# DRIED BLOOD SPOT ANALYSIS IN ROUTINE CLINICAL PRACTICE

by

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**Abstract**

Dried blood spots (DBS) are drops of capillary blood collected onto filter paper from a finger prick. They have many advantages compared with traditional phlebotomy and enable patients to take samples at home. A DBS collection device was developed and incorporated into a CE marked DBS collection kit. This was successfully used in an international direct access vitamin D DBS service. A random access DBS CRP method was established for use with the DBS collection device and a new microsampling device called the Mitra. The quality of DBS received and the impact of lancet type was assessed and the effect of blood spot characteristics on CRP and vitamin D concentration was examined. The vitamin D service uptake and the population using it was analysed. The vitamin D concentration and status of users was compared to serum samples received in the laboratory from the local GP population. Significant differences between the populations were seen, with DBS users showing higher levels of vitamin D. In addition, the response to vitamin D testing for both populations was analysed. A higher rate of high to toxic vitamin D levels was seen in the blood spot population and the reasons for this were explored.

**Dedication**

This thesis is dedicated to my father, Alan Shea, who never gave up on the idea of my becoming a doctor one day, and to my sister, Kerry Shea, who knew just how to keep me going when I needed it.

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**List of Abbreviations**

DBS – Dried Blood Spot
PKU – Phenylketonuria
NBS – Newborn screening
TSH – Thyroid stimulating hormone
LC/MS/MS – Liquid chromatography tandem mass spectrometry
LSD – Lysosomal storage disease
NSQAP – Newborn Screening Quality Assurance Program
CDC – Centers for Disease Prevention
SWBH – Sandwell and West Birmingham Hospitals
WRBC – Washed Red Blood Cells
PTAD – 4-phenyl-1,2,4-triazoline-3,5-dione
IS – Internal Standard
UP – Ultra-pure
FBC – Full blood count
QC – Quality control
25(OH)D – 25-Hydroxyvitamin D
PD – Pro-Diagnostics
$1,25(OH)_2D$ – Calcitriol, 1,25-Dihydroxyvitamin D
RA – Rheumatoid arthritis
CRP – C-reactive protein
EDTA – Ethylenediaminetetraacetic acid
CV – Coefficient of Variation
SD – Standard Deviation
IOM – Institute of Medicine
PSA – Prostate specific antigen
HbA1c – Haemoglobin A1c
FDA – US Food and Drug Administration
ISNS – International Society for Neonatal Screening

# CHAPTER 1 - INTRODUCTION

## 1.1. Dried Blood Spot Origins

The use of dried blood spots (DBS) – drops of capillary whole blood collected onto filter paper from a heel or finger prick – as a method for blood analysis is not a new concept. In fact, it was first mentioned in the literature over a century ago as a way of estimating blood glucose. (1) However, it was not until nearly 50 years later that this idea was further developed, initially as a way of screening for the presence of phenylketonuria (PKU) in mentally handicapped patients. (2) Guthrie then went on to use this method to screen newborn infants for PKU by collecting DBS taken around the $4^{th}$ day of life by heel puncture. (3)

The method of using a matrix of dried blood on filter-paper enabled samples to be sent to a central laboratory to be analysed more cheaply and easily compared with the typical method of collecting venous blood. Thus began the era of newborn screening (NBS) using DBS. (4) The state of Massachusetts in the United States in 1963 became the first place to make compulsory the testing of all infants for PKU. Soon many other areas of the world followed, including the rest of the United States, Australasia and most of Western Europe. (5)

Guthrie continued to develop methodology with DBS to allow rapid and large-scale testing of many children, and also worked on the organisational aspects of NBS. By 1980 he was able to offer 20 DBS screening tests in his laboratory. (6) Meanwhile, the development of robust immunoassay techniques in the 1970s enabled congenital hypothyroidism to be screened in newborns initially by the measurement of thyroxine (7) and then later thyroid stimulating hormone (TSH) in DBS. (8) Further important tests were added over time to NBS panels in many countries including congenital

adrenal hyperplasia, cystic fibrosis, sickle cell anaemia, biotinidase deficiency and the galactosaemias. (5, 9)

The potential for tests to be included in NBS panels was further increased during the 1990s by the development of liquid chromatography tandem mass spectrometry (LC/MS/MS) (10) and the application of DNA extraction and analysis to DBS. It is now possible to screen for over 40 diseases, and more than 90 metabolites, covering amino acid, organic acid and fatty acid metabolism disorders, urea cycle defects, and mitochondrial fatty-acid oxidation defects. Couple this with the number of potential genetic disorders that could be screened for using analysis of DNA from DBS, especially with the advent of next generation sequencing technologies, and potential NBS panels could be huge. (5, 11, 12)

Consequently, the disorders screened for in neonatal populations internationally still vary quite dramatically. (6, 13) In the UK only five diseases were screened for until January 2015 when a further four diseases were added, all using DBS as the means of getting the blood to the laboratory for analysis: congenital hypothyroidism, PKU, cystic fibrosis, sickle cell disease, medium chain acyl-CoA dehydrogenase deficiency, maple syrup urine disease, isovaleric acidaemia, glutaric aciduria type 1, and homocystinuria (pyridoxine unresponsive). (14) Despite the increase in the number of diseases screened for, this is still far fewer than the 32 specific conditions recommended to be looked for in first line screening by the Health Resources and Services Administration in the United States. However, not all states offer this panel, some offer more and some offer less showing that global NBS harmonisation still has some way to go. (12)

## 1.2. **Inherent Advantages in the Use of DBS**

Despite DBS not being commonly used as the mainstream method for sample delivery to the laboratory, there are many advantages to using DBS as opposed to taking venous samples (Table 1.1).

| Advantages for the laboratory | Advantages for the patient |
|---|---|
| Potential for storage at room temperature so lessening costs associated with refrigeration. (15) | Reduced sample volume. (16) |
| Prevents *in vitro* formation of some compounds e.g. gamma-hydroxybutyric acid. (17) | Time saving – patients do not have to take time out of work or school for phlebotomy or arrive early to clinic in order to be bled and have their sample tested before their appointment. |
| Safer for laboratory and postal workers as the infectivity of viruses such as HIV and hepatitis C is reduced. (18) | Clinically relevant samples e.g. therapeutic drug monitoring samples can be taken at peak or trough time, not just when the patient can be bled. (19) |
| No need for a trained healthcare professional to take the sample, so potentially reducing costs. | Convenient – patients can take the sample at home at a time that suits them. Important for people who do not always have easy access to transport. (15) |
| Easy to send in the post as no special packaging, cooling or licence requirements, and no risk of sample leakage. (20) | Timely return of sample means up-to-date results can be used in clinic. (21-23) |
| Improved stability for many analytes. (24, 25) | A trained phlebotomist is not required enabling home sampling. (19) |
| Potential reduction in waste in terms of plastic (large specimen tubes) and patient's blood (much is not used in analysis) which have to be disposed of. | Minimally invasive technique making it attractive to patients, especially children who have been anecdotally shown to prefer this technique to venesection. (25) |
| Compact so less space required for storage. | |
| Reduces the risk of needle stick injuries. (20) | |

**Table 1.1** – Advantages of using DBS for the laboratory and patient.

The reduced sample volume that DBS afford is especially important in premature neonates as there are only ~80 mL of blood for every kg in a newborn baby. A premature baby weighing 500 g will only have 40 mL of blood and therefore only a very limited amount is available for analysis. (16) The small sample size of DBS is also spurring the upsurge of their use in the pharmaceutical industry for drug development. DBS can be used for the measurement of drugs in as little as 4-12 µL of blood. (26) By using DBS as the method for collecting samples from animals used in toxicological and pharmacokinetic studies, only a small amount of blood needs to be taken. This reduced sample volume compared to traditional venous sampling means that more samples can be taken per animal and therefore overall fewer animals are required per study. Additionally, the benefits of using DBS as a sampling technique for clinical and pharmacokinetic studies has seen their use in multi-centre national and international trials and studies increase. (16)

DBS can improve the patient journey and make blood sampling more accessible to patients. For elderly or disabled patients, making a journey to hospital for a blood test can be inconvenient, may require transport, and may even be painful. Taking samples at home has advantages in such patient groups. Home testing means people do not have to take time out of work or school to have a blood test leading to economic and educational improvements, especially for patients with chronic disorders.

The lack of need for medical personnel to take a blood specimen means that roadside testing for driving whilst under the influence of drugs, when there is a limited time window for sampling due to rapid metabolism of the drugs, can be done in a quick and timely manner using DBS. (17)

DBS offer an attractive alternative to traditional venous sampling for a variety of reasons, not least that they are easier to handle, store and transport, as well as potentially improving the patient pathway by making blood tests patient centred and a more efficient process. DBS could even lead to a reduction in waste in terms of plastic (large specimen tubes), patient's blood (much is not used in analysis) and ultimately patient and staff time. The US Food and Drug Administration (FDA) are encouraging the scientific community to publish its finding on DBS and move the technology forward (27) and the ethical benefits of using DBS have been recognised by the Medicines and Healthcare products Regulatory Agency. They are strongly encouraging the pharmaceutical industry to implement DBS technology. (28, 29)

## 1.3. **Reasons for Limited Uptake in Routine Analysis**

| **Barriers to the uptake of DBS in routine clinical practice** |
|---|
| Technology not being sensitive enough to measure analytes in the volume of blood in a DBS. (25) |
| Laboratories being geared up to use liquid specimens. (15, 30) |
| The drive for using DBS not being forthcoming. What is practical for the laboratory or healthcare provider has often come before what is more practical for the patient. |
| Worries over the potential quality of spots produced by the public. |
| DBS collection devices not being fit-for-purpose for home collection of samples by patients. |
| Standardisation, calibration and reference range problems associated with using DBS. (15, 26, 31) |

**Table 1.2** – Barriers to the uptake of DBS in routine clinical practice.

There have been both historical and practical problems associated with the use of DBS and these have both contributed to the reasons why DBS are not more routinely used outside of NBS programmes (Table 1.2).

One of the advantages of DBS is the small volume of blood taken from the patient and this is vital when testing newborn babies: it is one of the reasons why DBS are used for neonatal screening programmes. However, this is also the reason why DBS

have not taken off in the past – the technology used routinely was not sensitive enough to measure analytes present in the small volume of blood available for analysis in a DBS. (25) Yet, with the advent of LC/MS/MS and the upsurge in its use in clinical laboratories, the number of analytes that can be measured in blood spots has increased dramatically, but these methods have yet to make their way into many routine laboratories.

Laboratories are traditionally geared up to deal with venous blood samples in test tubes. Specimens are expected to arrive like this and sample receptions are streamlined to cope with this sample type: dedicated centrifuges, either stand alone or as part of a track system; aliquoting stations, either as part of sample reception or now as part of automated pre-analytical track systems; refrigerators, specimen racks as part of the track system or stand alone. Automation and track systems have been designed to move test tubes around and analysers are geared up to sample volumes of liquid out of primary or secondary tubes. (15, 25) Consequently, most laboratories are not geared up to handle solid specimens such as DBS.

The small sample volume of DBS can lead to poor spots. DBS can arrive in a wide range of unusable forms, making analysis of the sample difficult or impossible. However, no sample matrix will ever be problem free (e.g. 24 hour urine collections) and therefore the potential for poor sample collection should not be a reason why DBS are not used in a mainstream setting. Appropriate training and information leaflets should ensure that the problems with inappropriate sample collection are minimised. Williams and McDade (32) showed that in a study they conducted that involved using DBS, 98.6% of blood spot cards collected were useable, although field professionals were used to collect the spots therefore the percentage of useable

cards would be expected to be lower than this if the spots were being collected by patients themselves.

NBS programmes have shown that DBS can work on a large scale as a matrix for blood testing and the issues surrounding blood-spot quality can be overcome. DBS just need to be seen to be fit for purpose and alternative matrices will always be available if DBS are deemed to be unsuitable for particular individuals. In fact, there will be certain situations where DBS are unsuitable, for example patients with poor circulation or with severely abnormal haematocrits, and it will be up to healthcare professionals ordering the tests to make sure that DBS are used appropriately. (25)

Some of the other practical problems relating to the widespread use of DBS are related to standardisation and calibration. Results from DBS can vary depending on the type of paper used, the volume of blood used to create the spots, haematocrit effects, where the spot is punched out from the original DBS, differences in reference ranges compared to serum/plasma etc. To overcome these issues, laboratories should validate their assays for the main types of filter paper on offer or be explicit about which paper they use, or use different calibrators depending on the sample type sent in. DBS assays should be compared to the current serum/plasma/whole blood assay on offer to see if reference ranges are comparable. If not then a decision should be taken as to whether to perform a reference range investigation and provide a new reference range with the results or use a factor to convert the results to serum based values and therefore use the reference range already established for that analyte in serum. (15) The assay should be thoroughly validated including an investigation of the effect of blood spot size and haematocrit as well as punch size and position on analyte concentration. This is discussed further in Chapter 3. By

properly validating assays and thoroughly explaining the procedures involved, the quality of DBS assays should improve and therefore become more accepted into routine practice.

A further reason why DBS have not yet taken off in routine clinical practice is because many of the devices available for DBS collection are not suitable for use outside of NBS programmes. By developing quality devices and providing a complete kit containing everything that is required for sample collection, DBS should become more attractive. The blood spot collection device should also be CE marked so that it can be used outside of the trust in which the analysing laboratory is based.

NBS programmes have paved the way for DBS use in the wider clinical setting. (13) NBS laboratories have shown how laboratories can easily gear up to handling large volumes of DBS. In fact, compared to traditional sample types, the automation required is much less. In addition no (or minimal) additional analytical equipment should be required as current methodologies should be able to measure DBS eluates with relatively little manipulation. We are just now beginning to see the signs of wider DBS implementation, however even within the pharmaceutical industry, due to issues surrounding validation and effort to introduce change, uptake of DBS has not been as swift as hoped, although it is definitely gaining momentum. (33)

## 1.4. **Clinical Situations where Blood Spots could be Advantageous**

NBS is the most successful and well known application of DBS. The limitations of sensitivity and specificity that came with using such small volumes of blood in a DBS (which may be as little as 5 µL) had restricted more wide-spread use in other areas. In the last couple of decades these problems could be overcome as a result of the

same improvements in technology that had seen the expansion of the repertoire of tests available to NBS panels.

The analytes that can be measured using DBS are vast (Table 1.3). Processes have been adapted or created so that DBS can work with a wide array of different technologies and platforms. It appears that in general, any analyte that can be measured in whole blood, serum or plasma can also be measured using DBS (24, 31) although exceptions do occur, such as ferritin which is best measured in dried serum spots as opposed to DBS.(34)

| Analyte | Analyte |
|---|---|
| Acarboxyprothrombin | Hemoglobin variants |
| Acylcarnitine | Hexosaminidase A |
| Adenine phosphoribosyl transferase | Human erythrocyte carbonic |
| Adenosine deaminase | anhydrase I |
| Albumin | 17 alpha-hydroxyprogesterone |
| Alpha-fetoprotein | Hypoxanthine phosphoribosyl |
| Amino Acid profiles | transferase |
| arginine (Krebs cycle) | Immunoreactive trypsin |
| histidine/urocanic acid | Lactate |
| homocysteine | Lead |
| phenylalanine/tyrosine | Lipoproteins |
| tryptophan | (a) |
| Andrenostenedione | B/A-1 |
| Antipyrine | ß |
| Arabinitol enantiomers | Lysozyme |
| Arginase | Mefloquine |
| Benzoylecgonine (cocaine) | Netilmicin |
| Biotinidase | Phenobarbitone |
| Biopterin | Phenytoin |
| C-reactive protein | Phytanic/pristanic acid |
| Carnitine | Progesterone |
| Carnosinase | Prolactin |
| CD4 | Prolidase |
| Ceruloplasmin | Purine nucleoside phosphorylase |
| Chenodeoxycholic acid | Quinine |
| Chloroquine | Reverse tri-iodothyronine (rT3) |
| Cholesterol | Selenium |

- Cholinesterase
- Conjugated 1-ß hydroxy-cholic acid
- Cortisol
- Creatine kinase
- Creatine kinase MM isoenzyme
- Cyclosporin A
- D-penicillamine
- De-ethylchloroquine
- Dehydroepiandrosterone sulfate
- DNA (PCR)
  - acetylator polymorphism
  - alcohol dehydrogenase
  - alpha 1-antitrypsin
  - cystic fibrosis
  - Duchenne/Becker muscular dystrophy
  - glucose-6-phosphate dehydrogenase
  - hemoglobinopathies
    - A,S,C,E
    - D-Punjab
    - beta-thalassemia
  - hepatitis B virus
  - HCMV
  - HIV-1
  - HTLV-1
  - Leber hereditary optic neuropathy
  - MCAD
  - RNA
  - PKU
  - Plasmodium vivax
  - sexual differentiation
  - 21-deoxycortisol
- Desbutylhalofantrine
- Dihydropteridine reductase
- Diptheria/tetanus antitoxin
- Erythrocyte arginase
- Erythrocyte protoporphyrin
- Esterase D
- Fatty acids/acylglycines
- Free ß-human chorionic gonadotropin
- Free erythrocyte porphyrin
- Serum pancreatic lipase
- Sissomicin
- Somatomedin C
- Specific antibodies
  - adenovirus
  - anti-nuclear antibody
  - anti-zeta antibody
  - arbovirus
  - Aujeszky's disease virus
  - dengue virus
  - Dracunculus medinensis
  - Echinococcus granulosus
  - Entamoeba histolytica
  - enterovirus
  - Giardia duodenalisa
  - Helicobacter pylori
  - hepatitis B virus
  - herpes virus
  - HIV-1
  - IgE (atopic disease)
  - influenza virus
  - Leishmania donovani
  - leptospira
  - measles/mumps/rubella
  - Mycobacterium leprae
  - Mycoplasma pneumoniae
  - Onchocerca volvulus
  - parainfluenza virus
  - Plasmodium falciparum
  - poliovirus
  - Pseudomonas aeruginosa
  - respiratory syncytial virus
  - rickettsia (scrub typhus)
  - Schistosoma mansoni
  - Toxoplasma gondii
  - Trepenoma pallidium
  - Trypanosoma cruzi/rangeli
  - vesicular stomatis virus
  - Wuchereria bancrofti
  - yellow fever virus
- Specific antigens
  - hepatitis B virus

| | |
|---|---|
| Free thyroxine (FT4) | HIV-1 |
| Free tri-iodothyronine (FT3) | Succinylacetone |
| Fumarylacetoacetase | Sulfadoxine |
| Galactose/gal-1-phosphate | Theophylline |
| Galactose-1-phosphate uridyltransferase | Thyrotropin |
| Gentamicin | Thyroxine (T4) |
| Glucose | Thyroxine-binding globulin |
| Glucose-6-phosphate dehydrogenase | Trace elements |
| Glutathione | Transferrin |
| Glutathione perioxidase | UDP-galactose-4-epimerase |
| Glycocholic acid | Urea |
| Glycosylated haemoglobin | Uroporphyrinogen I synthase |
| Halofantrine | Vitamin A |
| | White blood cells |
| | Zinc protoporphyrin |

**Table 1.3 –** Analytes measured from human blood collected and dried on filter paper. (24)

Despite DBS not being a mainstream method of delivering samples into the laboratory, since Bang (1) first mentioned the use of DBS for assessing blood glucose, there have been many published papers on the use of DBS in clinical settings. (19, 34-48) As the use of DBS for patient management, monitoring and diagnosis has become increasingly recognised as being a very useful adjunct to the current methods employed for these purposes, a number of DBS collection kits have appeared on the market.


**Figure 1.1 –** Blood spot collection kit available from ZRT for the assay of a wide range of analytes.

ZRT are a US company offering a wide range of blood spot testing services. They will send out a collection kit, complete with everything required to take the DBS, order

what tests are required and post the sample back to a laboratory for analysis (Figure 1.1). ZRT offer an extensive range of assays for DBS analysis including a wide variety of hormones (e.g. oestradiol, progesterone, cortisol, TSH, testosterone), some trace elements, Vitamin D, Prostate Specific Antigen (PSA), lipids, Haemoglobin A1c (HbA1c) and high-sensitivity C-Reactive Protein. They also offer dried urine and saliva test kits. (49)

Menarini were offering a direct to the public home testing kit for the measurement of HbA1c in the UK called Glucomen A1c. Everything that was required to take the sample and return it to the laboratory for analysis was included in the kit (Figure 1.2). Results were returned directly to the patient and a copy could also be sent to their doctor or nurse if the patient chose. However, no interpretation of the result would be available as part of the service and Menarini would not provide interpretive advice on the phone. However, since 2012 Menarini have withdrawn this service from the market. Other companies that were offering direct to the public DBS services included Genzyme (who offered DBS collection kits for the diagnosis of several lysosomal storage diseases (LSDs)) and Geonostics (another company offering home monitoring of HbA1c using DBS), however both of these companies no longer exist.

In the UK, DBS testing in the area of blood borne virus testing has grown. In 2008 the Association of Greater Manchester Primary Trusts launched a scheme to screen for Hepatitis C using DBS. DBS can either be collected in clinic or DBS collection kits can be sent out to interested clients. The packs contain everything required to collect a DBS sample, and they have designed their DBS collection card using Whatman #903 filter paper (Figure 1.3). In addition to screening for Hepatitis C virus via

antibody detection, Hepatitis C virus antibody confirmation can also be performed with the same set of DBS, and these can also be used for testing for Hepatitis B surface antigen, Hepatitis B core ar

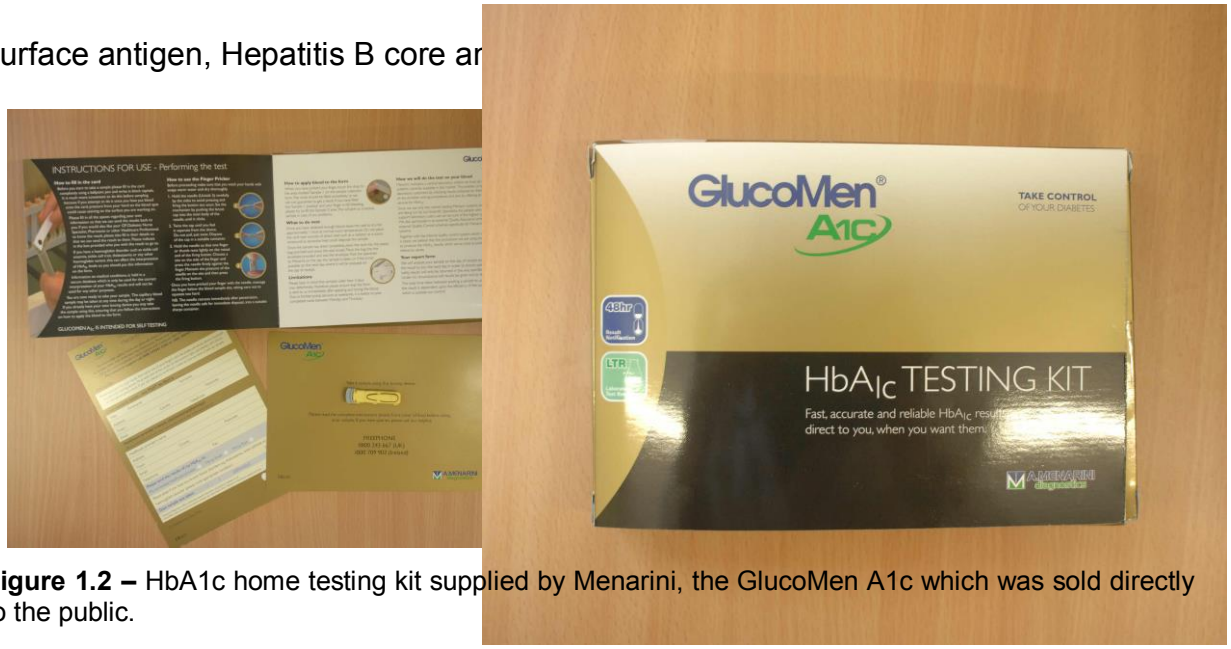



**Figure 1.2 –** HbA1c home testing kit supplied by Menarini, the GlucoMen A1c which was sold directly to the public.


**Figure 1.3 –** DBS collection card designed for Hepatitis C by the Association of Greater Manchester Primary Trusts.

Since the roll out in Greater Manchester of DBS testing for blood borne viruses, many other NHS trusts have adopted similar schemes, mainly due to the advantages that DBS testing has over venepuncture in the target group of drug injectors, e.g. poor venous access. Public Health Wales implemented DBS testing for hepatitis C, hepatitis B and HIV across the whole of Wales in 2010 and this service was run alongside venepuncture based testing. (51) NHS Greater Glasgow and Clyde offer DBS testing for HIV, hepatitis B and hepatitis C (52) as do Torbay and Southern

Devon Health and Care, (53) NHS Tayside, (54) Sandwell and West Birmingham Hospitals (SWBH) NHS Trust (55) and others. A private company in the UK, Alere toxicology, also offer blood borne virus testing via DBS (56) and a company called "just between us" are offering sexually transmitted infection testing using DBS as part of a paid for service. (57)

A number of DBS assays are available for patients as part of routine NHS laboratory testing but these are mainly for monitoring or diagnosis of inborn errors of metabolism (e.g. 17-hydroxyprogesterone, phenylalanine) or for diagnosis of LSDs (e.g. Hunter disease, Pompe disease). (58)

LSDs are caused by unique dysfunctional lysosomal enzymes or lysosome-associated proteins which lead to an accumulation of undigested macromolecules within lysosomes. (59) There are now treatments available for some of these disorders (enzyme replacement therapies for Gaucher, Fabry and Pompe disease and stem cell transplantation therapy for pre-symptomatic treatment of Krabbe disease) and this has led to an increased interest in screening for LSDs as part of some NBS programmes. There is also thought to be a high proportion of undiagnosed patients with LSDs in the general population and so selected screening e.g. for Fabry disease in hypertrophic cardiomyopathic, cryptogenic stroke and haemodialysis patients (60) may reveal a relatively high proportion of LSD sufferers. The ability to use DBS in these situations is highly advantageous. (59, 61-65)

Another clinical setting that is taking advantage of DBS and the opportunity it affords patients in home sampling is therapeutic drug monitoring. This can be done by measuring the drug itself, its metabolites or even by measuring the viral load after

drug administration. Many methods have been published describing TDM in routine clinical practice or in pharmacokinetic studies of the drugs. (30, 66-78)

The many advantages of DBS have led them to be used routinely in less developed countries. By the end of 2008, 33.4 million people had HIV/AIDS and there were over 4 million people receiving antiretroviral treatment for HIV/AIDS in low or middle income countries. Viral load measurements and drug resistance testing needs to be undertaken to detect drug resistance, but this traditionally required highly specialised laboratories and strict protocols for the storage and shipment of plasma. This is virtually impossible to undertake in countries that have limited resources. Consequently, drug resistance is often not detected until it is too late and the patient has developed severe immunodeficiency and widespread drug resistance. (79)

The aim of effective HIV/AIDS therapy is to suppress the viral load to undetectable levels by 24 weeks and maintain full viral suppression after this. Treatment failure needs to be detected early to prevent the build-up of drug-resistance mutations which could severely limit future drug options and to avoid the manifestation of the symptoms of HIV. The limited resources in developing countries restrict access to viral load monitoring. However, DBS can now be used to monitor patients on antiretroviral treatment in such settings as they can be used for viral load quantification, genotypic resistance testing and other HIV-related analyses without the need for the expensive equipment required to ship and store plasma. In addition to this, DBS can be used to screen for HIV in infants born to HIV-infected mothers by looking for HIV-1 DNA by PCR in DBS collected at the routine 6-week immunization clinic visit. This enables paediatric antiretroviral treatment to be started at an early stage, consequently reducing the mortality in this vulnerable age group. (79-81)

## 1.5. **Other Modern Day Applications of DBS**

Outside of the routine clinical setting DBS have found many areas where their inherent advantages make them a popular sample taking option. DBS have proven to be very popular in large-scale population-based research (Table 1.4), as they only require minimally trained field personnel who can collect samples whilst carrying out survey work. This coupled with the ease of transport, processing and storage makes it a cost-effective choice for such studies. (20) Using DBS can minimise participant burden and maximise participation in studies. (82) The use of DBS in such studies has been wide and varied, covering HIV infection surveillance, screening for drugs of abuse, (24) and for looking at the interaction between social, psychological and environmental domains and their impact on health, and the risk for development of chronic diseases. (32)

DBS samples are logistically easier to handle as they do not require centrifugation, separation or refrigeration. They can be batched and sent to the laboratory in a time scale suitable to the study, without the requirement of a cold chain. (15) It has been estimated that one trial that would have needed dry ice to ship samples if venepuncture had been used, saved tens of thousands of euros by using DBS and therefore not requiring dry ice. (26) At a DBS workshop held by the pharmaceutical industry in 2010 it was estimated that large organisations could save €1 million per year due to the ability to ship DBS in ambient conditions. (28) Approximately 30% of dry ice shipments used during clinical trials arrive with incorrect packaging or labelling and can therefore place samples at risk. By avoiding dry ice altogether a much higher proportion of specimens taken may get to be used in a study as a result of being shipped correctly. (83)

| Study | n[a] | Age Range (years) | Biomarkers in DBS |
|---|---|---|---|
| Great Smoky Mountains Study http://devepi.mc.duke.edu/GSMS.html | 1000[c] | 9-15 | Androstenedione, CRP, DHEA-S, cortisol, EBV antibodies, estradiol, FSH, LH, testosterone |
| Health and Retirement Study http://hrsonline.isr.umich.edu/ | 7000[b] | >50 | CRP, HbA1c, Total cholesterol, HDL |
| Los Angeles Family and Neighborhood Survey http://www.lasurvey.rand.org/ | 5000[b] | >3 | CRP, EBV antibodies, HbA1c, Total cholesterol, HDL |
| National Longitudinal Study of Adolescent Health http://www.cpc.unc.edu/addhealth | 17000[b] | 23-31 | CRP, HbA1c, Total cholesterol, HDL, EBV antibodies |
| National Social Life, Health, and Aging Project http://www.norc.org/Research/Projects/Pages/national-social-life-health-and-aging-project.aspx | 2000 | 57-84 | CRP, EBV antibodies, HbA1c, haemoglobin |
| Tsimane' Amazonian Panel Study (Bolivia) http://heller.brandeis.edu/sustainable-international-development/tsimane/ | 600[c] | 2-15 | CRP, transferrin receptor, leptin, EBV antibodies |
| Work and Iron Status Evaluation (Indonesia) http://ipl.econ.duke.edu/dthomas/WISE/ | 16000[b,c] | >1 | Transferrin receptor, CRP |
| Mexican Family Life Survey http://www.ennvih-mxfls.org/english/index.html | 17700[b,c] | >15 | CRP |
| Study of the Tsunami Aftermath and Recovery (Indonesia) https://dupri.duke.edu/research-project-group/study-tsunami-aftermath-and-recovery-star | 35000[b,c] | >1 | CRP, EBV antibodies |

[a] Sample sizes are approximate and refer to the number of participants providing DBS samples.

[b] These studies are in the process of collecting or analyzing DBS samples, and specific plans for analyzing biomarkers in DBS samples are subject to change.

[c] DBS samples are collected from the same respondents multiple times over several months or years.

**Table 1.4 –** Current Applications of DBS Sampling in Large Population-Based Studies (15)

The minimally invasive aspect of DBS is also preferable for studies as it can increase the participation of patients into research and may reach a wider range of participants by bringing sampling to people in the community, instead of just sampling those willing to come to the clinic or the laboratory. (15, 73) In one study, 83% of participants in a study that used DBS home sampling for monitoring HbA1c said that the filter paper method of collection should be brought into routine practice and that home collection in the future is desirable. (23)

Recently stored NBS DBS have been seen as a valuable resource as they represent a complete population and therefore allow historical comparisons to be made (as long as the analytes have been shown to be stable). Other valuable uses of historic DBS include using them for retrospective genetic diagnosis when the proband has died and no other material is available for testing. This is useful to help identify potential future problems in siblings. DBS have also been used for determining if the cause of deafness in older children was due to congenital cytomegalovirus infection. (13) A further use of stored DBS is for the forensic identification of human remains after accidents or natural disasters. The U.S. military keep stored DBS as a form of biological “dog tag” to identify military personnel killed in action. (84) Along a similar line, DBS have been used for identification of kidnapped children. Additional uses of residual DBS include epidemiologic surveys of infectious diseases, etiologic studies of birth defects and developmental disabilities, population-based studies of haplotype and allele frequencies for genetic disorders and potentially significant gene polymorphisms and population-based studies of environmental and pharmacologic exposures. (85) These kind of large-scale, population-based studies would be virtually impossible to carry out using liquid based samples.

Outside of the clinical arena, the pharmaceutical industry has embraced DBS as a way of increasing its drug discovery process, facilitating pharmacokinetic investigations and minimising the number of animals needed in its toxicological profile studies. (26, 28, 86)

## 1.6. **Practical Aspects of Analysing DBS**

DBS are a solid matrix and the way they are collected, handled and analysed in the laboratory is very different to liquid samples, with additional areas requiring consideration whilst setting up a DBS assay compared to a liquid sample

### 1.6.1. **Collection Paper**

During the process of sample collection for DBS, the skin is pierced with a sterile lancet and the resultant blood droplet is blotted onto filter paper. There have been many types of paper used for the purpose of collecting DBS, including glass fibre, cotton and cellulose based papers. These may or may not be treated with various reagents to aid analysis of different analytes. DBS filter paper must be free from impurities that may interfere with the quality of or composition of the sample. The paper should be of homogenous composition and well characterised, for example, the thickness, flow-rate, absorbency and purity should all be known. (87) If it is to be used for NBS programmes it must be validated in compliance with the requirements of the CLSI LA4-A5 (Clinical and Laboratory Standards Institute) consensus standard (formerly National Committee on Clinical Laboratory Standards) and the Newborn Screening Quality Assurance Programme (NSQAP) at the Centers for Disease Prevention (CDC) monitor the performance of filter paper blood collection products for neonatal screening programmes. (19)

There are many variables that affect the analysis of DBS and that can have an impact on the performance of assays, such as the volume of blood applied to form a blood spot, the haematocrit of the patient/control/calibrator blood and chromatographic effects of the paper. (24) These parameters can all influence the volume of blood contained within a punch taken from the DBS and as the type of filter paper used influences the total effect of all these parameters, it has an important role to play in DBS assay performance.

Many different types of paper have been used in DBS assays including Schleicher and Scheull #903 and #2992, (88) Macherey Nagel #818, Whatman (GE Healthcare) BFC #180, (89) FTA Classic, (90) #903 (also known as 903 Protein Saver), FTA®DMPK-A, FTA®DMPK-B, (25) #1, #3 MM, (20) #113, #17Chr, #160, #31 ET and ETCHR, Toyo Roshi type 1 and 545 paper, VEB #388, (19) and Perkin Elmer glass fibre filter paper. (91) Other filter papers available for DBS collection include Whatman FTA®DMPK-C, FTA®, FTA®Elute and Ahlstrom #226.

For many years the favoured filter paper for NBS programmes in Australia, Canada and the USA was the Schleicher and Scheull (S&S) #903 filter paper, which was incorporated as part of the Guthrie card and was the paper used by Guthrie in his first collection of DBS for the detection of phenylketonuria in newborns. (3) In 2004, Schleicher and Scheull were bought by Whatman and the S&S #903 paper is now referred to as Whatman #903. In recent years however, the Ahlstrom paper equivalent to Whatman #903, Ahlstrom #226, has been used for NBS programmes in Europe, distributed in partnership with ID Biological Systems. Prior to October 1999, S&S 2992 was the paper type favoured by European NBS programmes, but at this date S&S decided to promote only S&S 903 for screening programmes and

subsequently most countries switched to this paper. In Japan the favoured paper for neonatal screening is Toyo Roshi 545. A few other countries use Whatman BFC 180 paper for their NBS programmes.

Whatman #903 paper has a well-characterised performance and as a result the vast majority of published methods for DBS analysis have used this type of filter paper. (25) Whatman #903 (Figure 1.4) paper is manufactured from 100% pure cotton linters, has no wet-strength additives and the paper is left untreated making it suitable for assays used to detect proteins, enzymes or metabolites. It meets the specifications of the CLSI LA4-A5 consensus standard and has therefore been approved as a FDA Class II Medical Device. It is sold as a CE marked In-Vitro Diagnostic in Europe in compliance with 98/79/EC and is manufactured to ISO9001 and Good Manufacturing Practice guidelines. (92)

**Figure 1.4 –** Whatman #903 Protein Saver Card (93)

Only one other filter paper has been approved for use in neonatal screening programmes by the FDA and that is the Ahlstrom #226 paper. This is also a registered Class II Medical Device made from top quality cotton linters using ultra-pure, reverse-osmosis water and treated with dilute acid to remove any remaining organic and inorganic impurities. It also has no added wet-strength additives or reagents. The paper is made in a strictly controlled environment to ensure high

uniformity and high purity from filter to filter. In 2013, Perkin Elmer acquired the exclusivity to the Ahlstrom #226 paper and this is now marketed as the Perkin Elmer 226 sample collection device. (94)

NSQAP evaluated the comparability of the Ahlstrom #226 and Whatman #903 paper and found that the difference between the manufacturers could be at least 4-5% for comparability, which is equivalent to the lot-to-lot variance of a single manufacturer's filter paper products. NSQAP concluded that the performance of the two types of paper is essentially equivalent, (95) however when using DBS for analysis it is still important to state what filter paper has been used for collection of the DBS and use matrix matched calibrators if necessary, especially if other non-NSQAP papers are being used.

Some filter papers are available that are pre-treated with various reagents in order to optimise their use for different applications. One such example is the Whatman FTA® and FTA®Elute range of filter papers. These utilize patented Whatman FTA technology and contain chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidative and UV damage. The aim is to simplify the handling and processing of nucleic acids and the cards are aimed at markets that use DNA analysis such as forensics. It is stated that upon application of the sample to the card, cell membranes and organelles are lysed and the released nucleic acids become trapped in the fibres of the matrix. The immobilized nucleic acids are then preserved for transport, immediate processing or long-term storage at room temperature. (96)

GE Healthcare also manufacture Whatman FTA DMPK-A, B and C cards (Figure 1.5). FTA DMPK-C is an untreated card, but FTA DMPK-A and B contain chemicals

that lyse cells on contact with the matrix and inactivate endogenous enzymes by denaturing protein. These cards are recommended for use for pharmacokinetic and toxicological studies. (97) However, these papers need to be assessed thoroughly before implementation as some users have found that chemically treated papers can lead to substantial ion suppression issues compared with untreated cards and may lead to protein conformational change leading to lack of recognition of epitopes once eluted. (28)

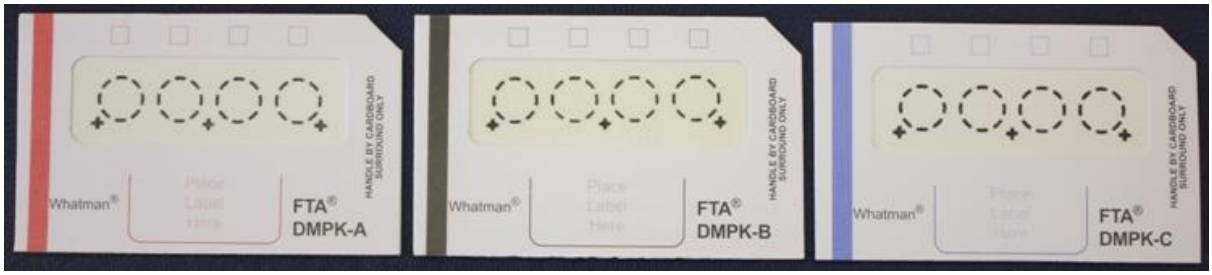



**Figure 1.5 –** Whatman DMPK-A, B and C FTA cards. (97)

In addition to the pre-treated commercially available papers, there are many examples of the collection paper being treated before sampling. A wide range of chemicals have been used including boric acid, (36) tartaric acid, dodecyl dimethyl ammonium bromide, (19) citrate buffer containing semicarbazide and aniline, (98) glucose oxidase (99) and ethylene glycol. (100)

Treating filter paper with an agent can affect the chromatography of the paper and therefore alter the volume of blood that may be contained in a punch taken from a pre-treated DBS compared with an untreated paper. For example boric acid treated paper contains less volume of blood per punch (~21.5 µL per 0.95 cm diameter punch) compared with untreated paper (~24 µL per 0.95 cm diameter punch). (101)

Treating the paper may also affect parameters other than chromatography. Elbin (90) found that the Whatman FTA Classic card lead to an increase in activity in some enzymes that are measured when investigating LSDs. Therefore, the potential

source of variation introduced by using treated cards should be investigated before their use is instigated.

### 1.6.2. **Production, Storage and Handling of DBS**

The collection of blood from a source of capillary blood (e.g. finger or heel) is a relatively straightforward process, especially when compared to traditional venesection. There are many published protocols providing advice on how to do it. (15, 19, 20, 24, 87) The sample can be collected either by the patient themselves in their own home, or by non-medically trained personnel such as when samples are required for epidemiological surveys. (32) However, if DBS are to be useful, an adequate volume of blood is required and so DBS need to be collected correctly.

Preparation for the collection may need to begin several days before the blood spot is taken if there is a chance that contamination of the blood spot with medication could occur. For example, if DBS will be used for steroid analysis and the patient is using topical steroids that they apply with their hands. Touching the filter paper should be kept to a minimum to avoid contamination with anything that the person taking the sample may have come into contact with, e.g. antiseptic, feeding formula, alcohol wipe, drugs. (19, 87)

When taking the sample, the skin should first be cleaned and disinfected with isopropanol and allowed to thoroughly dry to avoid haemolysis. (19) The skin should be warmed to encourage blood flow to the site (102) and ensure that the capillary blood is free-flowing for as long as possible. (32)

The first drop of blood formed after puncture should be wiped away (Figure 1.6A) as this may contain an excess of tissue fluid (103) although this is not mentioned in the guidance given to healthcare professionals in the UK performing NBS blood spot

collections. (104) A large drop of blood should then be allowed to form and gentle squeezing of the finger/heel to encourage blood flow (Figure 1.6B). Excessive squeezing or milking of the puncture should be avoided as this can lead to haemolysis and an increase in the proportion of tissue fluid in the specimen. The blood drop should be applied to the filter paper, without blotting, smearing or layering as this may adversely affect the uniform absorbing properties of the filter paper. Layering occurs when the blood spot is applied in multiple attempts one on top of the other, rather than in one smooth application, creating a jagged non-circular DBS. The filter paper should not be pressed against the puncture site, instead the blood should be freely dropped onto it. (15)

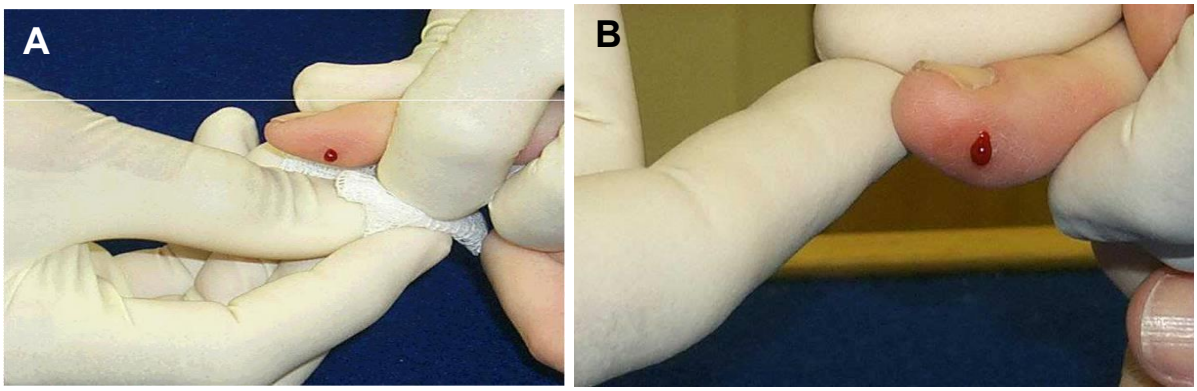



**Figure 1.6 –** The first drop of blood should be wiped away (A) and a large drop of blood allowed to form before being applied to filter paper (B). (105)

Several blood spots should be collected, ideally of similar size. Some filter papers come with pre-printed circles in order to help standardise the volume of blood collected (e.g. Guthrie cards used for neonatal screening). (106) Blood should only be applied to one side of the paper and spots should be spotted in a consecutive manner across the filter paper. There should also be sufficient blood in a drop so that it soaks all the way through to the back of the paper and therefore allows a uniform punch to be taken from the DBS. (32)

Depending on the collection device used, blood spots may need to dry for between three hours and overnight at room temperature before storage/posting, and they should not be heated, stacked, or allowed to touch other surfaces during the drying process. (90) Stacking of sample cards on top of one another could lead to cross contamination and should be avoided. (87)

Depending on the setting in which the DBS was taken and the analytes to be measured, the DBS will either be left at room temperature or frozen/refrigerated until it is time to ship it to the laboratory. The use of desiccant is often advocated during the drying, shipping and storage of DBS and may further improve stability, but this is often analyte dependent. (15, 84)

DBS can be sent through the postal system as long as they are appropriately packaged and labelled according to local rules. If postal regulations require the samples to be kept in sealed plastic containers, then it may be necessary to use desiccant to reduce exposure of the DBS to excessive moisture. Moisture can adversely affect the DBS by altering the elution time of the specimen or encouraging bacterial growth. (24) Care should be taken when choosing the type of packaging used for transport as different chemicals from plastic containers can leak onto the paper and interfere with analysis. (19) This will become increasingly important as more methods for LC/MS/MS analysis of DBS are developed.

For NBS, DBS should be sent to the laboratory as soon as possible to enable a fast turnaround of results and therefore pick up cases that need urgent treatment such as PKU. (87) During the validation of DBS assays the stability of the compounds being tested should be investigated and this should include testing at high temperatures, as

transportation of DBS in the post during the summer months could lead to such exposure. (25)

Due to the nature of DBS, they can be more easily stored for long periods of time than serum samples but there is wide variation in the length of time DBS are stored post analysis for NBS programmes. (85) In many programmes DBS are kept for quality assurance purposes – reanalysis of spots in false negative cases. (13) The filter paper matrix will act to stabilise most analytes in DBS, but the rate at which these samples degrade will vary by analyte and storage conditions. For example it has been shown that phenylalanine can be recovered from DBS stored at room temperature for up to 16 years, tyrosine from DBS stored for up to 5 years, but methionine, histidine and galactose are not stable in DBS stored in the same conditions. (107)

### 1.6.3. **Issues Unique to DBS Analysis**

As well as still having to tackle many of the issues facing traditional serum based assays, (9) there are additional factors that need to be considered when developing a DBS based assay and DBS calibrators. It has been stated that “the filter paper blood collection device has achieved the same level of precision and reproducibility that analytical scientists and clinicians have come to expect from standard methods of collecting blood, such as vacuum tubes and capillary pipettes”. (24) Therefore as the device used for collecting DBS samples can considered to be precise and reproducible (in the same way a vacuum tube is considered a reliable collection device), there is no reason why the assay using DBS cannot also be precise and reliable.

When a disk is punched from a DBS, it can be considered a volumetric measurement, similar to that of a liquid measuring device. (19) The disk can be of any size that is suitable for the assay (most common diameters are 3 – 6 mm) and sometimes multiple punches are used per test. The sample is then eluted from the disk and the DBS is effectively reconstituted as haemolysed liquid whole blood. (15) Using a punch is a way of ensuring that for each sample analysed the same volume of blood is used in the assay regardless of how much blood has been absorbed onto the filter paper. However, the exact volume of blood contained within that disk will be dependent on a number of variables, each of which need to be considered whilst validating an assay.

An alternative method to punching out a disk is to cut out the whole DBS. A situation where this would be useful would be when the sample had been collected from the prick site into a capillary and then the blood applied to the filter paper in a volumetric way using a capillary pipette. (19) Although this would lead to a known amount of sample being applied to the filter paper, it defeats one of the main objects of using DBS, mainly ease of sample collection without the need for specialist equipment.

The pharmaceutical industry often use capillary tubes coated in anticoagulant to collect blood from the puncture site and then use a suction bulb to apply all the blood collected to the filter paper. This aids the controlled application of sample to the paper and reduces risk of contamination of the card. (16)

Punches can be created using tools such as scissors or hand held manual hole punchers, or if a large number of samples need to be analysed then there are many automated machines available (Figure 1.7). By using tools such as manual hole

punches, accuracy of punch size should be improved and the use of automation should further improve the situation.

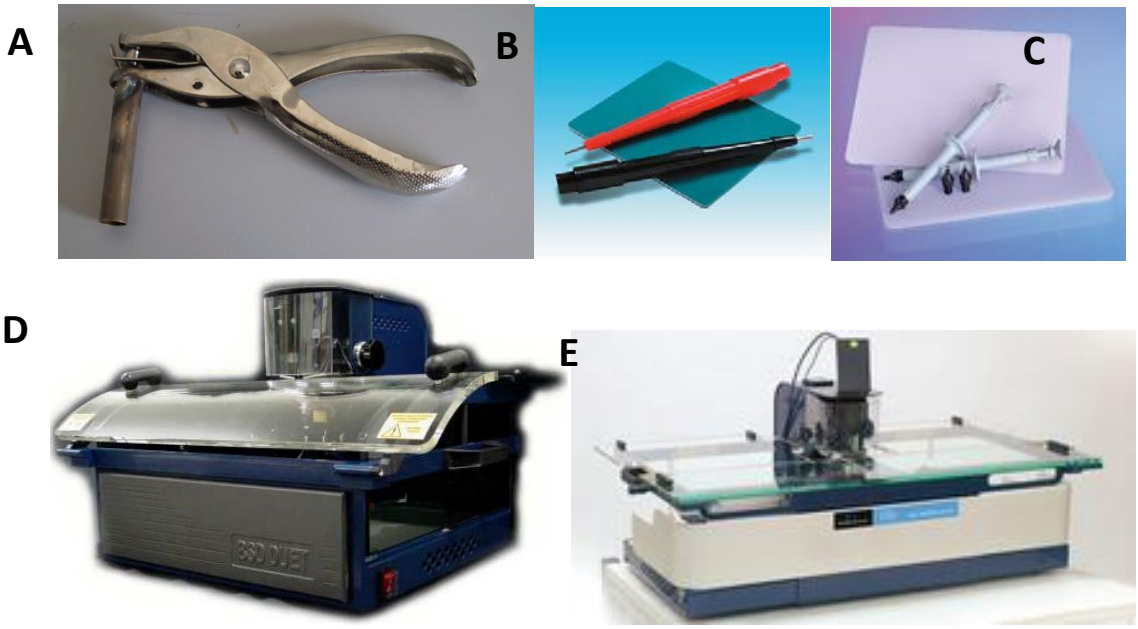



**Figure 1.7 –** Examples of tools for creating punches from DBS. A) Manual hole punch. B) Harris Uni-core punches and mat. C) Harris Micro punches, mats and replacement tips. D) BSD600 Duet Series II semi-automated punching system. E) Wallac MultiPuncher (Perkin Elmer) automated punching machine.

Technology is being developed, mainly in the pharmaceutical industry, which may dispose of the need to punch discs from DBS at all, and can allow direct analysis of the DBS. This includes technology where the mobile phase is passed directly through the DBS filter paper and onto the column for chromatographic methods, as well as automated sampling machines which would contain the whole DBS ready for extraction (Figure 1.8). (28, 78, 108) Increasing efficiency and the ability to process DBS in a high-throughput manner are major aims of the pharmaceutical industry. (109)

Before a laboratory begins the validation stage of a DBS assay, the suitability of DBS as a matrix for a particular analyte needs to be considered. In the first instance, whether or not the analyte of interest can be removed from the DBS in a form suitable for investigation needs to be assessed. (15) The process of DBS drying

needs to be investigated to make sure that DBS sampling is suitable and the compatibility of the eluting solution with the intended analytical technique should be considered (e.g. it is not recommended to use wetting agents with LC/MS/MS).

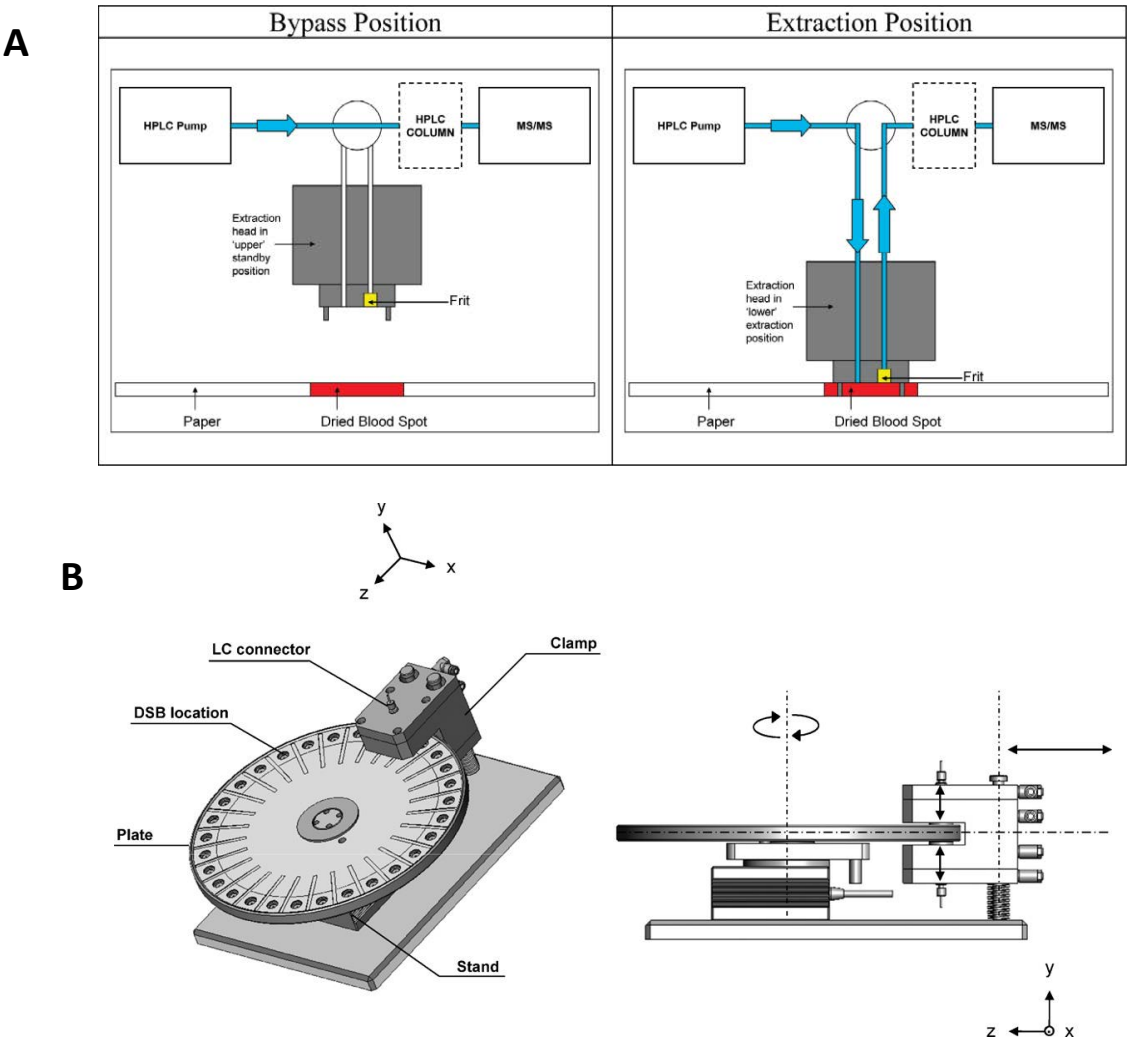



**Figure 1.8** – Examples of technology that is being developed to remove analytes from DBS without having to take a punch from the DBS first. A) Schematic diagram of direct extraction/analysis assembly and operation of the TLC-MS interface. (78) B) Schemas of the on-line DBS prototype. Black arrows illustrate the movements of the different components of the prototype. (108)

If the analyte is found to come off the filter paper, then the laboratory also needs to assess whether interferents in the assay have also been eluted, for example, will the presence of cellular components such as red or white blood cells interfere with the assay? This may be a problem for assays that are normally only used for

serum/plasma samples but are then adapted for use with DBS. Additional processing steps may be required to overcome this issue. (110)

If DBS are a suitable matrix (e.g. they are not suitable for ferritin because of the confounding effect of variable release of intracellular ferritin from red blood cells leading to higher results than expected) (111) then factors that can affect analysis and quality of results need to be considered. These include properties specific to the blood sampled, qualities of the paper, calibrators, use of anticoagulants and preservatives, and storage conditions amongst other things.

Properties relating to blood such as its haematocrit, the volume applied to create a DBS and its chromatography through the filter paper can all have an effect on the volume of blood (or effective serum volume) in a given size of punch. (24) Each of these parameters is altered to different extents depending on the paper used resulting in different volumes of blood per punch and analyte recovery, hence the reason that so much care is taken to ensure lot-to-lot consistency of filter paper. Therefore when setting up a DBS assay, filter paper type used should be chosen and assessed carefully. If samples from external sources using alternate paper types are likely to be received then the effect of the different type of filter paper on analysis should be assessed before results are reported. (90) The effects of haematocrit, chromatography, volume of blood used to make DBS and effect of haemolysis on results are further discussed in Chapter 3.

One of the aims when making standards and quality control (QC) material for use in DBS assays is to try and keep them as similar to the patient sample as possible. However, sometimes there are practical limits to what can be achieved. When validating a DBS assay it would be impossible to prepare all the material necessary

using samples collected by fingerprick. It is much easier and more practical to use whole blood that has been collected with anticoagulant. However, before using anti-coagulated blood as a substitute for capillary it must be assessed to ensure that there are no significant differences between the two. Hill and Palmer (36) found no difference in the spread of capillary blood (without anticoagulant) through filter paper compared with venous blood with ethylenediaminetetraacetic acid (EDTA) taken at the same time from the same person.

The choice of anticoagulant should also be investigated if possible. Olivova et al., (112) found that the use of EDTA in DBS led to α-galactosidase A enzyme activity being increased regardless of disease status. Elbin et al., (90) compared the effect on activity of five lysosomal enzymes when DBS were made using heparin or EDTA and found that EDTA gave more precise results. When considering which anticoagulant to use when preparing the first International Society for Neonatal Screening (ISNS) reference preparation for neonatal screening for TSH, phenylalanine and 17 α-hydroxyprogesterone (17-OHP) in DBS, the authors decided against the use of EDTA and citrate as these can affect some of the assays used for neonatal screening. As a result heparin was used as the anticoagulant. (113) Holtkamp et al., (114) investigated the affect that EDTA contamination of DBS had on TSH and 17-OHP measured by lanthanide fluorescence assays. They showed that 138 out of 190,000 NBS samples were contaminated with EDTA. Of these 138 samples, 27 went on to give false positive-results for 17-OHP, although no false positives for TSH were found. The authors showed that false negatives occurred when EDTA concentration was >2.0g/L for 17-OHP or >3.0g/L for TSH. The level of

EDTA present depended on how much blood was added to an EDTA coagulated container before the DBS spots were made.

### 1.6.4. **DBS Calibrators and Quality Control**

If calibrators are not standardised to a certain extent with regards to filter paper type, haematocrit, blood spot volume and the use of haemolysed or whole blood, then large variations in results can be found between calibrators purporting to contain the same concentration of analyte. (88, 115) However, even if these parameters are controlled as far as possible there are still a multitude of other decisions to make when considering how to make DBS calibrators and QC.

Matrix matching the calibrators and QC to the expected sample type is just as important for DBS assays as for the more conventional liquid based assays. However, as with conventional liquid based assays there are many options available for calibrating DBS assays (in addition to the parameters mentioned above). In fact, it is exactly because of these different options available to laboratories that many external quality assurance or proficiency testing programmes have encountered huge variation in results for the same DBS QC material. (116, 117)

One of the simplest ways to calibrate DBS assays is to not make DBS materials but to calibrate the assay using aqueous standards, however this would require post-analytical manipulation in order to account for dilution differences. Chuang et al., (118) used methanolic and aqueous standards for lactate and pyruvate, respectively and determined DBS levels by multiplying the results of the assay by a dilution factor. This method avoids having to make DBS calibrators and controls, however the assay is no longer matrix matched to patient samples. Using non-matrix matched calibrators

may be problematic for DBS assays, given the variation in patient samples being received for analysis in terms of haematocrit, volume, recovery, etc. (119)

DBS calibrators can be made using commercially available material, such as lyophilised calibrators. The material can be spotted to make dried spots (not technically DBS as they contain no blood) and then used in the DBS assay as a DBS. Due to its widespread availability, the commercial calibrator could lead to a reduction in inter-laboratory variation as has been seen in other settings. (9, 120) However, because of the physical difference between blood and the lyophilised calibrators, substantial quantitative differences will result as they will contain different volumes of material in a given sized punch. (25, 30) Hoogtanders et al., (121) found quantitative differences of up to 15% between ClinChek$^{(R)}$ QC material DBS and fresh blood DBS and therefore this would not be suitable as a calibrator. Yet once the expected concentration has been determined it could be used as QC material, alongside traditional DBS, if it had good reproducibility.

Paired serum and capillary blood spots collected simultaneously (or anticoagulated blood that is then used to prepare DBS) can be used as calibrators. The serum levels are quantitated and the paired DBS are assigned the serum values and used to calibrate the DBS assay. This method relies on there being a robust and accurate method available to measure the analyte in serum and the ability to collect samples over an analytical range that is clinically useful. This technique also does not allow control over the size of the haematocrit in the samples. Newman et al., (122) used this method to measure 25-hydroxyvitamin $D_2$ ($25(OH)D_2$) and 25-hydroxyvtiamin $D_3$ ($25(OH)D_3$) levels in DBS.

The most common methods for producing DBS calibrators and QC require the use of existing whole blood specimens. They can be divided into two broad categories: use of a whole blood specimen without separation of plasma/serum and use of washed red blood cells (WRBC) with a plasma type component added back in. Which type of method is used often depends on whether or not the analyte being measured is present endogenously or not.

For compounds that are not found endogenously, such as therapeutic drugs, the most popular method for making DBS calibrators is spiking whole blood. Some drugs would be measured in whole blood anyhow, so measuring them in DBS does not pose some of the challenges that face analytes that are usually measured in serum. van der Heijden et al., (123) measured everolimus in DBS using LC/MS/MS as part of a therapeutic drug monitoring procedure. They made an ethanolic stock solution which was then diluted with fresh everolimus-free EDTA blood to give a top standard. This was then further diluted to give the calibration standards. A similar approach was used by Saracino et al., (77) to measure clozapine and its metabolites and by a variety of other authors for drug measurement: etravine, (75) dextromethorphan and its metabolite dextrorphan, (124) cyclosporine A and tacrolimus. (30) These methods used the concentration spiked as the concentration of the calibrator.

Hoffman et al., (125) used DBS to measure PSA using the Immulite chemiluminescent immunoassay analyser. They spiked heparinised whole blood from women with various amounts of purified seminal plasma PSA to create DBS calibrators. They kept back some aliquots of the spiked whole blood calibrators and assayed them six times on the Immulite analyser against the manufacturer's own

calibrators. The average of the six replicates was then taken as the concentration of the DBS calibrator.

Sometimes whole blood samples are enriched with the target analyte to give a range of target concentrations. The actual concentration of the DBS calibrator material is then assigned afterwards. For example, in the preparation of the first European working standard for phenylalanine (EWS-Phe-01), Dhondt et al., (115) created a working solution of phenylalanine which was then used to spike heparinised whole blood to give a series of calibrators used to produce the DBS calibrators. An aliquot of unspiked blood was kept back, centrifuged and the plasma shipped to laboratories across Europe for amino acid analysis. The average of the phenylalanine analysis of the laboratories was taken as the concentration of the basal sample. The concentration of the calibrators was then taken as the basal level plus the concentration of the spike added. This method was used instead of just measuring the spiked blood and taking that as the concentration of the calibrators because there was no reference method for the measurement of phenylalanine and it was felt that the error in the weighing step and preparation of the stock solution was smaller than the error from the analytical method used to measure phenylalanine in blood. This method was also used by Elvers et al., (113) in the preparation of the first ISNS reference preparation for neonatal screening for TSH, phenylalanine and 17 α-hydroxyprogesterone in DBS.

Another way to make DBS calibrators involves the use of WRBC which are then combined with an artificial matrix. Shirtcliff et al., (126) mixed equal volumes of red blood cells with commercially available calibrators from the kits they used to measure gonadal hormones in serum with (Diagnostic Systems Laboratories) and Bio-Rad

(Anaheim, CA) lyphochek levels I, II and III, respectively. The resultant mixtures were used to make the DBS calibrators and controls. This method was also used to measure insulin, high-sensitivity CRP and triglycerides. (18)

Alternatively, WRBC can be combined with human serum instead of commercial calibrators. Spierto et al., (116) used WRBC and combined them with charcoal and dextran sulfate treated serum (to remove the endogenous phenylalanine). This mixture was then spiked with various levels of phenylalanine to make the DBS calibrator material. Janzen et al., (127) used WRBC combined with steroid-free serum from MP Biomedicals (Eschwege, Germany) as they were using DBS to quantify adrenal steroids by LC/MS/MS. Methanolic steroid standards were added to the whole blood pool at different concentrations to give a range of calibrators. This method has also been used for DBS drug analysis (moxifloxacin). (70) Alternatively, the alcoholic stock solutions can be dried down before being reconstituted with the whole blood pool before being spotted out to create DBS calibrators. This method was used in the analysis of protease inhibitors and non-nucleoside reverse transcriptase inhibitors by ter Heine et al. (76)

DBS calibrators can be made from animal material. However, as with liquid based assays, using non-human matrices can lead to calibration problems due to matrix differences. For example, in order to improve standardisation of thyroxine assays used in NBS programmes in the Netherlands, all five screening laboratories were asked to use DBS calibrators from one manufacturer that contained bovine serum albumin. Unfortunately, one of the laboratories experienced a decrease in reproducibility as their radioimmunoassay method required separation of the free and antibody-bound thyroxine fractions using dextran-coated charcoal and this is known

to be sensitive to the protein concentration of the incubation mixtures. Ultimately it was realised that the calibrators were not suitable for all methodologies. (89)

For most DBS assays (especially outside of NBS programmes) in-house calibrators will need to be produced due to lack of commercially available calibrators. In-house DBS calibrators that have been properly validated should serve their purpose. However, problems may arise when multiple laboratories offer DBS analysis for the same analyte because of the large number of additional variables that are present for DBS on top of the variables for liquid based assays. This problem has already been seen with the NBS programmes, but it is encouraging to see that coordinated international efforts have been made to try and minimise the problem.

### 1.6.5. **Reference Range Consideration**

There are many ways of approaching the issue of reference ranges for DBS. This may be by defining new reference ranges based on results obtained from DBS samples alone resulting in a situation similar to having different ranges for serum and plasma. Reference range determination is often a long and arduous process and is not routinely undertaken. Alternatively DBS results could be aligned with the serum results in order to use the original serum reference ranges and this can be achieved in a variety of ways.

The ideal situation is when there is no difference between whole blood values and DBS values, such as is seen for some drugs like tacrolimus and cyclosporine A. (30) This would be expected to be the case for analytes that are traditionally measured in whole blood, as measuring them in DBS should not alter the matrix considerably and therefore the same reference ranges can be used for both sample types.

An alternative is to calibrate the DBS calibrators to the levels of the analyte found in the paired serum/plasma. Newman et al., (122) used this approach in their DBS vitamin D assay. The technique worked for 25(OH)$D_3$ however, the correlation between paired serum and DBS samples for 25(OH)$D_2$ was not 1:1 therefore leading the authors to suggest that a conversion factor may be required. In fact, many authors found that because they were getting good correlation between paired DBS and serum/plasma samples that even if the relationship was not exactly 1:1 and therefore interchangeable, that at least the difference was consistent and so a conversion factor could be applied to bring the DBS results in line with serum/plasma results. (128) Some authors arrived at their conversion factor values based on average haematocrit values as they felt it was the space-filling effects of haematocrit in DBS that was leading to the difference in results. (77) Others used the linear regression equation that was produced from the plot of paired plasma/serum samples against DBS samples as a way of calculating what the expected serum level would be from a given level of analyte obtained from a DBS sample. (126) Many authors appear to favour the conversion factor route. (9, 19, 129)

The relationship between DBS and serum/plasma concentration is going to vary from analyte to analyte. Along with all the above parameters, this is an area that also needs close attention if the DBS assay being investigated is going to be of clinical use. If DBS and serum results do not agree and a conversion factor is not used, then a new reference range will need to be found specifically for the DBS assay with all that entails.

There are many different factors that need to be considered when setting up a DBS assay. However, other aspects can affect the accuracy of results as well as those

mentioned above such as the type of technique used. The main aim should be to assess whether or not the DBS assay is fit for purpose. (79) It may not be as good as a traditional liquid based assay, but the decrease in accuracy or precision may not be so great as to outweigh the benefits provided by using the DBS technique. (36) It may be that some of the problems posed by using DBS can be overcome.

## 1.7. **Aim of Thesis**

The initial aim of this work was to develop and implement a fit-for-purpose blood spot collection device and kit, and establish the first direct to the pubic vitamin D DBS service in the UK. The quality of DBS samples that could be obtained from the public and the impact of lancet type on "volume" collected was then investigated. In addition, the impact the characteristics of blood spots had on results was determined (see Chapter 3).

The use of DBS for the measurement of vitamin D status was examined by comparing DBS results from the public with results from inner-city Birmingham GP patients, measured using our routine NHS serum vitamin D service, and an attempt was made to characterise these two different populations. Attention was focused on the high to toxic results found in the DBS population in order to find out the reasons behind the high rate of hypervitaminosis D seen in our DBS population. A further aim was to find out the rate of repeat vitamin D testing in our GP and DBS populations, and investigate the response to initial results amongst those that have repeat tested. The impact of a direct to the public DBS service was assessed (see Chapter 4).

Another aim was to create a DBS CRP analytical method that could be performed on the Abbott Architect autoanalyser that gave results comparable to the Abbott Architect serum CRP method. This method was also used to investigate the

feasibility of using a new kind of dried blood collection device – the Mitra device (see Chapter 5).

