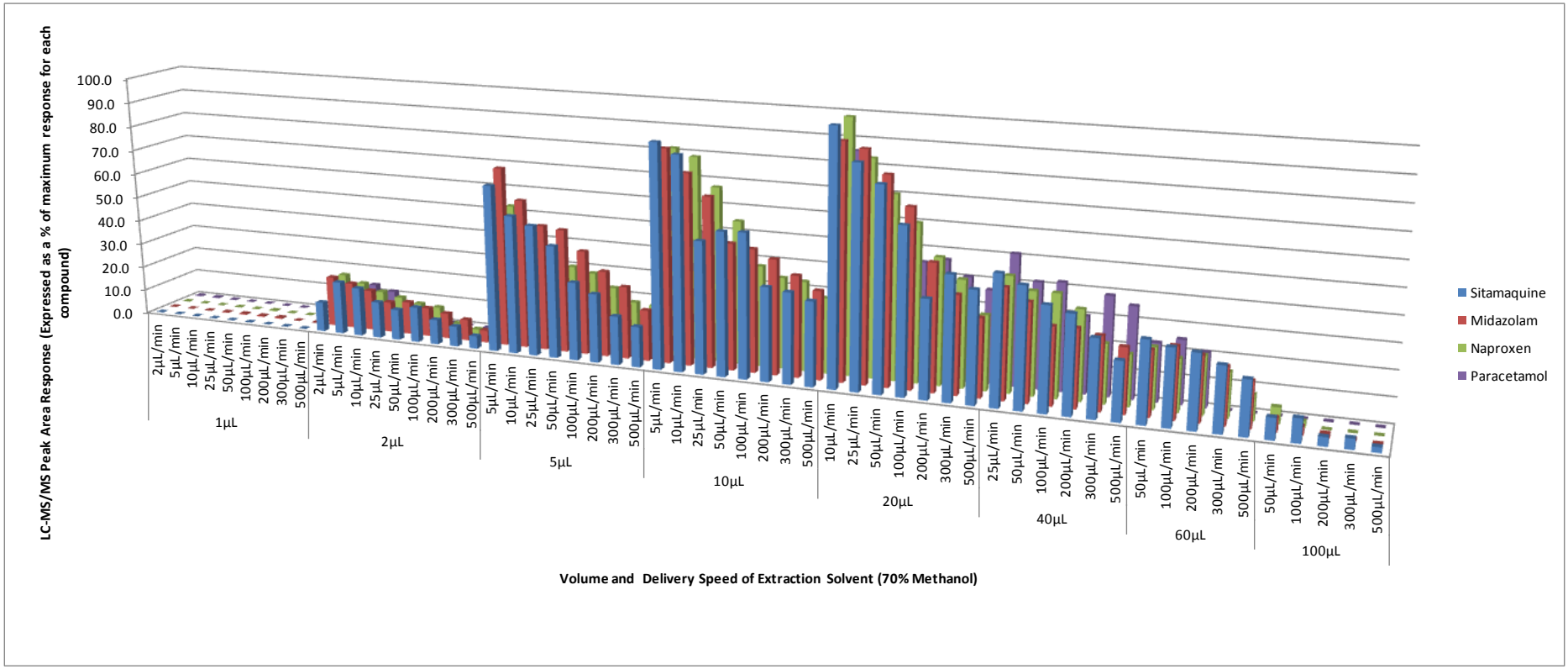


Figure S6.2: Whole-spot direct elution: Variation in LC-MS/MS peak area response with volume and delivery speed of optimised extraction solvent (70% methanol). Each value is a mean of n=6.

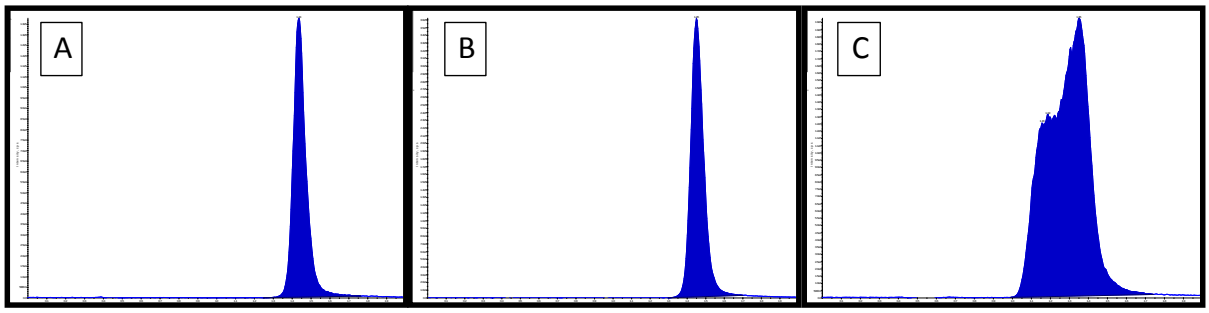


Figure S6.3: Representative LC-MS/MS chromatography for 2500 ng/mL paracetamol DBS using manual extraction and direct elution.
Fig 6.3A: Sub-sample manual extraction of a paracetamol sample using 70% methanol as the extraction solvent and an injection volume of 2 µL. This chromatography was deemed to be acceptable (number of theoretical plates, N = 2200).
Fig 6.3B: Sub-sample direct elution of a paracetamol sample using 10 µL of 70% methanol @ 5 µL/min. This chromatography was deemed to be acceptable (number of theoretical plates, N = 1926) and not significantly different to the chromatography obtained using conventional manual extraction.
Fig 6.3C: Sub-sample direct elution of a paracetamol sample using 20 µL of 70% methanol @ 5 µL/min. This chromatography was deemed to be unacceptable due to the very broad and asymmetric peak produced, and a significant decrease in the calculated number of theoretical plates (N = 184) compared to the chromatography obtained using conventional manual extraction.

Appendix F1

Equation used to calculate number of theoretical plates, N:

$$N = 5.54\left(\frac{t_r}{W_{0.5}}\right)^2$$

Where t_r = retention time, and $W_{0.5}$ = peak width at half height.