Developing analytical methods to determine physicochemical properties, protein and amino acid composition of African staple foods

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Submitted in fulfilment of the degree

Master of Science – Biochemistry

in the department of

Biochemistry, Genetics and Microbiology

at the

University of Pretoria

June 2022

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Declaration of Originality

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2. I declare that this MSc Biochemistry dissertation is my own original work. Where other people's work has been used (either from a printed source, internet, or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
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Acknowledgements

This present work is a testament to the intellectual capital leveraged from dedicated biochemistry and metrology experts. It is thus a pleasure to extend my earnest gratitude to my supervisor, Dr Precious Motshwene, whose commitment to the project and scholarly counsel has been instrumental in seeing the completion of this work. A debt of gratitude is also owed to my co-supervisor, Mrs Désirée Prevoo-Franzsen, who demonstrated exceptional expertise in the scientific method necessary to achieve the project objectives. Mr Sabelo Chamane is thanked for providing training on sample preparation techniques and instrument operation, and Dr Dennis Mkhize is recognised for the assistance with moisture determination.

This work was made possible by financial investments from the National Metrology Institute of South Africa (NMISA) which availed resources and infrastructure to conduct scientific analyses. Nuanced contributions from the team in the Organic and Bio-Analysis section at NMISA are also acknowledged as they have collectively ensured I could successfully navigate my way around a previously unfamiliar laboratory environment. My mother, Tebalelo Chiloane, and sister, Lerato Chiloane, have been pivotal to the completion of this work due to their continued support and encouragement. It is only through their resolute belief in education that I managed to muster the resilience to weather challenges present throughout the research project.

It was an extraordinary privilege to have you support this work, I thank you all unreservedly.

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Abbreviations

AAA	Amino acid analysis
AMQ	6-aminoquinolone
AOAC	Association of Official Analytical Chemists
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
GABA	Gamma-aminobutyric acid
HBr	Hydrobromic acid
MAM	Moderate acute malnutrition
NMISA	National Metrology Institute of South Africa
PDA	Photo-diode array
QC	Quality control
QPM	Quality protein maize
RSD	Relative standard deviation
RUTF	Ready-to-use therapeutic foods
SAM	Severe acute malnutrition
SSP	Seed storage protein
UN	United Nations
UNU	United Nations University
UHPLC	Ultra-high performance liquid chromatography
UV	Ultra-Violet
WFP	World Food Programme
WHO	World Health Organization
WTO	World Trade Organization

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Summary

Plant-derived staple foods are important sources of energy, nutrition and income in Sub-Saharan Africa as they are more accessible compared to animal sources of food. This study therefore sought to develop analytical methods to determine physicochemical properties (moisture and particle size distribution), protein and amino acid composition of five major African staple foods; namely, maize, sorghum, cassava, corn-soya and teff.

Moisture was determined by Karl Fischer titration, and it was found that corn-soya, sorghum, maize, and teff had moisture levels below the recommended maximum limits. However, cassava revealed a high moisture content which would increase the risk of fungal contamination. Particle size distribution was investigated by laser diffraction which showed corn-soya and teff had coarse particles distributed within a similar size fraction. Maize, cassava and sorghum showed relatively fine particles distributed between multiple size fractions.

The protein and amino acid composition was also determined to evaluate the nutritional content of staple foods, as well as to assign reference values for fit-for-purpose reference materials. To determine the amino acid composition, a 72-h hydrolysis method was developed and validated to quantify acid-stable amino acids. A separate hydrolysis protocol was optimised to quantify methionine and cysteine. The method included performic acid oxidation, followed by acid hydrolysis at 145 ºC for 4 h. Hydrolysates were analysed using UHPLC-UV and good recoveries between 87% and 117% were obtained. Despite several method optimisation attempts, a reproducible method could not be established for tryptophan analysis, and thus the residue was not quantified in this study.

The protein content was quantified using the Dumas combustion method, and the results showed a content of 12.6 g/100 g in corn-soya, 11.3 g/100 g in sorghum, 9.53 g/100 g in teff, 8.88 g/100 g in maize, and 1.13 g/100 g in cassava. Amino acid analysis showed a sum of 12.6 g/100 g in corn-soya, 11.5 g/100 g in sorghum, 9.29 g/100 g in teff, 8.16 g/100 g in maize, and 0.660 g/100 g in cassava, which matched protein results obtained from the Dumas method. Corn-soya revealed the highest composition of essential amino acids, followed by sorghum and teff, respectively. The results for corn-soya are not surprising as the preparation is distributed by food aid initiatives to combat malnutrition. Sorghum and maize showed low concentrations of the limiting amino acids lysine and methionine. Cassava revealed poor concentrations of all amino acids, providing evidence the food source is unlikely to meet nutritional demands in regions where it serves as a primary source of nutrition.

Chapter 1

Literature review

1.1. The African continent

Africa is the second largest continent in both land area and population size (Mboowa et al., 2021). The continent accommodates over 1.3 billion people as per recent United Nations (UN) population estimates (UN, 2019). In addition, the African mainland contains abundant natural resources such as precious minerals, oil, and natural gas reserves. However, most of the population has drawn marginal benefit from this natural wealth as the continent remains one of the poorest expanse in the world (Henry, 2019). This gross disconnect has been attributed to factors such as the legacy of colonialism, and post-colonial social ills including corrupt governments, civil conflict, and military warfare (Michalopoulos and Papaioannou, 2020; Mlambo et al., 2019; Adeyeye et al., 2021). This has led to a large proportion of the African population facing crippling poverty conditions, limited access to health services, and insufficient water supply. In recent years, Sub-Saharan Africa has experienced some semblance of economic prosperity, but this has been overshadowed by the scores of inhabitants still living below the poverty line. According to current poverty rates, more than 40% of the general population is forced to subsist on less than $1.90 per day (Schoch and Lakner, 2020). Economic developments driven by profit-centric industries have not always translated into lifting the poor out of poverty. Additionally, government policies are often poorly implemented leading to inadequate responses towards the incidence of poverty in rural areas where many people in Africa reside.

1.2. Malnutrition in the African population

Among the numerous issues present in Africa, poverty and the scarcity of nutritious food are among the most notable challenges which threaten the health and livelihood of many African inhabitants (Siddiqui et al., 2020). The most visible metric of poverty and insufficient nutrition, malnutrition, is prominent in Sub-Saharan Africa where it accounts for millions of child mortality cases (Ahinkorah et al., 2021; Alamu et al., 2020). The region has the second highest number of malnourished people, with over 200 million people reported to experience chronic hunger, ranking second to Asia owing to the larger population on the Asian continent (Bain et al., 2013). There are several factors contributing to the profound malnutrition observed in Africa and other parts of the world, with absolute poverty being the principal contributor (UNICEF and WHO, 2017). Poverty and malnutrition form a long-term cycle wherein poor children often suffer

malnutrition leading to permanent cognitive and physical impairment which render them unable to generate a practical income when they are older. This represents a widespread crises of malnourishment which oftentimes leads to irreparable physical, social, and economic erosion.

1.3. Food security in Africa

The problem of malnutrition is intertwined with food security in any population. Food security is a concept underscored by two main ideas – the availability of food and, subsequently, easy access to it. Food security is a significant global challenge, and the sentiment is even more pronounced in the African context. Chronic hunger and starvation driven by food insecurity are seemingly decreasing across the world, except in Africa as it remains the only continent where famines are an ongoing occurrence and malnutrition is steadily rising (Devereux, 2018; Adeyeye et al., 2021). Approximately 27% of the population was categorized as being critically food insecure as recent as 2017, which is almost four times higher than any other region (UNICEF and WHO, 2017).

Inadequate food security in Sub-Saharan African has resulted in the absolute increase of undernourished people from 181 million to 222 million during a six-year period starting from 2010 (Adeyeye et al., 2021). The issue of food insecurity therefore threatens the foundation of human and economic development if left unresolved. Achieving food security across the continent requires a multifaceted strategy that considers historical food demand and supply patterns and reducing dependence on inter-continental food imports. Moreover, aggressive government intervention is necessary in establishing and implementing effective policies as far as food security is concerned (Hedden et al., 2016; Garrity et al., 2010). All these strategies demand a holistic approach with participation across multiple disciplines ranging from policy makers, science and technology institutions, through to the common citizen.

1.4. Important staple foods relevant for the African continent

Staple foods refer to food routinely eaten by a specific group of people that constitutes a large part of their daily nutrition (FAO, 2017). Staple foods are different among the diverse communities found worldwide, but they all share the same general qualities of being inexpensive and easily accessible (Muyanga et al., 2005). Crops from which staple foods are derived also have an obligation to be drought-tolerant and thrive in soil with limited nutrients because most developing regions cannot afford advanced and expensive farming practices. The African continent has numerous staple foods that have served many generations, and the following section will discuss five of these staple foods, selected based on their importance in areas where nutrient deficiencies are commonplace.

1.4.1. Cassava

Manihot esculenta, commonly known as cassava, ranks as one of the most important sources of sustenance for many countries in Africa, Asia and Latin America (Okogbenin et al., 2013). It is the third-largest staple food on the African continent, preceded only by maize and rice, and its edible storage roots provide a basic diet for over 250 million people (Adenle et al., 2012). The bulky roots of the cassava plant are the primary source of food and the leaves are occasionally consumed for nutrients and medicinal benefit (Viduranga, 2018). As a staple food, cassava roots are peeled shortly after harvesting, dried and then processed into flour. Alternatively, the roots can be boiled and pound into a paste that is added to soup-based dishes. Cassava cultivars are classified as either sweet or bitter based on the cyanide content in the roots (Wahyuni et al., 2021). Sweet cassava has a cyanide content of 50 mg/kg, whereas bitter cassava contains a significantly higher cyanide content of 400 mg/kg. Therefore, cassava needs to undergo careful processing methods such as fermentation to reduce cyanide toxicity. Cassava is an ideal crop for farmers who have limited resources because it is known to tolerate stresses brought about by erratic rainfall and it can survive in soil with minimal nutrients (Akinpelu et al., 2011). The cassava crop requires relatively low-input farming practices, and it can remain in soil for up to three years while maintaining its agricultural viability (Legg et al., 2014). This places cassava amongst the most important crops to guard against food insecurity.

1.4.2. Corn-soya

Corn-soya features here because it is one of the supplementary products distributed by food aid initiatives to impoverished regions during famines (Rogers et al., 2017). The corn-soya formulation is a blend of corn (maize) flour and soybeans, fortified with several vitamins and minerals (Navarro-Colorado et al., 2008). The primary benefit of combining maize with soybeans is to improve the traditionally low lysine content in maize by supplementing maize proteins with legumes known to have a high lysine content (Kato et al., 2019; Leinonen et al., 2019). The corn-soya production process begins with heat-treated, milled corn that is blended with soybeans and a premix containing several other minerals and nutrients. The ingredients are then partially cooked or roasted. The formulation is further processed to deactivate trypsin inhibitors present in soybeans, which would interfere with the physiological functions of trypsin in the small intestine (Vagadia et al., 2017). Trypsin inhibitors form inactive complexes with trypsin and effectively impede protein digestion and absorption of essential dietary proteins. The finished product has a reported shelf-life of 18 months at approximately 27 ºC and can potentially retain its nutritional value for 12 months. Once distributed to the less-privileged, corn-soya only needs to be boiled for 5-10 minutes before consumption.

1.4.3. Teff

Eragrostis teff (Zucc.) Trotter, or teff, is a cereal grain originating from the east African country of Ethiopia (Assefa et al., 2015). The crop accounts for at least 28% of land used for agriculture, and 19% of the gross cereal grain production in the country (Assefa et al., 2011). Teff grass is grown for its tiny seeds, which are classified as white or brown (red) depending on the seed colour (Hagos et al., 2012). The seeds are ground into a fine flour and baked to make *injera* – the largest staple by consumption in east Africa (Baye et al., 2013). *Injera* is a soft and sour fermented flatbread widely consumed in the region because bread is culturally recognized as the major component of traditional dishes. In addition to bread, teff flour is also incorporated into cakes and porridge, and the seeds are fermented to make the native alcoholic beverage *talla* (Lacey and Llewellyn, 2005; Assefa et al., 2011). Teff is regarded as a health food since it does not contain gluten, making it ideal for people with celiac disease (Lacey and Llewellyn, 2005; Zhu, 2018). In South Africa, Kenya, Uganda and Mozambique, teff is mainly known as a forage crop (Assefa et al., 2011). This work can potentially shift the paradigm towards promoting teff as a viable food source for human consumption.

1.4.4. Maize

Maize (*Zea mays*) was first domesticated in present-day Mexico more than 7000 years ago (Hallauer and Carena, 2009). It has one of the highest production rates of any cereal, placed only second to wheat production on a global scale and first in developing economies such as Africa (Dowswell, 2019). The crop was first introduced to Africa *circa* AD 1500, and it has since become the dominant food crop on the continent – accounting for ~30% calorific intake in Sub-Saharan Africa (Ekpa et al., 2019). Maize is commonly used as food for human consumption, animal feed, and it has industrial uses as a substrate for biofuel production (Kaul et al., 2019; Choudhary et al., 2020). Maize as a source of food is primarily consumed as a milled flour that is prepared in boiling water to make porridge, which forms the base for many savoury dishes. The cob can also be boiled or roasted and eaten directly. South Africa is currently the second largest producer of maize in Africa. Therefore, a detailed scientific appraisal of the composition of maize bodes favourably for future agricultural and economic development in the country.

1.4.5. Sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is closely related to maize under the Panicoideae subfamily of grasses (Schober and Bean, 2008). The crop was first domesticated in central Africa, which boasts some of the largest sorghum production yields in modern times (Orr et al., 2016). The African continent accounts for 62% of global sorghum cultivation, making the crop

a major component of improving food security (Orr et al., 2016). Sorghum consumption varies based on the region, but it is commonly eaten as a milled flour that is used to prepare porridge similar to maize. The grain is also processed to make flat breads and home-brewed beer. Sorghum-based food products are a principal source of nutrition for many people in rural Asia and Africa because the plant can tolerate harsh, arid environments (Abdel-Ghany et al., 2020). Sorghum also does not require fertilizer supplements, which can be expensive for the many small-scale farmers that typically grow sorghum.

1.5. The physicochemical properties of staple foods

Staple foods have physicochemical properties that collectively contribute to their overall importance in the continental food economy. Moisture and particle size distribution are some of the properties that can directly and indirectly influence the value of staple foods. These properties may determine whether food is suitable for consumption (Zambrano et al., 2019; Ileleji et al., 2010). They may also influence the bioavailability of nutrients (Lyu et al., 2021), and also affect the appearance and desirability of foods which informs their economic viability (Malvern, 2015).

1.5.1. Moisture content

Post-harvest food losses in developing regions exacerbate the issue of food insecurity as these losses account for over 30% of fresh food that is unsuitable for the market (Gustavsson et al., 2011). These food losses are mainly a result of microbial contamination promoted by the moisture content of food products, which is a critical rate-limiting parameter governing microbial growth rates (Zambrano et al., 2019). Fungal species such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are primary microbial organisms affecting grain crops whose growth and proliferation is dependent on the moisture content (Daou et al., 2021; Hell and Mutegi, 2011). These fungal species produce secondary metabolites called mycotoxins which deteriorate grain quality and render food unsuitable for human consumption (Alshannaq and Yu, 2017; Daou et al., 2021). Approximately 25% of global carcinoma cases related to mycotoxin exposure are reported in Sub-Saharan African countries (Liu and Wu, 2010). The lives of poor communities are therefore disproportionally impacted by mycotoxins in grains with a moisture content that supports the growth of harmful organisms.

1.5.2. Particle size distribution

Staple foods are often processed into flours before consumption which results in food materials containing granules, or particles, of different sizes. The size and uniformity of these primary particles in processed food matrices is referred to as the particle size distribution (Stojanović

et al., 2012). Particle size and size distribution of food granules is an important physical quality that has a significant influence on functional properties of flours such as *in vitro* starch digestibility, water solubility, and oil holding capacity (de la Hera et al., 2014; Ahmed et al., 2016; Kim and Shin, 2014). Particle size distribution also has an impact on sensory traits of finished products made from flours. Sensory characteristics including appearance, hardness and softness, texture and overall quality have been shown to be significantly affected by the size and distribution of flour particles (Kim and Shin, 2014; Savlak et al., 2016). Particle size distribution is therefore a primary variable in determining consumer acceptability of milled foods, which would affect the economic success of food producers.

1.6. Proteins and amino acids

Proteins are arguably one of the most important nutrient groups present in food. Proteins are polymers of amino acids, and they are produced when the carboxyl group of one amino acid bonds with the amino group of the next amino acid in an interaction referred to as a peptide bond (Nissen et al., 2000). Amino acids link through peptide bonds to form long chains that fold into three-dimensional conformations giving rise to protein molecules (Onuchic and Wolynes, 2004). Proteins are indispensable for the proper functioning, maintenance, and development of all living organisms. They are involved in plentiful and diverse biological processes which could quite possibly span the length of this manuscript. For this reason, only a few, general protein functions are highlighted. Proteins are key factors in facilitating successful DNA replication (Sirbu et al., 2011), some are involved in digestion and nutrient absorption as mentioned in the case of trypsin (Vagadia et al., 2017), and other proteins are enzymes catalysing metabolic reactions that would otherwise occur at rates too slow to sustain life (Benkovic and Hammes-Schiffer, 2003). Additionally, certain protein groups such as antibodies are essential in immune responses to recognize foreign antigens and allow the body to mount effective defence responses (Wilson and Andrews, 2012). Another group of proteins functions to transport important molecules such as oxygen around the body (Mairbäurl and Weber, 2011). Lastly, there are structural proteins that give the cell its structure, without which it would not survive (Lundin et al., 2010).

Upon consumption of food, protein molecules are broken down to provide free amino acids which are incorporated into other proteins during protein synthesis, or they can be delegated to other metabolic processes. There are 20 common amino acids (Table 1.1). Humans can inherently produce 11 of these amino acids in sufficient quantities, and they are called non-essential amino acids (Choi and Coloff, 2019). The remaining 9 amino acids cannot be synthesized in adequate quantities, and they are aptly named essential amino acids.

The distinction between essential and non-essential amino acids does not suggest the superiority of certain amino acids over others since all 20 amino acids are required by the body. However, essential amino acids are more valuable because they must be obtained from the diet to supply the body with the full complement of amino acids. The composition of these amino acids in food therefore carries economic and humanitarian significance.

Table 1.1. The 20 common amino acids classified as essential or non-essential amino acids based on the ability of the body to synthesise them in sufficient quantities (Choi and Coloff, 2019).

Essential amino acids	Non-essential amino acids
Histidine	Alanine
Isoleucine	Arginine
Leucine	Asparagine
Lysine	Aspartic acid
Methionine	Cysteine
Phenylalanine	Glutamic acid
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline
	Serine

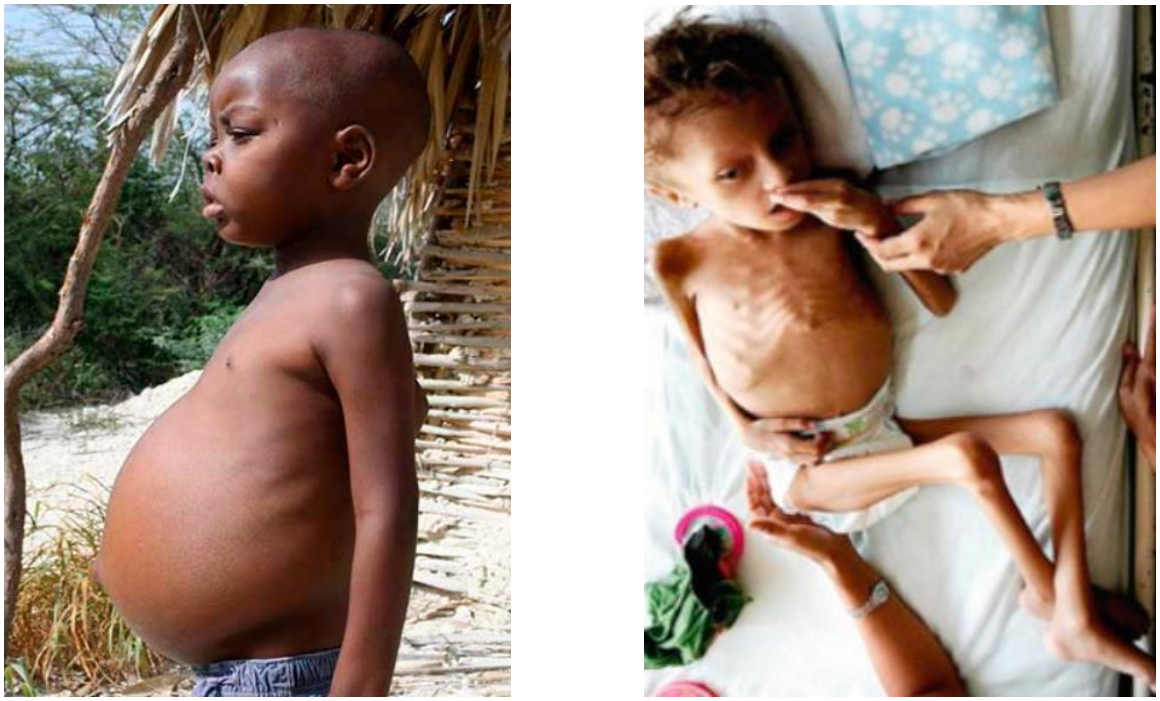
In addition to providing building blocks for protein synthesis, amino acids are also involved in important metabolic processes to support life. For example, serine plays a role in the proper functioning of the central nervous system and metabolism in the brain (Tabatabaie et al., 2010). Glycine is required for the synthesis of nucleic acids (building blocks for DNA), and bile acids which aid in the absorption of fat-soluble vitamins in the small intestines (Wang et al., 2013; de Aguiar Vallim et al., 2013). Glutamic acid is an abundant precursor of the gamma-aminobutyric acid (GABA) neurotransmitter that regulates neuron activity in the brain (Buddhala et al., 2012). Lysine is essential for calcium absorption, and thus bone development particularly in young children (Moya, 2016). Lysine is also required to repair damaged tissues (Telci and Griffin, 2006). The amino acids proline and arginine, along with hydroxyproline, assemble to form collagen, which is a major component of connective tissues (Li and Wu, 2018).