# CHAPTER 2 - MATERIALS AND METHODS

## 2.1. Reagents and Consumables

**Physiological saline**: 0.9% w/v.

**1.5 mL microtubes:** APEX tough microtubes (Alpha Laboratories, Eastleigh, UK).

**Ultra-pure (UP) water:** 18.2 Ω Millipore Milli-Q water system (Millipore, Billerica, USA).

**Ethyl acetate:** HPLC grade (Rathburn Chemicals, Walkerburn, Scotland).

**PTAD:** 4-phenyl-1,2,4-triazoline-3,5-dione (Sigma-Aldrich, Poole, UK).

**Acetonitrile:** HPLC grade (Rathburn Chemicals, Walkerburn, Scotland).

**Formic acid:** 99% ULC/MS Grade (Biosolve, Dieuze, France).

**Methanol:** LCMS grade (Fisher, Loughborough, UK).

**Hexane:** Glass distilled grade (Rathburn Chemicals, Walkerburn, Scotland).

**Isopropanol:** HPLC grade (Rathburn Chemicals, Walkerburn, Scotland).

**Ammonium acetate:** ≥ 99.9995% purity (Sigma-Aldrich, Poole, UK).

**25(OH)$D_3$ Internal Standard (IS):** 26,27-hexadeuterium-25-hydroxy Vitamin $D_3$ (Synthetica AS, Oslo, Norway).

**25(OH)$D_2$ IS:** 25(OH)$D_2$ (6,19, 19-d3) (Sigma-Aldrich, Poole, UK).

### 2.1.1. **Vitamin D**

**Mobile Phase A:** 154 mg ammonium acetate (2 mmol) and 1 mL formic acid (0.1%) in 1 L of UP water.

#### 2.1.1.1. *Serum Vitamin D*

**Mobile Phase B:** 385 mg ammonium acetate (2 mmol) and 2.5 mL formic acid (0.1%) in 2.5 L of methanol.

**Working IS:** 500 µL of 10,000 ng/mL 25(OH)$D_3$ IS and 50 µL of 100 µg/mL 25(OH)$D_2$ IS in 20 mL 80:20 methanol:isopropanol.

**Loading Reagent:** 70% methanol in UP water.

**0.2mol Zinc sulphate:** 7.16 g zinc sulphate in 200 mL UP water.

**Vitamin $D_2$ and $D_3$ Serum Calibrators and QC:** Level I and Level II, lyophilised powder (Chromsystems, Munich, Germany).

**Vitamin $D_2$ and $D_3$ Serum QC:** Level I and Level II Recipe ClinChek (Waters, Hertfordshire, England).

2.1.1.2. *DBS Vitamin D*

**Derivatising Agent:** 0.01 g PTAD in 100 mL ethyl acetate.

**Loading Reagent:** 40% acetonitrile in UP water.

**Mobile Phase B:** 0.1% formic acid in acetonitrile.

**Working IS:** 50 µL of 10,000 ng/mL 25(OH)$D_3$ IS and 5 µL of 100 µg/mL 25(OH)$D_2$ IS in 50 mL of UP water.

2.1.2. **CRP**

The standard MULTIGENT CRP Vario kit reagent (Abbott Laboratories, Illinois, USA) was used to analyse samples (serum and blood spot) for CRP. DBS were extracted into Abbott CRP Vario reagent R1 – a glycine buffer (pH 7.0, concentration 1.28%). The standard MULTIGENT CRP calibrator set (Abbott Laboratories, Illinois, USA) was used to make DBS calibrators. Randox Liquid Chemistry Premium Plus (Randox Laboratories Ltd, Crumlin, UK) Levels 1, 2 and 3 QC material was used to make DBS QC.

## 2.2. **Equipment**

**Sonicator:** Kerry ultrasonic bath (Guyson Ltd, Skipton, UK) or Ultrawave QS18 ultrasonic bath (Ultrawave, Cardiff, UK).

### 2.2.1. **Vitamin D**

**Nitrogen generator:** NN30LA-MS (Peak Scientific Instruments, Renfrewshire, Scotland).

#### 2.2.1.1. *Serum Vitamin D*

**LC/MS/MS:** Waters Acquity UPLC system with TQD mass spectrometer. Used in electrospray (ESI) positive mode.

**Column:** Waters Acquity UPLC BEH Phenyl 1.7 µm 2.1 x 50 mm.

#### 2.2.1.2. *DBS Vitamin D*

**DBS puncher:** Harris e-core hole punching device (GE Healthcare, Chalfont St. Giles, UK), using the 3 mm diameter cutter.

**LC/MS/MS:** Waters i-Class UPLC system with Xevo TQ-S mass spectrometer. Used in electrospray (ESI) positive mode.

**Column:** Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 50 mm.

### 2.2.2. **CRP**

All analysis was carried out on the Abbott Architect cSystem (Abbott Laboratories, Illinois, USA).

## 2.3. **Samples**

Blood spots were collected onto Whatman 903 filter paper (GE Healthcare, Whatman Plc, Maidstone, UK). Whatman 903 paper was chosen as this is one of two types of

paper approved by the CDC as being appropriate for NBS programmes, is very well characterised and has an acceptable lot to lot variation. In addition the laboratory was able to obtain large sheets of filter paper allowing us to develop the SWBH collection device and prepare calibrators and QC material.

### 2.3.1. **EDTA DBS Samples**

EDTA DBS samples were made by using paired full blood count (FBC – EDTA whole blood) and serum samples. The FBC samples were selected on the basis of the serum CRP or 25(OH)D levels measured during routine analysis. The FBC samples were then spotted out onto filter paper (using a pipette set to 20 µL unless otherwise stated) and left to dry overnight. Some FBC sample was retained and spun down in order to obtain plasma. The plasma was then reanalysed to compare with the serum value for confirmation. Development and most method validation work was performed using EDTA DBS as EDTA was readily available, unlike capillary DBS which could not be easily made to cover a range of values.

### 2.3.2. **Patient Samples**

Patient samples could consist of serum, EDTA whole blood or capillary finger prick samples. These were used for method comparison between serum and DBS assays and therefore required simultaneous collection or as close to simultaneous as possible. Patient samples were obtained by collecting matched serum, EDTA whole blood and capillary finger prick samples either during phlebotomy clinics (for CRP) or sent in by post from people using the blood spot vitamin D service (having been bled by a phlebotomist near them in order to obtain the serum and EDTA whole blood samples). The EDTA whole blood sample was spotted out as described above. The serum sample was measured for CRP or vitamin D. The capillary blood spot samples

were taken by patients with the assistance of laboratory staff when collected in phlebotomy clinics, or taken by the patient themselves for postal returns (and returned with the whole blood and serum samples).

The following procedure was used to collect capillary blood spot samples. A safety lancet was used to prick the patient's finger once it had been cleaned with an alcohol wipe. A blood drop was allowed to form on the patient's finger before it was gently touched to the filter paper contained within a blood spot collection device. Up to four drops were collected. The collection device was sealed and samples allowed to dry overnight or were posted to the laboratory.

### 2.3.3. **Manufactured Samples**

Sometimes samples such as calibrators, QC and other testing material had to be manufactured by mixing commercial material or patient serum with WRBC.

#### 2.3.3.1. *Washed Red Blood Cells*

Excess EDTA patient samples from FBC testing were collected, once all routine analysis had been completed. The volume collected depended on the final volume required. Approximately 0.45 mL of WRBC was required for every 1 mL of final manufactured material. Four times as much EDTA sample as WRBC required was collected, to allow for loses during processing. The EDTA whole blood was spun down at 3500 rpm for 5 minutes. The plasma was removed and replaced with the same volume of physiological saline. The sample was mixed gently by inversion before being placed on a rotary mixer for 5 minutes. The sample was spun down again, the saline removed and replaced with fresh saline. The sample was mixed gently by inversion before being placed on a rotary mixer for a further 5 minutes. The sample was spun down and the saline removed. The remaining WRBC were then

further spun down in microtubes at 6500 rpm for 5 minutes and any remaining supernatant removed. The WRBC were then combined and mixed.

#### 2.3.3.2. *Manufacturing Whole Blood Calibrators, Quality Control or other Samples*

To manufacture samples, WRBC were combined with serum or commercial material. The aim was to create a haematocrit of approximately 0.4 unless another level of haematocrit was required. A haematocrit of 0.4 was achieved by mixing WRBC with serum/commercial material in a ratio of 0.45:0.55. Once the WRBC and serum/commercial material was combined, the sample was mixed gently by inversion before mixing for 5 minutes on a rotary mixer. Once made, the manufactured whole blood was spotted out onto filter paper or used to make Mitra samples (Section 2.4.2).

## 2.4. **DBS Collection**

### 2.4.1. **DBS Collection Device – SWBH Device**

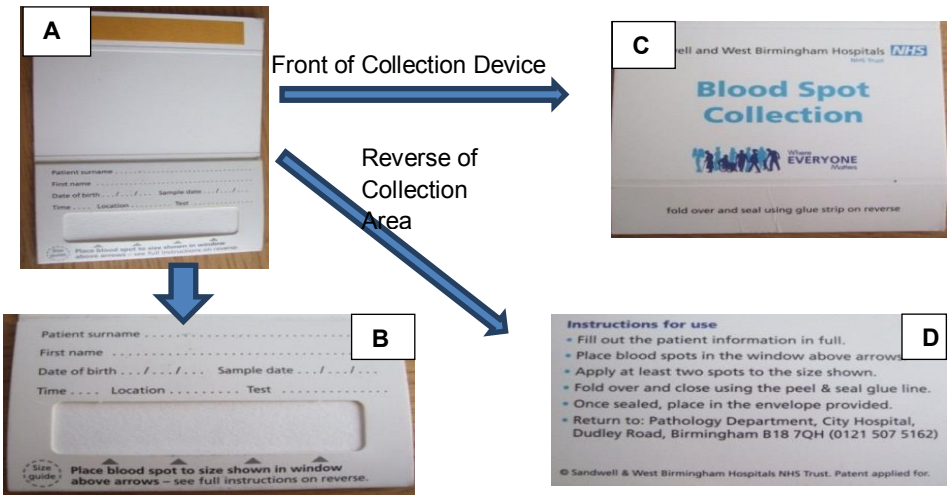



**Figure 2.1 –** A: Inside of collection device. B: Sample collection area with filter paper, size guide, placement guide and patient details. C: Front of collection device. D: Instructions printed on the reverse of the device.

The DBS collection device was developed in house (Figure 2.1) and consisted of filter paper (Whatman 903) within a cardboard frame and a window to allow application of blood by the patient. The cardboard folds around the filter paper and is sealed with a glue strip. There are no printed circles on the filter paper, but there is a size guide next to it.

### 2.4.2. **Dried Blood Collection Device – Mitra Device**

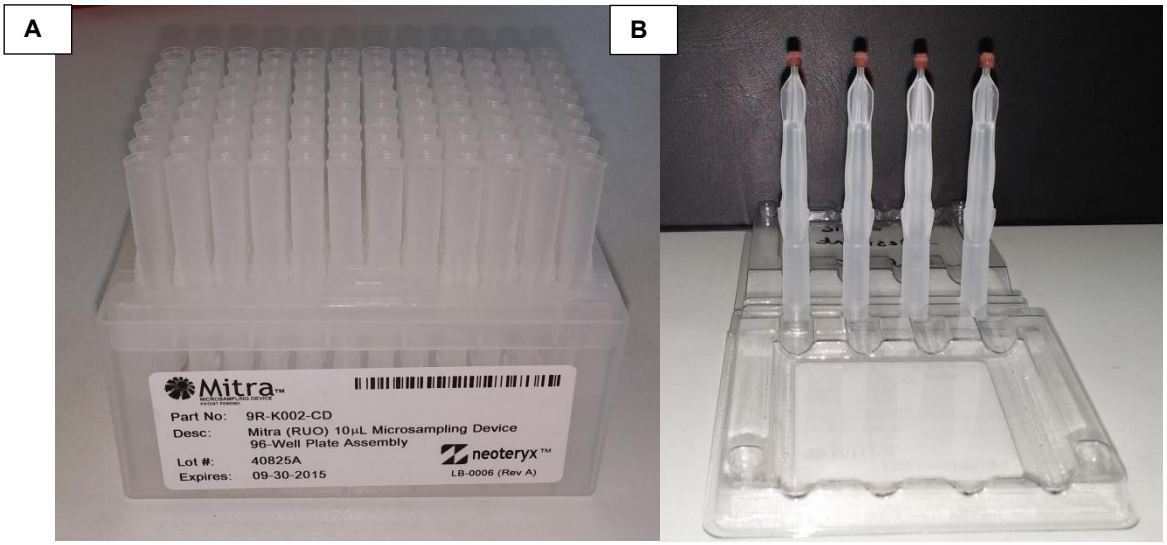



**Figure 2.2 –** Phenomenex Mitra device. A: 96 well plate. B: Clam shell with blood sampled.

Phenomenex (Torrance, USA) have recently introduced a novel approach to collecting dried blood for testing known as volumetric absorptive microsampling. The device (named Mitra) consists of a sponge like tip made of a porous substrate which sits on the end of a plastic stick, similar to a pipette tip (Figure 2.2). The tip wicks up a fixed volume of blood (10 µL). The Mitra devices can be acquired in “clamshell” packs of four, which would be used for taking patient samples, and in boxes of 96, which would be used for calibrators and QC. Samples are taken from patients by preparing and piercing the skin as for traditional DBS collection, but then instead of dripping drops of blood onto filter paper, the tip of the Mitra device is dipped into the

pool of blood until it just breaks the surface. The blood then wicks up the tip which is held in the pool of blood until the white of the tip has completely gone and the tip is entirely red. Alternatively, when making QC or calibrators, the tips are dipped into the pool of prepared QC and calibrator material as described above.

### 2.4.3. **DBS Collection Kit**

The DBS collection kit (Figure 2.3) contained everything required to take a DBS: a collection device, a consent form, instructions (Appendix 1), two blue BD Microtainer® Contact-Activated 1.5 mm x 2.0 mm lancets (BD, New Jersey, USA – in the final kit design), a plaster, an alcohol wipe and a pre-paid return envelope.

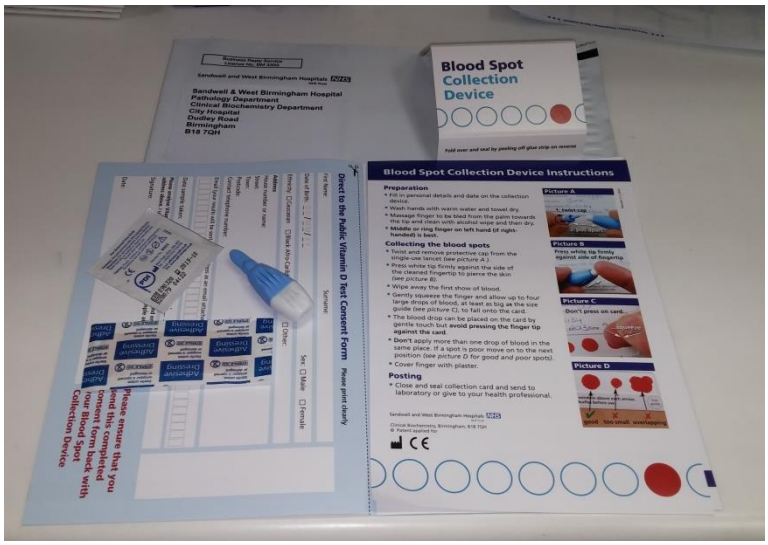



**Figure 2.3 –** SWBH collection kit contents.

### 2.4.4. **CE Marking**

The SWBH blood spot collection kit is an in vitro diagnostic medical device as its intended purpose is to act as a receptacle for specimens, i.e. blood. Thus the kit has to conform to the In Vitro Diagnostic Medical Device Directive 98/79/EC. A thorough examination of all processes and risks was undertaken, a technical file compiled and a declaration of conformity sent to the Medicines and Healthcare products Regulatory

Agency (MHRA). The MHRA accepted our registration of the DBS collection kit with them as an in vitro diagnostic medical device and granted us the right to CE mark our collection kit (Appendix 2).

### 2.4.5. **Direct-to-the-Public Vitamin D Service**

In July 2011 a direct-to-the-public DBS vitamin D service was introduced by the Clinical Biochemistry Department, part of SWBH NHS Trust. Members of the public phoned the department, ordered and paid for a kit, after using the website (www.vitamindtest.org.uk), through word of mouth or other laboratory communications. The patient received the kit in the post, took the sample and sent it back to the laboratory in a pre-paid envelope. Upon receipt, the sample was booked into the laboratory information system (Telepath) and batched for analysis. Results were reported to Telepath, a PDF report generated and emailed or occasionally posted to the patient. The report contained the patient's details, their results, their vitamin D status and the reference ranges (Appendix 3). If results were high to toxic (>220 nmol/L), users were contacted to discuss the implications before the results were released. No specific clinical advice regarding supplementation was provided. Users were always encouraged to discuss clinical queries with their GP or pharmacist.

## 2.5. **DBS Vitamin D Method**

### 2.5.1. **Method Overview**

The vitamin D DBS method measured the concentration of 25(OH)$D_2$ and 25(OH)$D_3$ in a 3 mm punch removed from a DBS. The sample was derivatised and then analysed using LC/MS/MS. 25(OH)$D_3$ and 25(OH)$D_2$ values were combined to give a

total vitamin D level. Initial developmental work, including linearity, paired serum and DBS comparison, limit of detection and quantitation, inter and intra assay variability and sample stability was completed before I started this PhD. Re-validation work has been undertaken during the course of the PhD. This work has not published due to reasons of confidentiality. The Technical Information Sheet that was available to users of the service can be seen in Appendix 4.

### 2.5.2. **Calibrators and Quality Control**

No commercial DBS calibrator or QC material was available so these were made in house by spotting 20 µL of EDTA whole blood onto Whatman 903 filter paper. QC was made by using excess patient EDTA samples that covered a range of 25(OH)$D_2$ and 25(OH)$D_3$. An aliquot of the whole blood was retained, spun down and the plasma retested using the traditional serum method enabling the target value for the DBS material to be assigned from the results.

Three calibrators and two QC samples were made by spiking blank whole blood with the required volume of ethanolic standards of 25(OH)$D_2$ and 25(OH)$D_3$. The plasma for each level was retested and the DBS material assigned values from the results. This procedure aligns the DBS method with the serum method, enabling the established reference range for serum currently used to be applied to DBS.

### 2.5.3. **Method**

3 mm punches were removed from calibrator, QC or patient samples and sonicated in 250 µL IS for 30 minutes. 500 µL of derivatising agent was added to the eluate and mixed for 1 minute before centrifugation at 4500 rpm for 5 minutes. 400 µL of the supernatant was transferred to another vial and incubated uncapped at 68°C for 30 minutes until dry. 80 µL of loading reagent was added and the vials mixed gently for

30 seconds. 35 µL was injected onto the LC/MS/MS and analysed. A gradient elution was used (Table 2.2) with a column temperature of 30°C and the MS/MS parameters shown in Table 2.1. Total run time was 4 minutes. Source temperature was set to 150°C and desolvation temperature 600°C. Capillary and cone voltage was set at 2.5 kV and 40 V respectively. Cone and desolvation gas flow was 150 L/h and 1000 L/h respectively. Software used was Masslynx v4.1.

| Compound | Parent Mass ($m/z^+$) | Daughter Mass ($m/z^+$) | Dwell Time (s) | Cone Voltage | Collision Energy |
|---|---|---|---|---|---|
| **25(OH)$D_3$-PTAD** | 558.3 | 298.3 | 0.05 | 40 | 15 |
| **25(OH)$D_3$ IS-PTAD** | 564.4 | 298.3 | 0.05 | 40 | 15 |
| **25(OH)$D_2$-PTAD** | 570.3 | 298.3 | 0.05 | 40 | 15 |
| **25(OH)$D_2$ IS-PTAD** | 573.3 | 301.3 | 0.05 | 40 | 15 |

**Table 2.1** – Parameters used in the mass spectrometer set up for blood spot vitamin D analysis.

| Time (min) | Flow rate (mL/min) | % Mobile Phase A | % Mobile Phase B | Curve |
|---|---|---|---|---|
| **Initial** | 0.4 | 60 | 40 | Initial |
| **1.9** | 0.4 | 20 | 80 | 6 |
| **2.5** | 0.4 | 10 | 90 | 6 |
| **3.7** | 0.4 | 60 | 40 | 6 |

**Table 2.2** – Parameters used in the gradient elution for blood spot vitamin D analysis.

The method is linear to at least 1,100 nmol/L for 25(OH)$D_3$ and 25(OH)$D_2$ with a limit of quantitation of 7.1 nmol/L and 2.8 nmol/L for 25(OH)$D_3$ and 25(OH)$D_2$ respectively. The inter-assay coefficients are shown in Table 2.3.

| | D3 | | | D2 | | |
|---|---|---|---|---|---|---|
| **In house QC** | **Level 1** | **Level 2** | **Level 3** | **Level 1** | **Level 2** | **Level 3** |
| **Mean (nmol/L)** | 18.0 | 40.3 | 158.0 | 16.2 | 51.3 | 153 |
| **SD (nmol/L)** | 1.2 | 2.8 | 8.5 | 2.0 | 4.3 | 13.6 |
| **CV (%)** | 6.6 | 7.0 | 5.4 | 12.1 | 8.5 | 8.9 |

**Table 2.3** – Inter-assay coefficients for blood spot vitamin D analysis.

## 2.6. **Serum Vitamin D Method**

### 2.6.1. **Method Overview**

Serum samples were analysed by LC/MS/MS after liquid-liquid extraction. The method used commercially available calibrators and QC. Samples were measured from inpatients, outpatients, GP patients and from sites all over the country, however only the results from GP samples were used in this study. We participated in an EQA scheme (DEQAS). 25(OH)$D_3$ and 25(OH)$D_2$ were measured and values combined to give a total vitamin D concentration. Validation data has not been included here as this is a Waters method and is used by multiple laboratories. This method was established for a number of years before this PhD and there was no merit in publishing data relating to the serum vitamin D methodology.

### 2.6.2. **Method**

To 150 µL of calibrator, QC or patient sample was added 20 µL IS, 150 µL zinc sulphate, 300 µL methanol and 700 µL hexane. Tubes were mixed for 1 minute before centrifugation at 4500 rpm for 5 minutes. 550 µL of the supernatant was transferred to a 96 well plate before evaporation with compressed air. 80 µL of loading reagent was added and the plate mixed gently for 30 seconds. 20 µL was injected onto the LC/MS/MS and analysed. A gradient elution was used (Table 2.4) with a column temperature of 35°C and the MS/MS parameters shown in Table 2.5. Total run time was 4.5 minutes and the inlet used a partial loop with needle overfill and a load ahead setup. Source temperature was set to 120°C and desolvation temperature 450°C. Capillary and cone voltage was set at 2.5 kV and 25 V

respectively. Cone and desolvation gas flow was 10 L/h and 900 L/h respectively. Software used was Masslynx v4.1.

| Time (min) | Flow rate (mL/min) | % Mobile Phase A | % Mobile Phase B | Curve |
|---|---|---|---|---|
| **Initial** | 0.45 | 35 | 65 | Initial |
| **3** | 0.45 | 15 | 85 | 6 |
| **3.1** | 0.45 | 2 | 98 | 6 |
| **3.6** | 0.45 | 35 | 65 | 11 |

**Table 2.4** – Parameters used in the gradient elution for serum vitamin D analysis.

The method is linear to at least 1,100 nmol/L for 25(OH)$D_3$ and 25(OH)$D_2$ with a limit of quantitation of 2.4 nmol/L and 1.8 nmol/L for 25(OH)$D_3$ and 25(OH)$D_2$ respectively. The inter-assay coefficients are shown in Table 2.6.

| Compound | Parent Mass (m/z+) | Daughter Mass (m/z+) | Dwell Time (s) | Cone Voltage | Collision Energy |
|---|---|---|---|---|---|
| **25(OH)D3** | 401.3 | 107.2 | 0.06 | 25 | 26 |
| **24(OH)D3 IS** | 407.3 | 159.1 | 0.06 | 25 | 26 |
| **25(OH)D2** | 413.3 | 355.3 | 0.06 | 25 | 9 |
| **25(OH)D2 IS** | 416.3 | 358.3 | 0.06 | 25 | 9 |

**Table 2.5** – Parameters used in the mass spectrometer set up for serum vitamin D analysis.

| | D3 | | | D2 | | |
|---|---|---|---|---|---|---|
| **Name** | **Chromsystems QC 1** | **Clin Chek 1** | **Clin Chek 2** | **Chromsystems QC 1** | **Clin Chek 1** | **Clin Chek 2** |
| **Target (nmol/L)** | 13.7 | 51.2 | 111 | 12.9 | 39.5 | 88.7 |
| **Mean (nmol/L)** | 13.6 | 47.1 | 108.6 | 13 | 41.6 | 98.2 |
| **SD (nmol/L)** | 0.8 | 1.5 | 4.8 | 1 | 1.1 | 4.7 |
| **CV (%)** | 6 | 3.2 | 4.4 | 8 | 2.7 | 4.8 |

**Table 2.6** – Inter-assay coefficients for serum vitamin D analysis.

## 2.7. **Vitamin D Status Categorisation**

The reference ranges reported related to total 25(OH)D concentrations, calculated by adding together the measured 25(OH)$D_2$ and 25(OH)$D_3$ concentrations. The ranges

used were those recommend by Pathology Harmony, the National Osteoporosis Society, (130) and the Institute of Medicine (IOM). (131)

Severely deficient: <15 nmol/L

Deficient: 15-30 nmol/L

Insufficient: 30.1-50 nmol/L

Adequate: 50.1-220 nmol/L

High to toxic: 220.1-500 nmol/L

Toxic: >500 nmol/L

## 2.8. **DBS CRP**

### 2.8.1. **Method Overview**

Dried blood CRP was measured using the automated analysers used to analyse routine serum samples for CRP. CRP is extracted from a punch from a DBS or from the Mitra sponge into the reagent that is used by the Abbott Architect for CRP analysis. The whole process, including analysis time on the machine, takes approximately 15 minutes.

### 2.8.2. **Abbott Architect Setup**

The Abbott CRP assay is a latex immunoassay. An antigen-antibody reaction occurs between CRP in the sample and anti-CRP antibody, which has been adsorbed to latex particles, resulting in agglutination. The agglutination is detected as an absorbance change (572 nm), with the rate of change being proportion to the quantity of CRP in the sample.

The standard Abbott CRP immunoturbidimetric method parameters were adapted for use with blood spots in the following way:

- The volume of R1 reagent volume added during analysis was reduced from 100 µL to 65 µL.
- The volume of sample added was increased from 2 µL to 35 µL. This maintains the total volume of R1 present during the reaction at 100 µL (as per the serum assay).
- All other method parameters were left as per the serum assay.

### 2.8.3. **Calibrators and Quality Control**

Calibrators were made by combining WRBC with the Abbott CRP serum calibrators (see 2.3.3.) in a ratio that would give an approximate haematocrit of 0.4. This was done for each level of standard (5, 10, 20, 40, 160 and 320 mg/L CRP). Once mixed the manufactured whole blood calibrators were then spotted onto filter paper (20 µL) and left to dry overnight to create DBS calibrators. Alternatively, Mitra devices were dipped into the whole blood and left to dry to create Mitra calibrators. These were stored at room temperature. Some manufactured whole blood calibrator was retained in order to centrifuge down and recapture the serum component for analysis by standard serum CRP measurement to ensure the anticipated levels of CRP were present in the DBS calibrators. The assay was then calibrated by extracting each calibrator and using the average of three replicate analyses to obtain a best fit spline curve. A blank value was obtained using a reagent blank measurement.

### 2.8.4. **SWBH Device - Extraction**

One 3 mm punch was removed from the DBS and extracted into 110 µL of R1 reagent in a microtube. The microtube was capped and the sample sonicated for 5 minutes. After sonication the punch was removed from the microtube and the extract

transferred to an Abbott insert cup. The cup was presented to the analyser and analysed using the modified DBS CRP method.

### 2.8.5. **Mitra Device - Extraction**

The sponge tip of the Mitra device was placed into a microtube containing 150 µL R1. The sample was sonicated for 5 minutes. After sonication the Mitra device was removed from the microtube and the extract transferred to an Abbott insert cup. The cup was presented to the analyser and analysed using the modified DBS CRP method.

## 2.9. **Statistical Analysis**

Data collection and manipulation was performed using Microsoft Excel (2010). IBM SPSS Statistics 20 was used to perform the statistical analyses. To test for data normality the Kolmogorov-Smirnov test was used. For non-parametric data the related samples Wilcoxon signed rank test (WSRT) was used to compare the median of differences between two related continuous data sets. This test was also used to compare the distribution of results for non-continuous related data sets. Friedman's ANOVA was used to test the difference between several related continuous data sets. The Mann-Whitney U test was used for non-parametric data to compare two independent sample population distributions and the Kruskal-Wallis test was used to compare more than two independent sample distributions. Bonferroni correction was used when Kruskal-Wallis was significant. When comparing proportions the binomial test or Pearson Chi-square test was used. For parametric data, one way ANOVA was used to compare the means of more than two independent sample populations. Tukey post-hoc analysis was used when ANOVA was significant and the sample

sizes were equal and the population variances similar (using the test of homogeneity of variances – Levene statistic). Games-Howell post-hoc analysis was used when ANOVA was significant, the sample sizes were equal and the population variances were unequal. Two independent samples t-test was used to compare the means of two independent sample populations. All tests were performed at a 5% level of significance.

Box plots drawn show the median as the centre line, the upper and low limits of the box represent the upper and lower quartiles and the whiskers represent the minimum and maximum data values. Outliers are represented as a circle and extreme outliers by a circle with an asterix.

# CHAPTER 3 - BLOOD SPOT QUALITY

## 3.1. Overview

The clinical biochemistry department at SWBH was interested in introducing DBS into the repertoire of sample types that were analysed in the laboratory. Although there are many different types of collection devices (and filter paper) available commercially for DBS collection, none of them appeared to be designed with patients taking samples in their home environment in mind. For example, the “Guthrie card”, used to collect DBS for NBS programmes, requires the sample to be left exposed to the air for at least three hours to enable it to dry before being stored and/or sent to the laboratory. Therefore it was necessary to develop an in-house, fit-for-purpose collection device.

Alongside the design of the DBS collection device, thought must be given to the way patients collect samples as this can effect results, for example if they layer spots (place two drops one on top of another), smear the blood or press down on the paper. How the instructions are written, the quality of the collection device and filter paper used, and lancet type can all have a bearing on the quality of the sample.

When a disk is punched from a DBS, it can be considered a volumetric measurement, similar to that of a liquid measuring device. (19) The disk can be of any size that is suitable for the assay (most common diameters are 3 – 6 mm) and sometimes multiple punches are used per test. The sample is eluted from the disk and is effectively reconstituted as haemolysed liquid whole blood. (15) Using a punch ensures that for each sample analysed the same volume of blood is used in the assay regardless of how much blood has been absorbed onto the filter paper. However, the exact volume of blood contained within that disk will be dependent on a

number of variables, such as the volume of blood applied to form a blood spot, the haematocrit of the patient/control/calibrator blood and chromatographic effects of the paper. (24)

Haematocrit (or packed cell volume) is the proportion of blood volume that is occupied by red blood cells (31) and it can have an effect on the volume of blood contained within a given sized punch. Haematocrit is directly proportional to blood viscosity, which affects the flux and diffusion properties of the blood once it has been applied to the filter paper. (19)

The chromatographic or distribution effect caused by the interaction of the blood and the analyte with the filter paper can lead to variation in the results obtained as it can affect the level of analyte found across it. (31) This is because when a DBS is collected, a piling of blood cells in the centre of the spot occurs with serum migrating to the periphery of the spot. As a result of this chromatographic effect, punches taken in different locations in the DBS may contain varying proportions of serum and analyte. The volume of DBS can also contribute to this phenomenon, even if haematocrit and punch size remain the same, and the effect may be more pronounced for analytes that reside mainly in the serum. (31, 113)

The use of whole blood or haemolysed blood to make calibrators and QC is another aspect that needs to be considered. Haemolysed blood will have a decreased viscosity and therefore will spread further through the filter paper when used to create DBS. (89) Alcoholic standards may be used for spiking whole blood when making calibrators and QC and this may also affect the way the blood interacts with the filter paper. Therefore these characteristics of preparing DBS should also be investigated before using DBS in routine clinical practice.

The aim of the work presented in this chapter was to determine if DBS received from the public were of high enough quality to enable DBS to be used as a viable sample matrix. To the best of our knowledge no such assessment of DBS collected by untrained members of the public has been performed before. In addition, the effect of the type of lancet used to collect DBS and the presence of printed circles on the filter paper on the size and quality of spots obtained was investigated. Finally, an investigation into the effect of blood spot characteristics such as size of spot, location of punch, presence of alcohol, haemolysis and haematocrit on results was undertaken.

## 3.2. **Method**

### 3.2.1. **Pro-Diagnostics Samples**


**Figure 3.1 –** PD collection kit showing instructions and DBS collection device.

After the establishment of a routine DBS method for 25(OH)D, the laboratory received DBS vitamin D test kits from a company called Pro-Diagnostics (PD). PD

began to supply direct to the public vitamin D testing kits around the same time as SWBH, but they could not measure 25(OH)D and therefore had to send the DBS to SWBH for analysis. These samples were also taken by members of the public and the kit used blue BD Microtainer® Contact-Activated 1.5 mm x 2.0 mm lancets (BD, New Jersey, USA) and Ahlstrom 226 paper (Figure 3.1). The DBS collection device in this kit differed from the SWBH kit in that it had no wrap around cardboard component to protect it whilst the spots were drying but it did have five printed circles on the filter paper. During the course of this PhD the company went into administration and therefore only a limited number of samples were received from PD.

### 3.2.2. **SWBH Samples**

The SWBH kit was as described in section 2.4.1. and 2.4.3. However during the first two years of the service, different lancets were included in the kits sent out to patients: Sarstedt Safety lancets (Nümbrecht, Germany) or blue BD Microtainer® Contact-Activated lancets 1.5 mm x 2.0 mm. With the Sarstedt Safety lancets, three types of lancets were distributed: Green (Normal, 1.8 mm penetration depth, 21 gauge), Yellow (Extra, 1.8 mm penetration depth, 18 gauge) and Purple (Super Blade, 1.5 mm, penetration depth 1.6 mm).

### 3.2.3. **DBS Quality Assessment**

DBS collected using the kits from SWBH and PD were assessed. The kits were pre-barcoded which allowed individual kits to be tracked and lancet type and source identified. Some patients sent in multiple samples so only the first attempt was analysed to avoid potential bias from people improving their technique with practice.

In total, 200 each of the green, yellow and purple Sarstedt lancets samples, 200 blue BD lancet SWBH samples and 200 PD samples were assessed. Different lancets were sent out at different time periods, therefore the first 200 samples received back for each lancet type were assessed. The sampling size of 200 was chosen as this represented a minimum of 13.5% of the population of samples for each lancet type. For each sample the patient's age and gender was recorded. As PD kits request five spots and SWBH request four, the 1$^{st}$ spot on the left hand side of the PD collection device was ignored to allow comparison. When more than four spots were collected on SWBH devices, the 1$^{st}$ spot on the left was ignored.

For every sample, the number of spots taken and how many of those spots provided a 3 mm or a 6 mm punch was recorded. The estimated number of 3 mm and 6 mm punches that could be collected in total from all the spots, regardless of quality, was noted to assess the "volume" of blood collected. If four ideal DBS were collected (the number SWBH request) then it would be possible to obtain at least 12 x 3 mm (three from each DBS) and 4 x 6 mm (one from each DBS) punches. Therefore, 12 and 4 punches were taken as the upper limits for 3 mm and 6 mm punches respectively.

The quality of the DBS was also assessed as some spots would not be fit for analysis even if a punch could be obtained. The following spot characteristics were recorded as being present or not present in a sample: incomplete saturation, double-spotting, smearing, overlapping with neighbouring spot, clotting or finger pressing onto the paper. Good technique was marked as present when at least one DBS in a sample displayed none of the above characteristics. The spots were graded from 0-4 for 3 mm and 6 mm punches in relation to the number of quality punches (complete saturation with no evidence of double spotting, smearing, overlapping or clotting) that

could be obtained: 0=insufficient for analysis, 1=1 punch, 2=2 punches, 3=3 punches and 4=4 punches available. An ideal sample would supply four quality spots that would each provide one quality punch but multiple quality punches could be supplied by one large quality spot.

The results of the quality DBS analysis for the different SWBH lancet types were compared to see if one lancet was significantly better than the others at providing DBS. In addition the blue lancet SWBH and PD results were compared to see if the presence of a target printed circle makes a difference to the quality of spots obtained.

### 3.2.4. **DBS Characteristics**

A range of DBS and Mitra samples were made that covered a range of DBS characteristics that could then be investigated using the DBS CRP or DBS vitamin D methods. These were within laboratory experiments.

#### 3.2.4.1. *Size of Spot*

SWBH devices were used for this. Ten spots were made for each of the following volumes of blood: 10 µL, 20 µL, 50 µL, 75 µL and 100 µL. 10µL was the minimum spot size investigated as this is the smallest size DBS that a 3mm punch can be consistently removed from. A centre punch was taken from each of the spots and analysed using the DBS vitamin D and CRP methods. Results were compared to those obtained for the 20 µL punches as that is the size used to make the calibrators for the assay. This was done for two different concentrations of CRP and 25(OH)D.

#### 3.2.4.2. *Location of Punch*

SWBH devices were used for this. Punches were taken from the ‘north’, ‘south’, ‘east’ and ‘west’ edges of the 75 µL spots made for section 3.2.4.1. Five 75 µL spots (20

punches) were used for the CRP assay and seven 75 µL spots (28 punches) were used for the vitamin D assay. Punches were extracted and analysed for 25(OH)D and CRP and results compared to centre punches from 20 µL spots made using the same blood used to make the 75 µL spots (see Section 3.2.4.1, n = 10). This was done for two different concentrations of CRP and 25(OH)D.

#### 3.2.4.3. *Haemolysed vs. Intact Blood*

An aliquot of blood was haemolysed by freezing at -80°C. For the SWBH device, ten 20 µL spots and ten 75 µL spots were made with the haemolysed blood. Centre punches were taken from all DBS, extracted and analysed for CRP and 25(OH)D. Results were compared with spots made from the same batch of intact blood. This was done for two different concentrations of CRP and 25(OH)D. This was carried out using the Mitra device as well, but samples were only analysed for CRP (n=8).

#### 3.2.4.4. *Presence of Ethanol*

Calibrators and QC can be made by spiking with material in alcohol, therefore it is useful to know if this can affect the DBS characteristics. An aliquot of the blood used in section 3.2.4.1 and 3.2.4.2 had 0.5% w/v ethanol added to it before DBS were spotted out as described in those sections. The experiments were then repeated for the DBS containing ethanol and results compared to those without ethanol. This was done for two different concentrations of CRP and 25(OH)D using the SWBH device.

#### 3.2.4.5. *Haematocrit*

Blood with a haematocrit of approximately 0.2, 0.3, 0.4, 0.5 and 0.6 was prepared using the method described in section 2.3.3. The actual haematocrit was measured using the Sysmex XP-300 automated haematology analyser before use. Ten 20 µL

blood spots and ten Mitra devices were made for each level of haematocrit. Samples were analysed for 25(OH)D and CRP and results compared to the 0.4 haematocrit samples. This was done for two different concentrations of CRP and 25(OH)D for the SWBH devices and for two different concentrations of CRP for the Mitra devices.

## 3.3. **Results**

### 3.3.1. **Blood Spot Quality Assessment**

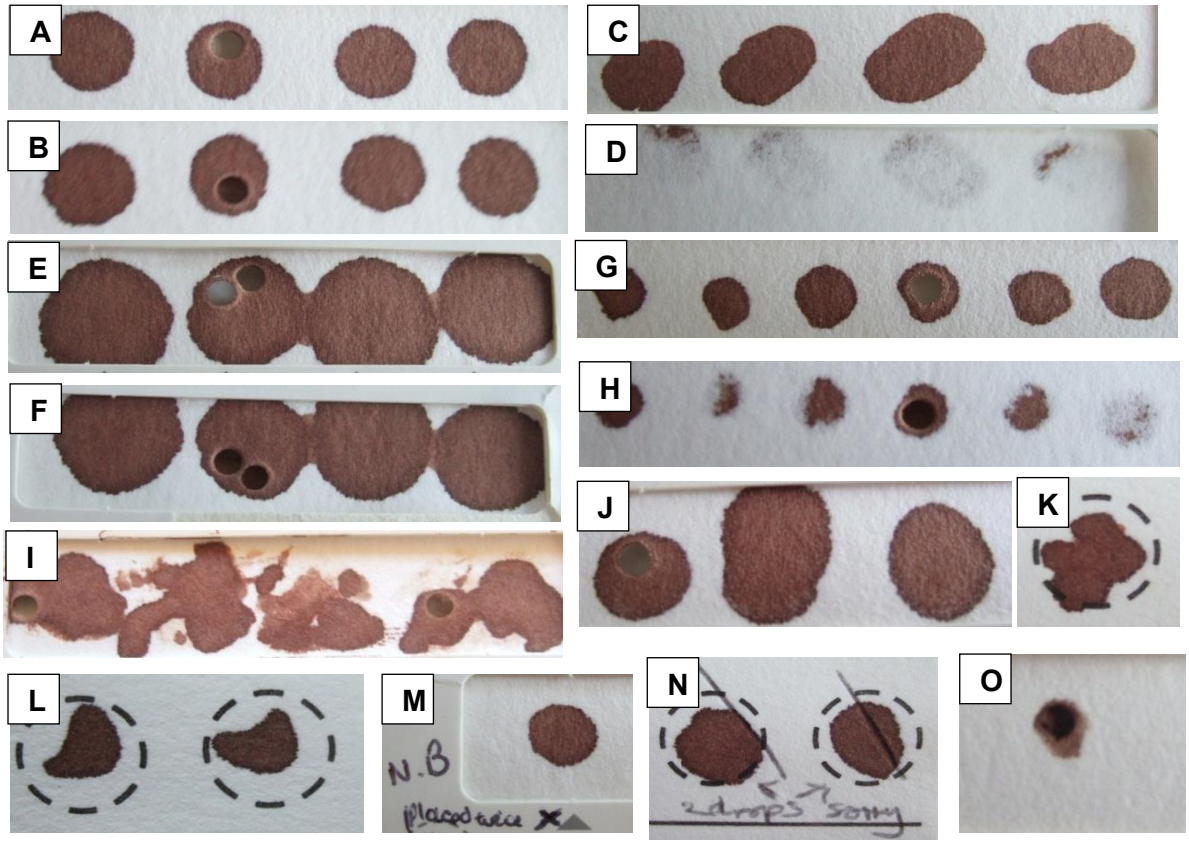



**Figure 3.2 –** Photos showing the different types of DBS that are sent to the laboratory. All punches taken from samples are 3 mm in size. A: The front of a good quality DBS. B: The back of A showing that the blood has soaked through the filter paper. C: A sample collected by pressing the finger onto the filter paper. D: The reverse of C showing how the blood has failed to soak through. E: Very large spots that have started to spread and overlap into the next spots. F: The reverse of E. G: Collection of more than 4 spots. H: The reverse of G, showing insufficient soaking, perhaps as a result of collecting more than 4 spots and not allowing blood to pool to form bigger spots. I: Very messy, smeared sample. J: Double spotting. This is not always so easy to spot. K: A more obvious sample of double spotting or layering. L: Finger pressed onto the paper whilst collecting a DBS. M and N: Double spotting only known as the patient annotated the sample, would be unable to tell otherwise. O: A clotted sample.

Many different types of spot were seen during the assessment of the 1000 samples and a selection can be seen in Figure 3.2.

3.3.1.1. *Demographics of Sample Populations*

| Lancet Type | PD | Yellow | Green | Purple | Blue |
|---|---|---|---|---|---|
| **Female (%)** | 49.2 | 62.3 | 65.5 | 59.9 | 58 |
| **Male (%)** | 50.8 | 37.7 | 34.5 | 40.1 | 42 |

**Table 3.1 –** Distribution of gender for the different lancet types assessed.

59.3% of the DBS assessed were from women which was not significantly different from the total population of all samples received of 62.9% (difference = 3.6%, 95% confidence interval = -0.25% to 6.2%). Table 3.1 shows the gender distribution for each of the lancet populations analysed. There is a significant difference (p=0.015) between the distribution of genders across the different lancet types due to the PD population (when PD are removed from analysis p=0.588). This is because the PD population is significantly different from the SWBH population in terms of gender population (difference = 13%, 95% confidence interval = 7% to 19%), as the PD population has an exactly equal split between the genders using the service and this is reflected in the PD sample population.

| Lancet Type | Normally Distributed? | Mean Age (years) | Median Age (years) | Minimum Age (years) | Maximum Age (years) | Interquartile Range (years) |
|---|---|---|---|---|---|---|
| **PD** | No | 44.9 | 43 | 1 | 83 | 29 |
| **Yellow** | Yes | 45.6 | 46 | 0 | 85 | 25 |
| **Green** | Yes | 45.3 | 45 | 0 | 91 | 23 |
| **Purple** | Yes | 45.3 | 47 | 2 | 86 | 24 |
| **Blue** | Yes | 47.7 | 49 | 1 | 81 | 24 |

**Table 3.2 –** Descriptive statistics for the distribution of age for the different lancet types assessed.

The distribution of age was not normally distributed for the total population assessed (p=0.002), however when tested for the individual lancet types only the PD population did not have a normal age distribution (p=0.02) (Table 3.2). The

distribution of age was the same across the different types of lancets assessed (p=0.411).

3.3.1.2. *Overall Quality of DBS*

| Number of spots in the sample | Attempted spots (%) | Useable spots (%) | Spots providing 1 x 3 mm punch (%) | Spots providing 1 x 6 mm punch (%) |
|---|---|---|---|---|
| **0** | 0 | 2.7 | 2.7 | 18.8 |
| **1** | 0 | 3.4 | 3.2 | 10.3 |
| **2** | 0.9 | 6.0 | 6.0 | 14.1 |
| **3** | 1.5 | 14.7 | 14.5 | 16.2 |
| **4** | 97.6 | 73.2 | 73.6 | 40.6 |

**Table 3.3 –** Table showing how many samples had 0-4 attempted spots, useable spots, 3 mm punches and 6 mm punches.

No samples assessed contained less than two attempted spots while the majority of samples contained four (97.6%). Despite all samples containing spots, 2.7% of samples had no useable spots. This was not significantly different to the total DBS population insufficient rate of 3.0% (difference = 0.3%, 95% CI for difference = -1% to 1.2%). 73.6% of samples contained four spots that could each provide a 3 mm punch, regardless of quality. 18.8% of samples had no spots that could provide a 6 mm punch and 40.6% had four spots that could each provide a 6 mm punch (Table 3.3).

The total number of 3 mm punches that could potentially be collected from a sample ranged from 0 to 12, with 2.7% providing no punches and the largest group of 38.6% of samples providing 12 punches. The total number of 6 mm punches that could be collected ranged from 0 to 4 with 18.8% of samples not providing any punches and the largest group of 42.7% providing four punches. When the quality of the spot provided was assessed, the number of samples that could not provide any 3 mm or 6 mm punches only increased by 0.3% to 3.0% and by 0.9% to 19.7%, respectively.

The largest proportion of samples could provide four quality punches for 3 mm (79.7%) and 6 mm (40.1%) punch sizes (Figure 3.3).

Good technique was present for at least one spot per sample in 94.6% of samples. The most common problem seen with the spots was the finger being pressed onto the filter paper, present in 51.5% of samples (Figure 3.4).

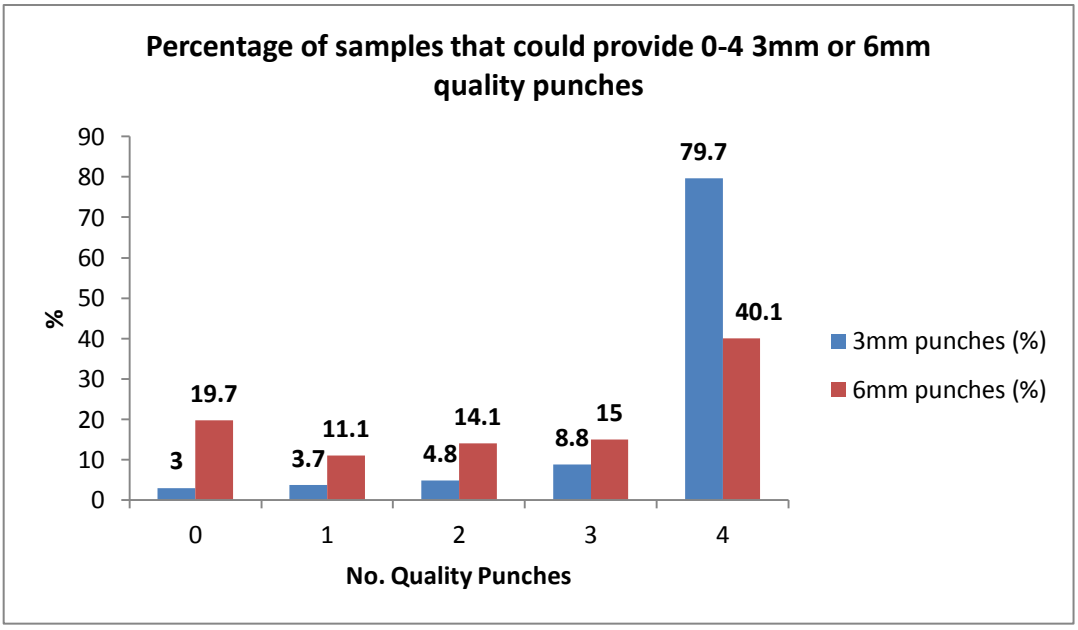



**Figure 3.3 –** Distribution of samples that can provide 3 mm or 6 mm quality punches.

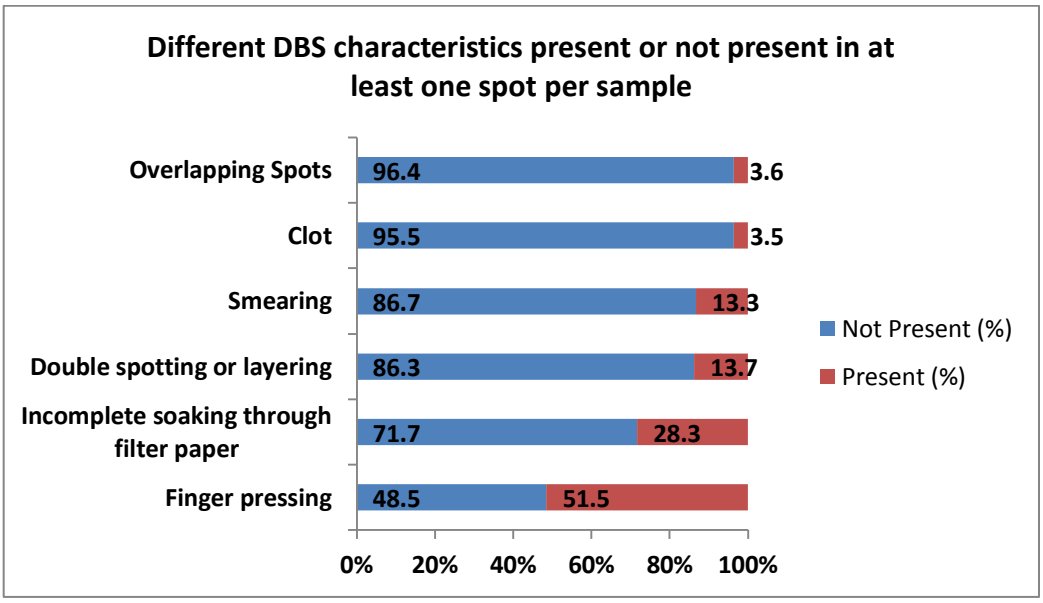



**Figure 3.4 –** Proportion of different characteristics that were or were not present in at least one spot per sample.

3.3.1.3. *Lancet Type Comparison*

Significant differences were looked for between the different lancet types used by SWBH (Table 3.4). As can be seen no significant difference was found for any of the characteristics examined at the 95% confidence level. When the makes of the lancets (Sarstedt for the yellow, green and purple lancets, and BD for the blue lancets) were compared the only significant difference found for the characteristics examined was for the presence of good technique, where 97.5% of BD lancet samples displayed good technique for at least one spot and only 93.5% of Sarstedt samples displayed good technique.

| | p-value | |
|---|---|---|
| **Characteristic** | **All lancet types compared** | **Manufacturers compared** |
| **No. of attempted spots** | 0.078[a] | 0.405[a] |
| **No. of useable spots** | 0.926 | 0.264 |
| **No. of spots per sample providing 3 mm punches** | 0.948 | 0.309 |
| **No. of spots per sample providing 6 mm punches** | 0.168 | 0.189 |
| **Estimated no. 3 mm punches per sample** | 0.357 | 0.055 |
| **Estimated no. 6 mm punches per sample** | 0.062 | 0.082 |
| **No. of quality 3 mm punches per sample** | 0.136 | 0.201 |
| **No. of quality 6 mm punches per sample** | 0.237 | 0.380 |
| **Good technique present** | 0.186 | 0.032 |
| **Double spotting present** | 0.731 | 0.390 |
| **Smearing present** | 0.712 | 0.528 |
| **Insufficient soaking through filter paper present** | 0.533 | 0.231 |
| **Finger pressed onto filter paper during collection present** | 0.984 | 0.743 |
| **Overlapping spots** | 0.677 | 0.369 |
| **Clot present** | 0.828 | 0.657 |

**Table 3.4 –** Statistical difference for characteristics of DBS collected using different SWBH lancets (yellow, green, purple and blue) and comparing manufacturers. [a]Several cells had an expected count less than 5 so statistic may not be valid.

Two characteristics relating to volume of blood (estimated number of 3 mm punches or 6 mm punches that could be obtained from a sample) were close to being

significantly different. When the number of estimated 3 mm punches obtained for the different manufacturers was looked at in more detail (Table 3.5) the difference between the two populations for estimated number of 12 x 3 mm punches (13.7%) appeared to be significant (95% confidence interval = 5.9% to 21.5%) with BD providing a higher "volume" of blood.

| Estimated number of 3 mm punches | Sarstedt (%) | BD (%) |
|---|---|---|
| **0** | 3 | 2.5 |
| **12** | 31.8 | 45.5 |

**Table 3.5 –** Proportion of samples that gave either no 3 mm punches or 12 x 3 mm punches for each manufacturer of lancet.

3.3.1.4. *Printed Circles on Filter Paper Comparison*

| Characteristic | p-value |
|---|---|
| **No. of attempted spots** | 0.366 |
| **No. of useable spots** | 0.494 |
| **No. of spots per sample providing 3 mm punches** | 0.343 |
| **No. of spots per sample providing 6 mm punches** | 0.007 |
| **Estimated no. 3 mm punches per sample** | 0.286 |
| **Estimated no. 6 mm punches per sample** | 0.007 |
| **No. of quality 3 mm punches per sample** | 0.073 |
| **No. of quality 6 mm punches per sample** | 0.001 |
| **Good technique present** | 0.188 |
| **Double spotting present** | 0.201 |
| **Smearing present** | 0.032 |
| **Insufficient soaking through filter paper present** | 0.099 |
| **Finger pressed onto filter paper during collection present** | 0.005 |
| **Overlapping spots** | 0.003 |
| **Clot present** | 0.778 |

**Table 3.6 –** p-values for characteristics of DBS collected using the PD kit and the SWBH BD blue lancet kit.

The same lancet was used by PD and SWBH when the BD blue lancet was used. The PD and SWBH collection kits that were sent out to people were different but the most substantial difference between the two packs was that the PD collection device filter paper had printed circles on it and the SWBH collection device filter paper did not, it just had a size guide on the cardboard next to it. The DBS characteristics that

were examined were compared for these two populations to see if the printing of circles makes a difference as this has not been established in the literature.

There were several characteristics that showed a significant difference between the two collection kits and every variable that involved 6 mm punches was significantly different (Table 3.6). The PD kit consistently provided a higher proportion of samples that could give more 6 mm punches than the SWBH kit (Table 3.7).

| | **No. of spots per sample providing 6 mm punches** | | **Estimated no. 6 mm punches per sample** | | **No. of quality 6 mm punches per sample** | |
|---|---|---|---|---|---|---|
| **No. of spots/punches** | **PD (%)** | **SWBH (%)** | **PD (%)** | **SWBH (%)** | **PD (%)** | **SWBH (%)** |
| **0** | 9.5 | 20.5 | 8.5 | 20.5 | 10 | 21.5 |
| **1** | 6 | 7.5 | 6 | 6.5 | 7 | 9 |
| **2** | 12.5 | 14 | 12 | 12.5 | 12 | 13 |
| **3** | 14 | 16 | 12 | 16 | 12.5 | 17.5 |
| **4** | 58 | 42 | 60.5 | 44.5 | 58.5 | 39 |

**Table 3.7 –** Proportion of samples for each kit type that give between 0 and 4 spots or punches for 6 mm characteristics.

Table 3.8 gives the details of the proportions of samples for each kit type for the other characteristics that were shown to be significantly different. The PD kits provided fewer smeared samples, fewer cases of finger pressing and fewer cases of overlapping spots.

| | **Present (%)** | |
|---|---|---|
| **Spot Characteristic** | **PD** | **SWBH** |
| **Smearing** | 7 | 13.5 |
| **Finger pressed onto the filter paper** | 39.5 | 53.5 |
| **Overlapping spots** | 0.5 | 5.5 |

**Table 3.8 –** Proportion of different characteristics that were present in at least one spot per sample for each kit type.

### 3.3.2. **DBS Characteristics**

#### 3.3.2.1. *Size of Spot*

See section 3.2.4.1 (page 64) for the method relating to this section.

3.3.2.1.1. Vitamin D

Examples of the different sizes of DBS that are created when different volumes of blood are used can be seen in Figure 3.5. Throughout this thesis, volume of spots refers to the volume of blood used to create the DBS. The results obtained from the centre punches for the different volume DBS containing the lower concentration of 25(OH)D can be seen in Table 3.9 and Figure 3.6.

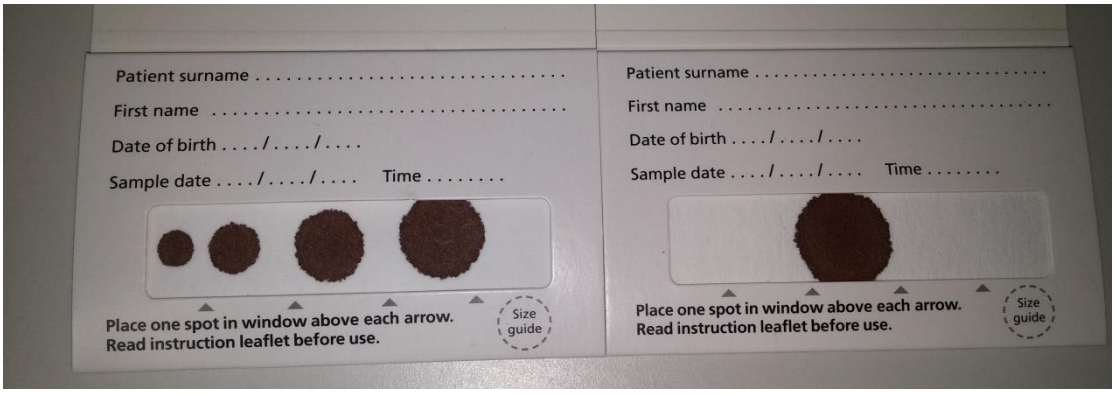



**Figure 3.5 –** DBS created using different volumes of blood. From left to right: 10 µL, 20 µL, 50 µL, 75 µL and 100 µL.

| | Volume of spots (µL) | | | | |
|---|---|---|---|---|---|
| **ID** | **10** | **20** | **50** | **75** | **100** |
| **1** | 39.0 | 41.6 | 58.7 | 47.7 | 70.3 |
| **2** | 37.5 | 38.3 | 55.9 | 44.4 | 58.5 |
| **3** | 36.0 | 45.6 | 54.6 | 70.4 | 46.8 |
| **4** | 43.1 | 40.0 | 52.6 | 54.4 | 47.1 |
| **5** | 36.5 | 45.5 | 48.8 | 62.6 | 49.2 |
| **6** | 45.5 | 39.9 | 49.8 | 58.1 | 57.6 |
| **7** | 43.6 | 48.5 | 48.2 | 49.5 | 54.2 |
| **8** | 42.6 | 50.2 | 48.2 | 48.2 | 58.0 |
| **9** | 46.7 | 40.4 | 41.2 | 48.2 | 59.3 |
| **10** | 40.8 | 48.9 | 39.2 | 63.1 | 69.4 |
| **Mean (nmol/L)** | **41.1** | **43.9** | **49.7** | **54.7** | **57.0** |
| **S.D. (nmol/L)** | **3.77** | **4.36** | **6.14** | **8.55** | **8.23** |
| **CV(%)** | **9.2** | **9.9** | **12.4** | **15.6** | **14.4** |

**Table 3.9 –** Results obtained from the centre punches for the different volume DBS made using the pool of blood containing the lower concentration of 25(OH)D (serum = 39 nmol/L).

The results showed that the mean concentration of 25(OH)D increased with increasing volume of blood spot. The data for each volume of DBS were normally distributed (all p>0.05). The means for the different DBS volumes were significantly different (p<0.001). Post-hoc analysis (variances equal p=0.091) showed that the 20 µL DBS were significantly different from the 75 µL (p=0.005) and the 100 µL DBS (p<0.001), but there was no significant difference seen between the 20 µL DBS and 10 µL (p=0.876) and 50 µL DBS (p=0.281).

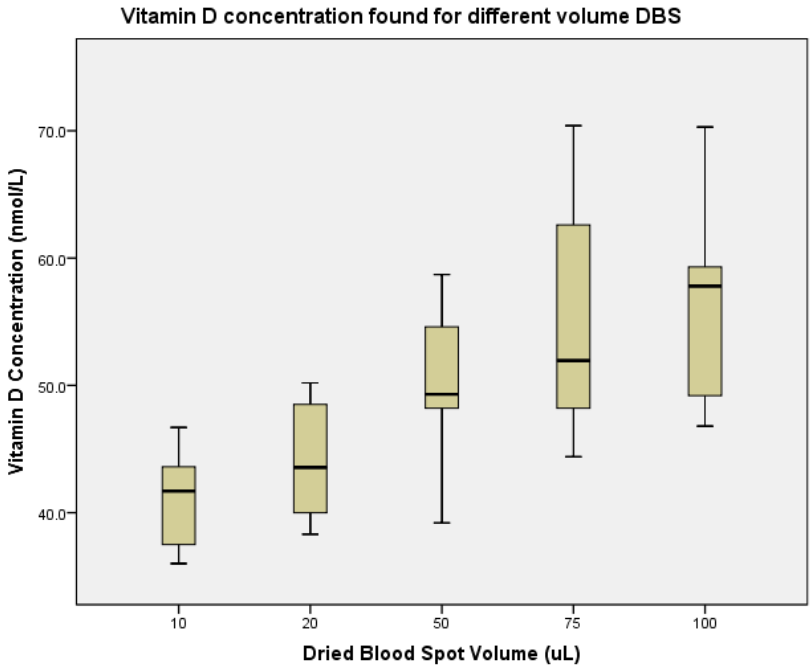



**Figure 3.6 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of 25(OH)D (n=10 for each volume of DBS).

The results of the centre punches for the different volume DBS containing the higher concentration of 25(OH)D can be seen in Table 3.10 and Figure 3.7. The same pattern was seen with the higher concentration of 25(OH)D – increasing DBS volume resulted in an increased mean concentration. The data were all normally distributed (all p>0.05) and the means for the different volume DBS were significantly different

(p<0.001). Post-hoc analysis (variances unequal p=0.013) showed that the 20 µL DBS were significantly different from the 75 µL (p=0.003) and the 100 µL DBS (p=0.002), but there was no significant difference seen between the 20 µL DBS and 10 µL (p=0.998) and 50 µL DBS (p=0.226).

| | Volume of Spots (µL) | | | | |
|---|---|---|---|---|---|
| **ID** | **10** | **20** | **50** | **75** | **100** |
| **1** | 105.5 | 97.7 | 121.6 | 158.2 | 147.7 |
| **2** | 112.3 | 120.8 | 130.4 | 124.4 | 183.9 |
| **3** | 101.3 | 102.9 | 109.0 | 131.4 | 142.0 |
| **4** | 99.2 | 91.0 | 137.4 | 126.3 | 124.6 |
| **5** | 106.7 | 109.5 | 102.0 | 167.4 | 158.0 |
| **6** | 98.3 | 109.3 | 103.2 | 148.4 | 115.9 |
| **7** | 103.8 | 109.3 | 127.5 | 115.0 | 121.3 |
| **8** | 104.3 | 129.1 | 117.1 | 125.7 | 154.5 |
| **9** | 111.6 | 100.8 | 126.0 | 148.3 | 136.9 |
| **10** | 112.3 | 96.8 | 109.3 | 124.3 | 136.5 |
| **Mean (nmol/L)** | **105.5** | **106.7** | **118.4** | **136.9** | **142.1** |
| **S.D. (nmol/L)** | **5.21** | **11.53** | **12.15** | **17.35** | **20.19** |
| **CV(%)** | **4.9** | **10.8** | **10.3** | **12.7** | **14.2** |

**Table 3.10 –** Results obtained from the centre punches for the different volume DBS made using the pool of blood containing the higher concentration of 25(OH)D (serum = 100 nmol/L).

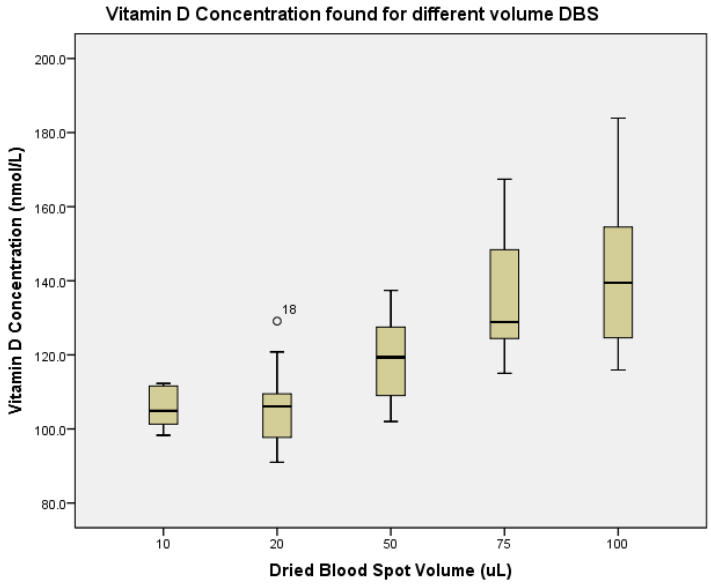



**Figure 3.7 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of 25(OH)D (n=10 for each volume of DBS).

3.3.2.1.2. CRP

The results obtained from the centre punches for the different volume DBS containing the lower concentration of CRP can be seen in Table 3.11 and Figure 3.8.

| | Volume of Spots (µL) | | | | |
|---|---|---|---|---|---|
| **ID** | **10** | **20** | **50** | **75** | **100** |
| **1** | 12.7 | 14.3 | 13.1 | 11.5 | 16.8 |
| **2** | 9.5 | 11.1 | 14.3 | 14.4 | 17.1 |
| **3** | 13.4 | 14.4 | 14.2 | 12.5 | 18.6 |
| **4** | 8.6 | 12.9 | 14.0 | 17.3 | 19.3 |
| **5** | 11.5 | 11.6 | 17.0 | 17.8 | 14.7 |
| **6** | 9.3 | 14 | 14.2 | 19.3 | 13.3 |
| **7** | 13.5 | 12.1 | 17.6 | 22.0 | 17.2 |
| **8** | 6.9 | 8.2 | 13.9 | 18.0 | 19.9 |
| **9** | 11.1 | 14.6 | 17.3 | 14.1 | 20.0 |
| **10** | 15.0 | 12.1 | 10.5 | 14.1 | 16.5 |
| **Mean (mg/L)** | **11.2** | **12.5** | **14.6** | **16.1** | **17.3** |
| **S.D. (mg/L)** | **2.6** | **2.0** | **2.2** | **3.3** | **2.2** |
| **CV(%)** | **22.9** | **15.8** | **14.9** | **20.5** | **12.7** |

**Table 3.11 –** Results obtained from the centre punches for the different volume DBS made using the pool of blood containing the lower concentration of CRP (serum = 14 mg/L). Variable CVs likely due to the presence of mushy spots which may have led to incomplete sampling by the analyser and potentially falsely low results.

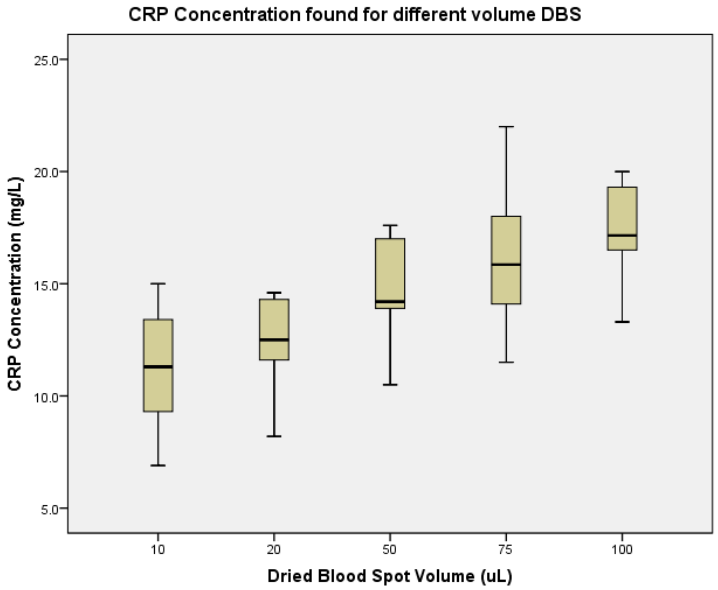



**Figure 3.8 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of CRP (n=10 for each volume of DBS).

The results showed that the mean concentration of CRP increased with increasing volume of blood spot. The data for each volume of DBS were normally distributed (all $p>0.05$). The means for the different DBS volumes were significantly different ($p<0.001$). Post-hoc analysis (variances equal $p=0.215$) showed that the 20 µL DBS were significantly different from the 75 µL ($p=0.020$) and the 100 µL DBS ($p=0.001$), but there was no significant difference seen between the 20 µL DBS and 10 µL ($p=0.727$) and 50 µL DBS ($p=0.347$).

