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Method development and validation using liquid chromatography-mass spectrometry to quantify thyroid hormones rT_3, T_3, T_4, and TSH.

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements of the degree of Master of Science in Medicine, 16 July 2024.

Declaration

I, Liezel Sephesu, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature: 

Date: 16 July 2024

Abstract

The thyroid gland produces three hormones namely: triiodothyronine (T_3), thyroxine (T_4) and Calcitonin. These play an important role in metabolism and development in the body. In addition to the thyroid hormones is reverse T_3 (rT_3), produced from the deiodination of T_4. The production of these thyroid hormones is regulated by the Hypothalamus-Pituitary-Thyroid-Axis via thyrotropin releasing hormone (TRH) and thyroid stimulating hormone (TSH). Even with this regulation, thyroid hormone imbalances can occur in the body. Immunoassays (IA), the method used routinely for thyroid hormone measurement, can be affected by abnormal concentrations of albumin and other serum proteins leading to erroneous results. Furthermore, IAs have also been shown to be inaccurate at lower concentrations, causing possible misdiagnosis and health complications.

Thus, alternative methods to measure thyroid hormone levels like liquid chromatography-tandem mass spectrometry (LC-MS/MS) are recommended as this method is not prone to interferences and has a better measuring range, particularly at the lower ranges.

In the current study, an LC-MS/MS method for measuring free T_3 (FT_3), free T_4 (FT_4) and rT_3, using commercially available standards was optimised and validated. The method was subsequently used to analyse 200 remnant samples previously measured by IA and the results from the two methods were compared. Samples with low thyroid hormone levels were prioritised when collecting remnant samples but the entire clinical measuring range was included.

The correlation between the two methods was only seen at low concentrations (2-5 pmol/L for FT_3 and 10-20 pmol/L for FT_4). It was observed that more patients (40.5%) were diagnosed as being euthyroid using the IA method of measurement versus euthyroid sick syndrome diagnosis by LC-MS/MS (72.5%).

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List of Abbreviations

Listed in order of appearance.

U.S.A	United States of America
LC-MS/MS	Liquid Chromatography-Tandem Mass spectrometry
TSH	Thyroid Stimulating Hormone
FT_3	Free Triiodothyronine
FT_4	Free Thyroxine
rT_3	Reverse Triiodothyronine
IA	Immunoassay
HPT axis	Hypothalamus-Pituitary-Thyroid-Axis
TRH	Thyrotropin Releasing Hormone
DIO 1	Deiodinase 1
DIO 2	Deiodinase 2
DIO 3	Deiodinase 3
TRβ	T Cell Receptor Beta Locus gene
TBG	Thyroxine Binding Globulin
TTR	Transthyretin
TRs	Thyroid Hormone Receptors
FDA	Food and Drug Administration
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
NHLS	National Health Laboratory Service
ECLIA	Electrochemiluminescence Immunoassay
ºC	Degrees Celsius
HPLC	High Pressure Liquid Chromatography
UPLC	Ultra Pressure Liquid Chromatography
mIU/L	Milli-International Units per Litre
pmol/L	Picomoles per Litre
T_4-C13	T_4 deuterated internal standard
T_3-C13	T_3 deuterated internal standard
kDa	kilodalton
LC	Liquid Chromatography
m	milli
M	molar
Min	minute
MS	Mass Spectrometer

m/z	Mass-to-charge ratio
ESI	Electron Spray Ionisation
CE	Collision Energy
DT	Dwell Time
s	seconds
µm	micrometre
mm	millimetre
Å	ångström
EP	Entrance Potential
CXP	Collision Cell Exit Potential
Psi	Pounds per square inch
IS	Internal Standard
DP	Declustering Potential
Q1	Quadrupole 1
Q2	Quadrupole 2
Q3	Quadrupole 3
Da	Dalton
EPI	Enhanced Product Ion
MS^3	MS/MS/MS
IDA	Information Dependent Acquisition
cps	counts per second
LIT	Linear Ion Trap
SD	Standard Deviation
CV	Coefficient of Variation
LOQ	Limit of Quantification
LOD	Limit of Detection
R^2	Regression coefficient
S/N	Signal to Noise ratio
PBS	Phosphate Buffer Saline
ANOVA	Analysis of Variance
rpm	Revolutions per minute
CAL	Calibrator
EQA	External quality control

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Chapter 1

Introduction

Thyroid hormones are involved in bodily functions such as metabolism, temperature regulation and oxygen consumption. Imbalances in these hormones lead to conditions such as hyperthyroidism (high levels of thyroid hormones) and hypothyroidism (low levels of thyroid hormones). Thyroid disorders are a recognized health condition around the world. The prevalence of hypothyroidism is between 0.06% and 1.2% in women, and between 0.13% and 4%, in men, in the United States (U.S.A), Japan and Europe (Vanderpump, 2011). Overt hypothyroidism occurs in approximately 5% of the population in developed countries such as the U.S.A and Europe (Welsh and Soldin, 2016). Hyperthyroidism is seen at an estimated prevalence of 0.3% in men and 3% in women globally (Goichot et al., 2016).

Kalk and Kalk (1989) found that Grave's disease made up 88% of hyperthyroidism cases seen in South Africa. According to Ogbera and Kuku (2011) thyroid disorders, such as, toxicosis was seen at 66%, whereas Grave's disease was at 34% in South Africa. Another common thyroid disorder in Africa is a swollen thyroid gland, also known as goitre. This was reported at 74.2% in 2006 in New Guinea, Northern Zaire, Cameroon, and Uganda (Ogbera and Kuku, 2011). To the best of my knowledge, there have been no recent studies documenting the prevalence of thyroid disorders in South Africa.

Thyroid hormone levels (and their subsequent disorders) have been analysed by techniques such as protein-bound iodine estimates, ultrafiltration, immunoassays (IA), and equilibrium dialysis (Welsh and Soldin, 2016; Koulouri et al., 2013; Gounden et al., 2014). These techniques can be affected by factors such as abnormal concentrations of albumin and other proteins in serum, protein leakage and absorption, binding competitors such as thyroid autoantibodies, and interference from thyroid antibodies which occur more frequently in pregnancy (Ekins, 1987; Jonklaas et al., 2009). Therefore, this has necessitated alternative techniques to

analyse these samples. Additionally, given the important functions that these hormones play, and the influence they have on other metabolic pathways, an accurate measurement of these hormones and an accurate diagnosis of their dysfunction are essential for patient health and care.

1.1 Study Aim

This study aimed to develop a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method for the detection and quantitation of thyroid hormones namely, thyroid stimulating hormone (TSH), free triiodothyronine (FT_3), free thyroxine (FT_4) and reverse triiodothyronine (rT_3).

1.2 Study Objectives

Objective 1

To develop an LC-MS/MS method to measure thyroid hormones in serum, namely TSH, FT_3, FT_4, and rT_3.

Objective 2

To validate the method by analysing variables such as linearity, accuracy, precision, as well as the limit of quantification and the limit of detection using commercially available standards.

Objective 3

To compare the results obtained for TSH, FT_3, and FT_4 measured by LC-MS/MS with that measured by immunoassay (IA) and compare results obtained by LC-MS/MS for rT_3 against external quality control standards.

Objective 4

To determine whether there are differences in thyroid function diagnoses in patients when analysing the hormone levels using the LC-MS/MS analytical method compared to the hormone levels measured by the IA method.

Chapter 2

Literature Review

2.1 Thyroid hormones physiology and functions

The hormones produced by the thyroid gland play an important role in the metabolism, growth, and development of the body. They are involved in embryogenesis all the way up to adult life as they are involved in metabolic effects such as changes in protein, carbohydrates, vitamins and lipids metabolism and they are also involved in oxygen consumption (Oetting and Yen, 2007).

The thyroid gland is a butterfly-shaped organ found at the front of the neck with its two lobes surrounding the trachea as illustrated in Figure 1. There are microscopic follicles called colloids in the thyroid gland, which contain glycoprotein thyroglobulin, which is the precursor of thyroid hormones namely, triiodothyronine, (T_3) and thyroxine (T_4) (British Thyroid Foundation, 2015). Thyroid hormones present in the body include T_3, T_4, Calcitonin, reverse T_3 and lastly thyroid stimulating hormone (TSH). The thyroid gland produces three of these hormones, namely, T_3, T_4 and Calcitonin (Kirsten, 2000). Calcitonin is secreted by the parafollicular cells or C cells in the thyroid gland (Marshall et al., 2020). Reverse T_3 (rT_3) is produced from the deiodination of T_4.

The production of the thyroid hormones is initiated by the Hypothalamus-Pituitary-Thyroid-Axis (HPT axis) as shown in Figure 2. This pathway is regulated by TSH which is secreted by the pituitary gland. The pituitary gland acts as a biosensor and senses the levels of free thyroxine (FT_4) and free triiodothyronine (FT_3) in the body. This means that the pituitary gland regulates the body's TSH levels according to the feedback from the thyroid hormones circulating in the body (Klee and Hay, 1997).

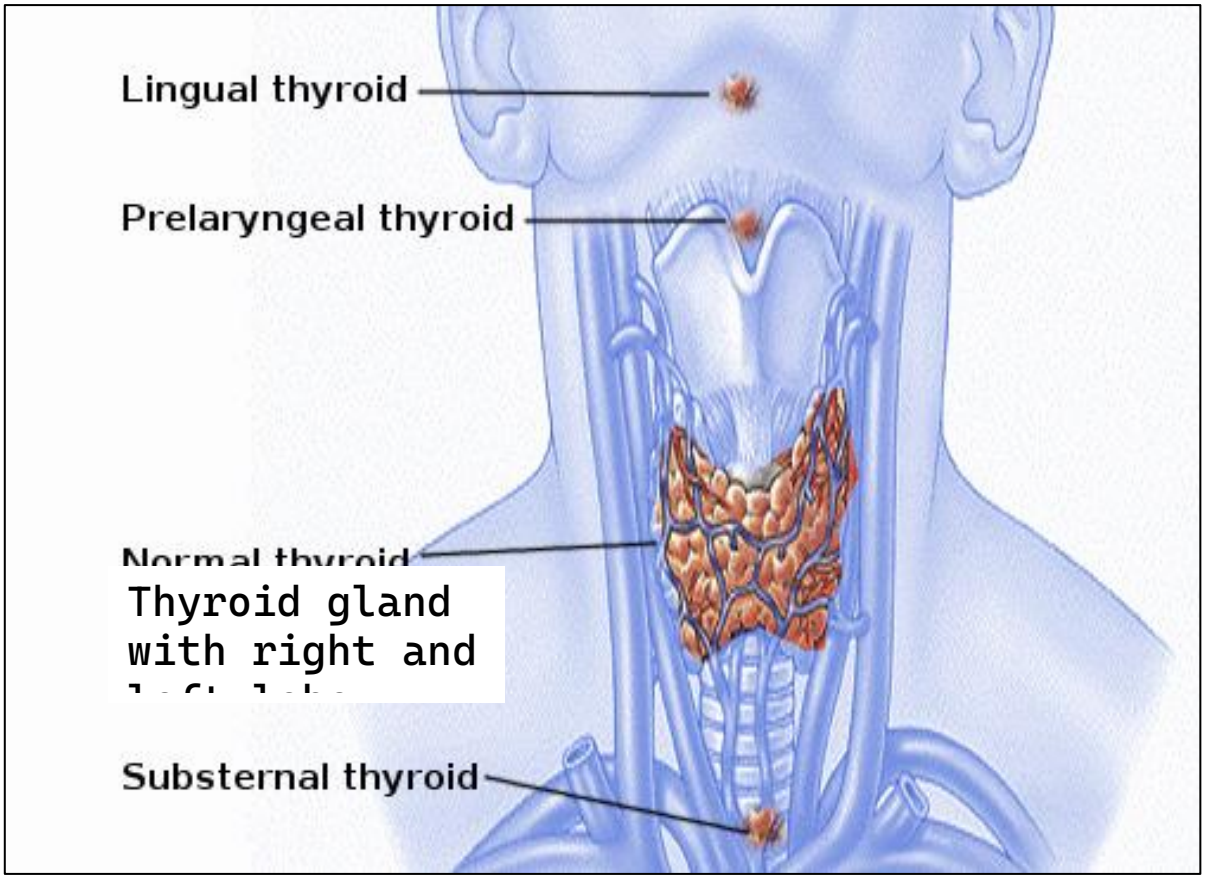


Figure 1: Anatomy of the thyroid gland.

Adapted from: Allen E, Fingeret A. Anatomy, Head and Neck, Thyroid. [Updated 2023 Jul 24]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470452/

TSH, in turn, is regulated by thyrotropin releasing hormone (TRH) from the hypothalamus. The production and secretion of these thyroid hormones involves a negative feedback loop in which the production of TRH and TSH is regulated by T_3 and T_4 circulating in the body. This is in patients with an intact or functioning HPT axis (Klee and Hay, 1997; Scanlon and Toft, 2005).

Figure 2 illustrates the feedback mechanism involved in the thyroid hormone pathway. Briefly, the hypothalamus exerts positive feedback or stimulates the pituitary gland via TRH. The pituitary gland then secretes TSH which acts on the thyroid gland by stimulating it. The thyroid gland then releases T_4, which gets

converted to T_3, the active product. The active T_3 then exerts its action on the peripheral tissues. High T_4 and T_3 levels can exert negative feedback on the TSH and TRH to reduce their levels in the body. T_3 accomplishes this by activating the thyroid hormone signalling pathway in the body.

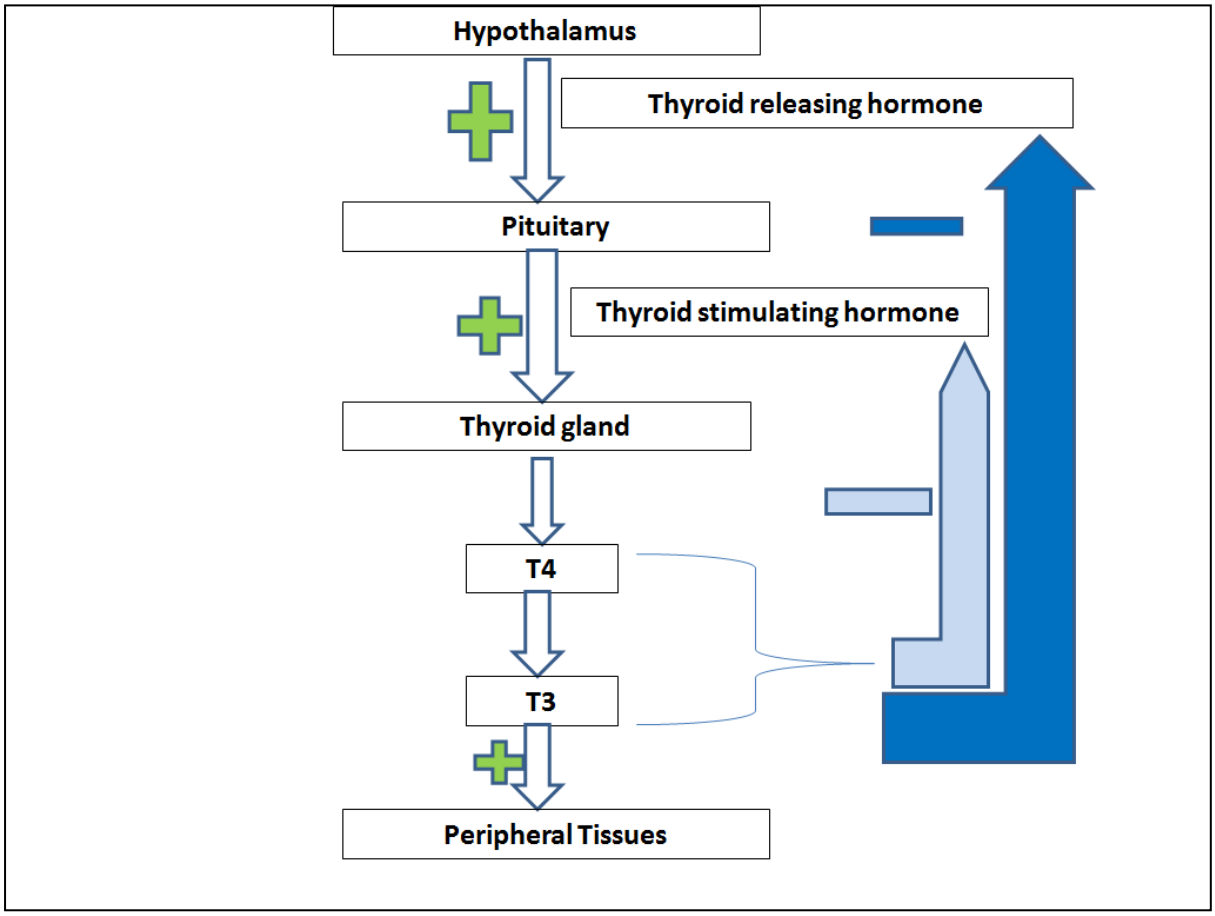


Figure 2: Thyroid hormone pathway.

Adapted from: Lahoti, A. and Frank, G.R., 2013. Laboratory thyroid function testing: do abnormalities always mean pathology? *Clinical Pediatrics*, 52(4), pp.287-296.

When T_4 and T_3 get close to the peripheral tissues, they move into the cells via the thyroid hormone receptors found on the membrane of the tissues. In the cytoplasm, T_4 is converted either to rT_3 or T_3 by deiodinase enzymes (DIO 1, 2, and 3). The

active T_3 then moves into the nucleus and reacts with the repressors and activators in the cell (Brent, 2012) (Figure 3).

Genes involved in regulating this signalling pathway include the TRβ (T Cell Receptor Beta Locus) gene. The TRβ gene is located on chromosome 3 and it encodes receptors such as thyroid hormone nuclear receptors TRα and TRβ. These receptors are involved in the binding of thyroid hormones on target cells and their subsequent action in the target cells (Cheng et al., 2010; Oetting and Yen, 2007).

