Development and optimisation of a gas chromatography time-of-flight mass spectrometry (GC-TOFMS) method for the quantification of amino acids in infant formula

by

Sabelo Wiseman Chamane

Submitted in partial fulfillment of the requirements for the degree
Master of Science (Chemistry)

In the

Department of Chemistry
Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

August 2018

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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by

Sabelo Wiseman Chamane

Supervisor	: Dr. Y. Naudé
Co-supervisor	: Mrs. D. Prevoo-Franzsen
Department	: Chemistry
University	: University of Pretoria
Degree	: Master of Science in Chemistry

X

SABELO CHAMANE

X

Dr. YVETTE NAUDE

X

DESIREE PREVOO-FRANZSEN

Declaration

I **SABELO WISEMAN CHAMANE** declare that the dissertation

“

Development and optimisation of a gas chromatography time-of-flight mass spectrometry (GC-TOFMS) method for the quantification of amino acids in infant formula

”

is my own work, that all the sources used or quoted in this report have been acknowledged by means of complete references and that this dissertation has never been submitted by me for the same degree at another university.

X

SABELO WISEMAN CHAMANE

August 2018

Acknowledgments

My deepest gratitude goes out to my supervisors Mrs. D Prevoo-Franzsen and Dr. Y. Naudé for working tirelessly putting this project together and ensuring that it is a success, without your efforts and guidance, this project would have been a huge failure and a tremendous waste of time. I would also like to thank the National Metrology Institute of South Africa (NMISA) for giving me an opportunity to work on this project in collaboration with the University of Pretoria, in a period of two years I have gained immeasurable amount of practical skills and I have also been able to grow my analytical knowledge and skills exponentially through various training opportunities offered and funded by NMISA. I would also like to pass my greatest gratitude to all those who contributed both in the project and on my personal growth as an analyst, be it through training or various other means:

Dr. L. Quinn

Mr. S.A. Nsibande

Mr. M. Brits

Dr. N. Nhlapo

Mr. W. Nxumalo

Mrs. R. Visser

Dr. M. Fernandes-Whaley

Mrs. M. Archer

Dr. C. Swiegelaar

Abstract

Improved infant food protein testing methods have become mandatory for testing laboratories around the world to ensure food safety and to curb infant food adulteration such as the melamine adulteration incident that occurred in China, 2008. In this study a speed optimised flow rate (SOF) gas chromatography time-of-flight mass spectrometry (GC-TOFMS) method for quantifying 14 N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) derivatised amino acids (AAs) viz. alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine, and tyrosine in infant formula was developed. Using this method, 14 target compounds together with additional analytes, namely, cysteic acid, methionine sulfone, taurine, ornithine, and tryptophan, were resolved in 12.5 minutes.

Using the GC-TOFMS method developed in this study, the above-mentioned analytes were quantified using two approaches, the external calibration approach, and the isotope dilution approach. An internal standard stock solution comprised of ^{13}C valine, ^{13}C isoleucine, ^{13}C proline and ^{13}C phenylalanine was used for the isotope dilution quantification method. Limits of detection (LODs) of between 0.0111 g/100g and 0.1064 g/100g were obtained by external calibration while LODs of between 0.01950 g/100g and 0.2456 g/100g were obtained by isotope dilution. Limits of quantification (LOQs) of between 0.0371 and 0.3548 g/100g were obtained by external calibration while LOQs of between 0.06510 and 0.8186 g/100g were obtained by isotope dilution. Linear regression correlation coefficients (r^2) of between 0.9988 and 1.0000 were obtained from the calibration curves generated by external calibration while r^2 values of between and 0.9959 and 0.9999 were obtained from the calibration curves generated using the isotope dilution approach.

The GC-TOFMS (external calibration and the isotope dilution) methods developed in this study were validated using the National Institute of Standards and Technology (NIST) infant/adult nutritional formula standard reference material (SRM 1849-a) that had been hydrolysed with hydrochloric acid to obtain protein hydrolysates. On analysis of the NIST SRM (1849-a) protein hydrolysates, analyte recoveries (accuracy) of between 61.13% and 103.99% were obtained by external calibration while analyte recoveries of between 73.31% and 104.76% were obtained using the isotope dilution method. With the external calibration approach, coefficients of variation (precision) ranging from 7.32% to 25.76% were obtained while coefficients of variation of between 2.99% and 41.53% were obtained by isotope dilution.

Method ruggedness was assessed by comparing the results obtained using the GC-TOFMS methods with the results obtained using the Waters Corporation's AccQ·Tag method on an ultra-performance liquid chromatography (UPLC) system with ultraviolet (UV) detection. Method transferability was assessed by comparing the results obtained with a GC-TOFMS (Pegasus III) system with the results obtained on an alternate GC-TOFMS (Pegasus IV) system. Additionally, the results obtained from the GC-TOFMS method using the HCl hydrolysis method were compared with the results obtained from the same instrument using the trifluoroacetic acid (TFA) hydrolysis method. The main purpose of using an additional hydrolysis method (the TFA hydrolysis method) and applying two independent analytical techniques (UPLC and GC-TOFMS technique), was to develop and validate two independent analytical methods for value assigning the amino acid content of infant formula reference material to be produced by the National Metrology Institute of South Africa (NMISA).

Using the Pegasus IV GC-TOFMS system, recoveries of between 50.85% and 101.62% were obtained through the isotope dilution method while recoveries ranging from 73.18% to 133.29% were obtained by external calibration. Additionally, using the Pegasus IV GC-TOFMS method, coefficients of variation ranging from 0.36% to 7.39% were obtained through the isotope dilution method while coefficients of variation ranging from 1.45% to 12.69% were obtained through the external calibration method. Although there were differences between the recoveries and the coefficients of variation obtained using the Pegasus III and the Pegasus IV GC-TOFMS systems, using the student's t-test, significant differences between the results obtained by these methods were only found between the experimental means of proline, threonine, phenylalanine, and histidine. Therefore, based on the t-test results both the external calibration and the isotope dilution methods were readily transferable between the Pegasus III GC-TOFMS system and Pegasus IV GC-TOFMS system with significant differences only found between the abovementioned analytes.

The Pegasus III GC-TOFMS results obtained by external calibration were comparable with the UPLC AccQ·Tag method results obtained by a similar calibration approach with significant differences found between alanine, leucine, isoleucine, proline, phenylalanine, and tyrosine. Most of the differences were observed between the results of the isotope dilution quantification method on the GC-TOMS system and the results of the internal standard method on the UPLC system. These include the experimental means of alanine, lysine, valine, leucine, isoleucine, proline, serine, histidine and tyrosine. Furthermore, the UPLC system yielded better precision compared to the GC-TOFMS methods. Using the UPLC method, coefficients of variation

ranging from 5.30% to 13.15% were obtained by the internal standard method while coefficients of variation ranging from 3.86% to 20.21% were obtained by external calibration. Analyte recoveries ranging from 73.01% to 142.90% were obtained by the internal standard method while analyte recoveries of between 59.51% and 104.49% were obtained by external calibration.

During method development, the guidelines provided in the Guide to Expression of Uncertainty in Measurement (GUM) were used to develop a cause and effect diagram which was subsequently used to identify experimental variables that may affect the accuracy and the uncertainty of measurements. Where possible, uncertainty contributions of the experimental variables identified through the cause and effect diagram were quantified mathematically using the GUM approach excluding the uncertainty contributions due to (1) the derivatisation temperature, (2) derivatisation period, (3) analyte reconstitution solvent type and (4) the stability of MTBSTFA derivatised amino acids. The uncertainty contributions due to the abovementioned variables could not be quantified mathematically due to complexity hence these variables were optimised experimentally to eliminate the need for their inclusion in the assessment of the uncertainty budget.

For the optimisation process, a two-way or one-way ANOVA in conjunction with a Tukey honest significant difference (HSD) post hoc test were used to statistically assess the significance of the differences of the optimisation results. From the derivatisation time and derivatisation temperature results, it was found that all amino acids (AA) of interest were completely derivatised after incubation at 100 ˚C for 4 hours. Furthermore, acetonitrile was identified as a better reconstitution (injection) solvent for the analysis of MTBSTFA derivatised amino acids compared to isooctane. Additionally, MTBSTFA derivatised AAs showed varying stability under the storage conditions (ambient temperature and 3 ˚C) tested in this study. Alanine, glycine, valine, leucine, lysine and tyrosine derivatives were stable under both storage conditions. In contrast, isoleucine, phenylalanine, aspartic acid and glutamic acid were only stable at room temperature while proline, serine, and threonine derivatives were only stable at 3 ˚C.

Analysis of MTBSTFA derivatised amino acids in infant formula by GC-TOFMS using both the external calibration and isotope dilution method gave results that were comparable to the results obtained through the routinely employed AccQ·Tag method as determined by (Bosch *et al.*, 2006a). The advantages of the GC-TOFMS methods over the routine LC method were quick analyte identification using mass spectral libraries, lower cost per analysis despite the

need for a longer sample preparation period, good stability of the MTBSTFA derivatised amino acids and the minimal use of organic solvents. On the other hand, the drawbacks of the GC-TOFMS method were longer sample preparation period due to the lengthy derivatisation procedure and the method's inability to quantify arginine as this analyte is degraded to ornithine during MTBSTFA derivatisation.

Research Outputs

Poster presentation

Chamane S. **W**, D. Prevoo-Franzsen, Dr. M. Fernandez-Whaley, Dr. Y. Naude., 2017, *Gas chromatography time of flight mass spectrometry (GC-TOFMS), an alternative method for quantification of amino acids in infant formula.* Presented at NMISA 7010 conference, CSIR, Pretoria.

Poster presentation

Chamane S. **W**, D. Prevoo-Franzsen, Dr. M. Fernandez-Whaley, Dr. Y. Naude., 2018, *Gas chromatography time of flight mass spectrometry (GC-TOFMS), an alternative method for quantification of amino acids in infant formula.* Presented at NMISA Food Safety Workshop, CSIR, Pretoria.

Oral presentation

Chamane S. **W**, D. Prevoo-Franzsen, Dr. M. Fernandez-Whaley, Dr. Y. Naude., 2017, *Gas chromatography time of flight mass spectrometry (GC-TOFMS), an alternative method for quantification of amino acids in infant formula*. Presented at Chromsaams Student Seminar, Pretoria.

Dedications

I dedicate this dissertation to my son **Mpilwenhle Ntandoyenkosi Chamane** and his beloved mother **Miss Mbali Zuma**; my family, my supervisors, my friends and anyone who will find this dissertation useful in their future studies.

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Abbreviations

AA	: Amino Acid
AAS18	: Sigma Aldrich Amino Acids Standard
ANOVA	: Analysis of Variance
AOAC	: Association of Analytical Communities
APCI	: Atmospheric Pressure Chemical Ionisation
AQC	: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BSTFA	: N,O-bis(trimethylsilyl)trifluoroacetic acid
BTO	: Bleed Temperature Optimised
˚C/min	: Degrees Celsius Per Minute
CITAC	: Community on International Traceability and Analytical Chemistry
CNBF	: Dinitrobenzotrifluoride
CV	: Coefficient of Variation
Da	: Daltons
DTT	: Dithiotreitol
ECF	: Ethyl chloroformate
eV	: Electron Volts
FAO	: Food and Agriculture Organisation
FDA	: Food and Drug Administration
FLD	: Fluorescence Detector
FLR	: Fluorescence
FMOC	: Flourenylmethyloxycarbonylchloride
GABA	: Gama Amino Butyric Acid
GC-MS	: Gas Chromatography-Mass Spectrometry
GC-MSD	: Gas Chromatography-Mass Selective Detector
GC-TOFMS	: Gas Chromatography Time-of-Flight Mass Spectrometry
GUM	: Guide to Expression of Uncertainty in Measurement
HAH	: Hydrochloric Acid Hydrolysis
HCl	: Hydrochloric Acid
HPLC	: High Performance Liquid Chromatography
HSD	: Honest Significant Difference
LC-MS	: Liquid Chromatography Mass Spectrometry

LC-MS/MS : Liquid Chromatography Tandem Mass Spectrometry
LC-UV : Liquid Chromatography Ultraviolet Detector
LODs : Limits of Detection
LOQs : Limits of Quantification
LPAH : Liquid Phase Acid Hydrolysis
M : Moles per Litre
m/z : Mass to Charge Ratio
MAAH : Microwave Assisted Acid Hydrolysis
mL/min : Millilitre per Minute
mol/µL : Moles per Microliter
MPa : Mega Pascals
MS : Mass Spectrometry
MSA : Methane Sulfonic Acid
MTBSTFA : N-tert-butyldimethylsilyl-N-Methyltrifluoroacetamide
NIST : National Institute for Standards and Technology
NMISA : National Metrology Institute of South Africa
OPA : *o*-Phthaldialdehyde
PCF : Propyl chloroformate
PITC : Phenyl isothiocyanate
pmol/µL : Picomole per Microliter
ppb : Parts per billion
RP-HPLC : Reverse-Phase High-Performance Liquid Chromatography
RSD : Relative Standard Deviation
S/N : Signal to Noise Ratio
SCW : Subcritical Water
SRM : Standard Reference Material
TBNS : Trinitrobenzesulfonic acid
TFA : Trifluoracetic Acid
UHPLC : Ultra High-Performance Liquid Chromatography
UN : United Nations
UNICEF : United Nations Children's Fund
UoM : Uncertainty of Measurements
UPLC : Ultra Performance Liquid Chromatography

USA	: United States of America
(*v/v*)	: Volume by Volume
WHA	: World Health Assembly
WHO	: World Health Organisation

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Introduction

1.1. Problem Statement

1.1.1. The context of the problem

Infant formula as defined in the Food and Drug Administration Board (FDA) Infant Formula Act of 1986, "*infant formula is any food that purports to be or is represented for special dietary use solely as food for infants by reason of its simulation of human-milk or its suitability as a complete or partial substitute for human-milk*". It is therefore imperative that any food produced for its purpose as an infant formula is safe to use and closely simulates breast milk. To ensure infant food safety, the Codex Alimentarius Commission of the World Health Organisation (WHO) and the Food and Agriculture Organisations (FAO) have developed a code known as the Codex STAN 72 (Koletzko *et al.*, 2005). This code as adopted by many nations, including South Africa, puts emphasis on the importance of proper food labeling practices and specification of nutritional ingredients including protein content in an infant formula, based on the test results obtained by an accredited laboratory.

Proteins are by far one of the most important nutritional components of infant foods due to the role these macromolecules play in infant growth and development (Dave *et al.*, 2016, Honda *et al.*, 2008). For decades, food protein has been quantified using an internationally accepted reference method known as the Kjeldahl's method, or the Dumas method as an alternative to the former (Jung *et al.*, 2003a). With these methods, food protein is quantified indirectly through the analysis of total nitrogen present in the sample. For this reason, these methods cannot distinguish between proteinogenic and non-proteinogenic nitrogen that might be present in the sample and they are therefore prone to food adulteration. In China (2008) about 54000 infants and young children were diagnosed with kidney stones that were thought to be due to consumption of infant formula contaminated with melamine, a nitrogen-rich compound that was used by manufacturers to unscrupulously increase their product's protein count (Tittlemier *et al.*, 2009a). Such food adulteration related incidents have compelled international governments, including the South African government, to amend their infant food labeling regulations in a quest to protect consumers from food adulteration and to ensure that products manufactured in all trading countries meet the international standards (Ismail, 2013). In this regard, it is imperative that alternative methods to the Kjeldahl or the Dumas methods for quantifying protein in infant food be developed.

1.1.2. Research Gap

As an alternative to the primitive protein analysis methods mentioned in the preceding subsection, various other methods based on amino acid analysis stemming from the technology developed by Moore and Stein in the 20th century have been developed and validated (Jajić *et al.*, 2013, Bosch *et al.*, 2006b). Moore and Stein's method involved using cationic exchange high-performance liquid chromatography (HPLC) columns for separation followed by post-column derivatisation with ninhydrin. Despite the excellent precision and accuracy, this method has to offer, it is not convenient for modern day analysis due to its cost and time inefficiency. Additionally, numerous other HPLC based methods that use other derivatising reagents such as flourenylmethyloxycaronylchloride (FMOC), *o*-phthaldialdehyde (OPA), 6- aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC) and phenylisothiocyanate (PITC) have been developed (Bosch *et al.*, 2006b, Kwanyuen and Burton, 2010, Roth, 1971).

The major drawbacks of these methods are (1) the use of organic solvents, (2) poor stability of derivatives when PITC is used, (3) inability to derivatise both primary and secondary amino acids when OPA is used and (4) poor derivatisation performance in the presence of salts and buffers where PITC and FMOC are used (Dorresteijn *et al.*, 1996). For these reasons, there is a need for development and assessment of other analytical techniques that may serve as reliable alternatives to both nitrogen content testing and HPLC based methods. Consequently, a GC-TOFMS based method was developed in this study. Due to its peak deconvolution capabilities, GC-TOFMS offers a worthwhile opportunity for quantitative analysis of analytes in complex matrices such as infant formula. Additionally, this technique requires a significantly reduced amount of organic solvents as compared to routine HPLC methods for the analysis of amino acids.

Furthermore, the National Metrology Institute of South Africa (NMISA) under the banner of the African Food and Feed project is in a quest to produce reference materials (RM) including infant food RM to be distributed to testing laboratories for proficiency testing schemes. This material will assist in evaluating the performance of local testing facilities to ensure that they meet global standards. Therefore, NMISA needs to establish and benchmark, two independent measurements techniques for value assignment of amino acids content in infant formula reference material. The two independent techniques would be (1) the trifluoroacetic acid (TFA) hydrolysis method followed by derivatisation with methyl-N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (MTBSTFA) for GC-TOFMS analysis and (2) the HCl hydrolysis method

followed by a 6-aminoquinolyl-n-hydoxylsuccinmidyl carbamate (AQC) derivatisation for UPLC-UV analysis.

1.2. Research Aim and Objectives

The aim of this study was to develop and validate a simplified speed optimised flow rate (SOF) GC-TOFMS method to quantify 14 amino acids, namely, alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine and tyrosine in infant formula. To achieve this aim, the following objectives were established:

1. To develop, optimise and validate as far as possible a GC-TOFMS method for quantification of amino acids in infant formula that meets the specifications provided by the Association of Analytical Communities (AOAC) for development of methods for analysis of amino acids content in infant formula based on the following parameters:

1.1. To develop a method with an analytical range of between 0.4 g/100g and 2.5 g/100g to ensure that the newly developed method can quantify all targeted amino acids concentrations specified in the NIST SRM (1849-a) certificate.

1.2. To obtain limits of quantification (LOQ) ≤ 0.4 g/100g as stipulated by the AOAC (Jacobs and Feng, 2015).

1.3. To obtain analyte recoveries (accuracy) of between 80% and 120% as proposed by (Green, 1996).

1.4. To obtain a spike recovery of between 90% and 110 % as proposed by the AOAC (Jacobs and Feng, 2015).

1.5. To obtain coefficients of variation (CV) ≤ 4% as proposed by the AOAC (Jacobs and Feng, 2015).

2. To calculate the uncertainty of measurements (UoM) in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) to evaluate whether GC-TOFMS method results differ significantly from the true values specified in the NIST SRM certificate.

1.3. Steps Followed to Achieve the Objectives

- Identification of experimental variables that might affect the accuracy of the results using the cause and effect diagram developed in accordance with GUM.

- Identification of experimental factors that could affect the accuracy of the GC-TOFMS method but which the uncertainty contributions could not be quantified mathematically.
- Optimisation of derivatisation time and temperature.
- Assessment of MTBSTFA derivatives stability.
- Comparison of acetonitrile and isooctane as injection solvents to determine the most efficient solvent for the analysis of MTBSTFA derivatised amino acids using the GC-TOMS method.
- Optimisation of the SOF method.
- Validation of the external calibration and the isotope dilution quantification methods through the analysis of the NIST SRM 1849-a.
- Determination of method selectivity by (1) retention times method, (2) ion ratios and (3) NIST library match percentage (70%) method.
- Determination of measurement uncertainty using the GUM principles.
- Assessment of method transferability by comparing the results obtained from the Pegasus III GC-TOFMS system with the results obtained from the Pegasus IV GC-TOFMS system.
- Method ruggedness assessment by comparing the results obtained from the Pegasus III GC-TOFMS methods with the results obtained using the AccQ·Tag method.
- Analysis of the commercial infant formula sample.

1.4. Dissertation Overview

Chapter 2: Literature Review

This chapter describes the current trends in the analysis of amino acids with emphasis on (1) analytical techniques, (2) sample preparation and (3) the results obtained by various analytical methods.

Chapter 3: Materials and Methods

Materials and methodologies used in the preparation of samples for method optimisation studies, infant formula hydrolysates, calibration standards for both the GC-TOFMS system and the ultra-performance liquid chromatography (UPLC) system are described in detail.

Additionally, this chapter details the mathematical models used in both method development and method validation.

Chapter 4: Results and Discussion

This chapter presents and discusses the results of both method development and validation focusing on the following:

- Optimisation of derivatisation and analytical conditions for analysis of amino acids using GC-TOFMS.
- Method validation using a NIST SRM (infant/adult formula standard reference material (SRM 1849-a).
- Comparison of the GC-TOFMS method with a routine UPLC (AccQ·Tag) method.
- Method's transferability between two GC-TOFMS systems.
- Calculation of Measurement of Uncertainty.

Chapter 5: Conclusion

This chapter provides conclusions and recommendations based on the results and discussion captured in Chapter 4.

Chapter 6: Appendices

Additional results figures and tables supporting the outcomes presented and discussed in **Chapter 4** can be found in this chapter.

2. Literature Review

2.1 Introduction

2.1.1 International regulations, standards, and codes for infant formula

Proteins are by far one of the most crucial components of human nutrition, particularly for the healthy growth and development of infants (Maubois, 1984, Friedman, 1996). These macromolecules and their building blocks, namely amino acids (AAs), facilitate countless fundamental bodily functions such as cell signalling, gene expression, metabolism and synthesis of hormones and other low molecular weight nitrogen-containing compounds (Delgado-Povedano *et al.*, 2016, Fazary *et al.*, 2006, Peace and Gilani, 2005, Senden *et al.*, 1992). For food safety, quality and enhancement of fair trade between countries, efforts have been made to develop reliable methods for quantification of total protein count in food (Moore *et al.*, 2010). Depending on the analysis requirements, total food protein can be quantified indirectly through a total nitrogen content test using Kjeldahl or Dumas methods followed by conversion of nitrogen content to protein content through the application of appropriate matrix dependent protein conversion factors (Jung *et al.*, 2003b, Moore *et al.*, 2010, Simonne *et al.*, 1997). Alternatively, food protein is also quantified through amino acids analysis using the method developed by Moore and Stein in the 20th century (Csapó *et al.*, 2008, Jajic *et al.*, 2013), which involves using cationic exchange high-performance liquid chromatography (HPLC) columns for separation followed by post-column derivatisation with ninhydrin.

Breast milk and infant formula by virtue of its simulation of breast milk are the main sources of nourishment for infants and young children (Johnston, 2011). As defined in the CODEX Alimentarius standard for infant formula and formulas for special medical purposes intended for infants, *"infant formula is a breast milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants for the first months of life up to the introduction of appropriate complementary feeding"* (Commission, 2007, Sullivan *et al.*, 2015). Due to its significance as a breast milk substitute, infant formula is one of the most highly regulated foods globally (Wargo, 2016). As a result, manufacturers are required to test if their products meet the standard nutritional requirements stipulated in the CODEX Alimentarius code (STAN-72), the international code on infant formula (Sharpless *et al.*, 2010, Owens *et al.*, 2014).

The Codex Alimentarius Committee was formed in 1963 by the World Health Organisation (WHO) and the Food and Agricultural Organisation (FAO) of the United Nations (UN) to develop food standards and related documents, such as codes of practices, in an effort to protect consumers against food adulteration such as the melamine adulteration in China, 2008 (Koletzko *et al.*, 2005, Moore *et al.*, 2010, Tittlemier *et al.*, 2009b, Fodey *et al.*, 2011). After its inception in 1981, the STAN-72 code was revised in 2005 and 2007, nutritional requirements for infant formula were agreed upon based on the scientific data that was available at that time (Koletzko *et al.*, 2005). It was agreed that infant formula prepared in accordance with the manufacturer's instructions should contain 60 to 70 kcal of energy per 100 mL and between 1.8 g and 3 g of protein per 100 kcal. Following its revision, further recommendations were made regarding the minimum amounts of specific amino acids required as shown in **Table 2-1**.

Table 2-1: Recommended amino acids concentrations per 100 kcal of energy (Koletzko *et al.*, 2005)

Amino Acids	g/100 g of protein	mg/100 kcal
Cystine	2.1	38
Histidine	2.3	41
Isoleucine	5.1	92
Leucine	9.4	169
Lysine	6.3	114
Methionine	1.4	24
Phenylalanine	4.5	81
Threonine	4.3	77
Tryptophan	1.8	33
Tyrosine	4.2	75
Valine	5	90

Furthermore, in the early 1970's, the WHO and the United Nations Children's Fund (UNICEF) initiated discussions regarding the marketing, promotion, and labeling of infant formula (Shubber, 1985). This came after the UNICEF and the WHO reported that there was a notable global decline in breastfeeding which was thought to be due to improper marketing of breast milk substitutes, especially in the third world countries (Baker, 1985, Shubber, 1985). A code for marketing of breast milk substitutes as it is known today was consequently discussed at the 31st World Health Assembly (WHA) in Geneva, 1978. Following the first discussions, the first and second drafts of the code were submitted and discussed further at similar gatherings

between 1979 and 1980. The final draft of the code was unanimously accepted by governmental and nongovernmental representatives at the 34th WHA in Geneva, 1981 (Organization, 1981).

The main objective of this code was to promote breastfeeding and provision of safe and adequate nutrition for infants (Mills, 2014, Brady, 2012). Through acceptance of this code, participating governments agreed to promote breastfeeding in their countries and to regulate marketing, promotion, and labeling of infant formula (Taylor, 1998). By the end of 1997, about 17 nations had adopted almost all the code's provisions as their statutory regulations (Taylor, 1998). By virtue of its UN and WHO membership, South Africa also accepted these provisions. However, it was not until 2012 that such provisions became a statute (Mills, 2014). On the 6th of December 2012, the South African national minister of health, Dr. Aaron Mostoaledi, published regulations (FOODSTUFFS, COSMETICS AND DISINFECTANTS ACT, 1972) regarding the marketing of breast milk substitutes. The regulations prohibit the marketing of infant formula to pregnant and lactating mothers and sponsorships from manufacturers to health workers and institutions, *etcetera*. Sections 4(b) and 6 of this act compel manufacturers to provide nutritional information on their products based on results obtained through laboratory tests performed by reputable (accredited) laboratories, on their product labels.

2.1.2 Evolution of protein testing techniques

Deliberate and nondeliberate chemical adulteration of food is undoubtedly one of the oldest and biggest health concerns in food production globally (Ellis *et al.*, 2012, Sharma and Paradakar, 2010). One of the world's renowned food adulteration cases is the case of melamine adulteration in China 2008 where melamine, a nitrogen-rich chemical, was deliberately added to infant formulae products to increase their protein content (Sharma and Paradakar, 2010, Tittlemier *et al.*, 2009b, Moore *et al.*, 2010, Azad and Ahmed, 2016, Kalaiyarasan *et al.*, 2017). Prior to the melamine incident, Kjeldahl and Dumas methods were widely accepted by the AOAC as plausible methods for quantifying food protein through total nitrogen content analysis (Mariotti *et al.*, 2008). However, lack of selectivity towards proteinogenic and nonproteinogenic nitrogen made these methods vulnerable to food adulteration by melamine and other nitrogen-containing chemicals (Moore *et al.*, 2010).

Despite its high accuracy and precision, the Kjeldahl and Dumas methods are vulnerable to the use of the incorrect protein conversion factors where sample protein percentage is not clearly defined (Lynch and Barbano, 1999). Complexity, toxicity, and lengthy sample preparation have also been cited as major draw backs of the Kjeldahl method (Bruhn *et al.*, 1980, Simonne *et*

al., 1997). Using the latter, samples are digested with sulfuric acid in the presence of potassium sulfate, nitrogen, and mercury or copper catalyst (Lynch and Barbano, 1999). Sodium hydroxide is added to the mixture to liberate nitrogen in the form of ammonium sulfate. The recovered ammonia is distilled in boric acid prior to titration with a standardised hydrochloric acid (HCl) solution and the results are multiplied by an appropriate protein conversion factor, based on the matrix/foodstuff being analysed. Due to recent developments, the Dumas method, also known as the combustion method, has become the preferred method for total nitrogen analysis rather than the Kjeldahl method (Simonne *et al.*, 1997). Using the Dumas method, a sample is combusted in a furnace in the presence of oxygen which converts nitrogen to nitrogen oxides followed by reduction of the nitrogen oxides back to nitrogen and quantifying the liberated nitrogen by thermal conductivity.

Since 2008, efforts to develop standard nutrients methods that meet voluntary consensus standard performance requirements established by the AOAC to assure quality and safety of infant formulas, regardless of manufacturer or country of origin, have been made (Wargo, 2016). As a result, to date a broad scope of methods all based on amino acid analysis, ranging from high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS), automated amino acid analysis and column electrophoresis mass spectrometry, have been developed and published by various laboratories. However, with the development of new methods came new challenges such as the need for complete protein hydrolysis, sample clean-up and amino acids derivatisation optimisation as discussed in the following sections.

2.2 Protein Hydrolysis

Proteins are polymers of amino acids that are formed as a result of reactions between the amine and carboxylic groups of amino acid monomers as shown in **Figure 2-1** (Bruice, 2006). For quantitative analysis of amino acids, peptide bonds must be hydrolysed to free amino acids monomers as depicted in **Figure 2-2**. This process can be accomplished through various techniques including enzymatic, acidic, basic and recently subcritical water hydrolysis (Tsugita and Scheffler, 1982, Powell *et al.*, 2017).

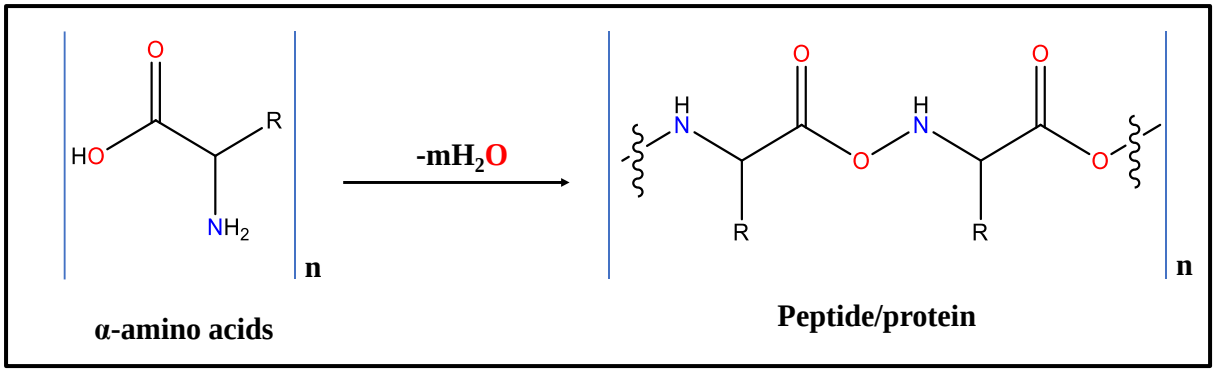


Figure 2-1: Formation of peptide bonds between amino acids to yield a protein or a peptide. n = the number of amino acid monomers, (-mH_2O) = the number of water molecules lost during formation of peptides bonds and (R) = unique functional group on the amino acid backbone (Bruice, 2006).

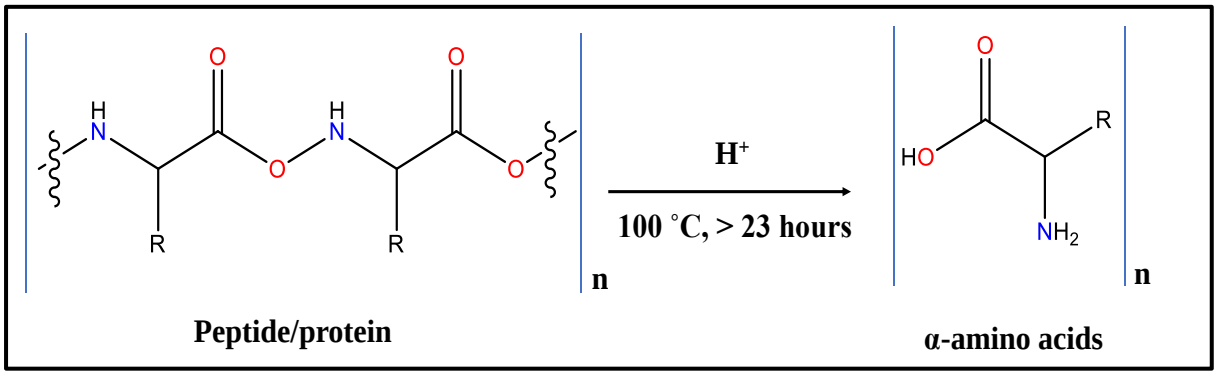


Figure 2-2: Acidic hydrolysis of peptide bonds to free amino acids monomers. (n) = the number of amino acid monomers and (R) = unique functional group on the amino acid side chains backbone (Bruice, 2006).

2.2.1 Hydrochloric acid hydrolysis

Hydrochloric acid hydrolysis (HAH) is the most frequently used hydrolysis method due to (1) simplicity, (2) convenience because HCl can be applied both in the gaseous and the liquid state; and (3) easy removal of this reagent at the end of the process (Simpson *et al.*, 1976). Typically, hydrolysis is carried out by placing a protein sample in a constantly boiling 6 M HCl at 110 ˚C for 18 to 24 hours (Tsugita and Scheffler, 1982, Simpson *et al.*, 1976). In the early days, 6 M sulfuric acid (H2SO4) was also tested as an alternative hydrolysis reagent. However, the acid removal process was long and resulted in poor amino acid (AA) recoveries (Pickering and Newton, 1990). With respect to hydrochloric acid hydrolysis, long hydrolysis periods, amino acid degradation and a lower number of samples that can be hydrolysed simultaneously were cited as the major drawbacks of the liquid phase acid hydrolysis (LPAH) technique, therefore, further improvements of this technique became imperative (Inglis *et al.*, 1971). After years of research, it was found that adding organic acids such as trifluoroacetic acid (TFA) to the

hydrolysis mixture, and the use of microwave-assisted acid hydrolysis (MAAH), could shorten the hydrolysis period significantly (Tsugita and Scheffler, 1982). Because of its ability to access hydrophobic regions of proteins, an organic acid can enhance bond cleavage and thereby reduce the hydrolysis time from 24 hours to ± 25 minutes for significantly hydrophobic proteins (Tsugita and Scheffler, 1982, Kroll *et al.*, 1998). Furthermore, a typical microwave system can operate at approximately 180 ˚C and 140 psi pressure reducing the hydrolysis period to approximately 10 minutes.

However, regardless of the improvements, progressive oxidative degradation of polar amino acids such as tyrosine, cysteine, glutamine, asparagine, methionine, serine, threonine and tryptophan remains a challenge (Blackburn, 1978). To reduce the degradation effect and to improve amino acid recoveries, modifications to the HAH method have been made (**Table 2- 2)**. The presence of carbohydrates during HAH is said to enhance degradation of cysteine and cystine to cysteic acid during the HAH procedure (Schram *et al.*, 1954). Similarly, methionine, another sulfur-containing AA, is also oxidised to methionine sulfoxide a derivative that is not stable under analytical conditions (Schram *et al.*, 1954). Therefore, to quantify cystine and methionine, protein samples have to be treated with performic acid which converts methionine, cysteine and cystine into methionine-sulfone and cysteic acid respectively as depicted in **Figures 2-3** and **2-4** (Schram *et al.*, 1954, Hirs, 1956, Moore, 1963, Bosch *et al.*, 2006b).

Alternatively, the addition of 1:2000 (*v*/*v*) mercaptoethanol to the hydrolysis mixture to convert methionine sulfoxide formed during hydrolysis back to methionine allowing methionine to be quantified in its original form has also been studied. The performic acid reagent is prepared by adding 1 mL of 30% H_2O_2 into 9 mL of formic acid. The mixture is allowed to stand at room temperature for 1 hour then cooled down to 0 ˚C (Moore, 1963). Oxidation is performed by adding 0.10 mL of AA or protein sample into a clean Pyrex ignition tube followed by addition of 2 mL of performic acid. The mixture is kept at 0 ˚C for 4 hours for oxidation of soluble proteins or overnight for less soluble proteins. At the end of the oxidation period, 0.30 mL of 48% hydrogen bromide (HBr) is added to the mixture then swirled in an ice bath to remove excess performic acid (Toran *et al.*, 1996). Alternatively, methanol can be used instead of HBr. Bromine is removed from the mixture by adding 20 mL of 1M NaOH followed by drying the sample using a rotary evaporator set to 40 ˚C for 30 min prior to acid hydrolysis (Hirs, 1956, Schram *et al.*, 1954).

Table 2-2: Acid hydrolysis modifications and other procedures used to minimise amino acid degradation during acid hydrolysis

AA	Decomposition Product	Correction	Converted Product	% Recoveries	Reference
Cysteine	Sulfo-cysteine	Performic acid oxidation	Cysteic acid	96 - 100	(Hirs, 1956, Inglis and Liu, 1970, Moore *et al.*, 2010, Schram *et al.*, 1954)
		Dithiothreitol and sodium tetrathionate	S-sulfocysteine	94 - 100	(Fountoulakis and Lahm, 1998)
		8 M urea	S-carboxymethyl cysteine	92 - 95	(Toran *et al.*, 1996)
		Methane sulfonic acid (MSA) and 3-(2-aminoethyl) indole	S-sulfocysteine, S-carboxymethylcysteine	84.6 - 95.5	(Simpson *et al.*, 1976)
Methionine	Methionine-sulfoxide	Performic acid oxidation	Methionine-sulfone	99.7 - 102	(Toran *et al.*, 1996, Hirs, 1956, Moore *et al.*, 2010)
		2-Mercapto-ethanol (0.2%)		<50	(Blackburn, 1978)
		MSA and 3-(2-aminoethyl) indole		93	(Simpson *et al.*, 1976)
		Zero-time extrapolation based on each analyte.		105	(Robel and Crane, 1972) (Downs and Pigman, 1969)
Tryptophan	Black humin	Thioglycolic acid		<50	(Simpson *et al.*, 1976)
		p-Toluenesulfonic acid		>90	(Simpson *et al.*, 1976)
		MSA and 3-(2-aminoethyl) indole		97	(Simpson *et al.*, 1976)
Threonine	Partially decomposed	Zero-time extrapolation		96	(Robel and Crane, 1972) (Downs and Pigman, 1969)
		Removing oxygen using nitrogen gas		95.3	(Bosch *et al.*, 2006b)
		MSA and 3-(2-aminoethyl) indole		99.7	(Simpson *et al.*, 1976)
Tyrosine	Partially decomposed	Phenol, thioglycolic acid		<80	(Blackburn, 1978)
		Zero-time extrapolation		97	(Robel and Crane, 1972) (Downs and Pigman, 1969)
		Removing oxygen using nitrogen gas		97.8	(Bosch *et al.*, 2006b)
		MSA and 3-(2-aminoethyl) indole		98	(Simpson *et al.*, 1976)
Serine	Partially decomposed	Zero-time extrapolation		100	(Robel and Crane, 1972) (Downs and Pigman, 1969)
		Removing oxygen using nitrogen gas		102	(Bosch *et al.*, 2006b)
		MSA and 3-(2-aminoethyl) indole		11	(Simpson *et al.*, 1976)

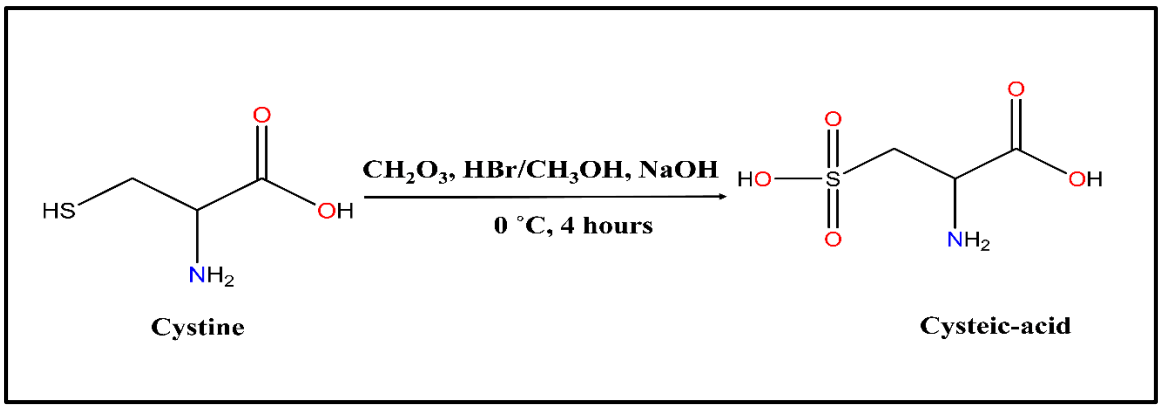


Figure 2-3: Oxidation of cysteine to cysteic acid through treatment with performic acid (CH_2O_3) (Zor *et al.*, 2015).

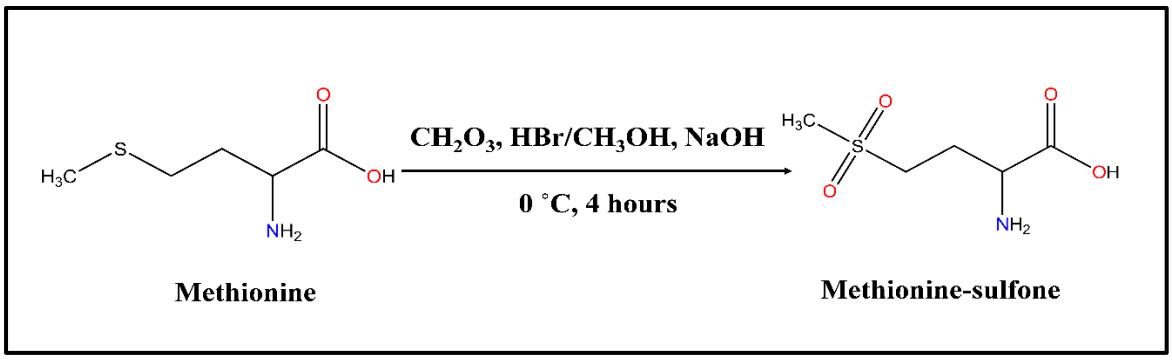


Figure 2-4: Oxidation of methionine to methionine-sulfone through treatment with performic acid (CH_2O_3) (Spindler *et al.*, 1984).

Additionally, the recoveries of methionine and cysteine, and other degradable amino acids have also been improved through the use of nonoxidative hydrolysis reagents such as methane sulfonic acid (MSA) or *p*-toluenesulfonic acid (Simpson *et al.*, 1976, Fountoulakis and Lahm, 1998, Liu and Chang, 1971). Alternatively, cysteine has also been quantified as s-sulfocysteine after it has been reduced with dithiothreitol (DTT) and excess sodium tetrathionate or as s-carboxymethyl cysteine after reduction with urea (Inglis and Liu, 1970).

During HAH, tyrosine is converted to 3-chlorotyrosine and 3-bromotyrosine due to hydrobromic acid impurities usually found in HCl (**Figure 2-5)**. The halogenation effect is thought to be enhanced in the presence of cysteine and cystine residues in the protein sample (Blackburn, 1978). Addition of 0.1% (*v/v*) mercapto acetic acid and 0.2% (*wt/v*) phenol to the hydrolysis mixture is said to improve the recoveries of tyrosine and serine (Blackburn, 1978). This procedure has also been used to improve the recoveries of cysteine and methionine. Alternatively, recoveries of tyrosine, threonine, and serine have also been improved by correcting the losses using the zero-time extrapolation method (Downs and Pigman, 1969,

Robel and Crane, 1972). This is achieved by hydrolysing protein samples at different time intervals while recording each AA recovery at each interval followed by extrapolation of amino acids concentration to zero time.

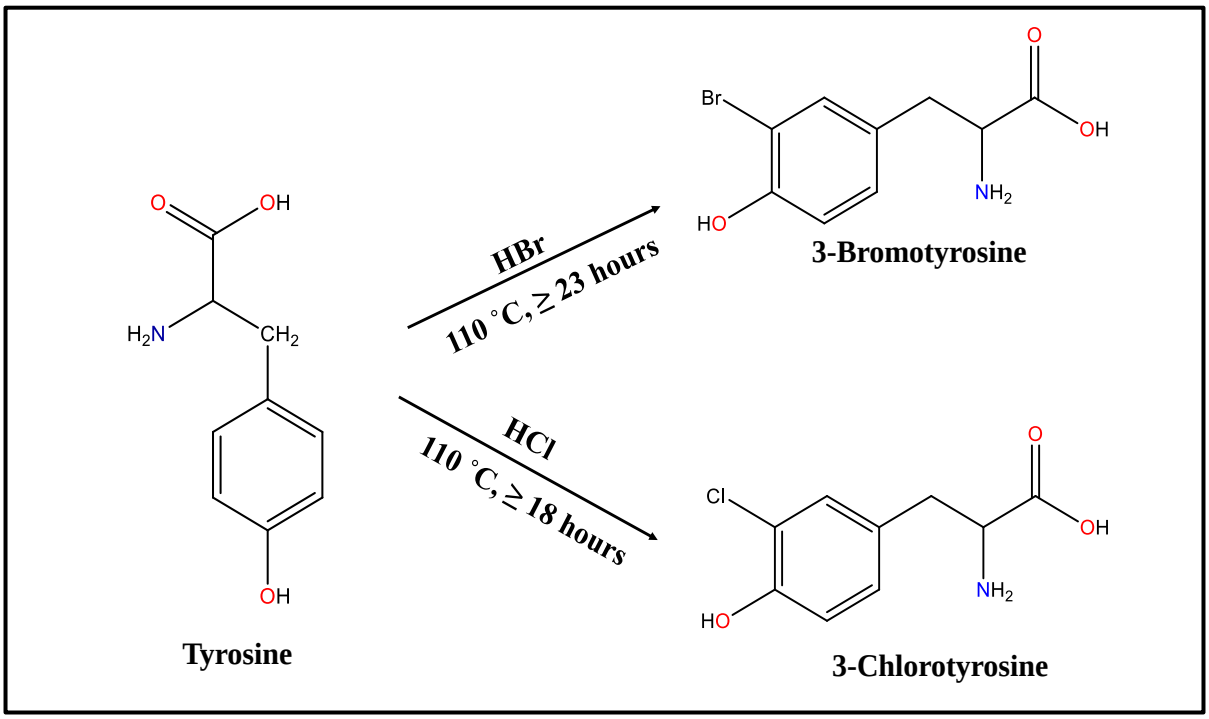


Figure 2-5: Schematic representation of the halogenation of tyrosine during hydrochloric acid hydrolysis (Blackburn, 1978)

2.2.2 Sodium hydroxide alkaline hydrolysis

Unlike other polar amino acids that are partially degraded during HAH, tryptophan, on the other hand, is completely destroyed during this process (Allred and MacDonald, 1987, Holm and Gortner, 1920, Matsubara and Sasaki, 1969, Miller, 1967). Consequently, a unique AOAC accredited alkaline hydrolysis method is required for determination of tryptophan in protein. Due to the inconvenience that comes with the need for an entirely separate hydrolysis method, tryptophan is often omitted from amino acid analysis data of protein in many instances (Yust *et al.*, 2004). Alkaline hydrolysis is carried out by dissolving a protein sample in 4 M NaOH in an evacuated hydrolysis tube followed by incubation at 100 ˚C for 4 hours. Hydrolysates are cooled in ice then neutralised to pH 7 with 2 M HCl followed dilution with a 1 M borate buffer and analysis by HPLC (Allred and MacDonald, 1987). Alternatively, tryptophan has also been determined through modification of the HAH method by adding reagents that limit degradation

such as mercaptoethanol, tryptamine, thioglycolic acid and phenol (Allred and MacDonald, 1987, Muramoto and Kamiya, 1990). Although these reagents do offer some improvements, their effectiveness depends on protein sample water solubility and the amount of tyrosine in the protein sample (Hanko and Rohrer, 2002). The higher the content of tyrosine in the protein sample the less effective these reagents become.

2.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis was routinely used in the production of protein hydrolysates of infant formulas for individuals who cannot digest intact proteins (Mahmoud *et al.*, 1992, Tello *et al.*, 1994, Society *et al.*, 1955). This method is renowned for its effective quantification of tryptophan in protein which is seemingly unmanageable by means of traditional acid hydrolysis (Edelhoch, 1967). Additionally, this method is regarded as very useful because it allows quantification of proteins using spectroscopic methods and derivatising reagents that are still used today including *o*-phthaldialdehyde (OPA) and trinitrobenzene sulfonic acid (TBNS) (Spellman *et al.*, 2003). Typically, enzymatic hydrolysates are prepared by hydrating the protein sample at room temperature for 1 hour followed by adjustment of pH to (7.0) with 2 M sodium hydroxide prior to addition of the enzyme and substrate (Spellman *et al.*, 2003). Some of the drawbacks of the enzymatic hydrolysis methods are (1) unavailability of a consensus method for the determination of the degree of hydrolysis, (2) the degree of hydrolysis depends on the enzyme used in the hydrolysis process and (3) assumptions that analyte responses are similar when OPA is used to determine the degree of hydrolysis (Rutherfurd, 2010).

2.2.4 Subcritical water hydrolysis

In recent years it has been shown that subcritical water (SWC) may be used for both protein extraction and hydrolysis equivalent to enzymatic hydrolysis (Powell *et al.*, 2017, Martínez-Maqueda *et al.*, 2013). Subcritical water is considered a greener solvent that predominantly exists at a temperature range of between 100 and 374 ˚C and at a pressure of 22.06 MPa. Under these conditions, water exists as hydronium (H_3O^+) and hydroxide (OH^-) ions, a property that allows water to act as both an acid and a base (Powell *et al.*, 2016). Using this method, water-soluble protein bovine serum albumin was reportedly converted to both amino and organic acids during a prolonged exposure to subcritical water in the temperature range between 275 and 300 ˚C (Abdelmoez and Yoshida, 2013).

2.3 Derivatisation of Amino Acids

Another challenge that arose with the development of new amino acid-based protein analysis methods was the need for derivatisation of amino acids to improve their detectability and separation. Over the years, numerous methods for both qualitative and quantitative analysis of amino acids in various matrices have been developed. Most of these methods are based on chromatographic separation of amino acids followed by detection using a number of detectors including mass spectrometry (MS), fluorescence detector (FLD) and ultraviolet (UV) detector. In almost all cases a detector dependent derivatisation step is mandatory to improve physical properties of amino acids for effective detection (Peace and Gilani, 2005, Orata, 2012). For GC-MS analysis, amino acids are derivatised to improve volatility, while for HPLC-UV/FLD amino acids are derivatised to improve photometric properties (see **subsections 2.3.1.** and **2.3.2** for further discussions on derivatisation procedures**)** (Orata, 2012).

2.3.1 Derivatisation for HPLC analysis

Analysis of amino acids by HPLC-UV/FLD requires AAs to be derivatised, either by means of pre-column or post-column derivatisation, to improve the light absorption properties of the analytes (Li *et al.*, 2012). The earlier AOAC accredited method for quantification of amino acids, which involved separation of amino acids by ion-exchange chromatography followed by post-column derivatisation with ninhydrin, was initially the go-to method for quantification of AAs in different matrices (Bosch *et al.*, 2006b). Although this method is reliable, lengthy analysis time or inefficiency remains a major drawback of this method (Sarwar and Botting, 1993, Bütikofer *et al.*, 1991).

Due to a growing need for better sensitivity and time efficiency, analytical methods based on reverse-phase high-performance liquid chromatography (RP-HPLC) with pre-column derivatisation have been developed (Li *et al.*, 2012, Aoyama *et al.*, 2004, Peace and Gilani, 2005). For this purpose, a range of frequently used derivatising reagents includes phenylisothiocyanate (PITC), *o*-phthaldialdehyde (OPA), dimethylaminonaphthalensulphonyl chloride (Dansyl-Cl), 9-fluorenylmethyl-chloroformate (FMOC) and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Bosch *et al.*, 2006b, Liu *et al.*, 1995, Li *et al.*, 2012). Additionally, derivatisation of amino acids by 4-chloro-3,5-dinitrobenzotrifluoride (CNBF)

has also been reported (Li *et al.*, 2012, Peace and Gilani, 2005, Senden *et al.*, 1992, Petritis *et al.*, 2002).

To prepare OPA and FMOC amino acids derivatives, a dilute protein sample hydrolysate is placed in a borate buffer at pH of 9.5 followed by addition of 2-mercaptoethanol prior to addition of OPA into the mixture and the later addition of FMOC inside the autosampler (Jørgensen and Jensen, 1997, Noctor *et al.*, 2007). OPA only reacts with primary amino acids while FMOC reacts with secondary amino acids as shown as in **Figure 2-6** and **Figure 2-7** respectively.

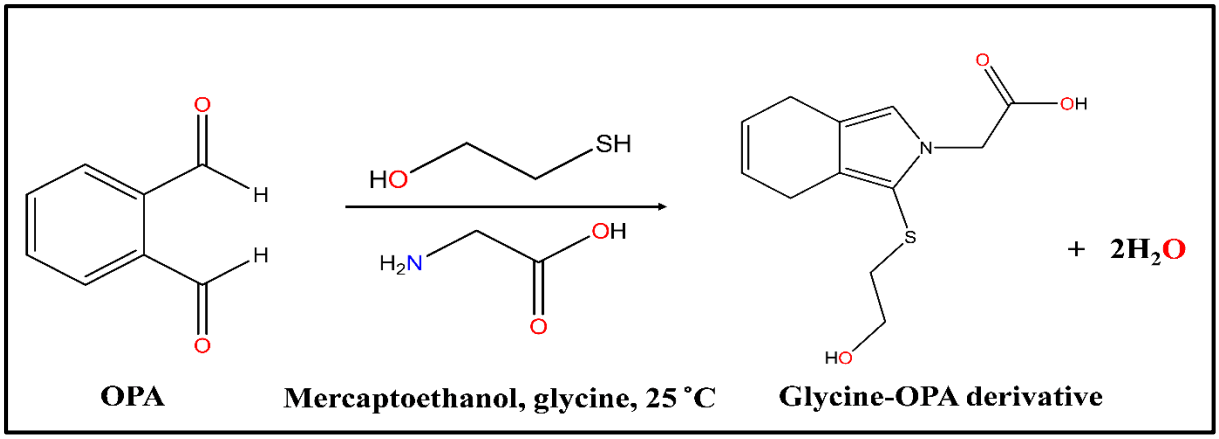


Figure 2-6: Schematic representation of the derivatisation of primary amino acid glycine with *o*-phthaldialdehyde (OPA) (Kim *et al.*, 2011)

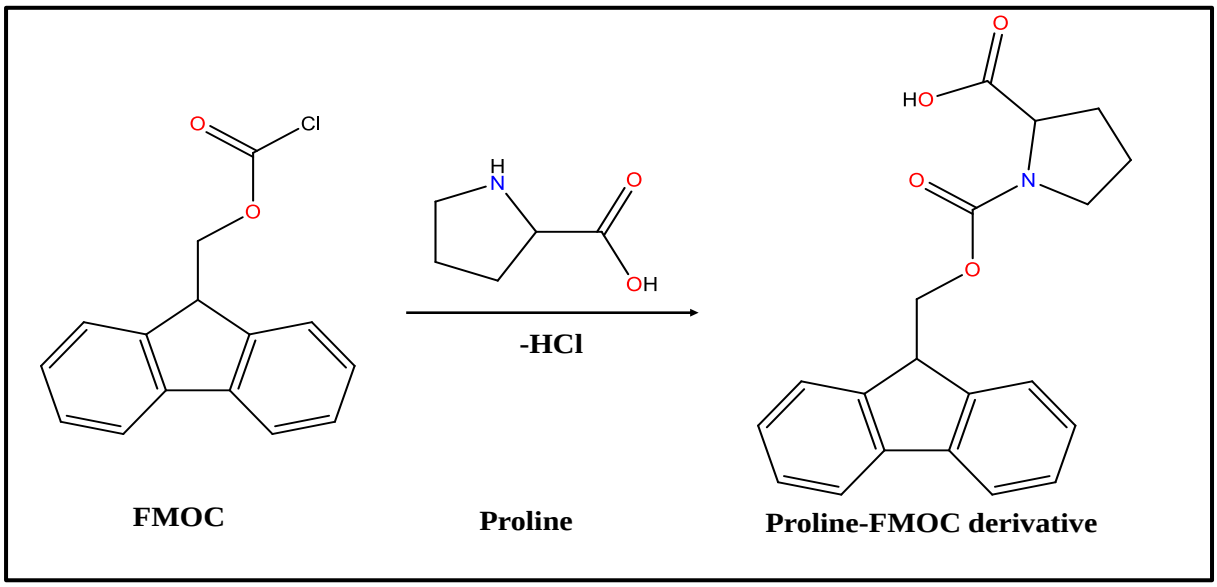


Figure 2-7: Schematic representation of the derivatisation of the secondary amino acid proline with 9-fluorenylmethyl-chloroformate (FMOC) (Fradi *et al.*, 2014).