The same pattern was seen with the higher concentration of CRP – increasing DBS volume resulted in an increased mean concentration (Table 3.12 and Figure 3.9). The data were all normally distributed (all $p>0.05$) and the means for the different volume DBS were significantly different ($p<0.001$). Post-hoc analysis (variances unequal $p=0.006$) showed that the 20 µL DBS were significantly different from the 50 µL ($p=0.004$), 75 µL ($p=0.003$) and the 100 µL DBS ($p<0.001$), but there was no significant difference seen between the 20 µL DBS and 10 µL DBS ($p=0.793$).

| | **Volume of spots (µL)** | | | | |
|---|---|---|---|---|---|
| **ID** | **10** | **20** | **50** | **75** | **100** |
| **1** | 91.3 | 85.3 | 127.3 | 93.7 | 111.6 |
| **2** | 90.0 | 83.7 | 107.2 | 96.4 | 125.9 |
| **3** | 77.2 | 88.3 | 115.3 | 101.3 | 104.6 |
| **4** | 72.4 | 84.1 | 96.6 | 113.6 | 133.3 |
| **5** | 90.1 | 88.4 | 119.9 | 113.1 | 113.4 |
| **6** | 79.1 | 82.3 | 108.0 | 123.6 | 133.1 |
| **7** | 85.8 | 94.6 | 101.2 | M | 138.1 |
| **8** | 92.5 | 88.3 | 91.6 | 108.0 | 116.6 |
| **9** | 75.6 | 80.3 | 99.6 | 132.9 | 125.3 |
| **10** | 76.8 | 86.0 | 89.4 | 130.0 | 109.4 |
| **Mean (mg/L)** | **83.1** | **86.1** | **105.6** | **112.5** | **121.1** |
| **S.D. (mg/L)** | **7.6** | **4.0** | **12.3** | **14.2** | **11.6** |
| **CV(%)** | **9.2** | **4.7** | **11.7** | **12.6** | **9.5** |

**Table 3.12 –** Results obtained from the centre punches for the different volume DBS made using the pool of blood containing the higher concentration of CRP (serum = 92 mg/L). M = mushy and unable to obtain result.

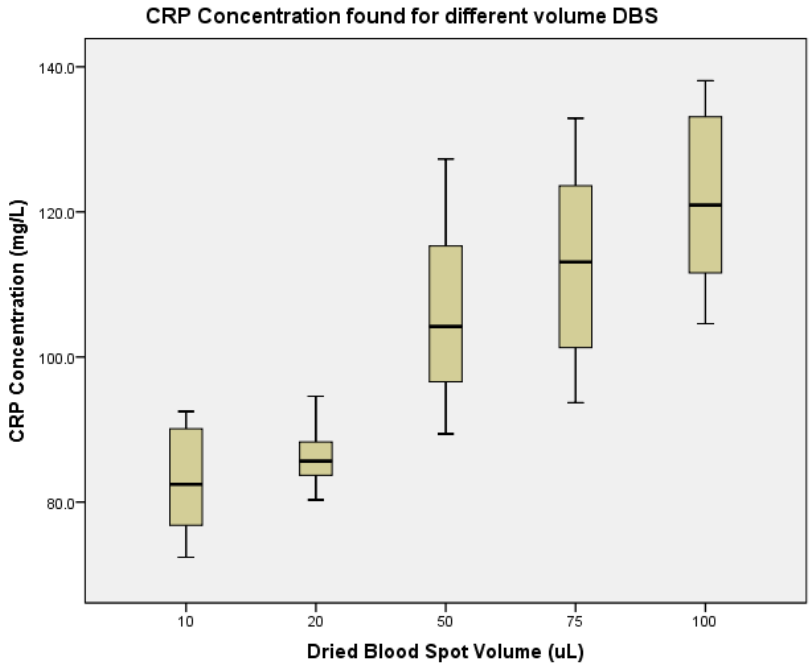



**Figure 3.9 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of CRP (n=10 for each volume of DBS).

3.3.2.2. *Location of Punch*

See section 3.2.4.2 (page 64) for the method relating to this section.

3.3.2.2.1. Vitamin D

| | Edge Punches (75 µL) | Centre Punches (20 µL) | Centre Punches (75 µL) |
|---|---|---|---|
| **Mean (nmol/L)** | 68.8 | 43.9 | 54.7 |
| **S.D. (nmol/L)** | 7.2 | 4.4 | 8.5 |
| **CV(%)** | 10.4 | 9.9 | 15.6 |

**Table 3.13 –** 25(OH)D concentration for centre and edge punches taken from DBS made using the pool of blood containing the lower concentration of 25(OH)D (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 39 nmol/L.

A summary of the 25(OH)D results for the 75 µL DBS outer punches for the lower concentration of 25(OH)D can be seen in Table 3.13 and Figure 3.10. The data were normally distributed for the edge punches (p=0.2) and the mean 25(OH)D concentration was significantly higher compared to the centre punches (variances equal p=0.334, p<0.001).

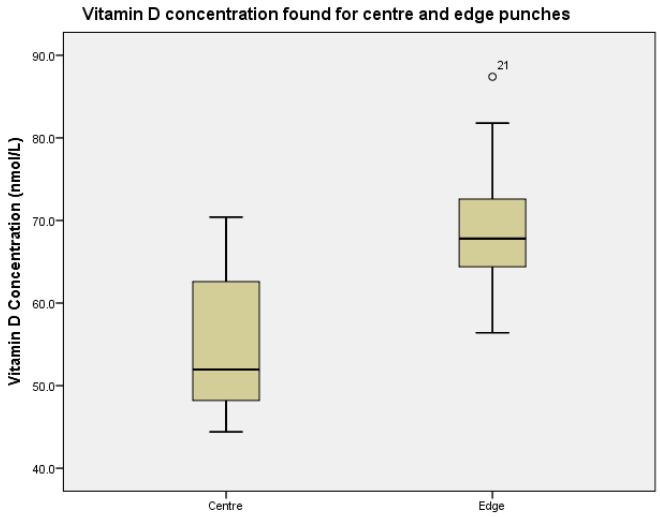



**Figure 3.10 –** Box and whisker plot showing the 25(OH)D concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the lower concentration of 25(OH)D.

| | Edge Punches (75 µL) | Centre Punches (20 µL) | Centre Punches (75 µL) |
|---|---|---|---|
| **Mean (nmol/L)** | 204.7 | 106.7 | 136.9 |
| **S.D. (nmol/L)** | 26.5 | 11.5 | 17.3 |
| **CV(%)** | 12.9 | 10.8 | 12.7 |

**Table 3.14 –** 25(OH)D concentration for centre and edge punches taken from DBS made using the pool of blood containing the higher concentration of 25(OH)D (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 100 nmol/L.

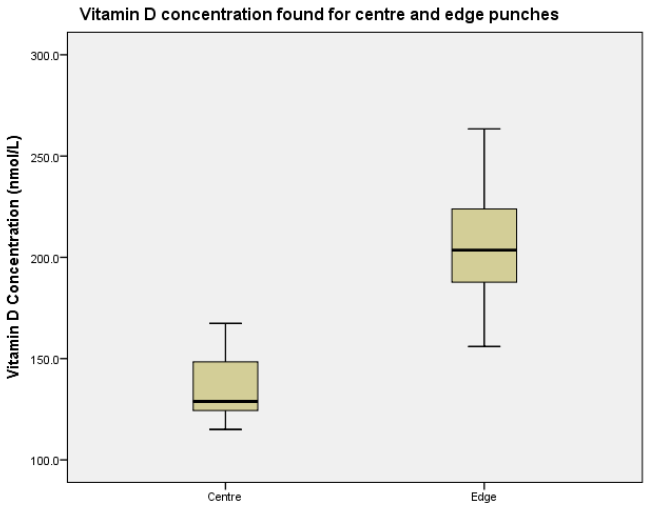



**Figure 3.11 –** Box and whisker plot showing the 25(OH)D concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the higher concentration of 25(OH)D.

The same pattern was seen with the higher concentration of 25(OH)D – punches taken from the edge of DBS resulted in a significantly increased mean concentration ($p<0.001$) compared to centre punches (Table 3.14 and Figure 3.11). The data were normally distributed ($p=0.2$) and variances equal ($p=0.288$).

3.3.2.2.2. CRP

A summary of the CRP results for the 75 µL DBS outer punches for the lower concentration of CRP can be seen in Table 3.15 and Figure 3.12. The data were normally distributed for the edge punches ($p=0.2$) and the mean CRP concentration, although higher compared to the centre punches, was not significantly different (variances not equal $p=0.015$, $p=0.096$).

| | **Edge Punches (75 µL)** | **Centre Punches (20 µL)** | **Centre Punches (75 µL)** |
|---|---|---|---|
| **Mean (mg/L)** | 18.1 | 12.5 | 16.1 |
| **S.D. (mg/L)** | 1.9 | 2.0 | 3.3 |
| **CV(%)** | 10.5 | 15.8 | 20.5 |

**Table 3.15 –** CRP concentration for centre and edge punches taken from DBS made using the pool of blood containing the lower concentration of CRP (n=28 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 14 mg/L.

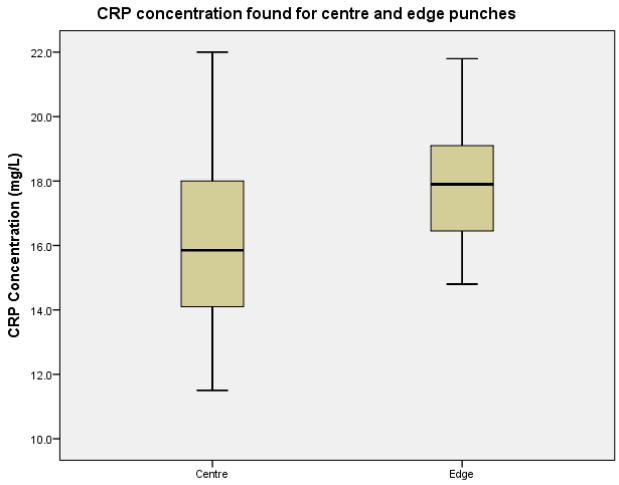



**Figure 3.12 –** Box and whisker plot showing the CRP concentration for centre (n=10) and edge (n=28) punches taken from 75 µL volume DBS, made from the pool of blood containing the lower concentration of CRP.

| | Edge Punches (75 µL) | Centre Punches (20 µL) | Centre Punches (75 µL) |
|---|---|---|---|
| **Mean (mg/L)** | 134.8 | 86.1 | 112.5 |
| **S.D. (mg/L)** | 25.8 | 4.0 | 14.2 |
| **CV(%)** | 19.2 | 4.7 | 12.6 |

**Table 3.16 –** CRP concentration for centre and edge punches taken from DBS made using the pool of blood containing the higher concentration of CRP (n=28 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 92 mg/L.

A similar pattern was seen with the higher concentration of CRP, however this time punches taken from the edge of DBS resulted in a significantly increased mean concentration (p=0.023) compared to centre punches (Table 3.16 and Figure 3.13). The data were normally distributed (p=0.2) and variances were equal (p=0.72).

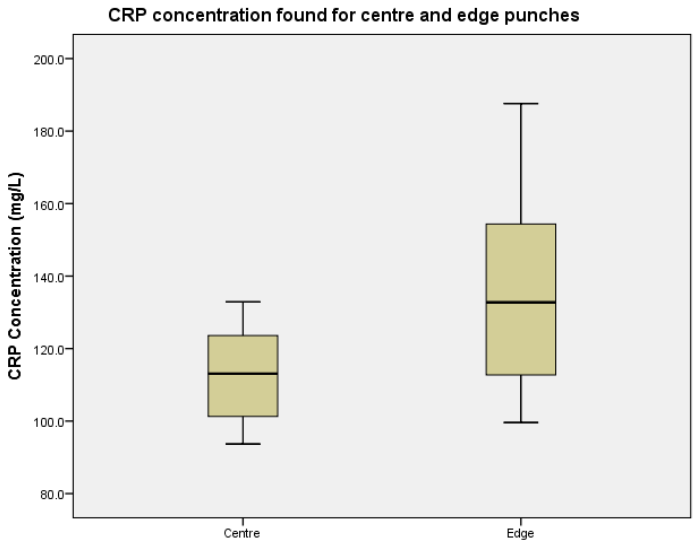



**Figure 3.13 –** Box and whisker plot showing the CRP concentration for centre (n=10) and edge (n=28) punches taken from 75 µL volume DBS, made from the pool of blood containing the higher concentration of CRP.

### 3.3.2.3. *Haemolysed vs. Intact Blood*

See section 3.2.4.3 (page 65) for the method relating to this section.

#### 3.3.2.3.1. Vitamin D

The results for punches taken from DBS made using intact and haemolysed blood containing the lower concentration of 25(OH)D can be seen in Table 3.17 and

Figures 3.14 and 3.15. The results showed that for both volumes of DBS, the mean concentration of 25(OH)D was significantly higher for DBS made from haemolysed whole blood (20 µL, variances equal p=0.052, p=0.002; 75 µL, variances not equal p=0.027, p=0.039). The data for each volume of haemolysed DBS were normally distributed (both p=0.2).

| | 20 µL | | 75 µL | |
|---|---|---|---|---|
| **ID** | **Haemolysed** | **Intact** | **Haemolysed** | **Intact** |
| **1** | 48.3 | 41.6 | 61.8 | 47.7 |
| **2** | 52.1 | 38.3 | 60.5 | 44.4 |
| **3** | 53.2 | 45.6 | 69.8 | 70.4 |
| **4** | 50.8 | 40.0 | 59.8 | 54.4 |
| **5** | 44.3 | 45.5 | 59.2 | 62.6 |
| **6** | 49.9 | 39.9 | 67.0 | 58.1 |
| **7** | 49.1 | 48.5 | 57.5 | 49.5 |
| **8** | 51.6 | 50.2 | 54.5 | 48.2 |
| **9** | 46.4 | 40.4 | 62.4 | 48.2 |
| **10** | 53.5 | 48.9 | 64.0 | 63.1 |
| **Mean (nmol/L)** | **49.9** | **43.9** | **61.7** | **54.7** |
| **S.D. (nmol/L)** | **2.97** | **4.4** | **4.48** | **8.5** |
| **CV(%)** | **5.9** | **9.9** | **7.3** | **15.6** |

**Table 3.17 –** 25(OH)D concentration found in centre punches taken from 20 µL and 75 µL DBS made from haemolysed and intact pools of blood containing the lower concentration of 25(OH)D. Serum concentration = 39 nmol/L.

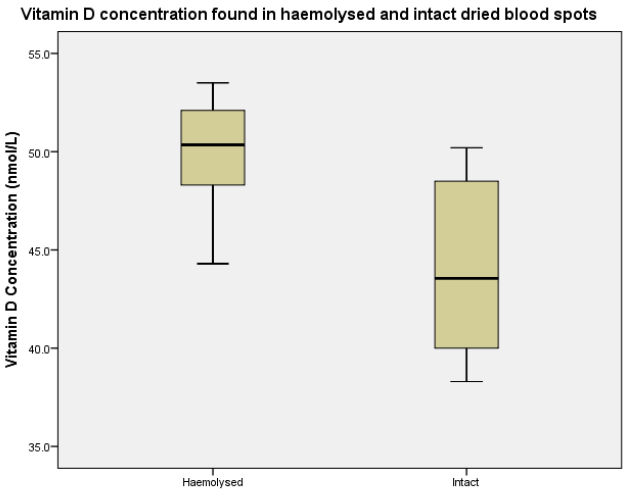



**Figure 3.14 –** Box and whisker plot showing the 25(OH)D concentration found in centre punches taken from 20 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the lower concentration of 25(OH)D.

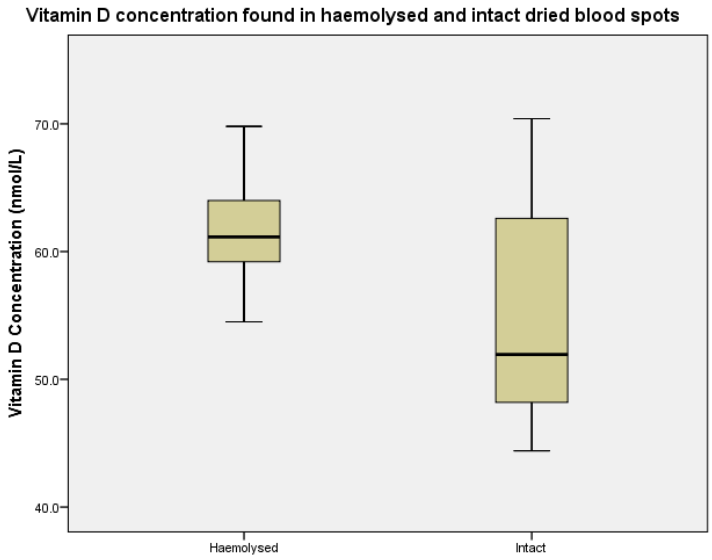



**Figure 3.15 –** Box and whisker plot showing the 25(OH)D concentration found in centre punches taken from 75 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the lower concentration of 25(OH)D.

| | 20 µL | | 75 µL | |
|---|---|---|---|---|
| **ID** | **Haemolysed** | **Intact** | **Haemolysed** | **Intact** |
| **1** | 145.9 | 97.7 | 177.6 | 158.2 |
| **2** | 138.4 | 120.8 | 161.4 | 124.4 |
| **3** | 140.9 | 102.9 | 168.8 | 131.4 |
| **4** | 142.4 | 91.0 | 168.6 | 126.3 |
| **5** | 151.6 | 109.5 | 154.5 | 167.4 |
| **6** | 143.6 | 109.3 | 140.6 | 148.4 |
| **7** | 144.6 | 109.3 | 174.5 | 115.0 |
| **8** | 139.4 | 129.1 | 187.7 | 125.7 |
| **9** | 139.0 | 100.8 | 194.3 | 148.3 |
| **10** | 161.3 | 96.8 | 172.1 | 124.3 |
| **Mean (nmol/L)** | **144.7** | **106.7** | **170.0** | **136.9** |
| **S.D. (nmol/L)** | **7.0** | **11.5** | **15.5** | **17.3** |
| **CV(%)** | **4.9** | **10.8** | **9.1** | **12.7** |

**Table 3.18 –** 25(OH)D concentration found in centre punches taken from 20 µL and 75 µL DBS made from haemolysed and intact pools of blood containing the higher concentration of 25(OH)D. Serum concentration = 100 nmol/L.

The same pattern was seen with the higher concentration of 25(OH)D – for both volumes of DBS, mean 25(OH)D concentration was significantly higher for DBS made from haemolysed blood (both $p<0.001$, Table 3.18 and Figures 3.16 and 3.17).

The data were normally distributed (20 µL p=0.133; 75 µL p=0.200) and variances were equal (20 µL p=0.147; 75 µL p=0.362).

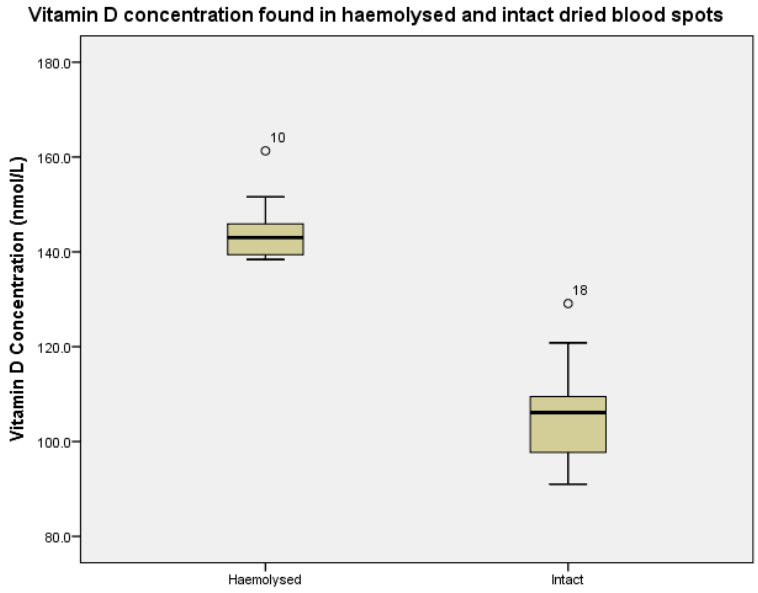



**Figure 3.16 –** Box and whisker plot showing the 25(OH)D concentration found in centre punches taken from 20 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the higher concentration of 25(OH)D.

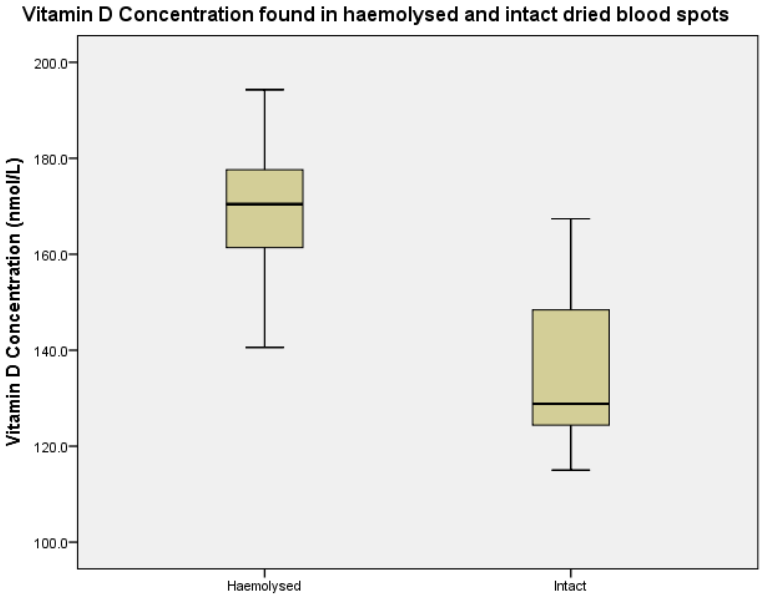



**Figure 3.17 –** Box and whisker plot showing the 25(OH)D concentration found in centre punches taken from 75 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the higher concentration of 25(OH)D.

3.3.2.3.2. CRP – SWBH Device

| | 20 µL | | 75 µL | |
|---|---|---|---|---|
| **ID** | **Haemolysed** | **Intact** | **Haemolysed** | **Intact** |
| **1** | 13.3 | 14.3 | 16.0 | 11.5 |
| **2** | 9.5 | 11.1 | 13.4 | 14.4 |
| **3** | 8.8 | 14.4 | 12.1 | 12.5 |
| **4** | 11.0 | 12.9 | 15.0 | 17.3 |
| **5** | 12.1 | 11.6 | 12.8 | 17.8 |
| **6** | 9.5 | 14.0 | 14.7 | 19.3 |
| **7** | 12.3 | 12.1 | 17.4 | 22.0 |
| **8** | 10.9 | 8.2 | 13.4 | 18.0 |
| **9** | 10.3 | 14.6 | 18.0 | 14.1 |
| **10** | 11.8 | 12.1 | 14.9 | 14.1 |
| **Mean (mg/L)** | **11.0** | **12.5** | **14.8** | **16.1** |
| **S.D. (mg/L)** | **1.4** | **2.0** | **1.9** | **3.3** |
| **CV(%)** | **13.1** | **15.8** | **13.1** | **20.5** |

**Table 3.19 –** CRP concentration found in centre punches taken from 20 µL and 75 µL DBS made from haemolysed and intact pools of blood containing the lower concentration of CRP. Serum concentration = 14 mg/L.

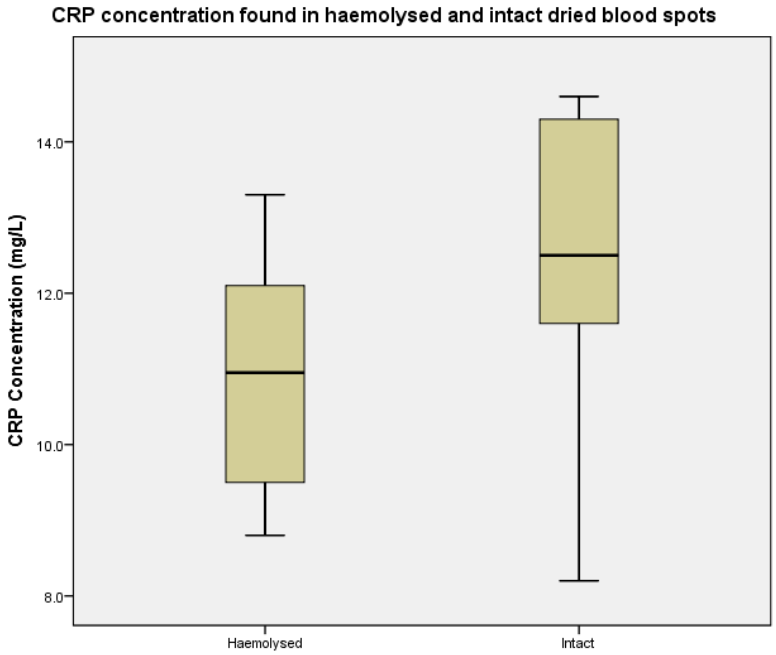



**Figure 3.18 –** Box and whisker plot showing the CRP concentration found in centre punches taken from 20 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the lower concentration of CRP.

The results for punches taken from DBS made using intact and haemolysed blood containing the lower concentration of CRP can be seen in Table 3.19 and Figures 3.18 and 3.19. The results showed that for both volumes of DBS, the mean concentration of CRP was lower for DBS made from haemolysed blood, but this difference was not significant (20 µL, variances equal p=0.430, p=0.056; 75 µL, variances not equal p=0.045, p=0.289). The data for each volume of haemolysed DBS were normally distributed (both p=0.2).

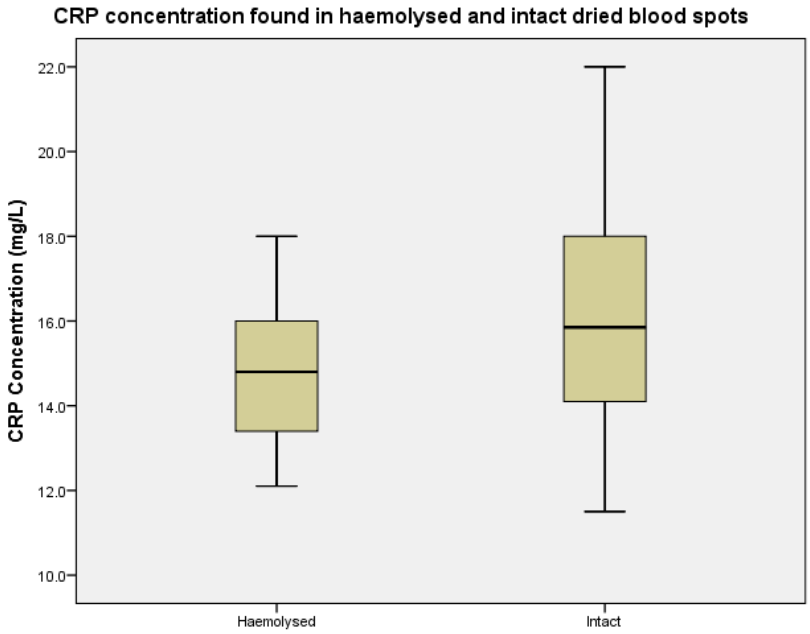



**Figure 3.19 –** Box and whisker plot showing the CRP concentration found in centre punches taken from 75 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the lower concentration of CRP.

The same pattern was seen with the higher concentration of CRP – for both volumes of DBS, mean CRP concentration was lower for DBS made from haemolysed blood although this difference was not significantly different (20 µL, variances not equal p=0.032, p=0.442; 75 µL, variances not equal p=0.023, p=0.081, Table 3.20 and

Figures 3.20 and 3.21). The data were normally distributed for both volumes of DBS (both p=0.2).

| | 20 µL | | 75 µL | |
|---|---|---|---|---|
| **ID** | **Haemolysed** | **Intact** | **Haemolysed** | **Intact** |
| **1** | 74.5 | 85.3 | 98.7 | 93.7 |
| **2** | 85.2 | 83.7 | 100.8 | 96.4 |
| **3** | 69.1 | 88.3 | 101.4 | 101.3 |
| **4** | M | 84.1 | 104.7 | 113.6 |
| **5** | 82.3 | 88.4 | 109.5 | 113.1 |
| **6** | 78.3 | 82.3 | 105.5 | 123.6 |
| **7** | 78.9 | 94.6 | 92.6 | M |
| **8** | 99.7 | 88.3 | 109.3 | 108.0 |
| **9** | 101.0 | 80.3 | 108.4 | 132.9 |
| **10** | 78.7 | 86.0 | 96.2 | 130.0 |
| **Mean (mg/L)** | **83.1** | **86.1** | **102.7** | **112.5** |
| **S.D. (mg/L)** | **10.8** | **4.0** | **5.8** | **14.2** |
| **CV(%)** | **13.0** | **4.7** | **5.6** | **12.6** |

**Table 3.20 –** CRP concentration found in centre punches taken from 20 µL and 75 µL DBS made from haemolysed and intact pools of blood containing the higher concentration of CRP. Serum concentration = 92 mg/L.

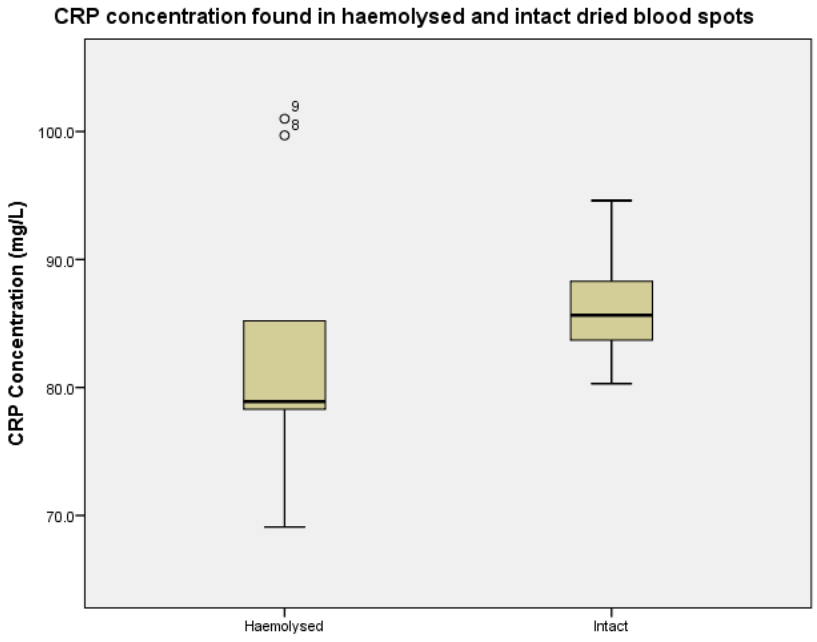



**Figure 3.20 –** Box and whisker plot showing the CRP concentration found in centre punches taken from 20 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the higher concentration of CRP.

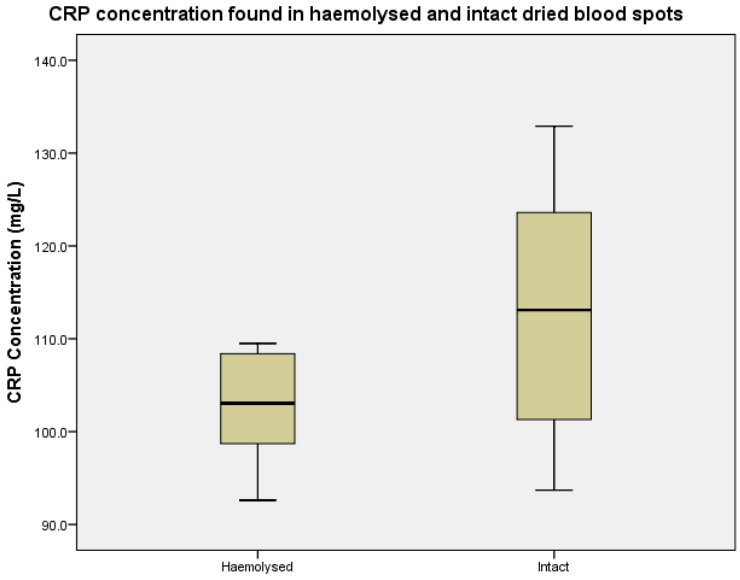



**Figure 3.21 –** Box and whisker plot showing the CRP concentration found in centre punches taken from 75 µL DBS made from haemolysed (n=10) and intact blood (n=10), using the pool of blood containing the higher concentration of CRP.

### 3.3.2.3.3. CRP – Mitra Device

| | Low | | High | |
|---|---|---|---|---|
| **ID** | **Haemolysed** | **Intact** | **Haemolysed** | **Intact** |
| **1** | 12.3 | 18.9 | 70.8 | 104.5 |
| **2** | 13.1 | 20 | 70.4 | 98.6 |
| **3** | 11.9 | 19 | 77.2 | 89.1 |
| **4** | 12 | 16.6 | 97.9 | 91 |
| **5** | 13.6 | 19.1 | 86.4 | 89.3 |
| **6** | 11.6 | 19.3 | 89.1 | 93.5 |
| **7** | 15.4 | 21.2 | 87.7 | 112.9 |
| **8** | 13.6 | 16.7 | 94 | 119.8 |
| **Mean (mg/L)** | **12.9** | **18.9** | **84.2** | **99.8** |
| **S.D. (mg/L)** | **1.3** | **1.6** | **10.3** | **11.6** |
| **CV(%)** | **9.7** | **8.2** | **12.2** | **11.6** |

**Table 3.21 –** CRP concentration found in Mitra devices made from haemolysed and intact pools of blood. Serum concentration = 14 mg/L and 92 mg/L.

The CRP results for Mitra devices made using intact and haemolysed blood can be seen in Table 3.21 and Figures 3.22 and 3.23. The results show that for both levels of CRP, the mean concentration of CRP was significantly lower for Mitra devices

made from haemolysed blood (low CRP, variances equal p=0.798, p<0.001; high CRP, variances equal p=0.742, p=0.013). The data for each concentration of haemolysed Mitra devices were normally distributed (both p=0.2).

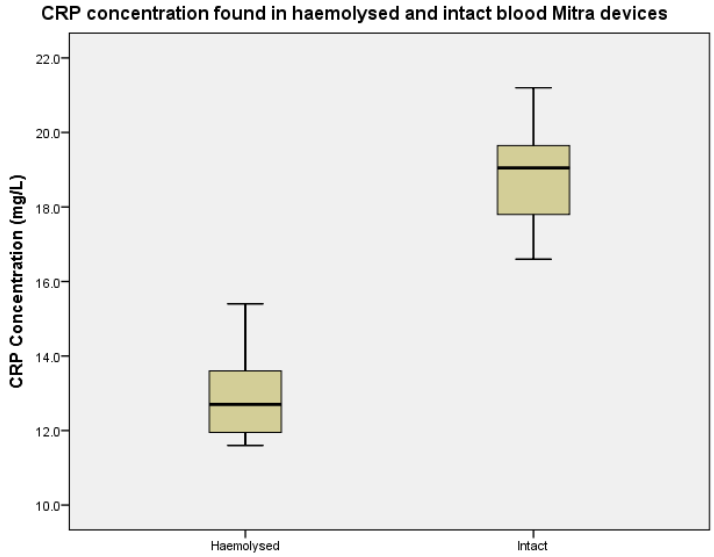



**Figure 3.22 –** Box and whisker plot showing the CRP concentration found in Mitra devices made from haemolysed (n=8) and intact blood (n=8), using the pool of blood containing the lower concentration of CRP.

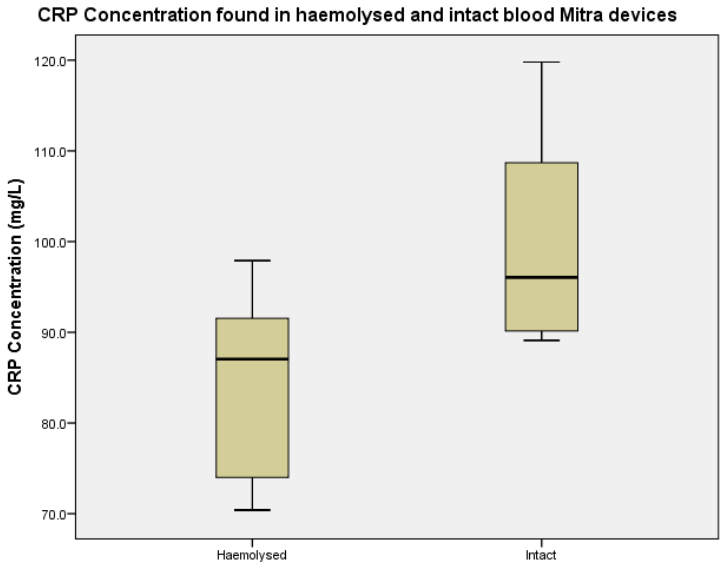



**Figure 3.23 –** Box and whisker plot showing the CRP concentration found in Mitra devices made from haemolysed (n=8) and intact blood (n=8), using the pool of blood containing the higher concentration of CRP.

3.3.2.4. *Presence of Ethanol*

See section 3.2.4.4 (page 65) for the method relating to this section.

3.3.2.4.1. Vitamin D

3.3.2.4.1.1. Size of Spot – Ethanol Containing Samples

| | Volume of Spots (µL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | | 20 | | 50 | | 75 | | 100 | |
| ID | No E | E | No E | E | No E | E | No E | E | No E | E |
| 1 | 39.0 | 37.3 | 41.6 | 34.6 | 58.7 | 40.6 | 47.7 | 49.6 | 70.3 | 48.2 |
| 2 | 37.5 | 43.5 | 38.3 | 36.4 | 55.9 | 30.4 | 44.4 | 49.9 | 58.5 | 64.3 |
| 3 | 36.0 | 42.2 | 45.6 | 38.0 | 54.6 | 34.4 | 70.4 | 41.2 | 46.8 | 47.3 |
| 4 | 43.1 | 41.2 | 40.0 | 41.4 | 52.6 | 43.6 | 54.4 | 37.3 | 47.1 | 46.7 |
| 5 | 36.5 | 40.7 | 45.5 | 30.5 | 48.8 | 36.9 | 62.6 | 47.2 | 49.2 | 45.1 |
| 6 | 45.5 | 43.9 | 39.9 | 37.4 | 49.8 | 49.1 | 58.1 | 41.2 | 57.6 | 41.4 |
| 7 | 43.6 | 38.7 | 48.5 | 44.0 | 48.2 | 49.5 | 49.5 | 48.4 | 54.2 | 50.3 |
| 8 | 42.6 | 38.7 | 50.2 | 43.7 | 48.2 | 43.1 | 48.2 | 49.0 | 58.0 | 58.2 |
| 9 | 46.7 | 40.8 | 40.4 | 38.3 | 41.2 | 42.7 | 48.2 | 57.7 | 59.3 | 40.9 |
| 10 | 40.8 | 24.6 | 48.9 | 44.4 | 39.2 | 40.6 | 63.1 | 52.6 | 69.4 | 61.1 |
| **Mean (nmol/L)** | **41.1** | **39.2** | **43.9** | **38.9** | **49.7** | **41.1** | **54.7** | **47.4** | **57.0** | **50.4** |
| **S.D. (nmol/L)** | **3.77** | **5.53** | **4.36** | **4.52** | **6.14** | **6.01** | **8.55** | **6.03** | **8.23** | **8.14** |
| **CV(%)** | **9.2** | **14.1** | **9.9** | **11.6** | **12.4** | **14.6** | **15.6** | **12.7** | **14.4** | **16.2** |

**Table 3.22 –** Results obtained from the centre punches for different volume DBS made using a pool of blood containing the lower concentration of 25(OH)D without ethanol (no E) and with ethanol (E) (serum = 39 nmol/L).

The results for centre punches taken from different volumes of DBS made using blood containing ethanol and no ethanol for the lower concentration of Vitamin D can be seen in Table 3.22 and Figure 3.24. The data were all normally distributed except for the 10 µL ethanol containing DBS (all $p=0.200$; 10 µL $p=0.040$). The results showed that the mean concentration of 25(OH)D increased with increasing volume of ethanol containing DBS. The mean concentration for the different DBS volumes was significantly different ($p<0.01$). Post-hoc analysis showed that the 20 µL ethanol

containing DBS was significantly different from the 100 µL ethanol containing DBS (p=0.011), but there was no significant difference seen between the 20 µL ethanol containing DBS and all other ethanol containing DBS (all p>0.05).

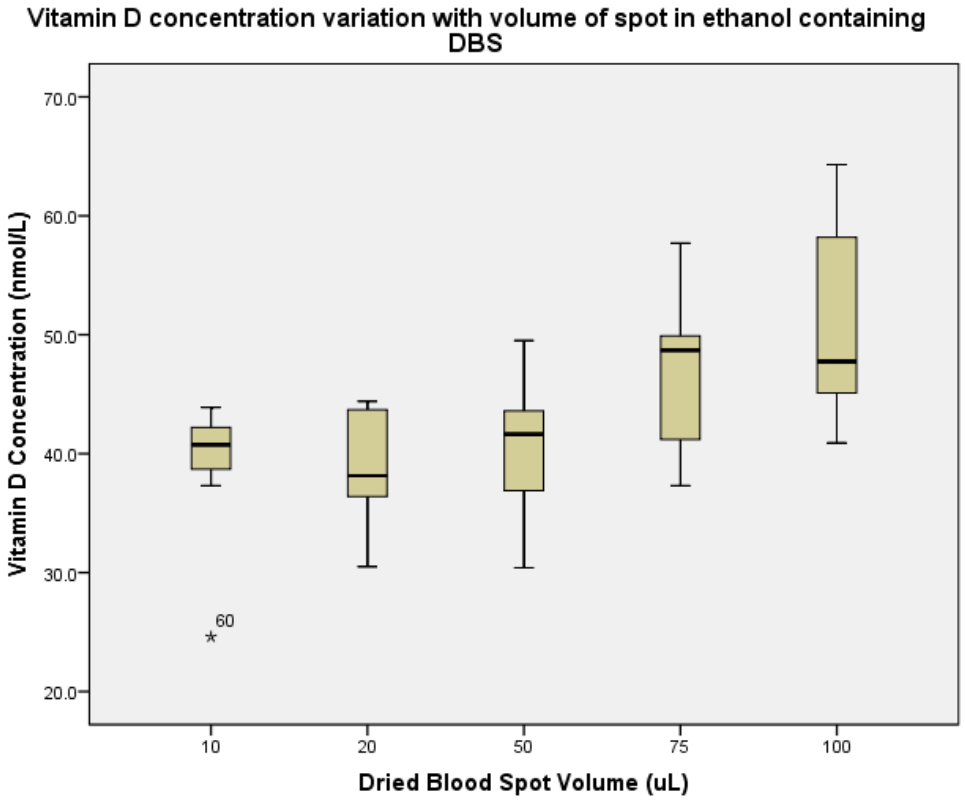



**Figure 3.24 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of 25(OH)D with ethanol (n=10 for each volume of DBS).

25(OH)D concentration found in different volumes of DBS made with ethanol containing blood.

The results also showed that for all volumes of DBS except 10 µL and 100 µL, there was a significant difference in 25(OH)D concentration between ethanol containing DBS and DBS containing no ethanol, with ethanol containing DBS showing lower levels of 25(OH)D (10 µL p=0.529; 20 µL p=0.021; 50 µL p=0.005; 75 µL p=0.042; 100 µL p=0.084). Where applicable variances were equal (all p>0.05, Figure 3.25).

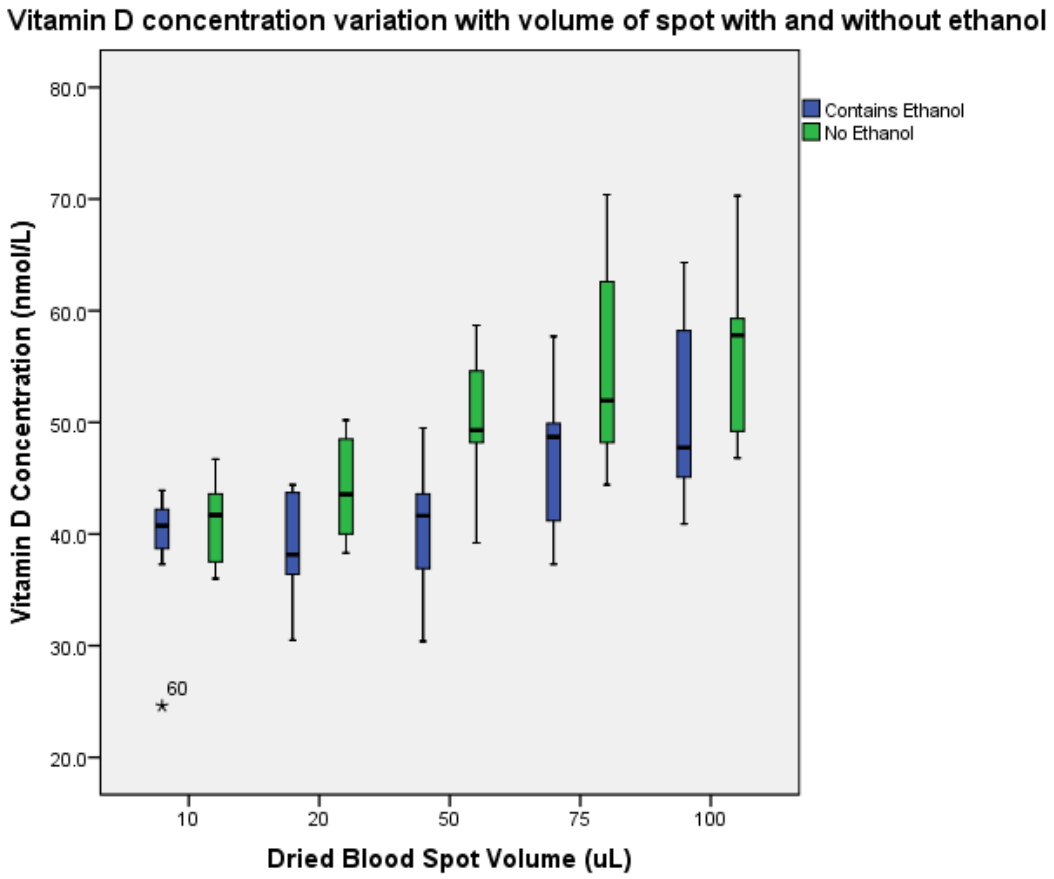



**Figure 3.25 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of 25(OH)D with ethanol and without ethanol (n=10 for each volume of DBS).

A similar pattern was seen with the higher concentration of 25(OH)D – the different volumes of ethanol containing DBS had significantly different mean concentrations of 25(OH)D ($p<0.001$, Table 3.23 and Figure 3.26). 25(OH)D concentrations increased with increasing DBS volume. All the data were normally distributed (all $p=0.200$). This time post-hoc analysis (variances were not equal, $p=0.043$) showed that the 20 µL ethanol containing DBS were significantly different from the 100 µL ($p=0.005$) and 75 µL ethanol containing DBS ($p<0.001$), but there was no significant difference seen between the 20 µL ethanol containing DBS and the 10 µL ($p=0.992$) and 50 µL ethanol containing DBS ($p=0.074$).

| | Volume of Spots (µL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | | 20 | | 50 | | 75 | | 100 | |
| ID | No E | E | No E | E | No E | E | No E | E | No E | E |
| 1 | 105.5 | 93.3 | 97.7 | 106.1 | 121.6 | 104.6 | 158.2 | 139.7 | 147.7 | 161.6 |
| 2 | 112.3 | 94.8 | 120.8 | 118.3 | 130.4 | 143.2 | 124.4 | 162.2 | 183.9 | 176.6 |
| 3 | 101.3 | 96.3 | 102.9 | 109.7 | 109.0 | 106.4 | 131.4 | 141.8 | 142.0 | 139.5 |
| 4 | 99.2 | 107.9 | 91.0 | 103.0 | 137.4 | 121.3 | 126.3 | 118.1 | 124.6 | 119.1 |
| 5 | 106.7 | 121.1 | 109.5 | 109.9 | 102.0 | 136.9 | 167.4 | 133.6 | 158.0 | 148.0 |
| 6 | 98.3 | 112.0 | 109.3 | 123.9 | 103.2 | 119.2 | 148.4 | 131.0 | 115.9 | 156.4 |
| 7 | 103.8 | 112.8 | 109.3 | 104.3 | 127.5 | 129.4 | 115.0 | 159.4 | 121.3 | 134.0 |
| 8 | 104.3 | 109.6 | 129.1 | 108.0 | 117.1 | 134.9 | 125.7 | 138.8 | 154.5 | 130.0 |
| 9 | 111.6 | 104.4 | 100.8 | 116.0 | 126.0 | 111.2 | 148.3 | 164.0 | 136.9 | 127.5 |
| 10 | 112.3 | 127.9 | 96.8 | 99.6 | 109.3 | 144.2 | 124.3 | 146.7 | 136.5 | 113.1 |
| **Mean (nmol/L)** | **105.5** | **108.0** | **106.7** | **109.9** | **118.4** | **125.1** | **136.9** | **143.5** | **142.1** | **140.6** |
| **S.D. (nmol/L)** | **5.21** | **11.30** | **11.53** | **7.50** | **12.15** | **14.75** | **17.35** | **14.8** | **20.19** | **19.97** |
| **CV(%)** | **4.9** | **10.5** | **10.8** | **6.8** | **10.3** | **11.8** | **12.7** | **10.3** | **14.2** | **14.2** |

**Table 3.23 –** Results obtained from the centre punches for different volume DBS made using a pool of blood containing the higher concentration of 25(OH)D without ethanol (no E) and with ethanol (E) (serum = 100 nmol/L).

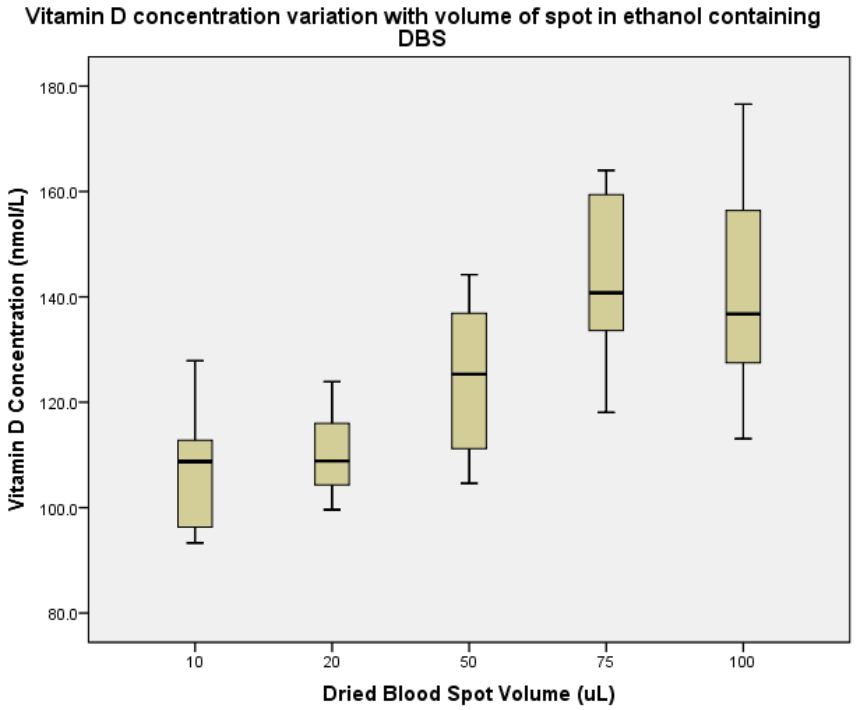



**Figure 3.26 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of 25(OH)D with ethanol (n=10 for each volume of DBS).

This time, for the higher level of 25(OH)D, the results showed that for all volumes of DBS, there was no significant difference in 25(OH)D concentration between ethanol containing DBS and DBS containing no ethanol, although ethanol containing DBS generally showed higher levels of 25(OH)D (10 µL p=0.537; 20 µL p=0.477; 50 µL p=0.277; 75 µL p=0.373; 100 µL p=0.865). The variances were equal for all DBS volumes (all p>0.05, Figure 3.27).

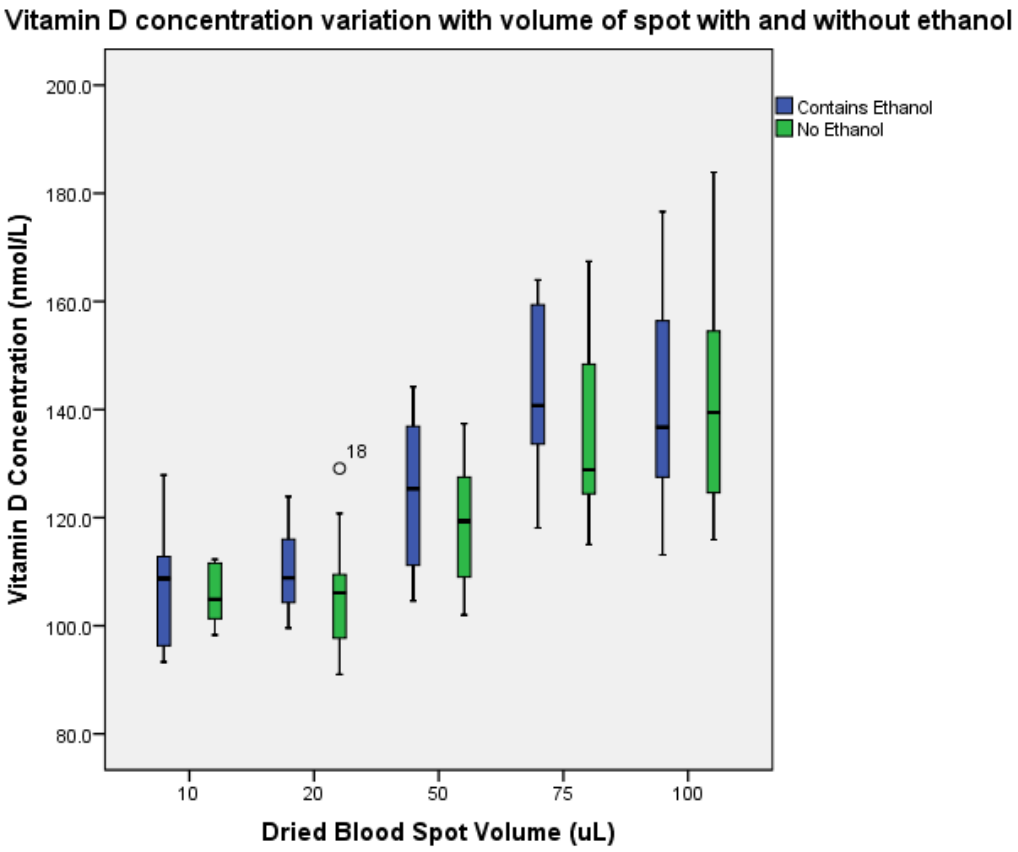



**Figure 3.27 –** Box and whisker plot showing the 25(OH)D concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of 25(OH)D with ethanol and without ethanol (n=10 for each volume of DBS).

3.3.2.4.1.2. Location of Spot – Ethanol Containing Spots

A summary of the 25(OH)D results for punches taken from the edge of 75 µL DBS made with ethanol containing blood for the lower concentration of CRP can be seen in Table 3.24 and Figure 3.28. The data were normally distributed for the edge

punches (p=0.2) and the mean 25(OH)D concentration was significantly higher for ethanol containing edge punches compared to ethanol containing centre punches (variances equal p=0.420, p<0.001).

| | Edge Punches (75 µL) | | Centre Punches (20 µL) | | Centre Punches (75 µL) | |
|---|---|---|---|---|---|---|
| | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **Mean (nmol/L)** | 68.8 | 74.1 | 43.9 | 38.9 | 54.7 | 47.4 |
| **S.D. (nmol/L)** | 7.2 | 6.96 | 4.4 | 4.52 | 8.5 | 6.03 |
| **CV(%)** | 10.4 | 9.4 | 9.9 | 11.6 | 15.6 | 12.7 |

**Table 3.24 –** 25(OH)D results found when punches were taken from the outer edges and centre of 75 µL DBS made with ethanol (E) containing blood and ethanol free (No E) blood, as well as centre punches for 20 µL DBS made with ethanol containing blood and ethanol free blood (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 39 nmol/L.

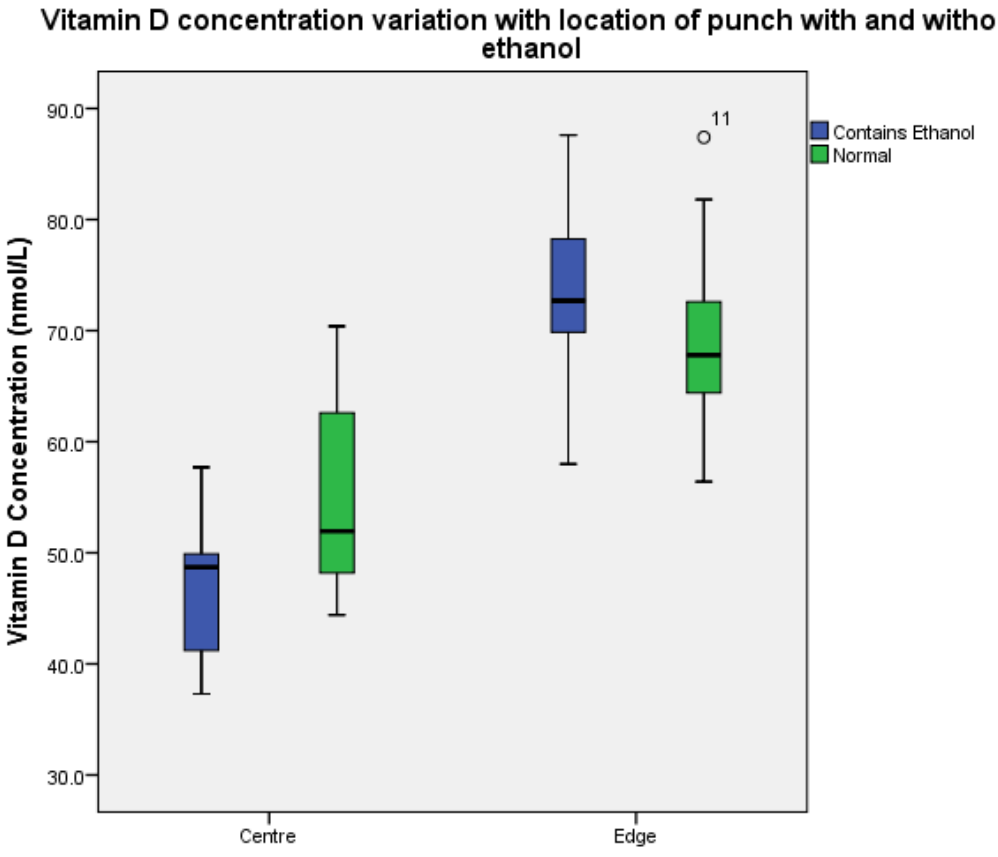



**Figure 3.28 –** Box and whisker plot showing the 25(OH)D concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the lower concentration of 25(OH)D containing ethanol and without ethanol.

The same pattern was seen for the higher concentration of 25(OH)D – the mean 25(OH)D concentration was significantly higher for ethanol containing edge punches compared to ethanol containing centre punches (variances equal p=0.075, p<0.001), Table 3.25 and Figure 3.29). The data were normally distributed (p=0.2).

| | Edge Punches (75 µL) | | Centre Punches (20 µL) | | Centre Punches (75 µL) | |
|---|---|---|---|---|---|---|
| | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **Mean (nmol/L)** | 204.7 | 206.3 | 106.7 | 109.9 | 136.9 | 143.5 |
| **S.D. (nmol/L)** | 26.5 | 22.76 | 11.5 | 7.50 | 17.3 | 14.8 |
| **CV(%)** | 12.9 | 11.0 | 10.8 | 6.8 | 12.7 | 10.3 |

**Table 3.25 –** 25(OH)D results found when punches were taken from the outer edges and centre of 75 µL DBS made with ethanol (E) containing blood and ethanol free (No E) blood, as well as centre punches for 20 µL DBS made with ethanol containing blood and ethanol free blood (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 100 nmol/L.

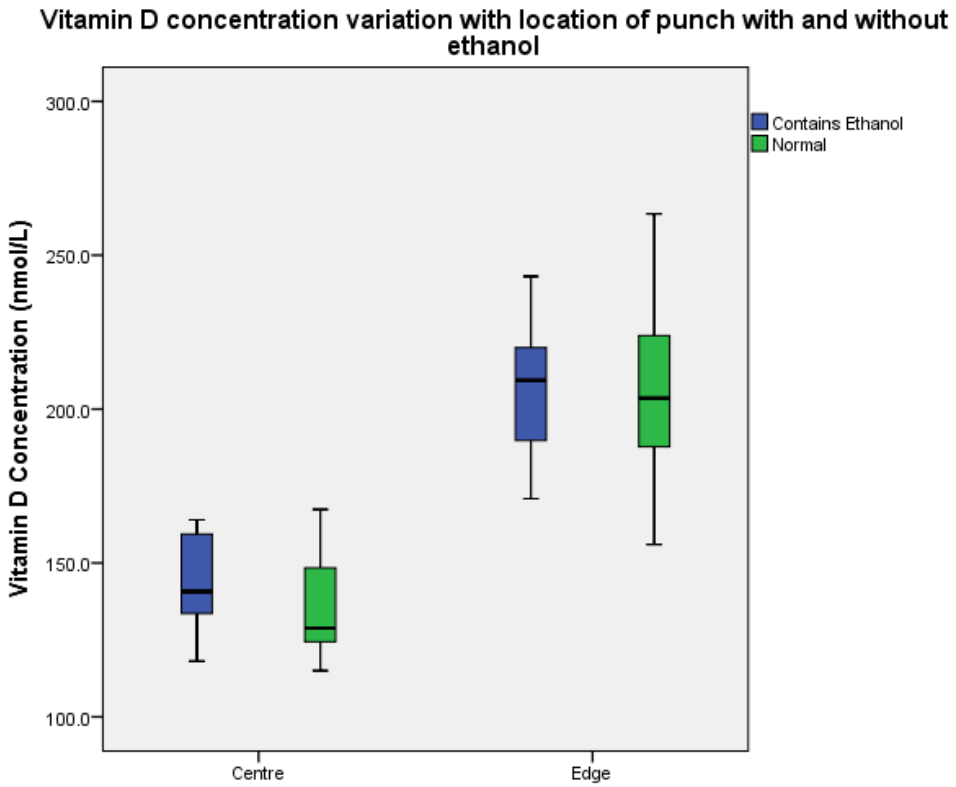



**Figure 3.29 –** Box and whisker plot showing the 25(OH)D concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the higher concentration of 25(OH)D containing ethanol and without ethanol.

3.3.2.4.2. CRP

3.3.2.4.2.1. Size of Spot – Ethanol Containing Samples

The results for punches taken from different volumes of DBS made using blood containing ethanol and no ethanol for the lower concentration of CRP can be seen in Table 3.26 and Figure 3.30. The data for ethanol containing DBS were all normally distributed except for 50 µL DBS (all $p>0.05$; 50 µL $p=0.038$). The results showed that for ethanol containing DBS, the mean concentration of CRP increased with increasing volume of DBS. The mean concentration for the different DBS volumes was significantly different ($p<0.01$). Post-hoc analysis showed that the 20 µL ethanol containing DBS was significantly different from the 100 µL ethanol containing DBS ($p<0.001$), but there was no significant difference seen between the 20 µL ethanol containing DBS and all other ethanol containing DBS (all $p>0.05$).

| | **Volume of Spots (µL)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **10** | | **20** | | **50** | | **75** | | **100** | |
| **ID** | **No E** | **E** | **No E** | **E** | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **1** | 12.7 | 9.2 | 14.3 | 13.1 | 13.1 | 13.6 | 11.5 | 16.9 | 16.8 | 16.8 |
| **2** | 9.5 | 9.2 | 11.1 | 11.1 | 14.3 | 12.1 | 14.4 | 16.3 | 17.1 | 15.6 |
| **3** | 13.4 | 10.5 | 14.4 | 10.4 | 14.2 | 10.5 | 12.5 | 15.8 | 18.6 | 19.8 |
| **4** | 8.6 | 8.7 | 12.9 | 7.8 | 14.0 | 14.4 | 17.3 | 13.5 | 19.3 | 18.7 |
| **5** | 11.5 | 10.9 | 11.6 | 9.9 | 17.0 | 13.5 | 17.8 | 11.6 | 14.7 | 18.7 |
| **6** | 9.3 | 12.2 | 14.0 | 12.8 | 14.2 | 13.8 | 19.3 | 12.1 | 13.3 | 14.6 |
| **7** | 13.5 | 13.4 | 12.1 | 9.6 | 17.6 | 13.1 | 22.0 | 10.1 | 17.2 | 13.7 |
| **8** | 6.9 | 10.5 | 8.2 | 12.4 | 13.9 | 13.4 | 18.0 | 15.0 | 19.9 | 18.0 |
| **9** | 11.1 | 9.3 | 14.6 | 7.2 | 17.3 | 9.2 | 14.1 | 14.0 | 20.0 | 18.7 |
| **10** | 15.0 | 13.1 | 12.1 | 14.8 | 10.5 | 14.4 | 14.1 | 14.5 | 16.5 | 16.7 |
| **Mean (mg/L)** | **11.2** | **10.7** | **12.5** | **10.9** | **14.6** | **12.8** | **16.1** | **13.9** | **17.3** | **17.1** |
| **S.D. (mg/L)** | **2.6** | **1.7** | **2.0** | **2.4** | **2.2** | **1.7** | **3.3** | **2.29** | **2.2** | **2.0** |
| **CV(%)** | **22.9** | **15.8** | **15.8** | **22.1** | **14.9** | **13.5** | **20.5** | **16.5** | **12.7** | **11.7** |

**Table 3.26 –** Results obtained from the centre punches for different volume DBS made using a pool of blood containing the lower concentration of CRP without ethanol (no E) and with ethanol (E) (serum = 14 mg/L).

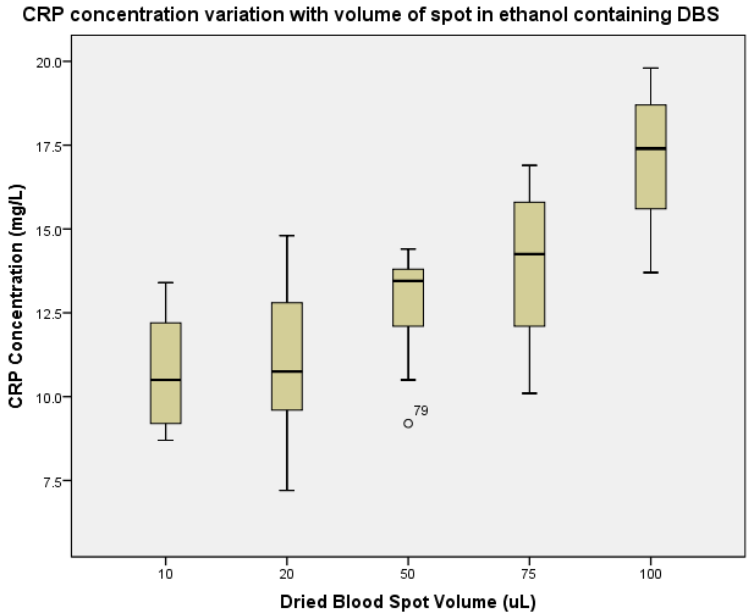



**Figure 3.30 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of CRP with ethanol (n=10 for each volume of DBS).

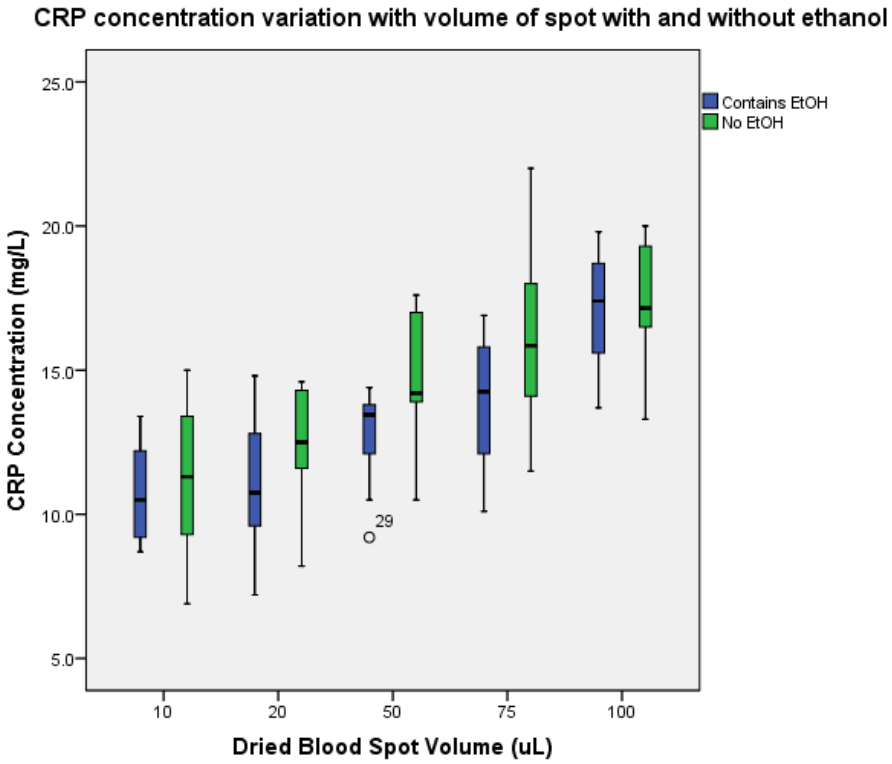



**Figure 3.31 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the lower concentration of CRP with ethanol and without ethanol (n=10 for each volume of DBS).