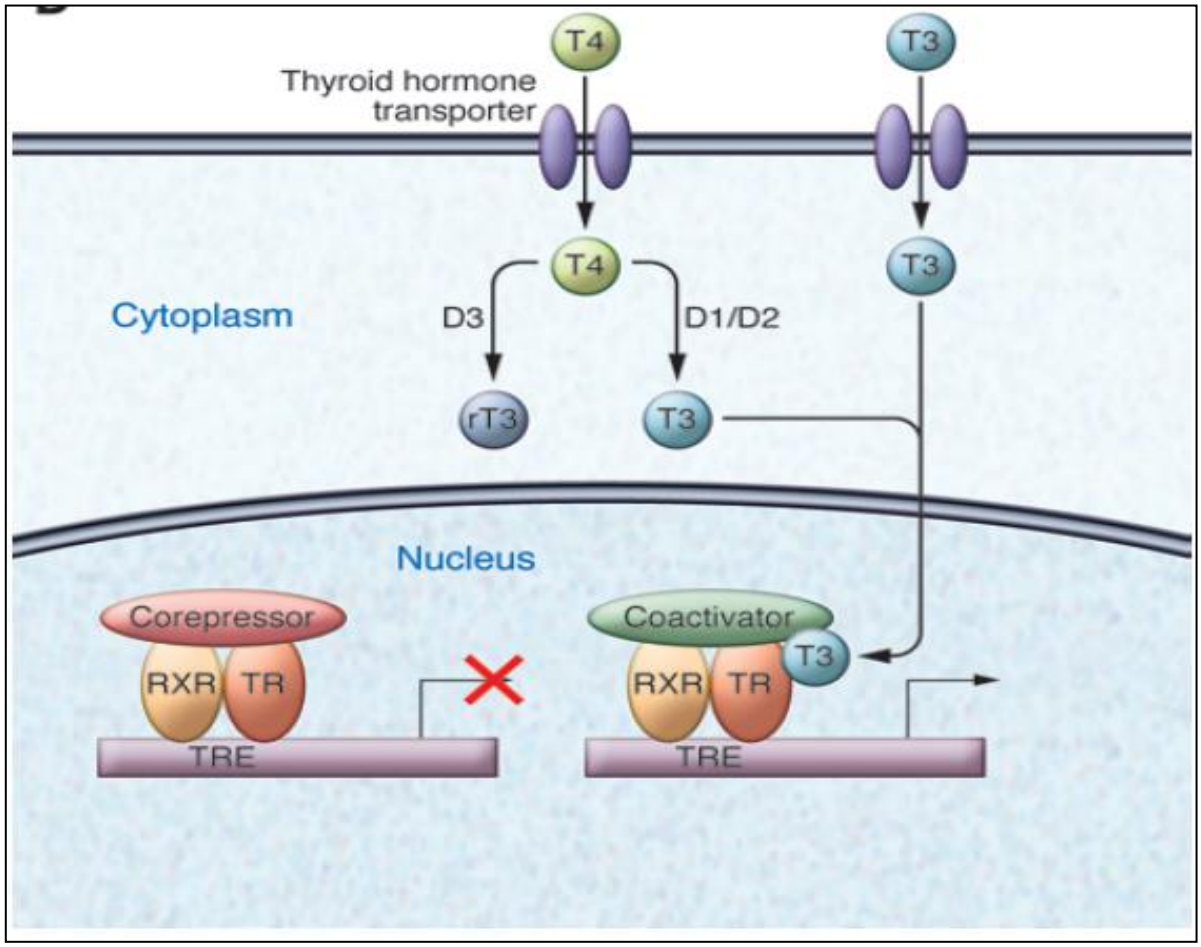


Figure 3: Thyroid hormone signalling pathways.

Taken from Brent, G.A., 2012. Mechanisms of thyroid hormone action. *The Journal of clinical investigation*, 122(9), pp.3035-3043.

Hormones depicted in Figure 3 are T_3, T_4 and rT_3. rT_3 also has an impact on the thyroid hormone regulatory pathways in the body. rT_3's first radioimmunoassay was

developed in 1974 (Halsall and Oddy, 2021). But this method was prone to interferences from substances in the sample that alter the binding of the assay antibodies such as auto-antibodies that compete with assay antibodies. They were also time consuming and used radio isotopes which require a license to run, and they also require safety measures in place to reduce the risk of radiation exposure (Halsall and Oddy, 2021) and so did not form part of current thyroid function screening.

Patients experiencing hypothyroid symptoms such as cold intolerance, fatigue, and constipation even when their T_3 levels are normal, attribute their hypothyroid symptoms to high levels of rT_3 (Gomes-Lima and Buurman, 2018). Therefore, rT_3 measurement is performed in patients who are on thyroid hormone replacement therapy but continue to have hypothyroid symptoms despite their TSH levels being within the normal ranges (Halsall and Oddy, 2021). The reciprocal changes in levels of T_3 and rT_3 suggest that T_4 degradation is regulated through activating T_3 or inactivating rT_3 pathways.

rT_3 impacts active T_3 levels in the body and in doing so may result in hypothyroid symptoms. This is because when rT_3 levels are elevated, it occupies the active T_3 binding sites prompting the body to detect this as high T_3 levels. The HPT axis results in the inhibition of TSH secretion causing less levels of TSH, subsequently leading to less FT_4 levels and low FT_3 levels. Thus, the measurement of rT_3 can help explain why patients still experience hypothyroid symptoms with normal FT_4 and TSH levels (Gomes-Lima and Burman, 2018). Therefore, assessing rT_3 levels could aid in treatment modification for patients on T_3 therapy (Halsall and Oddy, 2021).

When someone is ill or starving, the body's physiological response is to produce rT_3 in order to reduce energy consumption and to conserve energy in the body, as its production consumes less energy than the production of T_3. The body produces rT_3 because deiodinase 1 (DIO 1) is inhibited as the body's reaction to energy needs. DIO 1 normally converts T_4 to T_3. DIO 1 is also involved in the clearance of rT_3 to

ensure that more space is available on thyroid receptors for active T_3. This then ensures that there is more energy provision during starvation or illness as T_3 has more binding space on the receptors because rT_3 has been cleared away (Halsall and Oddy, 2021; Gomes-Lima and Burman, 2018).

rT_3 monitoring is important because drugs, such as amiodarone, which is used in patients with heart conditions, prevents the deiodination of T_4 by 5' deiodinase leading to an increase in the levels of T_4 and rT_3 that confounds the diagnosis of thyroid disorders (Halsall and Oddy, 2021). These levels of hormones then lead to a hypothalamic response that leads to the suppression of TSH and subsequent reduction in T_4 levels.

Conditions that cause an increase in rT_3 include non-thyroidal illness and carbohydrate depletion (Korhle, 2019). Elevated rT_3 has also been found in conditions of hyperthyroidism (Korhle, 2019). In conditions such as haemangioma's, deiodinase 3 (DIO 3) inactivates T_4 to rT_3 leading to overt hypothyroidism. Genetic influences on DIO 3 and levels of rT_3 includes T_3 nuclear hormone receptors THRα and THRβ (Halsall and Oddy, 2021; Persani and Campi, 2019). Conditions that also affect the concentration of rT_3 include caloric restriction, heart failure, HIV infections and major surgery (Korhle, 2019).

The measurement of rT_3 is thus used to optimize thyroxine replacement in hypothyroid patients. The results from analysing T_3, T_4 and rT_3 could help advise healthcare practitioners in terms of using combination therapy of T_3/T_4 for patients who still experience hypothyroid symptoms (Korhle, 2019; Gomes-Lima and Burman, 2018).

Furthermore, Burmeister (1995) suggested that proper clinical diagnosis of hypothyroid and euthyroid patients would be to conduct combined clinical assessment of FT_4, FT_3, TSH and rT_3. This would provide a differential diagnosis

between the sick patient with hypothyroidism and the sick patient with euthyroidism (Burmeister, 1995). For this reason, it would be beneficial for patients to provide measurement of FT_3 and FT_4, TSH and rT_3 for comprehensive thyroid function screening and maintenance (Gomes-Lima and Buurman, 2018). Because rT_3 is not part of the recommended profile for assessment of thyroid disorders, it can be added if and when clinically indicated.

2.2 Thyroid hormones transport in the body

The thyroid hormones produced by the thyroid gland are bound to the plasma proteins in the blood such as thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin (British Thyroid Foundation, 2015; Goodman, 2002). TBG accounts for about 70% of the total protein-bound hormone and has a molecular weight of 54 kilodaltons (kDa) (British Thyroid Foundation, 2015; Goodman, 2002). TTR binds about 10-15% of T_4 and 10% of T_3 and it has a molecular weight of 13.8 kDa (British Thyroid Foundation, 2015; Goodman, 2002). Albumin has a molecular weight of 66.5 kDa and it also binds 10-15% of T_4 and 10% of T_3 (British Thyroid Foundation, 2015; Goodman, 2002).

When the thyroid hormones are bound to proteins in serum, they are known as total T_3 (TT_3) and total T_4 (TT_4); and are biologically inactive (Almandoz and Gharib, 2012). When they are not protein-bound, after dissociation from the serum proteins, they become active, and are commonly known as free T_3 (FT_3) and free T_4 (FT_4) (Almandoz and Gharib, 2012).

T_4 is the principal or major hormone produced and secreted (Oetting and Yen, 2007). After T_4 is produced, it is shunted into the thyroid hormone pathway where it can be converted into either T_3 or rT_3 by deiodinase enzymes (DIO) 1, 2 and 3, these are also known as selenoproteins. Within the cell, T_3 is the more potent hormone as it binds to thyroid hormone receptors (TRs) with a higher affinity of up to 10-fold (Oetting and Yen, 2007).

DIO 1 is mostly expressed in the kidneys and liver which are peripheral tissues, and they convert circulating T_4 to T_3 (Oetting and Yen, 2007). DIO 2 is mostly expressed in the hypothalamus, skeletal muscle, and brown adipose tissue, and is mostly involved in thermogenesis which is heat production by converting T_4 to T_3 both in the intracellular and peripheral space (Muller et al., 2014). DIO 3 on the other hand is the third type of deiodinase enzyme found in the brain, placenta, and skin (Oetting and Yen, 2007). DIO 3 converts T_4 to rT_3. rT_3 is an inactive end product of thyroid hormone metabolism that binds to the thyroid hormone receptor. If rT_3 is produced in high amounts in an individual, rT_3 will bind weakly to the T_3 thyroid nuclear hormone receptors and occupy these receptors. Since these receptors will then be occupied by rT_3, this will send a signal to the body that T_3 levels are high as the receptors that bind T_3 will mostly be occupied by rT_3, which leads to the diverting of T_4 away from T_3 production (Halsall and Oddy, 2021). The conversion is illustrated in Figure 4.

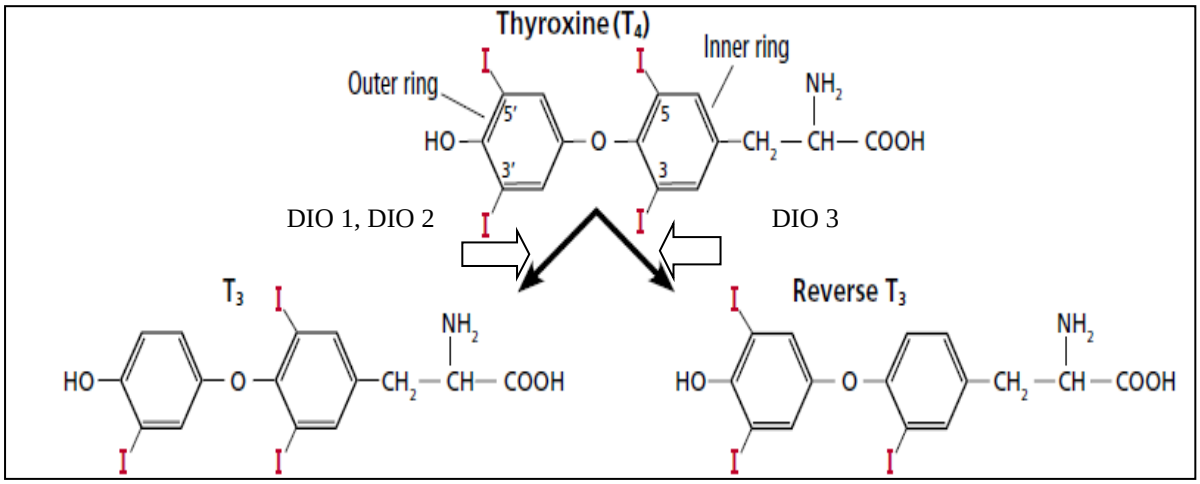


Figure 4: T_4 and its metabolites.

Adapted from Gomes-Lima, C., & Burman, K. D. (2018). Reverse T3 or perverse T3? Still puzzling after 40 years. *Cleveland Clinic journal of medicine*, 85(6), 450–455.

As the thyroid hormones are regulated and transported in the body, imbalances and dysfunction can arise in terms of these hormones and their levels in the body. These

imbalances can lead to different symptoms and health complications in patients.

2.3 Thyroid dysfunction

Imbalances of the thyroid hormones can occur if there is dysregulation of the negative feedback loop that results in either elevated or lowered thyroid hormone levels to the extent that they no longer fall within normal reference ranges (Devereaux and Tewelde, 2014).

It is crucial to measure maternal thyroid hormones in pregnancy (particularly in women with known thyroid disorders) as they are essential for adequate and optimal growth and development of the foetus, especially in the first half of the pregnancy (Korevaar et al., 2017). Thyroid disorders in pregnant women can lead to miscarriages, placental abruptions, pre-eclampsia, premature delivery, and also neonatal death (Krassas et al., 2010; Korevaar et al., 2017). In males, thyroid disorders may affect human reproductive health such as erectile dysfunction and abnormal sperm motility and in females may lead to menstrual disorders such as hypomenorrhea and oligomenorrhea (Krassas et al., 2010). Thus, thyroid hormone analysis is important across all ages and sexes.

Levels of a hormone are defined as (1) high, or (2) low when they (1) exceed, or are (2) less than, the reference range. Normal is defined as hormone levels being within the reference range. These terms will henceforth be used in the text to describe the hormone levels.

Hyperthyroidism is defined as the excess production and release of thyroid hormones by the thyroid gland, and this leads to high serum levels of these thyroid hormones (Devereaux and Tewelde, 2014). Symptoms of hyperthyroidism include constipation, night sweats, impaired memory, disturbed sleep, weakness, palpitations, weight loss, tremor of the extremities, tachycardia, and heat-related signs (Goichot et al., 2016). Hyperthyroidic patients can also develop conditions such as thyrotoxic storm that is defined as the life-threatening condition caused by

the exaggeration of the clinical manifestations of thyrotoxicosis, namely, high levels of T_3 and T_4 (Sarlis and Gourgiotis, 2003). This is mostly experienced following conditions such as Grave's disease, toxic multinodular goitre, and toxic adenoma's (Sarlis and Gourgiotis, 2003; Klubo-Gwiezdzinska and Wartofsky, 2018; Devereaux and Tewelde, 2014). Treatment of these conditions includes regulation of cardiovascular function, regulation of temperature and fluid and electrolyte balance (Sarlis and Gourgiotis, 2003).

A hyperthyroidism study analysing different symptoms associated with hyperthyroidism found that 15% of the black population in South Africa experienced weight loss compared to 46% of the white population. This study found that 27% of the black population complained of having a goitre compared to just 2% of the white population. This study also found that the black population experienced more cardiac complications compared to the white population (Kalk, 1980). These studies suggest that the symptoms differ by ethnicity.

In terms of diagnosis, subclinical hyperthyroidism is defined as a low TSH concentration with normal FT_4 and FT_3 hormone levels and is mostly not associated with clinical symptoms (Klee and Hay, 1997; Haarburger, 2012).

Hypothyroidism, on the other hand, is defined as an increased TSH concentration with normal FT_4 and FT_3 hormone levels (Haarburger, 2012). It is observed in patients who have undergone radiation therapy in the head and neck region at a prevalence of between 10% and 45% (Almandoz and Gharib, 2012). It can also develop due to primary causes such as chronic autoimmune (Hashimoto) thyroiditis (Almandoz and Gharib, 2012).

Symptoms include cold intolerance, fatigue, constipation, dry skin, muscle aches and vocal changes (Almandoz and Gharib, 2012). There are two main diagnoses namely, primary hypothyroidism and subclinical hypothyroidism. Primary, also known as,

overt hypothyroidism is when there is low FT_4 and or FT_3 with high TSH (Almandoz and Gharib, 2012). Subclinical hypothyroidism on the other hand is when FT_4 and/or FT_3 are within normal range but TSH is above its upper range. Subclinical hypothyroidism is mostly associated with the absence of clinical symptoms (Klee and Hay, 1997; Almandoz and Gharib, 2012). Patients with hypothyroidism can also develop a condition known as myxoedema coma. This occurs when there are low levels of thyroid hormones in the body and may be a life-threatening condition (Sarlis and Gourgiotis, 2003; Klubo-Gwiezdzinska and Wartofsky, 2018). It may also occur in patients that have a long-standing history of hypothyroidism (Sarlis and Gourgiotis, 2003).

As a consequence of the important roles that thyroid hormones play in the body, coupled with the imbalances that can arise in terms of thyroid hormones and the detrimental effects that these imbalances have on patients' lives, it is important to ensure that the hormone levels are accurately measured in patients.

2.4 Thyroid hormone measurement

Thyroid disorders have been analysed by techniques such as protein-bound iodine estimates, ultrafiltration, immunoassays, and equilibrium dialysis (Welsh and Soldin, 2016; Koulouri et al., 2013; Gounden et al., 2014; Haarburger, 2012). These techniques can be affected by factors such as abnormal concentrations of albumin and other proteins in serum, protein leakage and absorption, binding competitors such as thyroid autoantibodies, and interference from thyroid antibodies which occur more frequently in pregnancy (Ekins, 1987; Jonklaas et al., 2009).

Currently, TSH and FT_4 are measured simultaneously using IAs. FT_3 is measured when the FT_4 of a patient is within the reference range, while the TSH is below the lower reference limit. FT_3 is also measured when the patient is on T_3 replacement

therapy or if patients continuously experience hypothyroid symptoms regardless of treatment (Haarburger, 2012).

