# Development and validation of an LC-MS/MS Intact C-peptide method and a protocol for testing Dry Blood Spot samples for the diagnosis and management of Diabetes Mellitus.

A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Professional Doctorate in Biomedical Science

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**Abstract**

C-peptide, which is produced in the pancreatic β-cells, is widely accepted as a surrogate marker for insulin secretion and estimation assist in the diagnosis and management of Diabetes Mellitus. Currently, C-peptide is predominantly measured by immunoassay methods, which have a number of potential limitations such as interferences from biotin, proinsulin, and heterophilic antibodies. We have developed and validated an LC-MS/MS intact C-peptide method for serum and plasma samples that overcomes these analytical limitations. We have also developed a new approach for using dry blood spot (DBS) as an alternative sample type.

Assay calibrants and in-house internal quality control were prepared using Cerilliant C-peptide certified reference material spiked into charcoal stripped bovine serum. The internal standard used was (Tyr$^0$)-C-Peptide. Sample preparation was based on simple protein precipitation followed by solid phase extraction using the Oasis HLB µElution plate. LC separation was done by the Waters Acquity UPLC system.

The method was first validated for serum and plasma samples by the evaluation of accuracy, precision, recovery, carry over, sensitivity and interferences. DBS was verified against the serum method for systematic differences by method comparison studies and an evaluation of imprecision at medical decision points following CLSI guidance EP35.

The assay calibration curve for serum and plasma samples was linear, showing $R^2$ of >0.99, with a measurement range of 3.9 pmol/L to 8000 pmol/L. The within batch and between batch imprecision was <10%. Sample recovery was between 101.9% and 108.4% and carryover was <1%. There was no cross-reactivity with proinsulin or insulin, concentrations assessed was up to 5000 pmol/L and 100,000 pmol/L respectively. The Limit of Blank, Limit of Detection and Limit of Quantification were 0.0474 pmol/L, 0.08295 pmol/L, and 0.227 pmol/L respectively. There was no significant matrix effects. Measurement trueness using EQA samples, Z-scores were all within +/-2. Patient sample comparison $R^2$ was >0.99. DBS imprecision were all below the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation allowable limits of

8.3%. The systematic difference of DBS was -15.7%, -17.3% and -9.6% at 0 – 300pmol/L, 300-700pmol/L, and 700-1800pmo/L clinical decision target ranges respectively.

This intact C-peptide LC-MS/MS assay is sensitive with a low serum and plasma sample volume of 50 µL. DBS sampling and analytical workflow is convenient, and can support large scale clinical trials.

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**Glossary of Abbreviations**

| | |
|---|---|
| ACB | Association for Laboratory Medicine |
| ACN | Acetonitrile |
| ALTM | All Laboratory Trimmed Mean |
| APCI | Atmospheric pressure chemical ionisation source |
| ATP | Adenosine triphosphate |
| ATPase | Adenosine triphosphatase |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| BSPS | Berkshire and Surrey Pathology Services |
| CAP | College of American Pathology |
| CLSI | Clinical and Laboratory Standards Institute |
| C-peptide | Connecting peptide |
| CPE | Carboxypeptidase |
| CRM | Certified reference material |
| CV | Coefficient of variation |
| DBS | Dry blood spot |
| DKA | Diabetic ketoacidosis |
| DMSO | Dimethylsulfoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| Elisa | Enzyme-linked immunosorbent assay |
| ELSA | Early Surveillance for Autoimmune diabetes |
| EQA | External Quality Assurance |
| ESI | Electrospray spray ionisation |
| FTA | Flinders Technology Associates |
| GAD | Anti-glutamic acid decarboxylase |
| GST | Glucagon stimulation test |

| | |
|---|---|
| HAAA | Human anti-animal antibodies |
| HAMA | Human anti-mouse antibodies |
| HbA1c | Haemoglobin A1c |
| HPLC | High performance Liquid chromatography |
| ID-MS | Isotope-dilution mass spectrometry |
| IGF1 | Insulin growth factor I |
| IGFII | Insulin growth factor II |
| IQC | Internal quality control |
| JCTLM | The Joint Committee for Traceability in Laboratory Medicine |
| LADA | Latent autoimmune diabetes in adults |
| LCMS | Liquid chromatography mass spectrometry |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LLE | Liquid liquid extraction |
| LLOQ | Lower limit of quantification |
| LOB | Limit of blank |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LSG | Laparoscopic sleeve gastrectomy |
| m/z | Mass-over-charge ratio |
| MALDI | Matrix-assisted laser desorption ionisation |
| MLTM | Method Laboratory Trimmed Mean |
| MOYD | Maturity-onset diabetes of the young |
| MRM | Multiple reaction monitoring |
| mRNA | Messenger ribonucliec acid |
| MTT | Mixed meal tolerance test |
| NaCl | Sodium chloride |

| | |
|---|---|
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NBS | National Blood Service |
| NEQAS | National External Quality Assurance Scheme |
| NICE | National Institute of Clinical Excellence |
| NIPHS | Noninsulinoma pancreatogenous hypoglycemia syndrome |
| NMIJ | National Metrology Institute of Japan |
| OGTT | Oral glucose tolerance test |
| PBS | Phosphate buffered saline |
| PC | Prohormone convertase |
| PDIs | Protein disulfide isomerases |
| PEG | Polyethylene glycol |
| PGBH | Post-gastric bypass hypoglycemia |
| pI | Isoelectric point |
| PPT | Protein precipitation |
| $R^2$ | Coefficient of determination |
| RT | Retention time |
| SD | Standard deviation |
| SGs | Secretory granules |
| SPE | Solid phase extraction |
| SRM | Selective reaction monitoring |
| SST | System suitability Test |
| TFA | Trifluoroacetic acid |
| TFE | Trifluoroethylene acid |
| TOF | Time-of-flight |
| UKAS | United Kingdom Accreditation Service |
| UKPDS | United Kingdom prospective diabetes study |

| | |
|---|---|
| UPLC | Ultra performance liquid chromatography |
| WHO | World Health Organisation |

# Chapter 1.0 Research Background

## 1.1 Introduction

Measurement of C-peptide under standardised conditions provides a sensitive, well-accepted measure of insulin secretion for the evaluation of hyperinsulinemia, hypoglycaemia, hepatic insulin clearance, and pancreatic beta-cell (β-cell) function (Jones and Hattersley, 2013). Current diabetic research, facilitated by clinical trials to develop therapies to improve endogenous insulin, are designed with improvements in β-cell function as the main efficacy outcome. Several studies have confirmed that even the modest treatment effects on β-cell function in patients with type 1 diabetes mellitus (DM) are known to result in improved glycaemic control and reduced complications such as retinopathy and nephropathy (Basile *et al.,* 2022). In type 2 diabetes, changes in treatment requirements with time mainly relate to the degree of insulin resistance or the progressive loss of β-cell capacity at the later stages of the disease.

Despite this significant importance, measurement of C-peptide to assess β-cell function in the clinical care of diabetic patients is not frequent and with varied approach between countries. In fact, in the United Kingdom (UK), the National Institute of Clinical Excellence (NICE) only recommends that C-peptide be measured if there is difficulty distinguishing type 1 diabetes from other types of diabetes but not at initial presentation (NICE, 2017). Whereas in Scotland, C-peptide measurement is carried out in all type 1 diabetic patients 3 years after diagnosis.

The limited use of C-peptide may partly be due to the high cost of the test, limitations in sampling techniques that require special handling, and the reliability of test methods. In the UK, C-peptide is measured by immunoassay methods which are not highly sensitive assays, having just the necessary level of sensitivity and specificity for routine classification of Diabetes. Accurate C-peptide levels below 2.5 pmol/L are of interest in β-cell assessments which is hard to accurately achieve with most immunoassays due to interferences. (Venugopal *et al.*, 2023). Immunoassays are significantly affected by interference from heterophilic antibodies and proinsulin cross reactivity (Huynh, 2020). Currently, the lack of

traceability to a reference method causes the different immunoassay methods to have varied within-laboratory imprecision, and this makes C-peptide results lack predictive value (Little *et al.,* 2017).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an attractive approach for the analysis of C-peptide because the platform is sensitive and specific and can avoid certain limitations of immunoassays (Thomas *et al.,* 2020). Previously described LC-MS/MS methods for the quantification of C-peptide mostly relied on immunoaffinity enrichment, chemical modifications and enzymatic digestions (Thevis *et al.,* 2005; Taylor *et al.,* 2012; Owusu *et al.,* 2021). These approaches can be limited in terms of speed, sensitivity, and interference from autoantibodies. Current conventional sampling techniques are invasive and require special handling (Lei and Prow, 2019).

The measurement of intact C-peptide by LC-MS/MS using dry blood spots has the potential to make the assessment of C-peptide more reliable, have a better turnaround time, be less expensive, and be widely available to patients even from home. This is important at a time when there is an unprecedented rise in the prevalence of diabetes, especially in younger patients in the UK, which requires specific management and the development of relevant new therapies in clinical practice.

## 1.2 Overview of diabetes mellitus

Diabetes is a serious, chronic metabolic disease that impairs the body's ability to regulate blood glucose levels. This can either be a result of impaired insulin production or the inability of the body to respond to the insulin secreted. Health complications associated with poorly controlled diabetes include nerve damage, heart disease, chronic kidney failure, retinopathy, and other problems with feet, oral health, vision, hearing, and mental health (Marchetti *et al*., 2017).

Diabetes is a global health concern, with the prevalence rising from 108 million in the 1980s to about 422 million as of 2014, as shown in Table 1.1 below (Lancet, 2016; World Health Organisation, 2023). This is expected to continue to increase

to 693 million by 2045 (Cho *et al*., 2018). Statistics indicate that in 2017, over 3.6 million adults were diagnosed with diabetes in the United Kingdom (UK) alone (NICE, 2017). The total cost of diabetes care is estimated to account for up to 10% of the total NHS expenditure (NICE, 2017). The global healthcare expenditure linked directly to diabetes was estimated to be $850 billion in 2017 (Cho *et al.,* 2018).

Table 1. 1 World Health Organisation prevalence of diabetes by region.

| Region | Total Population (1980) | Prevalence (1980) | Total Population (2014) | Prevalence (2014) |
|---|---|---|---|---|
| **European** | 693,400,000 | 33,000,000 (4.5%) | 506,900,000 | 64,000,000 (11.2%) |
| **Americas** | 226,500,000 | 18,000,000 (7.4%) | 317,000,000 | 62,000,000 (16.4%) |
| **Eastern Mediterranean** | 107,200,000 | 6,000,000 (5.3%) | 214,600,000 | 43,000,000 (16.7%) |
| **South-East Asia** | 349,100,00 | 17,000,000 (4.6%) | 613,600,000 | 96,000,000 (13.5%) |
| **Western Pacific** | 1,128,700,000 | 29,000,000 (2.5%) | 1,554,500,000 | 131,000,000 (7.8%) |
| **African** | 481,500,000 | 4,000,000 (0.8%) | 1,170,300,000 | 25,000,000 (2.1%) |

### 1.2.1 The pathogenesis of diabetes mellitus

The hormone insulin plays a key role in maintaining circulating glucose levels within the physiologic range (Marchetti *et al*., 2017; Poitout *et al*., 2015). Higher glucose concentration following a meal (hyperglycaemia) is the main trigger for insulin secretion. Glucose is transported into the pancreatic β-cells through the glucose transporter GLUT1 and metabolised to pyruvate in the cytoplasm by phosphorylation and glycolysis (Dean and Matthews, 1968; Mitsuhisa *et al*., 2013).

Pyruvate is metabolised equally by two enzymes in the β-cells. The first enzyme is pyruvate dehydrogenase which converts pyruvate into adenosine triphosphate (ATP). The second is pyruvate carboxylase which leads to the generation of

oxaloacetate, an intermediate in the tricarboxylic acid cycle (Dean and Matthews, 1968; Mitsuhisa *et al*., 2013). ATP is a signalling molecule for insulin secretion in β-cells (Figure 1.1). Insulin targets cells such as skeletal muscle cells and adipocytes, to stimulate glucose uptake and hepatocytes to promote glycogen synthesis and storage (Suckale and Solimena, 2010; Weiss *et al*., 2014). Through these concerted actions, blood glucose is lowered to fasting levels.

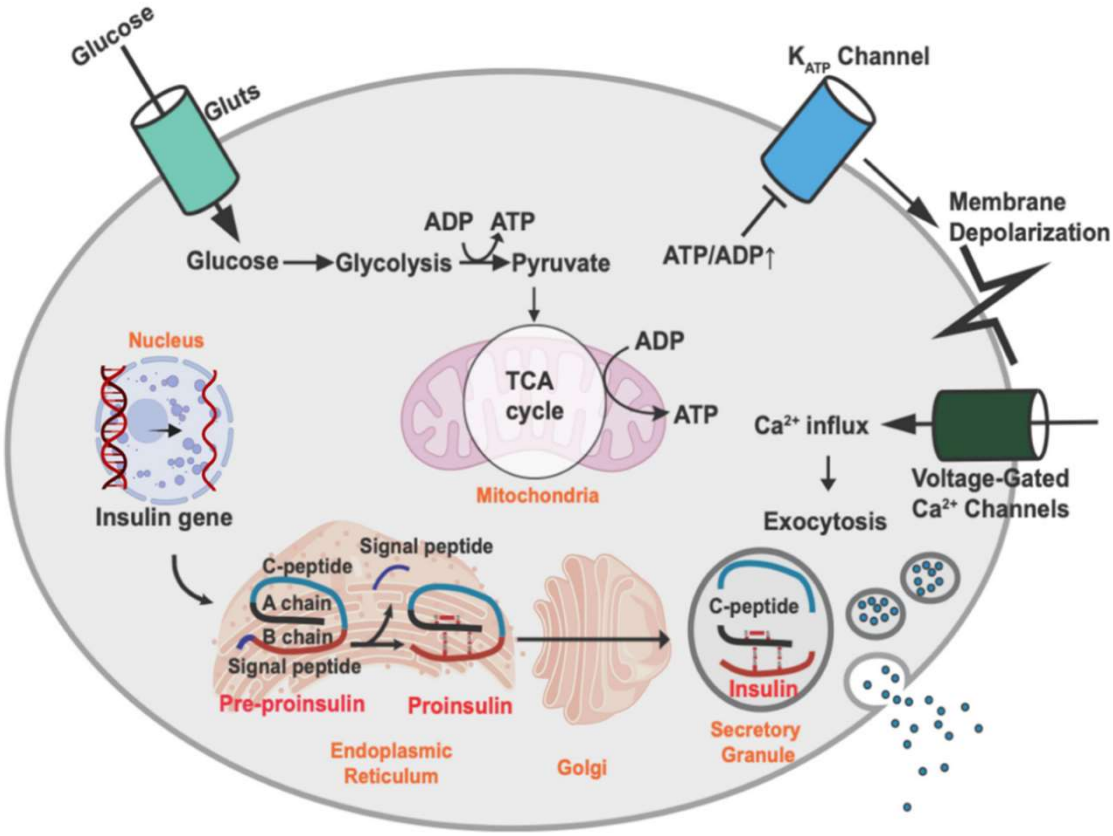



Figure 1. 1 ATP signals insulin secretion from β-cells into circulation.
The pancreatic β-cells are equipped with ATP-sensitive $K^+$ channels, which close when the cytoplasmic ATP or ATP/adenosine diphosphate ratio increases. This causes membrane depolarisation, resulting in the opening of L-type voltage-dependent calcium ion channels, followed by the influx of extracellular calcium ions into the β cells. The elevation of calcium ions induces the exocytosis of insulin (Lee and Lee, 2022).

### 1.2.2 Classification of diabetes

Most cases of diabetes can be categorised as either type 1 or type 2 diabetes. However, there are many fewer common aetiologies, such as monogenic diabetes (neonatal diabetes and maturity-onset *diabetes* of the young (MOYD)) gestational, and latent autoimmune diabetes in adults (LADA) (American Diabetes Association, 2017).

### 1.2.3 Type 1 diabetes

Type 1 diabetes can occur as an inherited polygenic disease characterised by absolute insulin deficiency. A combination of metabolic, environmental, and genetic risk factors acts together to promote the development of chronic autoimmune reactions to multiple autoantigens expressed by pancreatic β-cells. Over time, chronic autoimmunity leads to severe β-cell loss and, in turn, severe insulin deficiency (Eisenbarth, 1986; DiMeglio *et al*., 2018). Established features of type 1 diabetes include positivity for islet-specific autoantibodies, GAD autoantibody, insulinoma-associated antigen-2 autoantibody/Tyrosine phosphatase antibodies, and zinc transporter 8 autoantibody (Bingley, 2010).

Type 1 diabetes accounts for most of the diabetes in childhood (Hamman *et al*., 2014). Huopio *et al*., (2016) studied diabetes diagnosed below 12 months of age in Finland and demonstrated that type 1 diabetes was more likely in individuals diagnosed at 6 months of age and above, whereas monogenic diabetes was more likely in those diagnosed at less than 6 months of age (Huopio *et al*., 2016; Johnson *et al*., 2020). Type 1 diabetes accounts for about 10% of all cases of diabetes and 85% of all diabetes cases in youth individuals less than 20 years of age with an estimated annual increase of 3% worldwide (Kalliora *et al*., 2011; Chowdhury, 2015). It occurs most commonly in people of European descent and affects two million people in Europe and North America. The UK currently has the fifth highest rate of type 1 diabetes diagnosis in children in the world, with 24.5 per 100,000 children aged 0 to 14 diagnosed each year (Candler *et al*., 2018). There are currently about 31,500 children and young people with diabetes in the UK, under the age of 19 (Candler *et al*., 2018). The incidence of type 1 diabetes in

adults is lower than in children, although approximately 25% of people with type 1 diabetes are diagnosed as adults (Chiang *et al*., 2014; Lado and Lipman, 2016).

### 1.2.4 Type 2 diabetes

Type 2 diabetes on the other hand is caused by a combination of insulin resistance in target organs and an inadequate compensatory insulin secretory response due to the dysfunction of the pancreatic β-cell (Chatterjee *et al.,* 2017). Type 2 diabetes occurs in a two-step process in which insulin-resistant, normal glucose-tolerant individuals progress to prediabetes (impaired glucose tolerance and impaired fasting glucose), and then overt type 2 diabetes becomes established when the compensatory increase in insulin secretion is no longer sufficient to offset the underlying insulin resistance (Lyssenko *et al*., 2005; Kahn *et al*., 2014). Type 2 diabetes is associated with reduced quality of life and an increased risk of serious complications, such as blindness, kidney failure, and cardiovascular disease (Kanat *et al*., 2015). The life expectancy of individuals with type 2 diabetes may be shortened by as much as 10 years, with most dying of cardiovascular disease (Roper *et al*., 2001). It is estimated that approximately 90% of all adults with diabetes have type 2 diabetes, and there are a total of about 12.3 million people at risk of type 2 diabetes in the UK (NICE, 2017; International Diabetes Federation, 2015). The management of type 2 diabetes consumes around 10-12% of global healthcare expenditure, which is also expected to rise to 17% by 2036 (Hex *et al*., 2012; International Diabetes Federation, 2015).

Another less common type of diabetes with clinical pathogenesis, like type 2 diabetes, is gestational diabetes. This affects approximately 18% of pregnancies and usually disappears soon after delivery, but carries a significant risk if undiagnosed and untreated (Malamed and Or, 2015).

### 1.2.5 Latent autoimmune diabetes in adults

LADA is a subgroup of diabetes that develops in later life and shares genetic, metabolic, and immunologic features with both type 1 and type 2 diabetes (Leslie

*et al*., 2006). The three criteria conventionally used to describe LADA are age at diagnosis (greater than 35 years), autoantibody positivity to islet β-cells, and insulin independence for at least the first 6 months after initial diagnosis (Patterson *et al*., 2009; Rajkumar and Levine, 2022). There are geographic and ethnic differences in its incidence. In the United Kingdom Prospective Diabetes Study (UKPDS), the antibody positivity among those with a presumptive diagnosis of type 2 diabetes in adults was 12%. Similarly, studies from Norway showed a 10% incidence, whereas studies from the Middle East, Korea, and China showed between 4% and 6% (Rajkumar and Levine, 2022). LADA must be diagnosed in the early stages to ensure appropriate intervention to delay β-cell destruction and reduce complications.

### 1.2.6 Monogenic diabetes

Monogenic diabetes is an inherited form of diabetes that often develops before the age of 25 and is caused by single gene variants. Monogenic diabetes is associated with several different phenotypes; however, the two main types are MODY and neonatal diabetes (Shields *et al*., 2017). Clinical features of MODY overlap with those of type 1 diabetes, such as young onset and leanness, and those with type 2, such as preserved β cell function and family history. This has frequently led to its misdiagnosis (Zhang *et al*., 2021). It is, however, critical to identify individuals with MODY to guide treatment decisions, give patients information about prognosis, and define risk to family members. In the UK, it is estimated that the proportion of MODY is 3.6% of adults or children with diabetes diagnosed before age 30 (Shields *et al*., 2017).

Neonatal diabetes can be transient or permanent, is diagnosed in the first 6 months of life, and is, therefore, easier to diagnose when compared to MODY. The incidence of neonatal diabetes is around 1 in 100,000 live births (Iafusco *et al*., 2012). In recent years, the classification of monogenic diabetes has evolved into one based on genetic screening, which has improved diagnosis and management.

## 1.3 The structure and synthesis of human proinsulin and C-peptide

Connecting peptide (C-peptide) is a useful biomarker and an essential tool in the treatment and management of diabetes. It is synthesised in the β-cell of the pancreas at the same time as insulin (a peptide hormone key for glucose homeostasis, metabolism, and cell growth) (Tsiolaki *et al*., 2016). The biosynthesis of insulin and C-peptide begins within the cytosol, where insulin gene transcription is stimulated and mRNA is translated into the precursor preproinsulin (Poitout *et al*., 2006; Fu *et al*., 2013).

Preproinsulin (Figure 1.2) has a 110-amino acid sequence and consists of a N-terminal signal peptide, the B chain, the C-peptide, and the C-terminal A chain (Vasiljević *et al*., 2020). Preproinsulin is delivered by the signal peptide into the secretory pathway and translocated to the luminal side of the endoplasmic reticulum (ER) (Wolin and Walter, 1993; Huan *et al*., 2014). In the endoplasmic reticulum, the signal peptide is rapidly cleaved by signal peptidases, converting preproinsulin into proinsulin.

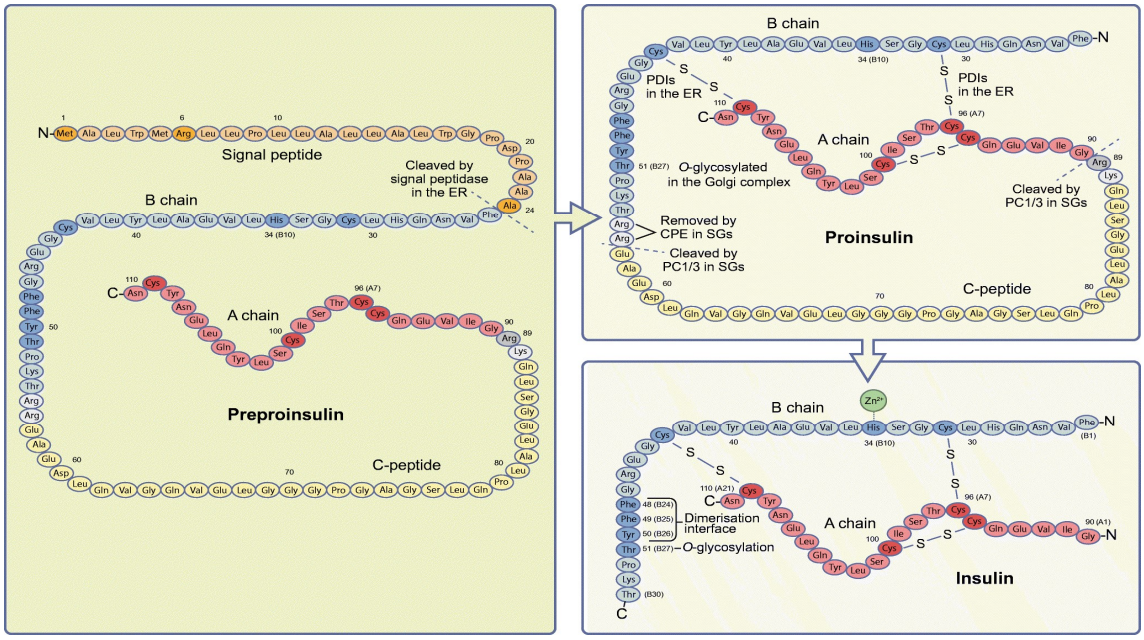



Figure 1. 2 Insulin and c-peptide production pathways

Preproinsulin is the first precursor and consists of a signal peptide (orange), a B chain (blue), a connecting peptide (C-peptide, yellow), and an A chain (red). Proinsulin is formed when the signal peptide is cleaved by the signal peptidase. (Vasiljević *et al*., 2020).

The role of the C-peptide chain in proinsulin is to ensure the correct alignment of A and B insulin chains through the formation of disulphide bonds between cysteine residues with the help of protein disulfide isomerases (PDIs), thus facilitating the correct folding of insulin (Tsiolaki *et al*., 2016). Intact proinsulin is transported out of the endoplasmic reticulum into secretory granules at the *trans*-Golgi network (Arvan and Halban, 2004). Endopeptidase cleavage of proinsulin can generate either 32, 33 split or 65, 66 split proinsulin conversion intermediates. Carboxypeptidase H rapidly cleaves these proinsulin intermediates to generate the more commonly seen processing intermediates des-31, 32 and des-64, 65 proinsulin (Rhodes *et al*., 1992). In the β-cell secretory granules (SGs), the enzymes prohormone convertases (PC) 1/3 and carboxypeptidase (CPE) H cleave the C-peptide chain and remove the residual C-terminal basic residues to generate the 5.8-kDalton mature insulin and 3.0 kDalton C-peptide molecules ready for secretion into circulation (Yang *et al*., 2010; Fu *et al*., 2013; Tsiolaki *et al*., 2016; Liu *et al*., 2018). Under normal circumstances only trace amounts of intact proinsulin or split proinsulin survive to be released into peripheral blood. In insulin resistance, this process becomes less efficient and increasing proportions of intact and split proinsulin appear in peripheral blood and cause assay cross-reactivity and account for some of differences between assays.

### 1.3.1 C-peptide as a surrogate marker of insulin secretion

The main goal in the treatment and management of diabetes is to prevent acute, potentially fatal diabetic complications such as retinopathy, neuropathy, kidney disease, cardiovascular disease and/or delay their progression if they occur. These chronic complications can impact the quality of life and are costly over time to manage. The correct type of diabetes must be established because different forms may be managed in different ways. Insulin therapy is a key element for the treatment of most forms, but an incorrect diagnosis can result in years of unnecessary insulin therapy (Brunton *et al*., 2005). In clinical practice, the distinction between the different types of diabetes is not always straightforward due to significant overlap between clinical features (Naik *et al*., 2009).

For example, type 1 and type 2 diabetes share common symptoms (weight loss, frequent urination, erection problems, itching, gum problems, and gum numbness)

(Diabetes UK, 2024). The only difference between the two is that the symptoms of type 1 diabetes often appear quickly but slowly in type 2 diabetes highlighting the need for biomarkers to aid classification.

In the differential diagnosis of diabetes, measurements of insulin levels to assess beta cell function is required. Measuring insulin in peripheral blood is challenging due to the pulsatile nature of its secretion and its short half-life in the circulation (Anoop, 2019; Misra *et al*., 2019). Insulin secreted from the pancreas passes through the liver, where about 80% is largely metabolised and eliminated. Only smaller levels eventually get into the systemic circulation (Leighton *et al*., 2017; Brands *et al*., 2013). Insulin also has a short half-life of about 3 to 5 minutes in plasma, so to monitor fluctuations in circulating levels, several samples would have to be taken (Larsen *et al*., 2017). Also, insulin-treated patients with diabetes typically have autoantibodies in the circulation, which can cause a cross-reaction when measurements are carried out using immunoassay or immunoaffinity LCMS methods (Leighton *et al*., 2017). Pre-analytical issues such as sample stability and the effect of haemolysis on the quantification of insulin also limit its widespread use. Furthermore, the establishment and implementation of traceable reference intervals for insulin is also challenging (Larsen *et al*., 2017) and is method dependent.

In contrast, C-peptide is widely accepted as a surrogate marker for insulin secretion and plays a significant role in the classification of diabetes (Palmer *et al*., 2004; Jones and Hattersley, 2013). The measurement of C-peptide overcomes a number of these limitations. Unlike insulin, C-peptide is metabolised and excreted by the kidney with a much longer half-life of about 30 minutes (De León *et al*., 2013; Katz *et al*., 1973; Jones and Hattersley, 2013). This makes C-peptide a more stable test window for fluctuating β-cell response and thus the best marker to estimate the function of the β-cell (Richards *et al*., 2013). Another advantage is that the production of C-peptide continues and remains functionally active for years after the onset of diabetes (Leighton *et al.,* 2017). Circulating C-peptide concentrations are also approximately 5 to 10-fold higher than insulin in venous blood (fasting). Fasting C-peptide can, therefore, be used to derive an estimate of insulin resistance, and high fasting C-peptide in the presence of hyperglycaemia may be suggestive of insulin resistance.

The limitation of C-peptide is that raised concentrations may occur in individuals with renal impairment, making random serum measurements inaccurate (Jones and Hattersley, 2013), but not in the case of mixed meal tests, oral glucose tolerance tests, or glucagon stimulation tests. C-peptide standardisation and harmonisation processes are far more advanced in comparison to insulin (Bonser and Garcia-Webb, 1984; Little *et al*., 2017b). This enables reliable and correct decisions concerning diabetes diagnosis (Leighton *et al*., 2017) and therapy, thereby directly increasing the patient's safety.

### 1.3.2 The Role of c-peptide in the classification of diabetes

C-peptide levels provide vital information on β-cell capacity, which determines the differences in glycaemic treatment requirements between type 1 and type 2 diabetes. Not only is C-peptide used in differentiating type 1 and type 2 diabetes, but it can also be used to aid diagnose of MODY (NICE, 2015). It is particularly important to identify MODY patients, as they are commonly misdiagnosed as having type 1 diabetes and treated with insulin instead of oral hypoglycaemic drugs such as sulphonylurea. Where diabetes is classified purely based on the presence or absence of autoantibodies, C-peptide remains a relatively good predictor with better performance than either age of diagnosis or BMI (Torn and Schersten 2001; Thunander *et al*., 2012).

In type 1 diabetes, insulin levels rapidly fall; therefore, the utility of C-peptide testing increases from 3 to 5 years post-diagnosis, where most patients with type 1 diabetes will have low C-peptide levels (Palmer *et al*., 2004; Besser *et al*., 2011). C-peptide levels taken within the first few years of diagnosis may be useful in confirming type 1 diabetes if results are low (non-fasting blood C-peptide < 0.2 nmol/L with hyperglycaemia confirms severe insulin deficiency; if C-peptide < 0.6 nmol/L, then type 1 diabetes is likely) (NICE, 2015). Individuals with low concentrations of C-peptide have a deficiency of endogenous insulin and, therefore, usually a total dependence on insulin preparations.

Substantial C-peptide secretion is consistent within an insulin-resistant state and a diagnosis of type 2 diabetes, although some individuals with type 2 diabetes have a degree of insulin deficiency as shown in Figure 1.3 below. In type 2

diabetes, changes in treatment requirements with time primarily relate to progressive loss of insulin secretion capacity (Oh *et al*., 2018; American Diabetes Association, 2017; Jones and Hattersley, 2013).

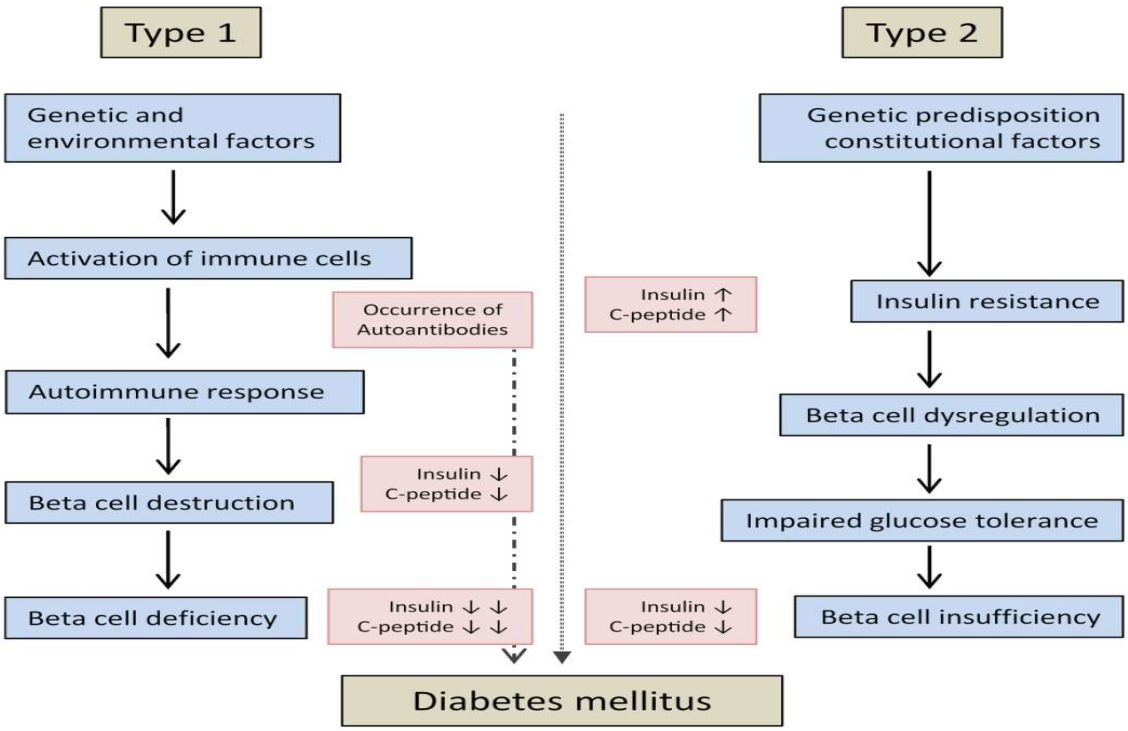



Figure 1. 3 The development of type 1 and type 2 diabetes.

In type 1 diabetes, the activation of immune cells leads to an autoimmune response and subsequent destruction of the β-cell, accompanied by insulin and C-peptide deficiency. In type 2 diabetes, there is insulin resistance accompanied by elevated insulin and C-peptide concentrations in the early stages, which progresses to β-cell insufficiency, leading to reduced insulin and C-peptide secretion and plasma concentrations (Hörber *et al.*, 2020).

### 1.3.3 Practical challenges in the diagnosis and classification of diabetes

Despite the wildly accepted role C-peptide plays in the diagnosis of diabetes, it is not widely adopted in practice. In children, the recommendation is to confirm type 1 diabetes using the World Health Organisation's (WHO) plasma glucose criteria and assume type 1 diabetes unless there are strong indications of type 2 diabetes. Diabetes symptoms in children can, however, be difficult to spot, and a study of

children aged 8 months to 16 years who had been diagnosed with type 1 diabetes found that by the time children under the age of 2 years received their diagnosis, about 80% of them had already developed diabetic ketoacidosis (DKA) (Usher-Smith *et al.*, 2012). In the UK, in an attempt to prevent the condition presenting as DKA, there is an ongoing Early Surveillance for Autoimmune diabetes (ELSA) screening study for an initial 20,000 children between 3-13 years to provide insight which could help lead to widespread type 1 diabetes screening (Diabetes UK, 2024).

Another study found that 16% of cases of new-onset type 1 diabetes among 335 children under the age of 17 had an initial incorrect diagnosis. Out of these, about 46.3% were diagnosed with respiratory tract infection, 16.6% with perineal candidiasis, 16.6% with gastroenteritis, 11.1% urinary tract infection, 11.1% stomatitis and 3.7% appendicitis (Pawlowicz *et al.,* 2008).
In the UK, C-peptide is currently predominantly measured in children if there is difficulty distinguishing type 1 diabetes from other types of diabetes, particularly MODY, but not at initial presentation because it is assumed that C-peptide concentrations have a better discriminative value the longer the interval between initial presentation and the test (NICE, 2015). A random blood C-peptide of ≥ 0.2 nmol/l in those with diabetes diagnosed under 30 years of age and > 3 years' duration has been suggested as a criterion for consideration of MODY (Thanabalasingham *et al*., 2012).

### 1.3.4 Investigations into non-diabetic associated hypoglycaemia

One of the complications of diabetes management is the development of hypoglycaemia due to insulin or oral anti-diabetic agents. However, hypoglycaemia can arise from other causes. C-peptide is a surrogate marker for insulin secretion and is also used as a biological marker in investigations into non-diabetic associated hypoglycaemia and in forensic studies to differentiate between an exogenous and endogenous source of insulin (Wallace *et al.,* 2004; Leighton *et al*., 2017; LiRalf *et al.,* 2018). The basis of the investigation is that both insulin and C-peptide levels will be raised if produced endogenously e.g. from an

insulinoma or sulphonylurea drug ingestion (Rui *et al.,* 2019; Klein-Schwartz *et al*., 2016).

To investigate the cause of non-diabetic associated hypoglycaemia, the Endocrine Society recommends the measurement of proinsulin, C-peptide and insulin. Endogenous hyperinsulinemia is characterised by the presence of hypoglycaemia with a raised insulin level of ≥18 pmol/L, C-peptide ≥200 pmol/L, and proinsulin concentrations of ≥5.0 pmol/L. The most common cause of endogenous hyperinsulinemia is insulinoma. Other causes include sulfonylurea-induced hypoglycaemia, non-insulinoma pancreatogenous hypoglycaemia syndrome, non-islet cell insulin-secreting tumours, and autoimmune insulin syndrome (Cryer *et al*., 2009; Endocrine Society, 2023).

In factitious hypoglycaemia (exogenous administration of insulin) there is a raised insulin level but low C-peptide and proinsulin concentrations. This could be self-harm, criminal intent, malicious, or self-administration for secondary gain (Rui et al., 2019; Schwartz *et al*., 2016). In very rare cases, hypoglycaemia can be mediated by insulin receptor antibodies, resulting in raised insulin levels but suppressed C-peptide and proinsulin concentrations. There are other causes of hypoglycaemia where insulin, C-peptide and proinsulin concentrations are all suppressed, but most of these have very distinctive clinical features, making diagnosis straightforward. Others require additional tests such as growth hormone, β-hydroxybutyrate, insulin-like growth factor 1, and insulin-like growth factor 2, as well as interpretation by a specialist (Table 1.2) (Labib, 2014).