Contrary to OPA/FMOC reacting with either primary or secondary AAs, PITC, on the other hand, reacts well with both primary and secondary amino acids as depicted in **Figure 2-8** (Kim *et al.*, 2011). To prepare PITC amino acids derivatives, a hydrolysed protein sample is placed into a mixture containing methanol (70% (*v/v*)), triethylamine (10% (*v/v*)), PITC (10% (*v/v*)) and water (10% (*v/v*)). The sample is stored at room temperature for 15 minutes prior to analysis by HPLC-UV. In recent years AQC derivatisation of amino acids has gained popularity due to (1) simplicity of the derivatisation procedure, (2) strong fluorescence and UV signals; and (3) relatively better stability of AQC derivatives (Pappa-Louisi *et al.*, 2007, Cohen and Strydom, 1988). Like PITC, AQC reacts with both primary and secondary amino acids as shown in **Figure 2-9**.

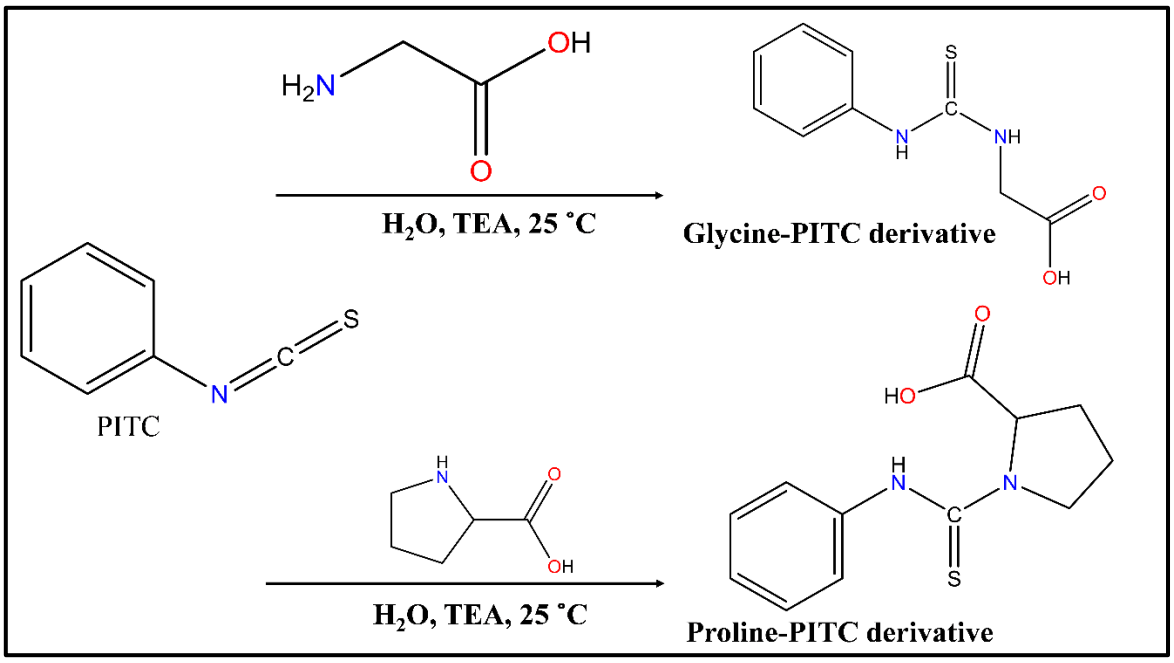


Figure 2-8: Schematic representation of the derivatisation of glycine with phenylisothiocyanate (PITC) (Kim *et al.*, 2011)

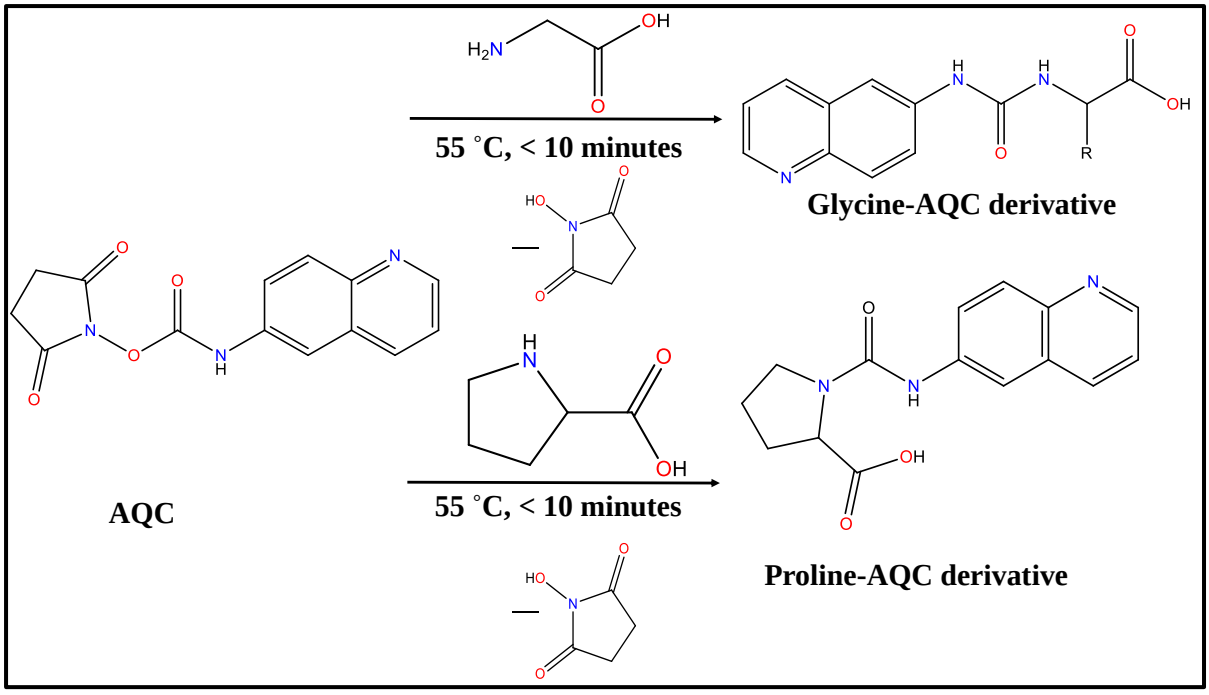


Figure 2-9: Schematic representation of derivatisation of glycine with 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) (Okamoto *et al.*, 2016)

For AQC derivatisation a Waters Corporation procedure is commonly used. With this procedure, 10 µL of a protein hydrolysate is added into a mixture containing 70 µL of borate buffer and 20 µL of AQC derivatising reagent. The mixture is heated to 55 ˚C for not more than 10 minutes and then analysed by HPLC (Bosch *et al.*, 2006b, Boogers *et al.*, 2008, Fiechter and Mayer, 2011, Fiechter *et al.*, 2013).

2.3.2 Disadvantages of HPLC derivatising reagents

Despite the usefulness of the above reagents, each has its own drawbacks, e.g. OPA only reacts with primary amino acids to form relatively unstable derivatives (Roth, 1971). To overcome this challenge, FMOC which reacts with secondary AAs has to be added to the derivatisation mixture after primary amino acids have been derivatised with OPA (Jámbor and Molnár-Perl, 2009). The biggest drawback with this procedure is that the unreacted FMOC is not removed from the sample prior to injection and therefore it may behave as a chromatographic interference during the analysis (Kwanyuen and Burton, 2009). In contrast to OPA or FMOC reacting with either primary or secondary amino acids, PITC reacts well with both primary and secondary amino acids and hence this method can be used as an alternative for the OPA/FMOC

method discussed previously (Gilani *et al.*, 2008, Boogers *et al.*, 2008). However, a longer derivatisation period and poor stability of PITC derivatives are the main disadvantages of the PITC method (Bosch *et al.*, 2006b). Another major shortfall of PITC derivatisation is the need for removal of the excess reagent at the end of the derivatisation because unreacted PITC may become a chromatographic interference during the analysis (Fiechter and Mayer, 2011).

2.3.3 Derivatisation for GC-MS analysis

One of the advantages of GC-MS over its counterparts such as LC-UV, lies in the availability of comprehensive analyte mass spectral databases (e.g. NIST library) that serve as a source of reference for analysts, making analyte identification simpler compared to other systems (Kaspar *et al.*, 2008). Additionally, GC-MS analysis requires a significantly reduced amount of organic solvent compared to HPLC. However, irrespective of these advantages, for an effective analysis of amino acids using GC-MS, amino acids must be derivatised to improve their volatility (Mudiam and Ratnasekhar, 2013, Husek and Simek, 2006). To accomplish this task, various reagents have been employed including reagents such as trimethyl silyl (TMS), pentafluoropropyl anhydride/isopropanol, trifluoroacetic anhydride/isopropanol *etcetera*.

When amino acids have to be derivatised directly in an aqueous medium, non-hygroscopic alkyl chloroformates, mainly ethyl chloroformate (ECF), is used as a derivatising reagent (Kaspar *et al.*, 2008, Wang *et al.*, 1994). To prepare ECF derivatives, aliquots of protein hydrolysates are transferred into clean reaction vessels followed by addition of ethanol and pyridine prior to addition of ECF (Qiu *et al.*, 2007, Mudiam *et al.*, 2012, Mudiam and Ratnasekhar, 2013). This reaction yields ECF amino acid derivatives as shown in **Figure 2-10**. Alternatively, silylating reagents are generally used for derivatisation of amino acids for GC-MS analysis. The biggest drawbacks of silylating reagents are that (1) they require longer derivatisation periods and (2) they are highly hygroscopic and therefore derivatisation has to be performed under anhydrous conditions (Kaspar *et al.*, 2008, Wang *et al.*, 1994, Mudiam and Ratnasekhar, 2013). Most commonly used silylating reagents include trimethylsilane (TMS), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) with BSTFA and TMS procedures being the primitive derivatisation techniques (Gehrke and Leimer, 1971). To prepare MTBSTFA derivatives, equivalent volumes of acetonitrile and MTBSTFA are added to the dried protein sample followed by derivatisation at 100 ˚C for 60 minutes (Jiménez-Martín *et al.*, 2012, Pérez-

Palacios *et al.*, 2014). From this reaction, amino acids are derivatised as shown in **Figure 2-11** (Mandalakis *et al.*, 2010).

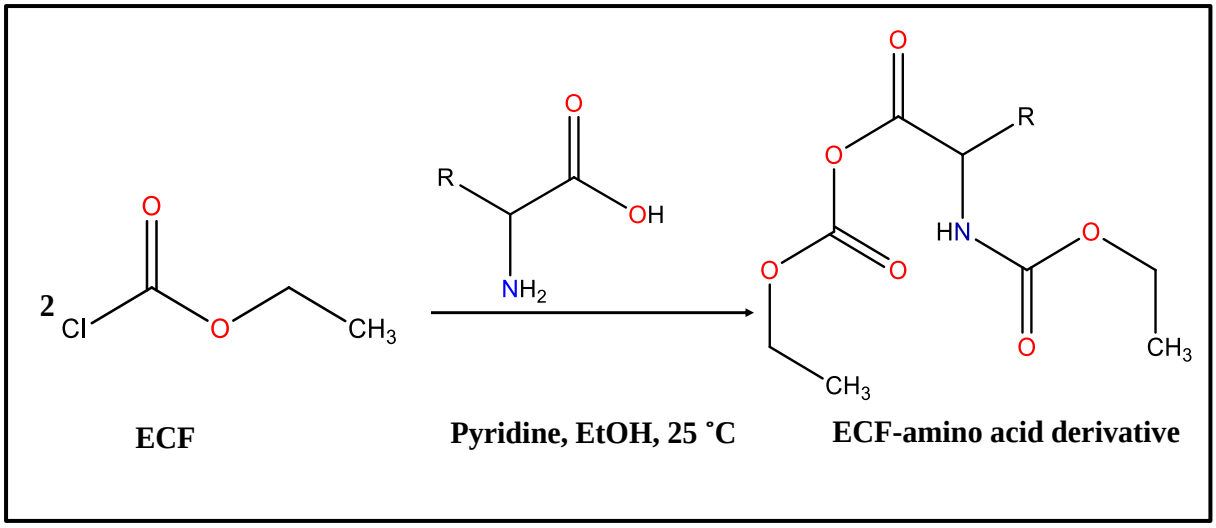


Figure 2-10: Schematic representation of the derivatisation of amino acids with ethyl chloroformate (ECF) (Mudiam *et al.*, 2012)

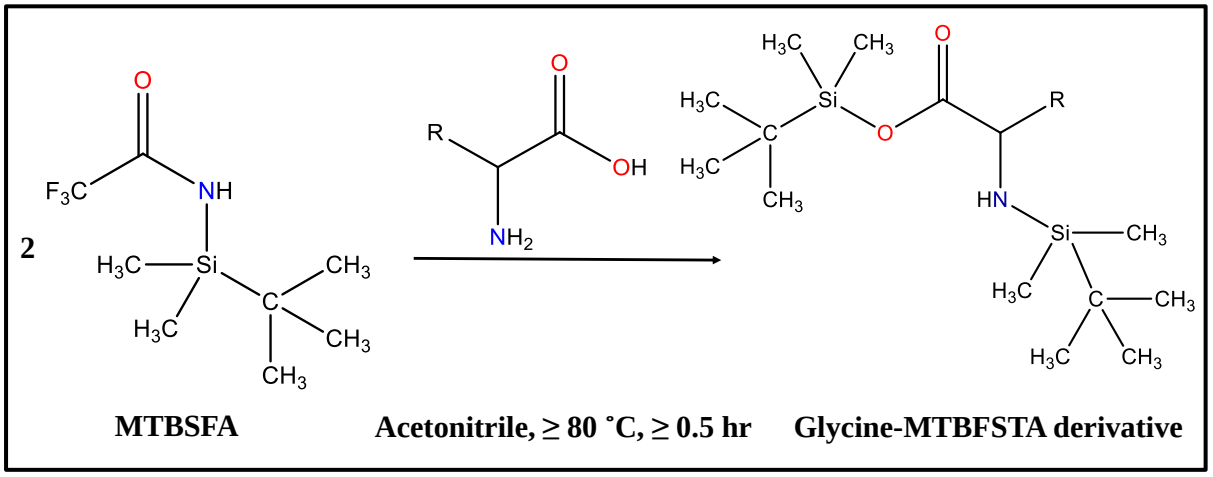


Figure 2-11: Schematic representation of the derivatisation of amino acids with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) (Mandalakis *et al.*, 2010)

2.3.4 Disadvantages of GC-MS derivatising reagents

Although alkylation is ideal for derivatisation of analytes for GC-MS analysis, alkylating reagents such as ECF are, however, generally toxic and their procedures sometimes require harsh reaction conditions (Orata, 2012). Similarly, BSTFA requires harsher reaction conditions

compared to MTBSTFA (Sobolevsky *et al.*, 2003). Furthermore, TMS and BSTFA derivatives are reportedly unstable and the extent of derivatisation of amino acids was found to be inconsistent when TMS was used as the derivatising reagent (Quéro *et al.*, 2014, Stalling *et al.*, 1968). Although MTBSTFA derivatives are reportedly 10 000 times more stable than TMS derivatives, some amino acids including arginine are reportedly not stable under MTBSTFA derivatisation conditions (Biermann *et al.*, 1986, Orata, 2012, Kaspar *et al.*, 2008).

2.4 Amino Acid Derivatives Detection and Quantification

Derivatisation is generally used to render amino acids both chromatographically separable and detectable. The choice of the derivatising reagent utilised depends on the type of detection being used, e.g. ninhydrin derivatives of amino acids would be detected by UV detection and OPA derivatives by FLD. Additionally, the type of detection and the derivatisation procedure would have different method validation parameters including selectivity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), linearity, reproducibility, and repeatability of results as shown in **Table 2-3** and **Table 2-4** (Diaz *et al.*, 1996).

2.4.1 HPLC detection and quantification of amino acids

Using AQC as the derivatising reagent and UV or FLD detection can produce LODs and LOQs in the picomole region as shown in **Table 2-3** (Bosch *et al.*, 2006b). However, the AQC LODs obtained under UV detection are lower than the LODs obtained with the same reagent with FLR detection (Bosch *et al.*, 2006b, Cohen and Strydom, 1988, Fiechter and Mayer, 2011, Pérez-Palacios *et al.*, 2014). As seen in **Table 2-3**, UHPLC-UV, HPLC-UV, and UPLC-UV can detect AQC derivatised AAs at an on-column concentration $\geq$ 0.08 pmol a LOD that is 10 times lower than the LOD obtainable by HPLC-FLD with the same derivatising reagent. Unlike the AQC derivatives with LODs in the femtomole (fmol) region, the LODs of OPA and FMOC with FLR detection are in the pmol region as shown in **Table 2-3** (Zheng *et al.*, 2017, Jing *et al.*, 2016). Furthermore, AQC LODs are100 times lower than the LODs obtained with PITC and dansylchloride as derivatising reagents.

Table 2-3: Comparison of method validation parameters obtained by different LC detectors and derivatising reagents used for amino acid derivatisation

Detector	Derivatising Reagent	LOD	LOQ	CV	r^2	References
HPLC-FLD	AQC	± 0.015 µM	± 0.17 µM	± 2.24	± 0.992	(Bosch *et al.*, 2006b)
HPLC-UV	AQC	± 180.52 fmol	Not reported	± 0.86	± 0.9994	(Cohen and Strydom, 1988)
UPLC-UV	AQC	± 0.421 pmol	± 1.21 pmol	Not reported	Not reported	(Fiechter and Mayer, 2011)
UHPLC-UV	AQC	± 0.080 pmol	± 0.80 pmol	± 3.57	Not reported	(Karger *et al.*, 1974)
LC/APCI-MS	Not Used	± 0.1 µg/mL	Not reported	<4.5	>0.99	(Zheng *et al.*, 2017)
LC-MS/MS	Not Used	± 9.56 ng/mL	Not reported	Not reported	± 0.999	(Fürst *et al.*, 1990)
LC-MS/MS	Not Used	± 1.7 fmol	± 5.6 fmol	± 2.5	± 0.9932	(Tuberoso *et al.*, 2015)
LC-MS/MS	N-phosphorylation	± 0.001 µM	± 0.003 µM	± 2.3	± 0.9930	(Özcan and Şenyuva, 2006)
HPLC-FLD	FMOC and OPA	± 0.12 µM	± 0.64 µM	± 6.35	± 0.9989	(Özcan and Şenyuva, 2006)
HPLC-FLD	FMOC and OPA	± 0.8 pmol	Not reported	± 2.2	Not reported	(Buiarelli *et al.*, 2013)
HPLC-UV	PITC	± 5.0 pmol	Not reported	± 1.9	Not report	(Buiarelli *et al.*, 2013)
HPLC-FLD	Dansyl-chloride	± 0.01 mg/L	± 0.1 mg/L	Not reported	0.9925	(Gao *et al.*, 2016)

LOD = Limit of detection, LOQ = limit of quantification, CV = coefficient of variation and (r^2) = squared correlation coefficient

On the other hand, liquid chromatography mass spectrometry systems (LC-MS) have been used to quantify underivatised amino acids (Özcan and Şenyuva, 2006). Since the derivatisation step is omitted, the total analysis time is shortened which is a big advantage compared to traditional methods (Buiarelli *et al.*, 2013). At a LOD of ± 1.7 fmol on column analyte concentration, LC-MS/MS systems can detect amino acids at a much lower concentration compared to all other systems presented in **Table 2-3**. Like the LODs, the coefficients of variation and correlation coefficients values obtained by different systems vary depending on the detection method and the derivatisation procedure used as seen in **Table 2-3**. This observation confirms the dependency of method validation parameters on the detector and the derivatising reagent used.

2.4.2. GC-MS detection and quantification of amino acids derivatives

In **subsection 2.3.3** derivatising reagents commonly used in the GC-MS analysis of amino acids were briefly discussed focusing mostly on silylating and alkylating reagents such as MTBSTFA and ECF respectively. As with liquid chromatography, GC-MS LODs of AAs depends largely on the derivatising reagent and the method of detection used during the analysis as shown in **Table 2-4**. Using propyl-chloroformate (PCF) as a derivatising reagent followed by GC-MSD analysis yields the lowest LOD compared to any of the derivatisation reagents presented in **Table 2-4** (Kaspar *et al.*, 2008). Typical LODs of AAs obtained from a GC-MSD system with PCF as the derivatising reagents are ± 10 times lower than the LOD obtained by a GC-TOFMS system with methoxyamine as the derivatising reagent. On the other hand, the LOD of methoxyamine derivatised AAs as seen in **Table 2-4** is ± 2 times lower than the LOD obtained by GC-MS with MTBSTFA as the derivatising reagent. Furthermore, the LODs of BSTFA, Isobutylcarboxy isobutyl ester and TMS obtained by GC-MS systems are about 100, 200 and 1000 times higher than the LODs obtained from a GC-MS system using MTBSTFA as a derivatising reagent as seen in **Table 2-4**.

Table 2-4: Comparison of method validation parameters obtained by GC-MS using various detectors and various derivatising reagents

Detector	Derivatising Reagent	LOD	LOQ	CV	r^2	References
GC-MSD	PCF	± 3 µM	Not reported	± 2.86	± 0.9987	(Kaspar *et al.*, 2008)
GC-MS (single quadrupole)	MTBSTFA	Not reported	± 0.15 mg/100g	± 12.00	± 0.9983	(Jiménez-Martín *et al.*, 2012, Roessner *et al.*, 2000)
GC-MS (single quadrupole)	MTBSTFA	± 2 pg/µL	Not reported	Not reported	Not reported	(Sobolevsky *et al.*, 2003)
GC-MS (single quadrupole)	BSTFA	± 0.1 ng/µL	Not reported	Not reported	Not reported	(Sobolevsky *et al.*, 2003)
GC-MS (single quadrupole)	Isobutylcarboxy isobutyl esters	± 0.2 ng/µL	Not reported	Not reported	Not reported	(Sobolevsky *et al.*, 2003)
GC-MS (single quadrupole)	TMS	± 1 ng/µL	Not reported	± 6.0	Not reported	(Sobolevsky *et al.*, 2003, Roessner *et al.*, 2000)
GC-TOFMS	Methoxyamine	± 18 µM	± 65 µM	± 6.44	± 0.9507	(Noctor *et al.*, 2007, Carrasco-Pancorbo *et al.*, 2009)

LOD = Limit of detection, LOQ = limit of quantification, CV = coefficient of variation and r^2 = squared correlation coefficient

2.5 Conclusion

Based on comparability of method validation parameters obtained by LC and GCMS including LODs, CV and correlation coefficients as seen in **Table 2-3** and **Table 2-4**, it is evident that GC-MS may indeed be a plausible alternative to the LC techniques for quantification of amino acids, particularly in complex matrices such as infant food, which has not been fully explored. There are currently very few reports on the analysis of amino acids by GC-MS, particularly in the area of food analysis, although GC-MS has proven to be excellent in the analysis of small organic compounds in the field of metabolomics (Gehrke and Leimer, 1971, Quéro *et al.*, 2014). Additionally, there are even fewer reports on the application of GC-TOFMS in the quantification of amino acids in food matrices despite its numerous advantages (Noctor *et al.*, 2007, Carrasco-Pancorbo *et al.*, 2009). For these reasons, a GC-TOFMS method for quantifying 14 (of the 20 naturally occurring AAs) amino acids, namely, alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine and tyrosine was developed in this study as an alternative to the routine AccQ·Tag UPLC method.

3. Materials and Methods

3.1 Reagents

An amino acid standard (AAS18) containing 17 of the 20 naturally occurring AAs (excluding glutamine, asparagine, and tryptophan) viz. alanine, glycine, valine, leucine, isoleucine, proline, serine, methionine, threonine, phenylalanine, aspartic acid, glutamic acid, arginine, lysine, histidine, tyrosine and cystine; N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), sodium hydroxide, phenol and tryptamine (indole), were sourced from Sigma-Aldrich (Riedstraße 2, 89555 Steinhem Albuch, Germany) and were used to prepare calibration standards for GC-TOFMS and UPLC. L-valine ($^{13}C_5$, ^{15}N), L-isoleucine ($^{13}C_6$), L- phenylalanine ($^{13}C_1$) and L-proline ($^{13}C_5$) ^{13}C-labelled amino acid isotopes used to prepare internal standard solutions for GC-TOFMS analysis were obtained from Cambridge Isotope Laboratories (3 Highwood Drive, Tewksbury, Massachusetts, United States of America (USA)). L-cysteic acid and L-methionine sulfone were sourced from Sigma-Aldrich. Although methionine sulfone and cysteic acid were among the reagents used in this study, these analytes were not quantified as they require an alternative sample preparation procedure (performic acid oxidation) to be carried out prior to the hydrolysis process. Therefore, these reagents were only used during the optimisation of the SOF method to ensure that these analytes are adequately resolved from other amino acids during the analysis.

UV grade acetonitrile and iso-octane used to test optimum sample and standards reconstitution solvent conditions for GC-TOFMS analysis were obtained from Romil LTD (Convert Dr, Waterbeach, Cambridge CB25 9QT, United Kingdom) and Honeywell – Burdick & Jackson (1953 Harvey Street, Muskegon, Michigan 49442, USA) respectively. National Institute for Standards and Technology (NIST) infant formula standard reference material (1849-a) containing 18 amino acids viz. alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine and valine used for GC-TOFMS method validation and UPLC robustness tests was sourced from NIST (100 Bureau Drive, Gaithersburg, Maryland, USA). One commercial infant formula sample used to assess the newly developed GC-TOFMS method was sourced from a local retailer (Glenfair Shopping Centre, Pretoria, South Africa (SA)). Hydrochloric acid (HCl) used for hydrolysis was sourced from Merck Chemicals (Feldbergstraße 80, 64293 Darmstadt, Germany). Trifluoroacetic acid (TFA), hydrogen peroxide and formic acid were sourced from Sigma-Aldrich.

The Waters AccQ·Tag amino acid analysis kit which included an amino acid standard containing the same 17 amino acids viz. alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine and derivatising reagents described only as AccQ·Tag reagent powder (2A) and reagent (2B); and HPLC eluents described only as eluent A (aqueous) and eluent B (organic) was sourced from Waters Corporation (34 Maple St, Milford, Massachusetts 01757, USA).

3.2 Sample Preparation

3.2.1 Preparation of samples for optimisation of derivatisation conditions

Experimental factors identified as those that could affect the accuracy and the uncertainty of measurements but for which uncertainty contributions may not be quantified mathematically due to complexity were optimised experimentally. These include derivatisation period; derivatisation temperature; sample reconstitution solvent type and sample stability conditions. To optimise derivatisation time and temperature, 80 µL aliquot of the AAS18 amino acid standard was transferred into a clean 1.5 mL vial and diluted to 800 µL with 0.1 M HCl. From the latter, 75 x 80 µL aliquots were transferred into clean 1.5 mL vials and dried under nitrogen at 35 ˚C. Into each of the dried sample, 20 µL of MTBSTFA and 80 µL of acetonitrile were added. The samples were sealed, vortexed and derivatised at 75 and 100 ˚C for 0.5, 1, 2.5, 4 and 6 hours. At the end of each derivatisation period, a set of 5 samples from both levels of temperature were transferred into 1.5 mL vials fitted with 250 µL glass inserts and analysed on the GC-TOFMS system. At the end of the analysis, a two-way ANOVA was used to identify significant differences across the levels of both derivatisation temperature and time.

To determine MTBSTFA derivatives stability, two sets of samples derivatised at 100 ˚C for 4 and 6 hours were stored under different environmental conditions. The 4-hour set of samples was stored in the refrigerator at 3 ˚C while the 6-hour set of samples was stored at ambient temperature. Each set of samples was analysed over a period of 5 days. At the end of the analysis period, a one-way ANOVA in conjunction with a Tukey honest significant difference (HSD) test was used to test for significant differences between the daily means of each set of samples (**NB** not across the sets) by applying **equations 3.3.5** and **3.3.6** for ANOVA and Tukey test respectively. To determine a plausible solvent for GC-MS analysis of MTBSTFA amino acids derivatives, acetonitrile and isooctane as commonly used reconstitution solvents in

similar studies were compared. To perform this test, samples were prepared as previously described by diluting 80 µL of AAS18 to 800 µL using 0.1 M HCl. From the latter, 10 x 80 µL aliquots were dried under the same conditions as described previously. Into each of the dried samples, 20 µL of MTBSTFA was added followed by addition of 80 µL of acetonitrile into a set of 5 samples and 80 µL of isooctane on the second set of samples. The samples were sealed, vortexed and derivatised at 100 ˚C for 4 hours then transferred into 1.5 mL vials fitted with 250 µL glass vials and analysed on the GC-TOFMS system. At the end of the analysis, the F-test and the student's t-test were used to test for significant differences between the variances and between sample means.

3.2.2 Preparation of hydrolysates for GC-TOFMS and UPLC analysis

All weighed standards and samples were weighed either on a Mettler Toledo AX 26 Delta range or Mettler Toledo XPE 205 mass balances both from Mettler-Toledo LLC (1900 Polaris Parkway, Columbus, Ohio 43240). Infant formula samples were hydrolysed in 18 mL Corning glass tubes heated on a Reactitherm III #TS018823 heating block from Thermo Fisher (168 Third Avenue, Waltham, Massachusetts 02451, USA). Post hydrolysis, unexpended HCl was removed by drying the hydrolysates on the Genevac EZ-2 plus system from Genevac Inc (SP Industries, 815 State route, 208 Gardiner, New York, USA). Hydrolysates were cleaned using Chrompure PTFE/L 0.22 µm syringe filters attached to disposable syringes which were sourced from Membrane Solutions (11088 Spring View Lane, Texas 75075, USA) and S-S Disposable Syringes (Shinwon Building 6th floor, 21 Teheran Road, 8-gil, Gangnam-gu Seoul, 135 – 935, South Korea) respectively.

To transfer reconstituted milk samples into Corning hydrolysis tubes before hydrolysis and filtered hydrolysates into 1.5 mL Restek screw cap vials sourced from Restek Corporation (110 Benner Circle, Bellefonte, Pennsylvania 16823, United States of America) after sample clean-up, gas-tight glass syringes from Hamilton Robotics (Via Crusch CH-7402 Bonanduz, Glarus, Switzerland) were used. Hydrolysate stock solutions used to prepare LC analytical samples were prepared in 15 mL Supelco screw top amber vials sourced from Sigma-Aldrich. In both GC and LC, injection samples were prepared in 1.5 mL screw cap vials fitted with 250 µL glass inserts from Macherey-Nage GmbH & Co. KG, Neumann-Neander street, 6 – 8, 52355, Düren, Germany).

For GC-TOFMS method validation, hydrolysates were prepared by (1) using the traditional HCl acid hydrolysis method as cited in various literature reports; and (2) modifying the traditional method by adding into the mixture, 50% *(v/v)* of TFA prior to purging samples with nitrogen as depicted in **Figure 3.1** (Simpson *et al.*, 1976, Tsugita and Scheffler, 1982). For LC method validation, the HCl hydrolysis method was utilised. To prepare NIST SRM hydrolysates, 1.2 g of SRM sample was dissolved in 12 mL of deionised water and homogenised on an orbital shaker at 145 rpm for 10 minutes. From the homogenised sample, 10 x 1 mL aliquots were transferred into Corning glass tubes and the content of each tube was dried overnight under nitrogen at 35 ˚C. Into each of the dried samples, 120 µL of 3.54 x 10^{-3} M phenol was added followed by addition of 6 mL of 6 M HCl. Into a set of 5 samples, 3 mL of TFA were added to each sample. All samples were purged with nitrogen to remove oxygen as means of preventing oxidative degradation of polar amino acids.

HCl samples were hydrolysed at 105 ˚C for 22 hours while TFA samples were hydrolysed at 165 ˚C for 57 minutes. At the end of the hydrolysis period, samples were transferred into 50 mL Falcon centrifuge tubes. The unexpended hydrochloric acid from each sample was removed by evaporation. Dried samples in Falcon centrifuge tubes were reconstituted in 6 mL of 0.1 M HCl. To prepare hydrolysates samples for LC analysis, 2.86 mL aliquots of HCl hydrolysate from all HCl samples were syringe filtered into 15 mL amber glass vails then diluted to 10 mL with 0.1 M HCl as shown in **Figure 3.1**. From each of the diluted samples, 6.35 mL aliquots were transferred into 15 mL amber glass vials then diluted to 10 mL with 0.1 M HCl. From each sample, 5 x 10 µL aliquots were transferred into 1.5 mL vials followed by addition of 10 µL of gamma-aminobutyric acid (GABA) (internal standard), 70 µL of AccQ·Tag borate buffer and 10 µL of AccQ·Tag derivatising reagent (not identified by the manufacturer). Samples were derivatised at 55 ˚C for 8 minutes then analysed on the LC system together with calibration standards which were prepared as outlined in **subsection 3.2.3**.

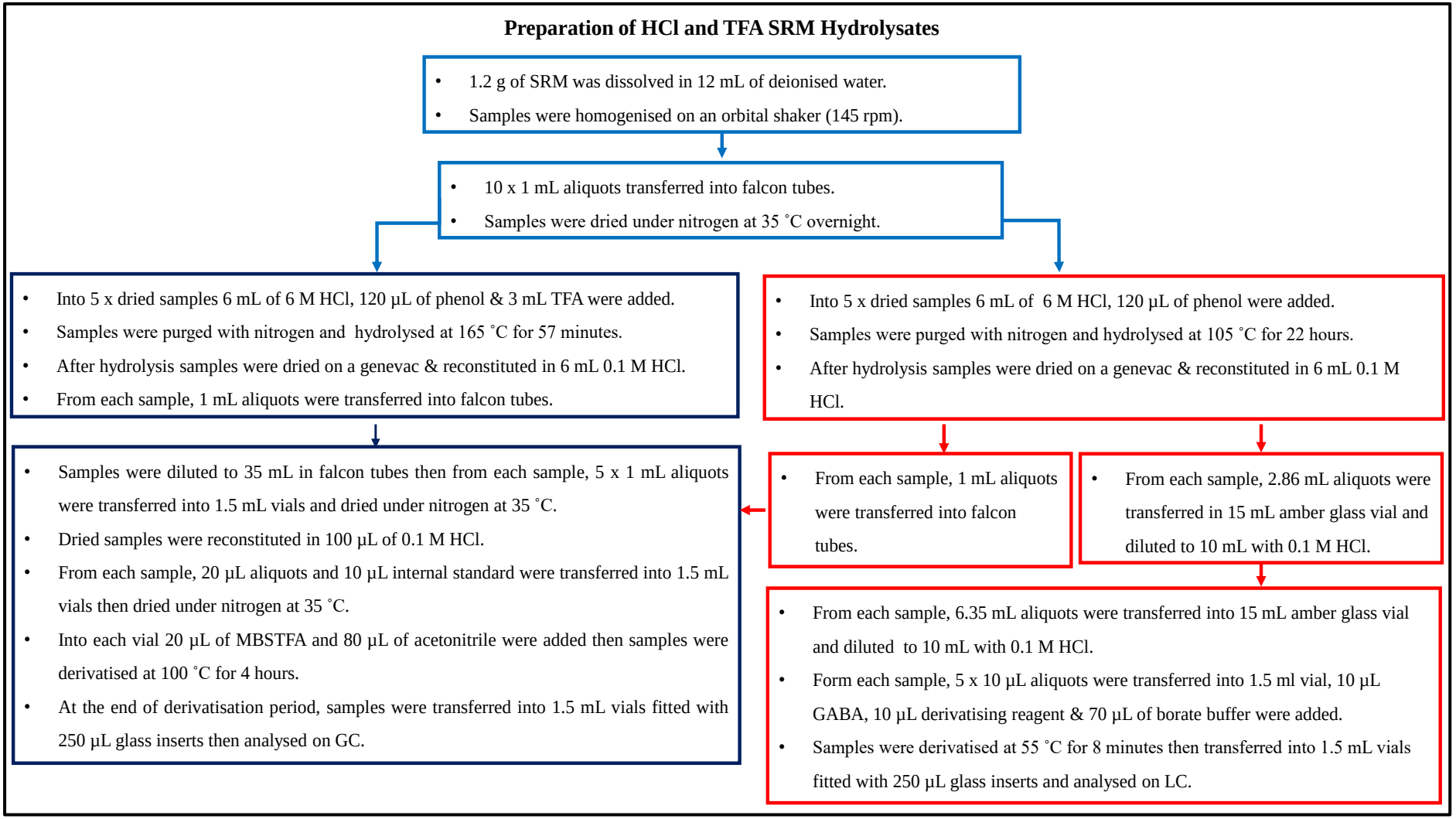


Figure 3-1: Flow diagram depicting the HCl and (HCl + TFA) hydrolysis of infant formula samples

To prepare TFA and HCl hydrolysates for GC-TOFMS analysis, a 1mL aliquot from each of the reconstituted samples was syringe filtered into a Falcon centrifuge tube then diluted to 35 mL with 0.1 M HCl. From each tube, 5 x 1 mL aliquots were transferred into 1.5 mL vials and dried under nitrogen. At the end of the drying period, each sample was reconstituted in 100 µL of 0.1 M HCl. From each sample, 20 µL aliquot was transferred into a 1.5 mL vial together with 10 µL of an internal standard comprising of four isotopically labeled amino acids namely valine, isoleucine, proline and phenylalanine prepared as outlined in **subsection 3.2.3** before the samples were dried under nitrogen at 35 ˚C. Into each dried sample 20 µL of MTBSTFA and 80 µL of acetonitrile were added and the samples were sealed and derivatised at 100 ˚C for 4 hours then transferred into 1.5 mL vials fitted with 250 µL glass inserts then analysed on the GC-TOFMS (Pegasus III and IV) systems together with calibration standards prepared in accordance with the procedure described in **subsection 3.2.3**.

3.2.3 Preparation of calibration and internal standards for GC-TOFMS and UPLC-PDA calibration

To prepare GC-TOFMS calibration standards, 80 µL of AAS18 was diluted to 800 µL with 0.1 M HCl to give a stock solution of 250 pmol/µL. From the latter 0, 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 µL aliquots were transferred into 1.5 mL vials. Into each standard, 10 µL (± 4 nmol/µL) of internal standard comprising of four ^{13}C isotopically labeled amino acids, namely, valine, isoleucine, proline, and phenylalanine was added. The standards were dried under nitrogen at 35 ˚C, 20 µL of MTBSTFA and 80 µL of acetonitrile were added into each vial, standards were then sealed and derivatised at 100 ˚C for 4 hours. At the end of the derivatisation period, standards were transferred into 1.5 mL vials fitted with 250 µL glass inserts then analysed together with infant formula samples on the Pegasus III GC-TOFMS system. Internal standard stock solution was prepared by weighing into a 15 mL amber glass vial 5.25, 4.8, 5.05 and 5.00 mg of the isotopically labelled valine, isoleucine, proline and phenylamine respectively followed by addition of 10 mL of 0.1 M HCl to give a concentration of 4.44, 3.63, 4.35 and 3.00 nmol/µL for valine, isoleucine, proline and phenylalanine respectively.

UPLC-PDA calibration standards were prepared by pipetting 0, 30, 40, 50, 60, 70 80, 90, 100 and 110 µL of AAS18 into 1.5 mL vials. Each sample was made up to 1 mL by adding a sufficient amount of 0.1 M HCl to give concentrations of 75, 100, 125, 150, 175, 200, 225, 250 and 275 pmol/µL. From each standard, a 10 µL aliquot was transferred into a 1.5 mL vial

followed by addition of 10 µL of GABA internal standard (0.299 nmol/µL), 70 µL AccQ·Tag borate buffer and 10 µL of AccQ·Tag derivatising reagent. The samples were sealed and derivatised at 55 ˚C for 8 minutes then transferred into 1.5 mL vials fitted with 250 µL glass inserts then analysed on the LC system. The LC internal standard stock solution was prepared by weighing 4.96 mg of GABA into a 15 mL amber glass vial followed by addition of 10 mL of 0.1 M HCl to give a concentration of 4.83 nmol/µL. The stock solution was vortexed, then a 62 µL aliquot from the latter was transferred into a 1.5 mL vial and made up to a volume of 1 mL using 0.1 M HCl to give a concentration of 0.299 nmol/µL. Ten microliter aliquots from this mixture were used to spike both infant formula and calibration standards before derivatisation.

3.2.4 Preparation of samples for interference evaluation by GC×GC-TOFMS

To prepare GC×GC samples, 300 mg of the SRM was weighed into a 7 mL amber glass vial, dissolved in 3 mL of deionised water and homogenised on an orbital shaker. From the homogenised sample, 2 x 1 mL aliquots were transferred into Corning tubes, dried overnight under nitrogen then hydrolysed using the HCl in accordance with the hydrolysis procedure described in **subsection 3.2.2**. At the end of the hydrolysis period, samples were dried to remove HCl then reconstituted in 6 mL of 0.1 M HCl. From each reconstituted sample, 5 x 1 mL aliquots were syringe filtered into 50 mL Falcon centrifuge tubes and diluted to 35 mL with 0.1 M HCl. From each of the sample, 1 × 10 µL aliquots were transferred into 1.5 mL glass vials, dried under nitrogen at 35 ˚C then derivatised with 100 µL of MTBSTFA at 100 ˚C for 4 hours. At the end of the derivatisation period, 20 µL aliquot of each sample was transferred into 1.5 mL vials fitted with 250 µL glass inserts followed by addition of 80 µL of acetonitrile prior to analysis of interferences by GCxGC-TOFMS. The expected concentrations of alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine and tyrosine in the test sample were 1.459, 9.172, 1.854, 2.747, 1.438, 2.966, 1.957, 1.535, 1.003, 2.297, 5.029, 1.974, 5.800, 8.042 fmol/µL respectively.

3.2.5 Preparation of spiked samples to determine recovery (accuracy)

To prepare spiked samples in order to determine analyte recoveries, 200 mg of infant formula SRM was weighed into a 7 mL amber glass vial followed by addition of 2 mL of 0.1 M HCl.

From the resuspended milk sample, 1 mL aliquots were transferred into two Corning glass hydrolysis tubes. Into 4 × 1.5 mL glass vials, 1 mg of each of the ^{13}C isotopically labeled amino acid (valine, isoleucine, proline, and phenylalanine) were dissolved in 2 mL of 0.1 M HCl to give a concentration of ≈ 4 nmol/μL of each isotope. The mixture was vortexed then each 1 mL aliquots of each solution were used to spike the infant formula sample in the Corning glass hydrolysis tube. The content of the tube was dried overnight at 35 ˚C under nitrogen. At the end of the drying period, the samples were hydrolysed using the method described in **subsection 3.2.2**. At the end of the hydrolysis period, the samples were cooled down and transferred into a Falcon centrifuge tube. The Corning hydrolysis tubes were rinsed with 0.1 M HCl and the content was transferred into the Falcon centrifuge tubes containing the hydrolysates. The content of each of the Falcon conical tubes was dried on the Genevac then resuspended in 6 mL of 0.1 M HCl to give a concentration of ≈ 0.7 nmol/μL. From the reconstituted samples, 1 mL aliquots were transferred into empty Falcon centrifuge tubes and diluted to 35 mL with 0.1 M HCl to give a concentration of ≈ 20 pmol/μL of each isotope. From the latter, 3 x 1 mL aliquots were syringe filtered into 1.5 mL screw cap glass vials and dried under nitrogen at 35 ˚C.

Into another set of 1.5 mL glass vials, 1 mg of each of the of the isotopically labeled amino acids namely, valine, isoleucine, proline, and phenylalanine was dissolved in 2 mL of 0.1 M HCl to give a concentration of ≈ 4 nmol/μL of each isotope. From each of the samples, 1 mL aliquots were transferred into two Falcon centrifuge tubes. The content of each tube was diluted to 6 mL with 0.1 M HCl to give a concentration of ≈ 0.7 pmol/μL which simulates the dilution achieved by adding 6 mL of 6 M HCl during the hydrolysis process. From each of the Falcon centrifuge tubes, 1 mL aliquots were transferred into another Falcon tube then diluted to 35 mL with 0.1 M HCl to give a concentration of 20 pmol/μL. From the latter, 3 x 1 mL aliquots were transferred into 1.5 mL screw cap glass vials and dried under nitrogen at 35 ˚C. Together with hydrolysed samples prepared in the previous step, all samples were derivatised in accordance with the derivatisation procedure used for derivatisation of samples for the GC-TOFMS analysis described in **subsection 3.2.2**. At the end of the derivatisation period, all samples were transferred into 1.5 mL glass vials fitted with 250 μL glass inserts then analysed by GC-TOFMS. The results from the hydrolysed and unhdrolysed samples were compared to evaluate the overall method recovery (accuracy).

3.3 Instrumentation

3.3.1 GC-TOFMS methods

GC-TOFMS method development and validation experiments were performed on an Agilent 6890 GC fitted with a 15 m Restek Rxi-5Sil MS column of 0.18 mm internal diameter (ID) and 0.18 µm film thickness (df) (Restek, Bellefonte, PA, USA). The GC oven was coupled to an Agilent 7683 B Series autosampler and a Leco Pegasus III TOFMS all from Leco Corporation (3000, Lakeview Avenue, Saint Joseph, Michigan 49085, USA). An Agilent 7890 GC fitted with a 30 m Restek Rxi-5Sil MS column of 0.25 mm ID and 0.25 µm df (Restek, Bellefonte, PA, USA), an Agilent 7683 B Series autosampler and a Leco Pegasus 4D TOFMS system (Leco Corporation, Michigan, USA) was used for GC-TOFMS robustness (ruggedness) experiments. Data acquisition and data processing on all GC-TOFMS systems were performed using ChromaTOF software version 2.0 and 5.0 for Pegasus III and Pegasus IV respectively.

The efficiency optimised flow rate (EOF) and speed optimised flow rate (SOF) GC-TOFMS methods used for separation of amino acids in one-dimensional gas chromatography during method development and method validation respectively were developed in accordance with recommendations described by Leonid M. Blumberg (Blumberg, 1999, Blumberg and Klee, 2000). In both the EOF and SOF methods, the inlet, ion source, and GC-MS transfer line temperatures were kept constant at 270, 250 and 300˚C respectively as depicted in **Figure 3-2** and **Figure 3-3**. EOF and SOF method oven temperature programs were also set as shown in **Figure 3-2** and **Figure 3-3** respectively. In both methods, high purity helium gas was used as the carrier gas.

For the SOF method, carrier gas flow was kept constant at 1.2 mL/min throughout the analysis while 0.72 mL/min flow rate was used in the EOF method in accordance with the theories of Blumberg (Blumberg, 1999). In both methods, mass spectrometry acquisition delay of 380s, an acquisition rate of 7 spectra/second and an acquisition mass range of between 50 m/z and 700 m/z were set. The detector voltage was set at 1651 V and the ionisation energy was 70 eV. For data processing, a signal to noise (S/N) ratio of 40 and a 70% NIST spectral library match criteria were used. For all GC-TOFMS experiments performed throughout this study, 1 µL aliquots of all analytical standards and samples were injected at a split ratio of 1:50. All GC-TOFMS inlets were fitted with dual vespel ring cross-disk inlet gold seals, premium non-stick bleed temperature optimised (BTO) septa and a 4 mm premium precision split liner with glass wool.

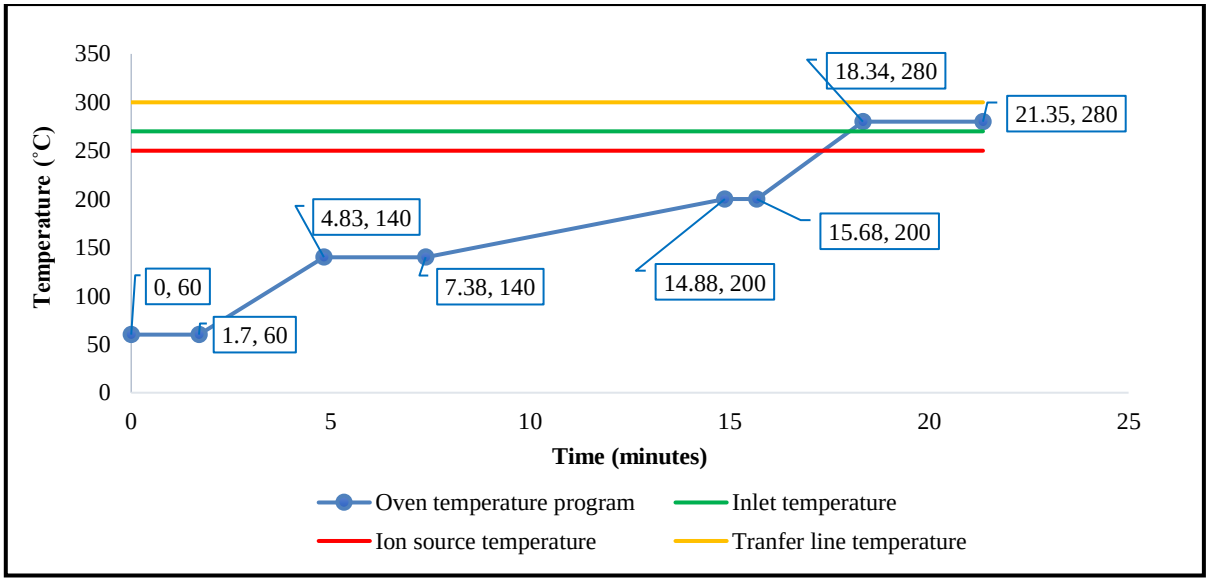


Figure 3-2: Graphical representation of the efficiency optimised flow rate (0.72 mL/min) method parameters including the oven temperature program, inlet, ion source and GC-MS transfer line temperatures used for the analysis of MTBSTFA-amino acids derivatives during GC-TOFMS method development. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.83; 7.38 to 14.88 and 15.68 to 18.34 minutes respectively. Numbers in blocks represent time and temperature coordinates at the beginning and the end of the GC oven temperature program; and each temperature ramp.

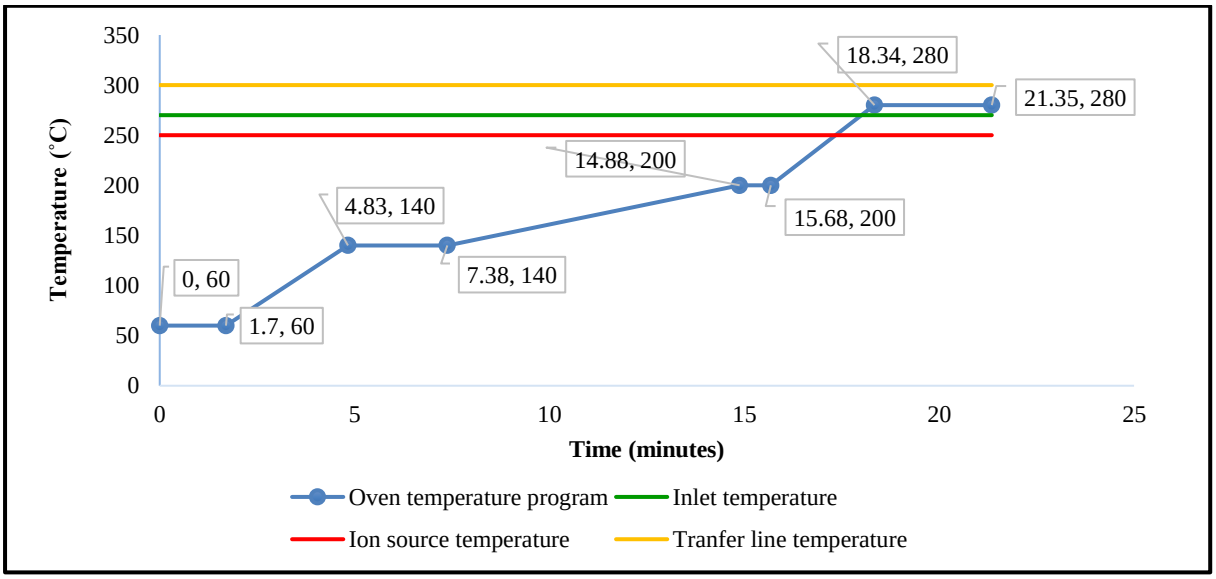


Figure 3-3: Graphical representation of the final speed optimised flow rate (1.2 mL/min (0 to 10.7 min) and 1.6 mL/min (10.70 min to 12.70 min)) method parameters including the oven temperature program, inlet, ion source and GC-MS transfer line temperatures used for qualitative and quantitative analysis of MTBSTFA-amino acids derivatives during GC-TOFMS method development and validation. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. Numbers in blocks represent time and temperature coordinates at the beginning and the end of the GC oven temperature program; time and temperature coordinates at the beginning and the end of each temperature ramp.

3.3.2 The GC×GC-TOFMS method used for interference evaluation

Qualitative analysis to test for presence of potential chromatographic interferences in the SRM hydrolysates samples was performed on a GC×GC (Agilent 7890) system fitted with a 15 m long Rxi-5Sil MS primary column (0.25 mm ID x 0.1 µm df) and a 1 m long Rxi-PAH (mid polarity) secondary column (0.25 mm ID and 0.1 µm df) (Restek, 110 Benner Circle, Bellefonte, Pennsylvania, USA) coupled to a Leco Pegasus 4D TOFMS (Leco Corporation, 3000 Lakeview Avenue, Saint Joseph, Michigan, USA) and Gerstel multipurpose autosampler (Eberhard-Gerstel-Platz 1, 45473 Müheim an der Ruhr, Germany). Data acquisition and data processing were performed using ChromaTOF software version 5.0. For this test, the inlet, ion source, and transfer line temperatures were kept constant at 280, 250 and 300 ˚C respectively as depicted in **Figure 3-4**.

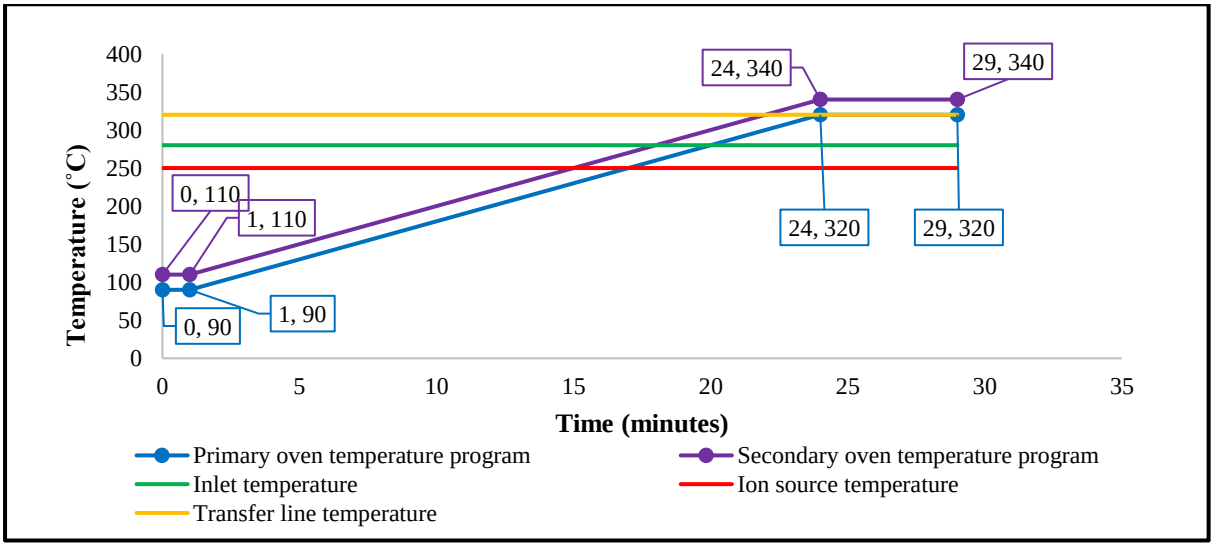


Figure 3-4: Graphical display of the GC×GC-TOFMS method parameters showing primary oven, secondary oven and modulator temperature, inlet, ion source and GC×GC-TOFMS transfer line temperature. Primary and secondary oven ramp rates were set at 10˚C/min between 1 and 24 min. The numbers in blocks represent time and temperature coordinates at the beginning and the end of the GC×GC oven temperature program. With respect to the secondary oven, modulator temperature offset was set at 15 ˚C.