2.4.1 Immunoassay measurement of thyroid hormones

As stated previously, IAs are widely used as the method for analysing thyroid hormones. Immunoassays function on the principle of antigen-antibody binding. The antigen in this case is the hormone. The hormones bind to and occupy the antibody binding sites (Ekins, 1987). In South Africa, thyroid disorders are conventionally diagnosed using TSH and FT_4 values measured by immunoassays. In general, patients are screened based on TSH measurements, if the TSH measurement is abnormal then FT_4 is measured and in some instances FT_3.

According to the American Thyroid Association, individuals with signs and symptoms of thyroid dysfunction and those with risk factors such as surgery or radiotherapy affecting the goitre, should have TSH levels measured frequently (Ladenson et al., 2000). The values expected in a euthyroid state for FT_3, FT_4, rT_3 and TSH are 3.1-6.8 picomoles per litre (pmol/L), 12-22 pmol/L, 0.77-1.5 pmol/L and 0.270-4.20 milli-international units per litre (mIU/L), respectively (Marshall et al., 2020; Baumgartner et al., 2017).

IAs can be affected by abnormal concentrations of albumin and other proteins in serum and binding competitors such as thyroid autoantibodies. These autoantibodies have been shown to occur more frequently in pregnancy (Jonklaas et al., 2009).

The measurement and subsequent interpretation of FT_4 and FT_3 is made difficult by high levels of TBG in serum, and this leads to falsely elevated levels of T_4 or T_3. This is because TBG has a high affinity for T_4 and T_3 even at low concentrations and this means that it binds approximately 75% of T_3 and 80% of T_4. This is the reason why slight changes in binding protein concentrations can affect the total thyroid concentrations significantly (Welsh and Soldin, 2016).

Frequently, patients are classified as having normal values for thyroid hormones when measured using IAs. However, these values were found to be low when measured by LC-MS/MS (Welsh and Soldin, 2016). Furthermore, immunoassays have also been shown to be inaccurate at ranges lower than 60.49 nmol/L for T_4, 9.78 pmol/L for FT_4 and 2.76 pmol/L for FT_3 (Jonklaas et al., 2014; Soldin and Soldin, 2011; Soldin et al., 2005). When patients are falsely diagnosed as having normal thyroid function by IA when they actually have low values, this can prevent the timely and correct diagnosis of thyroid disorders.

Additionally, Welsh and Soldin (2016) hypothesized that; patients who still experience symptoms despite treatment, may have low levels of T_3 that have been incorrectly classified as normal by IA methods. Thus, these patients may benefit from having their samples analysed by LC-MS/MS.

2.4.2 Measurement of thyroid hormones using LC-MS/MS

Chromatography is a technique that separates compounds in a mixture and makes use of a stationary and mobile phase. One such technique is liquid chromatography which can be coupled to tandem mass spectrometry (LC-MS/MS). Tandem mass spectrometry (MS/MS) is the name given to a group of mass spectrometric methods in which parent ions from a sample are fragmented by the mass spectrometer to yield daughter ions that are unique to the analyte. These are then detected and quantified by the mass spectrometer (Bateman and Hoyes, 2000).

The LC-MS/MS comprises a liquid chromatography system, an ion generation system, and a mass analyser (Subramani et al., 2014). LC-MS/MS generally separates compounds into individual compounds and quantifies the compound according to its mass/charge. The technique is highly specific, precise, and sensitive at measuring complex mixtures of analytes such as macromolecules and hormones

(Welsh and Soldin, 2016). It is also less prone to interferences from the biological matrix in the samples or interferences from the mixture of compounds analysed (Welsh and Soldin, 2016).

The workflow of High Pressure Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) is as follows: the sample is separated by the liquid chromatography system, sprayed into the ion source where it is converted into ions which are in a gas phase (Rifai et al., 2018). This spraying process causes liquid dispersion into fine aerosols (gas particles) that takes place when a strong electric field is exerted on the liquid (Hiraoka, 2013). These aerosols are charged either positively or negatively by electrochemical reactions that take place on the surface of the metal electrode in the analyser (Hiraoka, 2013; Ho et al., 2003). These ions then travel through the mass analyser sorting them according to their mass-to-charge ratios under the influence of the magnetic or electrical field in the analyser (Rifai et al., 2018; Ho et al., 2003). The photomultiplier of the detector then counts the charged ions emerging from the analyser and amplifies the signal generated. The uncharged ions will go undetected. Finally, a mass spectrum and chromatogram for the analyte is generated (Subramani et al., 2014).

LC-MS/MS can provide structural or qualitative information and quantitative information in the form of the molecular mass or concentration of the analyte (Ho et al., 2003). It also allows high numbers of samples to be analysed at a rapid rate (Ho et al., 2003). LC-MS/MS can also analyse analytes larger than the scanning range using the mass-to-charge ratio of the analytes (Ho et al., 2003).

There have been many studies that have utilised mass spectrometry to analyse thyroid hormones including Soukhova et al., 2004; and Soldin and Soldin, 2011. Jonklaas et al., (2009), showed that LC-MS/MS analysis was more robust when compared to the IA. Soldin et al., (2005), also analysed thyroid hormones using LC-MS/MS and found this to be faster, more efficient, and more precise compared to the IA. Based on these rational characteristics of LC-MS/MS, this project aimed to

develop an LC-MS/MS method for the measurement of thyroid hormones in a South African public hospital.

Chapter 3

Methods

3.1 Sample population

The sample population (N number) included two hundred remnant patient samples, across all ethnicities and ages, who had blood samples submitted to the National Health Laboratory Service (NHLS) chemistry laboratory for thyroid function testing at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). The N number was calculated following the guidelines outlined by Kadam, P., and Bhalerao, S. (2010). The NHLS utilise an Electrochemiluminescence Immunoassay (ECLIA) that uses the sandwich principle and Cobas 8000 Roche Hitachi automatic instrumentation (Basel, Switzerland). The remnant samples were collected after obtaining ethical clearance from the University of Witwatersrand Human Research Ethics Committee (Ethical clearance number M23/03/31). Refer to the appendix section for the ethics certificate.

3.2 Sampling

Convenience sampling was used since remnant samples were selected for use in the method comparison. Convenience sampling involves selecting clinical cases or participants that are available around a particular location (such as a hospital) (Nikolopoulou, 2022). In this case, CMJAH hospital was the site of collection. Samples were collected to cover the clinical measurement range; however, samples with lower T_4 results (as measured by IA) were prioritised for method comparison purposes.

The samples were collected from the CMJAH NHLS auto-laboratory after IA analysis and stored at -80ºC until further analysis, as suggested by Mannisto et al., 2010 and Gislefoss et al., 2015. According to a study conducted by Mannisto et al., (2007), thyroid concentrations in samples were stable after repeated freeze-thaw cycles and

they were also stable during long-term storage at temperatures below 4ºC. Samples were run in batches of between 40-60 samples for the duration of 5 days.

Samples were stratified and coded according to IA thyroid hormone results. These samples were classified as low, normal, or high for FT_3, FT_4 and TSH as per the sample classification used by the CMJAH NHLS auto-laboratory (Koulouri and Gurnell, 2013). The classification groups are shown in Table 1.

Table 1: Reference range classification table.

Analyte	Low	Normal	High
TSH	<0.27 mIU/L	0.27-4.20 mIU/L	>4.2 mIU/L
FT_4	<12.0 pmol/L	12.0-22.0 pmol/L	>22.0 pmol/L
FT_3	<3.1 pmol/L	3.1-6.8 pmol/L	>6.8 pmol/L

The units for measurement utilised by mass spectrometry were pmol/L for all the analytes and were therefore converted to mIU/L in order to make comparisons with the immunoassay results that are reported in mIU/L. This was performed by converting pmol/L to ng/mL and the ng/mL were subsequently converted to mIU/L as follows: $ng/mL = (mIU/L + 0.9137/50.31)^{1.35}$ (Schenck et al., 2011).

3.3 Materials

All chemicals and reagents used in this study were of analytical HPLC quality grade. The standards namely, T_4 deuterated internal standard (T_4-C13), T_3 deuterated internal standard (T_3-C13), rT_3, T_3, and T_4 were purchased from Industrial Analytical LGC Standards (Teddington, England). TSH was purchased from Merck (Darmstadt, Germany). 30 kilodalton (kDa) Pall's Microsep™ Advance Centrifugal devices were purchased from Cytiva (Massachusetts, United States). Acetonitrile and Methanol were purchased from Supelco (Sigma-Aldrich, U.S.A).

3.4 LC-MS/MS method optimization

The thyroid hormones were analysed by optimizing a method developed by Tanoue et al., (2018). The method was run using a Shimadzu Liquid Chromatography system coupled to a Sciex QTrap 5500 mass spectrometry system (Massachusetts, U.S.A) as depicted in Figure 5.



Figure 5: Shimadzu UPLC (Ultra Pressure Liquid Chromatography) coupled to Sciex QTrap 5500 Selexion mass spectrometer.

(Source: Chemical Pathology Development Laboratory, Wits Medical School)

Development of the LC method included optimizing the mobile phase conditions to reach the following: mobile phase A: 10mM Ammonium Formate, 0.1% Formic Acid in deionized water, mobile phase B: 5mM Ammonium Formate, 0.1% Formic Acid in Methanol. The mobile phases were run using gradient elution whereby the composition of the mobile phase is changed gradually during the elution process

(Robard and Ryan, 2021) (see Table 2). The gradient profile was optimized to provide the best chromatographic elution based on peak resolution.

Table 2: Gradient Parameters.

Time (minutes)	**Mobile phase A** (10mM Ammonium Formate, 0.1% Formic Acid in deionized water)	**Mobile phase B** (5mM Ammonium Formate, 0.1% Formic Acid in methanol)
0	80	20
2	50	50
3	20	80
4	20	80
6	80	20

Wash solvents comprised equal volumes of acetonitrile, methanol, and de-ionized water. Acquity C18 BEH UPLC column, 1.7 µm (2.1 x 50mm), Atlantis UPLC C18 column 5 µm (4.6 x 150mm), Acquity HSS column, 1.8 µm (2.1 mm x 150 mm), and Phenomenex F5 column 2.6 µm (50 x 2.1mm) were used and compared (data not shown) in order to obtain optimum peak height, shape and resolution. Ultimately, the Phenomenex F5 column, 2.6 µm (50 x 2.1mm), with a pore size of 100 Å (Phenomenex; Torrance, U.S.A) was used. The column temperature was set at 30 ºC and the flow rate at 0.1 millilitres per minute (mL/min). The MS was operated in positive electron spray ionisation mode (ESI), meaning that the ions were positively charged. Other MS parameters such as collision energy (CE - energy required to fragment the ions), dwell time (DT - the amount of time in milli seconds (ms) the instrument uses to collect data for a specific mass transition), and cone voltages (reduces the incidence of ion clusters), entrance potential (EP - the potential difference between the voltage of the collision cell), collision cell exit potential (CXP - focuses and accelerates the ions between the quadrupoles) are shown in Table 3. The transitions used for each analyte are represented in Table 4. The T_3 internal

standard was used for both T_3 and rT_3. Its transition along with the T_4 internal standard transition is depicted in Table 4. The curtain gas used was 30 pounds per square inch (psi); the collision gas was set at high. The ion spray voltage was set at 5500 volts and the temperature for the source was 500 °C. The ion source gases 1 and gas 2 were set at 60 psi and 70 psi, respectively.

Table 3: Mass Spectrometry Parameters.

Analyte	Dwell time (ms)	DP (volts)	CE (volts)	EP (volts)	CXP (volts)
T4	250	120	34	10	18
T3	250	121	47	10	14
rT3	250	120	51	10	34
TSH	250	120	24	10	13
T4 IS	250	56	39	10	18
T3 IS	250	121	33	10	14

IS – internal standard, DP – declustering potential, CE – collision energy, EP – entrance potential, CXP – exit potential, ms-milliseconds

Table 4: Mass spectrometry transitions.

Analyte	Q1 mass (Da)	Q3 mass (Da)
T_4	777.5	731.6
T_3	651.7	605.9
rT_3	651.7	478.7
TSH	608.7	371.2
T_4 IS	783.5	737.6
T_3 IS	657.7	611.7

IS – internal standard

3.5 Confirmation of thyroid hormone transitions

T_3 and rT_3 are positional isomers (Couldwell et al., 2005). This means that they co-elute as they have similar structures and sizes. Since FT_3 and rT_3 were shown to co-elute, it was important to confirm that the peaks that were appearing on the chromatogram were indeed the actual individual analytes. The transitions corresponding to each hormone were confirmed by conducting Enhanced Product Ion (EPI) scans. EPI scanning is used to obtain a high-quality MS/MS spectrum of fragments for a specific ion. This scan takes place in the second quadrupole (Q2) collision cell. After obtaining fragments from the EPI scan, these were used along with the transitions of each hormone to conduct the "granddaughter" ion scan also known as, MS/MS/MS (MS^3) scan. For this scan, the precursor ion is selected in the first quadrupole (Q1), then fragmented in the Q2 collision cell. The resulting fragments are then further fragmented by the linear ion trap and are thus the final product ions, granddaughter ions (Sciex Triple Quad 5500+ system user guide, 2019).

An IDA (Information Dependent Acquisition) scan was conducted. IDA scans enable compound identification based on MRM ratios (Sciex Triple Quad 5500+ system user guide, 2019). IDA criteria were set to acquire the MS/MS spectrum of the most intense peak of the compounds. This scan was only possible after the IDA criteria were met. Namely; a mass tolerance of 250 millidaltons and exceeding limit of 1000 counts per second (cps). The scan was completed using dynamic fill time. Dynamic fill time determines and controls the number of ions that fill the linear ion trap at a time so that good resolution and optimum sensitivity can be achieved across the analyte peak (Sciex Triple Quad 5500+ system user guide, 2019).

An EPI scan was also conducted to ascertain the abundant fragments for T_3, T_4, TSH and rT_3 (Figure 9). The EPI scan had a duration of 12 minutes (min(s)). The first scan started at 100 Daltons (da) and stopped at 297.144 da. The second scan started at 297.144 da and stopped at 800 da. The product was set to 30 da. This EPI scan allowed for the MS^3 scans to be obtained (Figure 10).

For the MS^3 scan, the multiple reaction monitoring parameters (MRM) had the same

transitions as the mass spectrometry transitions and MRM was run as the first experiment. These scans ran for a duration of 6 mins and an overall DT of 20 ms.

The MS^3 scans conducted for T_3, T_4, TSH and rT_3 were run as four separate experiments. The scan rate used for all the analytes was set to 10 000 da/second. The transitions used, and centre settings (the most abundant fragment from the EPI scans used as a central scanning point) are depicted in Table 5. The width of all the analytes was set to 2 da. The collision and excitation energy for all the analytes was 45 and 0.130 volts, respectively. The LIT (Linear Ion Trap) fill time (the time set for trapping the ions and ejecting them in order of size) was set to 250 ms. The analytes were subjected to Q0 trapping.

Table 5: MS^3 scanning parameters for the analytes

Analyte	Q1 (Da)	Q3 (Da)	Centre (Da)
T_3	651.5	605.7	478
T_4	777.5	731.6	604.8
TSH	608.7	371.2	256.8
rT_3	651.6	478.7	242

3.6 Method validation

Method validation was carried out according to the Food and Drug Administration (FDA) regulations (FDA, 2001). Limit of quantification (LOQ), linearity of the standard curve, limit of detection (LOD), accuracy, precision, sensitivity, and selectivity were determined. This validation was run for 5 days.

3.6.1 Accuracy

The closeness of the measured value to the assigned (actual) value is known as the accuracy (Harris, 2010). It describes the closeness of the calculated value obtained by the analytical method to the true concentration of the analyte and is expressed in percentages (FDA, 2001; European Medicines Agency, 2011). The accuracy of the

method was analysed using the standards and in-house controls. According to FDA, the mean value should be within 15% of the actual value (FDA, 2001; European Medicines Agency, 2011). The formula used to calculate accuracy was as follows:

% Accuracy: Measured concentration/True concentration x 100

3.6.2 Precision

Precision is defined as how well the repeated measurements correlate with each other and is usually assessed using the standard deviation between the measurements (Harris, 2010). Precision is described by the closeness of repeated individual measures of the analyte (European Medicines Agency, 2011). The precision was analysed using the coefficient of variation (CV) values for each of the standards and controls (European Medicines Agency, 2011). Methods are considered precise when CV values do not exceed 15% (FDA, 2001; European Medicines Agency, 2011).

Microsoft Excel was used for calculations using the following formulas:

1. For the Mean: =AVERAGE (Day 1: Day5) or Sum of numbers/number of numbers
2. For the standard deviation (SD): =STDEV (Day1:Day5) or square root (Σ (Measurement-mean) ²/ (number of measurement-1))
3. For the Coefficient of variation: =SD/Mean*100
4. For the relative standard deviation: =SD/Mean.

3.6.3 Linearity

Linearity shows the relationship between the points in the calibration curve and how well they form a straight line, thus showing that the peak response acquired is directly proportional to the quantity of the specific analyte (Harris, 2010). Methods are considered to be linear when the regression co-efficient (R^2) is close to one (1) (FDA, 2001).

3.6.4 Recovery

Recovery of a method in terms of an analyte is how well the method can recover the

analyte after an extraction process. Recovery is determined by comparing the assigned (actual) concentration of the analyte to the measured concentration (FDA, 2001). The formula to calculate recovery is as follows:
Measured concentration/True concentration *100 (FDA, 2001). This was assessed using calibrators of known concentration.

3.6.5 Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD is defined as the smallest quantity/concentration of the analyte that can be detected and is measured as the signal-to-noise ratio greater than a value of 3 (Harris, 2010). The LOQ is the lowest amount/ concentration of the analyte that can be measured or quantified with accuracy and precision and is measured as the signal-to-noise ratio (S/N) greater than a value of 10 (Harris, 2010; European Medicines Agency, 2011).

The S/N ratio is calculated using the signal or response of the specific analyte compared to the signal of the zero standard. A blank sample that does not contain any analyte was also included between samples to assess contamination and/or carry over. The blank sample then had the calibrators added to it to determine if the method had any ion suppression. This is because signal recovery studies using specimen extracts with added analyte are used to assess ion suppression (Annesley, 2003).