Table 1. 2 The Endocrine society guide for hypoglycaemia investigation.
First, glucose, C-peptide, insulin, proinsulin, β-hydroxybutyrate, and circulating oral hypoglycaemic agents are measured. The patient is then injected with 1.0 mg of glucagon intravenously to correct the hypoglycaemia and then the glucose response is measured. This result can be used to distinguish endogenous and exogenous hyperinsulinism in seemingly healthy patients, using the table as a guide. (Endocrine Society, 2023)

| Symptoms, signs, or both | Glucose (mg/dl) | Insulin (μU/ml) | C-peptide (nmol/L) | Proinsulin (pmol/L) | ß-Hydroxybutyrate (mmol/L) | Glucose after glucagon (mg/dl) | Circulating oral hypoglycemic agent | Antibody to insulin | Diagnostic interpretation |
|---|---|---|---|---|---|---|---|---|---|
| No | <55 | <3 | <0.2 | <5 | >2.7 | <25 | No | No | Normal |
| Yes | <55 | ≫3 | <0.2 | <5 | ≤2.7 | >25 | No | Neg (Pos) | Exogenous insulin |
| Yes | <55 | ≥3 | ≥0.2 | ≥5 | ≤2.7 | >25 | No | Neg | Insulinoma, NIPHS, PGBH |
| Yes | <55 | ≥3 | ≥0.2 | ≥5 | ≤2.7 | >25 | Yes | Neg | Oral hypoglycemic agent |
| Yes | <55 | ≫3 | ≫0.2a | ≫5a | ≤2.7 | >25 | No | Pos | Insulin autoimmune |
| Yes | <55 | <3 | <0.2 | <5 | ≤2.7 | >25 | No | Neg | IGFb |
| Yes | <55 | <3 | <0.2 | <5 | >2.7 | <25 | No | Neg | Not insulin (or IGF)-mediated |
| Neg, Negative; Pos, positive; PGBH, post gastric bypass hypoglycemia. | | | | | | | | | |
| a | Free C-peptide and proinsulin concentrations are low. | | | | | | | | |
| b | Increased pro-IGF-II, free IGF-II, IGF-II/IGF-I ratio. | | | | | | | | |

Most of the hypoglycaemic symptoms occur when the patient is at home which is why the use of DBS samples rather than visiting a clinic will be beneficial, speeding up diagnosis. The patient will not have to be in hospital for stimulated test such as glucose tolerance, thereby reducing patient inconvenience and also lowering healthcare costs.

### 1.3.5 Pancreatic and renal transplant suitability and monitoring

The only available clinical approach to restoring β-cell mass in patients with diabetes is the transplantation of β-cells (i.e., pancreas or islet transplantation). The goals of β-cell replacement are to restore glucose-regulated endogenous insulin secretion with normalisation of glucose levels, arrest the progression of the complications of diabetes, and improve quality of life. The number of pancreatic donors is limited. There is therefore a need to screen and select patients who will

respond to immunosuppression therapy to prevent graft rejection and recurrent β-cell autoimmunity. Most patients who undergo pancreas or islet transplantation are those with type 1 diabetes with complications such as recurrent severe hypoglycaemia or renal failure (Hering *et al*., 2016; Concepcion *et al*., 2020; Bellin and Dunn, 2020). Post-pancreatectomy diabetes and cystic fibrosis-related diabetes patients are sometimes also considered for pancreatic transplants (Usatin *et al*., 2016; Kandaswamy *et al*., 2020; Gruessner and Gruessner, 2016; Hering e*t al*., 2016). The criteria used to select eligible patients in most countries worldwide are that they must be between 18 and 65 years of age with undetectable C-peptide levels. It must have been over five years since the patient was diagnosed with type 1 diabetes and with complications such as severe hypoglycaemia, which is resistant to insulin therapy, and recurrent neuroglycopenia (Warnock *et al*., 2008; Peixoto *et al*., 2016).

In the UK, the general criteria are that the patient must be insulin-dependent for at least 5 years and have a negative C-peptide (<0.16 nmol/l with no increment at 6 min after 1 mg glucagon IV) (NHS England, 2019). C-peptide measurements during patient assessment for transplantation are often used as a baseline to compare post-transplantation results (British Transplantation Society Guidelines, 2019).

In the allogenic setting, both pancreas and islet transplantation require lifelong immunosuppression to prevent rejection of the graft and recurrence of the autoimmune process. Graft survival is defined as C-peptide positivity, rather than insulin independence. Current immunosuppressive regimens can prevent β-cell failure for months to years, but the agents used expose the patient to several side effects and increase the risk for specific malignancies (Geissler, 2015) and opportunistic infections (Helfrich and Ison, 2015).

A renal transplant is used to treat patients with kidney failure caused by diabetic complications and is often done together with a pancreatic transplant. C-peptide can be used to assist in patient selection for transplants based on severity (Ryan, 2005). C-peptide levels are also used to help define graft status following pancreatic and renal transplants. The criteria used in the UK are shown in Table 1.3 below (Barton, 2012).

Table 1. 3 NHS criteria for post pancreatic transplant classification.
The criteria include an assessment at 1, 3, and 12 months after transplantation and annually thereafter. This also includes metabolic monitoring (monitoring of graft function using mixed meal tolerance tests with paired glucose and C-peptide (Choudhary *et al*., 2016)).

| Graft status | New Criteria |
|---|---|
| Functioning | HbA1c < 6% [42 mmol/mol] on no anti-diabetic medication |
| Partial | HbA1c > 6% [42 mmol/mol]<br>OR<br>Requiring medication to maintain HbA1c < 6% C-peptide +ve [or C-peptide > pre-transplant C-peptide] |
| Graft Failure | C-peptide < 50 pmol/l<br>OR<br>C-peptide is lower than pre-test C-peptide [in the case of type 2 diabetes] |

### 1.3.6 Interventions to preserve and improve residual β-cell function.

Since the mid-1970s, C-peptide has been used as a surrogate marker in clinical research to provide insight into the physiology underlying both the clinical course of diabetes over time and determining the effects of interventions designed to preserve and improve residual β-cell function (Steiner *et al*., 1967; Matthews *et al*., 1985; Wahren, 2012; Leighton *et al*., 2017). Available data from research and clinical trials aimed at diabetes remission demonstrate that even relatively modest treatment effects on β -cell function, determined by C-peptide measurement as the primary efficacy outcome, will result in clinically meaningful benefits such as the prevention of diabetic complications such as hypoglycaemia, neuropathy, nephropathy, stroke, retinopathy, and heart attacks (Palmer *et al*., 2001; Lachin *et al*., 2014). It has been noted that adequate control of glucose levels can be achieved in some type 2 diabetic patients by lifestyle changes such as control of diet, weight reduction, exercise, and/or the use of hypoglycaemic agents without the need for insulin (American Diabetes Association, 2009).

Taylor *et al*. (2018) recently carried out a Diabetes Remission Clinical Trial (DiRECT) to assess whether intensive weight management, delivered in the

primary care setting, would achieve a sustained remission of type 2 diabetes. The findings confirmed that weight loss is sufficient to achieve remission of type 2 diabetes for up to 6 years' duration (Taylor *et al*., 2018). The conclusions were based on HbA1c target values but do not provide information on whether the β-cell function of the patients that achieved remission had been restored. Ideally, a series of C-peptide measurements would be an ideal marker to provide this vital information.

In the UK, NICE has issued guidelines for the management of type 2 diabetes in adults, which focus on patient education, dietary advice with a personalised diabetes management plan, including other aspects of lifestyle modification, managing cardiovascular risk, managing blood glucose levels, and identifying and managing long-term complications (NICE, 2015). Apart from diet and lifestyle changes, other interventions, such as laparoscopic sleeve gastrectomy (LSG), have been shown to improve glycaemic control in both diabetic and non-diabetic patients. Lee *et al*. (2010) found that elevated C-peptide is very important in predicting the success of type 2 diabetes remission after LSG in low BMI patients (Lee *et al*., 2010). Elevated C-peptide levels indicated the existence of insulin resistance and a compensated secretion of insulin by well-preserved pancreatic β-cells. Therefore, according to the study, a greater C-peptide level is usually associated with a higher type 2 diabetes remission rate after metabolic surgery (Lee *et al*., 2010; Yan *et al*., 2013). In severely obese patients with type 2 diabetes, bariatric surgery is considered the best treatment due to its effects on sustained weight loss and mortality reduction (Rubino *et al*., 2016; Cummings *et al*., 2016). A study of consecutive morbidly obese patients with type 2 diabetes enrolled in a surgically supervised weight loss programme with at least one year follow-up showed a marked reduction in C-peptide after a significant weight reduction one year after surgery with a type 2 diabetes remission rate of 78%, confirming bariatric surgery as the recommended treatment for obesity-related type 2 diabetes patients with elevated C-peptide (Lee *et al.*, 2010).

Chong *et al*. (2017) found that stimulated C-peptide levels was a predictor of type 2 diabetes remission (Chong *et al*., 2017; Souteiro *et al*., 2017). According to the study, adding Roux-en-Y gastric bypass (RYGB) to lifestyle and medical management was associated with a greater likelihood of remission of type 2

diabetes. Logistic regression revealed stimulated C-peptide (Odds ratio 2.22, $P = 0.02$) as a significant predictor of diabetes remission (Chong *et al*., 2017). A study was carried out to evaluate 100 severely obese patients to identify predictive factors (age, diabetes duration, insulin use, HbA1c, C-peptide plasma level, and basal insulinaemia) associated with diabetes remission after gastric bypass. The retrospective analysis identified only C-peptide level >3 ng/dL (>993.28 pmol/L) as a positive predictor of 3-year postoperative diabetes remission (Cleva *et al*., 2021).

The C-peptide level is a measurement of insulin production and indirectly reflects islet cell mass. Age and diabetes duration are also indirectly related to islet cell involvement in type 2 diabetes. C-peptide level may be a powerful predictor of diabetes remission since it reflects pancreatic reserve independent of age or diabetes duration (Cleva *et al*., 2021). It is important to note that the validity of such inferences from clinical research studies is contingent on the reliability of the measurement of C-peptide using an effective analytical method and a better sampling technique that offers better stability and supports large-scale clinical trials.

### 1.3.7 Antioxidant, anti-inflammatory, and anti-apoptotic effects

C-peptide is known to have an anti-oxidant, anti-inflammatory, and anti-apoptotic role (Wahren and Larsson, 2015). It reduces high glucose-induced reactive oxygen species formation via inhibition of NAD(P)H oxidase; inhibits pro-inflammatory gene activation by downregulating nuclear factor kappa-light-chain enhancement of β-cell activity (Luppi and Drain, 2016); diminishes pro-inflammatory cytokine and chemokine secretion; and reduces the expression of cellular adhesion molecules as well as a vascular endothelial growth factor, transforming growth factor, and plasminogen activator inhibitor-1 (Wahren and Larsson, 2015). Additionally, caspase-3 activity is inhibited, and apoptotic activity is retarded. C-peptide also elicits activation and induction of both endothelial nitric oxide synthase and sodium and potassium ATPase (Wahren and Larsson, 2015;

Luppi and Drain, 2016). Clinical trials investigating the effects of these properties in replacement therapy require several measurements of C-peptide to be made.

## 1.4 C-peptide laboratory sample stability

The widespread clinical application of C-peptide and insulin measurement is limited by practical restrictions associated with sample collection. C-peptide can currently be measured in urine (usually expressed as a urine C-peptide creatinine ratio), or in plasma or serum. Urinary C-peptide can be analysed with either a random urine sample or a 24-hour urine collection. Urinary C-peptide is non-invasive, and the samples are stable in boric acid for up to 3 days at room temperature (Besser *et al*., 2011). Compared to insulin, where only 0.1% is secreted, the amount of urinary C-peptide excreted in urine is reflective of 5 - 10% of the total C-peptide secreted by the pancreas in patients with normal renal function (Gjessing *et al*., 1989; Clare and McDonald, 2013). Previous studies suggest 24-hour urinary C-peptide concentrations accurately assess β-cell secretory capacity and correlate with fasting and stimulated serum insulin and C-peptide. However, 24-hour urine collection is a time-consuming method, cumbersome, often incomplete, and impractical in a busy clinic. Urine C-peptide creatinine ratio is measured on a post prandial sample taken approximately 2 hours after a meal stimulus making it a time-consuming, cumbersome, impractical in a busy clinic, which is not as convenient as a DBS sampling (Kim *et al*., 1986; Elzahar *et al*., 2020). Urinary testing, whether collected as a random or 24-hour collection, has been shown to be far less sensitive and is inaccurate in individuals with any degree of renal impairment (Clark and McDonald, 2013).

Although the most commonly used sample type in clinical practice, serum C-peptide, collected in serum gel or plain sample tubes, has traditionally been thought to be an inconvenient method as immediate laboratory analysis is required before degradation. Many laboratories recommend the immediate transport of C-peptide serum samples to the laboratory on ice, with rapid centrifugation, separation, and frozen storage if the sample cannot be immediately processed (Clark, 1999; Bolner, 2005). It is known that both insulin and C-peptide concentrations rapidly decrease because of protease activity present in the blood

(Palmer *et al*., 2001). C-peptide in whole blood collected in potassium ethylenediaminetetraacetic acid (EDTA) is stable at room temperature for at least 24 hours; in contrast, C-peptide in blood collected into serum gel or plain sample tubes is stable for 6 hours (McDonald *et al*., 2012; Oddoze *et al*., 2012; Ellis, 2003; Jones and Hattersley, 2013). The current sample stability for the measurement of C-peptide in serum at various storage temperatures is method-dependent and is shown in Table 1.4 below.

Table 1. 4 Serum sample storage stability for immunoassay (Mercodia, 2021; Siemens, 2005; Abbott, 2024; Roche 2018)

| Immunoassay method | Serum sample stability |
|---|---|
| Mercodia | can be stored 2-8 °C for 3 days |
| Siemens Centaur | freeze if not assayed within 24 hours |
| Abbott Architect | up to 24 hours at room temperature or 48 hours at 2-8 °C |
| Siemens Immulite 2000 | assay within 2-3 hours or store frozen for 1 week |
| Roche | 8 °C 4 hours at RT, 24 hours at 2 |

Serum sample stability issues also limit C-peptide measurement to healthcare settings that are suitably equipped with on-site laboratories or where immediate centrifugation and freezing of samples are possible.

C-peptide levels can be measured in a random, fasting, or stimulated state (Pozzan *et al*., 1997). Stimulation methods include using glucagon, intravenous or oral glucose, tolbutamide, sulfonylurea, glucose-like peptide 1, amino acids, or a mixed meal (Palmer *et al*., 2004; Greenbaum and Harrison, 2003). Random C-peptide has been shown to correlate with fasting C-peptide and post glucagon stimulation test (GST) samples in subjects with well-defined type 1 or type 2 diabetes (Berger and Stenström, 2000). Similarly, random C-peptide have been shown to correlate with 90 minute mixed meal tolerance test (MMTT) (Hope *et al*., 2016).

The MMTT and GST have been shown to provide a good measure of β-cell function in type 1 diabetes (Greenbaum *et al*., 2008). GST is the most widely used

of the provocation methods. GST is superior in sensitivity compared to the use of glucose or tolbutamide as substrates, with a twofold higher mean increase in C-peptide response (Mirel *et al*., 1980). Further studies have demonstrated that C-peptide levels following GST are a consistently sensitive measure of β-cell function, which may be associated with diabetes type and future use of insulin (Gjessing *et al*., 1989; Greenbaum *et al*., 2008; Madsbad *et al*., 1981; Koskinen *et al*., 1988). In patients with diabetes who were both insulin and non-insulin treated, GST demonstrated a 29% rise in C-peptide compared to a 19% rise postprandially (Koskinen *et al*., 1988; Leighton *et al*., 2017). The MMTT has been advocated as the "gold standard" of stimulation testing owing to its excellent sensitivity in detecting residual insulin secretion (Palmer *et al*., 2001; Greenbaum *et al*., 2008; Hope *et al*., 2016). In the MMTT, a weight-based liquid meal, such as Sustacal or Boost, is ingested over 5 minutes, and timed samples for C-peptide determination can be taken 10 minutes before ingestion (t = −10), at baseline (t = 0), and 15, 30, 60, 90, and 120 minutes.

C-peptide can also be measured across a 75 g oral glucose tolerance test (OGTT) (Breda *et al*., 2001). C-peptide sampling as part of the OGTT has been found to significantly correlate with insulin secretion in type 2 diabetes when samples are taken at 0, 30, 60, 90, and 120 minutes (with the possibility of extending this to include sampling at 150, 180, 240, and 300 minutes) (Okuno *et al*., 2013). Adding C-peptide determination to the protocol of an OGTT may therefore be a practical means of estimating β-cell function when the test is already being performed for diagnostic reasons if further interval sampling is taken between 0 and 120 minutes (Leighton *et al*., 2017).

Although sensitive, the MMTT is labour-intensive and requires admission to a clinical facility for several hours. It is therefore probably not the best test to obtain repeated measures of C-peptide over short periods of time. The GST is preferable owing to the short duration of the test, but still cumbersome and inconvenient to patients (Greenbaum *et al*., 2008).

## 1.5 Analytical methods for the measurement of C-peptide

The correct clinical interpretation of laboratory C-peptide test results is dependent on the accuracy of the measurement procedure and the sample integrity.
The earlier methods of measuring C-peptide were radio-immunoassays, which used radio isotopically labelled antigens. These were time-consuming, and prone to proinsulin interference (Jones and Hattersley, 2013). These methods are no longer common in clinical laboratories, especially due to the health and safety risk involved in using radioactive labels.

There are current enzyme immunoassays used to measure C-peptide in clinical laboratories. ELISA assays include the Mercodia C-peptide ELISA kit, which is a solid-phase, two-site enzyme immunoassay. The principle is based on a direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the C-peptide molecule (Mercodia, 2023).

Chemiluminescence immunoassays are available on most automated platforms. Examples of automated platforms include Abbott Architect and Roche Elecsys. The Architect C-Peptide assay is a two-step immunoassay using chemiluminescent microparticle immunoassay technology. The C-peptide present in the sample binds to anti-human C-peptide-coated microparticles, forming an antigen-antibody complex (Abbott, 2023). Roche Elecsys C-Peptide is also a sandwich method and uses two monoclonal antibodies specifically directed against human C-peptide (Roche, 2018).

Despite the continuous improvement in the analytical sensitivity of these automated methods by the manufacturers, C-peptide measurement using immunoassay technology has significant downfalls. Firstly, C-peptide immunoassay methods are costly. As of 2024, an RIA kit cost £730 for 125 tubes; £450 for 96 wells ELISA Mercodia kit; and an Abbott reagent kit cost £180 for 100 tests. These costs do not include instrumentation or staffing costs). Commercial development, optimisation of immunoassays and certification of assays take manufacturers several months/years and require significant investment. This is because several steps are involved in assay development. First, the antigen requires extraction from its matrix, purification, and inoculation to produce the

antibody. Depending on the antibody titre, another matrix may be required to improve sensitivity. The antibody and the antigen must also be compatible with other components of the method, such as the speed of the assay, reaction temperature, pH, and time, and all these components require optimisation to ensure a robust immunoassay method (Sheldon, 2020). Despite C-peptide immunoassays being a commercially available test, diagnostic laboratories still face challenges associated with reagent quality, stability, reliability, lot-to-lot changes, and availability.

Cross-reactivity is another problem in diagnostic immunoassays where endogenous molecules with a similar structure to C-peptide exist or where metabolites of the analyte have common cross-reactive epitopes and where there is administration of structurally similar medications (Kroll and Elin, 1994). Current C-peptide immunoassays suffer from cross-reactivity with insulin analogs, proinsulin and its partially processed forms such as Des 31-32 proinsulin, split 32-33 proinsulin, Des 64-65 proinsulin, and Split 65-66 proinsulin. Cross-reactivity occurs when they are present in much higher concentrations (Clark, 1999). The Elecsys C-Peptide assay monoclonal antibodies, for example, show cross-reactivity with the C-chain of human proinsulin. The effect of cross-reactivity is an overestimation or underestimation of C-peptide concentration. Cross-reactivity also has a negative effect on the limit of detection (LOD) (Table 1.5) (Stuart, 1992).

Table 1. 5 Sensitivity and Proinsulin Cross-reactivity of UK Assays (Mercodia, 2021; Siemens, 2005, Abbott, 2024; Roche 2018)

| Company name | Method | Limit of Detection | Proinsulin cross-reactivity |
|---|---|---|---|
| Abbott Architect | 2-step immunoassay | 3.3109 pmol/L (0.01 µg/L) | 12.8% with 1µg/L |
| Mercodia ELISA | Immunoassay | 25 pmol/L (0.07µg/l) | 2% |
| Mercodia Ultra-sensitive | Immunoassay | <2.5 pmol/L (0.0076 µg/l) | 5% with 0.317µg/L |
| Roche | Immunoassay | 7 pmol/L (0.0211µg/L); | 28.6 % with 100µg/L |
| Siemens Immulite 2000 | Competitive enzyme immunoassay. | 27 pmol/L (0.0815 µg/l) | 10% with 10 µg/L |

Heterophilic antibodies and other unsuspected binding proteins can cause interference in immunoassays, which can lead to erroneous interpretation of C-peptide results. This can lead to unnecessary investigations or the administration of inappropriate therapy (Sturgeon and Viljoen, 2011; Ghazal *et al*., 2021). Heterophile antibodies consist of natural antibodies and autoantibodies that are polyreactive against heterogeneous, poorly defined antigens of different chemical compositions and generally show low affinity and weak binding (Levinson and Miller, 2002; Bouvet and Quan, 2001). Heterophile antibodies interfere with immunoassays non-competitively by binding to the conjugate, enzyme, or other parts of the detection system. Assays using either polyclonal or monoclonal antibodies may be affected. Heterophilic antibodies in human serum can react with reagent immunoglobulins, interfering with in vitro immunoassays. In sandwich immunoassays, a heterophile antibody causes interference by forming a crosslink between capture and labelled antibodies (Hattore *et al*., 2016).

Human anti-animal antibodies (HAAA) can also compete with the test antigen by cross-reacting with reagent antibodies. Human anti-mouse antibodies (HAMA) are the most common, but antibodies to dogs, rats, cats, rabbits, goats, sheep, cattle, etc. can cross-react and are reported to occur in 30 to 40% of patient samples (Selby, 1999; Kricka, 1999). Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy, for example, commonly contain HAMA. Such specimens may show either falsely elevated or depressed values when tested with assay kits that employ mouse monoclonal antibodies.

Biotin has been shown to interfere with many streptavidin-biotin based immunoassays. It can cause falsely decreased results in sandwich immunoassays and falsely increased results in competitive immunoassays. A C-peptide test may exhibit interference when the sample is collected from a person who is consuming a supplement with a high dose of biotin (Ismail and Barth, 2001).

High-dose hook effect is another disadvantage of immunoassays, which occurs when the analyte concentration range is very high. This causes antigen-antibody reactions to go into antigen excess and can result in falsely low results, which can potentially lead to misdiagnosis (Ryall *et al*., 1982; Sturgeon and McAllister, 1998).

In two-site sandwich immunoassays, the excess antigen might prevent sandwich formation with the capture and signal antibodies separately bound to the antigen and the signal antibody being washed away, resulting in a lower recorded concentration (Ghazal *et al*., 2021). For two-site immunoassays in which both the capture and detection antibodies are added simultaneously, the free analyte and the analyte bound to the labelled antibody compete for the limited number of antibody-binding sites, and in the presence of a higher analyte concentration, the label bound to the solid phase will decrease rather than increase. The high-dose hook effect is usually averted in immunoassays by dilution of the sample (Tate and Ward, 2004).

C-peptide samples collected into EDTA tubes are known to be more stable than lithium heparin and serum separating gel tube samples. EDTA, however, can interfere with immunoassay reagent labels and is therefore not an accepted sample type for C-peptide immunoassay analysis.

### 1.5.1 Mass spectrometry-based methods

Alternative analytical techniques exist which are capable of overcoming the shortcomings of immunoassays. Mass spectrometry coupled with liquid chromatography is an essential tool with analytical capabilities and applications in the fields of proteomics, forensic toxicology, clinical research, metabolomics, and pharma/biopharma (Király *et al.,* 2016; Zhang *et al*., 2010). A wide range of sample types can be analysed within mass spectrometry, which includes proteins, nucleic acids, lipids, DBS, and fatty acids (Züllig and Köfeler, 2021).

A typical mass spectrometer converts molecules into ions, which are then manipulated by electric and magnetic fields using three main components; an ion source that ionises the sample, a mass analyser that separates the ions based on mass-to-charge ratio, and a detector that measures and displays the ions on a mass spectrum. (Bantscheff *et al*., 2007; Olshina *et al*., 2016).

Before using the mass spectrometer, a sample must be extracted and then ionised by utilising gas chromatography or liquid chromatography. In gas chromatography, the components of a mixture of gases are separated based on physical characteristics like size, shape, boiling point, and molecular weight, and after separation, the gases enter the mass spectrometer for analysis (Bourgogne *et al*.,

2015; Garg, 2023). In contrast, liquid chromatography separates samples based on interactions with a mobile and stationary phase (Rappold *et al*., 2022).

Most LCMS uses electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI), which leave the molecular ion largely intact. In ESI, charged droplets are formed from the liquid, eluting off a chromatographic column by using voltage, heat, and air. The charged droplets move towards the mass spectrometer inlet, generating analyte ions during evaporation and droplet fission (Tycova *et al*., 2021). In APCI, a combination of heat (used to completely vaporise the sample) and a corona discharge (which ionises the evaporated solvent) is used to form negative or positive ions (Pitman and LaCourse, 2020). Another ionisation commonly used in proteomics, is matrix-assisted laser desorption ionisation (MALDI), where samples are co-crystallised onto a sample plate with a small organic matrix compound that can absorb at the wavelength of the laser (Clark *et al*., 2013).

Mass spectrometry has gone through a period of rapid technological development, which has seen its introduction into clinical laboratories with dramatic improvements in automation (Wudy and Choi, 2016). There are now mass analysers that can provide higher resolution and increase sensitivity in one of several ways; an improvement of ion transmission efficiency, the selective enrichment of targeted ions, the improvement of the ion utilisation rate, or the improvement of the signal-to-noise ratio of the spectrum (Li *et al*., 2021). The three most common mass analysers are quadrupoles, time-of-flight (TOF), and ion trap mass analysers.

### 1.5.2 TOF Analysers

TOF analysers accelerate the ions by a short voltage gradient and measure the flight time of the ions. It is based on the principle that ions with the same kinetic energy and different mass-charge ratios move at different velocities in a constant electric field. The resolution and sensitivity of TOF are based on improving the ion utilisation rate. TOF-MS has the advantage of a theoretically unlimited mass range and is often combined with MALDI for structural identification (Qin *et al*., 2021). One disadvantage of TOF analysers is the uncertainty associated with the measurement of the ion flight time (Walker, 2021).

### 1.5.3 Ion-trap Analysers

Ion-trap mass spectrometers trap the ions in an electric field, where specific ions can be activated and ejected by manipulation of this electric field (Broek *et al*., 2008). They are physically compact, have modest vacuum requirements, can scan relatively quickly, and can perform multiple stages of MS/MS all within a single volume (Grebe and Singh, 2011). Ion-trap mass analysers use the strategy of selectively enriching targeted ions to improve resolution and sensitivity (Li *et al*., 2021). Ion traps have the advantage of having the ability to conduct tandem MS analysis with high sensitivity (Thomas, 2019).

### 1.5.4 Quadrupole Analysers

Quadrupoles are very suitable for quantitative measurements, because of their relative high ion transmission efficiency, large dynamic range, and high sensitivity. Several applications for the quantification of peptides have been described. (Broek *et al*., 2008) In quadrupole mass spectrometers, specific radio frequency and direct current voltages are applied to four parallel rods, allowing only ions with a specific mass-to-charge ratio value to move between the rods and reach the detector (Amoresano and Pucci, 2022). Sensitivity is based on the strategy of improving ion transmission efficiency (Li *et al*., 2021).

The technique of using two or more series of quadruples in a mass analyser is referred to as a Tandem mass spectrometer (Figure 1.4). The additional quadrupole in Tandem mass spectrometer has the benefit of an increased selectivity, less interference and less matrix effect because of double mass filtering compared to a single quadrupole (Antoniewicz, 2013). The strategy of improving the signal-to-noise ratio of the spectrum is mainly used in quadrupole tandem mass analysers (Li et al., 2021).

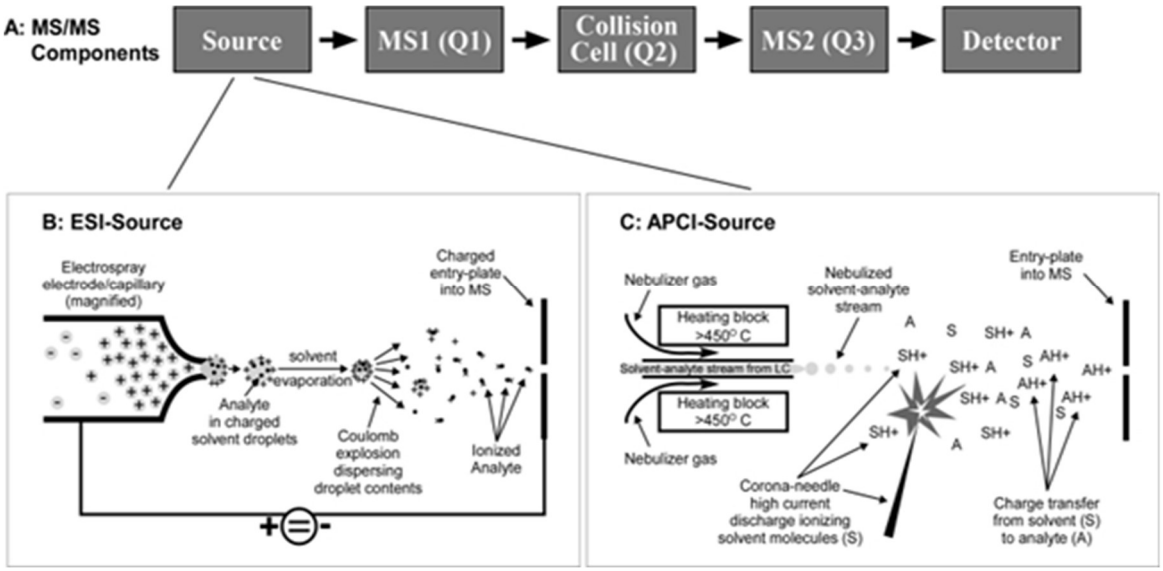



Figure 1. 4 Components of a Typical LC-MS/MS.

A typical LC-MS/MS instrument consists of an ESI source or an atmospheric pressure chemical ionisation source (APCI), a first mass-filtering device quadruple 1 (Q1), which leads into a collision chamber (Q2), followed by a second mass-filtering device (Q3), and finally an ion-impact detector (Grebe and Singh, 2011).

## 1.6 LC-MS/MS Assays in the Management of Diabetes

In the management of diabetes, LC-MS/MS has been useful in the search for diagnostic markers and in attempts to standardise currently used immunoassay analytic techniques. Recent applications include Brede *et al*. (2016) LC-MS/MS method developed to measure glycated albumin in serum and plasma in diabetic patients with end-stage renal disease who may have short-lived red blood cells due to haemodialysis, and thus a higher turnover of haemoglobin (Brede *et al*., 2016).

Table 1. 6 Summary of C-peptide LCMS Methods Available in the Literature
The table covers the matrix used, performance, limitations, and purpose (research or clinical use) of developing the method.

| Reference | Sample type | Sample Volume | Measuring range | Recovery or accuracy | Research or clinical use | Advantages | Disadvantages |
|---|---|---|---|---|---|---|---|
| Kippen *et al*., 1997 | Serum | 1ml | not stated | 80% | Reseach only | multiplexing insulin and Cpetide | Not validated. |
| Fierens et al 2000 | Urine | 1ml | 32 to 165 ng/ml. | 60% | Reseach only | Simple sample preparation | Limited to urine |
| Rogatsky et al., 2006 | Serum | 0.3 ml | 0.010 to 5 ng | 78%–89% | Reseach only | Accurate and sensitive | Large sample volume; Tedious and time-consuming extraction |
| Rogatsky et al., 2006 | Plasma | not stated | not stated | not stated | Reseach only | Accurate and sensitive | Tedious and time-consuming extraction, lower specificity in SIM mode. |
| Stoyanov et al., 2011 | Plasma | not stated | 0.010-3 ng | not stated | harmonisation efforts | semi-automatic SPE. JCTLM registered reference method | additional ion-exchange chromatography instrumentation |
| Kinumi et al., 2014 | Serum | Not stated | 0.003–2.9 ng | 99% -108% | harmonisation efforts | Good sensitivety; Calibrators are traceable; JCTLM registered reference method | Time consuming extraction |
| Taylor et al 2016 | Serum | Not stated | 0.11 to 27.2 ng/ml (36 to 9006 pmol/l) | 91%-104% | Reseach only | multiplexing insulin and Cpetide | Complex and tedious sample preparation |
| Thomas et al., 2020 | Plasma | 250ul | LOD (0.2 ng/mL), LOQ (0.6 ng/mL) | recovery (40–90%), accuracy (78–128%) | Reseach only | Simple SPE procedure, multiplexing insulin and Cpetide | long assay time; Poor precision |
| Owusu et al., 2021 | Serum | 200ul | 0.1 and 15 ng/mL | (accuracy):98.2 % (±9.1%) | clinical use | Low sample volume (200ul) | complicated and time consuming; 2 day assay; Poor recovery |
| Wan et al., 2020 | Serum | 100 and 500ul | 0.025–5 nmol/L; LOQ 25 pmol/L | Accuracy 39%-66% | harmonisation efforts | faster turnaround time (<4h); high-throughput. | Complex and tedious sample extraction; Poor recovery |
| Thomas et al., 2021 | Urine | 3ml | LOD (10–25 pg/mL | 25–100% | Reseach only | Sample collectin is noninvasive; multiplexing insulin and Cpetide | Results is qualitative; Limited to urines |

Several methods have been developed for C-peptide analysis, each with advantages and disadvantages, as shown in Table 1.7 above. Kippen *et al*. (1996), were one of the first researchers to develop an LCMS method for C-peptide. The method uses immunoaffinity and solid-phase extraction, achieving a recovery of about 80%. The method had a disadvantage of a large sample volume of 1 ml of serum and was also not validated for clinical use.

Fierens *et al*. (2000) also developed a quantitative LCMS method for C-peptide, but this was for urine samples only and achieved a very low average recovery of about 60%.

Rogatsky *et al*. (2006) developed a 2-dimensional liquid chromatography mass spectrometry C-peptide method using solid phase extraction. The extracts were filtered through an Ultrafree-MC centrifugation filter, making it a tedious and time-consuming process. The average recovery was 83.9% and had a narrow linear range (0.010 to 5 ng).

Taylor *et al*. (2016) developed a multiplexed LC-MS/MS assay for intact insulin and C-peptide, using monoclonal antibodies immobilized on magnetic beads, with an analytical measurement range of 36 pmol/L to 9006 pmol/L for C-peptide (Taylor *et al*., 2016).

Recently, Kinumi *et al*. (2014) developed an isotope-dilution mass spectrometry method for C-peptide using immunoaffinity purification and chemical modification with 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate, with a response in the 0.003-2.9 ng range and a limit of quantification of 0.003 ng. Mean recoveries range between 99 and 108 percent (Kinumi *et al*., 2014).

The University of Missouri-Columbia Diabetes Diagnostic Laboratory also developed an anion-exchange chromatographic method for C-peptide that utilises one-step cation exchange purification and ultrafiltration purification steps. The method's linearity range was 0.010 - 3 ng. Compared to an immunoassay, the ID MS results were on average 32% lower. (Stoyanov *et al*., 2011).

Thomas *et al.* (2020) developed a C-peptide method with 250µL of plasma utilising protein precipitation and a mixed-mode cation-exchange solid-phase extraction and subsequent detection by LC-high resolution MS. The limit of detection (0.2 ng/mL), the limit of quantification (0.6 ng/mL), recovery (40–90%), accuracy (78–128%). The analytical run was 14 minutes, with C-peptide eluting at about 7 minutes.

Owusu *et al.* (2021) used protein precipitation and solid-phase extraction with mixed anion exchange and proteolysis using Glu-C enzyme to detect C-peptide. The assay was linear between 0.1 and 15 ng/mL but had the disadvantage of a long turn-around time of two days.

Wan *et al*. (2020) developed a C-peptide method using immunoaffinity modified with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The LOD of this method was 0.025 nmol/L and had a linear range of 0.025-5 nmol/L. These studies suggest that an LC-MS/MS-based approach can quantify human C-peptide with high sensitivity and precision and could find potential use in the routine laboratory and reference measurement systems. Our method uses similar extraction principles (protein precipitation coupled with solid-phase extraction) as Thomas *et al.* (2020) and Owusu *et al.* (2021), but different extraction solutions and plates. While the existing LC-MS methods were used to quantify C-peptide in urine, serum, and plasma samples, to the best of our knowledge, this is the first LC-MS/MS method developed for the quantification of intact C-peptide in DBS samples.

### 1.7 Significant variation in C-peptide results due to a lack of assay standardisation

Standardisation of biomarkers is important for the comparability of laboratory results as it allows the definition of universal reference values and clinical decision limits. For decades, the use of non-harmonised C-peptide assays has caused confusion. Standardisation for the worldwide comparability of C-peptide will improve and increase confidence in this laboratory biomarker. Laboratory results are affected by the analytical procedure, i.e., if they are not standardised, wrong conclusions may be drawn for the diagnosis or the treatment, affecting patient safety (Miller *et al*., 2014). Standardisation of laboratory tests ensures that comparable results are obtained regardless of when and where the analysis is performed. At best, by standardising an assay, this indicates that, among other requirements, a well-defined reference standard together with a reference measurement system is available (Horber *et al*., 2019). There are several reference standards and reference measuring analysers now available for C-peptide, but immunoassay manufacturers are slow to catch up. This has made the chosen standard material, correct calibration, and appropriate specificity of an

immunoassay difficult to prove (Stöckl *et al*., 1997; Ashby and Frier, 1981; Little *et al*., 2017).

Currently, significant variability in C-peptide test results exists between laboratories in the UK due to the lack of standardisation (Larsen *et al*., 2017). As a result, values obtained with different assay methods cannot be used interchangeably. Only one assay method can be used consistently to monitor each patient's course of therapy. However there are now LCMS reference methods listed on the JCTLM website, (National Metrology Institute of Japan) NMIJ and University of Missouri-Columbia Diabetes Diagnostic Laboratory (UMC DDL) that can help achieve standardisation (Stoyanov *et al*., 2011; Kinumi *et al*., 2014)

Current C-peptide assays also have a variable dynamic range, a variable C-peptide reference range, and reporting units in clinical practice. Various factors influence the reporting reference ranges of C-peptide. These include obesity, renal impairment, age, ethnicity, assay type, and glucose levels. C-peptide reference ranges in clinical practice are determined using a limited number of healthy individuals who are not on insulin preparations. Although good practice dictates that each laboratory must establish its own expected range, assay manufacturers, using healthy individuals, provide assay ranges as a guide. It is worth noting that these reference ranges show significant variation among currently available immunoassay methods, as shown in Table 1.5. These reference ranges do not reflect assay bias or hypo- or hyperglycaemia states. The lack of population-specific reference ranges for C-peptide makes its interpretation in clinical care complex (Wiedmeyer *et al*., 2007; Kinumi *et al*., 2014

Table 1. 7 C-peptide Reference Range Guide for Immunoassay (Mercodia, 2021; Siemens, 2005; Abbott, 2024; Roche 2018)

| Immunoassay Methods | Reference range |
|---|---|
| Mercodia | 1.0 – 5.4 mg/L (343 – 1803 pmol/L) |
| Siemens Immulite 2000 | 0.9 – 7.1 mg/L (298 - 2350 pmol/L) |
| Abbott Architect | 0.78 - 5.19 mg/L (258 – 1718 pmol/L) |
| Roche | 1.1 – 4.4 mg/L (370 – 1470 pmol/L) |

## 1.8 The Emergence of DBS Sampling in Mass Spectrometry

Dried blood spot (DBS) microsampling is an innovative alternative means of obtaining venous blood samples on absorbent paper. The DBS card is dried at room temperature for preservation after a sample is taken and is then extracted and used for the measurement of biomarkers. The concept was first described in 1860 for glucose measurements (Grüner *et al*., 2015). In 1869, the technique was used for paediatric screening, to detect phenylketonuria in newborns (Party *et al*., 1971; Guthrie *et al*., 1963). Later in the 1960s heel-prick-derived DBS was used to screen for metabolic disorders in newborns (Guthrie and Susi, 1963). Since then, the technique has been used by many researchers and in mainstream medicine, finding uses such as HbA1c monitoring, maternal research, infectious disease diagnosis, and drug monitoring (Nguyen *et al*., 2014; Pollock *et al*., 2016; Aburu *et al*., 2006; Bakhireva *et al*., 2017; Basu *et al*., 2017; McCabe *et al*., 1987).