The results also showed that for all volumes of DBS, there was no significant difference between ethanol containing DBS and DBS containing no ethanol (all $p>0.05$, Figure 3.31). Where applicable variances were equal (all $p>0.05$).

A similar pattern was seen with the higher concentration of CRP – the different volumes of ethanol containing DBS had significantly different mean concentrations of CRP ($p<0.001$, Table 3.27 and Figure 3.32). CRP concentrations increased with increasing DBS volume. All the data were normally distributed (all $p>0.05$). This time post-hoc analysis (variances were equal, $p=0.443$) showed that the 20 µL ethanol containing DBS were significantly different from the 100 µL ($p<0.001$) and 75 µL ethanol containing DBS ($p=0.004$), but there was no significant difference seen between the 20 µL ethanol containing DBS and the 10 µL ($p=0.362$) and 50 µL ethanol containing DBS ($p=0.136$).

| | **Volume of Spots (µL)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **10** | | **20** | | **50** | | **75** | | **100** | |
| **ID** | **No E** | **E** | **No E** | **E** | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **1** | 91.3 | 63.8 | 85.3 | 82.3 | 127.3 | 128.6 | 93.7 | 94.5 | 111.6 | 143.2 |
| **2** | 90.0 | 85.2 | 83.7 | 90.2 | 107.2 | 90.0 | 96.4 | 100.2 | 125.9 | 112.7 |
| **3** | 77.2 | 68.6 | 88.3 | 90.2 | 115.3 | 96.0 | 101.3 | 94.5 | 104.6 | 113.4 |
| **4** | 72.4 | 68.7 | 84.1 | 68.8 | 96.6 | 85.1 | 113.6 | 113.4 | 133.3 | 121.1 |
| **5** | 90.1 | 86.7 | 88.4 | 80.4 | 119.9 | 88.6 | 113.1 | 109.0 | 113.4 | 115.1 |
| **6** | 79.1 | 60.0 | 82.3 | 76.8 | 108.0 | 99.3 | 123.6 | 108.3 | 133.1 | 120.9 |
| **7** | 85.8 | 77.3 | 94.6 | 89.3 | 101.2 | 73.2 | M | 107.4 | 138.1 | 130.8 |
| **8** | 92.5 | 78.5 | 88.3 | 76.3 | 91.6 | 85.4 | 108.0 | 114.7 | 116.6 | 107.9 |
| **9** | 75.6 | 85.6 | 80.3 | 94.0 | 99.6 | 99.5 | 132.9 | 102.6 | 125.3 | 130.2 |
| **10** | 76.8 | 72.6 | 86.0 | 92.5 | 89.4 | 116.8 | 130.0 | 89.1 | 109.4 | 102.1 |
| **Mean (mg/L)** | **83.1** | **74.7** | **86.1** | **84.1** | **105.6** | **96.3** | **112.5** | **103.4** | **121.1** | **119.7** |
| **S.D. (mg/L)** | **7.6** | **9.5** | **4.0** | **8.4** | **12.3** | **16.2** | **14.2** | **8.7** | **11.6** | **12.2** |
| **CV(%)** | **9.2** | **12.7** | **4.7** | **10.0** | **11.7** | **16.8** | **12.6** | **8.4** | **9.5** | **10.2** |

**Table 3.27 –** Results obtained from the centre punches for different volume DBS made using a pool of blood containing the higher concentration of CRP without ethanol (no E) and with ethanol (E) (serum = 92 mg/L).

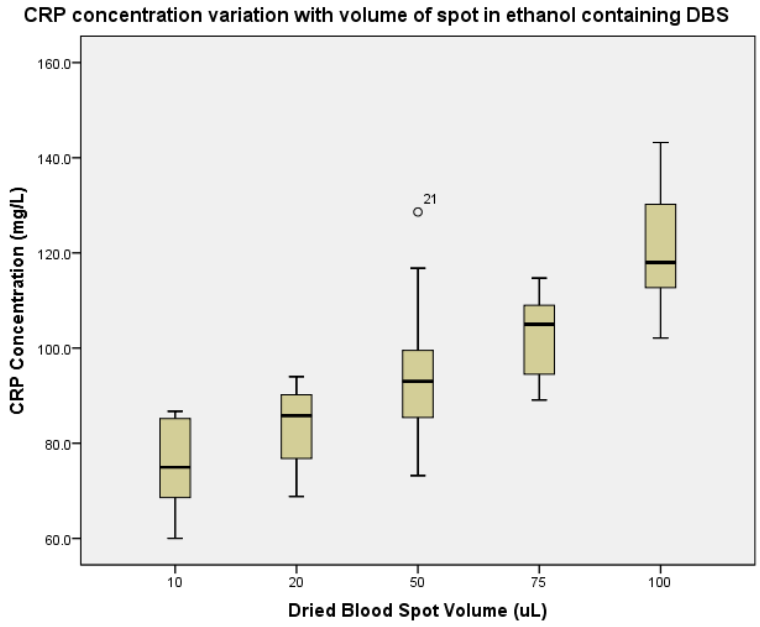



**Figure 3.32 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of CRP with ethanol (n=10 for each volume of DBS).

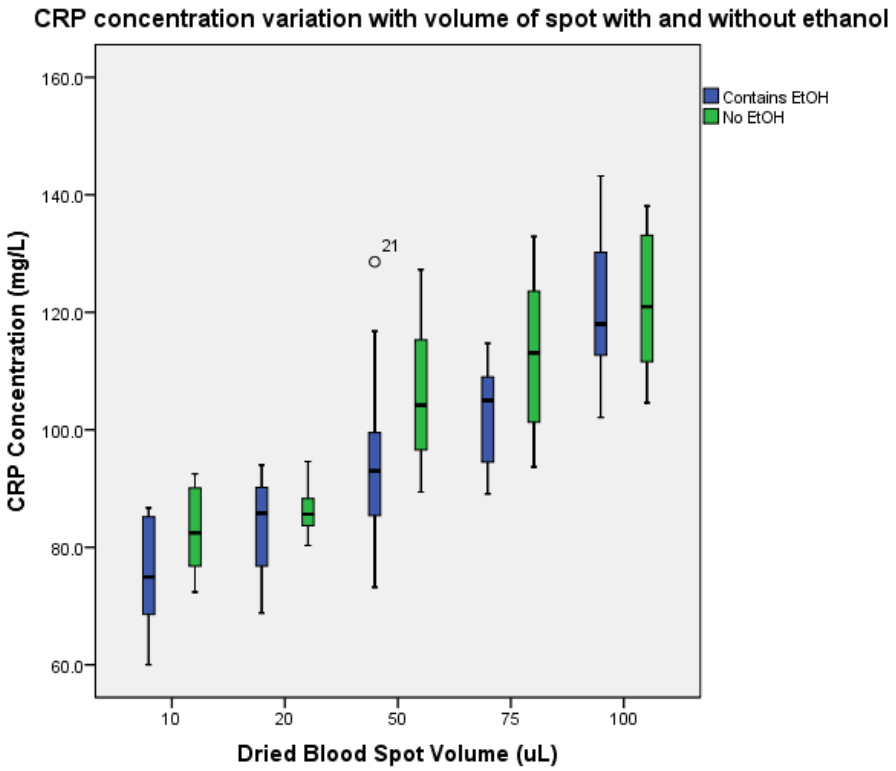



**Figure 3.33 –** Box and whisker plot showing the CRP concentration for punches taken from DBS made from different volumes of blood, using the pool of blood containing the higher concentration of CRP with ethanol and without ethanol (n=10 for each volume of DBS).

For the higher concentration of CRP there was also no difference between ethanol containing DBS and ethanol free DBS for all volumes of DBS except for the 10 µL DBS (all p>0.05 except 10 µL p=0.043, Figure 3.33). Variances were equal for all levels (p>0.05) except for 20 µL (p=0.009).

3.3.2.4.2.2. Location of Punch – Ethanol Containing Samples

| | Edge Punches (75 µL) | | Centre Punches (20 µL) | | Centre Punches (75 µL) | |
|---|---|---|---|---|---|---|
| | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **Mean (mg/L)** | 18.1 | 18.4 | 12.5 | 10.9 | 16.1 | 13.9 |
| **S.D. (mg/L)** | 1.9 | 3.35 | 2.0 | 2.4 | 3.3 | 2.29 |
| **CV(%)** | 10.5 | 18.2 | 15.8 | 22.1 | 20.5 | 16.5 |

**Table 3.28 –** CRP results found when punches were taken from the outer edges and centre of 75 µL DBS made with ethanol (E) containing blood and ethanol free (No E) blood, as well as centre punches for 20 µL DBS made with ethanol containing blood and ethanol free blood (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 14 mg/L.

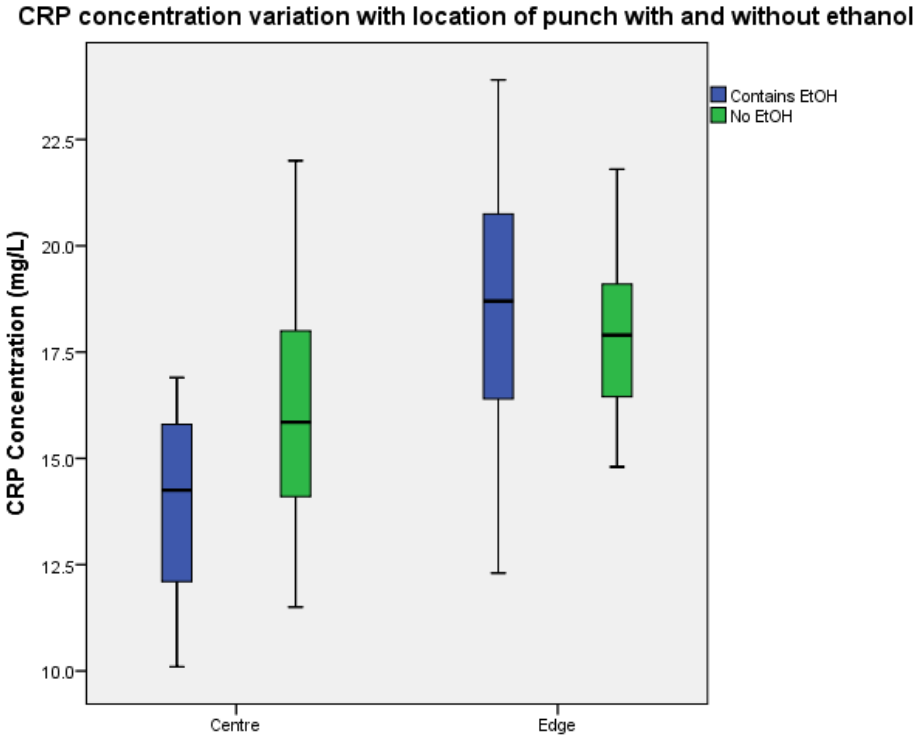



**Figure 3.34 –** Box and whisker plot showing the CRP concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the lower concentration of CRP containing ethanol and without ethanol.

A summary of the CRP results for punches taken from the edge of 75 µL DBS made with ethanol containing blood for the lower concentration of CRP can be seen in Table 3.28 and Figure 3.34. The data were normally distributed for the edge punches (p=0.2) and the mean CRP concentration was significantly higher for ethanol containing edge punches compared to ethanol containing centre punches (variances equal p=0.184, p=0.001).

| | Edge Punches (75 µL) | | Centre Punches (20 µL) | | Centre Punches (75 µL) | |
|---|---|---|---|---|---|---|
| | **No E** | **E** | **No E** | **E** | **No E** | **E** |
| **Mean (mg/L)** | 134.8 | 133.2 | 86.1 | 84.1 | 112.5 | 103.4 |
| **S.D. (mg/L)** | 25.8 | 21.8 | 4.0 | 8.4 | 14.2 | 8.7 |
| **CV(%)** | 19.2 | 16.4 | 4.7 | 10.0 | 12.6 | 8.4 |

**Table 3.29 –** CRP results found when punches were taken from the outer edges and centre of 75 µL DBS made with ethanol (E) containing blood and ethanol free (No E) blood, as well as centre punches for 20 µL DBS made with ethanol containing blood and ethanol free blood (n=20 for the edge punches and n=10 for the centre punches). The serum concentration for this pool of blood was 92 mg/L.

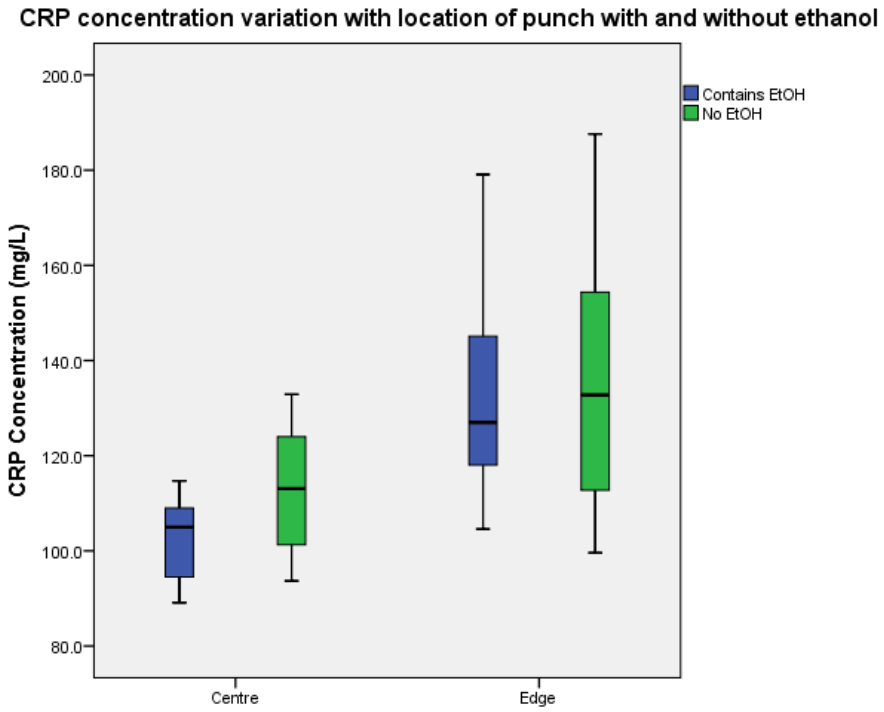



**Figure 3.35 –** Box and whisker plot showing the CRP concentration for centre (n=10) and edge (n=20) punches taken from 75 µL volume DBS, made from the pool of blood containing the higher concentration of CRP containing ethanol and without ethanol.

The same pattern was seen for the higher concentration of CRP – the mean CRP concentration was significantly higher for ethanol containing edge punches compared to ethanol containing centre punches (variances not equal p=0.021, p<0.001), Table 3.29 and Figure 3.35). The data were normally distributed (p=0.2).

### 3.3.2.5. *Haematocrit*

See section 3.2.4.5 (page 65) for the method relating to this section.

#### 3.3.2.5.1. Vitamin D

| | Haematocrit | | | | |
|---|---|---|---|---|---|
| **ID** | **0.206** | **0.3** | **0.408** | **0.49** | **0.62** |
| **1** | 39.4 | 42.0 | 42.6 | 39.8 | 40.0 |
| **2** | 46.5 | 52.4 | 43.8 | 47.6 | 39.4 |
| **3** | 56.4 | 50.8 | 45.2 | 39.3 | 37.7 |
| **4** | 49.0 | 47.3 | 44.7 | 44.8 | 40.6 |
| **5** | 51.8 | 44.7 | 43.2 | 49.5 | 36.1 |
| **6** | 50.1 | 54.6 | 42.7 | 45.0 | 37.0 |
| **7** | 55.4 | 51.4 | 48.8 | 38.3 | 35.1 |
| **8** | 39.3 | 49.4 | 65.3 | 50.1 | 34.9 |
| **9** | 49.3 | 44.6 | X | 47.5 | 40.0 |
| **10** | 31.5 | 45.3 | 43.4 | 41.7 | 38.0 |
| **Mean (nmol/L)** | **46.9** | **48.3** | **46.6** | **44.4** | **37.9** |
| **S.D. (nmol/L)** | **7.9** | **4.1** | **7.3** | **4.4** | **2.1** |
| **CV(%)** | **16.8** | **8.5** | **15.6** | **9.8** | **5.5** |

**Table 3.30 –** Concentration of 25(OH)D obtained from the centre punches for 20µL DBS made with different levels of haematocrit, using a pool of blood with a lower concentration of 25(OH)D. X = mistake in extraction, unable to obtain result. Serum 25(OH)D = 44 nmol/L.

A summary of the 25(OH)D results for DBS made with different levels of haematocrit for the lower concentration of 25(OH)D can be seen in Table 3.30 and Figure 3.36. The data were normally distributed for all levels of haematocrit (p>0.05) except for haematocrit = 0.408 (p=0.002). The mean 25(OH)D concentration was significantly different for the different levels of haematocrit (p=0.001). Post-hoc analysis showed that the 0.408 haematocrit DBS were not significantly different from the other levels

except for the 0.62 level (p=0.044), which showed a lower concentration of 25(OH)D. There does appear to be an overall trend of decreasing 25(OH)D concentration with increasing haematocrit and this may have been more apparent had the CVs for the lower concentration samples been better.

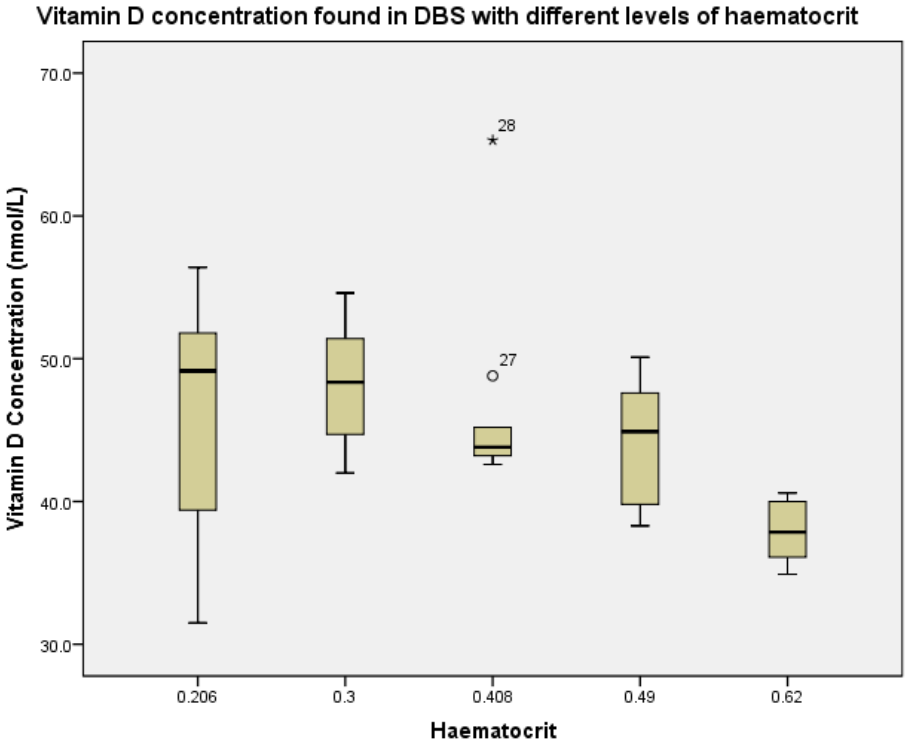



**Figure 3.36 –** Box and whisker plot showing the 25(OH)D concentration for centre punches taken from DBS made with different levels of haematocrit, using a pool of blood with a lower concentration of 25(OH)D (n=10 for each level of haematocrit).

A similar pattern was seen with the higher concentration of 25(OH)D – there was a significant difference between the different levels of haematocrit (p<0.001, Table 3.31 and Figure 3.37). The data were normally distributed (all p>0.05) and post-hoc analysis (variances unequal p=0.046) showed that the 0.4 haematocrit DBS were not significantly different from the 0.326 haematocrit DBS (p=0.093) and the 0.51 haematocrit DBS (p=0.856) but they were significantly different from the 0.22 haematocrit DBS (p=0.004) and the 0.62 haematocrit DBS (p=0.20). For this level of

25(OH)D, there was an overall decrease in concentration in 25(OH)D with increasing levels of haematocrit.

| | Haematocrit | | | | |
|---|---|---|---|---|---|
| **ID** | **0.22** | **0.326** | **0.4** | **0.51** | **0.62** |
| **1** | 157.7 | 151.3 | 123.4 | 133.0 | 129.3 |
| **2** | 169.6 | 163.6 | 158.2 | 148.5 | 150.0 |
| **3** | 176.4 | 161.4 | 137.6 | 130.5 | 117.3 |
| **4** | 176.5 | 166.3 | 158.9 | 153.6 | 145.4 |
| **5** | 177.9 | 157.3 | 140.3 | 143.7 | 129.8 |
| **6** | 184.5 | 157.1 | 137.9 | 152.7 | 116.3 |
| **7** | 176.0 | 168.2 | 158.7 | 143.9 | 119.2 |
| **8** | 171.7 | 164.7 | 155.8 | 154.4 | 125.3 |
| **9** | 158.5 | 159.1 | 142.5 | 137.9 | 118.3 |
| **10** | 167.2 | 169.6 | 169.9 | 132.3 | 129.9 |
| **Mean (nmol/L)** | **171.6** | **161.9** | **148.3** | **143.1** | **128.1** |
| **S.D. (nmol/L)** | **8.6** | **5.7** | **14.1** | **9.2** | **11.7** |
| **CV(%)** | **5.0** | **3.5** | **9.5** | **6.4** | **9.1** |

**Table 3.31 –** Concentration of 25(OH)D obtained from the centre punches for 20µL DBS made with different levels of haematocrit, using a pool of blood with a higher concentration of 25(OH)D. Serum 25(OH)D = 151 nmol/L.

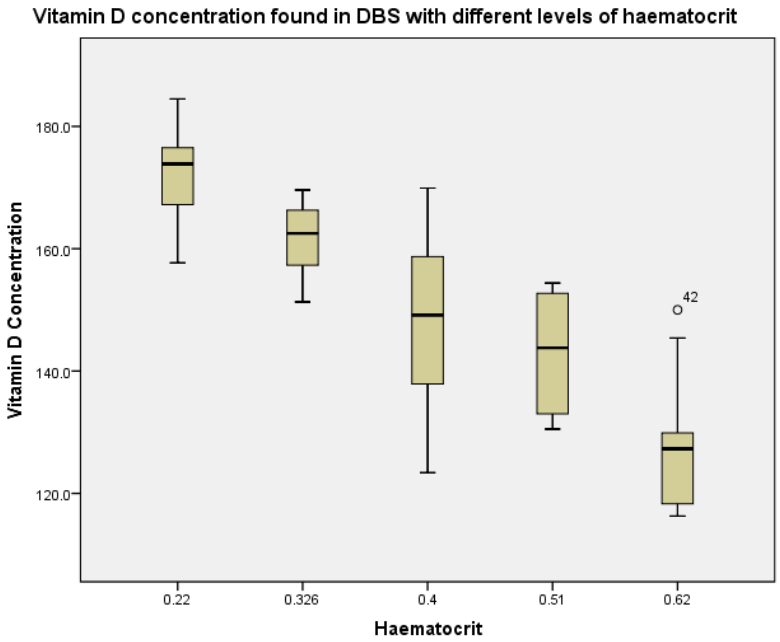



**Figure 3.37 –** Box and whisker plot showing the 25(OH)D concentration for centre punches taken from DBS made with different levels of haematocrit, using a pool of blood with a higher concentration of 25(OH)D (n=10 for each level of haematocrit).

3.3.2.5.2. CRP – SWBH Device

| | Haematocrit | | | | |
|---|---|---|---|---|---|
| **ID** | **0.192** | **0.302** | **0.42** | **0.505** | **0.604** |
| **1** | 21.5 | 20.7 | 13.0 | 11.0 | 13.9 |
| **2** | 18.4 | 16.9 | 18.6 | 20.4 | 12.7 |
| **3** | 24.4 | 21.7 | 16.7 | 11.5 | 6.4 |
| **4** | 23.6 | 19.6 | 16.6 | 9.1 | 13.0 |
| **5** | 16.3 | 21.8 | 18.6 | 11.9 | 10.6 |
| **6** | 18.5 | 21.6 | 18 | 12.2 | 9.0 |
| **7** | 19.7 | 20.5 | M | 11.5 | 4.5 |
| **8** | 21.1 | 21.1 | 13.8 | 11.4 | 12.3 |
| **9** | 25.1 | 24.1 | 21.1 | 20.2 | 15.1 |
| **10** | 20.5 | 23.9 | 22.9 | 23.7 | 17.3 |
| **Mean (mg/L)** | **20.9** | **21.2** | **17.7** | **14.3** | **11.5** |
| **S.D. (mg/L)** | **2.8** | **2.1** | **3.2** | **5.1** | **3.9** |
| **CV(%)** | **13.6** | **9.7** | **17.8** | **35.6** | **34.2** |

**Table 3.32 –** Concentration of CRP obtained from the centre punches for 20µL DBS made with different levels of haematocrit, using a pool of blood with a lower concentration of CRP. M = mushy and unable to obtain result. Serum value = 20 mg/L.

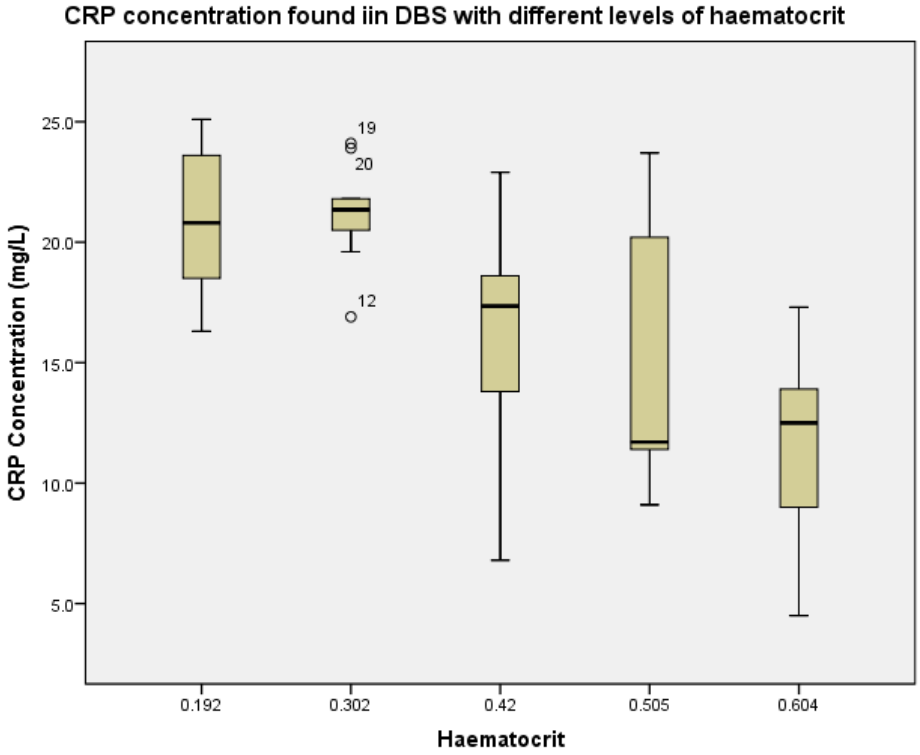



**Figure 3.38 –** Box and whisker plot showing the CRP concentration for centre punches taken from DBS made with different levels of haematocrit, using a pool of blood with a lower concentration of CRP (n=10 for each level of haematocrit).

A summary of the CRP results for DBS made with different levels of haematocrit for the lower concentration of CRP can be seen in Table 3.32 and Figure 3.38. The data were normally distributed for all levels of haematocrit ($p>0.05$) except for the 0.505 haematocrit ($p=0.001$). There was a general trend of decreasing CRP with increasing haematocrit. The mean CRP concentration was significantly different for the different levels of haematocrit ($p<0.001$). Post-hoc analysis showed that the 0.42 haematocrit DBS were not significantly different from the other levels of haematocrit (all $p>0.05$). The significant difference was due to differences between the higher levels of haematocrit and lower levels of haematocrit.

| | **Haematocrit** | | | | |
|---|---|---|---|---|---|
| **ID** | **0.209** | **0.29** | **0.408** | **0.496** | **0.615** |
| **1** | 22.4 | 23.5 | 45.5 | 41.8 | 28.6 |
| **2** | M | 22.3 | 32.2 | 38.1 | 48.9 |
| **3** | 17.2 | 20.5 | 43.2 | 32.6 | 41.6 |
| **4** | 20.1 | 23.0 | 38.7 | 39.4 | 39.7 |
| **5** | 23.7 | 16.9 | 40.0 | 38.3 | 40.3 |
| **6** | 25.0 | 15.2 | 42.5 | 32.6 | 37.9 |
| **7** | 25.7 | 23.3 | 37.4 | M | 41.2 |
| **8** | 23.4 | 24.7 | 41.1 | 50.6 | 40.4 |
| **9** | 28.3 | 17.7 | 34.1 | 43.0 | 38.5 |
| **10** | 16.1 | 22.7 | 42.4 | 39.8 | 44.3 |
| **Mean (mg/L)** | **22.4** | **21.0** | **39.7** | **39.6** | **40.1** |
| **S.D. (mg/L)** | **4.0** | **3.3** | **4.2** | **5.5** | **5.1** |
| **CV(%)** | **17.8** | **15.5** | **10.5** | **13.8** | **12.8** |

**Table 3.33 –** Concentration of CRP obtained from the centre punches for 20µL DBS made with different levels of haematocrit, using a pool of blood with a higher concentration of CRP. M = mushy and unable to obtain result. Serum CRP = 44 mg/L.

A different pattern was seen with the higher concentration of CRP – a higher level of CRP was found with higher levels of haematocrit (Table 3.33 and Figure 3.39). The data were normally distributed for all levels of haematocrit ($p>0.05$) and the mean CRP concentration was significantly different for the different levels of haematocrit ($p<0.001$). Post-hoc analysis (variances equal $p=0.96$) showed that the 0.408

haematocrit DBS were significantly different from the lower levels of haematocrit (both $p<0.001$) but were not significantly different to the other levels of haematocrit (both $p=0.99$).

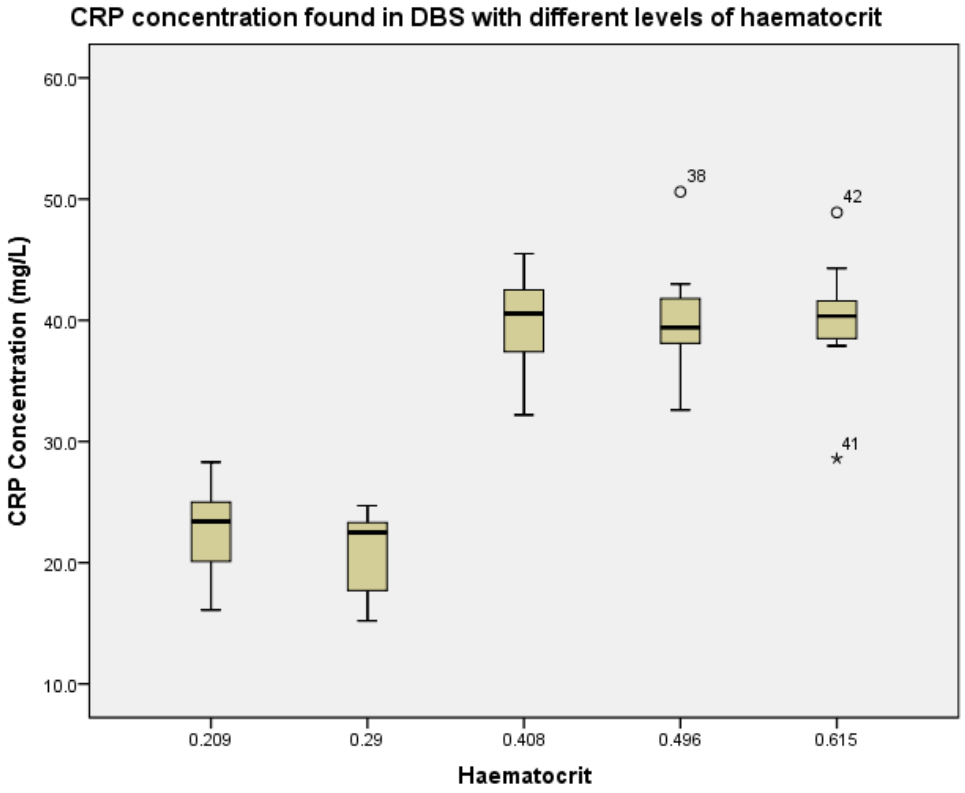



**Figure 3.39 –** Box and whisker plot showing the CRP concentration for centre punches taken from DBS made with different levels of haematocrit, using a pool of blood with a higher concentration of CRP (n=10 for each level of haematocrit).

3.3.2.5.3. CRP – Mitra Device

A summary of the CRP results for Mitra devices made with different levels of haematocrit for the lower concentration of CRP can be seen in Table 3.34 and Figure 3.40. The data were normally distributed for all levels of haematocrit ($p>0.05$). There was a general trend of decreasing CRP with increasing haematocrit. The mean CRP concentration was significantly different for the different levels of haematocrit ($p<0.001$). Post-hoc analysis (variances unequal $p<0.01$) showed that there was a

significant difference in CRP concentration between all levels of haematocrit ($p<0.001$ for all pairs except between 0.192 and 0.302 haematocrit, $p=0.02$).

| | Haematocrit | | | | |
|---|---|---|---|---|---|
| **ID** | **0.192** | **0.302** | **0.42** | **0.505** | **0.604** |
| **1** | 33.5 | 25.7 | 21.2 | 18.9 | 13.8 |
| **2** | 32.7 | 26.8 | 20.9 | 17.3 | 13.2 |
| **3** | 28.8 | 25.7 | 20 | 17.9 | 13 |
| **4** | 28 | 23.6 | 21.4 | 20.3 | 12.8 |
| **5** | 27.6 | 26.2 | 20.4 | 18.1 | 12.7 |
| **6** | 28 | 26.4 | 19.2 | 18.7 | 14 |
| **7** | 27.8 | 24.7 | 19.5 | 18.1 | 13.9 |
| **8** | 25.9 | 25.8 | 20.3 | 17.5 | 12.3 |
| **9** | 31.8 | 27.8 | 20.2 | 17.4 | 14.9 |
| **10** | 26.3 | 23 | 22.1 | 19.1 | 13 |
| **Mean (mg/L)** | **29.0** | **25.6** | **20.5** | **18.3** | **13.4** |
| **S.D. (mg/L)** | **2.7** | **1.4** | **0.9** | **0.9** | **0.8** |
| **CV(%)** | **9.2** | **5.7** | **4.3** | **5.1** | **5.8** |

**Table 3.34 –** Concentration of CRP obtained Mitra devices made with different levels of haematocrit, using a pool of blood with a lower concentration of CRP. Serum CRP = 20 mg/L.

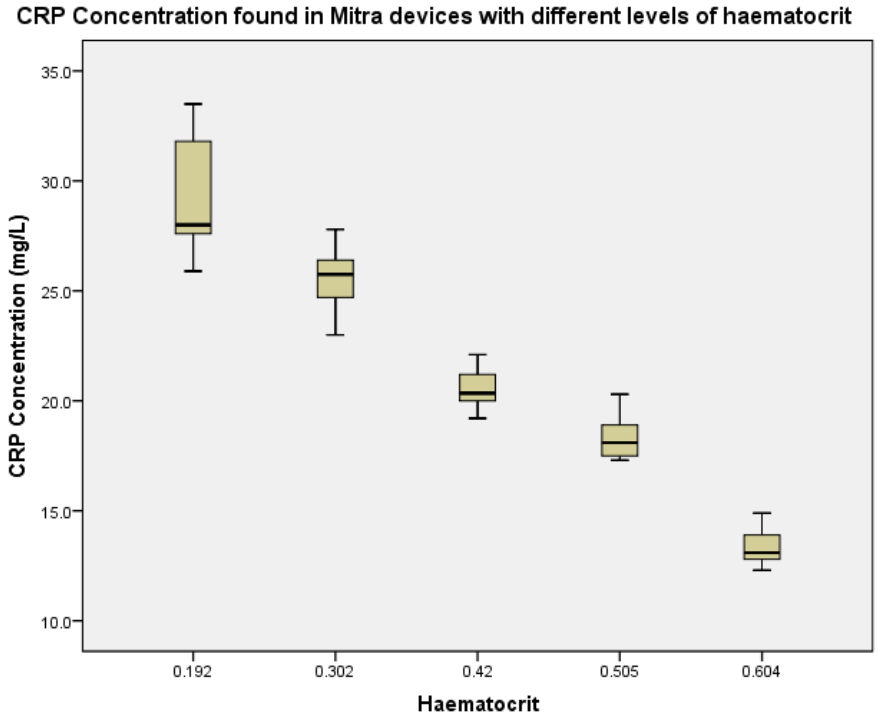



**Figure 3.40 –** Box and whisker plot showing the CRP concentration for Mitra devices made with different levels of haematocrit, using a pool of blood with a lower concentration of CRP (n=10 for each level of haematocrit).

| | Haematocrit | | | | |
|---|---|---|---|---|---|
| **ID** | **0.206** | **0.3** | **0.408** | **0.49** | **0.62** |
| **1** | 26.2 | 28.1 | 46.9 | 44.0 | 28.1 |
| **2** | 29.2 | 28.2 | 47.7 | 36.4 | 33.5 |
| **3** | 30.6 | 26.0 | 45.9 | 38.1 | 29.7 |
| **4** | 29.0 | 25.6 | 44.3 | 39.8 | 29.8 |
| **5** | 28.8 | 27.3 | 46.5 | 37.3 | 31.0 |
| **6** | 30.2 | 29.2 | 44.9 | 41.8 | 31.8 |
| **7** | 27.9 | 28.3 | 48.6 | 29.3 | 29.6 |
| **8** | 28.3 | 27.2 | 47.9 | 39.7 | 29.3 |
| **9** | 28.7 | 28.7 | 47.4 | 37.9 | 33.4 |
| **10** | 27.1 | 25.5 | 46.5 | S | 33.5 |
| **Mean (mg/L)** | **28.6** | **27.4** | **46.7** | **38.3** | **31.0** |
| **S.D. (mg/L)** | **1.3** | **1.3** | **1.3** | **4.1** | **2.0** |
| **CV(%)** | **4.6** | **4.8** | **2.9** | **10.7** | **6.4** |

**Table 3.35 –** Concentration of CRP obtained Mitra devices made with different levels of haematocrit, using a pool of blood with a higher concentration of CRP. Serum CRP = 44 mg/L.

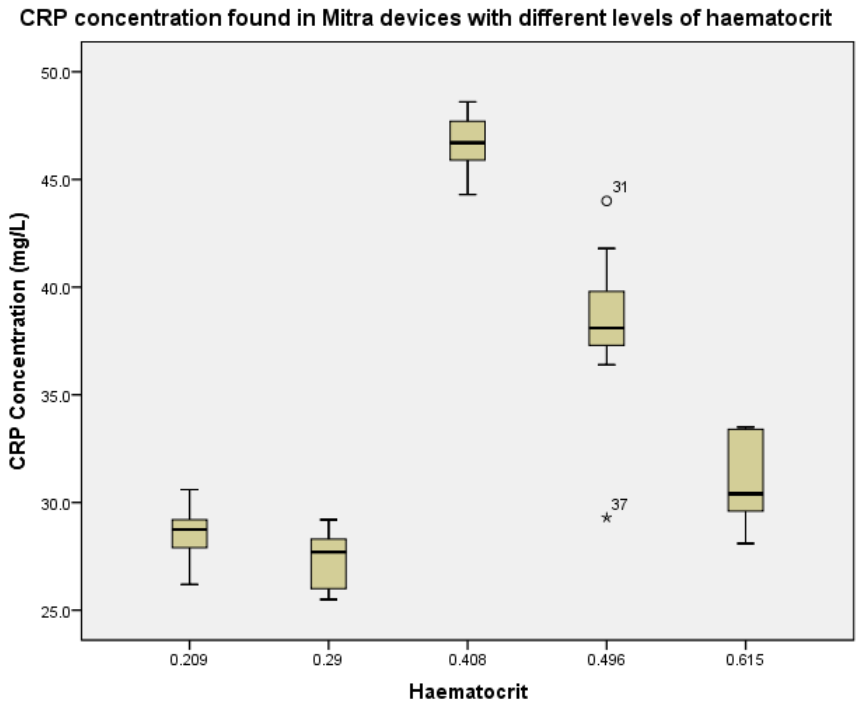



**Figure 3.41 –** Box and whisker plot showing the CRP concentration for Mitra devices made with different levels of haematocrit, using a pool of blood with a higher concentration of CRP (n=10 for each level of haematocrit).

A different pattern was seen with the higher concentration of CRP – the highest level of CRP was seen with the 0.408 haematocrit, all other haematocrits showed a lower

level of CRP (Table 3.35 and Figure 3.41). The data were normally distributed for all levels of haematocrit ($p>0.05$) and the mean CRP concentration was significantly different for the different levels of haematocrit ($p<0.001$). Post-hoc analysis (variances equal $p=0.058$) showed that the 0.408 haematocrit devices were significantly different from all the other levels of haematocrit (all $p<0.001$).

## 3.4. Conclusion

### 3.4.1. Blood Spot Quality Assessment

The work undertaken here has shown that the public are able to successfully take quality DBS, with an insufficient rate that is acceptable. We have shown that different makes of lancet can lead to a difference in the quality of DBS received. In addition, we have shown that the design of the collection device, in terms of the presence of printed circles and size of collection guide, can lead to a difference in the characteristics of the spots collected.

The DBS collection device has been successfully used in a direct to the public setting producing good quality DBS. Our unique design of collection device has been proven to work in a non-clinical environment. Although there are other commercially available devices, all have the same feature of the filter paper being open to the environment and require the blood to be left exposed to air for at least three hours. This was also true of the collection device in the PD kit and is the main reason we chose to develop our own fit for purpose device.

There were some precedents set relating to the use of DBS as a means of the public getting samples to the laboratory without the aid of a healthcare professional, with several DBS collection kits appearing on the market offering direct to the public

services before us around the world. The most comprehensive service was ZRT, (49) who supply a DBS collection kit that can be used for a wide range of assays for DBS analysis.

Our direct to the public DBS vitamin D service was the first DBS vitamin D method in the UK and was the first to be made widely available to the public. The insufficient rate for DBS was relatively low at <3.0%, probably because the method only requires one 3 mm punch. This would not be possible without the derivatisation step used in our method, which increases the sensitivity of the assay when using LC/MS/MS. Vitamin D metabolites have low ionisation efficiencies in ESI sources due to the lack of easily charged functional groups. By derivatising the 25(OH)D, more easily charged functional groups are added into the molecule, increasing the ionisation efficiency of the molecule. (132) Eyles et al. (133) also measured $25(OH)D_2$ and $25(OH)D_3$ by LC/MS/MS using PTAD as a derivatising agent.

Newman et al., (122) published a method for measuring $25(OH)D_2$ and $25(OH)D_3$ in DBS without derivatisation, but this requires four 6 mm punches. According to our data, using the PD kit which provides the most amount of sample for 6 mm punches, only 58% of samples would be sufficient, rendering the method impractical to use. The use of so much of the sample would mean it would be unlikely that any sample would be left for repeat analysis, should the assay fail or a result require checking.

The rate of insufficient sample seen for our DBS quality assessment (2.7%) is similar to that found in a study by Williams and McDade. (32) They found that 1.4% of DBS samples collected by field interviewers were insufficient for analysis. Approximately 1% of GP serum samples in our laboratory could not be analysed for vitamin D because there was insufficient for analysis, the sample was lost in transit or had

leaked. In one year 0.15% of missing results were due to similar problems at the laboratory of San Raffaele Hospital in Italy. (134)

The number of DBS samples that cannot be analysed is higher than for serum, but it is not enough to make DBS unusable for our DBS methods. The inconvenience to the patient caused by insufficient DBS may be less than for serum, as the patient can repeat the sample in their own time at home. If a 6 mm punch is required for analysis, our DBS quality assessment shows that the lowest rate of 6 mm insufficient samples (using the PD kit) was 8.5%. This is an unacceptably high rate, made worse when quality of spots are taken into account (10%), and so it may be advantageous to optimise methods to use less sample or use multiple smaller punches instead. This is particularly pertinent given that the PD kits were designed to collect larger volumes of blood (larger guide area) compared with the SWBH devices.

The results clearly demonstrate that the public have the potential to take quality DBS. Some of the parameters investigated, such as double spotting and finger pressing, have subjective elements and so these may have been over or under reported. The most common problem was finger pressing with more than half of the samples appearing to show some evidence of it; however this is a minor problem and does not necessarily mean a quality punch cannot be obtained, as long as the blood has soaked all the way through the paper. Designing DBS assays that use as few small punches as possible will ensure that the majority of DBS samples received by the lab can provide quality punches for analysis.

The type of lancet used may have an effect on the quality of DBS obtained. The BD lancet appeared to make it easier for people to collect DBS with good technique and a larger “volume” of blood was collected using the BD lancet compared with the

Sarstedt lancets. This has also been reproduced in the laboratory, when we have had to collect DBS samples for method evaluation onto SWBH devices – more blood is obtained more easily with the BD lancets than with the Sarstedt lancets. As a result of this work, all further SWBH kits produced contained the blue BD safety lancets.

The PD kits appeared to provide a greater “volume” of blood than the SWBH kits using the BD lancet. As there was no statistical difference between whether or not good technique was present, it may be that the difference seen between the two kits is due to the presence of the printed circles.

There has been speculation in the literature that printing on the filter paper collection area could lead to compression of the filter paper fibres leading to variation in the volume of blood contained in it. (135) Holub et al., (136) investigated this potential source of error by pipetting 70 µL of blood onto printed and unprinted areas of filter paper and then measuring the width of the blood spot created. They could not detect any significant differences between the printed (mean = 13.38 mm, SD = 0.53) and unprinted (mean = 13.42 mm, SD = 0.51) areas of the paper. Therefore the actual printing on the PD filter paper itself is unlikely to be cause of the difference in the “volume” of blood collected between the SWBH and PD kits.

The difference between the two devices may be because the printed circles on the PD kits may help people to collect larger spots as the size guide is more apparent and larger. This may also explain the higher rate of overlapping spots for SWBH samples compared to PD. The collection area is larger for the PD kit and so spots can be more spaced out and the risk of overlapping is reduced. As we only require 3 mm punches and our calibrator and QC material is based on a spot size that is similar to the majority of patient samples we receive, there is a better matching of

sample size to calibrator and QC size for SWBH kits than for PD kits. We therefore do not need printed circles on our collection device. However, if larger spots are required, it would be advisable to use collection devices with printed circles on the filter paper and match QC and calibrators to this larger size.

We have clearly demonstrated that the public are capable of taking quality DBS in their own home and that DBS are a feasible pathway in the patient journey, showing that they can be used in routine clinical practice.

### 3.4.2. **DBS Characteristics**

The work undertaken in this chapter has shown how variable results can be due to DBS characteristics. We have shown for both 25(OH)D and CRP that increasing the volume of DBS leads to an increase in concentration and that punches taken from the edge of DBS lead to higher concentrations. We also found that using haemolysed blood leads to significantly higher levels of 25(OH)D and lower levels of CRP, although this was only significant for Mitra devices. The presence of ethanol caused variable results for 25(OH)D and CRP but did not remove the patterns seen with volume of spots or location of punch. Finally, we showed that haematocrit can have a significant effect on the results obtained for both 25(OH)D and CRP. It is important to have an understanding of these issues before launching a DBS assay so that best practice can be undertaken in terms of matching calibrator and QC DBS to likely patient DBS size and haematocrit, and to not unnecessarily manipulate calibrator blood.

For our 25(OH)D assay we use 20 µL of blood to make our calibrator and QC material. The DBS quality work and the experience gained from running a direct to the public vitamin D service has shown that this size is similar to the majority of DBS

received from the public. This is important as the DBS characteristic work showed that increasing DBS volume results in an increased concentration of 25(OH)D. The difference only becomes significant between the 20 µL DBS and the 75 µL and 100 µL DBS. This held true for both concentrations of 25(OH)D investigated. These differences however were quite substantial, therefore wherever possible the laboratory must make sure that if a selection of DBS are available for analysis, ones in size most similar to the calibrators should be used.

The same pattern was seen for the CRP DBS samples – increasing volume leads to an increase in CRP concentration. However, at higher concentrations the 20 µL DBS was significantly different to all other volumes except the 10 µL. Whether or not this degree of difference is acceptable or not will depend on the clinical application of the assay. The DBS CRP assay at SWBH was developed for use with rheumatoid arthritis (RA) patients to aid with monitoring the efficacy of treatment (see Chapter 5). It is unlikely that patients will have such high CRP concentrations as seen with the higher CRP concentration pool used in this blood spot characteristic work and if they do, this may be due to something other than their RA and will need further investigation in any case. Therefore the difference between 20 µL DBS and the larger volume DBS would not be so important in this context. The alternative would be to take a very strict approach to selecting DBS of the appropriate size when sampling DBS for analysis, however this is likely to lead to an increase in the rejected sample rates.

Kvaskoff et al., (137) investigated the effect of DBS volume on 25(OH)D concentrations on their DBS 25(OH)D assay. They used 50 µL DBS calibrators in their assay made from whole blood spiked with ethanol. They also found that the

higher the DBS volume the higher the concentration of 25(OH)D. They found no significant difference between 50 µL and 100 µL DBS, but they did find a significant difference between 50 µL DBS and 25 µL and 10 µL DBS. They concluded that "smaller spot volumes lead to spuriously lower results" and that the likely reason for this was due to diminished blood absorption in spots that are less than 50 µL. They gave no evidence for this however and were basing their conclusions on an assay that used 50 µL calibrators. They went on to recommend that DBS samples be sourced from a minimum volume of 50 µL – I would argue that it is far better to base a DBS assay on the likely size of samples you will be receiving, as we have done for our 25(OH)D assay and where we see no significant difference in results for the smaller blood spot volumes which we most commonly receive.

The volume of blood used to make DBS and the effect this has on results was examined whilst the standardisation of phenylalanine and TSH material used for international NBS programmes was being investigated. Dhondt et al., (88) and Elvers and Loeber (89) showed how increased blood volume used to prepare the DBS standards resulted in significantly increased concentrations of phenylalanine (88) and TSH. (89) Both sets of authors (as well as others (113)) concluded that it would be advantageous to use standards and QC that were prepared with volumes of blood likely to be seen in DBS made by the target population e.g. 50-60 µL for NBS, the volume laid out by the printed circles on Guthrie cards.

Other researchers have found that the volume of blood used to create DBS has minimal effect on results obtained. Vu et al., (70) assessed the impact of varying blood spot volume (30–100 µL) on concentration of moxifloxacin in DBS and found that although the volume of blood used was directly proportional to the concentration,

the effect was less than 15% and deemed to be acceptable. Elbin et al., (90) found that differences in activities amongst five lysosomal enzymes (acid α-glucosidase, acid α-galactosidase, acid β-glucocerebrosidase, acid sphingomyelinase and galactocerebrosidase) in DBS made using different volumes of blood (25 µL-125 µL) were minor compared to the mean activity of the control DBS (75 µL), although they also found that increasing activity was found with increasing DBS blood volume. Liang et al., (124) found that although there was some variation in results obtained for dextromethorphan and dextrorphan in DBS when different blood volumes were used to make the DBS (18.5% and 18.8% respectively when volume went from 10 to 50 µL), this was not enough of a variation to require that samples be collected using an accurate volumetric technique.

O'Broin (138) noted that when using an isotopic method to estimate punch volume, apparent punch volume increased with increasing size of blood spot. Mei et al., (24) similarly showed that increasing the volume of blood used to create DBS led to an increase in the apparent volume of serum contained within punches. A 13% increase in serum volume was seen when blood spot volume increased from 25 µL to 125 µL. This may help to account for some of the differences in concentration seen with different DBS volume, alongside differences due to chromatographic effects.

Variation in results do seem to occur when different volumes of blood are used to create DBS, but the differences are not so great that they preclude the use of DBS. As with the other parameters, this phenomenon needs to be assessed as part of assay validation to establish what variation may result so that appropriately sized calibrators and QC can be made and so that users can be advised that certain sized spots need to be collected, or else alert laboratory staff to reject samples that are

above or below a certain size. Alternatively it may be found that this parameter is not an issue at all or that variation can be minimised by ensuring calibrators are made that reflect average patient sample DBS size.

A further parameter that needs investigation before a DBS assay can be put into routine use is location of punch. This is under the control of the laboratory therefore once the effect has been established, best practice in relation to the handling of patient samples, QC and calibrator material can be put into practice.

The work done here showed that for both 25(OH)D and CRP, at both concentrations investigated, punches taken from the edges of DBS led to higher results. Apart from CRP at lower concentrations, this effect was significant. Given that larger volume DBS will also lead to an increase in concentration, by the time an edge punch is taken the results can be substantially different from what would have been obtained from an “ideal” DBS. In the case of the higher concentration 25(OH)D DBS, edge punches taken from a 75 µL DBS were on average almost twice that of 20 µL DBS. As a result we consistently sample all patient, QC and calibrator material from the centre of the spot. This is less of an issue with the generally smaller spots that we receive into our laboratory as part of the routine vitamin D DBS service.

Kvaskoff et al., (139) also found that punches taken from the edge of DBS contained significantly higher levels of 25(OH)D compared to central punches. They found that outer punches were significantly heavier and concluded that the outer punches must therefore contain more blood, resulting in higher levels of 25(OH)D. However, they also felt that chromatographic effects – the way 25(OH)D interacted with and moved across the filer paper – could come into play. If serum moves to the edges of DBS at a greater rate than red blood cells, resulting in a relatively higher level of serum at the

outer edges compared to the centre, then as 25(OH)D is found in serum and not red blood cells it would follow that 25(OH)D would be found at higher concentrations at the periphery of DBS.

This chromatographic phenomenon is a likely explanation as to why not all analytes show an increase in concentration in peripheral punches relative to central punches. For example, Holub et al., (136) found that only 14 out of the 31 analytes they tested (mainly amino acids and acylcarnitines), showed an increased concentration in peripheral punches from DBS compared with central punches, and this effect was haematocrit dependent for 10 of those analytes. Liang et al., (124) investigated the use of DBS for analysis of dextromethorphan and its metabolite dextrorphan by LC/MS/MS. Punches were taken from central and peripheral locations from DBS containing four different concentrations of the two drugs and analysed. The authors could find no significant difference between central and peripheral punches. Given the variable nature of the chromatographic effect and its influence on the concentration found in central and peripheral punches taken from DBS, each analyte should be investigated before an assay is put into routine use. It may be that no effect is seen, however if it is, steps should be put into place to minimise variation, either by always sampling from a central (or peripheral) location or by collecting spots that are small enough to prevent edge spots from being taken.

The use of whole blood or haemolysed blood to make calibrators and QC is another aspect of DBS preparation that needs to be considered. Haemolysed blood has a decreased viscosity and therefore will spread further through the filter paper when used to create DBS. (89) A punch from a DBS prepared with haemolysed blood may

contain less blood than a DBS prepared with intact whole blood and the chromatographic effects may be different. (9)

We found for our SWBH CRP assay that the mean concentration was lower in haemolysed DBS compared with intact DBS but not significantly so. When the Mitra device was used, significantly lower levels of CRP were found in haemolysed blood. Interestingly, we found the opposite for 25(OH)D, with significantly higher levels found in haemolysed DBS compared with intact.

Slazyk et al., (135) investigated the variation in results for TSH, thyroxine and phenylalanine for calibrators made with intact or haemolysed whole blood. They found that results for thyroxine and TSH were generally higher when intact whole blood was used, whereas phenylalanine results were slightly higher for some of the lysed DBS specimens than for the intact DBS specimens. This was accounted for by the different distributions of the different analytes in whole blood. Phenylalanine is thought to be more evenly distributed through whole blood whereas TSH and thyroxine are thought to be mainly associated with the serum component of blood. However, this does not explain our 25(OH)D results as 25(OH)D is exclusively found in the serum and therefore following Slazyk et al.'s reasoning, we should have found a similar pattern to their TSH and thyroxine results whereas we found the opposite. It may be that by haemolysing the blood, the way 25(OH)D spreads across the filter paper was altered and led to a higher proportion being found in the centre of the spot compared with when intact whole blood was used.

This may also explain the significant differences seen for CRP for the Mitra device, except instead of the CRP flowing differently across the filter paper, the haemolysed and whole blood may be flowing into the Mitra sponge differently. It is likely that the

same volume of liquid is being taken up regardless of whether the sample is intact or haemolysed, but potentially the haemolysed samples are effectively diluting the CRP in the serum component and so relatively less CRP is being taken up by the Mitra. It would be interesting to investigate this phenomenon further with the Mitra devices for other analytes.

Dhondt et al., (88) also found that differences in phenylalanine concentration occurred when calibrators were made with intact or lysed red blood cells, however they found that concentrations were significantly higher when intact red blood cells were used compared with haemolysed blood. They were investigating this as the CDC had recommended the use of lysed red blood cells in the preparation of DBS materials in order to avoid the potential variability introduced by uncontrolled lysis of red blood cells during the drying process. However, it was the opinion of several authors (113, 115) that it would be preferable to use intact red blood cells in the preparation of standards as this would most closely mimic the type of sample that would be obtained in NBS programmes. That is why the first European working standard for phenylalanine (EWS-(115)Phe-01) was made using whole blood. (115) In fact the CDC appeared to change their mind on the use of haemolysed material to make DBS calibrators and QC as they went on to use whole blood for the Accuracy-based Amino Acid Reference Materials used in NBS programmes in the USA. (119)

There are many examples of published papers where DBS assays have been calibrated by DBS calibrators that have been made from whole blood spiked with alcoholic standards. (77, 123, 127) The 25(OH)D DBS method used by Kvaskoff et al., (133, 137) is calibrated by DBS made using whole blood spiked with ethanolic standards (0.5%, v/v). Although this is not a method used by us in the preparation of

our 25(OH)D DBS standards (the ethanolic standard is first dried down under compressed air before being reconstituted in the required volume of whole blood to give the desired concentration), we were interested in seeing what effect the presence of ethanol in the whole blood used to make DBS material had, and if it could negate the effects seen on results by the volume of blood used to make DBS and the location of punch.

We found that the presence of ethanol resulted in slightly lower levels of 25(OH)D compared with non-spiked blood at lower concentrations of 25 (OH)D although this was only significant for 20 µL, 50 µL and 75 µL volume DBS. At the higher concentration of 25(OH)D we found no significant difference between DBS made with and without ethanol for all volumes of DBS tested, although in general ethanol containing DBS had slightly higher concentrations of 25(OH)D. The trend of increasing 25(OH)D with increasing DBS volume remained when ethanol was present in the DBS, as did the fact that edge punches resulted in significantly higher 25(OH)D compared with central punches.

Similarly to us, Kvaskoff et al., (137) found variable results when looking at the effect of the presence of ethanol on 25(OH)D results. They found that mean 25(OH)D was higher in central punches from ethanol spiked DBS compared to unspiked DBS for samples made from Whatman 903 paper, however with Whatman FTA paper there were slightly lower levels of 25(OH)D in central punches from spiked DBS compared with unspiked. There is no comment as to whether these difference were significant or not.

Kvaskoff et al., (137) found that the increase in 25(OH)D concentration seen with punches taken from the edges of DBS compared with central punches did not occur

when DBS were made with blood containing ethanol – in fact no difference was seen between central and peripheral spots, including in terms of the weight of punches. This is in contrast to what we found – significantly higher 25(OH)D in edge punches compared with central punches. It is unclear why Kvaskoff et al., found a different pattern to us, but we found that the pattern of edge punches containing higher levels remained when alcohol was present for our SWBH CRP assay as well.

Although we found that the presence of alcohol did not alter the 25(OH)D results significantly in most of the areas examined, patient DBS will not contain alcohol, therefore calibrating a 25(OH)D DBS assays using ethanol spiked whole blood could lead to a mismatch in behaviour between patient samples and calibrators and potentially increase the likelihood of reporting incorrect results.

We found that for our CRP DBS assay, the presence of ethanol did not substantially change the way the CRP DBS behaved. Edge punches from ethanol containing DBS were still significantly higher compared with central punches and there was still a significant difference between 20 µL DBS and 100 µL DBS made with ethanol containing whole blood (and 75 µL DBS at higher CRP concentrations). Overall ethanol containing DBS did appear to have a slightly lower concentration of CRP compared with alcohol free DBS, however no significant difference was found except for 10 µL DBS at the higher concentration of CRP.

Location of punch, presence of ethanol and the use of intact or haemolysed blood in the production of calibrators and QC are all parameters that are under the control of the laboratory. In addition, the size of spots obtained from patients can be influenced by the laboratory too, through the use of appropriate lancets and the design of the collection device. However, haematocrit is not under the control of the laboratory and

therefore the degree to which it affects an assay and the kind of haematocrit that can be expected from the target population that will be using the assay needs to be considered.

We found that at lower levels of 25(OH)D, only the very high haematocrit DBS had a significantly lower concentration compared with the target haematocrit of approximately 0.4, although there did appear to be an overall decrease in 25(OH)D as haematocrit increased. A more obvious trend was seen at the higher concentration of 25(OH)D, where we found that the higher the haematocrit the lower the 25(OH)D, but still a significant difference was only found for the very low and very high levels of haematocrit relative to the 0.4 haematocrit calibrator. As our DBS direct to the public service is going to be mainly used by generally healthy people, the likely haematocrit range will be 0.3-0.54 – covering the reference range for most children and adults.

For our SWBH CRP assay a similar pattern was found at the lower concentration of CRP, with decreasing CRP for increasing haematocrit. However, no significant difference was found when compared to the reference 0.4 haematocrit. The CV was high for the two highest haematocrit levels. This may be due to the haematocrit affecting the way the CRP flows across the filter paper or it could be due to inconsistent sampling due to mushy spots.

A different pattern was found for the higher level of CRP. It almost looks like two populations of DBS were present, with higher CRP found for the three highest haematocrit levels and no significant difference between them, but the two lowest levels of haematocrit had significantly lower CRP levels. In fact the CRP concentration looks like the levels found in the lower concentration CRP DBS and the

overall pattern shown by changes in haematocrit does not follow the pattern seen for 25(OH)D or the lower level CRP. It would be interesting to repeat this work at the higher concentration of CRP, in case a mistake was made when making up the different haematocrit level pools (e.g. the wrong pool of serum mixed with the WRBC). As it stands however, there appears to be a significant difference between the 0.4 haematocrit and lower levels of haematocrit at higher CRP levels. For the lower level of CRP, as no difference was found between the haematocrit levels relative to the 0.4 target level, it implies that haematocrit variation in our target user population will not be an issue.

Interestingly the same pattern was seen for the Mitra devices, with a consistent pattern of higher haematocrit leading to lower levels of CRP for the lower concentration CRP pool. This pattern is partly followed for the higher CRP pool with CRP decreasing with increasing haematocrit from a level of 0.4, but the two lowest levels of haematocrit have CRP concentrations similar to that of the lower CRP pool. The Mitra and SWBH devices were made with the same pools of whole blood, so if a mistake was made, it would follow that both devices show a similar pattern. It would be interesting to repeat this work for the Mitra device too. In any case, for the Mitra device, a significant difference was found between all levels of haematocrit, for both high and low CRP concentrations, relative to the 0.4 haematocrit. This has implications for the future use of the Mitra device and may make it unusable for routine use with CRP. This drawback needs to be weighed against the benefits of using it when compared with serum results and see what impact it has in a real population. It would be interesting to see if this pattern is seen for other analytes as well.

Several authors have investigated the impact of variations in haematocrit on DBS punch volume and analyte recoveries. Mei et al., (24) looked at the effect of haematocrit variation (0.3 to 0.7) on serum volume in 6 mm punches. They spiked five whole blood pools with varying haematocrits with $^{125}I$-$_L$-thyroxine, created DBS and, using their isotopic method, compared the mean volumes with QC material that had a haematocrit of 0.55. They found that increasing haematocrit decreased the serum volume in a punch – 0.3 haematocrit punches contained ~47% more serum volume than 0.7 haematocrit punches.

The work by Mei et al., (24) helps to explain our findings, however their results disagreed with work performed by O'Broin (138) who investigated the effect of haematocrit on punch volume using $^{125}I$ to investigate the serum absorbency. He also looked at erythrocyte absorbency by using labelled sodium chromate ($^{51}Cr$) which binds predominantly to haemoglobin. The results showed that haematocrit does affect the volume of blood contained in punches and that serum absorbency increased with increasing haematocrit, whereas erythrocyte absorbency remained relatively constant no matter what the haematocrit. (138) This has implications for assays depending on whether they are measuring analytes that are mainly found in the serum, in cells or both. The authors also noted that the spread of the sample decreased with increasing haematocrit which would explain why an increase in blood volume in punches was seen with increasing haematocrit – the blood has not travelled so far it is more concentrated in a smaller area leading to a higher punch volume.

Dhondt et al., (88) looked at the effects of haematocrit on phenylalanine recovery in terms of size of spot made and found higher haematocrits resulted in higher

phenylalanine recoveries. But other authors suggest that haematocrit does not seem to play a major role in the results obtained when using DBS. When validating a DBS assay for 25(OH)D, Newman et al., (122) found that across the haematocrit levels often found in the healthy population (defined by them as being 0.4-0.6) there was little change in concentration. We did find a difference across this range but only for the 0.6 level. Other examples of haematocrit having little or no effect on the concentration of analytes measured in DBS can be found in the literature, for example PSA, (125) IGF-I and IGF-binding protein III (140) and cyclosporine A. (68) Sometimes the variation caused by haematocrit could be adjusted for by the use of a constant correction factor based on the population's mean haematocrit. (79)

The effect of haematocrit on the concentration of an analyte found in a DBS is not straight forward and is very much dependent on the analyte in question. However, having an understanding of the partitioning of the analyte between plasma and erythrocytes will help to estimate the kind of impact that variations in haematocrit may cause. (26) Despite this, it is still important for every assay using DBS that the effect of haematocrit is thoroughly investigated. This is even more important when the target population of the assay is likely to be diseased as these patients are more likely to have extremes of haematocrit. The uncertainty introduced by haematocrit should not deter laboratories from using DBS as their usefulness has been overwhelmingly demonstrated by the worldwide use of DBS in NBS, and the newborn population can show some of the largest variation in haematocrit (0.28-0.67 for 0-1 year olds) that is likely to be seen in a target population. (31) If variations in haematocrit are deemed to lead to an unacceptable bias in samples outside of a range of haematocrits, then it may be feasible to measure haematocrits in samples

as well as the target of interest (141) and try to correct for variation in haematocrit using previously determined regression analysis. (70)

Blood spot characteristics can lead to large variations in results obtained, but this does not mean that DBS assays should not be used. Instead, before going live with an assay, it should be thoroughly validated including an investigation of the effect of blood spot size and punch size and position on analyte concentration. This approach will help to minimise variation and improve accuracy of the results obtained from DBS by using best practice. A haematocrit reflecting the average of the population likely to be tested should be selected for the preparation of calibrators in order to minimise haematocrit affects. (26) A range of haematocrit values outside of which results from DBS assays are no longer valid should be determined and communicated to the healthcare professionals and users ordering the tests so that an informed decision on whether or not to use DBS can be made.

The work done here demonstrates key variations in using DBS for quantitative analysis for two analytes. We have demonstrated that variation can exist and that with knowledge of this we can control and, to some extent, mitigate this as we optimise DBS assays.

# CHAPTER 4 - DIRECT TO THE PUBLIC VITAMIN D TEST

## 4.1. Overview

Vitamin D is an essential nutrient required for bone health and calcium homeostasis. It is also described as a pro-hormone as it is the biologically inactive precursor to the active secosteroid hormone 1,25-dihydroxyvitamin D (calcitriol, $1,25(OH)_2D$). (142)

Vitamin D is found in two forms: vitamin $D_2$ (ergocalciferol) and vitamin $D_3$ (cholecalciferol). Both forms are available as supplements and in a small number of foodstuffs, naturally occurring or fortified. The main source of vitamin D, around 90%, is through endogenous synthesis in the skin with the conversion of 7-dehydrocholesterol, via UVB radiation from the sun, into vitamin $D_3$. Vitamin $D_2$ or $D_3$ are hydroxylated, first in the liver to form 25(OH)D and secondly in the kidney to form the active hormone $1,25(OH)_2D$. The first hydroxylation step is unregulated and 25(OH)D levels therefore depend on the availability of the vitamin D substrate. The second hydroxylation step is tightly controlled via parathyroid hormone and through a variety of negative feedback mechanisms including calcium and phosphate levels as well as $1,25(OH)_2D$ itself. (143)

The best marker of vitamin D status is 25(OH)D. Even though vitamin D can be made endogenously and is available through dietary sources, vitamin D deficiency is extremely common. (144) If vitamin D insufficiency is defined as <75 nmol/L then it has been estimated that 1 billion people worldwide could be vitamin D deficient or insufficient. (145) Deficiency of vitamin D is most commonly linked with rickets in children and osteomalacia in adults, but in the last few years it has been associated with a range of other areas such as diabetes, immune function, cardiovascular disease and cancer.

It is very hard to predict what a person's vitamin D status is, even if you take into account factors such as age, supplement use, season, sun exposure, race and body mass index. (146) However, there are a range of risk factors and these can often help to identify people who are vitamin D deficient, e.g. people with pigmented skin, lack of sun exposure, obesity, malabsorption and people taking certain drugs such as anticonvulsants. (130, 144, 147, 148)

Routine population screening is not advocated by most of the literature. Neither of the guidelines from the Endocrine Society (144) or the National Osteoporosis Society recommend routinely testing 25(OH)D in asymptomatic individuals who may be at higher risk of vitamin D deficiency, but suggest that these individuals should have a higher intake of vitamin D. (130) They recommend only testing in a limited number of clinical scenarios. The drawback of this approach is that the recommended daily allowance will not be enough to correct severe deficiency, and giving higher loading doses to those already replete may put them at risk of vitamin D intoxication.