3.7 Sample preparation

The FT_4, FT_3 and rT_3 standards were prepared using 100% methanol. For FT_4, a stock solution of 300 pmol/L was used to prepare a standard curve ranging from 5 to 100 pmol/L. Similarly, a stock solution of 100 pmol/L was used to prepare a standard curve ranging from 2.5 to 50 pmol/L for FT_3. The rT_3 stock solution was 30 pmol/L and the standard curved ranged from 0.5 to 10 pmol/L. These concentration ranges were chosen to correlate with those of the IA method and to ensure coverage of the reference range of the analytes.

For TSH, the lyophilized powder was reconstituted with 0.05 M phosphate-buffered saline (PBS) as per the instructions on the package insert. The stock solution had a concentration of 0.58 pmol/L and was used to prepare a standard curve ranging from 0.01 to 0.29 pmol/L. TSH was then subjected to tryptic digestion as described by Illiano et al., 2022.

Methanol was used to reconstitute the internal standards to a concentration of 56.175 pmol/L for T_4 and T_3.

Patient samples were prepared for analysis by LC-MS/MS using an adaption of the method described by Jonklaas et al., 2014. In summary, 400 µL of serum was centrifuged in a 30 kDa ultrafiltration device (Pall's Microsep™ Advance Centrifugal Devices,) at 3600 revolutions per minute (rpm) at 37 ºC for 30 mins. The ultrafiltration device was used to separate and extract the free hormone. In addition, this step was also used to reduce matrix effects by cleaning up the sample. The ultra-filtrate (150 µL) was mixed with 75 µL T_4 -C_{13} and 75 µL T_3-C_{13} internal standards at a concentration of 0.025 µg/mL. The samples were vortex mixed for 30 seconds and then centrifuged for 10 mins at 3000 rpm. Finally, 100µL of the supernatant was placed into an amber vial and 10 µL was injected onto the LC/MS/MS and analysed. This process is summarized in Figure 6.

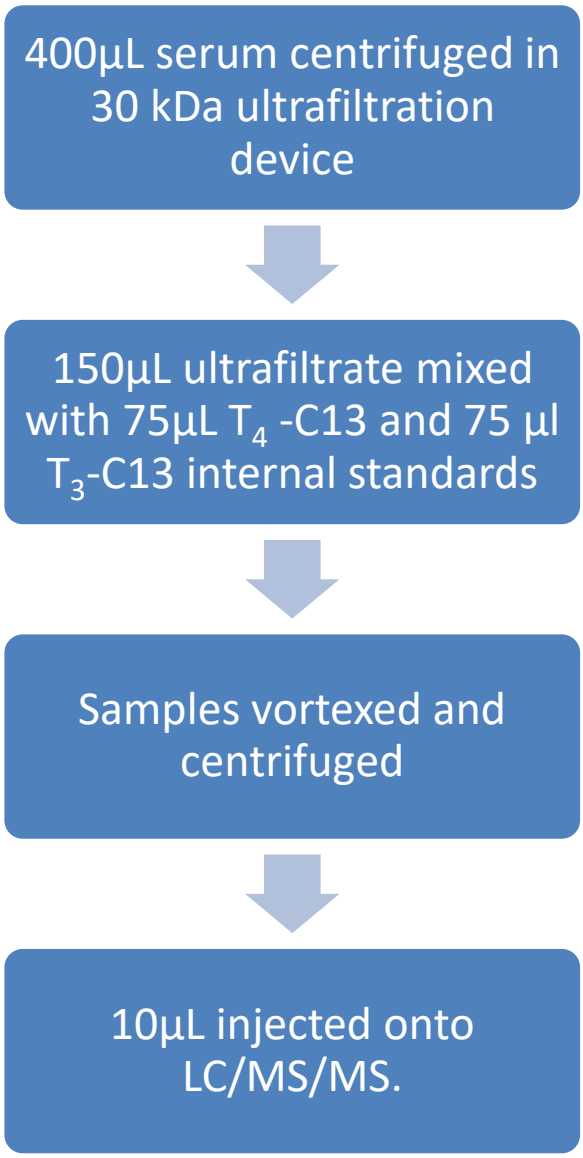


Figure 6: Sample preparation flow diagram.

3.8 Statistical analysis and method comparison

Ethnicity for the sample population was only available for 67% (133/200) of the samples. Thus, a "Hot Deck Imputation" technique that was developed by the National Cancer Registry was used to impute the ethnicities of the patients (Little et al., 1990). This technique compares the surname of an individual of unknown ethnicity to a database of known cases and then assigns the individual to a population group. This approach correctly classifies 90% of cases (Little et al., 1990).

Statistical analyses were performed using STATA 18 software (College Station, TX, U.S.A). In addition, Microsoft Excel was used to illustrate some of the statistical results namely, the box-and-whisker plots and bar graphs. The normality of data distribution was checked using Shapiro-Wilk tests. Normally distributed data was presented by means (Standard deviation; SD) and non-normally distributed data was presented by medians (Inter-Quartile Range (IQR)). The baseline characteristics were compared between age categories, genders and ethnicity. For the data that was normally distributed, student T-tests were utilised to analyse the data. Data that was not normally spread was analysed with nonparametric tests, namely, Wilcoxon Signed rank test. Thyroid hormones measured by IA and LC-MS/MS were compared between age categories by Analysis of variance (ANOVA) for normally distributed data.

Before the data was assessed for normality, outliers were removed from the data. Outliers were defined as single measurements which highly deviate from the rest of the measurements in question, and they differ significantly from the other observations (Rifai et al., 2018; Wang et al., 2023). The outliers were identified by observing scatter produced in normality plots, as suggested by Ludbrook, 2008. These values were removed from the data. Once the data points were removed, they were treated as missing values (Ludbrook, 2008). The data was then assessed for normality.

Pearson regression analyses were conducted using the MedCalc 22 statistical software, and subsequently used to graphically represent the agreement of the IA and the LC-MS/MS methods of analysis. Pearson regression is a technique used to estimate the line of best fit for y on x and inserts the predicted y-values for the given x-values and thus checks the correlation between x and y (Ludbrook, 2008). The regression analysis was used to measure systematic differences between the two measurement methods, namely, LC/MS/MS and IA.

To determine the differences in diagnoses in patients when using an LCMS/MS analytical method compared to an IA method, Kappa statistics, namely, Cohen's Kappa coefficient was utilised. Statistical significance was defined as a p-value ≤ 0.05.

Chapter 4

Results

4.1 Method development and optimization

The Phenomenex F5 column using Mobile phase A: 10mM Ammonium Formate, 0.1% Formic Acid in deionized water, and Mobile phase B: 5mM Ammonium Formate, 0.1% Formic Acid in methanol produced the best peak shapes and separation of the analytes. This differed from the parameters described by Tanoue et al., (2018) in terms of the column (Phenomenex F5 column used in this study versus the Ascentis F5 column used in the reference study) and mobile phases (Ammonium formate and Methanol used in this study versus acetic acid and Methanol used in the reference study). The source gas parameters were also different from the ones described by Tanoue et al., (2018) (ion source gases 1 and gas 2 were set at 60 psi and 70 psi, respectively versus 60 psi and 80 psi used in the reference method). After optimization of the method, the retention time for FT_3 was 4.79 mins, rT_3 4.77 mins, FT_4 4.86 mins and TSH 2.14 mins (Figure 7). The internal standard retention times were 4.77 mins for T_3-$^{13}C_6$ and 4.85 mins for T_4-$^{13}C_6$ (Figure 8). Chromatographic analysis produced peak shapes that were symmetrical with acceptable signal responses from the detector.

4.2 Confirmation of transitions

Since FT_3 and rT_3 were shown to co-elute it was important to confirm that the peaks that were appearing on the chromatogram were indeed the actual analytes. After infusing the pure standards of each analyte, they were scanned using EPI (see section 3.5) and subsequently analysed in MS^3 (see section 3.5) mode.

Figure 9 is a representation of the EPI scan obtained for FT_3 (A), rT_3 (B), FT_4, (C) and TSH (D). Figure 10 is a representation of the MS^3 scans obtained for FT_3 (A), rT_3 (B), FT_4, (C) and TSH (D). These two scan types provided a means to confirm the transitions used for the analytes. For the EPI scans T_3 is different to that of rT_3 since the second transition was 605.9 and 478.7 for T_3 and rT_3, respectively.

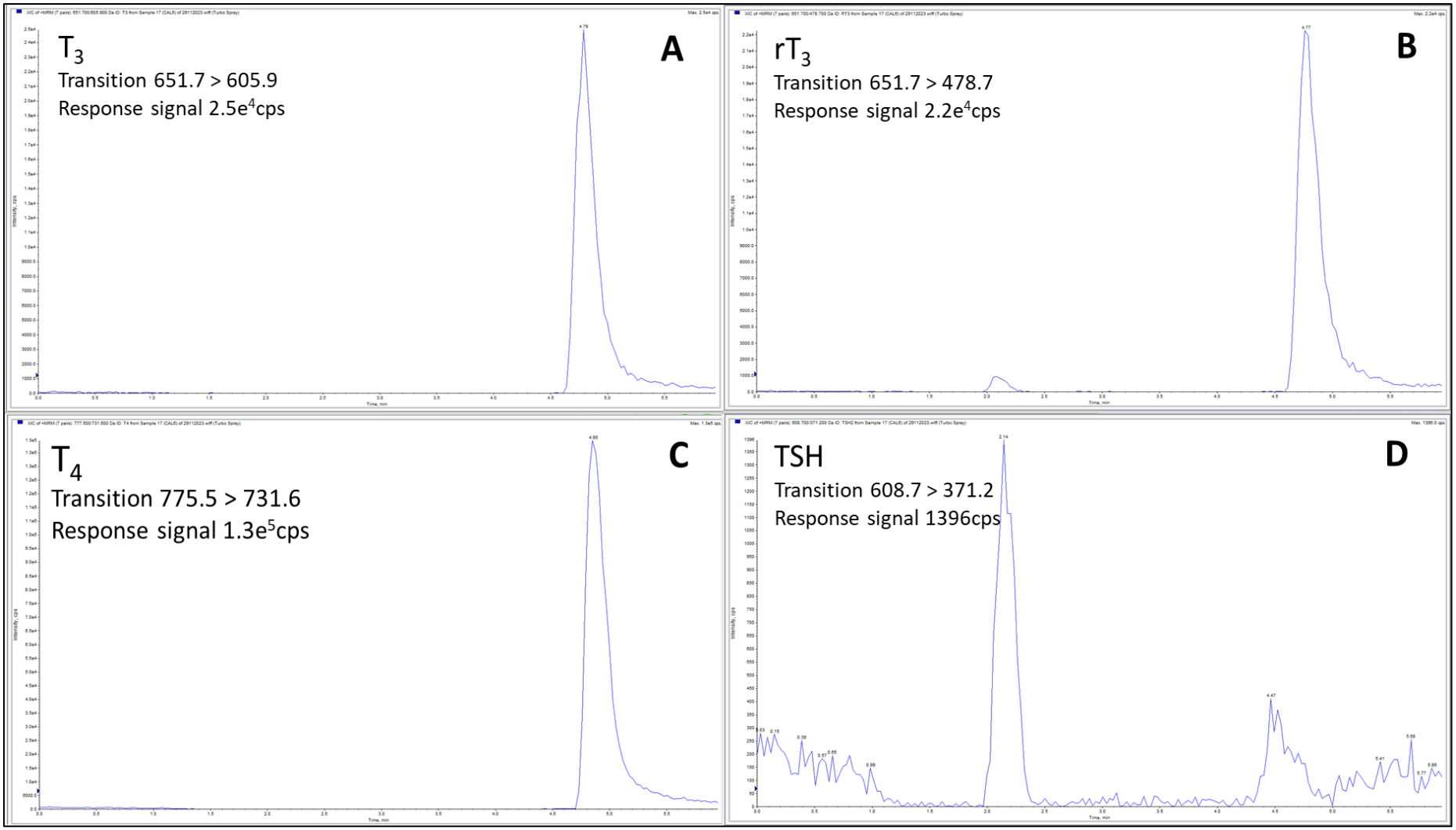


Figure 7: Chromatograms, transitions, and signal response (cps) for FT_3 (A), rT_3 (B), FT_4 (C) and TSH (D).

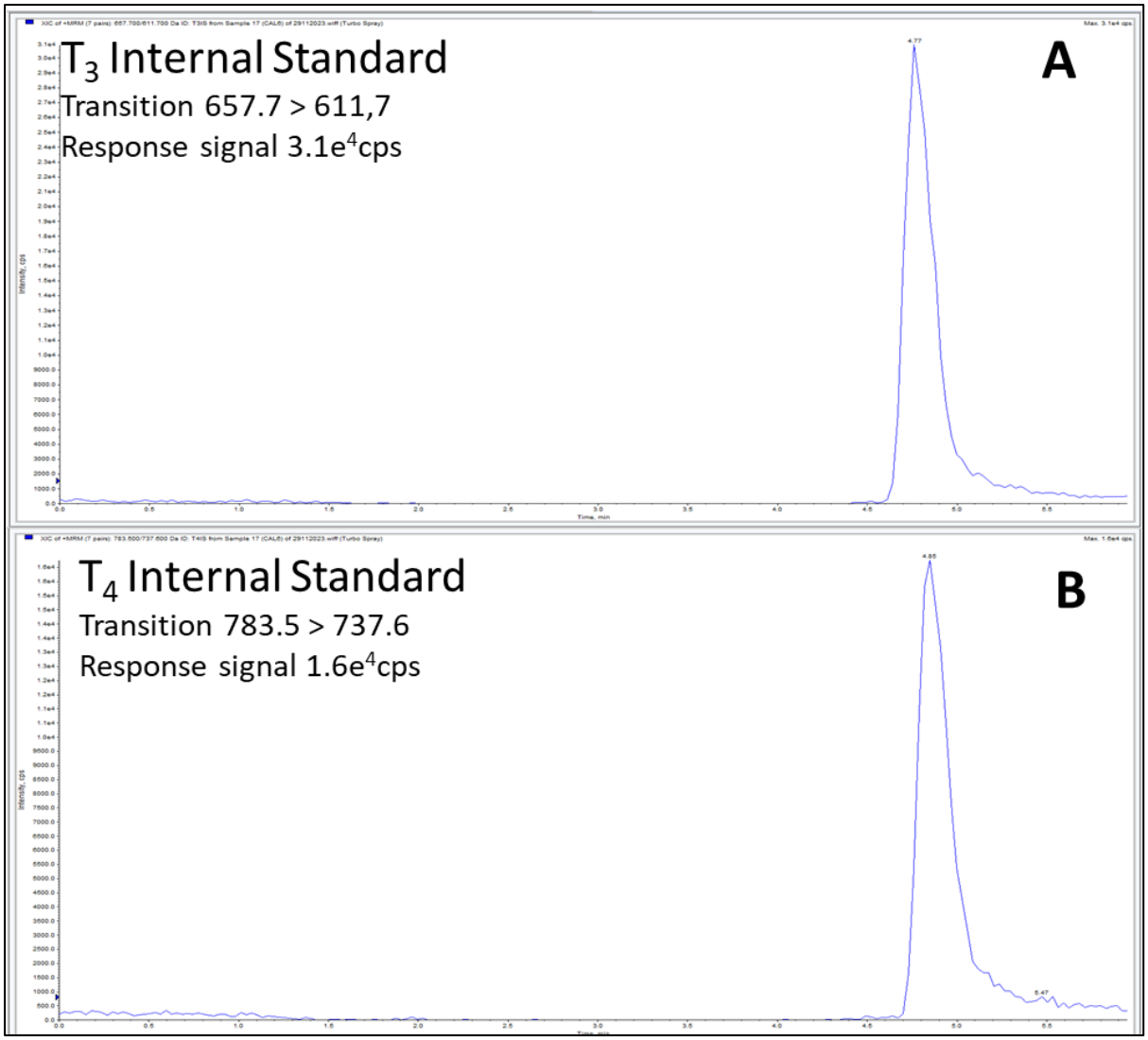


Figure 8: Chromatograms, transitions, and signal response (cps) for internal standards T_3-$^{13}C_6$ (A) and T_4-$^{13}C_6$ (B).