DBS can be considered the sample of choice for paediatrics, the elderly, and in situations where a series of C-peptide measurements are required. The collection of DBS samples is normally conducted by pricking the finger, heel, or toe with a lancet, and the blood drops are then spotted onto pre-printed circles, ideally one drop per spot, on specially manufactured paper cards.

There are several DBS cards available on the market. One of the popular DBS formats is the Whatman 903 card, which is composed of cotton-based filter paper within a rigid cardboard frame for handling and labelling. Flinders Technology Associates (FTA) cards utilise Whatman FTA technology. FTA cards contain chemicals that lyse cells, denature proteins, and protect nucleic acids from nucleases or oxidation damage, while most potential pathogen present in the sample is completely inactivated (Arca-Lafuente *et al*., 2022; Picard-Meyer *et al.,* 2007). FTA Classic and FTA Elute have been shown to assure the stability of nucleic acids from whole blood for up to 36 months when stored in a dry atmosphere at -15 °C (Arca-Lafuente *et al*., 2022).

The matrix of 903 Protein Saver cards is not chemically treated and can preserve proteins and peptides from whole blood and plasma at room temperature. 903 Protein Saver cards are the FTA alternative technology for conventional DBS (Party *et al*., 1971; Guthrie, 1963; Arca-Lafuente *et al*., 2022). The cost of a protein

saver card (£234 per pack of 100 in 2024) is about a half of the cost of Whatman FTA cards (£478 per pack of 100 in 2024), making them a more economically feasible option.

The combination of DBS and mass spectrometry in the targeted analysis of small molecules and proteins or peptides in biological samples has become increasingly popular in biomedical research and neonatal screening programmes. This is attributed to the fact that the sensitivity and selectivity of analytical methods have improved, especially with mass spectrometry (Li *et al*., 2010). Mass spectrometric platforms permit both surveying and preliminary fractionation of a complex biological matrix for the quantitative measurement of peptides and proteins eluted from DBS (Johansson *et al*., 2010). Particularly, this approach has been adapted to measure ceruloplasmin for neonatal screening of Wilson's disease (Kroll *et al*., 2006). Moreover, with support from a multiplex system, the mass spectrometry platform has the potential to quantify a vast number of protein analytes from a small volume of biomaterial. For example, using this approach, Chambers *et al*. (2012) effectively quantified a panel of 40 serum proteins eluted from the DBS surface (Chambers *et al.,* 2012).

In the last decade, the use of DBS sampling has grown and gained popularity in the pharmaceutical, clinical, toxicological, and forensic industries. Simoes *et al.* (2018), used DBS combined with UPLC-MS/MS method for the simultaneous determination of drugs of abuse in forensic toxicology. Heussner *et al.* (2017) used the technique for steroid measurement via LC-MS/MS in rats. Barfield *et al*. (2008) used DBS combined with LC-MS/MS for the quantification of acetaminophen.
McDonald and colleagues have demonstrated the feasibility of a retrospective diagnosis of neonatal diabetes using stored NBS cards and that elevated glucose levels may be reliably detected in various storage conditions at day five of life (McDonald *et al*., 2017).

A recent study successfully proved in principle that C-peptide can be measured in DBS using an immunoassay method. Even though the DBS method slightly overestimated C-peptide levels, there was a good correlation between the DBS and plasma C-peptide levels (Willemsen *et al*., 2018). LC-MS/MS has the potential to offer a better sensitivity for C-peptide measurements using DBS compared to immunoassay. No study has developed an LC-MS/MS procedure to measure C-

peptide from DBS samples possibly because of the challenges of sample extraction and the difficulty in achieving the level of sensitivity required with low volume samples. C-peptide is susceptible to enzymatic proteolytic cleavage, which is why samples must be transported on ice to slow down the enzymatic activity that causes degradation. To the best of our knowledge there are no studies comparing the stability of C-peptide in serum and DBS. However, DBS is generally well known to offer significantly improved stability because drying samples can put a halt to specific and non-specific enzymatic degradative events (Björkesten *et al*., 2017; Elbin *et al*., 2011). DBS therefore has the potential to offer more stability than serum or plasma and was investigated in this study.

## 1.9 Aims and objectives of the research

The primary aim of the study is to develop and validate a LC-MS/MS C-peptide method for the determination of C-peptide for clinical use in the UK. Building on this and taking advantage of the smaller sampling requirement, this research proposes that C-peptide measurement via LC-MS/MS will have an increased impact with DBS sampling. This will permit frequent assessment of C-peptide to investigate causes of hypoglycaemia, monitor ß-cell function in pre-diabetes, monitor disease course in newly diagnosed type 1 and type 2 diabetic patients, obtain valuable information on glucose variability, the risk of hypoglycaemia, and ketoacidosis in children, as well as carry out an assessment of β-cell function in clinical trials following interventions.

- The objectives of the research are as follows:
    - Develop and validate the method to meet International Standard Organisation (ISO) 15189 standards for quality and competence in medical laboratories for conventional sampling (serum or plasma).
    - Develop a protocol for the analysis of DBS and verify this method against the serum method using CLSI EP35 guidelines for the verification of alternative sample types.
    - Carry out a cost-benefit analysis against the existing method (Mercodia ELISA) used at the Berkshire and Surrey pathology services (Mercodia) with the aim of replacing this method.

The next chapter, chapter 2, describes the methods and materials, used to develop this method as well as the validation parameters measured to assess performance characteristics. Chapter 3 covers the results data and discussion for the serum and plasma C-peptide method. The results data and discussion of the DBS C-peptide method is covered in Chapter 4. The conclusion of the research including future work and limitations is covered in Chapter 5.

# Chapter 2.0 Methodology

## 2.1 Material and Reagents

The column for this research was a Waters Ltd (Borehamwood, UK) Ultra high-performance liquid chromatography (UPLC) CORTECS C18 1.6µm 2.1x50mm column. LCMS grade water, LCMS-grade methanol, and LCMS-grade acetonitrile (ACN) were purchased from Rathburn (Walkerburn, Scotland). Formic acid, phosphoric acid, and acetic acid were purchased from Biosolve (Valkenswaard, The Netherlands). PerkinElmer DBS filter paper, trifluoroethanol (TFE), trifluoroacetic acid (TFA), dimethylsulfoxide (DMSO), and ammonium hydroxide solution were purchased from Merck Life Science UK Limited (Dorset, UK). Try(C)-peptide internal standard, Cerilliant C-peptide Certified Reference Material (CRM), and rat plasma were also purchased from Merck Life Science UK Limited (Dorset, UK). Stable isotope-labelled C-peptide internal standard [D8] Val7·10], was purchased from Bachem (Bubendorf, Switzerland). Charcoal-stripped bovine serum and equine whole blood were purchased from TSC Biosciences (Buckingham, UK). Semi-automated DBS puncher was purchased from JYH Scientific (Pennsylvania, United States). Low protein-bind 1.5 mL tubes and deep-well 2ml collection plates were purchased from Eppendorf (Stevenage, UK). Insulin and proinsulin standards were purchased from NIBSC. EQA samples were from UK NEQAS Guildford Peptide Hormones and College of American Pathology (CAP) EQA schemes, haemoglobin, Intralipid, bilirubin and creatinine standards were purchased from MERCK.

## 2.2 LC-MS/MS Equipment

The LC-MS/MS equipment used was the Xevo TQ-XS tandem quadrupole atmospheric pressure ionisation (API) mass spectrometer from Waters ltd (Borehamwood, UK) (Figure 2.1). The Xevo TQ-XS was compatible with the following API sources: standard multi-mode electrospray ionisation, atmospheric pressure chemical ionisation, and combined electrospray ionisation and atmospheric pressure chemical ionisation (ESCI). The ACQUITY UPLC (Ultra-

performance high liquid chromatography) H-Class Series system was from Waters and included a PLUS Series quaternary solvent manager, sample manager, 30-cm column heater, sample organiser, detectors, a specialised ACQUITY UPLC column, and MassLynx software v4.2, for system control. The nitrogen generator was sourced from Peak Scientific (Inchinnan, Scotland, UK) and was used to generate sheath gas. The tandem mass spectrometer used a low-pressure argon gas (V-Argon N6.0 200-bar cylinder) purchased from BOC Online UK (Woking, UK). Argon gas was used at low pressure in the collision cell for the fragmentation of precursor ions into product ions.


Figure 2. 1 The Waters Xevo TQSX Tandem Quadrupole Mass Analyser
The components are a sample manager, Binary pump, an Acquity UPLC column manager, quaternary pump, and a mass analyser.

## 2.3 Preparation of Reagents and Consumables

### 2.3.1 Tuning Solution and Mobile Phases

The Mobile phase combinations were screened first by direct infusion and then by combined mode. The tuning solution was prepared in Methanol/Acetic Acid/water in the ration of 6:3:1. A selection of additives was then added to form various mobile phase combinations and investigated at concentrations of between 0.1%v/v to 0.5%v/v. The additives tested were TFA, TFE, FA, and DMSO.

Cerilliant C-Peptide (CRM) solution (2000 ng/mL), Try(C)-peptide internal standard (2000 ng/mL) and C-peptide internal standard [D8] Val7·10] (2000 ng/mL) tuning solutions were prepared in the various mobile phase-additive combinations for screening as shown in table 2.2 below.

Table 2. 1 Mobile Phase Combinations screened during LC Development

| Mobile phase | Additive | Estimated pH |
|---|---|---|
| 50:50 ACN/H2O | 0.1%FA | 2.8 |
| 50:50 ACN/H2O | 0.1%TFA | 1.8 |
| 50:50 ACN/H2O | 0.1%TFE | 7.4 |
| 50:50 ACN/H2O | 0.5%DMSO | 7.2 |

The final selected mobile phase solvent A, (0.1%v/v formic acid in LCMS-grade water) was prepared by adding 1 mL of formic acid to 1000 mL of LCMS-grade water. Mobile phase solvent B (0.1%v/v formic acid in acetonitrile) was prepared by adding 1 mL of formic acid to 1000 mL of acetonitrile. Acetonitrile was replaced with methanol for mobile phase B and was also tested and compared in the chromatographic and sample extraction development steps. The selected sample manager and seal wash after optimisation were made up of 20% LCMS grade methanol in 80% LCMS-grade water. The selected sample manager purge solution was 50:50 LCMS grade ACN and LCMS-grade water.

2.3.2 Preparation of Calibrators and Quality Control Material (IQC)

In the absence of human serum or human whole blood free from C-peptide, Cerilliant C-Peptide CRM solution was added into Charcoal-stripped bovine serum, which has a similar protein and ionic strength as human serum to produce in-house calibration standards and internal IQC material. Equine whole blood, was selected and used to prepare the calibration standards and IQC for the DBS method. The target analytical measuring range of the C-peptide assay was 0 to 8000 pmol/L (0 -24.16 ng/mL). A 12-point calibration curve was prepared by spiking the surrogate matrix with a series of different concentrations of the Cerilliant C-peptide CRM (serial dilution standards). The working standard concentration (8000 ng/mL) was prepared from the purchased 100 µg of CRM by adding 40 µL and 0.05%v/v rat plasma to a (6:3:1) methanol, water, and acetic acid solution. The working standard was then spiked into a 2 mL matrix to form an 8000 pmol/L top standard (calibrator 12). Calibration solutions 1-11 were prepared by serial dilution of calibrator 12 to form the target concentrations shown in Table 2.2 below.

Table 2. 2 Target 12-point Calibration Concentrations

| Cal | Cal 0 | Cal 1 | Cal 2 | Cal 3 | Cal 4 | Cal 5 | Cal 6 | Cal 7 | Cal 8 | Cal 9 | Cal 10 | Cal 11 | Cal 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ng/ml | 0 | 0.012 | 0.024 | 0.047 | 0.091 | 0.183 | 0.378 | 0.76 | 1.51 | 3.02 | 6.04 | 12.08 | 24.16 |
| pmol/L | 0 | 3.91 | 7.81 | 15.63 | 30.25 | 60.5 | 125 | 250 | 500 | 1000 | 2000 | 4000 | 8000 |

Similarly, in-house internal quality controls were prepared by spiking bovine serum or equine whole blood with Cerilliant C-peptide CRM to form concentrations at 100 pmol/L, 400 pmol/L, 900 pmol/L and 1400 pmol/L. Additional serum IQC was prepared using pooled patient serum samples at the concentrations of approximately 35 pmol/L, 250 pmol/L, 500 pmol/L, 700 pmol/L and 1500 pmol/L. The final concentrations of the patient pools were determined using the Mercodia C-peptide Elisa Assay. The whole blood calibrators and IQC material were spotted onto a DBS card, and dried for at least 24 hours before being packed together with desiccants for storage at -20 °C until analysis.

External quality control samples were sourced from the UK NEQAS Guildford Peptide Hormones Scheme and the College of American Pathologist Scheme (CAP).

## 2.4 Research Ethics

The research received favourable ethical approval from the University of the West of England's research ethics committee and a favourable opinion for conduct in the NHS by the Research Ethics Committee for the use of anonymised patient surplus samples (REC reference: 24/LO/0104).

### 2.4.1 Sample Recruitment

The samples used for this research were anonymised surplus serum aliquots obtained from the Berkshire and Surrey Pathology Services, Peptide Hormones laboratory at the Royal Surrey NHS Foundation Trust.
The second stage involved the validation of DBS samples against the validated serum method. The samples that were used at this stage were C-peptide certified reference material spiked into purchased charcoal-stripped Equine whole blood at various concentrations. These were spotted on a DBS card and paired with a similarly spiked charcoal-stripped bovine serum for comparison studies. This research initially sought ethical approval to obtain a paired serum collected into a gel tube and a DBS sample taken from a finger prick onto a DBS card from diabetic patients' volunteers on a visit to diabetic clinics. Spiking purchased serum and whole blood as an alternative means to verify the accuracy of the DBS method was agreed upon to prevent disruption of the workflow of the diabetic clinics. This research will therefore prove, in principle, the performance of the DBS method. Further verification with actual patient samples will be required for clinical application.

A statistical comparison was essential for this research, and therefore a formal calculation was used to determine the sample size for serum and plasma method validation as well as DBS method verification (Pearson's correlation tests). A

sample size of 84 achieves 90% power to detect a difference of -0.05000 between the null hypothesis correlation of 0.90000 and the alternative correlation of 0.95000 using a two-sided hypothesis test with a significant level of 0.05000. In using the power calculation as shown in Table 2.1 below, it was noted that it was not convincing enough if the correlation achieved was low and was with a smaller sample number. For example, if a correlation of 0.85 is achieved, then a sample size of 48 will give a 90% power of rejecting the null hypothesis. The sample size goes down with a higher correlation but will not be representative enough to prompt a change in practice.

For this reason, a minimum of 84 samples each, were used for method validation and validation for the serum and DBS methods respectively. This sample size was large enough to statistically prove the relationship between methods. A minimum of 84 samples was also considered to be manageable and deliverable, and it was enough to cover the measuring range and clinical decision points.

Table 2. 3 Method Comparison Sample size estimation.
(Power is the probability of rejecting a false null hypothesis. It should be close to one. N is the size of the sample drawn from the population. To conserve resources, it should be small. Alpha is the probability of rejecting a true null hypothesis, which also should be small. Beta is the probability of accepting a false null hypothesis. It should be small. P0 is the value of the population correlation under the null hypothesis. P1 is the value of the population correlation under the alternative hypothesis).

| Power | N | Alpha | Beta | P0 | P1 |
|---|---|---|---|---|---|
| **0.80532** | 64 | 0.05000 | 0.19468 | 0 .90000 | 0.95000 |
| **0.85054** | 72 | 0.05000 | 0.14946 | 0.90000 | 0.95000 |
| **0.900 97** | 84 | 0.05000 | 0.09903 | 0.90000 | 0.95000 |

## 2.5 C-peptide LC-MS/MS method development steps

### 2.5.1 Mass spectrometry optimisation steps

Mass spectrometry optimisation was carried out by tuning the mass analyser with the selected tuning solution (0.1%v/v formic acid in 50:50 ACN/ $H_2O$) first by direct infusion and then combined with the mobile phase at an initial flow rate of 5 µL/min and 10 µL/min. The direct infusion experiment was repeated at least 10 times over several days to select consistent transitions with the best sensitivity. The parameters adjusted were the capillary voltage, cone voltage, desolvation temperature, desolvation gas flow, cone gas flow, nebuliser gas flow, low-mass and high-mass resolution settings, and collision gas flow.

0.5%v/v formic acid in 50:50 ACN/ $H_2O$ was also optimised to assess the impact on sensitivity with an increase additive concentration. 0.5%v/v formic acid gave a slightly better signal-to-noise ratio sensitivity however 0.1%v/v formic acid was still selected. This was primary based on the mobile phases already in use on the analyser for other assays with the goal to harmonise mobile phases where possible. The selection of 0.1%v/v formic acid was also based on the solubility of the C-peptide standard in the mobile phase combination, consistent transition ratios, good sensitivity (signal-to-noise ratio), good peak shape and peak height in the mass spectrum, and daughters corresponding to b and y ions in a software predicted C-peptide fragmentation pattern. The final selected mobile phase and additive (Mobile phase A: 0.1%v/v formic acid in Water and Mobile phase B: 0.1 formic acid in Acetonitrile) were then tuned in a combined mode to optimise the final mass spectrometry conditions by manually adjusting the parameters in incremental steps. The mass spectrum was reviewed at each stage to select the best parameters using the peak shape, height, and signal-to-noise ratio as a guide.

The selected mobile phase and mass analyser conditions must aid fragmentation to allow identification of C-peptide (3020.29 Daltons) which falls outside the mass range of the analyser (2-2400 Daltons) through the formation of multiply charged ions. The potential multiply charged precursor ions and the daughter ions (Table 2.3) were predicted using a software tool to serve as a starting guide. The table shows the possible parent ions screen for the first quadrupole with 3+ precursor ion selected.

Table 2. 4 Predicted precursor ions using Biolynx software.

| Precursor Ions | Average Mass (Positive ESI Mode) | Monoisotopic Mass | Average mass (Negative ESI Mode) |
|---|---|---|---|
| 1+ | 3021.2693 | 3019.5224 | 3017.51 |
| 2+ | 1511.1386 | 1510.2651 | 1508.25 |
| 3+ | 1007.7617 | 1007.1793 | 1005.16 |
| 4+ | 756.0733 | 755.6365 | 753.62 |
| 5+ | 605.0602 | 604.7107 | 602.70 |

### 2.5.2 UPLC optimisation steps

A systematic approach to chromatography method development was employed, where selectivity factors such as pH, organic modifiers, and column chemistry were evaluated to determine the parameters needed to achieve the best separation and desired resolution. This was then optimised (gradient slope and temperature) to obtain the final desired results. The steps were as follows:
Using a 50 mm x 2 mm C18 column, C-peptide pure standard was screened for its retention time employing a 5 to 95% organic gradient over 2 minutes at both low pH (formic acid) and high pH (ammonium acetate). Both methanol and acetonitrile were screened as organic modifiers.
The four solvent inlets of the UHPLC was used to manipulate the organic solvents and pH, and the gradient that gave the best retention time, sensitivity, and selectivity were selected and optimised. The temperature was adjusted in 15°C increments between 10°C to 50°C to investigate the effect on separation components.

Chromatographic performance characteristics such as the peak asymmetry, capacity factor, and plate height were also estimated to assess the effects of the mobile phase, sample injection volume, and column quality on the chromatographic separation.

Peak Asymmetry (As) was calculated using the equation (2.1) As = B/A, where (B) is the distance from the peak's midpoint to its trailing edge, and (A) is the distance from the peak's midpoint to its leading edge.

Capacity factor (k) was calculated using the equation (2.2) K = (Tr – To)/To, where Tr is the retention time and To is the void time.

Void time was calculated using equation (2.3): To = Void volume/Flow rate

Void volume was calculated using equation (2.4) = $\pi(r)^2 * L * 0.70$, where r is the column internal radius and L is column length. The Waters uPLC column is very porous, with about 70% (0.7) of the solid phase still accessible to the mobile phase.

Plate height, which is the height equivalent to a theoretical plate (HETP), was calculated using the equation (2.5) HETP = L/N, where L is the length of the column and N is the number of theoretical plates.

N was calculated using equation (2.6): $N = 16(t_R/w_b)^2$ where $t_R$ is the retention time and $w_b$ is the peak width at its base in units of time.

### 2.5.3 Sample extraction method development

Three of the commonly used sample extraction procedures were assessed for cost, complexity, throughput, recovery, and matrix effect. These were: liquid-liquid extraction, solid phase extraction, and antibody affinity extraction methods.
Due to the poor recovery and matrix effects of liquid-liquid extraction and the high cost and complexity of the antibody affinity extraction methods, a combination of protein precipitation followed by solid phase extraction (SPE) was selected and optimised in this research.

Protein precipitation was assessed using methanol, acetonitrile and a 50:50 methanol-acetonitrile combination. The internal standard was spiked into the selected extraction solution (methanol-acetonitrile) with a target concentration in the middle of the calibration curve (2000 pmol/L to 4000 pmol/L) to give a good

response signal but also avoid high concentrations that could cause interference of the analyte ionisation. For protein precipitation, 100 µL of the extraction solution was added to 50 µL of serum (1-2 ratio, v/v) in a low protein-bind Eppendorf tube, vortex mixed for 20 seconds, and then spun for 10 minutes at 16089 g. After centrifugation, the supernatant was acidified with 4%v/v phosphoric acid before solid-phase extraction.

The Waters Oasis HLB prime SPE plate was optimised for the solid-phase extraction procedure based on the properties of the SPE plate absorber material and the theoretical properties of C-peptide. Increasing concentrations of methanol and ACN in $H_2O$, with additives such as formic acid and acetic acid were screened as washing and eluting organic buffers. The final selected solutions were 200 µL 5% methanol for wash 1, 200 µL 5% formic acid for wash 2, and 50 µL 5% ammonium hydroxide in methanol eluting buffer. The final eluent was diluted with Methanol/Water/Acetic acid (6:3:1 ratio) before analysis.

DBS samples were eluted from the DBS card before protein precipitation and solid phase extraction. The spots, 8 mm diameter, were punched out of the DBS card using a JYH scientific semi-automated puncher into low protein-binding Eppendorf tubes and 500 µL 20% Bovine serum albumin (BSA/PBS) eluting buffer was added. This was incubated at 37°C for 30 minutes before agitation on a plate mixer for a further 3 hours. The extraction solution was then added and spun for 15 minutes at 16089 g. The supernatant was acidified with 400 µL of 4% phosphoric acid and then taken through the same solid phase extraction steps as detailed for the serum method.

## 2.6 Validation of the Method for Serum and Plasma Samples

The serum and plasma LC-MS/MS method developed was validated in accordance to the CLSI C62 guidance to provide objective evidence to declare the method fit-for-purpose for clinical use, as well as exhibiting appropriate performance characteristics to be a candidate reference method (The Clinical & Laboratory Standards Institute, 2022). Being a quantitative in-house developed method, the validation parameters measured covered the minimum requirements

of ISO 15189 medical laboratory method validation standards and included accuracy, imprecision, specificity, quantification limits, linearity, recovery, dilution efficiency, matrix effect, and stability studies.

### 2.6.1 Assessment of Method Linearity

Assay linearity was assessed using a patient serum sample with a high concentration (5251.6 pmol/L), which was then diluted with the appropriate diluent to cover the analytical range of C-peptide. A series of dilutions (neat, 80%, 60%, 40%, and 20%) was prepared and analysed in duplicate (due to limited sample volume) within the same batch, and the correlation coefficient ($R^2$) was calculated. Linearity was also confirmed using the mean correlation coefficient of at least five standard calibration curves using linear regression analysis.

### 2.6.2 Imprecision Studies

In-house prepared quality control material (100 pmol/L, 400 pmol/L, 900 pmol/L and1400pmol/L and pooled patient samples (35 pmol/L, 250 pmol/L, 500 pmol/L, 700 pmol/L and 1500 pmol/L) were analysed repeatedly 10 times within the same batch and 5 times between batch to assess the precision of the method.

### 2.6.3 Method Sensitivity Studies

The Limit of blank (LoB) was assessed using Calibrator zero (blank sample), which was analysed repeatedly 10 times within a batch, and the mean response was calculated. The LoB was then calculated using the formula;
LoB = mean [blank] + 1.645 (SD [blank]) (Armbuster and Pry, 2008).
The Limit of Detection (LoD) was determined using data from the limit of blank and precision data of calibrator 1 and applying the following equation;
LoD = mean [blank] +3.3 (SD [blank]) + 1.645 (SD [Cal 1 concentration]) (Armbuster and Pry, 2008).

The Limit of quantitation (LoQ) was calculated using data from the Limit of blank using the formula;
LOQ = Mean blank + 10 (SD [blank]) (Armbuster and Pry, 2008)

### 2.6.4 An assessment of accuracy and measurement trueness

To assess accuracy, patient sample comparison studies were carried out with the Mercodia ELISA C-peptide method, which was the method used at the Peptide Hormones laboratory, Berkshire and Surrey Pathology services. At least eighty four surplus samples, covering the analytical range of 7.8 pmol/L to 8000 pmol/L, which had already been processed for C-peptide levels using the Mercodia C-peptide ELISA, were analysed using the LC-MS/MS method. The samples were analysed in duplicate within small batches, which span assay re-calibrations.
The results between the two methods were compared using the Association for Laboratory Medicine Excel method comparison spreadsheet.
Measurement trueness was assessed using EQA material from two EQA schemes: past EQA distributions from the UK NEQAS Guildford Peptide Hormones scheme based in the United Kingdom and enrolment in the CAP quality-based EQA scheme in the USA. The appropriate samples were obtained from the relevant EQA scheme and analysed by the LC-MS/MS method. The results from the UKNEQAS Guildford peptide hormones were compared with the ALTM (All Laboratory Trimmed Mean), as there was no LCMS method group in the scheme. The results for the CAP EQA were submitted to the scheme, and the evaluation report was received at the close of the distribution.

### 2.6.5 Assessment of carryover and recovery

A sample with a very low concentration of 35 pmol/L C-peptide and a high concentration of 4500 pmol/L was used for the carryover experiment. Three aliquots of a high concentration sample were measured, followed by three aliquots of a low concentration sample. This set of six measurements was repeated several times, each time using fresh aliquots of the sample, and the carryover was calculated. Percentage recovery was assessed by spiking a patient sample of

concentration 218 pmol/L at low (100 pmol/L), medium (1000 pmol/L), and high (2000 pmol/L) levels with Cerrilliant C-peptide standard. Each spiked aliquot was run in triplicate, and the recovery was calculated.

### 2.6.6 Dilution Integrity Studies

Serial dilutions of a sample with a high C-peptide concentration of 3089 pmol/L were used to assess the dilution integrity of patient C-peptide concentrations that fell outside the analytical range during clinical use. Serial dilutions of x5, x10, x20, and x50 were carried out in LCMS grade water; samples were analysed and the dilution integrity was estimated by calculating the percentage difference.

### 2.6.7 Assessment of matrix effects

Both ion suppression and enhancement are problems that have to be dealt with because they can be unpredictable depending on the sample from patient to patient. An assessment of the matrix effect was carried out both qualitatively and quantitatively. The qualitative matrix effect was assessed by injecting 30 µL mobile phase (0.1 formic acid in 50:50 ACN/ $H_2O$) followed by a concurrent direct infusion of 30 µL of Calibrator 11, which contained 4000 pmol/L C-peptide standard with a 2000 pmol/L Try(C)-peptide internal standard. The chromatograms were inspected for areas of ion suppression or enhancement.

The quantitative matrix effect was assessed by pre-spike recovery experiments using purchased human serum, bovine serum, and PBS/SBA solutions spiked at a low concentration of 100 pmol/L and a high concentration of 4000 pmol/L C-peptide. This was analysed, and the percentage matrix effect (percentage difference in concentration levels) and normalised matrix effect (percentage difference in the responses) were calculated. Further assessment of the matrix effect was carried out by standard addition to assess if bovine serum was suitable for preparing the calibrators. A sample with an unknown concentration was spiked at various concentrations with the C-peptide standard and analysed. The peak

area count of the neat sample and spiked sample was used in a standard addition curve to determine the concentration of the sample and was compared to the sample concentration determined using a calibration curve.

### 2.6.8 Interference studies

Specificity was confirmed by assessing the effect of interference due to common, known, or potential interfering substances. The effects of lipaemia (L), icterus (I), haemolysis (H), and high levels of proinsulin, insulin, and creatinine were evaluated using surplus patient samples spiked with either PBS/SBA buffer or an increasing concentration of potential interferent, as shown in the table 2.5 below.

Table 2. 5 Concentrations of Interferons used in the Interference Studies

| Interferon | Spike 1 concentration | Spike 2 concentration | Spike 3 concentration |
|---|---|---|---|
| **Lipaemia** | 33.9 mmol/L | 16.95 mmol/L | 8.48 mmol/L |
| **Icteric** | 42.76 µmol/L | 85.52 µmol/L | 171.04 µmol/L |
| **Haemoglobin** | 5 g/L | 10 g/L | 20 g/L |
| **Creatinine** | 1.105 mmol/L | 2.210 mmol/L | 4.420 mmol/L |

For proinsulin interference studies, 5 samples with concentrations of 86 pmol/L, 328 pmol/L, 764 pmol/L, 991 pmol/L, and 1803 pmol/L were each spiked with 5000 pmol/L First international Standard for human Pro-insulin (09/296). For assessment insulin interference, two samples with C-peptide concentrations of 300 pmol/L and 1000 pmol/L were each spiked with 5000 pmol/L, 15000 pmol/L, 25000 pmol/L, 50000 pmol/L and 100000 pmol/L Second International Standard for Insulin (11/212). For each of the samples analysed, the bias was calculated to assess interference.

### 2.6.9 Stability Studies

Stability studies assessed the stability of samples in different tube types (serum separator tubes, EDTA plasma, and Lithium heparin plasma) stored in an ultralow frozen temperature freezer (−80°C), in a freezer (−20°C), in a fridge (4°C), and at room temperature (21°C) in the short term (24hrs and 48hrs) and over the long term (2 months and 6 months). Samples collected in the three collection tubes were split into several 50 µL aliquots, and at least 10 of each were stored at the various temperatures. The serum sample was analysed on the first day of collection to serve as the baseline. Aliquots were also analysed on day two, day four, month two, and month six. Each sample was run in triplicate, and the percentage bias was calculated.

## 2.7 DBS Method Verification

CLSI has provided guidance for the introduction of new specimen types with dissimilar matrices into clinical laboratories (The Clinical & Laboratory Standards Institute, 2019). This ensures that studies focus on verifying that performance is consistent with appropriate clinically based performance goals specific to the new specimen type, such that the laboratory would not necessarily need to undertake full method validation. Parameters assessed include the calibration curve, LOQ, evaluation of the systematic difference, and comparison of precision at each medical decision point (300, 700, 900, and 1800 pmol/L).

### 2.7.1 Comparison of Precision between Serum and DBS

Imprecision studies were carried out using samples with concentrations covering the clinical decision points (350, 700, 900, and 1800 pmol/L). The precision of each specimen type was obtained from the results of at least five replicates. The $SD_{DBS}/SD_{SERUM}$ (SD ratio) was calculated with a ratio of less than or equal to 1.00 being acceptable. Ratios over 1.00 were only acceptable if the CV of the imprecision was within the allowable bias of 8.3%, which was set using the desirable specification from the Westgard Biological Variation database.

### 2.7.2 Evaluation of Systematic Differences between DBS and Serum

Comparative analysis between serum and DBS samples was carried out to evaluate systematic differences, ensuring samples covered the medical decision concentration levels. In the absence of paired patient serum and DBS samples, a minimum of 84 samples each of spiked bovine serum and equine whole blood at various concentrations were used for comparison studies. This was done by adding 200 µL of bovine serum to 84 Eppendorf tubes. Similarly, 200 µL of equine whole blood was added to 84 Eppendorf tubes.

A standard C-peptide spiking solution was prepared with concentrations of 4000 pmol/L, 16000 pmol/L and, 24,000 pmol/L. The standard was spiked into samples by adding the same volume to the bovine serum and equine whole blood. The volume added was from 2 µL to 20 µL with an incremental volume of 0.5 µL (i.e., 2 µL was added to the bovine serum, 2 µL added to equine whole blood as the first paired sample, 2.5 µL was added to the second pair, 3 µL was added to the third pair, etc.). The generated samples range from 50 pmol/L to 400 pmol/L.

Similarly, 6 µL to 20 µL of the 16,000 pmol/L standard were spiked, an incremental volume of 0.5 µL to form samples from 480 pmol/L to 1600 pmol/L. To cover a high concentration range, 14 µL to 32 µL of the 24000 pmol/L C-peptide solution were added in an incremental volume of 0.5 µL to form samples from 1680 pmol/L to 6000 pmol/L.

The equine whole blood samples were spotted on the DBS card, dried, and stored at -20°C until analysis. The bovine serum was also stored at -20°C in aliquots of 50 µL until analysis. The bovine serum samples were run as a batch, as were the equine whole blood samples, all in duplicate. Systemic differences were calculated using Passing-Bablok regression and compared to the total allowable error.

### 2.7.3 Effect of hematocrit on DBS samples

Whole blood samples with hematocrit values of about 20 to 80% were prepared by removing and adding plasma to equine whole blood with a known haematocrit concentration. This was done by first spiking 20 mL of equine whole blood with Cerrilliant C-peptide standard to a concentration of 1000 pmol/L, and then aliquots were taken. The red cells of the 20 mL equine whole blood were allowed to settle in a universal container, and 10 mL of the plasma at the top was removed. The remaining whole blood sample was mixed, and aliquots were taken as sample two. 5mL of the plasma was added back to the remaining whole blood in the universal container, mixed, and aliquots taken as sample 3. Finally, 2 mL of the remaining plasma was added to the whole blood and again mixed and aliquots were taken as sample 4. The modified hematocrit values were verified by analysis using the Abbott Alinity Haematology Analyser. The samples were then spotted on a DBS card and analysed for recovery. A mean deviation of less than 15% was considered acceptable.

### 2.7.4 Stability of DBS samples

The stability of C-peptide was evaluated over time under different storage conditions (room temperature, 4°C, -20°C, and -80°C). DBS cards spotted with whole blood at various C-peptide concentrations were stored at room temperature, in the fridge (4°C), freezer (-20°C), and -80°C freezer. A set of samples was processed immediately after preparation to serve as the control, and then different stored sets were extracted and run weekly for room-temperature storage. Samples stored at -20°C, and -80°C were run at the end of each month for a total of 4 and 6 months, respectively. Each sample was run in triplicate, and the bias was calculated.

## 2.8 Cost-benefit analysis

Cost implications using cost-outcome models (old cost vs. new cost) and the impact were carried out to assess the cost-effectiveness of the LC-MS/MS method. Modelling was carried out with two scenarios: full implementation using serum or plasma as sample types and full implementation using DBS samples only. Both cost models were compared to the cost of running a C-peptide clinical service using the Mercodia ELISA test method.

## Chapter 3.0 Results and Discussion: Serum and Plasma Method

### 3.1 C-peptide LC-MS/MS method development and validation Report

#### 3.1.1 Analyte tuning and optimised mass spectrometry conditions

Unlike small molecules, which form singly charged precursors, C-peptide has a molecular weight of 3020.29 Daltons, which falls outside the mass range of a typical mass spectrometer (usually 500 to 2000 Daltons) and therefore can only be detected as a multiply charged species. The multiply charged ions ($(M+2H)^{2+}$ $(M+3H)^{3+}$ $(M+4H)^{4+}$) were initially selected as precursor ions for tuning, and for greater specificity and sensitivity, the precursor and fragments with the highest signal-to-noise under the optimal conditions (Table 3.1) were selected.

Table 3. 1 Mass spectrometry optimised conditions

| Source (ES+) | Settings |
|---|---|
| Capillary (kV) 3.32 | 3.2 |
| Cone (V) 130.90 | 45 |
| Source Offset (V) | 30 |
| Source Temperature (°C) | 150 |
| Desolvation Temperature (°C) | 600 |
| Cone Gas Flow (L/Hr) | 150 |
| Desolvation Gas Flow (L/Hr) | 1000 |
| Collision Gas Flow (mL/Min) | 0.16 |
| Nebuliser Gas Flow (Bar) | 6.7 |
| LM 1 Resolution | 1.9 |
| HM 1 Resolution | 15.2 |
| Ion Energy 1 | 0.6 |
| MS Mode Collision Energy | 48 |
| LM 2 Resolution | 2.9 |
| HM 2 Resolution | 15.2 |
| Ion Energy 2 | 1.2 |

The $(M+3H)^{3+}$ protonated ions were selected as the parent ions for the C-peptide standard and (Tyr$^{o}$)-C-Peptide internal standard. Table 3.2 below shows the qualifier and quantifier transition ions consistent throughout the experiment selected for the chromatographic method development.

Table 3. 2 Selected transitions showing the quantifier and qualifier ions

| | Parent ions (Da) | Daughter ions (Da) | Dwell(s) | Cone voltage(V) | Collision energy (eV) |
|---|---|---|---|---|---|
| Quantifier ion | 1007.46 | 147.19 | 0.195 | 45 | 20 |
| Qualifier ion | 1007.46 | 130.08 | 0.195 | 45 | 22 |
| Internal Standard | 1062.16 | 260.2 | 0.195 | 45 | 34 |

The method gave well-resolved initial tuning peaks for the Cerilliant C-peptide standard, with a typical mass spectrum represented in Figures 3.1 and 3.2 below. The mass spectrum for the (Tyr$^{o}$)-C-Peptide internal standard is shown in Figures 3.3 and 3.4 The mass spectrum shows the peaks and masses of selected parent and daughter ions, as well as the signal-to-noise ratio.

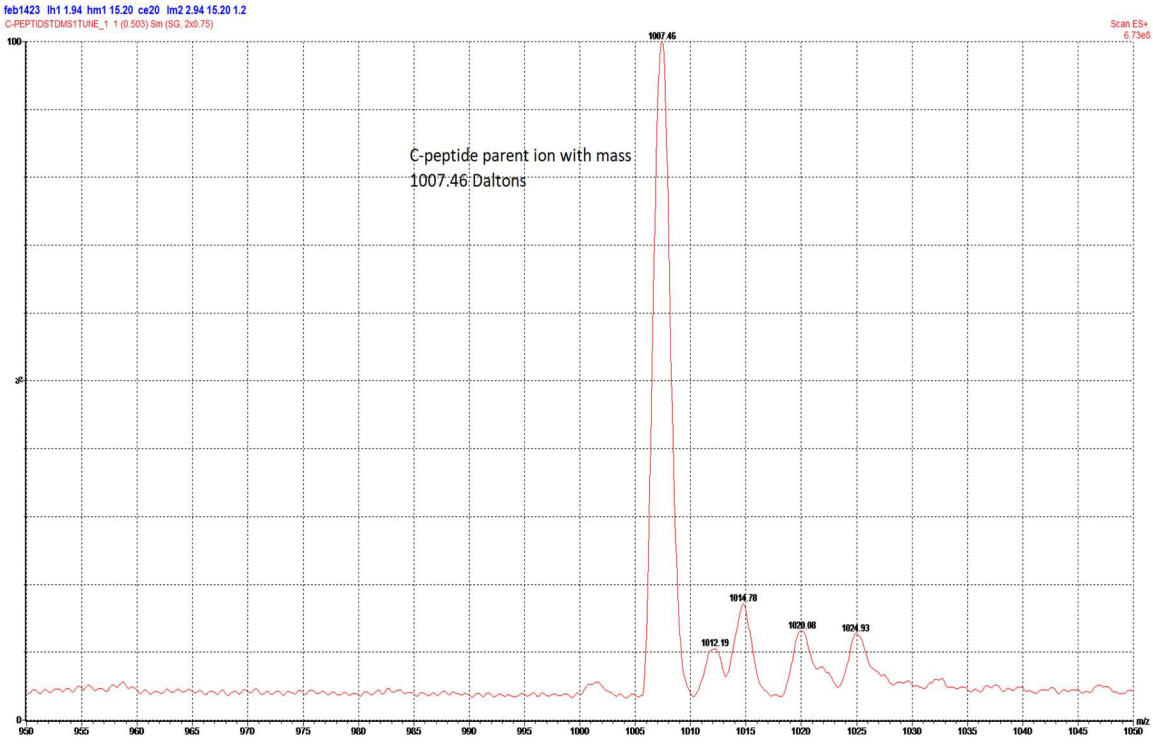



Figure 3. 1 Representation of mass spectrum for C-peptide

The figure shows the parent ion with a mass of 1007.46 Daltons for the Cerilliant C-peptide standard.