1.7. Diseases associated with protein malnutrition

Protein malnutrition is categorically one of the leading causes of child and adolescent mortality, being responsible for over 200 000 fatalities (Kyu et al., 2016). Insufficient protein consumption from the diet can lead to several, adverse health complications. Kwashiorkor is a notable example of protein malnutrition observed almost exclusively in children living in regions facing

famines (Garrett, 2013). The mortality rate of acute kwashiorkor has remained relatively unchanged since it was first identified in the early 1930's, with approximately half of the children who are diagnosed with the disease succumbing to it (Coulthard, 2015). The disease is characterised by hypoalbuminemia leading to a condition known as oedema. Albumin modulates the safe distribution of fluids in bodily organs, and in hypoalbuminemia, the albumin concentration is critically low leading to swelling of body parts such as the stomach as shown in Figure 1.1 (A) (Ahmed et al., 2009; Fanali et al., 2012; Osorio, 2011).

Marasmus is another disease condition resulting from a diet lacking adequate nutrients including proteins (Müller and Krawinkel, 2005). Its clinical symptoms include severe wasting of muscle, tissue and fat giving rise to a withered and wrinkled appearance as shown in Figure 1.1 (B) (Müller and Krawinkel, 2005). Children with the condition are typically weak with little appetite and heightened food intolerance (Prada et al., 2011).



A **B**

Figure 1.1. Diseases associated with protein malnutrition. (A) Kwashiorkor type malnutrition is characterised by a protruding stomach (Burnette, 2015). (B) Marasmus symptoms include loss of fat, tissue and reduced muscle mass (González-Torres et al., 2014).

1.8. Recommendations for protein and amino acid requirements

Dietary protein and amino acid requirements have been discussed and debated substantially throughout the years. These requirements were established to outline minimum levels of daily intake to support maintenance, growth, and reproduction (WHO, 2007). A joint consultation

between the World Health Organisation (WHO), the Food and Agriculture Organization (FAO) and the United Nations University (UNU) was held in Geneva, Switzerland from the 9th-16th April 2002. Expert opinion and empirical data were presented to offer guidelines and recommendations for protein and amino acid requirements in human nutrition. Establishing protein and amino acid requirements takes into consideration protein digestibility and amino acid biological value (WHO, 2007). Protein digestibility refers to a measure of the protein intake that is available for absorption relative to the total protein ingested (Sá et al., 2020). Amino acid biological value specifies a fraction of amino acids absorbed by the body, and how well the absorbed amino acids match with nutritional requirements (WHO, 2007).

A report of the WHO/FAO/UNU joint consultation sought to review and update intake requirements for children and adults. Presented below are tables adapted from the joint consultation report with recommendations for dietary protein and amino acid intake. Protein requirements were calculated by first determining the requirement per kilogram (kg), and thereafter multiplying the value with the median weight per age group. Table 1.2 highlights protein requirements in infants, children and adolescent boys and girls to promote healthy development. The recommendations show that safe levels of daily protein intake directly correlate to age in both boys and girls, where protein requirements increase as the age increases to match growing protein demands.

Table 1.2. Recommended protein intake for infants, children and adolescent boys and girls (WHO, 2007).

	Boys		Girls	
Age (years)	Weight (kg)	Minimum protein intake (g/day)	Weight (kg)	Minimum protein intake (g/day)
0.5	7.8	10.2	7.2	9.4
1	10.2	11.6	9.5	10.8
2	12.3	11.9	11.8	11.4
3	14.6	13.1	14.1	12.7
4-6	19.7	17.1	18.6	16.2
7-10	28.1	25.9	28.5	26.2
11-14	45.0	40.5	46.1	41.0
15-18	66.5	57.9	56.4	47.4

Protein requirements for adult men and women, in relation to body weight, are shown in Table 1.3. Dietary protein requirements in adults (> 18 years) are considered to be the same across all adult ages, and in both sexes (WHO, 2007). As per the consultation report, the minimum level of daily protein intake for adults is accepted to be 0.83 g/kg, which is multiplied by weight to calculate intake requirements on a body weight basis. Therefore, an adult with a

weight of 40 kg requires a minimum daily protein intake of 40 kg x 0.83 g/kg/day, which is equal to 33 g/day (Table 1.3). Safe levels of protein intake increase proportionally to the increase in weight in adult men and women, an indication for higher protein requirements to serve the needs of more considerable body masses.

Table 1.3. Daily dietary protein requirements for adult men and women (> 18 years) with various body weights (WHO, 2007).

Body weight (kg)	Safe level of protein intake (g/day)
40	33
45	37
50	42
55	46
60	50
65	54
70	58
75	62
80	66

The WHO/FAO/UNU joint consultation report also provided recommendations for essential amino acid requirements. Minimum requirements for lysine have received widespread scientific interrogation because it is the first limiting nutrient in most cereal grains such as wheat and maize (WHO, 2007). Lysine requirements were derived from oral tracer studies using isotopically labelled lysine (^{13}C-lysine) where the findings proposed a minimum lysine requirement of 30 mg/kg/day (El-Khoury et al., 2000). Recommendations for isoleucine and valine requirements were set to 20 mg/kg/day and 26 mg/kg/day, respectively (WHO, 2007). Leucine requirements were based on ^{13}C-leucine tracer investigations which reported a minimum leucine requirement of 40 mg/kg/day (Kurpad et al., 2003). The requirement for threonine was determined by two independent studies which outlined a minimum daily intake of 15 mg/kg/day (Borgonha et al., 2002; Kurpad et al., 2002).

Recommendations for phenylalanine requirements were derived from nitrogen balance studies which reported a requirement of 25 mg/kg/day (Tolbert and Watts, 1963). Tryptophan is a nutritionally important amino acid that participates in metabolic processes such as redox reactions and post-translational modifications of proteins (Fukuwatari and Shibata, 2013). Tryptophan is a limiting amino acid in most maize cultivars, and the recommended daily intake is 4 mg/kg/day (WHO, 2007). Requirements for sulphur-containing amino acids assume that methionine, an indispensable amino acid, serves as a continuous donor of sulphur to support

cysteine synthesis, a non-essential amino acid (WHO, 2007). Therefore, the requirement for sulphur-containing amino acids is a sum of individual methionine and cysteine requirements; i.e., 10.4 mg/kg/day of methionine + 4.1 mg/kg/day of cysteine ≈ 15 mg/kg/day (WHO, 2007). The minimum intake recommendation for histidine was endorsed by the joint consultation to be 10 mg/kg/day (WHO, 2007).

1.9. Codex Alimentarius food standards

The Codex Alimentarius is a set of internationally accepted standards, guidelines and codes of practice relating to food safety and fair trade of food commodities (CAC, 2007). The African continent has been identified by the Codex committee to be amenable to economic expansion through the development of reference food standards (FAO and WHO, 2007). These food standards underpin accurate measurement practices and are indispensable in verifying food products comply with legislation set in place to support food safety and fair trade. Laboratories on the African continent are faced with challenges related to funding, infrastructure, and availability of skilled personnel. Therefore, cost-effective reference food standards are critical to ensure a sustainable testing environment is maintained, effectively guaranteeing Africa participates meaningfully in the global food trade. Harmonious food trade backed by scientific evidence would undo many barriers impeding trade, allowing farming communities to benefit from thriving economic activity and thus alleviate hunger and poverty.

1.10. The importance of characterising physical properties and nutrition of staple foods

Plant-derived staple foods are important sources of energy and nutrition in the developing world because they are easily accessible to most people. Furthermore, food crops are relatively less restrictive to produce compared to animal sources of food thus serving as an essential source of income. This highlights the importance of investigating the nutritional offering of major African staple foods, as well as properties that would improve marketability and profitability. The rationale for this research is thus to characterise the moisture content and particle size distribution of the aforementioned staple foods. Food producers are in an increasingly competitive global economy which requires improved control of product quality. Describing these physicochemical properties would provide tangible economic benefits such as the ability to charge a premium on food products, increased customer acceptability, as well as compliance with regulations.

The present study also sought to characterise the protein and amino acid content of staple foods which would give insight into their capacity to provide adequate protein and amino acid nutrition to the scores of people dependent on them for nourishment. Characterising these

nutritional parameters also has the added benefit of facilitating improved trade of food commodities as discussed below.

This work is a collaboration between the University of Pretoria (UP) and the National Metrology Institute of South Africa (NMISA). NMISA is an entity of the South African government with many duties related to measurement standards, including the provision of internationally recognized reference materials. Reference materials have uses in quality control, and they are also used in proficiency testing schemes. Analytical laboratories participate in these schemes for accreditation purposes, or to obtain independent evaluation of their analytical competency. In responding to the need for reference materials in Africa, NMISA launched the African Food and Feed Reference Material Programme to provide fit-for-purpose reference materials relevant to the African continent.

Characterising proteins and amino acids in African staple foods contributes to this mandate because this work seeks to develop measurement methods needed for value assignment of the protein and amino acid content of reference materials. The reference materials will be disseminated to testing laboratories as part of proficiency testing schemes or quality control materials. Laboratories participating in proficiency testing schemes would conduct testing on the materials and report their results to the NMISA which is accredited under the International System of Organisation (ISO) 17043 as a proficiency testing scheme provider, and ISO 17034 as a reference material producer. Results submitted by participating laboratories would then be compared to the reference value obtained in the current testing. Testing laboratories can thus assess whether their current analytical methods are fit-for-purpose. This effectively assists in achieving international comparability and acceptance of measurement results, supporting the “measured once, accepted everywhere” aim. Trade in food commodities would thus be improved because trade partners will have confidence in test results from food testing laboratories that have demonstrated their competence through participation in testing schemes or the use of accurate and reliable reference materials.

1.11. Aim and Hypotheses

Aim:

This work set out to characterise the moisture content, particle size distribution, and the protein and amino acid composition of staple foods. The findings will highlight the value or deficiencies of specific food staples and may facilitate improved food trade through the provision of reliable food reference standards.

Hypotheses:

- The moisture content of staple foods is below the recommended moisture limit
- The particle size distribution of milled food samples comprises uniformly sized fine particles
- Plant-derived food sources have a high protein and amino acid composition

1.12. Project Objectives

- Quantify the moisture content of staple foods
- Describe the particle size distribution of milled food samples
- Quantify the total protein content using the Dumas method
- Hydrolyse food proteins into constituent amino acids
- Determine the amino acid composition by liquid chromatography
- Reference material value assignment

Chapter 2

The physicochemical properties of staple foods

2.1. Introduction

The current study investigated the moisture content and particle size distribution of the following African staple foods: White maize (sourced from a local farmer), brown teff grains (Local Village Foods, Midrand, South Africa), cassava flour (sourced from a local informal market, origin unknown), King Korn brand of milled sorghum (local supermarket), and corn-soya (Zambia). The corn-soya preparation was manufactured according to the United Nations Industrial Development Organisation (UNIDO) guidelines established by the FAO to foster innovation, promote economic growth, and build capacity in the industrial and manufacturing sector (Ward, 2019). These guidelines address the needs and challenges of developing countries by providing expertise and technical assistance to support sustainable growth through industrial development.

2.2. Materials and methods

2.2.1. Moisture content analysis by Karl Fischer titration

2.2.1.1. Theory

Measurement of the moisture content was performed based on the coulometric titration method proposed by the German chemist Karl Fischer in 1935 (Fischer, 1935). Karl Fischer titration is based on two reactions: In the first reaction, an alcohol, usually methanol or ethanol (ROH), reacts with sulphur dioxide (SO_2) and a base (RN) to produce an alkylsulfite intermediate as shown in Reaction 2.1.

$$\mathrm{ROH} + \mathrm{SO_2} + \mathrm{RN} \rightarrow (\mathrm{RNH}) \cdot \mathrm{SO_3R} \qquad \text{Reaction 2.1}$$

In the second reaction, the alkylsulfite intermediate is oxidised by iodine (I_2) to produce an alkylsulfate salt and the reaction consumes water (H_2O) present in the test sample as shown in Reaction 2.2.

$$(\mathrm{RNH}) \cdot \mathrm{SO_3R} + 2\mathrm{RN} + \mathrm{I_2} + \mathrm{H_2O} \rightarrow (\mathrm{RNH}) \cdot \mathrm{SO_4R} + 2(\mathrm{RNH})\mathrm{I} \qquad \text{Reaction 2.2}$$

The two reactions occur in a titration cell comprising an anode compartment that contains negatively charged iodide ions. The iodide ions release electrons to form iodine, which in turns reacts with water in equimolar amounts. When all the available water has reacted, the titration

endpoint is reached and the Karl Fischer instrument measures the time taken and current consumed to generate iodine, which is proportional to the amount of water in the test sample (Bruttel and Schlink, 2003). The titration endpoint is determined by bivoltametric indication, where a constant current is applied to a double-platinum pin electrode and the current remains unchanged when iodine reacts with water. When water is depleted, free iodine is detected in the anode compartment which causes a sudden drop in current signifying the titration endpoint where the moisture content can be inferred.

2.2.1.2. Protocol

The moisture content was determined on a Metrohm 874 Oven Sample Processor (Metrohm, Herisau, Switzerland). A preliminary temperature gradient experiment was first performed with 100 mg of each test sample heated to 250 ºC at a rate of 2 ºC/min. The preliminary experiment was performed to develop a suitable method for moisture determination by establishing the optimal oven temperature at which moisture is released. The expected water content was also obtained from the experiment, which was applied to calculate the mass of sample required to ensure the determined moisture content would be within the moisture range of the QC sample. Results from the temperature gradient experiment also assisted in selecting a suitable reference material necessary for quality control purposes.

For moisture determination, three replicates of 60 mg corn-soya, 50 mg maize, and 30 mg sorghum, teff, and cassava were weighed on a Mettler Toledo XPE 205 analytical balance (Mettler Toledo, Ohio, USA). The samples were added to 6 mL crimp-top vials (part number: 6.2419.007), sealed with an aluminium septum cap (part number: 6.1448.057) supplied by Metrohm. Three empty vials were also analysed to determine the blank value indicating the amount of residual moisture. Five replicates (75 mg) of a Hydranal™ 140-160 ºC, 5% water standard (Honeywell, North Carolina, USA), traceable to the National Institute of Standards and Technology SRM 2890 (NIST, Maryland, USA), were added to clean 6 mL vials for analysis. Samples were queued, and Tiamo™ software was used to program suitable methods for each of the samples. Cassava was heated at an optimal oven temperature of 156 ºC to release vapourised moisture, teff was heated at 180 ºC, corn-soya was heated at 165 ºC, and sorghum and maize were heated at 170 ºC. The moisture vapour was transported by dried nitrogen gas to a Karl Fischer titration cell where the water content was extrapolated from the titration endpoint.

2.2.2. Particle size distribution analysis by laser diffraction

2.2.2.1. Theory

Laser diffraction relies on a laser beam that is passed through a representative particulate sample (Malvern, 2015). The sample scatters the light beam in various directions, and the scattered light is subsequently detected by a multi-element light detector. Particle size measurements from laser diffraction are based on the inverse relationship between the diffraction angle and particle size (Malvern, 2015). Incident light produces small diffraction angles in large particles, whilst in small particles it produces large diffraction angles relative to the laser beam (Figure 2.1). The information on the angle of diffraction and intensity of scattered light is consolidated and transformed using complex Mie mathematical models in the instrument software to calculate the particle size distribution (Stojanović et al., 2012). Particle size results are then presented as a volume-equivalent sphere diameter. This means that laser diffraction assumes particles are perfectly spherical, and thus the diameter of the sphere has the same volume as the particle being analysed (Malvern, 2015).

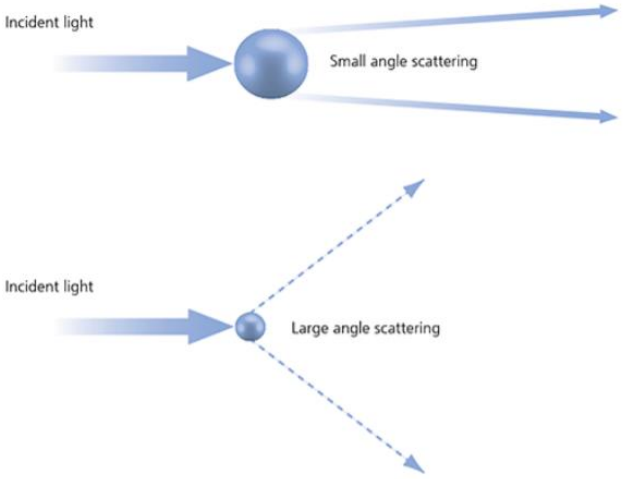


Figure 2.1. Laser diffraction theory: Light scattering in large particles produces small diffraction angles, and light scattering in small particles produces large diffraction angles relative to the laser beam (Malvern, 2015).

2.2.2.2. Protocol

Particle size distribution measurements were performed on a Malvern Mastersizer 3000 with a working range of 0.01-3500 µm (Malvern Instruments Ltd, Worcestershire, UK). The instrument has an optical unit housing a helium–neon laser light source operating at 632.8 nm, and a Hydro LV dispersion unit to evenly disperse the sample. The dispersion unit also has a stirrer set to 2500 rpm to prevent sedimentation. The measurement cell contains a multi-element detector to measure the diffraction angle and intensity of light scatter to generate particle size data. To perform the measurement, a test sample was slowly added to isopropanol (Romil,

Leicestershire, UK) in the dispersion unit, until the obscuration reading was ~10%. The obscuration reading should ideally be low to prevent multiple light scattering. This phenomenon would result in light reflected from one particle to bounce on other particles leading to undesired repeat measurements of the same particle. The resultant light scattering data was processed by the Mastersizer Version 3.00 software which reported the results under four classifications: $D_{4.3}$ (mean), D_{10}, D_{50}, and D_{90}. The D_{10} percentile, for example, means that 10% of particles in the sample have a diameter size smaller than the D_{10} value, and 90% have a size larger than the value. The same principle is applicable for the remaining percentiles.

2.3. Results

2.3.1. The moisture content of staple foods

The moisture content was obtained by Karl Fischer titration which provided absolute moisture values. To determine the true moisture content, residual moisture from blank samples was first subtracted from the absolute moisture content, and the resulting moisture was converted to percentage units using Equation 2.1.

$$\text{Moisture content} = \frac{\text{Absolute moisture (g)} - \text{Blank value (g)}}{\text{Sample mass (g)}} \text{ x } 100\% \qquad \text{Equation 2.1}$$

The accuracy of moisture determination was confirmed with a Hydranal™ 5% water standard. Five replicates of the water standard were analysed, and the true moisture content was calculated as described. The mean moisture content of the water standard was within the certified moisture range of 5.11 ± 0.04% provided on the certificate of analysis, verifying accuracy of the measured moisture content (Table 2.1). The high precision of the moisture measurements was demonstrated by the low relative standard deviation (%RSD).

Table 2.1. The moisture content of five replicates of a Hydranal™ water standard to demonstrate measurement accuracy and precision.