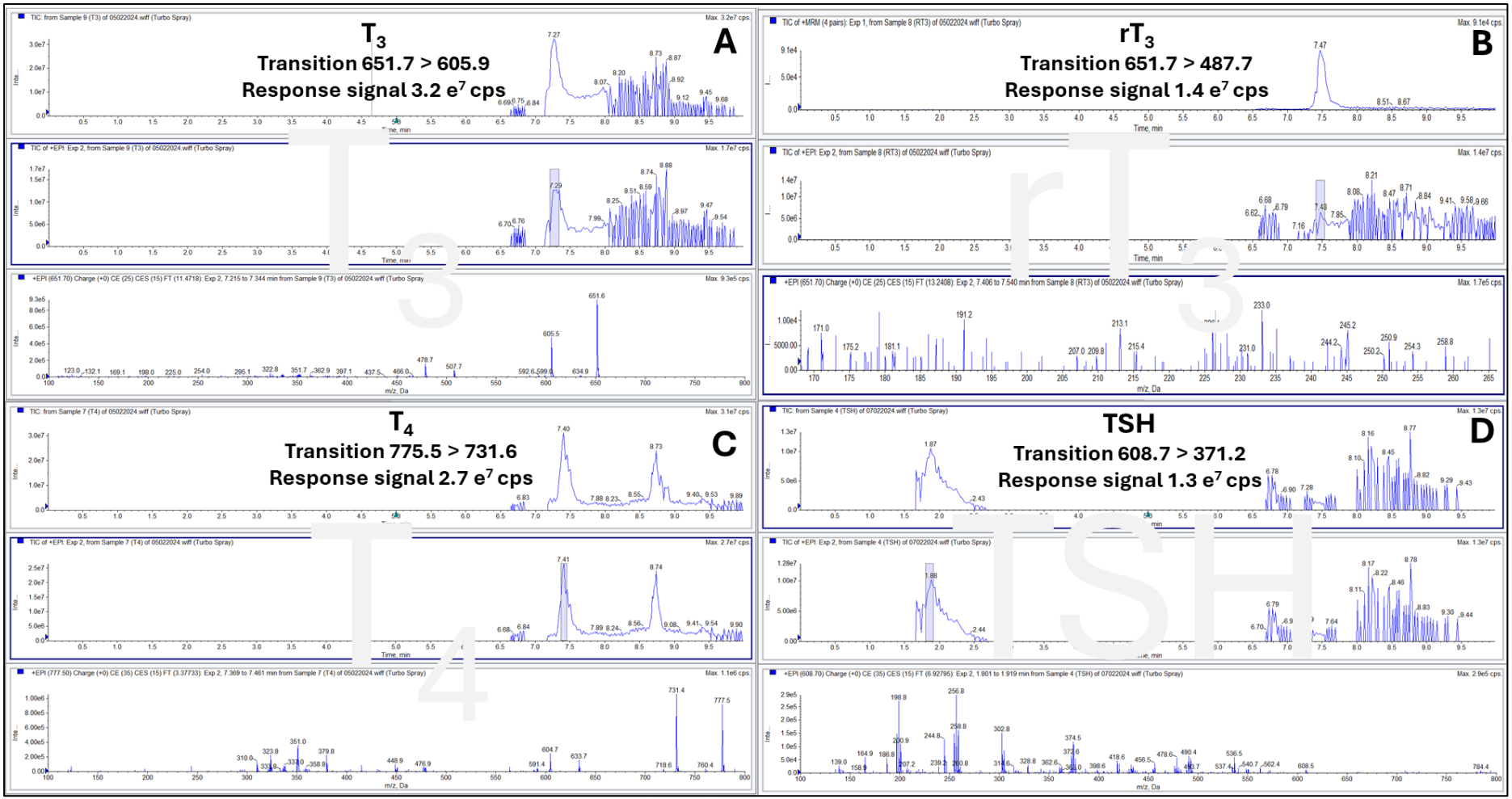


Figure 9: EPI scans of FT_3 (A), rT_3 (B), FT_4, (C) and TSH (D).

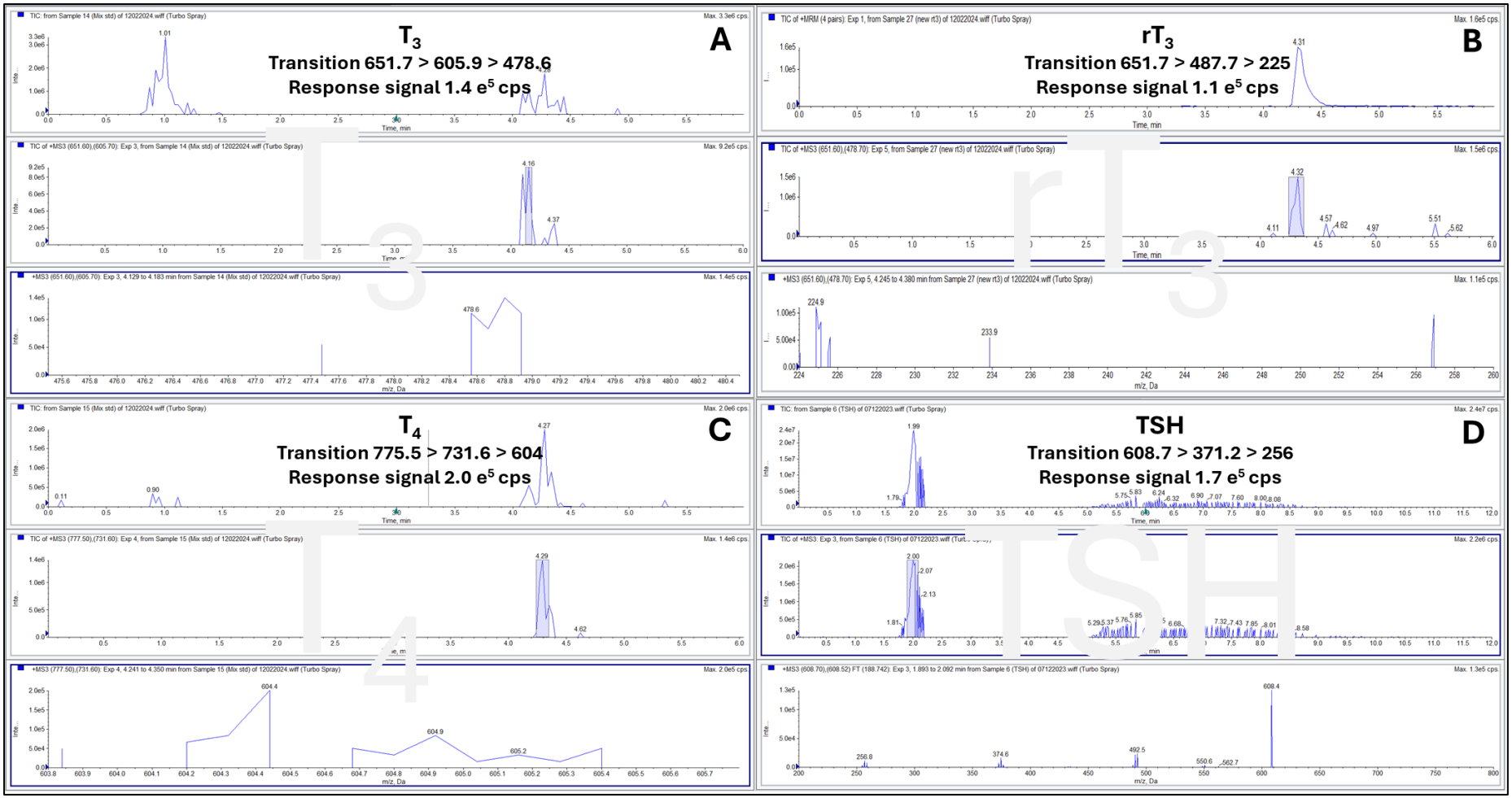


Figure 10: Chromatograms showing transitions for FT_3 (A), rT_3 (B), FT_4, (C) and TSH (D).

The MS^3 scans showed that the granddaughter ion was different between the two analytes since T_3 was 478.6 m/z and rT_3 was 225 m/z. It allowed for the elucidation of the unique make-up of each of the analytes in the study. These scans verified the transitions used and that the chromatographic separations obtained were unique to each of the analytes. These transitions and the response signal for each of the analytes are summarized in Table 6. The transitions were finalised and used in the method validation.

Table 6: Analyte transitions according to EPI and MS^3 scans

Scan type	**Analyte**	**Transitions**	**Response signal**
EPI scans	T_3	651.7>605.9	$3.2e^7$ cps
	rT_3	651.7>478.7	$1.4e^7$ cps
	T_4	775.5>731.6	$2.7e^7$ cps
	TSH	608.7>371.2	$1.3e^7$ cps
Scan type	**Analyte**	**Transitions**	**Response signal**
MS^3 scans	T_3	651.7>605.9>478.6	$1.4e^5$ cps
	rT_3	651.7>478.7>225	$1.1e^5$ cps
	T_4	777.5>731.6>604	$2.0e^5$ cps
	TSH	608.7>371.2>256	$1.7e^5$ cps

4.3 Method validation

4.3.1 Linearity

The standard curves are shown in Figure 11. The graphs were constructed using the mean response obtained from the analysis of CAL 1- CAL 6 and the mean concentrations of the analytes over a period of five days. The mean standard curve

regression coefficients (R^2) values (Table 7) for FT_4, FT_3, rT_3 were close to 1, this means that the method was linear for the analytes. This means that as the concentration of the analyte increases, the response from the detector also increased accordingly. However, for TSH the R^2 value was 0.95, this is not as close to 1 as required for linearity (See section 3.6.3). Furthermore, CAL 3 for the TSH curve with a concentration of 0.04 pmol/L was excluded due to its concentration being measured above the acceptable range on all days.

Table 7: Regression coefficients for FT_4, FT_3 and rT_3

Analyte	**Regression co-efficient (R^2)**
FT_4	0.99
FT_3	0.99
rT_3	0.98
TSH	0.95

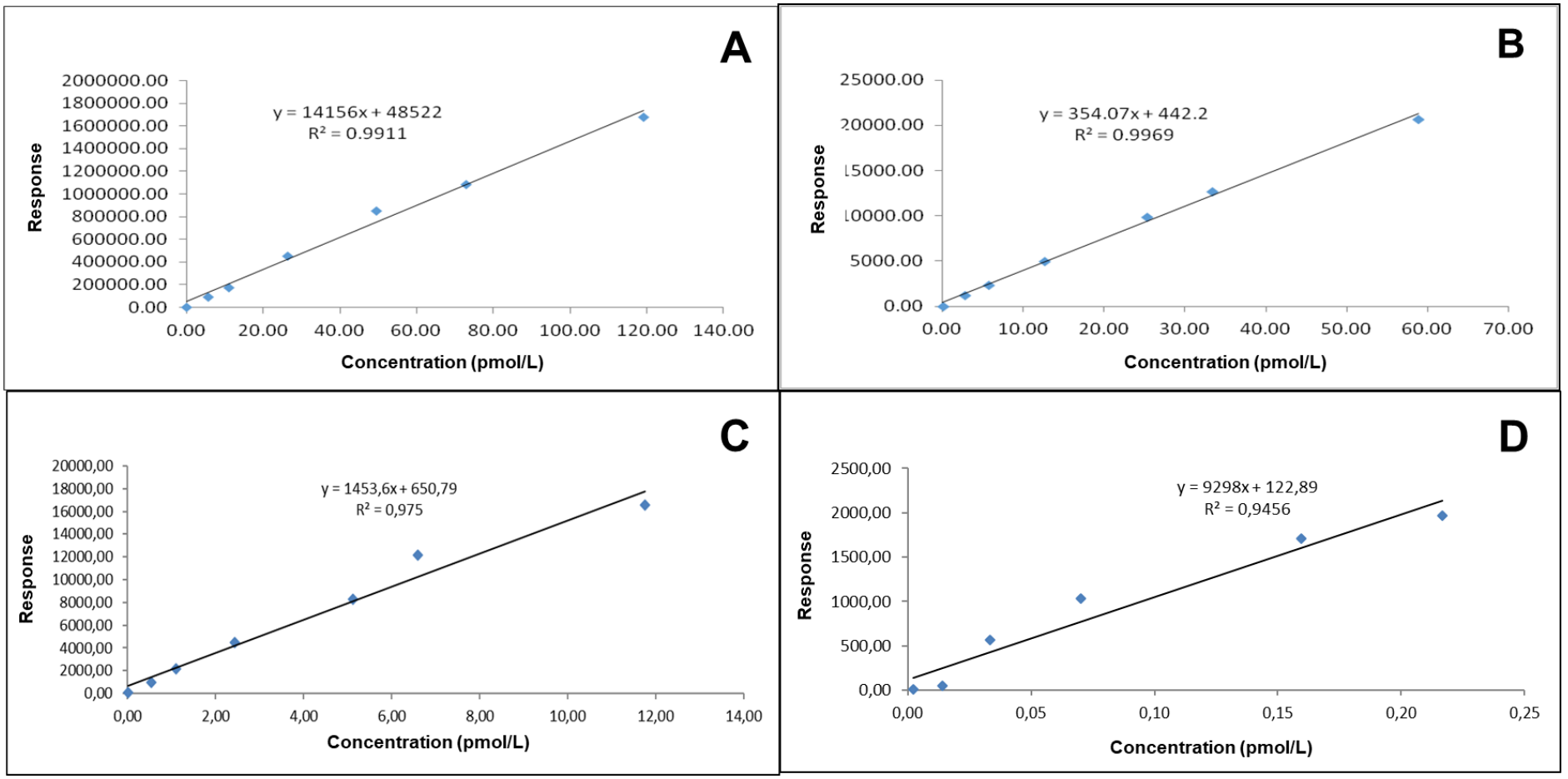


Figure 11: Standard curves for FT_4 (A), FT_3 (B), rT_3 (C) and TSH (D).

4.3.2 Accuracy

The measured concentrations for FT_4, FT_3, rT_3 and TSH from calibrator (CAL) 1 to 6 are depicted in Table 8. The measured accuracy of the analytes FT_4, FT_3 and rT_3 were acceptable as they were all within the stipulated limits (FDA, 2001). These analytes passed the validation according to the FDA guidelines as per the accuracy values presented in Table 8.

Accuracy was calculated as an average of the curves run three times per day. The accuracy values for FT_4, FT_3 and rT_3 were all within the allowable range (80-120%) (FDA, 2001). They ranged from 96.85-119.15% for FT_4; 88.80-117.75% for FT_3 and 87.98-117.63% for rT_3. For TSH, the accuracy values for CAL 1-3 were above 120%. This is possibly because CAL 1-3 had low concentrations of 0.01 pmol/L to 0.04 pmol/L. This meant that the method did not have a good accuracy at these low concentrations for TSH.

Table 8: FT_4, FT_3, rT_3 and TSH intraday accuracy.

FT_4	**Concentration (pmol/L)**	**Measured concentration mean (pmol/L)**	**Accuracy (%)**
CAL1	5.00	5.62	112.39
CAL2	10.00	10.08	100.81
CAL3	25.00	24.33	97.31
CAL4	50.00	49.39	98.77
CAL5	75.00	72.64	96.85
CAL6	100.00	119.15	119.15
FT_3			
CAL1	2.50	2.80	111.93
CAL2	5.00	5.31	106.22
CAL3	12.50	11.76	94.05
CAL4	25.00	23.56	94.23
CAL5	37.50	33.30	88.80
CAL6	50.00	58.88	117.75
rT_3			
CAL1	0.50	0.48	95.11
CAL2	1.00	1.09	109.40
CAL3	2.50	2.44	97.79
CAL4	5.00	4.78	95.63
CAL5	7.50	6.60	87.98
CAL6	10.00	11.76	117.63
TSH			
CAL1	0.01	0.01	140.00
CAL2	0.02	0.03	166.67
CAL3	0.04	0.07	150.83
CAL4	0.07	0.07	100.00
CAL5	0.15	0.16	106.22
CAL6	0.29	0.23	78.16

4.3.3 Interday Precision

For the precision of the method, the curves (CAL 1 - CAL 6) were run daily for 5 days, and a mean was calculated. The mean value was used to calculate the CV of each calibrator across the five days (Table 9). The CV values are shown in Table 9.

The analytes FT_4, FT_3 and rT_3 had CV values ranging from 0 to 14.07 (Table 9). However, for TSH, the CV value for CAL 1 and CAL 2 were 17.32% and 18.18% respectively. This is above the allowable limit of 15% (FDA, 2001). As previously stated, this could be due to the low concentration of CAL 1 of only 0.01 pmol/L, a value below the LOD of 0.02 pmol/L.

Table 9: Inter-day measured values for all analytes.

FT_4	Target Concentration (pmol/L)	Measured concentration mean (pmol/L)	SD	CV (%)
CAL1	5.00	5.35	0.66	12.34
CAL2	10.00	11.21	1.15	10.25
CAL3	25.00	26.02	1.50	5.77
CAL4	50.00	51.82	0.94	1.82
CAL5	75.00	67.55	8.10	11.99
CAL6	100.00	99.69	4.66	4.68
FT_3				
CAL1	2.50	3.12	0.44	14.07
CAL2	5.00	5.30	0.34	6.39
CAL3	12.50	12.62	1.00	7.90
CAL4	25.00	26.46	3.25	12.29
CAL5	37.50	34.20	2.48	7.26
CAL6	50.00	49.77	4.10	8.24
rT_3				
CAL1	0.50	0.59	0.05	8.14
CAL2	1.00	1.12	0.06	5.41
CAL3	2.50	2.52	0.17	6.58
CAL4	5.00	4.86	0.22	4.51
CAL5	7.50	6.54	0.91	13.85
CAL6	10.00	9.87	0.99	10.05
TSH				
CAL1	0.01	0.03	0.01	17.32
CAL2	0.02	0.03	0.01	18.18
CAL3	0.04	0.07	0.01	6.90
CAL4	0.07	0.08	0.01	7.21
CAL5	0.15	0.15	0.01	8.25
CAL6	0.29	0.24	0.01	6.23

4.3.4 Intraday precision

Table 10 depicts the intraday precision values and the corresponding SD and CV values. All analytes had CV values that were acceptable as the values were below 15%. However, the CV value for TSH CAL 1 and CAL 2 were high and once again may be attributed to the very low concentration of CAL 1 and CAL 2 (0.01 pmol/L and 0.02 pmol/L respectively).

Table 10: Measured values for the analytes in the study

FT_4	**Target Concentration (pmol/L)**	**Measured mean (pmol/L)**	**SD**	**CV (%)**
CAL1	5.00	5.35	0.66	12.34
CAL2	10.00	11.21	1.15	10.25
CAL3	25.00	26.02	1.50	5.77
CAL4	50.00	51.82	0.94	1.82
CAL5	75.00	67.55	8.10	11.99
CAL6	100.00	99.69	4.66	4.68
FT_3				
CAL1	2.50	3.12	0.44	14.07
CAL2	5.00	5.30	0.34	6.39
CAL3	12.50	12.62	1.00	7.90
CAL4	25.00	26.46	3.25	12.29
CAL5	37.50	34.20	2.48	7.26
CAL6	50.00	49.77	4.10	8.24
rT_3				
CAL1	0.50	0.59	0.05	8.14
CAL2	1.00	1.12	0.06	5.41
CAL3	2.50	2.52	0.17	6.58
CAL4	5.00	4.86	0.22	4.51
CAL5	7.50	6.54	0.91	13.85
CAL6	10.00	9.87	0.99	10.05
TSH				
CAL1	0.01	0.03	0.01	15.38
CAL2	0.02	0.03	0.01	21.07
CAL3	0.04	0.07	0.01	6.90
CAL4	0.07	0.07	0.01	7.40
CAL5	0.15	0.15	0.01	7.21
CAL6	0.29	0.24	0.01	6.23

4.3.5 LOD and LOQ

The LOD and LOQ values for each of the analytes are depicted in Table 11. According to FDA guidelines (2001) and the European Medicines Agency (2011) the signal of the lowest calibrator, in this case, CAL 1 should be at least 5 times the signal of a blank.