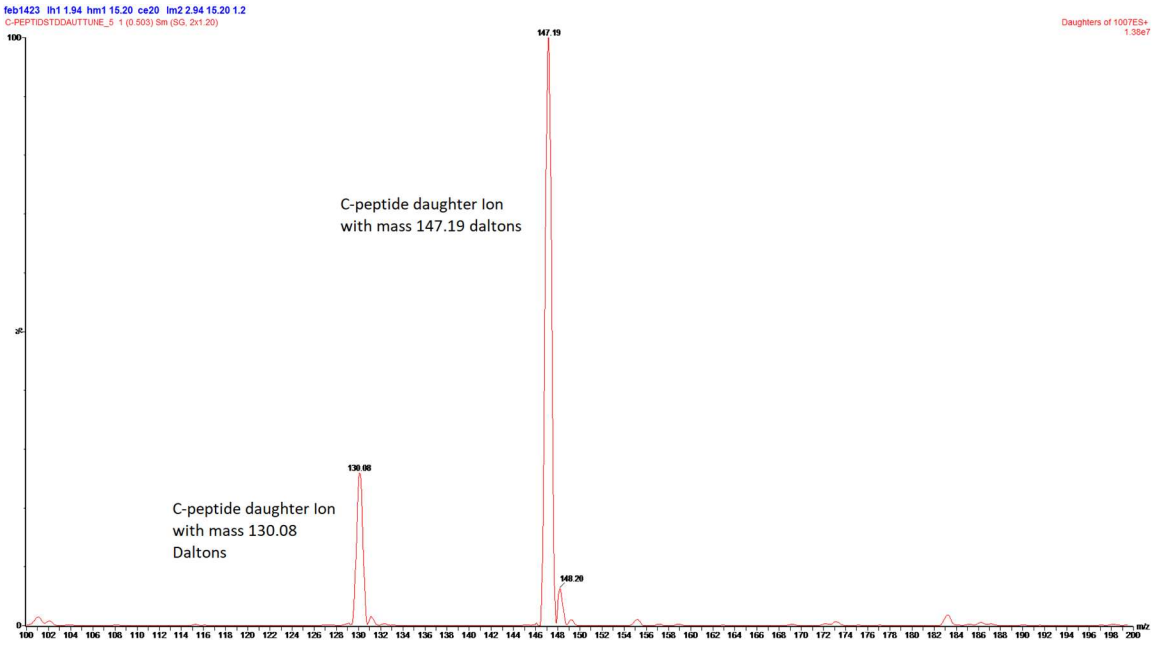



Figure 3. 2 Mass spectrum of C-peptide daughter ions

The figure is a representation of the masses of the quantifier and qualifier daughter ions 147.1 and 130.08 respectively from analysis of the Cerilliant C-peptide standard.

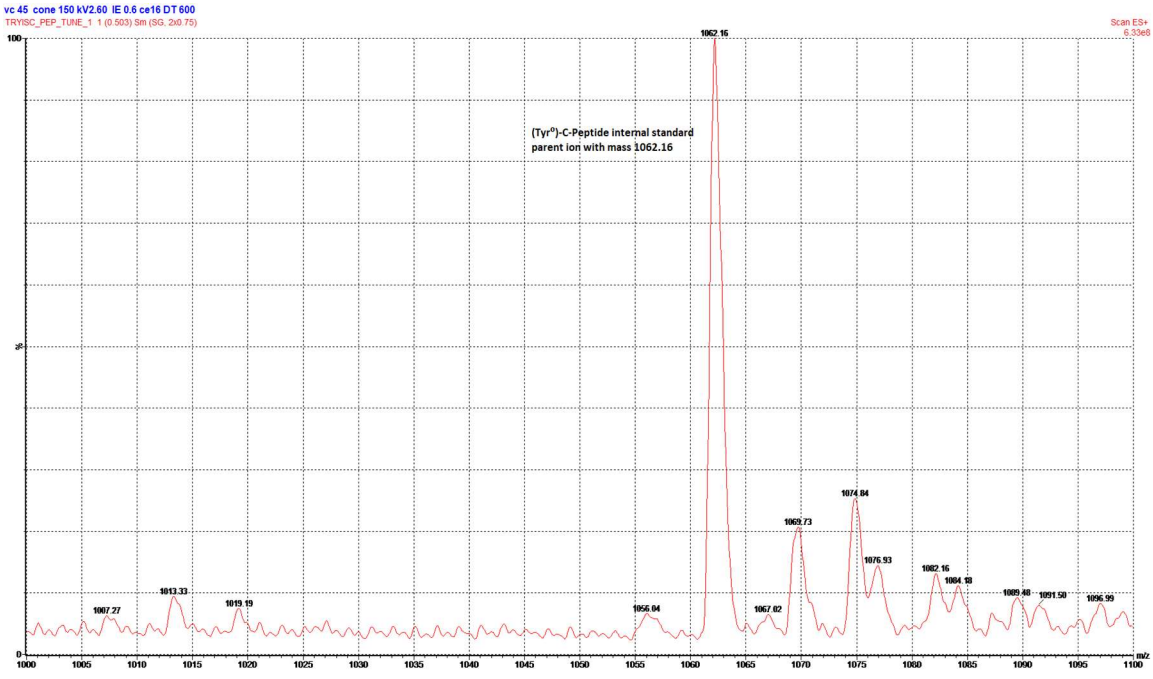



Figure 3. 3 Mass spectrum of the (Tyr⁰)-C-Peptide internal standard parent ion

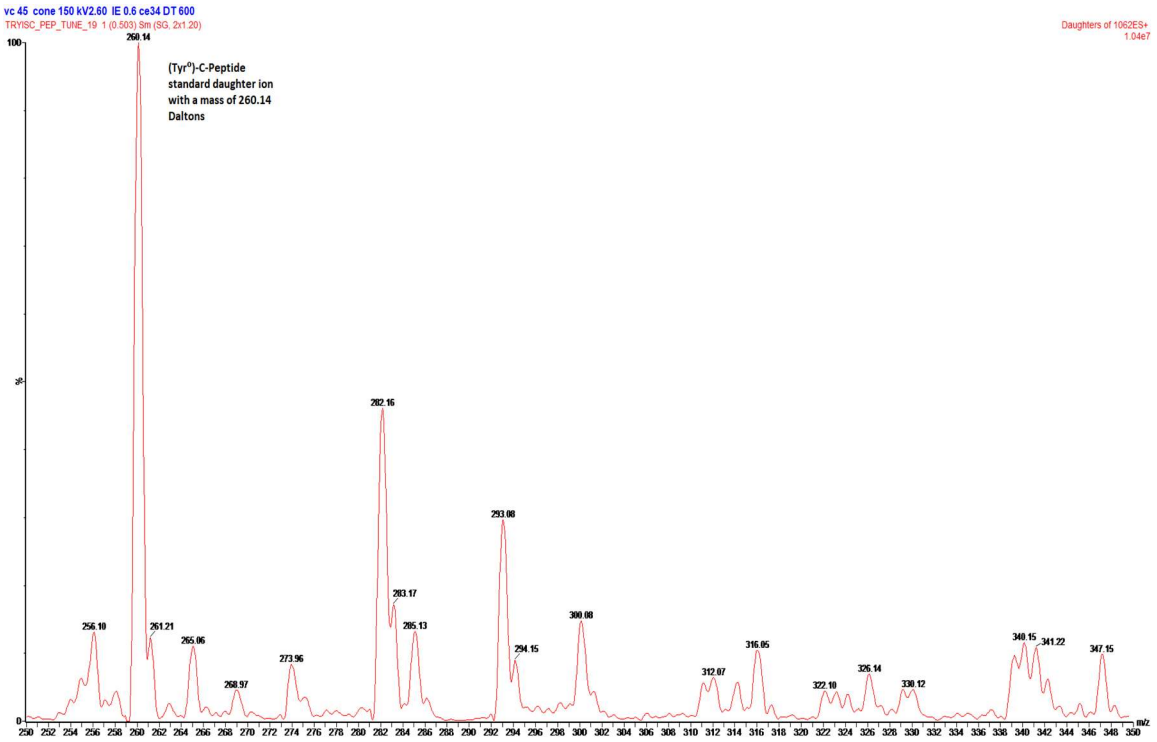



Figure 3. 4 Mass spectrum of (Tyr⁰)-C-Peptide internal standard daughter ion

### 3.1.2 Chromatographic conditions

Consistent transitions and high sensitivity were found with 0.1%v/v formic acid as an additive for water (mobile phase A) and acetonitrile (mobile phase B) compared to other additives such as DMSO, TFE and TFA. The chromatographic gradient slope was manipulated by changing the percentage of organic at start and finish to achieve the desired conditions shown in Table 3.3. A steeper gradient slope resulted in a reduction in resolution and an increase in sensitivity, while a shallower gradient slope resulted in an increase in resolution and a decrease in sensitivity. The flow rate was optimised followed by temperature. A flow rate of 0.250 mL/minute and a column temperature of 60°C improved the analyte peak shape, as shown in the chromatogram below (Figure 3.5). The chromatogram shows the achieved retention times for the C-peptide standard (6.40 minutes) and internal standard (6.41 minutes).

Table 3. 3 The optimal gradient and LC conditions

(Mobile phase A is 0.1v/v formic acid in $H_2O$ and Mobile phase B is 0.1v/v formic in ACN)

| Gradient | Time (min) | Flow rate (mL/min) | % Mobile phase A | %Mobile Phase B |
|---|---|---|---|---|
| 1 | initial | 0.250 | 85 | 15 |
| 2 | 4.0 | 0.250 | 85 | 15 |
| 3 | 4.3 | 0.250 | 75 | 25 |
| 4 | 5.0 | 0.250 | 65 | 35 |
| 5 | 5.3 | 0.250 | 55 | 45 |
| 6 | 5.5 | 0.250 | 35 | 65 |
| 7 | 5.7 | 0.250 | 15 | 85 |
| 8 | 8.0 | 0.250 | 85 | 15 |

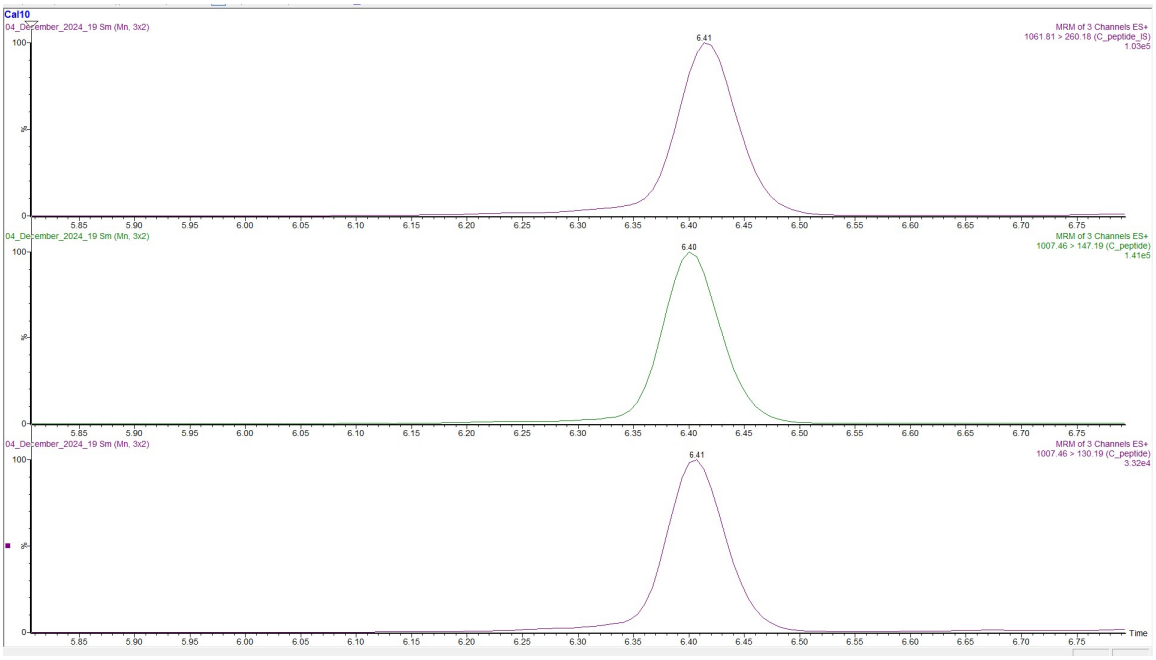



Figure 3. 5 Typical chromatograms of the expected transition ions from the infusion of C-peptide standard in a 6:3:1 ratio of methanol/ $H_2O$/acetic acid.

The figure shows (Tyr$^{0}$)-C-Peptide internal standard with transitions 1062.16 >120.2 and retention time 6.45 minutes (top peak) and C-peptide standard with transitions 1007.46 > 147.19 and retention time 6.43 minutes (bottom peak).

Figure 3.6 below shows how the performance parameters ($A_s$, N and K) was calculated using the chromatographic peaks.

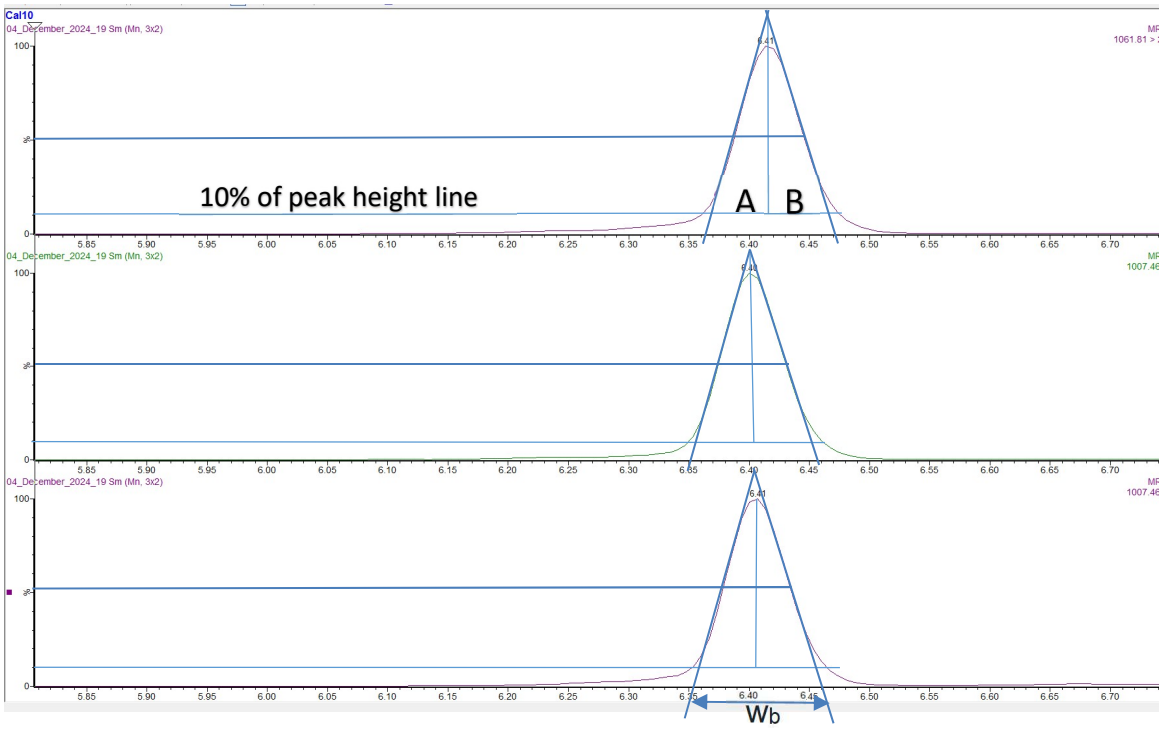



Figure 3.6 Estimation of $A_s$, N, and K from the chromatographic peak.

(The figure shows that the distance from the peak's midpoint to its trailing edge (B) was 2 cm, and the distance from the peak's midpoint to its leading edge (A) was 1.95 cm. $w_b$ was 0.108 minutes (6.46-6.352)).

$A_s$ using equation (2.1) = 2/1.95 = 1.0256, which was within the acceptable limits of 1.0 -1.2, which confirms the absence of peak tailing or fronting.

The Cortecs C18 1.6 µM 2.1 x 100 mm column internal radius (r) was 1.05 mm with a column length (L) of 100 mm. The flow rate and retention time were 0.25 ml/min and 6.40, respectively.

Void volume using equation (2.4) = 3.142 $(1.05)^2$ x 100 x 0.70 = 242.48 µl = 0.242 ml.

Void time using equation (2.3) = 0.242/0.25 = 0.968 minutes

K using equation (2.2) = (6.40 - 0.9699) / 0.9699 = 5.59, which was greater than the acceptable minimum of 2. A high K value of 5.59 indicates that the sample was highly retained with a good separation.

N using equation (2.6) = 16 $(6.40/0.108)^2$ = 56,186. A high number of theoretical plates of 56,186 indicates an efficient column.

Plate height using equation (2.5) = 100/56,186 = 0.00178. A low plate height of 0.00177 indicates a high-efficiency separation. The chromatographic peak asymmetry, capacity factor, and plate height were all acceptable, as shown in Table 3.4 below.

Table 3.4 Chromatographic performance parameters of the C-peptide method.

| Chromatographic Parameter | C-peptide Method |
|---|---|
| Column temperature | 40 °C |
| Retention time | 6.40 |
| Retention factor | 5.59 |
| Theoretical plates | 56.186 |
| Peak Asymmetry | 1.0256 |
| Plate Height | 0.00178 |
| Sample volume | 30µL |

## 3.2 Method validation report for serum and lithium heparin plasma

### 3.2.1 Calibration curve for serum and plasma

Table 3.5 below highlights the data for the calibration study, which shows the concentrations used for a 12-point calibration curve. The percentage difference between the expected and observed concentrations was within 10% for all the calibration points, which was acceptable for both serum and plasma. Figures 3.7

below represent a typical calibration curve that shows good linearity across the concentration range of the standard curve with an $R^2$ of 0.9999.

Table 3. 5 Calibration expected and observed standard concentrations.

| Quantify Compound Summary Report | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Printed Sat Apr 15 16:04:22 2023 | | | | | | | | | | | | | |
| Compound 1: C_peptide | | | | | | | | | | | | | |
| # | Name | Type | Sample Text | ID | Std. Conc | RT | Area | IS Area | Response | Primary | pmol/L | %Dev | S/N |
| 1 | 14_April_2023_16 | Standard | Calibrator0 | Calibrator0 | 0 | | | 5611.19 | | | | | |
| 2 | 14_April_2023_17 | Standard | Calibrator1 | Calibrator1 | 3.91 | 6.42 | 29.903 | 6212.68 | 0.005 | db | 3.7 | -5 | 7.15 |
| 3 | 14_April_2023_18 | Standard | Calibrator2 | Calibrator2 | 7.81 | 6.43 | 43.572 | 5573.83 | 0.008 | bb | 8 | 2.2 | 21.403 |
| 4 | 14_April_2023_19 | Standard | Calibrator3 | Calibrator3 | 15.63 | 6.43 | 83.604 | 6301.39 | 0.013 | db | 15.7 | 0.6 | 12.826 |
| 5 | 14_April_2023_20 | Standard | Calibrator4 | Calibrator4 | 31.25 | 6.43 | 141.836 | 6413.48 | 0.022 | bb | 28.3 | -9.5 | 27.455 |
| 6 | 14_April_2023_21 | Standard | Calibrator5 | Calibrator5 | 62.5 | 6.43 | 317.807 | 6563.09 | 0.048 | db | 65.7 | 5.1 | 138.934 |
| 7 | 14_April_2023_22 | Standard | Calibrator6 | Calibrator6 | 125 | 6.43 | 580.777 | 6844.45 | 0.085 | bb | 117.4 | -6.1 | 197.36 |
| 8 | 14_April_2023_23 | Standard | Calibrator7 | Calibrator7 | 250 | 6.43 | 1091.67 | 5923.73 | 0.184 | bb | 258.6 | 3.5 | 110.303 |
| 9 | 14_April_2023_24 | Standard | Calibrator8 | Calibrator8 | 500 | 6.43 | 2313.43 | 6190.86 | 0.374 | bb | 527.7 | 5.5 | 570.646 |
| 10 | 14_April_2023_25 | Standard | Calibrator9 | Calibrator9 | 1000 | 6.43 | 4564.36 | 6458.74 | 0.707 | bb | 1000.7 | 0.1 | 700.882 |
| 11 | 14_April_2023_26 | Standard | Calibrator10 | Calibrator10 | 2000 | 6.43 | 8690.97 | 5989.63 | 1.451 | bb | 2057.9 | 2.9 | 2521.546 |
| 12 | 14_April_2023_27 | Standard | Calibrator11 | Calibrator11 | 4000 | 6.43 | 18173.6 | 6209.27 | 2.927 | bb | 4154.2 | 3.9 | 6271.43 |
| 13 | 14_April_2023_28 | Standard | Calibrator12 | Calibrator12 | 8000 | 6.43 | 36657.6 | 6708.59 | 5.464 | bb | 7758.3 | -3 | 7650.552 |

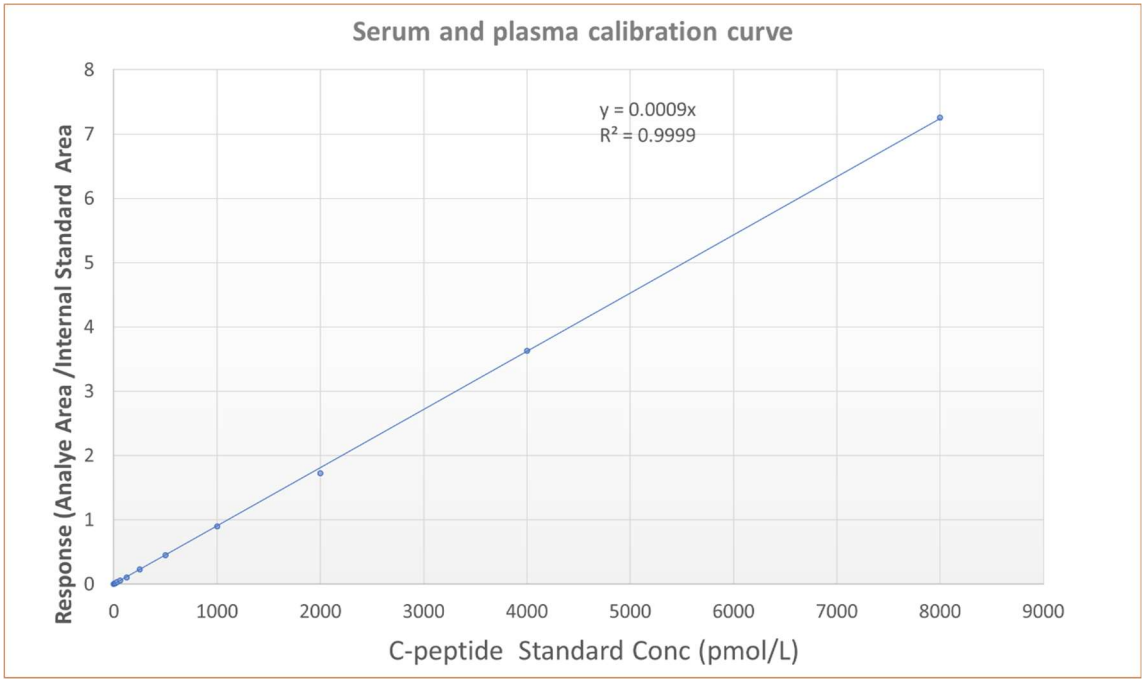



Figure 3. 7 Representation of a typical C-peptide calibration curve

### 3.2.2 Linearity

The linearity of the serum and lithium heparin standard curve was demonstrated by the mean correlation coefficient ($R^2$) values for five curves from separate runs, as shown in Table 3.6. The mean $R^2$ value for C-peptide was greater than 0.99 and demonstrated the linearity of the concentration range of the standards.

Table 3. 6 Correlation Coefficient Values for Five Standard Curves.

| CURVE | C-peptide ( $R^2$) |
|---|---|
| **1** | 0.9994 |
| **2** | 0.9999 |
| **3** | 0.9997 |
| **4** | 0.9998 |
| **5** | 0.9989 |
| **MEAN** | 0.9995 |

The linearity using a high-concentration patient sample of 5251 pmol/L, with a series of dilutions (neat, 80%, 60%, 40%, and 20%) (Table 3.7) also demonstrated a correlation coefficient ($R^2$) value of 0.9994 (Figure 3.8), which was within the acceptable limit of >0.99, therefore, the assay was considered linear across the range tested. The analytical range of the assay was confirmed as 3 pmol/L – 8000 pmol/L. This was considered to cover the clinically relevant range of C-peptide.

Table 3. 7 Linearity data using a high C-peptide concentration patient sample

| | pepide | Observed C-peptide (pmol/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (pmol/L) | Rpt 1 | Rpt 2 | Rpt 3 | MEAN | SD | Mean (0dp) | % difference | Abs difference | |
| Neat | 5251.6 | 5255.2 | 5221.2 | 5278.4 | 0.0 | 28.8 | 0 | | | |
| 1 in 2 | 2625.8 | 2553.9 | 2571.7 | 2567.1 | 2564.2 | 9.2 | 2564 | -2% | -62 | |
| 1 in 3 | 1750.5 | 1767 | 1747.4 | 1790.1 | 1768.2 | 21.4 | 1768 | 1% | 18 | |
| 1 in 4 | 1312.9 | 1325.5 | 1329.6 | 1313.6 | 1322.9 | 8.3 | 1323 | 1% | 10 | |
| 1 in 5 | 1050.32 | 1057.8 | 1073 | 1092.5 | 1074.4 | 17.4 | 1074 | 2% | 24 | |

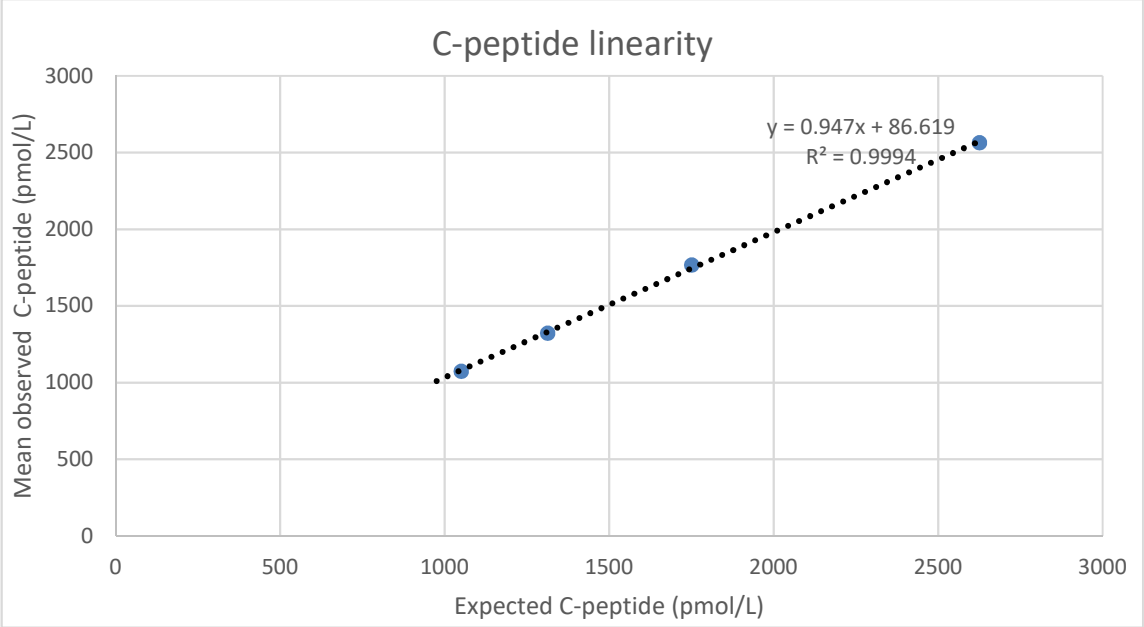



Figure 3. 8 Linearity using serial dilution of a high-concentration sample

Calibration verification using CAP EQA scheme samples was also carried out. The CAP programme satisfies the requirements for scheduled calibration verification and verification of the analytical measurement range as specified in the CAP Laboratory Accreditation Programme and Current Clinical Laboratory Improvement Amendments (CLIA) Regulations Section 493.1255 for most analytes. Acceptable impression and non-linearity are specified using the goal for total error and the mean of the included results. The results (Figure 3.9) show an acceptable linear evaluation from 0.08 to 30.325 ng/mL. (26.49 to 10,040 pmol/L)

EVALUATION
REVISION

**LN46-B 2023 C-Peptide/Insulin Calibration Verification/Linearity**
**C-Peptide ng/mL Linearity Evaluation**

**Evaluation Result: Linear from 0.080 to 30.325**
Method: OTHER

Evaluation Type: Standard
Goal for Total Error (TE): 25%

| Specimen | Assay 1 | Assay 2 | Your Mean | Best-fit Target | Relative Concentration |
|---|---|---|---|---|---|
| LN46-08 | 0.09 | 0.07 | 0.080 | 0.005 | 0.000 |
| LN46-09 | 0.39 | 0.41 | 0.400 | 0.307 | 0.010 |
| LN46-10 | 7.25 | 7.65 | 7.450 | 7.563 | 0.250 |
| LN46-11 | 14.46 | 15.41 | 14.935 | 15.122 | 0.500 |
| LN46-12 | 22.48 | 22.97 | 22.725 | 22.680 | 0.750 |
| LN46-13 | 30.67 | 29.98 | 30.325 | 30.238 | 1.000 |

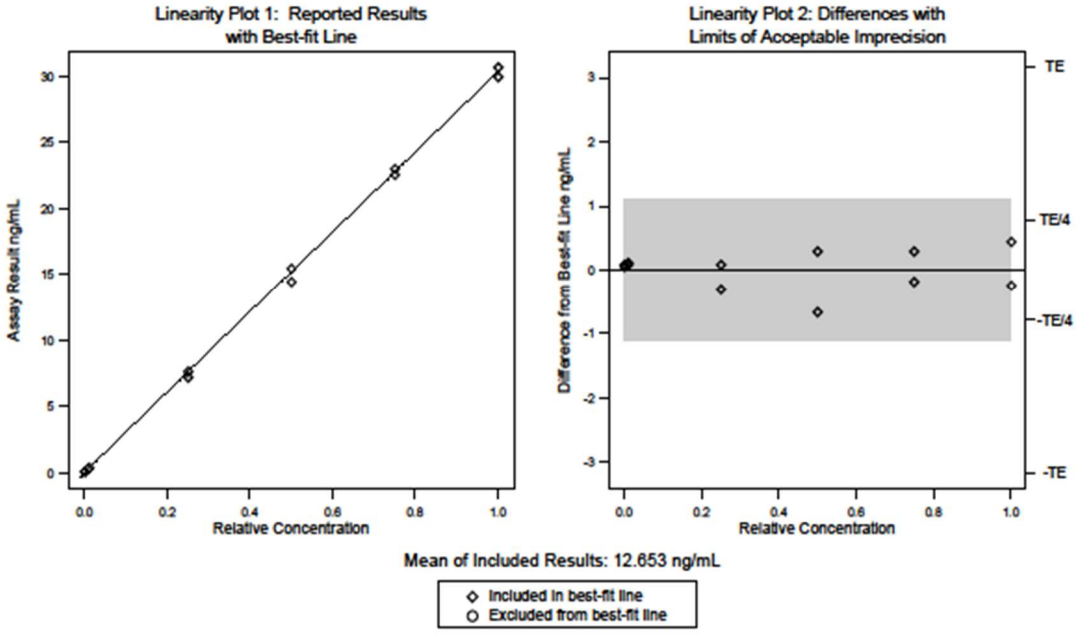



Figure 3. 9 CAP EQA calibration verification and linearity evaluation report.

### 3.2.3 Measurement accuracy and assessment of trueness

Patient samples (91) that had previously been analysed for C-peptide using the Mercodia C-peptide Elisa assay were re-analysed using the new LC-MS/MS method. The Deming and linear regression graph (Figure 3.10) shows a linear relationship and dispersion of results for both methods, with an $R^2$ value of 1.0 and a slope of 0.9313. The Bias plots show an average relative bias of -1.46% between the assays (Figure 3.11). The partitioning of the results into three concentration intervals of 0-300 pmol/L (12), 300-1000 pmol/L (44), and 1000-5000 pmol/L (34) shows a bias of -2.0%, -1.4%, and -0.3%, respectively, which was within the accepted limit of +/- 5%.

The bias indicates a review of reference ranges will not be needed to replace the Mercodia Elisa assay.

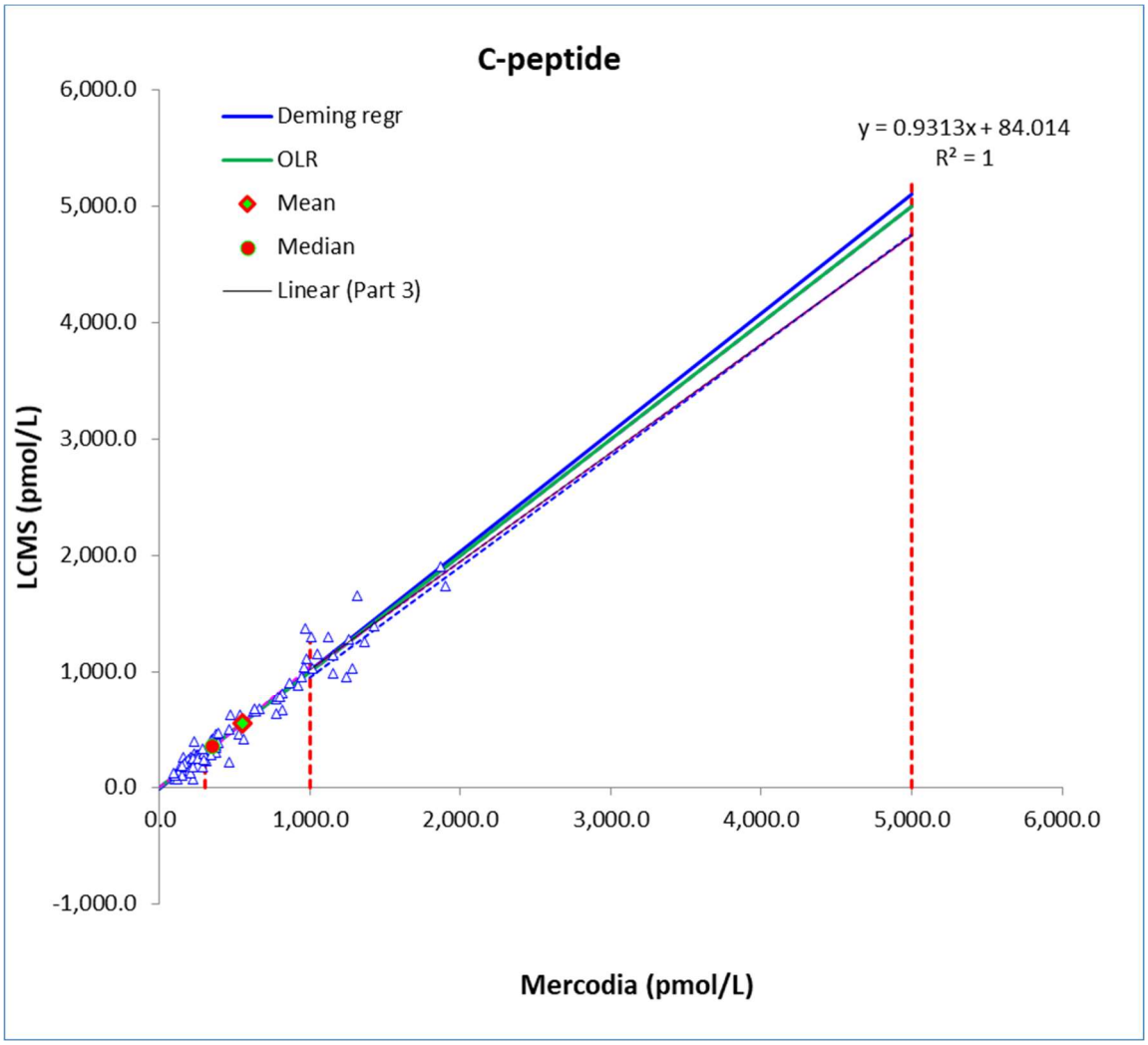



Figure 3. 10 Patient sample comparison between Mercodia and LC-MS/MS assays. Agreement has been assessed by Deming and linear regression.

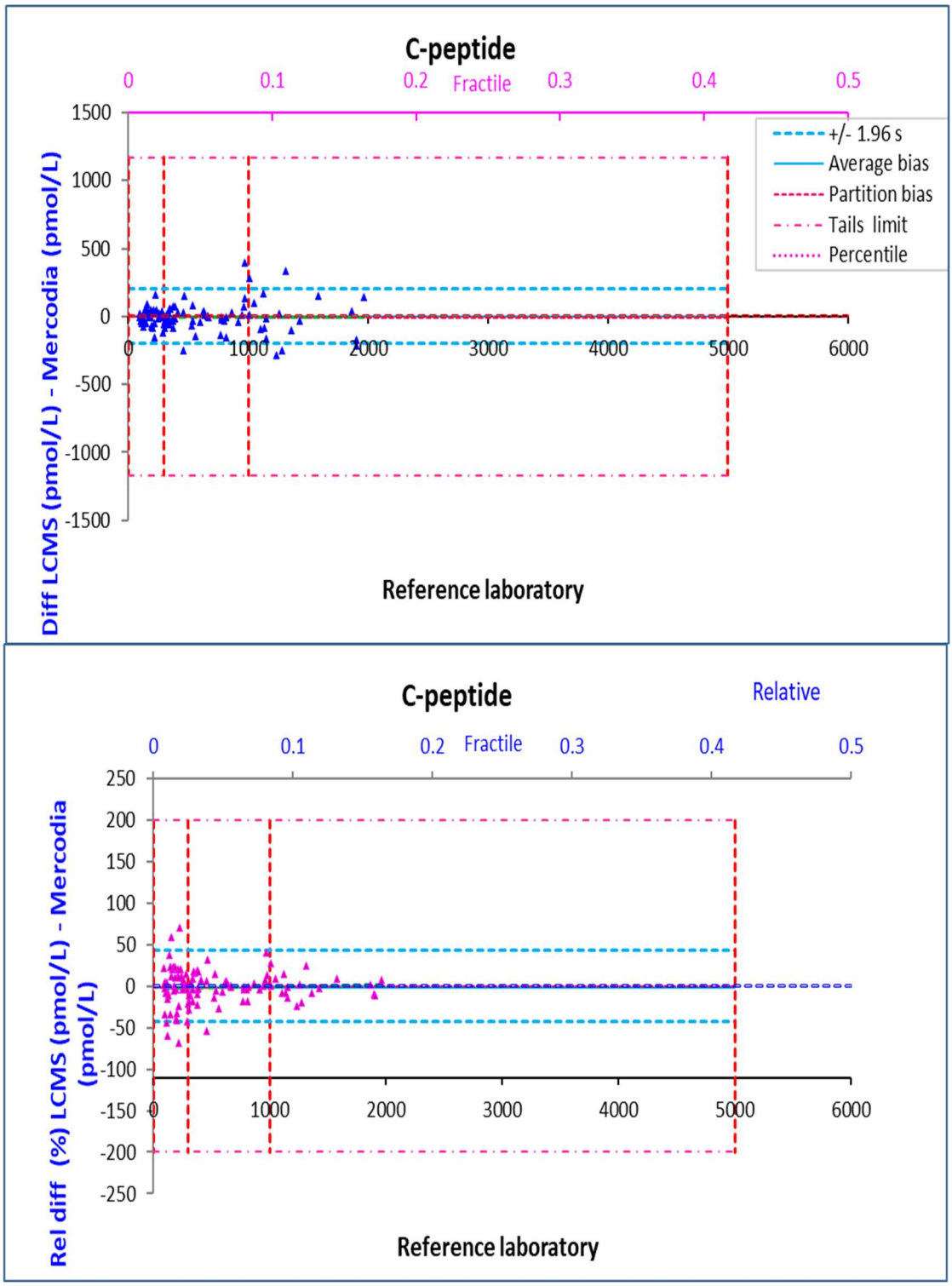



Figure 3. 11 Difference plots showing patient sample bias between methods

The plot shows the mean bias (top graph) and relative percentage difference (bottom graph) between the LC-MS/MS method and the Mercodia ELISA kit.