Relative to the secondary oven, the modulator temperature offset temperature was set at 15 ˚C. A modulation period of 2 seconds was set. Hot and cold pulses were set at 0.6 and 0.4 seconds respectively. A carrier gas (helium) flow rate of 2 mL/min was kept constant throughout the analysis. From each of the test samples, 1 µL aliquots were injected using a splitless injection method. The detector voltage was set to 1651 V and the ionisation energy was 70 eV. An acquisition delay of 180 s, a 50 spectra/second acquisition rate and an acquisition range of 50

to 700 m/z were used. For data processing, a signal to noise ratio of 150 and a 70% NIST library match criterion was used for identification of analytes.

3.3.3 UPLC PDA method

HPLC analyses were performed on a Waters Acquity UPLC H-Class system (Waters Inc., Milford, Massachusetts, USA) fitted with a Waters AccQ·Tag ultra amino acid analysis reverse phase column with a stationary phase particle size of 1.7 µm df x and 2.1 mm I.D. x 100 mm long which was coupled to a diode array (PDA) detector. Mass Lynx software version 4.0 was utilised for LC data acquisition and data processing. For Acquity UPLC analysis, four mobile phase solvents were prepared, A, B, C and D where A was 100% eluent A (described by the manufacturer as aqueous), B was 90% double deionised water and 10% eluent B (described by the manufacturer as organic), C was 100% deionised water and D was 100% eluent B (described by the manufacturer as organic). For all Acquity UPLC amino acids analysis experiments performed, the mobile phase flow rate, sample and standard injection volume, sample temperature, column temperature and PDA wavelength were set to 0.7 mL/min, 1.0 µL, 20 ˚C, 43 ˚C and 260 nm respectively. The solvent ratios at given times during the analysis were as shown in **Table 3-1**

Table 3-1: Mobile phase program used for UPLC AccQ·Tag amino acid analysis of the infant formula reference material and samples

Time (min)	%A (aqueous)	%B (water: organic (90:10))	%C (water)	%D (organic)
initial	10.0	0.0	90.0	0.0
0.29	9.9	0.0	90.1	0.0
5.49	9.0	80.0	11.0	0.0
7.10	8.0	15.6	57.9	18.5
7.30	8.0	15.6	57.9	18.5
7.69	7.8	0.0	70.9	21.3
7.99	4.0	0.0	36.3	59.7
8.59	4.0	0.0	36.3	59.7
8.68	10.0	0.0	90.0	0.0
10.2	10.0	0.0	90.0	0.0

3.4 Mathematical Models

3.4.1 Chemometrics equations used in method development and method validation

The mathematical models used to quantify amino acids in the infant formula samples using the protocols described in this chapter are shown in **Equation 3-1** for the external calibration method and **Equation 3-2** for the isotope dilution method. Based on the mathematical models presented in **Equation 3-1** and **Equation 3-2**, a fishbone diagram in **Figure 3-5.** was developed in accordance with the principles for calculation of measurement uncertainty as described in the Guidelines to Expression of Uncertainty in Measurement (GUM) and its simplified version (Eurachem /CITAC Guide CG 4) namely Quantifying Uncertainty in Analytical Measurements (Magnusson, 2014).

$$[\boldsymbol{AA}] = \left(\frac{\boldsymbol{y - a}}{\boldsymbol{b}}\right) \times \boldsymbol{DF} \times \boldsymbol{Mr} \times \left(\frac{\mathbf{100}}{\mathbf{0.0000166}}\right) \boldsymbol{g/100\,g}$$

Equation 3-1: The mathematical model used for the quantification of amino acids using the external calibration method. [AA] = amino acids concentration, y = instrument response, a = intercept of the regression line, b = slope of the regression line; Mr = molecular mass of the analyte, DF = dilution factor, (100/0.0000166) = conversion factor (obtained by dividing 0.1g of infant formula by 6000 µL of 0.1M HCl), 100 = the numerator obtained when converting 100 g/100 g to g/100g the final concentration units and g/100g = measured concentration units.

$$[\boldsymbol{AA}] = \left(\frac{\boldsymbol{y - a}}{\boldsymbol{b}}\right) \times \boldsymbol{DF} \times [\mathbf{IS}] \times \boldsymbol{Mr} \times \left(\frac{\mathbf{100}}{\mathbf{0.0000166}}\right) \boldsymbol{g/100\,g}$$

Equation 3-2: The mathematical model used for the quantification of amino acids using the isotope dilution method. [AA] = amino acids concentration, y = instrument response ratio (native response /isotope response), a = intercept of the regression line, b = slope of the regression line; [IS] = concentration of the internal standard, Mr = molecular mass of the analyte, DF = dilution factor, (100/0.0000166) = conversion factor (obtained by dividing 0.1g of infant formula by 6000 µL of 0.1M HCl), 100 = the numerator obtained when converting 100 g/100 g to g/100g the final concentration units and g/100g = measured concentration units

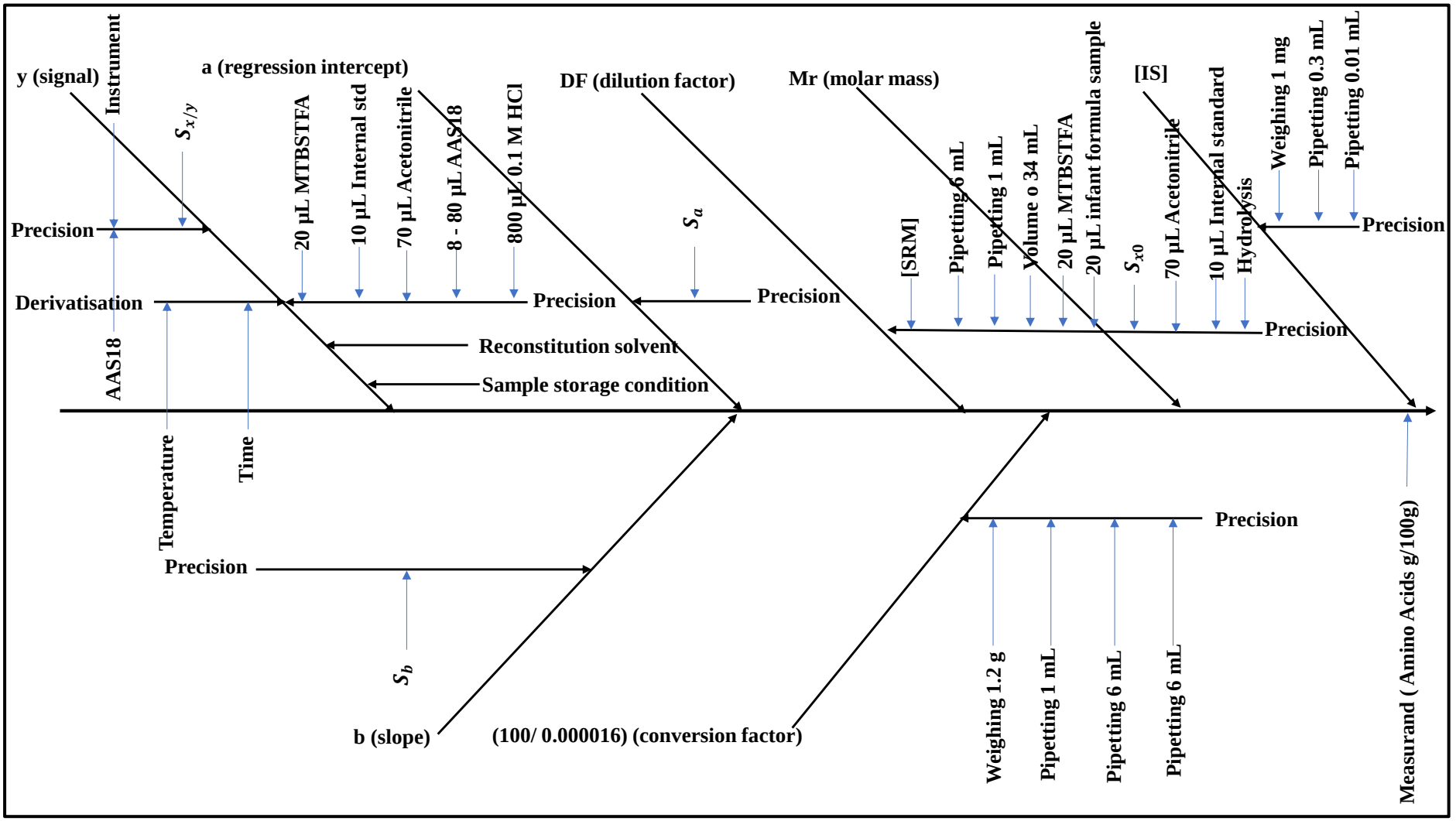


Figure 3-5: Fishbone diagram used to identify factors that may affect the accuracy and precision of amino acid measurements

The fishbone diagram (**Figure 3-5**) was used to identify experimental factors or experimental variables that may readily affect the accuracy and uncertainty of measurements of each analyte being quantified. Using this diagram derivatisation temperature and derivatisation period; reconstitution solvent type, and sample storage conditions were identified as experimental variables that are likely to have an impact on measurements accuracy but the uncertainty contributions of which may not be quantified mathematically due to the complexity of assigning a numerical value to their measurement uncertainty contributions. Therefore, these factors were optimised experimentally as outlined in **subsection 3.2.1.** During experimental optimisation of the abovementioned factors and validation of the GC-TOFMS method developed in this study, chemometrics **Equation 3-3** to **Equation 3-8** were used for statistical analysis of the experimental results. Where applicable, chemometrics **Equation 3-3** was used to compare sample variances prior to application of the student's t-test using of either **Equation 3-4** or **Equation 3-5** (Miller and Miller, 2005). **Equation 3-4** was used to perform students t-test where sample variances did not differ significantly while **Equation 3-5** was applied where the opposite was true (Miller and Miller, 2005).

In conjunction with **Equation 3-7**, chemometrics **Equation 3-6** (one-way ANOVA) was used to test for the general significant differences between means while chemometrics **Equation 3-7** was used to identify means that differed significantly. **Equation 3-8** was used to determine analytes recoveries (accuracy) in accordance with the guidelines provided in The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics published by the Eurachem (Magnusson, 2014). Further details on the application of all equations briefly described under this subsection are discussed in **Chapter 4**. The limit of detection and the limit of quantification were calculated using **Equation 3-8** and **3-9** respectively (Miller and Miller, 2005). The standard errors of the slope (S_b) of the regression line, intercept (S_a), the standard error of the samples (S_{x0})were calculated using **Equation 3-11**, **Equation 3-12**, **Equation 3-13** respectively (Miller and Miller, 2005).

$$\boldsymbol{t(experiental)} = \frac{(\boldsymbol{mean\ x_1 - mean\ x_2})}{\boldsymbol{S_{pooled}}\sqrt{\frac{\boldsymbol{1}}{\boldsymbol{n_1}} + \frac{\boldsymbol{1}}{\boldsymbol{n_2}}}}$$

Equation 3-3: The chemometrics expression for a two-sided t-test used when sample variances do not differ significantly, $mean\ x_1$= sample 1 mean, $mean\ x_2$ = sample 2 mean, n_1 = number of replicate measurement

taken of sample 1, n_2 = number of replicate measurement taken of sample 2 and S_{pooled} = pooled standard deviations of sample 1 and sample 2.

$$\boldsymbol{t(experimental) = \frac{(mean\, x_2 - mean\, x_1)}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}}$$

Equation 3-4: The chemometrics expression for a two-sided t-test used when sample variances differ significantly, $mean\, x_2$ = mean of sample 2, $mean\, x_1$ = mean of sample 1, S_1^2 = variance of sample 1, S_2^2 = variance of sample 2, n_1 = number of replicate measurements in sample 1 and n_2= number of replicate measurements in sample 2.

$$\boldsymbol{F(Experimental) = \frac{S_1^2}{S_2^2}}$$

Equation 3-5: The chemometrics expression for comparing two sample variances, S_1^2 = the variance of sample 1 and S_2^2 = the variance of sample 2, this equation is applied provided the numerator is greater than the denominator.

$$\boldsymbol{F(ANOVA) = \frac{S^2_{within\ sample\ variance}}{S^2_{between\ sample\ variance}}}$$

Equation 3-6: The chemometrics expression for one-way analysis of variance (ANOVA)

$$\boldsymbol{HSD = q\sqrt{\frac{Msw}{n_k}}}$$

Equation 3-7: Tukey honest significant difference (HSD) equation used for post hoc analysis when the null hypothesis of the one-way ANOVA has been rejected, $\boldsymbol{q}$ = quantile for the students range probability distribution, $\boldsymbol{MSw}$ = mean square error within and $\boldsymbol{n_k}$ = the number of degrees of freedom within samples

$$\%\boldsymbol{Recovery} = \left(\frac{\boldsymbol{mean\ x}}{\boldsymbol{mean\ SRM}}\right) \times \mathbf{100}\%$$

Equation 3-8: The chemometrics expression for analyte percentage recovery used to determine method bias as part of the method validation procedure, $\boldsymbol{mean\ x}$ = experimental mean value and $\boldsymbol{mean\ SRM}$ = certified value

$$\boldsymbol{LOD} = \boldsymbol{a} + \mathbf{3}\ \boldsymbol{S_{x/y}}$$

Equation 3-9: The chemometrics expression for calculating the limit of detection, a = regression line intercept, $\boldsymbol{S_{x/y}}$= the standard error of the regression line.

$$\boldsymbol{LOQ} = \boldsymbol{a} + \mathbf{10}\ \boldsymbol{S_{x/y}}$$

Equation 3-10: The chemometrics expression for calculating the limit of quantification, a = regression line intercept and $\boldsymbol{S_{x/y}}$= standard deviation of the regression line.

$$\boldsymbol{S_b} = \frac{\boldsymbol{S_{x/y}}}{\sqrt{\sum_i (\boldsymbol{x_i} - \boldsymbol{meanx_i})^2}}$$

Equation 3-11: The chemometrics expression for calculating the standard error of the slope, $\boldsymbol{S_{x/y}}$ = the standard of the regression line, $\boldsymbol{x_i}$ = values of x, $\boldsymbol{n}$ = number of calibration points and $\boldsymbol{x_i}$ = average value of x.

$$\boldsymbol{S_a} = \boldsymbol{S_{x/y}} \sqrt{\frac{\sum_i \boldsymbol{x_i^2}}{\boldsymbol{n} \sum_i (\boldsymbol{x_i} - \boldsymbol{meanx_i})^2}}$$

Equation 3-12: The chemometrics expression for calculating the standard error of the intercept where $\boldsymbol{S_{x/y}}$ = the standard error of the regression line, $\boldsymbol{x_i}$ = values of x, $\boldsymbol{n}$ = number of calibration points and $\boldsymbol{x_i}$ = average value of x.

$$S_{x_0} = \frac{S_{y/x}}{b}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{(y_0 - mean\ y)^2}{b^2 \sum_i (x_i - meanx_i)^2}}$$

Equation 3-13: The chemometrics expression for calculating the standard error of the samples, $S_{x/y}$ = the standard error of the regression line, x_i = values of x, n = number of calibration points, *m* = number of measurements from which y_0 is calculated, *b* = slope and x_i = average value of x.

3.4.2 Chemometrics equations used to evaluate the uncertainty of measurements

As part of method validation, the uncertainty of measurement (UoM) is key to the reliability of any analytical measurement results. As defined in the International Standardisation Organisation's Guide to Uncertainty in Measurement (GUM) and its simplified version namely Quantifying Uncertainty in Analytical Measurement (QUAM) published by the Cooperation on International Traceability in Analytical Chemistry (CITAC), "*uncertainty is a parameter associated with the result of measurement that characterises the dispersion of the values that could be attributed to the Measurand*". Furthermore, GUM emphasises that it is obligatory that uncertainty of measurement be indicated so that those who will use the results can assess its reliability. In this study, the uncertainty of measurement was quantified in accordance with the guidelines provided in both GUM and QUAM. To do this, following steps specified in GUM were followed:

1. **Specification and Modelling**: the Measurand was identified as amino acids in infant formula, evaluated using the mathematical model in **Equation 3-1** and **Equation 3-2**.
2. **Identification of uncertainty sources**: from the mathematical model, a fishbone diagram or a cause and effect diagram in **Figure 3-5.** was developed and used as a guide for assignment of uncertainty sources on each branch of the fishbone diagram.
3. **Quantification of uncertainty sources**: uncertainty sources were divided into two categories type A and type B with type A being sources that comes directly from the measurements performed in this study and type B being those obtained from other sources such as analytical balances calibration certificates, SRM certificates, and volumetric apparatus certificates. These assignment categories were then used to evaluate the standard uncertainties of each uncertainty source.

4. **Calculation of the combined uncertainties**: in this step sensitivity coefficients were calculated using **Equation 3-14** and the results thereof are presented in **Table 4-10**. Sensitivity coefficients were used to assess the change brought about changing the value of any of the uncertainty contributions to the value of the measurand. Furthermore, sensitivity coefficients were used to calculate the uncertainty contributions of each uncertainty source subbranch (subbranches of the fishbone diagram) using **Equation 3-14**. Each uncertainty contribution was used to calculate the combined uncertainty using **Equation 3-15**.

$$\boldsymbol{c_i = \frac{\partial y}{\partial xi}}$$

Equation 3-14: The uncertainty of measurement equation used to calculate sensitivity coefficients where c_i = sensitivity coefficient, $\partial y =$ partial change in analyte concentration and $\partial xi =$ partial change in any uncertainty source.

$$\boldsymbol{u(y_i) = c_i \times u(x_i)}$$

Equation 3-15: The uncertainty of measurement equation for calculating uncertainty contribution. Where u ($\boldsymbol{y_i}$) is the uncertainty contribution, $\boldsymbol{c_i}$ is the sensitivity coefficient and $\boldsymbol{u(x_i)}$ is the standard uncertainty (an uncertainty that has been converted into a standard deviation).

$$\boldsymbol{u_c(y) = \sqrt{\sum u_c^2\ (y)}}$$

Equation 3-16: The uncertainty of measurement equation for the calculation of combined uncertainty where $\boldsymbol{u_c(y)}$is the combined standard uncertainty and $\boldsymbol{u_c^2\ (y)}$ is the square of each uncertainty contribution.

To calculate the total combined uncertainty, **Equation 3-1** and **Equation 3-2** were simplified by combining all uncertainties of the (b) and (a) branches of the fishbone into $\sum (b - a)$.

Uncertainty contributions of (a) and (b) were equated to (p) such that **Equation 3-1** can be represented as **Equation 3-17**. Therefore, the combined uncertainty of (y-a) in terms of p could be calculated using **Equation 3-18** and the total combined uncertainty calculated from **Equation 3-19**.

$$[\boldsymbol{AA}]\left(\frac{\boldsymbol{g}}{\boldsymbol{100g}}\right) = \left(\frac{\boldsymbol{p}}{\boldsymbol{b}}\right) \times \boldsymbol{Mr} \times \boldsymbol{DF} \times \left(\frac{\boldsymbol{100}}{\boldsymbol{0.000016}}\right)\frac{\boldsymbol{g}}{\boldsymbol{100g}}$$

Equation 3-17: Representation of **Equation 3-1** as **Equation 3-17** by converting (y-a) to $\boldsymbol{p}$ in order to simplify **Equation 3-1** for calculation of total combined standard uncertainty**.** $\boldsymbol{p = (y - a)}$**,** y = instrument response, a = intercept of the regression line, b = slope of the regression line; Mr = molecular mass of the analyte, DF = dilution factor, (100/0.0000166) = conversion factor (obtained by dividing 0.1g of infant formula by 6000 µL of 0.1M HCl), 100 = the numerator obtained when converting 100 g/100 g to g/100g the final concentration units and g/100g = measured concentration units.

$$\boldsymbol{u_c(p) = \sqrt{u_c^2(a) + u_c^2(y)}}$$

Equation 3-18: The uncertainty equation for calculation of the combined standard uncertainty of (y-a) in terms of p

$$\boldsymbol{u_c([AA]) = \sqrt{\left(\frac{u_c(p)}{p}\right)^2 + \left(\frac{u_c(b)}{b}\right)^2 + \left(\frac{u_c(DF)}{DF}\right)^2 + \left(\frac{u_c(conversion\ factor)}{conversionfactor}\right)^2}}$$

Equation 3-19: The uncertainty of measurement equation for calculation of the total combined standard uncertainty.

5. **Determination of the expanded uncertainty**: the expanded uncertainty was calculated from **Equation 3-20**. The effective number of degrees of freedom at a 95% confidence level was calculated from Satterthwaite equation (**Equation 3-21).**

$$\boldsymbol{U = k \times u_c([AA])}$$

Equation 3-20: The uncertainty of measurement equation for calculating the expanded uncertainty where U is the expanded uncertainty, $\boldsymbol{k = 2}$ is the coverage factor at a 95% confidence level and $\boldsymbol{u_c([AA])}$ is the total combined uncertainty with respect to each analyte.

$$\boldsymbol{\upsilon_{eff} = \frac{u_c^4([AA])}{\sum_{i=1}^{N} \frac{u_i^4([AA])}{\upsilon_i}}}$$

Equation 3-21: The uncertainty of measurement equation for calculating the effective number of degrees of freedom where $\boldsymbol{u_c([AA])}$ is the total combined uncertainty, $\boldsymbol{u_i}$ is the standard uncertainty per contribution and $\boldsymbol{\upsilon_i}$ is the number of degrees of freedom per contributor in each branch.

$$\boldsymbol{percentage\ uncertainty\ contribution = \frac{u_c(y)}{u_c[AA]} \times 100\%}$$

Equation 3-22: The uncertainty of measurement equation for calculating the percentage uncertainty contributions of each uncertainty source (branch) where $\boldsymbol{u_i(y)} =$ the combined uncertainty of each uncertainty source and $\boldsymbol{u_c[AA]}$ = total combined uncertainty.

6. **Reporting uncertainty of measurements:** Uncertainty calculations were done at a 95% confidence level and a coverage factor ($k = 2$) was used. As seen in **Table 4-14**, analyte concentrations and expanded uncertainties were rounded up to two significant digits in accordance with GUM.

4. Results and Discussion

4.1 GC-TOFMS Method Development

The fishbone diagram or the cause and effect diagram presented in **Chapter 3 (Figure 3-5)** was developed in accordance with the guidelines provided in the Guide to Expression of Uncertainty in Measurement (GUM) and it indicates experimental variables that might have an impact on analytes recovery (accuracy) and on the uncertainty of measurement (Guide, 1998, Metrology, 2008, Magnusson, 2014). Derivatisation period, derivatisation temperature, sample reconstitution solvent, the stability of amino acids derivatised with MTBSTFA and sample storage conditions are some of the variables for which the uncertainty contribution cannot be evaluated mathematically due to complexity. Therefore, these derivatisation factors were optimised experimentally as outlined in **Chapter 3** and the results thereof are discussed in this chapter.

Early subsections (**4.1.1** to **4.1.4**) of this chapter (**Chapter 4**) discuss the results obtained from the experimental optimisation of the above-mentioned variables. Additionally, these subsections also discuss the results of the optimisation of separation of target compounds using GC-TOFMS. Subsections **(4.2.1 to 4.2.6)** focuses on method validation including the estimation of measurement uncertainty and the determination of the accuracy (recovery) of the optimised method by hydrolysing and analysing the NIST SRM 1849-a infant formula reference material and comparing the values determined experimentally to those presented on the reference material certificate. The results obtained through the optimised GC-TOFMS method were subsequently compared with the results from the UPLC-UV method and the results obtained using an alternate GC-TOFMS to study method's transferability and methods ruggedness. Furthermore, results obtained from the comparison of two hydrolysis approaches (HCL and TFA) were compared towards the goal of optimising two independent methods to value assign a reference material for an NMISA proficiency testing scheme is also discussed in this chapter.

Additionally, the results from statistical tests such as ANOVA and the Tukey honest significant difference tests used to assess the significance of the differences during the optimisation of derivatisation factors are also presented. Prior to the application of statistical procedures, experimental results were initially visually assessed using paired means plots similar to those in **Figure 4-5** and **Figure 6-2** in **Appendix A2**. In instances where a two-way ANOVA was

utilised, interactions between the levels of the factors being evaluated were initially assessed using interactions plots such as those in **Figures 4-6** and **4-7**; and in **Figures 6-3** to **6-9** in **Appendix A3**. In cases where significant interactions were found between the variables, no further statistical evaluation was performed.

4.1.1 The utilisation of the EOF method for optimisation of separation

All optimisation experiments were performed using the EOF method by injecting amino acids standards (2.5 nmol/mL) that had been derivatised with MTBSTFA as outlined in **Chapter 3**. Using the efficiency optimised flow rate method described in **Chapter 3**, 14 analytes of interest namely, alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine and tyrosine; together with methionine and ornithine a by-product of arginine degradation that is reportedly formed during derivatisation of arginine with MTBSTFA were resolved in 20 minutes as shown in **Figure 4-1**. Cystine an additional amino acid present in the AAS18 amino acids standard was excluded from the chromatogram because it eluted outside the range of interest. Additionally, cysteine is normally quantified as cysteic acid which elutes before lysine presented in **Figure 4-11**. Using a reconstructed ion chromatogram at 73 m/z which is an ion with the highest abundance in all amino acids, an acceptable baseline resolution was obtained between all analytes except glutamic acid and ornithine. These analytes could only be resolved by peak deconvolution using unique ions 272 and 330 m/z for glutamic acid; 184 and 285 m/z for ornithine as depicted on the exploded view of **Figure 4-1**.

The absence of ornithine in the AAS18 standard used to prepare optimisation samples and its presence after derivatisation, is commonly encountered when a sample of amino acids containing arginine is derivatised with MTBSTFA. It has been previously suggested that ornithine is likely a degradation by-product formed during the derivatisation of arginine with MTBSTFA (Corso *et al.*, 1993). This theory explains the absence of arginine on the chromatogram and the appearance of ornithine as seen in **Figure 4-1**. To verify this hypothesis a pure arginine powder was subjected to sample preparation followed by GC-TOFMS analysis. As depicted in **Figure 4-2**, in addition to the analyte tentatively identified as ornithine, a second by-product of arginine tentatively identified as citrulline was also found after a pure arginine standard was derivatised with MTBSTFA. As depicted in **Figure 4-3**, an 87% NIST library mass spectral match for ornithine was found. Meanwhile, the citrulline mass spectra in **Figure 6-1** in **Appendix A1**, did not meet the 70% NIST library mass spectral match criterion

as required for identification of analytes in this study as stipulated in **Chapter 3**. **N**o further attempts to elucidate citrulline were made.

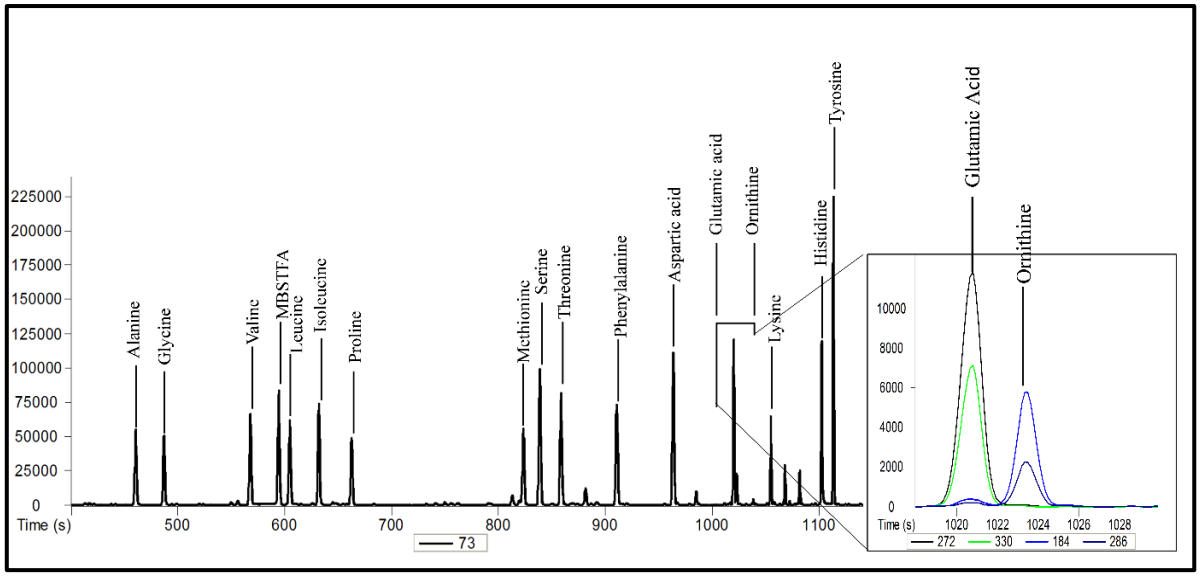


Figure 4-1: A reconstructed ion chromatogram (RIC) at 73 m/z (exploded view of **Figure 4.1**, RIC at 272, 330, 184 and 286 m/z) obtained using the EOF method when a pure amino acids standard (AA-S-18) (2.5nmol/mL) derivatised with MTBSTFA was analysed using the Pegasus III GC-TOFMS system. The flow rate was set to 0.72 mL/min. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.83; 7.38 to 14.88 and 15.68 to 18.34 minutes respectively. The run time was 20 minutes.

However, additional attempts to verify the formation of ornithine during derivatisation or arginine with MTBSTFA by comparing fragmentation patterns of MTBSTFA arginine derivative with those of other MTBSTFA derivatised amino acids were made. In general, MTBSTFA amino acid derivatives were identified by a characteristic loss of radical ions, 159 m/z and 57 m/z which by applying the Stevenson-Audier rule these ions corresponds to the fragments shown in **Figure 4-4** (De Oca *et al.*, 2012). The Stevenson-Audier rule states that after the analyte has been fragmented, the positive charge will remain on the ion (fragment) with a lower ionisation energy that is generally the most substituted or effectively the most stable ion (De Oca *et al.*, 2012). Therefore, provided arginine was fully derivatised a molecular ion mass of 516 m/z would be expected and by invoking the Stevenson-Audier rule, arginine would fragment into a set of ions including two unique daughter ions at 357 m/z and 300 m/z.

However, as depicted in **Figure 4-3**, 184 m/z and 286 m/z ions were instead the most prominent unique ions on the mass spectra suggesting that arginine was effectively converted to its by-products during derivatisation or prior to injection.

Additionally, the above-mentioned ions were consistent with ornithine fragmentation patterns observed by (Philip *et al., 2010*). However, since the rate of conversion of arginine to ornithine during derivatisation is not known and due to unavailability of the ornithine standard that could be used to quantify arginine as its by-product ornithine, arginine was not quantified in this study but will be considered in future studies that will be carried out to improve the current method. In addition, arginine, tryptophan, taurine, cystine, and methionine were not quantified in this study because these analytes also require additional experiments to be carried out such as oxidation of methionine and cysteine to methionine sulfone and cysteic acid respectively before the hydrolysis process and derivatisation process are carried out. As discussed in **Chapter 2**, to quantify tryptophan a separate alkaline hydrolysis approach is required while acid hydrolysis is required for the analysis of other amino acids. Additionally, analysis of MTBSTFA derivative of tryptophan with GC-MS has not been extensively reported in literature therefore requires additional method development and method validation which was beyond the scope of this study. Furthermore, the concentration of taurine (0.0366 g/100g) in the NIST SRM is very low, its analysis thereof requires the development of methods to determine the free amino acids using larger samples which is not economically feasible.

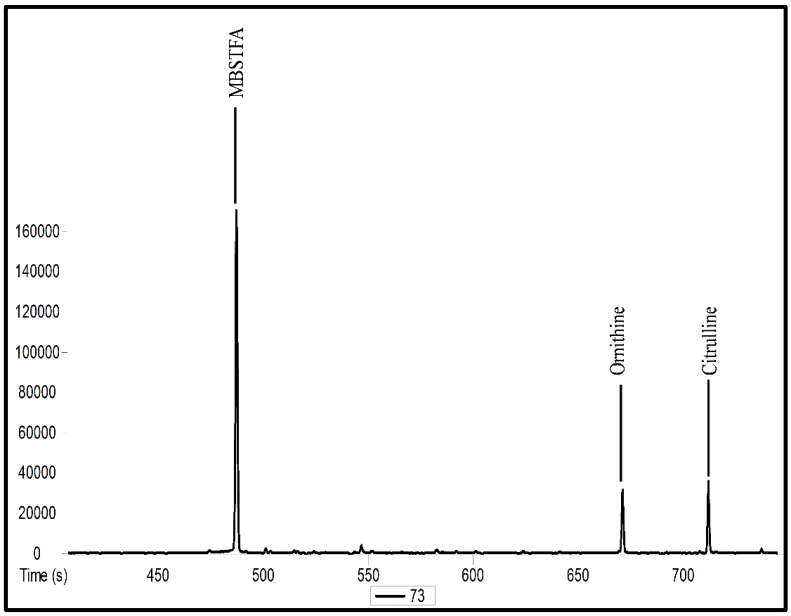


Figure 4-2: A reconstructed ion chromatogram (RIC) at 73 m/z obtained using the SOF method when a pure arginine standard (4.89 nmol/mL) derivatised with MTBSTFA was analysed on the Pegasus III GC-TOFMS system. By-products of arginine, i.e. ornithine and citrulline, formed after MTBSTFA derivatisation. The run time was 12.5 minutes.

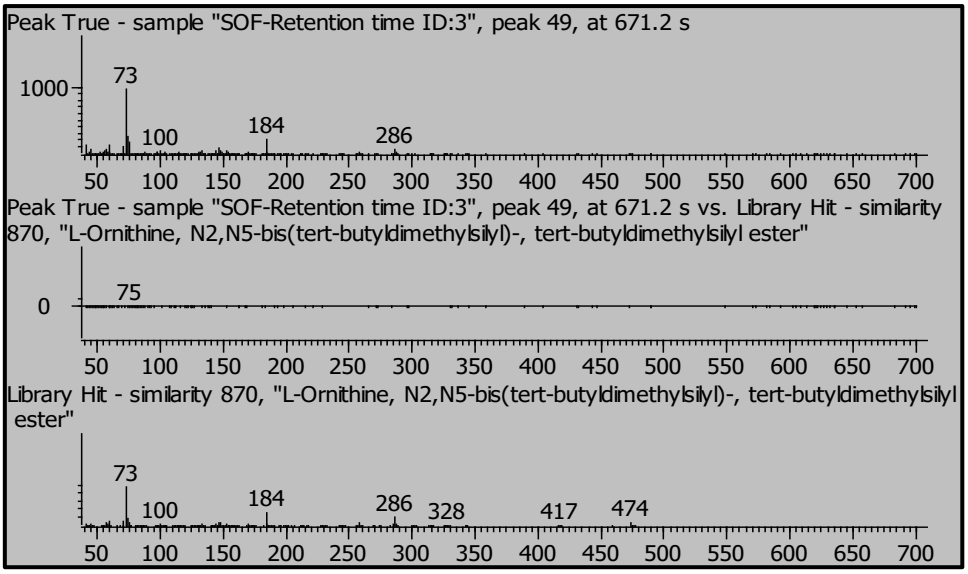


Figure 4-3: L-Ornithine mass spectrum obtained when a neat arginine sample was analysed on the Pegasus III using the SOF method to verify the conversion of arginine to ornithine during derivatisation with MBSTFA. The top mass spectrum is the spectrum of ornithine. The bottom mass spectrum is the library match spectrum of ornithine while the spectrum in the middle represents the differences between the library and experimentally obtained spectra of ornithine.

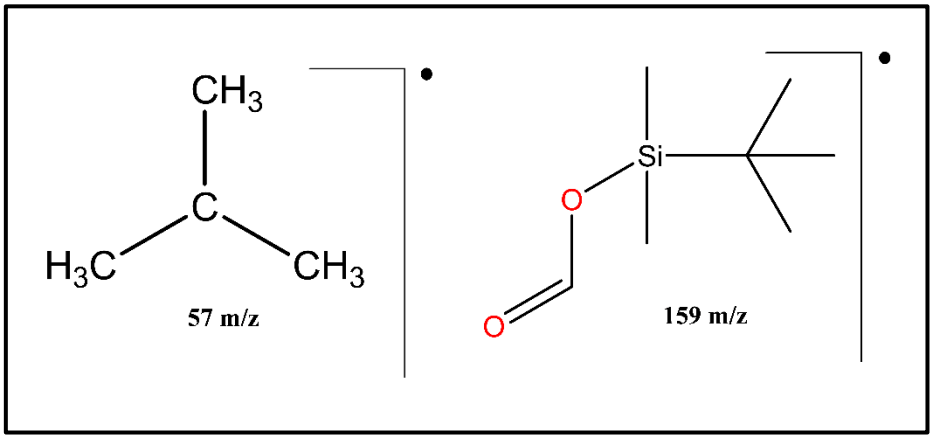


Figure 4-4: Predicted radical ions lost during fragmentation of MBSTFA-amino acid derivatives at 70 eV.

4.1.2 Optimisation of derivatisation temperature and derivatisation period using the EOF method

Derivatisation temperature optimisation studies were performed at two temperatures, 75 and 100 ˚C across five intervals of time (t) = 0.5, 1, 2.5, 4 and to 6 hours. During this study, other experimental variables such as solvent type, solvent volume and the volume of MBSTFA were kept constant as outlined in **Chapter 3**. Instrument response (peak area) of each amino acid at

the two temperatures across all time intervals were compared as depicted in **Figure 4-5** and in **Figure 6-2** in **Appendix A2.**

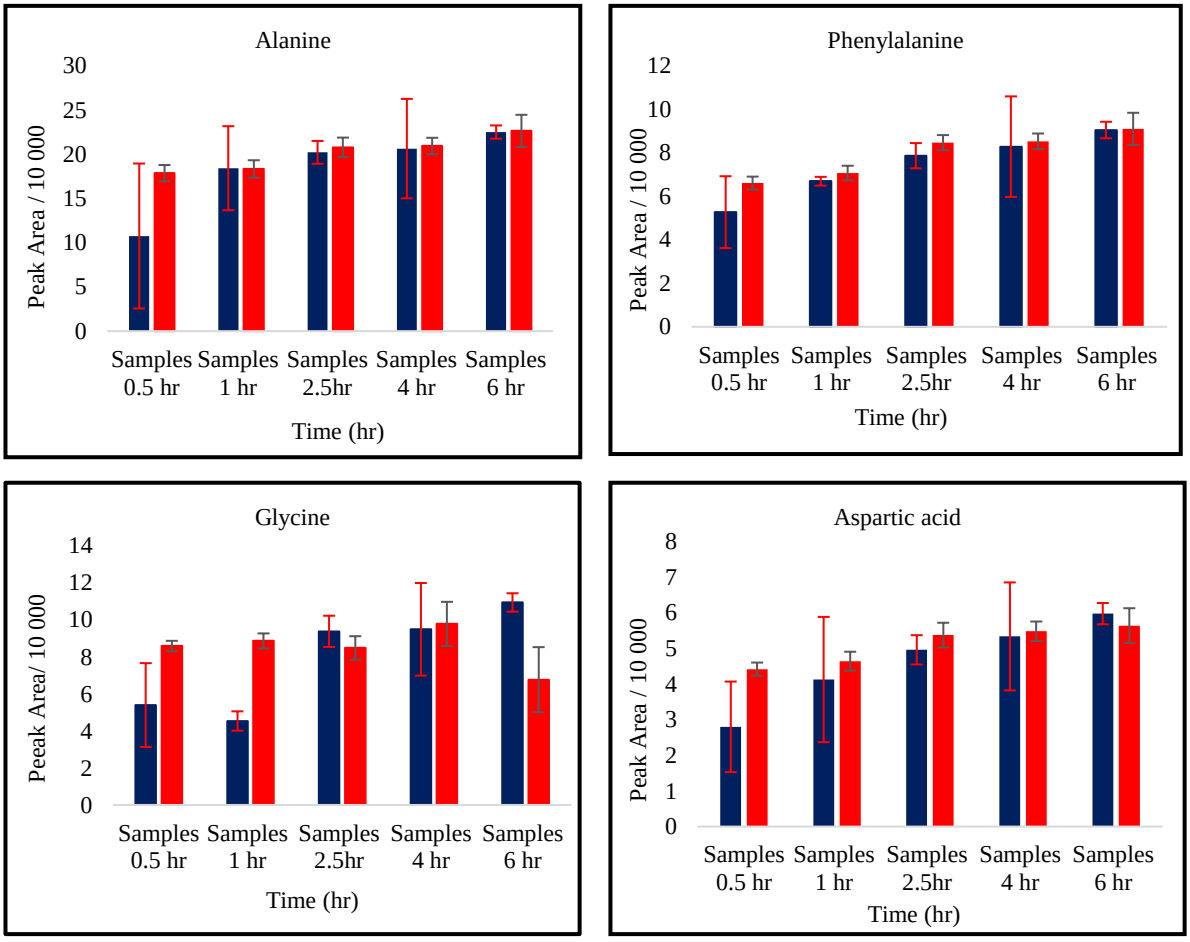


Figure 4-5: Paired means (n = 5) plots to compare average amino acids responses between 75 and 100˚C, at derivatisation time intervals of 0.5, 1, 2.5, 4 and 6 hours to determine the optimum derivatisation temperature and the derivatisation incubation period required for complete derivatisation of amino acids using MTBSTFA. Included in **Figure 4.5** are paired means plots of alanine, glycine, phenylalanine and aspartic acid. The blue bars represent derivatisation at 75 ˚C while the red bars represent derivatisation at 100 ˚C.

As seen in **Figure 4-5** and **Figure 6-2**, at all intervals of time, amino acid responses at 100 ˚C were on average greater than responses of the same analyte at 75 ˚C. This observation was true for all analytes except for glycine, lysine, histidine, and tyrosine where some of the responses observed at 75 ˚C were higher than the responses observed at 100 ˚C as depicted in **Figure 4-5** and **Figure 6-2**. Additionally, at any given derivatisation time, derivatisation at 75 ˚C resulted in a much larger uncertainty (standard deviation represented by error bars in both **Figure 4-5** and **Figure 6-2**) compared to derivatisation at 100 ˚C. Therefore, performing derivatisation at 75 ˚C during quantitative analysis would result in a large between sample of variation compared

to derivatisation at 100 ˚C. Of the two temperatures investigated, the lowest uncertainty was observed at 100 ˚C and time (t) = 4 hours. Consequently, derivatisation at 100 ˚C for 4 hours was identified as the optimum condition for the complete derivatisation of AAs using MTBSTFA based on visual inspection of **Figure 4-5 and Figure 6-2**.

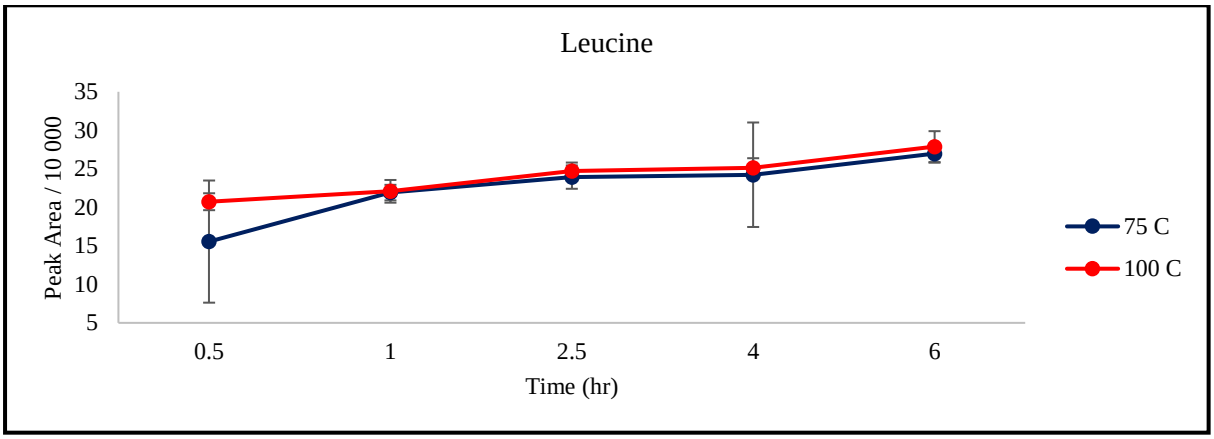


Figure 4-6: Derivatisation period interactions plots used to assess interactions between derivatisation period and temperature for leucine.

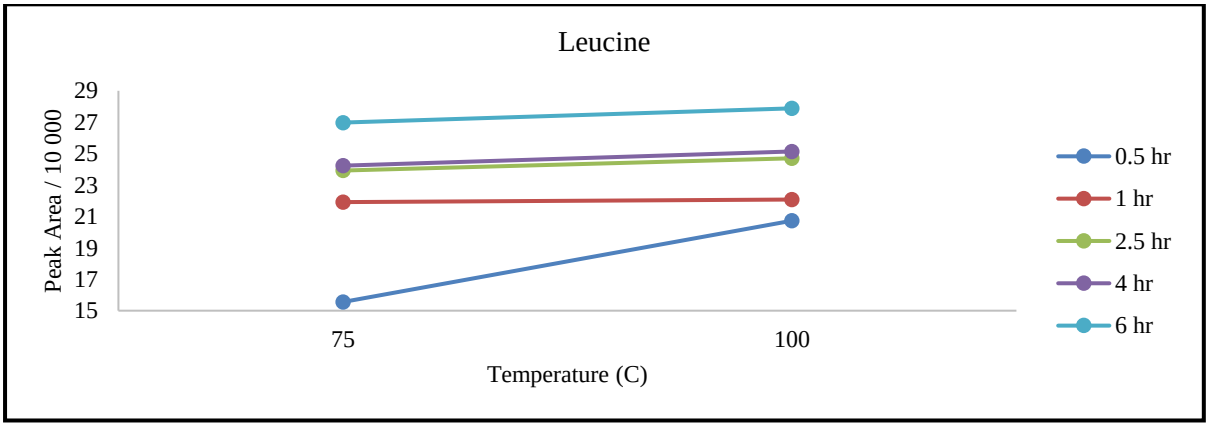


Figure 4-7: Leucine temperature interactions plots used to assess interactions between time and temperature.

It is important to note that in all parts of this study, all visual observations were verified statistically. This was done to ensure that, (1) all visual observations are significant enough to have not occurred by chance and (2) to ensure that well informed conclusions are made about each result. In the case of derivatisation time and derivatisation temperature, because these two factors were optimised simultaneously a two-way ANOVA was proposed for the assessment of these results. Initially, interactions between the levels of the factors in question had to be assessed using interactions plots in **Figure 4-6** and **Figure 4-7**; and in **Figures 6-3** to **Figure 6- 9** in **Appendix A3** prior to the application of a two-way ANOVA. Based on the

interactions plots of all amino acids, responses between the time and temperature levels were not parallel which signified that the two factors were interdependent or in simple terms, there were interactions between these factors.

Table 4-1: F-interactions values for all amino acids obtained by a two-way ANOVA

Amino Acids	$F\ (Interactions)$	$F\ (Critical)$	v
Alanine	7.41	2.61	4
Glycine	12.76	2.61	4
Valine	5.85	2.61	4
Leucine	5.98	2.61	4
Isoleucine	5.63	2.61	4
Proline	8.28	2.61	4
Serine	3.27	2.61	4
Threonine	6.00	2.61	4
Phenylalanine	5.34	2.61	4
Aspartic Acid	6.84	2.61	4
Glutamic Acid	6.45	2.61	4
Lysine	6.69	2.61	4
Histidine	5.73	2.61	4
Tyrosine	6.18	2.61	4

v = the number of degrees of freedom

To confirm this observation, the significance of these interactions was further assessed statistically at a 95% confidence level, the results thereof are presented in **Table 4-1**. For this test, the following null and alternative hypotheses were made:

H_0: there are no significant interactions between the derivatisation time and the derivatisation temperature.

H_a: there are signification interactions between the derivatisation time and derivatisation temperature.

As seen in **Table 4-1**, all the values of F (interactions) for all amino acids were greater than F (critical) meaning all the observed interactions were significant. Therefore, the null hypothesis was rejected, the alternative hypothesis was accepted. For this reason, further statistical analysis was not applicable due to the interlinked effects of time and temperature. Therefore, to select the best derivatisation temperature and derivatisation period we revisited

the paired means plots that were initially used to visually examine the effects of the factors in question. As previously discussed, derivatisation of amino acids at 100 ˚C for 4 hours yielded the lowest uncertainty compared to other conditions. Therefore, these conditions were identified as the optimum derivatisation conditions and were in turn used throughout the study.

4.1.3 Reconstitution solvent optimisation

To find the most appropriate reconstitution solvent between acetonitrile and isooctane for analysis of MTBSTFA derivatised amino acids by GC- TOFMS, solvent optimisation samples were prepared as outlined in **Chapter 3**. As in the previous section, paired means plots (**Figure 4-8)** were initially used to visually examine the differences between analyte responses in acetonitrile and in isooctane.

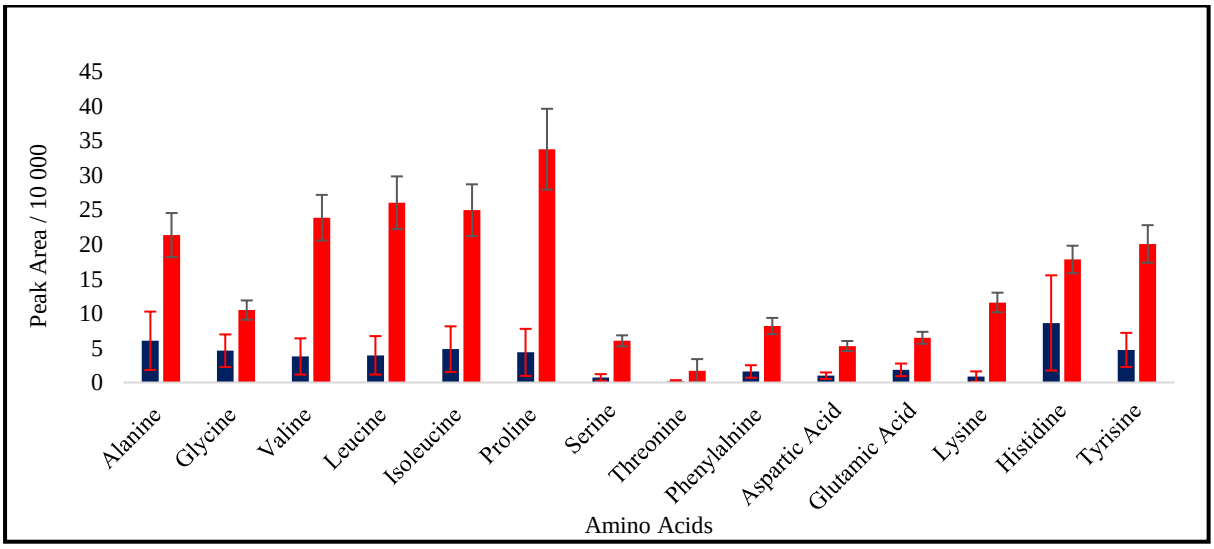


Figure 4-8: Paired means (n = 5) plots used to compare the responses of MTBSTFA-amino acids derivatives reconstituted in acetonitrile and isooctane to determine the most appropriate reconstitution solvent for analysis of amino acids on a GC-TOFMS system. The blue bars represent mean responses of amino acids in isooctane while the red bars represent mean responses of amino acids in acetonitrile.

Based the visual inspection of **Figure 4-8**, acetonitrile was seemingly the most appropriate reconstitution solvent for analysis of MTBSTFA derivatised amino acids because it gave a better analyte response compared to isooctane which in turn resulted in signal suppression. To confirm this observation, at a 95% confidence level a two-sided students t-test was used to

statistically assess the significance of the visually observed differences using **Equation 3-2** where sample variances did not differ significantly and **Equation 3-3** where the opposite was true. Prior to the application of the student's t-test, an F-test was performed to assess the significance of differences between sample variances using **Equation 3-4**.

Table 4-2: F-test and t-test results used to support the use of either acetonitrile or isooctane as reconstitution solvents

Amino Acids	$F\ (Experimental)$	$t\ (Experimental)$	$v\ (effective)$
Alanine	$F(4,4) = 1.76\ p < 0.05$	6.46	8
Glycine	$F(4,4) = 2.84\ p < 0.05$	4.79	8
Valine	$F(4,4) = 1.59\ p < 0.05$	10.64	8
Leucine	$F(4,4) = 1.88\ p < 0.05$	10.44	8
Isoleucine	$F(4,4) = 1.29\ p < 0.05$	9.02	8
Proline	$F(4,4) = 2.95\ p < 0.05$	9.71	8
Serine	$F(4,4) = 2.83\ p < 0.05$	12.82	8
Threonine	$F(4,4) = 102.09\ p < 0.05$	2.03	4
Phenylalanine	$F(4,4) = 1.69\ p < 0.05$	9.96	8
Aspartic Acid	$F(4,4) = 2.40\ p < 0.05$	11.09	8
Glutamic Acid	$F(4,4) = 1.11\ p < 0.05$	8.26	8
Lysine	$F(4,4) = 3.58\ p < 0.05$	14.94	8
Histidine	$F(4,4) = 12.02\ p < 0.05$	2.93	4
Tyrosine	$F(4,4) = 1.49\ p < 0.05$	9.33	8

$v\ (effective)$ = the effective number of degrees of freedom

For the F-test, the following null and alternative hypotheses were made.

H_0: $S^2_{analyte\ in\ acetonitrile} = S^2_{analyte\ in\ isooctane}$

H_a: $S^2_{analyte\ in\ acetonitrile} \neq S^2_{analyte\ in\ isooctane}$

Where S^2 = the variance of each analyte in the sample.

For the t-test, the following null and alternative hypotheses were made:

H_0: $\mu_{analyte\ in\ acetonitrile} = \mu_{analyte\ in\ isooctane}$

H_a: $\mu_{analyte\ in\ acetonitrile} \neq \mu_{analyte\ in\ isooctane}$

μ = the mean of each analyte in the sample.

At a 95% confidence level and with respect to all amino acids, the critical value of (F) was 9.61 while the critical values of (t) were 2.31 and 2.78 for 8 and 4 effective degrees of freedom respectively as shown in **Table 4-2**. As seen in **Table 4-2**, all experimental (F) values were below the critical value except for the experimental (F) values of threonine and histidine. Therefore, the F-test null hypothesis was accepted with respect to all amino acids except for threonine and histidine for which the alternative hypothesis was accepted. From the t-test results, all experimental t-values were greater than critical (t) values except for the experimental t-value of threonine. Therefore, all the observed differences between the two solvent systems were significant except with respect to threonine. The (t) test results in **Table 4-2**, were in good agreement with the visual observations from **Figure 4-8**. Acetonitrile was identified as the most appropriate solvent for analysis of MTBSTFA derivatised amino acids compared to isooctane. Consequently, acetonitrile was used throughout this study.