Sample	Moisture (%)
Sample 1	5.14
Sample 2	5.10
Sample 3	5.11
Sample 4	5.13
Sample 5	5.18
Mean	5.13
Standard deviation	0.032
%RSD	0.61
Reference value (from certificate of analysis)	5.11 ± 0.04

The moisture content results obtained for staple foods are presented in Table 2.2. Corn-soya had the lowest moisture content with a value of 6.30%, followed by maize with the second lowest moisture content of 7.36%. Sorghum showed a comparatively higher moisture content of 12.9%. Cassava revealed an even higher moisture content with a value of 13.5%, and teff had the overall highest moisture content with a value of 13.9%. The results showed good precision as indicated by low %RSD values.

Table 2.2. The moisture content of five staple food samples as determined by Karl Fischer titration (n = 3).

Staple foods	Moisture (%)	Standard deviation	%RSD
Corn-soya	6.30	0.14	2.26
Maize	7.36	0.15	2.00
Sorghum	12.9	0.32	2.48
Cassava	13.5	0.04	0.26
Teff	13.9	0.09	0.64

2.3.2. Particle size and size distribution

Laser diffraction provided particle size distribution plots of the staple food particulate material (Figure 2.2). Particle sizes in corn-soya and teff showed a monomodal distribution pattern (single peak), indicating the abundance of a single size fraction with an average diameter of 310 µm and 272 µm, respectively. Maize particle sizes showed a bimodal distribution pattern (two peaks), revealing two abundant size fractions with average diameters of 0.872 µm and 14.5 µm. Particle sizes in cassava showed a trimodal distribution pattern (three peaks), showing the abundance of multiple size fractions with average diameters of 0.767 µm, 16.4 µm, and 98.1 µm. Sorghum particle sizes also revealed a trimodal distribution pattern with multiple size fractions having an average diameter of 0.767 µm, 18.7 µm, and 144 µm.

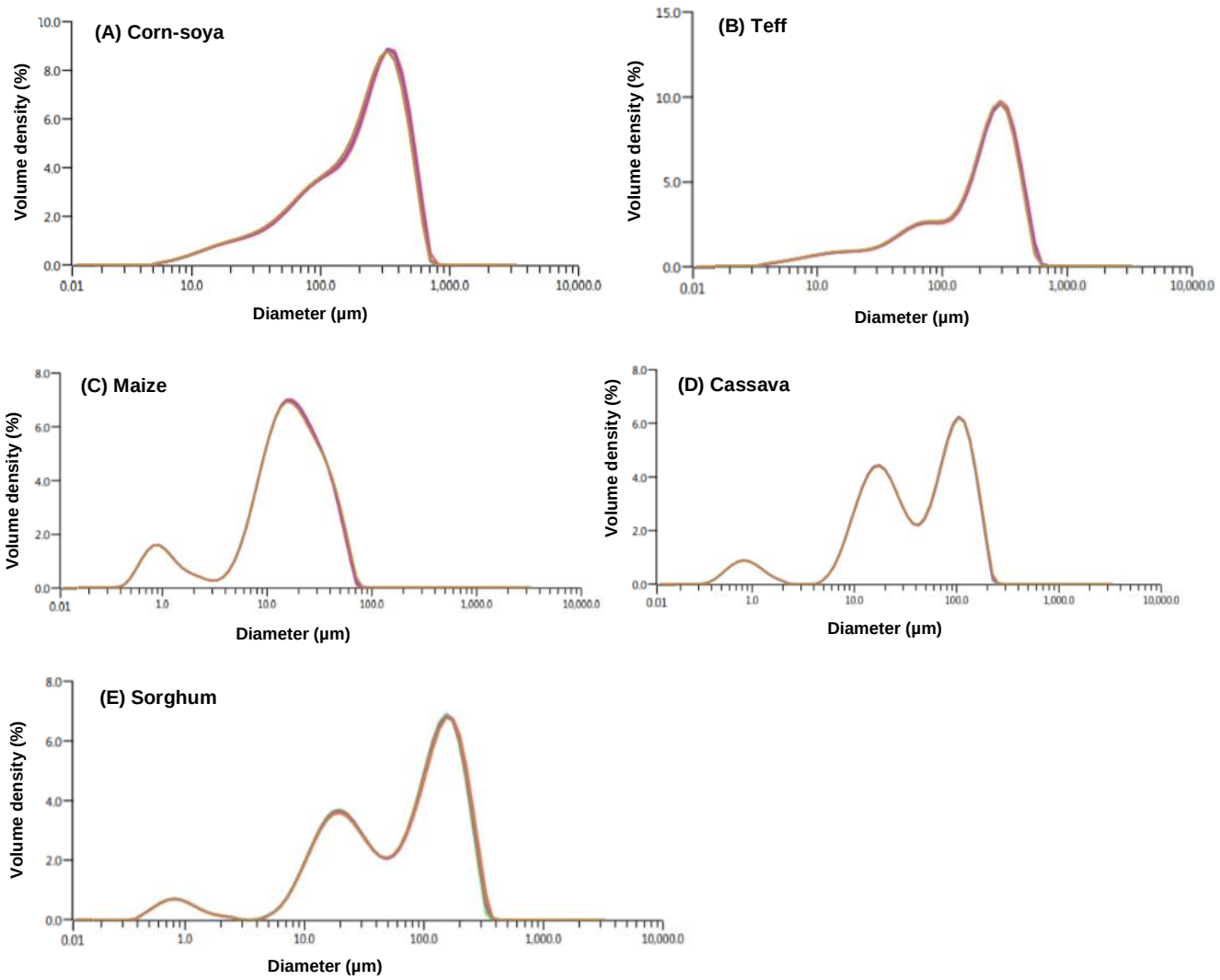


Figure 2.2. Particle size distribution plots from laser diffraction analysis of (A) corn-soya, (B) teff, (C) maize, (D) cassava, and (E) sorghum.

Particle size measurement results were also classified by four categories: $D_{4.3}$ (mean), D_{10}, D_{50}, and D_{90} (Table 2.3). Maize showed the smallest particle size with a mean diameter of 18.7 µm. Cassava revealed the second smallest average particle diameter size with a value of 59.4 µm. Sorghum was found to have a relatively small average particle size at 92.5 µm. Teff showed a significantly larger particle diameter size of 210 µm, and corn-soya had the largest mean particle diameter size with a value of 235 µm.

Table 2.3. Particle size distribution of five staple foods categorised by the $D_{4,3}$, D_{10}, D_{50}, and D_{90} (n = 6).

Sample	$D_{4,3}$ (µm)	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)
Maize	18.7	1.59	15.6	39.0
Cassava	59.4	8.54	41.5	136
Sorghum	92.5	10.4	76.8	207
Teff	210	32.6	210	391
Corn-soya	235	40.3	219	459

2.4. Discussion

2.4.1. The moisture content of staple foods

The current work reports the moisture content of five African staple foods as analysed by Karl Fischer titration. The first staple that was analysed is corn-soya which revealed a moisture content of 6.3%. According to the United States Department of Agriculture (USDA), the moisture content of corn-soya should be below 10% to maintain good quality and safety standards (USDA, 2017). The results from this study indicate that corn-soya meets quality control standards. The moisture content of maize was also analysed, and it was found to be 7.36%, which matches the previously reported value of 7.65% (Abiose and Ikujenlola, 2014). Previous studies concluded a content of 14% or below would be suitable to safely store maize (Suleiman and Kurt, 2015). Therefore, moisture in the maize sample analysed in this work was well below the recommended moisture limit. Moisture in sorghum was found to be 12.9%, which is higher than the content observed in corn-soya and maize. However, the moisture in sorghum is below the recommended maximum limit of 13.5% for safe storage (McNeill and Montross, 2003).

Teff was determined to contain the highest amount of moisture with a content of 13.9%. The moisture value compares to a previously reported content of 12.6% (Sadik, 2015). The relatively high moisture content could be a result of the traditional practice of field-drying teff in piles for several months following the harvest season. Field-drying has been adopted by most teff farmers because it improves the appearance and flavour of *injera*, and also leads to increased teff masses (Gizaw et al., 2018). Although the content is relatively high compared to corn-soya, maize, and sorghum, it has been reported that teff can be safely stored at a moisture level of 14% or lower, indicating the moisture content determined in this study would not lead to significant storability challenges (Alemneh et al., 2022).

Corn-soya, maize, sorghum and teff thus have reduced chances of spoilage during storage at the determined moisture content. This is important because moisture levels in cereal grains directly influence microbial contamination (Hell and Mutegi, 2011). Staple foods such as maize are highly susceptible to contamination from fungal species known to produce dangerous levels of mycotoxins (Suleiman and Kurt, 2015). Considering these staple foods are essential elements of the local food economy where access to suitable storage facilities may be limited. The low spoilage potential is important in providing a secure, long-term source of food and income for consumers and food producers.

Moisture analysis in cassava showed a content of 13.5%, which is higher than previously reported values of 11.4% and 11.6% (Chukwu and Abdullahi, 2015; Oladunmoye et al., 2014). The moisture differences could be a result of differences in harvesting seasons and post-harvest handling practices which significantly influence the moisture content (Chukwu and Abdullahi, 2015). The higher moisture content could also signal the hygroscopicity of cassava flour, which is the high potential of the flour to absorb moisture from the environment (Carmo and Pena, 2019). The recommended moisture content in cassava flour should be ≤ 11.3% to limit microbial contamination (Chisté et al., 2015). The sample analysed in this work showed a moisture content above the recommended limit, which may lead to high perishability of cassava during storage, therefore impacting food security. Cassava flour is thus recommended to be stored at an ambient temperature of 38 ºC in cost-effective low-density polyethylene sheets to allow for safe, long-term storage (Carmo and Pena, 2019).

2.4.2. Particle size distribution

Particle size measurements by laser diffraction provided size distribution plots and particle size diameters of milled food samples. Laser diffraction analysis is limited in that it cannot distinguish between primary particles and agglomerates on the distribution plots (Hackley et al., 2004). Agglomerates are particle clusters that arise due to small particles having a larger surface area which facilitates particle bonding by weak Van der Waals forces to form large particles (Kim et al., 2019; Shah et al., 2017). Agglomeration was not a significant cause for concern in this study because samples were continuously subjected to mechanical forces from a stirrer in the dispersion unit. These mechanical forces can dissociate the weak interaction forces with relative ease (Hackley et al., 2004). Therefore, the multiple size fractions observed in some distribution plots could be confidently identified as primary particles.

The distribution plots for corn-soya and teff showed a monomodal distribution pattern, which is an indication that most particles fell in a similar size interval. Particulate material with similarly sized particles would likely result in more homogenous milled foods (Djuragic et al., 2009). Homogeneity is essential to ensure uniform dispersion of all ingredients in a sample which would affect the physical and chemical characteristics of food. On the other hand, multimodal distribution patterns were observed for maize, cassava, and sorghum indicating particles were distributed over multiple size fractions. Although this study did not interrogate the relationship between particle size distribution and homogeneity; generally, a sample containing significantly different particle size fractions would be susceptible to separation. Small particles would percolate between large particles and settle at the bottom, and large particles would settle on top possibly reducing homogeneity, and ultimately product quality (Gough, 2021).

In addition to the distribution curves, laser diffraction analysis provided particle size data. The analysis showed that corn-soya comprised the largest particle sizes, which could be contributions from hard soybeans leading to flours that are coarser than other grain flours (Belorio et al., 2019). Teff had the second largest particle sizes, and this observation agrees with previous studies that have reported similarly large particle sizes (Assefa et al., 2018; Abebaw, 2020). The relatively large particle sizes may be a result of the hard, outer bran layer that is difficult to remove from the small teff kernels, which would reduce efficiency of the milling process (Galassi et al., 2020). Coarse flour particles such as the ones observed in corn-soya and teff have been demonstrated to improve the hardness and texture of finished food products leading to positive consumer reception (Rao et al., 2016).

Sorghum, maize, and cassava revealed finer particle sizes compared to corn-soya and teff. The mean particle size of sorghum found in this study was significantly lower than previously reported particle size values (Bolade and Buraimoh, 2006). The cited study conducted particle size measurements using the sieve shaker technique as opposed to laser diffraction which would explain the discrepancy between the reported results. Cassava had finer particles and maize showed the finest particle distribution, consistent with previous studies (Chisenga et al., 2019; Budacan et al., 2013). Fine particle sizes have shown positive correlation with a desirable taste profile and improved starch digestibility (Lv et al., 2019; Mahasukhonthachat et al., 2010). Small particle sizes have also been associated with a higher quantifiable protein content in soybeans (Xing et al., 2018). Therefore, the particle size distribution of sorghum, maize, and cassava may be advantageous with regard to these properties. However, other studies have obtained lower protein concentrations in fine particles (Ahmed et al., 2016), whilst others have reported no universally applicable correlation between particle size and functional properties of food (Ahmed, 2014; Ahmed et al., 2015). Therefore, patterns between particle size and food properties are product specific, and each milled food type requires detailed study to elaborate the effect of particle size on food quality.

2.5. Chapter conclusions

Measurement of the moisture content revealed that moisture in corn-soya, maize, teff, and sorghum would theoretically not lead to microbial instability. However, moisture in cassava is likely to exceed recommended limits most likely due to the hygroscopic nature of cassava flour. Particle size measurements demonstrated that staple food flours have significantly different particles size distributions that would potentially offer advantages and drawbacks depending on the desired end product. The current findings contribute towards providing a complete profile of staple food characteristics.

Chapter 3

The protein content of staple foods

3.1. Introduction

Protein analysis is normally performed using indirect nitrogen determination techniques such as the Dumas combustion method (Hayes, 2020). The principle of the Dumas method is illustrated in Figure 3.1, and it operates as follows: A homogenous organic sample is accurately weighed onto a ceramic crucible and placed in a preheated furnace set to ~1350 °C, with an atmosphere rich in 99.99% pure oxygen. The sample is flash combusted and releases combustion gases which are carbon dioxide (CO_2), water (H_2O), nitrogen gas (N_2) and nitrogen oxides (NO_x) (Saint-Denis and Goupy, 2004). The gases are collected and passed through a series of gas traps that retain all the other gases and only allow nitrogen gas and nitrogen oxides to flow through. Nitrogen oxides are carried by 99.99% pure helium gas over a copper catalyst which reduces the oxides to N_2. The nitrogen gas mixture is then transported to a thermal conductivity detector (TCD) which emits an electrical signal equivalent to the nitrogen intensity. The nitrogen value, expressed as a percentage, is subsequently multiplied by a standard conversion factor of 6.25, i.e., N x 6.25 to obtain the protein yield of a sample (Mæhre et al., 2018). The conversion factor assumes that every 1000 g of plant and animal protein contains 160 g of nitrogen; that is, 1000 g/160 g = 6.25.

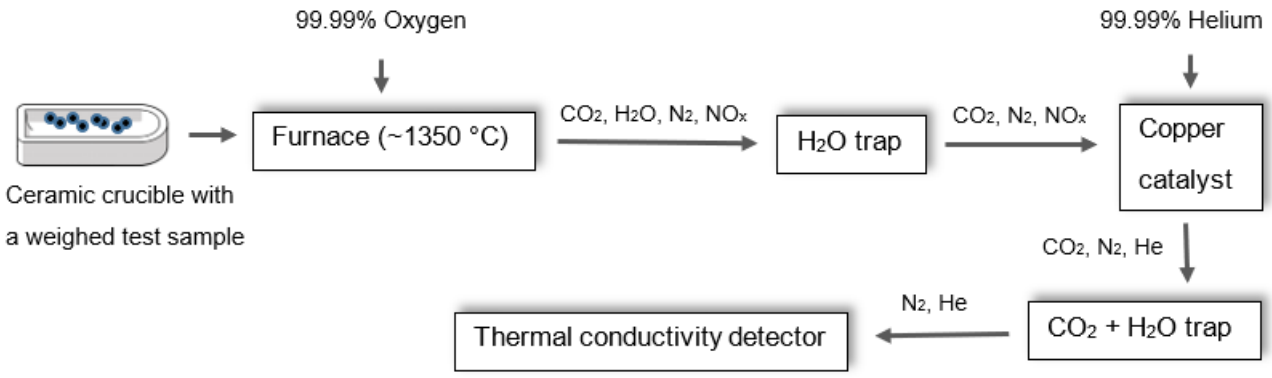


Figure 3.1. Schematic diagram illustrating the principle of the Dumas combustion method to determine the nitrogen content for total protein quantification in food.

3.2. Materials and methods

3.2.1. Determining the protein content by the Dumas combustion method

A 500 mg mass of each test sample was weighed (n = 5) into a ceramic boat crucible (Part No: 528-203-250, LECO, Michigan, USA) using a Mettler Toledo XPE 205 analytical balance (Mettler Toledo, Ohio, USA). Six replicates of 500 mg ethylenediaminetetraacetic acid (EDTA, LECO, Part No: 502-092-250) were also weighed. EDTA served as quality control to ensure measurement accuracy. It is a certified reference material traceable to the SI unit of measurement through the National Institute of Science and Technology (NIST). Test samples were analysed by the Dumas method (N x 6.25) on a TruMac® CN analyser (LECO). Prior to conducting nitrogen determination, operational gases: 99.99% pure oxygen for combustion, 99.99% pure helium carrier gas, and 99.99% pure synthetic air to operate pneumatics of the instrument were checked and confirmed to be 2.8 bar (2.4 bar for helium). A leak test was subsequently performed to ensure the operational gases did not leak.

Thereafter, a system check was performed to confirm parameters including instrument communications, temperature, pressure, and the detector cell were operating optimally. Following the system check, empty crucibles were analysed until the nitrogen %RSD was ≤ 5% to purge nitrogen from previous analyses and blank the instrument. The EDTA standard was analysed to calibrate the instrument, and thereafter the test samples were analysed. Nitrogen analysis settings on the TruMac® instrument were programmed as shown in Tables 3.1 – 3.3.

Table 3.1. TruMac® instrument conditions.

Parameter	Setting
Furnace temperature	1350 ℃
Thermo-electric cooler temperature	5 ºC
Dehydration time	0 min
Purge cycles	2
Lance flow	1.8 L/min
Purge flow	4.2 L/min
Ballast equilibration time	30 sec
Ballast not filled time out	300 sec
Aliquot loop equilibration pressure time	4 sec
Precision	High

Table 3.2. TruMac® element parameters.

Parameter	Nitrogen	Carbon	Range
Baseline delay time	6 sec	0 sec	0 – 60 (10 -120 Nitrogen)
Minimum analysis time	35 sec	15 sec	0 – 400 sec
End line time	2 sec	2 sec	0 – 5 sec
Conversion factor	1.00	1.00	0.01 – 99.9
Significant digits	5	5	1 – 5.5

Table 3.3. TruMac® burn cycle parameter settings.

Burn cycle	Lance flow	Purge flow	Time (s)
1	Off	On	5
2	On	On	35
3	On	Off	End

3.2.2. Statistical assessment

The protein content is reported as the mean ± expanded uncertainty. Expanded uncertainty was calculated as outlined by the Joint Committee for Guides in Metrology (JCGM, 2008). Uncertainty reflects the margin of doubt on the results by accounting for sources that contribute to uncertainty of the measurement results. The various factors contributing to measurement uncertainty of the protein results were identified and they are listed in Table 3.4.

Table 3.4. Factors contributing to measurement uncertainty in protein analysis.

Factors contributing to measurement uncertainty in protein analysis
The purity of the EDTA calibrant
The precision of weighing EDTA samples
The precision of the nitrogen content in EDTA
The precision of the blank as obtained through the analysis of blank crucibles
The precision of weighing test samples
The precision of the nitrogen content in test samples
The error of the analytical balance as provided on the calibration certificate.

To calculate the expanded uncertainty (U), the standard uncertainty (u) was first calculated for each factor contributing to uncertainty using Equation 3.1, where s denotes the standard deviation and n is the number of replicates.

$$u = \frac{s}{\sqrt{n}} \qquad \text{Equation 3.1}$$

Thereafter, the combined standard uncertainty (u_c) was determined by squaring the standard uncertainties of the uncertainty components, followed by calculating the sum, and lastly calculating the square root of the sum (Equation 3.2).

$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 + \cdots u_n^2} \qquad \text{Equation 3.2}$$

The expanded uncertainty (U) was calculated by multiplying u_c with a standard coverage factor (k) that is equal to two (2) at a 95% level of confidence (Equation 3.3).

$$U = u_c \times k \qquad \text{Equation 3.3}$$

A Tukey test was also used to test for outliers. Repeatability and reproducibility were tested using %RSD. Microsoft (MS) Excel was used to perform a single-factor analysis of variance (ANOVA) to test for significance. Statistical significance was determined at $p < 0.05$ at a 95% level of confidence.