To assess the trueness of this method, EQA material from the UK NEQAS Guildford Peptide Hormones scheme was analysed, and the results obtained compared to the All Lab Trimmed Mean (ALTM) value. There was no LC-MS/MS method group within the scheme, so the method was in addition compared to an immunoassay, the Siemens Immulite 2000 method group which had the highest number of participants. The results were compared using Bland Altman and Passing Bablok plots. The data obtained from 14 UK NEQAS distributions is shown in Table 3.8. These results showed no significant difference between the expected UK NEQAS ALTM and the obtained result using the LC-MS/MS method, despite a slight negative bias (Figures 3.12 to 3.15**)**. The Z-scores (Figure 3.16) were all within +/-2, which was acceptable. The LC-MS/MS results against the Siemens Immulite 2000 method group showed an $R^2$ of 0.9588, showing the methods were in good agreement (Figure 3.17).

Table 3. 8 EQA data comparison to the ALTM.

| EQA Distribution number | Xevo result 1 | Xevo result 2 | Xevo result Mean | Immulite 2000 Method mean | ALTM | Target SD | Deviation from Target | % CV | N | Standard uncertainty | Z-scores |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EQA N286 | 159.9 | 151.7 | 155.8 | 141.6 | 177.7 | 51.2 | -0.43 | 28.8 | 61 | 9.3 | -0.36 |
| EQA N305 | 530.7 | 519.6 | 525.15 | 649.7 | 727 | 107.9 | -1.87 | 14.8 | 54 | 20.8 | -1.92 |
| EQA N306 | 187 | 188.4 | 187.7 | 242.6 | 297.8 | 71.8 | -1.53 | 24.1 | 53 | 14 | -1.53 |
| EQA N307 | 481.4 | 456.2 | 468.8 | 651.3 | 678.5 | 91.3 | -2.30 | 13.4 | 55 | 17.4 | -2.31 |
| EQA N311 | 971.4 | 930.1 | 950.75 | 1130.5 | 1236.6 | 149.9 | -1.91 | 12.1 | 52 | 29.6 | -1.91 |
| EQA N313 | 322.2 | 322.2 | 322.2 | 358.3 | 416.3 | 90.9 | -1.04 | 21.8 | 55 | 17.3 | -1.04 |
| EQA N315 | 515.5 | 515.5 | 515.5 | 584.4 | 668.3 | 97.9 | -1.56 | 14.6 | 54 | 18.9 | -1.57 |
| EQA N317 | 274.8 | 273.7 | 274.25 | 379.2 | 435.3 | 80.4 | -2.00 | 18.5 | 54 | 15.5 | -2.00 |
| EQA N307 | 490.9 | 505.8 | 498.35 | 651.3 | 678.5 | 91.3 | -1.97 | 13.4 | 55 | 17.4 | -1.98 |
| EQA N310 | 552.4 | 576.6 | 564.5 | 634 | 726.2 | 122.8 | -1.32 | 16.9 | 48 | 24.9 | -1.20 |
| EQA N312 | 1377 | 1398.3 | 1387.65 | 1421.7 | 1571.2 | 180.7 | -1.02 | 11.5 | 52 | 35.7 | -1.02 |
| EQA N313 | 328.7 | 322 | 325.35 | 358.3 | 416.3 | 90.9 | -1.00 | 21.8 | 55 | 17.3 | -1.00 |
| EQA N317 | 313.6 | 314.5 | 314.05 | 379.2 | 435.3 | 80.4 | -1.51 | 18.5 | 54 | 15.5 | -1.51 |
| EQA N318 | 643.3 | 663 | 653.15 | 836.2 | 876.4 | 108.7 | -2.05 | 12.4 | 50 | 21.5 | -2.02 |

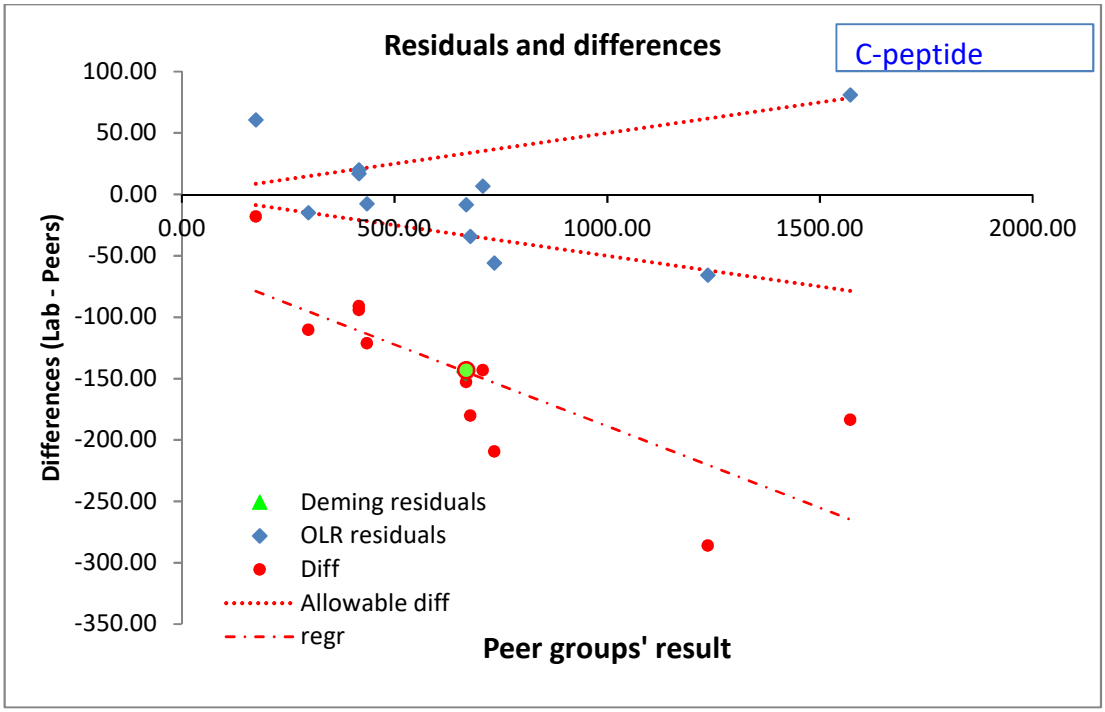



Figure 3. 12 Residuals and difference plots for UK NEQAS material using the ALTM as the performance target.

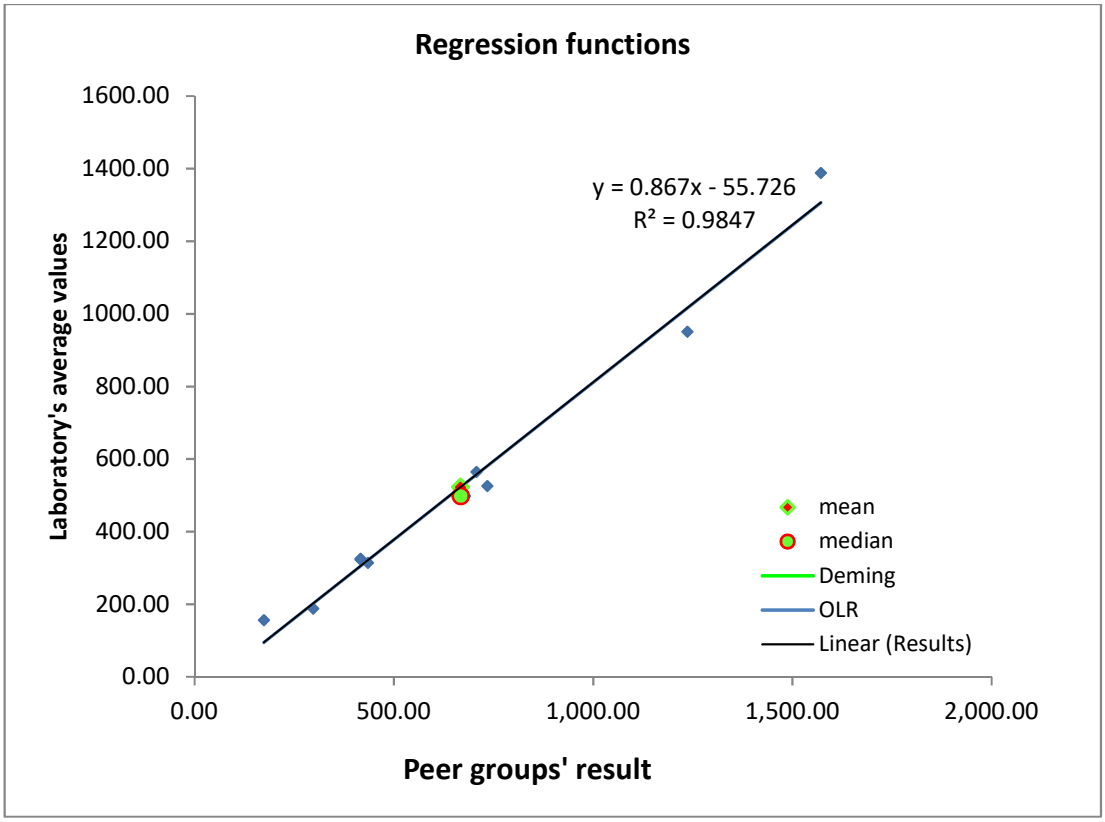



Figure 3. 6 Bland-Altman plot for UK NEQAS samples using the ALTM as the peer group

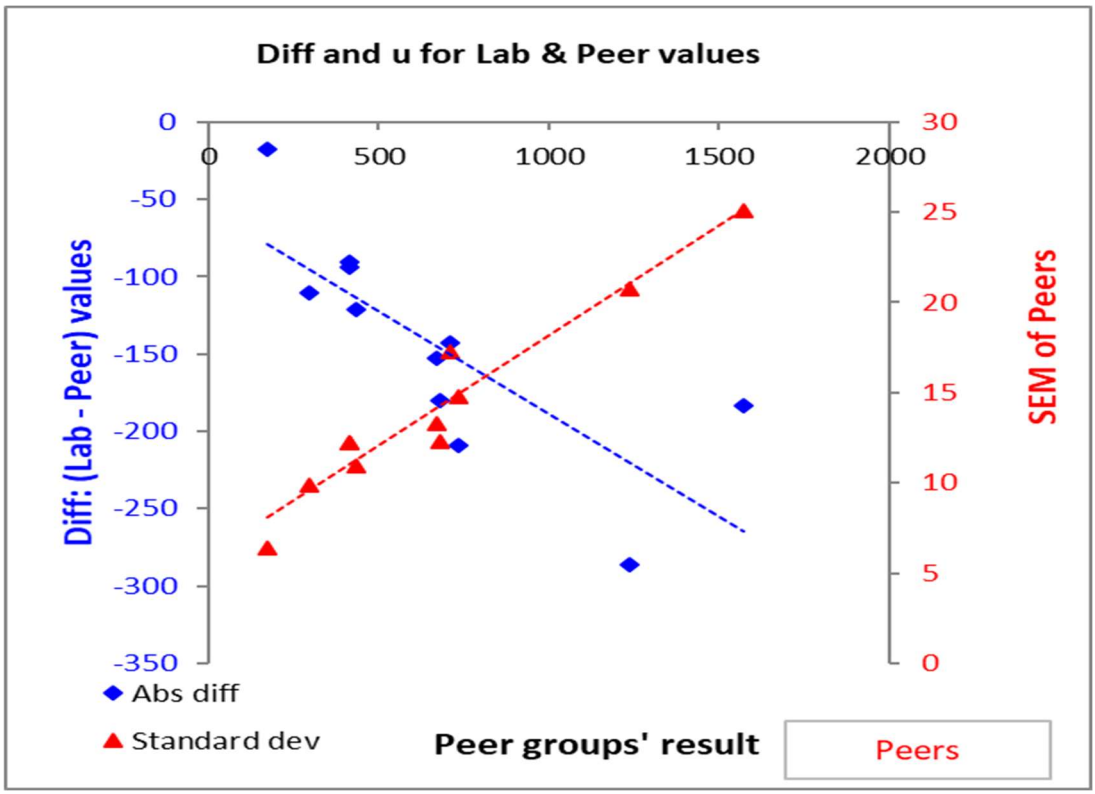



Figure 3. 7 Absolute difference and standard deviation of the UK NEQAS samples using the ALTM as the peer group.

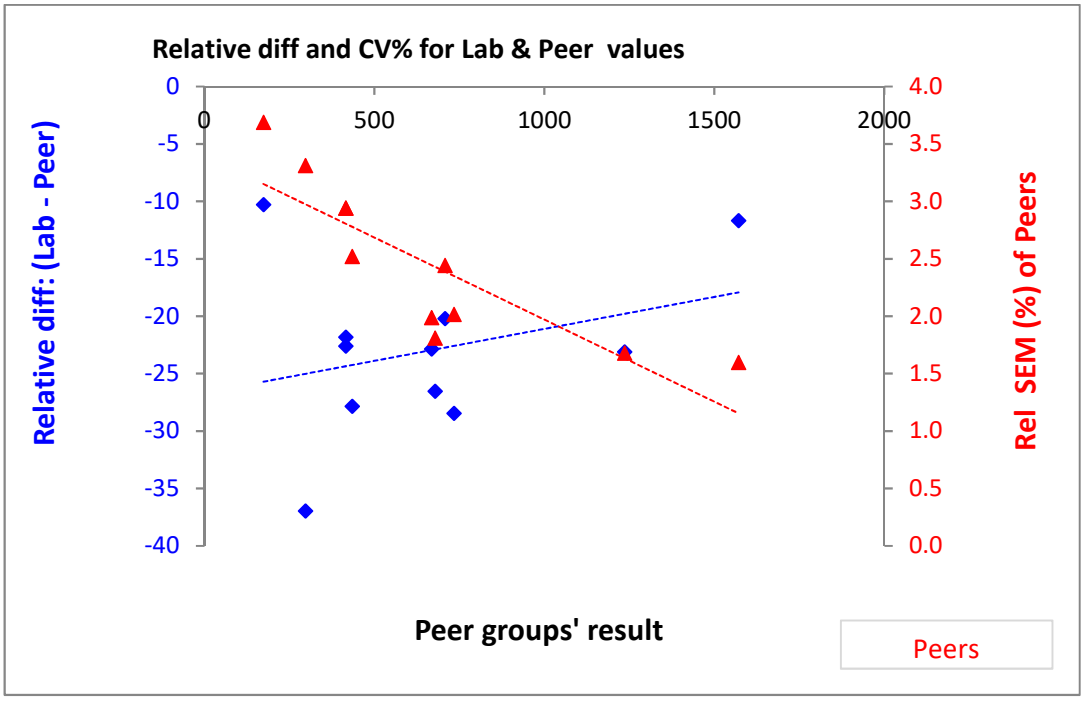



Figure 3. 8 Relative difference and %CV of the NEQAS samples

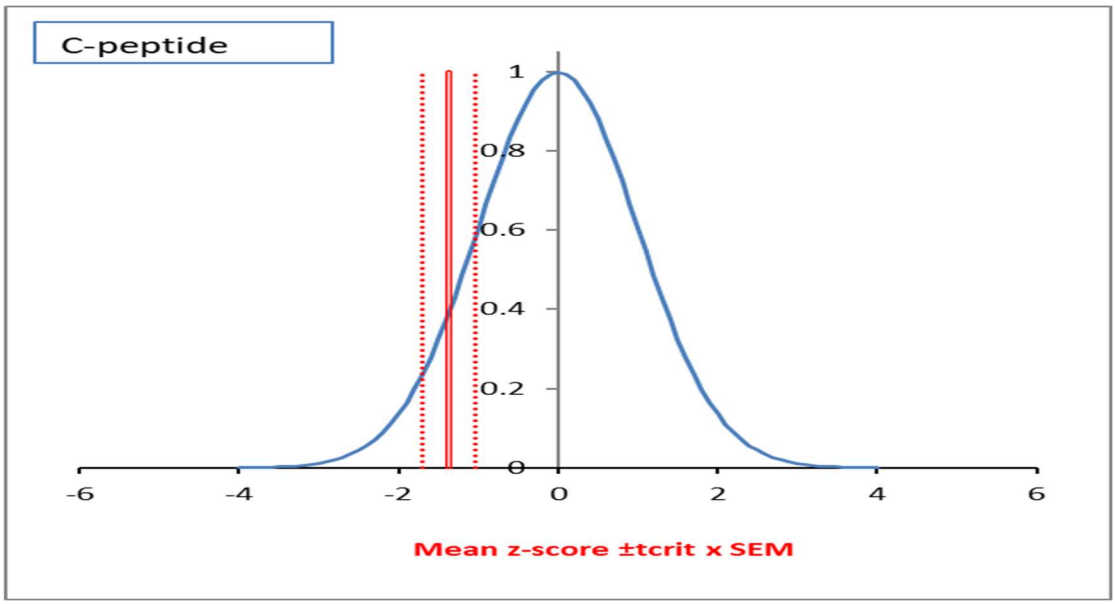



Figure 3. 9 Mean Z-scores of the LC-MS/MS NEQAS results

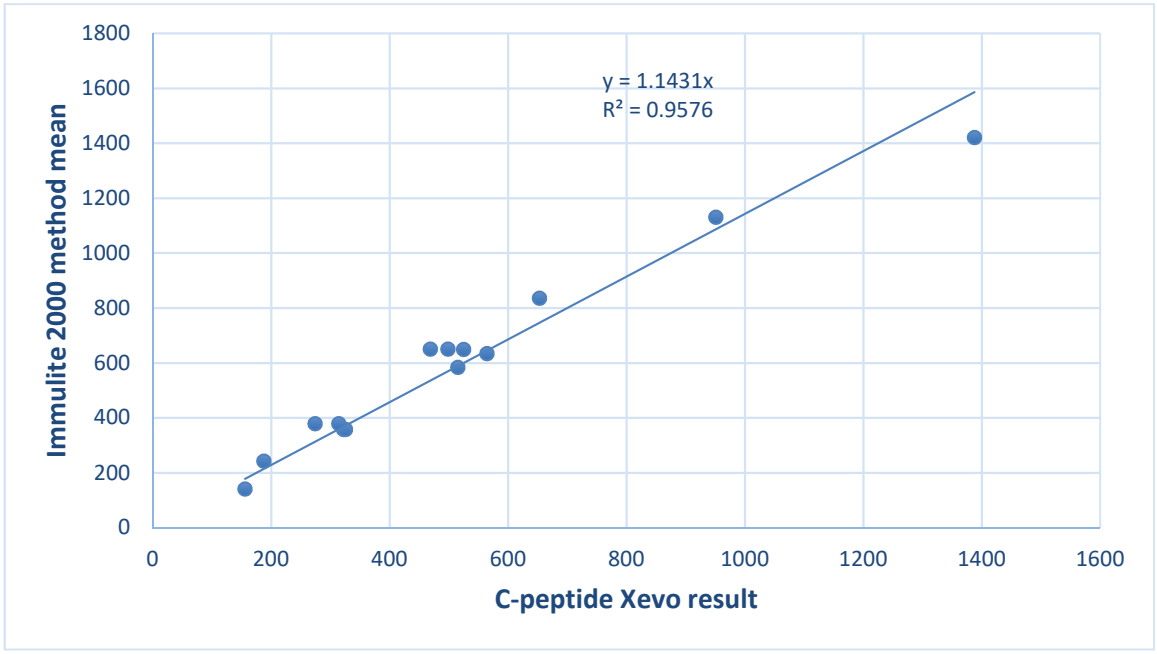
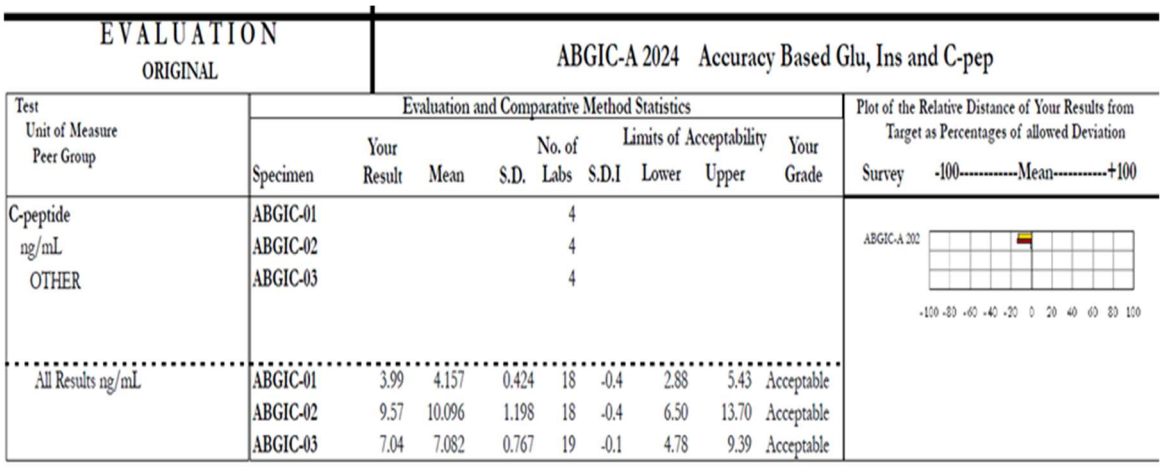



Figure 3. 10 LC-MS/MS against the Siemens Immulite 2000 method group.

CAP Accuracy-Based Scheme samples were also used to assess the accuracy of the LC-MS/MS C-peptide assay. The target values for these specimens were established using an IDMS reference method, which is crucial for result interpretation for labs that receive samples from various sources. The evaluation report (Figure 3.18) shows a standard deviation of the three samples as -0.4, -0.4, and -0.1. The relative distance of the LC-MS/MS method results from the target as

a percentage of the allowable deviation shows an acceptable performance of the LC-MS/MS method.



EVALUATION
ORIGINAL

ABGIC-A 2024 Accuracy Based Glu, Ins and C-pep

| Test / Unit of Measure / Peer Group | Specimen | Your Result | Mean | S.D. | No. of Labs | Limits of Acceptability S.D.I | Lower | Upper | Your Grade |
|---|---|---|---|---|---|---|---|---|---|
| C-peptide | ABGIC-01 | | | | 4 | | | | |
| ng/mL | ABGIC-02 | | | | 4 | | | | |
| OTHER | ABGIC-03 | | | | 4 | | | | |
| All Results ng/mL | ABGIC-01 | 3.99 | 4.157 | 0.424 | 18 | -0.4 | 2.88 | 5.43 | Acceptable |
| | ABGIC-02 | 9.57 | 10.096 | 1.198 | 18 | -0.4 | 6.50 | 13.70 | Acceptable |
| | ABGIC-03 | 7.04 | 7.082 | 0.767 | 19 | -0.1 | 4.78 | 9.39 | Acceptable |

Plot of the Relative Distance of Your Results from Target as Percentages of allowed Deviation

Survey -100----------Mean----------+100

ABGIC-A 202

-100 -80 -60 -40 -20 0 20 40 60 80 100

Figure 3. 11 LC-MS/MS CAP accuracy based EQA results summary

### 3.2.4 Imprecision Studies

To assess inter-assay imprecision, a minimum of five replicates were tested on different days using both spiked matrix and patient pools at various concentrations of the analyte. The mean, standard deviation, and CV were then calculated for the replicates. The results are shown below in Table 3.9. These results demonstrated good inter-assay imprecision, with CVs all within 10%.

Table 3. 9 Between batch IQC precision

| Between Batch Precision | 1st run | 2nd run | 3rd run | 4th run | 5th run | 6th run | Mean | SD | CV | Uncertainty |
|---|---|---|---|---|---|---|---|---|---|---|
| IQC patient Pool Level 1 | 34.1 | 34.1 | 33.8 | 33.8 | 35.8 | | 34.32 | 0.84 | 2.45% | 1.7 |
| IQC patient Pool Level 2 | 266.7 | 253.3 | 236 | 236 | 266.5 | | 251.70 | 15.33 | 6.09% | 30.7 |
| IQC patient Pool Level 3 | 498 | 505.8 | 469.1 | 469.1 | 506.9 | | 489.78 | 19.19 | 3.92% | 38.4 |
| IQC patient Pool Level 4 | 711.1 | 826.1 | 764.7 | 764.7 | 762.3 | | 765.78 | 40.75 | 5.32% | 81.5 |
| IQC patient Pool Level 5 | 1655.7 | 1547.8 | 1430.8 | 1430.8 | 1563.9 | | 1525.80 | 95.99 | 6.29% | 192.0 |
| | | | | | | | | | | |
| IQCspike Level 1 | 104.6 | 106.9 | 111 | 91.3 | 104.6 | 107.1 | 104.25 | 6.76 | 6.49% | 13.5 |
| IQCspike Level 2 | 410.9 | 393.8 | 394.7 | 392.2 | 364.7 | 333.9 | 381.70 | 27.76 | 7.27% | 55.5 |
| IQCspike Level 3 | 887.4 | 866.1 | 850.3 | 815.1 | 741 | 746.7 | 817.77 | 61.96 | 7.58% | 123.9 |
| IQCspike Level 4 | 1403.2 | 1350.5 | 1381 | 1389.5 | 1197.3 | 1276.8 | 1333.05 | 80.38 | 6.03% | 160.8 |

Intra-assay precision was assessed by running 10 replicates of in-house prepared IQC material within one batch, and over a range of concentrations. The results are shown in Table 3.10 below. The CV was within 10% for all analytes at all concentrations, which demonstrates good reproducibility within the assay.

Table 3. 10 Within Batch IQC precision

| Within Batch Precision | IQC Level 1 | IQC Level 2 | IQC Level 3 | IQC Level 4 |
|---|---|---|---|---|
| RPT 1 | 107.1 | 413.1 | 802.3 | 1394.1 |
| RPT 2 | 118.9 | 397.3 | 829.1 | 1394.8 |
| RPT 3 | 120.5 | 396 | 800.6 | 1438.6 |
| RPT 4 | 115.1 | 405.6 | 839.6 | 1382.1 |
| RPT 5 | 118.5 | 392.9 | 837.4 | 1385.7 |
| RPT 6 | 115.1 | 404 | 799 | 1369 |
| RPT 7 | 117.1 | 398.6 | 825.2 | 1381.8 |
| RPT 8 | 108.7 | 396.1 | 820.1 | 1398.3 |
| RPT 9 | 107 | 409.1 | 840.2 | 1382.9 |
| RPT 10 | 113 | 394.7 | 820.2 | 1425.8 |
| **Mean** | 114.1 | 400.74 | 821.37 | 1395.31 |
| **SD** | 4.99 | 6.79 | 16.04 | 21.37 |
| **CV** | 4.37% | 1.70% | 1.95% | 1.53% |

### 3.2.5 Recovery and carryover

Recovery was assessed using a patient sample with a known low concentration of C-peptide which was spiked with C-peptide standard at low, medium, and high concentrations. Each spiked aliquot was run in triplicate, and the mean was calculated. Recovery was calculated using the equation (3.1) below:

Equation (3.1) Recovery (%) = ((spiked result - base result) x 100)/ spike added, and the results are shown in Table 3.11 below. The percentage recovery was 101.9% to 108.4%, which was within the acceptance range of 80% to 120%.

Table 3. 4 Data showing C-peptide percentage recovery

| Recovery | Rpt 1 | Rpt 2 | Rpt 3 | Mean | Mean (0dp) | "base result" | "spike added" | % recovery |
|---|---|---|---|---|---|---|---|---|
| Neat (No spike) | 218.2 | 228 | 217.4 | 221.2 | 221 | 221 | 0 | #DIV/0! |
| Low (100pmol) | 316.9 | 339.7 | 331.7 | 329.4333 | 329 | 221 | 100 | 108.4% |
| Medium (1000pmol) | 1245.2 | 1251.6 | 1221.8 | 1239.533 | 1240 | 221 | 1000 | 101.9% |
| High (2000pmol) | 2270.1 | 2252.8 | 2261.7 | 2261.533 | 2262 | 221 | 2000 | 102.0% |

To assess carryover, a patient sample was spiked with a C-peptide standard to give a high concentration of around 4000 pmol/L. This sample was run in triplicate, followed by a sample with a low concentration of C-peptide (around 100pmol/L), run in triplicate, and the sequence was repeated five times. Percentage carryover was calculated using equation (3.2) below.

Equation (3.2) % carryover (K) = (L1-L3)/(H1-H3) x 100, and the results are shown in Table 3.12 below. The mean percentage carryover was <1% and was therefore considered acceptable.

Table 3. 5 Data showing C-peptide percentage carryover.

| Sample set | H1 | H2 | H3 | L1 | L2 | L3 | L1-L3 | H1-L3 | Percentage carryover (K) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3921.5 | 3936.7 | 4031.5 | 116.8 | 110.4 | 108.1 | 8.7 | 3813.4 | 0.2% |
| 2 | 3898.5 | 4029.2 | 3942.2 | 101.7 | 103.5 | 108.9 | -7.2 | 3789.6 | -0.2% |
| 3 | 3897.7 | 3886.9 | 3893.6 | 103.8 | 108.3 | 108.9 | -5.1 | 3788.8 | -0.1% |
| 4 | 3767.9 | 3956.6 | 3891.6 | 111.3 | 117.3 | 116.2 | -4.9 | 3651.7 | -0.1% |
| 5 | 4016.4 | 3911.7 | 3817.2 | 112.5 | 106.7 | 105.2 | 7.3 | 3911.2 | 0.2% |
| | | | | | | | | MEAN | -0.07% |

### 3.2.6 Dilution integrity

To assess dilution integrity, serial dilutions of a sample in LCMS-grade water and stripped serum were performed, and the results are shown below in Table 3.13, and demonstrated that samples can be diluted in water as good linearity was achieved. All dilutions gave results within 10% of the nominal value; therefore, dilution integrity was confirmed.

Table 3. 6 Assessment of dilution integrity using patient sample

| | Expected C-peptide (pmol/L) | Observed C-peptide (pmol/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Rpt 1 | Rpt 2 | Rpt 3 | MEAN | SD | Mean (0dp) | Calculated concentration (pmol/L) | % difference | Abs difference |
| **Neat** | 3089 | 3084.4 | 3104.9 | 3078.4 | 3089.23 | 13.8954 | 3089 | - | - | - |
| **DilutionX5** | 617.8 | 664.5 | 676.3 | 685.1 | 675.3 | 10.3363 | 675 | 3375 | 9.26% | 286 |
| **Dilutionx10** | 308.9 | 302.7 | 293.6 | 301.6 | 299.3 | 4.96689 | 299 | 2990 | -3.20% | -99 |
| **Dilutionx20** | 154.45 | 155.1 | 153.6 | 155.5 | 154.733 | 1.00167 | 155 | 3100 | 0.36% | 11 |
| **Dilutionx50** | 61.78 | 60.1 | 56.3 | 59.2 | 58.5333 | 1.98578 | 59 | 2950 | -4.50% | -139 |
| | | | | | | | **Mean** | **3103.75** | | |
| | | | | | | | **SD** | **191.63** | | |
| | | | | | | | **CV** | **6.2%** | | |

3.2.7 Analytical sensitivity

To assess the Lower Limit of Quantitation (LLOQ), calibrators 1 to 5 were run 10 times in a single batch, and the %CV and %accuracy were calculated and assessed against the nominal concentration. The results are shown in Tables 3.14 and 3.15 below. Since calibrator 1 (concentration 3.91 pmol/L) achieved a CV of 17.80%, which was <20%, this was considered acceptable as the LLOQ. The percentage accuracy against the nominal concentration was <±20%.

Table 3. 7 LLOQ assessment data: calibrator 1 and calibrator 2

| LLOQ: | Calibrator 1 | | | LLOQ: | Calibrator 2 | |
|---|---|---|---|---|---|---|
| | C-peptide result (pmol/L) | Accuracy | | | C-peptide result (pmol/L) | Accuracy |
| Nominal concentration (umol/L) | | 3.91 | | Nominal concentration (umol | | 7.81 |
| Cal 1 rpt 1 | 4.20 | 0.074169 | | Cal 2 rpt 1 | 8.10 | 0.0371 |
| Cal 1 rpt 2 | 5.10 | 0.304348 | | Cal 2 rpt 2 | 9.10 | 0.1652 |
| Cal 1 rpt 3 | 5.00 | 0.278772 | | Cal 2 rpt 3 | 7.20 | -0.0781 |
| Cal 1 rpt 4 | 3.50 | -0.10486 | | Cal 2 rpt 4 | 9.90 | 0.2676 |
| Cal 1 rpt 5 | 3.60 | -0.07928 | | Cal 2 rpt 5 | 7.10 | -0.0909 |
| Cal 1 rpt 6 | 4.20 | 0.074169 | | Cal 2 rpt 6 | 8.90 | 0.1396 |
| Cal 1 rpt 7 | 4.50 | 0.150895 | | Cal 2 rpt 7 | 6.50 | -0.1677 |
| Cal 1 rpt 8 | 3.20 | -0.18159 | | Cal 2 rpt 8 | 8.20 | 0.0499 |
| Cal 1 rpt 9 | 3.10 | -0.20716 | | Cal 2 rpt 9 | 9.60 | 0.2292 |
| Cal 1 rpt 10 | 3.60 | -0.07928 | | Cal 2 rpt 10 | 9.80 | 0.2548 |
| MEAN | 4 | 0.023018 | | MEAN | 8.44 | 0.0807 |
| SD | 0.712 | | | SD | 1.213 | |
| CV% | 17.80% | | | CV% | 14.37% | |

Table 3. 8 LLOQ assessment data: calibrator 3 to calibrator 5

| LLOQ: | Calibrator 3 | | LLOQ: | Calibrator 4 | | LLOQ: | Calibrator 5 | |
|---|---|---|---|---|---|---|---|---|
| | C-peptide result (pmol/L) | Accuracy | | C-peptide result (pmol/L) | Accuracy | | C-peptide result (pmol/L) | Accuracy |
| Nominal concentration | | 15.63 | Nominal concentrati | | 30.25 | Nominal concentration | | 62.5 |
| Cal 3 rpt 1 | 13.70 | -0.12348 | Cal 4 rpt 1 | 30.20 | -0.00165 | Cal 5 rpt 1 | 64.80 | 0.0368 |
| Cal 3 rpt 2 | 14.90 | -0.04671 | Cal 4 rpt 2 | 30.40 | 0.004959 | Cal 5 rpt 2 | 58.50 | -0.064 |
| Cal 3 rpt 3 | 17.00 | 0.087652 | Cal 4 rpt 3 | 31.00 | 0.024793 | Cal 5 rpt 3 | 60.10 | -0.0384 |
| Cal 3 rpt 4 | 12.60 | -0.19386 | Cal 4 rpt 4 | 32.90 | 0.087603 | Cal 5 rpt 4 | 62.20 | -0.0048 |
| Cal 3 rpt 5 | 17.00 | 0.087652 | Cal 4 rpt 5 | 30.60 | 0.01157 | Cal 5 rpt 5 | 62.80 | 0.0048 |
| Cal 3 rpt 6 | 14.00 | -0.10429 | Cal 4 rpt 6 | 32.70 | 0.080992 | Cal 5 rpt 6 | 61.50 | -0.016 |
| Cal 3 rpt 7 | 16.40 | 0.049264 | Cal 4 rpt 7 | 29.00 | -0.04132 | Cal 5 rpt 7 | 64.70 | 0.0352 |
| Cal 3 rpt 8 | 16.20 | 0.036468 | Cal 4 rpt 8 | 28.70 | -0.05124 | Cal 5 rpt 8 | 64.90 | 0.0384 |
| Cal 3 rpt 9 | 14.20 | -0.09149 | Cal 4 rpt 9 | 32.20 | 0.064463 | Cal 5 rpt 9 | 62.00 | -0.008 |
| Cal 3 rpt 10 | 16.40 | 0.049264 | Cal 4 rpt 1 | 31.50 | 0.041322 | Cal 5 rpt 10 | 59.70 | -0.0448 |
| MEAN | 15.24 | -0.02495 | MEAN | 30.92 | 0.022149 | MEAN | 62.12 | -0.00608 |
| SD | 1.559 | | SD | 1.437 | | SD | 2.248 | |
| CV% | 10.23% | | CV% | 4.65% | | CV% | 3.62% | |

To assess the limit of blank (LOB), a blank sample (calibrator 0) was assayed 10 times within a batch, and the mean response was calculated. The limit of blank was calculated using the equation mean [blank] + 1.645 (SD [blank]), and the results are shown in Table 3.16 below.

Table 3. 9 LOB assessment data using calibrator zero.

| **Extracted Blank Matrix** | C-peptide result (pmol/L) |
|---|---|
| Nominal concentration (µmol/L) | |
| Cal 0 rpt 1 | 0.00 |
| Cal 0 rpt 2 | 0.00 |
| Cal 0 rpt 3 | 0.00 |
| Cal 0 rpt 4 | 0.00 |
| Cal 0 rpt 5 | 0.04 |
| Cal 0 rpt 6 | 0.00 |
| Cal 0 rpt 7 | 0.00 |
| Cal 0 rpt 8 | 0.06 |
| Cal 0 rpt 9 | 0.02 |
| Cal 0 rpt 10 | 0.00 |
| MEAN | 0.012 |
| SD | 0.021499354 |

LOB = Mean blank + 1.645 (SD [blank]) (Armbuster and Pry, 2008)
= 0.012 + 1.645 (0.0215) = 0.0474pmol/L

The limit of Detection (LoD) was assessed using the data from the limit of blank and precision data on calibrator 1 and the limit of Quantification (LoQ) was calculated using the data from the limit of blank as shown below.

LOD = Mean blank + 3.3 (SD [blank]) (Armbuster and Pry, 2008)

= 0.012 + 3.3 (0.0215) = 0.08295 pmol/L

LOQ = Mean blank + 10 (SD [blank]) (Armbuster and Pry, 2008)

= 0.012 + 10 (0.0215) = 0.227 pmol/L

An assessment of the effect of sample volume (50 µL and 100 µL) on sensitivity was carried out to see if 50 µL or 100 µL of a sample can be used in situations where 250 µL samples cannot be obtained, especially in the case of paediatric samples. For this study, a calibration curve was built with 50 µL and 100 µL sample volumes and used to analyse past UK NEQAS and CAP EQA distribution samples. The UKNEQAS distribution samples was prepared using the new international standard (First International Standard, human, C-peptide 13/146). A sensitivity check was also carried out to confirm the analytical range when a 50 µL sample volume was used. The results show the method has the same level of accuracy (Table 3.17) and precision (Table 3.18) when 100 µL (Figure 3.19) or 50 µL (Figure 3.20) of sample were used. A sensitivity check (Table 3.18) confirms the analytical range when 50 µL of sample volume is used to be 7-8000 pmol/L.