The method was assessed for carryover and ion suppression due to matrix effects. Carryover is defined as the contamination of a specimen by the previous one. It occurs when the previous sample contained the analyte of interest and the following blank or low-level sample produces a signal comprising some fraction of the signal from the previous injection. This was assessed by analysing blank samples after the analysis of the highest calibrator. A blank sample is a sample that is extracted in the same way as all the other samples but that does not contain any analyte. It is however, comprised of the same matrix as the other samples, in this case, serum. Signal response below the signal to noise of the method were observed and therefore, it could be said that no carryover was observed.

Matrix effects are the suppressing or enhancing properties of a co-eluting compound from a liquid matrix on the primary signal response of the target analyte. To test for matrix effects a blank sample is analysed. The analysis of the blank revealed that no ion suppression nor ion enhancement occurred.

Table 11: FT_4, FT_3 and rT_3 LOQ and LOD values:

Analyte	Mean LOQ (pmol/L)	Mean LOD (pmol/L)
FT_4	1.73	0.52
FT_3	0.53	0.16
rT_3	0.18	0.06
TSH	0.08	0.02

The method for FT_3, FT_4 and rT_3 passed the validation according to the assessed factors such as accuracy, precision, linearity, LOD and LOQ. TSH however, failed to perform as required and was therefore, not utilised to assess TSH levels in the patient samples.

4.4 Statistical analysis of the sample population

The proportions of the samples in each group (low, normal, or high) are shown in Table 12. These samples were run on the Cobas 8000 Roche Hitachi automated instrumentation.

Table 12: The sample population proportions per analyte.

Analyte	Low	Normal	High	Total
TSH	8.5%	65.5%	26%	100%
FT_4	23%	53.5%	20.5%	97%
FT_3	56%	36.5%	7.5%	100%

4.4.1 Method comparison

Reverse T_3 was not monitored against external quality control standards due to the inaccessibility of external quality assurance (EQA) samples. Thus, rT_3 run on the LC-MS/MS could not be verified nor compared to the IA method.

Table 13 shows the mean or median thyroid hormone levels measured by IA and LC-MS/MS. There was a significant difference between the two methods (p-value was less than 0.0001 for all analytes). This meant that there was a difference in the values measured by IA and LC-MS/MS.

Table 13: Method comparison between IA and LC-MS/MS.

Analyte	IA (n =200)	LC-MS/MS (n= 200)	Significance level (p-value)
FT_3 (pmol/L)	3.82 ± 1.30	0.51 (0.26; 1.36)	< 0.0001
FT_4 (pmol/L)	16.23 ± 5.71	2.91 (1.59; 4.08)	<0.0001

The values in the table for the LC-MS/MS method were lower than those for the IA method. This shows that the LC-MS/MS method was measuring at a lower concentration as compared to the IA method.

4.4.1.1 Method comparison using Cohen kappa statistics

Using the coding method described in section 3.2; the measured hormone levels were used to biochemically diagnose the patients. Cohen's Kappa Statistic was used to compare the differences seen between the two methods of measurement (Table 14 and Table 15). TSH measurements by IA were used to diagnose the patients in combination with IA and LC-MS/MS measurements as per the table below.

Table 14: Diagnosis: comparison between IA and LC-MS/MS.

Diagnosis categories	Diagnosis by IA N (%)	Diagnosis by LC-MS/MS N (%)
Euthyroid Sick Syndrome (ESS)	32 (16)	145 (72.5)
Subclinical Hyperthyroidism	33 (16.5)	3 (1.5)
Primary Overt Hyperthyroidism	14 (7)	10 (5)
Euthyroid	81 (40.5)	0 (0)
Primary Overt Hypothyroidism	23 (11.5)	42 (21)

The calculated Cohen's kappa statistic, K was found to be 0.0323. According to Cohen's kappa categorization scheme (Table 15), the level of agreement between the IA and the LC-MS/MS is considered slight as it is between the levels of 0.00-0.20 (Landis and Koch, 1977).

Table 15: Cohen's Kappa Categorization scheme.

Kappa Statistic	Strength of the Agreement
<0.00	Poor
0.00-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Almost Perfect

This table was adapted from: Landis, J.R. and Koch, G.G., 1977. The measurement of observer agreement for categorical data. *biometrics*, pp.159-174.

4.4.1.2. Method comparison using regression analysis

The two methods had different values for the analytes and lead to different diagnoses. To better understand the relationship between the two methods a Pearson regression analysis between the analytes measured by IA versus LC-MS/MS was conducted and is depicted in Figure 12. The n value depicts the number of observations used to construct the plots for each analyte. The numbers were not equal as outliers were removed before the regression analysis was conducted. The r value is the correlation coefficient and it depicts the relationship between an independent variable (the analytes measured on IA) and a dependent variable (the analytes measured on LC-MS/MS). Lastly the p-value reported in the plots gives an

overall test for the significance of the regression models. The difference is not significant as the p-value was >0.05.

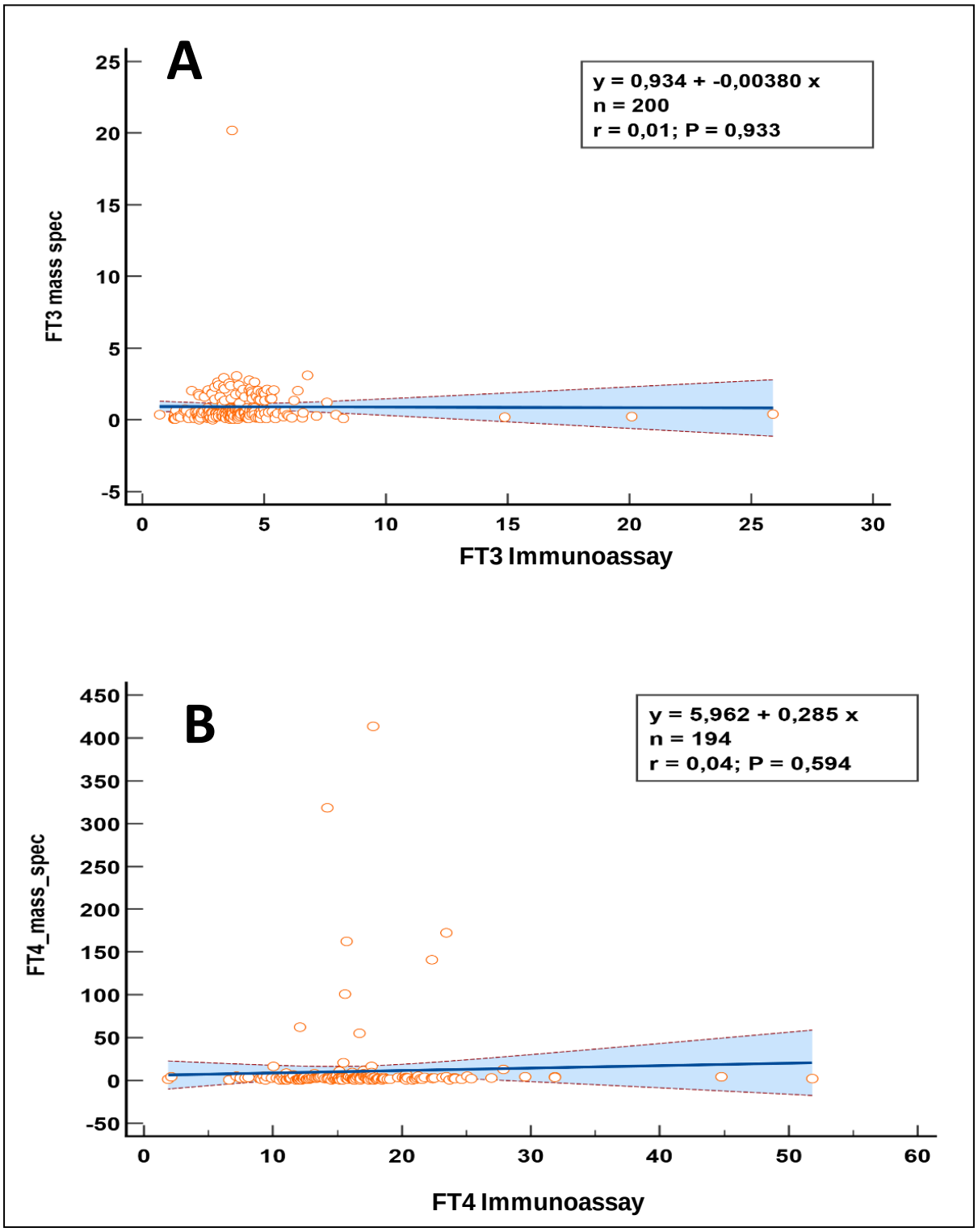


Figure 12: Regression analysis for FT_3 (A) and FT_4 (B).

There is better correlation between the two methods at the lower concentration range of 2-5 pmol/L for FT_3 and 10-20 pmol/L for FT_4 as it is observed that at these concentration ranges the data points are closer to each other. The r-value shows the relationship between the independent and dependent value. The closer the value is to 1, the better the correlation between the two methods. The r-value is close to 0 and shows that the methods do not correlate with each other.

4.4.1.3. Method comparison with respect to TSH

Studies conducted by Jonklaas and Soldin, 2008; Jonklaas et al., 2009, evaluated the relationship between log TSH and FT_4 measured on IA. They found that log transformed TSH had a better correlation compared to non-log transformed TSH values when compared to FT_4 on IA.

Figure 13 shows the relationship between log TSH and FT_4 measured by IA in this study (A), and the relationship between non-log transformed TSH and FT_4 measured by IA in this study (B). They both show that there was a better correlation with the log transformed TSH compared to the non-log transformed TSH. This is in agreeance with the above studies and can be interpreted as the log TSH measured correlating better to the FT4 measured. It better represents the relationship between the TSH levels in the body and the subsequent FT4 response from the body.

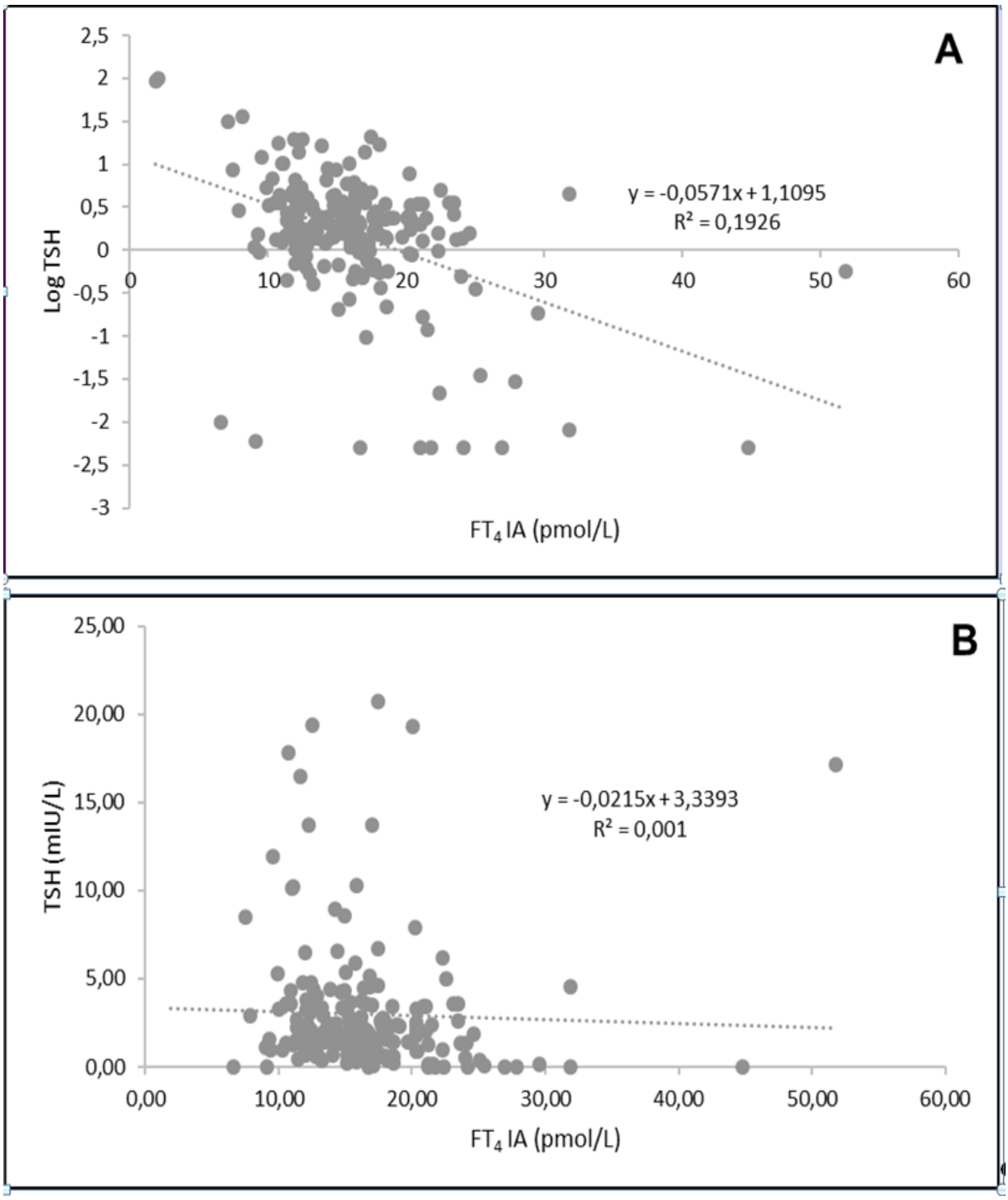


Figure 13: (A) Relationship between log TSH versus FT_4 measured by Immunoassay and (B) Relationship between non-log transformed TSH and FT_4 measured by Immunoassay.

The studies by Jonklaas and Soldin, 2008; Jonklaas et al., 2009 also assessed the relationship between the hormones measured by IA and LC-MS/MS. They found that

LC-MS/MS had a broader measuring range, especially at lower concentrations, compared to IA. Box and whisker plots, Figure 13 A-F, depict the correlations between IA and LC-MS/MS using TSH ranges measured by IA. These ranges were: suppressed TSH (<0.27 mIU/L), normal TSH (0.27-4.20 mIU/L) and elevated TSH (>4.2 mIU/L). The IQR for the two measurement techniques are therefore different. This means that LC-MS/MS could pick up lower hormone levels compared to IA for all the TSH ranges. The box in the plots depicts the IQR range of the data with the median values in the middle of the boxes. The whiskers are IQR ±1.5 on either side of the boxes. The outliers of the data are represented by the dots above the boxes. According to the boxes, the IQR range of the LC-MS/MS method was much lower than that of the IA method.

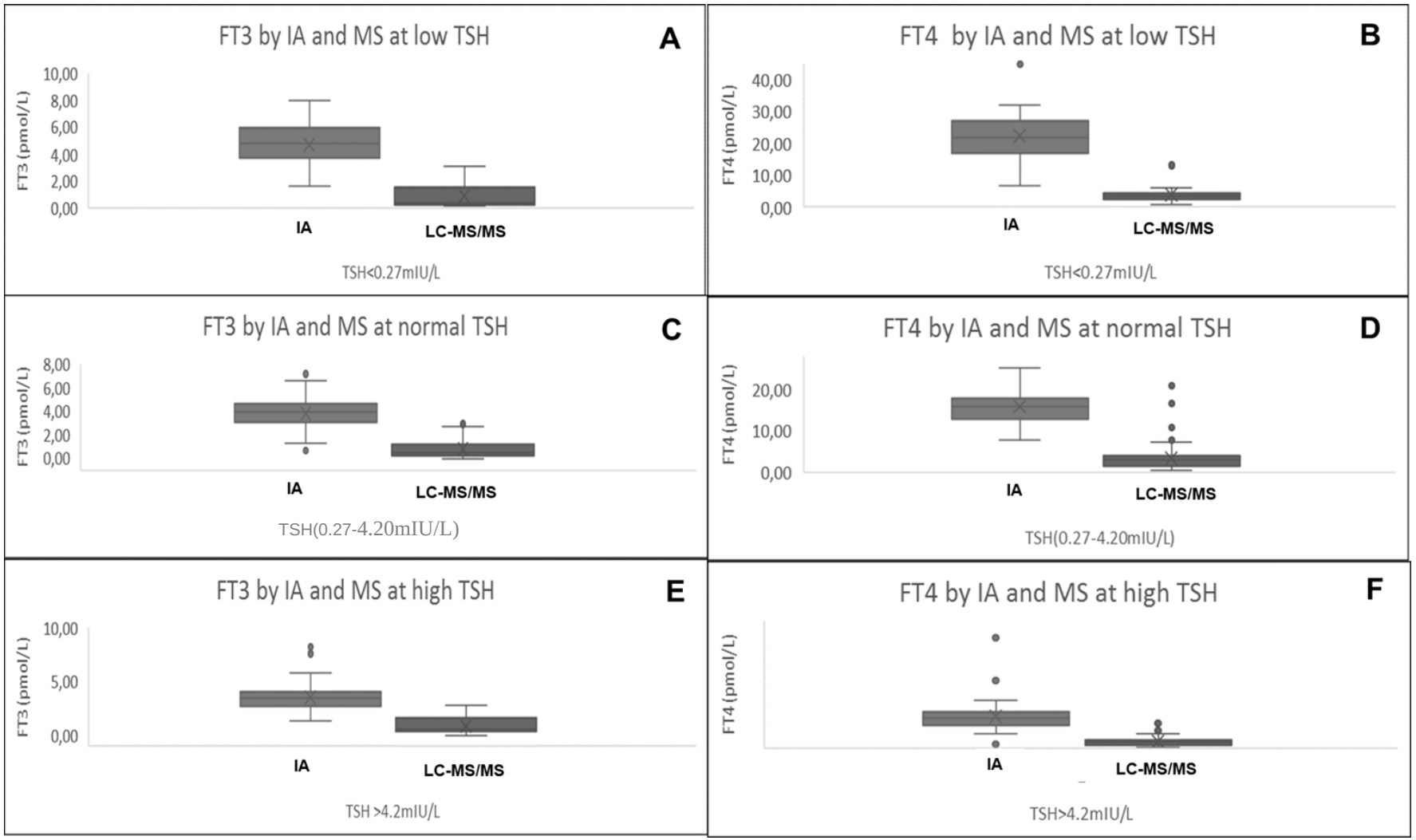


Figure 14: Box and whisker plots representing the difference between IA and LC-MS/MS for all analytes measured at different TSH ranges.