4.1.4 MTBSTFA Derivatives Stability Tests

For the MTBSTFA derivatised amino acids stability test samples were prepared and analysed as outlined in **Chapter 3**. As in the previously discussed optimisation studies, here means plots depicted in **Figure 4-9, Figure 4-10** and in **Figures 6-10** to **6-13** in **Appendix A4** were also initially used to visually assess the differences between daily means prior to the application of a one-way ANOVA in conjunction with Tukey HSD post hoc multicomparison statistics to assess the significance of the observed differences using **Equation 3-5** and **Equation 3-6** respectively. As depicted in **Figure 4-9**, day 2 to day 5 mean responses of proline and alanine under both storage conditions were visually different from the mean responses observed on day 1. This observation was true for all analytes except for glycine under cool storage conditions where the difference between day 1 mean response and other daily mean responses was much smaller compared to other analytes as seen in **Figure 6-10** in **Appendix A4**. Other than day 1 mean responses, the differences between daily mean responses for all derivatives were minimal except for histidine in the refrigerated samples and tyrosine in both the refrigerated and non-refrigerated samples as depicted in **Figure 4-10**.

From **Figure 4-10**, the day 5 mean response of histidine in the refrigerated sample was visibly lower than the responses observed on any other day. Similarly, as also seen in **Figure 4-10**, day 5 mean responses of tyrosine in both the refrigerated sample and the sample stored at ambient temperature were visibly lower than responses observed on any other day. By visual

inspection of the figures (**Figures 4-9** and **4-10**) presented and discussed under this subsection and the additional **Figures 6-10** to **6-13** presented in the appendices section, most MTBSTFA derivatives were stable over a period of 5 days except for histidine and tyrosine. As briefly discussed earlier in this subsection, the significance of the differences between the daily means was assessed using a one-way ANOVA and the results thereof are presented in **Table 4-3**.

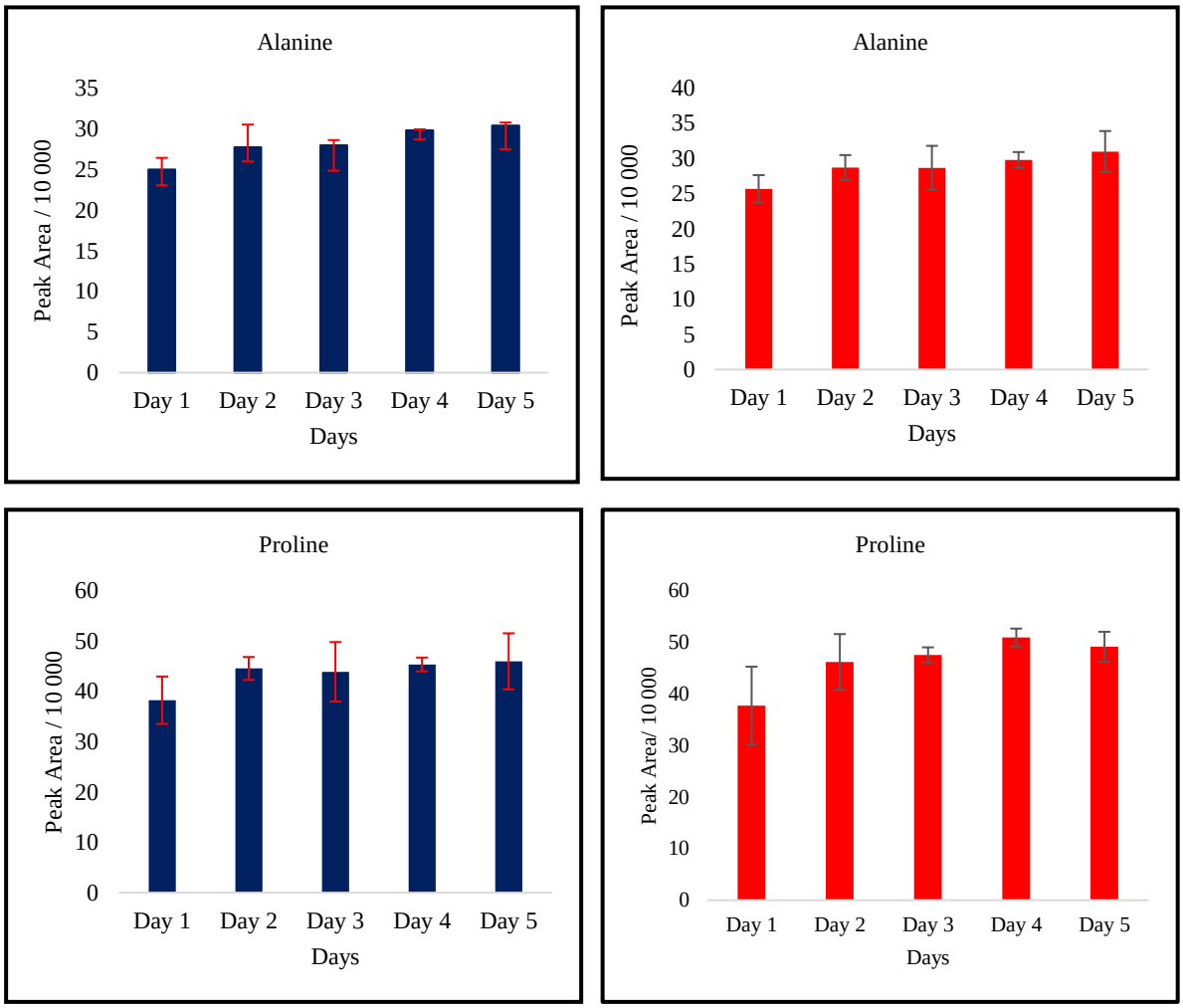


Figure 4-9: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure includes the observations made from proline and alanine stored at ambient and a cool temperature of 3 ˚C. The blue bars represent samples stored at ambient temperature while the red bars represent samples stored at 3 ˚C.

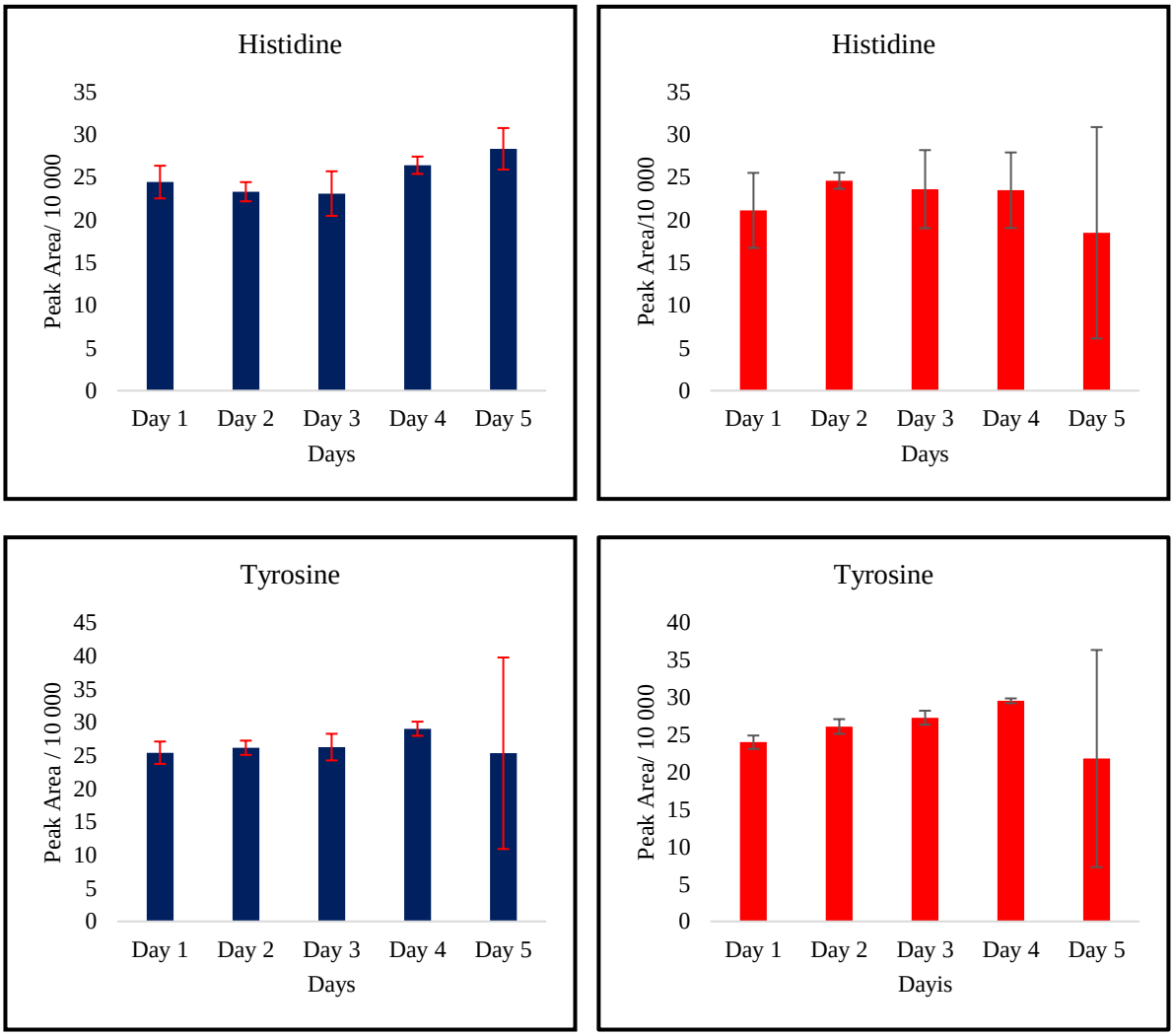


Figure 4-10: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure includes the observations made from histidine and tyrosine stored at ambient and a cool temperature of 3 ˚C. The blue bars represent samples stored at ambient temperature while the red bars represent samples stored at 3 ˚C.

In the derivatives stability test the single-factor ANOVA was applied as we were interested only in the variation that was due to one factor which was the storage condition. The variation due to the day factor was not assessed since the samples were different as discussed in **Chapter 3**. Therefore, samples stored under the two storage conditions discussed herein were treated independently of each other. The ANOVA test was performed at 95% significance level based on the following null and alternative hypotheses:

For the analysis of variance, the following null and alternative hypotheses were made:

H_0: $\mu_{day1} = \mu_{day\ 2} \ldots = \mu_{day\ n}$

H_a: at least $\mu_{day\ i} \neq \mu_{day\ k}$

$\mu_{day\ n}$ = the mean of the last analysis day, $\mu_{day\ i}$ and $\mu_{day\ k}$ = variables representing any of the daily means that might differ significantly.

Table 4-3: Single-factor ANOVA and Tukey (HSD) results used to determine the stability of MTBSTFA derivatives over a period of 5 days

Amino Acids	$F\ (ANOVA)\ Ambient$	$HSD\ Ambient$	$F\ (ANOVA)\ Refrigerated$	$HSD\ Refrigerated$
Alanine	$F(4,20) = 3.66\ p < 0.05$	4.34	$F(4,15) = 8.70\ p < 0.05$	3.04
Glycine	$F(4,20) = 0.77\ p < 0.05$	3.26	$F(4,15) = 0.51\ p < 0.05$	3.81
Valine	$F(4,20) = 7.28\ p < 0.05$	4.29	$F(4,15) = 8.86\ p < 0.05$	3.68
Leucine	$F(4,20) = 7.40\ p < 0.05$	4.92	$F(4,15) = 9.56\ p < 0.05$	3.93
Isoleucine	$F(4,20) = 7.46\ p < 0.05$	4.81	$F(4,15) = 10.83\ p < 0.05$	3.65
Proline	$F(4,20) = 2.51\ p < 0.05$	8.25	$F(4,15) = 5.24\ p < 0.05$	9.44
Serine	$F(4,20) = 9.48\ p < 0.05$	1.19	$F(4,15) = 18.33\ p < 0.05$	0.86
Threonine	$F(4,20) = 6.53\ p < 0.05$	1.99	$F(4,15) = 4.12\ p < 0.05$	2.52
Phenylalanine	$F(4,20) = 6.85\ p < 0.05$	1.47	$F(4,15) = 15.95\ p < 0.05$	0.82
Aspartic Acid	$F(4,20) = 6.03\ p < 0.05$	0.91	$F(4,15) = 13.17\ p < 0.05$	0.66
Glutamic Acid	$F(4,20) = 6.12\ p < 0.05$	1.27	$F(4,15) = 16.82\ p < 0.05$	0.67
Lysine	$F(4,20) = 1.52\ p < 0.05$	2.78	$F(4,15) = 1.82\ p < 0.05$	4.08
Histidine	$F(4,20) = 6.65\ p < 0.05$	3.66	$F(4,15) = 12.02\ p < 0.05$	13.84
Tyrosine	$F(4,20) = 0.26\ p < 0.05$	12.47	$F(4,15) = 0.83\ p < 0.05$	13.84.

HSD = honest significant difference

At a 95% confidence level, the critical values of F-ANOVA were 2.87 and 3.06 for samples stored at ambient temperature and at 3 ˚C respectively. From the ANOVA results presented in **Table 4-3**, results from both storage conditions showed that there were significant differences between some of the daily means for all amino acids except for lysine, glycine, proline, and tyrosine. Consequently, the null hypothesis was accepted with respect to the above-mentioned analytes and respect with respect to all others. Using a Tukey honest significance test, all means were compared to identify the sources of the differences. From the Tukey test, the calculated differences between mean pairs were compared with the honest significant difference (HSD) values also shown in **Table 4-3.** Additionally, Tukey test results were also graphically presented on "HSD charts" in **Figure 4-10** and **Figures 6-14 to 6-20** in **Appendix A5**. On "HSD charts" daily means were compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Therefore, each point of the "HSD chart" represents

the differences between a pair of means starting from the left to right. Additionally, all points appearing above the HSD line shows pairs of means that differ significantly.

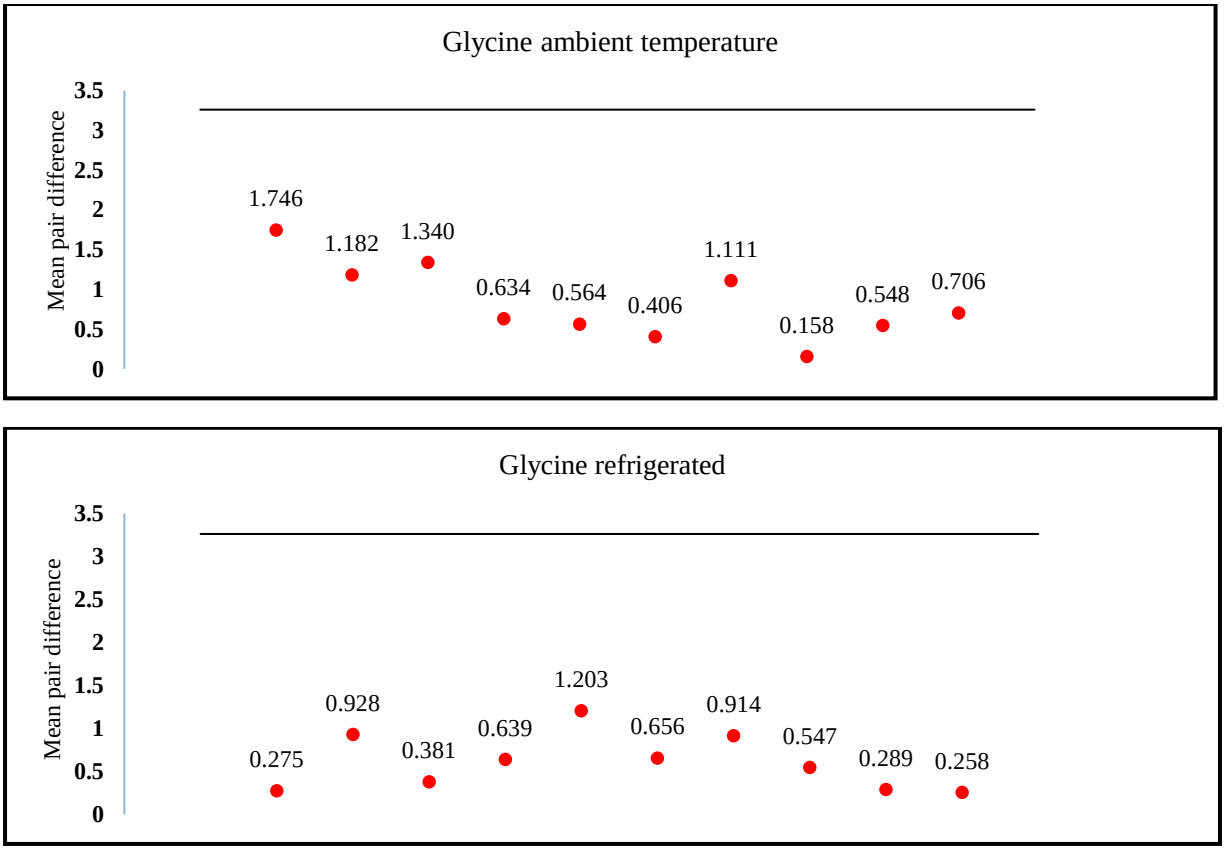


Figure 4-11: Honest significant difference (HSD) chart used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared.

In general, most significant differences were found between day 1 and day 4; day 1 and day 5 as were also seen on the means plots **(Figures 6-14 to 6-20).** From HSD charts, some amino acids showed varying stability under different storage conditions. Glycine, lysine, and tyrosine were stable under both storage conditions (e.g. **Figure 4-11**). Alanine, valine, and leucine were stable under ambient and cooled storage conditions, while isoleucine, proline, phenylalanine, aspartic acid and glutamic acid were stable under ambient conditions. Serine, threonine, and proline showed better stability at 3 ˚C. In general, storing MTBSTFA derivatives at ambient temperature over a period of 5 days will result in losses of proline, threonine, and serine. However, a loss of a greater number of compounds is incurred when derivatives are stored at 3 ˚C. Therefore, MTBSTFA derivatives are more stable at room temperature than at 3 ˚C and should be analysed within 5 days after preparation to ascertain the accuracy of the method.

4.2 GC-TOFMS Method Validation

As defined in the Eurachem's Laboratory Guide to Method Validation and Related Topics, *"method validation is confirmation through the provision of objective evidence, that the method's requirements for a specific intended use or application have been fulfilled*". As outlined by (Tarveniers *et al.*, 2004) before the method is validated, the scope of validation must be defined and fixed comprising of both analytical system and analytical requirements. In this study, method validation was performed in accordance with Eurachem's guide to method validation as per the validation scope outlined in **Chapter 1** (Magnusson, 2014, Jacobs and Feng, 2015). For this purpose, method performance characteristics such as instrument and method linearity (working range); limit of detection (LOD), limit of quantification (LOQ), precision, bias, specificity, the uncertainty of measurement and method's ruggedness were evaluated. The instrument working range was assessed by plotting the calibration standards concentrations against the instrument response followed by visual inspection of the calibration plots to identify the concentration at which effects, such as plateauing, occurred as depicted in **Figure 4-15**. To assess regression line bias and to confirm linearity, y-residuals plots such as **Figure 4-16** were used.

Limits of detection and limits of quantification were calculated in accordance with the guidelines provided in Statistics and Chemometrics for Analytical Chemistry 6th edition using **Equation 3-8** and **Equation 3-9** respectively (Miller and Miller, 2005). As stipulated in the AOAC guide for the development of methods for quantifying amino acids in infant formula, the limit of quantification should not exceed 0.4 g/100g (Jacobs and Feng, 2015). Precision and bias parameters namely relative standard deviation %RSD or coefficient of variation (CV) and % recovery (accuracy) were calculated and evaluated in accordance with the AOAC specifications stipulated in the *Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance*; and the AOAC specification for the development of methods for quantification of amino acids in infant formula published in 2015 (Taverniers *et al.*, 2004, Jacobs and Feng, 2015). In general, % recoveries of between 80 and 120% are mandatory for any analytical method (Green, 1996). As outlined in **Chapter 1**, according to the AOAC specifications for development of methods for quantifying amino acids in an infant formula the coefficient of variation should not exceed 4% (Jacobs and Feng, 2015). As part of method validation, analyte selectivity was evaluated using 3 methods, (1) by comparing samples and pure standards in terms of analyte retention times, (2) comparing ion ratios of

samples to that of the pure standard and (3) using the 70% percentage NIST MS library match criteria as specified in **Chapter 3**. Furthermore, GC×GC-TOFMS was used to test the NIST SRM for interferences.

Also, as part of method validation method ruggedness was assessed. To do this, the results obtained using the GC-TOFMS method developed in this study were compared with the results obtained using the Waters AccQ·Tag method and the results obtained using an alternative Pegasus IV GC-TOFMS system as outlined in **Chapter 3**. The results obtained using the Pegasus IV system were used to assess GC-TOFMS method transferability i.e. to test if the results obtained using one GC-MS system can be reproduced using any other GC-MS system. There were two major differences between the Pegasus III GC-TOFMS system used to develop the method and the Pegasus IV GC-TOFMS used to assess method's transferability, (1) Pegasus IV system as newer model is much more sensitive compared to the Pegasus III and (2) the column used in the Pegasus IV was 30 m long with 0.25 mm internal diameter and 0.25 μm film thickness while the column used on the Pegasus III system was 15 m long with 0.18 mm internal diameter 0.18 μm film thickness. On the other hand, the Waters AccQ·Tag method was used to assess the GC-TOFMS method's performance against one of the commonly used methods for quantification of protein via amino acids analysis.

4.2.1 Optimisation of the SOF method for method validation

For all method validation studies performed in this study, the speed optimised flow rate method discussed in **Chapter 3** was used for the separation of analytes of interest. For method validation purposes, analyte separation on a speed optimised flow rate method was optimised by injecting an AAS18 standard (2.5 nmol/mL) derivatised at 100 ˚C for 4 hours using MTBSTFA and had been reconstituted in acetonitrile. Using this method, ornithine and 15 amino acids namely, alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, methionine, phenylalanine, aspartic acid glutamic acid, lysine, histidine and tyrosine together with 4 additional analytes taurine, methionine sulfone, tryptophan and cysteic acid were resolved within 12.5 minutes as shown in **Figure 4-12**. As discussed in **Chapter 2**, because cystine and methionine are normally quantified as cysteic acid and methionine sulfone respectively; and taurine is a nonproteinogenic "amino acid" also found in milk, these additional analytes together with tryptophan were deliberately added into the test sample to test

if their presence in a real sample during quantitative analysis could result in any coelutions. From **Figure 4-12**, all additional analytes including cysteic acid, taurine and tryptophan were well resolved except for methionine sulfone which eluted close to glutamic acid and co-eluted with ornithine. As seen in the exploded view in **Figure 4-12**, ornithine and methionine sulfone peaks could only be separated by mass deconvolution using unique masses 184 and 352 m/z for ornithine and methionine sulfone respectively.

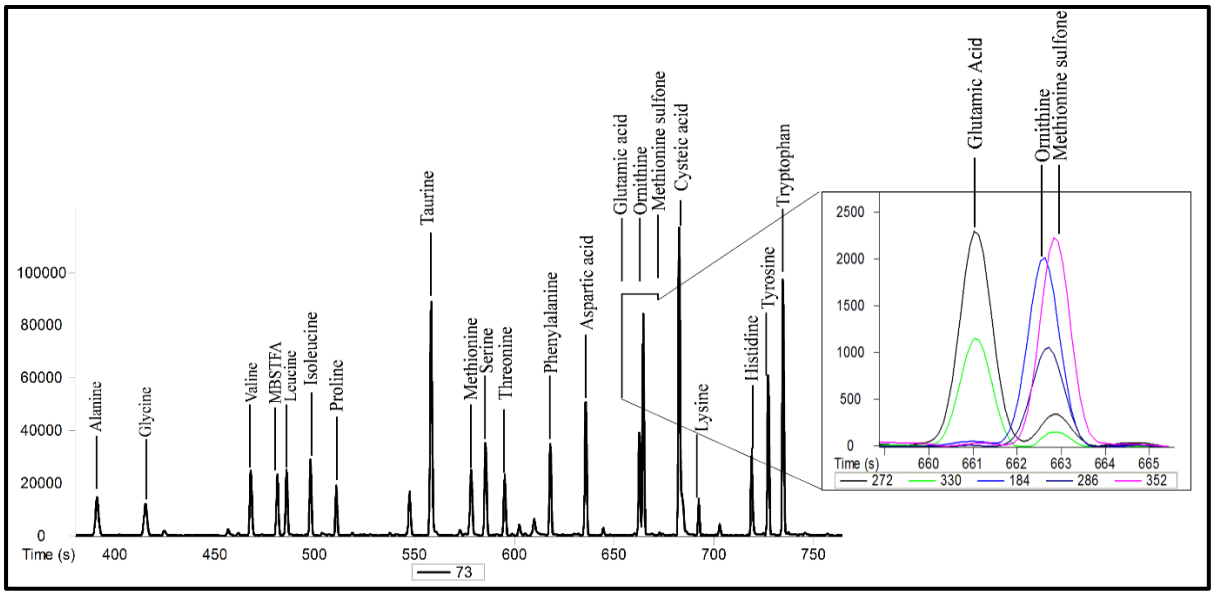


Figure 4-12: Reconstructed ion chromatogram (73 m/z) (exploded view of Figure 4-12 RIC at, 272, 330, 244, 286 and 184 m/z) obtained using the SOF method when a pure amino acid standard (AA-S-18) (2.5 nmol/mL) derivatised with MTBSTFA was analysed on the Pegasus III GC-TOFMS system. The optimised flow rate of 1.2 mL/min was used. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. The run time was 12.5 minutes

As in the preceding section (**section 4-1**) visual inspection together with statistical verification of the visual observations were used to analyse method validation results. Where applicable, method validation results were compared with the values specified in the method development specification published by the AOAC for the development of methods to be used for quantification of amino acids in infant formula (Jacobs and Feng, 2015). In addition, the uncertainty of measurement of the GC-TOFMS method developed in this study was also evaluated. This was done as the last step in gauging the performance of the GC-TOFMS method developed in this study against the certificate values on the SRM certificate.

4.2.2 Method accuracy and precision tests using the NIST SRM

Table 4-4: Pegasus III GC-TOFMS method calibration parameters obtained by external calibration approach

Amino Acids	Linear regression equation	Quant Ion (m/z)	r	r^2	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}(g/100g)$	$Sa\ (g/100g)$	Sb (g/100g)
Alanine	$y = 1095.3x - 12435$	158	0.9998	0.9997	0.01780	0.0594	0.0805	0.0799	0.0746
Glycine	$y = 567.02x - 1658.4$	218	0.9994	0.9988	0.03740	0.1246	0.0287	0.0250	0.0163
Valine	$y = 1341.2x - 15672$	186	0.9996	0.9992	0.04370	0.1456	0.1156	0.1122	0.1012
Leucine	$y = 1290.8x - 16750$	200	0.9997	0.9994	0.04080	0.1359	0.1392	0.1375	0.1258
Isoleucine	$y = 1273.4x - 14543$	200	0.9999	0.9997	0.03290	0.1097	0.1215	0.1193	0.1106
Proline	$y = 1877.9x - 6270.4$	184	0.9995	0.9990	0.06130	0.2045	0.0488	0.0420	0.0285
Serine	$y = 243.9x - 2302.7$	288	0.9997	0.9994	0.03340	0.1112	0.0843	0.0821	0.0733
Threonine	$y = 116.52x + 863.11$	303	0.9990	0.9979	0.09350	0.3116	0.0340	0.0438	0.0649
Phenylalanine	$y = 373.68x - 2601.5$	234	0.9990	0.9981	0.10640	0.3548	0.1204	0.1141	0.0852
Aspartic Acid	$y = 230.02x - 1815.9$	302	1.0000	1.0000	0.01110	0.03710	0.0813	0.0806	0.0776
Glutamic Acid	$y = 263.31x - 306.37$	432	0.9994	0.9989	0.07910	0.2638	0.0390	0.0305	0.0128
Lysine	$y = 411.62x - 465.91$	198	0.9998	0.9995	0.05470	0.1825	0.0305	0.0244	0.0244
Histidine	$y = 998.85x - 1720.2$	196	0.9995	0.9991	0.06290	0.2098	0.0407	0.0332	0.0199
Tyrosine	$y = 900.37x - 842.11$	302	0.9997	0.9995	0.06770	0.2258	0.0351	0.0281	0.0127

Quant ion = ion used for quantification, r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard error of the regression line slope.

Table 4-5: Pegasus III GC-TOFMS method calibration parameters obtained by isotope dilution approach

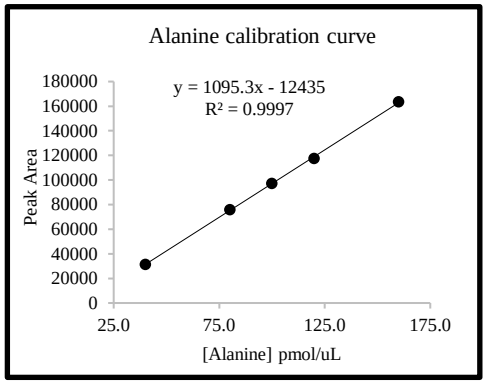
Amino Acids	Linear regression equation	Quant Ion (m/z)	Internal standard	r	r^2	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}\ (g/100g)$	$Sa\ (g/100g)$	$Sb\ (g/100g)$
Alanine	$y = 0.0446x + 0.0079$	158	Valine	0.9993	0.9986	0.02710	0.0902	0.0250	0.0274	0.0298
Glycine	$y = 0.0256x + 0.0042$	218	Valine	0.9996	0.9992	0.01970	0.0656	0.0198	0.0224	0.0238
Valine	$y = 0.0595x + 0.0018$	186	Valine	0.9990	0.9981	0.06340	0.2113	0.0134	0.0059	0.0013
Leucine	$y = 0.0543x + 0.0074$	200	Isoleucine	0.9994	0.9987	0.05340	0.1779	0.0258	0.0258	0.0326
Isoleucine	$y = 0.0551x + 0.007$	200	Isoleucine	0.9990	0.9979	0.06850	0.2283	0.0128	0.0195	0.0282
Proline	$y = 0.0515x + 0.0105$	184	Proline	0.9999	0.9997	0.1434	0.4779	0.2913	0.3100	0.3232
Serine	$y = 0.0075x + 0.0017$	288	Proline	1.0000	0.9999	0.08170	0.2725	0.3099	0.3196	0.3289
Threonine	$y = 0.0225x + 0.005$	303	Phenylalanine	0.9986	0.9973	0.1478	0.4927	0.1284	0.1335	0.1533
Phenylalanine	$y = 0.052x + 0.0403$	234	Phenylalanine	0.9989	0.9979	0.08820	0.2939	0.2454	0.2566	0.2653
Aspartic Acid	$y = 0.039x + 0.125$	302	Phenylalanine	0.9995	0.9990	0.1290	0.4300	0.2457	0.2577	0.2724
Glutamic Acid	$y = 0.0494x + 0.0136$	432	Phenylalanine	0.9999	0.9999	0.01950	0.06510	0.0805	0.0827	0.0849
Lysine	$y = 0.0754x - 0.0173$	198	Phenylalanine	0.9997	0.9995	0.0431	0.1436	0.0578	0.0638	0.0638
Histidine	$y = 0.4648x + 0.0640$	196	Phenylalanine	0.9980	0.9959	0.07320	0.2441	0.0271	0.0179	0.0464
Tyrosine	$y = 0.0284x + 0.0096$	302	Phenylalanine	0.9987	0.9973	0.2456	0.8186	0.1986	0.2277	0.2557

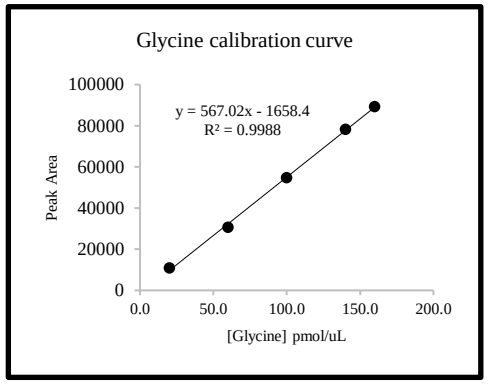
Quant ion = ion used for quantification, r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard error of the regression line slope.

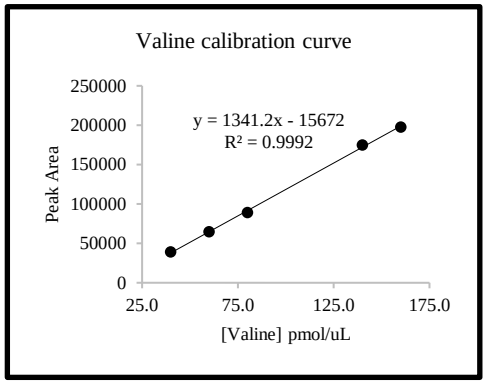
Method validation using the NIST SRM was performed by both external and isotope dilution calibration approaches as outlined in **Chapter 3**. From the results of each calibration approach, performance parameters such LODs, LOQs and coefficients of variation were evaluated as outlined at the beginning of this section and the results thereof are presented in **Table 4-4** for external calibration and **Table 4-5** for isotope dilution. Additionally, calibration curves and y-residuals plots from which external calibration results presented in **Table 4-4** are based, are presented in **Figure 4-13** and **Figure 6-21** in **Appendix B1** respectively. The calibration curves and y-residuals plots from which the isotope dilution results are based are presented in **Figure 6-22** and **Figure 6-23** in Appendix B1 in the appendices section.

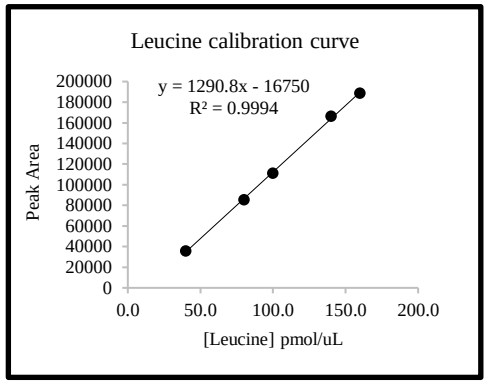
As depicted in **Figure 4-13** and **Figure 6-22**, calibration curves obtained by both calibration approaches were linear. Correlation coefficients ranging from 0.9988 to 1.0000 were obtained by external calibration while correlation coefficients ranging from 0.9959 to 0.9999 were obtained by isotope dilution as seen in **Table 4-4** and **Table 4-5** respectively. By visual inspection of the y-residuals plots in **Figure 6-21** and **Figure 6-23**, there was no bias in any of the regression curves since all points are randomly scattered about the origin (zero line). Based on the results presented in **Table 4-4** and **Table 4-5**, the external calibration method yielded better linearity compared to the isotope dilution method. The differences between the correlation coefficients obtained through these methods may be attributed to the fact that isotope dilution linearity is isotope dependent i.e. two different isotopes with equal concentration may yield different correlation coefficients depending on how closely each isotope mimics the native analyte. This is because instrument response may differ due to the identity of the isotope irrespective of the isotope concentration. For best results, the best practice would be to use the isotopically labeled form of each of the native analytes. Using the isotopically labeled form of the native analyte will ascertain the best comparability in terms of (1) retention of both the native and the isotopically labeled analyte on the stationary phase of the column and (2) instrument response with respect to the change in concentration with respect to both native and the isotopically labeled analytes.

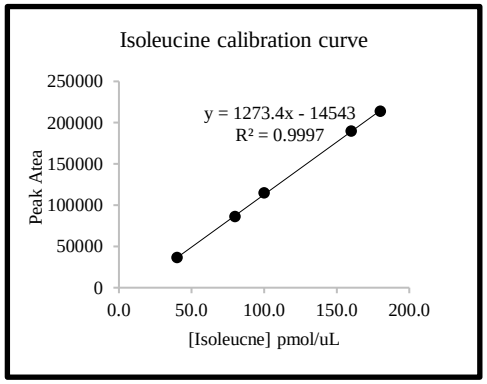
As specified by the AOAC, the limits of quantification for methods developed for the purpose of quantifying amino acids in an infant formula should not exceed 0,4 g/100g. As seen in **Table 4-4**, **Table 4-5** and in **Figure 4-14**, the LOQs obtained using both the isotope dilution method and the external calibration method were generally lower than the AOAC specified limit except the LOQs of proline, threonine, aspartic acid and tyrosine obtained by isotope dilution.

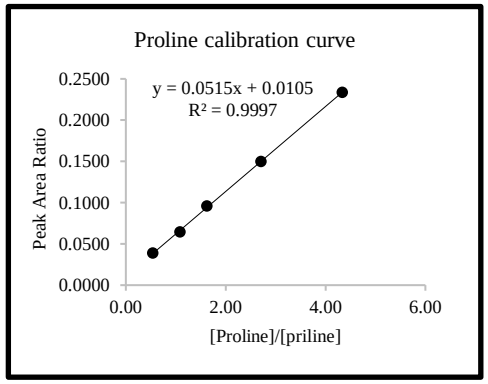

Alanine calibration curve
y = 1095.3x - 12435
R² = 0.9997
Peak Area
[Alanine] pmol/uL

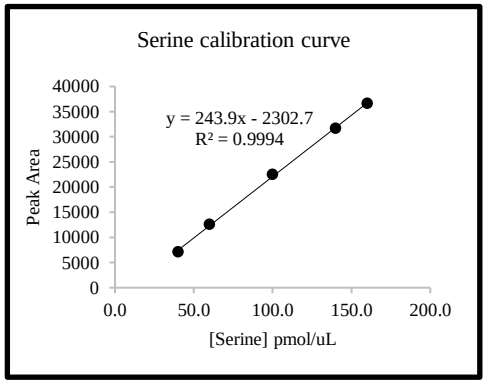

Glycine calibration curve
y = 567.02x - 1658.4
R² = 0.9988
Peak Area
[Glycine] pmol/uL

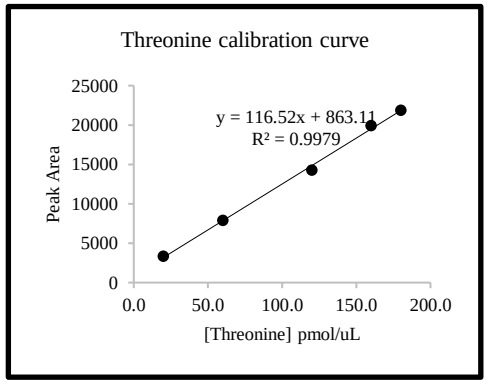

Valine calibration curve
y = 1341.2x - 15672
R² = 0.9992
Peak Area
[Valine] pmol/uL

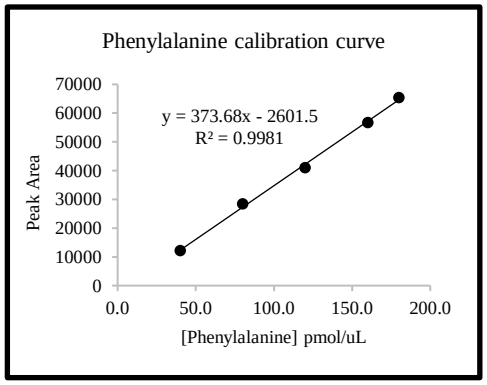

Leucine calibration curve
y = 1290.8x - 16750
R² = 0.9994
Peak Area
[Leucine] pmol/uL

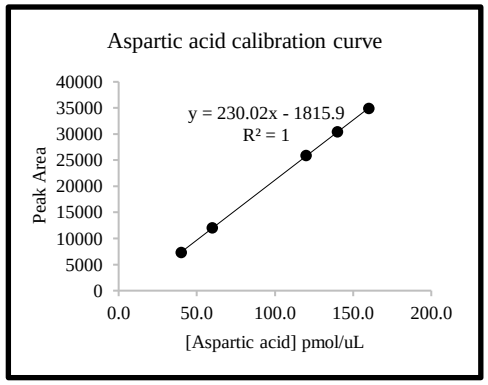

Isoleucine calibration curve
y = 1273.4x - 14543
R² = 0.9997
Peak Atea
[Isoleucne] pmol/uL

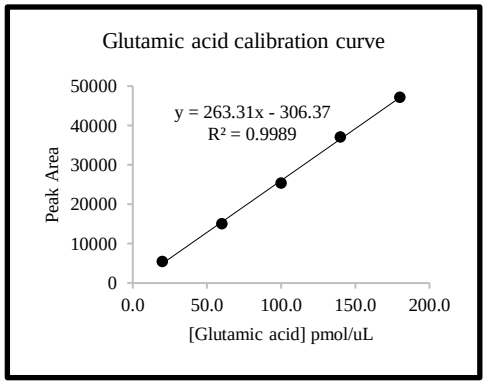

Proline calibration curve
y = 0.0515x + 0.0105
R² = 0.9997
Peak Area Ratio
[Proline]/[priline]

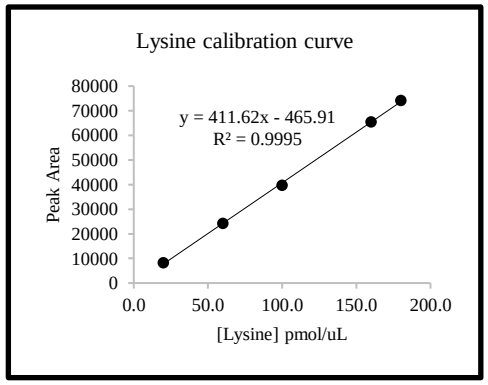

Serine calibration curve
y = 243.9x - 2302.7
R² = 0.9994
Peak Area
[Serine] pmol/uL


Threonine calibration curve
y = 116.52x + 863.11
R² = 0.9979
Peak Area
[Threonine] pmol/uL


Phenylalanine calibration curve
y = 373.68x - 2601.5
R² = 0.9981
Peak Area
[Phenylalanine] pmol/uL


Aspartic acid calibration curve
y = 230.02x - 1815.9
R² = 1
Peak Area
[Aspartic acid] pmol/uL


Glutamic acid calibration curve
y = 263.31x - 306.37
R² = 0.9989
Peak Area
[Glutamic acid] pmol/uL


Lysine calibration curve
y = 411.62x - 465.91
R² = 0.9995
Peak Area
[Lysine] pmol/uL

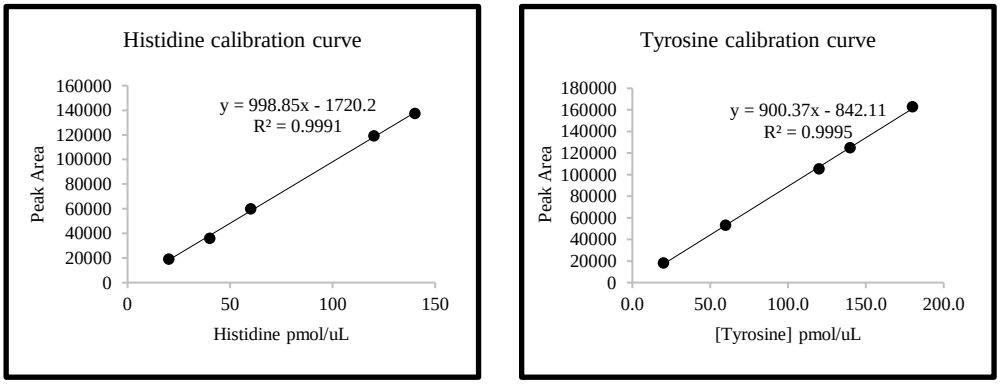


Figure 4-13: External calibration regression curves obtained by the SOF method also used for method linearity test. Calibration points are based on quantification ions presented in Table 4-3 and 4-4 i.e.158, 218, 186, 200, 200, 184, 288, 303, 234, 302, 432, 198, 196 and 302 m/z for alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, histidine and tyrosine respectively. The optimised flow rate of 1.2 mL/min was used. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. The run time was 12.5 minutes.

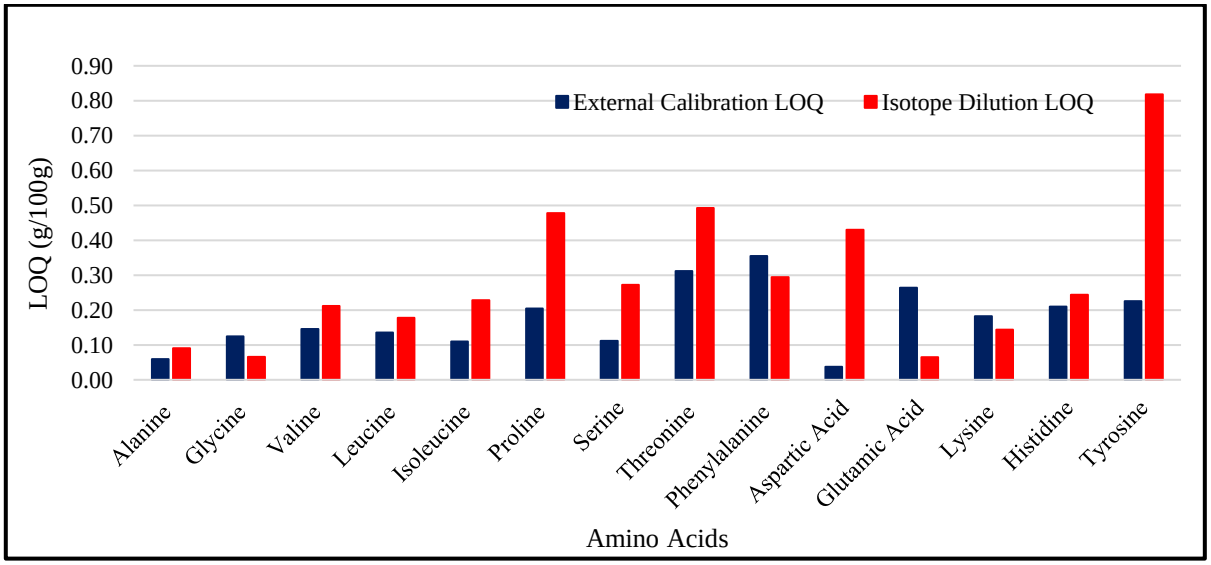


Figure 4-14: Comparison of LOQs obtained by external calibration against those obtained by isotope dilution methods. LOQs in Figure-414 were compared with the LOQ value of ≤ 0.4 g/100g specified by the AOAC for the development of methods for quantifying amino acids in infant formula.

Additionally, the LOQs obtained by external calibration were lower than LOQs obtained by isotope dilution as depicted in **Figure 4-14**. As seen in **Table 4-4**, using the external calibration method, LODs and LOQs ranging from 0.01110 g/100g to 0.1064 g/100g and from 0.03710 g/100g to 0.3348 g/100g were obtained respectively. Alternatively, from the isotope dilution method, LODs of between 0.01950 g/100g and 0.2456 g/100g and LOQs of between 0.06510 and 0.8186 g/100g were obtained. However, as seen in **Figure 4-14**, although the LOQs of

proline, threonine and aspartic acid obtained by isotope dilution are higher than the AOAC specified limit. These LOQs were lower than the concentrations of the above-mentioned analytes and therefore could still be used to quantify these analytes. On the other hand, the LOQ of tyrosine obtained through the isotope dilution method was higher than the concentration of this analyte in the NIST SRM.

Table 4-6: Pegasus III GC-TOFMS method performance parameters obtained by isotope dilution

Amino Acids	[AA] (g/100g)	S_{x0} (g/100g)	%Recovery	RSD	CI (g/100g)
Alanine	0.4669	0.1363	102.62	29.20	0.08660
Glycine	0.2100	0.031	87.30	14.71	0.03800
Valine	0.6758	0.1789	88.92	26.47	0.1137
Leucine	1.321	0.3684	104.76	27.88	0.2340
Isoleucine	0.6014	0.1530	91.12	25.43	0.09718
Proline	1.089	0.3402	90.87	31.32	0.2161
Serine	0.7119	0.2476	98.87	34.78	0.1573
Threonine	0.6531	0.0.2158	102.05	33.04	0.1371
Phenylalanine	0.4659	0.2525	80.32	54.21	0.1605
Aspartic Acid	1.0051	0.1868	93.94	18.59	0.1187
Glutamic Acid	2.3608	0.7390	91.15	31.30	0.4696
Lysine	0.740	0.307	73.31	41.53	0.382
Histidine	0.376	0.079	123.81	2.99	0.073
Tyrosine	0.4974	0.05179	97.53	10.41	0.03291

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by **Equation 3.7**, RSD = relative standard deviation and CI = confidence interval of the mean.

As a measure of method precision, the coefficients of variation obtained by both the external calibration method and the isotope dilution method were also assessed and the results thereof are presented in **Table 4-6** and **Table 4-**7 for the isotope dilution method and the external calibration method respectively. As seen in **Table 4-6** and **4-7**, %RSD obtained through the external calibration approach ranged from 6.28 to 25.76% while %RSDs ranging from 2.99 to 41.53% were obtained using the isotope dilution method. On average, %RSD obtained by isotope dilution were lower than coefficients of variation obtained by external calibration. However, using both calibration approaches the coefficients of variation were on average greater than 4% failing the limit specified by the AOAC for the development of similar methods except for the relative standard deviation of histidine obtained by isotope dilution as seen in **Table 4-6** (Jacobs and Feng, 2015). Because the internal standard was added after the hydrolysis process had been completed and the samples were derivatised under optimised

conditions. Variation due to the instrument may be ruled out as one of the key contributing factors to the observed between sample variation. Therefore, the plausible reason for precision not meeting the permissible value may be attributed to the volumetric preparation of analytical samples and the utilisation of small sample sizes. Therefore, in the future precision may be improved by (1) gravimetric preparation of analytical samples and (2) if economically viable the addition of the internal standard before hydrolysis in order to cater for variations due to the hydrolysis process.

Table 4-7: Pegasus III GC-TOFMS method performance parameters obtained by external calibration

Amino Acids	[AA] (g/100g)	S_{x0} (g/100g)	%Recovery	RSD	CI (g/100g)
Alanine	0.4732	0.05209	103.99	11.01	0.03310
Glycine	0.2133	0.02465	88.51	11.56	0.01566
Valine	0.6604	0.05582	86.89	8.45	0.03546
Leucine	1.214	0.1091	96.32	8.98	0.06930
Isoleucine	0.6113	0.1551	92.62	25.38	0.09857
Proline	0.7305	0.07675	61.13	10.50	0.04876
Serine	0.6068	0.03808	84.27	6.28	0.02419
Threonine	0.5864	0.08004	91.63	7.32	0.05086
Phenylalanine	0.6998	0.1803	120.66	25.76	0.1146
Aspartic Acid	0.9740	0.2082	92.09	21.38	0.1323
Glutamic Acid	2.4417	0.5098	94.28	20.88	0.3425
Lysine	0.809	0.328	80.11	40.59	0.408
Histidine	0.234	0.042	101.59	10.42	0.039
Tyrosine	0.4322	0.06655	84.74	15.40	0.04228

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by **Equation 3.7**, RSD = relative standard deviation and CI = confidence interval of the mean.

Also shown in **Table 4-6** and **Table 4-7** are analyte recoveries (accuracy). Recoveries are the measure of trueness or closeness of the experimental values to the true values reported in the NIST SRM certificate. Analyte recoveries ranging from 73.31% and 104.76% were obtained using the isotope dilution method while analyte recoveries of between 80.11% and 103.99% were obtained by external calibration as seen in **Table 4-6** and **Table 4-7** respectively. As seen in **Table 4-6** and **Table 4-7**, analyte recoveries obtained by the both the external calibration method and the isotope dilution method were well within the range of between 80% and 120% as specified by (Green, 1996) except for the recoveries of proline (61.13%) and histidine (120.66%) obtained through the external calibration method and lysine (73.31%) and phenylalanine (123.81%) obtained through the isotope dilution method as shown in **Table 4-6**

and **Table 4-7** respectively. Based on the AOAC spike recovery requirements, a spike recovery percentage of between 90 and 110% is expected. The experimental spike recoveries obtained in this study were 94.45%, 93.56%, 69.41% and 98.32% for ^{13}C isoleucine, ^{13}C valine, ^{13}C proline and ^{13}C phenylalanine respectively. These spike recoveries were well within the AOAC specified range of 90% and 110% except for the recovery of ^{13}C proline for an unknown reason.

Table 4-8: F and t-test results used to assess the significance of the differences between external and isotope dilution methods recoveries

Amino Acids	$F\ (Experimental)$	$F\ (Critical)$	$t\ (Experimental)$	$t\ (critical)$	v
Alanine	$F(11,11) = 6.85\ p < 0.05$	2.82	0.14	2.15	14
Glycine	$F(6,11) = 1.26\ p < 0.05$	3.20	0.19	2.12	16
Valine	$F(11,11) = 10.27\ p < 0.05$	2.82	0.28	2.16	13
Leucine	$F(11,11) = 11.41\ p < 0.05$	2.82	0.96	2.16	13
Isoleucine	$F(11,11) = 2.82\ p < 0.05$	1.03	0.15	2.07	22
Proline	$F(11,11) = 19.645\ p < 0.05$	2.98	3.38	2.20	11
Serine	$F(11,10) = 42.27\ p < 0.05$	2.94	1.45	2.18	12
Threonine	$F(9,9) = 7.27\ p < 0.05$	3.18	0.92	2.20	11
Phenylalanine	$F(11,11) = 1.96\ p < 0.05$	2.82	2.61	2.07	22
Aspartic Acid	$F(10,10) = 2.75\ p < 0.05$	2.98	0.30	2.08	20
Glutamic Acid	$F(11,10) = 2.10\ p < 0.05$	2.94	0.30	2.08	21
Lysine	$F(4,4) = 1.41\ p < 0.05$	9.61	0.34	2.31	8
Histidine	$F(4,4) = 5.04\ p < 0.05$	9.61	3.24	2.31	8
Tyrosine	$F(11,11) = 1.65\ p < 0.05$	2.82	2.68	2.07	22

$F(Experimental)$ = test the significance of differences between sample variances for isotope dilution and external calibration samples, $t\ (experimental)$ = t-test results for the differences between isotope dilution and external calibration means, $F\ (critical)$ = critical F value for the given number of degrees of freedom, $t\ (critical)$ = critical t value for the given number of degrees of freedom and v = number of degrees of freedom used in the t test.

To assess the significance of the differences between the isotope dilution and external calibration results, a two-sided students t-test was used, the results thereof are presented in **Table 4-8**. Prior to the application of the t-test, an F-test was performed to assess the significance of the differences between the variances of the two methods based on following null and alternative hypotheses:

H$_0$: $S^2_{external\ calibration} = S^2_{isotope\ dilution}$

$\mathbf{H_a}$: $S^2_{external\ calibration} \neq S^2_{isotope\ dilution}$

Where S^2 is the variance of each method.

To assess the significance of the differences between the means of these methods, the following null and alternative hypotheses were made:

$\mathbf{H_0}$: $\mu_{external\ calibration} = \mu_{isotope\ dilution}$

$\mathbf{H_a}$: $\mu_{external\ calibration} \neq \mu_{isotope\ dilution}$

Table 4-9: T-test results used to assess the significance of the differences between analyte recoveries obtained by external calibration and isotope dilution and the true values provided in the NIST SRM certificate

Amino Acids	$t\ (IS)$	$t\ (IS - critical)$	$t\ (NOIS)$	$t\ (NOIS - critical)$
Alanine	0.29	2.20	1.16	2.20
Glycine	6.51	2.78	3.89	2.20
Valine	1.63	2.20	6.18	2.23
Leucine	0.56	2.20	1.48	2.20
Isoleucine	1.33	2.20	1.09	2.20
Proline	1.06	2.23	20.07	2.23
Serine	0.11	2.20	9.86	2.23
Threonine	0.19	2.26	2.12	2.26
Phenylalanine	1.57	2.20	2.20	2.20
Aspartic Acid	1.15	2.23	2.18	2.23
Glutamic Acid	1.07	2.18	0.96	2.20
Lysine	1.46	2.78	1.84	2.78
Histidine	5.42	2.78	3.24	2.78
Tyrosine	0.84	2.20	4.05	2.20

$t\ (IS)$ = isotope dilution experimental t-value, $t\ (IS - critical)$ = critical t-value for the isotope dilution based on the number of degrees of freedom provided in **Table 4-8**, t (NOIS) = external calibration experimental t-value and , $t\ (NOIS - critical)$ = critical t-value for the external dilution method based on the number of degrees of freedom provided in **Table 4-8.**

Furthermore, experimental means obtained by both the isotope dilution method and the external calibration method were compared with the true values reported on the NIST SRM and the results thereof are shown in **Table 4-9**. To assess the significance of the differences between

experimental means obtained through the external calibration approach and the true values provided in the SRM certificate, the following null, and alternative hypotheses were made:

H_0: $\mu_{external\ calibration} = \mu_0$

H_a: $\mu_{external\ calibration} \neq \mu_0$

Where μ_0 is the true value

To assess the significance of the differences between experimental means obtained through the isotope dilution calibration approach and the true values provided in the SRM certificate, the following null, and alternative hypotheses were made:

H_0: $\mu_{isotope\ dilution} = \mu_0$

H_a: $\mu_{isotope\ dilution} \neq \mu_0$

Where μ_0 is the true value

All the above significance tests were performed at a 95% confidence level based on the number of degrees of freedom shown in **Table 4-8**. In instances where significant differences were found between the variances e.g. valine, **Equation 3-3** was used to perform the t-test. Alternatively, **Equation 3-2** was used in instances where the opposite was true. As seen from the F-test results in **Table 4-8**, significant differences between the variances were only found on alanine, valine, leucine, isoleucine, proline, serine, and threonine when the external calibration method and the isotope dilution method were compared. Therefore, the F-test null hypothesis was rejected with respect to these analytes and the alternative hypothesis was accepted. Consequently, **Equation 3-2** was used to calculate the experimental t-values of the previously mentioned analytes while **Equation 3-3** was used to calculate the experimental t-values for the rest of the analytes.