The level at which vitamin D toxicity can occur is contentious, as indeed is the level at which a person can be considered to have an adequate level of vitamin D and how much vitamin D can be safely taken. This has resulted in multiple guidelines with varying recommendations. (130, 131, 142, 144, 145, 149-152). Reports of vitamin D toxicity can be regularly seen in the literature (153-157) and this is a very real, and often avoidable, problem. Hypervitaminosis D can lead to hypercalcaemia as a result of increased intestinal calcium absorption and bone resorption which can ultimately lead to kidney injury. (158) The effects of long term high dose vitamin D supplementation are not well known, however it appears that not only is hypercalcaemia and hypercalciuria a risk but other detrimental side effects such as

increased falls (159) are being acknowledged with some suggesting that there may be a U-shaped curve for 25(OH)D levels, with potential harm being associated with low levels of vitamin D as well as with very high levels of vitamin D. (131, 160, 161)

Vieth stated that hypercalcaemia due to vitamin D intoxication is always associated with 25(OH)D concentration >220 nmol/L, (162) although more recently other reports have revised this level upwards to 250 or 375 nmol/L. (163, 164) There is no consensus on what level vitamin D toxicity should be defined at so we have taken 220 nmol/L as the lower limit of our high to toxic range as this is the lowest level reported to be associated with toxicity in the literature.

One of the aims of the work in this chapter was to compare the results of 25(OH)D concentration and status for members of the public using our DBS service with GP samples received from our inner-city Birmingham population over the same time period, in order to see how different this new population is compared with the traditional patients encountered in routine clinical practice. We also wanted to see how different the rate of repeat testing was between these two groups and if there was a difference in the way they responded to the initial results they obtained. A further aim was to establish what supplementation regimes were being followed by users of our DBS service causing them to have high to toxic 25(OH)D concentrations. Finally we wanted to assess the overall impact of a direct to the public test for Vitamin D.

## 4.2. **Method**

### 4.2.1. **Vitamin D Concentration and Status**

The DBS population is a novel population and we wanted to see what the characteristics of this population were and what the 25(OH)D concentration and status of the DBS population were. We then wanted to compare this population with the local GP population as this is much more well understood and defined population. The characteristics of the populations using the serum GP vitamin D service and those using the direct to the public vitamin D DBS service were investigated and the concentration of 25(OH)D compared (June 2012 to July 2013). The age (patients <1 year were classed as age 0) and gender distribution of both types of populations were compared and a map showing where requests for DBS testing were coming from in the UK was compiled. Where available, ethnic origin and 25(OH)D concentration was also investigated. The ethnicity of patients was grouped in the following way: Caucasian, Asian, Black, mixed or any other ethnic group. Ethnicity data were only available on DBS since January 2013, therefore a large proportion of the ethnicity data were missing (56%) compared with the serum samples (23%). The proportions of the different populations falling into different statuses as described above were also compared.

The population characteristics and results were found by querying Telepath which had been populated with the information received from request forms. In total, 28,660 GP serum samples and 4,480 DBS samples were analysed.

### 4.2.2. **High to Toxic Patient Investigation**

#### 4.2.2.1. *Population Selection and Telephone Interview*

Between January 2013 and September 2015, all users of our direct to the public vitamin D testing service whose total 25(OH)D levels were >220 nmol/L were contacted by telephone. This was to appropriately inform users of their potentially toxic result and suggest how to respond to it. After explaining the reason for the phone call, users were asked if they were happy to discuss their supplementation regime in more detail. If so, we ascertained the extent of medical supervision and vitamin D supplementation, formulation, brand, source of supplements, and finally the length of time supplemented for. If further information was volunteered (e.g. reason for supplementation, additional supplements being taken), this was also recorded (Appendix 5). The option of confirming their result using a serum sample at no charge was also offered. This option was given as some users had said that GPs wanted to confirm the vitamin D results but were unable to do so with their local service. Other users questioned the validity of the DBS result and so we confirmed the result using paired serum and DBS samples.

All users were encouraged to discuss their results with their GP and advised to immediately stop all supplements containing vitamin D, unless these had been prescribed to them in which case they were urged to discuss the results with the prescriber. Telephone interviews lasted between 5 and 30 minutes.

#### 4.2.2.2. *Data Analysis*

Results of the telephone interview were tabulated along with any previous results on the patient and the initial high result. The age and gender of the patient and the date the sample was taken were also recorded. Trends relating to whether or not patients

were under medical supervision and the amount, formulation and where the supplement was obtained from were identified. General themes relating to reasons for supplementing and what other supplements were taken were also identified. Brands of supplements were checked to see if they existed as some users had difficulty recalling what they were taking. If the quoted brand could not be found this was recorded as being unverified.

#### 4.2.2.3. *Daily Supplementation rates*

Not all patients were taking daily supplements, but their equivalent daily supplementation rate was calculated based on the information provided. For example, if patients were taking a weekly supplement, the amount of supplement taken was divided by seven to provide a daily supplement rate. Some patients changed their regimes over time and so their most recent regime leading to the elevated result was taken as the daily supplementation rate. The data relating to supplementation patterns were self-reported and unconfirmed.

### 4.2.3. **Impact of a Direct to the Public Service**

326 questionnaires (Appendix 6) were sent out to all individuals who bought the kit (not to companies, pharmacies or children) who had results sent to them in April 2013. There was no pre-selection, the questionnaires were sent out to represent one month's work and to give a snap shot in time. This was done in order to assess how people found using DBS, what they thought of our service and their reasons for using it. Completed questionnaires were either sent back to us electronically or printed out and posted back.

### 4.2.4. **Repeat Testing**

#### 4.2.4.1. *Data Gather*

Data regarding patient name, date of birth, date of receipt of sample and 25(OH)D results for both GP serum samples and DBS was gathered from Telepath, from 1st May 2011 until 31st March 2013. In addition, GP location and ethnicity data were recorded for serum samples. Ethnicity was defined as Asian, Black, Mixed, Caucasian, any other ethnic group and not stated. Duplicate and multiple records were searched for using the Excel conditional formatting function that highlights duplicate records which were identified by first name, surname and date of birth. If all three parameters did not agree then the record was not regarded as a duplicate.

#### 4.2.4.2. *Samples*

In total, 48,538 serum samples were received from GPs for analysis of 25(OH)D during the time frame being investigated. During the same period, 5,534 DBS were received from the public for 25(OH)D analysis. Using the technique described above, duplicate records were identified and data analysed.

Repeat samples may have occurred due to a previously insufficient sample (serum or DBS) being received by the laboratory. When that occurs users are sent another DBS kit in order to collect a specimen that can be used, or patients may be re-bled. This would result in at least two samples coming in from the same person, however data would only be available from one specimen. Pairs of samples where only one out of the pair had concentration information available were ignored. If an insufficient sample came at the beginning, middle or end of a set of repeat samples but concentration data were available for two or more specimens, then that repeat data was included.

During this time frame a demand management system for GP serum vitamin D testing was in place (introduced in April 2010) whereby only one vitamin D test per patient per year could be requested. If a second sample for a GP patient was received within the one year time frame the request would go into Telepath but would be overruled. This would result in at least two samples coming in from the same person, however data would only be available from one specimen. Pairs of samples where only one out of the pair had concentration information available were ignored. The demand management rule could be overruled if required e.g. for patients <16 years old, if the GP contacted us to request the test be performed or if there was only a short period of time before the year time frame was met. This meant that GP patients could still have more than one result within a one year time frame.

#### 4.2.4.3. *Data Analysis*

For both populations, the data were investigated to look at the change in concentration and status of individuals with repeat data. Only the first two results were investigated. The time period between repeat testing was also investigated. The demographics of the population sending in repeat samples were identified.

## 4.3. **Results**

### 4.3.1. **Vitamin D Concentration and Status**

See section 4.2.1 (page 133) for the method relating to this section.

#### 4.3.1.1. *Vitamin D Patient Demographics*

There has been a steady rise in the use of the vitamin D DBS service since its inception (Figure 4.1) with 9,589 vitamin D DBS kits ordered and 7,098 analysed (by July 2013, numbers do not include PD kits). The figures differ as people often order

several kits, for immediate and future testing, introducing a lag phase. The DBS numbers were relatively small when compared with the number of serum samples analysed for vitamin D over the same period, 88,188 of which 59,513 were GP specimens.

DBS samples were received from all over the world as well as all over the UK (Figure 4.2), including Africa, Australia, New Zealand, China, mainland Europe, and Scandinavia. GP serum samples were received from our local population in inner city Birmingham.

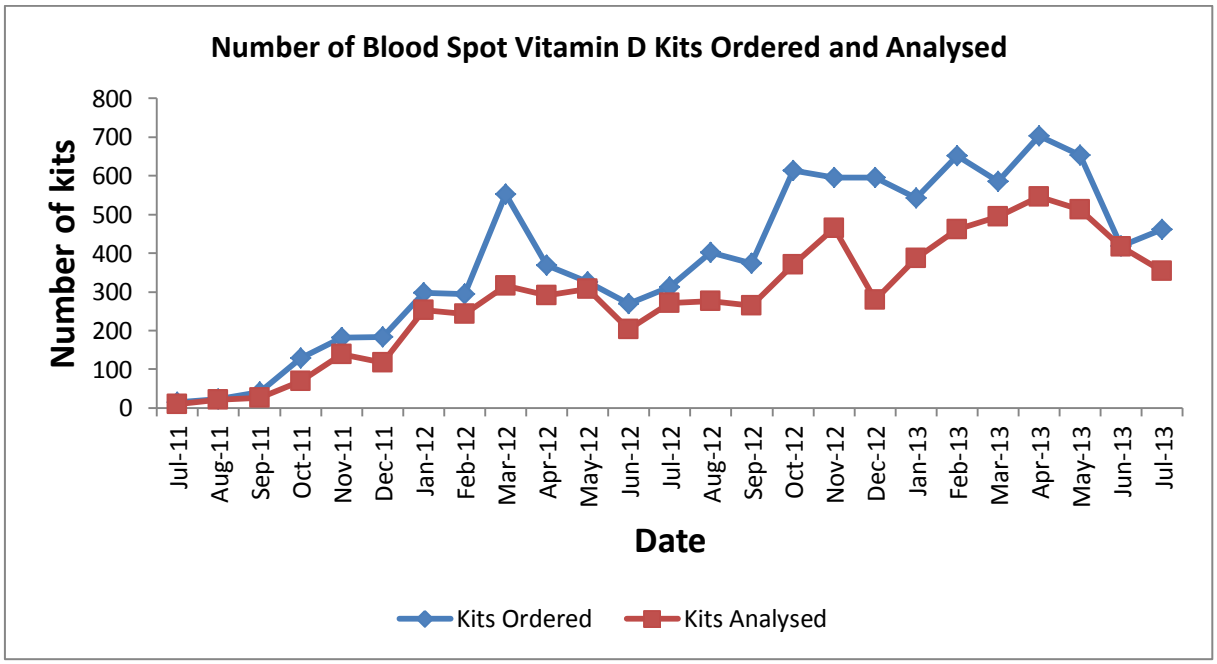



**Figure 4.1 –** The number of DBS kits ordered and analysed every month since service launch.

Significantly more samples were received for DBS vitamin D analysis from women (63.2%) than men (36.8%) ($p<0.001$). A similar pattern was seen for the serum samples with 69.1% of requests from female patients ($p<0.001$) (Figure 4.3). Although the trend was similar for both sample types, the difference between sample types was significant ($p<0.001$).

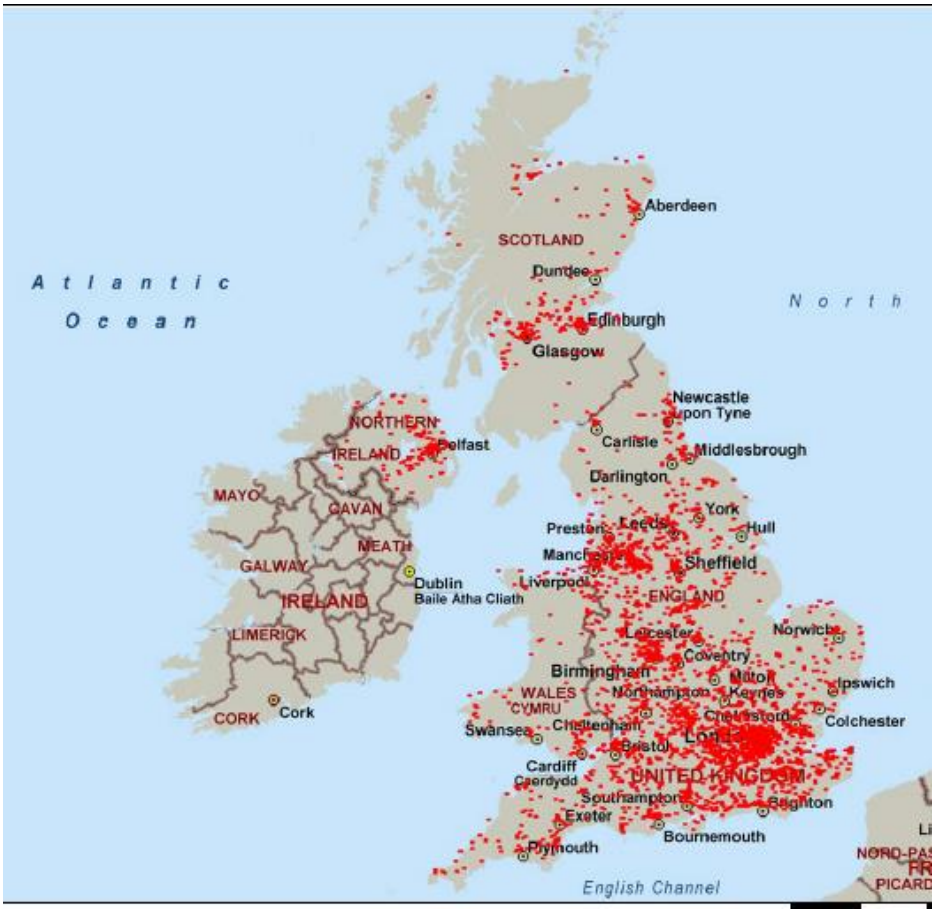



**Figure 4.2 –** Distribution of samples received from the public for vitamin D DBS testing in the UK. Each red dot represents one sample. Test kits were also received from around the world.

The descriptive statistics for the age of patients that used the two vitamin D services is given in Table 4.1. The distribution of age for both sample types was not normal ($p<0.001$ for both sample types) and the distribution of age was significantly different between the two sample types ($p<0.001$).

| Sample Type | Mean Age (years) | Median Age (years) | Minimum Age (years) | Maximum Age (years) | Interquartile Range (years) |
|---|---|---|---|---|---|
| **DBS** | 46.9 | 48 | 0 | 99 | 25 |
| **Serum** | 43.3 | 42 | 0 | 100 | 28 |

**Table 4.1 –** Descriptive statistics for the ages of patients for serum and DBS samples.

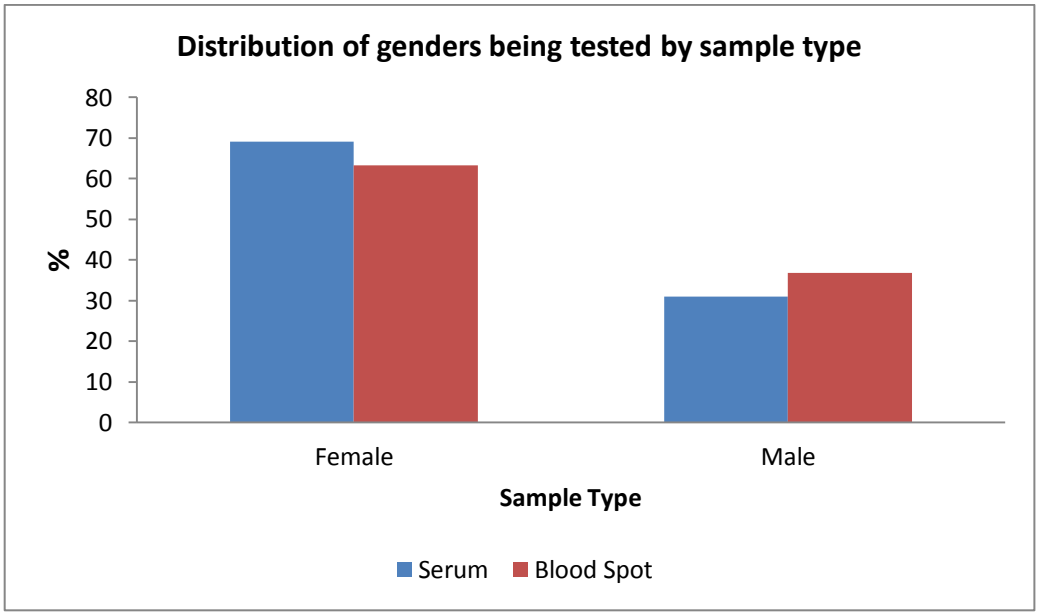



**Figure 4.3 –** Proportion of samples from males and females for serum and DBS samples.

The ethnicity of the patients (when provided) that used both types of service is shown in Table 4.2. The distribution of the ethnicities of patients across serum and DBS samples was significantly different ($p<0.001$ and $p<0.001$), with Asian being the most common ethnicity for serum samples and Caucasian the most common ethnicity for DBS samples. The distribution of ethnicity was significantly different between the two sample types ($p<0.001$).

| Sample Type | Caucasian (%) | Asian (%) | Black (%) | Mixed (%) | Any other (%) |
|---|---|---|---|---|---|
| **DBS** | 90.2 | 4.4 | 1.1 | 0.4 | 4 |
| **Serum** | 22.8 | 57.8 | 14.4 | 1.8 | 3.2 |

**Table 4.2 –** Descriptive statistics for the ethnicities of patients for serum and DBS samples.

4.3.1.2. *Vitamin D Concentration and Status*

| Sample Type | Median (nmol/L) | Minimum (nmol/L) | Maximum (nmol/L) | Interquartile Range (nmol/L) |
|---|---|---|---|---|
| **DBS** | 53.1 | 10.3 | 701 | 43.2 |
| **Serum** | 27.7 | 10.3 | 735 | 28.3 |

**Table 4.3 –** Descriptive statistics for total 25(OH)D for DBS and serum samples.

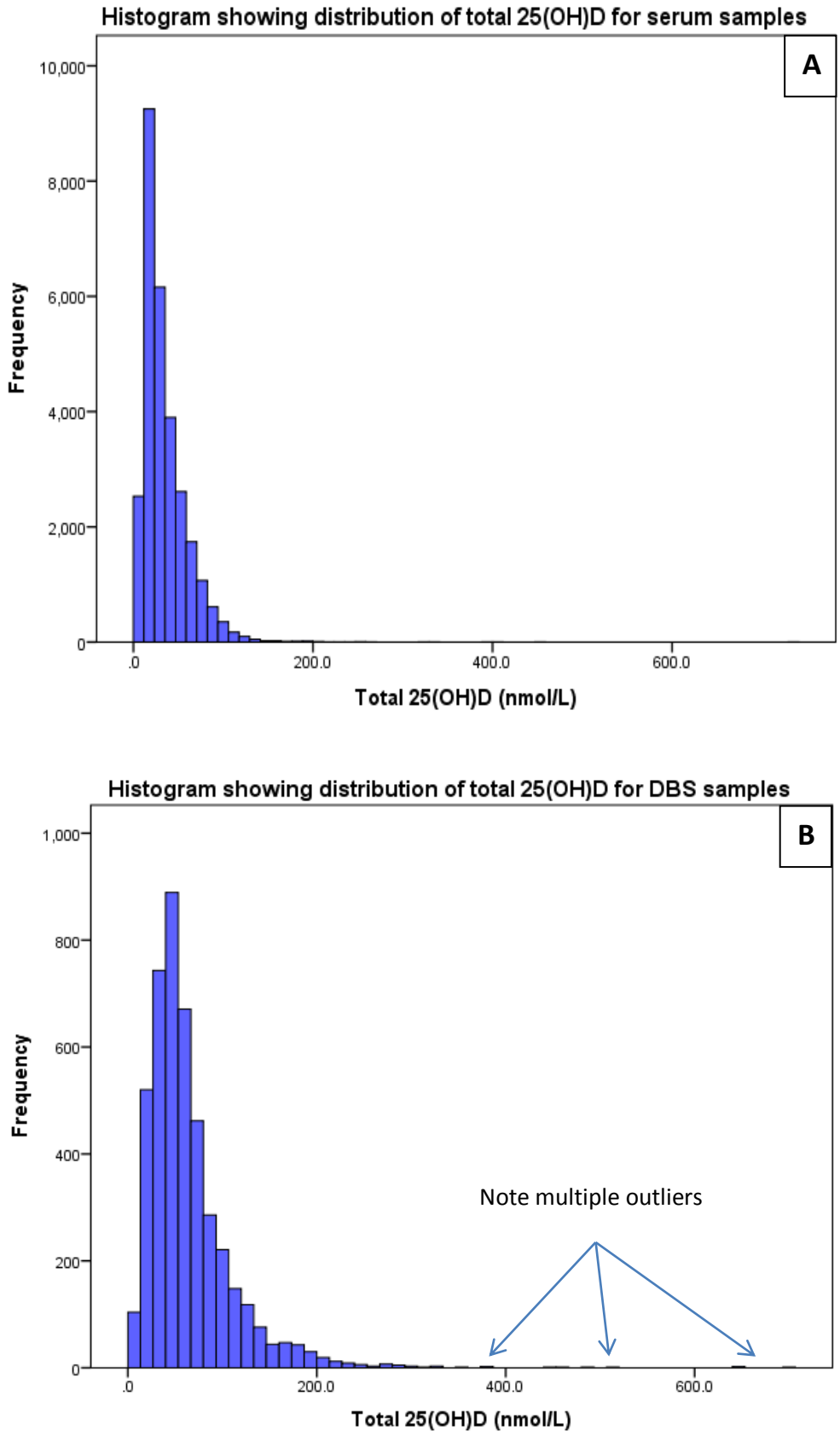



**Figure 4.4 –** Distribution of total 25(OH)D results for A: serum and B: DBS samples.

The data for total 25(OH)D for both sample types were not normally distributed ($p<0.001$). The lower reporting limits for both sample types for $25(OH)D_2$, $25(OH)D_3$ and total 25(OH)D were 2.8, 7.5 and 10.3 nmol/L respectively. This may have contributed to the lack of normality (Figure 4.4). The distribution of results for total 25(OH)D for serum and DBS samples was significantly different ($p<0.001$) (Table 4.3).

The median total 25(OH)D for the DBS samples was higher every month compared with the serum samples (Figure 4.5). Serum samples appear to follow a seasonal variation with higher results in the summer months and lower results in the winter months and early spring, however the same pattern is not so apparent for the blood spot samples. The serum samples were all from local GP patients and therefore geographical location was known. The vast majority of DBS samples in this time frame came from people based in the UK and therefore the geographical location was known.

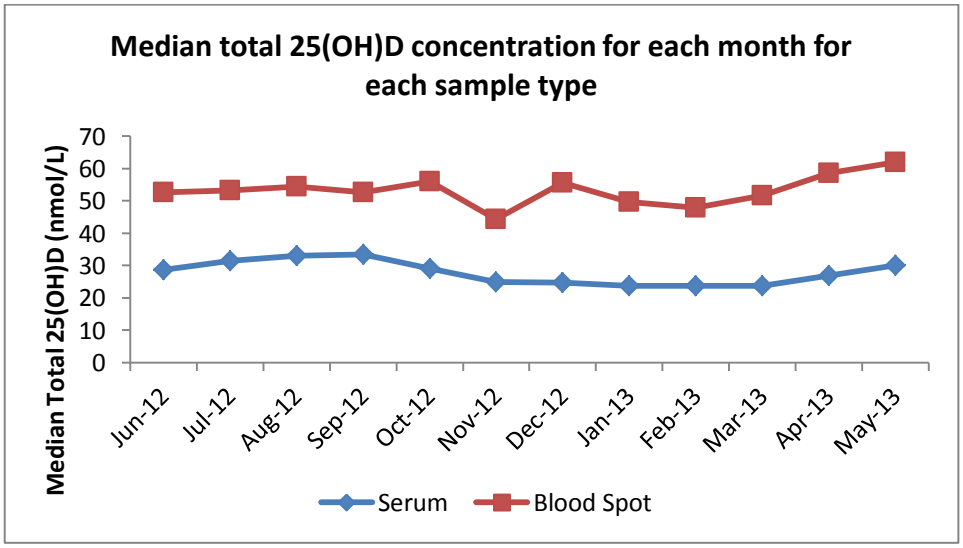



**Figure 4.5 –** Variation in median total 25(OH)D for serum and DBS samples.

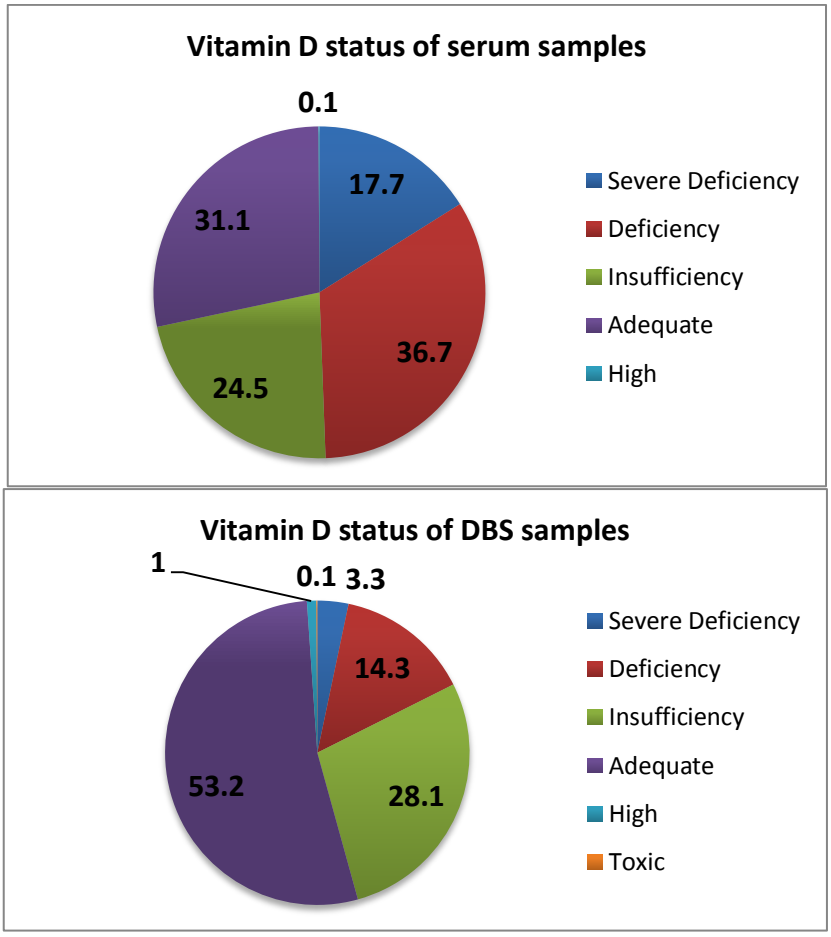



**Figure 4.6 –** Percentage of cases in different categories of 25(OH)D status for serum and DBS samples.

The majority of serum GP samples fell into the deficient status category, with only 21.1% of patients tested displaying adequate levels of 25(OH)D. A significantly different pattern was found for the DBS samples, with 53.2% of patients showing adequate levels of 25(OH)D with only 3.3% of samples in the severely deficient category (Figure 4.6) ($p<0.001$).

The high and toxic samples were not included in the following statistical analysis as the numbers in this time period were very small and a separate investigation in 4.3.2. looked at this area in greater detail. There was a difference between the genders for serum vitamin D status ($p<0.001$) but there was no difference between genders for DBS samples status ($p=0.155$) (Table 4.4).

| | | % | | | |
|---|---|---|---|---|---|
| **Sample Type** | **Gender** | **Severely Deficient** | **Deficient** | **Insufficient** | **Adequate** |
| **Serum** | Female | 18.5 | 35.2 | 23.7 | 22.5 |
| | Male | 15.7 | 39.9 | 26.3 | 18.0 |
| **DBS** | Female | 3.2 | 14.3 | 29.3 | 52.5 |
| | Male | 3.7 | 14.1 | 26.1 | 54.3 |

**Table 4.4 –** Percentage of men and women that fell into the different vitamin D status categories for serum and DBS samples.

The distribution of age across the different categories was significantly different for serum and DBS samples ($p<0.001$ and $p<0.001$) with older patients appearing to be more likely to have an adequate status (Table 4.5).

| | Median Age (years) | | | |
|---|---|---|---|---|
| **Sample Type** | **Severely Deficient** | **Deficient** | **Insufficient** | **Adequate** |
| **Serum** | 38 | 40 | 44 | 49 |
| **DBS** | 41 | 44 | 47 | 49 |

**Table 4.5 –** Median age of patients for the different status categories for both sample types.

| | | % | | | |
|---|---|---|---|---|---|
| **Sample Type** | **Ethnicity** | **Severely Deficient** | **Deficient** | **Insufficient** | **Adequate** |
| **Serum** | **Caucasian** | 5.6 | 24.4 | 30.6 | 39.3 |
| | **Asian** | 23.1 | 39.9 | 21.3 | 15.6 |
| | **Black** | 10.8 | 40.9 | 28.3 | 19.9 |
| | **Mixed** | 11.0 | 41.2 | 28.7 | 19.0 |
| | **Other** | 18.8 | 37.1 | 24.0 | 20.0 |
| **DBS** | **Caucasian** | 3.3 | 15.6 | 25.5 | 54.2 |
| | **Asian** | 10.3 | 33.3 | 18.4 | 35.6 |
| | **Black** | 4.8 | 4.8 | 33.3 | 57.1 |
| | **Mixed** | 0.0 | 14.3 | 42.9 | 42.9 |
| | **Other** | 2.5 | 19 | 25.3 | 51.9 |

**Table 4.6 –** Percentage of the ethnicities that fell into the different vitamin D status categories for serum and DBS samples.

There was a relationship between vitamin D status and ethnicity for serum and DBS samples ($p<0.001$ and $p<0.001$ respectively) (Table 4.6) with Caucasians more likely to have an adequate status for serum samples and Asian samples most likely to be severely deficient for both sample types.

### 4.3.2. **High to Toxic Patient Investigation**

See section 4.2.2 (page 134) for the method relating to this section.

#### 4.3.2.1. *Population*

Between January 2013 and September 2015, we analysed 14,806 DBS. Of these, 454 (3.1%) samples had 25(OH)D >220 nmol/L. These samples came from 372 users. The age range of this group went from 19 weeks to 87 years, with a median age of 48 years, and 51% were women. For the same period we analysed 99,122 serum samples from our local GP population and 0.06% (n=63) were found to have a 25(OH)D level >220 nmol/L.

#### 4.3.2.2. *Vitamin D Results*

The results ranged from 221-1,235 nmol/L with a median concentration of 274 nmol/L (Table 4.7). 35 patients chose to confirm their high results by sending us paired blood spot and plasma samples. The results showed good agreement between the two sample types (Figure 4.7).

| Range 25(OH)D (nmol/L) | n (%) |
|---|---|
| **220.1-375** | 310 (83.3) |
| **375.1-500** | 30 (8.1) |
| **500.1-1000** | 31 (8.3) |
| **>1000** | 1 (0.3) |

**Table 4.7 –** Breakdown of 25(OH)D blood spot results found in the DBS population whose results were >220 nmol/L.

We had previously received a DBS from 153 of the users who were found to subsequently have a 25(OH)D concentration >220 nmol/L when using the direct to the public service. The majority of those 153 users (n=109; 71%) had received a previous adequate result, ranging from 50 nmol/L to 217 nmol/L. The people who had formerly been deemed less than adequate had results ranging from 11-49 nmol/L.

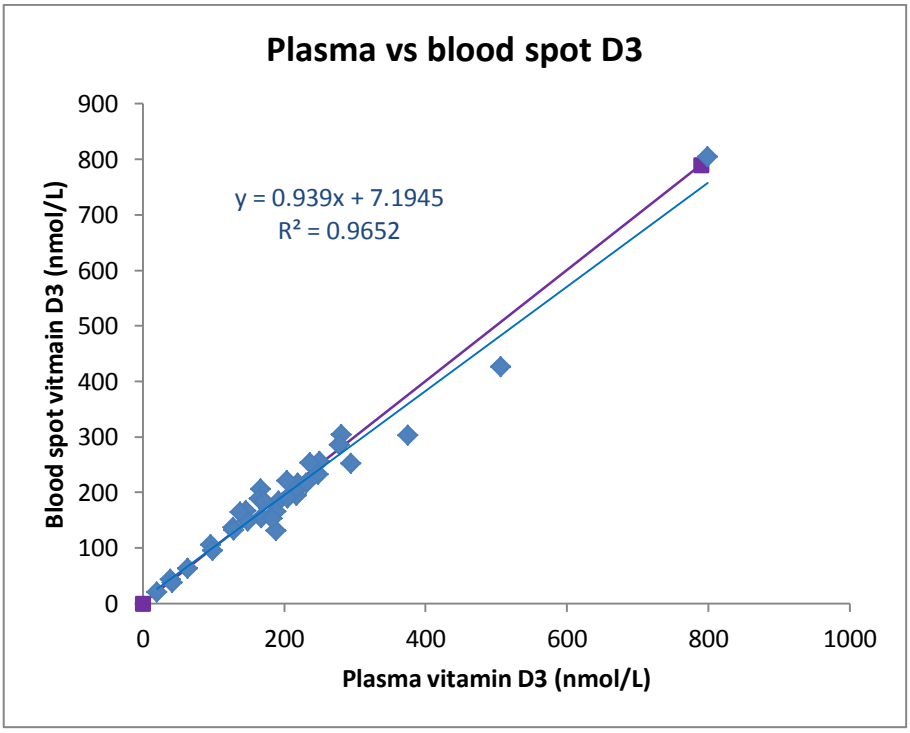



**Figure 4.7 –** Relationship between paired plasma and blood spot samples for measurement of 25(OH) D, n=35. These were samples taken between January 2013 and September 2015 and were sent to us by users of the service when further investigating results or confirming high results.

4.3.2.3. *Supplementation Patterns*

Of the 372 users who had results >220 nmol/L, three could not be contacted and five did not wish to discuss their results. The data from the remaining 364 users was analysed below.

4.3.2.3.1. Daily Supplementation

The high to toxic levels of 25(OH)D seen were a result of regular supplementation in 361 users, but 11 could not recall how much they were taking (results ranged from 226 to 446 nmol/L). Stated supplementation rates ranged from 1,000-120,000 IU/day (Table 4.8). One user claimed not to be taking supplements, but had achieved a level of 239 nmol/L through sunshine exposure alone. One person had taken 300,000 IU in the form of a liquid supplement as they “wanted to do an experiment to see how rapid the increase would be”. Another member of the public had taken 900,000 IU of

a liquid supplement as a bolus dose because they worked for a company that produced vitamin D supplements and they decided to take it “for a laugh”. The two bolus doses led to levels of 355 nmol/L and 581 nmol/L respectively.

Some users also stated that they had recently been on holiday/sunbathing/using sun beds near the time they took the test.

| IU/day | n (%) | 25(OH)D range (nmol/L) | 25(OH)D median concentration (nmol/L) |
|---|---|---|---|
| **1,000-9,000** | 148 (42.3) | 221-643 | 255 |
| **10,000** | 89 (25.4) | 221-684 | 274 |
| **11,700-16,000** | 23 (6.6) | 223-589 | 264 |
| **20,000** | 43 (12.3) | 223-1235 | 277 |
| **22,000-40,000** | 19 (5.4) | 229-796 | 438 |
| **50,000-80,000** | 24 (6.9) | 228-855 | 388 |
| **100,000-120,000** | 4 (1.1) | 496-716 | 613 |

**Table 4.8 –** Daily supplementation patterns in users who achieved a 25(OH)D result >220 nmol/L and who could recall how much they were supplementing with.

| Age (years) | IU/day | 25(OH)D (nmol/L) |
|---|---|---|
| **0** | ? | 231 |
| **3** | 3,500 | 225 |
| **3** | 1,000 | 309 |
| **4** | 1,500 | 236 |
| **5** | 1,000 | 498 |
| **5** | 2,000 | 221 |
| **5** | 3,750 | 370 |
| **6** | 3,500 | 244 |
| **6** | 5,000 | 232 |
| **7** | 2,000 | 264 |
| **8** | 2,000 | 246 |
| **8** | 4,000 | 410 |
| **15** | ? | 276 |
| **16** | 5,000 | 246 |

**Table 4.9 –** Daily supplementation patterns in users who achieved a 25(OH)D result >220 nmol/L and who were 16 years old or less. One user’s mother was unable to recall the amount she was supplementing her child with and another did not wish to discuss the results.

There were 14 users of the DBS service who were aged 16 years or less and who achieved a high 25(OH)D concentration in the time frame. One user’s mother was

unable to recall the amount she was supplementing her child with and another did not wish to discuss the results. Table 4.9 shows the amount of daily supplements taken by these children and the concentration of 25(OH)D achieved. None of the children were under medical supervision.

4.3.2.3.2. Brand

| Brand | n (%) | Brand | n (%) |
|---|---|---|---|
| Healthy Origins | 77 (27.7) | Nature's Remedy | 2 (0.7) |
| Solgar | 22 (7.9) | Seeking Health | 2 (0.7) |
| Credence | 22 (7.9) | Osteocaps | 2 (0.7) |
| Nutri Advanced | 17 (6.1) | Puritan's Pride | 1 (0.4) |
| Better You | 13 (4.7) | Oxford Vitality | 1 (0.4) |
| Biotics Research Corporation | 12 (4.3) | Linden's apothecary | 1 (0.4) |
| Nature's Answer | 11 (4) | Bioconcepts | 1 (0.4) |
| Biotech | 9 (3.2) | Boots contract manufacture "special" | 1 (0.4) |
| Doctor's Best | 6 (2.2) | Country Life | 1 (0.4) |
| Life Extension | 6 (2.2) | Biovital | 1 (0.4) |
| Holland and Barrett | 5 (1.8) | Fultium | 1 (0.4) |
| Costco | 4 (1.4) | Healthspan | 1 (0.4) |
| Nature's Plus | 4 (1.4) | Health Aid | 1 (0.4) |
| Swanson | 3 (1.1) | Kirkland | 1 (0.4) |
| Now Foods | 3 (1.1) | Nature's Own | 1 (0.4) |
| Nature's Best | 3 (1.1) | ProHealth | 1 (0.4) |
| Solaray | 3 (1.1) | Protocol for Life Balance | 1 (0.4) |
| Source of Life Garden | 3 (1.1) | St. George's Medical Ltd. | 1 (0.4) |
| Thorne Research | 3 (1.1) | Hux D3 | 1 (0.4) |
| Naturewise | 2 (0.7) | Immiflex | 1 (0.4) |
| Vitamin Research Products | 2 (0.7) | Synergy Biologics Pro-D3 | 1 (0.4) |
| Innopure | 2 (0.7) | Vigantol Merck | 1 (0.4) |
| Troo | 2 (0.7) | Vitabiotics | 1 (0.4) |
| Bioceuticals | 2 (0.7) | Vitashine | 1 (0.4) |
| Blackburn Distributions | 2 (0.7) | Lamberts | 1 (0.4) |
| Vitalady | 2 (0.7) | Carlson | 1 (0.4) |
| Kiron Health | 2 (0.7) | Emulsi D3 Synergy | 1 (0.4) |
| BioCare BioMulsion D | 2 (0.7) | | |

**Table 4.10 –** Different vitamin D supplement brands used by the members of the public.

Of the 363 users who had taken supplements, 292 (80%) gave a brand when asked. Nine brands could not be verified and in five cases the website that the supplements were obtained from were stated as the brand even though these websites did not manufacture vitamin D supplements. The remaining 278 members of the public used a total of 55 different brands with the most popular being Healthy Origins with 77 (27.7%) people using it (Table 4.10).

#### 4.3.2.3.3. Formulation

The range of formulations described by 342 members of the public covered capsules (including gel capsules and soft gels), drops, powder, spray, under tongue lozenge, and tablets. There were 21 users who did not state what formulation their supplements came in. The most popular formulation was capsule with 200 (58.5%) people taking their supplements in this form (Table 4.11).

| Formulation | n (%) |
|---|---|
| **Capsules** | 200 (58.5) |
| **Drops** | 73 (21.3) |
| **Tablets** | 44 (12.9) |
| **Spray** | 15 (4.4) |
| **Powder** | 7 (2) |
| **Under tongue lozenge** | 3 (0.9) |

**Table 4.11 –** Formulation of the supplements taken by users.

#### 4.3.2.3.4. Where the Supplements were Sourced

| Source | n (%) |
|---|---|
| **Internet** | 248 (74) |
| **Health shop** | 38 (11) |
| **Health care practitioner** | 22 (7) |
| **Pharmacy** | 16 (5) |
| **Shop** | 8 (2) |
| **Other** | 5 (1) |

**Table 4.12 –** Where supplements were sourced from.

The source the supplements were obtained from could be recalled by 337 users. The most popular route for obtaining supplements was via the internet (248 users, 74%) (Table 4.12).

#### 4.3.2.3.5. Length of Time Supplemented for

Many people could only give rough estimates for how long they had supplemented for, with 345 people giving a figure, including the two who took bolus doses. Some people only started supplementing after receiving a 25(OH)D result from their GP or our service. The supplementation time varied from eight days to “years”. One person stated that they had been taking 5700 IU/day for 10 years and their 25(OH)D was 361 nmol/L. The user who supplemented for the shortest stated time of eight days had taken 50,000 IU every day, resulting in a 25(OH)D concentration of 238 nmol/L. Another user who had been supplementing with 20,000 IU/day for 10 days because they were “experimenting” had a 25(OH)D concentration of 450 nmol/L. Another user had a “tingling forehead” and so took 26,000 IU/day for 12 days which resulted in a 25(OH)D level of 284 nmol/L. The user with the highest daily supplementation rate took 120,000 IU a day for 1 month, resulting in a concentration of 716 nmol/L. The member of the public with the highest concentration seen of 1,235 nmol/L had been most recently supplementing with 20,000 IU/day for 9 months and prior to that had been taking 40,000 IU/day for 4-5 months.

#### 4.3.2.3.6. Extent of Medical Supervision

Out of the 361 users who were taking regular supplementation, only 23 (6.3%) were under medical supervision. Of the 28 users whose results were >500 nmol/L, just one was under medical supervision. Only seven (6.2%) users out of the 113 taking >10,000 IU/day were under medical supervision. 14 patients had started

supplementing on the advice of a GP after having a previous low 25(OH)D result. Two of these patients had apparently low 25(OH)D measured by their local laboratory (one NHS laboratory in the UK, one in the Czech Republic) and were prescribed high dose supplementation by their GP. When the sample was retested by a mass spectrometric method the patients were actually found to have high to toxic levels. Another member of the public was prescribed Osteofos for their osteoporosis and their dietician had then added in vitamin D drops of 4,000 IU/day. Six other patients had an initial prescription from the GP which they then topped up with over-the-counter supplements. One person was taking at least 50,000 IU/day (often up to 100,000 IU) against the advice of their medical consultant.

#### 4.3.2.4. *Reasons for Supplementation*

The reason for supplementation, while not asked, was volunteered by 243 (67%) users. The reasons for taking vitamin D were very varied, with many taking it for medical conditions and some because they were worried about having low levels as they avoided the sun. Some users took vitamin D supplements because they were "experimenting" and others had read books or information on the internet advocating vitamin D. Table 4.13 tabulates the many reasons behind why people decided to supplement, with some having multiple reasons.

Although not directly asked, many people with severe diseases reported that their conditions had improved after they had started taking vitamin D supplements, although other people felt that there had not been any improvement. Some people (n=45) stated that they were co-administering Vitamin K2 as they had read or been told that taking vitamin K2 would minimise any side effects, namely hypercalcaemia,

caused by taking high doses of vitamin D. Often people remarked that they were also taking other supplements as well and in particular magnesium.

| Reason | n (%) |
|---|---|
| Multiple sclerosis or ?multiple sclerosis | 48 (15) |
| Health care practitioner advice* | 47 (15) |
| Previous low result | 32 (10) |
| Other (wide variety of medical conditions) | 29 (9) |
| Family/friends advice or to support them | 18 (6) |
| GP/consultant advice/prescription | 14 (4) |
| Books/internet advice advocating vitamin D e.g. Jeff T Bowles | 14 (4) |
| Arthritis/osteoarthritis/osteoporosis/joint aches | 13 (4) |
| Always ill/tired/lethargic/aches and pains | 12 (4) |
| Cancer – type not stated | 12 (4) |
| No sunlight exposure or allergic to sunlight | 8 (3) |
| Breast cancer | 8 (3) |
| Experiments/for a laugh | 8 (3) |
| Autoimmune disease/boost immune system/multiple immune disorders/ allergies | 6 (2) |
| Other cancer (basal cell carcinoma, brain, skin, leukaemia, non-Hodgkin's lymphoma) | 6 (2) |
| Psoriasis | 5 (2) |
| Chronic fatigue syndrome/ME | 5 (2) |
| Cluster Headaches | 4 (1) |
| Depression | 4 (1) |
| Autism | 3 (1) |
| Olympic weight lifter/ strength training | 3 (1) |
| Rheumatoid arthritis | 2 (1) |
| Fibromyalgia | 2 (1) |
| Family history of melanoma | 2 (1) |
| Prostate cancer | 2 (1) |
| Fungal infection | 2 (1) |
| Lyme's disease | 2 (1) |
| Feet and bone problems | 2 (1) |
| Vegan | 2 (1) |

**Table 4.13 –** Reasons users of our direct to the public vitamin D blood spot testing service decide to take vitamin D supplements. *Health care practitioner covers osteopath, nutritionist, dietician

A few people wanted to tell us that they felt our NHS reference ranges were inappropriate and some had disease specific reference ranges that they were aiming for. For example, some patients with multiple sclerosis were following the

"overcoming multiple sclerosis programme" (http://www.overcomingmultiplesclerosis.org) which recommends aiming for a 25(OH)D concentration of 150-225 nmol/L.

Many of the people questioned were remarkably knowledgeable about vitamin D and often informed by websites. Some people felt very frustrated by the apparent lack of interest and importance placed on vitamin D by mainstream medical professionals, especially when many of them felt they had benefited from taking vitamin D.

### 4.3.3. **Impact of a Direct to the Public Service**

See section 4.2.3 (page 135) for the method relating to this section.

Of the 326 questionnaires sent out, 68 responses were received (20.9%). Table 4.14 summarises the demographics of the people who returned questionnaires. Table 4.15 summarises the yes/no answers, and the scale answers are summarised in Table 4.16. Some people chose to use the option to add free text at the points in the questionnaire where it was permitted to do so (3 points). These comments were collated and can be seen in Appendix 7.

| **Question** | **Gender[a]** | | **Age Group (years)** | | | | **Service awareness** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Male** | **Female** | **18-25** | **26-40** | **41-60** | **>60** | **Word of mouth** | **Internet** | **Other [b]** |
| **% of responses** | 30.9 | 67.6 | 1.5 | 10.3 | 22.1 | 66.2 | 35.3 | 35.3 | 29.4 |

**Table 4.14 –** Summary of the answers to the questions: what is your gender, what is your age group, and how did you hear about our service? [a]1.5% of respondents declined to give their gender. [b]When responders chose "other" and gave the method in the free text box, their comments were collated and can be seen in Appendix 7. n=68.

The majority of replies came from women, which replicates the trend seen in the demographics of who is using the DBS service. More replies were seen from the older age groups and this may be because the median age of testing is 47 years

(with an interquartile range of 25 years). It is also interesting to see that just as much work is generated from word of mouth as it is from the internet.

| Question | | Yes (%) | No (%) | Missing (%) |
|---|---|---|---|---|
| **Were the instructions easy to understand?** | | 100 | 0 | 0 |
| **Were the results easy to understand?** | | 98.5 | 1.5 | |
| **Why did you use our service?** | **Not available through GP** | 26.5 | 23.5 | 50 |
| | **Convenient** | 70.6 | 1.5 | 27.9 |
| | **Cheap** | 33.8 | 17.6 | 48.5 |
| | **Recommended by health care worker e.g. nutritionist** | 27.9 | 30.9 | 41.2 |
| | **Underlying medical reasons** | 44.1 | 20.6 | 35.3 |
| | **Less blood used** | 11.8 | 27.9 | 60.3 |
| | **Ability to take control of health** | 77.9 | 22.1 | |
| | **Less invasive than traditional venous sampling** | 42.6 | 11.8 | 45.6 |
| | **Other reason[a]** | 17.6 | 0 | 82.4 |
| **Would you like to see other DBS tests available?** | | 91.2 | 2.9 | 5.9 |
| **Would you like to be able to purchase the kits via the website?** | | 82.4 | 10.3 | 5.9 |
| **Are you satisfied with our turnaround time?** | | 95.6 | 2.9 | 1.5 |
| **Are you satisfied with your experience of contacting us by telephone e.g. for ordering kits and advice?** | | 86.8 | 0 | 13.2 |

**Table 4.15 –** Summary of responses to questions that had a yes/no answer. [a]If other reasons were given in the free text box, then these were collated and can be found in Appendix 7. n=68.

The results in Table 4.15 demonstrate that the instructions were easy to understand, and this helps to explain why only 3.0% of samples have to be rejected due to an insufficient volume of blood being provided (see Section 3.3.1.). The data also shows that the majority of people found it easy to take DBS samples (Table 4.16) and to understand the results. This may in part explain why 95.5% of the people that answered the question would choose to have a DBS test rather than a traditional blood test at the GP/hospital, and why 96.9% of people that answered would like to see other DBS tests available.

| | Response (%) | | | | |
|---|---|---|---|---|---|
| **Question** | **1** | **2** | **3** | **4** | **5** |
| **On a scale of 1. Very easy to 5. Very hard, how did you find DBS collection with filter paper:** | 42.6 | 38.2 | 11.8 | 7.4 | 0 |
| **On a scale of 1. Terrible to 5. Excellent, how would you rate our service overall?** | 0 | 0 | 0 | 11.8 | 88.2 |

**Table 4.16 –** Summary of responses to scale questions. n=68.

The most common reason given for choosing to use our DBS service was because people wanted to take control of their own health, and the second most popular reason was because it was convenient. Only 26.5% of people said they could not get a vitamin D test from their GP (although 50% did not answer). The least common reason given for choosing to use our service was because less blood was used, which is commonly cited in the literature as being a reason for using DBS.

Overall our service was rated very highly and this was reflected in the free text comments that were returned back with the questionnaires (Appendix 7).

### 4.3.4. **Repeat Testing**

See section 4.2.4 (page 136) for the method relating to this section.

#### 4.3.4.1. *Direct to the Public DBS*

Of the 5,534 samples received during the period of interest, 1,394 (25.2%) samples came from people who had sent in more than one sample (including the initial sample). A total of 476 people sent in more than one sample and the total number of samples sent in by these people during the time frame investigated ranged from two to ten (Table 4.17). There were a total of 258 samples (18.5% of repeat specimens) that were ignored due to being part of a pair or group of samples that were insufficient. Taking this into account, in total, 12% of all samples received in the time frame were genuine repeat specimens.

| | Number of multiple samples sent in | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 8 | 10 |
| **Number of people** | 372 | 78 | 18 | 5 | 1 | 2 |

**Table 4.17 –** The number of people who sent in multiple samples, excluding those who had to send in a second repeat sample as an earlier one had been insufficient for analysis.

#### 4.3.4.1.1. Population Demographics of Repeat Testers

The majority of people that sent in repeat specimens were women (58.6%). The data for the age of the patients in the repeat data set were not normally distributed (p=0.022). The median age was 48 years and ranged from 2 to 89 years. Ethnicity data were not collected on request forms until January 2013, therefore ethnicity data is not available for the majority of DBS samples looked at in this time frame.

#### 4.3.4.1.2. Vitamin D Status

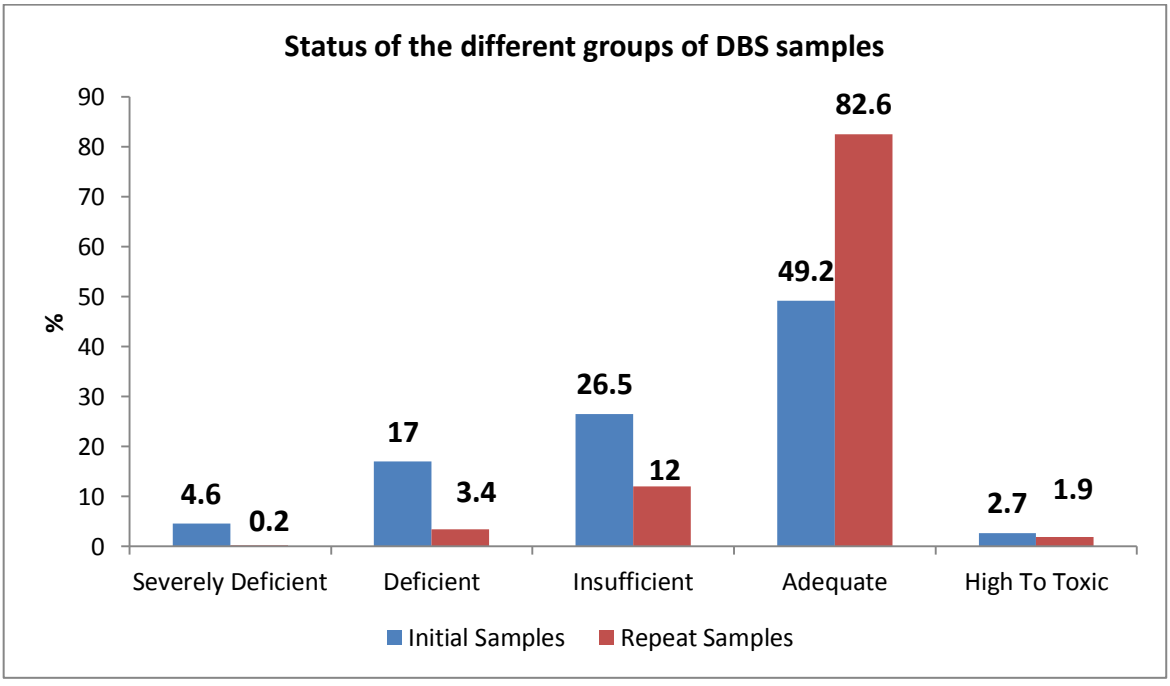



**Figure 4.8 –** Status of the initial and repeat DBS samples, figures are percentages.

The initial samples showed the highest rate of severely deficient people and also the highest rate of people with a high to toxic vitamin D status (Figure 4.8). The repeat samples showed a vast improvement in the number of people in the adequate

category. The distribution of statuses for the initial samples was significantly different from that of the paired repeats ($p<0.001$).

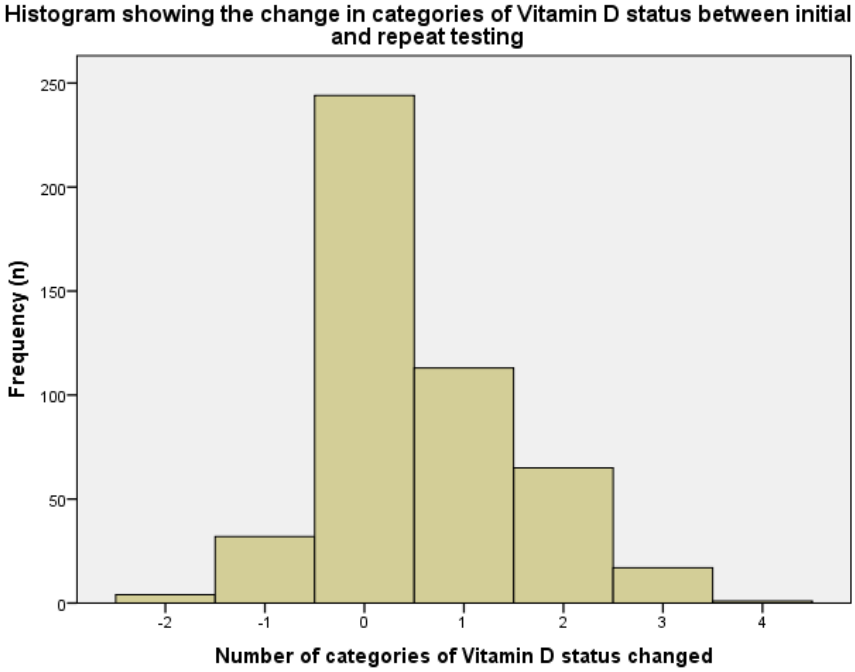



**Figure 4.9 –** Histogram showing how many categories of 25(OH)D status people changed by after their initial result. The negative numbers indicate a deterioration in Vitamin D status, although this will also include people who were high to toxic initially and needed to decrease their levels. Positive numbers indicate an increase in Vitamin D status and 0 indicates no change from initial status.

Upon repeat testing, the majority of people, 244 (51.2%), did not change vitamin D status compared with their initial result (Table 4.18). The second largest combined group, 196 (41.2%), increased their vitamin D status after retesting. Only a small group found that their status decreased after retesting (n=36, 7.6%, Figure 4.9). Of this last group, one third needed to decrease their status from high to toxic and did so by becoming adequate, so only 24 people (5.0%) actually saw a deterioration in their vitamin D status upon repeat testing. One person remained in the high to toxic category after repeat testing. Their total 25(OH)D concentration went from 256 nmol/L to 240 nmol/L after 98 days.

Of the 22 people that were severely deficient according to their initial results, none were severely deficient by their repeat test. The majority (77.3%) went on to have an

adequate vitamin D status and one went on to have a high to toxic level (14.7 to 440 nmol/L) 53 days later. That individual had a further repeat test 214 days later resulting in an adequate status (62 nmol/L).

| | | Repeat Status n (%) | | | | | Initial Status Total (n) |
|---|---|---|---|---|---|---|---|
| | | Severely Deficient | Deficient | Insufficient | Adequate | High to Toxic | |
| Initial Status n (%) | Severely Deficient | 0 (0) | 2 (9.1) | 2 (9.1) | 17 (77.3) | 1 (4.5) | 22 |
| | Deficient | 1 (1.2) | 9 (11.1) | 11 (13.6) | 60 (74.1) | 0 (0) | 81 |
| | Insufficient | 0 (0) | 1 (0.8) | 26 (20.6) | 96 (76.2) | 3 (2.4) | 126 |
| | Adequate | 0 (0) | 4 (1.7) | 18 (7.7) | 208 (88.9) | 4 (1.7) | 234 |
| | High to Toxic | 0 (0) | 0 (0) | 0 (0) | 12 (92.3) | 1 (7.7) | 13 |
| Repeat Status Total (n) | | 1 | 16 | 57 | 393 | 9 | 476 |

**Table 4.18 –** Results of how people responded to their initial result. Boxes highlighted in grey indicate no change in status between initial result and first repeat result. Boxes to the left of the grey boxes indicate a lowering in vitamin D status and boxes to the right of the grey boxes indicate an increase in vitamin D status.

The largest number of people, 208 (43.7%), were in the adequate category and remained adequate upon repeat testing. A small number of people (22, 9.4%), who initially had an adequate status, went on to have a less than adequate status, although no one dropped into the severely deficient category. Four of the people with an initial status of adequate went on to have high to toxic levels.

A substantial amount of people had a less than adequate status initially (229, 48.1%). The majority of these, 173 (75.5%), went on to improve their vitamin D status and became adequate upon repeat testing. However, 52 (22.7%) remained less than adequate, although only two (0.9%) went on to have a worse status than they initially started with (deficient to severely deficient, insufficient to deficient). The remaining four people that initially had a less than adequate status went on develop high to toxic levels on retesting.

4.3.4.1.3. Vitamin D Concentration

The concentration for both groups of samples (initial and repeat) was not normally distributed ($p<0.001$ for both data sets). The median and ranges for both groups is shown in Table 4.19.

| | Initial Samples | Repeat Samples |
|---|---|---|
| **Median (nmol/L)** | 51.3 | 82.7 |
| **Range (nmol/L)** | 10.3-386 | 12-449 |

**Table 4.19 –** Median and range of 25(OH)D concentration for initial and repeat DBS samples.

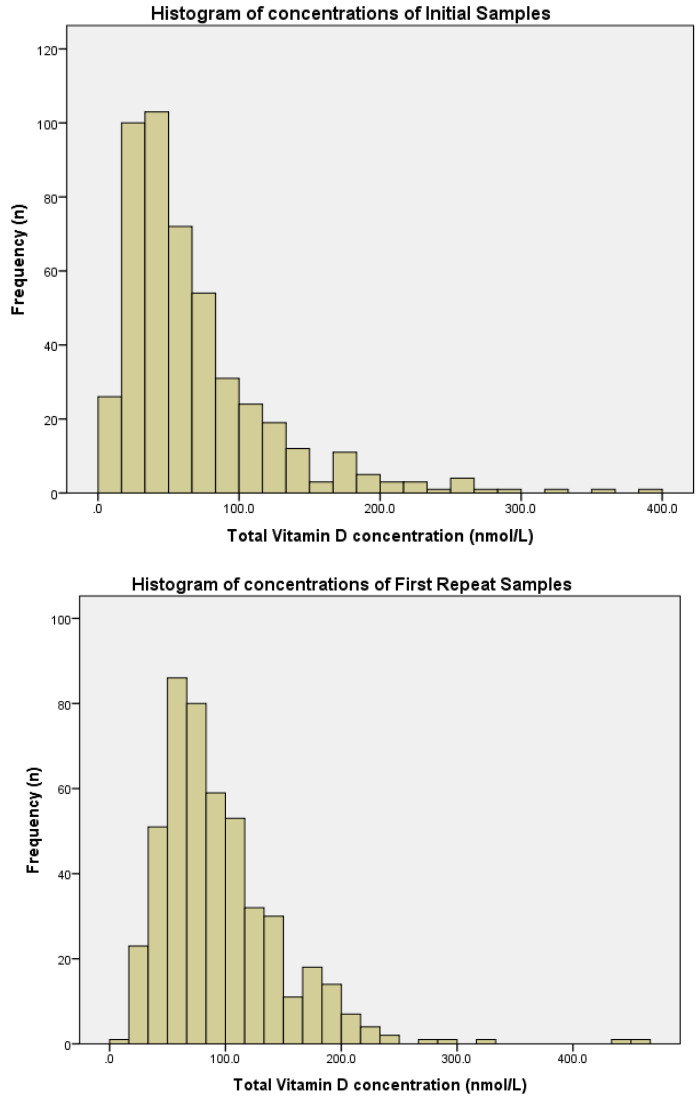



**Figure 4.10 –** The distribution of total 25(OH)D concentration for initial and repeat DBS samples.

As was seen for the status results, the distribution of results for the initial samples were significantly different from the repeat samples ($p<0.001$, Figure 4.10). Overall,

the 25(OH)D concentration was higher for the repeat samples compared to the initial samples.

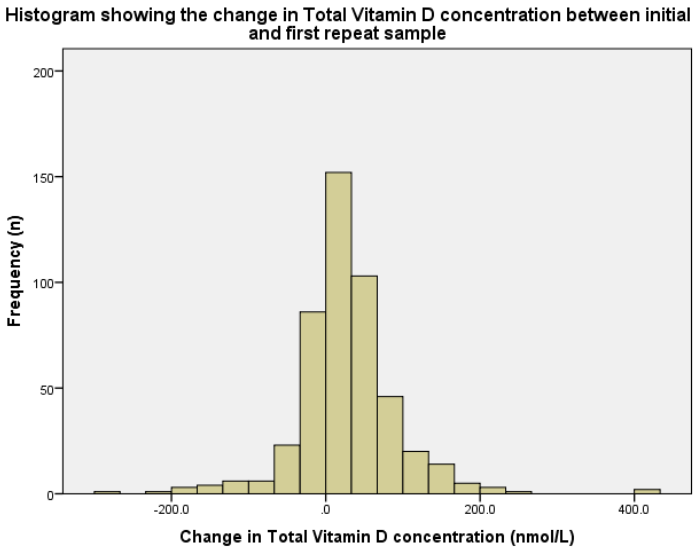



**Figure 4.11 –** Change in total 25(OH)D concentration between initial and repeat samples.

The change in concentration from initial sample to repeat was not normally distributed ($p<0.001$). Median change in total concentration from the initial sample to repeat was an increase of 21.5 nmol/L. The largest decrease in concentration seen was 289 nmol/L and the largest increase in concentration was 425 nmol/L. Most people, 346 (72.7%), increased in concentration from initial to first repeat sample by a median of 39.5 nmol/L (range 0.1 to 425 nmol/L). The other 130 people decreased in concentration from initial to repeat sample by a median of 20.7 nmol/L (range 0.1 to 289 nmol/L, Figure 4.11).

#### 4.3.4.1.4. Repeat Testing Timing

There was huge variation in the time taken for repeat tests (Figure 4.12). This was not normally distributed ($p<0.001$), with a range of 1-526 days and a median of 132 days. Individuals who increased their concentration sent in their repeat sample between 11-480 days with a median of 115 days. People who saw a decrease in

their concentration sent in their repeat sample between 1-526 days with a median of 193 days.

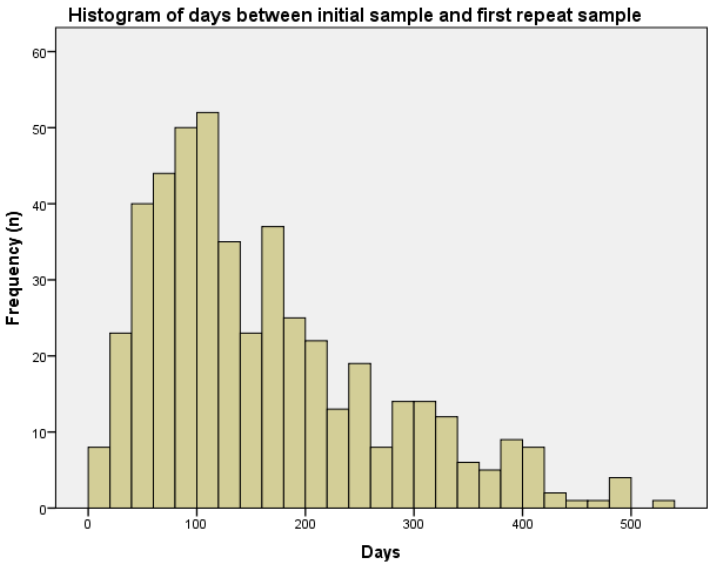



**Figure 4.12 –** Time taken for people to perform a repeat DBS vitamin D test.

4.3.4.2. *GP Serum Samples*

Of the 48,538 samples received during the period of interest, 11,238 (23.2%) samples came from people whose GP had requested more than one sample (including the initial sample). A total of 2,519 people had more than one serum sample analysed and the total number of samples from these people during the time frame investigated ranged from two to four. Two samples from the same person were analysed for 2,504 people. Three repeat samples were received for 14 people and only one person had four samples analysed in the time frame. There were a total of 6,186 samples (55% of repeat specimens) that were ignored as they were part of a pair or group of samples that were overridden due to the demand management rule. Taking this into account, in total, 5.2% of all samples received in the time frame were genuine repeat specimens (not including the initial specimen). It should be noted that what has been taken as the “initial” sample in this data set may not actually be the

first time vitamin D has been measured in that particular patient, but is the "initial" sample for the time frame investigated.

#### 4.3.4.2.1. Population Demographics of Repeat Testers

The majority of people that had repeat serum vitamin D analysed were women (77.4%). The age of the patients in the repeat data set was not normally distributed ($p<0.001$) and the median age was 44 years with a range from 2 to 95 years. The ethnicity was not available for 15.2% of people and the distribution for the rest is summarised in Table 4.20. The most common type of ethnicity was Asian, with the least common mixed. The patients were represented by 118 different GP locations and there were 162 (6.4%) people who had a different location for their first repeat test compared to their initial test.

| | Any other ethnic group | Asian | Black | Mixed | Caucasian |
|---|---|---|---|---|---|
| % | 3.4 | 70.8 | 13 | 1.4 | 11.4 |

**Table 4.20 –** Ethnicity data for GP patients who had repeat testing performed.

#### 4.3.4.2.2. Vitamin D Status

The initial samples group had the highest proportion of severely deficient samples, however the highest rate of people with a high to toxic vitamin D status was seen in the repeats cohort (Figure 4.13). The repeats showed a vast improvement in the number of people in the adequate category, with this category more than doubling in size compared with the initial results, and the severely deficient category halved. However, by the time patients had a second test, 64.5% were still less than adequate. The distribution of statuses for the initial samples was significantly different from that of the repeats ($p<0.001$).

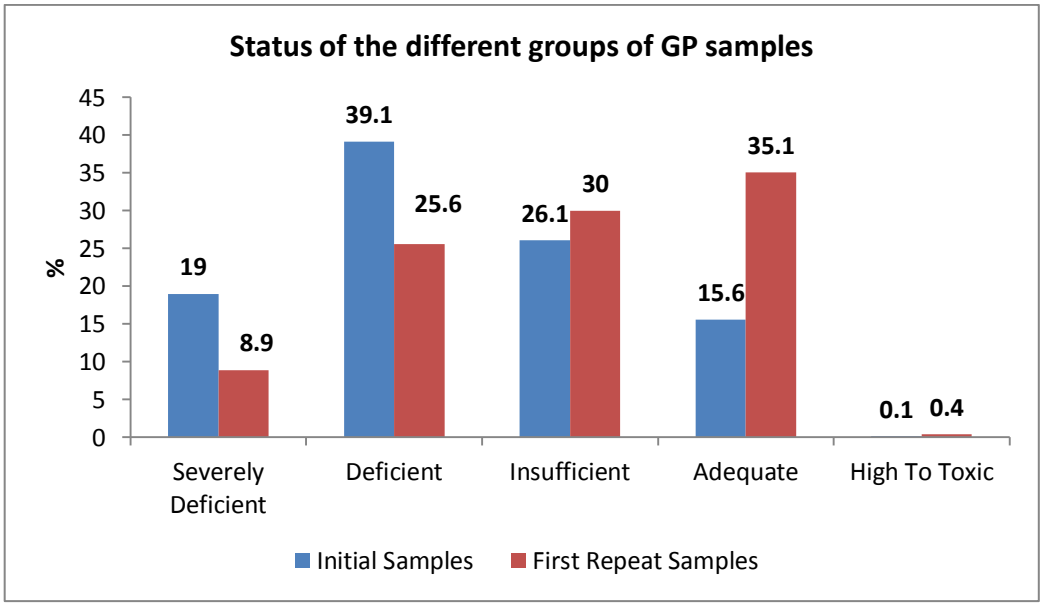



**Figure 4.13 –** Status of the initial and repeat GP samples, figures are percentages.

| | | Repeat Status n (%) | | | | | Initial Status Total (n) |
|---|---|---|---|---|---|---|---|
| | | **Severely Deficient** | **Deficient** | **Insufficient** | **Adequate** | **High to Toxic** | |
| **Initial Status n (%)** | **Severely Deficient** | 106 (22.1) | 118 (24.6) | 103 (21.5) | 150 (31.3) | 2 (0.4) | 479 |
| | **Deficient** | 87 (8.8) | 306 (31.0) | 272 (27.6) | 316 (32.0) | 5 (0.5) | 986 |
| | **Insufficient** | 18 (2.7) | 156 (23.7) | 241 (36.6) | 240 (36.5) | 3 (0.5) | 658 |
| | **Adequate** | 13 (3.3) | 64 (16.2) | 139 (35.3) | 177 (44.9) | 1 (0.3) | 394 |
| | **High to Toxic** | 0 (0) | 0 (0) | 0 (0) | 2 (100) | 0 (0) | 2 |
| **Repeat Status Total (n)** | | 224 | 664 | 755 | 885 | 11 | 2519 |

**Table 4.21 –** Results showing how GP patients responded to their initial result. Boxes highlighted in grey indicate no change in status between initial result and repeat result. Boxes to the left of the grey boxes indicate a lowering in vitamin D status and boxes to the right of the grey boxes indicate an increase in vitamin D status.