3.3. Results

The Dumas combustion method was used to quantify the protein content. Briefly, test samples were rapidly combusted at 1350 ºC under a flow of pure oxygen. The resulting combustion gases were passed through a series of gas traps to isolate nitrogen gas, which was transported to a TCD cell. The detector emits a signal based on the intensity of nitrogen, which is interpreted by software that reports the final nitrogen content as a percentage. The percentage nitrogen was multiplied by a protein factor of 6.25 to obtain the total protein yield as per (Mæhre et al., 2018).

3.3.1. Accuracy of the Dumas combustion method for protein analysis

Measurement accuracy of the Dumas method was determined by analysis of six replicates of an EDTA certified reference material on five different days during a 12-month period. A Tukey outlier test was performed on the data set, and outliers were not identified. The instrument reported a mean nitrogen content of 9.57%, and thus accuracy of the Dumas method was within specification since the nitrogen results were within the reference range of 9.56 ± 0.04% reported on the EDTA certificate of analysis (Table 3.5).

Table 3.5. Measurement accuracy of the Dumas combustion method as determined by analysis of six replicates of an EDTA certified reference material on five different days (n = 4 on day 4).

Parameter	Nitrogen (%)
Mean (n = 28)	9.57
Standard deviation	0.050
%RSD	0.53
Reference value (from certificate of analysis)	9.56 ± 0.04

3.3.2. Repeatability of the Dumas method

Repeatability measures the closeness of results under identical experimental conditions. To determine method repeatability, six replicates of an EDTA standard were analysed on a single day. The criteria for repeatability is based on an in-house criteria derived from published method validation criteria including the Eurachem Guide (Magnusson and Örnemark, 2014), and the South African National Accreditation System (SANAS, 2012; SANAS, 2008). The parameter for repeatability was outlined as $\%RSD_r \leq 5\%$. Table 3.6 shows data from the six replicates and a $\%RSD_r$ of 0.29 was obtained, which satisfied the criteria for repeatability.

Table 3.6. $\%RSD_r$ from six replicates of an EDTA reference material to demonstrate repeatability of the Dumas method.

EDTA	Nitrogen (%)
Sample 1	9.61
Sample 2	9.58
Sample 3	9.53
Sample 4	9.57
Sample 5	9.56
Sample 6	9.55
Mean	9.57
Standard deviation	0.03
$\%RSD_r$	0.29

3.3.3. Reproducibility of the Dumas method

Reproducibility refers to repeatability of the method on different days. Six replicates of the EDTA standard were analysed on five different days during a 12-month period (n = 4 on day 4). The parameter for reproducibility was outlined as $\%RSD_R \leq 5\%$. Table 3.7 shows the $\%RSD_R$ from the five days of analysis was 0.53%; therefore, the Dumas method satisfied requirements for reproducibility.

Table 3.7. $\%RSD_R$ from six replicates of an EDTA reference material on five different days over a 12-month period to demonstrate reproducibility of the Dumas method (n = 4 on day 4).

Day of analysis	Mean nitrogen (%)
Day 1	9.57
Day 2	9.57
Day 3	9.57
Day 4	9.57
Day 5	9.57
Mean	9.57
Standard deviation	0.050
$\%RSD_R$	0.53

3.3.4. The protein content of staple foods

Staple food samples were similarly analysed on five different days during a 12-month period, with five replicates on each day (n = 3 on day 1). A single-factor ANOVA statistical test was performed on the data set and the test indicated the variation in results from the five different days was statistically significant ($p < 0.05$). The variation in the results may be a result of factors related to sample homogeneity and variations in temperature and humidity during the 12-month analysis period. The protein content is therefore reported together with the expanded measurement uncertainty (U), which represents the upper and lower limit of the mean value. The protein values obtained on the five different days fell within limits of the expanded uncertainty and thus they were accepted as being reliable.

Protein analysis results are reported in Table 3.8, together with the within sample precision ($\%RSD_r$) and between-day reproducibility ($\%RSD_R$). Corn-soya had the highest mean protein content with a value of 12.8 g/100 g, followed by sorghum with an average protein content of 11.3 g/100. Teff showed a lower protein content with a value of 9.53 g/100 g. Maize had the second lowest protein amount with a content of 8.88 g/100 g. Cassava revealed a significantly lower protein content with a value of only 1.13 g/100 g. The within-sample precision ($\%RSD_r$) was below 5% for all the test samples, which represents high measurement precision. However, the between-day reproducibility ($\%RSD_R$) was higher than 5%, but below 10% in food samples, except for cassava with the highest $\%RSD_R$ of 55.6% representing poor reproducibility.

Table 3.8. The mean protein content of five staple foods measured using the Dumas combustion method. Measurements were performed on five different days with five replicates on each day (n = 3 on day 1). Results are reported with the expanded uncertainty (U) at a 95% confidence level.

	Protein (g/100 g)			
Sample	Mean	U	$\%RSD_r$	$\%RSD_R$
Corn-soya	12.8	5.92	1.32	5.38
Sorghum	11.3	5.93	0.79	5.20
Teff	9.53	5.93	2.18	8.90
Maize	8.88	5.92	1.79	6.83
Cassava	1.13	5.93	3.73	55.6

3.4. Discussion

The Dumas combustion method was implemented to determine the protein content of five staple foods. The analysis was performed on five different days and a significant between-day variation ($p < 0.05$) was obtained, with the highest variation observed in cassava as reflected by an $\%RSD_R$ of 55.6%. The variation could be a result of the change in moisture during the 12-month testing period. Moisture measurements conducted in this work (Chapter 2) were not performed alongside protein analysis to confirm this working theory, however, it is known that moisture is dependent on temperature and relative humidity which naturally fluctuate owing to seasonal variations (Aqil, 2020). Changes in moisture may have influenced the amount of sample weighed, which would have had an impact on protein analysis. Cassava had the highest between-day variation, which is likely a result of the observed low protein content since samples with a reduced content are associated with poor reproducibility (Miller et al., 2007). Nonetheless, the results were valid because the variation fell within limits of the expanded measurement uncertainty.

Corn-soya is a supplementary food product distributed by food aid initiatives to address malnutrition in poverty-stricken regions. A minimum protein content of 13.3 g/100 g (with an allowance provided for < 5% of the specified value) has been set for any corn-soya preparation to be approved for use in supplementary feeding programmes (USDA, 2017). A protein content of 12.8 g/100 g was obtained in this study, which compares to a previously reported content of 10.4 g/100 g (Mangani et al., 2015). The protein content does not vary significantly from the minimum allowable limit, which, when considered alongside previously reported values, suggests corn-soya manufacturers may be targeting the minimum allowable protein content. This practice is likely to minimize production costs as protein-rich ingredients would be more expensive to source. The current findings for corn-soya show that the food product would likely meet minimum protein requirements presented in Chapter 1 based on WHO recommendations.

Indeed, corn-soya is an effective supplementary food source which aided the recovery of at-risk infants and children, although, the efficacy was lower compared to the more expensive ready-to-use therapeutic foods (RUTFs) as demonstrated in Malawian children (Matilsky et al., 2009).

Sorghum is the second staple food analysed for its protein content. This work reports an average protein content of 11.3 g/100 g, which matches the previously reported content of 10.2 g/100 g (Abdelhalim et al., 2019). Sorghum thus represents a relatively good source of proteins when benchmarked against WHO protein requirements (WHO, 2007). The combination of favourable agronomy and relatively good protein content places sorghum at the forefront to accelerate economic development as it has widespread applications in the food and beverage industry.

Although teff is indigenous to east Africa, it has gained a foothold in the Netherlands, Canada, and Switzerland among other global economies (Gebru et al., 2020). Continued introduction and cultivation of teff in other parts of the world necessitate a valid scientific evaluation of the nutritional content. The brown teff variety tested in this study revealed a protein content of 9.53/100 g, which is in agreement with the previously reported content of 9.37 g/100 g (Gebru et al., 2020). The protein content in teff represents a relatively good source of plant proteins that can satisfy protein requirements for several demographics (WHO, 2007). As the global cost of food prices rises above budgetary constraints of many people in developing countries, protein-rich food sources such as meat, poultry and dairy products become even more elusive. Alternative protein sources are therefore important to meet dietary demands. Teff promises a healthy supply of plant proteins, and thus increased research on the underexploited grain would contribute significantly towards food security.

Maize was also analysed in this work and the protein content was found to be 8.88 g/100 g, which corresponds with literature values (7.82 g/100 g) (Qamar et al., 2017). Although the maize cultivar analysed in this work showed a relatively good protein content, other maize cultivars such as yellow maize and quality protein maize (QPM) have a superior nutritional offering (Pillay et al., 2013; Nuss and Tanumihardjo, 2011). The nutritional benefits offered by these improved maize cultivars may go unrealised owing to traditional eating practices favouring the texture, colour, and taste of conventional white maize. Therefore, white maize varieties would benefit from bioengineering studies to further improve the protein content while preserving traditional eating perceptions.

Cassava is classified into bitter and sweet cultivars based on the cyanide content, but the cultivar used in this study was unknown. The protein content of 1.13 g/100 g obtained in this study is consistent with the content of bitter cassava that has poor protein quantities compared to the sweet variety (Chisenga et al., 2019; Obueh and Ekanah, 2016). Bitter cassava comprises 90% of cassava production owing to its favourable agronomic properties such as high yields and disease resistance (Steenkamp and McCrindle, 2014). However, the limited protein content in the bitter variety diminishes its economic value because it would fetch lower prices compared to other food crops such as maize, thus generating less income for producers. Cassava is among the most important foods relied upon by many people because it is an affordable, energy-dense food source (Morgan and Choct, 2016). However, widespread consumption of bitter cassava poses health risks for communities relying on the crop as it cannot reasonably meet protein requirements outlined by WHO guidelines.

The cost of producing food commodities, and their retail price are important factors to consider in addition to the nutritional composition to classify the true value of staple foods. The protein content, production costs and retail price of the five staple foods under study are compared in Table 3.9. It must be noted that these costs are not universally applicable because production costs and retail prices are influenced by variables such as environmental conditions, infrastructure costs, labour laws, and a host of other factors which vary significantly on a continental and regional scale.

Corn-soya and sorghum showed the highest protein content, but they also constitute two staple foods with the highest production costs. Corn-soya is the most expensive to produce owing to the combination of two different crops requiring independent farming inputs. However, the food source is provided to consumers at no cost, and producers are typically afforded government subsidies (Bridges, 2015). Corn-soya is therefore a nutritionally valuable staple food with a reduced financial burden on producers, and it is accessible to consumers. Sorghum showed the second highest protein content, with the second highest production costs at R12 700/hectare (Wilde, 2021). However, sorghum produces higher yields on average at approximately 2.05 tonnes/hectare (Assefa, 2019). Producers would therefore receive higher returns on their initial investments. Sorghum has s retail price of R11.65/kg representing an affordable food source (Betaprice, 2021).

Production costs for teff are estimated to be R6253/hectare, which are lower than sorghum and maize (R10 000/hectare) (Bekele et al., 2019; Van Zyl, 2017). However, teff produces lower yields at 1.28 tonnes/hectare, and this has been attributed to inability of the crop to successfully compete for nutrients (Berhe et al., 2011). In contrast, maize and sorghum have

higher average yields with maize yields being approximately 2.95 tonnes/hectare (Assefa, 2019). Teff is therefore more expensive to produce compared to other common staple foods. The retail price for teff is normally higher due to its nutritional composition and gluten-free qualities, and thus it typically sells for R17.81/kg which is higher than the price for maize at R11.99 (Teka, 2021; IFPRI, 2021). The higher prices have resulted in teff consumption being more prominent among affluent consumers, and comparatively low in poor communities (Zhu, 2018). Teff is therefore less accessible compared to maize, particularly in rural areas where most of the population lives under poverty conditions.

The production costs for cassava are among the lowest at R3000/hectare (Kosemani and Bamgboye, 2009). These lower production costs can be linked to cassava being drought-tolerant, thus negating the need for expensive irrigation systems. Moreover, cassava can grow in soil with minimal nutrients without requiring extensive fertilisation which collectively reduce production costs. Cassava also has higher accessibility to most consumers with a retail price of only R3.11 (Akinfenwa, 2021). However, the low retail price is an attribute of the cyanide toxicity, as well poor protein quality that cannot sufficiently meet nutritional demands.

Table 3.9. A comparison of the protein content, production costs and retail price of five African staple foods.

Staple foods	Protein* (g/100 g)	Literature values[a] (g/100 g)	WHO minimum[b] intake (g/kg/day)	Production costs[c] (per hectare)	Retail price[d] (per kg)
Corn-soya	12.8	10.4		R10 000 and R13 000[#]	NA[Ψ]
Sorghum	11.3	7.9		R12700	R11.65
Teff	9.53	9.37	10.2 – 66	R6253	R17.81
Maize	8.88	7.82		R10 000	R11.99
Cassava	1.13	1.36		R3000	R3.11

*Obtained in this study using the Dumas combustion method (N x 6.25). [a, b, c, d]Referenced in-text.

[#]Cost/hectare for maize and soybean (Bourlion et al., 2013), respectively.

[Ψ]Retail price not applicable for corn-soya because it is not a commercial product.

3.5. Chapter conclusions

This section of the work reports protein quantities in five staple foods determined using the Dumas combustion method. The method uses a protein factor of 6.25 to convert percentage nitrogen to protein yield (Mæhre et al., 2018). Protein factors have been a subject of numerous studies, and it has been demonstrated that protein quantification based on these factors is prone to overestimate the actual protein quantity (Mariotti et al., 2008). The 6.25 protein factor assumes the amount of nitrogen in living organisms is conserved, enabling application of the value in most food types. This practice may not be precise because food naturally has varying amino acid compositions with differing nitrogen compositions. Additionally, non-protein

nitrogen from other compounds such as nucleic acids and alkaloids may be categorised as protein-nitrogen leading to an overestimation of the protein content. Numerous research efforts have suggested species-specific protein factors for some sample types to make total protein quantification using nitrogen more precise (Mariotti et al., 2008; Lourenço et al., 2002).

Despite efforts to improve method precision, the 6.25 factor is almost universally applied in both industry and routine laboratory work. It has been demonstrated that more than half of the studies on protein determination in seaweeds apply a protein factor of 6.25 despite several other studies documenting the correct value to be lower (Angell et al., 2016). Some explanations for continued use of the 6.25 factor are related to tradition, that is, continuing with methods well-established in a laboratory even when there is evidence they may be inaccurate. High costs associated with initial setup and implementation of more precise analytical methods also contribute to the issue.

A relevant case study demonstrating the significance of accurate nutritional testing practices is the 2008 milk scandal that occurred in China. Producers of infant milk formula added melamine to the milk preparation to increase the perceived nitrogen content and pass quality control checks (Ingelfinger, 2008; Pei et al., 2011). The increased nitrogen resulted in higher protein yields reported following protein analysis, which improperly increased the economic value of infant formula. This practice also presented explicit health risks on infants consuming less than the required daily dietary protein and introduced the harmful effects of ingesting melamine to children. As a result, 6 infant fatalities were reported along with hospitalisation of over 50 000 children, and a further 250 000 children experienced mild urinary and kidney problems (Pei et al., 2011).

Since indirect protein quantitation based on nitrogen values cannot distinguish between different nitrogen sources, food adulteration incidents such as the one described here could not be avoided. The risk of food adulteration extends to food material such as corn-soya, teff, cassava, sorghum, and maize. Additives included in staple food products designed to misleadingly increase the protein content cannot be identified using nitrogen-based analysis methods that manufacturers may apply. These limitations therefore provide motivation to verify the protein content with more selective techniques. Amino acid analysis is less susceptible to interferences and thus satisfies requirements for selectivity, and this technique will be discussed in detail in the next chapter.

Chapter 4

The amino acid composition of staple foods

4.1. Introduction

Proteins are, at their basic level, chains of amino acids that fold into functional molecules. Analysis of these building units can therefore provide a wealth of knowledge in the nutritional composition of different food types. Additionally, amino acid analysis would provide detailed information to produce well-characterised reference materials. Amino acid analysis, as performed by chemical hydrolysis, considers the diverse chemistries of the 20 different amino acids. It is for this reason that this study endeavoured to optimize several methods derived from the Association of Official Analytical Chemists (AOAC), NMISA SOPs, manufacturer application manuals, and literature recommendations to reliably quantify the full spectrum of amino acids. Various parameters were optimised including sample pre-treatment (milling and freezing steps), the time applied for hydrolysis, hydrolysis temperature, and the hydrolysis volume which will be discussed in detail in the results section.

The first step in amino acid analysis involves heating samples in highly concentrated hydrochloric acid (HCl) to hydrolyse proteins and peptides into their constituent amino acids. Additives such as phenol, indole, and thioglycolic acid are normally to the HCl reagent to prevent the chlorination of tyrosine which would form 3-chlorotyrosine that is highly susceptible to destruction (Whiteman and Spencer, 2008). Hydrolysed amino acids are then derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) to produce highly stable urea derivatives (Waters, 2007). AQC also imparts ultraviolet (UV) properties to facilitate detection using a photo-diode array (PDA) detector. Most amino acids lack a chromophore element in their structure and thus would not produce a signal for detection if derivatization is not performed (Stepanenko et al., 2011). The derivatization reaction is illustrated in Figure 4.1: AQC first reacts with the amine group of primary and secondary amino acids to form stable urea derivatives (Waters, 2007). Excess AQC reacts with water to form the by-product 6-aminoquinolone (AMQ). AMQ in turn reacts with residual AQC to form bis-aminoquinoline urea, which is another by-product of the derivatization reaction.

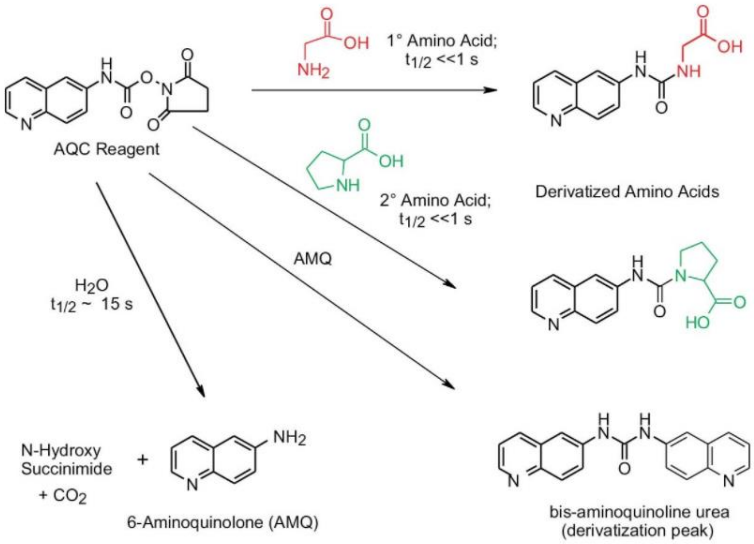


Figure 4.1. Schematic illustration of the AQC derivatization reaction to derivatize primary and secondary amino acids into stable urea derivatives. AMQ and bis-aminoquinoline urea are the major by-products of the derivatization reaction (Waters, 2007).

4.2. Materials and methods

The current work is based on the principle of gravimetry, which determines the quantity of a sample by measuring the change in mass. The gravimetric method relies on analytical balances and internationally accepted mass pieces that are routinely calibrated and verified, which establishes a traceability link to the SI unit of measurement. Traceability to international standards assists in achieving measurement comparability and improving measurement accuracy. Laboratory work was performed under the following conditions as prescribed by ISO 17025 and ISO 17043: Ambient temperature of 20 ± 2 ºC and relative humidity of 45 ± 20%. Test samples were corn-soya, cassava flour, sorghum, white maize, and brown teff seeds. Teff seeds were processed on a Retsch ZM 200 mill (Retsch GmbH, Haan, Germany) fitted with a 0.25 µm sieve to obtain sufficiently milled samples. All five food samples were thoroughly mixed prior to subsampling to achieve a homogenous sample material as homogeneity is essential to ensure subsamples are representative of the whole sample.

4.2.1. Hydrolysis of acid-stable amino acids

The current analysis method is suitable to determine the 15 amino acids shown in Table 4.1, collectively referred to as acid-stable amino acids. To make the 6 M HCl/phenol required for hydrolysis, 1 g phenol crystals (Sigma-Aldrich, Missouri, USA) were first dissolved in 500 mL Milli-Q water (Milli-Q® Academic®, Merck Millipore, Massachusetts, USA). Phenol was added to prevent tyrosine degradation. Thereafter, 500 mL 37% HCl (VWR Chemicals, Pennsylvania, USA) was added, and the mixture was inverted at least 32 times to thoroughly mix the solution.