As also seen in **Table 4-8**, although differences were found between the variances of some of the analytes. Significant differences between the means obtained by external calibration and

isotope dilution were only found on proline, phenylalanine, histidine, and tyrosine. Therefore, the t-test null hypothesis was rejected with respect to the above-mentioned analytes while the opposite was true for other analytes namely, alanine, glycine, valine, leucine, isoleucine, serine, threonine, aspartic acid, glutamic acid, and lysine. Additionally, the experimental means of proline, histidine, and tyrosine obtained by external calibration also differed significantly from the true values provided in the certificate as shown in **Table 4-9**. Similarly, experimental means of glycine, valine, and serine obtained by external calibration also differed significantly from true values. Therefore, the t-test null hypothesis for comparing experimental means obtained by external calibration with the true values was rejected with respect to glycine, valine, proline, serine, histidine, and tyrosine.

With respect to other analytes obtained by external calibration where differences between the true values and the experimental mean values were not significant, the t-test null hypothesis was accepted and the alternative hypothesis was rejected. Those analytes include alanine, leucine, isoleucine, threonine, phenylalanine, aspartic acid, glutamic acid, and lysine. With respect to the isotope dilution method, significant differences between the true value and experimental mean were only observed on glycine and histidine as seen in **Table 4-9**. In this regard, the t-test null hypothesis was rejected with respect to the above-mentioned analytes while accepted it was accepted with respect to the rest of the remaining analytes. These observations suggest that the results generated from the isotope dilution quantification approach generated more accurate values.

4.2.3 GC-TOFMS method selectivity (analyte identification)

It goes without saying that for any analytical method to be reliable and useful it should be able to quantify the analytes of interest selectively. Therefore, as part of method validation, methods selectivity must be assessed. As mentioned earlier in this chapter, method selectivity was studied using three methods. In this study method selectivity tests were performed in accordance with the guidelines provided in the European Commission Directorate-General for Health and Food Safety's Guide on Analytical Quality Control and Method Validation Procedures for Pesticides Analysis in Food and Feed due to a lack of a better alternative guide for development of analytical method for analysis of amino acids in infant formula using a GC-MS (Hill and Reynolds, 1999). The first method involved comparing the retention times of the analytes in an infant formula sample with the retention time of the same analytes in a pure

standard. The rejection criterion with regards to retention times test was that the retention time of each analyte in the sample should fall within the retention time confidence interval (CI) of the same analyte in a pure standard, or at most fall within 6 seconds of the pure standard's analyte retention time. The second method involved comparing analyte's mass spectrum with the spectrum of the same analyte on the NIST mass spectral library. The rejection criterion was that a library match of ≥70% was mandatory for identification of analytes as outlined in **Chapter 3**. The third approach used to assess selectivity was the ion ratios approach. Based on one of the fundamental theories of mass spectrometry, relative abundances of ions of an analyte in mass spectrometry remain unchanged within a relatively small range of concentrations provided (1) ionisation energy is not changed and (2) the detector is not overloaded. Therefore, ion ratios may be a used to assess analyte selectivity provided these conditions are not violated. For ion ratios test, the rejection criterion was that the percentage difference between ion ratios of the sample should not exceed 30% of the average ion ratios of the same analyte in a pure standard as required by the AOAC.

Table 4-10: Identification of analytes in the SRM by retention time and percentage MS library match results

	Pure Standard		SRM sample		
Analytes	Mean t_r (s)	CI (s)	Mean t_r (s)	CI (s)	% MS Similarity
Alanine	457.605	0.062	457.580	0.072	90.700
Glycine	471.623	0.060	471.622	0.065	91.300
Valine	509.717	0.066	509.708	0.065	75.900
Leucine	524.026	0.060	524.035	0.073	92.600
Isoleucine	534.588	0.062	534.567	0.067	84.800
Proline	548.249	0.068	548.281	0.063	74.300
Serine	626.089	0.064	626.060	0.079	85.100
Threonine	634.553	0.063	634.503	0.068	75.100
Phenylalanine	653.564	0.067	653.478	0.070	83.200
Aspartic Acid	666.773	0.127	666.597	0.145	87.300
Glutamic Acid	690.421	0.244	690.094	0.278	82.200
Lysine	710.837	0.311	710.395	0.362	80.900
Histidine	745.989	0.435	745.375	0.515	88.500
Tyrosine	755.785	0.482	755.104	0.569	91.700

Mean t_r = average retention time (n=5), CI = confidence interval and %MS Similarity = SRM mass spectral library match sample = NIST standard reference material sample

For the first and second selectivity test methods, the test results are presented in **Table 4-10** while for the third method the results are presented in **Table 4-11**. As seen in **Table 4-10**, all

the sample retention times fell within the confidence interval of the pure standard retention times. Therefore, using the retention times comparison, there was no evidence to suggesting false identification of analytes in a sample. With respect to the MS library match test, all analytes library match percentages were greater than 70% (**Table 4-10)**. Therefore, none of the analytes could be rejected as a false positive. For the third test, the results are presented in **Table 4-11**. As seen in **Table 4-11**, all analyte ion ratio percentage differences were below 30% except for the ion ratio of phenylalanine. This observation suggested that an interference might be present. To verify this hypothesis a GC×GC-TOFMS system was used as outlined in **Chapter 3** and the results thereof are discussed in the following subsection (**subsection 4.2.4**).

Table 4-11: Identification of analytes in the SRM by ion ratios

Analytes	Standard Average Ion Ratio	SRM Average Ion Ratio	% Difference
Alanine	0.448	0.444	0.787
Glycine	0.516	0.534	3.338
Valine	0.334	0.336	0.367
Leucine	0.322	0.322	0.016
Isoleucine	0.335	0.331	0.989
Proline	0.215	0.205	4.664
Serine	0.831	0.867	4.322
Threonine	0.062	0.058	6.499
Phenylalanine	0.653	0.366	43.966
Aspartic Acid	0.049	0.049	0.494
Glutamic Acid	0.532	0.531	0.142
Lysine	0.390	0.411	5.567
Histidine	0.159	0.198	24.674
Tyrosine	0.169	0.155	8.583

SRM sample = NIST standard reference material sample

4.2.4 Analyte verification by GC×GC-TOFMS (interference test)

GC×GC-TOFMS is renowned for its ability to resolve coelutions that may not be obvious or may be difficult to resolve by one dimensional GC. This ability stems from the use of two columns with different polarity stationary phases which allow analytes to be separated in two dimensions as shown in **Figure 4-15**. In this study, a Pegasus IV GC×GC-TOFMS was used to test the NIST SRM for interferences as part of method validation focusing on analytes that showed poor accuracy. Those being analytes with recoveries lower than 80% or higher than 120% under either external calibration or isotope dilution method, namely proline,

phenylalanine, lysine, and histidine. This was done to verify whether the observed analyte responses were only due to the analytes of interest and not interferences. As seen in **Table 4-6**, the recoveries of lysine and histidine obtained through the isotope dilution method were 73.31 and 123.81% respectively while the recoveries of proline and phenylalanine obtained by external calibration approach were 61.13 and 120.66% respectively as seen in **Table 4-7**. As depicted in **Figures 4-16** to **4-19**, there were no interferences found on all the amino acids except for proline as depicted in **Figure 4-18**.

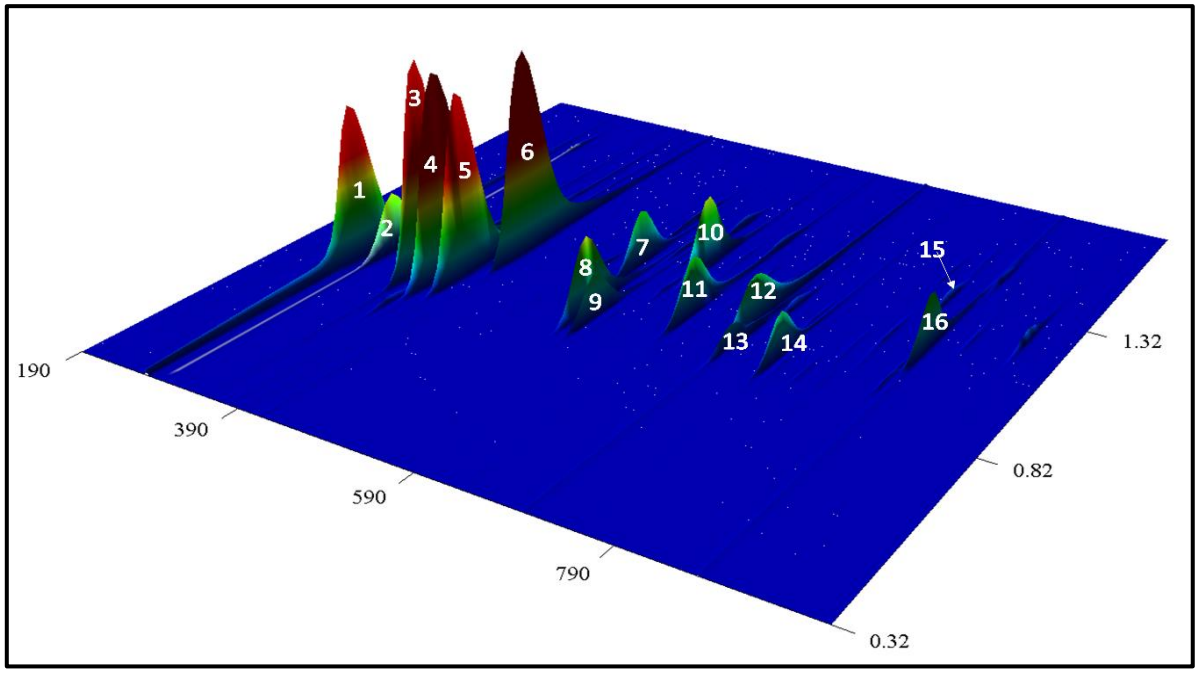


Figure 4-15: GC×GC-TOFMS surface plot obtained from a NIST SRM. Extracted masses were 158, 218, 186, 200, 184, 288, 303, 234, 302, 198, 196, 302 and 130. Peak area numbers 1 to 16 represent alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, glutamic acid, ornithine, lysine, histidine, and tyrosine respectively.

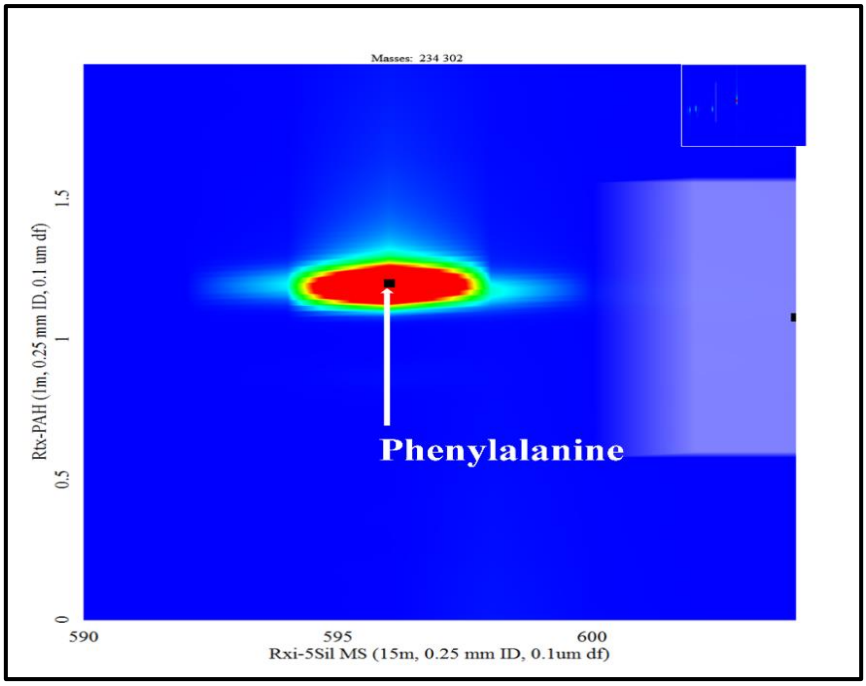


Figure 4-16: Contour plot of phenylalanine obtained by GC×GC-TOFMS when a NIST SRM was tested for interferences that could have caused poor recovery of phenylalanine.

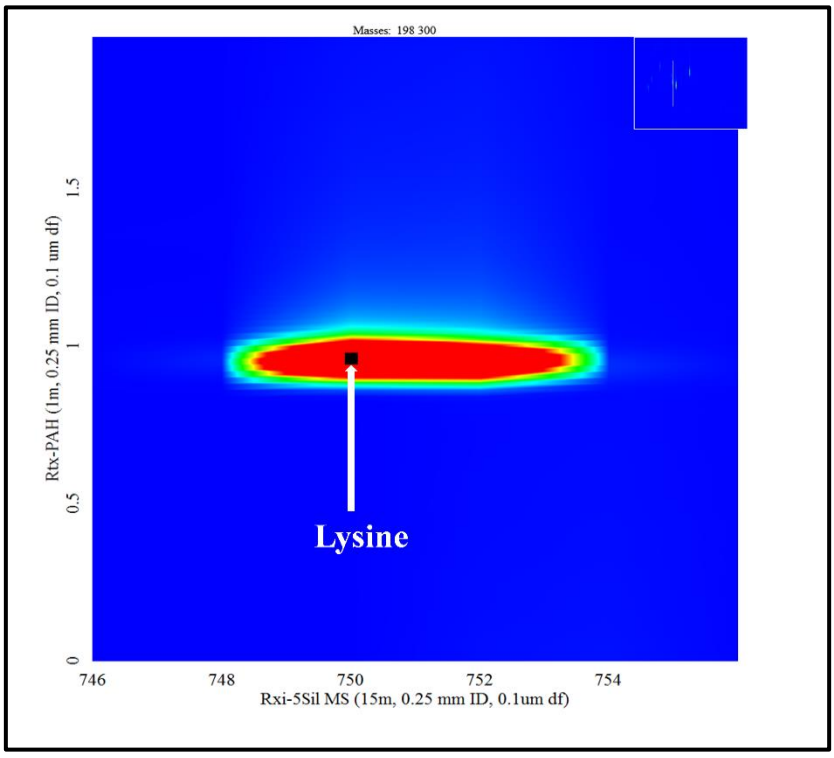


Figure 4-17: Contour plot of lysine obtained by GC×GC-TOFMS when a NIST SRM was tested for interferences that could have caused poor recovery of lysine.

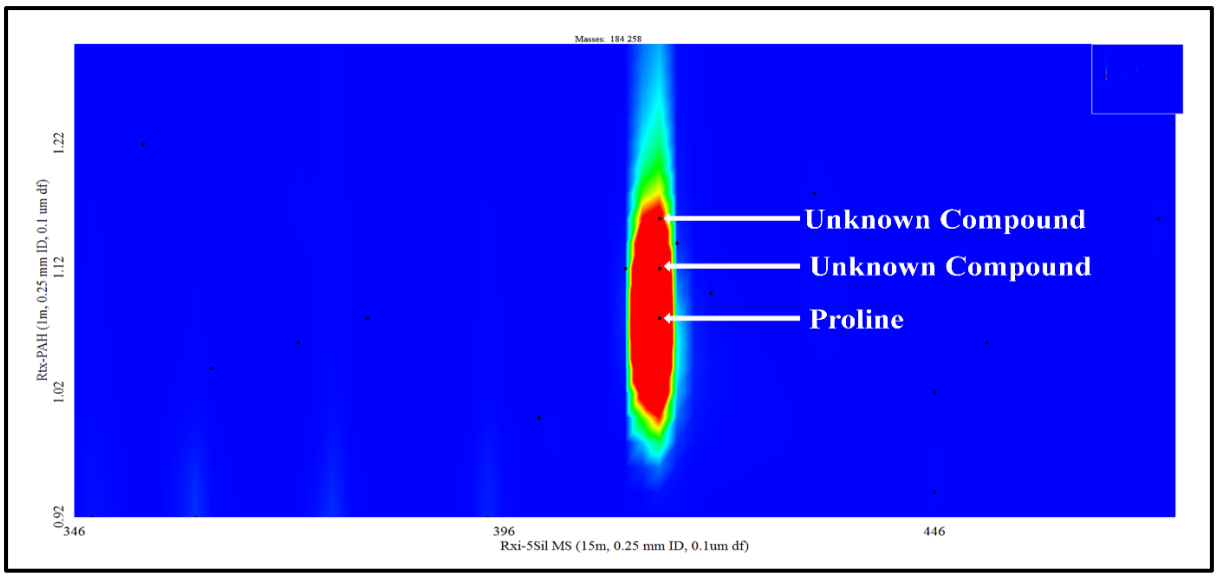


Figure 4-18: Contour plot of proline obtained by GC×GC-TOFMS when a NIST SRM was tested for interferences that could have caused poor recovery of proline.

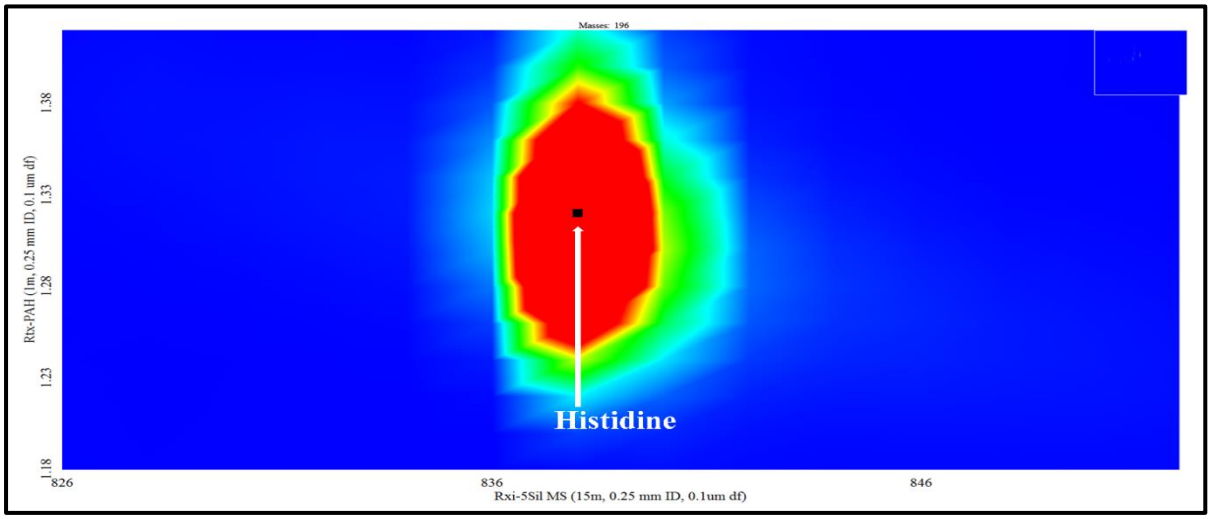


Figure 4-19: Contour plot of histidine obtained by GC×GC-TOFMS when a NIST SRM was tested for interferences that could have caused poor recovery of histidine.

4.2.5 Estimation of the uncertainty of measurement of the GC-TOFMS method

The uncertainty of measurement is one of the key aspects of method validation. This is because in some instances when using chemometrics statistics one might draw a conclusion that the experimental recoveries differ significantly from the true value and therefore the newly developed method is less useful. Although this might be true, however, it might happen that

the expanded uncertainty of the experimental results overlaps with the expanded uncertainty of the true value signifying that the two values do not really differ significantly as one might have thought. As defined in the International Standardisation Organisation's Guide to Uncertainty in Measurement (GUM) and its simplified version namely Quantifying Uncertainty in Analytical Measurement (QUAM) published by the Cooperation on International Traceability in Analytical Chemistry (CITAC), "*uncertainty is a parameter associated with the result of measurement that characterises the dispersion of the values that could be attributed to the Measurand*". Furthermore, GUM emphasises that it is obligatory that uncertainty of measurement be indicated so that those who will use the results can assess its reliability. In this study, the uncertainty of measurement was quantified in accordance with the guidelines provided in both GUM and QUAM. The initial step in the evaluation of uncertainty of measurement was to determine the sensitivity coefficients with respect to each of the uncertainty sources (fishbone diagram main branches) using **Equation 3-14**. Sensitivity coefficients were calculated to determine the change in the value of the measurand with respect to an infinitesimal change on any of the values of the uncertainty sources and the results thereof are shown in **Table 4-12**.

Table 4-12: Sensitivity coefficients used to calculate the combined uncertainty

Amino Acids	$C_i(y)$	$C_i(b)$	$C_i(a)$	$C_i(DF)$	$C_i([IS])$	$C_i(conversion\ factor)$
Alanine	5.99×10^{-6}	-4.53×10^{-4}	-5.99×10^{-6}	0.0405	0.0900	-4543.64
Glycine	9.77×10^{-6}	-3.34×10^{-4}	-9.77×10^{-6}	0.0155	0.0900	-996.94
Valine	6.45×10^{-6}	-5.99×10^{-4}	-6.45×10^{-6}	0.0656	0.140	40202.95
Leucine	7.49×10^{-6}	-9.90×10^{-4}	-7.49×10^{-6}	0.104	0.0500	-7705.98
Isoleucine	-1.22×10^{-4}	-5.92×10^{-4}	-7.60×10^{-6}	0.0616	0.0500	37314.89
Proline	4.52×10^{-6}	-5.77×10^{-4}	-4.52×10^{-6}	2.11×10^{-3}	0.0900	-1836.11
Serine	3.18×10^{-5}	-2.87×10^{-3}	-3.18×10^{-5}	5.67×10^{-4}	0.0900	-4486.18
Threonine	7.54×10^{-5}	-7.78×10^{-3}	-7.54×10^{-5}	7.34×10^{-4}	-0.0100	3805.54
Phenylalanine	3.26×10^{-5}	-1.72×10^{-3}	-3.26×10^{-5}	5.22×10-4	0.0300	-5179.86
Aspartic acid	-4.27×10^{-5}	-4.46×10^{-3}	-4.27×10^{-5}	8.30×10^{-4}	0.110	-4785.55
Glutamic acid	4.12×10^{-5}	-9.65×10^{-3}	-4.12×10^{-5}	0.208	0.260	-1065.28
Lysine	2.62×10^{-5}	-1.56×10^{-3}	-2.62×10^{-5}	0.0524	-0.0400	-811.36
Histidine	1.14×10^{-5}	-2.33×10^{-4}	-1.15×10^{-5}	0.0191	0.0400	-1213.61
Tyrosine	1.48×10^{-5}	-2.23×10^{-3}	-1.48×10^{-5}	0.0253	0.0400	-807.857

$\boldsymbol{C_i\ (y)}$ = sensitivity coefficients of the y-branch, $C_i(b)$ = sensitivity coefficients of the slope, $C_i(a)$ = sensitivity coefficients of the intercept, $C_i(DF)$ = sensitivity coefficients of the dilution factor and $C_i\ (conversion\ factor)$ = sensitivity coefficients of the conversion factor.

Table 4-13: Analyte dependent standard uncertainties used to calculate the effective number of degrees of freedom

Amino Acids	u_{SRM}	u_{ESDM}	$u_{Sx/y}$	u_{Sa}	$\mu_{[IS]}$	u_{Sb}
Alanine	0.0091	0.0021	0.0360	0.0799	0.0093	0.0746
Glycine	0.0082	0.0194	0.0128	0.0112	0.0191	0.0073
Valine	0.0476	0.0178	0.0517	0.0502	0.0191	0.0452
Leucine	0.0216	0.0316	0.0623	0.0615	0.0102	0.0562
Isoleucine	0.0307	0.0085	0.0543	0.0533	0.0102	0.0495
Proline	0.0372	0.1970	0.0218	0.0188	0.0192	0.0128
Serine	0.0130	0.0204	0.0377	0.0367	0.0127	0.0328
Threonine	0.0095	0.0346	-0.0152	-0.0196	0.0218	-0.0290
Phenylalanine	0.0091	0.0040	0.1203	0.0510	0.0069	0.0381
Aspartic acid	0.0247	0.0459	0.0363	0.0806	0.0044	0.0776
Glutamic acid	0.1169	0.0786	0.0174	0.0136	0.0205	0.0057
Lysine	0.0710	0.0350	0.0136	0.0109	0.105	0.0055
Histidine	0.0156	0.0134	0.0182	0.0148	0.0087	0.0089
Tyrosine	0.0186	0.0167	0.0157	0.0126	0.0087	0.0057

μ_{SRM} = standard uncertainty of the SRM, μ_{ESDM} = standard uncertainty of the mean, $\mu_{Sx/y}$ = standard uncertainty of the regression line, μ_{Sa} = standard uncertainty of the regression line intercept and S_b= standard uncertainty of the slope

Table 4-14: Analyte independent standard uncertainties used to calculate the effective number of degrees of freedom

Equation Branch	Source	Uncertainty type	Distribution	Precision	u_i	v_i
	800 µL MBSTFA	B	Triangular	unknown	****	infinite
	AA-S-18	B	Rectangular	0.0200	0.012	infinite
Y (Signal)	Instrument precision	A	Normal	0.0074	0.007	29
	80 µL AA-S-18	A	Normal	unknown	****	infinite
	Acetonitrile	A	Normal	0.0034	0.001	4
	Pipetting 35 mL	B	Triangular	0.5000	0.204	infinite
	Pipetting 1 mL	A	Normal	0.0198	0.007	4
DF (Dilution factor)	Pipetting 20 µL	A	Normal	0.0071	0.003	4
	Pipetting 80 µL Acetonitrile	A	Normal	0.0034	0.001	4
	Pipetting 6mL 0.1 M HCl	A	Normal	0.0500	0.016	5
	Pipetting 10 µL Acetonitrile	A	Normal	0.0319	0.011	4
Conversion Factor	1.20 g	A	Normal	0.00002	0.00002	infinite
	Weighing 1 mg	B	Normal	0.000006	0.00003	infinite
[IS]	300 µL	A	Triangular	0.21	0.12	9
	10 µL	A	Normal	0.15	0.0374	16

**** represents undefined or unknown standard uncertainty.

Table 4-15: The results of the uncertainty of measurements

Amino Acids	$[AA]_1\ (g/100g)$	$[AA]_2\ (g/100g)$	$U\ (g/100g)$	$v_{effecive}$
Alanine	0.48	0.47	0.078	9
Glycine	0.22	0.22	0.048	11
Valine	0.67	0.68	0.27	14
Leucine	1.22	1.33	0.15	9
Isoleucine	0.62	0.61	0.26	17
Proline	0.74	1.09	0.40	11
Serine	0.61	0.72	0.091	7
Threonine	0.59	0.66	0.080	15
Phenylalanine	0.70	0.47	0.25	12
Aspartic Acid	1.00	1.01	0.13	15
Glutamic Acid	2.45	2.37	0.17	12
Lysine	0.81	0.75	0.076	10
Histidine	0.24	0.38	0.046	10
Tyrosine	0.44	0.50	0.047	12

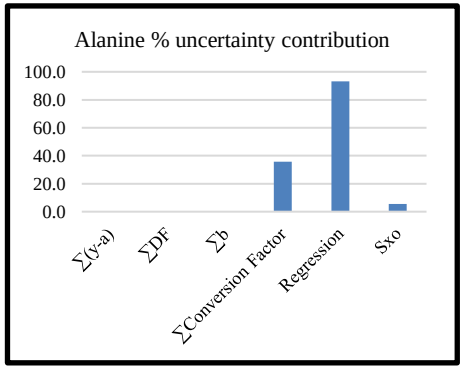
$[AA]_1$ = amino acids concentrations obtained by external calibration, $[AA]_2$ = amino acids concentrations obtained by isotope dilution, U = the expanded uncertainty and $v_{effective}$ = the effective number of degrees of freedom

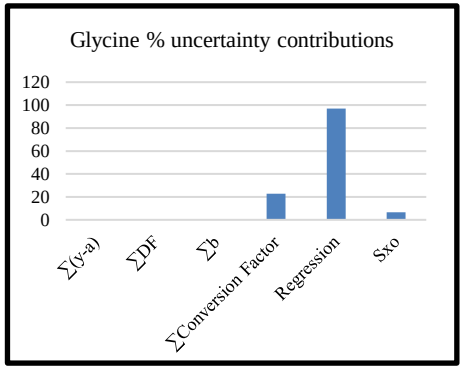
Standard uncertainties **(Tables 4-13** and **4-14)** together with sensitivity coefficients were used to calculate the uncertainty contributions of each of the fishbone subbranches using **Equation 3-15**. The uncertainty contributions of the subbranches were subsequently used to calculate the combined uncertainty of each branch using **Equation 3-16**. To calculate the total combined uncertainty **Equation 3-17** was used. The total combined uncertainty was multiplied by the coverage factor (k = 2) (**Equation 3-20**) to obtain the expanded uncertainty of each analyte and the results thereof are presented in **Table 4-15**. Also shown in **Table 4-15**, are the effective number of degrees of freedom calculated using **Equation 3-21**.

In accordance with the guidelines provided in GUM, all concentrations obtained using the GC-TOFMS **(Tables 4-6 and 4-7)** method were rounded up to 2 significant figures after the comma as shown in **Table 4-15**. To evaluate the contributions of each uncertainty branch to the total combined uncertainty, **Equation 3-22** was used and the results thereof are graphically represented in **Figure 4-20**. This type of representation of the percentage uncertainty contribution allowed us to determine the major uncertainty contributing factors which in turn assisted in identifying experimental factors that may be improved in the future. As seen in **Figure 4-20**, on average, the largest contributors to the uncertainty were (1) between sample standard deviation S_{x0} and (2) regression line standard deviation. The between sample standard deviation may be improved by changing the sampling technique and ensuring that samples

used in the preparation of hydrolysates are homogeneous and representative (e.g. using larger volumes/subsamples).

Similarly, the regression line standard deviation may be improved by gravimetric preparation of the calibration standards. To assess the uncertainty compliance of the GC-TOFMS method developed in this study, the expanded uncertainty was graphically represented as depicted in **Figure 4-21**. By so doing, we were able to distinguish between poorly recovered analytes, analytes with expanded uncertainty values (represented by error bars) that includes the true value and analytes with an expanded uncertainty (represented by error bars) that overlaps with the upper or lower limits of the expanded uncertainty of the true value. Using both the external and the isotope dilution methods, the recoveries of lysine including its expanded uncertainty were below the lower limit of the expanded uncertainty reported on the SRM certificate as depicted in **Figure 4-21**. On the other hand, the expanded uncertainties of proline, serine, and histidine obtained by external calibration crossed the lower limit of the expanded uncertainty of the SRM reported on the certificate. However, the expanded uncertainties of the above-mentioned analytes did not include the true value. Similarly, the expanded uncertainty of histidine obtained by isotope dilution crossed the upper limit of the expanded uncertainty reported on the SRM certificate but the expanded uncertainty did not include the true value. Except for the above-mentioned analytes, expanded uncertainties of other analytes included the true value. Therefore, the GC-TOFMS method may be used to quantify AAs in infant formula.


Alanine % uncertainty contribution
100.0
80.0
60.0
40.0
20.0
0.0
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Glycine % uncertainty contributions
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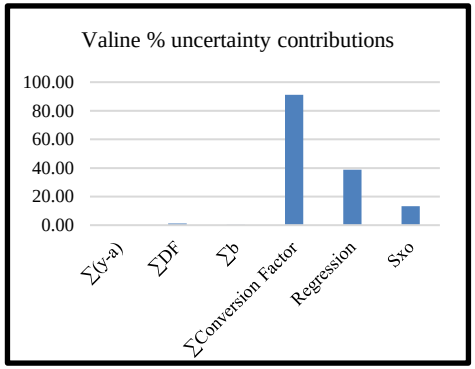
100
80
60
40
20
0
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Valine % uncertainty contributions
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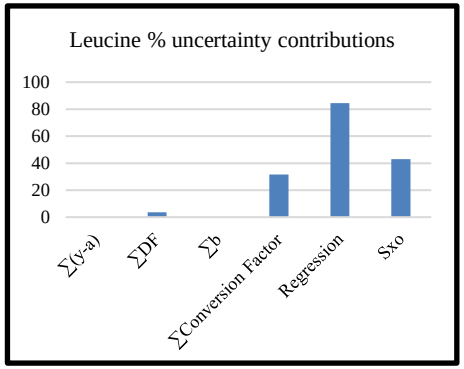
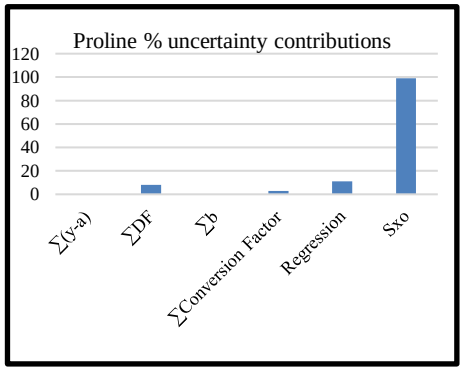
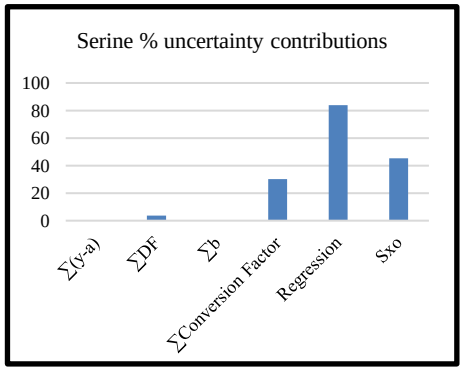
0.00
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Leucine % uncertainty contributions
100
80
60
40
20
0
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Proline % uncertainty contributions
120
100
80
60
40
20
0
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Serine % uncertainty contributions
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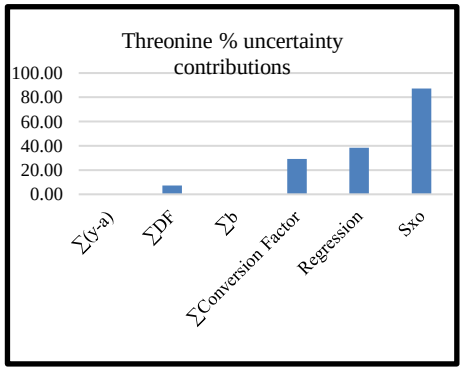
80
60
40
20
0
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Threonine % uncertainty contributions
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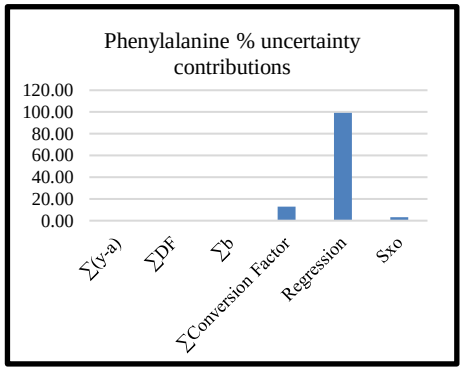
60.00
40.00
20.00
0.00
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Phenylalanine % uncertainty contributions
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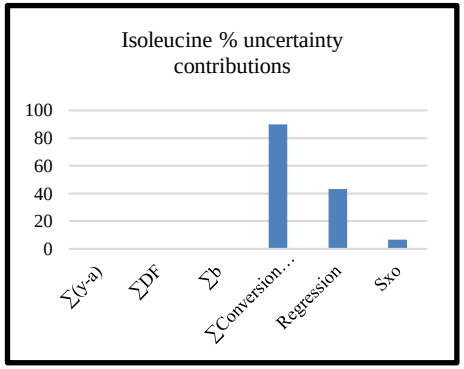
100.00
80.00
60.00
40.00
20.00
0.00
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Isoleucine % uncertainty contributions
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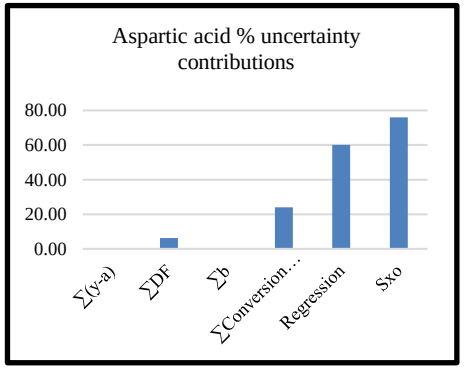
80
60
40
20
0
∑(y-a)
∑DF
∑b
∑Conversion…
Regression
Sxo


Aspartic acid % uncertainty contributions
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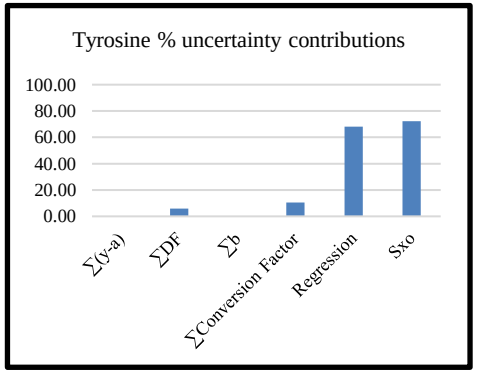
40.00
20.00
0.00
∑(y-a)
∑DF
∑b
∑Conversion…
Regression
Sxo


Tyrosine % uncertainty contributions
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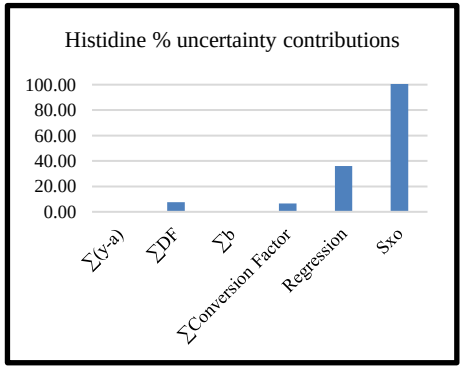
80.00
60.00
40.00
20.00
0.00
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo


Histidine % uncertainty contributions
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80.00
60.00
40.00
20.00
0.00
∑(y-a)
∑DF
∑b
∑Conversion Factor
Regression
Sxo

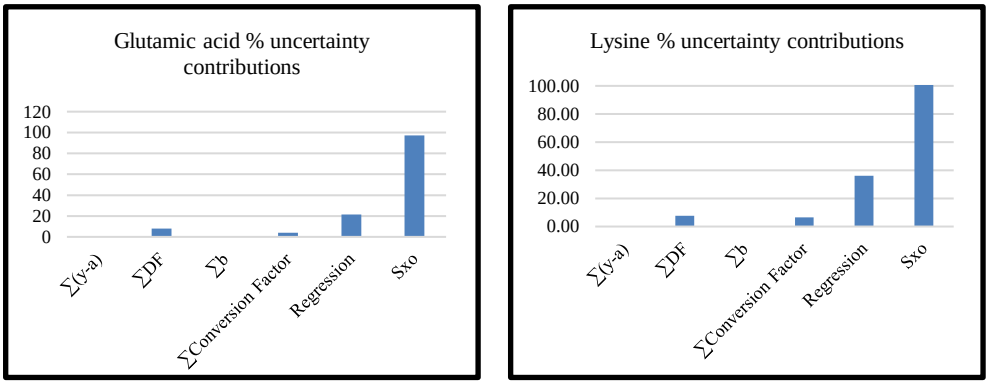
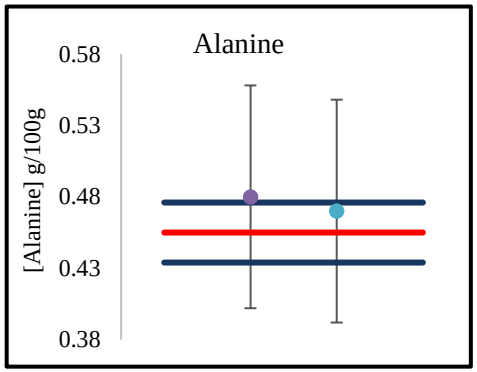
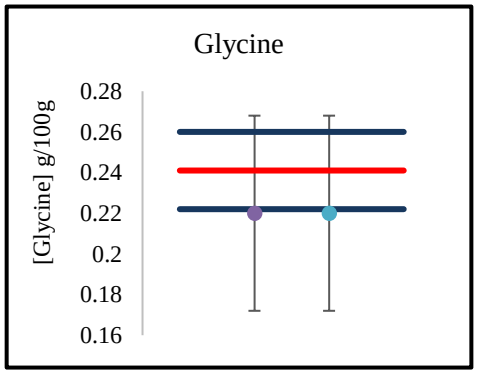
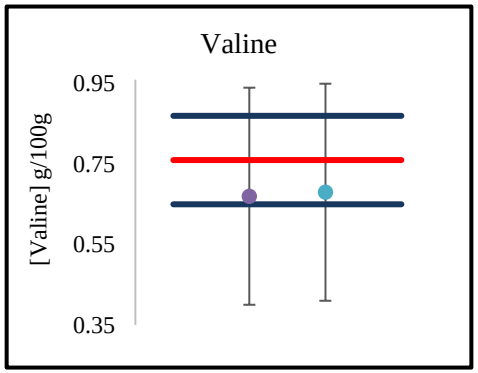
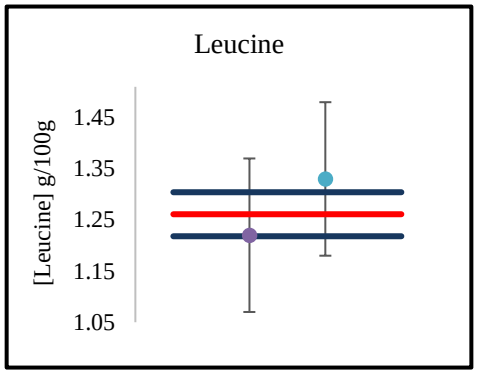


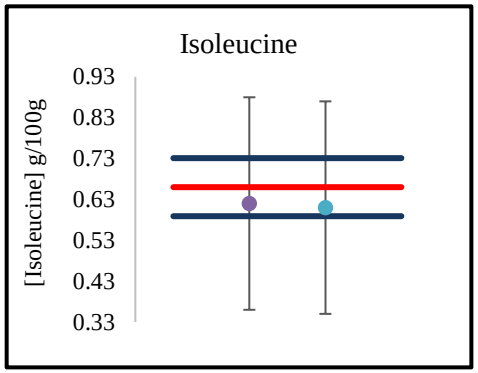
Figure 4-20: Graphical representation of the percentage uncertainty contributions of the branches of the fishbone diagram **(Figure 3-5)**. $\sum(y-a) =$ the combined contribution of the (y) and (a) branches, $\sum b =$ contribution from the regression line slope, $\sum DF =$ contribution from the dilution factor, $\sum conversion\ factor =$ contribution from the dilution factor, Regression ($S_{x/y}$)= contribution from the regression line and S_{x_0} = contribution from the sample standard deviation.

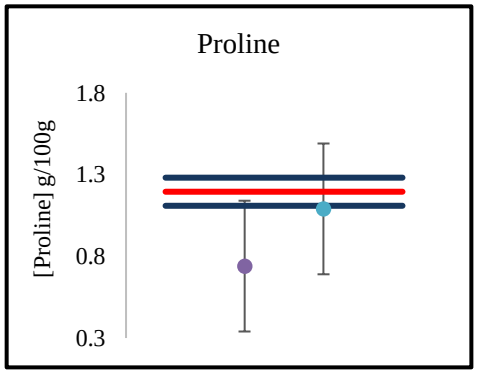

Alanine
[Alanine] g/100g
0.58
0.53
0.48
0.43
0.38


Glycine
[Glycine] g/100g
0.28
0.26
0.24
0.22
0.2
0.18
0.16


Valine
[Valine] g/100g
0.95
0.75
0.55
0.35


Leucine
[Leucine] g/100g
1.45
1.35
1.25
1.15
1.05


Isoleucine
[Isoleucine] g/100g
0.93
0.83
0.73
0.63
0.53
0.43
0.33


Proline
[Proline] g/100g
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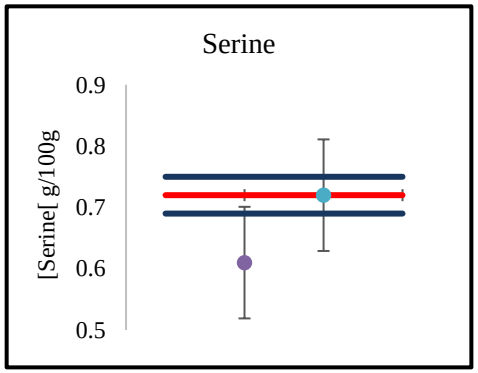
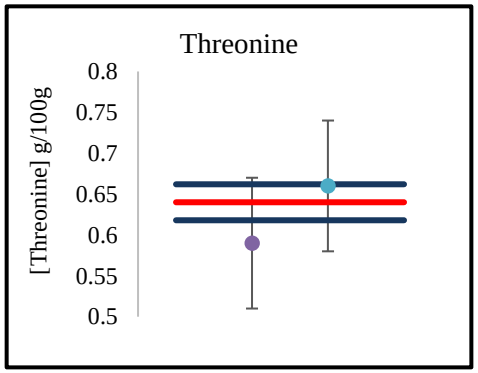
1.3
0.8
0.3


Serine
[Serine] g/100g
0.9
0.8
0.7
0.6
0.5


Threonine
[Threonine] g/100g
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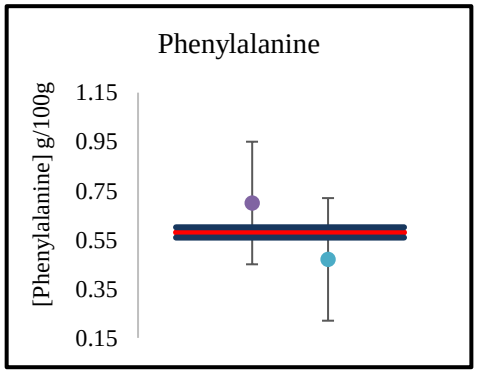
0.75
0.7
0.65
0.6
0.55
0.5


Phenylalanine
[Phenylalanine] g/100g
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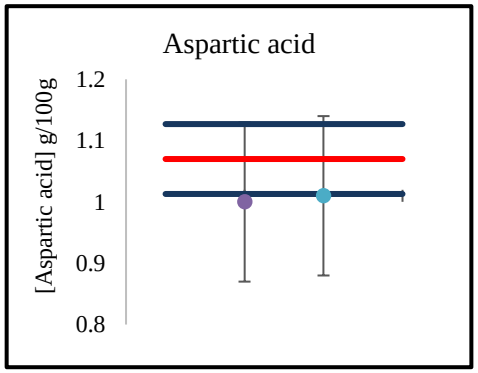
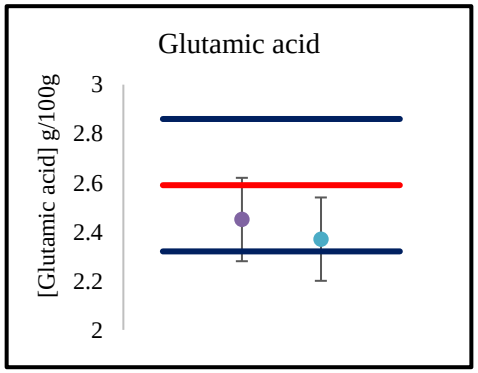
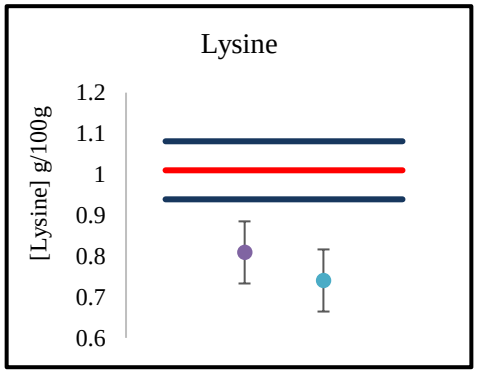
0.95
0.75
0.55
0.35
0.15


Aspartic acid
[Aspartic acid] g/100g
1.2
1.1
1
0.9
0.8


Glutamic acid
[Glutamic acid] g/100g
3
2.8
2.6
2.4
2.2
2


Lysine
[Lysine] g/100g
1.2
1.1
1
0.9
0.8
0.7
0.6

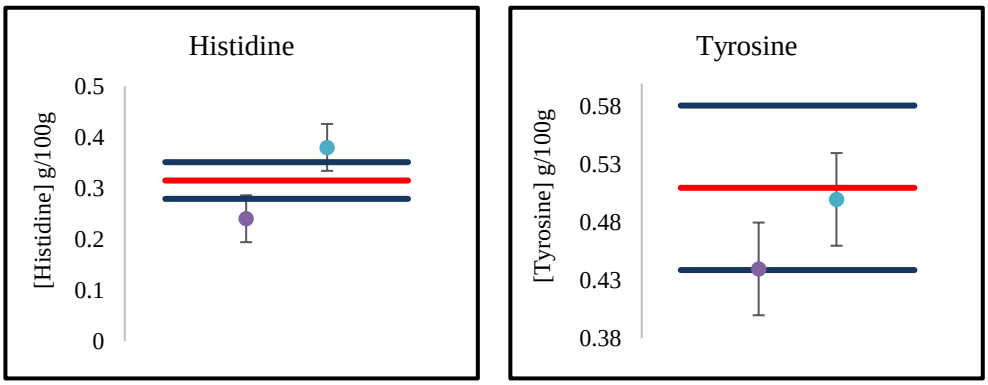


Figure 4-21: Graphical representation of the the expanded uncertainty of measurement. The blue lines represent the upper and lower limits of expanded uncertainty provided in the SRM certificate. The red line represents the true value provided on the SRM certificate. The purple and the blue dots represent experimental means obtained by external calibration and by isotope dilution respectively. The error bars represent the calculated expanded uncertainty**.**

4.2.6 Method ruggedness test by GC-TOFMS and UPLC

As briefly discussed in the beginning of this chapter, method ruggedness was assessed by comparing the results obtained from the Pegasus III GC-TOFMS with the results from the Waters AccQ·Tag UPLC method and the results obtained from the Pegasus IV GC-TOFMS system (see **Table 6-1** to **Table 6-8** in **Appendix C1** for UPLC and Pegasus IV results). The AccQ·Tag UPLC method was used to assess the GC-TOFMS method's performance while the Pegasus IV system was used to study GC-TOFMS method's transferability. Prior to the application of the statistical methods, differences between analyte recoveries were visually examined as depicted in **Figure 4-22** and **Figure 4-23**. The recoveries obtained through the external calibration using the Pegasus III GC-TOFMS system were between 84.72% and 103.99%. The recoveries obtained by external calibration on the Pegasus IV GC-TOFMS ranged from 73.18% and 110.98% while the recoveries obtained through the same method using the UPLC system ranged from 59.51% to 104.49% as shown in **Table 6-4** and **Table 6-8**.

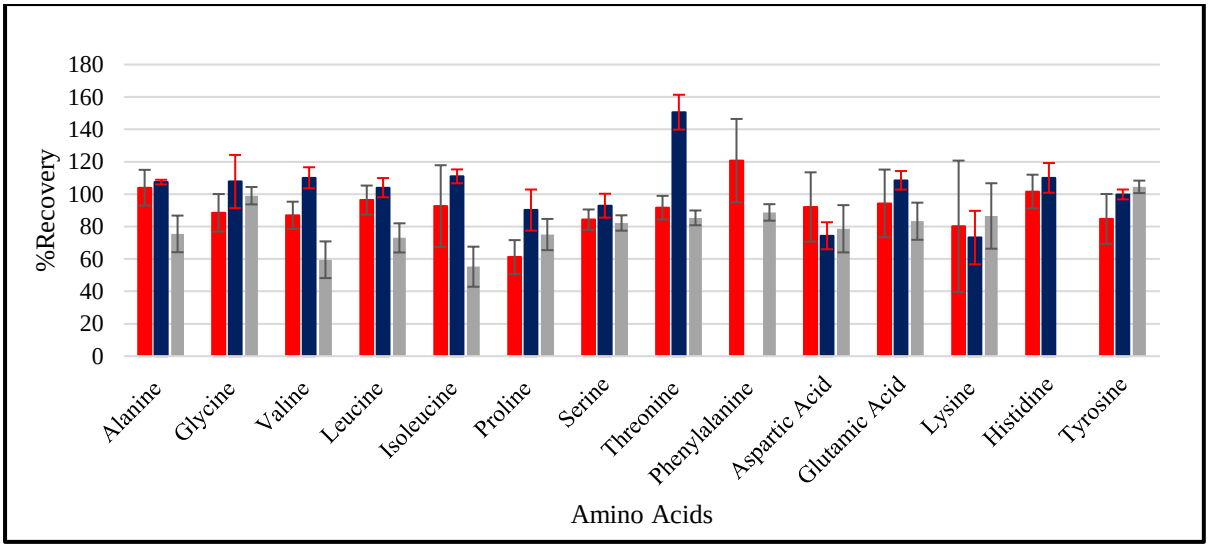


Figure 4-22: % Recoveries (n = 5) obtained by Pegasus III GC-TOFMS, Pegasus IV GC-TOFMS, and the UPLC system through the external calibration method. These recoveries were used to visually assess the differences between the results obtained by the three systems. The red bars represent Pegasus III %recoveries, dark blue bars represent the Pegasus IV %recoveries, the grey bars represent UPLC %recoveries and the error bars represent the relative standard deviations of each method.

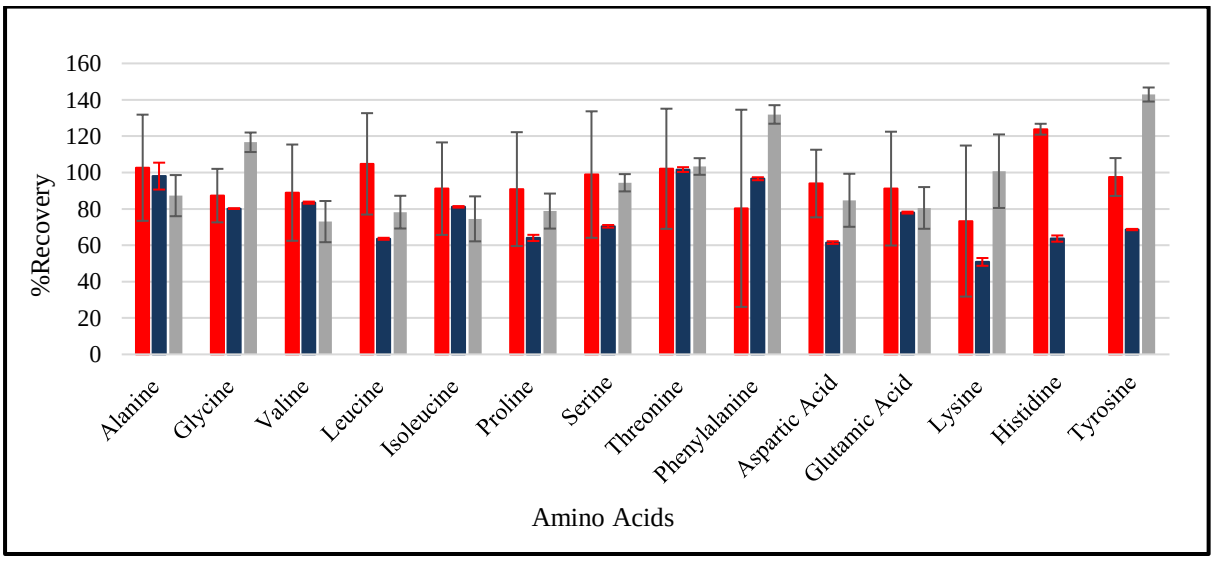


Figure 4-23: Isotope dilution %recoveries (n = 5) obtained by Pegasus III GC-TOFMS, Pegasus IV GC-TOFMS, and the UPLC system. These recoveries were used to visually assess the differences between the results obtained by the three systems. The red bars represent Pegasus III %recoveries, dark blue bars represent the Pegasus IV %recoveries, the grey bars represent UPLC %recoveries and the error bars represent the relative standard deviations of each method.