Upon repeat, the largest group of patients, 1210 (48%), improved their vitamin D status compared with their initial result (Table 4.21, Figure 4.14). The second largest combined group, 830 (33%), did not change status. The smallest group, 479 (19%) decreased their vitamin D status. Of this last group, only two (0.4%) needed to decrease their status from high to toxic and did so by becoming adequate.

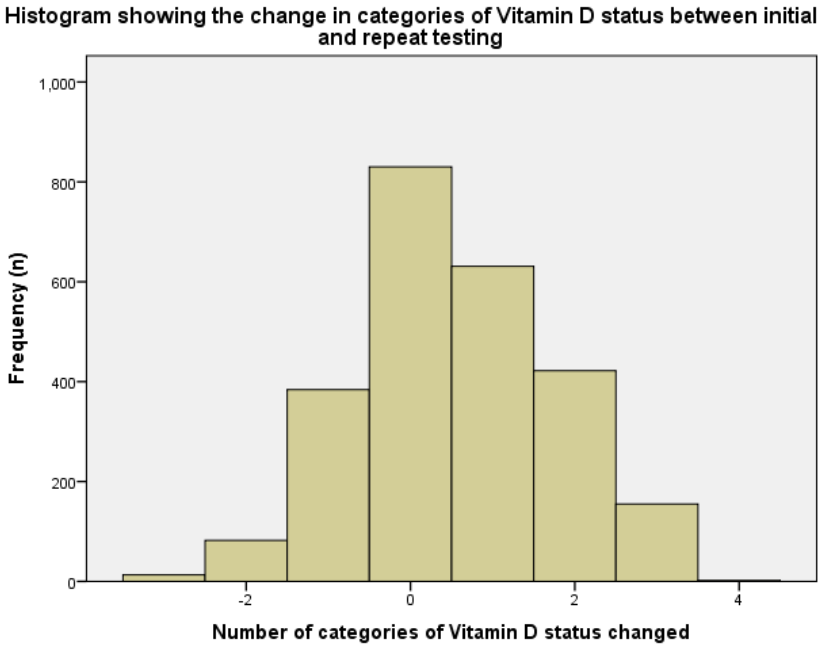



**Figure 4.14 –** Histogram showing how many categories of Vitamin D status patients increased or decreased by after their initial result. The negative numbers indicate a deterioration in Vitamin D status, positive numbers indicate an increase in Vitamin D status and 0 indicates no change from initial status.

People became high to toxic starting from all categories of vitamin D status except those that were initially found to be high to toxic, both of whom became adequate upon retesting. One person became high to toxic despite having an adequate status initially.

A substantial amount of people were severely deficient on their initial test and only 31.3% of them were found to be adequate on retesting. The majority (68.3%) continued to have a less than adequate status with 22.1% of them remaining severely deficient.

There were 2,123 people who had an initial result showing them to be less than adequate. Of these, 653 (30.8%) showed no change in status and 493 (23.2%) improved but remained with a less than adequate status. A large proportion did reach an adequate status (n=706, 33.3%), whilst a smaller proportion actually saw a deterioration in their status (n=261, 12.3%). Of the small group of patients who were

adequate to begin with, the majority actually saw a deterioration in their status (n=216, 54.8%) while 44.9% maintained their adequate status.

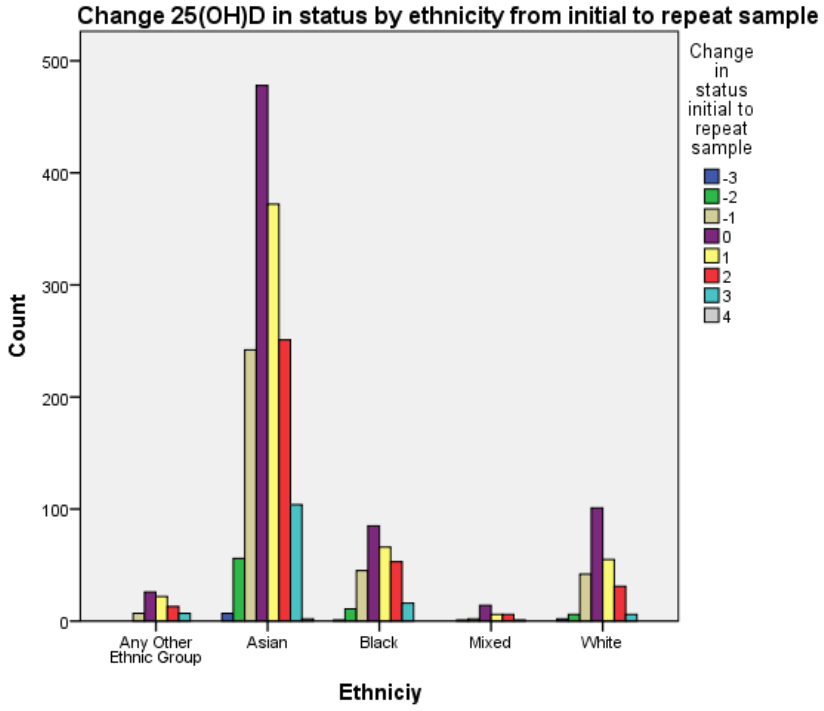



**Figure 4.15 –** Change in 25(OH)D status by ethnicity. The negative numbers indicate a deterioration in Vitamin D status, positive numbers indicate an increase in Vitamin D status and 0 indicates no change from initial status.

When the change in status was looked at in terms of ethnicity (Figure 4.15), there was no significant difference between the different ethnicities (p=0.306). When the change in status was compared between Asian and Caucasians (the two largest groups), there was a significant difference in the change in status (p=0.017). A larger percentage of white patients had no change in status and a greater proportion of Asian patients showed an increase in status compared to white patients.

#### 4.3.4.2.3. Vitamin D Concentration

The concentration for both groups of samples (initial and repeat) was not normally distributed (p<0.001 and p<0.001). The median and ranges for each of the groups of results are shown in Table 4.22.

| | Initial Samples | Repeat Samples |
|---|---|---|
| **Median (nmol/L)** | 25.9 | 39.5 |
| **Range (nmol/L)** | 10.3-245 | 10.3-399 |

**Table 4.22 –** The median and ranges of 25(OH)D concentration for both groups of samples.

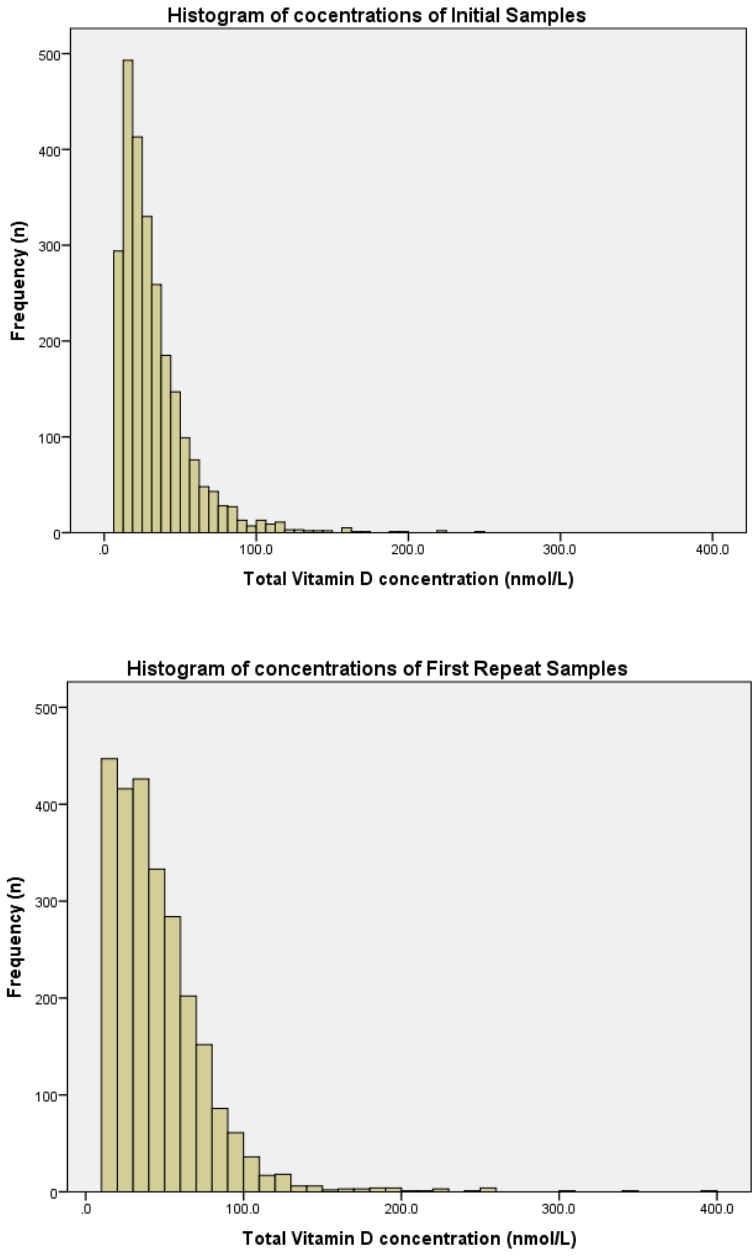



**Figure 4.16 –** Histograms showing the distribution of total Vitamin D concentration for the initial samples and first repeat samples.

As was seen for the status results, the distribution of concentration for the initial samples are significantly different from the repeat samples ($p<0.001$) with the repeat samples generally showing a higher vitamin D concentration (Figure 4.16).

The change in concentration from initial sample to repeat was not normally distributed (p<0.001) (Figure 4.17). Median change in total concentration from the initial sample to repeat sample was an increase of 8 nmol/L. The largest decrease in concentration was 167 nmol/L and the largest increase in concentration was 388 nmol/L. Most patients, 1,636 (64.9%), increased in concentration from initial to first repeat sample by a median of 21 nmol/L (range 0.1 to 388 nmol/L). There were 24 patients who showed no change (15 of which had levels of 10.3 nmol/L, the lowest reportable concentration). The other 859 patients decreased in concentration from initial to repeat sample by a median of 11 nmol/L (range 0.1 to 167 nmol/L).

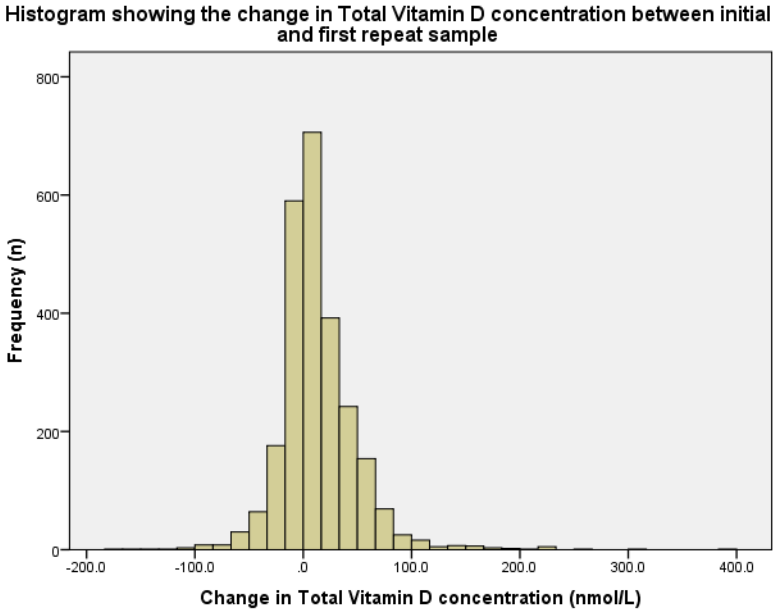



**Figure 4.17 –** Histogram showing the change in total 25(OH)D concentration between initial and repeat samples.

There was no statistically significant difference between the different ethnic groups and the way concentration changed between initial and first repeat samples (p=0.267). The lack of statistical significance was maintained when just the Asian and Caucasian populations were selected (p=0.305).

#### 4.3.4.2.4. Repeat Testing Timing

There was huge variation in the time taken for repeat tests (Figure 4.18). The time taken between initial test and repeat was not normally distributed ($p<0.001$), with a range of 0-687 days and a median of 409 days. A substantial number of samples (n=696, 27.6%) had the demand management rule overridden and were analysed within 365 days of the initial test. For these samples the time taken between repeat tests was still not normally distributed ($p<0.001$), with a range of 0-365 days and a median of 325 days. Individuals who increased their concentration had a repeat test performed between 0-687 days after their initial test with a median of 403 days. Patients who saw a decrease in their concentration had their first repeat sample between 4-678 days with a median of 426 days.

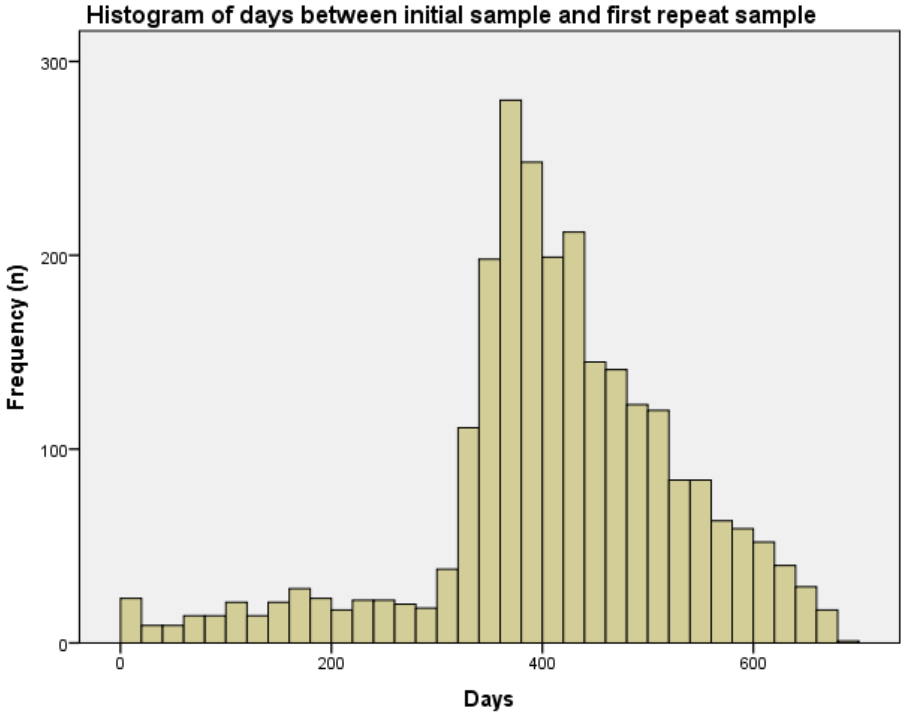



**Figure 4.18 –** Histogram showing the time between GP repeat tests. Some of the very short intervals were due to the request intervention failing due to no results being available for the original request e.g. when there was a backlog due to analytical failure. Samples were therefore sometimes analysed when they should have been rejected. Other samples would have had the request intervention over-ruled e.g. if the patient was <16 years old or for clinical reasons.

## 4.4. Conclusion

### 4.4.1. Vitamin D Concentration and Status

Our data on the direct to the public DBS service and the GP serum samples showed that these two populations have different demographic profiles. More women were tested for vitamin D than men by GP's and significantly more women were tested by GP's than used the DBS service. This may be a result of the fact that significantly more women use healthcare services than men and so are more likely to be tested for vitamin D. (165) More men may use the DBS service than the GP service because they may feel more inclined to test themselves than to ask the GP for a vitamin D test.

The results also showed that a wide age range of patients were being tested in both populations, but the age distribution was different. The median age of patients using the DBS service was older and it may be that older patients find it easier to take a DBS sample in the comfort of their own home and post it into the laboratory, rather than make a trip to the GP to get tested. There is no literature regarding this, however 97.9% of people responding to our survey question asking if convenience was a reason why they used our service said it was. The majority of people answering this survey were in the >60 years age group category.

There was shown to be a huge difference in the ethnic make-up of both populations. This is likely to be because the GP serum samples were from patients in the area surrounding City and Sandwell Hospitals in Birmingham, which is a multi-ethnic population, while the DBS samples came from all around the country, and indeed the world. The distribution of ethnicity of the DBS samples more closely resembles that seen in the 2011 census estimate of the ethnicity of England and Wales (Figure

4.20), although the DBS population did include some samples from other countries. (166)

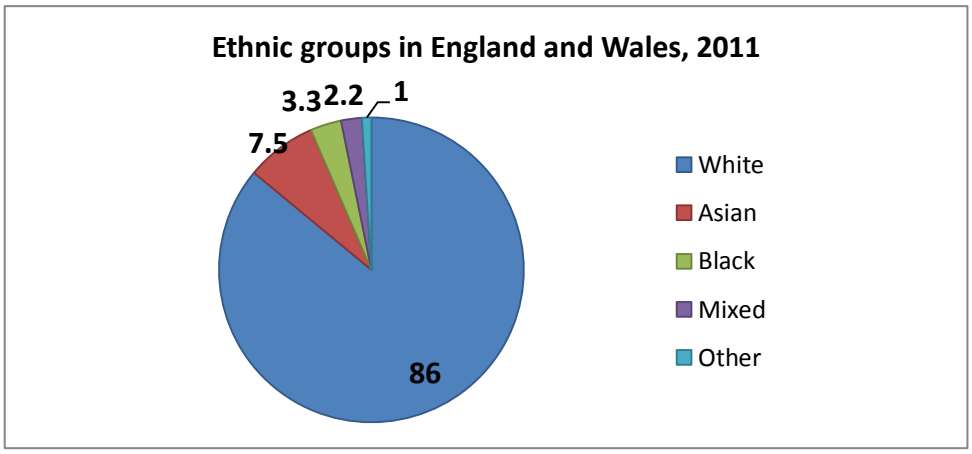



**Figure 4.20 –** Ethnic distribution of the population of England and Wales in 2011. (166)

Not only were the demographics of the two populations markedly different, but the there was also a significant difference between the 25(OH)D concentrations for the two sample types, with the majority of GP patients less than adequate and the majority of DBS patients adequate. This difference between the two populations could be due to the differences in age and gender distribution seen between the populations. However, although there was an association between gender and vitamin D status for GP samples, it was not an obvious trend and no such relationship existed for the DBS population.

There was an association between age and vitamin D status for both populations, with older patients tending to have a higher vitamin D status. As the DBS population was older than the GP population, this may explain some of the differences seen between the two populations in terms of vitamin D status and concentration.

A further difference between the two populations could be their socioeconomic status. People have paid to use the DBS service, this is how the service is funded, whereas the GP population had their levels measured for free. This may imply that if

people can afford the kit they may be able to afford supplements more readily than the GP population. A study in Canada showed that the prevalence of supplement use was positively associated with high household income. (167) Black et al. looked at the predictors of vitamin D supplement use in an Australian population and they also found that higher socio-economic status was a predictor. (168) However our GP population may be able to get vitamin D supplements free of charge and therefore their socioeconomic status may not be such a large factor in supplement use (e.g. Healthy start scheme etc.). This is an interesting area that requires further research.

The greatest recorded difference between the populations was the distribution of ethnicity which is known to affect people's susceptibility to vitamin D deficiency. Ethnicity is likely to play an important role in explaining the differences in vitamin D concentration between the two populations as the proportion of non-Caucasian samples is much higher for the serum samples than for the DBS samples. It has been shown in our local GP population that 1 in 4 Blacks and 1 in 3 Asians were deficient, compared with 1 in 8 Caucasians, using a definition of deficiency of 25 nmol/L.(169)

Another potential reason for the difference in concentration between the two populations could be due to why people are testing for vitamin D in the first place. GPs will test when they suspect a deficiency due to the presence of symptoms or risk factors and therefore this population is more likely to have deficient results. Some of the DBS feedback we receive suggests that many people using the service could be classed as the "worried well", as they are often on supplements and are testing to see if the supplements are having an effect. Alternatively people may be testing themselves and their families to check their status to ensure that they are replete, not

necessarily because they are symptomatic. If they are deficient, they rectify their deficiencies before rechecking, meaning more DBS samples will be from replete patients.

Work performed using the same local population as our GP serum samples involved testing randomly chosen serum from outpatient samples for 25(OH)D. (169) Deficiency using different cut-offs to those used here was reported and when severe deficiency was defined as 20 nmol/L, 14% of the total randomly selected population was found to be severely deficient. By applying the same cut-off to our serum GP samples, 32% of patients would be classified as severely deficient, even though the ethnic make-up of the two populations is likely to be similar. If the same cut-off is applied to our DBS samples it would mean that 7.3% of DBS patients were severely deficient.

This large difference in proportion of population being found severely deficient could be because the original study (169) was done at the end of summer when deficiency should be at its lowest and our data set includes results from the whole year. However, the results suggest that a random population could have a prevalence of deficiency in between our two diverse populations. GPs could be appropriately testing for deficiency, resulting in this population having lower concentrations, while the DBS population includes a large proportion of the “worried well” who just want to check that they are replete in vitamin D leading to a higher average concentration of vitamin D in the DBS population. This is borne out by Figure 4.5 which shows that the GP serum samples display seasonal variation (suggesting minimal supplementation) and the DBS population displays a flatter trend over the year (suggesting supplementation is overcoming the seasonal variation).

### 4.4.2. **High to Toxic Patient Investigation**

The rate of 25(OH)D results >220 nmol/L in our direct to the public vitamin D DBS population was high at 3.1%, 52 times greater than that seen in our GP population at 0.06%. The majority of results were <375 nmol/L, however a substantial proportion (8.6%) were >500 nmol/L. Although we were unable to show toxicity in our users as we were only able to measure 25(OH)D and not urine or serum calcium, 500 nmol/L is a level most would consider is required for toxicity to occur. (130, 131, 156, 170, 171) Surprisingly, only one of the users whose level was >500 nmol/L was under medical supervision.

Paired DBS and plasma samples showed how well the two sample types agreed, so the high rate of results >220 nmol/L found in the DBS population compared to the GP population was not due to methodological differences, but was a genuine difference.

The difference between the two populations may be due to the interest in vitamin D shown by the direct service users. This interest is highlighted by the varied reasons why people were taking vitamin D supplements. One of the most common reasons given was because people had multiple sclerosis and many of them stated that they were following the “overcoming multiple sclerosis” programme. (172) This programme encourages people to aim for 25(OH)D levels >150 nmol/L and this may explain why so many people following this programme were contacted by us. Of the 48 people who listed MS as a reason for supplementing with vitamin D, four had a previous low result and 15 had a previous adequate result – all were trying to achieve a 25(OH)D concentration >150 nmol/L. The use of vitamin D in the treatment of multiple sclerosis has caused some controversy in the literature (173, 174) however many of the users we spoke to did feel that they had gained benefit from having an

“adequate” level of vitamin D. There were 10 other people who had had a previous result from us who went on to have a high result because they were purposefully aiming high.

This data was gathered on a novel population that to the best of our knowledge has not been studied in this way before. This population may be more representative of the general public than a population who have had their vitamin D levels tested by their GPs, who may be predominantly looking for deficiency. However this population is likely to be “biased” as it is self-selecting because users have chosen to monitor their vitamin D levels at a financial cost to themselves. This population may be more likely to take vitamin D supplements than the general public because they have chosen to have their levels measured, implying that they have knowledge of vitamin D. The data presented here is only representative of the cohort of patients using our service who were found to have high levels and does not necessarily reflect the practice of those using our service whose 25(OH)D levels have remained <220 nmol/L.

We encountered two users who had taken large bolus doses of vitamin D (300,000 and 900,000 IU) without medical supervision. Such cases have been reported in the literature, both intentional and unintentional, with and without side effects, in acute and non-acute settings. (158, 174-177) We have no evidence of toxicity in our cases, only that high levels of 25(OH)D were achieved. None of the cases mentioned here had their levels of calcium (either serum or urine) measured by us and therefore we cannot comment on whether or not the levels of 25(OH)D caused toxicity or how safe the amount of vitamin D taken by our users was. However, we have shown that high levels are occurring, intentionally or otherwise, at levels that the wider literature has

deemed to be toxic. Further work looking at the general public that are routinely taking high doses of vitamin D and linking it to the presence or absence of toxicity would be an interesting area of further study.

All of the data except for the 25(OH)D results was self-reported, therefore we cannot confirm the accuracy of the amount of vitamin D people were claiming to be taking or the length of time they had been supplementing for. This may in part explain the over-lapping and wide range of concentrations of 25(OH)D obtained by the users taking very different amounts of vitamin D. This work however highlights the wide-ranging regimens people are using in everyday life with regards to vitamin D supplementation and how these can all potentially lead to hypervitaminosis D.

The IOM's 2011 report on Vitamin D (131) generated debate as to whether their no observed adverse effects level of 10,000 IU/day and their upper intake level of 4,000 IU/day was too low. (164, 178) The Endocrine Society published their own guidelines in 2011 (144) which recommended higher cut-offs for adequacy compared with the IOM report (75 nmol/L vs. 50 nmol/L respectively) and they also recommended higher daily requirements and an upper intake level of up to 10,000 IU/day in some groups, not to be exceeded without medical supervision. However, we found that just under a third of our high level population were regularly taking more than 10,000 IU/day and only 6% of those were doing so under medical supervision.

Not all users that obtained a high 25(OH)D did so through supplementing. One person had a result of 239 nmol/L not by supplementing but by spending as much time as possible outside, without sunscreen and avoiding washing. This is similar to levels mentioned in the literature, obtained by people working in sun rich environments and who were not taking vitamin D supplements. (162)

Due to the high cost of testing 25(OH)D, it is recommended that populations are not screened and that routine monitoring of patients for 25(OH)D is not required. (131, 144) The National Osteoporosis Society suggest routine monitoring is generally unnecessary except in patients with symptomatic deficiency or malabsorption and where there is poor compliance with medication. (130) Nearly half of our high population had measured their 25(OH)D concentrations with us before receiving their first high result and three-quarters of those had an adequate result (although what we considered adequate was not always what the users considered adequate). Our work suggests that just because someone has once obtained an adequate result whilst on supplementation, it does not mean that they will remain within that range and therefore in certain other populations retesting may be required. This is not just the case for people taking above 10,000 IU/day as we found users taking as little as 1,000 IU/day could also be at risk of having high levels.

We found a number of children who had high levels of 25(OH)D. The IOM recommended in 2011 (131) that the upper tolerable limit for children was: 1,000 IU/day for infants 0-6 months old, 1,500 IU/day for 6-12 month old infants, 2,500 IU/day for 1-3 year old, 3,000 IU/day for 4-8 year olds, and 4,000 IU/day for children 9 years and above (same as adults). The Endocrine Society's guidelines (144) recommended higher upper tolerable limits of 2,000 IU/day for children up to 12 months and 4,000 IU/day for children from 1 to 18 years. Of the 12 children in our study who gave the amount of vitamin D they were supplementing, six were exceeding the IOM's guidelines and two were exceeding the Endocrine Society's guidelines, whilst none were under medical supervision. The variability in regimens followed by these children (amount taken, length of time taken, brand and formulation

taken) could all have contributed to the variable responses seen to the levels of vitamin D taken. A recent review by Vogiatzi et al., (158) describes how many studies have been undertaken in children following a variety of vitamin D dosing regimens within the IOM or Endocrine Society's guidelines, and the majority of those studies reported no documented harm although some subjects did attain high levels of 25(OH)D.

Of particular concern in our study were the two children who were only taking 1,000 IU/day, well within both sets of recommendations mentioned above. Despite this they both had levels >300 nmol/L (309 and 498 nmol/L). We do not know whether the children were hypercalcaemic or hypercalciuric, however there have been cases reported where paediatric patients have become hypercalcaemic after receiving vitamin D well within the accepted guidelines (179) highlighting the need for caution when administering vitamin D to children.

Many factors should be considered when looking at vitamin D supplementation. Vitamin D is available as a licensed product in the UK via prescriptions and in 2014 190,000 prescriptions for vitamin D were issued each month. (180) However, our data reveal that most direct service users obtained vitamin D without the use of a medical professional by purchasing unlicensed products themselves, usually through the internet. Unfortunately, although over the counter unlicensed supplements may be cheaper or more convenient to obtain, the quality cannot be guaranteed as it would be with licensed prescription vitamin D. LeBlanc et al., (181) found that the potency of unlicensed vitamin D, when compared to that quoted on the bottle, ranged from 9% to 146%. In addition, they found that potency varied within batches as well as between batches, a problem highlighted in other work where unlicensed vitamin D

supplements have been tested (182, 183) This may also explain some of the variability in concentration of 25(OH)D seen in our study even when users were taking similar amounts of vitamin D.

Formulation of vitamin D can also affect a person's response to vitamin D supplements. Work has been done that suggests that vitamin D in an oil based vehicle can produce a greater response of 25(OH)D compared with vitamin D in powder or ethanol based vehicles. (184)

A further factor that should be considered when examining people's response to vitamin D supplementation is their genetic makeup. Recently a number of reports have highlighted the role of CYP2R1 and CYP24A1 in the metabolism of vitamin D. Mutations in these genes can lead to both 25(OH)D deficiency (185) and excess, (186, 187) therefore the vastly different responses to the supplement regimens in our cohort of patients could have in some way been influenced by genetics.

Our study has been enlightening in regards to showing how members of the public approach vitamin D supplementation. The rate of vitamin D supplement use is on the rise (160, 188) and it has been shown that 70% of patients do not report the use of alternative treatments to their doctors. (175) It is therefore important that clinicians consider the use of vitamin D supplementation in the differential diagnosis of hypercalcaemia, especially given how common hypervitaminosis D may be in the general public based on the work done here. This work demonstrates that there are a considerable number of people supplementing to potentially toxic vitamin D levels and brings the idea that standard doses of vitamin D can be applied across the board into question.

### 4.4.3. **Impact of a Direct to the Public Service**

The people surveyed by our questionnaire had chosen to use our service and therefore a positive response regarding DBS may be expected from them, however the responses we received were overwhelmingly positive. The vast majority of people felt that the instructions and the results were easy to understand and most people found it easy to collect a DBS. Our service was rated either good or excellent by all customers and people were generally satisfied with our turnaround times and customer handling. Many people would like to be able to buy kits on the internet, and this is something that will hopefully be introduced in the future.

A common theme in the literature relating to DBS is that one of the principal benefits of using DBS is the smaller volume of blood collected from the patient (100-150 µL of capillary blood collected compared to mLs of blood from venous collections). (15) However, for the cohort of patients questioned by us, this was not a major concern. People were much more interested in the fact that they could take control of their own health and that it was convenient. With the direction that the NHS is taking in allowing patients access to their blood results and encouraging patients to more actively manage their health, DBS may be a way that can enable this to happen in certain settings. The response to our questionnaire strongly indicates that the public would be interested in seeing other DBS tests made available.

Time spent on phlebotomy is an important aspect of the patient pathway that is often overlooked. It is interesting to note that the second most popular reason for people choosing to use the DBS service was because it was convenient. Some literature that discusses pre-analytical errors in laboratory medicine mentions the length of time that patients have to wait for phlebotomy. It has been reported (189) that the

average patient wait time was 21(±3) minutes before improvements were made to Brigham and Women's Hospital outpatient phlebotomy service. Another hospital reported a wait time of between 37.4(±21) and 40.7(±25.1) minutes (190) for patients requiring tests before elective procedures. These times do not include the time taken to get to phlebotomy. Our own unpublished data found that, when travel time is included, patients can spend between 5 minutes and 4 hours 15 minutes on a phlebotomy visit. If DBS could help to eliminate this, especially for patients who need regular phlebotomy, then it would be of great benefit to the patient journey. Our DBS collection kit has demonstrated the feasibility of using DBS in a non-clinical environment and this could lead to substantial time savings for patients.

The direct to the public DBS vitamin D service offered by SWBH has been well received and continues to grow. We have shown that using DBS as a means of testing is viable and is appreciated by the public. Some work into user satisfaction with the use of DBS for home testing $HbA_{1c}$ found 96% of people were satisfied with home collection and 83% indicated that home collection in the future was desirable and that the filter paper method of collection should be brought into practice. (23)

The work done here demonstrates that demand for the use of DBS by the public is there and this has been seen by the great uptake of our vitamin D DBS service and the fantastic feedback we receive from the public about it.

### 4.4.4. **Repeat Testing**

As stated previously, many things in the area of vitamin D lack consensus, such as what is the optimal level of serum vitamin D. However as far as the rate of testing goes the evidence is unequivocal: vitamin D testing is growing at a staggering rate. Our laboratory has seen a 70% increase in serum vitamin D testing since 2012 and

measured just short of 80,000 serum samples between April 2013-March 2014 (Figure 4.21). This is even after demand management was introduced in 2010, whereby GPs could only have one vitamin D test per patient per year, unless there were strong clinical indications for measurement. Other laboratories have noticed an annual 80-90% growth in 25(OH)D test rates. (191) One report suggested that Quest laboratories in the USA were receiving 500,000 requests for 25(OH)D analysis per month. (192)

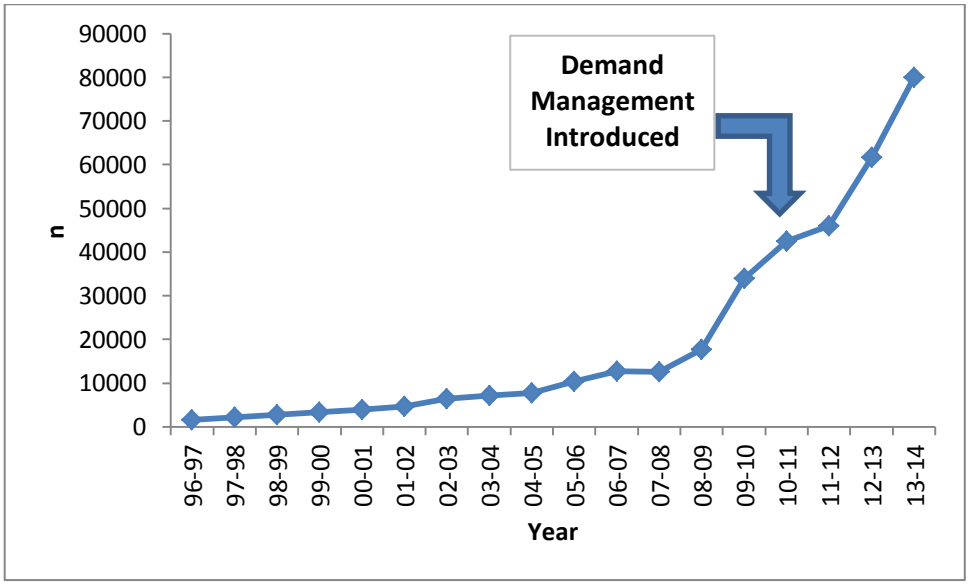



**Figure 4.21 –** Growth in 25(OH)D testing since records began for City Hospital, Birmingham, UK. Years run from April to March. Data starts when records began. Demand management was introduced in April 2010 meaning that GPs could only order one 25(OH)D test per patient per year.

Growth has come from testing in the GP population as well as hospital based testing. In addition to this, our DBS Vitamin D service has also grown steadily since its introduction in 2011 with requests being received from all over the world as well as the UK.

Health professionals test vitamin D for a variety of reasons but we have found that the reasons the general public want to test are very different (see 4.3.2.4. and 4.3.3.). The different reasons behind testing, as well as population differences such as ethnic

distribution, have led to a significantly different distribution of vitamin D statuses in the two populations. Given this difference in statuses, it is unsurprising that the initial concentrations of the repeat testers were also so different. The data also showed that on repeat testing there was an overall improvement in status and an increase in median concentration for both populations. What is less apparent is why the response to the initial result was so different, with DBS users showing a far greater improvement in vitamin D status and concentration.

For both populations, if a severely deficient level is uncovered then the aim would normally be to improve that vitamin D concentration and achieve an adequate status. This appears to be happening in the majority of cases for the DBS patients, but the bulk of GP patients are remaining less than adequate on repeat testing with an unacceptably high proportion remaining severely deficient. Maintaining adequate levels also seems to be a problem for GP patients, but not for the users of our DBS services. As far as we are aware, there has been no data published on repeat testing rates or response to initial results in GP patient populations and certainly no data published on how the public manage their own vitamin D levels.

Unfortunately, we have no information on the treatment regimens of either population or their reasons for repeat testing. Our work on the high to toxic patients using the DBS service has shown that many members of the public are happy to supplement with high levels of vitamin D without medical supervision. However, without specific information on what GPs are doing when they receive an initial vitamin D result we are unable to say for definite why GP patients are not achieving adequate levels of vitamin D on repeat testing when the public are.

Based on overall population data, there are massive differences between the two populations in their ethnic make-up. This is likely to contribute to the differences between the initial results for both of the populations. However, there are no published guidelines that suggest different ethnicities should follow different treatment regimes in order to achieve an adequate status, once a less than adequate status has been found. That implies that ethnicity should not have an effect on the way people respond to their results.

Other contributory factors may be that the GPs are treating the patients initially but not prescribing a maintenance therapy, so by the time the repeat testing is performed over a year later, the patient has reverted to where they were originally. The GP patients may not be particularly interested or concerned about their vitamin D status and so may not be compliant with any treatment prescribed. There may be differences in social-economic status, making it more likely that the DBS service users, who have paid to be tested in the first place, will be able to afford to buy supplements “over-the-counter” and so treat themselves when a deficiency is made apparent.

The reason for repeat testing may also be playing a role. GPs may only be testing patients they suspect have not been compliant, or who are still likely to be vitamin D deficient. In which case the data would suggest they are picking up on the right patients and we need to find out why the treatment, that they may or may have not given to deal with the hypovitaminosis in the first place, has not been successful or why GP patients are not able to maintain an adequate status.

Another difference could be that GPs may feel constrained by the available guidelines. The recommended daily allowance according to the IOM (131) is 600-800

IU/day depending on age. There are no reference nutritional intakes for adults in the UK who are deemed to have an adequate dietary intake or exposure to sunshine. For those who are deemed at risk (pregnant women, people over the age of 65, housebound) then the reference nutritional intake is 400 IU/day (Committee on medical Aspects of Food and Nutrition Policy - COMA, 1991). The Scientific Advisory Committee on Nutrition (SACN) are currently reviewing the dietary reference values for vitamin D intake. There are no NICE guidelines available relating to vitamin D supplementation, although there are a variety of other guidelines available and a NICE guideline was published in 2014 on how to increase supplement use in at-risk groups. (193) GPs will therefore have to decide for themselves how best to treat patients and this will lead to a large variation in practice and may explain why some GP patients do improve their statuses while others do not. Given the conservative nature of the COMA recommendations, it may be that some GPs are being conservative in their treatment of vitamin D deficiency and so may not be able to improve their patient's vitamin D status greatly as a result. Also, until recently, there were very few prescription options available in the BNF and most that were available also contained a calcium component, which is not always appropriate.

Whatever the reasons for repeat testing, it comes against a backdrop of rocketing levels of vitamin D testing generally amidst a tightening NHS budget. Laboratories are having to restrict further and further the availability of vitamin D testing to GPs. In this context, we need to make sure that when a patient is seen to have vitamin D deficiency that the test result is used and the patient is treated appropriately. It looks like this is not occurring for our GP patients for those that are being re-tested. Not only is this leading to a cost to the NHS for having to repeat the vitamin D testing, but

long term health costs could also occur. Yet, the DBS service users show that it is possible to respond to a vitamin D test appropriately and maintain adequate levels of vitamin D. Until there is a national strategy, led by NICE, on how GPs should use vitamin D testing services and how they should treat patients, and until a less conservative approach to vitamin D supplementation is taken, this variation in response to an initial vitamin D result is likely to continue.

# CHAPTER 5 - DBS C-REACTIVE PROTEIN

## 5.1. Overview

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that results in inflammation of the joints in the body causing pain, swelling and stiffness (synovitis) and affects approximately 1% of the adult population. (194) The disease course and presentation can be highly variable within and between patients, but it is crucial to detect RA as early as possible in order to suppress disease activity and therefore minimise loss of function and damage of joints. Early detection and treatment can have a significant impact on the subsequent course of the disease. (195)

Once a patient has been diagnosed by a rheumatologist as having RA, a DAS28 (Disease Activity Score looking at 28 joints) is used to measure the disease activity. The DAS28 is a composite outcome measure that combines four types of assessment to give a score which measures the extent of disease activity. It requires a clinical assessment of the patient assessing the number of joints that are tender and the number that are swollen (out of 28 potential joints) together with a C-reactive protein (CRP) measurement (an erythrocyte sedimentation rate can be used instead) and a patient's "global assessment of health" using a visual analogue score. The visual analogue scale score is a simple scale (a 100 mm line) upon which the patient indicates how they are feeling by marking a point between 0 (very good) and 100 (very bad). (196)

CRP is a nonspecific acute phase protein synthesised in the liver that increases rapidly as a result of inflammation, infection and injury. (197) CRP can be used to assess the inflammation status of a patient with RA, in particular increased levels are

associated with decreased functional ability, increased disease activity and radiological progression, (195) hence its inclusion in the DAS28 calculation.

NICE recommends the monthly measurement of CRP and DAS28 in people with recent-onset RA until treatment has controlled the disease. Once that status has been achieved, it should then be measured regularly to inform decisions on whether treatment should be increased or decreased. (198) The DAS28 helps in this decision making process as it measures disease activity (Table 5.1).

| DAS | Explanation |
|---|---|
| **<2.6** | Disease remission |
| **2.6-3.2** | Low disease activity |
| **3.2-5.1** | Moderate disease activity |
| **>5.1** | High disease activity |

**Table 5.1 –** The Disease Activity Score (DAS) – DAS28

In order for the DAS28 to be calculated the patient must arrange to be bled before their clinic appointment so that the CRP result can be available in time. This can result in multiple visits to phlebotomy in addition to clinic visits and so in reality, the patient may only go to phlebotomy after the clinic appointment. Consequently, the CRP result may not be available and so the DAS28 is either not calculated or results that are at least a month old need to be used instead. As a result the DAS28 does not always reflect the current inflammation status of the patient. Discussion with our Trust's consultant rheumatologist has revealed that this is often the case at SWBH.

Using DBS, patients could avoid having to make extra trips to phlebotomy in addition to clinic appointments, because they could send in a sample to the laboratory for measurement before their appointment. (15) This would also enable better management of patients based on knowledge of current inflammatory status.

The aim of the work in this chapter is to establish a DBS method for the measurement of CRP using the SWBH device and also see if the Mitra could be used to measure CRP in dried blood.

## 5.2. **Method**

Please also see Chapter 2 for general CRP DBS, Mitra and serum methodology including sample preparation and the final method used for DBS and Mitra CRP analysis.

### 5.2.1. **SWBH Device**

The SWBH device was described in Chapters 2 and 3. The aim for CRP measurement using DBS was to use methodology enabling analysis to be done quickly and easily. We attempted to set up a DBS assay using the Abbott Architect analysers that are used to measure serum samples routinely in our department.

#### 5.2.1.1. *Method Development*

##### 5.2.1.1.1. Extraction

The length of time spots should be sonicated for was investigated. The machine was calibrated using spots that had been sonicated for five minutes and then the punches removed before analysis. Another five sets of calibrators were prepared, each having different lengths of time in the sonicator (5, 10, 20, 30 and 60 minutes). After sonication the punches were removed from the extraction buffer and extracts presented to the machine and analysed. In addition the length of time spots could be left *in situ* was investigated.

#### 5.2.1.2. *Method Validation*

Unless otherwise stated the DBS preparation and extraction methods are as described in Chapter 2.

##### 5.2.1.2.1. Analyser Variation

The aim of this experiment was to sample from a single cup and see what the CV of the assay was when the extraction of the punches and DBS variation was not part of the procedure i.e. to obtain the CV of the analyser component of the assay. The reason for doing this was to assess the impact of the "alternative sample type" of the DBS sample extract on the assay, in comparison to the usual sample type of serum. This was done by obtaining the CV of the assay when the DBS extracts were combined into one cup (large volume of extract required for 10 replicate analysis therefore punches were extracted and the eluates combined) and the analyser sampled multiple times out of one cup. This was then compared to the CV obtained when a serum sample is analysed multiple times from one cup.

Two samples with different levels of CRP were used. Five punches were removed from each sample, extracted and combined into one cup. The machine then analysed 10 replicates from one cup for each DBS sample using the DBS CRP method. Five serum samples with different levels of CRP were placed into cups and the machine analysed 10 replicates of each sample from the same cup using the standard CRP method.

##### 5.2.1.2.2. Inter and Intra Assay Performance

The CV data for the standard CRP serum method is known from the kit insert. For the DBS CRP method intra assay variation, three DBS samples were extracted 10 times each and analysed in one batch. The inter assay variation was assessed by

analysing three DBS samples on 10 consecutive occasions. The mean, SD and CV were calculated to give the intra and inter assay variation.

#### 5.2.1.2.3. Extract Stability

Three DBS EDTA samples were used. Each had five punches removed, extracted and the extracts pooled. The extracts were analysed immediately and then stored at 4°C. They were then analysed daily for the next four days.

#### 5.2.1.2.4. DBS Sample Stability

Three EDTA DBS samples with different levels of CRP were used. Each sample was split into six batches and stored in one of the following ways:

- At room temperature without desiccant
- At room temperature with desiccant
- At 4°C without desiccant
- At 4°C with desiccant
- At -80°C without desiccant
- At -80°C with desiccant

A baseline measurement was taken for each of the three DBS samples (in singleton). All 18 samples (three different samples split into six storage conditions) were measured in singleton on 14 consecutive days (day six and 12 missing data) plus one further measurement at day 24 when the analyser was re-calibrated. The mean of all storage types for each sample was calculated for each measurement day and plotted. Statistical analyses were performed to investigate the impact different storage conditions made on the results and whether the results changed over time.

#### 5.2.1.2.5. Linearity

Five samples that had a high serum CRP and paired FBC were used. Plasma was obtained from the EDTA whole blood sample and measured using the traditional Abbott serum method. The rest of the EDTA sample was used to make DBS (20 µL spots). Two 3 mm punches were extracted as described above and the extracts combined. The extracts were double diluted with R1 to a final 1:64 dilution. The extracts were analysed using the DBS CRP method.

#### 5.2.1.2.6. Limit of Detection and Quantitation

The limit of detection can be defined as the mean + 2SD of a series of blank measurements. To determine the limit of detection 10 replicate measurements of blank R1 buffer was made. In addition DBS were made from an EDTA sample with a concentration <1 mg/L for its paired serum sample. Ten punches were extracted and analysed.

The limit of quantitation was determined to be the level at which the CV of the assay was ≤ 20%. Therefore 10 punches were taken from DBS samples with CRP concentrations of 1, 2, 3 and 5 mg/L in order to determine what the intra assay CV was at those levels.

#### 5.2.1.2.7. Carryover

To assess whether or not carryover could occur, two punches were taken from very high DBS followed by five punches from blank filter paper. The DBS puncher was not cleaned between punches being taken. Carryover was deemed to have occurred if a blank filter paper punch gave a value greater than the limit of detection. This was done for three different high DBS samples.

5.2.1.2.8. Patient Samples

Patient samples were collected during phlebotomy clinics taking place during the rheumatology clinics. Ethics approval had been granted to collect extra samples from patients in addition to the routine samples that were going to be collected (Appendix 8). Every patient had a serum and EDTA blood sample taken as part of their post-clinic workup. After consent had been obtained, a capillary finger-prick blood spot sample was collected by a member of laboratory staff. In total, 41 samples were collected and prepared as described in Section 2.3.2. The DBS samples (EDTA and capillary) were extracted and analysed after being allowed to dry overnight. Serum samples were analysed using the Abbott serum CRP method. Samples were collected over a month.

5.2.2. **Mitra Device**

The DBS CRP methodology was established before the Mitra came onto the market. Once available we wanted to see if the same methodology that was used for the SWBH device could be used with the Mitra. All experiments below were carried out using Mitra devices made from EDTA whole blood or manufactured samples as described in Chapter 2, except for section 5.3.2.2.7 where capillary Mitra devices were also used.

5.2.2.1. *Method Development*

The method was established using the SWBH device. None of the analyser settings needed adapting for use with the Mitra device. The extract stability was assumed to be the same as the extraction conditions were the same for both sample types. The length of time samples could be left *in situ* was not investigated as the cups cannot be presented to the analyser with the Mitra device in place due to the presence of the

sticks. Carryover was not investigated as no punching device was needed and therefore there was no source of carryover to investigate.

#### 5.2.2.1.1. Extraction

The length of time devices should be sonicated for was investigated. The machine was calibrated using four week old Mitra calibrators that had been sonicated for five minutes and the devices removed immediately after sonication. Five sets of seven EDTA patient samples were prepared. After drying overnight, each set of seven samples were sonicated for different lengths of time (5, 10, 20, 30 and 60 minutes). After sonication the devices were removed from the extraction buffer and extracts presented to the machine and analysed.

#### 5.2.2.1.2. Age of Sample

The extraction experiment was repeated when the samples were 13 days old. Analysis was first performed using the same calibration curve used when the patient samples were one day old (for Section 5.2.2.1.2.). The machine was then recalibrated using the same calibrators used for the initial calibration curve two weeks earlier and the extraction extracts reanalysed. The results obtained for the one day old and 13 day old samples were compared. The results for the 13 day old samples for both calibration curves were compared.

In addition 44 paired serum and EDTA Mitra samples were made. The technique to make the Mitra devices involved dipping the devices into the EDTA whole blood (Section 2.4.2). The Mitra samples were left to dry overnight before being analysed the next day. They were then left for a further 11 days before being analysed again. Results for both ages of samples were compared.

#### 5.2.2.2. *Method Validation*

Unless otherwise stated the Mitra device preparation and extraction methods are as described in Chapter 2.

##### 5.2.2.2.1. Analyser Variation

The aim of this experiment was to sample from a single cup and see what the CV of the assay was when the extraction of the Mitra devices and Mitra variation was not part of the procedure i.e. to obtain the CV of the analyser component of the assay. The reason for doing this was to assess the impact of the "alternative sample type" of the Mitra sample extract on the assay, in comparison to the usual sample type of serum. This was done by obtaining the CV of the assay when the Mitra extracts were combined into one cup (large volume of extract required for 10 replicate analysis therefore multiple devices were extracted and the eluates combined) and the analyser sampled multiple times out of one cup. This was then compared to the CV obtained when a serum sample is analysed multiple times from one cup.

To assess the CV of the Abbott analyser when the machine samples from the same cup, two samples with different levels of CRP were used. Two EDTA Mitra devices were extracted and combined into one cup. The machine then analysed six replicates of each sample using the DBS CRP method.

##### 5.2.2.2.2. Inter and Intra Assay Performance

The CV data for the standard CRP serum method is known from the kit insert. For the DBS CRP method intra assay variation, four EDTA Mitra samples were extracted 10 times and analysed in one batch. The inter assay variation was assessed by analysing four EDTA Mitra devices on eight consecutive occasions. The mean, SD and CV were calculated to give the intra and inter assay variation.

#### 5.2.2.2.3. Mitra Stability

To investigate the stability of CRP with Mitra devices, two pools of whole blood with different levels of CRP were used to create Mitra samples. Each pool was split into eight batches and stored in one of the following ways:

- At room temperature without desiccant
- At room temperature with desiccant
- At 4°C without desiccant
- At 4°C with desiccant
- At -80°C without desiccant
- At -80°C with desiccant
- At 37°C without desiccant
- At 37°C with desiccant

A baseline measurement was taken for each Mitra sample. All 16 samples (two different samples split into eight storage conditions) were measured in singleton on 11 consecutive days (not at weekends) plus three further measurements at day 24, day 35 and day 43. The analyser was re-calibrated on day 24 and 43 with the same calibrators used for the first part of the stability measurements. The 37°C storage results were analysed separately to the other storage types as this method of storage was investigated to see if sample deterioration would occur. The other storage conditions were investigated to see how stable the Mitra devices are. The mean of all other storage types for each sample was calculated for each measurement day and plotted. Statistical analyses were performed to investigate the impact different storage conditions made on the results and whether the results changed over time.

#### 5.2.2.2.4. Mitra Absorbency Consistency

To assess how consistently the Mitra devices absorb blood, eight Mitra device tips were weighed. The tips were then weighed after blood had been absorbed and the amount of blood taken up calculated by subtracting the blank tip weight away from the total tip weight post blood absorption. In addition, a 20 µL pipette was used to weigh out 10 µL of the same blood pool absorbed by the Mitra devices eight times.

#### 5.2.2.2.5. Linearity

Five samples that had a high serum CRP and paired FBC were used. Plasma was obtained from the EDTA whole blood sample and measured using the traditional Abbott serum method. The rest of the EDTA sample was used to make Mitra devices. Two Mitra devices were extracted as described above and the extracts combined. The extracts were double diluted with R1 to a final 1:64 dilution. The extracts were analysed using the DBS CRP method.

#### 5.2.2.2.6. Limit of Detection and Quantitation

The limit of detection can be defined as the mean + 2SD of a series of blank measurements. To determine the limit of detection 10 replicate measurements of blank R1 buffer was made. In addition 10 Mitra samples were made from an EDTA sample with a concentration <1 mg/L for its paired serum sample, and these were extracted and analysed.

The limit of quantitation was determined to be the level at which the CV of the assay was ≤ 20%. Therefore 10 Mitra devices with CRP concentrations of 1, 2, 3 and 5 mg/L were analysed in to determine what the intra assay CV was at those levels.

#### 5.2.2.2.7. Patient Samples

Patient samples were collected during phlebotomy clinics taking place during the rheumatology clinics. Ethics approval had been granted to collect extra samples from patients in addition to the routine samples that were going to be collected (Appendix 7). Every patient had a serum and EDTA blood sample taken as part of their post-clinic workup. After consent had been obtained, a Mitra device sample was collected. In total, 30 samples were collected and prepared as described in Section 2.4.2. The Mitra devices were extracted and analysed in one batch when samples were at least seven days old. Serum samples were analysed using the Abbott serum CRP method. Samples were collected over a month.

## 5.3. **Results**

### 5.3.1. **SWBH Device**

#### 5.3.1.1. *Method Development*

See section 5.2.1.1.1 (page 188) for the method relating to this section.

#### 5.3.1.1.1. Extraction

It was decided to extract into R1 reagent of the CRP Vario Abbott kit as this was a buffer into which it was hoped CRP should elute from the filter paper and as the normal serum reaction occurs in this medium. Also by using R1 reagent the reaction conditions could be kept as close to the original serum method as possible as no additional buffers/solvents would be added into the reaction mix. The volume was taken as 110 µL because the largest sample volume that the Architect will use is 35 µL, therefore 110 µL should allow two sample injections to be taken whilst leaving some sample spare as dead volume. Additionally, by extracting into such a small

volume, dilution of the relatively small amount of serum present in a 3 mm DBS punch will be kept to a minimum.

Five minutes was initially used as the sonication time as this worked well. When sonicated for this time or longer using the Guyson Kerry sonicator, some punches had a tendency to disintegrate entirely or become mushy. This resulted in aspiration errors occurring when the samples were presented to the analyser. Additionally if the sample was taken up by the machine it was noted that samples that had mushy punches would give variably lower results when compared with non-mushy punches, potentially explained by the full 35 µL not being taken up by the analyser. The Ultrawave QS18 sonicator was purchased at a later date and has the "Frequency LEAP technology" which minimises the appearance of hot and cold spots of sonication (where the waves are concentrated or cancelled out) by randomly changing the frequency of sonication between a wider frequency range, reducing standing waves and leading to a more homogeneous sonication. Since using this sonicator there have been reduced instances of mushy spots.

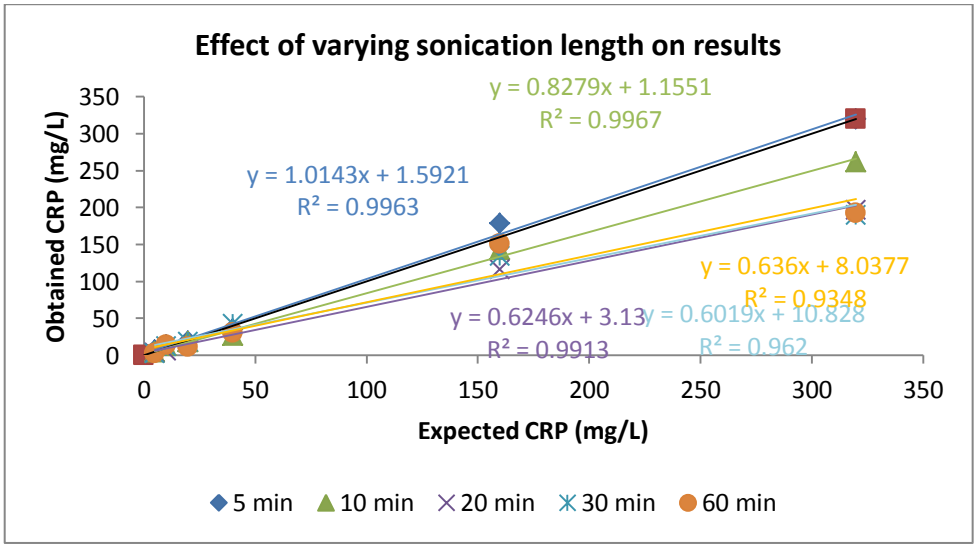



**Figure 5.1 –** The effect of varying sonication time on results of DBS CRP concentration. In each case centre punches were extracted and results were obtained using a calibration curve made from punches that had been sonicated for 5 minutes.

Five minutes sonication was also chosen as it appeared that there was actually a decrease in measured CRP if samples were sonicated for longer than five minutes using the Guyson Kerry sonicator (Figure 5.1).

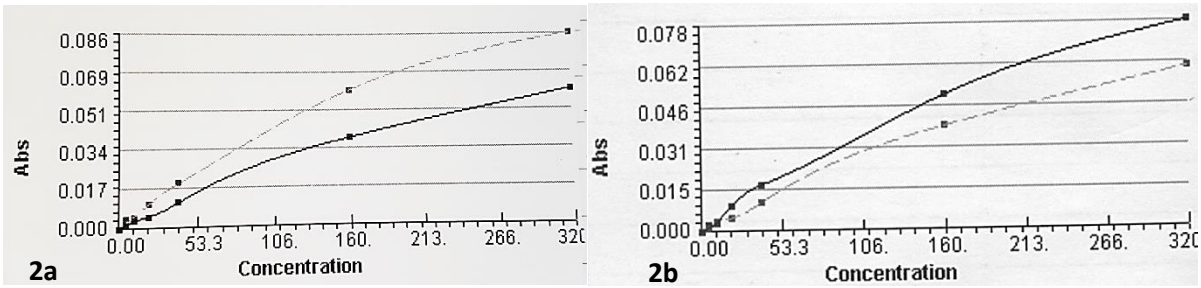



**Figure 5.2 –** Calibration curves produced when the same DBS calibrators were prepared in different ways. 2a shows a calibration curve prepared after 5 minutes sonication and punch left *in situ* for 1 hour (dotted line) and a calibration curve prepared after 5 seconds of mixing and immediate presentation to analyser (solid line). 2b shows the 5 second mixing calibration curve (dotted line) and a calibration curve prepared after 5 minute sonication and immediate removal of punches (solid line).

The length of time the spots were left *in situ* was investigated to see if their removal from the extraction buffer was required. There was an increase in absorbance when the spots were left *in situ* for one hour before analysis compared with five seconds of mixing (Figure 5.2a). There was also an increase in absorbance when spots were left sonicating for five minutes before immediate removal of punches and analysis compared with five seconds of mixing (Figure 5.2b).

Due to the increase in response when spots are left *in situ*, presumably as more CRP is extracted from the punch, and an adequate response being seen after five minutes sonication, it was decided that punches should be immediately removed post-sonication to keep variation to a minimum. This was further demonstrated when calibration of the analyser was performed using DBS that had been mixed for five seconds and the punches left *in situ*. The calibrators were then re-presented to the analyser as samples. The results showed a vast increase in concentration as a result of the spot being left *in situ*, so much so that the top two calibrators gave results >320

mg/L (Figure 5.3). Ten punches from three samples were extracted and left *in situ* and analysed. The CVs obtained from this method were very poor and further reinforced that punches should be removed after sonication is complete (Table 5.2).

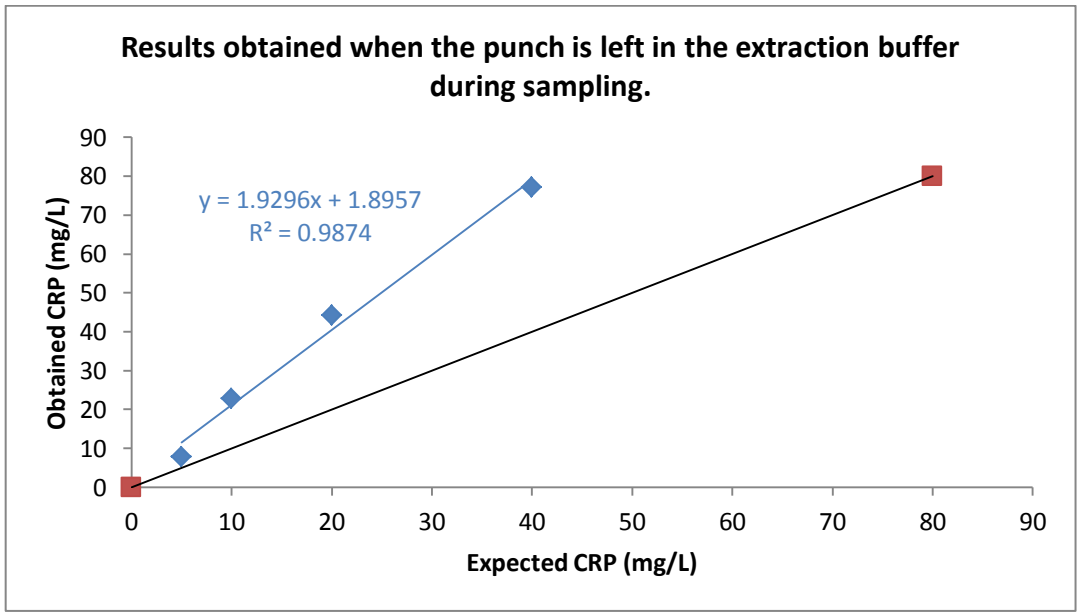



**Figure 5.3 –** Results of calibrators when re-analysed as samples with punches left *in situ*. The calibration curve had been created using punches that had undergone 5 seconds of mixing and had then been left *in situ* during calibration. The calibrators were then represented to the analyser as samples after the punches had been left *in situ*. The top two calibrators gave results >320 mg/L.

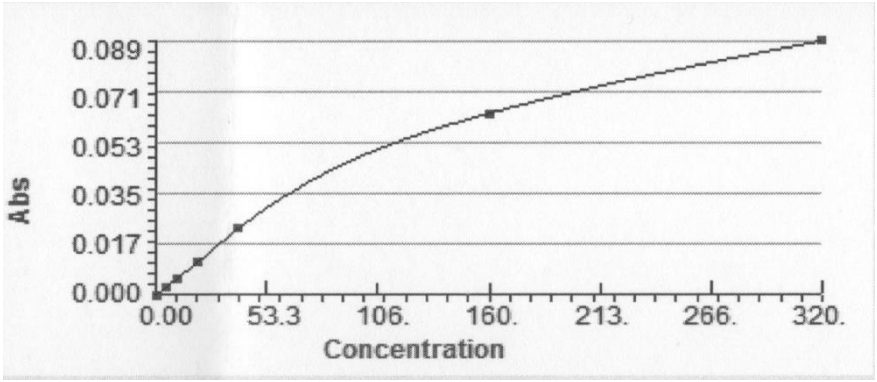
| Sample | Blank | Sample A | Sample B | Sample C |
|---|---|---|---|---|
| **Mean (mg/L)** | 2.6 | 4.5 | 9.6 | 96.5 |
| **SD (mg/L)** | 2.0 | 2.1 | 2.8 | 42.6 |
| **CV (%)** | 76.2 | 45.8 | 29.7 | 44.2 |

**Table 5.2 –** Mean concentration of CRP, SD and CV found when 10 extracts for each sample were prepared and punches left *in situ* before analysis.

5.3.1.1.2. Calibration

The correlation between expected serum calibrator concentrations and the concentrations found when the serum calibrator has been obtained from spun down whole blood DBS calibrator was very good (Figure 5.4 and 5.5). It shows that mixing with WRBC has minimal effect on the calibrator concentration.

A




B

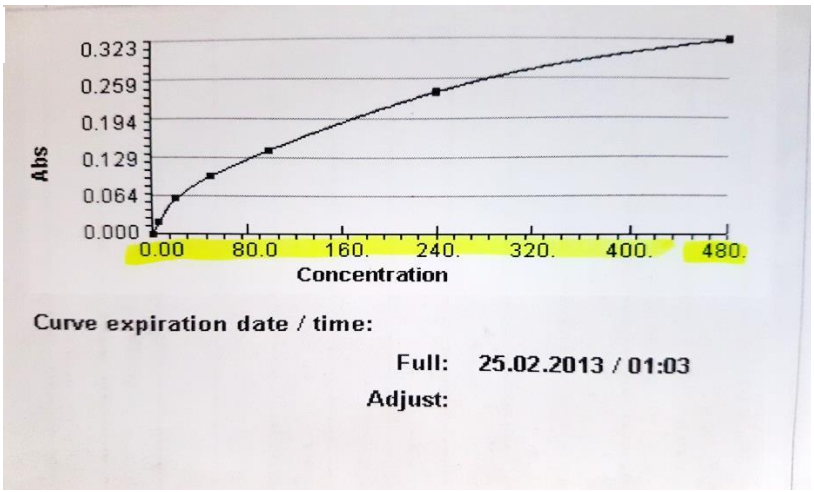



**Figure 5.4 –** A) Calibration curve produced using the accepted protocol for DBS – three punches extracted separately three times, extracts pooled and three replicate measurements taken by the analyser. B) Calibration curve obtained for the serum CRP assay. This curve was obtained for a wider range of calibrators (top calibrator was 480 mg/L, not 320 mg/L as for the DBS curve). Note the much higher absorbance achieved. Due to the different scales it is hard to assess the comparability of the curves at the lower end of the concentration range.

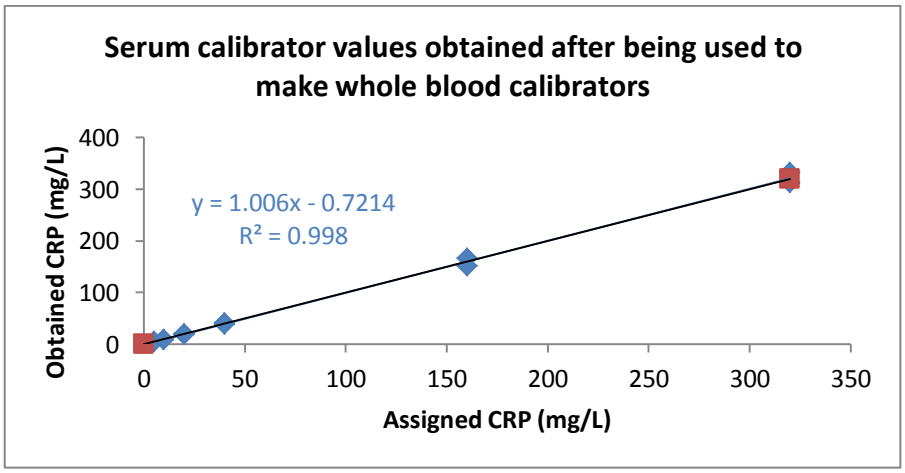



**Figure 5.5 –** Correlation between assigned calibrator values and obtained CRP values of serum calibrators recovered from the liquid whole blood made using the serum calibrators and WRBC.

5.3.1.2. *Method Validation*

See section 5.2.1.2 (page 189) for the method relating to this section.

5.3.1.2.1. Analyser Variation

The CVs for both sample types are very similar when sampling is done from the same cup, showing that the method modifications made for the DBS CRP method have not affected the CRP reaction adversely (Table 5.3).

| **Sample Type** | **DBS** | | | **Serum** | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sample ID** | **1** | **2** | **3** | **1** | **2** | **3** | **4** | **5** |
| **Mean (mg/L)** | 24 | 49.8 | 147 | 6.5 | 18.8 | 70.7 | 96.6 | 131.3 |
| **SD (mg/L)** | 0.5 | 0.8 | 1 | 0.5 | 0.4 | 0.8 | 1.0 | 0.9 |
| **CV (%)** | 2.2 | 1.7 | 0.7 | 8.1 | 2.3 | 1.2 | 1.0 | 0.7 |

**Table 5.3 –** CVs obtained when replicates (n=10) were sampled from the same cup for the DBS and serum method. Five punches were extracted and pooled into the same cup for the DBS experiment in order for there to be enough volume to undertake ten replicate analyses. The serum results are the intra assay variation obtained for the serum assay.