Test samples were prepared by adding 100 mg of each staple food sample (n = 3) into pre-weighed 18 mL vacuum hydrolysis tubes (Thermo-Fisher, Massachusetts, USA). The quality control sample used with each batch of analysis was a finely milled pasta reference material from the *Bureau Interprofessionnel d'Etudes Analytiques* (BIPEA, Paris, France) which was weighed in triplicate (100 mg) and added to clean hydrolysis tubes. A 10 mL volume of freshly prepared 6 M HCl/phenol was added to the samples and vortexed for 30 sec on a Vortex-Genie® 2T timed mixer (Scientific Industries, New York, USA).

Table 4.1. List of acid-stable amino acids quantified by conventional acid hydrolysis.

Acid-stable amino acids				
Histidine (HIS)	Glycine (GLY)	Threonine (THR)	Lysine (LYS)	Isoleucine (ILE)
Serine (SER)	Aspartic acid (+asparagine) (ASP)	Alanine (ALA)	Tyrosine (TYR)	Leucine (LEU)
Arginine (ARG)	Glutamic acid (+glutamine) (GLU)	Proline (PRO)	Valine (VAL)	Phenylalanine (PHE)

The hydrolysis tubes were connected to a glass vacuum manifold (supplied by the University of Kwazulu-Natal glassblowing workshop) as pictured in Figure 4.2. The vacuum manifold was connected to a Welch MPC 301 Z vacuum pump (Gardner-Denver-Thomas, Fürstenfeldbruck, Germany) at one end and a nitrogen source at the other end. The caps on the hydrolysis tubes were loosened, and the tube headspace was purged with nitrogen for 3 min. Thereafter, the hydrolysis tubes were evacuated using the vacuum pump for 1 min. The tubes were sealed shut, disconnected from the vacuum manifold, and samples were hydrolysed at 110 ºC for 72 h on a pre-heated TS-18823 Reacti-Therm™ (Thermo-Fisher) under a fume hood. Following hydrolysis, test samples were removed from the heating block and allowed to equilibrate to room temperature. The hydrolysates were decanted into clean and labelled 50 mL Falcon tubes, and a 1 mL volume of the hydrolysate was aliquoted into 1 L amber Boston round bottles. Hydrolysates were diluted with Milli-Q water using dilution values obtained from a preliminary amino acid analysis experiment performed using amino acid concentrations available in literature. Optimised dilution values were calculated to ensure on-column amino acid concentrations fell within the linear range of the liquid chromatography instrument.

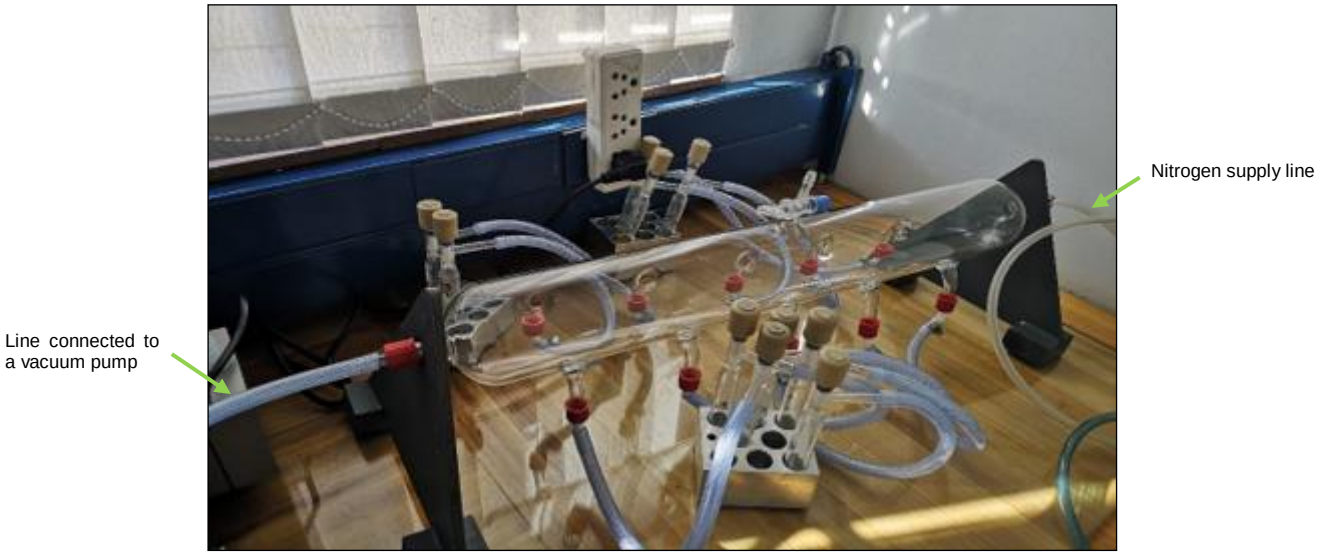


Figure 4.2. Vacuum manifold setup to purge and evacuate the hydrolysis tube headspace. The gas used for purging was nitrogen.

4.2.2. Hydrolysis of methionine, cysteine, and tryptophan

Traditional acid hydrolysis poses challenges for the analysis of tryptophan, methionine, and cysteine. These three analytes are unstable under hydrolysis conditions with 6 M HCl leading to amino acid losses. As a result, modifications to the hydrolysis protocol are necessary for accurate amino acid quantification. Tryptophan is destroyed by the HCl hydrolysis agent, and thus alkaline hydrolysis is normally performed using sodium hydroxide (NaOH) to prevent losses (Çevikkalp et al., 2016). Sulfhydryl groups on methionine and cysteine are unstable under classical acid hydrolysis conditions and require a preparatory oxidative step using performic acid to produce stable sulphur derivatives (Phillips et al., 2020). The derivatives are referred to as methionine sulfone and cysteic acid. All references to cysteine/cysteic acid encompass both cysteine and its oxidised dimer cystine.

4.2.2.1. Methionine and cysteine hydrolysis

The performic acid required for methionine and cysteine oxidation was prepared by adding 1 part 30% hydrogen peroxide (Sigma-Aldrich) to 9 parts 88% formic acid (Sigma-Aldrich). The mixture was incubated at room temperature for 1 h while swirling at 65 rpm on an IKA® Roller 6 (IKA, Staufen, Germany). The performic acid solution was incubated in an ice bath for 15 min. A 10 mL volume of the freshly prepared cold performic acid was added to 100 mg of the test samples and incubated at 0-5 °C overnight. Following incubation, 1 mL hydrobromic acid (HBr) (Sigma-Aldrich) was added to quench the oxidation reaction, and samples were kept in an ice bath for 30 min. Thereafter, samples were allowed to equilibrate to room temperature and incubated on an unheated Reacti-Therm™ module for 1 h under a stream of nitrogen.

The temperature was increased to 65 ºC and samples were left to dry overnight. A 10 mL volume of 6 M HCl was added to the dried samples, which were hydrolysed for 4 h at 145 ºC. Thereafter, hydrolysate samples were decanted into 50 mL Falcon tubes, and 1 mL was aliquoted into 1 L amber Boston round bottles. Hydrolysates were diluted with Milli-Q water using optimised dilution values to obtain on-column amino acid concentrations that fell within the linear range of the liquid chromatography instrument.

4.2.2.2. Tryptophan hydrolysis

For tryptophan hydrolysis, 100 mg of each test material (n = 3) was added to clean and labelled hydrolysis tubes. A 50 mg mass of ascorbic acid (Click's pharmacy brand of ground 1000 mg Vitamin C tablets) was added to the samples to prevent oxidative degradation of tryptophan. A 5 mL volume of 4.2 M NaOH (Sigma-Aldrich) was added, followed by 50 µL 1-octanol (Sigma-Aldrich) to prevent foaming. Samples were purged with nitrogen for 3 min and evacuated for 1 min. The mixture was vortexed for 2 min and incubated at -20 ºC for 2 h. Samples were equilibrated to room temperature before hydrolysis at 110 ºC for 22 h in a fume hood. Thereafter, hydrolysate samples were decanted into 50 mL Falcon tubes, and 1 mL (2 mL for cassava) was aliquoted into 1 L amber Boston round bottles. The hydrolysates were diluted with Milli-Q water using optimised dilution values to obtain on-column amino acid concentrations that fell within the linear range of the liquid chromatography instrument.

4.2.3. Preparation of the calibration series

4.2.3.1. Calibration series for acid-stable amino acids

Calibration blends contain known concentrations of the amino acids under study. They were gravimetrically prepared to plot a calibration standard curve for use in determining the unknown concentration of amino acids in test samples. The calibrator used in this work was a TraceCert® certified reference material (Sigma-Aldrich). The TraceCert® standard is a high-purity composite solution produced in compliance with ISO 17034 and ISO 17025 requirements. It also has an established traceability chain to NIST reference standards, ensuring reliable calibration of the analytical method. To prepare calibration blends, a weighed aliquot was obtained from the TraceCert® standard and diluted in 0.1 M HCl to prepare a stock solution with a final concentration of 8000 ng/g. The ng/g concentration unit is the standard convention of reporting concentration values as a mass fraction (g/g) instead of volume (g/mL). From this 8000 ng/g stock solution, ten working standards were prepared in 0.1 M HCl, and they were evenly distributed between 100 ng/g and 3000 ng/g.

4.2.3.2. Calibration series for methionine sulfone, cysteic acid, and tryptophan

To prepare the calibration blends, 6 mg was weighed from individual tryptophan, methionine sulfone and cysteic acid standards (Sigma-Aldrich) to make a final concentration of 200 µg/g of each standard in 0.1 M HCl. A composite stock solution was prepared from the three standards to a final concentration of 8000 ng/g in 0.1 M HCl for each amino acid. A 10-point calibration series was prepared in 0.1 M HCl from the composite stock solution and evenly distributed between 100 ng/g and 3000 ng/g.

4.2.4. Amino acid derivatization

Amino acids were derivatised to produce highly stable urea derivatizes, and to impart UV properties on the analytes to facilitate detection by the PDA detector. A 500 µL volume of the diluted test samples, 100 µL of the acid-stable amino acid calibration series, and 300 µL of the methionine, cysteine and tryptophan calibration series was transferred into clean and labelled 1.5 mL amber vials. The caps were removed, and vials were dried in a Genevac EZ-2 Plus centrifugal evaporator (Genevac, Ipswich, United Kingdom) using the “aqueous” settings pre-programmed by the manufacturer. Dried vials were removed from the evaporator, and amino acid samples were derivatized using an AccQ·Tag™ Ultra Derivatization kit (Waters). The kit contains a vial labelled borate buffer, and a vial labelled 2A containing the AQC derivatization reagent in dried powder form to ensure reagent stability before use. The kit also contains a vial labelled 2B which contains acetonitrile to reconstitute the dried derivatization reagent. To reconstitute the reagent, 1 mL acetonitrile from solution 2B was injected into the dried derivatizing powder and the solution was vortexed for 10 sec before incubation on a pre-heated Reacti-Therm™ at 55 ºC for ≤ 10 min and allowed to cool before use.

A 10 µL volume of 0.1 M HCl was added to the dried samples in amber vials and thoroughly vortexed to reconstitute the dried down amino acids. This was followed by the addition of 70 µL borate buffer to maintain optimum pH for derivatization, and lastly 20 µL of the freshly prepared acetonitrile/AQC derivatization solution was added. The mixture was vortexed for 10 sec and incubated at 55 ºC for ≤ 10 min to facilitate the derivatization reaction. After incubation, samples were cooled and 100 µL of the derivatized amino acids was transferred into 250 µL glass inserts which were placed back into corresponding amber vials for UHPLC analysis. Samples were transferred into glass inserts to allow for consistent sampling of small sample volumes (1 µL) by the UHPLC sampling needle.

4.2.5. UHPLC-UV analysis and amino acid quantification

Following hydrolysis and amino acid derivatization, test samples and calibration standards were analysed on a binary pump UHPLC-UV system from Waters. The instrument was fitted with a reversed-phase 2.1 × 100 mm, C_{18}, 1.7 µm AccQ·Tag™ Ultra column, and coupled to a PDA detector. Before data acquisition, the instrument was primed with 100% Milli-Q water and 100% acetonitrile for 3 min. The seal wash was primed with 80:20 Milli-Q water and methanol for at least 1 min. The needle and syringe were also primed before ramping up the flow rate to 0.7 mL/min of Eluent A (95:5 Milli-Q/Eluent A concentrate) and Eluent B in a 99.9:0.01 ratio. Eluent A concentrate and Eluent B were provided in the AccQ·Tag™ Ultra kit, and they served as the aqueous and organic mobile phases, respectively. Amino acid analysis was carried out under the conditions outlined in Tables 4.2 – 4.4. Following data acquisition, QuanLynx™ software (Waters) was used to integrate the area under the peaks of the chromatograms. The peak area data was transferred to an MS Excel spreadsheet for further processing to quantify the amino acid composition of staple foods.

Table 4.2. PDA detector settings for the AccQ·Tag™ analysis protocol.

Parameter	Value
3D Data	Not Enabled
Resolution	4.8 nm
Sampling Rate	10 points/sec
Time Constant	0.200 sec
Exposure time	Auto (msec)
2D Channels – Data Mode	Absorbance
2D Channels – Wavelength	260 nm
2D Channels – Resolution	4.8 nm

Table 4.3. UHPLC system gradient conditions and method flow rates.

Time (min)	Flow (mL/min)	%A	%B	Curve
0.54	0.7	99.9	0.1	6
5.74	0.7	90.9	9.1	7
7.74	0.7	78.8	21.2	6
8.04	0.7	40.4	59.6	6
8.05	0.7	10	90	6
8.64	0.7	10	90	6
9.50	0.7	99.9	0.1	6

Table 4.4. Binary sample manager settings.

Parameter	Value
Loop option	Partial loop with needle overfills
Weak needle wash solvent name	5% acetonitrile
Weak needle wash volume	600 µL
Strong needle wash solvent name	95% acetonitrile
Strong needle wash volume	200 µL
Target column temperature	55 ℃
Target sample temperature	20 ℃
Syringe draw rate (Advanced setting)	30 µL
Needle placement (from bottom) (Advanced setting)	4.00 mm
Needle overfill flush	3.0 µL

4.2.6. Statistical assessment

Amino acid concentrations are presented as the mean ± standard deviation. Repeatability and reproducibility were tested using $\%RSD_r$ and $\%RSD_R$, respectively. MS Excel was used to perform student's t-tests, single-factor ANOVA, and linear regression analyses to test for significance. Statistical significance was determined at $p < 0.05$ at a 95% level of confidence.

4.3. Results

4.3.1. Method development for the quantification of acid-stable amino acids

The method described here is based on optimization of the NMISA SOP ORG-MTD-0043: Amino acids in dried food matrices, that was derived from the Association of Official Analytical Chemists (AOAC) Official Method 2012.08 and Official Method 982.30. Successful method optimization was dependent on amino acid recovery, representing accuracy, between 80-120% in a chosen QC sample as previously outlined (Green, 1996), which is still regarded as being representative of high accuracy (Huang et al., 2018; Chen et al., 2021). The QC material for the current work was a pasta sample from the *Bureau Interprofessionnel d'Etudes Analytiques* (BIPEA). Therefore, experimental amino acid quantities in pasta would be within 80-120% of the true value for the analysis results to be accepted as being accurate.

To determine amino acid concentrations, pasta and staple food samples were initially hydrolysed at 110 ºC for 24 h according to the SOP. The resulting hydrolysates and TraceCert® calibration blends were derivatised and thereafter analysed by liquid chromatography to obtain amino acid chromatograms. Illustrated in Figure 4.3 is a chromatogram of the highest concentrated calibration blend (3000 ng/g) in the amino acid calibration series.

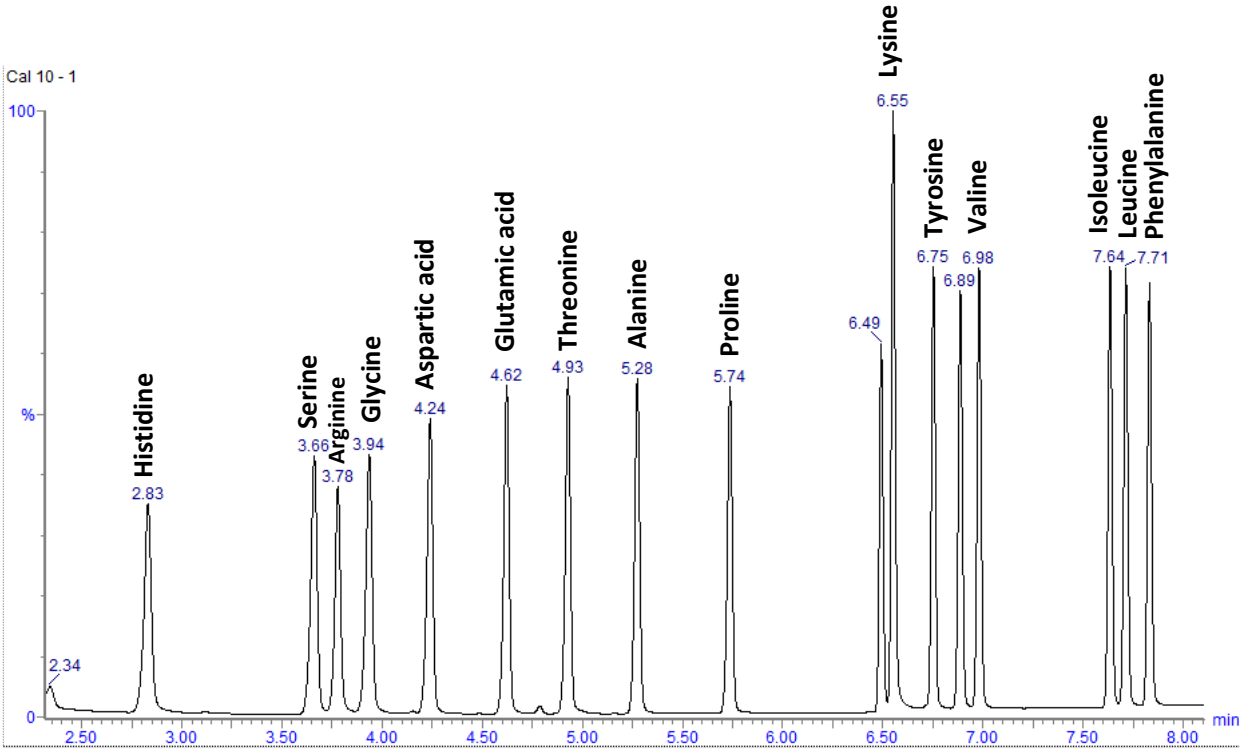


Figure 4.3. A chromatogram showing retention times for 15 acid-stable amino acids in a TraceCert® calibration blend prepared in 0.1 M HCl (concentration: 3000 ng/g). The gravimetrically prepared calibration standards were derivatized using the AccQ·Tag™ Ultra Derivatization kit and amino acids were separated by gradient elution on a UHPLC-UV platform.

Amino acid chromatograms provided by UHPLC-UV analysis (such as the one shown in Figure 4.3) were processed using QuanLynx™ software to integrate the area under the peak – this simply means the total peak area was calculated by the software. Peak area data was transferred to an MS Excel spreadsheet for further processing. Firstly, a 10-point calibration curve was plotted for each amino acid using peak areas of the calibration blends on the y-axis and the gravimetrically prepared concentrations on the x-axis. An example of a histidine standard curve is plotted in Figure 4.4. The calibration curves for all remaining amino acids can be found in Appendix A. The calibration curves had good linearity as indicated by the squared coefficient of variation (r^2). The tolerance for linearity was $r^2 \geq 0.9900$.