4.4.2 Population characteristics

For the sample population baseline characteristics, the mean age for the overall population was 51 years. The sample population had 128 (64%) females and 72 (36%) males. In terms of ethnicity, 139 (69.5%) individuals were black, 6 (3%) individuals were coloured, 8 (4%) individuals were indian and 47 (23.5%) were white.

4.4.2.1 Method comparison using patient population baseline characteristics

The male and female data was normally distributed as determined by the Shapiro-Wilk test. A T-test was used to analyse the data. There was a significant difference between measuring methods (IA versus LS-MS/MS) for FT_3 in males and females with p-values of 0.0152 and 0.0330, respectively. However, there was no significant difference in thyroid hormone levels between the genders.

Table 16: Gender specific thyroid hormone measurements.

Gender	Thyroid Hormone	Immunoassay (pmol/L) (n =194)	LC-MS/MS (pmol/L) (n=200)	Significance level (p-value)
Females (n=128)	FT_3	3.92 ± 1.46	0.49 (0.28, 0.68)	0.0330*
	FT_4	17.47 ± 8.44	2.75 ± 1.53	<0.0001
Males (n=72)	FT_3	4.08 ± 1.45	0.48 (0.24, 0.76)	0.0152 *
	FT_4	16.01 ± 4.96	2.74 (1.55, 4.01)	<0.0001

The sample database was stratified into different age categories. Then a t-test was conducted in each of the categories to see if the difference between the two measuring methods was significantly different. No significant differences were observed between most of the age groups such as 0-29 for FT_4; 30-39 for FT_3 and FT_4; 50-59 for FT_4 and 60-69 for FT_4 (Table 17).

Table 17: Age baseline characteristics by thyroid measuring method.

Ages	Analyte	IA (pmol/L)	LC-MS/MS (pmol/L)	Significance level (p-value)
0-29 (n=30)	FT_3	4.53±1.31	0.54 (0.35; 1.47)	0.0418
	FT_4	15.71±4.63	2.40 ± 1.30	0.7537
	TSH	2.25±1.69	14.67 ± 5.14	0.0001
30-39 (n=26)	FT_3	3.99±1.09	0.69 (0.37; 1.61)	0.2889
	FT_4	15.74±3.47	3.10 ± 1.97	0.4821
	TSH	2.03±1.31	15.98 ± 3.35	0.0004
40-49 (n=42)	FT_3	3.93±1.40	0.53 (0.26; 1.38)	0.0063
	FT_4	14.37±3.09	3.51 ± 2.52	<0.0001
	TSH	2.04±1.60	15.86 ± 3.02	<0.0001
50-59 (n=35)	FT_3	3.77±1.26	0.52 (0.21; 1.37)	0.0298
	FT_4	16.08±5.77	3.33 ± 2.29	0.3492
	TSH	2.15±2.11	16.40 ± 3.07	0.0001
60-69 (n=37)	FT_3	3.48±1.20	0.40 ± 0.20	0.0001
	FT_4	16.53±4.99	2.87 ± 1.26	0.5906
	TSH	2.08 (0.58; 4.39)	15.63 ± 3.68	0.0085
Over 70 (n=30)	FT_3	3.31±1.17	0.38 ± 0.21	0.0003
	FT_4	16.82±4.77	3.42 ± 2.11	<0.0001
	TSH	2.54±1.83	15.17 ± 3.05	0.0004

In Tables 18-23, the difference in significance level between the different age groups, with respect to the thyroid hormones for FT_3, FT_4, rT_3 and TSH, was assessed using ANOVA. The significant values are indicated by asterisks (*). These include age groups such as 40-49, 50-59, 60-69 and over 70 for FT_3 analysed by IA; 50-59, 60-69 and over 70 for FT_3 analysed by LC-MS/MS; 60-69 and over 70 for FT_4 by LC-MS/MS and lastly 40-49, 50-59, 60-69 and over 70 for rT_3 by LC-MS/MS.

Table 18: ANOVA Comparison of thyroid hormones between all age groups for FT_3 by IA.

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	1.83				
	0.51				
40-49	2.91	0.90			
	0.03*	1.00			
50-59	3.15	1.22	0.40		
	0.01*	1.00	1.00		
60-69	4.19	2.24	1.53	1.07	
	<0.01*	0.19	0.95	1.00	
Over 70	4.24	2.37	1.71	1.27	0.26
	<0.01*	0.13	0.65	1.00	1.00

Table 19: ANOVA Comparison of thyroid hormones between all age groups for FT_3 by LC/MS/MS

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	0.16				
	1.00				
40-49	0.25	0.08			
	0.07	1.00			
50-59	0.45	0.28	0.20		
	0.00*	0.025*	0.16		
60-69	1.03	0.87	0.79	0.59	
	0.00*	0.00*	0.00*	0.00*	
Over 70	1.84	1.68	1.59	1.39	0.81
	0.00*	0.00*	0.00*	0.00*	0.00*

Table 20: ANOVA Comparison of thyroid hormones between all age groups for FT_4 by IA.

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	0.62				
	1.00				
40-49	-2.54	-3.16			
	1.00	0.43			
50-59	-2.58	-3.20	-0.04		
	1.00	0.50	1.00		
60-69	-3.43	-4.04	-0.88	-0.84	
	0.34	0.10	1.00	1.00	
Over 70	-2.28	-2.89	0.26	0.31	1.15
	1.00	0.95	1.00	1.00	1.00

Table 21: ANOVA Comparison of thyroid hormones between all age groups for FT_4 by LC-MS/MS.

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	-0.15				
	1.00				
40-49	-0.57	-0.42			
	1.00	1.00			
50-59	-1.46	-1.31	-0.89		
	0.68	1.00	1.00		
60-69	0.76	0.91	1.33	2.22	
	1.00	1.00	0.59	0.02*	
Over 70	0.92	1.08	1.49	2.38	0.17
	1.00	1.00	0.38	0.01*	1.00

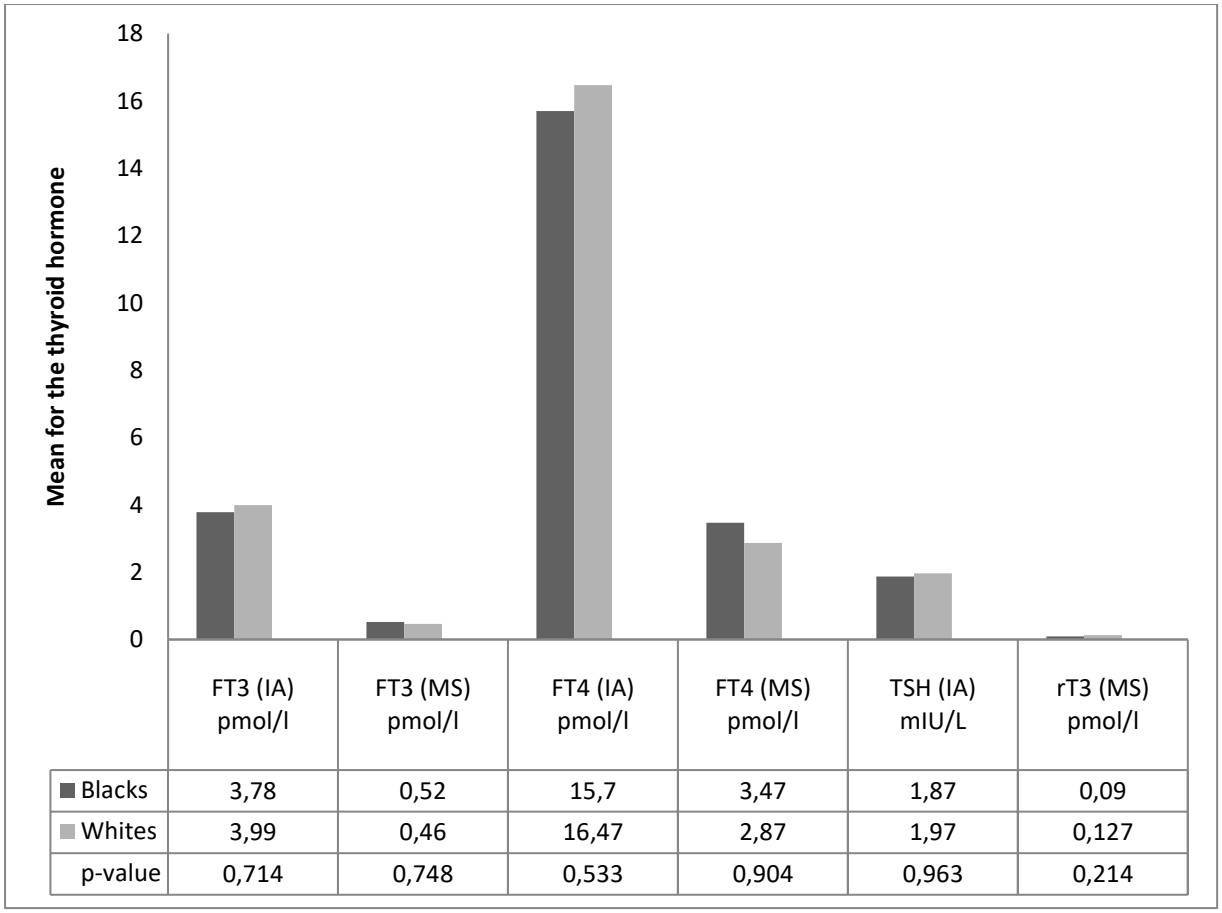
Table 22: ANOVA Comparison of thyroid hormones between all age groups for TSH by IA.

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	1.29				
	1.00				
40-49	0.31	-0.98			
	1.00	1.00			
50-59	0.29	-1.0	-0.02		
	1.00	1.00	1.00		
60-69	0.59	-0.71	0.27	0.29	
	1.00	1.00	1.00	1.00	
Over 70	1.20	-0.09	0.89	0.90	0.61
	1.00	1.00	1.00	1.00	1.00

Table 23: ANOVA Comparison of thyroid hormones between all age groups for rT_3 by LC-MS/MS.

Age groups	0-29	30-39	40-49	50-59	60-69
30-29	0.03				
	0.43				
40-49	0.05	0.02			
	0.00*	0.25			
50-59	0.09	0.06	0.04		
	0.00*	0.00*	0.00*		
60-69	0.18	0.15	0.13	0.09	
	0.00*	0.00*	0.00*	0.00*	
Over 70	0.39	0.36	0.34	0.30	0.21
	0.00*	0.00*	0.00*	0.00*	0.00*

The population was stratified according to ethnicity and the variables for the black and white population were analysed. The sample numbers for the coloured and Indian patient populations were low with 6 and 7 samples, respectively. Therefore, no statistical analysis could be performed. The mean age for the black participants was 48 and for the white participants was 58. Figure 15 illustrates the difference between the black and white participants in the study according to their mean thyroid hormone levels.



	FT3 (IA) pmol/l	FT3 (MS) pmol/l	FT4 (IA) pmol/l	FT4 (MS) pmol/l	TSH (IA) mIU/L	rT3 (MS) pmol/l
Blacks	3,78	0,52	15,7	3,47	1,87	0,09
Whites	3,99	0,46	16,47	2,87	1,97	0,127
p-value	0,714	0,748	0,533	0,904	0,963	0,214

Figure 15: Differences in the mean thyroid hormone levels between the black and white populations measured by IA and LC-MS/MS.

There was no significant difference between the two ethnicities when it came to the hormone levels (FT_3, FT_4, rT_3 and TSH) in each of the populations determined by the p-values from the t-test conducted. The N number for each of the populations was nevertheless, low and statistical significance was not attainable.

Chapter 5

Discussion

This study aimed to develop a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method for the detection and quantitation of thyroid hormones TSH, FT_3, FT_4 and rT_3. The objectives of the study were: to develop an LC-MS/MS method to measure thyroid hormones in serum. Then, to validate the method by analysing variables such as linearity, accuracy, and precision as well as the limit of quantification and the limit of detection. To compare the results obtained for FT_3 and FT_4 measured by LC-MS/MS with that measured by IA. And lastly, to determine whether there are differences in thyroid function diagnoses in patients when analysing the hormone levels using the LC-MS/MS analytical method compared to the hormone levels measured by the IA method.

5.1 Method optimization

The LC/MS/MS method for the detection and quantitation of thyroid hormones FT_3, FT_4, and rT_3 was optimized and validated according to FDA guidelines (FDA, 2001). There were notable differences between the reference method (Tanoue et al., 2018) and the LC-MS/MS method in this study. Different mobile phases were used. The reference method made use of acetic acid and Methanol whereas the current study utilised ammonium formate and Methanol. The main difference between acetic acid and ammonium formate is that acetic acid is an acetate buffer that prevents pH changes that might affect the biochemical activity of the compounds of interest (Cajka et al., 2023). Ammonium formate is a weak base and provides a different pH that may have enhanced the analytes interaction with the column. It also assists in the ionisation of the analytes in the form of adduct ions.

Since thyroid hormones are hydrophobic (Hulbert, 2000) and the Phenomenex F5 columns have hydrophobic functional groups and a carbon ring skeleton, in non-acetonitrile mobile phases, the π-π electrons of the carbon ring interact with the analytes π-π electrons and this encourages hydrophobic analyte retention and results in positive retention increases (Kinetex Core shell Technology, 2018). This

meant that since the functional groups of both the column and the analytes are similar, this interaction was better and resulted in optimal compound separations.

The source gas parameters (refer to section 3.4) were also different to the reference method. This may be due to the difference in the flow rate used. The reference method had a flow rate of 0.25 mL/min compared to the current method of 0.1 mL/min. The reason a lower flow rate was used was due to the observation that better peak shapes occurred at the lower flow rate. Lower flow rates allow for the analytes to have more time to interact with the functional groups on the column and thus translates into peak intensity and height improvements.

During the initial stages of the analyte optimisation, it was observed that T_3 and rT_3 had very similar if not identical masses and retention times, as confirmed by literature. Tanoue et al. (2018); Tai et al. (2004); Richards et al. (2019) and; Bråtveit. (2017), showed the closeness in elution of T_3 and rT_3 in terms of their retention times such that they were basically co-eluting. Literature also indicated that the analytes shared a Q1 mass as they were positional isomers.

Therefore, the transitions for these analytes were further investigated by use of different MS scanning modes, as discussed in section 4.2. These scans verified that the transitions used, and the chromatographic separations obtained were unique to each of the analytes. The studies mentioned above did not however, confirm the transitions used by running MS^3 scans. Richards et al. (2019) did perform MS^3 scans on other iodothyronines in human serum but did not include rT_3, FT_3 and FT_4. Therefore, to the best of my knowledge this is the first study to utilise MS^3 scans to verify the transitions used in an LC-MS/MS quantitation method for analytes rT_3, T_3, T_4, and TSH.

To differentiate the two positional isomers T_3 and rT_3, Couldwell et al., (2005) used transitions of 359.7 and 604.5 for rT_3 to differentiate it from T_3. They found that these

fragments were unique to rT_3. However, this study found that the 604.5 transition was attributed mostly to T_3. This may be explained by this study analysing the samples in positive ionisation mode and Couldwell et al., (2005) utilising negative ionisation mode using a Quattro Micro Triple quadrupole mass spectrometer (Waters Corp., Milford, Massachusetts, U.S.A). Regardless, Couldwell helped to highlight the importance of differentiating the two positional isomers (rT_3 and T_3, Figure 4) from each other and a means to do so.

For TSH, the lyophilized powder was reconstituted with 0.05 M PBS. PBS was recommended by the supplier according to the package insert because phosphate is mostly used for high recovery and better resolution of peptides (Rivier, 1978). However, it can lead to crystal formation and is thus not well tolerated on MS (Shibue et al., 2005). Phosphate is an anion and a salt derived from phosphoric acid (Shibue et al., 2005). Phosphate can neutralize hydrophobic positively charged groups in the peptides, thus decreasing overall peptide hydrophobicity. This leads to less retention of the target analyte on the stationary phase (Shibue et al., 2005). This would lead to decreased optimal separation of the target analyte. According to Holčapek et al. (2004), using ion pair reagents such as PBS decreases MS signal intensity and therefore, although present in low concentrations this may have impacted the intensity of TSH, especially at the lower more difficult to detect concentrations as seen during the validation.

Furthermore, Illiano et al., (2022) analysed TSH as a smaller peptide, namely, β-TSH. TSH was subjected to tryptic digestion as described by Illiano et al. (2022) and the digested products were then infused into the mass spectrometer to pick up the peptides. However, the TSH digestion did not work because the digestion products were not detected on the mass spectrometer. This could have been because the method conditions used caused the tryptic enzyme to cleave at different sites, or it underwent partial digestion due to method conditions. Illiano et al. (2022) used a Xevo TQ-S equipped with an Ion-key UPLC micro flow source, this is a system that physically integrates a UPLC separation into the source of the mass spectrometer (Shan and Jones, 2022), this may have provided a better measuring means for

detecting the peptides. Since, the digestion methods were unsuccessful non-conventional “top down” mass spectrometry was used and so this study attempted to analyse TSH as an “intact” molecule. This entails the protein being ionized directly without being digested prior to analysis (Catherman et al., 2014). Injecting intact, undigested, proteins into the mass spectrometer allows for better characterization of post-translational modifications and avoids interferences associated with peptide-based proteomics (Catherman et al., 2014). This is possible because the mode of MS analysis, namely, ESI, allows for the analysis of high-molecular-weight compounds through the production of multiply charged ions in the gas phase (Polo and Limbach, 2000). TSH has a molecular mass of 28 000 Da. The maximum range that a triple quadrupole mass spectrometer can analyse is 1250 Da in triple quadrupole mode and 1000 in linear ion trap mode (Sciex Triple Quad 5500+ system user guide, 2019). This is because mass spectrometers record mass- to- charge ratios (m/z) and electrospray analysers typically produce a spectrum in the range of 500-2000 when analysing proteins (Sciex Triple Quad 5500+ system user guide, 2019) as used in this study for TSH. An ion with a single charge appears at a mass equivalent to the molecular weight, as per FT_3, FT_4 and rT_3 as shown in Figure 7 (Sciex Triple Quad 5500+ system user guide, 2019). However, if the ion of the same mass is doubly charged this means that it will appear at half of the apparent mass (Sciex Triple Quad 5500+ system user guide, 2019). This could help to explain why we observed a Q1 mass of only 608.7 for TSH due to its high mass. According to Polo and Limbach (2000), the presence of multiple charges on the molecule will result in a decrease in the m/z values and therefore, the Q1 mass will be less than the known molecular weight of the compound. This allows for the characterization of large molecules using mass analysers with limited m/z ranges.