On the other hand, the percentage recoveries obtained by the isotope dilution method using the Pegasus III and the Pegasus IV systems ranged from 73.31% to 104.76% and from 50.85 to 101.00% respectively while the recoveries obtained through the internal standard method using the UPLC system ranged from 73.01 to 142.90% as shown in **Table 6-3** and **Table 6-7** shown

in **Appendix C1** in the appendices section. As seen in **Figure 4-22** and **Figure 4-23**, the error bars representing the range of possible recovery values with respect to both the isotope dilution and the external calibration method using the Pegasus III and Pegasus IV GC-TOFMS systems tend to overlap which signifies that the recoveries from these systems do not differ significantly. This was true with respect to all analytes except for leucine, aspartic acid, histidine and tyrosine obtained using the external calibration method and proline, threonine, and phenylalanine with respect to the isotope dilution method.

Table 4-16: One-way ANOVA results used to test the significance of the differences between the Pegasus III, Pegasus IV and the UPLC results

Amino Acids	$F(ANOVA)$	HSD
Alanine	$F(5,24) = 9.12\ p < 0.05$	0.095
Glycine	$F(5,24) = 5.33\ p < 0.05$	0.055
Valine	$F(5,24) = 50.26\ p < 0.05$	0.10
Leucine	$F(5,24) = 20.75\ p < 0.05$	0.21
Isoleucine	$F(5,24) = 32.20\ p < 0.05$	0.092
Proline	$F(5,24) = 18.89\ p < 0.05$	0.24
Serine	$F(5,24) = 16.08\ p < 0.05$	0.15
Threonine	$F(5,24) = 10.46\ p < 0.05$	0.19
Phenylalanine	$F(5,24) = 42.67\ p < 0.05$	0.13
Aspartic Acid	$F(5,24) = 5.12\ p < 0.05$	0.22
Glutamic Acid	$F(5,24) = 3.23\ p < 0.05$	0.67
Lysine	$F(5,24) = 31.37\ p < 0.05$	0.41
Histidine	$F(5,24) = 31.37\ p < 0.05$	0.48
Tyrosine	$F(5,24) = 36.39\ p < 0.05$	0.09

HSD = honest significant difference

Similarly, in both **Figure 4-22** and **Figure 4-23**, the error bars from both the isotope dilution method and the external calibration method obtained using the Pegasus III GC-TOFMS system and through external calibration and the internal standard method using the UPLC system also overlapped except with respect to glycine, histidine and tyrosine obtained through the external calibration method and alanine, valine, leucine, isoleucine, phenylalanine and histidine with respect to the isotope dilution and the internal standard method. Therefore, based on the above observation, the results obtained from the Pegasus III using the isotope dilution method were most likely to differ significantly from the results obtained using the internal standard method on the UPLC system. Using a single factor ANOVA in conjunction with the Tukey honest

significance test, the external calibration, isotope dilution and internal standard method results obtained from the Pegasus IV and UPLC were each compared with isotope dilution and external calibration results from the Pegasus III system and the results thereof are shown in **Table 4.16**.

With regards to the experimental means obtained through external calibration method and the isotope dilution method on the Pegasus III and the experimental means obtained through the external calibration method and the internal standard method on the UPLC, the following null and alternative hypothesis were made:

$\mathbf{H_0}$: $\mu_{PegIII-external\ calibration} = \mu_{UPLC-external\ calibration}$

$\mathbf{H_0}$: $\mu_{PegIII-isotope\ dilution} = \mu_{UPLC-internal\ standard}$

$\mathbf{H_a}$: $\mu_{PegIII-external\ calibration} \neq \mu_{UPLC-external\ calibration}$

$\mathbf{H_a}$: $\mu_{PegIII-isotope\ dilution} \neq \mu_{UPLC-internal\ standard}$

Similarly, the following null and alternative hypotheses were made with respect to experimental means obtained through the external and isotope dilution method using the Pegasus III GC-TOFMS system and the Pegasus IV GC-TOFMS system.

$\mathbf{H_0}$: $\mu_{PegIII-external\ calibration} = \mu_{PegIV-external\ calibration}$

$\mathbf{H_0}$: $\mu_{PegIII-isotope\ dilution} = \mu_{PegIV-isotope\ dilution}$

$\mathbf{H_a}$: $\mu_{PegIII-external\ calibration} \neq \mu_{PegIV-external\ calibration}$

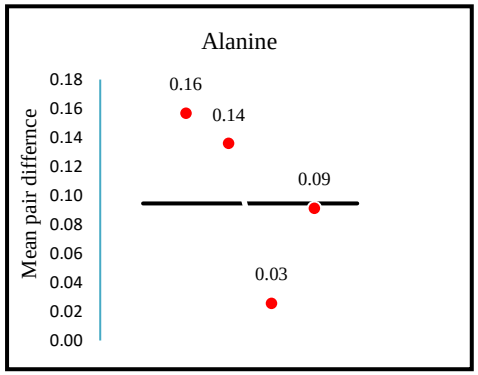
$\mathbf{H_a}$: $\mu_{PegIII-isotope\ dilution} \neq \mu_{PegIV-isotope\ dilution}$

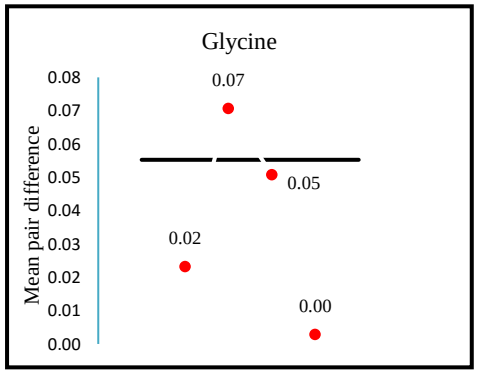
In all the above hypotheses, μ represents an experimental mean of each method.

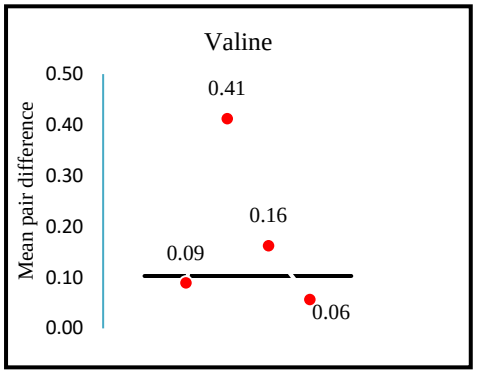
The above statistical tests were performed at a 95% confidence level. The critical value of F(ANOVA) was 2.62 with respect to all the analytes. As shown in **Table 4-16**, all experimental F(ANOVA) values were greater than F-critical signifying that some of the means differed

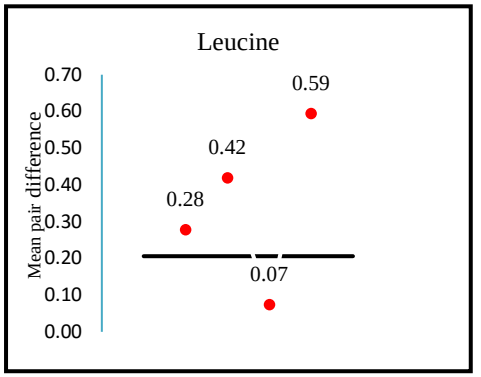
significantly. A Tukey test was then used to identify the means that differed significantly, the results thereof are shown on "HSD charts" in **Figure 4-24**. On the HSD charts in **Figure 4-24**, experimental means were compared from left to right. From left, experimental means obtained by external calibration using the Pegasus III GC-TOFMS system and the UPLC system were compared, followed by experimental means obtained through the isotope dilution method on the Pegasus III GC-TOFMS and the internal standard method using the UPLC system. Thirdly, experimental means obtained through the external calibration method using the Pegasus III GC-TOF system were compared with experimental means obtained using the same method on the Pegasus IV GC-TOFMS system. Lastly, experimental means obtained through the isotope dilution method using the Pegasus III GC-TOFMS system were compared with the results obtained using the same method on the Pegasus IV GC-TOFMS system. Therefore, each point on each on the "HSD charts" represents the differences between the experimental means in the previously described order and all the points that appear above the HSD line signifies means differed significantly.

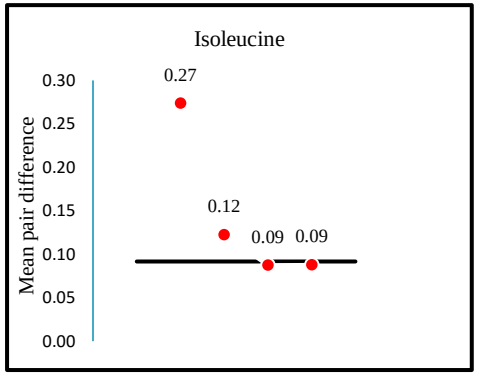
As depicted in **Figure 4-24**, some of the means differed significantly which is in good agreement with the data present in **Table 4-16**. With respect to the experimental means obtained by external calibration using the Pegasus III GC-TOFMS system and the UPLC system, significant differences were found on alanine, leucine, isoleucine, proline and tyrosine as depicted in **Figure 4-24**. In this regard, the null hypothesis was rejected, the alternative hypothesis accepted with respect to the above-mentioned analytes. On the other hand, using the isotope dilution method on the Pegasus III GC-TOFMS and the internal standard method on the UPLC system, significant differences were found on alanine, glycine, valine, leucine, isoleucine, proline, serine, lysine, histidine, and tyrosine as shown in **Figure 4-24**. Similarly, the null hypothesis was rejected, the alternative hypothesis accepted with respect to all the above-mentioned analytes. Because there were significant differences found between some of the means obtained using the UPLC system and GC-TOFMS system as previously discussed. The best practice would be to use both methods concurrently and use the results that produce the best recovery for each of the methods e.g. the recovery of histidine is much better on the GC-TOFMS system than on the UPLC system as seen in **Figure 4-23** and **Figure 4-24**.

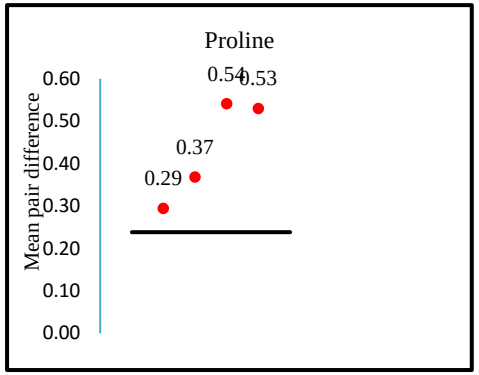

Alanine
Mean pair difference
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.16
0.14
0.09
0.03

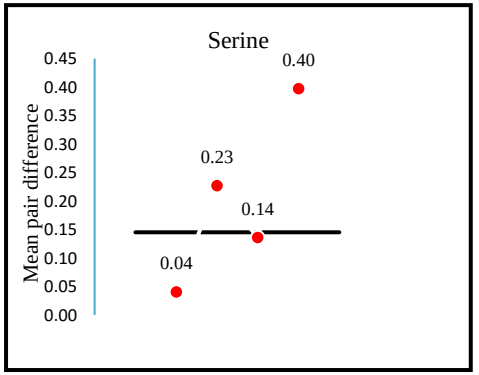

Glycine
Mean pair difference
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.07
0.05
0.02
0.00

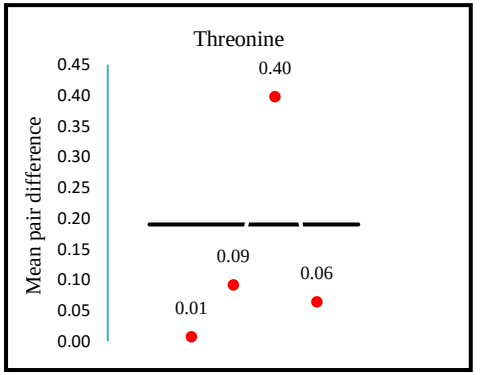

Valine
Mean pair difference
0.50
0.40
0.30
0.20
0.10
0.00
0.41
0.16
0.09
0.06

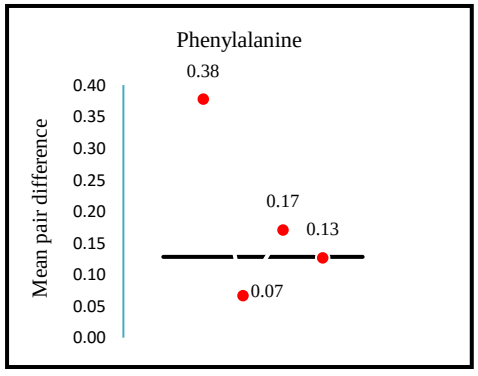

Leucine
Mean pair difference
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.59
0.42
0.28
0.07

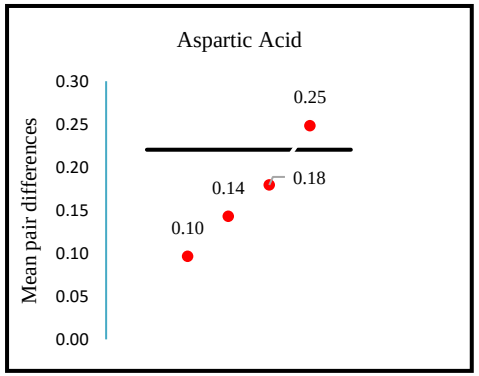

Isoleucine
Mean pair difference
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.27
0.12
0.09
0.09

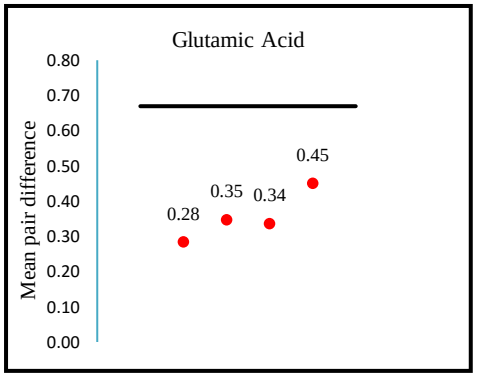

Proline
Mean pair difference
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.54
0.53
0.37
0.29

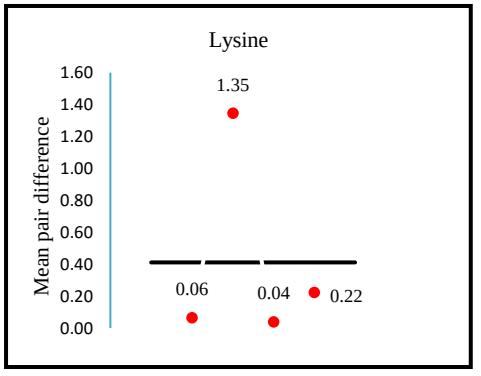

Serine
Mean pair difference
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.40
0.23
0.14
0.04


Threonine
Mean pair difference
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.40
0.09
0.06
0.01


Phenylalanine
Mean pair difference
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.38
0.17
0.13
0.07


Aspartic Acid
Mean pair differences
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.25
0.14
0.18
0.10


Glutamic Acid
Mean pair difference
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.45
0.35
0.34
0.28


Lysine
Mean pair difference
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1.35
0.06
0.04
0.22

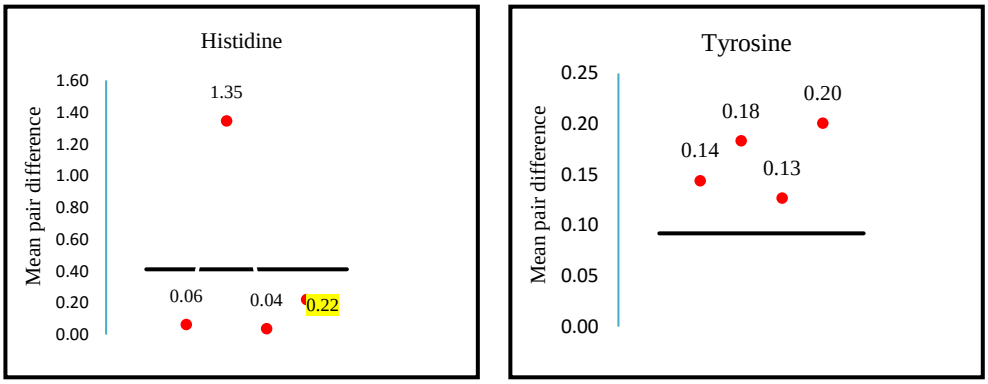


Figure 4-24: HSD charts used to identify differences between means obtained through the external calibration, isotope dilution and the internal standard method using the Pegasus III GC-TOFMS, Pegasus IV GC-TOFMS and the UPLC system. The red dots represent the differences between the experimental means. The first red dot represents the difference between the means obtained through the external calibration method using the Pegasus III GC-TOFMS system and the UPLC system. The second red dot represents the differences between the experimental means obtained through the isotope dilution method using the Pegasus III GC-TOFMS system and the UPLC system. The third red dot represents the differences between experimental means obtained through the external calibration method using the Pegasus III GC-TOFMS system while the fourth red dot represents the differences between the experimental means obtained through the isotope dilution method using the Pegasus III GC-TOFMS system and the Pegasus IV GC-TOFMS system. The numbers above each red dot represent the absolute difference between the means being compared. The black line represents the Tukey HSD value.

With respect to the experimental means obtained through external calibration method using the Pegasus III GC-TOFMS system and the Pegasus IV GC-TOFMS system, significant differences were found on valine, proline, threonine, phenylalanine, and tyrosine. Therefore, the null hypothesis was rejected, the alternative hypothesis accepted with respect to the above-mentioned analytes. On the other hand, using the isotope dilution on both the Pegasus III and the Pegasus IV GC-TOFMS systems, significant differences were found on leucine, proline, serine, aspartic acid, and tyrosine. Similarly, the null hypothesis was rejected, the alternative hypothesis accepted with respect to the above-mentioned analytes. Based on these observations, only a few analytes under both external calibration and isotope dilution differed significantly between the two systems. Tyrosine was the only analyte that differed significantly using both the external calibration method and the isotope dilution method. Therefore, the GC-TOFMS method showed good transferability between different GC-MS systems.

4.3 Commercial Infant Formula Analysis Using The GC-TOFMS Method

Finally, the GC-TOMS method developed in this study was used to analyse a commercial infant formula sample obtained from a local retailer and the results thereof are presented in **Table 4-17**. Target compounds were identified as outlined in **subsection 4.2.3** and the results thereof are presented in **Table 4-18** and **Table 4-19**. Uncertainty calculations were performed at a 95% confidence level using a coverage factor of $k = 2$. Out of eleven amino acids identified by the CODEX Alimentarius as vital amino acids as seen in **Table-2.1**, eight of those namely histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tyrosine and valine were quantified with the newly developed GC-TOFMS method. According to the manufacturer's label, the infant formula contains 14.7 g of protein per 100g grams of powdered milk. By conversion, the recommended minimum concentrations of the previously mentioned analytes are as shown in **Table-4.19**. Based on the analysis results, all analytes in the commercial infant formula, except tyrosine, agreed well with CODEX STAN 72 infant formula code recommendations.

Table 4-17: The results of analysis of the commercial infant formula sample

Amino Acids	[AA] (g/100g)	U (g/100g)	S_{x0} (g/100g)	CV	CI (g /100g)	υ_{eff}
Alanine	0.53	0.12	0.030	5.69	0.13	18
Glycine	0.20	0.01	0.001	0.41	4.60×10^{-3}	0
Valine	0.81	0.30	0.047	5.85	0.21	79
Leucine	1.30	0.21	0.084	6.49	0.38	8
Isoleucine	0.76	0.27	0.022	1.48	0.10	41
Proline	1.086	0.75	0.521	18.37	2.34	75
Serine	0.71	0.56	0.355	3.54	1.59	33
Threonine	0.72	0.15	0.092	12.89	0.41	43
Phenylalanine	0.67	0.29	0.033	4.97	0.01	5
Aspartic acid	1.021	0.21	0.122	11.92	0.55	4
Glutamic acid	2.67	0.30	0.208	7.81	0.93	27
Lysine	0.72	0.17	0.093	12.92	0.42	24
Histidine	0.28	0.070	0.035	12.70	0.16	20
Tyrosine	0.47	0.050	0.044	9.31	0.20	6

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, CV = relative standard deviation and CI = confidence interval of the mean. U = expanded uncertainty of measurement and υ_{eff} = effective number of degrees of freedom.

Table 4-18: Commercial infant formula: analytes identification by comparison of retention time to that of calibration standards and by percentage MS library match

	Pure Standard		Commercial Infant Formula Sample		
Analytes	Mean t_r (s)	CI (s)	Mean t_r (s)	CI (s)	% MS Similarity
Alanine	457.605	0.062	457.648	0.200	90.70
Glycine	471.623	0.060	471.666	0.218	91.30
Valine	509.717	0.066	509.794	0.173	75.90
Leucine	524.026	0.060	524.121	0.159	92.60
Isoleucine	534.588	0.062	534.665	0.190	84.80
Proline	548.249	0.068	548.374	0.190	74.30
Serine	626.089	0.064	626.128	0.112	85.10
Threonine	634.553	0.063	634.577	0.148	75.10
Phenylalanine	653.564	0.067	653.546	0.175	83.20
Aspartic Acid	666.773	0.127	666.636	0.288	87.30
Glutamic Acid	690.421	0.244	690.103	0.547	82.20
Lysine	710.837	0.311	710.381	0.726	80.90
Histidine	745.989	0.435	745.405	1.081	88.50
Tyrosine	755.785	0.482	755.116	1.170	91.70

Mean t_r = average retention time (n = 5), CI = confidence level and %MS Similarity = mass spectral library match

Table 4-19: Commercial infant formula: analyte identification by ion ratios

Analytes	Pure Standard Average Ion Ratio	CIF Average Ion Ratio	% Difference
Alanine	0.448	0.447	0.079
Glycine	0.516	0.503	2.59
Valine	0.334	0.333	0.52
Leucine	0.322	0.323	0.19
Isoleucine	0.335	0.328	2.07
Proline	0.215	0.204	4.94
Serine	0.831	0.837	0.73
Threonine	0.062	0.052	16.80
Phenylalanine	0.653	0.823	26.11
Aspartic Acid	0.049	0.047	4.55
Glutamic Acid	0.532	0.527	0.98
Lysine	0.390	0.410	5.14
Histidine	0.159	0.131	17.68
Tyrosine	0.169	0.155	8.12

CIF = commercial infant formula sample

Table 4-20: Comparison of Codex Alimentarius Commission's recommended amino acid concentrations against experimental concentrations

Analyte	Recommended in 14.7 g protein	Experimental in 14.7 g protein	U in 14.7 g protein
Histidine	0.34	0.28	0.070
isoleucine	0.75	0.76	0.27
Leucine	1.4	1.30	0.21
Lysine	0.93	0.72	0.17
Phenylalanine	0.66	0.67	0.29
Threonine	0.63	0.72	0.15
Tyrosine	0.62	0.47	0.050
Valine	0.74	0.81	0.30

U = expanded uncertainty of measurement

4.4 Comparison of TFA and HCl Hydrolysis

In **Chapter 2**, literature reports suggested that addition of TFA in HCl during acid hydrolysis can shorten the protein hydrolysis period from 22 hours to approximately 1 hour. With such an improvement, analysis time could be reduced from days to merely hours. As part of NMISA's quest to develop two independent methods for value assigning the content amino acids in infant formula for proficiency testing schemes, it was therefore imperative to compare the performance of hydrochloric acid hydrolysis method and the TFA hydrolysis method to determine whether the TFA hydrolysis method may be used as an alternative to the most commonly used HCl hydrolysis method. For this purpose, TFA hydrolysates were prepared as outlined in **Chapter 3** then analysed using both the external calibration and the isotope dilution method the results thereof are presented in **Table 4-21** and **Table 4-22**. As seen in **Table 4-21**, using the external calibration method recoveries ranging from 86.73% to 132.98% were obtained while recoveries of between 19.14% and 123.67% were obtained through the isotope dilution method as seen in **Table 4-22**. Furthermore, using the external calibration method coefficients of variation ranging from 1.71% to 19.16% were obtained while coefficients of variation ranging from 1.20% to 26.21% were obtained through the isotope dilution method. As seen in **Table 4-21** and **Table 4-22**, on average the coefficients of variation obtained using TFA hydrolysis method were lower than %RSDs obtained by HCl acid hydrolysis as seen in **Table 4-6** and **Table 4-7** in **subsection 4.2.2**.

Table 4-21: TFA hydrolysis method performance parameters obtained through the external calibration

Amino Acids	[AA] $(g/100g)$	S_{x0} $(g/100g)$	%Recovery	CV	CI (g/100g)
Alanine	0.4950	0.0267	108.79	5.40	0.0332
Glycine	0.2153	0.0329	89.35	15.29	0.0409
Valine	0.7869	0.0185	103.54	2.35	0.0230
Leucine	1.268	0.0458	100.52	3.61	0.0569
Isoleucine	0.7466	0.0214	113.12	2.87	0.0266
Proline	1.036	0.1199	86.73	11.57	0.1489
Serine	0.9062	0.1137	125.86	12.54	0.1411
Threonine	0.8777	0.0754	132.98	8.59	0.0936
Phenylalanine	0.6437	0.0110	110.98	1.71	0.0137
Aspartic Acid	1.006	0.0496	93.98	4.93	0.0616
Glutamic Acid	2.495	0.0615	96.35	2.47	0.0764
Lysine	0.6166	0.1181	57.63	19.16	0.1467
Histidine	0.6166	0.1181	195.76	19.16	0.1467
Tyrosine	0.4602	0.0160	90.23	3.47	0.0198

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by **Equation 3.7**, CV = relative standard deviation and CI = confidence interval of the mean**.**

Table 4-22: TFA hydrolysis method parameters obtained through the isotope dilution method

Amino Acids	[AA] $(g/100g)$	S_{x0} $(g/100g)$	%Recovery	CV	CI $(g/100g)$
Alanine	0.3787	0.0307	83.23	8.12	0.0382
Glycine	0.0871	0.0418	19.14	17.36	0.0520
Valine	0.6088	0.0300	80.11	4.93	0.0373
Leucine	1.426	0.0172	113.12	1.20	0.0213
Isoleucine	0.5891	0.0256	89.26	4.35	0.0318
Proline	1.478	0.0824	123.67	5.58	0.1023
Serine	0.5515	0.1132	76.60	20.53	0.1406
Threonine	0.7248	0.1533	109.82	21.16	0.1904
Phenylalanine	0.3531	0.0097	60.87	2.74	0.0120
Aspartic Acid	0.8569	0.0731	80.08	8.53	0.0907
Glutamic Acid	2.628	0.1519	101.47	5.78	0.1886
Lysine	0.5483	0.1437	51.24	26.21	0.1785
Histidine	0.5483	0.1437	174.05	26.21	0.1785
Tyrosine	0.2986	0.0215	58.55	7.19	0.0266

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by **Equation 3.7**, CV = relative standard deviation and CI = confidence interval of the mean**.**

As in the preceding subsections, the differences between the TFA hydrolysis and the hydrochloric acid hydrolysis methods were initially visually assessed as shown in **Figures 4-25** and **Figure 4-26** prior to the application of the statistical methods to assess the significance of the observed differences. Using both the external calibration and the isotope dilution method, differences were found on various amino acids as depicted in **Figure 4-25** and **Figure 4-26**. However, in using both the external calibration method and the isotope dilution method most error bars overlapped suggesting that some recoveries did not differ significantly. With respect to the external calibration method, all error bars overlapped except for the error bars of valine, serine, threonine, and histidine. Similarly, using the isotope dilution method, all error bars overlapped except for the error bars of glycine, proline, phenylalanine, histidine, and tyrosine. A two-sided t-test was used to assess the significance of the observed differences and the results thereof are shown in **Table 4-23** and **Table 4-24**. Prior to the application of the student's t-test an F-test was used assess the significance of the differences between the variances of the TFA hydrolysis method and the hydrochloric acid hydrolysis method, and the results thereof are also shown in **Table 4-23** and **Table 4-24** for the external calibration method and the isotope dilution method respectively.

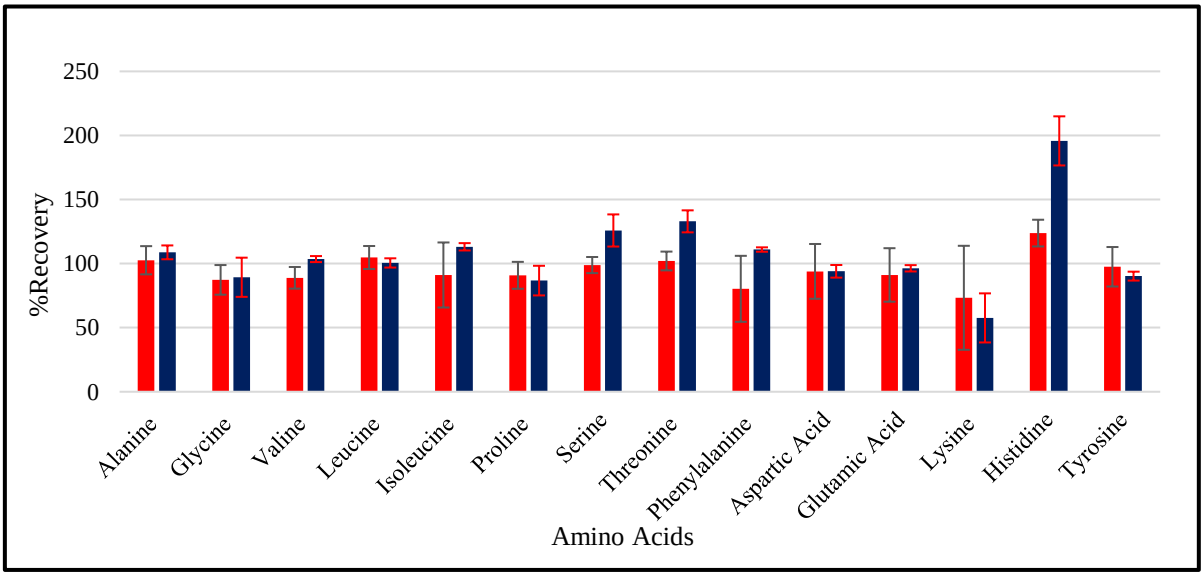


Figure 4-25: External calibration %recoveries used to compare the TFA hydrolysis (blue bars) method and the hydrochloric acid hydrolysis method (red bars). The error bars represent the coefficients of variation.

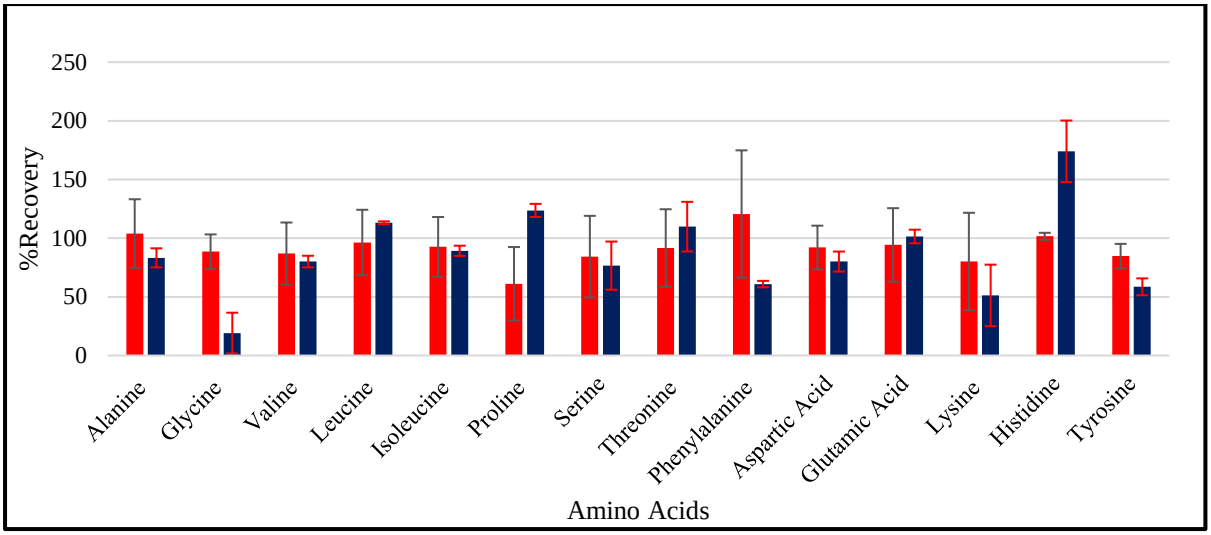


Figure 4-26: Isotope dilution %recoveries used to compare the TFA hydrolysis (blue bars) method and the hydrochloric acid hydrolysis method. (red bars). The error bars represent the coefficients of variation.

Table 4-23: External calibration method F-test and t-test results used to compare the TFA hydrolysis method and the hydrochloric acid hydrolysis method

Amino Acids	F (experimental)	F(critical)	t(experimental)	t(critical)	v
Alanine	$F(4,4) = 6.89\ p < 0.05$	6.39	0.15	2.57	5
Glycine	$F(4,4) = 1.09\ p < 0.05$	6.39	4.27E-03	2.31	8
Valine	$F(4,4) = 17.27\ p < 0.05$	6.39	4.02	2.77	4
Leucine	$F(4,4) = 12.31\ p < 0.05$	6.39	0.922	2.57	5
Isoleucine	$F(4,4) = 10.88\ p < 0.05$	6.39	3.28	2.57	5
Proline	$F(4,4) = 2.05\ p < 0.05$	6.39	4.63	2.31	8
Serine	$F(4,4) = 3.76\ p < 0.05$	6.39	3.43	2.31	8
Threonine	$F(4,4) = 4.09\ p < 0.05$	6.39	4.44	2.31	8
Phenylalanine	$F(4,4) = 68.50\ p < 0.05$	6.39	6.05	2.77	4
Aspartic Acid	$F(4,4) = 16.18\ p < 0.05$	6.39	0.03	2.77	4
Glutamic Acid	$F(4,4) = 100.03\ p < 0.05$	6.39	0.20	2.77	4
Lysine	$F(4,4) = 7.73\ p < 0.05$	6.39	1.23	2.57	5
Histidine	$F(4,4) = 7.73\ p < 0.05$	6.39	1.23	2.57	5
Tyrosine	$F(4,4) = 30.12\ p < 0.05$	6.39	1.78	2.77	4

v = the number of degrees of freedom

Table 4-24: Isotope dilution method F-test and t-test results used to compare the TFA hydrolysis method and the hydrochloric acid hydrolysis method

Amino Acids	F (experimental)	F(critical)	t(experimental)	t(critical)	v
Alanine	$F(4,4) = 6.43\ p < 0.05$	6.39	4.12	2.57	5
Glycine	$F(4,4) = 1.83\ p < 0.05$	6.39	5.30	2.31	8
Valine	$F(4,4) = 7.77\ p < 0.05$	6.39	2.13	2.57	5
Leucine	$F(4,4) = 86.15\ p < 0.05$	6.39	0.28	2.77	4
Isoleucine	$F(4,4) = 6.57\ p < 0.05$	6.39	0.80	2.57	5
Proline	$F(4,4) = 5.78\ p < 0.05$	6.39	1.75	2.31	8
Serine	$F(4,4) = 2.92\ p < 0.05$	6.39	0.66	2.31	8
Threonine	$F(4,4) = 1.34\ p < 0.05$	6.39	1.71	2.31	8
Phenylalanine	$F(4,4) = 45.129\ p < 0.05$	6.39	11.74	2.77	4
Aspartic Acid	$F(4,4) = 5.87\ p < 0.05$	6.39	0.60	2.31	8
Glutamic Acid	$F(4,4) = 12.37\ p < 0.05$	6.39	0.78	2.57	5
Lysine	$F(4,4) = 4.58\ p < 0.05$	6.39	1.26	2.31	8
Histidine	$F(4,4) = 4.58\ p < 0.05$	6.39	1.27	2.31	8
Tyrosine	$F(4,4) = 4.30\ p < 0.05$	6.390	11.16	2.31	8

v = the number of degrees of freedom

For the F-test, the following null and alternative hypotheses were made:

H_0: $S^2_{TFA\ hydrolysis\ external\ claibration\ method} = S^2_{HCl\ hydrolysis\ external\ calibration\ method}$

H_0: $S^2_{TFA\ hydrolysis\ isotope\ dilution\ method} = S^2_{HCl\ hydrolysis\ isotope\ dilution\ method}$

H_a: $S^2_{TFA\ hydrolysis\ external\ claibration\ method} \neq S^2_{HCl\ hydrolysis\ external\ calibration\ method}$

H_a: $S^2_{TFA\ hydrolysis\ isotope\ dilution\ method} \neq S^2_{HCl\ hydrolysis\ isotope\ dilution\ method}$

Where S^2 is the variance of the hydrolysis method obtained by external calibration of through the isotope dilution method.

For the students t-test the following null and alternative hypotheses were made:

H_0: $\mu_{TFA\ hydrolysis\ extrenal\ calibration\ method} = \mu_{HCl\ hydrolysis\ external\ calibration\ method}$

H_0: $\mu_{TFA\ hydrolysis\ isotope\ dilution\ method} = \mu_{HCl\ hydrolysis\ isotope\ dilution}$

H_0: $\mu_{TFA\ hydrolysis\ extrenal\ calibration\ method} \neq \mu_{HCl\ hydrolysis\ external\ calibration\ method}$

H_0: $\mu_{TFA\ hydrolysis\ isotope\ dilution\ method} \neq \mu_{HCl\ hydrolysis\ isotope\ dilution}$

Where μ is the experimental mean of the hydrolysis method obtained by external calibration or through the isotope dilution method.

Using the external calibration method, the experimental F-values of alanine, valine, leucine, isoleucine, phenylalanine, aspartic acid, glutamic acid, histidine, and tyrosine were greater than the critical F-values as seen in **Table 4-23**. Therefore, the null hypothesis was rejected with respect to the above-mentioned analytes hence **Equation 3-3** was used to calculate the experimental t-values of these analytes. Furthermore, using the isotope dilution method, the experimental F-values of alanine, valine, isoleucine, phenylalanine and glutamic acid also exceeded the critical F-value as seen in **Table 4-24**. Similarly, the null hypothesis was also rejected with respect to the above-mentioned analytes and **Equation 3-3** was also used to calculate the experimental t-values of thereof.

Using the external calibration method, the experimental t-values of valine, isoleucine, proline, threonine, serine, and phenylalanine were greater than the critical t-values as seen in **Table 4-23**. Therefore, the null hypothesis was rejected with respect to all the above-mentioned analytes. In addition, using the isotope dilution method, the experimental t-values of alanine, glycine, phenylalanine, and tyrosine were greater than the critical t-values as shown in **Table 4-24**. Similarly, the null hypothesis was rejected with respect to the above-mentioned analytes. As seen from **Table 4-23** and **Table -24**, only phenylalanine differed significantly under both the external calibration method and the isotope dilution method. Therefore, the TFA method may be used as an alternative hydrolysis method for quantification of amino acids in infant formula.

4.5 GC-TOFMS and UPLC Methods' Summary

Table 4-25: Comparison of methods for the determination of AAs in infant formula/CRM

Parameter	GC-TOFMS External Calibration	GC-TOFMS Isotope Dilution	UPLC External Calibration	UPLC Internal Standard Method	Meet AOAC Criteria
Accuracy (%Recovery)	61.13% to 103.99%	73.31% to 104.76%	55.21% to 104.49%	73.01% to 142.90%	Yes, with few exceptions such as proline and histidine.
Precision (CV)	7.32% to 25.76%	2.99% to 41.53%	3.86% to 20.21%	5.30% to 13.15%	No, on average coefficients of variation were greater than 4%
Sample Preparation	30 hours	30 hours	28 hours	28 hours	N/A
Instrumental Analysis Time	12.5 minutes	12.5 minutes	10 minutes	10 minutes	N/A
Total Cost Per Analysis	R 478.93	R 569.93	R 491.61	R 502.61	N/A
Advantages	• Quick analyte identification using MS libraries. • Lower cost per analysis. • Good stability of the derivatives. • Minimal use of organic solvents.	• Quick analyte identification using MS libraries. • Good stability of the derivatives. • Minimal use of organic solvents.	• Shorter sample preparation time. • Can be used to quantify cysteine, methionine, and tryptophan. • Shorter instrumental analysis time.	• Shorter sample preparation time. • Can be used to quantify cysteine, methionine, and tryptophan. • Shorter instrumental analysis time.	N/A
Disadvantages	• Longer sample preparation time. • Cannot be used to quantify cysteine, methionine, and tryptophan. • Longer instrument analysis time. • Degradation of arginine.	• Longer sample preparation time. • Cannot be used to quantify cysteine, methionine, and tryptophan • Longer instrument analysis time. • Degradation of arginine	• Use of organic solvent. • Higher total cost per analysis. • Reagents can only be sourced from one supplier	• Use of organic solvent. • Higher total cost per analysis. • Reagents can only be sourced from one supplier	N/A

N/A = not applicable

5. Conclusion

Our literature search suggested that, of all the existing methods used for quantification of protein in different matrices, no single analytical method can quantify all the amino acids simultaneously because some amino acids such as methionine, cysteine, and tryptophan either require a separate hydrolysis method to be used or additional sample preparation steps to be carried out. The AOAC accredited methods namely the Kjeldahl and Dumas methods currently used in protein analysis are vulnerable to errors stemming from food adulteration with nitrogen-containing compounds. There are also shortcomings when using more advanced analytical techniques such as high-performance liquid chromatography. Most HPLC methods are prone to errors arising from poor amino acids derivatives stability (e.g. OPA amino acid derivatives) and other issues relating to instrument sensitivity. The evidence presented in our literature review strongly suggests that other analytical techniques such as GC-MS should be tested in order to (1) find alternative solutions to the shortcomings of the currently certified methods or (2) to improve the currently existing amino acids testing methods. Consequently, a GC-TOFMS method for quantifying 14 of the 20 naturally existing amino acids was developed in this study.

Using the GC-TOFMS method developed in this study, analytes recoveries were well within the permissible range of between 80% and 120% as specified by (Green, 1996) with few exceptions namely phenylalanine (120.66%) and histidine (123.84%). Additionally, spike recoveries of between 90% and 110% as specified in the AOAC requirements for the development of methods for quantifying amino acids in infant formula were obtained except for the recovery of ^{13}C proline. Furthermore, GC-TOF MS LOQs were well below the maximum AOAC permissible LOQ value of 0.4 g/100g with a few exceptions namely proline (0.4779 g/100g), threonine (0.4927 g/100g), aspartic acid (0.4300 g/100g) and tyrosine (0.8186 g/100g) with tyrosine being the only analytes whose LOQ was greater than the sample concentration. Although LOQs and spike recoveries met the AOAC's specifications described in the call for the development of methods for quantification of amino acids in infant formula, the repeatability coefficients of variation (RSD_r) were on average greater than the AOAC specified value at 4%. This deviation may be attributed to (1) volumetric preparation of samples and (2) use of a small number of subsamples. Therefore, in future this method may be improved by exploring some of the following options, (1) gravimetric preparation of both calibration standards and samples in larger volumes as this will ensure better homogeneity thereby

improving between-sample variation, (2) if economically feasible, increasing the number of replicate subsamples from 5 to 10 subsamples per sample in order to reduce the between sample-variation, and (3) also if it is economically feasible spiking, samples before the samples are hydrolysed in order to cater for the variation due to the hydrolysis process.

Using the fishbone diagram to identify experimental variables that may have an impact on the outcomes of this study played a significant role in both method development and method validation. If applied correctly, this approach can easily improve method development and minimise wasteful consumption of expensive laboratory consumables. Furthermore, optimising variables likely to have an impact on the measurement result is likely to reduce uncertainty. Additionally, the use of unoptimised conditions would have also resulted in a considerable number of unaccounted for uncertainty contributions which would have led to the rejection of correct experimental results (means that have expanded uncertainties that include the true value).

Similarly, using statistical techniques during both method development and method validation allowed us to identify significant differences that were not obvious from visual inspection of the raw data and thereby enabling us to identify the optimum experimental conditions for analysis of amino acids using a GC-MS method in the case of method validation. Additionally, only statistical data was able to indicate whether the differences between analyte recoveries obtained using different instruments were significant or not in the case of method validation. This observation suggests that it is vital to apply simple chemometric statistics on experimental results in order to make informed decisions. Similarly, the inclusion of uncertainty of measurement as part of method validation is also key to making informed decisions. Without the uncertainty of measurement, we would have incorrectly deemed some analytes such as threonine, phenylalanine, and histidine as poorly recovered while the uncertainty of these measurements suggests that the spread of possible results include the reference values of these analytes specified in the SRM certificate.

In conclusion, the GC-TOFMS method developed in this study was able to quantify 14 of the 20 naturally occurring amino acids specified in **Chapter 3** namely alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, lysine, histidine, and tyrosine. With this method, some analytes are best recovered using the external calibration approach while others are better recovered using the isotope dilution method. Alanine, leucine, isoleucine, aspartic acid, glutamic acid, and histidine recoveries were better with using the external calibration approach while glycine, proline, phenylalanine,

serine, threonine, lysine and tyrosine were better recovered using the isotope dilution quantification approach. In contrast, UPLC AccQ·Tag yielded better results for lysine, threonine. Furthermore, given that HPLC methods for quantifying methionine, cysteine, cystine, and tryptophan are well established, combining the two GC-TOFMS calibration methods for total analysis of amino acids infant formula could yield positive results and should be considered in future studies.

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6. Appendices

6.1 Appendix A1

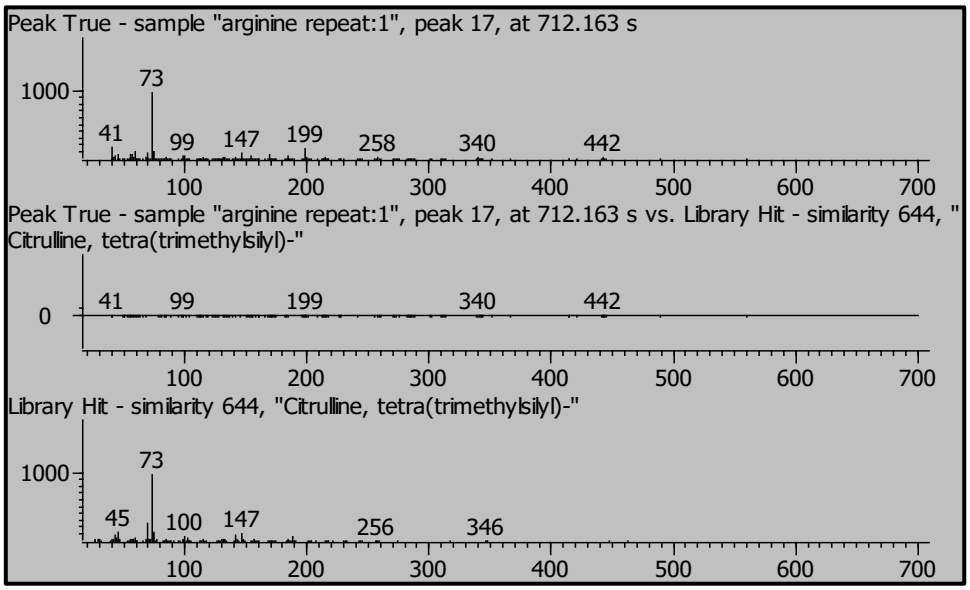
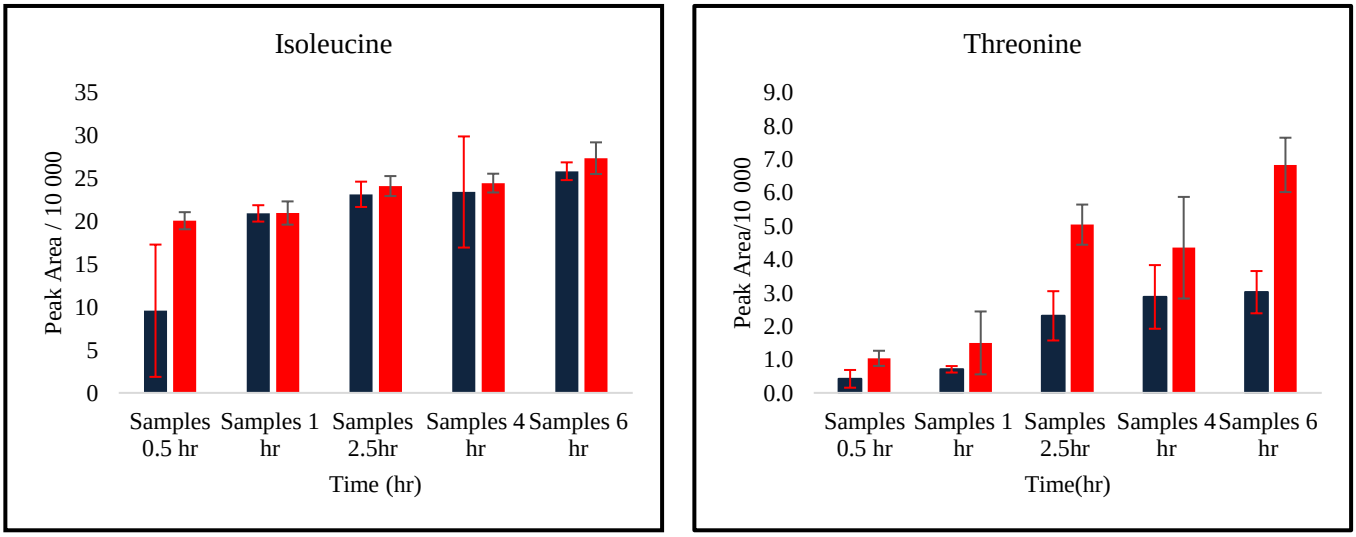
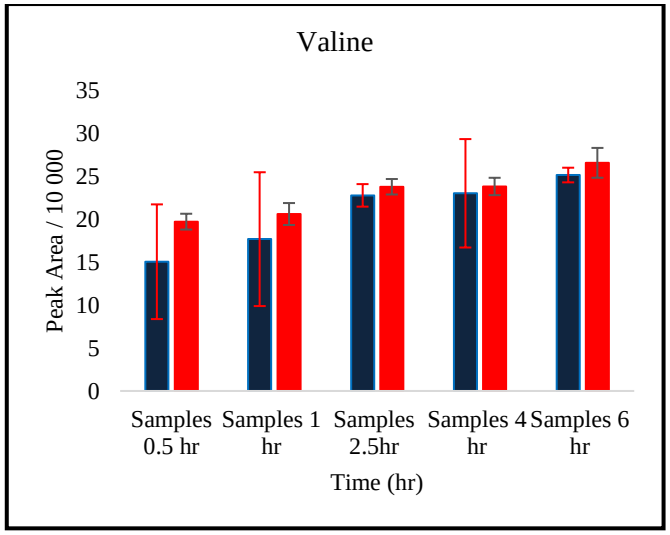


Figure 6-1: Mass spectra of citrulline that was detected in the sample of pure arginine after derivatisation with MTBSTFA. The top mass spectrum is the spectrum of citrulline. The bottom mass spectrum is the library match spectrum of ornithine while the spectrum in the middle represents the differences between the library match spectrum and the spectrum of citrulline (64.40%).