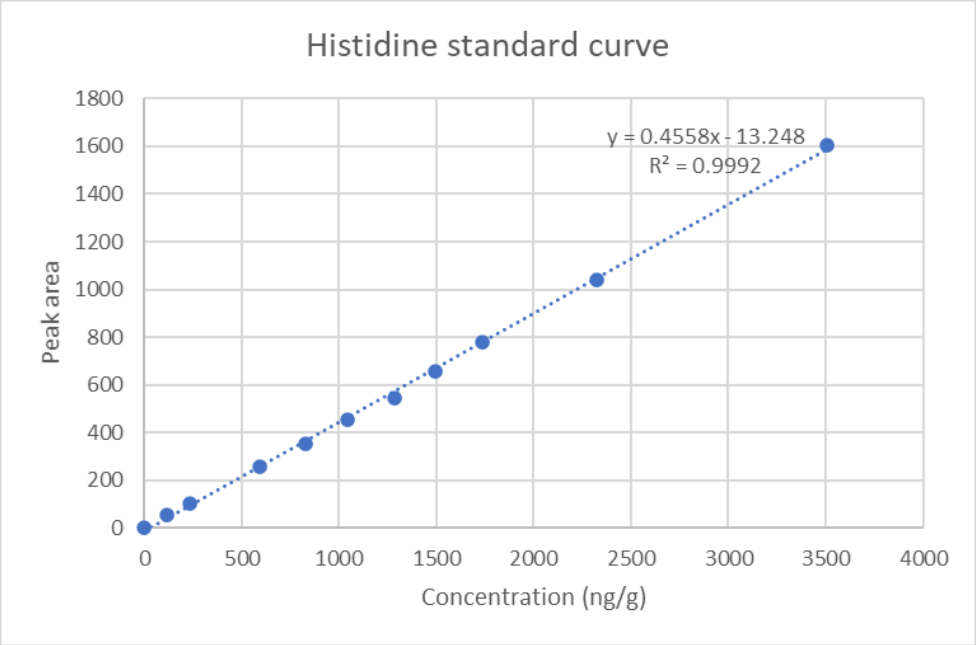


Figure 4.4. A 10-point calibration curve to determine the quantity of histidine in test samples. Calibration curves for the remaining amino acids are plotted in Appendix A. Linear concentration range: 100-3000 ng/g.

The unknown amino acid concentration in pasta and staple food samples was extrapolated from the standard curve using the linear equation shown in Equation 4.1.

$$y = mx + c \qquad \text{Equation 4.1}$$

Where:

$y =$ Peak area of unknown sample

$m =$ Gradient of the slope

$x =$ Unknown amino acid concentration

$c =$ y-intercept

Once the unknown amino acid concentration was calculated, it was multiplied by a dilution factor calculated during sample preparation, to obtain a final amino acid concentration reported in g/100 g as customarily reported on food nutritional labels. Thereafter, amino acid recovery in pasta was calculated to determine method accuracy. To calculate recovery, peak areas were obtained from a chromatogram of the pasta QC (Figure 4.5), and they were applied to calculate amino acid concentrations as described. The concentrations were substituted into the equation for recovery as shown in Equation 4.2.

$$\text{Recovery} = \frac{\text{Experimental [amino acid]}}{\text{Theoretical [amino acid]}} \text{ x } 100\% \qquad \text{Equation 4.2}$$

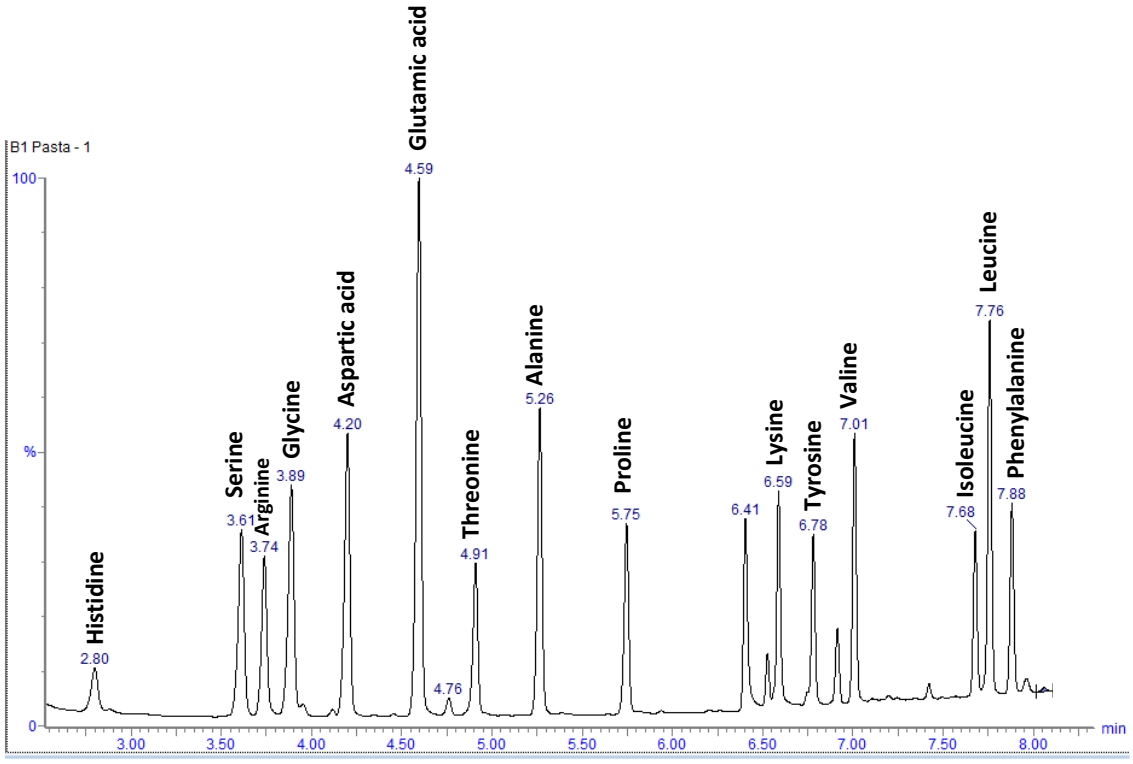


Figure 4.5. A chromatogram for pasta to evaluate the accuracy of amino acid analysis (concentration range: 100-3000 ng/g). The sample was hydrolysed using 6 M HCl at 110 ºC for 24 h and thereafter derivatized on a UHPLC-UV platform.

The amino acid recoveries calculated from analysis of the pasta QC were used for recovery correction of amino acid concentrations in staple food samples. For example, if histidine recovery in pasta was 105%, an assumption is made that histidine in all staple food samples was over-recovered by 5%. Therefore, 5% is subtracted from the calculated amino acid concentrations in staple foods to obtain 100% histidine recovery. The amino acid value that is corrected for recovery is therefore the accepted quantity of an amino acid in a sample.

The SOP (ORG-MTD-0043) was initially interrogated on the material significance of a freezing step, where samples should be incubated at -20 ºC overnight before hydrolysis. Official Method 982.30 recommends rapidly freezing samples in an ethanol/dry-ice bath. Dry ice was not available in the laboratory; therefore, the SOP modified the freezing step to be performed overnight at -20 ºC. The modified freezing step made the method less time efficient, and Official Method 2012.08 does not specify a freezing step. Omitting the freezing step would save on analysis time and also increase the life span of costly hydrolysis tubes against breakage which was regularly observed due to temperature shifts from -20 ºC to 110 ºC. An experiment was therefore designed to determine whether freezing test samples could be omitted while maintaining the integrity of amino acid analysis.

Two sample sets (n = 4) of pasta were prepared with one set subjected to the freezing step and the second set was not frozen. The inclusion of multiple replicates per sample set was to eliminate measurement error by reducing chances of random events, leading to greater confidence in the results. The resulting amino acids were quantified, and a student's t-test showed there was no significant difference ($p > 0.05$) in amino acid concentrations of samples frozen before hydrolysis and samples that were not frozen (Table 4.5). All subsequent experiments were therefore carried out without the freezing step, effectively making the protocol more time efficient.

Table 4.5. Student's t-test results evaluating the impact of freezing pasta samples before hydrolysis compared to when samples were not frozen (n = 4). Where $p > 0.05$, there is no significant difference in amino acid analysis results from the two sample sets.

	Frozen pasta samples		Non-frozen pasta samples			
Amino acids	Concentration (g/100 g)	σ	Concentration (g/100 g)	σ	t-critical (df = 3)	p
Histidine	0.211	0.006	0.264	0.056	3.18	0.15
Serine	0.488	0.021	0.608	0.113	3.18	0.13
Arginine	0.683	0.025	0.857	0.161	3.18	0.12
Glycine	0.410	0.017	0.534	0.114	3.18	0.12
Aspartic acid	0.814	0.047	1.022	0.183	3.18	0.12
Glutamic acid	1.474	0.063	1.842	0.321	3.18	0.11
Threonine	0.315	0.005	0.397	0.071	3.18	0.11
Alanine	0.508	0.021	0.635	0.116	3.18	0.12
Proline	0.420	0.012	0.521	0.095	3.18	0.13
Lysine	0.329	0.010	0.409	0.079	3.18	0.14
Tyrosine	0.397	0.004	0.487	0.084	3.18	0.12
Valine	0.344	0.010	0.439	0.082	3.18	0.11
Isoleucine	0.245	0.009	0.308	0.048	3.18	0.08
Leucine	0.670	0.008	0.840	0.161	3.18	0.13
Phenylalanine	0.427	0.013	0.528	0.086	3.18	0.10

σ = Standard deviation. df = degrees of freedom.

The data from evaluating the freezing step was used to calculate amino acid recovery in pasta as described. The results showed excellent recovery for most of the amino acids, with recovery values falling within the 80-120% tolerance (Figure 4.6). However, the data also revealed valine and isoleucine were poorly recovered with recoveries between 64% and 75% in both sample sets, which is below the lower limit for recovery. Valine and isoleucine have previously been reported to be difficult residues to hydrolyse (Lamp et al., 2018; Mustățea et al., 2019), which shifted the focus for method development towards optimising the analysis method to obtain suitable recovery of these two analytes.

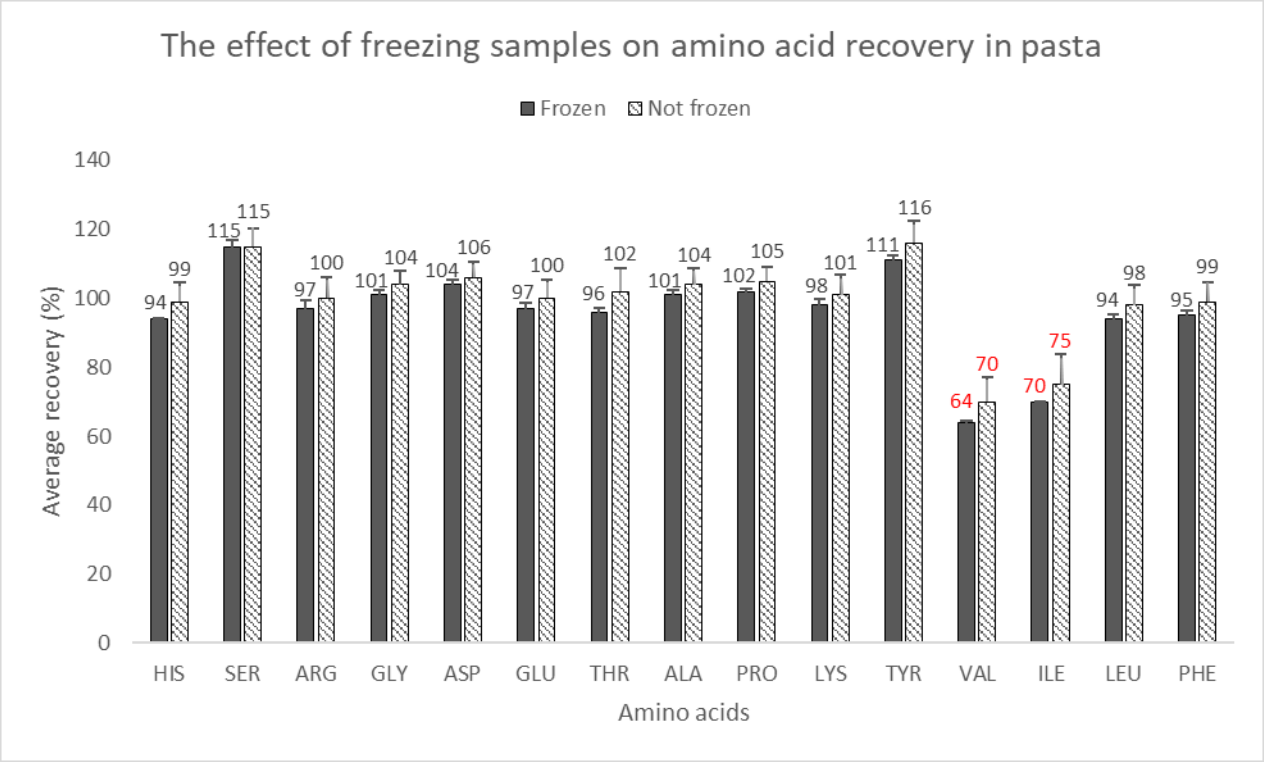


Figure 4.6. Amino acid recovery in pasta hydrolysed using 6 M HCl at 110 ºC for 24 h to test the effect of freezing samples prior to acid hydrolysis. The variation in recovery from samples which were frozen before hydrolysis (black) and samples that were not frozen (striped) was not statistically significant ($p > 0.05$). Valine and isoleucine recoveries in both sample sets was below the limit for recovery (highlighted in red). Error bars represent the standard deviation of independent measurements (n = 4).

To optimise the method to improve valine and isoleucine concentrations, pasta samples were milled on a rotor mill. A working theory was established that hydrolysis of finer sample material would positively influence recovery by increasing the particle surface area exposed to the acid to ensure complete hydrolysis of proteins in the material. Teff seeds were also ground on the rotor mill since they were received as whole seeds. Corn-soya, cassava, maize, and sorghum were sufficiently milled upon receipt. The concentration of valine in pasta was 0.354 g/100 g before milling, and 0.385 g/100 g after the milling process (Figure 4.7). The concentration of isoleucine was 0.261 g/100 g before pasta samples were milled, and 0.268 g/100 g after milling. A t-test evaluation showed the concentration of both valine and isoleucine was not significantly improved ($p > 0.05$) following the milling process, and thus the analysis method required further interrogation to significantly improve the recovery of valine and isoleucine.

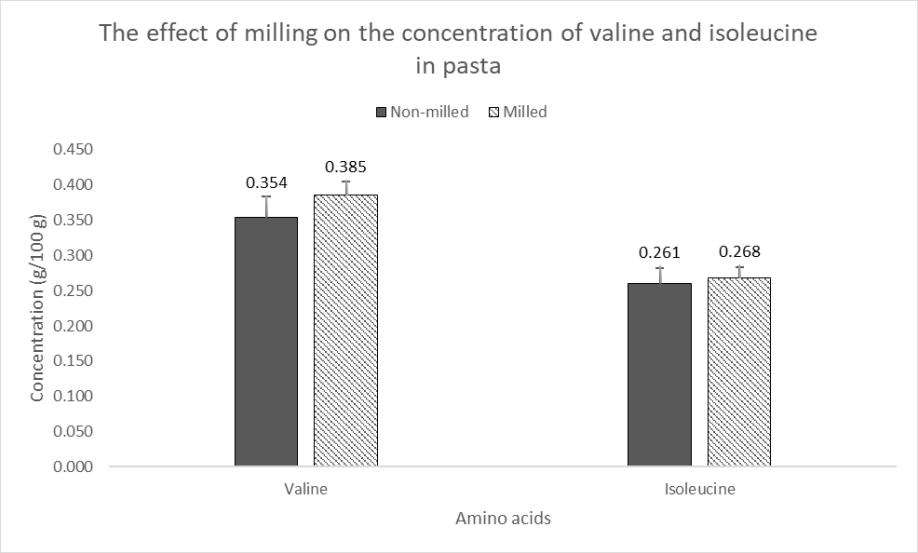


Figure 4.7. The effect of milling on valine and isoleucine recovery in pasta. The concentrations obtained in non-milled samples (black) did not significantly improve ($p > 0.05$) compared to the recovery in milled samples (striped). Error bars represent the standard deviation of independent measurements ($n = 4$).

Teff seeds were also milled to determine the effect of analysing finely ground sample material on the concentration of valine and isoleucine. Following hydrolysis and UHPLC analysis, amino acids were quantified, and the results are shown in Figure 4.8. Whole teff seeds had a valine concentration of 0.302 g/100 g, and the concentration was 0.425 g/100 g in milled seeds. Isoleucine in whole seeds showed a concentration of 0.213 g/100 g, and the content in milled seeds was 0.311 g/100 g. Milling teff seeds significantly improved the concentration of valine and isoleucine (t-test, $p < 0.05$) and thus further analyses on teff were conducted with milled samples to improve accuracy.

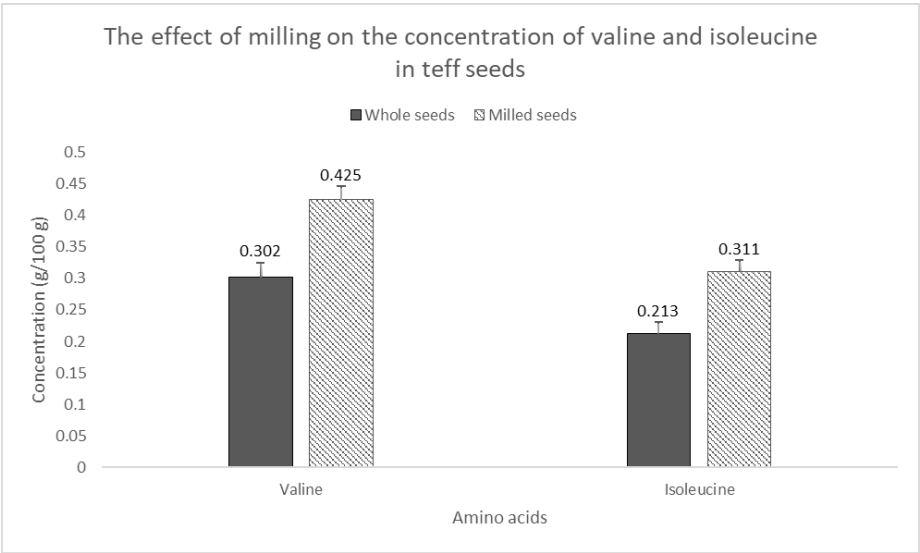


Figure 4.8. The effect of milling on the concentration of valine and isoleucine in whole teff seeds (black) and milled seeds (striped). The concentration significantly increased ($p < 0.05$) following sample milling. Error bars represent the standard deviation of independent measurements ($n = 4$).

Milling pasta samples was ineffective in improving valine and isoleucine concentrations, which necessitated an interrogation of other parameters to obtain good recoveries. Increasing the time or temperature applied for hydrolysis are alternative techniques to improve amino acid concentrations (Mustățea et al., 2019; Rivas-Vela et al., 2021). Both these approaches require caution because they can potentially lead to the continuous destruction of amino acids such as serine and threonine (Rowan et al., 1992). This study resolved to increase the time applied for hydrolysis instead of temperature. It was reasoned that an increase in temperature could promote an even higher degree of amino acid degradation compared to an increase in the time applied for hydrolysis at a relatively low temperature. All previous test results were obtained following a hydrolysis time of 24 h. An increased hydrolysis time of 30 h was tested, and the results are shown in Figure 4.9. Valine recovery was 73% after hydrolysis for 24 h, and 77% after 30 h. The average recovery for isoleucine was 74% after a 24-h hydrolysis period and 79% after 30 h. A student's t-test showed the increase in recovery for both amino acids was not statistically significant ($p > 0.05$), and thus further method optimization was necessary.

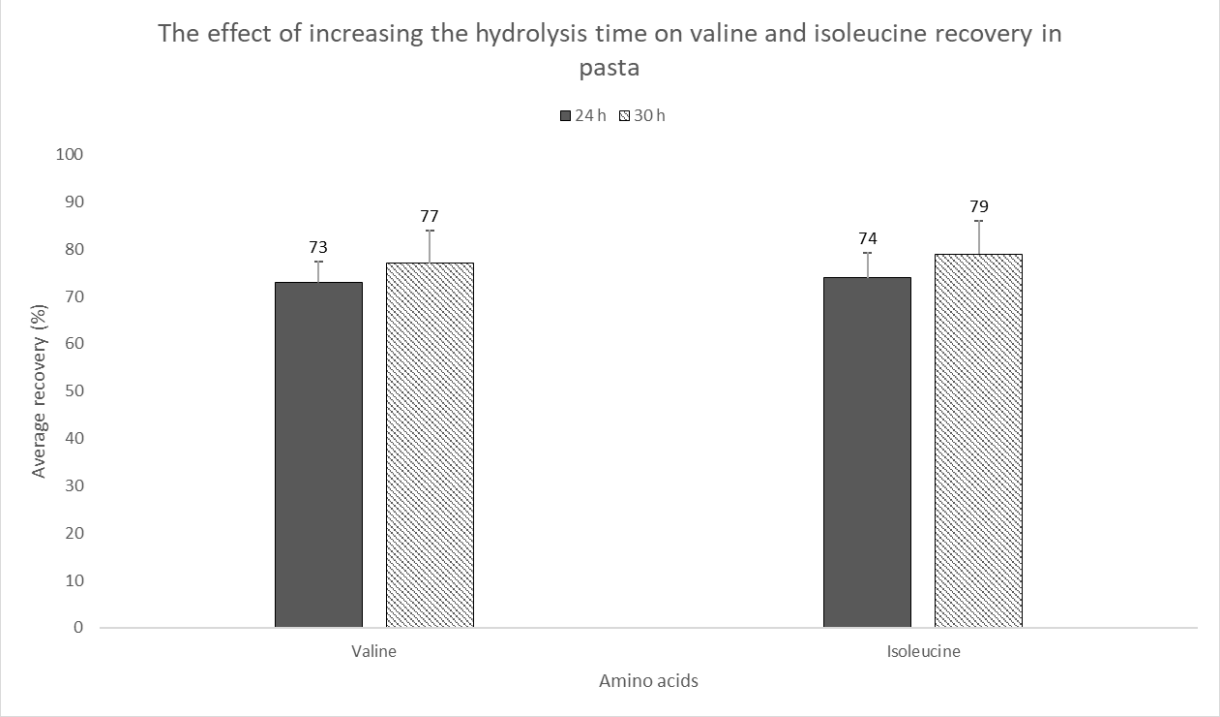


Figure 4.9. The effect of increasing the hydrolysis time to 30 h on valine and isoleucine recovery in pasta. Recovery in samples hydrolysed with 6 M HCl at 110 ºC for 24 h (black) was not significantly different ($p > 0.05$) from recovery in a 30-h hydrolysis (striped). Error bars represent the standard deviation of independent measurements ($n = 2$).