5.2 Method Validation

Method validation was used to demonstrate the reliability of this method for the determination of thyroid hormone concentrations in serum (European Medicines Agency, 2011). The method developed was able to detect and quantify FT_3, FT_4 and rT_3 in pmol/L using the criteria specified by the FDA guidelines (FDA, 2001).

The LC-MS/MS method developed in this study had lower LOQ’s versus that of the

IA method of analysis. This is a similar finding to that of Jonklaas et al. (2009). However, their study reported a higher LOQ for FT_4 and FT_3 compared to that reported here. Jonklaas (2009) utilised a Supelco LC-18-DB (3.0 µm, 3.3cm × 3.0 mm) column as opposed to the F5 and this could have altered the binding on the analytes to the column. Yue et al. (2008) utilised equilibrium dialysis to isolate free thyroid hormones, this is a separation technique that is based on the movement of low molecular weight molecules through a membrane with a predetermined cut-off (Shea et al., 2014). Equilibrium dialysis was used by Yue et al. (2008) in order to analyse FT_3 and FT_4. They reported higher LOQ values for both analytes as compared to this study. This implies that this study had a broader measuring range at lower concentrations compared to others. It is important to note that Yue et al. (2008) used a population comprised of pregnant women. Pregnancy influences the levels of thyroid hormones such that it can lead to falsely elevated values (Ekins, 1987; Jonklaas et al., 2009).

The validation of the method met the required criteria for all the analytes (FDA, 2001) except for TSH. TSH had a LOQ of 0.08 pmol/L but was required to have a measuring range as low as 0.01 pmol/L. This meant that the method could not accurately quantify values below 0.08pmol/L. This is why CAL1 was not accurately quantified and had a CV value that did not fall within the acceptable range (FDA, 2001). Additionally, the TSH method was not linear and therefore, was not used to quantify TSH in the patient samples.

5.3 Method Comparison

The difference found between the two measuring methods in this study is consistent with what was found by studies conducted by Jonklaas et al. (2014), Soldin and Soldin, (2011) and Soldin et al. (2005). These researchers found that LC-MS/MS had a lower measuring range compared to the measuring range of IA.

Patient hormone measurements obtained using IA and LC-MS/MS methods were compared as described in section 4.4.1 and illustrated in figures 12-15 and Tables 13-23. The regression analysis (section 4.4.1.2 and Figure 12) showed that there is

a greater correlation between IA and LC-MS/MS at low concentration of 2-5 pmol/L for FT_3 and 10-20 pmol/L for FT_4. This correlation is lost at higher and lower concentrations (figure 12). This confirmed the results reported by Jonklaas et al. (2014), who also found that the agreement between the two techniques (IA and LC-MS/MS) was greatest at the lower extremes (2-8 pmol/L for FT_3 and 10-30 pmol/L for FT_4).

Box and whisker plots of the data better revealed the relationship between LC-MS/MS and IA for thyroid hormone measurement. These plots (Figure 14) revealed that LC-MS/MS is measuring at a lower range compared to the IA method. Similarly, the box and whisker plots of Jonklaas et al. (2014) depicted the correlation of the measurements between IA and LC-MS/MS and found that lower T_4 and T_3 values were more frequently reported for TSH levels above 3.5 mIU/L when measured by LC-MS/MS as compared to IA. Yue et al. (2008) and Gu et al. (2007) also found that the IA and LC-MS/MS had greater correlations with each other at lower concentrations (2-7 pmol/L for FT_3 and 10-26 pmol/L for FT_4). They also found that lower concentrations of FT_4 and FT_3 were more closely associated for IA and LC-MS/MS. Furthermore, the LC-MS/MS was able to measure lower concentrations as compared to IA. As the concentrations increased above 10 pmol/L for FT_3 and above 30 pmol/L for FT_4, the divergence between the points became larger and thus the correlation was lost.

Soldin et al. (2005) found that IA for FT_3 correlated very poorly with LC-MS/MS at high concentrations. This is similar to this study as the FT_4 and FT_3 IA results both correlated better to the LC-MS/MS at lower concentrations, however, as mentioned previously, the correlation was lost at lower and higher concentrations (Figure 12).

Jonklaas et al. (2009) compared log TSH versus FT_4 analysed by both, IA and LC-MS/MS. They found a better correlation between the log TSH and FT_4 measured by LC-MS/MS as compared to that of IA. It can be said that the closer relationship between serum TSH and each of the thyroid hormones measured by LC-MS/MS reflects the greater specificity of LC-MS/MS. This was also the relationship observed

by the results in this study (see section 4.4.1.3 and Figure 13). It is also evident from this study and others (Soldin et al., 2005; Welsh and Soldin, 2016) that LC-MS/MS performs better at low (2-5 pmol/L for FT_3 and 10-20 pmol/L for FT_4) and high (>50 pmol/L) thyroid hormone concentrations and is thus better at diagnosing, in particular, hypothyroid conditions.

5.4 Patient data

5.4.1. Overall diagnosis using IA versus LC-MS/MS

Samples were collected to cover the clinical measurement range; however, samples with lower T_4 results were prioritised. This was done in order to test the measuring range of mass spectrometry as it is known that this instrumentation is able to detect and quantify thyroid hormone levels more accurately at lower ranges of less than 12 pmol/l for FT_4 and less than 3 pmol/l for FT_3 (Jonklaas et el., 2014) and also above 19.31 pmol/l for FT_4 and 12.29 pmol/l for FT_3 (Gu et al., 2007).

According to the diagnosis code scheme described by a study done by Koulouri and Gurnell, (2013), patients were diagnosed differently when measuring samples using IA versus LC-MS/MS (See Table 14). In this study the two different methods also diagnosed the patients differently with respect to biochemical diagnoses. This is because 40.5% of patients were euthyroidic when diagnosed by IA measurement versus 21% diagnosed as having primary hypothyroidism when using LC-MS/MS measurement.

The levels of the analytes for each of the genders and the analytical techniques used were found to be significantly different for the two methods of analysis (Table 13). As mentioned previously, the difference found between the two measuring methods in this study is consistent with what was found by studies conducted by Jonklaas et al. (2014), Soldin and Soldin, (2011), Soldin et al. (2005). These researchers found that LC-MS/MS had a lower measuring range compared to IA.

5.4.2. Method dependent diagnoses trends between genders

A study conducted by Vanderpump (2011), found that in areas where there is a sufficient supply of iodine, the prevalence of spontaneous hypothyroidism is between 1 and 2%, and it is more common in older women and 10 times more common in women compared to men. Salih et al. (2021) in a sample population of 88 patients showed that there was a difference in T_3, T_4 and TSH values between males and females. This is different from what this study found as no significant difference in terms of diagnosis of hypothyroidism was found between the genders (see Table 16). This may be explained by the sampling method of favoring low thyroid hormone level samples. However, this study found that only FT_3 has a significant difference level ($p<0.005$), between males and females (Table 16). There is mandatory iodization of table salt produced in South Africa (Jooste et al., 2001) and therefore, iodine supply to the population should be sufficient and can be ruled out as a possible cause for low thyroid function. According to this study, patients are mostly diagnosed as hypothyroid when using LC-MS/MS measurement. This corresponds to studies conducted by Jonklaas et al. (2014); Soldin and Soldin, (2011) and Soldin et al. (2005) who also found that most hypothyroid patients are falsely diagnosed by IA as euthyroid and that LC-MS/MS is able to more accurately diagnose these patients. Since most countries continue to analyse thyroid hormone levels in patient samples using IA as opposed to LC-MS/MS, and since LC-MS/MS has been shown to identify hypothyroid cases more easily and accurately, it could be said that the status quo of patients still experiencing symptoms of hypothyroid even with normal thyroid hormone measurements is likely the result of utilizing an inferior assay, namely IA.

5.4.3. Age related method dependent diagnoses trends

Mirjanic-Azaric et al. (2017), analysed the difference between thyroid hormones measured on IA (Roche, ECLIA) using 250 euthyroid patient samples and found that there was a significant difference in TSH levels in the age groups 30-50 years and a

significant difference in FT_3 levels in ages 30 and 50. They found a statistically significant difference observed in thyroid hormone levels between males and females for FT_3 and FT_4. This study however, revealed that the differences observed in thyroid hormone levels between the ages and the genders were not significantly different (see Table 17). Again, this could be due to the sampling method of favouring samples with low thyroid hormone levels measured by IA. Chen et al. (2019) conducted a study to determine the impact of age and gender on thyroid hormone levels in a large Chinese population with sufficient iodine intake. They found that there was a significant difference ($p<0.001$) between TSH values across the ages. And there was also a significant difference between the genders ($p<0.001$) for TSH. In this study FT_3 and rT_3 values decreased as age increased, whereas FT_4 values increased as age increased. The TSH value stayed the same over the age groups. This does not correspond to what was found previously. Klee and Hay, (1997) found a significant difference in the levels of TSH between genders in a population of 625 people using IA. This study had a skewed patient population in order to target hypothyroid patients. Hence the TSH values between the populations stayed the same. This was similar to the findings of a study by Mirjanic-Azaric et al. (2017) who used ECLIA, on 250 euthyroid patients as they only found a difference in FT_3, FT_4 values and not TSH values, this finding according to them, could not be explained. Looking at the thyroid hormone control feedback mechanism, FT_4 increases, whiles FT_3 is on a constant decrease as one ages (Salih et al., 2021). This implies that although the body is releasing FT_4 its active form, FT_3, is not being produced. This can also be attributed to the rT_3 value that is also decreasing over time, since rT_3 functions to bind to the FT_3 receptors and inhibit the release of FT_4 (Hennemann et al., 1993). As one ages, the peripheral metabolism of thyroid hormone drops because of the reduction in the activity of the deiodinase enzymes in the liver and kidneys which are responsible for converting FT_4 into active FT_3 and into inactive rT_3 (Chen et al., 2019 and Silvestri et al., 2008). Thus, an overall decline in FT_3 and rT_3 levels can occur in the presence of high FT_4. They also found a difference between thyroid hormone diagnoses between age groups in that hyperthyroidism was seen in the 40-49 years age group and hypothyroidism was seen in the 30-39 years.

5.4.4. Method dependent diagnoses based on ethnicity

Following the gender and age stratification of the populations, the population was stratified according to ethnicity. The variables for blacks and whites only were analyzed because the coloured and Indian patient population had small samples number of 6 and 7, respectively (see Figure 15). A 10-fold lower annual incidence of hyperthyroidism in a black population (0.09 per 1000 women and 0.007 per 1000 men) compared to in a white population from Johannesburg, South Africa was reported by Vanderpump (2011). The overall diagnosis by IA, found in this study, is euthyroid as the FT_3, FT_4 and TSH were within the normal ranges. This is not surprising given the limitations of the IA assay as compared to LC-MS/MS. Consequently, the overall diagnosis according to LC-MS/MS, across both the ethnicities, was Euthyroid Sick Syndrome as the FT_3 and FT_4 were suppressed. This is a similar finding compared to those by Jonklaas et al. (2014) and Welsh and Soldin (2016) who reported that LC-MS/MS was able to diagnose more hypothyroid cases compared to IA owing to the lower measuring range of LC-MS/MS.

5.4.5. Utility of rT_3 measurement for thyroid function

A study conducted by Chopra et al. (1979), measured serum rT_3 concentrations in patients with low T_4 and non-thyroidal illnesses in order to assess the clinical utility of rT_3 measurements and its usefulness in thyroid dysfunction diagnosis. They analysed thirty-two patient samples using IA, and sixteen of the samples corresponded to patients who had systemic or severe chronic illnesses. These samples had low T_4 and for this reason the rT_3 was measured. rT_3 was shown to be high (983.1 to 7219.7 pmol/L) in eleven patients and normal (522.3 to 921.7 pmol/L) in the remaining five patients (normal range established as 414.8 to 952.4 pmol/L) (Chopra et al., 1979). This study found that rT_3 values were lower on average between all the patients regardless of whether they had low T_4 or not. Low concentrations of T_3 usually occur in non-thyroidal illnesses and are attributable largely to reduced conversion of T_4 to T_3. This health condition is also mostly associated with reduced rT_3 clearance that causes the rT_3 to be high in the serum (Chopra et al., 1983). Notably, the measuring range of rT_3 selected for the current study was lower compared to others (Chopra et al., 1979; Richards et al., 2019 and

Halsall and Oddy, 2021). This measuring range is more in line with the relationship between serum levels of T_3 compared to rT_3 that are ten-fold lower (Halsall and Oddy, 2021). This may have impacted the ability to make clinically relevant distinctions regarding thyroid function. Furthermore, a statistical meaningful correlation between TSH, FT_3, FT_4 compared to rT_3 was not evident from this sample population. It can therefore be said that measuring serum rT_3 simultaneously with other thyroid hormones does not appear to have clinical value. This is in agreement with the review article regarding the clinical and laboratory aspects of measuring rT_3 published in 2021 by Halsall and Oddy.

5.5 Strengths and limitations of the study

This study contributes to the knowledge of the techniques and methods used to analyse thyroid hormones. It also strengthens the knowledge regarding the different diagnosis outcomes when using IA and LC-MS/MS, more specifically in a South African population. The results found correlate well to previous studies that have also found differences in diagnosis when comparing IA and LC-MS/MS methods of thyroid hormone analysis in other population groups. Furthermore, this study re-enforces the recommendation of the use of LC-MS/MS as the preferential method of analysis of thyroid hormones.

The limitations of this study include the sample size of 200. This sample size resulted in some ethnical and age groups having small numbers and being under-represented. This meant that this study could not make any statistical meaningful correlations with respect to some sample populations i.e., coloured and Indian populations in South Africa relating to thyroid hormone function and/or dysfunction.

Using remnant samples did not require patient recruitment but this meant that patient histories and possible comorbidities could not be recorded and used to better interpret the data. If samples came from patients who had undergone thyroidectomies or who were/are on thyroid therapy, this was not taken into consideration as this information was not available and could not be used to better interpret the data. Furthermore, using remnant samples from hospital patients

implies these patients were more than likely ill and so did not represent that of a normal healthy population. This however, did not prevent method comparison. Selecting samples with thyroid hormone levels that were low, appeared to have skewed the data in terms of diagnosis. However, this allowed us to evaluate the measuring power of the LC-MS/MS at the lower range.

rT_3 LC-MS/MS EQA material was unfortunately not available and so could not be used to verify this method of analysis. Since no other laboratories in South Africa currently analyse rT_3 using LC-MS/MS, inter-lab comparisons were not possible.

Chapter 6

6.1 Conclusion

In conclusion, a mass spectrometric method has been developed, optimized and validated to run FT_3, FT_4 and rT_3, simultaneously. The LC-MS/MS method had lower measuring ranges when compared to IA. Differences in diagnosis were evident with IA diagnosing most patients as euthyroid whereas LC-MS/MS differentiated these into euthyroid sick syndrome. Therefore, this study also concludes that LC-MS/MS could be used as an alternative method of analysis of thyroid hormones in particular groups of patients such as patients who are on thyroid hormone treatment but still complain about hypothyroid-like symptoms and in patients that are diagnosed as euthyroid but consistently display symptoms synonymous with hypothyroid.

6.2 Recommendations

Future studies should be directed at looking at a larger sample size especially for ethnical groups such as the South African coloured and Indian populations. This will permit more valid inferences regarding the influence of ethnicity on thyroid function in the South African population.

The utility of measuring rT_3 could be further considered and it may be beneficial to investigate genetic correlations, if any, of thyroid disorders in the different ethnic groups in a South African population and how they may be related to levels of rT_3. Studies done by Moran et al., (2017) and Persani and Campi, (2019) highlight the genetic aspects of rT_3 such as contrasting phenotypes affecting T_3 nuclear hormone receptors THRα and THRβ and the deiodinase enzymes, especially DIO1 that has been shown to influence rT_3 levels (Halsall and Oddy, 2021). These receptors and enzymes affect the levels of rT_3 circulating in the body. This could be further explored by not only measuring the levels of rT_3 in serum by LC-MS/MS but also simultaneously analysing the corresponding patients' phenotypes.

Chapter 7

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Chapter 8

Appendix

8.1 Ethics certification

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Ms LK Sephesu
School of Pathology
Department of Chemical Pathology
Medical School
University

E-mail: sephesu.liezel@gmail.com

CC: Supervisor: Dr D Legg-E'Silva
<Derryn.Legg@wits.ac.za>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2023/06/26

REF: R14/49

PROTOCOL NO: **M230331** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Thyroid hormone measurement in South Africa*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000\Iain0007/Clearscan.wps

2023/10/02

Ms LK Sephesu
School of Pathology
Department of Chemical Pathology
Medical School
University

Sent by e-mail to: sephesu.liezel@gmail.com

Dear Ms Sephesu

Re: **Protocol Ref No:** M230331
Protocol Title: *Thyroid hormone measurement in South Africa*
Principal Investigator: Ms LK Sephesu

Thank you for your letter of 2023/09/15.

We note and approve of your proposal to add de-identified data on patient age, sex and race to your study.

Thank you for keeping us informed.

Yours Sincerely

I Burns

.....

Mr I Burns
For the Human Research Ethics Committee (Medical)