5.3.1.2.2. Intra and Inter Assay Variation

Four of the samples measured for the intra assay variation produced mushy spots. The CVs have been calculated with and without these (Table 5.4). The CVs for the intra and inter assay variation are within acceptable limits (Table 5.5).

| **Sample** | **X** | **Y** | **Z** |
|---|---|---|---|
| **Mean (mg/L)** | 17.9 | 71.9 | 144.1 |
| **SD (mg/L)** | 0.6 | 17 | 13.5 |
| **CV (%)** | 3.2 | 23.6 | 9.4 |
| **Mean (mg/L)** | 17.9 | 81 | 146.4 |
| **SD (mg/L)** | 0.6 | 6.6 | 12.2 |
| **CV (%)** | 3.2 | 8.1 | 8.3 |

**Table 5.4 –** Intra assay variation for the DBS CRP method. Results highlighted in yellow are those obtained when mushy spot results are excluded. n = 10.

| **Target** | **21.1** | **47.5** | **80** |
|---|---|---|---|
| **Mean (mg/L)** | 15.3 | 47 | 82.9 |
| **SD (mg/L)** | 2.0 | 4.8 | 8.8 |
| **CV (%)** | 12.8 | 10.3 | 10.7 |

**Table 5.5 –** Inter assay variation for the DBS CRP method obtained over 10 consecutive occasions.

The Abbott serum methods quoted intra and inter assay CVs are shown in Table 5.6. The DBS CRP CVs are higher than the standard serum CVs. This is likely to be due to issues relating to the use of DBS, rather than assay imprecision. The serum quoted CVs are considerably lower than the CVs we found when analysing several serum replicates from the same cup (Table 5.3).

| | | Level 1 | Level 2 | Level 3 | Level 4 |
|---|---|---|---|---|---|
| | **Mean (mg/L)** | 5.1 | 18.3 | 73.3 | 319.4 |
| **Intra Assay** | **SD (mg/L)** | 0.1 | 0.1 | 0.4 | 2.1 |
| | **CV (%)** | 2.0 | 0.6 | 0.5 | 0.7 |
| **Inter Assay** | **SD (mg/L)** | 0.04 | 0.12 | 0.1 | 1.1 |
| | **CV (%)** | 0.8 | 0.7 | 0.2 | 0.4 |

**Table 5.6 –** Intra and Inter assay variation quoted by Abbott for the standard serum CRP assay.

5.3.1.2.3. Extract Stability

The CVs obtained when extracts are analysed over several days are acceptable, although one flyer for sample 1 has made the CV for that sample fairly high (Table 5.7). There is no decrease in CRP concentration for any of the samples implying that the extracts are stable.

| Day | Sample 1 (mg/L) | Sample 2 (mg/L) | Sample 3 (mg/L) |
|---|---|---|---|
| **0** | 15.6 | 74 | 149.9 |
| **1** | 21.9* | 77.6 | 145.8 |
| **2** | 16.6 | 76.1 | 150.9 |
| **3** | 17.1 | 75.5 | 149.5 |
| **4** | 16.8 | 75 | 151.1 |
| **Mean (mg/L)** | **17.6** | **75.6** | **149.4** |
| **SD (mg/L)** | **2.5** | **1.3** | **2.1** |
| **CV (%)** | **14.0** | **1.8** | **1.4** |

**Table 5.7 –** Stability of extracts over time. Five punches were extracted, pooled and then stored at 4°C until analysis was undertaken. *Flyer.

5.3.1.2.4. DBS Sample Stability

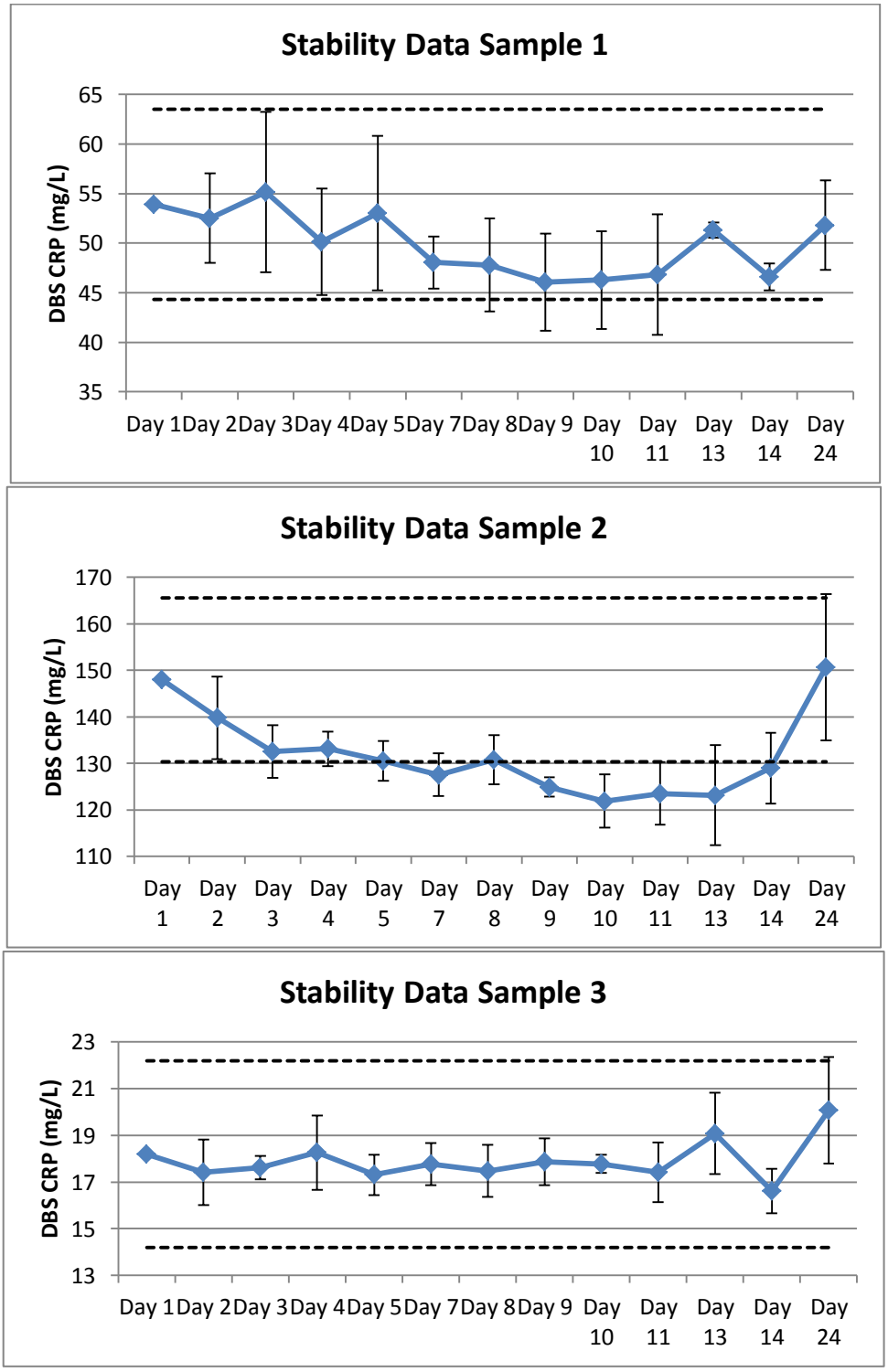



**Figure 5.6 –** Stability data for three DBS samples covering a 24 day period. The dotted lines show ±2SD calculated using the inter assay variation CVs from Table 5.5. Each blue dot represents the average of all six storage conditions for that sample on that day. The error bars represent 1 SD calculated from all results obtained for the six different storage conditions on that particular day. The first time point on the graph is just a single measurement taken on the day the DBS were freshly made and therefore had not undergone any of the different storage conditions.

The mean stability data for all storage conditions for three DBS samples can be seen in Figure 5.6. Out of the 219 results (3 initial time points, then 6 storage conditions for each of the 12 subsequent time points for each of the three samples) that should have been collected over the course of this stability study, 28 (13%) were not obtained due to mushy samples causing errors. Overall there appears to be a drop off in concentration for the samples but a definite rise in concentration when the assay is re-calibrated (on day 24). This implies that the samples are not going off but that the assay may need to be re-calibrated more often. The majority of the results were within 2SD of the first result.

| | p-value | | | Mean CRP (mg/L) | | Median CRP (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Sample 1 | | Sample 2 | | Sample 3 | |
| **Sample** | **1** | **2** | **3** | **D** | **No D** | **D** | **No D** | **D** | **No D** |
| **-80°C** | 0.532 | 0.203 | 0.168 | 50.1 | 51.7 | 132.8 | 131.9 | 18.2 | 18.2 |
| **4°C** | 0.434 | 0.022[a] | 0.479 | 48.4 | 48.5 | 134.9 | 127.2 | 17.6 | 17.9 |
| **Room Temp** | <0.001[a] | 0.310 | 0.919 | 52.7 | 47.9 | 133 | 127.9 | 17.5 | 17.4 |

**Table 5.8 –** p-values obtained when the distributions of results for samples stored with desiccant (D) and without desiccant (No D) were compared. Sample 1: -80°C desiccant n = 13, -80°C no desiccant n = 11, 4°C desiccant n = 8, 4°C no desiccant n = 8, room temperature desiccant n = 11, room temperature no desiccant = 13. Sample 2: -80°C desiccant n = 12, -80°C no desiccant n = 11, 4°C desiccant n = 13, 4°C no desiccant n = 11, room temperature desiccant n = 10, room temperature no desiccant = 11. Sample 3: -80°C desiccant n = 11, -80°C no desiccant n = 13, 4°C desiccant n = 13, 4°C no desiccant n = 13, room temperature desiccant n = 12, room temperature no desiccant = 12. [a] Statistical significance.

| | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| **p-value** | 0.054 | 0.064 | 0.086 |

**Table 5.9 –** p-values obtained when the distribution of data for each storage condition was compared for each sample in order to assess the impact of temperature on stability. Data were grouped for each sample when desiccant made no difference resulting in three groups for sample 3 and four groups for samples 1 and 2.

The data for the different storage conditions were normally distributed for sample 1 (p=0.2), but not normally distributed for samples 2 and 3 (p=0.001 and <0.001 respectively). Generally there was no significant difference between desiccated and

non-desiccated storage, but in two cases there was a difference, and then it was found that desiccated storage gave a higher concentration of CRP (Table 5.8).

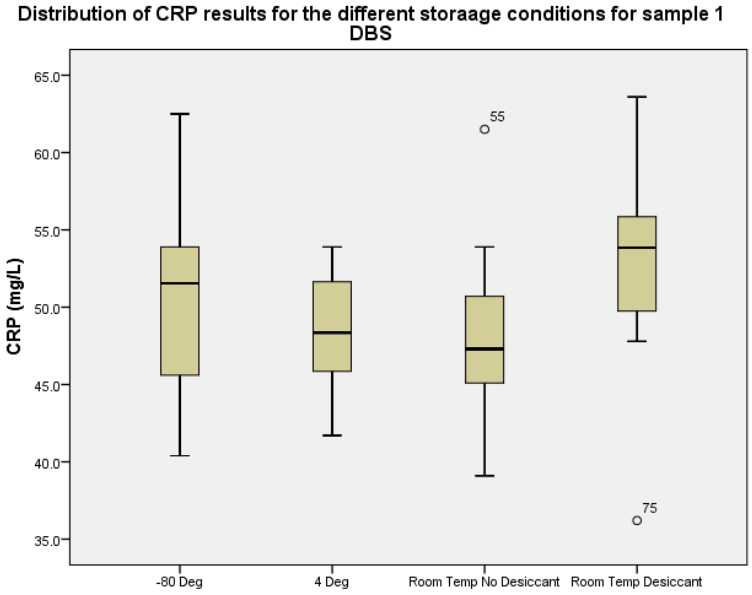
To assess the impact of the different storage temperatures on stability, the data were grouped for each sample when the use of desiccant made no difference (resulting in three groups for sample 3 and four groups for samples 1 and 2) and the distribution of the data for the different storage conditions for each sample assessed (Table 5.9).

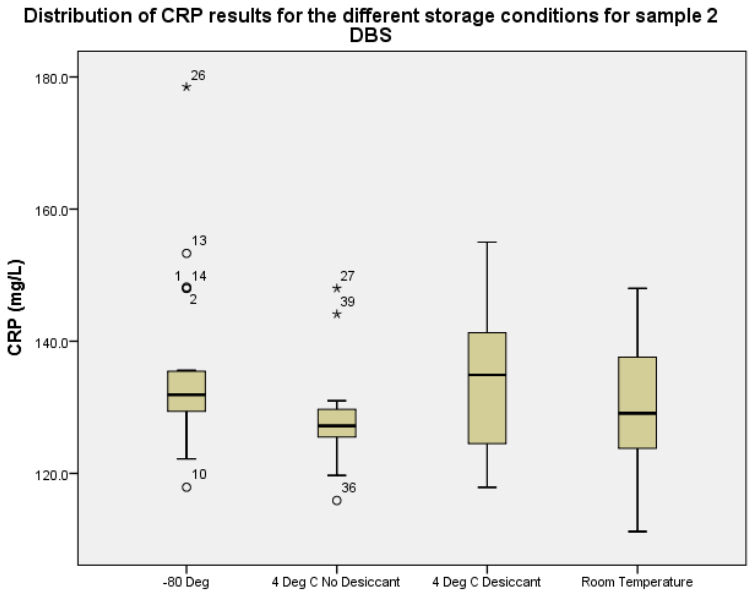
| Sample 1 | | | Sample 2 | | | Sample 3 | | |
|---|---|---|---|---|---|---|---|---|
| **Groups** | **p-value** | **Mean (mg/L)** | **Groups** | **p-value** | **Median (mg/L)** | **Groups** | **p-value** | **Median (mg/L)** |
| **-80°C and 4°C** | 0.021 | -80°C = 50.9 | **-80°C and 4°C ND** | 0.068 | -80°C = 131.9 | **-80°C and 4°C** | 0.044 | -80°C = 18.2 |
| **-80°C and RTND** | 0.135 | 4°C = 46.6 | **-80°C and 4°C D** | 0.308 | 4°C ND = 127.2 | **-80°C and RT** | 0.032 | 4°C = 17.7 |
| **-80°C and RTD** | 0.283 | RTND = 47.9 | **-80°C and RT** | 0.14 | 4°C D = 134.9 | **4°C and RT** | 0.651 | RT = 17.4 |
| **4°C and RTND** | 0.893 | RTD = 54.2 | **4°C ND and 4°C D** | 0.022 | RT = 129.1 | | | |
| **4°C and RTD** | 0.015[a] | | **4°C ND and RT** | 0.484 | | | | |
| **RTND and RTD** | <0.001[a] | | **4°C D and RT** | 0.114 | | | | |

**Table 5.10 –** p-values obtained when pairwise comparisons of the different storage conditions were conducted for each of the samples with a Bonferroni correction applied. RT = room temperature, D = desiccant, ND = no desiccant. Sample 1: -80°C n = 23, 4°C n = 15, room temperature no desiccant n = 13, room temperature desiccant n = 11. Sample 2: -80°C n = 22, 4°C no desiccant n = 11, 4°C desiccant n = 13, room temperature n = 19. Sample 3: -80°C n = 23, 4°C n = 25, room temperature n = 23. [a]Statistical significance at p<0.017

In some cases only seven data points were available for this statistical analysis because there were a high number of missing results due to the disintegration of samples. No statistically significant difference was found but the p values were close to 0.05; therefore pairwise comparisons of the different storage conditions for each sample was conducted with a Bonferroni correction applied, resulting in a significance level set at p<0.017. The median or mean for each of the new grouped storage conditions was also assessed (Table 5.10). Overall there were only

statistically significant differences seen for sample 1 between room temperature with desiccant compared with 4°C and room temperature without desiccant storage conditions but these differences were not seen for the other samples. No one condition appeared to be better than the others although there seemed to be more outliers for -80°C storage which may suggest increased problems with mushy spots for that storage condition (Figure 5.7).



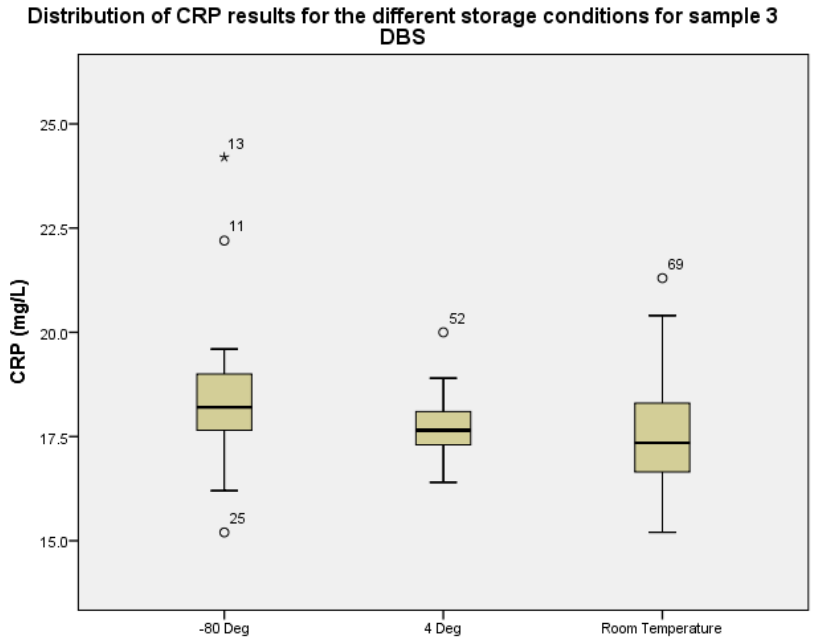



**Figure 5.7 –** Distribution of CRP results for the different storage conditions for DBS samples 1-3 over the 24 day time frame. Ideally each storage condition would have 13 (if desiccant and non-desiccant are separated) measurements or 25 measurements (when desiccant and non-desiccant are combined). However due to the mushy samples leading to results not being able to be obtained there were often fewer samples for each storage condition. Sample 1: -80°C n = 23, 4°C n = 15, room temperature no desiccant n = 13, room temperature desiccant n = 11. Sample 2: -80°C n = 22, 4°C no desiccant n = 11, 4°C desiccant n = 13, room temperature n = 19. Sample 3: -80°C n = 23, 4°C n = 25, room temperature n = 23.

To determine if the samples had significantly deteriorated compared with the starting value, the mean of the data from the different storage conditions was compared to the starting concentration for each sample (Table 5.11).

| Sample 1 | | Sample 2 | | Sample 3 | |
|---|---|---|---|---|---|
| **Storage condition** | **p-value** | **Storage condition** | **p-value** | **Storage condition** | **p-value** |
| **-80°C** | 0.017[a] | **-80°C** | 0.001[a] | **-80°C** | 0.751 |
| **4°C** | 0.003[a] | **4°C ND** | 0.005[a] | **4°C** | 0.002[a] |
| **RTND** | 0.003[a] | **4°C D** | 0.005[a] | **RT** | 0.085 |
| **RTD** | 0.816 | **RT** | <0.001[a] | | |

**Table 5.11 –** Results of statistical analyses comparing the data for the different storage conditions with the starting concentration for each sample in order to see if the samples had deteriorated over time. RT = room temperature, D = desiccant, ND = no desiccant. [a]Statistical significance

Overall, the data imply that there may have been some deterioration of samples with time with no one storage condition shown to be preferable, however when the assay

was re-calibrated the results increased again. The data may have been confounded by the large number of missing points making it hard to draw firm conclusions from this data. However, if any deterioration was seen it was gradual and not dramatic.

5.3.1.2.5. Linearity

Linearity was assessed using five different samples (Figure 5.8). The DBS assay was linear up to the top standard of 320 mg/L.

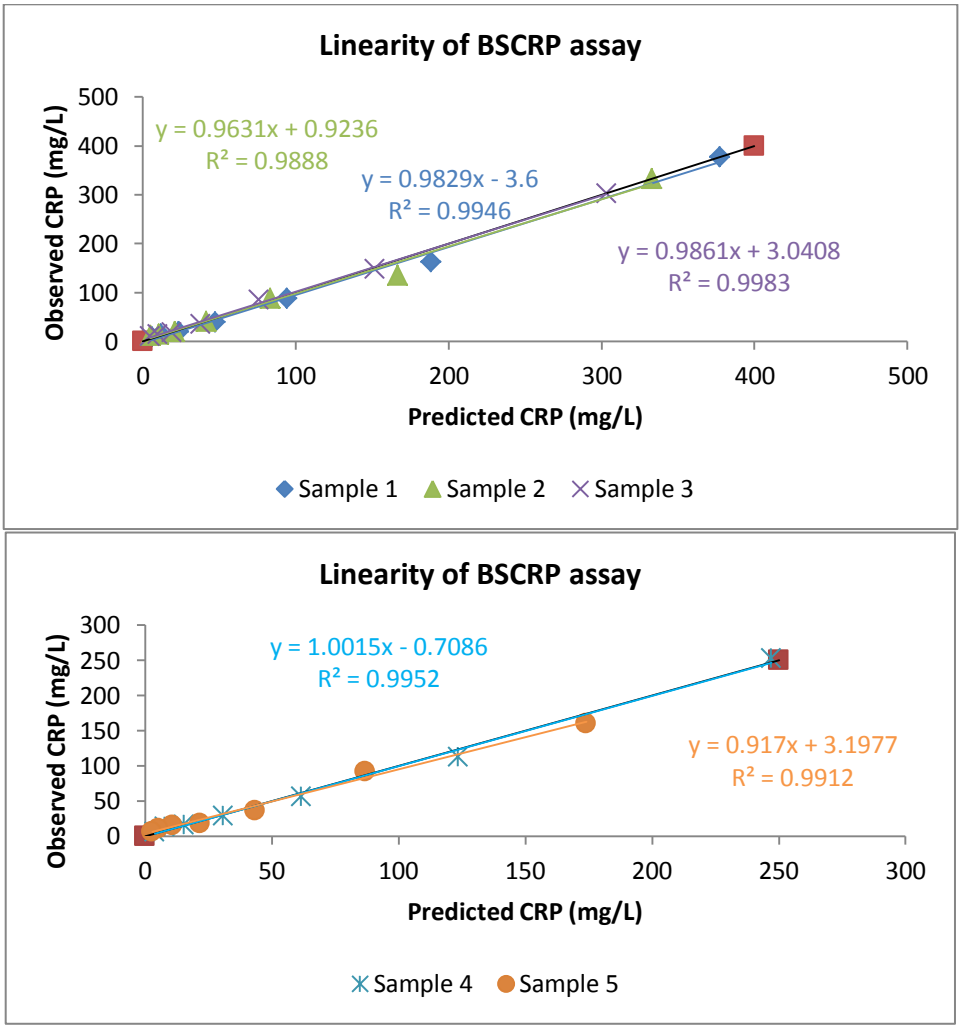



**Figure 5.8 –** Linearity results for the DBS CRP assay for five different samples.

5.3.1.2.6. Limit of Detection and Quantitation

The ten blank replicates all gave values of <0.2 mg/L. The results for the other levels of DBS EDTA can be seen in Table 5.12. The blank measurements cannot be used

to calculate a mean 2SD, therefore the results of the <1 mg/L DBS were used. The limit of detection was found to be 2.4 mg/L. The limit of quantitation was between 3 and 5 mg/L and therefore the limit of quantitation was taken as 5 mg/L. Results found to be less than 5 mg/L were reported as < 5 mg/L.

| | DBS CRP Concentration (mg/L) | | | | |
|---|---|---|---|---|---|
| **Replicate** | **<1** | **1** | **2** | **3** | **5** |
| **1** | 2.1 | 2.1 | 1.8 | 2.1 | 3.4 |
| **2** | 0.4 | <0.2 | 2.2 | 1.1 | 4.3 |
| **3** | 1.7 | 2.3 | 1.6 | 3.8 | 3.2 |
| **4** | <0.2 | 1.3 | 1.3 | 1.1 | 2.6 |
| **5** | 1.3 | 1.2 | 1.5 | 2.5 | 2.8 |
| **6** | M | 1.9 | 0.6 | 2.5 | 3.2 |
| **7** | 1.3 | 1.5 | 1.4 | 2.1 | 3.8 |
| **8** | 1.5 | 0.6 | 1.1 | 2.7 | 4.2 |
| **9** | 0.7 | 1.6 | 1.2 | 3.1 | 3.1 |
| **10** | 1.7 | 0.6 | 3.7 | 1.2 | 2.8 |
| **Mean (mg/L)** | **1.2** | **1.3** | **1.6** | **3.1** | **3.3** |
| **SD (mg/L)** | **0.6** | **0.7** | **0.8** | **0.9** | **0.6** |
| **CV (%)** | **53.1** | **52.1** | **51.2** | **28.9** | **17.6** |

**Table 5.12 –** Results of replicates of samples at the low end of the DBS CRP measuring range in order to determine the limit of quantitation for the DBS CRP assay.

5.3.1.2.7. Carryover

All blank results following the high samples gave values less than the limit of detection, therefore carryover was deemed to not occur (Table 5.13).

| CRP (mg/L) | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| **High CRP** | >320/>320 | >320/>320 | 149.6/142 |
| **1st blank** | 0.7 | 0.5 | M |
| **2nd blank** | 0.5 | 1.2 | 1.5 |
| **3rd blank** | 0.4 | <0.2 | M |
| **4th blank** | 1.2 | 0.3 | 0.5 |
| **5th blank** | <0.2 | <0.2 | 0.4 |

**Table 5.13 –** Results of carryover experiment. A very high sample was extracted followed by 5 blank samples. M = mushy

5.3.1.2.8. Patient Samples

Of the 41 patient samples collected, 21 had serum CRP >5 mg/L. For all 20 samples that had serum CRP <5 mg/L, all DBS (capillary and EDTA) were also <5 mg/L. Patient haematocrits varied from 0.28-0.51 with an average of 0.39.

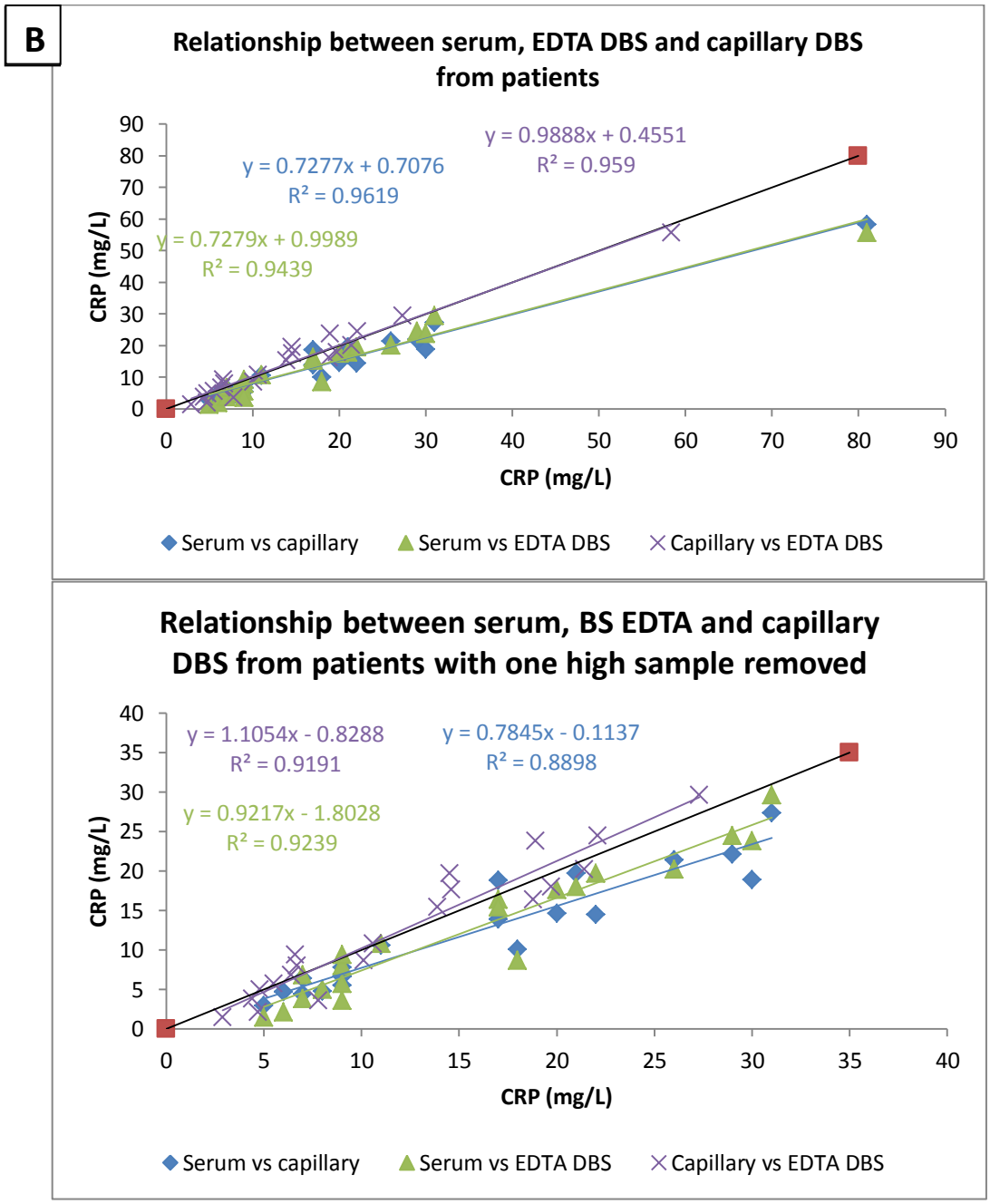



**Figure 5.9 –** Relationship between the three sample types collected from rheumatology patients. Graph A shows the relationship between all samples that were obtained that had measureable levels of CRP, n = 21. Graph B shows the same data but with the one sample with very high levels of CRP removed, n = 20.

The results showed that there was a very good agreement between serum capillary DBS and EDTA DBS, therefore EDTA DBS can act as a surrogate for capillary DBS. Serum CRP and DBS CRP correlated very well, however the DBS samples displayed a proportional negative bias (Figure 5.9-5.12). Even when the one high sample was removed the good correlation between the different sample types remained and the negative bias became much less (shown by Figure 5.9B and 5.11). The average difference between serum and capillary DBS samples was 4 mg/L for all data. For serum CRP samples <30 mg/L (most common area for RA patients) the bias was still present but not quite as large (average difference 3 mg/L). There were two serum CRP results (5 and 7 mg/L) that would have been reported as <5 mg/L by the DBS assay (measured at 3 and 4 mg/L respectively).As the results showed good correlation, further work on the calibration of the assay could be performed or a conversion factor could be used to bring the DBS results into line with the serum results.

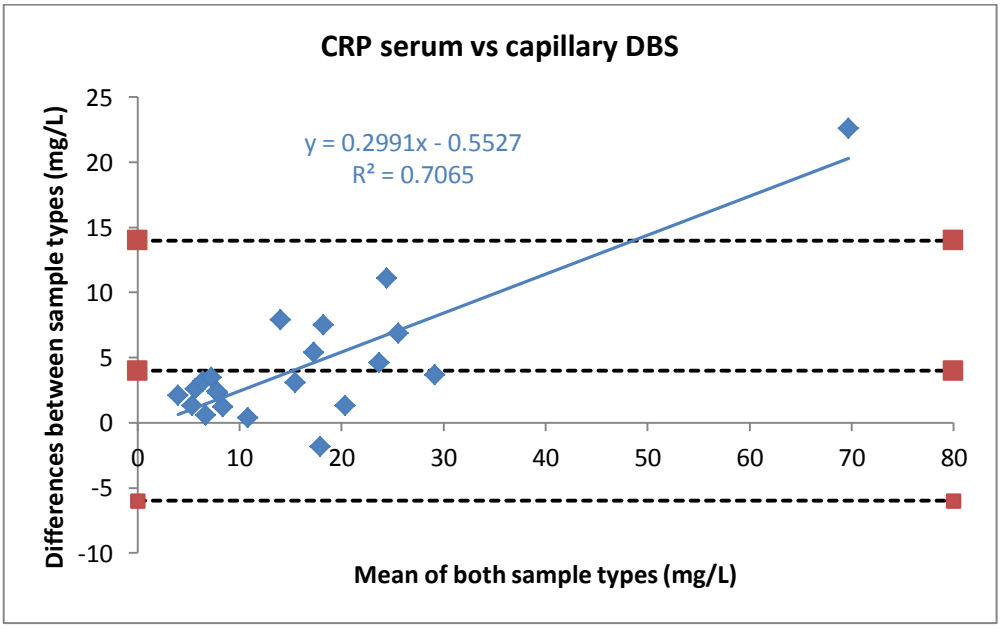



**Figure 5.10 –** Bland and Altman plot of the data obtained from 21 paired serum and capillary DBS samples. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus capillary DBS. n = 21

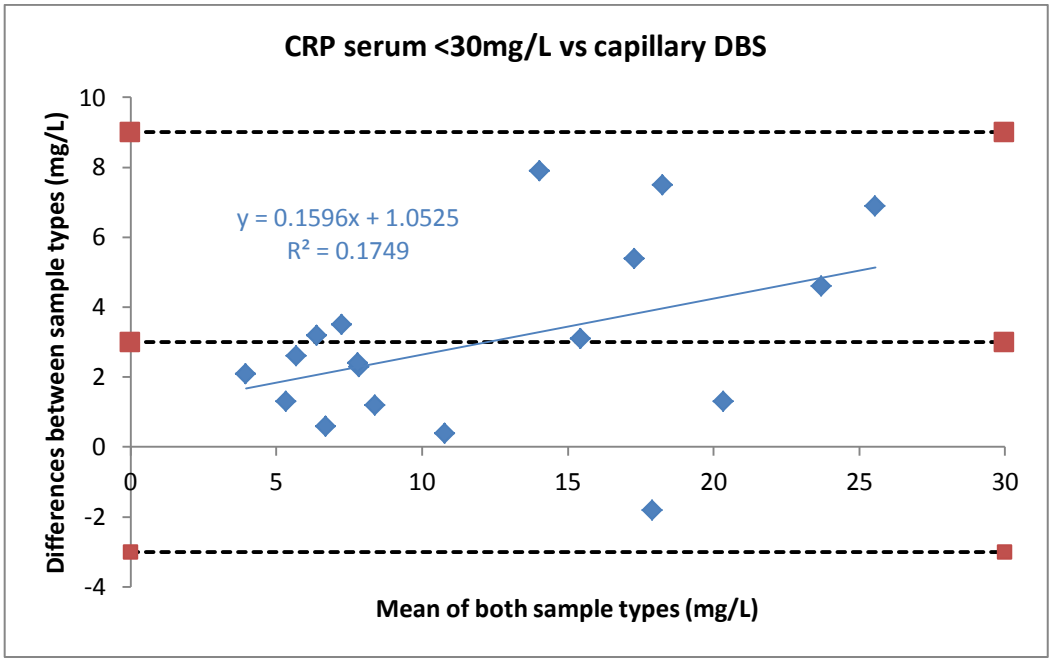



**Figure 5.11 –** Bland and Altman plot of the data obtained from paired serum and capillary DBS samples, whose serum CRP was <30 mg/L. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus capillary DBS. n = 18

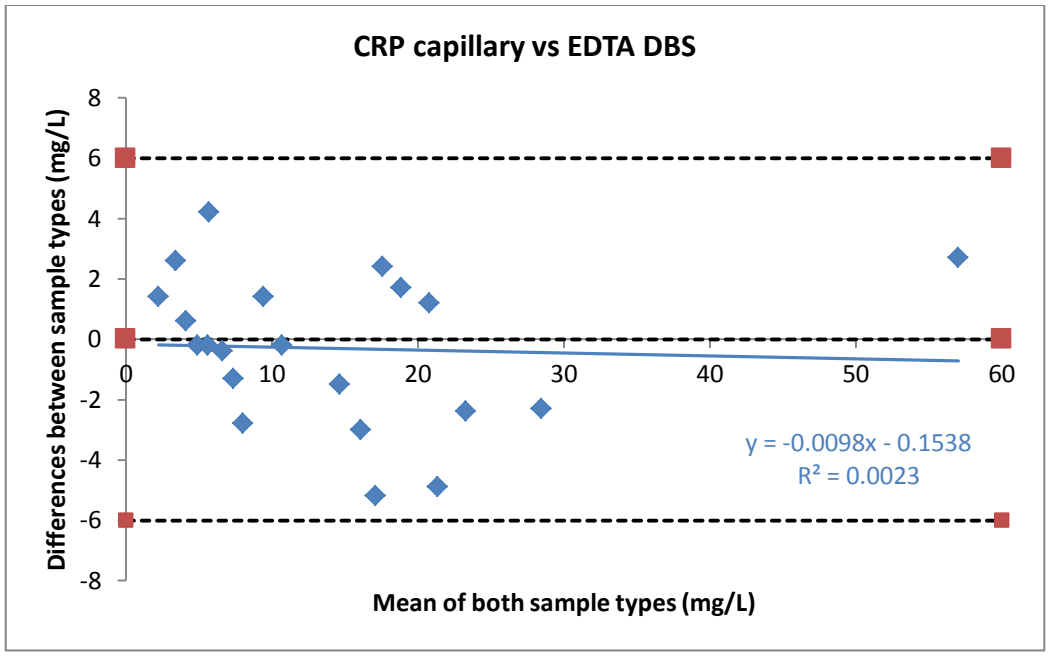



**Figure 5.12 –** Bland and Altman plot of the data obtained from 21 paired capillary and EDTA DBS samples. Mean and ± 2SD of differences represented by dotted lines. The y-axis is capillary DBS minus EDTA DBS.

### 5.3.2. **Mitra Device**

#### 5.3.2.1. *Method Development*

See section 5.2.2.1 (page 192) for the method relating to this section.

##### 5.3.2.1.1. Calibration

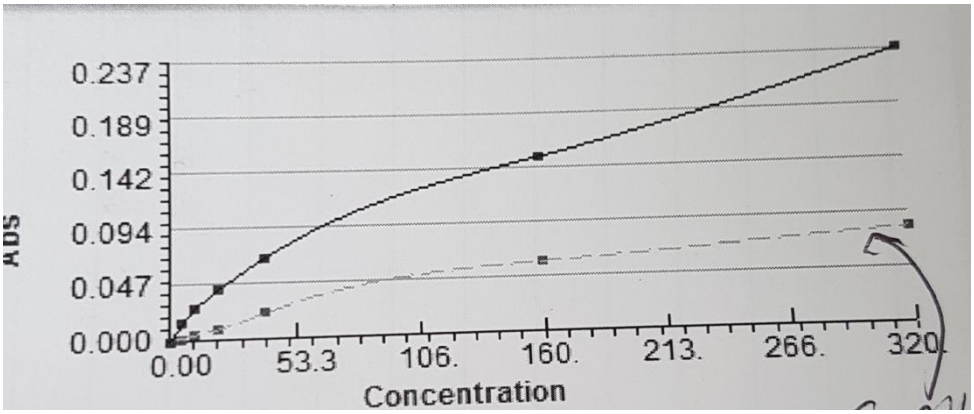



**Figure 5.13 –** Calibration curve produced using Mitra devices (solid line). Dotted line shows the calibration curve obtained when DBS are used.

The calibration curve obtained when Mitra devices were used is shown in Figure 5.13. In the same figure, using the same conditions, can be seen the calibration curve for SWBH filter paper DBS. A much higher response is achieved with the Mitra devices and this is due to more blood being present during the extraction process. The Mitra device contains 10µL of blood and it has been estimated that a 3mm punch contains approximately 3µL of blood and 1.5µL of serum (haematocrit dependent). (24, 106)

##### 5.3.2.1.2. Extraction

As the Mitra device holds 10 µL of blood, more than a 3 mm DBS punch, the sponges were extracted into 150 µL R1 instead of 110 µL. This increased the amount of sample available for analysis without reducing sensitivity, reduced the chances of the Abbott analyser misampling and meant fewer calibrators were required to

calibrate the assay. The Mitra devices did not appear to suffer from disintegration as the DBS did.

Five minutes sonication was chosen as there was no effect on concentration obtained when the samples were sonicated for longer (Figure 5.14). There was a proportional positive bias with EDTA Mitra devices showing higher levels of CRP compared with the serum results. This was further explored in Section 5.3.2.1.3.

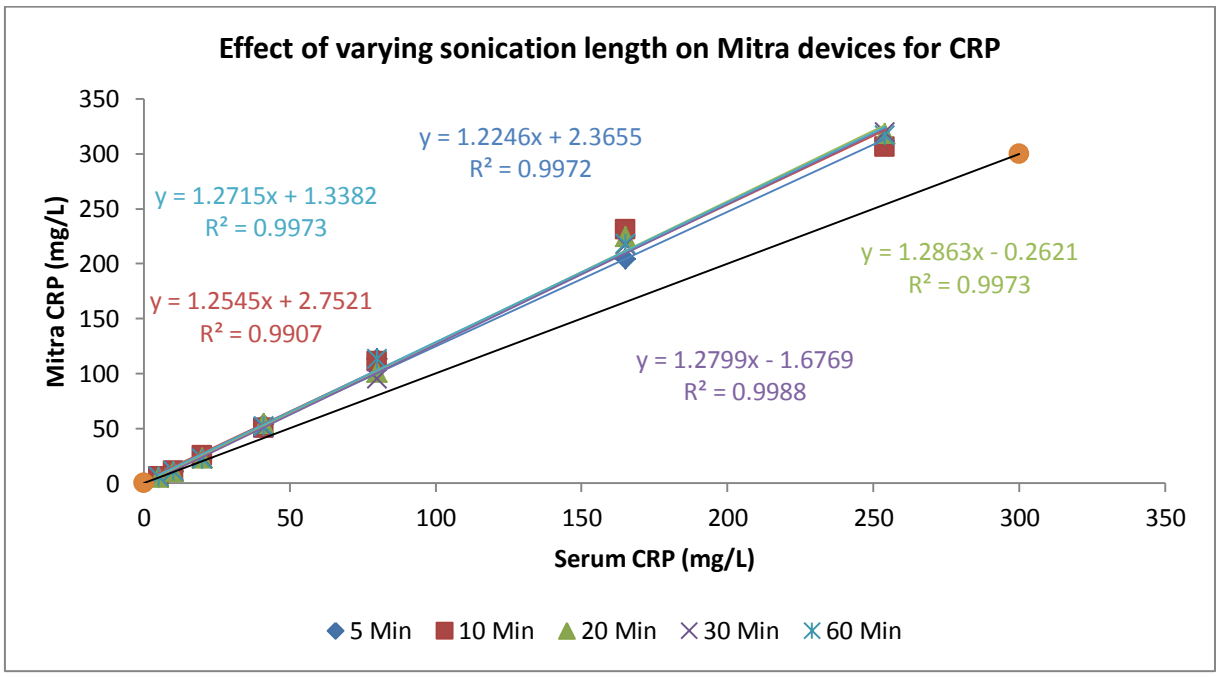



**Figure 5.14 –** The effect of varying sonication time on EDTA Mitra CRP concentration. Results were obtained using a calibration curve made from Mitra devices that had been sonicated for 5 minutes.

5.3.2.1.3. Age of Sample

The results found when the samples from Section 5.3.2.1.2. were extracted (sonicated for five minutes) when they were 13 days old can be seen in Figure 5.15. The results were lower than those found when the samples were one day old and closer to the serum values. The serum and plasma samples showed an excellent agreement and are therefore not the cause of the discrepancy. When the analysis was repeated with a new calibration curve, no difference was found in CRP

concentration for the 13 day old Mitra devices (Figure 5.16), therefore the age of the calibration curve was not the cause of the discrepancy of the results and deterioration of the samples (leading to falsely low results) cannot be the cause either, otherwise recalibrating with deteriorated calibrators would have led to a change in results. The calibrators were six weeks old at this point and gave good results for the older Mitra devices.

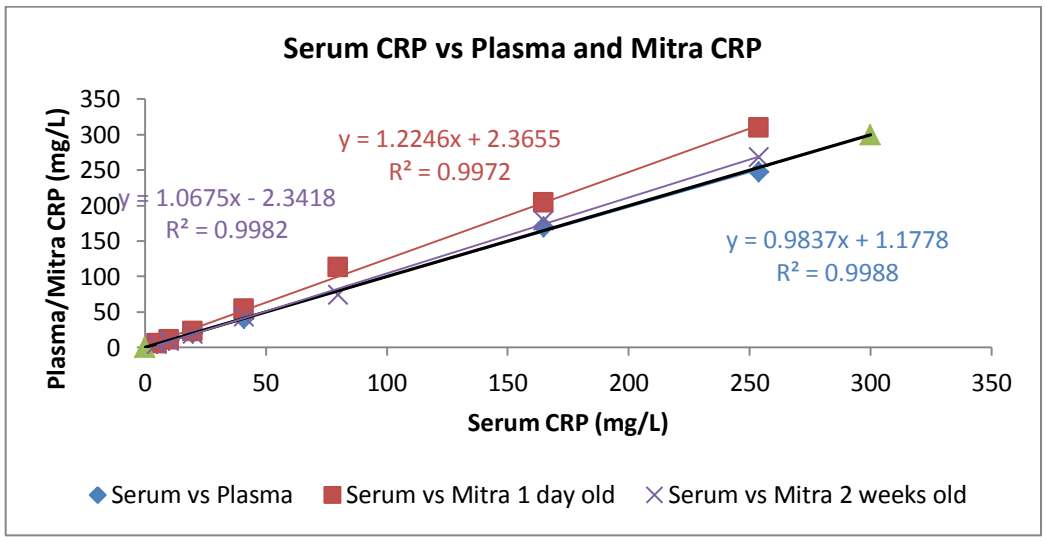



**Figure 5.15 –** Effect of age on the results obtained from the Mitra devices. The samples were those used in Figure 5.14,

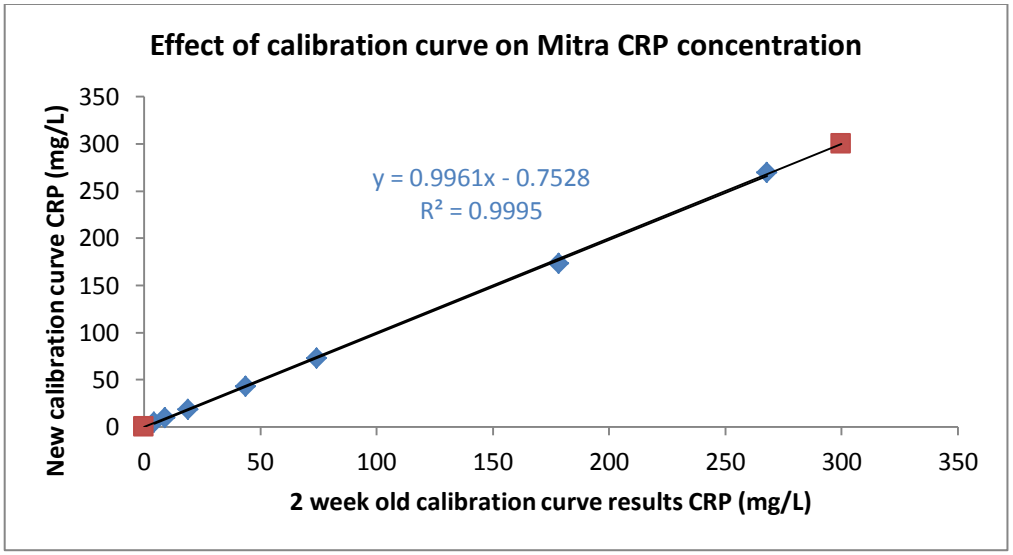



**Figure 5.16 –** Results obtained when 13 day old Mitra device extracts were analysed with a calibration curve made two weeks prior and a calibration curve extracted on the same day as the patient samples.

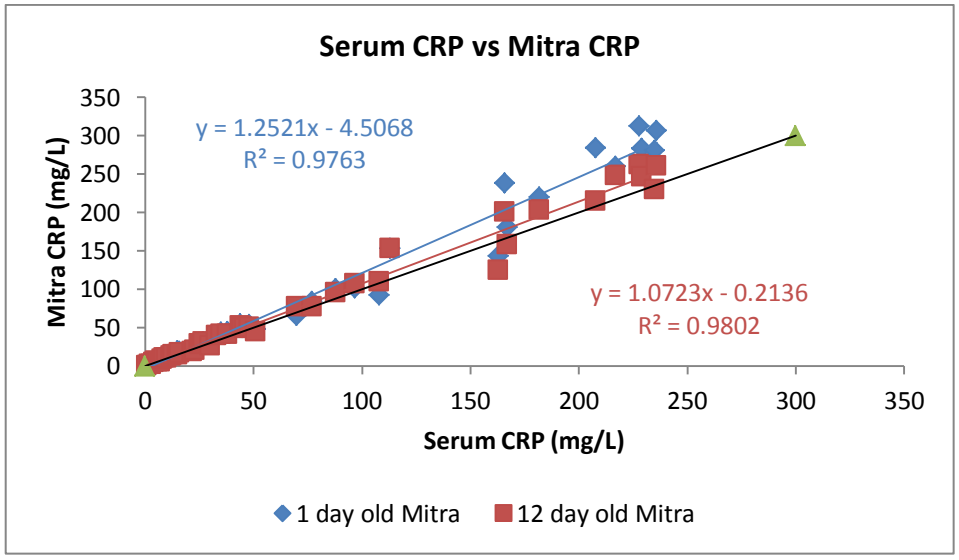



**Figure 5.17 –** Results obtained when 44 paired serum and 1 day old and 12 day old Mitra devices were analysed.

On the basis of this data, it appeared that in order to achieve results closer to that of the serum values, the Mitra devices had to be more than a day old. This was confirmed when another cohort of paired specimens were analysed (Figure 5.17-5.19), which also showed that the older Mitra devices were much closer to the expected serum levels (but still showing a slight proportional positive bias) compared with the one day old samples. Haematocrit varied from 0.24-0.46.

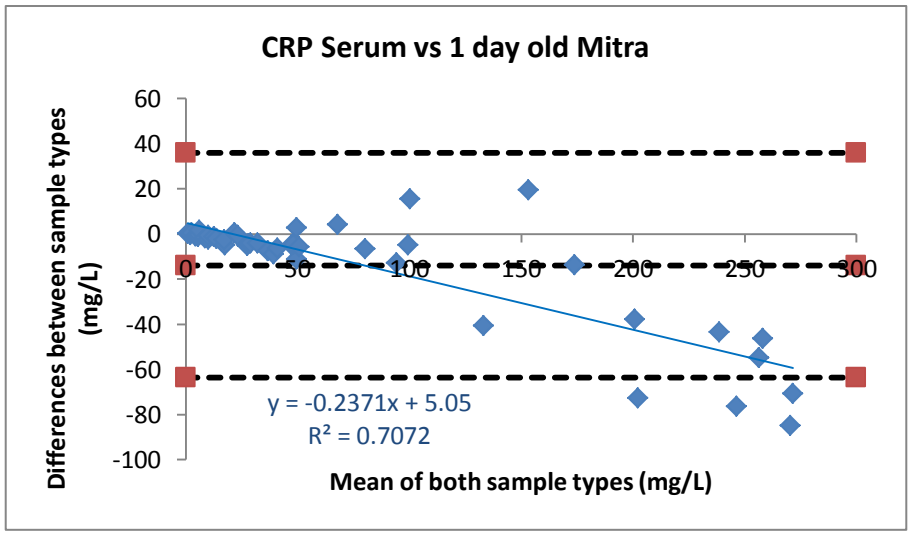



**Figure 5.18 –** Bland and Altman plot of the data obtained from 44 paired serum and 1 day old EDTA Mitra devices. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus 1 day old Mitra devices.

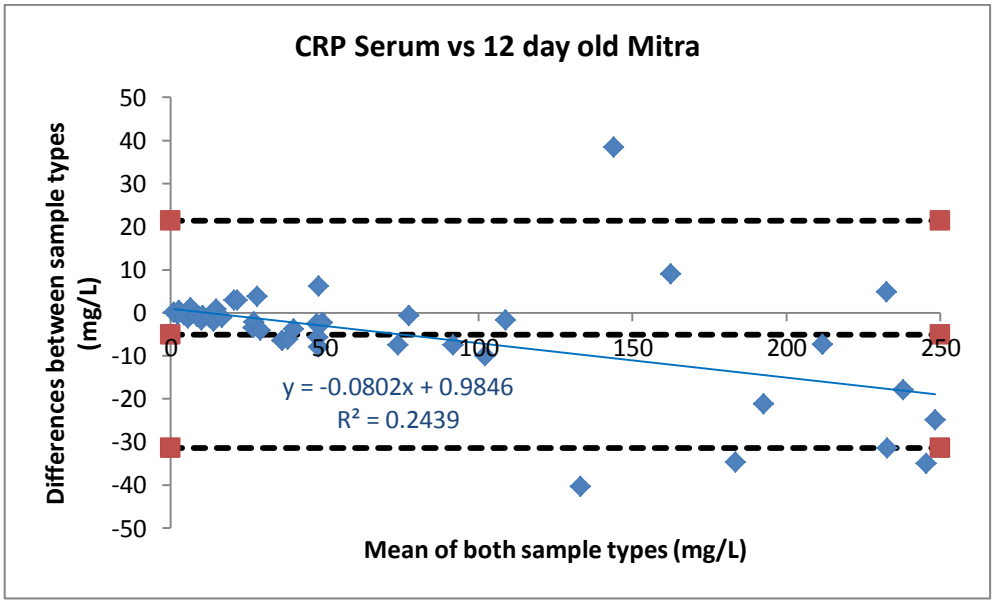



**Figure 5.19 –** Bland and Altman plot of the data obtained from 44 paired serum and 12 day old EDTA Mitra devices. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus 12 day old Mitra devices.

In this cohort of samples, there were a further three 1 day old Mitra samples that had a CRP >320 mg/L but that then gave measureable results when the Mitra devices were 12 days old. There was one sample that was <1 mg/L for the serum and the paired Mitra device gave results <1 mg/L for both Mitra sample ages (Table 5.14).

| | | CRP (mg/L) | | |
|---|---|---|---|---|
| **ID** | **Haematocrit** | **Serum** | **Mitra 1 day old** | **Mitra 12 day old** |
| **1** | 0.35 | 239 | >320 | 245 |
| **2** | 0.36 | 316 | >320 | 303 |
| **3** | 0.5 | 357 | >320 | 295 |
| **4** | 0.4 | <1 | 0.3 | 0.2 |

**Table 5.14 –** Results of very high and very low paired serum and 1 day old and 12 day old Mitra samples.

At serum CRP <30 mg/L the proportional bias virtually disappeared when the Mitra samples were 12 days old (mean of differences = 0.3 mg/L, whole range mean of differences = 5 mg/L). The proportional bias for Mitra devices made from serum samples <30 mg/L was present in the 1 day old samples (mean of differences = 1.7

mg/L), but this was a lot less than that seen when the whole measuring range was analysed (mean of differences = 13.8 mg/L, Figure 5.20-5.21).

There did appear to be some correlation between CRP difference between serum and Mitra devices and haematocrit of samples. The 12 day old Mitra devices made from samples with lower haematocrits tended to show a higher result compared with the paired serum sample whereas samples with a higher haematocrit tended to give a lower result compared with the serum sample. This was not a consistent pattern however (Figure 5.22).

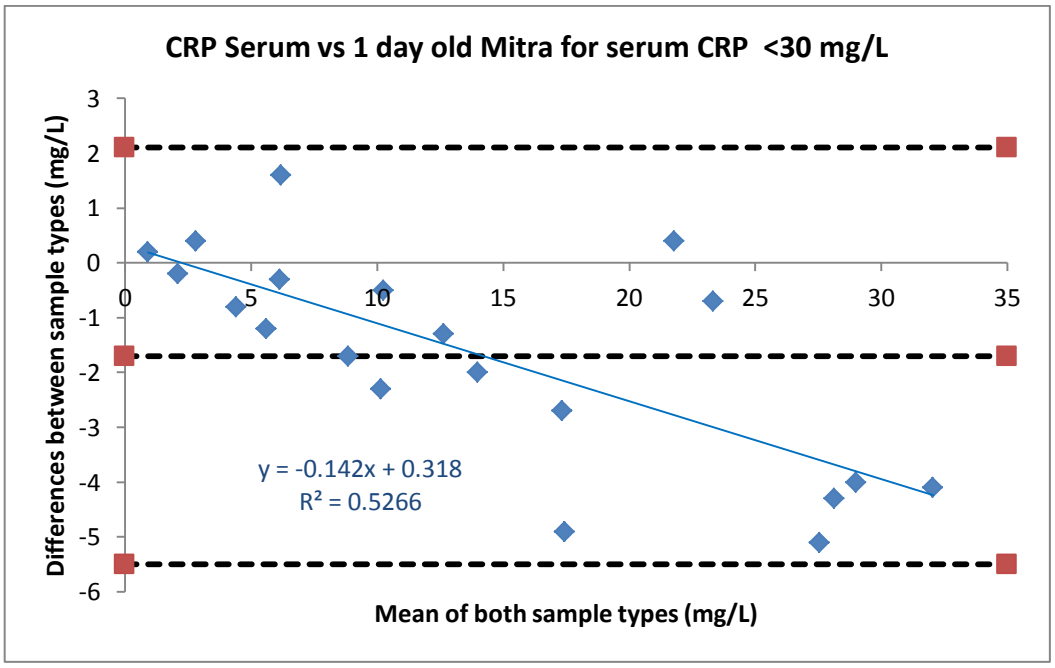



**Figure 5.20** – Bland and Altman plot of the data obtained from paired serum and 1 day old EDTA Mitra devices when the serum result was <30 mg/L. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus 1 day old Mitra devices. n = 20.

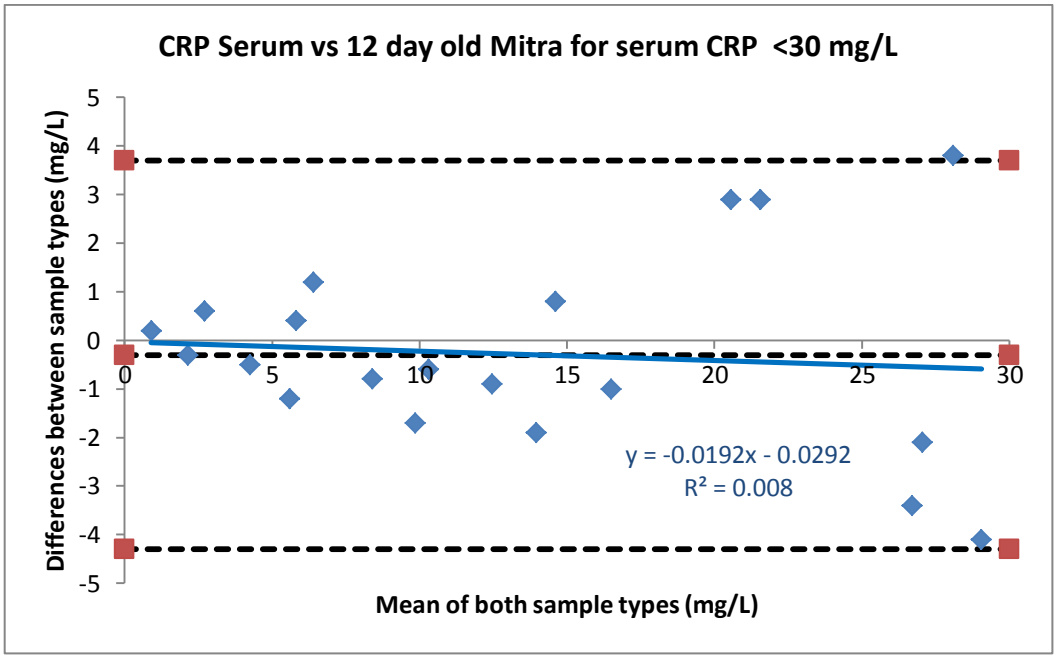



**Figure 5.21** – Bland and Altman plot of the data obtained from paired serum and 12 day old EDTA Mitra devices when the serum result was <30 mg/L. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus 12 day old Mitra devices. n = 20.

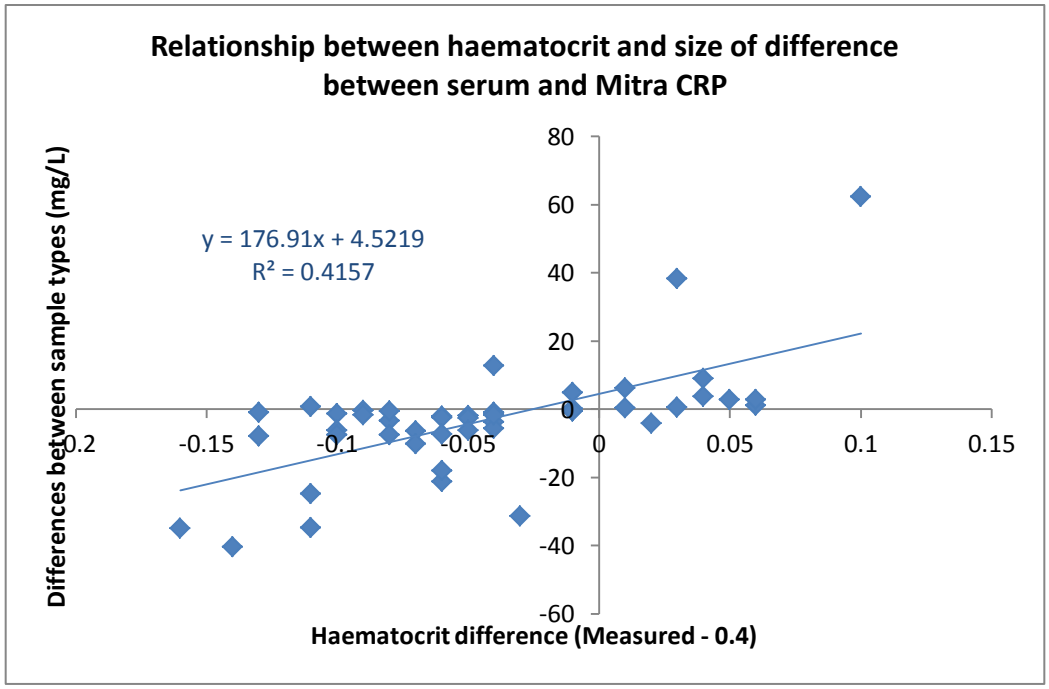



**Figure 5.22** – Data showing how Mitra CRP concentration appeared to decrease with increasing haematocrit. The x-axis shows the measured patient haematocrit minus the haematocrit of the Mitra calibrators (0.4). The actual haematocrit was not plotted as the characteristics work in Chapter 3 showed that increasing haematocrit led to decreasing concentration. The y-axis shows the serum CRP concentration minus the 12 day old Mitra CRP concentration. The data show that when the haematocrit is low (to the left of the central axis) then the Mitra CRP concentration is greater than the serum, but when the haematocrit is higher the Mitra CRP concentration is less than that of serum. n = 47.

### 5.3.2.2. *Method Validation*

See section 5.2.2.2 (page 194) for the method relating to this section.

#### 5.3.2.2.1. Analyser Variation

The results for the analyser variation of the Mitra samples (Table 5.15) were much better than those found for the DBS and serum assay. This was performed at a later date and therefore the analyser performance itself may have improved, but also the performance of the assay will have improved as more blood was present for extraction and therefore more CRP was available for analysis.

| **Sample ID** | **1** | **2** |
|---|---|---|
| **Mean (mg/L)** | 18.3 | 123.7 |
| **SD (mg/L)** | 0.16 | 0.44 |
| **CV (%)** | 0.89 | 0.36 |

**Table 5.15 –** CVs obtained when replicates (n=6) were sampled from the same cup. Two Mitra devices were extracted and pooled into the same cup in order for there to be enough volume to undertake six replicate analyses.

#### 5.3.2.2.2. Inter and Intra Assay Performance

The CVs for the intra and inter assay variation were within acceptable limits and better than those seen for the DBS assay (Table 5.16 and 5.17).

| **Sample** | **A** | **B** | **C** | **D** |
|---|---|---|---|---|
| **Mean (mg/L)** | 2.0 | 26.3 | 51.9 | 82.1 |
| **SD (mg/L)** | 0.14 | 1.01 | 1.71 | 1.78 |
| **CV (%)** | 7.4 | 3.8 | 3.3 | 2.2 |

**Table 5.16 –** Intra assay variation for DBS CRP method for Mitra devices. n = 10 for each sample.

| **Target** | **2** | **21.1** | **47.5** | **80** |
|---|---|---|---|---|
| **Mean (mg/L)** | 2.0 | 22.1 | 44.9 | 78.1 |
| **SD (mg/L)** | 0.2 | 3.2 | 2.9 | 3.7 |
| **CV (%)** | 10.7 | 14.3 | 6.5 | 4.8 |

**Table 5.17 –** Inter assay variation for DBS CRP method for Mitra devices obtained over 8 consecutive occasions.

5.3.2.2.3. Mitra Stability

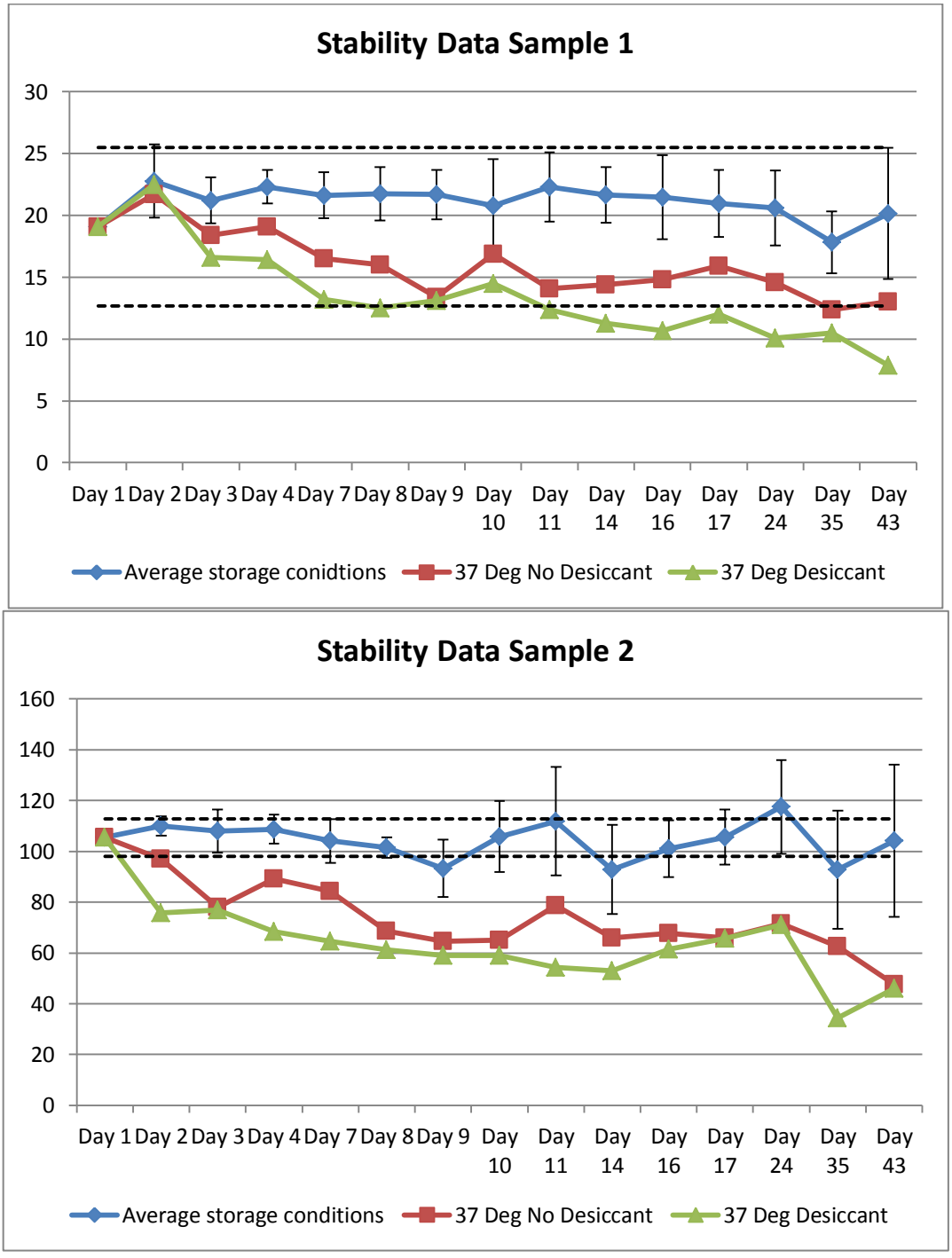



**Figure 5.23 –** Stability data for two Mitra samples covering a 43 day period. The average storage conditions refer to all storage condition results (six different storage conditions) except 37°C results, which are shown separately. Each blue point on the graph is the average concentration of six storage conditions. The dotted lines show ±2SD calculated using the inter assay variation CVs from Table 5.17. The error bars represent 1 sd calculated from all results obtained for the six different storage conditions on that particular day. The first time point on the graph is just a single measurement taken on the day the Mitra devices were freshly made and therefore had not undergone any of the different storage conditions.

The mean stability data for all storage conditions (except 37°C) for two Mitra samples can be seen in Figure 5.23. The majority of the results for the average storage conditions were within 2SD of the first result and there did not appear to be sample deterioration with time. Both samples stored at 37°C with and without desiccant showed deterioration straight away and they continued to decline over time. In both cases storage at 37°C with desiccant appeared to increase the deterioration seen. For sample 2 all results for Mitra devices stored at 37°C were less than -2SD.

The data for the different storage conditions were normally distributed for sample 1 (all $p>0.05$), and all but -80°C storage with desiccant for sample 2 were normally distributed (all $p>0.05$, -80°C with desiccant $p=0.038$). Generally there was no significant difference between desiccated and non-desiccated storage, except for -80°C storage for sample 2 when it was found that desiccated storage gave a higher median concentration of CRP (Table 5.18).

| | p-value | | Mean/Median CRP (mg/L) | | | |
|---|---|---|---|---|---|---|
| | | | Sample 1 | | Sample 2 | |
| **Sample** | **1** | **2** | **D** | **No D** | **D** | **No D** |
| **-80°C** | 0.658 | 0.030[a] | 22.8 | 22.5 | 115.2 | 107.8 |
| **4°C** | 0.690 | 0.388 | 22.2 | 22.0 | 105 | 102.7 |
| **Room Temp** | 0.328 | 0.271 | 18.9 | 18.1 | 97.4 | 93.1 |

**Table 5.18 –** p-values obtained when the distributions of results for samples stored with desiccant (D) and without desiccant (No D) were compared. For each storage condition n = 15. [a] Statistical significance.