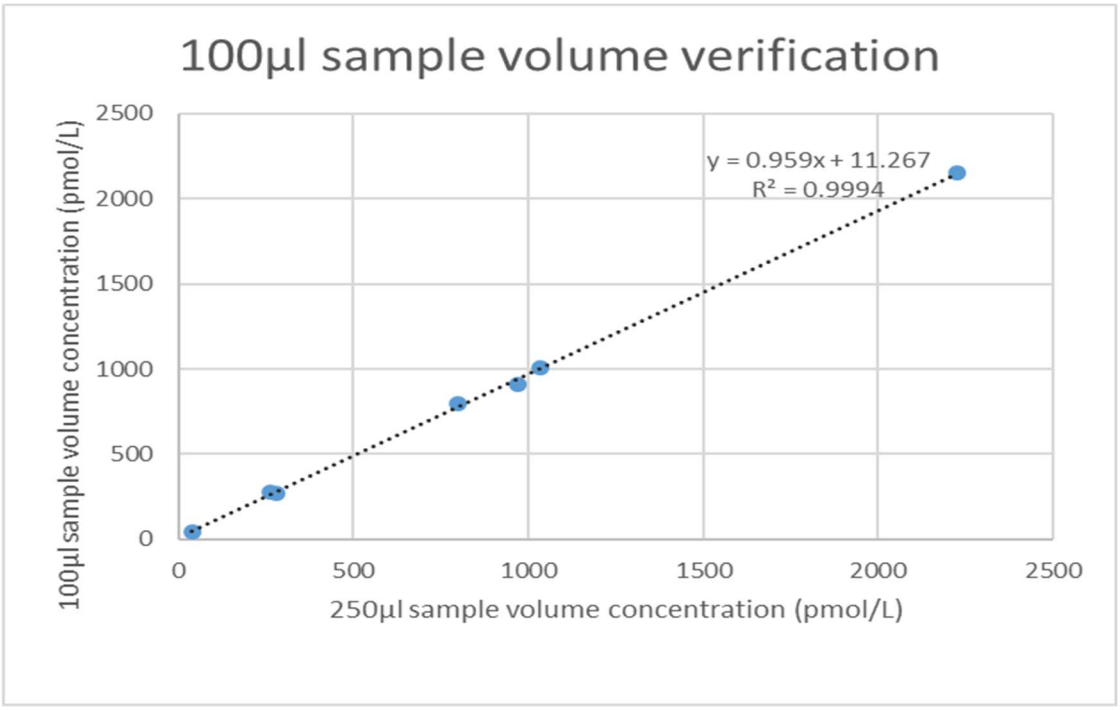



Figure 3. 19 Verification of 100 µL sample volume

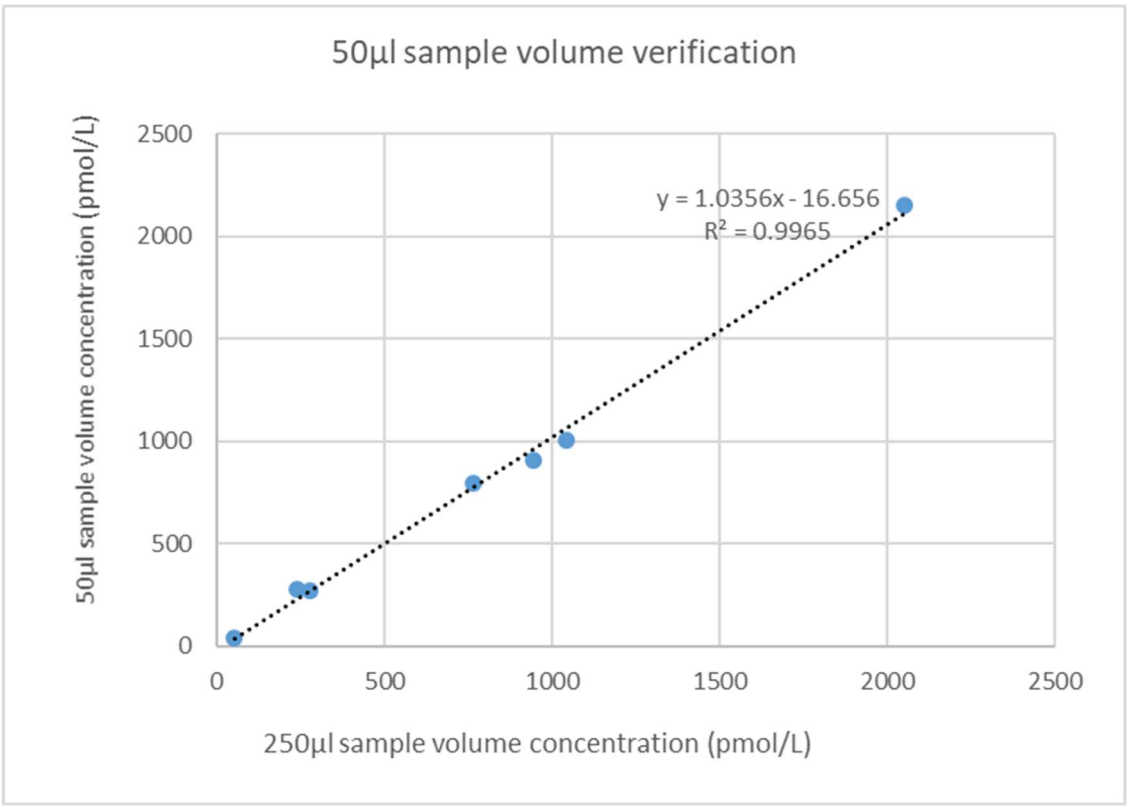



Figure 3. 20 Verification of 50 µL sample volume

Table 3. 10 UK NEQAS and CAP EQA for 50 µL sample volume verification

| Scheme | Sample | LCMS run | LCMS run | LCMS Mean | ALTM | % Difference |
|---|---|---|---|---|---|---|
| NEQAS | EQA N311 | 1139.9 | 1122.1 | 1131 | 1236.6 | -9% |
| NEQAS | EQA N312 | 1444.4 | 1422.1 | 1433.25 | 1571.2 | -9% |
| CAP | ABGIC 01 | 1271.7 | 1306.2 | 1288.95 | 1376.36 | -6% |
| CAP | ABGIC02 | 3029.4 | 3124 | 3076.7 | 3342.73 | -8% |
| CAP | ABGIC03 | 2350 | 2376 | 2363 | 2344.81 | 1% |

Table 3. 11 Sensitivity check for 50 µL sample volume

| Calibrator 2 | | Calibrator 3 | | Calibrator 4 | | Calibrator 5 | |
|---|---|---|---|---|---|---|---|
| | 7.2 | | 17.3 | | 31.7 | | 72.4 |
| | 8.1 | | 16.5 | | 31.1 | | 63.1 |
| | 5.5 | | 14 | | 34.2 | | 61.7 |
| | 6 | | 13.5 | | 36.9 | | 65.6 |
| | 6.4 | | 16.3 | | 36 | | 65.8 |
| | 6.6 | | 14.8 | | 34.2 | | 68.5 |
| | 8.7 | | 13 | | 36.2 | | 63.7 |
| | 7.4 | | 14.9 | | 32.5 | | 61.5 |
| | 6.2 | | 15.5 | | 32.8 | | 70.2 |
| | 8.5 | | 14.9 | | 34.5 | | 67.9 |
| Mean | 7.06 | | 15.07 | | 34.01 | | 66.04 |
| SD | 1.101716 | | 1.363859 | | 1.971153 | | 3.667939 |
| CV | 15.61% | | 9.05% | | 5.80% | | 5.55% |

3.2.8 Quantitative and qualitative matrix effects

The qualitative matrix was assessed by the injection of blank matrices with a concurrent direct infusion of the C-peptide standard and the internal standard. Visual inspection of chromatograms shows no areas of ion suppression or enhancement in the region of the retention time of C-peptide and the internal standard, as shown in Figure 3.21 and 3.22 below.

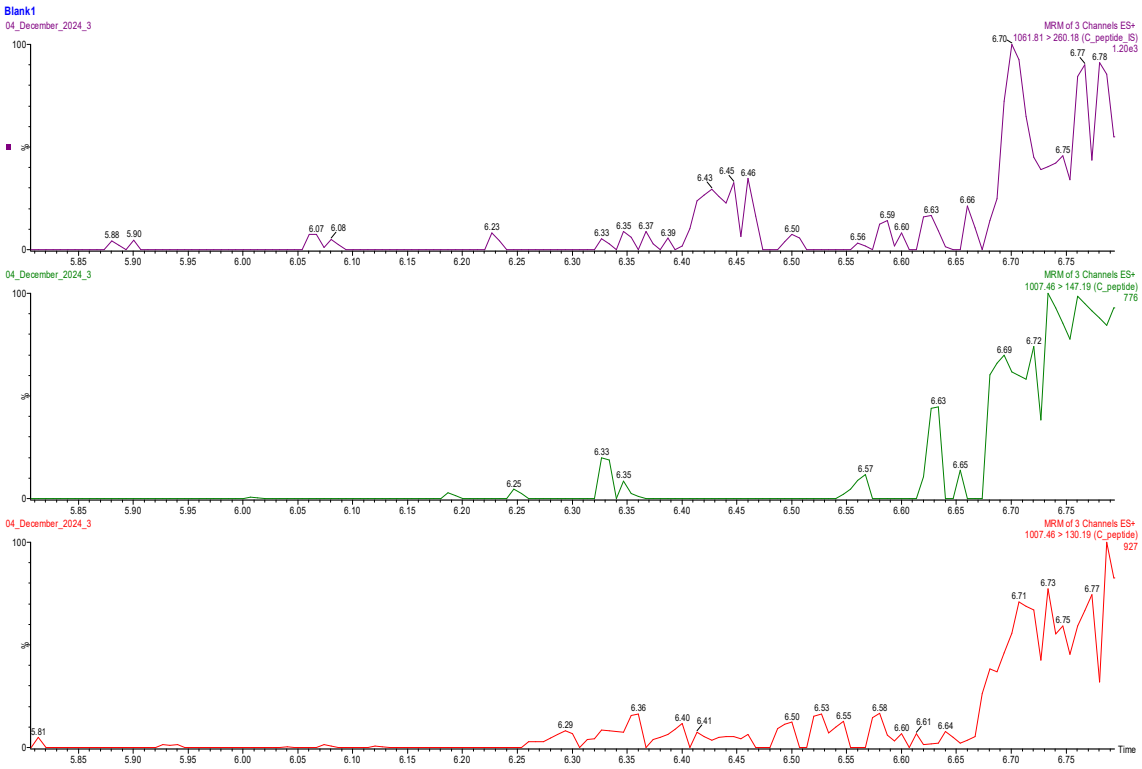



Figure 3. 21 Qualitative matrix effect chromatograms showing the blank matrix injection

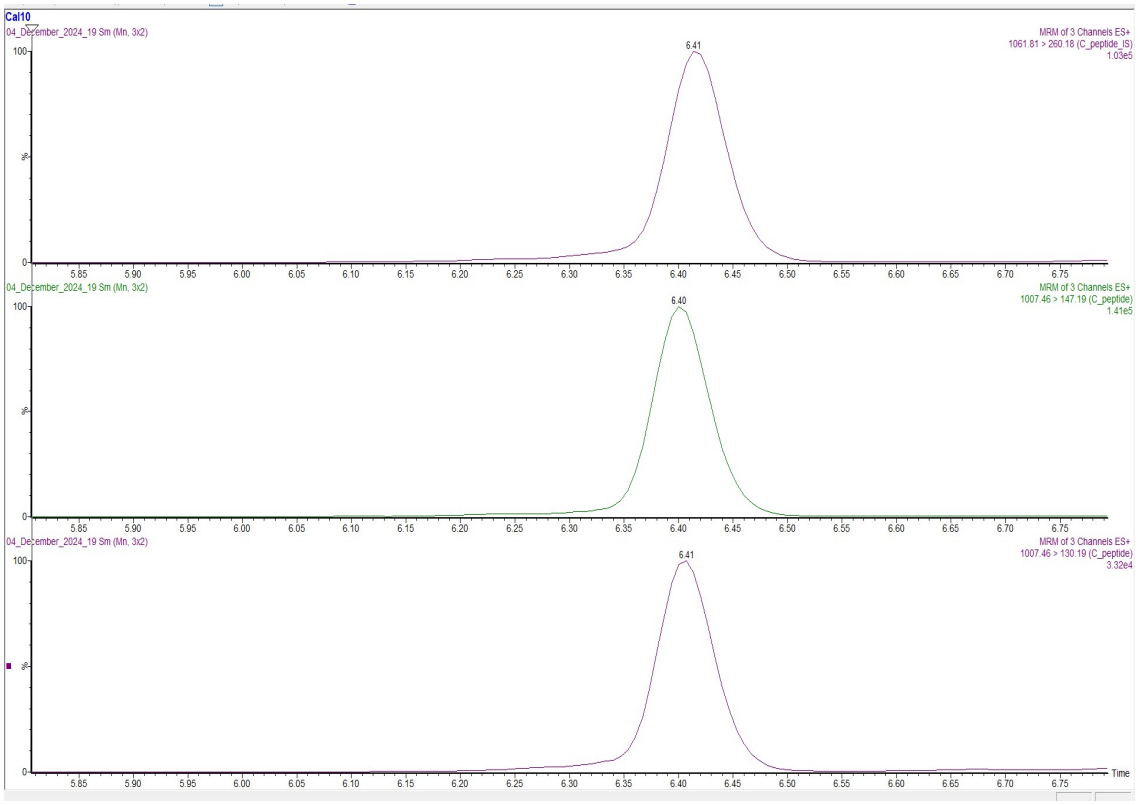



Figure 3. 22 Qualitative matrix effect chromatograms showing C-peptide and Internal standard injection

The quantitative matrix effect was assessed using three independent samples: purchased human serum, bovine serum matrix, and PBS/SBA spiked at a low C-peptide concentration of 100 pmol/L and a high concentration of 4000 pmol/L. Each spiked sample was analysed in triplicate, and the percentage matrix effect (%ME) and percentage normalised matrix effect (normalised %ME) were calculated and shown in Tables 3.19 (using peak area) and 3.20 (using response) below. The quantitative matrix effect of the internal standard is shown in Table 3.21. The %ME for all three patient matrices was <15%, apart from the high C-peptide standard spike, which was >15% but this was corrected for by the internal standard.

Table 3. 12 Matrix effect using peak area of the C-peptide standard

| | Peak area | | | | | |
|---|---|---|---|---|---|---|
| **Quantitative C-peptide standard** | | | | | | |
| **Sample** | **Spike** | **Rpt 1** | **Rpt 2** | **Rpt 3** | **Mean** | **%ME** |
| **PBS/BSA** | 100pmol/L Low spike | 571.719 | 545.878 | 514.36 | 544 | |
| **Bovine serum** | 100pmol/L Low spike | 456.199 | 483.134 | 483.532 | 474 | -13% |
| **Human Serum** | 100pmol/L Low spike | 499.779 | 518.983 | 540.095 | 520 | -4% |
| **Sample** | **Spike** | **Rpt 1** | **Rpt 2** | **Rpt 3** | **Mean** | **%ME** |
| **PBS/BSA** | 4000pmol/L High spike | 13998.3 | 14461.01 | 13884.56 | 14115 | |
| **Bovine serum** | 4000pmol/L High spike | 10605.44 | 11135.47 | 11204.72 | 10982 | -22% |
| **Human Serum** | 4000pmol/L High spike | 10346.43 | 10367.8 | 10284.4 | 10333 | -27% |

Table 3. 13 Matrix effect using the response of the C-peptide standard

| | Response | | | | | |
|---|---|---|---|---|---|---|
| **Quantitative C-peptide standard** | | | | | | |
| **Sample** | **Spike** | **Rpt 1** | **Rpt 2** | **Rpt 3** | **Mean** | **Normalised %ME** |
| **PBS/BSA** | 100pmol/L Low spike | 0.051 | 0.054 | 0.046 | 0.050 | |
| **Bovine serum** | 100pmol/L Low spike | 0.047 | 0.056 | 0.049 | 0.051 | 1% |
| **Sample** | **Spike** | **Rpt 1** | **Rpt 2** | **Rpt 3** | **Mean** | **Normalised %ME** |
| **PBS/BSA** | 4000pmol/L High spike | 1.675 | 1.624 | 1.492 | 1.597 | |
| **Bovine serum** | 4000pmol/L High spike | 1.92 | 1.93 | 1.826 | 1.892 | 18% |

Table 3. 14 Matrix effect using peak area of the C-peptide internal standard

| | Peak area | | | | | |
|---|---|---|---|---|---|---|
| Quantitative Internal standard | | | | | | |
| Sample | Spike | Rpt 1 | Rpt 2 | Rpt 3 | Mean | Normalised %ME |
| PBS/BSA | 2000pmol spike | 5698.417 | 5918.895 | 6016.775 | 5878 | |
| Bovine serum | 2000pmol spike | 5253.99 | 4183.581 | 4541.527 | 4660 | -21% |
| Human Serum | 2000pmol spike | 4881.514 | 5013.906 | 4901.798 | 4932 | -16% |

Further assessment of the matrix effect was carried out by standard addition to determine if bovine serum was suitable for preparing the calibrators. A sample with a concentration of 171.1 pmol/L using the calibration curve was spiked with 1 ng/mL, 2 ng/mL, 4 ng/mL, 6 ng/mL and 8 ng/mL of C-peptide standard and analysed. The peak area count of the neat sample and spiked sample is noted in Table 3.22 and the standard addition curve is shown in Figure 3.23. The sample concentration using standard addition was 167 pmol/L with a difference of 2.5% from the known concentration.

Table 3. 15 Quantitative matrix effect by standard addition.

| Matrix effect by standard Addition | | | | | | | |
|---|---|---|---|---|---|---|---|
| Spike Conc | 22B21501896 | Spike Conc pmol/l | Area Count | | | | |
| 0 ng/ml | Patient1SPIKE0 | 0 | 420.284 | Slope | 2.956748126 | 493.67 | Intercept |
| 1ng/ml | Patient1SPIKE1 | 331.094 | 1439.899 | SD of Slope | 0.040983197 | 55.95 | sd of intercept |
| 2ng/ml | Patient1SPIKE2 | 662.1881 | 2542.06 | r2 | 0.999423958 | 85.82 | |
| 4ng/ml | Patient1SPIKE3 | 1324.3761 | 4477.897 | | 5204.955186 | 3.00 | |
| 8ng/ml | Patient1SPIKE4 | 2648.7523 | 8272.656 | | 38334638.45 | 22095.08 | |
| | | | | unknown Conc (pmol/L) | Intercept/slope | 166.9652574 | |
| **Repeat Standard Addition curve** | | | | | | | |
| Spike Conc | 22B21501896 repeat 1 | Spike Conc pmol/l | Target Mean | | | | |
| 0 ng/ml | Patient1SPIKE0 | 0 | 467.994 | Slope | 3.072543628 | 506.31 | Intercept |
| 1ng/ml | Patient1SPIKE1 | 331.094 | 1547.339 | SD of Slope | 0.02729625 | 37.26 | sd of intercept |
| 2ng/ml | Patient1SPIKE2 | 662.1881 | 2604.4 | r2 | 0.999763284 | 57.16 | |
| 4ng/ml | Patient1SPIKE3 | 1324.3761 | 4515.4 | | 12670.39488 | 3.00 | |
| 8ng/ml | Patient1SPIKE4 | 2648.7523 | 8655.939 | | 41396042.83 | 9801.44 | |
| | | | | unknown Conc (pmol/L) | Intercept/slope | 164.7858823 | |

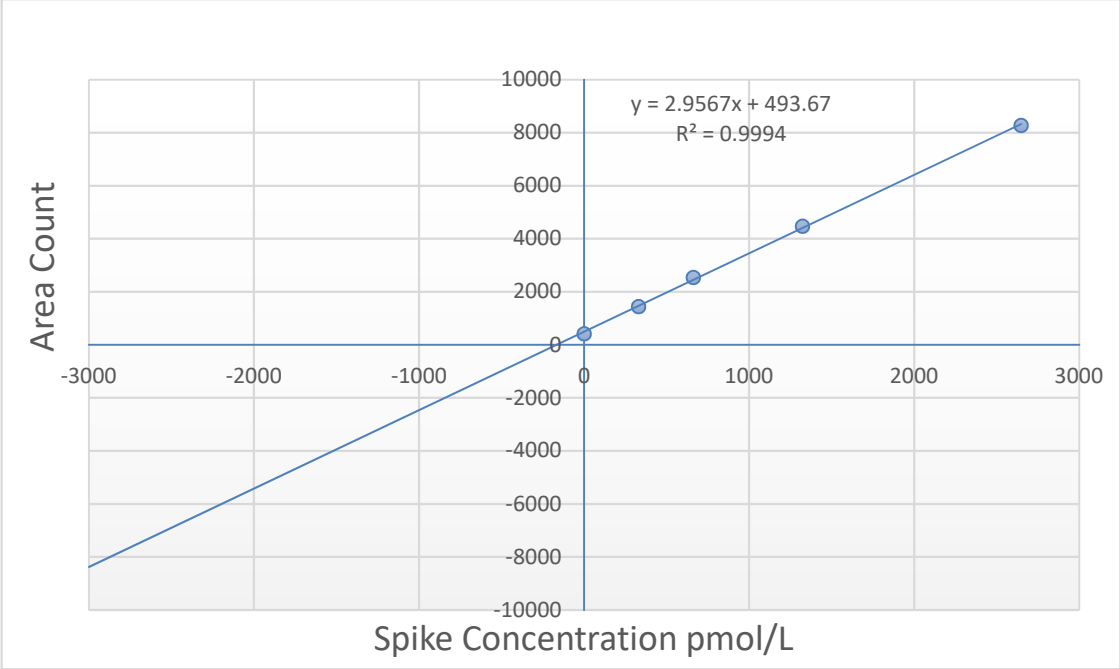



Figure 3.23 Method of multiple standard additions curve

(Matrix effect was carried out by standard addition using the peak area of a sample spiked at various concentrations to determine the unknown concentration of 164.75 pmol/L using linear regression analysis and compared to the concentration determined using a calibration curve, which was 171.1 pmol/L)

### 3.2.9 Interference studies

The effects of common interferences on the assay, such as lipaemia, icterus, haemolysis, proinsulin, and insulin, were evaluated using surplus patient samples spiked with either PBS/SBA buffer or an increasing concentration of potential interferent. The sample aliquots spiked with PBS/BSA to account for the dilutional effect and separate aliquots spiked with a high level of potential interferent were quantified, and the percentage interference was calculated and compared for significant changes to the expected concentration. Chromatograms were also analysed for the presence of other peaks. The results for proinsulin interference studies (5000 pmol/L proinsulin (First International Standard, 09/296) spiked at different C-peptide concentrations are shown in Table 3.23 below.

Table 3. 16 Effect of 5000 pmol/L proinsulin international standard (09296) on C-peptide quantitation.

| Neat | Rpt 1 | Rpt 2 | Rpt 3 | Mean | Neat-Mean | % Bias |
|---|---|---|---|---|---|---|
| 86.116 | 79.94 | 87.48 | 86.87 | 84.76333 | -1.353 | -1.57 |
| 328.33 | 341.68 | 327.08 | 347.45 | 338.7367 | 10.407 | 3.17 |
| 763.856 | 761.67 | 741.85 | 710.53 | 738.0167 | -25.839 | -3.38 |
| 991.268 | 927.57 | 927.07 | 945.29 | 933.31 | -57.958 | -5.85 |
| 1802.748 | 1875.41 | 1816.51 | 1724.87 | 1805.597 | 2.849 | 0.16 |

An insulin interference study was carried out to assess the effect on samples with C-peptide concentrations of 350 pmol/L, 1000 pmol/L, and 2000 pmol/L. The samples were each spiked with increasing concentrations of the Second International Standard for Insulin (11/212), and the percentage interference was calculated. The results for insulin interference studies for up to 100,000 pmol/L are shown in Tables 3.24 to 3.26 below.

Table 3. 17 Effect of insulin (IS 11/212) on C-peptide (350 pmol/L) quantitation.

| Spike conc | Neat + diluent | | | | Neat + Insulin spike | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (pmol/L) | Rpt1 | Rpt2 | Rpt3 | Mean (X1 | Rpt1 | Rpt2 | Rpt3 | Mean (X2 | X1-X2 | % Interference |
| 5000 | 344.8 | 343.2 | 344.5 | 344.17 | 355.7 | 362.7 | 357.2 | 358.53 | 14.3667 | 4.17 |
| 15000 | 365.5 | 362.1 | 356 | 361.20 | 362.2 | 359.4 | 347.4 | 356.33 | -4.8667 | -1.37 |
| 25000 | 341.4 | 356.6 | 333.7 | 343.90 | 358.2 | 354.1 | 340.1 | 350.80 | 6.9 | 2.07 |
| 50000 | 328.3 | 327.5 | 325.3 | 327.03 | 350.9 | 332.1 | 332.5 | 338.50 | 11.4667 | 3.52 |
| 100000 | 327.7 | 318.4 | 323.4 | 323.17 | 345.2 | 347 | 325.1 | 339.10 | 15.9333 | 4.93 |
| | | | | | | | | | Average | 2.66 |

Table 3. 18 Effect of insulin (IS 11/212) on C-peptide (1000 pmol/L) quantitation.

| Spike conc (pmol/L) | Neat + diluent | | | | Neat + Insulin spike | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2 | X1-X2 | % Interference |
| 5000 | 1087 | 1046.1 | 1071 | 1068.17 | 1024.9 | 1028 | 1068.8 | 1040.43 | -27.733 | -2.59 |
| 15000 | 1025 | 1008.3 | 963.2 | 998.97 | 1035.1 | 1048 | 1035.3 | 1039.33 | 40.3667 | 4.19 |
| 25000 | 1016 | 1006.8 | 1020 | 1014.03 | 1078.1 | 1032 | 1042.7 | 1050.90 | 36.8667 | 3.62 |
| 50000 | 988.8 | 1005.2 | 1036 | 1010.00 | 988.5 | 1023 | 987.1 | 999.40 | -10.6 | -1.02 |
| 100000 | 932 | 934.1 | 966.6 | 944.23 | 990.9 | 1003 | 972.5 | 988.80 | 44.5667 | 4.61 |
| | | | | | | | | | Average | 1.76 |

Table 3. 19 Effect of insulin (IS 11/212) on C-peptide (2000 pmol/L) quantitation.

| Spike conc (pmol/L) | Neat + diluent | | | | Neat + Insulin spike | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | % Interference |
| 15000 | 2006.6 | 1993.7 | 2028.5 | 2009.60 | 2029.3 | 2064.5 | 2103.6 | 2065.80 | -56.20 | -2.77 |
| 25000 | 1926.7 | 1932 | 1948 | 1935.57 | 2009.1 | 2048.8 | 2001.1 | 2019.67 | -84.10 | -4.32 |
| 50000 | 2007.2 | 2037.2 | 2026.8 | 2023.73 | 1996.9 | 1998.5 | 1998.2 | 1997.87 | 25.87 | 1.28 |
| 100000 | 1882.7 | 1939.6 | 1839.4 | 1887.23 | 1930.7 | 2009.2 | 1972.2 | 1970.70 | -83.47 | -4.54 |
| | | | | | | | | | Average | -2.59 |

For the lipaemia interference study, a sample with a C-peptide concentration of approximately 3000 pmol/L was fortified with 8.48 mmol/L, 16.95 mmol/L and 33.9 mmol/L of intralipid and analysed in triplicate. The percentage interference was calculated and shown in Table 3.27.

Table 3. 20 Effect of lipaemia on C-peptide quantitation.

| Lipemia | Neat + diluent | | | | Neat + spike | | | | | % Interference |
|---|---|---|---|---|---|---|---|---|---|---|
| spike conc | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | |
| 8.48 mmol/L | 3005.5 | 2913.8 | 2901.5 | 2940.2667 | 2845.4 | 2801.1 | 2826.3 | 2824.2667 | -116 | -3.95 |
| 16.95 mmol/L | 2843.2 | 2858.5 | 2806.2 | 2835.9667 | 2878.4 | 2981.4 | 3043.4 | 2967.7333 | 131.7667 | 4.65 |
| 33.9 mmol/L | 2768.5 | 2888.4 | 2807 | 2821.3 | 2936.7 | 2993.8 | 2903.4 | 2944.6333 | 123.3333 | 4.37 |
| | | | | | | | | | Average | 1.69 |

For the icteric interference study, a sample with a C-peptide concentration of approximately 3000 pmol/L was spiked with 42.76 µmol/L, 85.52 µmol/L and 171.04 µmol/L of bilirubin and analysed in triplicate. The percentage interference was calculated and shown in Table 3.28.

Table 3. 21 Effects of icterus on C-peptide quantitation.

| Bilirubin | Neat + diluent | | | | Neat + spike | | | | | % Interference |
|---|---|---|---|---|---|---|---|---|---|---|
| spike conc | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | |
| 42.76 µmol/L | 2898.4 | 2727.3 | 2844.1 | 2823.2667 | 2809.1 | 2763.6 | 2727.3 | 2766.6667 | -56.6 | -2.00 |
| 85.52 µmol/L | 2898.4 | 2727.3 | 2844.1 | 2823.2667 | 2944.7 | 2872.4 | 2890.2 | 2902.4333 | 79.16667 | 2.80 |
| 171.04 µmol/L | 2898.4 | 2727.3 | 2844.1 | 2823.2667 | 2512.5 | 2646.5 | 2589.4 | 2582.8 | -240.467 | -8.52 |
| | | | | | | | | | Average | -2.57 |

For the haemolysis interference study, a sample with an approximately C-peptide concentration of 3000 pmol/L was spiked with 0.5 g/L, 10 g/L and 20 g/L of haemoglobin and analysed in triplicate. The percentage interference was calculated and shown in Table 3.29.

Table 3. 22 Effect of Haemolysis on C-peptide quantification.

| Haemolysis | Neat + diluent | | | | Neat + spike | | | | | % Interference |
|---|---|---|---|---|---|---|---|---|---|---|
| spike conc | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | |
| 0.5 g/L | 3022.9 | 2930.5 | 2931.7 | 2961.7 | 2992.1 | 2948.3 | 2943.2 | 2961.2 | -0.5 | -0.02 |
| 10 g/L | 3022.9 | 2930.5 | 2931.7 | 2961.7 | 3065.6 | 2914.5 | 2832.7 | 2937.6 | -24.1 | -0.81 |
| 20 g/L | 3022.9 | 2930.5 | 2931.7 | 2961.7 | 2881.6 | 2863.7 | 2799.2 | 2848.1667 | -113.533 | -3.83 |
| | | | | | | | | | Average | -1.55 |

For the high creatinine level interference study, a sample with an approximately C-peptide concentration of 3000 pmol/L was spiked with 1.105 mmol/L, 2.21 mmol/L and 4.4201 mmol/L of creatinine and analysed in triplicate. The percentage interference was calculated and shown in Table 3.30 below.

Table 3. 23 Assessment of high creatinine interference

| Creatinine | Neat + diluent | | | | Neat + spike | | | | | % Interference |
|---|---|---|---|---|---|---|---|---|---|---|
| spike conc | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | |
| 1105.02 µmol/L | 2892.5 | 2935.2 | 2902.2 | 2909.9667 | 2950.6 | 2927.3 | 2929.4 | 2935.7667 | 25.8 | 0.89 |
| 2210.04 µmol/L | 2892.5 | 2935.2 | 2902.2 | 2909.9667 | 3014.4 | 2931 | 2978.8 | 2974.7333 | 64.76667 | 2.23 |
| 4420.08 µmol/L | 2892.5 | 2935.2 | 2902.2 | 2909.9667 | 2878.1 | 2900.8 | 2945.4 | 2908.1 | -1.86667 | -0.06 |
| | | | | | | | | | Average | 1.02 |

The results of the interference studies indicate that samples from patients with bilirubin concentrations up to 171.04 µmol/L, haemoglobin concentrations up to 20 g/L, triglyceride concentrations up to 33.9 mmol/L, creatinine concentrations up to 4.420 mmol/L, insulin concentrations up to 100,000 pmol/L and proinsulin concentrations of up to 5000 pmol/L did not substantially interfere with the quantification of C-peptide. The percentage interference was all less than 5%.

## 3.3 Stability studies

The stability of C-peptide over time was evaluated by using a patient sample collected into a serum separating gel tube, an EDTA plasma tube, and a lithium heparin plasma tube, which were then aliquoted and stored. The storage conditions assessed were room temperature (21°C), fridge temperature (4°C), freezer temperature (-20.0°C), and ultralow freezer temperature (-80.0°C). Freeze-thaw stability was evaluated using the same patient serum samples. The samples were first analysed immediately after separation, and the results (2370 pmol/L) served as a baseline for the patient sample. The results of the stability study are shown in Table 3.31 below.

Table 3. 24 Stability data based on storage Conditions

| Time Analysed | Temp | Sample type | Run 1 | Run 2 | Run 3 | Mean (X2) | X1-X2 | %Bias |
|---|---|---|---|---|---|---|---|---|
| 24hrs | 21°C | Serum | 2321.4 | 2314.8 | 2318.5 | 2318.23 | 51.77 | 2.23 |
| 24hrs | 21°C | EDTA | 2325.8 | 2319 | 2331.6 | 2325.47 | 44.53 | 1.92 |
| 24hrs | 21°C | Lit Heparin | 2283.9 | 2296.3 | 2278 | 2286.07 | 83.93 | 3.67 |
| 48hrs | 4°C | Serum | 2132.4 | 2258.8 | 2282.8 | 2224.67 | 145.33 | 6.53 |
| 48hrs | 4°C | EDTA | 2178.5 | 2286.7 | 2262.3 | 2242.50 | 127.50 | 5.69 |
| 48hrs | 4°C | Lit Heparin | 2193.5 | 2205.6 | 2196 | 2198.37 | 171.63 | 7.81 |
| 2 months | −20 °C | Serum | 2210.7 | 2252.2 | 2222.6 | 2228.50 | 141.50 | 6.35 |
| 2 months | −20 °C | EDTA | 2282.8 | 2295.5 | 2271.7 | 2283.33 | 86.67 | 3.80 |
| 2 months | −20 °C | Lit Heparin | 2272.3 | 2265.7 | 2281.6 | 2273.20 | 96.80 | 4.26 |
| 6 months | −80 °C | Serum | 2294.1 | 2285.7 | 2281.9 | 2287.23 | 82.77 | 3.62 |
| 6 months | −80 °C | EDTA | 2362.1 | 2316.8 | 2326.8 | 2335.23 | 34.77 | 1.49 |
| 6 months | −80 °C | Lit Heparin | 2311.3 | 2309.7 | 2325.5 | 2315.50 | 54.50 | 2.35 |

There was also little impact when the tubes were stored for 24 hours at room temperature, 48 hours at 4°C, 2 months at -20°C and 6 months at -80°C (bias ranged from 1.49% to 7.81%). The bias values were all <8.3%, which was within the acceptable bias for C-peptide.

To assess whether EDTA plasma and lithium heparin were acceptable sample types for the assay, the recovery of C-peptide spiked into EDTA plasma and lithium heparin was compared to analyte fortified into control serum samples, and the results are shown in Table 3.32 below.

Table 3. 25 Effect of tube types on C-peptide quantification.

| Tube type | serum (Gel tube control) | | | | Alternative tube type | | | | | % difference |
|---|---|---|---|---|---|---|---|---|---|---|
| stability | Rpt1 | Rpt2 | Rpt3 | Mean (X1) | Rpt1 | Rpt2 | Rpt3 | Mean (X2) | X1-X2 | |
| Lit heparin 1 | 3571.8 | 3592.5 | 3548.2 | 3570.833 | 3553.5 | 3531.3 | 3549.7 | 3544.833 | -26 | -0.73 |
| Lit heparin 2 | 1351.9 | 1332.5 | 1315 | 1333.133 | 1328.4 | 1317.9 | 1317.1 | 1321.133 | -12 | -0.91 |
| EDTA 1 | 3571.8 | 3592.5 | 3548.2 | 3570.833 | 3401.9 | 3469.4 | 3396.5 | 3422.6 | -148.233 | -4.18 |
| EDTA 2 | 1351.9 | 1332.5 | 1315 | 1333.133 | 1458.9 | 1381.4 | 1360.9 | 1400.4 | 67.26667 | 5.12 |

There was no substantial bias observed for EDTA anticoagulated plasma, lithium-heparin anticoagulated plasma collected in a gel separator tube, or for serum collected in a gel separator tube. The percentage differences were all <8.3%, which was within the acceptable bias for C-peptide.

## 3.4 Discussion: Serum and Plasma Method

### 3.4.1 Method validation

Clinical LC-MS/MS assays are sensitive and offer high specificity but require special considerations as they are often developed in-house. The three key caveats to be considered when replacing an immunoassay with a clinical LC-MS/MS assay are: first, an acceptable performance that can be demonstrated following a validation study that conforms to the relevant laboratory standards. Secondly, the assay must facilitate the recapture of resources, including an improved turnaround time, high sample throughput, ease of use, and significant cost savings. In this research, we have developed an LC-MS/MS C-peptide method and performed full validation to conform to the requirements of ISO 15189.

### 3.4.2 Representative chromatograms

The results of the method validation illustrated good chromatogram resolution of peaks of the C-peptide analyte and internal standard, with no background noise as shown in Figure 3.5. This highlighted an effective and robust extraction process. An effective sample extraction process should be able to remove any interfering contaminants, such as proteins, phospholipids, and salts, to prevent poor ionisation efficiency and low sensitivity (Bugyi *et al*., 2022). In this method, the combination of protein precipitation using methanol and acetonitrile, followed by solid-phase extraction, and a final dilution of the eluent has effectively removed interfering matrix components with minimal ion suppression or enhancement. Protein precipitation has a significant advantage over other extraction methods due to its low cost, simplicity, and speed (Yuan, 2019). Solid phase extraction has several parameters that can be optimised, such as analysis time, reproducibility, binding capacity, or desalting efficiency, and as such makes it the preferred method in peptide analysis (Tubaon *et al*., 2017; Bladergroen and Burgt, 2015; Callesen *et al*., 2009).

### 3.4.3 Calibration curve and linearity

The 12-point calibration curve using bovine serum was linear, with $R^2$ values greater than 0.99 and a slope of 0.9948 response/pmol/L. Monitoring the slope during method validation can provide valuable information on the quality of the method. A variable slope highlights issues such as variations in internal standard concentration, sample preparation errors, and variations in instrumentation (Zhao *et al*., 2014). These variations are usually caused by matrix effects and mass calibrations following instrumentation service maintenance. It is therefore important that the transitions are checked following an instrument service.

The linearity of the standard curve was demonstrated using the mean $R^2$ value for five calibration curves from separate runs and also by using a high-concentration patient sample with a C-peptide concentration of approximately 5200 pmol/L. An $R^2$ of 0.9994 and >0.99 was achieved, respectively, which demonstrated a linear assay. The analytical range of the assay was confirmed as 3 pmol/L – 8000 pmol/L. This covers the clinically relevant range of C-peptide and was an extended analytical range compared to other LC-MS/MS C-peptide methods in the literature. Taylor *et al*. (2016) and Owusu *et al*. (2021) developed C-peptide methods with an analytical range of 36 - 9006 pmol/l and 33.1 – 4966 pmol/L respectively (Owusu *et al*., 2021; Taylor *et al*., 2016).