Although increasing the time for hydrolysis to 30 h did not significantly improve valine and isoleucine recovery, the marginal increase prompted further investigation into whether an even longer hydrolysis time may be effective in improving recovery. Extended hydrolysis times of 48 h and 72 h were therefore tested. Following hydrolysis for 48 h, valine had an average recovery of 87% and the recovery for isoleucine was 90% (Figure 4.10). After the 72-h hydrolysis, the recovery of valine was 93%, and isoleucine recovery was 96%. A student's t-test showed that valine and isoleucine recovery at both hydrolysis times significantly improved ($p < 0.05$) compared to recovery under the conventional 24 h hydrolysis time. The variation in recovery between the 48 h and 72 h hydrolysis times was not statistically significant ($p > 0.05$).

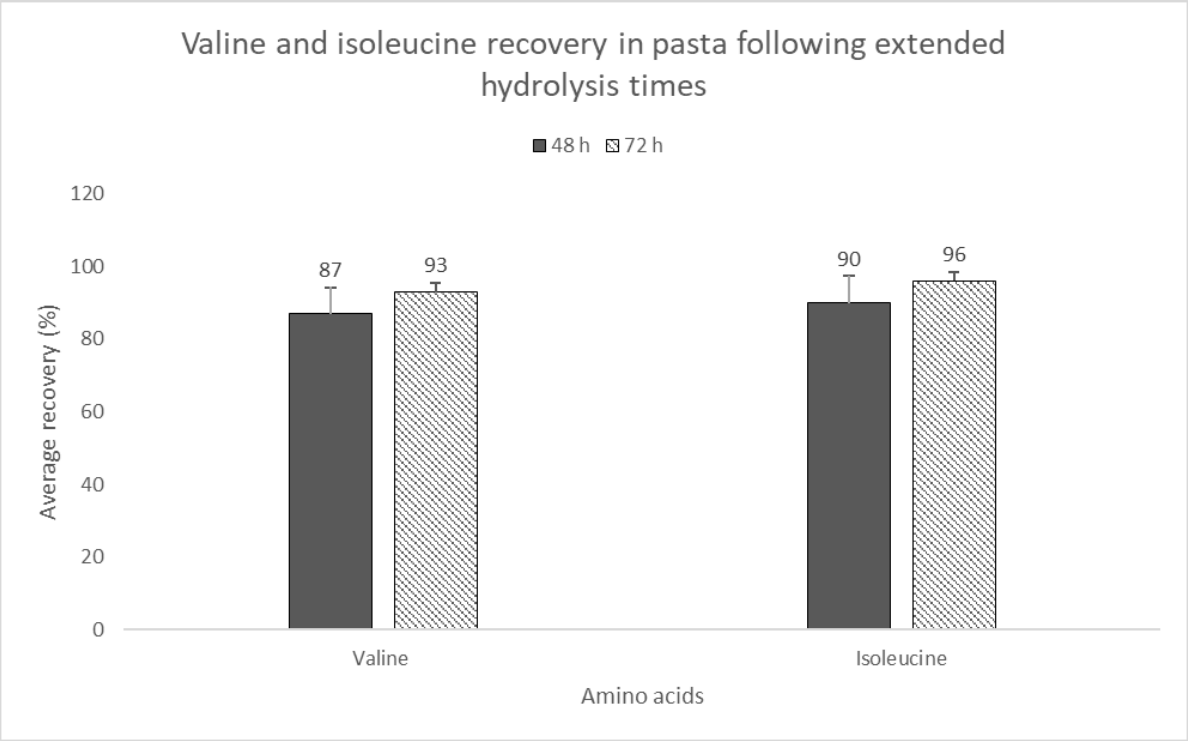


Figure 4.10. The effect of increasing the hydrolysis time to 48 h and 72 h on valine and isoleucine recovery in pasta. Recoveries in samples hydrolysed at 110 ºC for 48 h (black) and 72 h (striped) fell within the 80-120% tolerance. Error bars represent the standard deviation of independent measurements (n = 4).

Prolonged hydrolysis times were expected to lead to significant losses of some amino acids such as serine and threonine as previously reported (Rowan et al., 1992). A t-test appraisal of the results showed the two amino acids were not significantly degraded ($p > 0.05$) when hydrolysed for an extended period. The increased hydrolysis time was therefore suitable to hydrolyse all 15 acid-stable amino acids. Figure 4.11 shows the combined average recovery for the full complement of acid-stable amino acids following the prolonged hydrolysis time. Results from the 48 h and 72 h hydrolysis times were averaged together because they were not significantly different. Amino acid recovery in pasta ranged from 87% for valine to 117% for tyrosine, which fell within parameters of the 80-120% recovery tolerance.

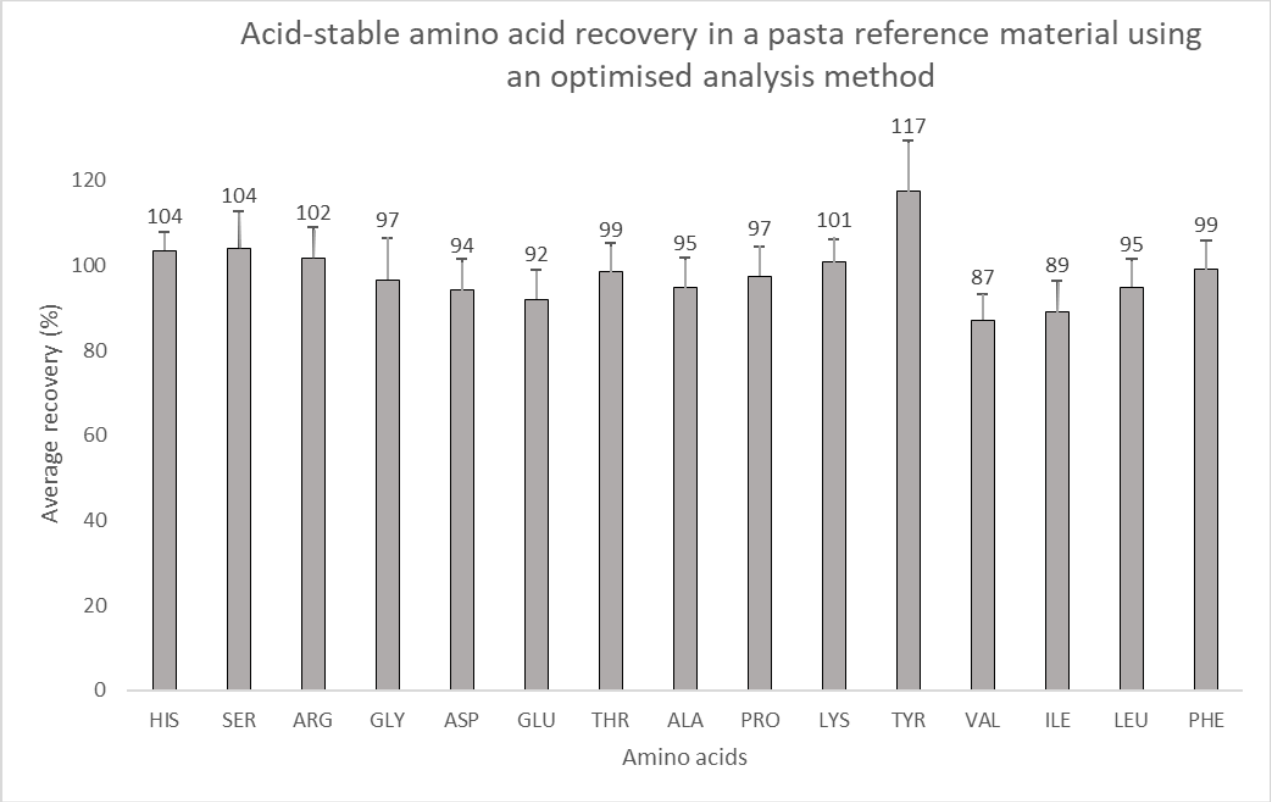


Figure 4.11. The average recovery of 15 acid-stable amino acids in a pasta reference material following acid hydrolysis at 110 ºC for 48 h and 72 h. Recovery values from the two hydrolysis times were averaged together and recovery fell within the 80-120% range for all the analytes. Error bars represent the standard deviation of independent measurements (n = 12).

The presence of sulphur-containing amino acids provides a high degree of stability to the protein structure by forming disulphide bonds, potentially reducing the efficiency of acid hydrolysis (Liu et al., 2016). This part of the work therefore investigated whether staple food samples contained a high content of disulphide bonds which would be evident through a significant increase in amino acid yield at increasingly longer hydrolysis times. Staple food samples were hydrolysed at three hydrolysis times: 24 h, 30 h, and 72 h. The non-recovery corrected amino acid concentrations were summed to obtain total amino acid concentrations at each hydrolysis time and the results are compared in Figure 4.12. The sum of amino acids in all test samples was not significantly different under all three hydrolysis times (single-factor ANOVA, $p > 0.05$). Therefore, the presence of disulphide bonds, if any, did not have a significant impact on the efficiency of acid hydrolysis.

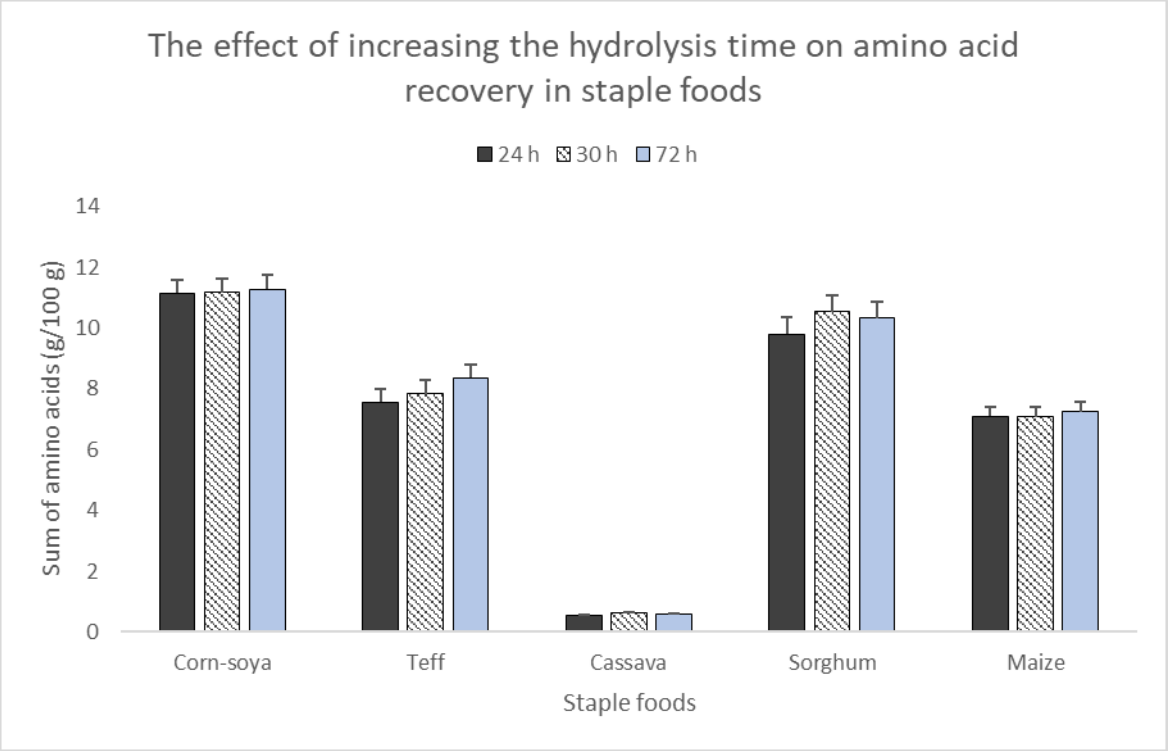


Figure 4.12. Amino acid concentrations in five staple foods hydrolysed with 6 M HCl at 110 ºC for 24 h (black), 30 h (striped), and 72 h (blue) to determine the impact of disulphide bonds on acid hydrolysis. Amino acid concentrations did not change significantly ($p > 0.05$) at progressively longer hydrolysis times, indicating disulphide bonds, if any, did not have a significant impact on the hydrolysis efficiency. Error bars represent the standard deviation of independent measurements (n = 2).

4.3.2. Validation of the optimised acid-stable amino acid analysis method

The method developed to quantify acid-stable amino acids was validated through analysis of a pasta reference material from BIPEA. The validation criteria shown in Table 4.6 were based on method performance parameters as recommended by the Eurachem Guide (Magnusson and Örnemark, 2014). Method validation was conducted to provide confirmation that the optimized method satisfied requirements for use in amino acid analysis.

Table 4.6. Parameters evaluated for validating the optimised method based on analysis of a pasta reference material from BIPEA (Magnusson and Örnemark, 2014).

Parameter	Description
Confirmation of identity	%RSD ≤ 5% between samples and calibrant retention times
Accuracy	80-120% amino acid recovery
Repeatability (%RSD_r)	%RSD ≤15% for measurement results obtained on a single day
Reproducibility (%RSD_R)	%RSD ≤15% for measurement results obtained on different days

4.3.2.1. Confirmation of identity

Confirmation of identities for the 15 acid-stable amino acids was carried out by comparing amino acid elution profiles of a pasta chromatogram obtained in this study (Figure 4.13: A), with a reference chromatogram presented in the AccQ·Tag™ Ultra manual supplied with the derivatization kit (Figure 4.13: B). The two chromatograms illustrate all amino acids share similar elution profiles on both chromatograms with histidine eluting first and phenylalanine eluting last.

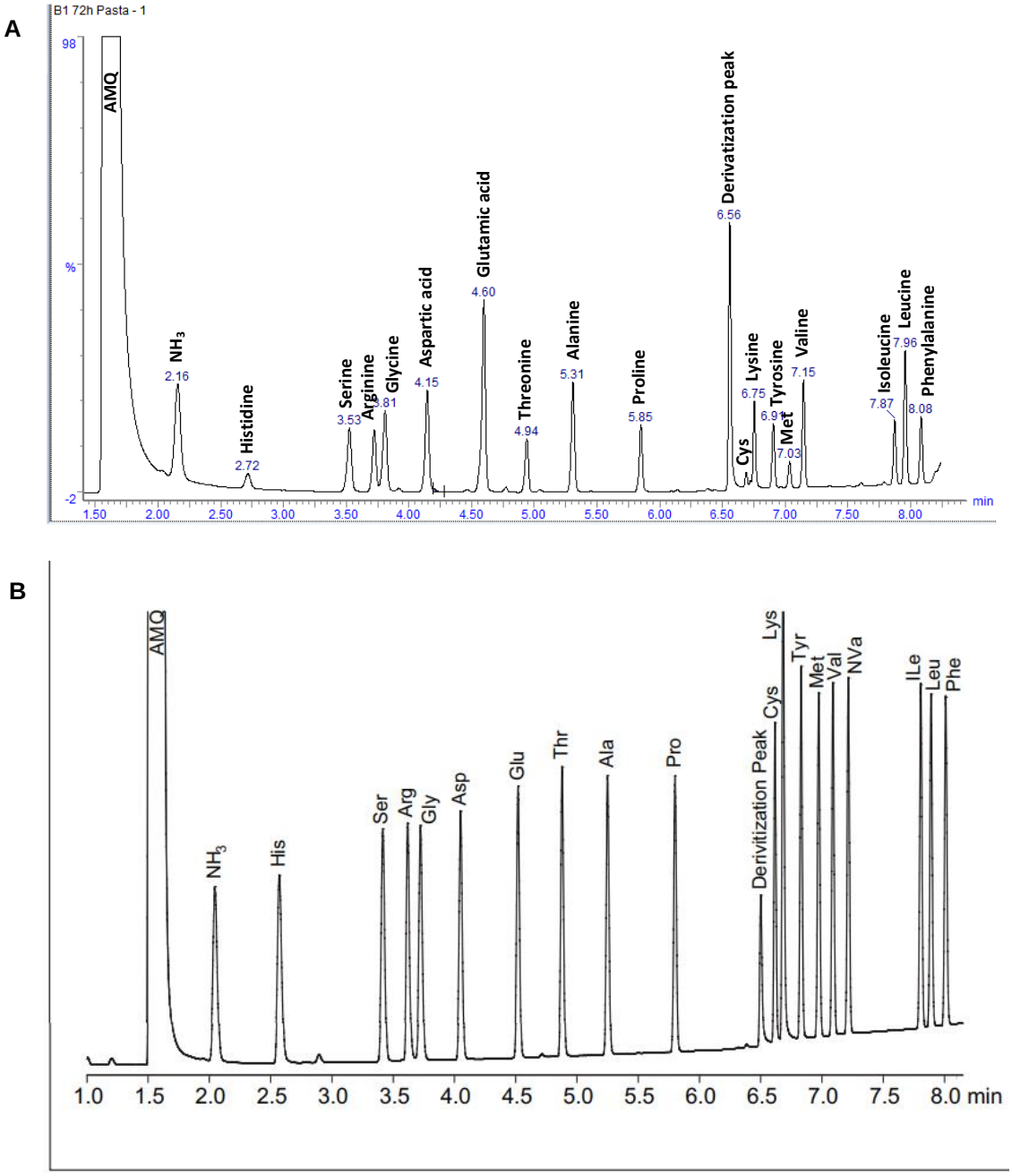


Figure 4.13. Confirmation of amino acid identity by comparison of elution profiles for (A) a chromatogram of a pasta QC sample obtained in this study and (B) a representative chromatogram supplied with the AccQ·Tag™ Ultra kit manual. The amino acid elution profiles match when inspected visually.

In addition to inspecting the chromatograms visually, amino acid identities were confirmed by comparison of the retention time %RSD of the TraceCERT™ calibrator, pasta QC, and the five staple food samples. %RSD ≤ 5% in retention times between all samples and the calibrator served as the parameter to confirm amino acid identity. Retention times are shown in Table 4.7 and all %RSD values were below 5% which further confirmed identity of the analytes.

Table 4.7. Retention time %RSD to demonstrate selectivity and specificity of the optimised amino acid analysis method (n = 2, n = 6 for pasta).

	Retention time (min)							
Amino acids	TraceCERT™	Pasta	Corn-soya	Teff	Cassava	Sorghum	Maize	%RSD
Histidine	2.89	2.95	2.82	2.82	2.99	2.80	2.99	2.90
Serine	3.68	3.73	3.73	3.62	3.75	3.61	3.75	1.60
Arginine	3.81	3.85	3.75	3.75	3.88	3.74	3.88	1.65
Glycine	3.95	3.99	3.90	3.90	4.01	3.89	4.02	1.43
Aspartic acid	4.25	4.30	4.21	4.21	4.32	4.20	4.32	1.27
Glutamic acid	4.64	4.68	4.60	4.60	4.70	4.60	4.70	1.04
Threonine	4.94	4.99	4.91	4.91	5.01	4.91	5.01	0.95
Alanine	5.29	5.34	5.26	5.27	5.36	5.26	5.36	0.83
Proline	5.76	5.82	5.74	5.75	5.83	5.75	5.83	0.73
Lysine	6.56	6.64	6.58	6.59	6.65	6.59	6.65	0.56
Tyrosine	6.78	6.83	6.78	6.78	6.84	6.78	6.84	0.47
Valine	7.00	7.06	7.00	7.01	7.06	7.01	7.06	0.41
Isoleucine	7.67	7.73	7.67	7.68	7.73	7.68	7.73	0.37
Leucine	7.75	7.81	7.75	7.76	7.82	7.76	7.82	0.41
Phenylalanine	7.87	7.93	7.87	7.88	7.94	7.88	7.94	0.43

4.3.2.2. Accuracy

To evaluate accuracy of the proposed method, the pasta reference standard was analysed on four different days (n = 3). Amino acid recoveries obtained on the four days were averaged and they are shown in Figure 4.14. Recovery for the 15 acid-stable amino acids fell within the 80-120% tolerance interval. Valine had the lowest recovery with 87% and tyrosine showed the highest recovery with 117%. Therefore, all the amino acids being studied satisfied requirements for accuracy detailed on the method validation criteria.