6.2 Appendix A2




Valine
Peak Area / 10 000
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15
10
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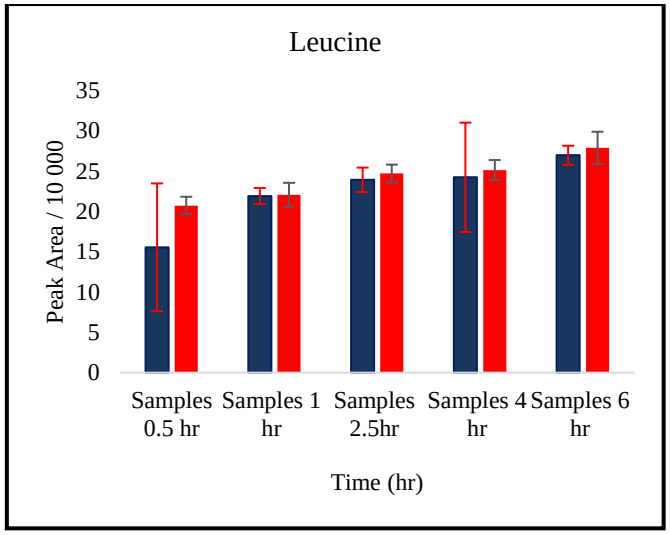
Samples 1 hr
Samples 2.5hr
Samples 4 hr
Samples 6 hr
Time (hr)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Samples 1 hr
Samples 2.5hr
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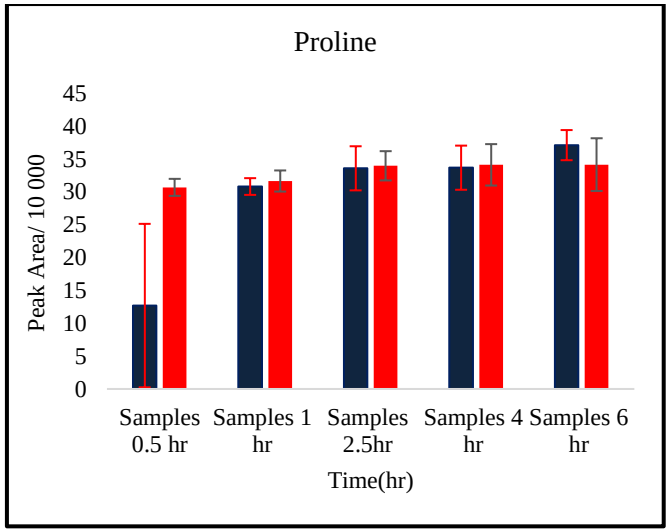
Samples 6 hr
Time (hr)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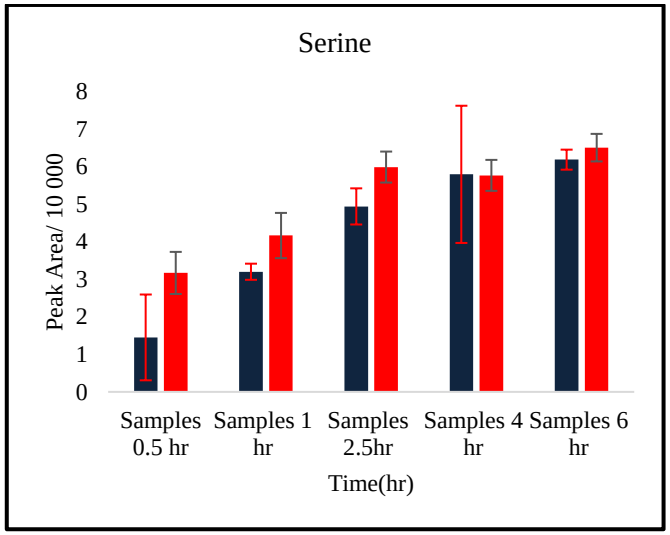
Samples 6 hr
Time(hr)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Peak Area/ 10 000
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Samples 2.5hr
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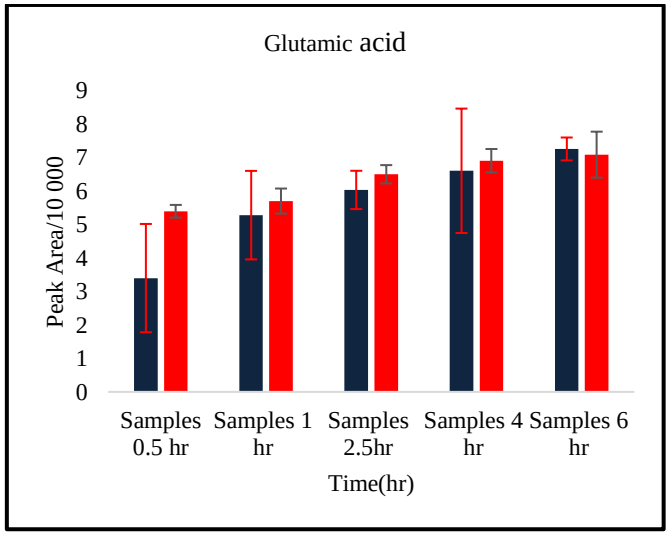
Samples 6 hr
Time(hr)


Glutamic acid
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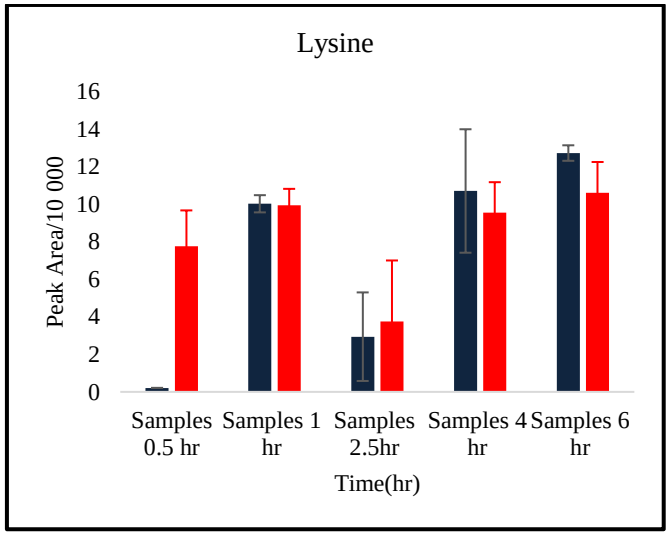
Samples 6 hr
Time(hr)


Lysine
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12
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8
6
4
2
0
Samples 0.5 hr
Samples 1 hr
Samples 2.5hr
Samples 4 hr
Samples 6 hr
Time(hr)

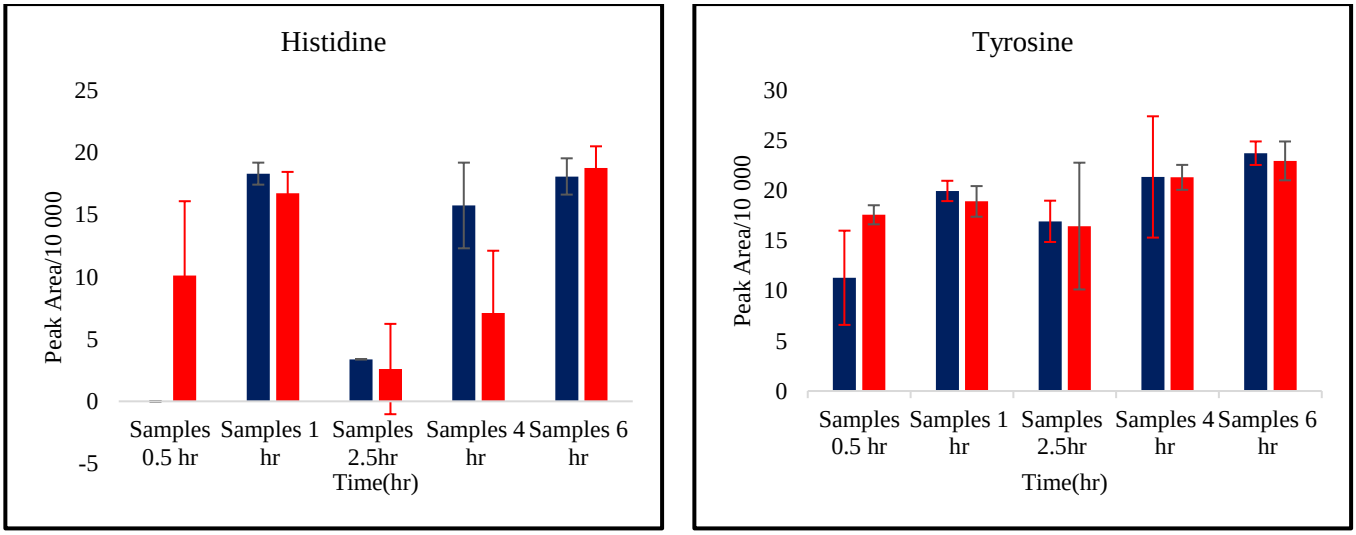


Figure 6-2: Paired means (n=5) plots comparing average amino acids responses between 75 and 100˚C, at **derivatisation** times of 0.5, 1, 2.5, 4 and 6 hours, to determine the optimum **derivatisation** temperature required for complete **derivatisation** of amino acids using MBSTFA. included in Figure 6.2 are paired means plots of valine, serine, leucine and proline, isoleucine, threonine, glutamic acid, lysine, aspartic acid, histidine, tyrosine. The blue bar graph represents **derivatisation** at 75 ˚C and the red bar graph represents **derivatisation** at 100 ˚C.

6.3. Appendix A3

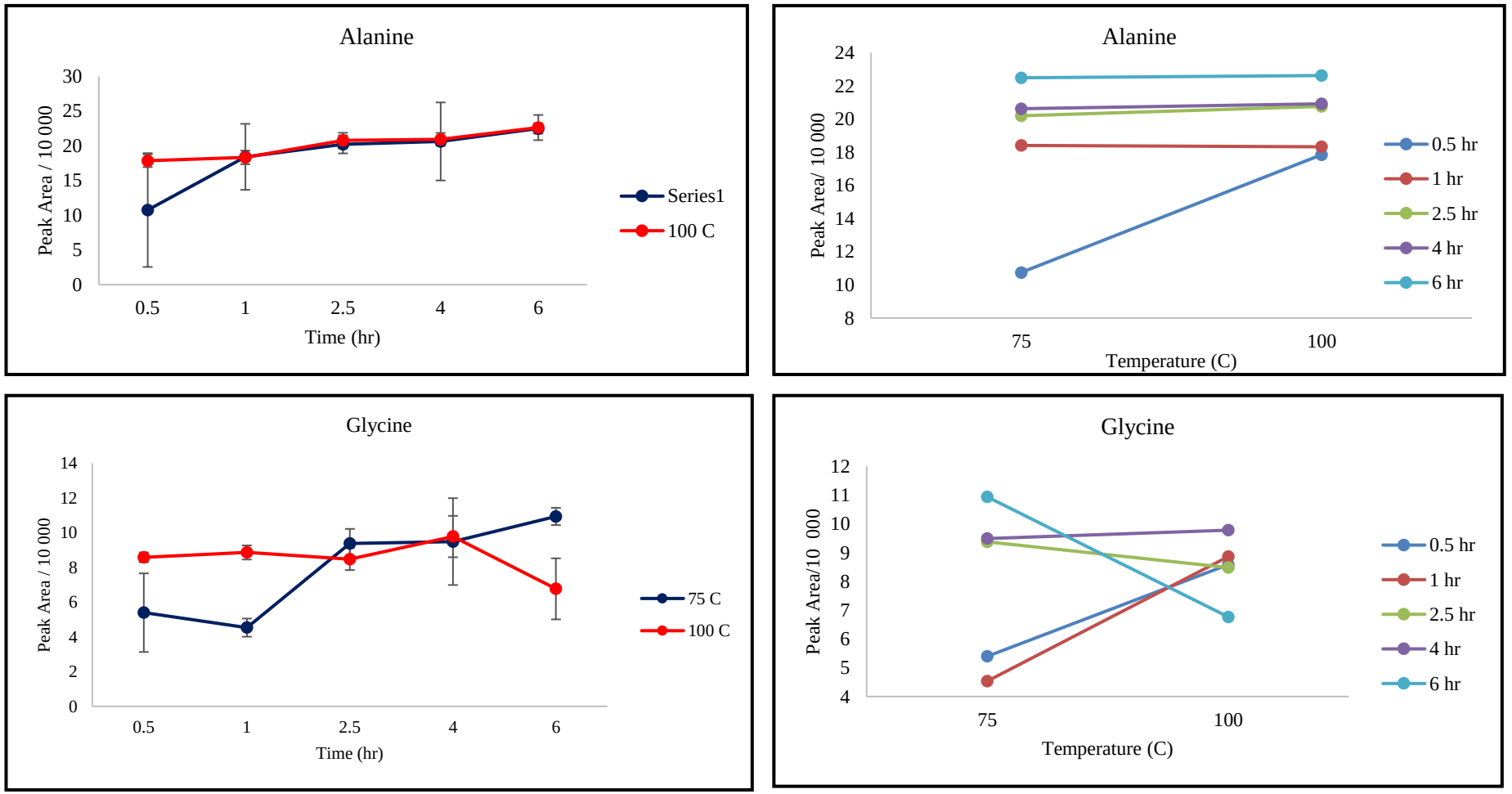


Figure 6-3: Derivatisation period and derivatisation temperature interactions plots of alanine and glycine used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represents derivatisation temperature interactions plots.

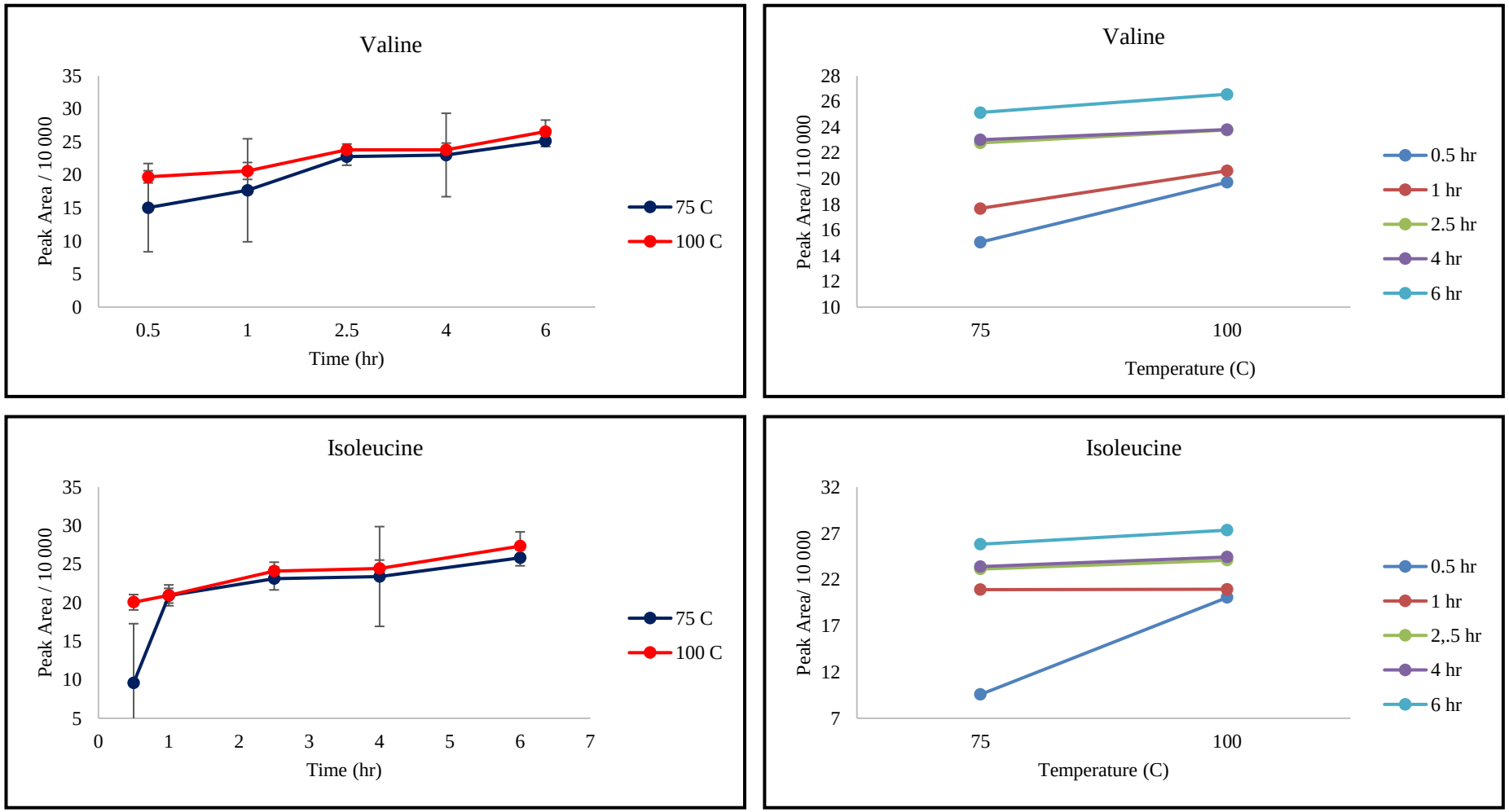


Figure 6-4: Derivatisation period and derivatisation temperature interactions plots of valine and isoleucine used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represents derivatisation temperature interactions plots.

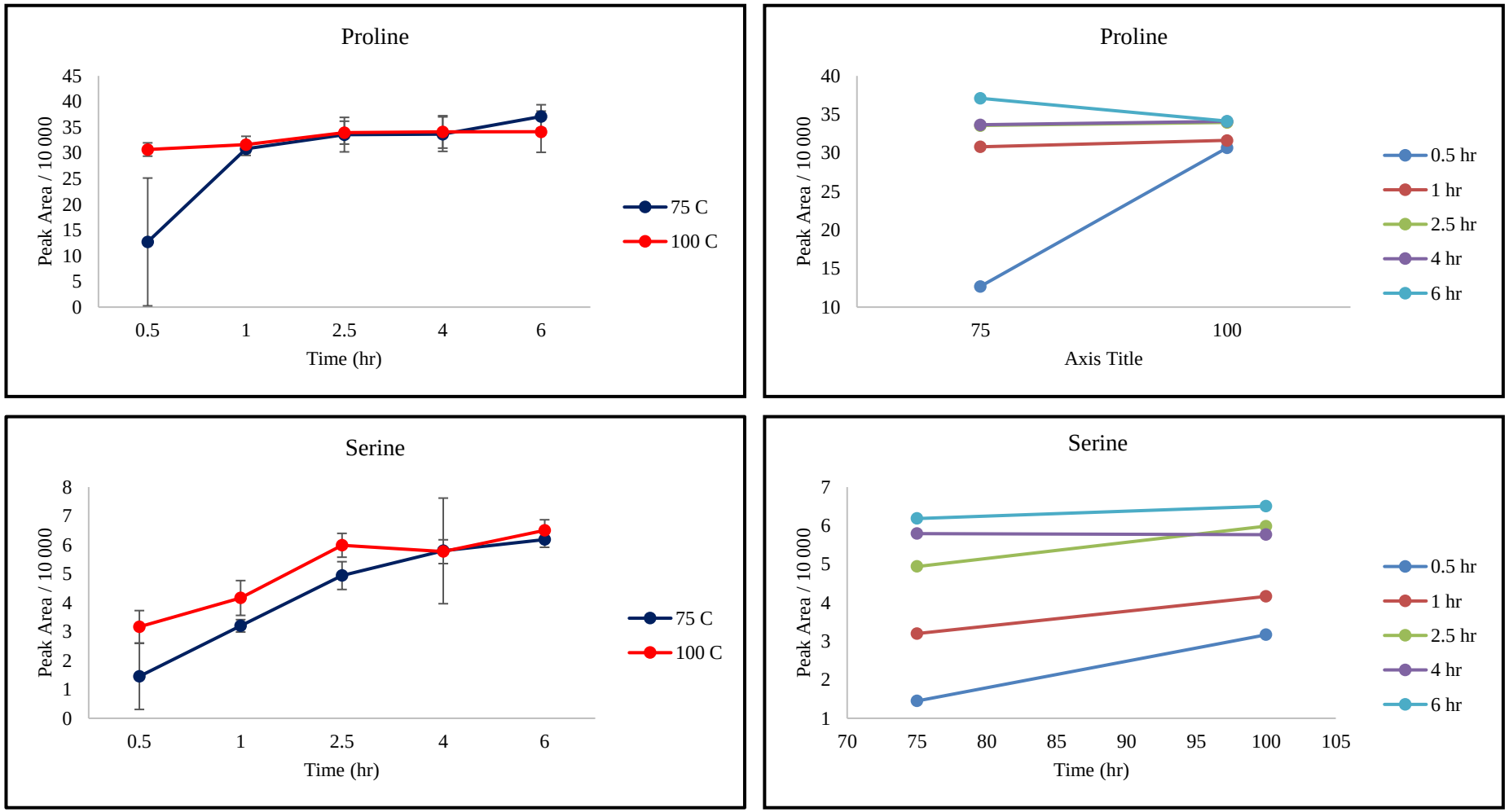


Figure 6-5: Derivatisation period and derivatisation temperature interactions plots of proline and serine used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represents derivatisation temperature interactions plots.

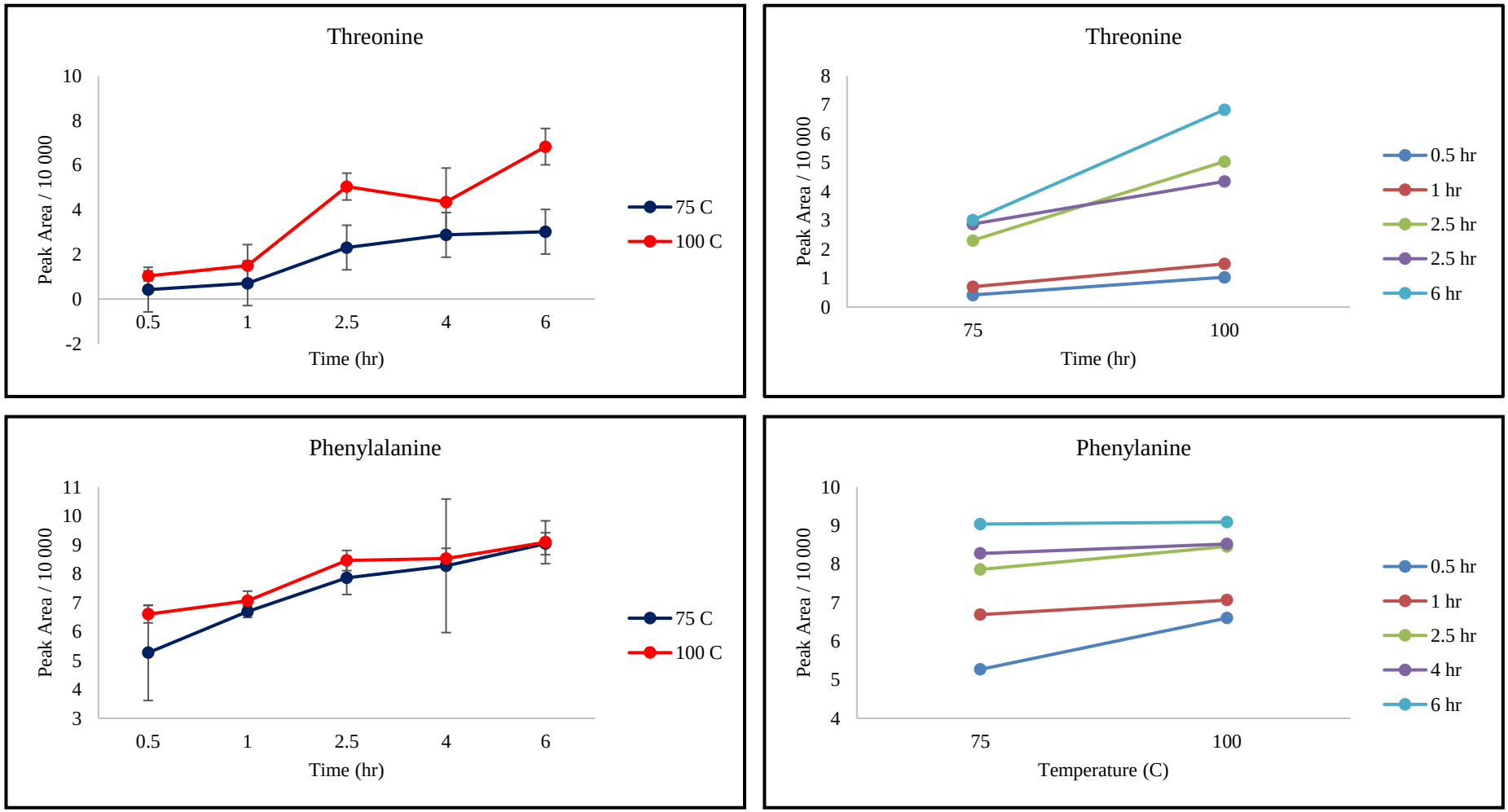


Figure 6-6: Derivatisation period and derivatisation temperature interactions plots of threonine and phenylalanine used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represents derivatisation temperature interactions plots.

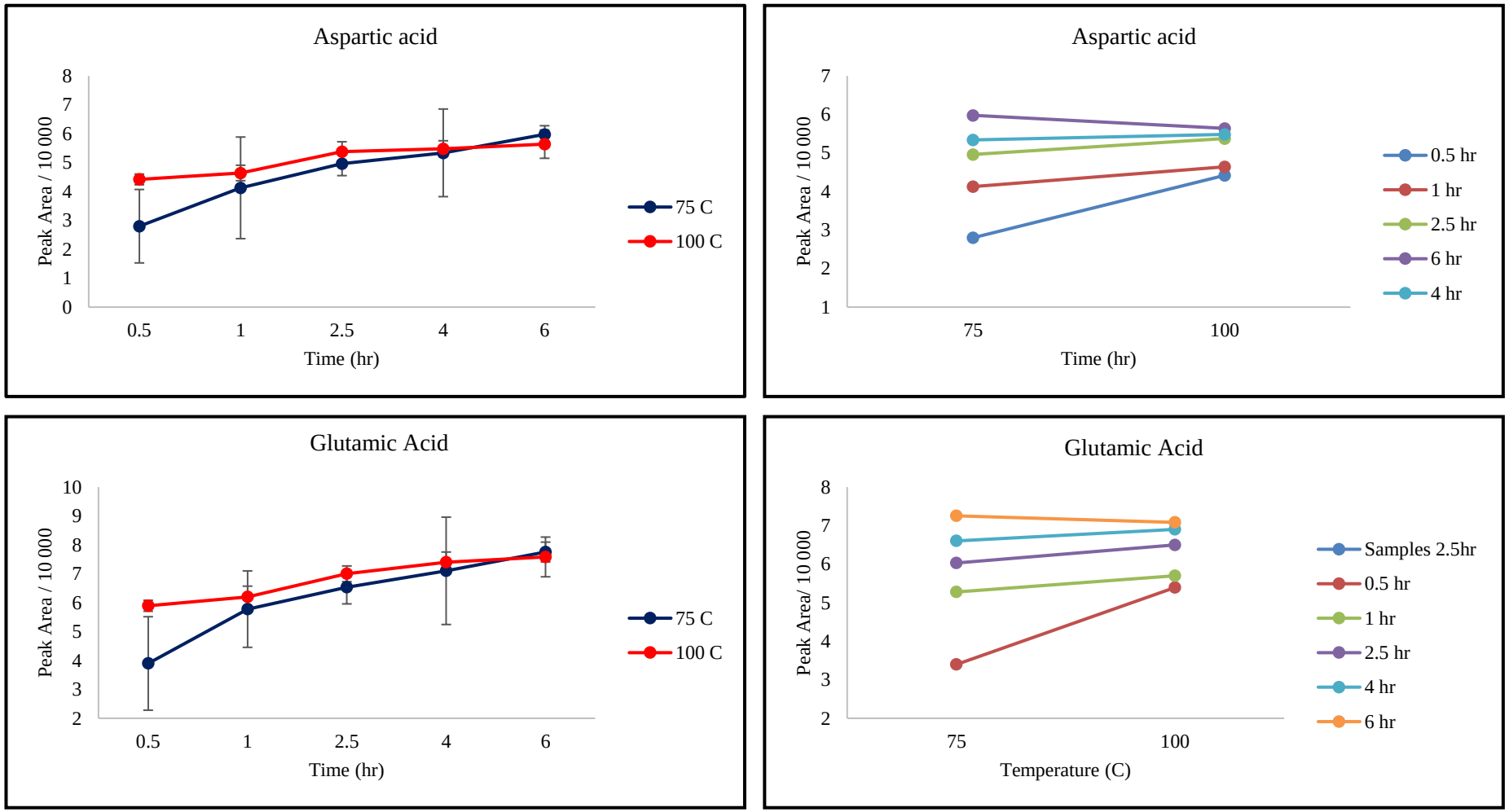


Figure 6-7: Derivatisation period and derivatisation temperature interactions plots of aspartic acid and glutamic acid used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represents derivatisation temperature interactions plots.

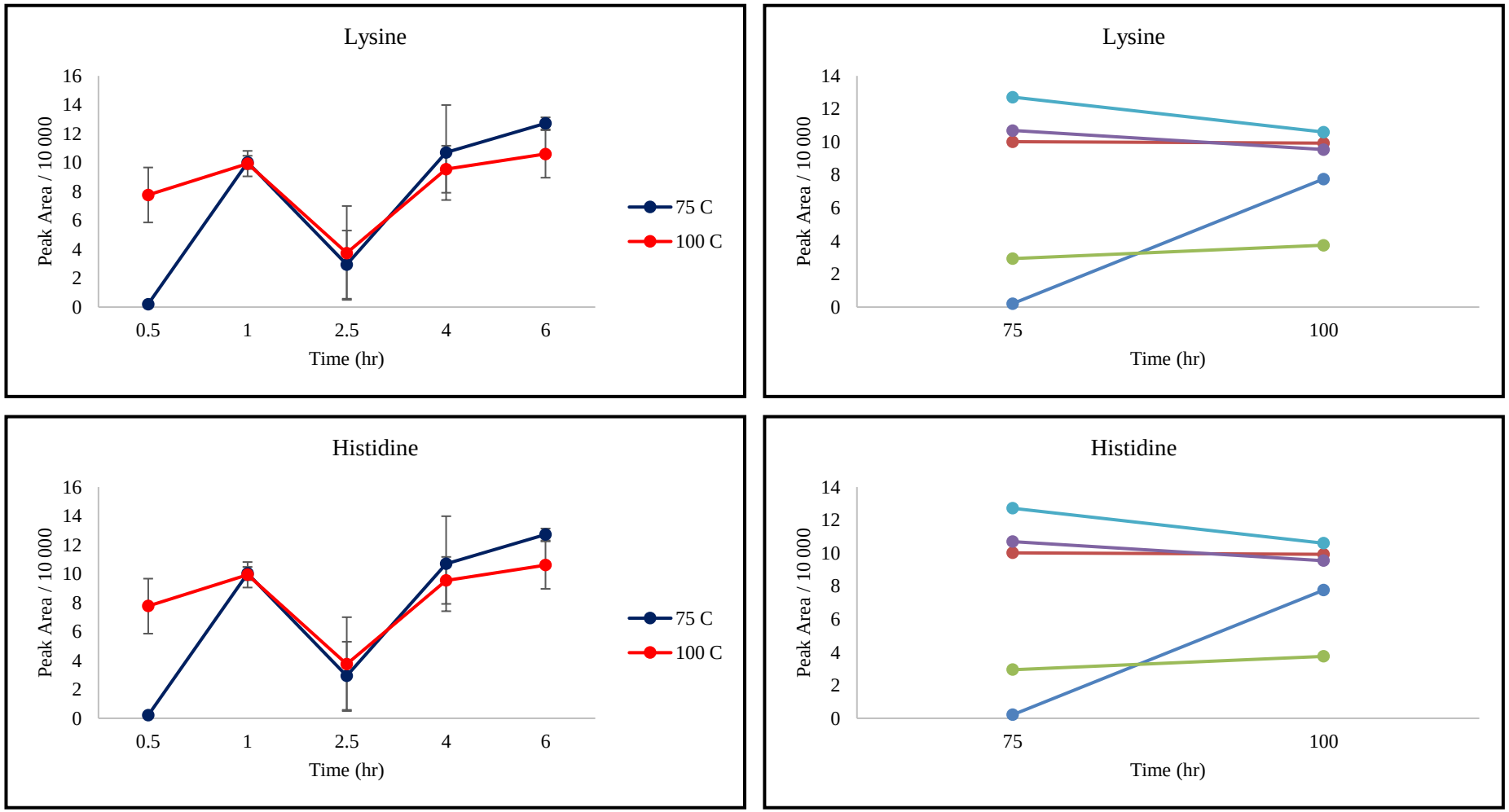


Figure 6-8: Derivatisation period and derivatisation temperature interactions plots of lysine and histidine acid used to assess interactions between derivatisation time and temperature. The interactions plots of the on the left-hand side represent derivatisation period interactions plots while the interactions plots on the right-hand side represent derivatisation temperature interactions plots.

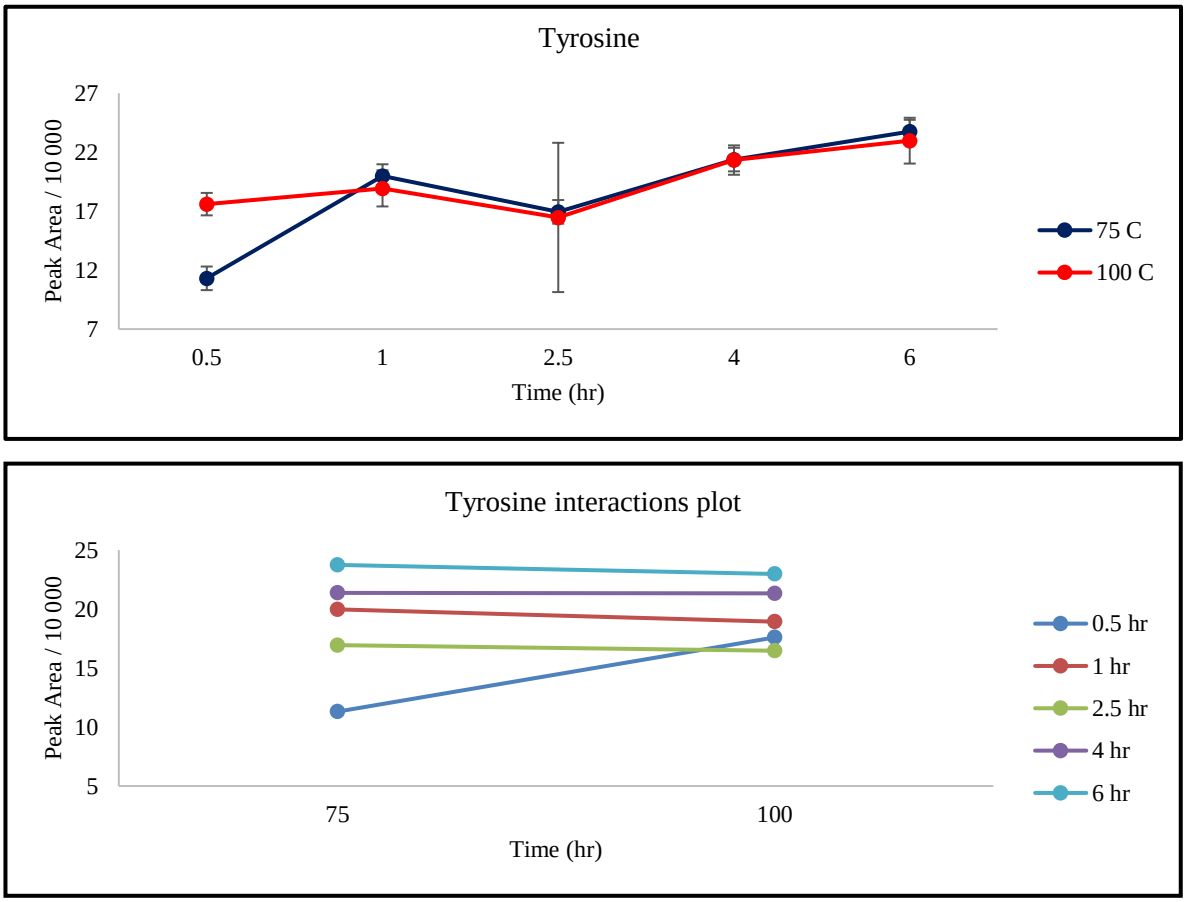


Figure 6-9: Derivatisation period and derivatisation temperature interactions plots of tyrosine used to assess interactions between derivatisation time and temperature. The top interactions plot represents derivatisation period interactions plots while the bottom interactions plot represents derivatisation temperature interactions plots.

6.4 Appendix A4

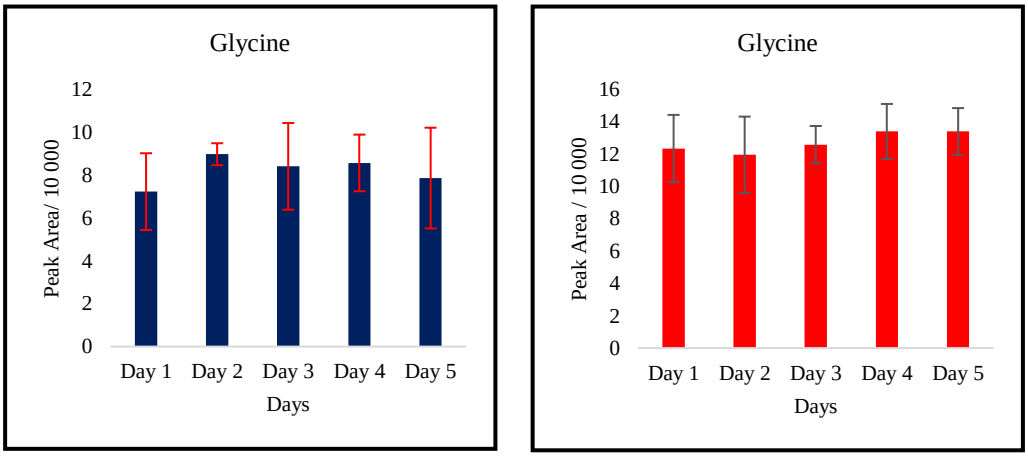


Figure 6-10: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure presents observation made on glycine stored at ambient (blue bars) and a cool temperature of 3 ˚C (red bars).

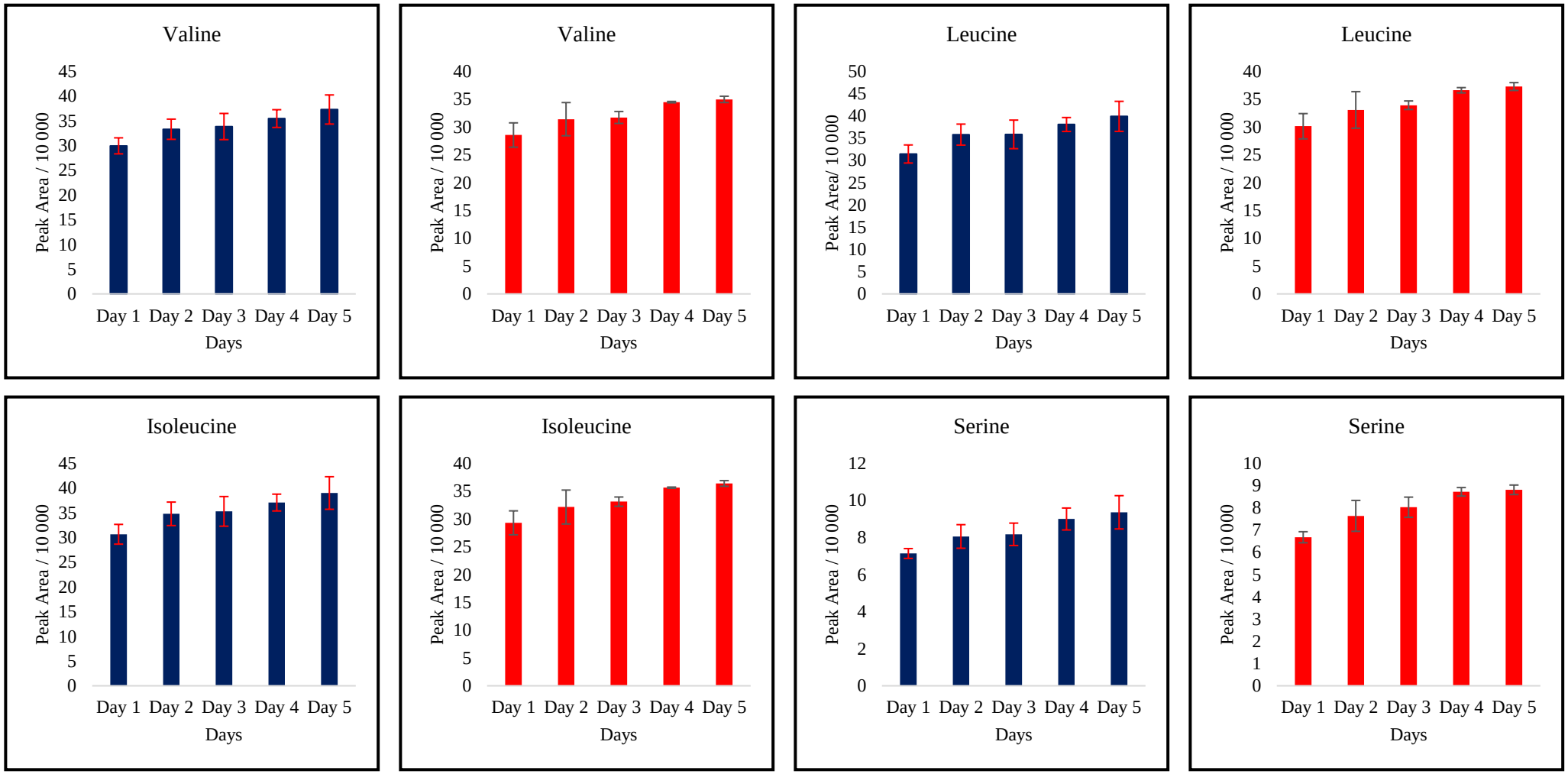


Figure 6-11: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure includes the observations made from valine, leucine, isoleucine, and serine stored at ambient (blue bars) and a cool temperature of 3 ˚C (red bars).

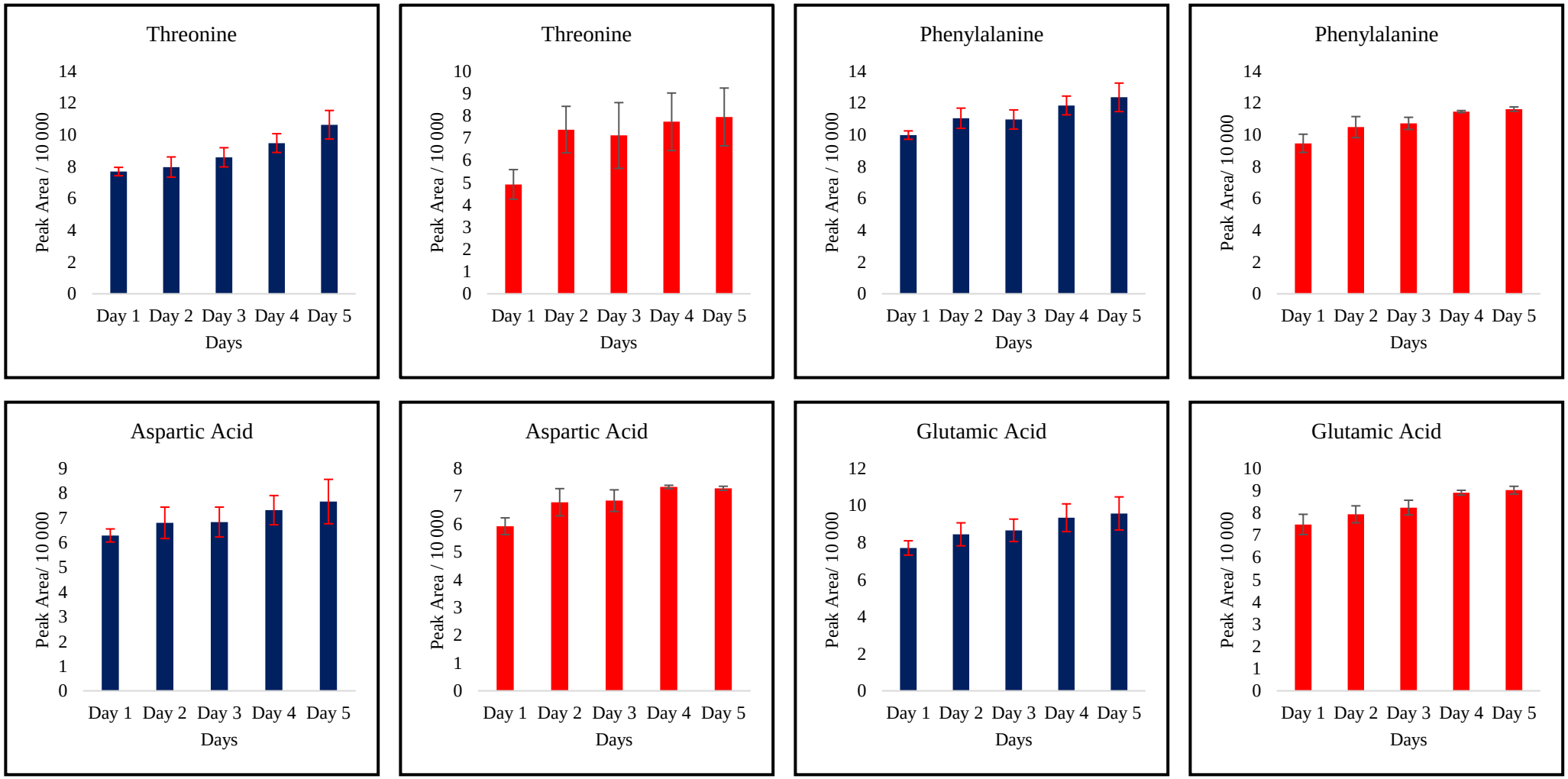


Figure 6-12: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure includes the observations made from threonine, phenylalanine, aspartic acid, and glutamic acid stored at ambient (blue bars) and a cool temperature of 3 ˚C (red bars).

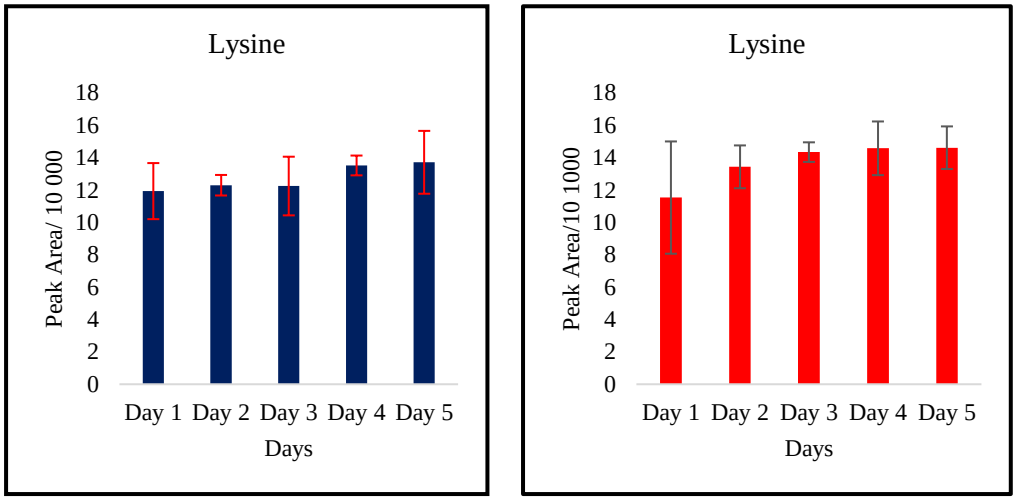


Figure 6-13: Graphical representation of the observed changes in the response of MBSTFA derivatives over a period of 5 days to determine the stability of MBSTFA derivatives. This figure includes the observations made from lysine at ambient (blue bars) and a cool temperature of 3 ˚C (red bars).

6.5 Appendix A5

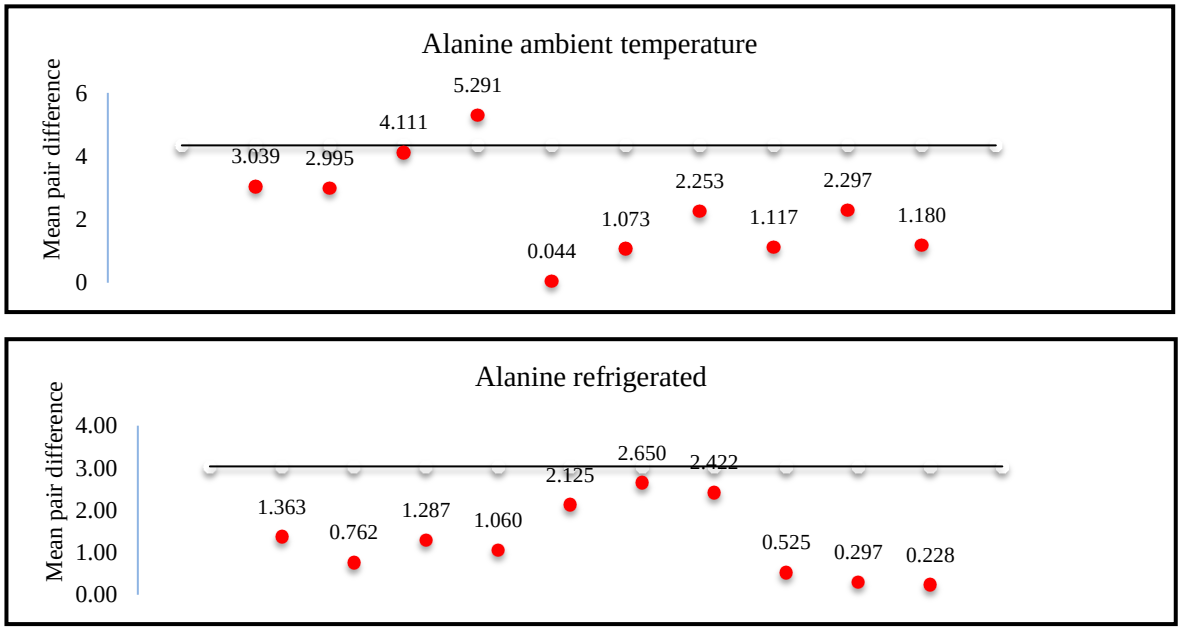


Figure 6-14: Honest significant difference (HSD) chart used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

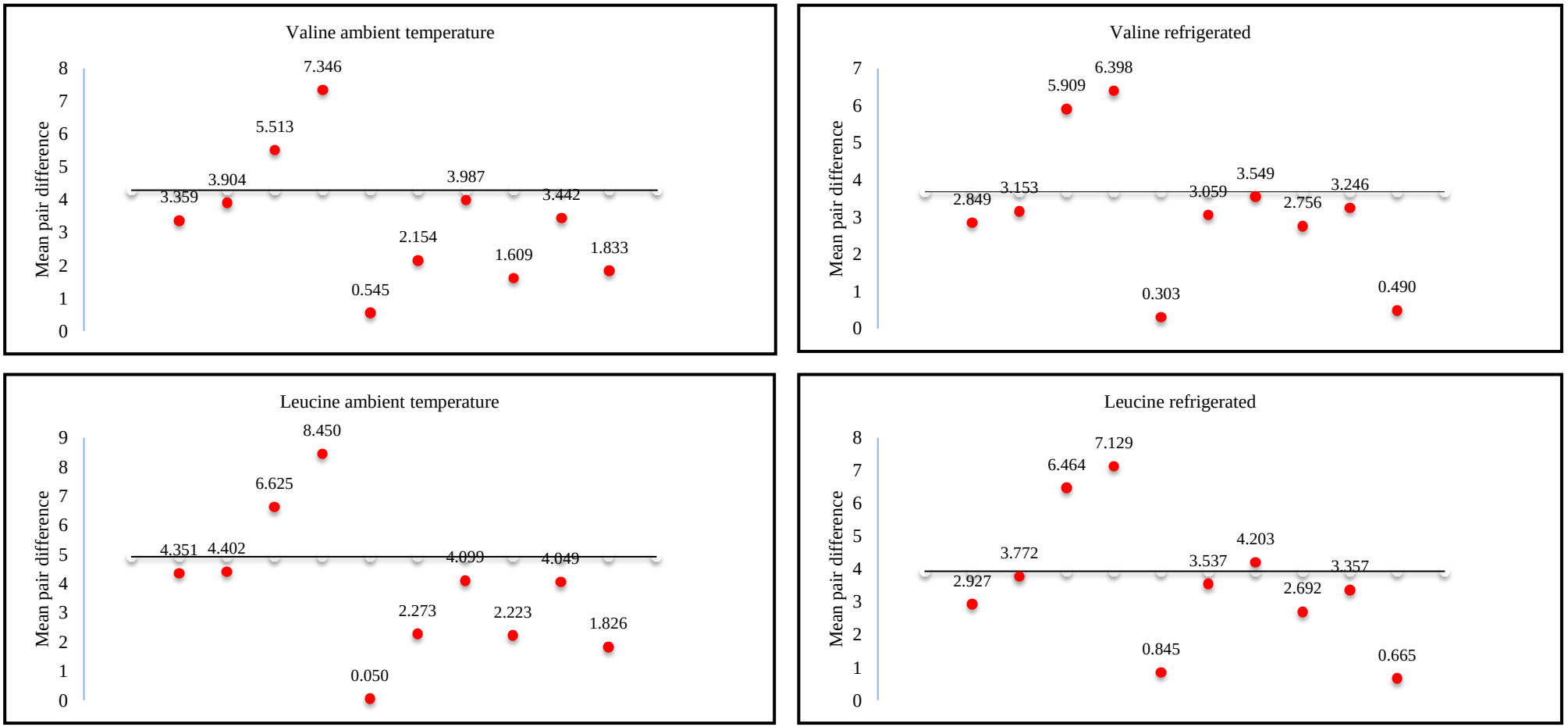


Figure 6-15: Honest significant difference (HSD) charts of valine and leucine used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

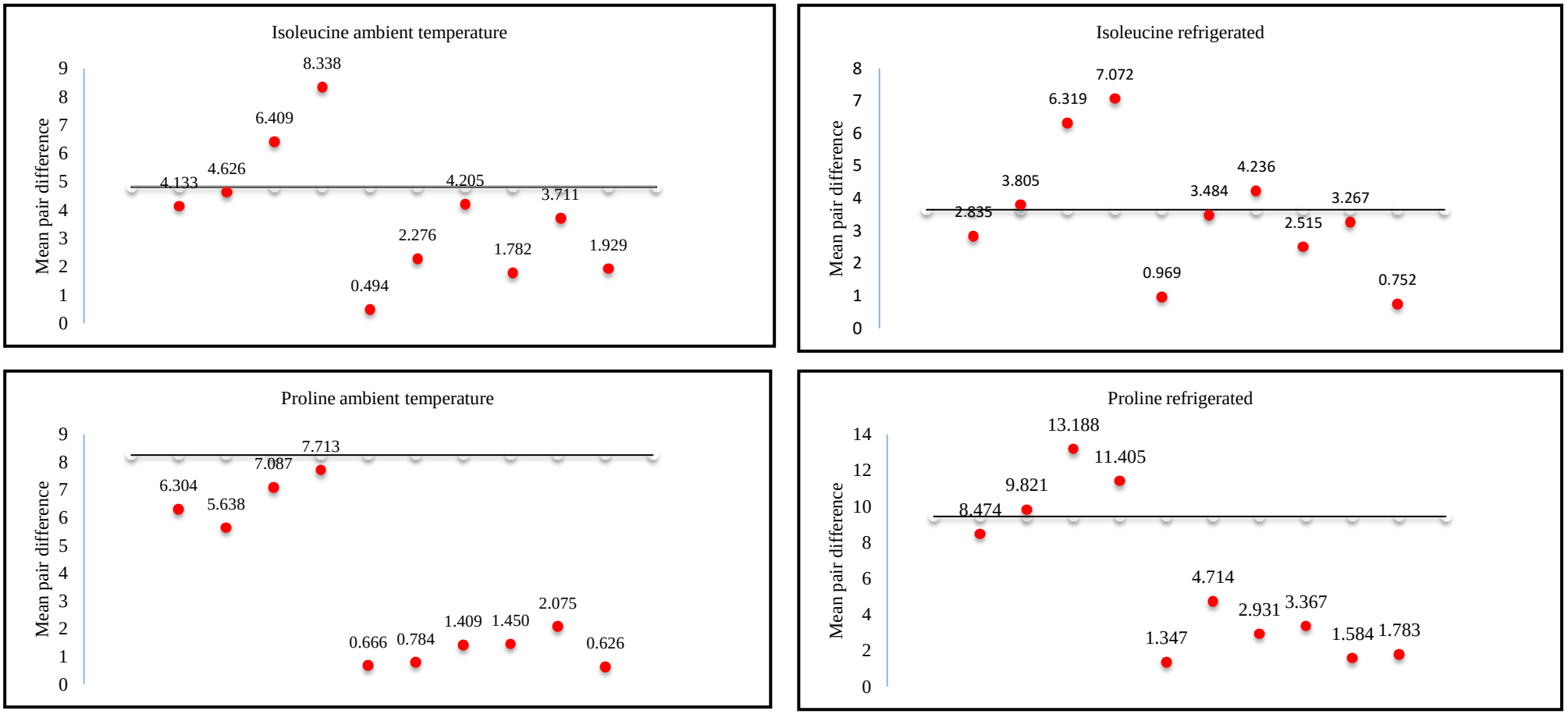


Figure 6-16: Honest significant difference (HSD) charts of isoleucine and proline used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

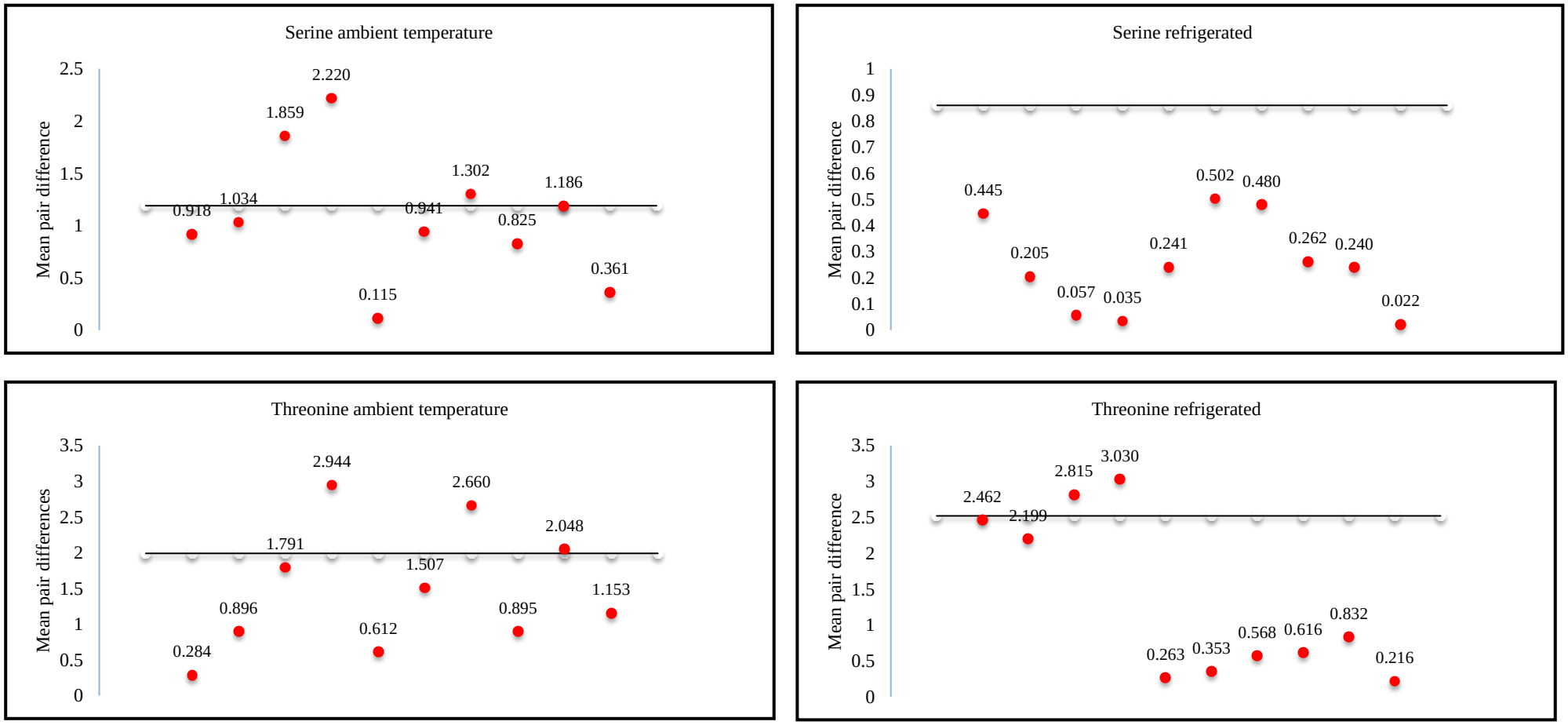


Figure 6-17: Honest significant difference (HSD) charts of serine and threonine used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