Calibration verification of the reportable range before clinical application is important when a surrogate matrix is used to prepare standard calibration curves. Samples from an external quality assessment scheme can be used. Alternatively, verification can be done by analysing the calibrators as unknown samples or analysing patient samples of known C-peptide concentrations (Killeen *et al*., 2014). Calibration and analytical range verification for this method were carried out using the CAP calibration verification programme, which showed an acceptable linear evaluation of the range (25 pmol/L to 10000 pmol/L). This confirmed bovine serum as an acceptable matrix for the calibration curve. Deng *et al*. (2023) also used bovine serum to prepare calibrators and found that the properties were similar to those of serum samples (Deng *et al*., 2023).

All dilutions in an assessment of dilution integrity were <10%, confirming that the dilution of samples containing high concentrations of C-peptide above the range of the calibration curve (8000 pmol/L) with water can give an accurate result.

### 3.4.4 Imprecision and accuracy

This method within and between batch precision using spiked IQC and patient pools was all <7.6%, which was within the desirable biological variation specifications of <8.3% for C-peptide measurement for routine assays (González-Lao *et al*., 2019). This was a better performance compared to other methods in the literature. Owusu et *al.* (2021) C-peptide method achieved a within-batch imprecision of 11.2% and 11.8% for between-batch imprecision (Owusu, *et al.,* (2021). A precise C-peptide assay will allow a consistent quality of results for patients and provide accurate results and reproducibility when monitoring patient levels. This also means that patient pooled samples can be used as internal quality control material between assays, which offers significant cost savings.

Accuracy is the most important aspect of method validation for the assessment of systematic errors. In this method, the comparison of UK NEQAS Guildford Peptide Hormones samples demonstrates an acceptable performance, with values within the acceptable range. There was no LC-MS method group within the UK NEQAS Guildford Peptide Hormones scheme, so the assays were compared to the Immulite 2000 method group, which shows a 20% negative bias performance of the Immulite 2000, and this was consistent with the performance of the method group on the EQA report.

Assessment of accuracy using the CAP accuracy-based proficiency testing C-peptide scheme shows the assay can be considered accurate with a bias close to zero, which was acceptable performance. The patient comparison experiment demonstrated a good positive linear relationship between the LC-MS/MS assay and the Mercodia ELISA C-peptide method, with an insignificant negative bias of approximately -1.46 % across the concentration range. This means the existing Mercodia method can be replaced with the LCMS/MS assay at Berkshire and Surrey Pathology without the need to revise the existing clinical decision points.

Foulon *et al*. (2022) also observed a substantial mean bias of 29.5% between an LC-MS/MS C-peptide assay and the Beckman immunoassay (Foulon *et al*., 2022). Deng *et al*. (2023) compared an LCMS assay to Roche, Abbott, Mindray, and Snibe immunoassays and observed a bias of 49.9%, 35.4%, 32.2%, and 81.7%, respectively (Deng *et al*., 2023). This highlights the current issue of C-peptide immunoassay standardisation. Calibration of immunoassay methods by

manufacturers with an IDMS-certified reference material will help achieve standardisation and prevent variations in results.

### 3.4.5 Recovery and carryover

The recovery experiment was carried out to investigate any C-peptide loss during sample preparation and analysis. The percentage recovery was between 101.9% and 108.4%, which was within the acceptable criteria of 80% to 120% and was therefore considered acceptable. This demonstrated that the extraction process was effective. This was also comparable to other methods in the literature. Kinumi *et al*. (2014) and Owusu *et al*. (2021) developed C-peptide methods that achieved a recovery of 99% to 108% and 89.1% to 107.3%, respectively (Kinumi *et al*., 2014; Owusu *et al*., 2021).

Matrix effect and nonspecific binding are well known causes of analyte losses in peptide analysis (Jenkins *et al*., 2015). The result of this experiment demonstrated that the strategies implemented to minimise matrix effects and nonspecific binding were effective. C-peptide has absorptive properties and hence can stick to the components of the instrumentation and can also suffer significant carryovers, particularly on the column or guard column if used. In this assay, the average percentage carryover was <1% and was therefore considered acceptable. This means the column used, the column temperature, and the wash solutions were all effective in minimising carryovers. In laboratories where autosamplers are used, it is important to use wash solutions with at least 10% organic content to wash the sample needle and injection loop, which are good candidates for carryover. In this assay, a 50/50 acetonitrile and water wash solution and a longer dwell time were effective in further preventing carryovers.

### 3.4.6 Method sensitivity

A sensitive LC-MS/MS method is key to reducing sample volume and producing accurate results, especially at low concentration levels. The ability to measure low levels of C-peptide below 33 pmol/L in patients with advanced diabetes and in the

assessment of β-cell capacity in diabetic remission interventions and clinical trials is critical in the management of diabetes (Wang *et al.*, 2012). Sample preparation, instrument setting optimisation, and ionisation efficiency are three key components of method development that can have a significant impact on assay sensitivity. In this assay, using a 250 µL sample volume, calibrator 1 (concentration 3.91 pmol/L) achieved a CV of 17.80%, which was below the desirable specification for allowable total error of 20.8% and therefore considered acceptable as the LLOQ (González-Lao *et al.*, 2019). The percentage accuracy against the nominal concentration was also less than 20.8%. The LLOQ was below the lowest standard on the calibration curve of this assay. The ability to accurately quantify concentrations as low as 3.91 pmol/L will ensure this assay is suitable for the investigation of patients with very low levels of C-peptide, such as in type-1 diabetes, and the assessment of β-cell remission following interventions. In clinical practice, where very low sample volumes are received in the laboratory or where samples must be split for another test. This method has demonstrated that sample volumes as low as 50 µL can be used to accurately quantify C-peptide within the analytical range of 7 pmol/L to 8000 pmol/L. This offers this method a significant advantage over other methods in the literature that require large sample volumes, as shown in Table 1.7. The assay sensitivity gives its clinical utility for the assessment of hypoglycaemia and diabetes diagnosis and management.

### 3.4.7 Matrix effects

Matrix effects are a major concern in peptide assay development, especially when analysing in ESI mode (Zhou *et al.*, 2017). In this method, visual inspection of chromatograms shows no areas of ion suppression or enhancement in the region of the retention time of C-peptide and the internal standard (Figure 3.21 and 3.22). For the quantitative matrix effect, the %ME for all three patient matrices was <15%, apart from the high C-peptide standard spike, which was >15% but corrected for by the internal standard. Matrix effects were also assessed by the method of multiple standard addition method. The aim was to assess if bovine serum was a suitable matrix for preparing the calibrators. There was a percentage difference of 2.5% from the known concentration, demonstrating a good recovery was achieved. This confirmed that the strategies used in this assay to reduce matrix

effects in the extraction steps, including the 1:2 dilution of the final eluent, were effective. Other studies have confirmed that sample dilution may be considered an easy and effective method to reduce matrix effects in LC-MS assays (Ferrer *et al*., 2011; Zhou *et al*., 2017).

### 3.4.8 Interference and stability studies

The presence of high levels of insulin, proinsulin, creatinine, haemolysis, lipaemia, and icteric can cause significant interference in immunoassay methods. In this study, we have assessed the effect of high levels of each interferant on the assay. The results demonstrated that this method was highly specific. The results indicated that samples with bilirubin concentrations up to 171.04 µmol/L, haemoglobin concentrations up to 20 g/L, triglyceride concentrations up to 33.9 mmol/L, creatinine concentrations up to 4.420 mmol/L, insulin concentrations up to 100,000 pmol/L and proinsulin concentrations up to 5000 pmol/L do not cause cross-reactivity or substantially interfere with the quantification of C-peptide. The inability of high insulin levels to cross-react with the assay will be particularly beneficial in cases of insulin overdose and for the diagnosis and management of insulin autoimmune syndrome (IAS). In IAS, there is spontaneous hyperinsulinemic hypoglycemia due to insulin-binding autoantibodies in individuals not receiving insulin therapy. Hyperinsulinemic hypoglycemia with high C-peptide usually indicates endogenous insulin hypersecretion (Cryer *et al*., 2009). High C-peptide immunoreactivity has, however, been reported in IAS when measured with immunoassays, even in the face of frequent profound hypoglyacemia (Church *et al*., 2018; Saxon *et al*., 2016; Cavaco *et al*., 2016). This has the potential to confuse managing clinicians since normal endogenous C-peptide production by the pancreas is inhibited in response to exogenous insulin-induced hypoglycemia (Service et *al*., 1977; Scarlett *et al*., 1977; Kay *et al*., 2021).

This study has also demonstrated that serum samples from gel tubes or plasma samples from EDTA or lithium heparin can be used to quantify C-peptide using this method. The data on the stability of serum is available in the literature and is well-established (Nkuna *et al*., 2023; McDonald *et al*., 2012). This study therefore compared stability among the different tube types. Samples are stable in tubes for up to 24 hours at room temperature, 48 hours at 2 – 4 ℃, and 2 months in the

freezer (-20°C). Serum C-peptide was stably preserved at −80 ℃ for 6 months, which was the duration of the study. This finding was consistent with other stability studies, which found no substantial bias observed for EDTA-anticoagulated plasma, lithium-heparin anticoagulated plasma collected in a gel separator tube, or serum collected in a gel separator tube (Foulon *et al*., 2022; Owusu *et al*., 2021).

## 3.5 General Discussion

### 3.5.1 C-peptide LC-MS/MS method development steps

Compared to immunoassays, before LC-MS/MS test results are released, valuable data that can be reviewed to assure the quality of the analysis. For example, the internal standard in the sample must recover the same level as calibrators and controls. The peak shape must also be appropriate, and the transition ratios must agree with the calibrators and controls. In contrast, it is difficult to identify spurious results when immunoassay steps are not working properly until a clinician notices a disparity between the results and the clinical presentation of the patient. This makes C-peptide analysis by LC-MS/MS superior to immunoassays.

In developing an LC-MS/MS assay for clinical use, there are two key factors to consider. First of all, an assay developed for clinical use requires full method validation to meet regulatory requirements. Secondly, the ability to use a low sample volume is also key for paediatric studies. Developing an LC-MS/MS C-peptide assay for clinical use can, therefore, be challenging. Rogatsky *et al*. (2006) developed a method that was not validated for clinical use, has a large sample volume, and is tedious and time-consuming to extract. Thomas *et al*. (2020) developed a method with a long assay time, poor precision, and poor recovery. Wan *et al*. (2020) also developed a method that had a complex and tedious sample extraction process and poor recovery. This highlights the complexity of LC-MS method development, which this method has overcome.

### 3.5.2 Mass analyser and ionisation technique

The first step in method development is the selection of the mass analyser and ionisation technique. C-peptide methods have been reported whereby mass analysers such as high resolution mass spectrometry (HR-MS) and Time of flight (TOF) were used (Wan *et al*., 2020; Thomas *et al.,* 2020). However, most laboratories prefer the use of a triple quadrupole mass analyser for quantitative peptide analysis because of its ease of use, lower cost, relatively high ion transmission efficiency, large dynamic range, and high sensitivity compared to other mass analysers (Dillen *et al.*, 2012). This C-peptide method was developed using a Waters Xevo TQSX tandem mass spectrometer in ESI-positive mode. C-peptide is a polar, labile, non-volatile, and high molecular mass peptide, hence requiring mild ionisation (Banerjee and Mazumdar, 2012). The Waters Xevo TQSX tandem mass spectrometer in ESI-positive mode offered mild ionisation, enabling the identification of the intact peptide and avoiding additional procedures such as enzymatic digestion, which can be tedious, imprecise, operator dependent and expensive. ESI allows C-peptide, with a molecular mass outside the mass range of a typical mass analyser, to be detected in a lower mass-to-charge range by the formation of multiply charged ions such as (M+2H)2+, (M+3H)3+, or (M+4H)4+). The precursor and fragments with the highest signal-to-noise under the optimal conditions (Table 3.2) were the (M+3H)3+ protonated ions and hence were selected as the parent ions for the C-peptide standard. This method used positive ESI ionisation in multiple reaction monitoring mode (MRM). MRM eliminates the interference of co-eluting contaminants more efficiently than SIM, especially for analytes with high molecular masses such as C-peptide (van den Broek *et al*., 2008).

Optimisation of the resolution settings of a triple quadrupole mass analyser is key for c-peptide analysis, as it can greatly enhance the intensity of ion transmission more than interfering substances. It also helps to clearly distinguish the analyte from the internal standard. This method applied lower LRM and HRM resolutions to enhance product ion response and monitored the transitions of 1007.46 > 147.19 Daltons and 1007.46 > 130.08 Daltons for C-peptide. Fierens *et al*. (2003) and Rogatsky *et al*. (2006) monitored the transitions 1507 > 1320 Daltons and 1007.7 > 147.2 Daltons, respectively, for C-peptide.

### 3.5.3 Selection of an internal standard

In developing an LC-MS/MS C-peptide method, there are some limitations that can have an impact on selectivity, sensitivity, speed, and cost. The aim is to balance all these, but there will always be trade-offs. However, unlike immunoassays, LC-MS/MS has an external calibration curve with internal standardisation, which can correct for these trade-offs. An internal standard properly selected can correct for injection errors, ionisation efficiency, variabilities in recovery, dilution, adsorption, evaporation, degradation, and most importantly, MS detection. MS response may vary largely due to several parameters that are difficult to control, such as matrix effects, which can cause ion suppression of the analyte, especially when ESI is employed. An ideal internal standard should have the same characteristics as the analyte, showing similar extraction recovery but also similar retention time for identical ESI conditions and similar MS ionisation. Stable-labelled isotopes are commonly used as internal standards for bioanalytical peptide LC-MS assays because these compounds are stable and differ from the analyte only by mass, thereby allowing simultaneous MS detection. Stable-labelled isotopes provide confidence for assays developed for clinical use and have been used by several methods. Stenov *et al.* (2011) used C-peptide $^{13}$C-labelled at two leucines (Stenov *et al.*, 2011). The synthesis of stable isotope compounds is very expensive, costing thousands of pounds, and this makes the use of structural analogues a useful and cheaper alternative.

This research assessed the deuterated internal standard ([$D_8$]Val$^{7,10}$)-C-Peptide from Bachem and compared it to the structural analogue (Tyr$^0$)-C-Peptide (human) as an internal standard, and there were no significant differences observed apart from a slight change in retention time. Several studies also observed matrix effects in spite of using a deuterated internal standard. The deuterated internal standard caused a slight change in the retention time, resulting in different ion suppression between the internal standard and the analyte in some studies (Wang *et al*., 2007; Stokvis *et al*., 2005). (Tyr$^0$)-C-Peptide structural analogue differs from C-peptide by the addition of tyrosine. The selection of a structural analogue was based on cost, fitness for purpose, availability, and assay needs. The performance of (Tyr$^0$)-C-Peptide showed a good level of sensitivity. The concentration of the internal standard was aimed at 2000 pmol/L (middle of the calibration curve). Unlike isotope-labelled internal standards, which have identical physicochemical

properties and are therefore lost at exactly the same rate as the endogenous analyte, structural analogues can sometimes show different absorptive losses and therefore might not account for peptide loss (Oe *et al*., 2006; Ciccimaro and Blair, 2010). During the clinical validation process, any issue with the structural analogue internal standard would be obvious. In selecting a structural analogue, the certificate of analysis should be checked for purity greater than 95%.

### 3.5.4 Chromatography optimisation

A detailed understanding of the chemical properties of C-peptide was an essential first step in LC-MS/MS method development, as it guided the selection of the chromatographic technique. C-peptide ionisation efficiency is correlated with many factors, but most importantly pH. Nine different amino acids form the building blocks of C-peptide (Figure 3.24), as shown in Figure 3.25.

| **C-peptide Amino Acid Sequence** |
| --- |
| **H-Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val-Glu-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu-Gly-Ser-Leu-Gln-OH** |

Figure 3. 24 Structure of C-peptide sequence with the 31 amino Acids

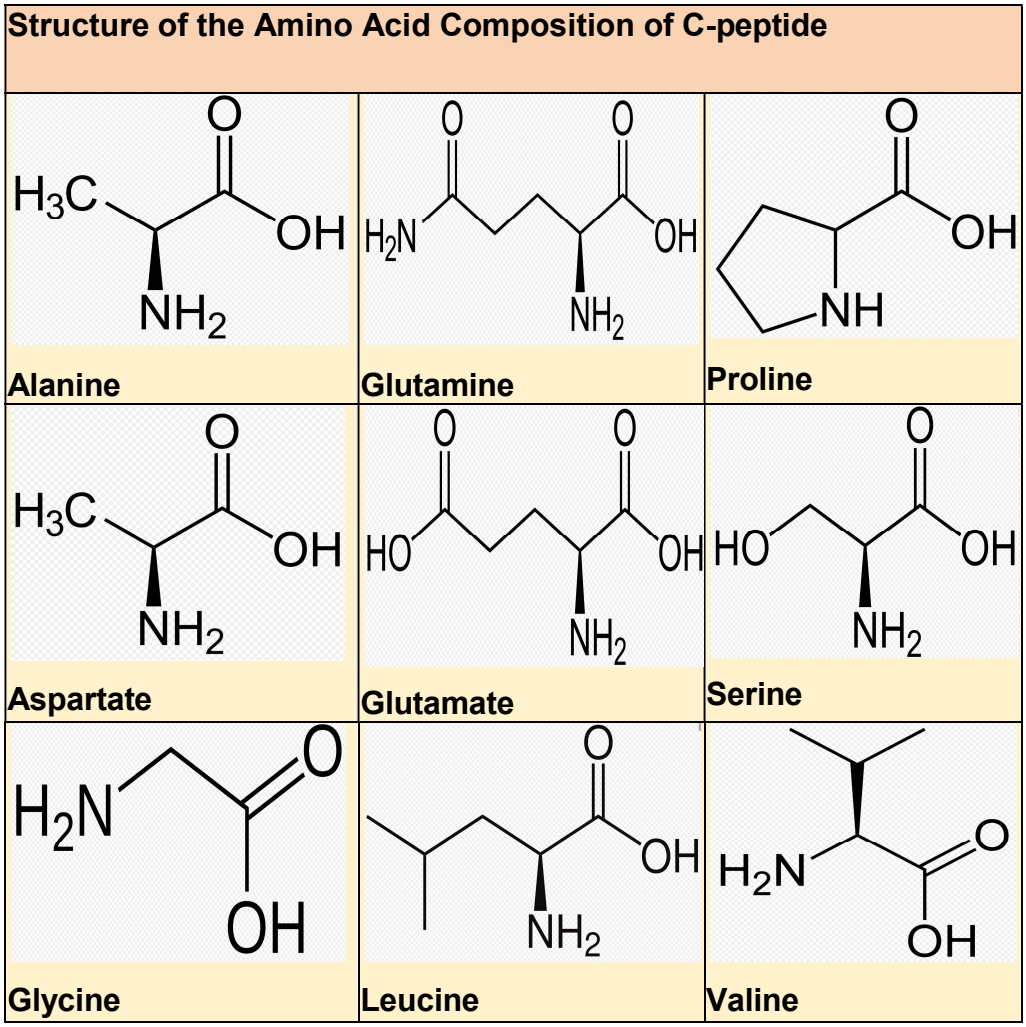



Figure 3. 12 The structure of C-peptide sequence amino acids composition

The figure shows the amine group, the carboxyl group, and the R side chain of each amino acid (Alanine, Glutamine, Proline, Aspartate, Glutamate, Serine, Glycine, Leucine, and Valine).

These amino acids that make up the C-peptide chain are broadly divided by charge, hydrophobicity, and polarity (Table 3.33), which influence the way they interact and guide the selection of chromatographic solvents.

Table 3. 26 The Properties of the amino acid composition of C-peptide

The table shows the single-letter code and three-letter code, their charges, and side chain polarity, and their pKa, pKb, and pKx values. pKa is the acidity of the carboxyl group, and the lower this is, the easier it is for the group to be deprotonated at a neutral pH. The pKb is the basicity of the amine group, and the higher this is, the easier it is for the group to be protonated (accept additional proton). The pKx is the acidity or basicity of the side group.

| **Amino Acid** | **Single letter code** | **Three letter code** | **Charge (+/+/Neatral)** | **Polarity** | **pKa** | **pKb** | **pKx** |
|---|---|---|---|---|---|---|---|
| Alanine | A | Ala | Neutral | Nonpolar | 2.34 | 9.69 | - |
| Aspartate | D | Asp | -ve | Polar | 1.88 | 9.60 | 3.65 |
| Glycine | G | Gly | Neutral | Nonpolar | 2.34 | 9.60 | - |
| Glutamine | Q | Gln | Neutral | Polar | 2.17 | 9.13 | - |
| Glutamate | E | Glu | -ve | Polar | 2.19 | 9.67 | 4.25 |
| Leucine | L | Leu | Neutral | Nonpolar | 2.36 | 9.60 | - |
| Proline | P | Pro | Neutral | Nonpolar | 1.99 | 10.60 | - |
| Serine | S | Ser | Neutral | Nonpolar | 2.21 | 9.15 | - |
| Valine | V | Val | Neutral | Nonpolar | 2.32 | 9.62 | - |

The two most useful optimisation parameters in LCMS analysis of peptides are the organic modifier concentration and the pH of the mobile phase (Sanz-Nebot *et al*., 2000). As shown in Table 3.33, the amino acids of the C-peptide molecule with ionisable acidic side groups are aspartic acid and glutamic acid, with pKx values of 3.65 and 4.25. There are a total of four glutamine acid residues (-4), and one aspartic acid residue (-1), making the net charge of the C-peptide molecule -5. Peptides are separated based on the adjustment of the pH below or above the pKa value. Changes in mobile phase pH can have a dramatic effect on the retention of the compound. C-peptide is an acidic molecule and is retained under acidic pH conditions. Other proteins and peptides have different pKa values and hence varied retention. The pKa for insulin and human proinsulin, two analytes

with the potential to interfere with the C-peptide assay is 5.4 and 5.5, respectively (Mikiewicz *et al*., 2017; Farinas *et al*., 2007). The estimated pKa for C-peptide and other properties of C-peptide used in the method development are shown in Table 3.34 below.

Table 3. 27 C-peptide chemical properties guide for method development

(C-peptide has a molecular weight of 3018 Daltons, making it a large macromolecule. The HPLC index provides a sense of the relative hydrophobicity of the peptide, and the larger and more hydrophobic a peptide, the more you start to see issues with solubility, nonspecific binding, and protein binding. The pI helps determine how to solubilise the peptide).

| Description | C-peptide Chemical Properties |
|---|---|
| Molecular formula | $C_{129}H_{211}N_{35}O_{48}$ |
| Molecular Weight | 3020.3 g/mol (Daltons) |
| Exact Mass | 3019.517928 g/mol |
| Monoisotopic Mass | 3018.514574 g/mol |
| Hydrophobic amino acids | 22.58% |
| Hydrophilic amino acids | 41.94% |
| Neutral amino acid | 35.48% |
| Isoelectric point | pH 3.45 |
| Charge at pH 7 | -5 |
| peptide status | Acidic |
| Instability index | 51.27 |
| Aliphatic index | 103.87 |
| hydropathicity | -0.081 |
| Solubility | Good water solubility |
| Polarity | Polar molecule |

### 3.5.5 Mobile phase and column selection

During method development, the analytical column and mobile phase selection were based on the properties of C-peptide and the matrix, which was human plasma, serum, and whole blood. The choice of mobile phase additives is important to achieve a good chromatographic separation. The mobile phase selected must be compatible with the ESI source, and the additives must enhance or at least not suppress the ionisation of the analyte. There are a significant

number of variables that make the scope of LC optimisation wide, and hence this research took the approach of exploring a large amount of solvent chemistry in a 96-well plate and using a hypothesis-driven experiment. A lower pH mobile phase was generally beneficial for C-peptide ionisation (Table 3.34). In general, methanol is known to offer better retention, but in this method, acetonitrile offered better separation and lower back pressures. Formic acid was also selected over DMSO, TFA, and TFE, similar to other C-peptide methods but with slightly varied additive concentrations (Thomas *et al*., 2021; Deng et *al*., 2021). For acidic compounds, a decrease in pH of the mobile phase below the pKa by the addition of 0.1% formic acid prevents dissociation, enhances polarity, and achieves more retention. 0.1% formic acid as the additive in this assay improved the chromatographic separation by forming ion pairs with the charged groups and thereby increased the hydrophobicity of C-peptide. This affects interaction with the column. Foulon *et al*. (2022) used 0.1% formic acid and 2% dimethyl sulfoxide. Other LCMS applications published for peptide analysis used a water-acetonitrile mobile phase, mostly acidified with TFA. However, in this assay, the use of TFA caused ion suppression and lower sensitivity compared to formic acid.

In general, the smaller the particle size of the column packing, the more likely it is to clog. uPLC columns, however, allow for small solvent volumes while maintaining good chromatography efficiency. The Waters Cortex C18 column with 1.6 µM 2.1 x 100 mm column size material is more appropriate for the C-peptide analysis because it allows for stronger interactions with the hydrophobic regions, enabling efficient separation and retention. C18 also has the advantage of allowing for slight changes in sensitivity to detection and accommodating variability among samples (Ali *et al*., 2022; DeStefano *et al*., 2014; Broeckhoven *et al*., 2017). Depending on the goal of the method, efforts must be made to prolong the lifespan of the column as much as possible. There must be a balance between the time and cost of sample preparation and the cost of replacing the column more frequently. One of the best ways to prolong the lifespan of columns in peptide analysis is by using a guard column. This method did exploit the use of a guard column but caused issues with carryovers and hence was not used. Other studies used an analytical column fitted with a vanguard pre-column (Foulon *et al*., 2022). A study to compare carryover with or without the use of a guard column found that carryover was

reduced in the LC-MS system lacking a guard column by as much as 2.15% (Yamagaki and Yamazaki, 2019).
As an alternative, this method used a shallow gradient and prolonged gradient re-equilibration times, blanks, and long, strong wash steps between injections to help prolong the lifespan of the column. This slightly increased the analysis time to 6.5 minutes but was still significantly quicker than most assays reported in the literature. Peterson *et al*. (2009) demonstrated that shallow gradients enhance the identification of hydrophilic peptides (Peterson *et al*., 2009). Stoyanov *et al.* (2013) also used a shallow linear gradient during the first dimension separation prior to reverse phase column separation to reduce carryover.

During clinical use, retention times, and the area counts of the analytes, and the internal standard must be monitored for reproducibility between runs, with a target of ideally +/- 0.09 seconds and a 20% difference, respectively. A drop in area count signifies a drop in sensitivity, which should prompt additional maintenance procedures such as replacing the ion block, cleaning the cone and step wave, and possibly mass calibration or retuning to check the transitions. This method introduced a system suitability test (SST) using a low concentration standard and internal standard to check system performance before analysis of patient samples. The use of SST ensures the retention time, peak width, peak area, and signal-to-noise ratio are consistent.
An early, late, or variable retention time outside of acceptable limits could indicate a problem with the sample preparation, method design, and HPLC system or column contamination.

In LC gradient optimisation, there should be a balance between resolution and sensitivity. Retention factor can be optimised using the gradient to produce better separation. For this method, the gradient slope was manipulated by changing the percentage of the acetonitrile organic component at the start and finish, and the effect on the area count was assessed. A shallower gradient improved the C-peptide area count and reduced carryover for this method. A mobile phase with 85% organic (acetonitrile) was introduced for an additional minute after the analyte had eluted in order to push off late-eluting contaminants off the column and equilibrate the column for the next injection by calculating the equilibration time for the system dwell volume to wash out.

Temperature optimisation improves sensitivity and recovery in reverse phase HPLC (Martosella *et al*., 2005). An increase in temperature of the C-peptide method offered adequate separation, which was evaluated by the assessment of retentivity, peak shape, and resolution. An elevated temperature of 45 °C improved resolution and decreased retention time, because of lower mobile phase viscosity. After the temperature was optimised, it was important to keep the temperature of the HPLC column constant or controlled during each analysis to achieve reproducible results, especially in situations where the temperature in the room is higher than the column temperature (Chen, 2019). A change in the temperature of the column can affect the back pressure, which in turn affects separation.

The flow rate can also influence sensitivity (Page *et al.,* 2007). C-peptide can stick to surfaces and is therefore prone to column carryover which, can be reduced by decreasing the amount of sample injected. More time is needed to get in and out of the chromatographic pores. In this method, slowing the flow rate down to 0.23ml/min for a prolonged period improved diffusivity, overall sensitivity, and minimised carryover, which was consistent with other studies that found lower flow rates increase peak capacity of peptides in reverse phase chromatography (Wang *et al*., 2006).

To ensure that the chromatographic performance is acceptable after optimisation, characteristics such as the peak asymmetry, capacity factor, and plate height were estimated. Peak asymmetry was assessed to investigate the mobile phase used, sample diluent, dead volume, injection volume, and the quality and absorptive effects of the column, which could cause chromatographic peak fronting or tailing. The peak asymmetry was 1.0256, which confirms a Gaussian shape peak was achieved (Figure 3.5). The retention factor was calculated to assess the retention of the C-peptide on the column used. C-peptide must be retained on the column long enough to allow a lot of contaminant in the sample, such as salts and other polar compounds, to elute at the void time. A high retention factor of 5.59 indicates that the analyte was highly retained on the column and achieved a good separation.

Plate height was calculated to assess the column efficiency. The quality of the column packing, the particle size, and the column dimensions all contribute to the

column efficiency (de Villiers *et al*., 2006). For efficiency separations, the theoretical plate number must be high and the plate height low, as was achieved in this research (Table 3.4). The theoretical plate number for uPLC is expected to be greater than 2,000 (Dolan, 2016). The ideal plate height for uPLC columns is also between 0.0014 and 0.0025 mm (Gritti and Wahab, 2018). Each plate is the distance over which the sample components achieve one equilibration between the stationary and mobile phase in the column. Therefore, the more theoretical plates available within a column, the more equilibrations are possible, and the better quality the separation. Column performance over time can be monitored and replaced when the plate number falls below a predetermined value, usually approximately 30% loss (Dolan, 2016).

### 3.5.6 Fundamental challenges during method development

The primary issues during MS tuning, column selection, and chromatographic optimisation included solubility, specificity, protein binding, nonspecific binding, and chemical instability. There were other issues, but they were symptoms caused by one or more of the primary causes, such as peptide loss, poor sensitivity, and chromatography (inadequate retention, peak shape, carryover, and low extraction recovery).

### 3.5.7 Assessing solubility

C-peptide can stick to parts of the LC-MS/MS system, as indicated by the pI, HPLC index, and amino acid sequence. Problems with solubility were expected and investigated by dissolving and injecting the pure standard in different mobile phase combinations and organic modifiers such as acetic acid, DMSO, TFA, TFE, and formic acid. An increased area count was used as an indication of good sensitivity. Methanol, water, and acetic acid in a ratio of 6:4:1 improved the solubility of C-peptide. The final eluent after solid-phase extraction was therefore diluted in this mobile phase combination before injection without the need for a drying down step. Sample eluent diluted in a buffer that is not the same as the mobile phase starting conditions can have an impact on the peak shape and sensitivity. Some of the

steps that can be taken to reduce the impact include ensuring that the diluting buffer has a lower solvent strength compared to the mobile phase. Reducing the injection volume to less than 10% of the nominal flow rate also helps to balance solubility and peak shape. Other studies reconstituted the eluent in 10% acetonitrile/water containing 0.4% formic acid for LC-MS/MS analysis but required a drying down step (Deng *et al*., 2023). This may improve the chromatography but increase the TAT and cost due to requiring a drying step and a vacuum centrifuge.

### 3.5.8 Issues with nonspecific binding

When working with neat standard solutions or matrixes that have very low protein content, you often encounter issues with nonspecific binding. C-peptide has a high polarity and is hence prone to ionic interactions and absorptive properties, hence leading to issues with nonspecific binding and protein binding. The most effective way to prevent this was by using the carrier protein, rat plasma and 0.05% rat plasma was added to the working C-peptide and internal standard solutions. The 0.05% rat plasma enhanced solubility and prevented nonspecific binding by holding the C-peptide or the internal standard in solution before spiking into the sample matrix. Carrier proteins also act as transporters and preferentially coat the sides of vessels. The impact of a carrier protein can be assessed by comparing the peaks with and without a carrier protein. In this method, there was a loss of C-peptide (low peak detection) during sample preparation and LC stages without a carrier protein. The addition of 0.05% rat plasma to neat solutions helped to minimise binding to parts of the LC system, such as the column and other LC tubing. Other researchers had used carrier proteins such as bovine serum albumin (BSA) as non-specific binding competitors to prevent C-peptide loss during analysis (Fierens *et al*., 2003; Fierens *et al*., 2000; Vanplaterink *et al*., 2006). Our method used rat plasma because it was easier to prepare compared to BSA.

## 3.6 Sample extraction optimisation

There are numerous proteins, lipids, salts, and other components present in serum, plasma, and whole blood that can cause assay interference. An effective LCMS extraction process helps purify and concentrate the analyte of interest, promoting sensitivity and reducing matrix effects. This is a significant advantage

over an immunoassay, whereby it is almost impossible to isolate sufficiently ‘clean’ analytes free from interferences such as autoantibodies that may compete for binding sites (Kay *et al*., 2021).

The development of an effective sample extraction process is the final and most challenging part of the method development. Generally, to achieve sufficient enrichment and purification, it is essential to combine multiple preparation methods. Ultimately, the selection of an extraction method is based on cost, complexity, throughput, recovery, and matrix effects.

This method used a combination of protein precipitation and the Oasis PRiME HLB SPE cartridge in a reversed-phase sample matrices clean-up steps. The protein precipitation protocol and the solid phase extraction provided more opportunity to remove interferences, reducing the complexity of the sample. Protein precipitation in this method involved the use of a combination of acetonitrile and methanol, followed by centrifugation. It is simple but requires further sample processing to avoid the risk of contamination or ion suppression from the remaining matrix components. Deng *et al*. (2023) also used protein precipitation, but in contrast, with $ZnSO_4$ solution (Deng *et al*., 2023). This research found that $ZnSO_4$ caused significant ion suppression when added to the precipitation solution.

SPE is based on the interaction between C-peptide and the sorbent material in the SPE cartridges, the choice of which is based on the polarity and charge, which makes it a selective, sensitive, and versatile technique. SPE offers selective extraction using cartridges packed with silica-based or polymer-based sorbents (Badawy *et al*., 2022). Many SPE options are available, such as reversed phase, ion exchange, normal phase, and mixed mode phases (Sentellas *et al*., 2020). The SPE used in the method was semi-automated using the Water's positive pressure manifold and multichannel pipettes to reduce manual steps, resulting in a less laborious and less time-consuming method. Some methods have been reported in the literature using ion-exchange chromatography, two-dimensional liquid chromatography, and derivatisation for sample clean-up (Deng *et al.,* 2021). Rogatsky *et al.* (2006) used a two-step chromatographic process to purify C-peptide from most interferences. LLE is suited for nonpolar compounds, but because C-peptide exhibits ionic activity, the usefulness of this method was considered limited and not explored in this research. Another technique is enzymatic digestion, which involves the cleavage of C-peptide into smaller

fragments using a proteolytic enzyme under specific conditions, and the fragment is quantified to serve as a representation of the intact peptide (Deng *et al*., 2021). Several applications of enzymatic digestion for the quantification of C-peptide have been reported. Owusu *et al*. (2021), developed an LCMS measurement procedure for C-peptide based on endoproteinase Glu-C digestion. However, the sample preparation process was tedious and required up to two days to complete due to an 8-hour incubation step. Connolly *et al*. (2023) compared the enzymatic digestion workflow to the MRM quantitation of intact C-peptide and found that both methods showed no significant matrix effects, but the determination of intact C-peptide was simpler in clinical practice to implement due to a smaller number of separation steps.

Owing to C-peptide sharing its amino acid sequence with proinsulin, it is also necessary to consider the contribution of proinsulin when quantifying C-peptide following enzymatic digestion, adding additional complications. Another sample clean-up technique is immunoaffinity, which is based on high-specificity binding reactions between antigens and antibodies (Deng et al., 2021). There are two commonly used immunoaffinity purification approaches: magnetic beads and column-based procedures. This technology has recently been used for the immunoaffinity purification of C-peptide (Thomas *et al.*, 2020). However, the significant downsides of immunoaffinity purification are the high cost of antibodies, the time-consuming nature of the procedure and issue of autoantibodies in sample affecting the interaction.

## 3.7 Clinical application of the C-peptide method

This research has developed a cost-effective, sensitive C-peptide LC-MS/MS assay for serum and plasma with vast clinical applications such as diabetic patient screening and management, hypoglycaemia investigation, clinical trials, assay harmonization/standardisation, and the potential to offer an accuracy based external quality assessment scheme.

### 3.7.1 Diabetic patient screening and management

The method is expected to replace the Mercodia ELISA method for diabetes diagnosis/management and for the investigation of causes of hypoglycaemia. The factors to consider in the introduction of an assay for patient sample analysis are sample type, sample stability, cost, time, appropriate analytical and clinical performance specifications. The current limitations of the Mercodia Elisa assay this method would overcome include high cost, and laborious assay procedures (Shah and Maghsoudlou, 2016; Hosseini *et al*., 2018).

### 3.7.2 Assay standardisation efforts

This method can also help with ongoing assay standardisation efforts. Currently, results reported from one laboratory or clinical trial to the next are not comparable. Insulin and C-peptide assays are not yet standardised so there is the need for the laboratory to derive own cut-offs for new LC-MS assays before clinical application. This is particularly important as current guidelines such as the Endocrine Society guidelines for the investigation non-diabetic associated hypoglycaemia, do not state which assays were used to determine cut-offs, and cut-offs are assay dependent.

Harmonisation of current immunoassays is also a problem because antibodies are heterogeneous and their affinity for the antigen of interest may vary over time, even from batch to batch (Hörber *et al*., 2020).

Standardisation of C-peptide is achievable as reference measurement procedures and reference materials that can be traced to the SI have been developed. The JCTLM database shows there are two reference procedures for C-peptide based on ID-LC-MS/MS. With the help of isotopic dilution LC-MS/MS, the primary reference materials tracing to SI units (NMIJ CRM6901-b; NMIJ CRM6901-c) are also listed in the JCTLM database and can be used for calibration (Little *et al*., 2017). C-peptide measurement can be traced to higher-order reference measurement procedures by calibrating manufacturers' commercial systems with a panel of native clinical samples assigned by accredited reference laboratories performing reference measurement procedures. There is currently no accredited

laboratory that provides reference services for C-peptide measurements listed in the JCTLM database (Little *et al*., 2017). This LC-MS/MS method can contribute to and play a significant role in the standardisation of C-peptide measurement by registering as a reference measurement procedure and then providing reference services to manufacturers and clinical laboratories. JCTLM registration requires a comparison between two reference laboratories. This method can help with ensuring comparability across different methods and different laboratories. In addition, this method can be used to enhance the UK NEQAS Guildford Peptides EQA program by setting target values, which will provide a useful tool to evaluate the performance of routine measurements and promote the standardisation of C-peptide measurement in clinical laboratories in the UK and overseas.

## 3.8 Cost-benefit analysis

The most expensive part of an LC-MS/MS assay in the laboratory is the initial high cost of the equipment. The equipment can be purchased outright (average cost £250,000), but most laboratories often acquire the equipment on a lease contract with the total cost expected to depreciate, sometimes as much as 50% over time, offering cost savings in the long term. The cost of the reagents used to run the instrument as well as the cost of the extraction is, however, significantly lower than that of the immunoassay reagents.

The factors affecting the cost per test and the impact of providing a C-peptide clinical service were considered in this research, as shown in tables 3.35 and 3.36 below. A detailed costing data for the LC-MS/MS method and the Mercodia Elisa assay can be found in Appendix 1 and 2 respectively.

Cost implications using cost-outcome models (old cost vs. new cost) show that the LC-MS/MS method cost per sample is 53.4% (£8.09) lower than the Mercodia ELISA assay (£20.55).