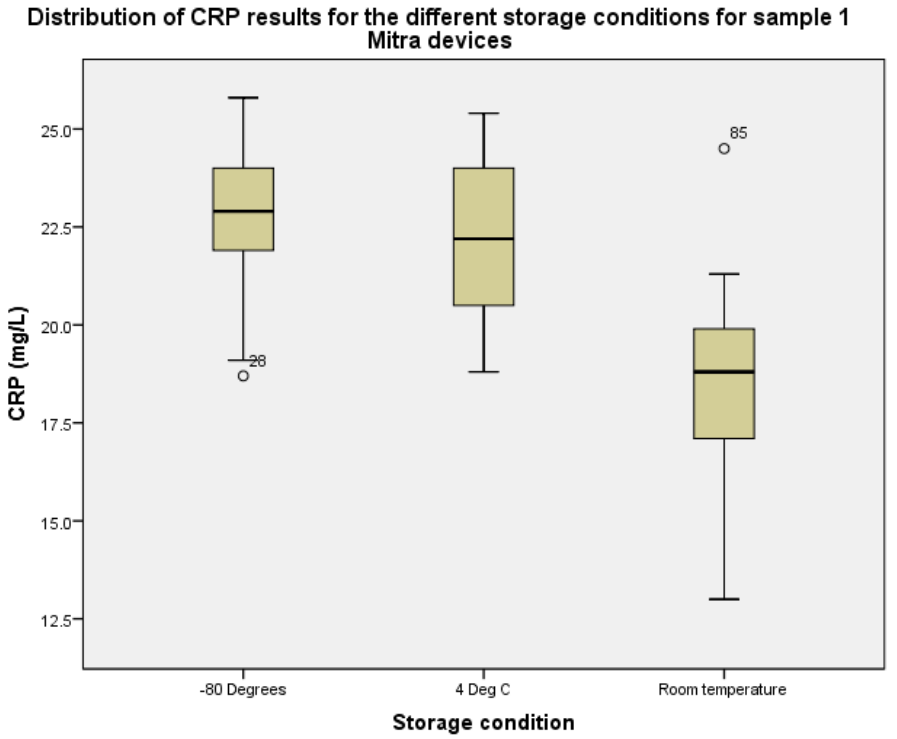
| | Sample 1 | Sample 2 |
|---|---|---|
| **p-value** | <0.001 | <0.001 |

**Table 5.19 –** p-values obtained when the distribution of data for each storage condition was compared for each sample in order to assess the impact of temperature on stability. Data were grouped for each sample when desiccant made no difference resulting in three groups for sample 1 and four groups for sample 2.

| | Sample 1 | | | Sample 2 | |
|---|---|---|---|---|---|
| **Groups** | **p-value** | **Mean (mg/L)** | **Groups** | **p-value** | **Mean (mg/L)** |
| **-80°C and 4°C** | 0.598 | -80°C = 22.8 | **-80°C ND and -80°C D** | 0.030 | -80°C ND = 107.8 |
| **-80°C and RT** | <0.001[a] | 4°C = 22.2 | **-80°C ND and 4°C** | 0.124 | -80°C D = 119.3 |
| **4°C and RT** | <0.001[a] | RT = 18.5 | **-80°C ND and RT** | 0.013[b] | 4°C = 102.7 |
| | | | **4°C and -80°C D** | 0.002[b] | RT = 93.1 |
| | | | **RT and -80°C D** | 0.001[b] | |
| | | | **4°C and RT** | 0.011[b] | |

**Table 5.20 –** p-values obtained when pairwise comparisons of the different storage conditions were conducted for each of the samples with a Bonferroni correction applied. When desiccant and no desiccant groups were combined n = 29, otherwise n = 15. [a]Statistical significance at p<0.05. [b]Statistical significance at p<0.017

The data were then grouped for both samples when the use of desiccant made no difference (resulting in three groups for sample 1 and four groups for sample 2) and the distribution of the data for the different storage conditions for each sample assessed (Table 5.19). Both samples showed that there was a statistically significant difference between storage conditions (variances equal for sample 1, p=0.483). Post hoc analysis using Tukey correction was performed for sample 1, and for sample 2 pairwise comparisons of the different storage conditions was conducted with a Bonferroni correction applied, resulting in a significance level set at p<0.017. The median or mean for each of the storage conditions was also assessed (Table 5.20). For both samples the room temperature storage condition was significantly different to all other storage conditions, showing a lower mean concentration in comparison. Storage at -80°C gave the highest mean for both samples and for sample 2 the use of desiccant appeared to lead to a significantly higher mean CRP concentration. This may be due to the presence of three high outliers. Storage at room temperature leads to a lower concentration of CRP compared with storage at -80°C (Figure 5.24).



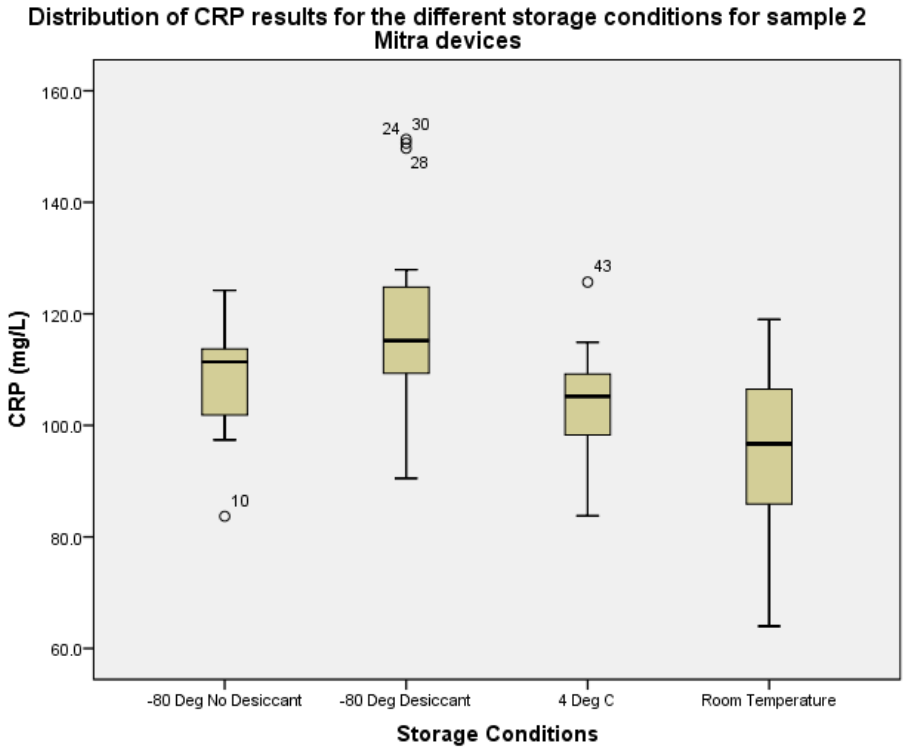




**Figure 5.24 –** Distribution of CRP results for the different storage conditions for Mitra samples 1 and 2 over the 43 day time frame. When desiccant and no desiccant groups were combined n = 29, otherwise n = 15.

To determine if the samples had significantly deteriorated compared with the starting value, statistical tests were performed comparing the mean of the data from the different storage conditions to the starting concentration for each sample (Table 5.21). For sample 1, only room temperature storage showed no significant difference from the starting concentration. The 4°C and -80°C storage conditions had mean concentrations that were increased in relation to the starting value, showing that there had not actually been any deterioration in concentration. The samples stored at 37°C were also significantly different to the starting concentration, however in this case that was due to sample deterioration. The pattern was similar for sample 2, with a significant difference for the -80°C desiccant samples due to an increase in concentration compared with the starting concentration. However, for sample 2 room temperature samples were significantly different from the starting concentration and this was due to a decrease in concentration. On closer inspection of the data for both samples, this deterioration looked like it only started to occur after day 24 and by day 35. The 37°C storage conditions for sample 2 also showed a significant deterioration.

| Sample 1 | | Sample 2 | |
|---|---|---|---|
| **Storage condition** | **p-value** | **Storage condition** | **p-value** |
| **-80°C** | <0.001[a] | **-80°C ND** | 0.300 |
| **4°C** | <0.001[a] | **-80°C D** | 0.009[a] |
| **RT** | 0.180 | **4°C** | 0.368 |
| **37°C ND** | <0.001[a] | **RT** | 0.002[a] |
| **37°C D** | <0.001[a] | **37°C ND** | 0.001[a] |
| | | **37°C D** | 0.001[a] |

**Table 5.21 –** Results of statistical analyses comparing the data for the different storage conditions with the starting concentration for each sample in order to see if the samples had deteriorated over time. RT = room temperature, D = desiccant, ND = no desiccant. [a]Statistical significance

Overall, the data imply that the stored samples are very stable at -80°C and 4°C. The samples appear to be relatively stable for at least three weeks at room temperature

and deteriorate quickly at 37°C. It may be that samples need to be left at room temperature for a short while, or for 24 hours at 37°C to bring patient Mitra samples into the serum range (see Section 5.3.2.1.3), but for long term storage Mitra samples should be stored at 4°C or -80°C where they appear to be stable for at least 43 days.

### 5.3.2.2.4. Mitra Absorbency Consistency

| | Blank Dry Tip | 10 µL Pipette | Mitra 10 µL |
|---|---|---|---|
| **Mean (mg)** | 15.5 | 11 | 12 |
| **SD (mg)** | 0.05 | 0.33 | 0.45 |
| **CV (%)** | 0.34 | 3.0 | 3.8 |

**Table 5.22 –** Results showing variation of the weight of blank Mitra tips and variation seen when 10 µL of blood was pipetted or absorbed by Mitra devices.

The data showed that the blank Mitra tips were very consistent in weight. The Mitra absorbency consistency was very similar to that obtained with a pipette (Table 5.22). The results were similar to those obtained by Phenomenex who found that when water was wicked, the average wicking volume was 11.2 µL and the CV was 4.8%.

### 5.3.2.2.5. Linearity

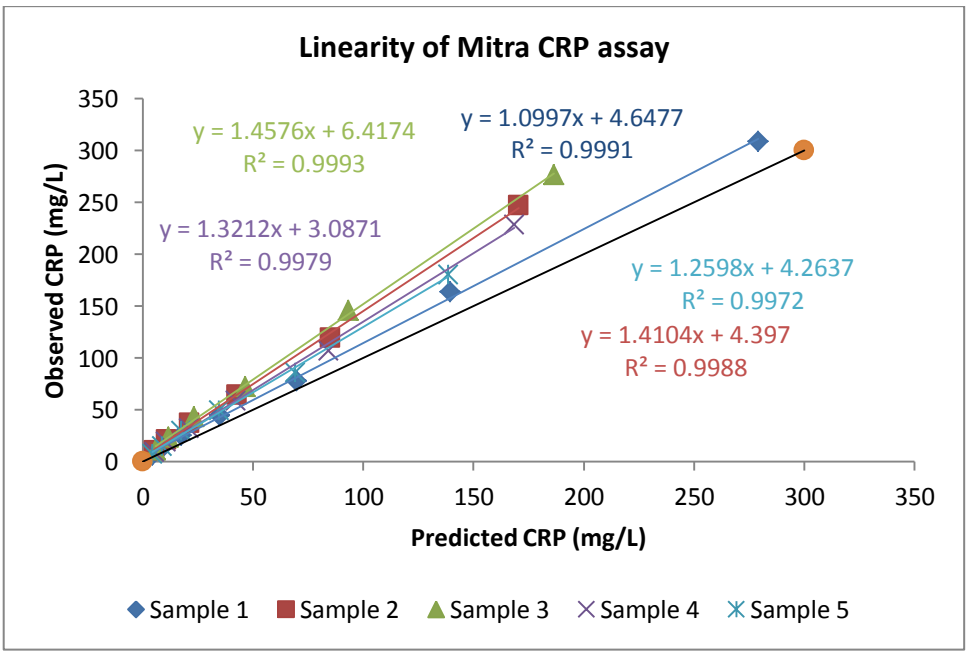



**Figure 5.25 –** Linearity results for the Mitra CRP assay for five different samples.

Linearity was assessed using five different samples (Figure 5.25). The Mitra assay was linear up to the top standard of 320 mg/L, showing excellent correlation with the predicted serum CRP concentration, however as the Mitra samples were only two days old there was a significant positive bias shown by the Mitra samples (see Section 5.3.2.1.3).

5.3.2.2.6. Limit of Detection and Quantitation

| | DBS CRP Concentration (mg/L) | | | | |
|---|---|---|---|---|---|
| **Replicate** | **<1** | **1** | **2** | **3** | **5** |
| **1** | 0.5 | 0.9 | 1.9 | 3.2 | 5 |
| **2** | 0.3 | 0.8 | 1.8 | 2.9 | 4.7 |
| **3** | 0.5 | 1 | 1.9 | 2.9 | 5.4 |
| **4** | 0.5 | 1 | 1.8 | 3 | 5.3 |
| **5** | 0.3 | 0.9 | 1.8 | 3.2 | 5.4 |
| **6** | 0.4 | 0.9 | 2 | 3 | 5 |
| **7** | 0.4 | 0.9 | 2 | 2.9 | 5 |
| **8** | 0.4 | 0.9 | 2.1 | 2.8 | 5.3 |
| **9** | 0.5 | 0.8 | 1.8 | 2.7 | 5.5 |
| **10** | 0.4 | 0.9 | 1.9 | 3.1 | 5.1 |
| **Mean (mg/L)** | **0.4** | **0.9** | **1.9** | **3.0** | **5.2** |
| **SD (mg/L)** | **0.1** | **0.1** | **0.1** | **0.2** | **0.3** |
| **CV (%)** | **18.8** | **6.6** | **5.5** | **5.4** | **5.1** |

**Table 5.23 –** Results of replicates of samples at the low end of the DBS CRP measuring range in order to determine the limit of detection for Mitra devices.

The ten blank replicates all gave values of <0.2 mg/L. The results for the other levels of Mitra EDTA can be seen in Table 5.23. The blank measurements cannot be used to calculate a mean 2SD, however the results of the <1 mg/L Mitra gave a CV of <20% and all reported a result. Therefore the limit of detection was taken as <1 mg/L and the limit of quantitation was 1 mg/L. Results found to be less than 1 mg/L were reported as <1 mg/L.

5.3.2.2.7. Patient Samples

Of the 30 patient samples collected, 28 had serum CRP >1 mg/L. The paired capillary Mitra samples for the samples that had serum CRP <1 mg/L were both <1 mg/L. Patient haematocrits varied from 0.33-0.47 with an average of 0.41. The results for the rest of the 28 paired samples are shown in Figures 5.26-5.27.

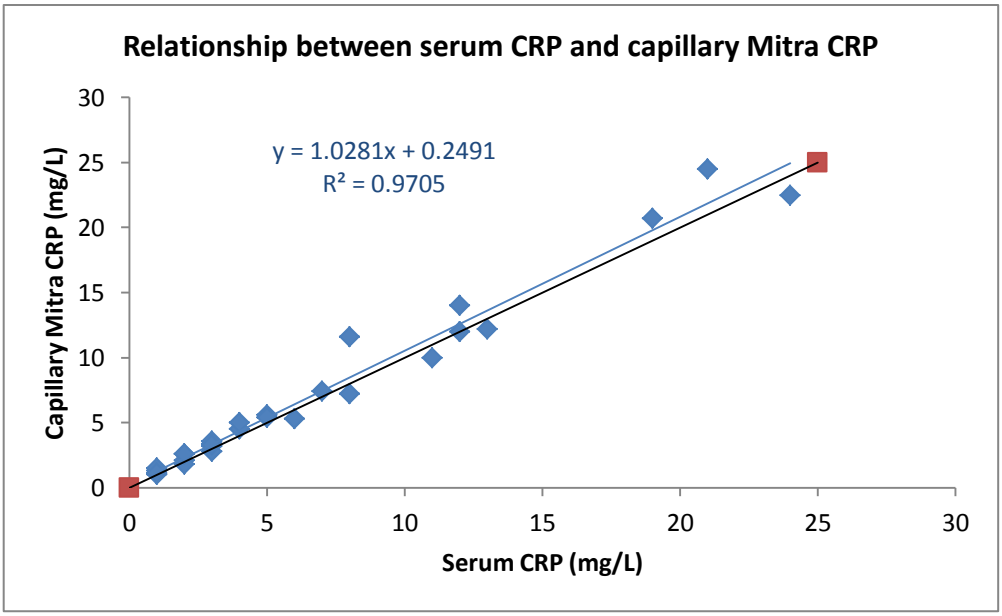



**Figure 5.26 -** Relationship between serum CRP and Mitra samples collected from rheumatology patients. Samples were at least seven days old at the time of analysis. n = 28.

The results showed that there was a very good agreement between serum capillary Mitra samples and serum CRP, with the Mitra devices showing very little positive bias. This was probably because the samples were all more than 7 days old at the time of analysis. The largest difference between the two sample types was 4 mg/L. The Mitra calibrators used for the Mitra analysis were two months old at the time of analysis and showed that the Mitra devices are relatively stable at room temperature (as the calibrators had been stored at room temperature).

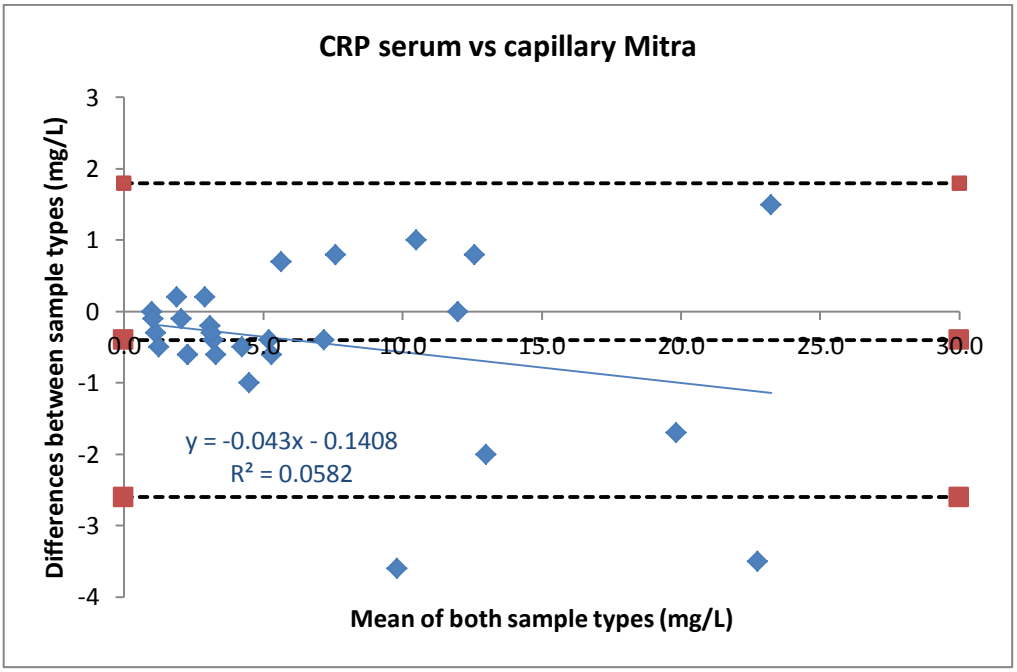



**Figure 5.27 –** Bland and Altman plot of the data obtained from 28 paired serum and capillary Mitra samples. Mean and ± 2SD of differences represented by dotted lines. The y-axis is serum CRP minus capillary Mitra devices. n = 28

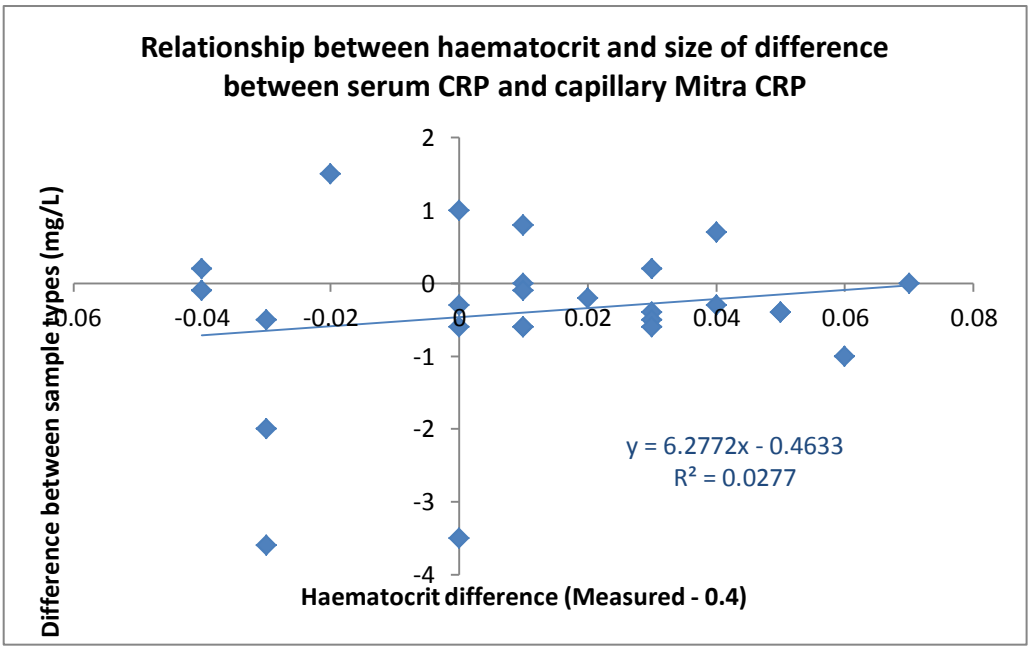



**Figure 5.28 –** Relationship between haematocrit and difference between serum and capillary Mitra samples. The x-axis shows the measured patient haematocrit minus the haematocrit of the Mitra calibrators (0.4). The actual haematocrit was not plotted as the characteristics work in Chapter 3 showed that increasing haematocrit led to decreasing concentration. The y-axis shows the serum CRP concentration minus the capillary Mitra CRP concentration. The data do not show a very strong correlation unlike that seen in Figure 5.22, potentially because the patient haematocrits did not vary far from 0.4.

The patient haematocrits were not very different to the haematocrit used to produce the Mitra calibrators and this may be why there was no real correlation seen between difference in haematocrit and difference between serum CRP and capillary Mitra CRP (Figure 5.28).

## 5.4. **Conclusion**

The work done here has shown that both DBS and Mitra samples can be used to measure CRP and that both assays performed well. This is the first time to the best of our knowledge, that DBS have been analysed using this type of random access methodology. The assay for both sample types has been shown to be linear with a good limit of quantitation. The Mitra device has a lower limit of quantitation which is in line with the serum assay, however before the laboratory moved over to the Abbott serum method, the limit of quantitation was 5 mg/L which is the limit of quantification for the DBS assay. Both Mitra and DBS samples displayed acceptable inter and intra assay variation. The DBS assay showed no signs of carryover and the Mitra devices showed good consistency of absorbtion.

There are several DBS CRP methods published, (18, 40, 45, 129, 199, 200) however these all involve manual ELISA assays and all take a considerable amount of time to perform. The DBS CRP assay described here is the first to be performed on an automated analyser and the whole process (from punching out the spot or extracting the Mitra device to obtaining the result) takes approximately 15 minutes. Many of the other published methods require lengthy or overnight extraction and shaking for 30-120 minutes before analysis even begins, at which point further lengthy incubations may be required. (18, 45, 199, 200) With the method reported here, multiple samples can be analysed at the same time and there is no need to batch samples as the

Abbott analyser is open access, providing further advantages over the reported ELISA plate methods.

CRP has gained considerable interest as a biomarker for stress and immune function (201) and has been measured in the Chicago Health, Aging, and Social Relations Study. (202) This involved the measurement of 229 participants CRP levels in DBS samples collected at baseline, year 2 and year 3. CRP is increasingly being used for large scale epidemiological research (interest in the lower measuring range) and measuring it in DBS makes it logistically and economically more feasible for large scale research to be carried out. By using an automated analyser instead of a manual ELISA, costs and turn-around times will be further driven down.

Making calibrators from WRBC and mixing with serum calibrators is a common method which has been published for other ELISA assays. (199, 200) The DBS calibrators are then assigned the same CRP concentration as the serum calibrators. In theory this should enable DBS samples to give CRP results equivalent to that of paired serum samples. However, several groups have found that DBS samples (both capillary and EDTA spotted DBS) give slightly lower values than serum or plasma (199-201) on a scale similar to the results presented here for the SWBH DBS assay, although others have found no statistically significant difference. (40) For the SWBH DBS assay, it may be that the RA clinicians would be happy to use the assay as it stands, providing they know what the relationship between serum and DBS samples is, because it would give them a better idea as to the CRP status of the patient when the alternative is no result or results that are a least one month old. It would give them a discussion point for the clinic visit, which could then be confirmed with a serum CRP result later. Alternatively, the calibration could be amended to give

results consistent with the serum assay or a correction factor applied. The difference does not appear to be so extreme in the range in which the RA clinicians would be interested (<30 mg/L) and it may be that calibration can be amended to concentrate on that area to improve performance. Further work, in discussion with the clinicians needs to be performed in this area.

Interestingly the Mitra assay showed very good agreement with the serum assay once the samples had been allowed to age. Verbal discussion with Phenomenex revealed that the problem of overestimation in “young” samples had been seen with other assays, but they were not able to give a reason why. If a quick turnaround time is required for the Mitra results, then waiting a week or more to get results closer to the expected serum CRP result would not be acceptable. Alternatively, samples could be incubated overnight at 37°C and then analysed which should give results closer to the expected serum values. Further work investigating this option should be performed.

Like the DBS data presented here on stability, the published data are conflicting over the stability of CRP in DBS. Skogstrand et al., (203) found that DBS CRP showed no change in concentration (in reference to a control sample stored frozen immediately after collection) when samples were stored at room temperature for up to 30 days, however there was decline seen at 30 days when stored at 35°C, a decline at 7 days but not 30 days when stored at 4°C (not protecting against humidification) and no decline seen at 4°C for up to 30 days when the samples were protected against humidification.

Beesley et al,. (129) found that samples stored at 4°C, room temperature and 37°C, with and without desiccant showed very varying results with both increases and

decreases in concentration seen (from 40% to 180% original concentration). This may have had more to do with assay variability than stability (they suggested the DBS elution step may have been the cause), but by the end of 12 weeks the concentrations for all storage conditions were near the starting concentration except for storage at 37°C which appeared to degrade by 8 weeks. They also concluded that desiccant had little effect on CRP stability.

The results of the study performed by McDade et al., in 2004 (199) seem to suggest that DBS CRP samples are less stable than that as they found DBS CRP was only stable for 3 days at 37°C. McDade et al., did find that samples were stable at room temperature and 4°C for at least 14 days, which is similar to what we found for both DBS and Mitra assays. They also found that 5 cycles of freezing and thawing caused no deterioration of CRP concentration. Cordon et al., (40) found that DBS CRP was stable for at least 21 days when stored at -70°C, -20°C, 4°C and room temperature and also found that posting samples to the laboratory had no effect on mean CRP concentration.

Brindle et al., (200) suggest that CRP is less stable than previously thought. They found that CRP in DBS degraded after just 12 h storage at 37°C followed by 12h storage at 15°C. They also found significant degradation after DBS were stored for 14 days at room temperature or for 7 days at 37°C. DBS were stable for at least 42 days when stored at -20°C. We found similar patterns for storage at 37°C.

The variability of DBS CRP stability in the literature may be partly due to assay variability as well as the stability of the DBS. Our data appeared to show that SWBH DBS CRP are fairly stable and that more frequent calibration would be beneficial. It does not appear that DBS for CRP are susceptible to sudden deterioration. Due to

the problems with mushy spots however, it was hard to draw firm conclusions for the SWBH devices. It may be that storing the DBS frozen actually increases the chances of the samples becoming mushy and so any advantage in stability from storing frozen may be lost.

The Mitra devices appear to be more stable than DBS CRP, especially when stored at 4°C or frozen. The data also showed that the samples do show signs of deterioration fairly quickly when stored 37°C. A major advantage of the Mitra devices are that they are not subject to disintegration when sonicated and so the stability data has not been confounded by that. For both sample types, calibrators and QC should be stable at room temperature for at least one month, probably longer, especially for the Mitra devices.

The disintegration of DBS samples could lead to the reporting of falsely low results. The use of a different type of sonicator did appear to reduce the number of mushy spots obtained (although it did not eliminate it altogether and it is not always obvious when the spot has slightly disintegrated) but investigating alternative methods of extracting the CRP (e.g. shaking the sample) may lead to more consistent analysis. If put into routine use the DBS method should involve measuring samples in duplicate, as is done for the vitamin D DBS assay, to minimise the risk of inappropriately low results being reported. The quality of DBS work shown in Chapter 3 and our experience of the routine vitamin D DBS assay, has shown that analysing DBS in duplicate does not pose a problem.

The DBS and Mitra CRP methods have shown excellent linearity and correlation with serum samples and good intra and inter assay variation. The Mitra devices show improved methodological performance compared with the DBS assay and patients

appear to prefer taking Mitra device samples compared with DBS samples (unpublished data). The better intra and inter assay variation shown by the Mitra devices is likely to be due to the fact that the Mitra devices contain 10μL of blood and all of this is added to the extraction buffer. There is therefore more CRP available for extraction compared with a 3mm punch from a DBS. As there is more CRP available for analysis, the assay is not being pushed to its limits and therefore performs better. It may also be that CRP is extracted more easily from the Mitra device compared with filter paper. In addition, the presence of mushy spots will increase the variation seen with the DBS CRP assay and this is not a problem seen for the Mitra. Although the Mitra device has better reproducibility compared with DBS, the Mitra device has two major drawbacks – the time required before a result similar to the serum CRP assay is obtained and the influence of haematocrit on results. The DBS assay does not suffer from these problems, however it does have a large negative bias compared to the serum assay and has issues with disintegrating spots. Both sample types however use a quick and convenient method and the drawbacks could be overcome either through adaptation of the method or through discussion of the limitations with the clinicians (e.g. explaining influence of haematocrit on Mitra results). As the method is so quick and simple it should easily fit into the routine repertoire of laboratory tests. However, further work needs to be done in conjunction with the rheumatologists to explore the benefit to patients of using DBS CRP as a way of routinely testing their CRP before clinic appointments.

# CHAPTER 6 - SUMMARY AND FURTHER WORK

## 6.1. Introduction

The work presented in this thesis demonstrates the viability of DBS as a method of sample delivery to the laboratory. DBS have been successfully used in the direct to the public vitamin D service and the DBS samples obtained from this service have shown that the public are capable of taking quality DBS. The work has shown that there are multiple areas to consider when validating a DBS assay, but that as long as these issues are appreciated and the setting that the DBS will be used in are understood then DBS can be a viable sample collection technique. The work has also shown how direct access analysers can be adapted for use with traditional DBS and new microsampling devices in the form of the Mitra.

The use of DBS in a direct to the public vitamin D testing service has opened up a new population for study that has been shown to be significantly different from the local GP population, including displaying a much higher rate of high to toxic levels of vitamin D. In addition this new DBS population have also been shown to be able to respond to their vitamin D results in a much better way than the local GP population.

## 6.2. Research Outcomes

### 6.2.1. DBS Quality

The work undertaken in Chapter 3 has shown that DBS can be used as a vehicle for samples to reach the laboratory and that the public are capable of taking quality DBS. (204) We have shown that the rate at which insufficient DBS are taken by the public is acceptably low at <3% as long as techniques analysing DBS have been set up to use one 3mm punch. Our work has shown that more blood could be obtained if

the right lancet is selected, and if larger size guides or printed circles are used on the collection device. Whatever size of DBS is required, it is important that DBS calibrators are produced that have characteristics, such as size of spot and haematocrit, that are similar to those that will be sent in by patients. This is because we have demonstrated that size of spot and haematocrit, two factors least in control of the laboratory, can influence the concentration of analytes. Other factors that need to be considered when setting up a DBS assay, such as location of punch, the use of haemolysed blood and the presence of ethanol in calibrators should also be thoroughly explored as these factors can also affect results.

In particular we found that increasing the size of DBS increased 25(OH)D and CRP concentration and that punches taken from the edges of DBS significantly increased the concentration of CRP and 25(OH)D found. (205, 206) The use of haemolysed blood to make DBS resulted in a significantly increased concentration of 25(OH)D but a decreased concentration of CRP – significantly lower for the Mitra device. This highlights how investigation of the influence of DBS characteristics on results should be investigated for each analyte as the patterns may not always be the same. The presence of ethanol on the concentration of CRP and 25(OH)D was variable and shows how it is best to make calibrators from blood as similar to that as the users blood as possible in order to avoid matrix differences. Very low and very high levels of haematocrit were shown to potentially cause significant differences in 25(OH)D and CRP results, in particular for the Mitra devices, and again highlighted how important it is to know the target population blood characteristics, to determine if the variation in results would be acceptable or not. The Mitra device overcomes some of the DBS characteristic problems – location of punch and size of DBS – however it

appears that it has some other problems, such as haematocrit variation, that need to be addressed instead.

### 6.2.2. **Direct to the Public Vitamin D**

The work undertaken in Chapter 4 has shown that the users of the vitamin D DBS service had generally higher levels of 25(OH)D compared to our inner city Birmingham GP population, with the majority of DBS users having an adequate status whilst the majority of the GP population were less than adequate. (207, 208) The DBS population were also found to have a significantly higher rate of high to toxic results, 3.1% compared to 0.06% in the GP population, with concentrations ranging from 221-1,235 nmol/L. (209) Further investigation of this phenomenon revealed that this was mainly a result of regular vitamin D supplementation, although in two cases it was due to bolus ingestion of liquid vitamin D supplements. The supplementation regimes undertaken were very varied, ranging from a daily supplementation pattern of 1,000-120,000 IU/day spanning days to years. (210) Toxicity could not be proven, but there was surprisingly little medical supervision, with only 6.2% of users taking above the IOMs no observed adverse effect level of 10,000 IU/day being medically supervised. There was no real correlation between amount of supplementation and concentration of 25(OH)D achieved, but this may have been due to a variety of reasons such as length of time supplemented for, starting 25(OH)D concentration, brand, formulation, genetic makeup of users and the fact that the levels supplemented with were self-reported and therefore unconfirmed. (211)

The work reported in Chapter 4 also demonstrated the impact that the direct to the public vitamin D service had, with 95.5% of respondents to our survey stating that

they would rather have a DBS test than a traditional phlebotomy test. The survey also showed that the instructions were easy to understand, it was easy to take a DBS sample and that they could also understand the results. This work demonstrated the very real desire for DBS assays from the general public. One of the popular reasons stated for using the DBS service was the ability it provided to take control of their health, with 77.9% of users stating this as a reason for using the service. This may explain the huge difference seen in how the DBS service users responded to initial vitamin D results compared with the GP population. Our work showed that 75.5% of DBS users that needed to improve their vitamin D status after an initial result of <50 nmol/L went on to improve their vitamin D status and became adequate on repeat testing. (212) Only 33.3% of GP patients who had an initial result <50 nmol/L went on to achieve adequate vitamin D status upon repeat testing, and in fact 54.8% of patients who were initially adequate were subsequently found to be less than adequate upon repeat testing. (213)

### 6.2.3. **DBS CRP**

The work performed in Chapter 5 has shown for the first time that routine random access analysers can be adapted to analyse CRP in dried blood in a method that takes only 15 minutes compared to the days often required by other published methods for CRP analysis of DBS. We have shown that this method can be used for traditional filter paper DBS as well as the Mitra device. The assay has been shown to have acceptable intra and inter assay variation for both devices, with good linearity. The limit of quantitation is acceptable for the SWBH device, but is the equivalent of serum for the Mitra device. The SWBH assay did not suffer from carryover and extract stability was shown to not be an issue. For both sample types, CRP

deterioration was shown to not occur for at least 3 weeks at room temperature. Room temperature storage may be better for SWBH devices as it may lead to less instances of punches disintegrating. Very little deterioration was seen for Mitra devices stored at -80°C or at 4°C. Samples stored at room temperature also showed good stability, however Mitra devices stored at 37°C started deteriorating almost straight away.

Both sample types displayed excellent correlation with patient samples, however the SWBH method did suffer from a large negative bias, although this was not as great in the range in which RA patients would be likely to fall (<30 mg/L). The Mitra device also displayed good correlation with patient samples, but the agreement only improved once the samples had been allowed to age. In addition the Mitra assay appeared to be influenced by haematocrit, with lower levels of CRP found with higher haematocrit levels. However, in the patient cohort analysed haematocrit did not appear to be an issue.

## 6.3. **Future Directions**

The work presented here has opened up many avenues for further research. We have only looked at the use of DBS for two analytes, vitamin D and CRP. It would be interesting to extend the repertoire further, for example looking at the use of DBS to measure HbA1c in diabetics. It would also be useful to expand the repertoire of the Mitra devices past CRP and the vitamin D assay would be a sensible place to start.

The DBS characteristics work showed how the size of the DBS and the location of the punch taken from the DBS can lead to large variations in the concentration of the analyte, however the concentration profile of vitamin D and CRP found across the DBS was not investigated. Future work should investigate the concentration profile

across the DBS and investigate whether this is consistent when the size of the DBS varies.

The Mitra device proved popular with users of the direct to the public DBS vitamin D service (unpublished data) who found it easier to collect samples with the Mitra compared with filter paper collection. However, the Mitra had other problems which became apparent with the CRP assay, namely that the samples needed to age before they gave results in line with serum results and that haematocrit had quite a strong influence on results. It would be interesting to see if these issues were present with other analytes, such as vitamin D. It would also be useful to examine the impact haemolysed samples have on Mitra results further, to find out the reason for CRP concentration differences between haemolysed and non-haemolysed samples and if differences are seen for other analytes too.

We have established a good CRP assay for both the SWBH and the Mitra device. Future work exploring the routine use of these devices for RA patients would be very valuable, in particular looking at whether the use of DBS improved the patient journey and clinical outcomes. Further work should also be undertaken to see if dexterity issues caused by RA has an impact on the patient's ability to take DBS samples. The issues remaining with the DBS CRP assay, namely the negative bias for the SWBH device and the influence of haematocrit and age of sample on the Mitra results would need to be further explored to see what impact they had in a routine setting. In addition it would be very useful to obtain further paired EDTA, capillary and serum samples for higher levels of CRP to see if the relationships are maintained at higher levels of CRP.

The Mitra CRP work undertaken so far did not include the collection of Mitra samples by patients themselves in the field. A future area of work should look at the ability of patients to collect Mitra samples in the field and in particular how patients with RA manage to collect Mitra samples and whether or not they are able to collect Mitra samples more easily than DBS.

The vitamin D worked showed how different the GP serum and DBS populations were and it would be interesting to find out the reasons for these differences. For example, what impact does socioeconomic status have on the ability of someone to improve their vitamin D levels after an initial vitamin D result? It would also be illuminating to work with GPs to find out what protocols they are following when they find a patient is vitamin D deficient.

It was fascinating to find out the wide variety of vitamin D supplementation regimes that DBS users were undertaking to obtain such high levels of 25(OH)D. However, it would also be interesting to find out what supplementation patterns non-toxic users of the DBS vitamin D testing service were using, to see how different the regimes are from those that are causing the high results. It would also be interesting to see how much of a role genetics plays in causing those users to achieve such high results. We were unable to show whether or not those high levels resulted in toxic sequelae and it would be very useful to show how often this was occurring and at what concentrations of 25(OH)D.

## 6.4. **Conclusion**

In summary, DBS are a valuable addition to the repertoire of sample types analysed by the laboratory. We have shown that the methodology is valid and useful for the public and can lead to an improvement in the patient pathway, with massive scope

for future work. DBS are another way that laboratories can link directly with the public and potentially enable clinicians to provide a better clinical service too.

# APPENDIX

**Appendix 1** – Instructions on how to collect a DBS contained within the kit sent out to patients.

## Blood Spot Collection Device

### Self test instructions

#### Preparation

- Fill in personal details and date on the collection device.
- Wash hands with warm water and towel dry.
- Massage finger to be bled and clean with alcohol wipe.
- **Middle or ring finger on left hand (if right-handed) is best.**

#### Collecting the blood spots

- Twist and remove protective cap from the single-use lancet *(see picture A )*.
- Press it firmly against the side of the cleaned finger tip then press the button *(see picture B)*.
- Wipe away the first show of blood.
- Gently squeeze the finger and allow up to four large drops of blood, at least as big as the size guide *(see picture C)*, to fall onto the card.
- The blood drop can be placed on the card by gentle touch but **avoid pressing the finger tip against the card.**
- **Don't** apply more than one drop of blood in the same place. If a spot is poor move on to the next position *(see picture D for good and poor spots)*.
- Cover finger with plaster.

#### Posting

- Close and seal collection card and sent to laboratory or give to your health professional.

Sandwell and West Birmingham Hospitals NHS
Clinical Biochemistry, Birmingham, B18 7QH
© Patent applied for

CE

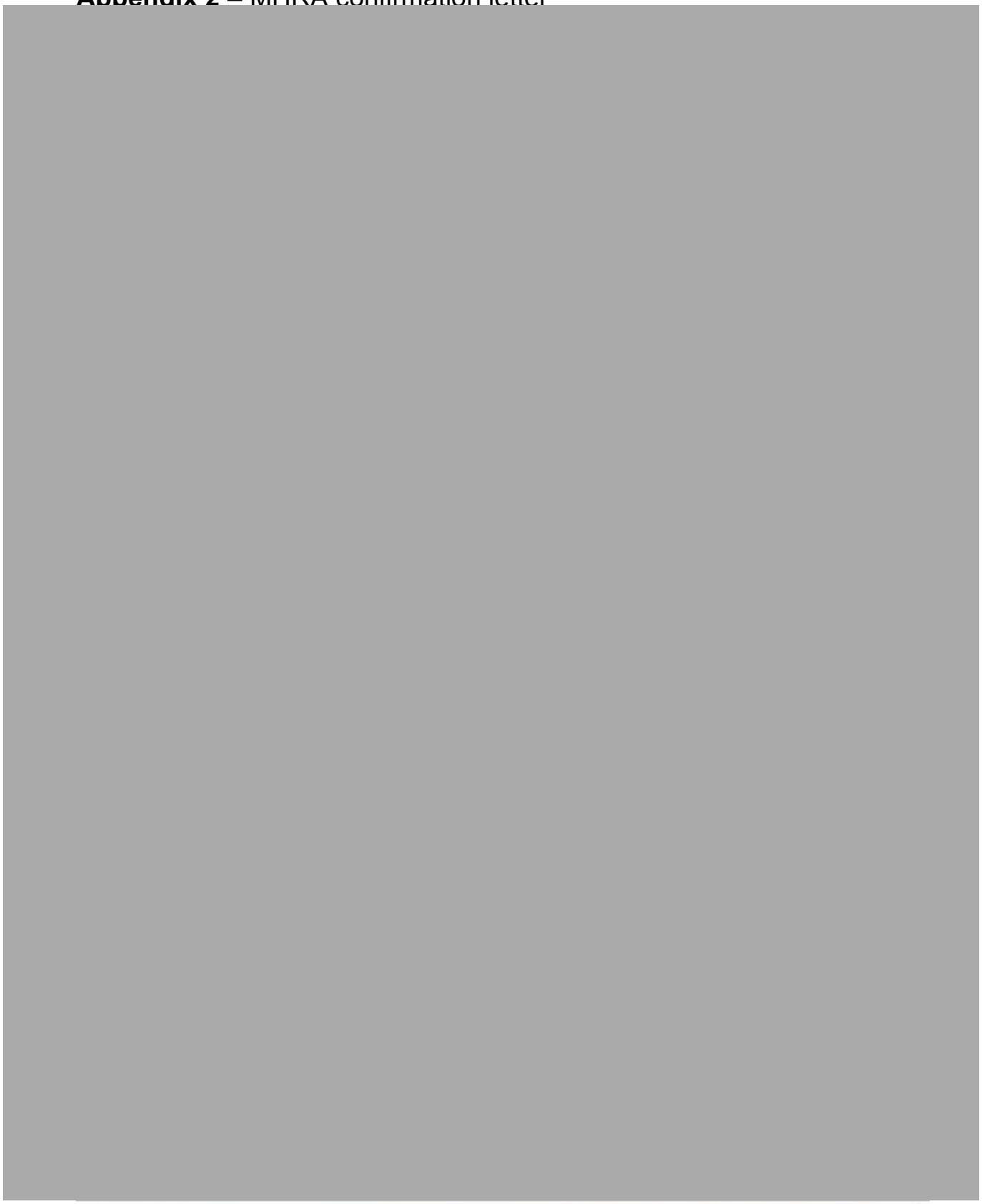
Picture A

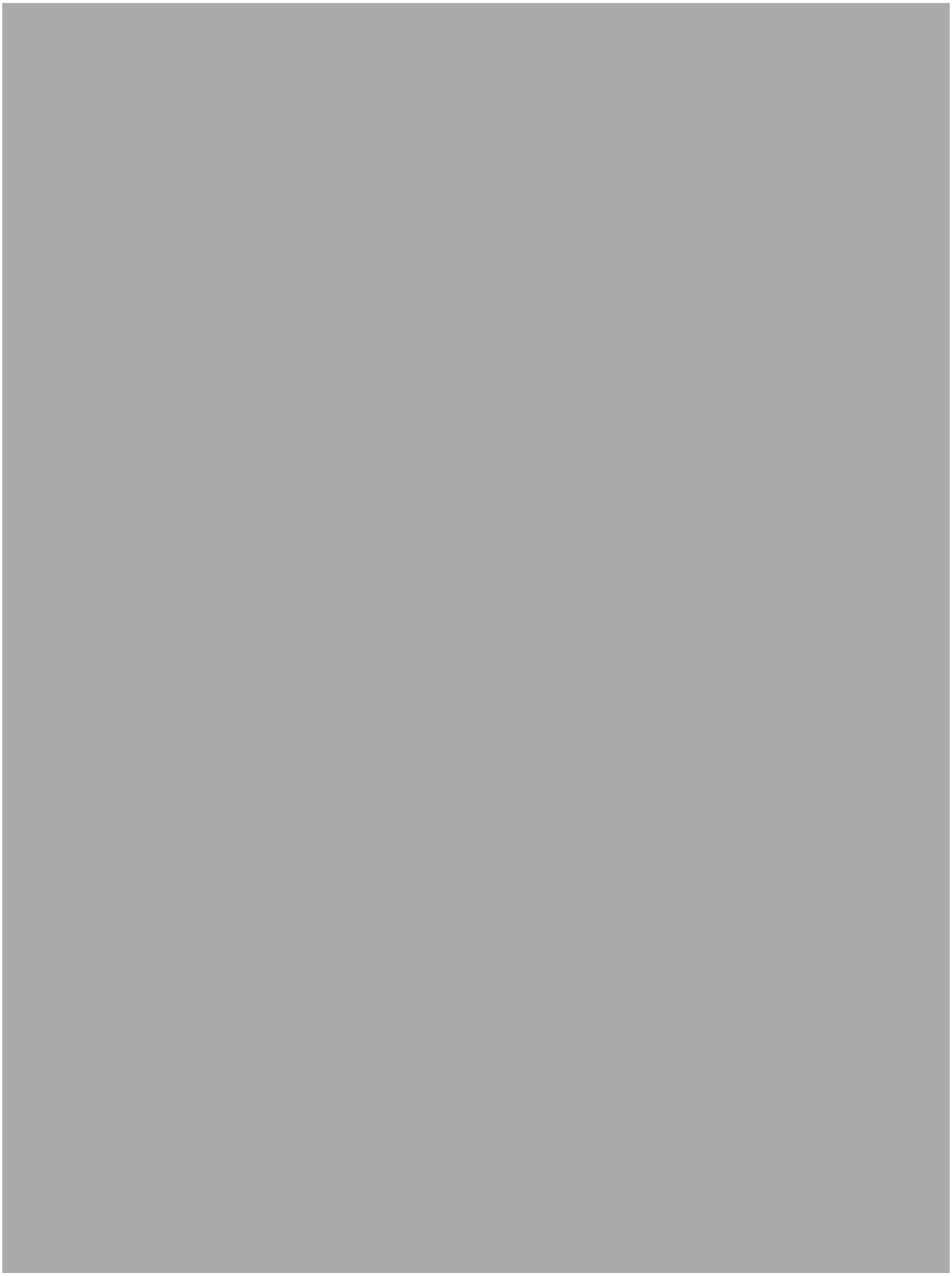

Picture B


Picture C


Picture D

**Appendix 2 –** MHRA confirmation letter





**Appendix 3** – Report PDF that is generated and sent out by post or email to the user

Sandwell and West Birmingham Hospitals NHS

**Department Clinical Biochemistry**
City Hospital, Dudley Road Birmingham

## Vitamin D

| | | | |
|---|---|---|---|
| First Name: | Rs | Date Sample Taken: | 14/08/2016 |
| Surname: | TE | Date/Time Received: | 15/08/2016 12:10 |
| Date Of Birth: | NK | Date Tested: | 15/8/2016 |
| Sample Number: | C,16.0001649.T | | |

### Your Results:

**Total Vitamin D : 102.4 nmol/L**

**Status: ADEQUATE**

(25-hydroxyvitamin $D_3$ : 96.8 nmol/L, 25-hydroxyvitamin $D_2$ : 5.6 nmol/L)

### Interpretive Guide:

| Total Vitamin D Reference Interval (nmol/L) | Vitamin D status |
|---|---|
| Less than 15 | Severe Deficiency |
| 15 – 30 | Deficiency |
| 30.1 – 50 | Insufficiency |
| Greater than 50 | Adequate |

**Total vitamin D levels above 220 nmol/L are considered 'High' and increase the risk of vitamin D toxicity.**



For more information visit our website: www.vitamindtest.org.uk

Where EVERYONE Matters

**Appendix 4 –** DBS technical service information sheet

**Sandwell and West Birmingham Hospitals** NHS Trust

**Technical Service Information Sheet**

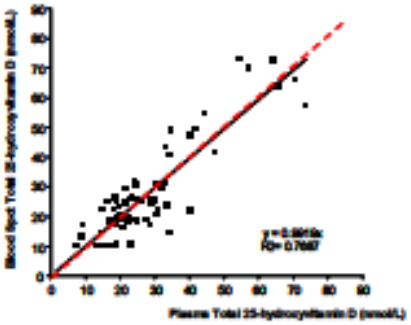
## Determination 25-Hydroxyvitamin $D_3$ & $D_2$ in Dried Blood Spots by LC-MS/MS

**Sample(s)**

- Our assay can receive dried blood spots collected on Whatman 903 (GE Healthcare) and Ahlstrom 226 (ID Biological Systems). The type of filter paper must be clear upon receipt. Send devices appropriately packaged to; Clinical Biochemistry, City Hospital, Dudley Road, Birmingham B18 7QH
- Stable for 6 months stored dry at RT

**Method:**
The method is standardised against our conventional 25-hydroxyvitamin $D_3$ and $D_2$ LC-MS/MS service plasma/serum using specific blood spot calibrators. Blood spot results are equivalent to the serum/plasma level if a venous sample had been collected. Our laboratory participates in DEQAS external quality assurance.

***Figure 1 Comparison of Total 25-hydroxyvitamin D results for capillary blood spots with conventional LC-MS/MS method (n=65)***



**Performance:**

| Characteristic | Blood Spot |
|---|---|
| %Recovery | 88.4% |
| Limit of Quantification (Signal to Noise >10) | 3.3 nmol/L |
| Intra-assay variation (*n*=10) | <10% |
| Inter-assay variation (*n*=10) | <15% |

**Reference Ranges:**

| **Total 25-Hydroxyvitamin D** | **(nmol/L)** |
|---|---|
| Severe Deficiency | <15 |
| Deficiency | 15 - 30 |
| Insufficiency | 30 – 50 |
| Adequate Status | >50 |

*We also report the individual values for 25-hydroxyvitamin $D_2$ & $D_3$ in nmol/L*

***Target Turnaround time results***
2-3 working days

**Results Return**
First class post or PDF reports to secure identified email address.

*DBSVITD_SWBH2011_VS1.0*

**Appendix 5** – Form used to collect information on patients that have DBS total vitamin D >220 nmol/L

# High Level Vitamin D Patient Information Form

This form to be used when contacting a patient regarding a high level of vitamin D that has been found in a blood spot sample.

| | |
|---|---|
| **Date** | |
| **Lab Number** | |
| **Name** | |
| **Result** | |
| **Are they under medical supervision?** | |
| **If yes, what have they been prescribed and route?** | |
| **If no, what have they taken (brand and amount)?** | |
| **Where did they get it from?** | |
| **How long have they been taking it for?** | |
| **Any other information?** | |

**Appendix 6** – Questionnaire sent out to users of the vitamin D DBS service

# Questionnaire to Audit the Impact of our Dried Blood Spot Service

Thank you for using our Dried Blood Spot Vitamin D service. I am doing a PhD on Dried Blood Spots in Routine Clinical Practice and as part of this work I am trying to audit the impact that using dried blood spots as an alternative to venous sampling has on people using the service.

I would greatly appreciate it if you would take the time to fill out this short questionnaire and then email it back to us at [redacted]

**All answers will be treated with confidentiality and anonymity.**

**Please select your gender:**

Male: ☐ Female: ☐

**Please select your age group:**

18 – 25 years: ☐ 26-40 years: ☐ 41- 60 years: ☐ >60 years: ☐

**How did you hear about our service?**

Word of mouth: ☐ Internet: ☐ Other: ☐

If other, please state (box expands): ☐

1. **Were the instructions easy to understand?**

   Yes: ☐ No: ☐

2. **On a scale of 1. Very easy to 5. Very hard, did you find blood spot collection with filter paper:**

   1. Very Easy: ☐ 2. Easy: ☐ 3. Neutral: ☐ 4. Hard: ☐ 5. Very Hard: ☐

3. **Were the results easy to understand?**

   Yes: ☐ No: ☐

4. **Why did you use our service?**

   Not available through GP: Yes: ☐ No: ☐

   Convenient: Yes: ☐ No: ☐

   Cheap: Yes: ☐ No: ☐

   Recommended by health care worker e.g. nutritionist: Yes: ☐ No: ☐

   Underlying medical reasons: Yes: ☐ No: ☐

   Less blood used: Yes: ☐ No: ☐

   Ability to take control of health: Yes: ☐ No: ☐

   Less invasive than traditional venous sampling: Yes: ☐ No: ☐

   If you have other reasons please state them here (box expands):

   ☐

5. **If you could choose between either having a traditional blood test at the GP/hospital or doing a blood spot test, which would you prefer?**

   Traditional blood test: ☐ Blood spot test: ☐

6. **Would you like to see other blood spot tests available?**

   Yes: ☐ No: ☐

7. **On a scale of 1. Terrible to 5. Excellent, how would you rate our service overall?**

   1. Terrible: ☐ 2. Poor: ☐ 3. Average: ☐ 4. Good: ☐ 5. Excellent:☐

8. **Would you like to be able to purchase the kits via the website?**

   Yes: ☐ No: ☐

9. **Are you satisfied with our turnaround time?**

   Yes: ☐ No: ☐

10. **Are you satisfied with your experience of contacting us by telephone e.g. for ordering kits and advice?**

    Yes: ☐ No: ☐

---

**Please use this space to leave any other comments you wish to make.**

☐

**Thank you for participating in the survey. Please save the document and email to one of the addresses above.**

**Appendix 7** – Comments received as part of the feedback from the questionnaire.

| ID | How did you hear about our service | Why did you use our service? | Other comments |
|---|---|---|---|
| 2 | In a sun-tanning salon | | Excellent service except that an incorrect report was sent to me showing that an even lower vit D value was sent to me. This despite 2 months of UVB narrow band treatment. This made me see my GP and consider using extreme medical solution. Luckily the incorrect name on the report was spotted (refer your letter (email) of 17th April 2013 15.49hr |
| 3 | I attended a Philip Day seminar who told me about Vit D deficiency, then Eliz Roddick Chemist taking blood samples. Tests needs to be wider known | | Blood prick samples taken at New Life Chemist, Glasgow |
| 4 | My wife attended a seminar by Philip Day in Glasgow | Lack of energy and feeling tired all of the time | Blood prick samples taken at New Life Chemist, Glasgow |
| 5 | | | Should have secure website payment |
| 6 | | | TV |
| 7 | From a talk on nutrition given at our local U3A monthly meeting | I'm nosey | If you are doing any other kinds of blood spot test then let me know |
| 8 | Dr referred | | |
| 10 | | The kit was | The links to the email address for returning the questionnaire did not work |

| | | | |
|---|---|---|---|
| | | given to me as a gift | |
| 11 | | | Some of the marking is lower because of the delay, but no complaints about service once you became aware results had not been received. Will readily use service for family if can be sure results are accurate. |
| 12 | Recommended at a vitamin D seminar I attended by Nutri Advanced Ltd | I am a nutritional therapist so I use them to check my clients vitamin D levels before supplementation | |
| 14 | | | Very speedy and efficient, thank you |
| 15 | Margaret Hills Clinic | | |
| 17 | Philip Day, Credence | | Thank you! |
| 19 | Nutritionist | | I did find it quite painful afterwards to do via this method! Probably more so than venepuncture. Though v convenient and quick! |
| 22 | | Just to check that the levels were right, to stay on top of health | Can't find anything to improve! Wonderful service. Just brilliant! |

| | | | |
|---|---|---|---|
| 24 | | | Either traditional or blood spot tests. I appreciate that toxicity levels is a variable but it may be worth adding to the scale with 2 additional levels - High adequate - could be at risk of toxicity High- Concern of toxicity. Maybe with information leaflet that explains the concerns of low Vitamin D along with symptoms and problems but how beyond optimum levels could be undesirable along with the why's and the effects of too much and becoming toxic, poisonous. This would also allow to include that the test should be used as a guide as could be slightly higher or lower than indicated results. |
| 29 | My firms doctor, John Briffa | | |
| 30 | | | Excellent service. Great customer service. Thank you very much. |
| 31 | | Health website recommended | |
| 33 | | | From a point of interest when I tested back in Sept-12 Insufficient. On the April-13 test I was adequate. This after taking 1000Ius a day between tests. |

| | | | |
|---|---|---|---|
| 36 | | | Following on from Question 6, as complex organisms, it would be helpful if all serum measurements could be derived from the blood spot test because it would help us the patients to see if other things besides Vit D are out of ideal ranges e.g. Boron which in a male reduces the effects of SHBG which converts free testosterone into bound testosterone. Magnesium isn't so important considering the great lengths the body goes too to maintain magnesium serum levels, and when the variability of other substances in the body/blood can vary throughout the day like cortisol or even testosterone, whilst it's not perfect due to the daily swings, it could still show trends over time when currently no trends can be found due to the absence of any data in the first place. In my IT world, I always work on the basis you can never have enough data, so "if it's not measured, how are we to know?" is the principle I try to adhere to all the time. After all knowledge is power and all that so if a service existed where I could give a blood sample and then get results back showing me everything that could possibly be measured I think it would be useful to me and others, after all in some problem solving situations, Dr's start off diagnosis with a FBC, when can't the patient do this if they want to? With the ability to access the latest reports as well as historical scientific studies online with some help from Google, after overcoming the terminology used in health it's not hard for someone with some logic to form their own conclusions so yes having the facility to measure as much as possible from the blood could be useful (hard to quantify the exact benefits though) for those who don't have to go to the doctors but do want to pay attention to their health. |
| 38 | Nutritional therapist | | Would be beneficial to give suggestions/recommendations as to how to increase level of vitamin D if it is low. |

| | | | |
|---|---|---|---|
| 42 | | No medical reason for requesting test by GP but having had breast cancer nearly ten years ago am determined to keep vit d levels as high as possible | I was disappointed that my result wasn't higher as I have been taking 5000IU of vit D3 throughout winter. I had a tooth infection at the time of doing the test and wonder if that could have affected my results. I was also wondering why my d2 count was so high as I am not aware that I getting any d2 from anywhere |
| 43 | | | We can get a blood test at our doctors but your service is quicker and more convenient |
| 44 | From Phillip Day - Credence | GP agreed to do the first test, but then didn't agree to do the following ones (eventually said that it was due to costs!!) | I have found out via other (not GP) sources the importance of adequate levels of Vit D in my body and how damaging can be low levels, as they impact on other vitamins etc. if they are absorbed into the body. That made me decide to take it in "my hands", make tests on monthly bases and monitor VD while taking different strength supplements, using SAD lamp, spending time in the sun. Results will allow me use the best combination of supplements in the future. I also feel that regular screening for Vit D would help improve populations health enormously. |
| 45 | | | A company called Myrios already has cheap kits for hypothyroidism etc. http://www.myrios.co.uk/en/buy-online/myrios/ |
| 46 | Mother | Gift | Altogether excellent |

| | | | |
|---|---|---|---|
| 48 | | Trying to conceive without miscarrying and hear Vit D plays an important role | |
| 49 | Dentist | | |
| 55 | | | Wanted explanation of D2 and D3 difference and normal guidelines etc. |
| 56 | | | It would be good to have a reduction in price for retests, within a certain time scale. After taking the necessary supplements to increase Vitamin D levels, then checking to monitor levels, 1 or 2 follow tests within 4-8 weeks may be needed which starts to bump up the price. I have also recommended this test to 6 other people who have purchased the test, on has had one retest already and will be having a third test in a couple of weeks. This will be the same for me and the other people who have had their results back. I will continue to recommend this test to my clients as it is an important one in my consideration, however it would be lovely to say to them that consecutive tests will be cheaper, to encourage them to keep monitoring their levels. |
| 57 | | Testing only available through my GP at a cost of nearly £70, which for me is | I cannot understand why this service is not available on the NHS but at the very least patients should be made aware of the service. I have fibromyalgia and need repeat testing to ensure that I am supplementing at the correct level to improve my health, after being found to be severely deficient but this inly |

| | | | |
|---|---|---|---|
| | | not affordable and also 16 days to await the result! | |
| 60 | Nutritionist | | The service was really excellent. The only issue is perhaps getting a drop of blood sufficient for each spot with getting it all over the place. However, the style of kit is great, easy and the results were indeed very prompt. Thank you |
| 61 | Attended a presentation with a pharmacy colleague at another pharmacist's shop | | The testing was carried out by a pharmacist colleague but I would be able/happy to use the blood spot test system if it was available. |
| 62 | | Reliable NHS service | |
| 63 | Attended Phillip Day seminar | | Pleased to have this information to enable me to make decisions about vitamins etc. |
| 64 | Attended Phillip Day seminar | | Pleased to have this information so I can take appropriate vitamins |
| 65 | | | I would have liked to have paid for the kit over the phone when I ordered it |
| 67 | Dr Briffa | | |

## **Appendix 8 –** Ethics approval

Ethics approval was sought and granted in 2010 to allow the collection of a dried blood spot sample at the same time as a venous sample (RC10/1202/76). An extension to this was sought and granted in 2014. The ethics application related to patients who were already having blood taken for other clinical purposes. The sample collection would take place at phlebotomy clinics where patients awaiting phlebotomy would be asked if they were willing to provide an additional sample. A patient information leaflet (see below) was provided and on agreement the consent form (see below) was signed. The additional blood spot sample was collected during the phlebotomy session whilst serum and/or EDTA samples were collected. The DBS was collected by a member of staff in order to minimise the disruption to the phlebotomy service.

Confirmation of whether or not ethics was required for discussing high vitamin D results obtained using the DBS direct to the public service with users of the service was sought from SWBH R&D department and it was decided that ethics were not required as this was part of the service and could be classified as service development.

**Consent Form**

Identification Number: _____

# CONSENT FORM: Addition blood sample

Research Title**:**

*Investigating the use of capillary blood sampling as an alternative to venous blood sampling for routine analysis*

**Name of Researcher Co-ordinator**: *Dr Jonathan Berg, Consultant Clinical Biochemist*

Please initial box

1. I confirm that I have been offered a copy the information sheet dated **November 2010 (Version 1.3)** for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and involves the donation of an addition blood sample which can be used at the discretion of the laboratory throughout the completion of the above project. ☐
3. I understand that there will be no additional results reported to myself or my doctor following testing of the blood spot sample and the results of the tests will be matched and compared to the results of my routine blood tests. ☐
4. I understand that the blood spot sample collection will involve pricking of a finger with blood spotted on to a collection card. ☐

## Declaration

**I agree to donate an additional blood spot sample as outlined above to be used at the discretion of the laboratory and data will be paired to the results of my routine blood test.**

☒ _____ ☒ _____ ☒ _____

***Name*** ***Signature*** ***Date***

*Name of the person taking consent*: _____

# Can You Help Us Test Out A New Way of Collecting Blood?

- We would like to take a few drops of blood from your finger today onto a card as well as the normal bloods from your arm.
- By participating you will be helping us bring a new way of taking blood into use.

**Contacts for further information & background details**

If you have any questions, or there are any aspects of this project you wish to discuss, please contact any of the following members of the research group via City Hospital switch board:

[redacted],

[redacted]

[redacted]

If you have any concerns and wish to contact someone independent, you may telephone the Patient Advice and Liaison Service on [redacted]. The work has been approved by the Research and Development departments of the Sandwell and West Birmingham NHS Trust and the Birmingham and Solihull Mental Health NHS Foundation Trust. It has also been approved by the Black Country Regional Research Ethics Committee.

## **Appendix 9** – Papers and posters

### Papers

**Shea, R.L.** and Berg, J.D. Self-administration of Vitamin D supplements in the general public can result in high to toxic 25-hydroxyvitamin D levels. Annals of Clinical Biochemistry (2016) epublication ahead of print

*Research Article*

The Association for Clinical Biochemistry & Laboratory Medicine
*Better Science, Better Testing, Better Care*

Annals of Clinical Biochemistry
0(0) 1–7

DOI: 10.1177/0004563216662073
acb.sagepub.com
SAGE

# Self-administration of vitamin D supplements in the general public may be associated with high 25-hydroxyvitamin D concentrations

Robyn L Shea and Jonathan D Berg

**Abstract**

**Introduction:** Our dried blood spot vitamin D testing service enables members of the public to assess their vitamin D status. Vitamin D has become popular with the media and the general public. We noticed that our direct access service had a higher rate of high to toxic 25-hydroxyvitamin D levels compared with our GP population and we wanted to know why.

**Methods:** Between January 2013 and September 2015 we contacted all direct access users who had 25-hydroxyvitamin D >220 nmol/L measured using LC/MS/MS. We investigated the amount, type and length of supplementation used and whether or not users were medically supervised.

**Results:** A total of 372 service users had 25-hydroxyvitamin D concentrations >220 nmol/L. Of 14,806 direct access samples received, 372 (2.5%) were from users with 25-hydroxyvitamin D concentrations ranging from 221 to 1235 nmol/L. Only 0.06% of GP patients had results >220 nmol/L over the same time frame. There were 361 direct access users regularly supplementing, taking between 1000 to 120,000 IU/day. Two users took bolus doses of 300,000 and 900,000 IU. Only 23 users taking supplements (6.4%) were under medical supervision. There were 28 users with levels >500 nmol/L, but only one was under medical supervision. The internet was the main source of supplements (74%).

**Conclusions:** A high proportion of the direct access users had a high to toxic concentration of vitamin D. Many people were taking more than the Institute of Medicine's NOAEL of 10,000 IU/day, yet only a few were being medically supervised. Clinicians should be aware that patients may be self-administering very high concentrations of vitamin D, especially when investigating unexplained hypercalcaemia.

**Keywords**

Vitamin D, dried blood spot, supplementation, hypervitaminosis, general public

Accepted: 10th July 2015

**Shea, R.L.** and Berg, J.D. The rise and rise of Vitamin D. Clinical Laboratory International (2014) **38** (Feb/March): 10-12

**Posters**

National Meeting of the Association for Clinical Biochemistry – Focus 2013

**Shea, R.L.** and Berg, J.D. Are the public capable of taking viable dried blood spots for quantitative analysis? Annals of Clinical Biochemistry (2013) **50** (Suppl. 1): 103

**Shea, R.L.** and Berg, J.D. Self referral vitamin D testing: are we just testing the worried well or making an important contribution to healthcare? Annals of Clinical Biochemistry (2013) **50** (Suppl. 1): 34

National Meeting of the Association for Clinical Biochemistry – EuroMedLab Focus 2014

**Shea, R.L.** and Berg, J.D. How do GP patients respond to a vitamin D result? Clinical Chemistry and Laboratory Medicine (2014) **52** (11) eA205-eA379

**Shea, R.L.** and Berg, J.D. How do members of the public respond to a vitamin D result? Clinical Chemistry and Laboratory Medicine (2014) **52** (11) eA205-eA379

Vitamin D and Human Health – 2014

**Shea, R.L.** and Berg, J.D. Direct-to-the-public vitamin D testing compared to GP referrals. Nutrients (2014) **6** (7) 2759-2919

**Shea, R.L.** and Berg, J.D. Incidences of high to toxic 25-hydroxyvitamin D levels amongst users of a direct-to-the-public blood spot Vitamin D testing service. Nutrients (2014) **6** (7) 2759-2919 – **Certificate of Young Investigator Award**

National Meeting of the Association for Clinical Biochemistry – Focus 2015

**Shea, R.L.** and Berg, J.D. Direct-to-the-public vitamin D service provides evidence of over-supplementation to toxic levels in the general public. Annals of Clinical Biochemistry (2015) **52** (4) (Abstracts from the ACB National Meeting 2015, Thursday)

National Meeting of the Association for Clinical Biochemistry – Focus 2016

**Shea, R.L.** and Berg, J.D. Supplementing our children with vitamin D: how easy is it to over-D it? Annals of Clinical Biochemistry (2016) **53** (3) (Abstracts from the ACB National Meeting 2016, Wednesday)

**Shea, R.L.** and Berg, J.D. Effect of size and location of punch on dried blood spot CRP analysis. Annals of Clinical Biochemistry (2016) **53** (3) (Abstracts from the ACB National Meeting 2016, Wednesday)

**Shea, R.L.** and Berg, J.D. Size and location matters for dried blood spot 25-hydroxyvitamin D analysis. Annals of Clinical Biochemistry (2016) **53** (3) (Abstracts from the ACB National Meeting 2016, Wednesday)

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