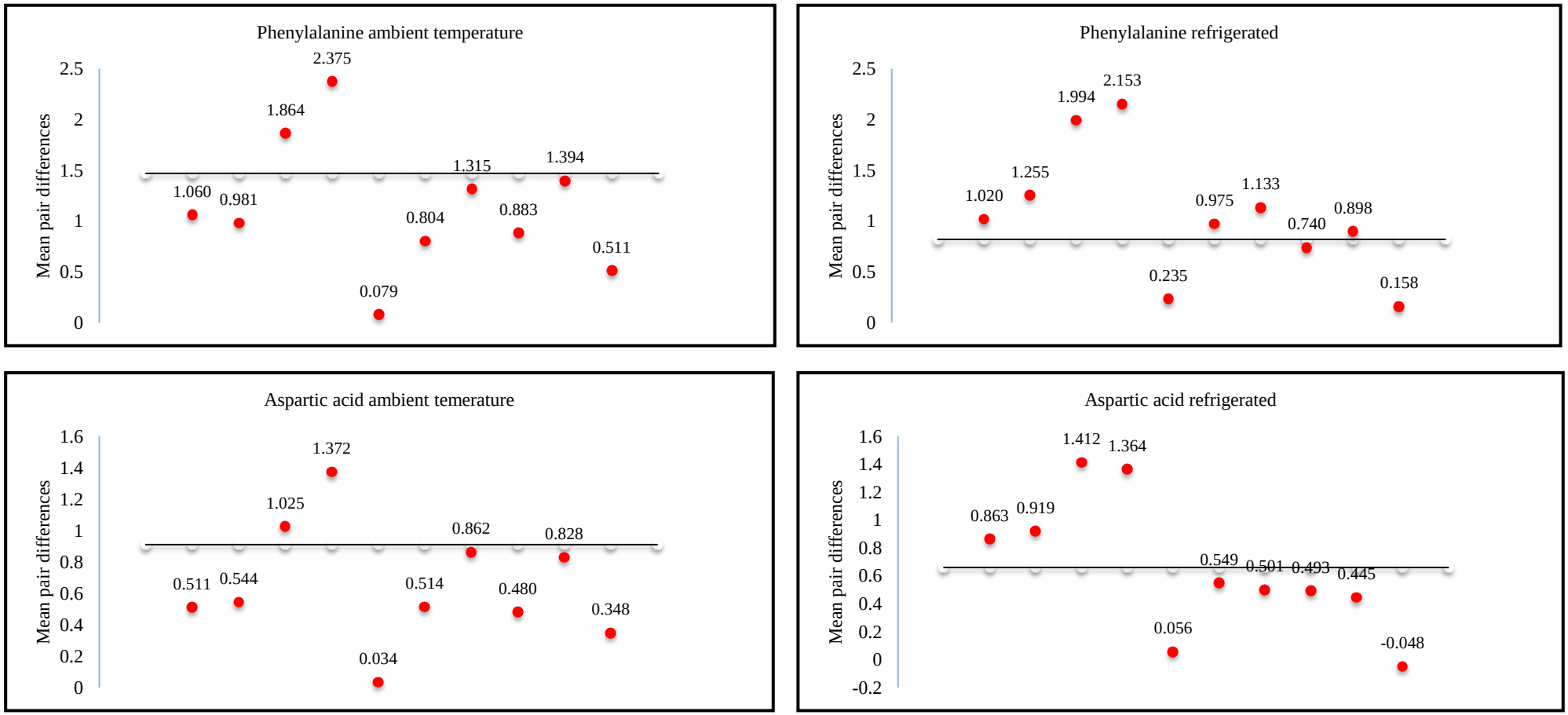


Figure 6-18: Honest significant difference (HSD) charts of phenylalanine and aspartic acid used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

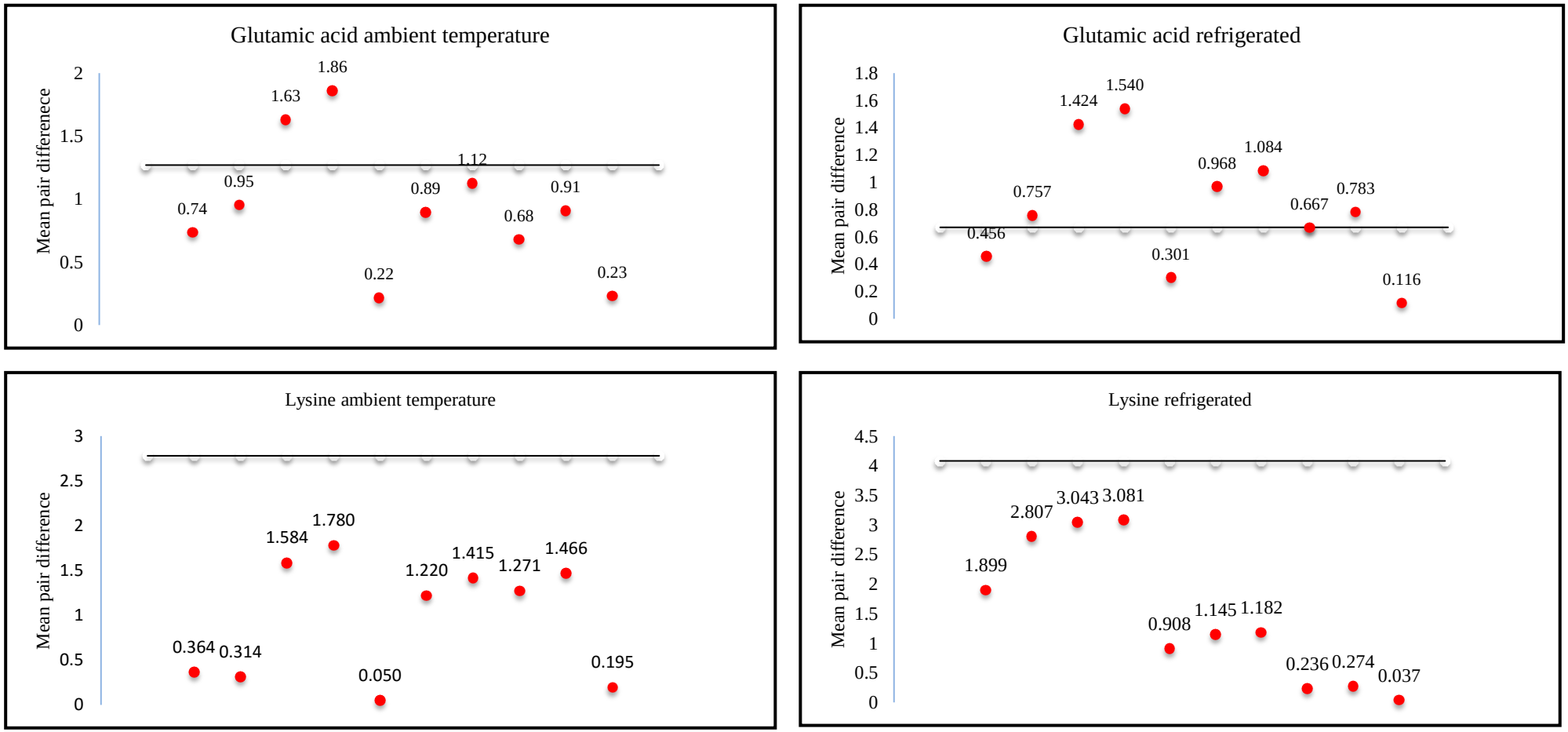


Figure 6-19: Honest significant difference (HSD) charts of glutamic acid and lysine used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

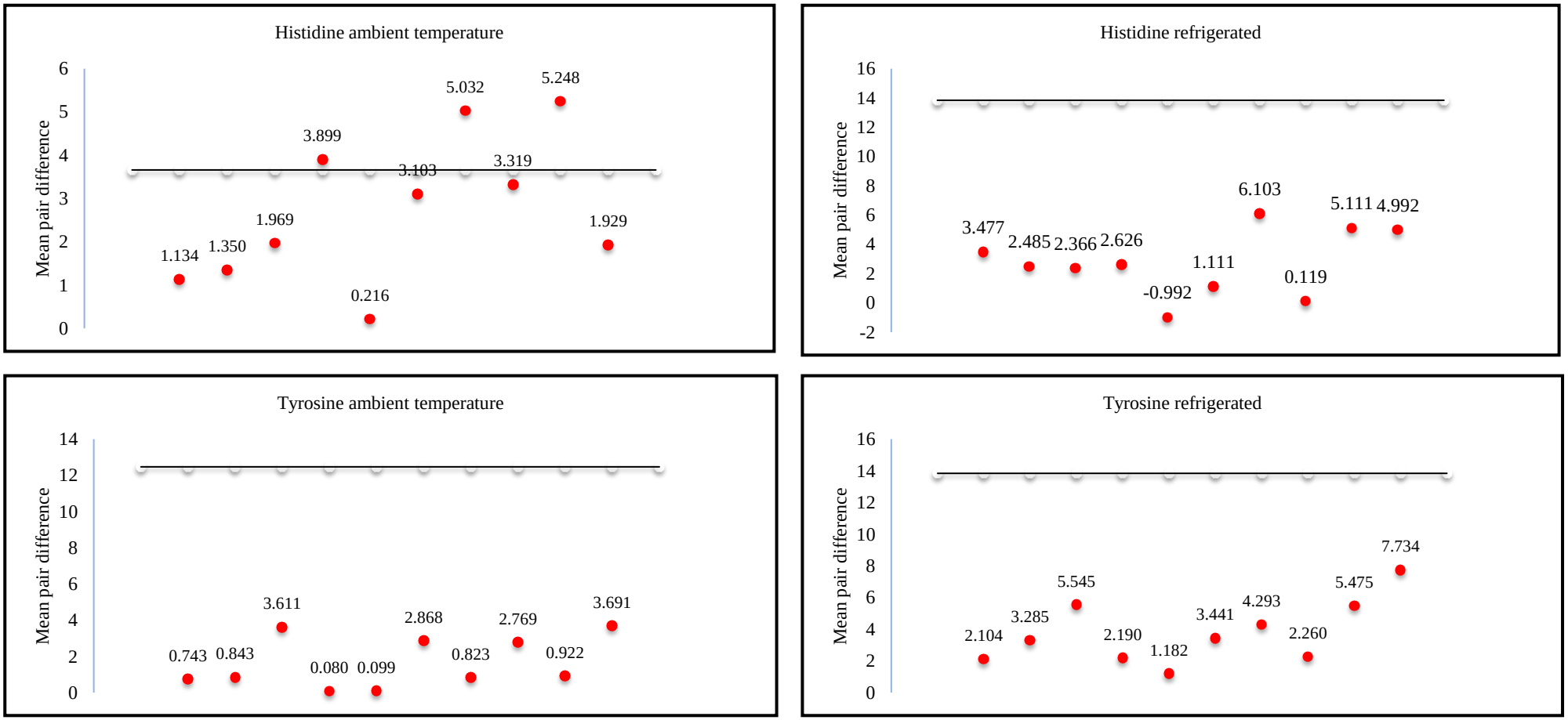
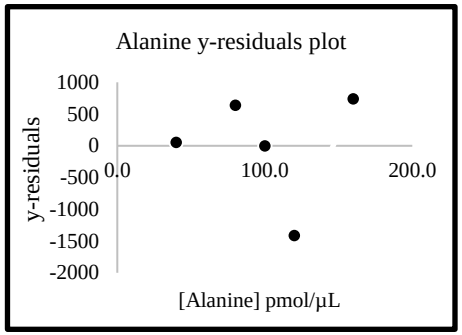
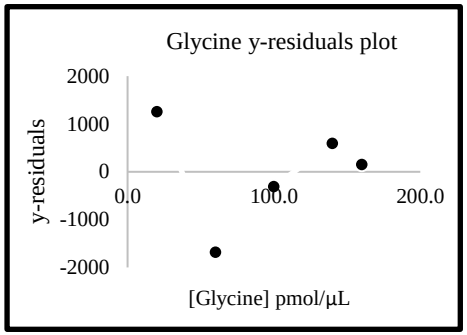
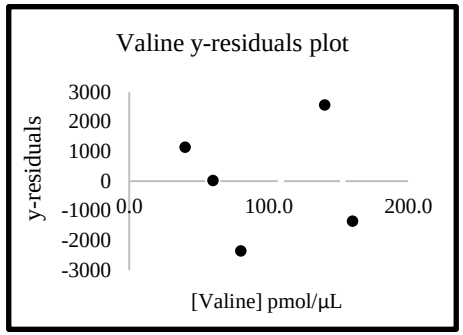


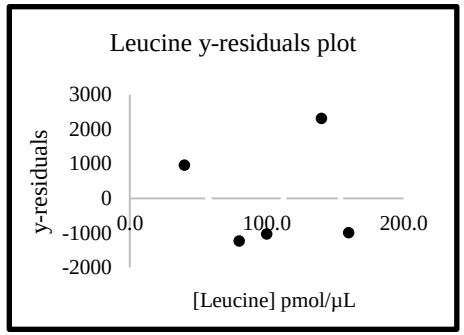
Figure 6-20: Honest significant difference (HSD) charts of histidine and tyrosine used to identify significant differences between daily means. The red dots represent mean differences while the black line represents the HSD value. The number above each point represents the difference between the pair of means being compared. Daily means are compared from left to right, that is, mean day 1 to day 2, day 1 to day 3, day 1 to day 4, day 1 to day 5, day 2 to day 3, day 2 to day 4, day 2 to day 5, day 3 to day 4, day 3 to day 5 and day 4 to day 5. Each point of the chart represents the differences between a pair of means starting from the left to right. Points above the HSD line show pairs of means that differ significantly.

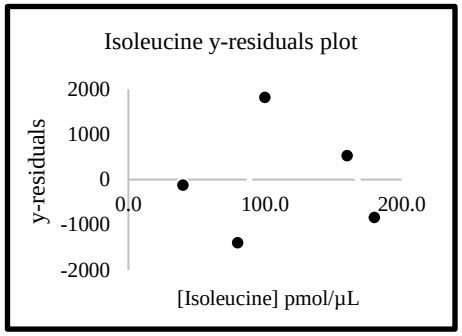
6.6 Appendix B1

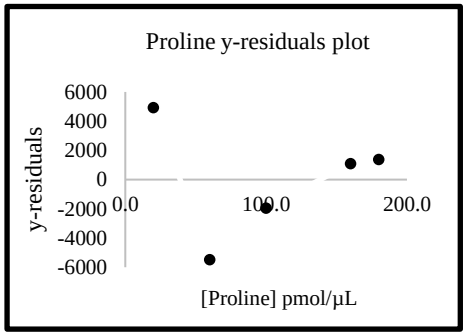


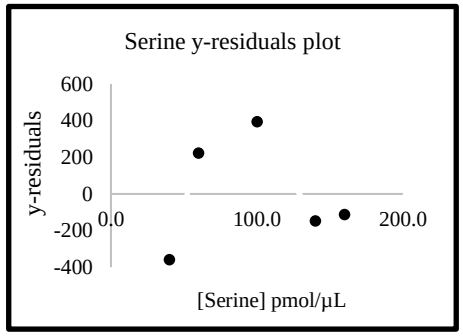


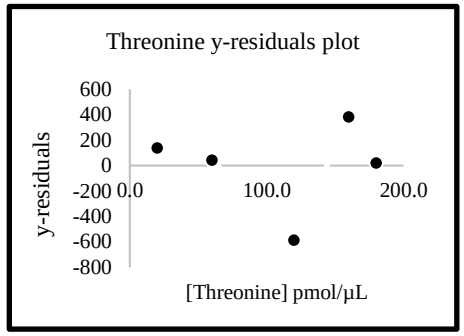


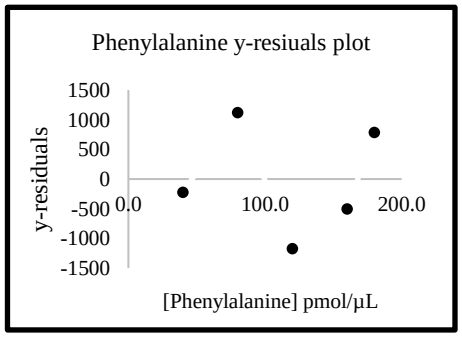


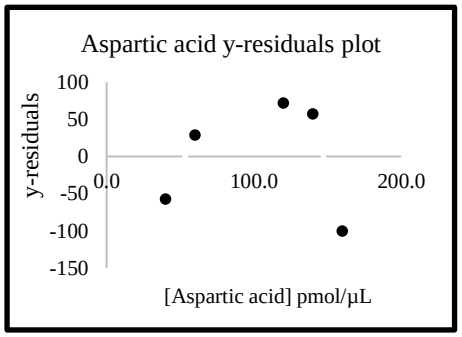


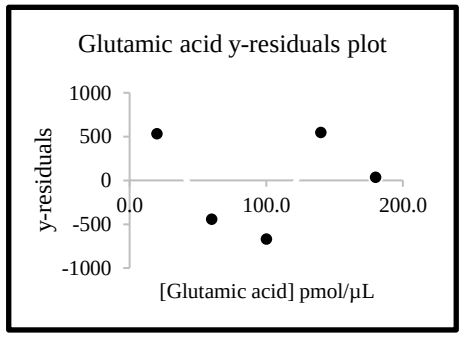


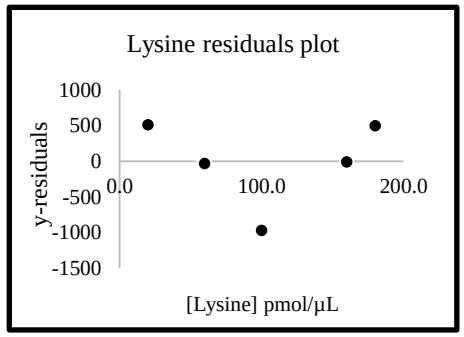












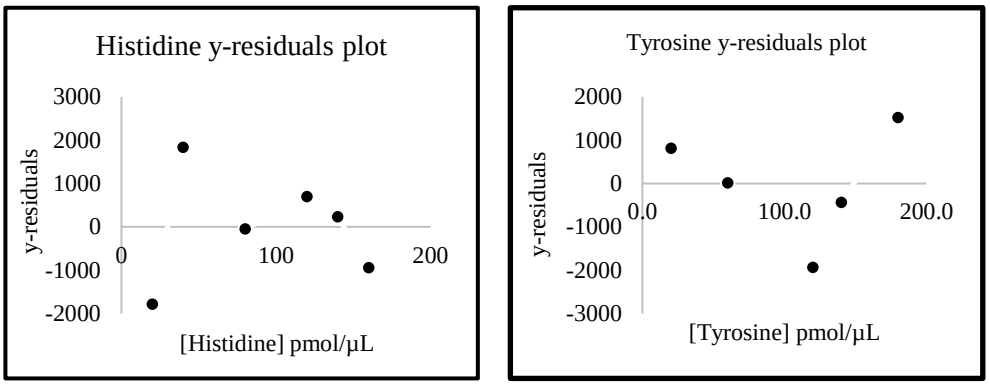
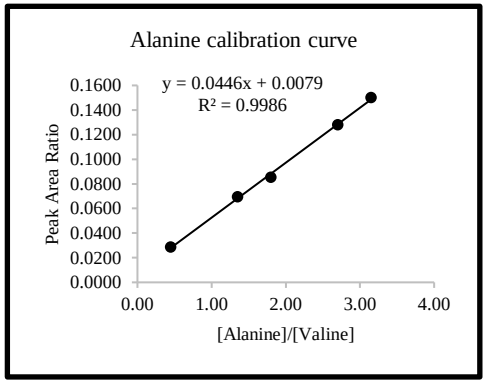
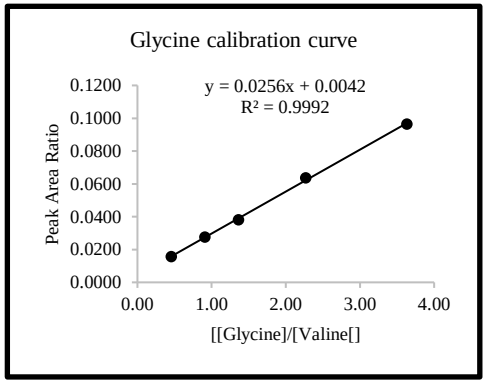
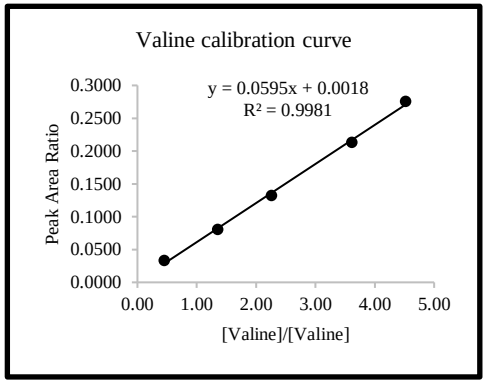
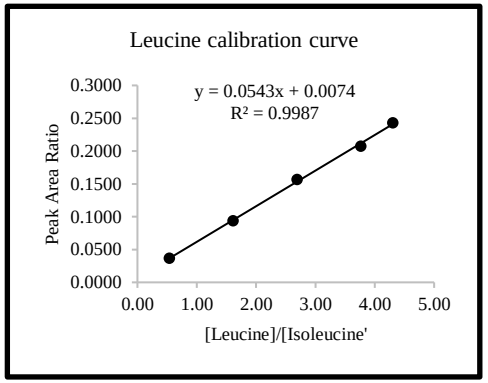


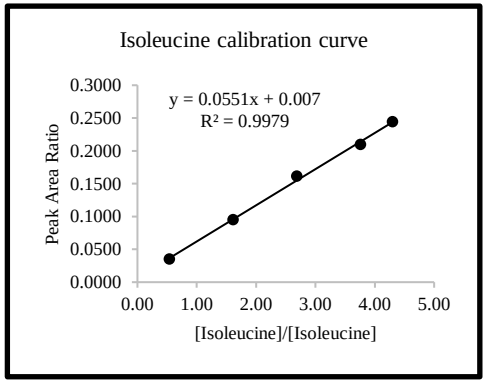
Figure 6-21: Amino acids y-residuals plots obtained by external calibration used to test for bias and to confirm instrument linearity. The y-residual plots presented herein are based on quantification ions presented in **Table 4-3** and **4-4** i.e.158, 218, 186, 200, 200, 184, 288, 303, 234, 302, 432, 198, 196 and 302 m/z for alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, histidine and tyrosine respectively. The optimised flow rate of 1.2 mL/min was used. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. The run time was 12.5 minutes. The black dots represent the calculated y-residual values.

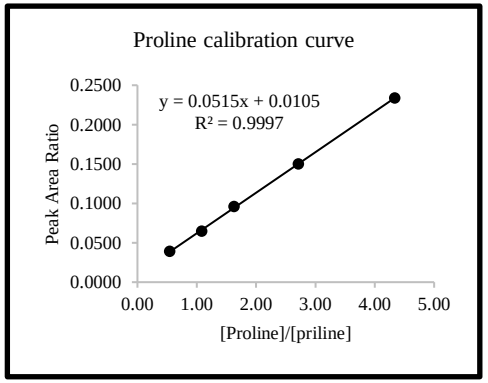

Alanine calibration curve
y = 0.0446x + 0.0079
R² = 0.9986
Peak Area Ratio
[Alanine]/[Valine]

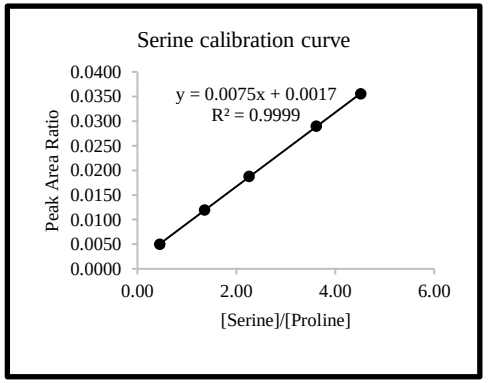

Glycine calibration curve
y = 0.0256x + 0.0042
R² = 0.9992
Peak Area Ratio
[[Glycine]/[Valine[]

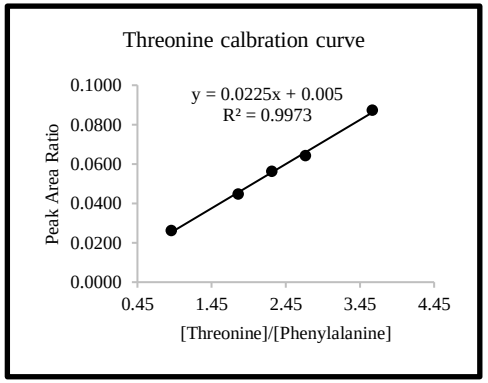

Valine calibration curve
y = 0.0595x + 0.0018
R² = 0.9981
Peak Area Ratio
[Valine]/[Valine]

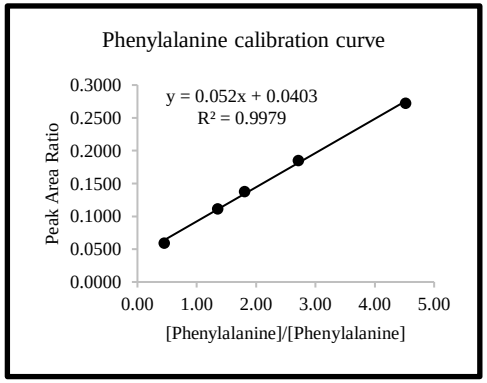

Leucine calibration curve
y = 0.0543x + 0.0074
R² = 0.9987
Peak Area Ratio
[Leucine]/[Isoleucine'

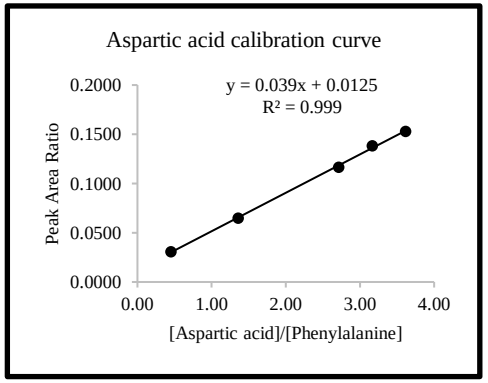

Isoleucine calibration curve
y = 0.0551x + 0.007
R² = 0.9979
Peak Area Ratio
[Isoleucine]/[Isoleucine]

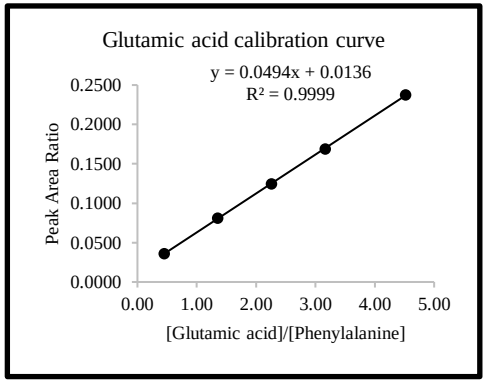

Proline calibration curve
y = 0.0515x + 0.0105
R² = 0.9997
Peak Area Ratio
[Proline]/[priline]

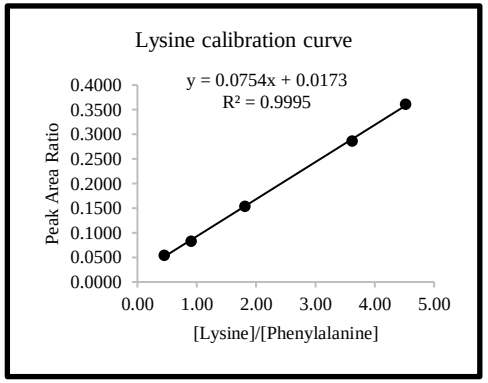

Serine calibration curve
y = 0.0075x + 0.0017
R² = 0.9999
Peak Area Ratio
[Serine]/[Proline]


Threonine calbration curve
y = 0.0225x + 0.005
R² = 0.9973
Peak Area Ratio
[Threonine]/[Phenylalanine]


Phenylalanine calibration curve
y = 0.052x + 0.0403
R² = 0.9979
Peak Area Ratio
[Phenylalanine]/[Phenylalanine]


Aspartic acid calibration curve
y = 0.039x + 0.0125
R² = 0.999
Peak Area Ratio
[Aspartic acid]/[Phenylalanine]


Glutamic acid calibration curve
y = 0.0494x + 0.0136
R² = 0.9999
Peak Area Ratio
[Glutamic acid]/[Phenylalanine]


Lysine calibration curve
y = 0.0754x + 0.0173
R² = 0.9995
Peak Area Ratio
[Lysine]/[Phenylalanine]

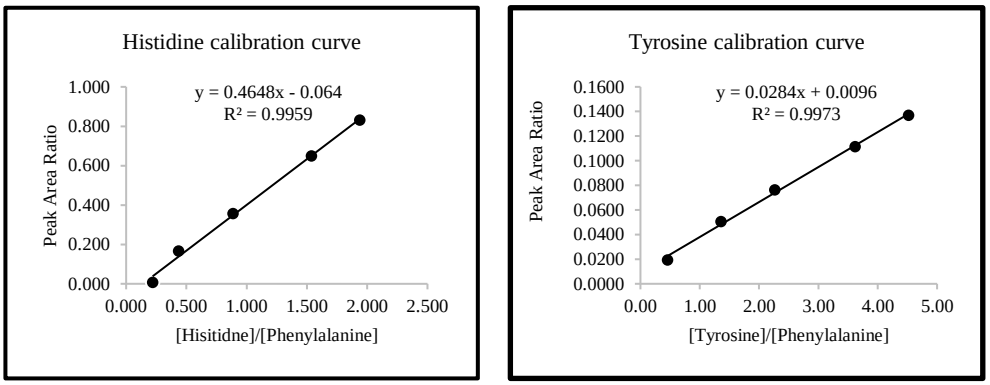
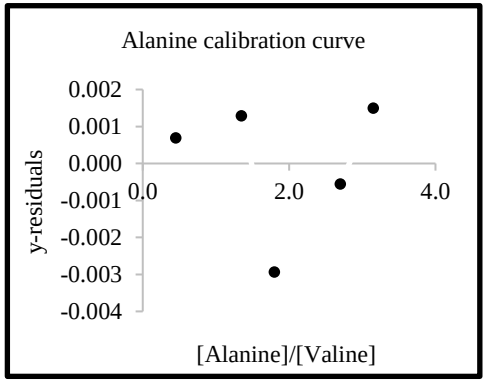
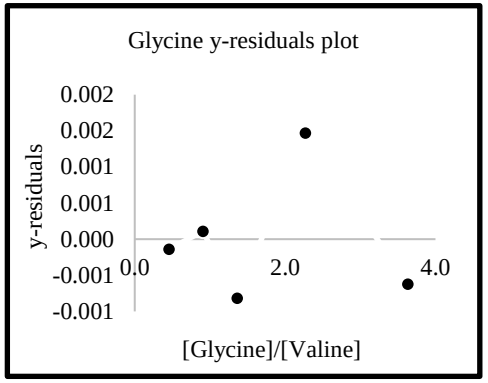
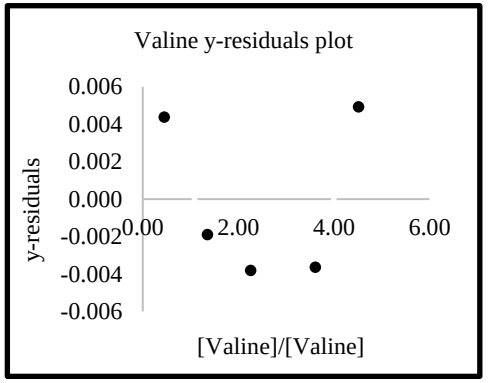
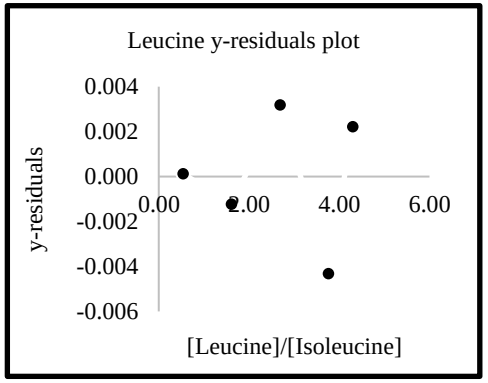


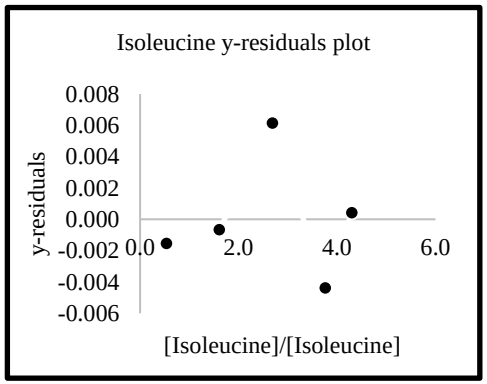
Figure 6-22: Isotope calibration regression curves obtained by the SOF method also used for method linearity test. Calibration points are based on quantification ions presented in Table 4-3 and 4-4 i.e.158, 218, 186, 200, 200, 184, 288, 303, 234, 302, 432, 198, 196 and 302 m/z for alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, histidine and tyrosine respectively. The optimised flow rate of 1.2 mL/min was used. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. The run time was 12.5 minutes.

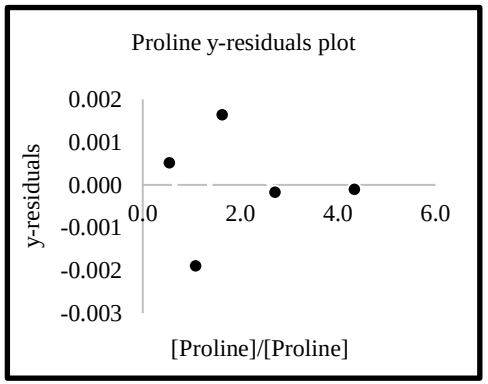

Alanine calibration curve
y-residuals
0.002
0.001
0.000
-0.001
-0.002
-0.003
-0.004
0.0
2.0
4.0
[Alanine]/[Valine]

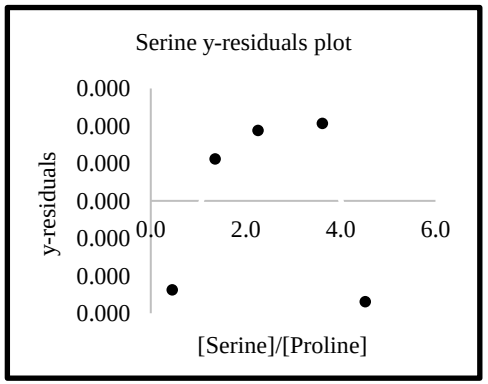

Glycine y-residuals plot
y-residuals
0.002
0.002
0.001
0.001
0.000
-0.001
-0.001
0.0
2.0
4.0
[Glycine]/[Valine]

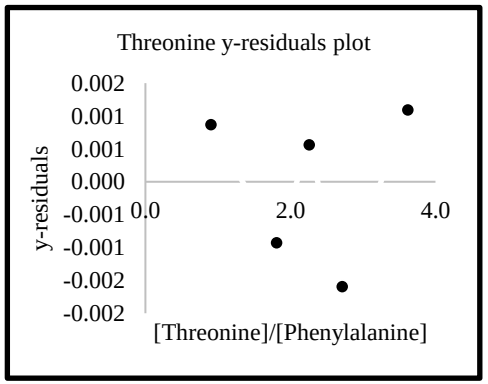

Valine y-residuals plot
y-residuals
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
0.00
2.00
4.00
6.00
[Valine]/[Valine]

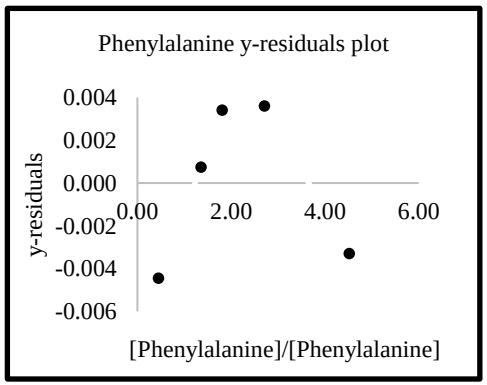

Leucine y-residuals plot
y-residuals
0.004
0.002
0.000
-0.002
-0.004
-0.006
0.00
2.00
4.00
6.00
[Leucine]/[Isoleucine]

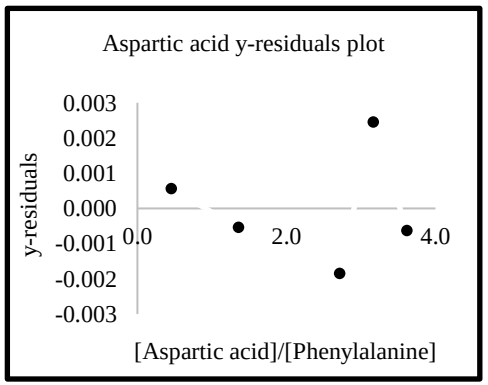

Isoleucine y-residuals plot
y-residuals
0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
-0.006
0.0
2.0
4.0
6.0
[Isoleucine]/[Isoleucine]

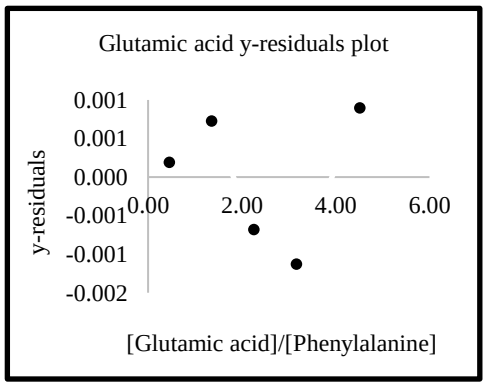

Proline y-residuals plot
y-residuals
0.002
0.001
0.000
-0.001
-0.002
-0.003
0.0
2.0
4.0
6.0
[Proline]/[Proline]

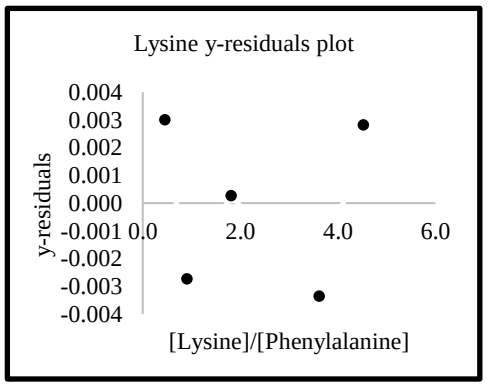

Serine y-residuals plot
y-residuals
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.0
2.0
4.0
6.0
[Serine]/[Proline]


Threonine y-residuals plot
y-residuals
0.002
0.001
0.001
0.000
-0.001
-0.001
-0.002
-0.002
0.0
2.0
4.0
[Threonine]/[Phenylalanine]


Phenylalanine y-residuals plot
y-residuals
0.004
0.002
0.000
-0.002
-0.004
-0.006
0.00
2.00
4.00
6.00
[Phenylalanine]/[Phenylalanine]


Aspartic acid y-residuals plot
y-residuals
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
0.0
2.0
4.0
[Aspartic acid]/[Phenylalanine]


Glutamic acid y-residuals plot
y-residuals
0.001
0.001
0.000
-0.001
-0.001
-0.002
0.00
2.00
4.00
6.00
[Glutamic acid]/[Phenylalanine]


Lysine y-residuals plot
y-residuals
0.004
0.003
0.002
0.001
0.000
-0.001
-0.002
-0.003
-0.004
0.0
2.0
4.0
6.0
[Lysine]/[Phenylalanine]

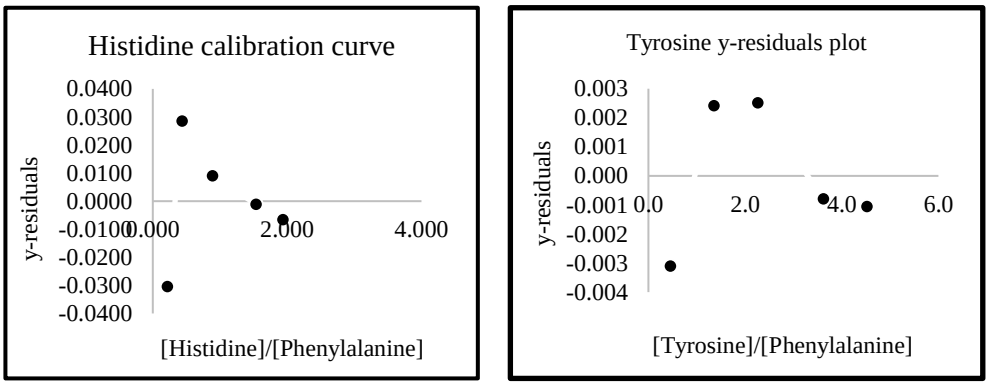


Figure 6-23: Amino acids y-residuals plots obtained by isotope dilution calibration used to test for bias and to confirm instrument linearity. The y-residual plots presented herein are based on quantification ions presented in Table 4-3 and 4-4 i.e.158, 218, 186, 200, 200, 184, 288, 303, 234, 302, 432, 198, 196 and 302 m/z for alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartic acid, glutamic acid, histidine and tyrosine respectively. The optimised flow rate of 1.2 mL/min was used. Oven temperature ramp rates were set to 25, 8 and 30 ˚C/min between 1.7 to 4.28; 6.83 to 8.31 and 9.19 to 11.77 minutes respectively. The run time was 12.5 minutes. The black dots represent the y-residual values.

6.7 Appendix C1

Table 6-1: UPLC method calibration parameters obtained by external calibration

Amino Acids	Linear regression equation	r	$r2$	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}\ (g/100g)$	$S_a\ (g/100g)$	$S_b\ (g/100g)$
Alanine	$y = 39.272x + 61.801$	0.9997	0.9994	0.0042	0.0139	0.0090	0.0090	0.0102
Glycine	$y = 59.084x - 71.485$	0.9993	0.9985	0.0059	0.0196	0.0087	0.0087	0.0068
Valine	$y = 49.933x + 99.349$	0.9996	0.9991	0.0079	0.0262	0.0146	0.0148	0.0171
Leucine	$y = 49.28x + 105.86$	0.9992	0.9985	0.0112	0.0373	0.0171	0.0175	0.0206
Isoleucine	$y = 49.1515x + 104.48$	0.9993	0.9985	0.0109	0.0168	0.0168	0.0172	0.0202
Proline	$y = 45.247x + 91.352$	0.9999	0.9998	0.0035	0.0118	0.0160	0.0160	0.0171
Serine	$y = 68.48x - 98.512$	0.9984	0.9968	0.0118	0.0394	0.0151	0.0151	0.0114
Threonine	$y = 69.747x - 117.93$	0.9985	0.9970	0.0131	0.0436	0.0192	0.0192	0.0151
Phenylalanine	$y = 63.645x - 23.534$	0.9980	0.9960	0.0237	0.0791	0.0116	0.0116	0.0116
Aspartic Acid	$y = 33.473x + 116.79$	0.9997	0.9994	0.0070	0.0232	0.0320	0.0320	0.0341
Glutamic Acid	$y = 35.68x + 93.517$	0.9993	0.9986	0.0117	0.0389	0.0246	0.0248	0.0282
Lysine	$y = 33.064x + 182.38$	0.9942	0.9884	0.0304	0.1013	0.0494	0.0495	0.0588
Histidine	$y = 45.735x - 424.46$	0.9991	0.9981	0.0133	0.0443	0.1107	0.1111	0.1066
Tyrosine	$y = 75.553x - 208.02$	0.9981	0.9962	0.0230	0.0768	0.0445	0.0445	0.0373

r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard uncertainty of the regression line gradient.

Table 6-2: UPLC method calibration parameters obtained by the internal standard calibration method

Amino Acids	Linear regression equation	r	$r2$	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}\ (g/100g)$	$S_a\ (g/100g)$	$S_b\ (g/100g)$
Alanine	$y = 1.5144x - 0.0847$	0.9992	0.9984	0.0360	0.1201	0.0456	0.0444	0.0476
Glycine	$y = 2.2115x - 0.3449$	0.9967	0.9933	0.0622	0.2072	0.0998	0.0977	0.1031
Valine	$y = 2.0268x - 0.2168$	0.9984	0.9968	0.0665	0.2218	0.1068	0.1046	0.1104
Leucine	$y = 2.0047x - 0.1959$	0.9985	0.9969	0.0712	0.2373	0.1103	0.1079	0.1150
Isoleucine	$y = 2.0135x - 0.199$	0.9985	0.9970	0.0704	0.2348	0.1111	0.1086	0.1157
Proline	$y = 1.8256x - 0.1683$	0.9987	0.9973	0.0590	0.1966	0.0914	0.0894	0.0950
Serine	$y = 2.8003x - 0.7785$	0.9988	0.9976	0.0494	0.1645	0.2138	0.2137	0.2174
Threonine	$y = 2.8436x - 0.8228$	0.9990	0.9979	0.0531	0.1769	0.2506	0.2504	0.2539
Phenylalanine	$y = 2.8361x - 0.9139$	0.9994	0.9988	0.0570	0.1900	0.3787	0.3785	0.3818
Aspartic Acid	$y = 1.3547x - 0.039$	0.9977	0.9955	0.0903	0.3008	0.0560	0.0529	0.0610
Glutamic Acid	$y = 1.444x - 0.0829$	0.9988	0.9977	0.0712	0.2375	0.0808	0.0783	0.0848
Lysine	$y = 1.7737x - 0.4632$	0.9961	0.9922	0.1117	0.3725	0.2952	0.2949	0.3085
Histidine	$y = 1.7294c - 0.882$	0.9993	0.9987	0.0508	0.1693	0.5516	0.5534	0.5566
Tyrosine	$y = 3.0412x - 0.982$	0.9994	0.9988	0.0621	0.2069	0.4160	0.4158	0.4194

r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard uncertainty of the regression line gradient.

Table 6-3: UPLC method performance parameters obtained by the internal standard method

Amino Acids	$[AA]$ ($g/100g$)	S_{x0} ($g/100g$)	%Recovery	CV	CI ($g/100g$)
Alanine	0.397	0.038	87.29	9.55	0.047
Glycine	0.281	0.032	116.63	11.50	0.040
Valine	0.555	0.060	73.01	10.76	0.074
Leucine	0.986	0.117	78.21	11.87	0.145
Isoleucine	0.492	0.051	74.52	10.37	0.063
Proline	0.942	0.101	78.80	10.76	0.126
Serine	0.679	0.089	94.37	13.15	0.111
Threonine	0.661	0.082	103.31	12.34	0.101
Phenylalanine	0.765	0.086	131.93	11.21	0.107
Aspartic Acid	0.907	0.060	84.73	6.63	0.075
Glutamic Acid	2.086	0.204	80.53	9.80	0.254
Lysine	1.017	0.054	100.72	5.30	0.067
Histidine	1.108	0.172	351.84	15.48	0.213
Tyrosine	0.729	0.070	142.90	9.55	0.086

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by Equation 3.7, CV = relative standard deviation and CI = confidence interval of the mean.

Table 6-4: UPLC method performance parameters obtained by the external calibration

Amino Acids	$[AA]$ ($g/100g$)	S_{x0} ($g/100g$)	%Recovery	CV	CI ($g/100g$)
Alanine	0.343	0.039	75.46	11.31	0.048
Glycine	0.239	0.013	99.03	5.36	0.016
Valine	0.452	0.051	59.51	11.33	0.064
Leucine	0.921	0.083	73.00	9.00	0.103
Isoleucine	0.364	0.045	55.21	12.37	0.056
Proline	0.897	0.086	75.07	9.63	0.107
Serine	0.592	0.028	82.19	4.75	0.035
Threonine	0.546	0.025	85.35	4.57	0.031
Phenylalanine	0.515	0.026	88.71	5.10	0.033
Aspartic Acid	0.841	0.123	78.64	14.58	0.152
Glutamic Acid	2.157	0.247	83.28	11.46	0.307
Lysine	0.874	0.177	86.53	20.21	0.219
Histidine	1.064	0.081	337.68	7.60	0.100
Tyrosine	0.533	0.021	104.49	3.86	0.026

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by Equation 3.7, CV = relative standard deviation and CI = confidence interval of the mean.

Table 6-5: Pegasus IV method calibration parameters obtained by isotope dilution

Amino Acids	Linear regression equation	Quant Ion (m/z)	Internal standard	r	r^2	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}\ (g/100g)$	$S_a\ (g/100g)$	$S_b\ (g/100g)$
Alanine	$y = 8.7898x + 0.9207$	158	Valine	0.9936	0.9872	0.0820	0.2734	0.0780	0.0839	0.0391
Glycine	$y = 0.5549x - 0.0316$	218	Valine	0.9996	0.9992	0.0133	0.0442	0.0527	0.0517	0.0621
Valine	$y = 1.3082x - 0.0761$	186	Valine	0.9993	0.9985	0.0280	0.0933	0.0862	0.0842	0.1062
Leucine	$y = 1.3391x + 0.0101$	200	Isoleucine	0.9999	0.9997	0.0139	0.0465	0.0066	0.0082	0.0034
Isoleucine	$y = 1.325x - 0.0546$	200	Isoleucine	0.9998	0.9996	0.0157	0.0523	0.0662	0.0651	0.0774
Proline	$y = 2.1336x - 0.0204$	184	Proline	0.9996	0.9992	0.0293	0.0978	0.0222	0.0193	0.0336
Serine	$y = 0.2391x - 0.0086$	288	Proline	0.9994	0.9988	0.0216	0.0719	0.0498	0.0482	0.0662
Threonine	$y = 0.1068x + 0.0004$	303	Phenylalanine	0.9994	0.9988	0.0168	0.0560	0.0000	0.0056	0.0268
Phenylalanine	$y = 0.3703x + 0.0776$	234	Phenylalanine	0.9974	0.9948	0.0975	0.3251	0.3585	0.3690	0.3132
Aspartic Acid	$y = 0.314x - 0.0106$	302	Phenylalanine	0.9997	0.9995	0.0242	0.0806	0.0587	0.0566	0.0706
Glutamic Acid	$y = 0.2457x - 0.0072$	432	Phenylalanine	0.9995	0.9991	0.0325	0.1082	0.0594	0.0564	0.0780
Lysine	$y = 0.54142x - 0.008$	198	Phenylalanine	0.9997	0.9993	0.0266	0.0887	0.0347	0.0319	0.0319
Histidine	$y = 1.4492x - 0.0375$	196	Phenylalanine	0.9994	0.9988	0.0353	0.1177	0.0572	0.0090	0.0344
Tyrosine	$y = 1.2677x - 0.0241$	302	Phenylalanine	0.9989	0.9978	0.0599	0.1997	0.0588	0.0526	0.0947

Quant ion = ion used for quantification, r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard error of the regression line slope.

Table 6-6: Pegasus IV method calibration parameters obtained by external calibration

Amino Acids	Linear regression equation	Quant Ion (m/z)	r	r^2	$LOD\ (g/100g)$	$LOQ\ (g/100g)$	$S_{x/y}\ (g/100g)$	$S_a\ (g/100g)$	$S_b\ (g/100g)$
Alanine	y = 1131.6x - 18074	158	0.9998	0.9996	0.0243	0.0809	0.1130	0.1117	0.1050
Glycine	y = 604.03 - 13942	218	0.9988	0.9976	0.0591	0.1970	0.1476	0.1433	0.1280
Valine	y = 1407.3x - 32088	186	0.9992	0.9984	0.0616	0.2060	0.2177	0.2129	0.1973
Leucine	y = 1408x - 30627	200	0.9998	0.9995	0.0393	0.1310	0.2177	0.2215	0.2106
Isoleucine	y = 1423.6x - 26527	200	0.9996	0.9992	0.0471	0.1570	0.1961	0.1927	0.1805
Proline	y = 2361.1x - 37195	184	0.9999	0.9999	0.0173	0.0577	0.1382	0.1370	0.1325
Serine	y = 234.1x - 1705.9	288	0.9995	0.9985	0.0648	0.2160	0.0781	0.0715	0.0567
Threonine	y = 110.47x - 501.86	303	0.9997	0.9993	0.0380	0.1270	0.0526	0.0500	0.0400
Phenylalanine	y = 447.3x + 16708	234	0.9996	0.9991	0.0715	0.2380	0.4311	0.4396	0.4547
Aspartic Acid	y = 290.91x + 1934.6	302	0.9992	0.9984	0.0881	0.2940	0.0439	0.0439	0.0651
Glutamic Acid	y = 250.82x - 2872.7	432	0.9999	0.9999	0.0218	0.0727	0.1316	0.1304	0.1244
Lysine	y = 548.07x - 5315.1	198	0.9993	0.9986	0.0786	0.2620	0.1308	0.1249	0.1049
Histidine	y = 1627.7x - 29504	196	0.9997	0.9994	0.0449	0.1500	0.2225	0.2210	0.2077
Tyrosine	y = 1307.6x - 1343.7	302	0.9994	0.9988	0.1040	0.3470	0.1721	0.1585	0.1377

Quant ion = ion used for quantification, r = correlation coefficient, r^2 = squared correlation coefficient, LOD = limit of detection, LOQ = limit of quantification, $S_{x/y}$ = standard error of the regression line, S_a = standard error of the regression line intercept and S_b = standard error of the regression line slope.

Table 6-7: Pegasus IV method performance parameters obtained by isotope dilution

Amino Acids	$[AA]$ ($g/100g$)	S_{x0} ($g/100g$)	%Recovery	CV	CI ($g/100g$)
Alanine	0.446	0.033	98.02	7.39	0.030
Glycine	0.193	0.030	80.15	0.03	0.027
Valine	0.634	0.026	83.43	0.59	0.024
Leucine	0.801	0.031	63.56	0.55	0.028
Isoleucine	0.535	0.016	81.12	0.42	0.014
Proline	0.765	0.092	64.03	1.71	0.085
Serine	0.507	0.026	70.41	0.72	0.024
Threonine	0.650	0.060	101.62	1.31	0.055
Phenylalanine	0.560	0.032	96.58	0.81	0.030
Aspartic Acid	0.658	0.033	61.49	0.72	0.031
Glutamic Acid	2.019	0.075	77.96	0.53	0.069
Lysine	0.514	0.077	50.85	2.14	0.071
Histidine	0.201	0.024	63.68	1.72	0.022
Tyrosine	0.347	0.009	68.63	0.36	0.008

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by Equation 3.7, CV = relative standard deviation and CI = confidence interval of the mean.

Table 6-8: Pegasus IV method performance parameters obtained by external calibration

Amino Acids	[AA] SRM (g/100g)	S_{x0} (g/100g)	% Recovery	CV	CI (g /100g)
Alanine	0.49	0.0071	107.43	1.45	0.007
Glycine	0.26	0.0426	107.79	16.41	0.039
Valine	0.84	0.0545	110.09	6.51	0.050
Leucine	1.31	0.0777	104.00	5.93	0.072
Isoleucine	0.73	0.0315	110.98	4.29	0.072
Proline	1.08	0.1367	90.17	12.69	0.046
Serine	0.67	0.0493	92.87	7.37	0.046
Threonine	0.96	0.1037	150.58	10.76	0.096
Phenylalanine	1.10	0.0983	189.83	8.93	0.091
Aspartic acid	0.80	0.0664	74.31	8.35	0.061
Glutamic acid	2.81	0.161	108.52	5.74	0.149
Lysine	0.74	0.122	73.18	16.51	0.113
Histidine	0.35	0.0318	110.05	9.17	0.029
Tyrosine	0.51	0.0155	99.82	3.05	0.014

[AA] = experimental concentration of each amino acid, S_{x0} = sample standard deviation, %Recovery = experimental analyte recovery as calculated by Equation 3.7, CV = relative standard deviation and CI = confidence interval of the mean.