Table 3. 28 Cost-per-test of using the LC-MS/MS method.

| Total cost of C-peptide (Average run 50 samples) | | |
|---|---|---|
| | | Cost |
| Calibrators and Controls | | 0.01373 |
| Extraction | | 0.8761 |
| Column+SPE plate | | 204.78 |
| Mobile phases | | 5.12 |
| Other Consumables | | 28.36 |
| Nitrogen gas | | 3.04 |
| Argon | | 2.14 |
| Instrumentation cost | | 35.66 |
| Band 6 running cost | | 124.50 |
| | Total | 404.50 |
| | | |
| Cost per test C-peptide (£) | | 8.09 |

Table 3. 29 Cost-per-test using the Mercodia ELISA test kit

| **Total cost of C-peptide (Average run 34 samples)** | Cost(£) |
|---|---|
| Cost of kits | 550.80 |
| Cost of tips | 2.96 |
| Cost of IQC | 1.75 |
| Cost of EQA | 0.08 |
| Instrumentation | 18.53 |
| Staff costs | 124.59 |
| **Total** | 698.71 |
| | |
| Cost per patient | 20.55 |

As shown in the table above, the day-to-day running costs are mostly from the SPE plate and chromatography column for the LCMS assay. The solvent volumes and costs are significantly low. The SPE plates can be used to run 96 samples, and a C18 column can provide over 1000 separations before it needs replacing.

The extraction process can also be automated to further decrease staff cost which can be beneficial considering the current difficulties in recruitment and retention of biomedical scientists.

A cost-benefit analysis can be used to evaluate the economic value of a new laboratory test against its alternatives before implementation. The test value must include the level of accuracy and improvement in turnaround time. This LCMS method match the required TAT and can offer a same day result if required. A good test performance is associated with high accuracy and a short turnaround time (Bogavac-Stanojevic and Jelic-Ivanovic, 2017). In addition, healthcare decisions should be based on a broad range of variables, such as priorities, accessibility, and ease of use, when deciding whether to adopt a new laboratory test (Wiseman *et al.*, 2016).

## Chapter 4.0 Results for the DBS Method

### 4.1 Dry Blood Spot (DBS) method verification

DBS sampling was verified against the serum LC-MS/MS method following the Clinical and Laboratory Standards Institute (CLSI) guidelines for confirming the suitability of an alternative candidate specimen, EP35 ED1. This required an assessment of precision at clinical decision points and a sample comparison to evaluate systemic differences. Parameters assessed include the calibration curve, LOD, LOQ, systematic difference, and precision at each medical decision level.

#### 4.1.1 DBS Calibration curve and linearity

C-peptide standard was spiked into calf whole blood, and aliquots 50 µL were spotted on a Whatman DBS card and dried. A calibration curve was performed using the extraction protocol following sample elution from the DBS card. Figure 4.1 below represents a typical calibration curve that shows good linearity across the concentration range of the standard curve. The calibration curve $R^2$ was 0.999477, which was acceptable.

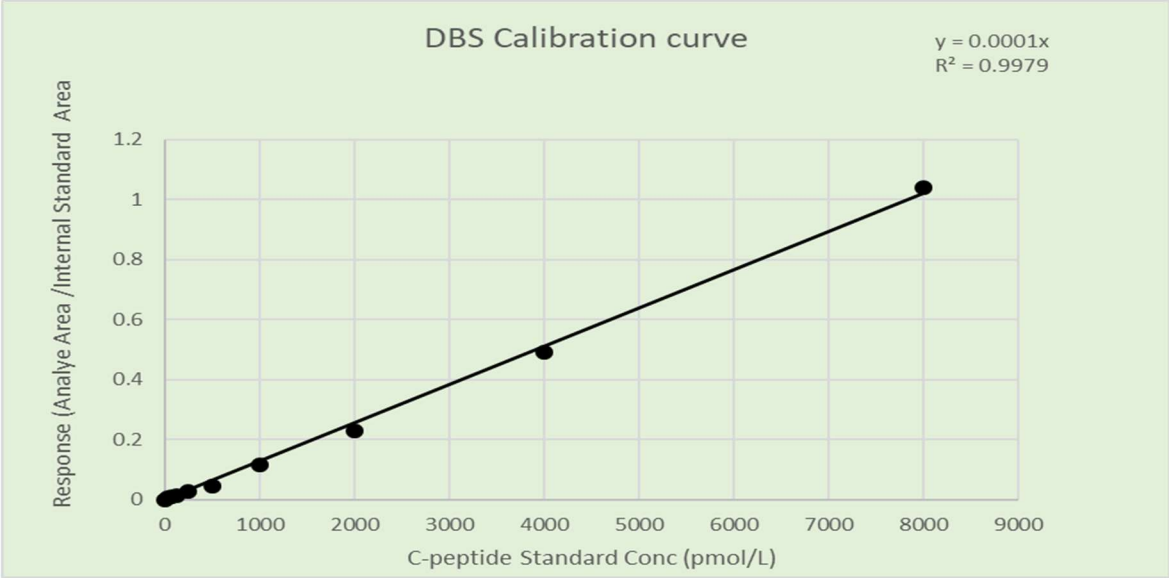



Figure 4. 1 Representation of a typical C-peptide calibration curve for DBS.

To assess the Lower Limit of Quantitation (LLOQ), calibrators 2 to 5 were run 10 times in a single batch, and the %CV were calculated. The results are shown in Table 4.1 below. Calibrator 3 (concentration 62.5 pmol/L) achieved a CV of 8.66%, which was less than the acceptable total error of 20.8%, this was considered acceptable as the LLOQ.

Table 4. 1 Sensitivity for DBS C-peptide quantitation

| **Sensitivity check** | Cal 2 | Cal 3 | Cal 4 | Cal 5 |
|---|---|---|---|---|
| | 31.5 | 66.7 | 128.2 | 248.6 |
| | 40 | 70.1 | 120.9 | 239.5 |
| | 28.3 | 69.2 | 130.5 | 264.3 |
| | 29.7 | 59.3 | 135.4 | 250.8 |
| | 27.6 | 54.7 | 125.8 | 256.6 |
| | 39.5 | 63.7 | 119.8 | 229.5 |
| | 25.8 | 72.5 | 129.4 | 248.4 |
| | 37.5 | 59.5 | 132.5 | 260.3 |
| | 44.2 | 64.3 | 116.9 | 258.3 |
| | 25.8 | 68.2 | 123.8 | 238.5 |
| **Mean** | 32.99 | 64.82 | 126.32 | 249.48 |
| **SD** | 6.708278 | 5.614624 | 5.947698 | 10.99614 |
| **CV** | 20.33% | 8.66% | 4.71% | 4.41% |

4.1.2 Comparison of imprecision between DBS and serum

A comparison of imprecision between serum and DBS was carried out using samples that cover C-peptide clinical decision points. The intervals were set at 0 – 200 (100 pmol/L), 300-400 (350 pmol/L), 500-800 (700 pmol/L), 800-1200 (900 pmol/L, and 1500 - 2000 (1800 pmol/L). The precision of each specimen type was obtained from the results of at least 5 replicates, and the allowable CV was set using the desirable specification from Biological Variation. The $SD_{DBS}/SD_{SERUM}$ (SD ratio) was also calculated, and the results of the precision study are shown in Tables 4.2 and 4.3 below.

Table 4. 2 DBS precision at C-peptide clinical decision points

| Conc | 87.80 | 320.32 | 666.28 | 1028.69 | 1711.69 |
|---|---|---|---|---|---|
| (pmol/L) | 86.13 | 348.29 | 580.62 | 998.02 | 1931.68 |
| | 79.21 | 325.57 | 744.51 | 958.03 | 1918.83 |
| | 86.97 | 330.13 | 628.22 | 932.55 | 1800.34 |
| | 90.47 | 313.90 | 601.28 | 1102.31 | 2041.83 |
| **Mean** | 86.12 | 327.64 | 644.18 | 1003.92 | 1880.87 |
| **SD** | 4.19 | 13.03 | 64.59 | 66.20 | 127.51 |
| **%CV** | 4.87% | 3.98% | 10.03% | 6.59% | 6.78% |

Table 4. 3 Serum C-peptide precision at clinical decision points

| Conc | 88.78 | 339.94 | 628.54 | 1004.81 | 1800.61 |
|---|---|---|---|---|---|
| (pmol/L) | 95.09 | 328.12 | 654.90 | 1011.52 | 1834.88 |
| | 90.75 | 320.74 | 687.40 | 979.33 | 1820.39 |
| | 88.27 | 332.99 | 647.29 | 955.70 | 1820.46 |
| | 91.31 | 319.86 | 664.06 | 1004.98 | 1737.40 |
| **Mean** | 90.84 | 328.33 | 656.44 | 991.27 | 1802.75 |
| **SD** | 2.70 | 8.45 | 21.69 | 23.39 | 38.51 |
| **%CV** | 2.97% | 2.57% | 3.30% | 2.36% | 2.14% |

Table 4. 4 SD Ratios between serum and DBS for C-peptide clinical decision points (pmol/L)

| Clinical Decision points | Performance | Serum(Primary) | DBS | $SD_{serum}/SD_{DBS}$ |
|---|---|---|---|---|
| | | | | |
| 100 | Mean | 90.84 | 86.12 | 0.64 |
| | SD | 2.70 | 4.19 | |
| | %CV | 2.97 | 4.87 | |
| 350 | Mean | 328.33 | 327.64 | 0.65 |
| | SD | 8.45 | 13.03 | |
| | %CV | 2.57 | 3.98 | |
| 700 | Mean | 656.44 | 644.18 | 0.42 |
| | SD | 21.69 | 51.59 | |
| | %CV | 3.30 | 10.30 | |
| 900 | Mean | 991.27 | 1003.92 | 0.35 |
| | SD | 23.39 | 66.20 | |
| | %CV | 2.36 | 6.59 | |
| 1800 | Mean | 1802.75 | 1880.87 | 0.30 |
| | SD | 38.51 | 127.51 | |
| | %CV | 2.14 | 6.78 | |

The calculated ratio of $SD_{DBS}/SD_{SERUM}$ (SD ratio) (Table 4.4) were all below 1 at all subintervals which was considered clinically acceptable. The precision CVs were also less than <8.3%.

4.1.3 Evaluation of systematic differences between DBS and serum

The systematic difference between serum and DBS across medical decision levels was calculated using Deming regression analysis and compared to the total allowable error (TEa) of 20.8%.

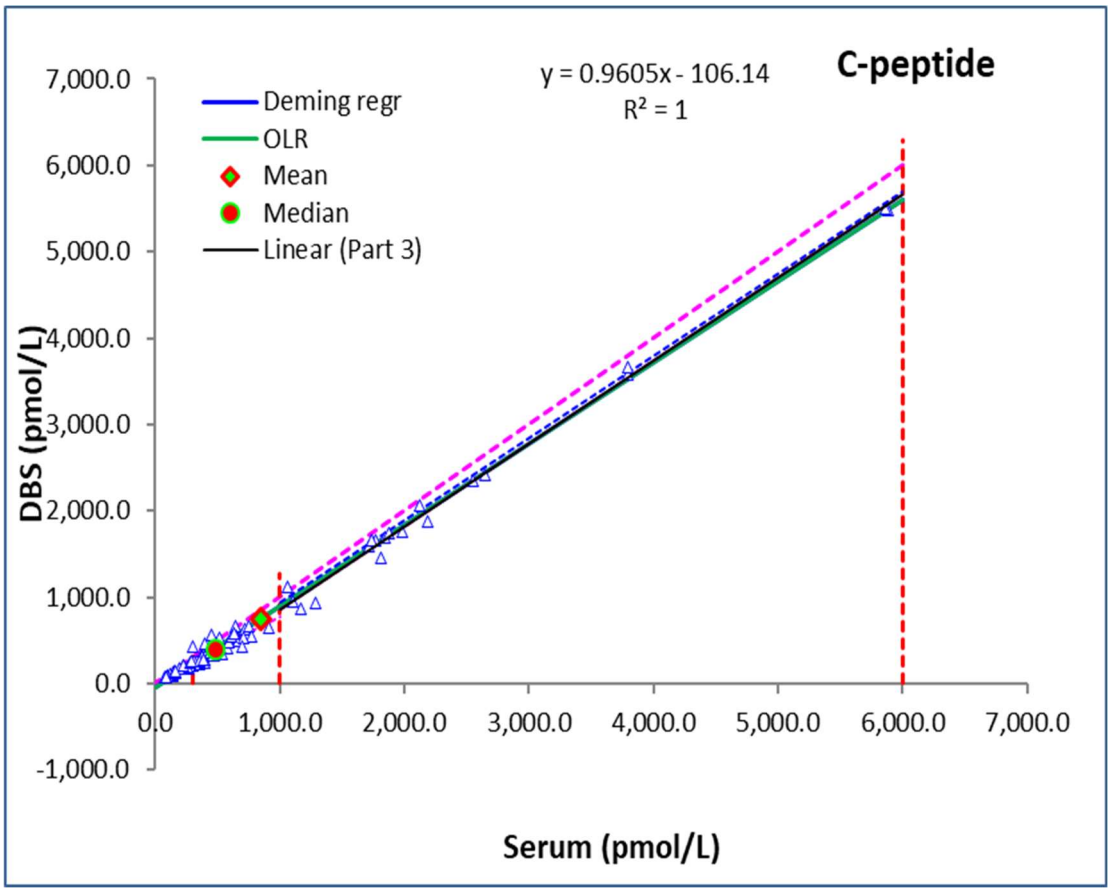



Figure 4. 2 Patient sample comparison between serum and DBS

Systematic differences across the medical decision levels were investigated according to CLSI EP35-ED1. The serum C-peptide compared with DBS C-peptide levels are illustrated in Figures 4.2 and 4.3. The Deming regression analysis gave a slope of 0.96. The $R^2$ value was 1. These results show a good correlation between the two methods. There is a considerable negative bias, however, of approximately -14.2%. Compared to the serum results using the LC-MS/MS, the systematic differences in DBS were -15.7%, -17.3%, and -9.6% at 0 - 300, 300-1000, and 1000-6000 pmol/L C-peptide results partitioning, respectively (Figure 4.3). When the allowable total error criteria (20.8% for C-peptide) were applied, the systematic differences compared to serum were acceptable when a DBS specimen was used on the LC-MS/MS.

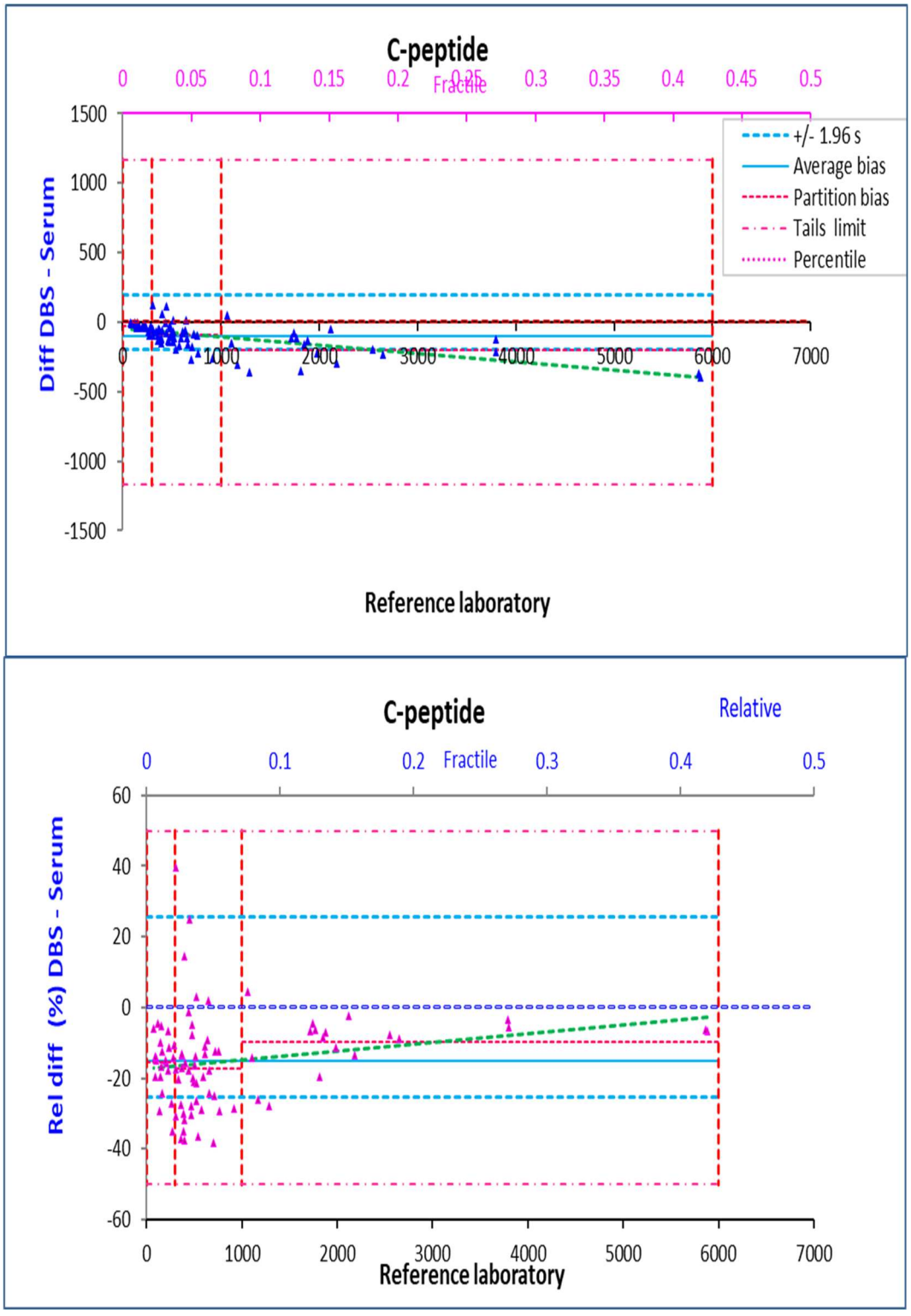



Figure 4. 3 Systematic difference plot between DBS and serum

4.2 Stability studies of DBS sample storage

DBS cards spotted with whole blood at various concentrations were stored at room temperature (21°C), fridge (4°C), freezer (-20.0°C), and ultralow freezer temperature (-80.0°C). A set of samples was extracted at regular intervals, and the results were analysed (Table 4.5). The results show an acceptable bias below 8.3% at the storage temperatures and durations covered in the study.

Table 4. 5 Effect of DBS Sample Storage on C-peptide (pmol/L)

| **Room Temp (21°** | **Run 1** | **Run 2** | **Run 3** | **Mean** | **%Bias** |
|---|---|---|---|---|---|
| week 1 | 1249.5 | 1254.8 | 1251.9 | 1252.07 | 1.30% |
| week 2 | 1239.6 | 1241.6 | 1229.7 | 1236.97 | 2.49% |
| Week 3 | 1225.8 | 1219.5 | 1226.3 | 1223.87 | 3.52% |
| Week 4 | 1212.5 | 1215.7 | 1210.9 | 1213.03 | 4.37% |
| | | | | | |
| **Fridge (4°C)** | **Run 1** | **Run 2** | **Run 3** | **Mean** | **%Bias** |
| week 1 | 1255.5 | 1254.8 | 1248.5 | 1252.93 | 1.23% |
| week 2 | 1253.8 | 1241.4 | 1252.8 | 1249.33 | 1.51% |
| Week 3 | 1227.8 | 1231.6 | 1218.9 | 1226.10 | 3.34% |
| Week 4 | 1216.8 | 1211.6 | 1217.5 | 1215.30 | 4.19% |
| | | | | | |
| **Freezer (-20°C)** | **Run 1** | **Run 2** | **Run 3** | **Mean** | **%Bias** |
| Month 1 | 1257.3 | 1247.4 | 1252.8 | 1252.50 | 1.26% |
| Month 2 | 1241.5 | 1245.2 | 1236.7 | 1241.13 | 2.16% |
| Month 3 | 1230.7 | 1229.6 | 1228.6 | 1229.63 | 3.06% |
| Month 4 | 1223.7 | 1221.5 | 1218.9 | 1221.37 | 3.72% |
| | | | | | |
| **Ultra Low (-80°C** | **Run 1** | **Run 2** | **Run 3** | **Mean** | **%Bias** |
| Month 1 | 1263.8 | 1256.9 | 1254.6 | 1258.43 | 0.79% |
| Month 2 | 1259.8 | 1255.8 | 1249.2 | 1254.93 | 1.07% |
| Month 3 | 1247.5 | 1252.3 | 1258.3 | 1252.70 | 1.25% |
| Month 4 | 1224.3 | 1238.2 | 1248.3 | 1236.93 | 2.49% |
| Month 5 | 1244.8 | 1238.6 | 1241.5 | 1241.63 | 2.12% |
| Month 6 | 1230.8 | 1228.6 | 1235.9 | 1231.77 | 2.90% |

4.3 Effect of haematocrit on accuracy

The effect of haematocrit was accessed by spiking C-peptide standards into Equine whole blood with different haematocrit concentrations by diluting with plasma, and the recovery was calculated (Table 4.6).

Table 4. 6 Effect of haematocrit (HCT) levels on DBS C-peptide quantification (pmol/L)

| HCT(L/L) | 0.476 | 0.422 | 0.341 | 0.303 | 0.241 |
|---|---|---|---|---|---|
| HCT %diff | 0.00% | 11.34% | 28.36% | 36.34% | 49.37% |
| Repeat 1 | 1171.7 | 1134.9 | 1252.5 | 1368.4 | 1492.8 |
| Repeat 2 | 1157.3 | 1217.5 | 1280.3 | 1363.2 | 1547.4 |
| **Mean** | 1164.5 | 1176.2 | 1266.4 | 1365.8 | 1520.1 |
| **%Diff** | 0.00% | -1.00% | -8.75% | -17.29% | -30.54% |

The percentage difference was all less than the total allowable error of 20.8% when there was a variation of up to 36.34% in haematocrit levels.

## 4.4 Discussion: DBS Method

### 4.4.1 Verification of DBS against serum sample type

DBS analysis showed good chromatogram resolution of peaks with no background noise following an effective sample elution and extraction process. Whole blood on the DBS card precipitates the proteins, and during elution, C-peptide and smaller molecules were eluted into solution using PBS/BSA. The addition of methanol and acetonitrile further cleaned the sample before SPE following the sample extraction protocol as for serum samples. The 10-point calibration curves were linear, with $R^2$ values greater than 0.99. The analytical range of our DBS assay was confirmed as 60 pmol/L – 8000 pmol/L. This was considered to better cover the clinically relevant range of C-peptide and the assessment of beta cell function in type 1 diabetes. Johansson *et al*. (2010) evaluated C-peptide from DBS samples on the Siemens Immulite 2000 platform. The detection limit of the assay was 440 pmol/L which can support general diabetes screening (Johansson *et al*., 2010). Most type 1 diabetic patients, however, have impaired beta cell function with low C-peptide values below 200 pmol/L. Therefore, our assay has superior clinical utility.

The imprecision of the DBS LC-MS method was compared to serum, the primary specimen, and the SDserum/SDdbs ratio was all less than 1.00. The CVs of intra-batch precision were all less than 8.3% at all clinically relevant concentrations, which was consistent with the acceptable criteria for imprecision based on biological variability and better compared to what Butter *et al*. (2001) reported for their DBS insulin assay. Their reported intra-assay and inter-assay variability were 14% and 25%, respectively (Butter *et al*., 2001).

Biological factors such as haematocrit level may lead to variation in sample quality because there is an inverse relationship between blood haematocrit and spot area (Butter *et al*., 2001; Zakaria *et al*., 2016). The study to investigate the effect of haematocrit and homogeneity on DBS samples demonstrated a mean deviation of less than 17.29% when samples were diluted up to 36.34%, which was considered acceptable. Our method used a large punch (8 mm), which may partly overcome the variety caused by the haematocrit concentration. The study only covered the haematocrit range of 0.34 to 0.58L/L. Haematocrit may affect values in different

populations, such as children, the elderly, or populations with various diseases. The average limits for haematocrit concentration are approximately 0.45 to 0.50L/L in adults but tend to be higher in neonates (approximately 0.56L/L) for the first 3 days of life (Dworkin, 1992; Hoffman and Pettit, 1993; Butter *et al*., 2001). Samples with expected haematocrit above the range covered in this research may require further study and correction factors applied if needed before clinical application.

The results of this study are similar to what other researchers found. A study to assess the effect of haematocrit on the measurement of vitamin $D_3$ concentrations found that within a haematocrit range of 0.4 to 0.6L/L, very little change in levels was seen (Man *et al*., 2019). Experimental results from another study indicated that there were no significant differences when the concentration of insulin was measured in bloodspots that had a haematocrit of 0.50 or 0.55L/L (Diamandi *et al*., 1998; Butter *et al*., 2001).

### 4.4.2 Comparison between DBS and serum sample types

In the absence of paired serum and DBS patient samples, Cerrillant C-peptide was spiked into bovine serum and equine whole blood samples to represent patient samples. The concentrations spiked into paired bovine serum and equine blood were the same, and each set was taken through the extraction and quantification steps for serum and DBS, respectively. The comparison was carried out using the Bland Altman and Passing-Bablok plots. The DBS specimens showed comparable performance with the serum sample type for C-peptide testing. The evaluation of the systematic difference among DBS and serum specimen types at clinical decision points shows an acceptable correlation coefficient. This is similar to other studies reported in the literature. Willemsen *et al*. (2018) demonstrated a correlation of 0.91 between DBS samples and plasma. Using a chemiluminescence assay, a pilot study also found that C-peptide can be accurately measured from capillary blood samples collected using either dried blood spots or a volumetric absorptive microsampling (VAMS) device with an overall agreement of 0.929 (Jones *et al*., 2018). The slopes and the intercepts of this method were acceptable using the Passing-Bablok regression analysis. The DBS was about -14.2 % negatively average biased against the serum for the

patient comparison. Negative differences were consistent at all levels. In general, DBS performance is consistent with appropriate clinically based performance goals specific to the new specimen type but would require a separate reference range from serum samples due to the apparent bias.

This study assessed the stability of the DBS samples, which were considered stable for up to 4 weeks at room temperature or 4°C, 4 months at -20°C and 6 months at -80°C. In a stability study at room temperature of DBS and VAMS samples, Jones *et al*. (2018) observed that there were decreases of 11.6% and 0.1% observed after 48 hours of storage at room temperature for DBS and VAMS, respectively. Johansson *et al*. (2010) demonstrated that C-peptide is stable on DBS filter paper cards for 6 months.

### 4.4.3 Cost-benefit analysis of DBS sampling

The laboratory cost per test of DBS sampling is similar to that of the serum method because both methods use the same sample extraction procedure after DBS elution. There is an extra cost associated with the elution of the DBS sample. The cost of DBS sample elution is £0.00366, as shown in table 4.7 below, which highlights that there is no significant cost per test difference between serum (£8.09) and DBS (£8.094) using this LCMS assay as shown in Appendix 3.

Table 4. 7 Cost of DBS and serum sampling pathway

| **DBS sampling pathway cost** | **Cost (£)** |
|---|---|
| Sample collection cost | 2.9562 |
| Sample postage cost | 2.0629 |
| Sample elution cost | 0.003666 |
| LCMS cost per test | 8.09 |
| Total DBS | 13.11277 |
| | |
| **Serum sampling pathway cost** | **Cost(£)** |
| Sample collection cost | 9.2515 |
| Sample separation and storage | 0.465483 |
| LCMS cost per test | 8.09 |
| Total Serum | 17.80698 |

There are other costs associated with the DBS sampling pathway that were estimated to assess the full cost benefit over conventional sampling. The cost of

DBS sampling included the cost of the sampling kit, which consists of DBS cards, a lancet, a desiccant pouch to remove any moisture, a zip-lock bag to protect the blood sample during shipment, hand wipes, and one small bandage (Appendix 2.0). A DBS card can be sent to the laboratory using regular first class Royal Mail. The cost of the serum sampling pathway in a clinical setting included the cost of blood collection (a gel tube, syringe, tourniquet, alcohol hand wipe, hand gloves, and a small plaster bandage). In addition, the cost of sample separation and storage, as well as staff time, was included in the cost (Appendix 3). The results show that DBS sampling and LC-MS testing (£13.11) offered 24% savings per sample compared to serum sampling and analysis (£17.81).

The cost of taking a DBS sample is 48.35% lower than conventional sampling. This is similar to what other studies have found. A study compared the total costs (healthcare cost, patient related costs, and staff cost) involved in conventional sampling versus DBS home sampling in renal transplant patients and hemato-oncology patients and found that DBS home sampling was associated with a total cost reduction of 43% for hemato-oncology patients and 61% for nephrology patients per blood draw (Martial *et al*., 2016). Another study evaluated the costs of different sampling methods for the assessment of prenatal alcohol exposure and concluded that DBS sampling was cheaper compared to conventional venous sampling in terms of personnel involved in venous sampling and shipping costs (Bakhireva *et al*., 2013).

There are additional benefits of DBS sampling, such as cost savings on patient travel costs, which were not included in this research and were difficult to estimate. There is a possible cost associated with distributing the filter cards, depending on the patient population and where the patient is being treated. DBS filter cards can be posted by regular mail to patients or to different clinics within the country. A second option is to provide the patients with DBS filter cards when they are discharged from the hospital or during their visit to the outpatient clinic. Finally, the DBS filter cards can be distributed to the patients through the pharmacy by providing patients with a combined prescription for DBS filter cards along with the drugs (Francke *et al*., 2022).

## 4.5 General discussion DBS analysis

In this study, we have developed a protocol for the analysis of DBS samples as an alternative sample type. LC-MS/MS offered the sensitivity to accurately detect C-peptide in DBS samples. These findings provide useful information for institutions facing challenges with conventional sampling methods where sample stability is affected by the type of collection tube, storage temperature, and time delay before separation and analysis (Ellis *et al*., 2003; McDonald *et al*., 2012; Oddoze *et al.,* 2012).

DBS analysis has integrated well into the bioanalytical workflow, providing low-cost, fast quantitative results in clinical settings where collection and storage of samples are limited to ambient conditions. Willemsen *et al*. (2018) developed an approach for the frequent assessment of C-peptide to monitor β-cell function at home. There was a median number of 24 DBS cards received per participant, which indicated that the method was feasible in the home setting (Willemsen *et al*., 2018). Another study also shows that DBS home sampling in a clinical study among female adult cancer patients was successful with about 70% participation (Sakhi *et al*., 2015). Bingley *et al*. (2015) demonstrated that patients would prefer DBS home sampling if this would avoid visits to the clinic, in an islet autoantibody screening study in patients aged 12–38 years (Bingley *et al*., 2015).

The 50 µL sample volume in utilising dried blood spots is particularly advantageous when looking at paediatric studies. The biggest driving force in the move to DBS is the potential for very large cost savings. The savings include sampling, processing of the sample (for example, centrifugation), storage of the sample (-20 °C or -70 °C), and shipment of the sample (usually on dry ice) (Geyer *et al.*, 2017).

DBS sampling is much more accepted and convenient in developing countries, which lack infrastructure for quality-assured venepuncture and also allow far greater numbers of samples to be taken and assayed, dramatically increasing population data in disease studies (Fonjungo *et al*., 2017). The use of DBS can extend from healthcare services or research and surveillance studies to harder-to-reach populations and help overcome healthcare inequalities. Compared with liquid blood, DBSs are relatively more stable and less reactive in the dried matrix

since enzymes are deactivated during the drying process (Deglon *et al.*, 2012; Waterman *et al*., 2005; Alfazil *et al*., 2008). Such studies should be replicated for peptide analysis.

In addition to the convenience of minimally invasive sampling, DBS can be easily shipped on the filter paper card without risk of infection since pathogenic agents are deactivated on the paper during the drying process. It is often difficult to obtain standard blood specimens for analysis during hypoglycaemia because the hypoglycaemic episodes may happen when the patient is at home or in another location where no facilities are available for phlebotomy. This could delay the diagnosis. The ability to carry out analysis on DBSs would mean that a specimen could be obtained when the patient is symptomatic and hypoglycaemic. This would be more convenient for the patient, as it could potentially save on the need for the 72-hour fasting tests or mixed meal tests and standard phlebotomy. In diabetic forensic cases, it is even easier to sample a victim casualty by pricking the finger instead of requesting a serum or urine sample (Aviram *et al*., 2018).

This LC-MS/MS C-peptide assay using DBS can therefore support screening for type 2 diabetes. With a relatively cheaper cost of £9.2 per test, conducting the screening and providing ongoing disease management to those diagnosed are outweighed by the cost savings of preventing future diabetic complications, mortality, and associated treatment costs (Najafi *et al*., 2016). In the management of Type 1 diabetes, the ability to monitor changes in β-cell function over the short and long term may be critical in the evaluation of potentially useful interventions to prevent the development and progression of the disease (Insel *et al*., 2016). This method can support studies aimed at preserving and restoring residual β-cell function in type 1 diabetic patients as well as help in epidemiological studies of insulin resistance in neonates and children (Todd *et al*., 2016). Some of the ongoing studies that could benefit from an LCMS DBS method include immunotherapy studies for the application of diabetic drugs such as thiazolidinediones and glucagon-like peptide 1 receptor agonists for the management of paediatric type 1 diabetes (Tafuri *et al*., 2013; Raman *et al.,* 2010; Herold *et al*., 2005; Ludvigsson *et al*., 2011; Gitelman *et al*., 2016; Mastrandrea *et al.,* 2009). Finally, the DBS method may be useful in monitoring β-cell function in

autoantibody-positive at-risk patients, a feature of type 1 diabetes research receiving increasing attention (Ziegler *et al*., 2016).

Several challenges are associated with DBS sampling that can affect downstream analytical measurements (Youhnovski *et al*., 2011). The weakest link for sensitivity within a bioanalytical workflow is the quality of the specimen (Lim *et al*., 2011). Commercially available DBS cards are not designed for the minimally resourced environments typical of remote health settings. Most DBS are susceptible to contamination by the user, patient, environment, insects, equipment, or contact with other cards. The impact of these variables on data quality needs to be assessed through careful studies simulating the pre-analytical workflow.
It was shown that haematocrit and blood viscosity can lead to a change in the size of the spots and an uneven distribution of analytes. This contributes to measurements of uncertainty (Wilcken and Wiley, 2008). Thus, punching and extracting a fixed spot size can sometimes entail an unacceptable bias in the analysis results (Ren *et al*., 2010; (Denniff and Spooner, 2010). High or low haematocrit has two primary issues. First, haematocrit can affect the blood-to-plasma ratio for target analytes, which can alter their measurements and bias attempts at conversion from DBS to plasma (Meesters and Hooff, 2013). Second, haematocrit directly affects the viscosity of blood, which affects how a spot spreads and saturates filter paper, which in turn limits volumetric extraction from a set diameter punch and extraction recovery (Sharma *et al*., 2014; Shi and Jiang, 2011).

The haematocrit effect is more substantial when a disc punch is analysed rather than the whole DBS specimen. Hence, this method validation included an examination of the impact of haematocrit, variation on measurement, and assay performance (Timmerman *et al*., 2014).

Aside from the traditional DBS microsampling methods, newer platforms have been developed that provide solutions to some of the challenges listed above. For example, pre-cut DBSs can reduce the haematocrit effect, improve sample utilisation, and minimise carryover issues between the punches (Youhnovski *et al*., 2011). Volumetric absorptive microsampling overcomes haematocrit effects by collecting precise volumes of the biofluid into an absorbent tip with reproducible

internal porous volume (Denniff and Spooner, 2014; D'Urso *et al*., 2021) Recently, the biofluid sampler approach was introduced, where the cellulose fabric substrate is coated with porous sol-gel sorbents to decrease the maximum sample volume, reduce the haematocrit effect, and improve the physical adsorption of the sample (Locatelli *et al*., 2020). Like the traditional DBS microsampling methods, these new methods also require several hours of drying in ambient air and are subject to dilution since several steps are required to recover and extract analytes for offline analysis.

# Chapter 5.0 Conclusion

## 5.1 Serum and plasma sampling for C-peptide analysis

Diabetes is a public health concern that needs to be effectively diagnosed and managed in the preliminary stages. C-peptide is one of the most frequently ordered diabetes-associated laboratory tests and plays a significant role in the treatment and management of diabetes. We have developed a serum LCMS method that is more accurate and specific compared to immunoassays and other LCMS methods. The method quantifies intact C-peptide and avoids the use of enzymatic digestion, immunoaffinity enrichment, and multidimensional chromatography and instead uses simple protein precipitation and a single solid-phase extraction step. Given its comparatively low cost, accuracy of measurement, and range of potential applications, measurement of C-peptide in clinical practice using this LCMS method may be a valuable and cost-effective tool for the management of diabetes in clinical settings. Serum C-peptide testing can easily be incorporated into an outpatient clinic setting and could be a cost-effective intervention. This method will now be incorporated into the test repertoire at the Peptide Hormones Laboratory for investigations. It will also be used to enhance the UK NEQAS Guildford Peptides Hormones Schemes to enhance the scheme towards the standardisation of C-peptide by using the method to set target values to replace the current used All laboratory trimmed mean. The method has a low detectable limit and hence can be used for type 1 diabetics, in hypoglycaemic investigations and to assess β-cell function in newly diagnosed type 2 diabetic patients on diet treatment to predict those who have the ability to reverse diabetes and those who will need medication or other interventions. The benefit will be the ability to obtain vital information on the progress of treatment, which will serve to motivate the patient.

## 5.2 DBS sampling for C-peptide analysis

The current means of measuring C-peptide using serum samples is ideal for clinical settings but imperfect, laborious, and expensive in remote settings. Vacutainers are needed to collect blood samples, and a centrifuge is needed to

separate serum/plasma from whole blood. Aside from specialised equipment, freezers are required to store the samples optimally prior to analysis, adding to the cost. Due to the expensive nature of the handling (i.e., storage, electricity) of blood samples, clinical trials are often problematic in a resource-limited setting. DBS have been proven to be a possible alternative, as they require a less complex procedure to collect and have been proven to be possible to collect at home. We have developed and validated a method for the determination of C-peptide in DBS using protein precipitation and offline solid-phase extraction with several potential clinical, research, external quality assessment, and reference services uses. In order to use the serum method for reference laboratory activities, registration on the JMLTC database will be needed. This requires a sample comparison with another reference laboratory. To serve as a reference method, it would be ideal to introduce a stable isotopic internal standard to guarantee reproducibility and stability before registration. Although it can be expensive, $^{13}C$ internal standard is preferred as it can be obtained with high isotopic purity (99.5%) and is chemically stable. This research has proven that BDS can be used as an alternative sample type with a significant cost-per-test savings. Further studies are needed to evaluate potential challenges and factors that have the potential to introduce variability to the DBS sample quality, such as the type of DBS card and collection techniques.

## 5.3 Limitations and future work

One of the limitations of this method is that the reference range of results found under normal circumstances needs to be established before introduction of DBS for clinical use. Although the systematic differences compared to serum LCMS/MS method were acceptable when a DBS specimen was used, the DBS assay is negatively biased across the analytical range. The reference interval for the assay must be determined using samples collected from at least 100 healthy volunteers (50 males and 50 females) between 18 and 65 years old. The inclusion criteria should be apparently healthy, ambulatory, community-dwelling, and nonmedicated adults. The exclusion criteria should include any endocrine disorders, fasting glucose >5.55 mmol/L, and abnormally high insulin >120 pmol/L.

Another limitation of this study is the use of spiked samples for the comparison between DBS and serum. This research therefore proves, in principle, the performance of the DBS method. Further verification with actual patient samples is required for clinical application. The encouraging results from the verification studies justify performing a full-method comparison study using patient samples. At least 64 patient-paired samples must be run to establish validity for clinical application.

In conclusion, an LC-MS/MS method have been developed and validated for the determination of intact C-peptide in serum and plasma samples and verified for DBS sampling. This method overcome the limitations of immunoassays. It is cost effective and can support large scale population screening.

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