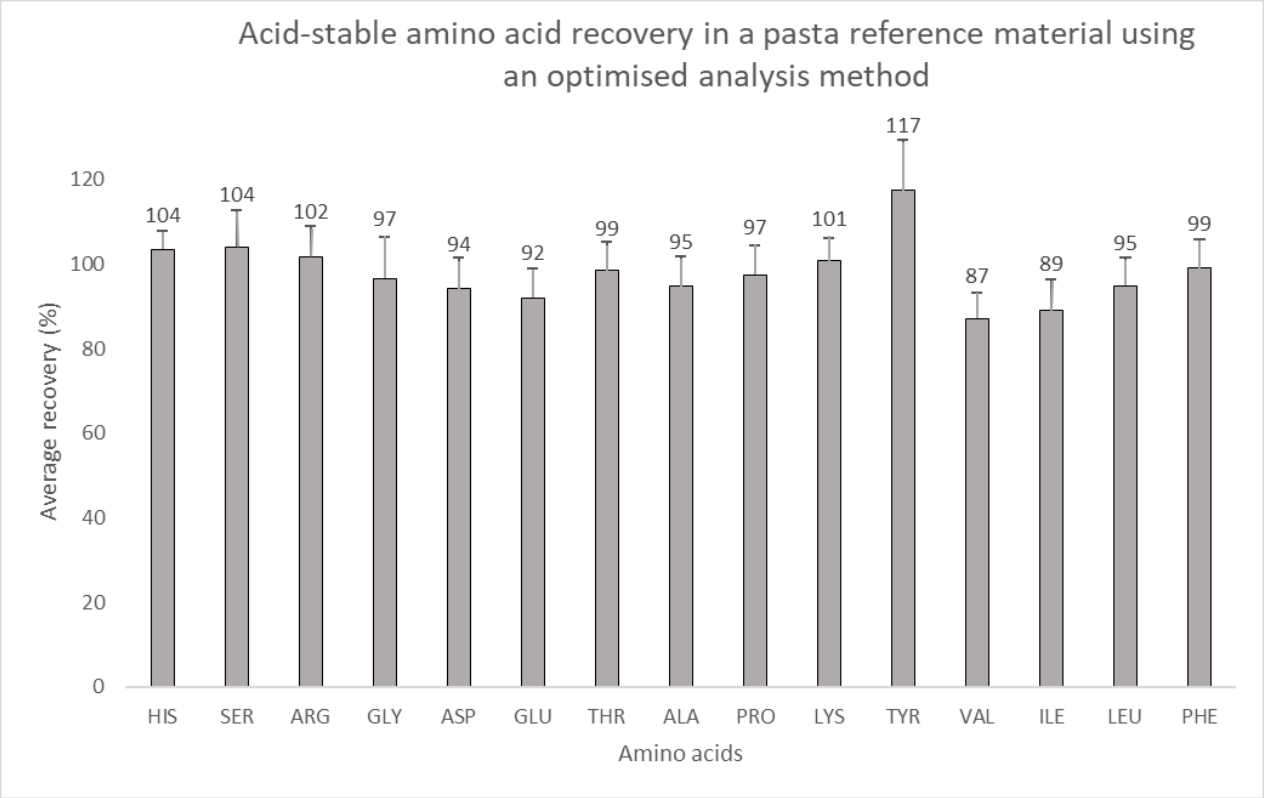


Figure 4.14. The recovery of 15 acid-stable amino acids in pasta analysed on four different days (n = 3). The recovery fell within the 80-120% range for all amino acids. Error bars represent the standard deviation of 12 independent measurements.

4.3.2.3. Repeatability

Repeatability measures the closeness of results under identical experimental conditions. To determine repeatability of the method, three replicates of the pasta sample were analysed under the same conditions on the same day. The method validation criteria outline the parameter for repeatability to be $\%RSD_r \leq 15\%$. Table 4.8 shows the data following analysis of the pasta QC, and all analytes presented $\%RSD_r$ values below 15%, with the highest variation observed in isoleucine with 6.74%. Therefore, all acid-stable amino acids satisfied the criteria for repeatability.

Table 4.8. $\%RSD_r$ from three replicates of a pasta sample to demonstrate repeatability of the method developed in this study.

	Amino acid concentration (g/100 g)					
Amino acids	Sample 1	Sample 2	Sample 3	mean	σ	$\%RSD_r$
Histidine	0.221	0.233	0.233	0.229	0.006	2.55
Serine	0.449	0.447	0.421	0.439	0.013	2.97
Arginine	0.715	0.715	0.724	0.718	0.004	0.60
Glycine	0.366	0.370	0.388	0.374	0.010	2.58
Aspartic acid	0.748	0.761	0.734	0.748	0.011	1.47
Glutamic acid	1.355	1.409	1.385	1.383	0.022	1.58
Threonine	0.322	0.325	0.306	0.318	0.008	2.55
Alanine	0.468	0.477	0.466	0.470	0.005	0.98
Proline	0.398	0.395	0.388	0.394	0.004	1.04
Lysine	0.334	0.356	0.352	0.347	0.010	2.80
Tyrosine	0.369	0.396	0.392	0.386	0.012	3.09
Valine	0.419	0.457	0.478	0.451	0.025	5.45
Isoleucine	0.283	0.307	0.324	0.305	0.017	5.51
Leucine	0.655	0.666	0.653	0.658	0.006	0.85
Phenylalanine	0.436	0.442	0.439	0.439	0.002	0.51

σ = Standard deviation.

4.3.2.4. Reproducibility

Reproducibility refers to repeatability of the method across different days. Three replicates of the pasta sample were analysed on four different days. The method validation criteria outline the parameter for reproducibility to be $\%RSD_R \leq 15\%$. Table 4.9 shows the repeatability results following analysis of the pasta standard and all the analytes presented $\%RSD_R$ values below 15%, with the highest variation observed in glycine with a value of 10.93%. Therefore, all 15 amino acids satisfied requirements for reproducibility.

Table 4.9. %RSD_R from analysis of pasta on four different days to demonstrate reproducibility of the method developed in the current study (n = 3).

Mean amino acid concertation (g/100 g)					
Amino acids	Day 1	Day 2	Day 3	Day 4	%RSD_R
Histidine	0.229	0.233	0.229	0.218	2.81
Serine	0.469	0.496	0.439	0.411	8.08
Arginine	0.741	0.784	0.718	0.679	6.01
Glycine	0.433	0.441	0.374	0.352	10.93
Aspartic acid	0.757	0.843	0.748	0.707	7.47
Glutamic acid	1.482	1.548	1.383	1.312	7.29
Threonine	0.333	0.346	0.318	0.295	6.76
Alanine	0.508	0.528	0.470	0.447	7.51
Proline	0.421	0.438	0.394	0.373	7.08
Lysine	0.327	0.359	0.347	0.330	4.44
Tyrosine	0.443	0.450	0.386	0.376	9.15
Valine	0.491	0.465	0.451	0.435	5.12
Isoleucine	0.348	0.328	0.305	0.296	7.34
Leucine	0.708	0.725	0.658	0.622	6.96
Phenylalanine	0.474	0.482	0.439	0.415	6.96

4.3.3. Method development for the quantification of methionine and cysteine

As a direct consequence of their chemistry, the sulphur-containing amino acids, methionine and cysteine cannot be reliably quantified using the analysis protocol for acid-stable amino acids. This is because the two analytes are unstable in highly concentrated HCl. A suitable method has not yet been established in our laboratory; therefore, various protocols sourced from several research articles were investigated as part of the project. The aim was to optimize and apply these methods for the characterisation of all the relevant amino acids, to enable a holistic review of staple foods with respect to the amino acid content.

To overcome challenges with methionine and cysteine stability in HCl, a preparatory oxidation step is normally performed using performic acid. Methionine was therefore quantified in its oxidised form as methionine sulfone and cysteine as cysteic acid. The initial method for oxidation and hydrolysis was adapted from a Waters application guide (Waters, 2020). Briefly, freshly prepared performic acid was added to test samples which were incubated on a heating block at 50 ºC for 1 h. Performic acid was evaporated under nitrogen at 50 ºC and traditional hydrolysis with 6 M HCl was carried out at 110 ºC for 22 h. Hydrolysed amino acids in test samples and calibration blends were derivatized and analysed by liquid chromatography to obtain amino acid chromatograms. A chromatogram for the highest concentrated calibration

blend (3000 ng/g) in the calibration series is presented in Figure 4.15, and it shows excellent peak separation for cysteic acid and methionine sulfone.

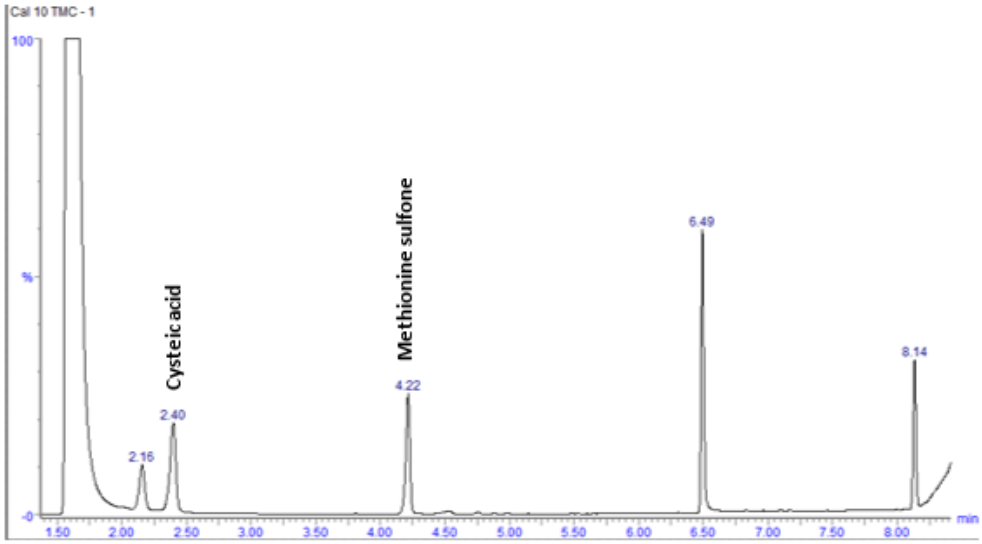


Figure 4.15. A chromatogram for the highest concentrated calibration blend of a cysteic acid and methionine sulfone calibration standard from Sigma-Aldrich prepared in 0.1 M HCl (concentration: 3000 ng/g). The prepared calibration standards were derivatized using the AccQ·Tag™ Ultra Derivatization kit and the amino acids were separated by gradient elution on a UHPLC-UV instrument.

Peak areas obtained from chromatograms of the calibration blends were used to plot calibration standard curves for cysteic acid and methionine sulfone (Figure 4.16). Standard curves for both amino acids had good linearity with an r^2-value that meets the set tolerance for linearity ($r^2 \geq 0.9900$). Amino acid concentrations in test samples were quantified as previously described. Briefly, unknown amino acid concentrations were calculated using the linear equation, and thereafter multiplied by the dilution factor. Experimental amino acid concentrations in the pasta standard were compared to theoretical values to determine recovery of each amino acid under study, thereby evaluating accuracy of the analysis method.

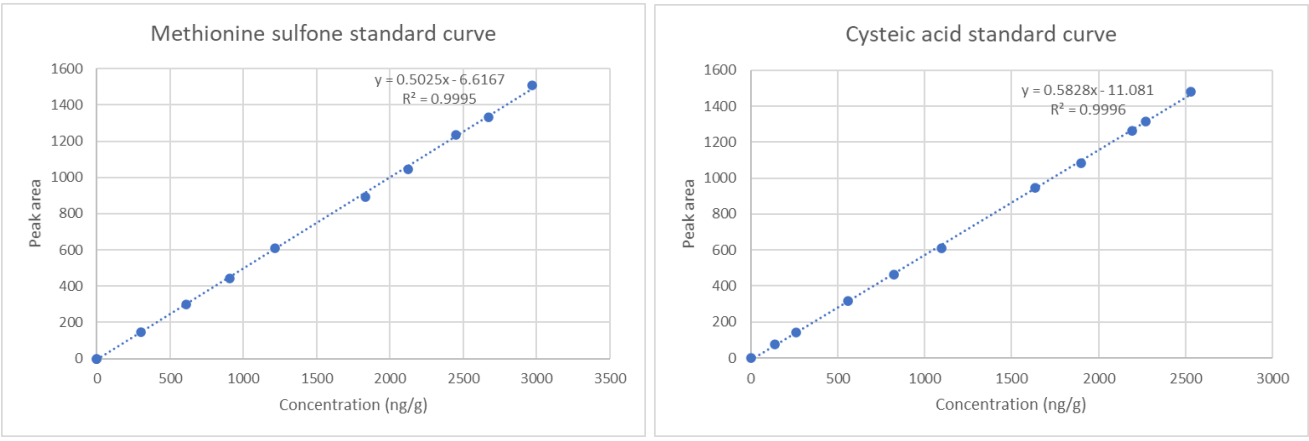


Figure 4.16. Calibration curves for methionine sulfone and cysteic acid used for amino acid quantification (concentration range: 100-3000 ng/g).

To calculate experimental amino acid concentrations in pasta, a chromatogram was first obtained from analysis of a pasta sample by liquid chromatography. Excellent peak separation was obtained for cysteic acid at 2.42 min (Figure 4.17). The peak for methionine sulfone showed coelution with another amino acid suspected to be aspartic acid based on the retention time. Although methionine sulfone showed obvious coelution, peak integration parameters were adequately applied to allow for accurate quantification of the analyte, which will be demonstrated by results of the pasta reference material.

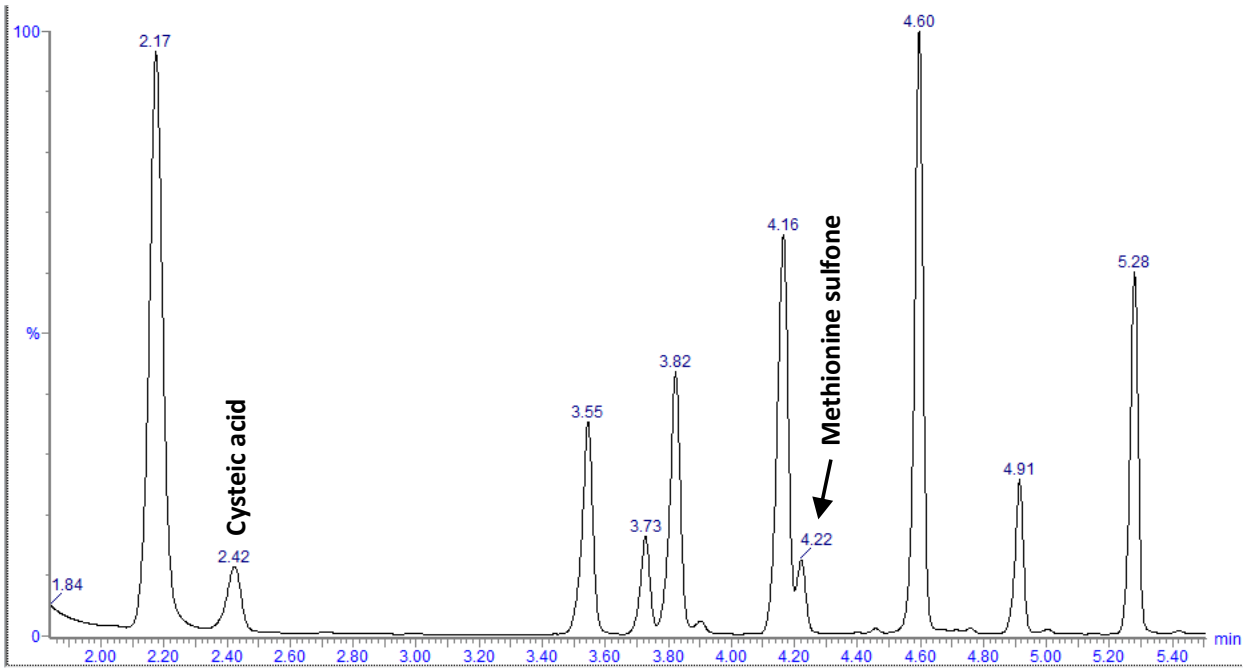


Figure 4.17. A chromatogram for cysteic acid and methionine sulfone in a pasta reference material. The pasta sample was subjected to performic acid oxidation, followed by acid hydrolysis using 6 M HCl at 110 ºC for 22 h. Amino acids were derivatized using the AccQ·Tag™ Ultra Derivatization kit, and thereafter separated by gradient elution using UHPLC. Excellent peak separation was obtained for cysteic acid, and coelution was observed for methionine sulfone. On-column concentration range: 100-3000 ng/g.

Peak areas from the chromatogram were substituted into the linear equation and multiplied by the dilution factor to calculate methionine sulfone and cysteic acid concentrations in pasta. Thereafter, the equation for recovery was applied to determine amino acid recovery. Recovery values from initial implementation of the Waters method are shown in Figure 4.18. The protocol produced good results for methionine sulfone with an average recovery of 108%. However, cysteic acid recovery was 154%, which was higher than the acceptable recovery range for amino acid determination.

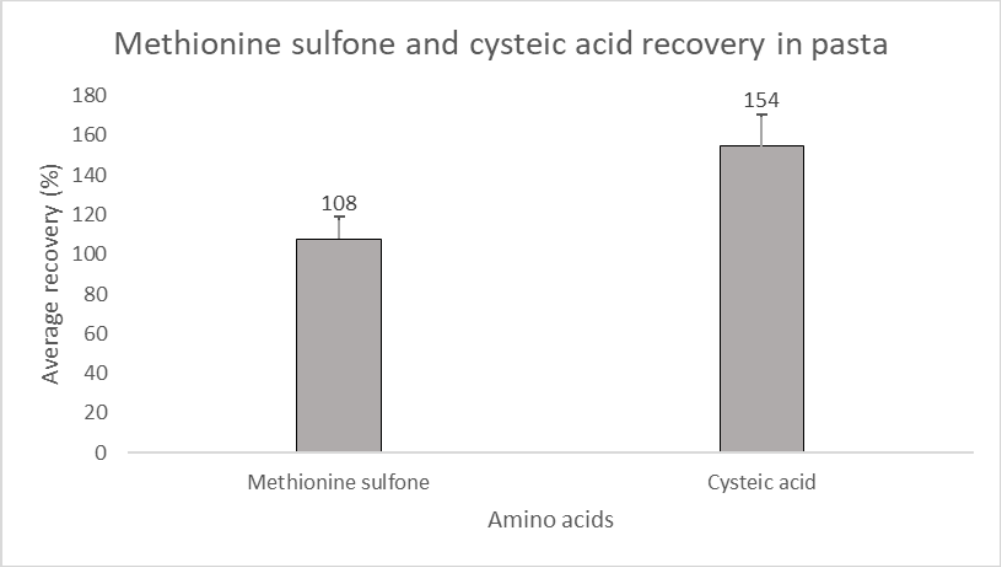


Figure 4.18. The recovery of methionine sulfone and cysteic acid in pasta following performic acid oxidation and acid hydrolysis at 110 ºC for 22 h. Error bars represent the standard deviation of independent measurements (n = 2).

The experiment was repeated with inclusion of a secondary milk powder QC sample to determine whether it was the nature of the pasta material that may have been the contributing factor to the over recovery of cysteic acid. In the repeat analysis, methionine sulfone recovery was 116% in pasta and 90% in milk powder as shown in Figure 4.19. Cysteic acid recovery was 150% in pasta and 141% in milk powder. Results from the two reference materials provided further evidence that the Waters method was suitable for quantifying methionine in its oxidised form, but it was unable to produce acceptable recoveries for cysteic acid.

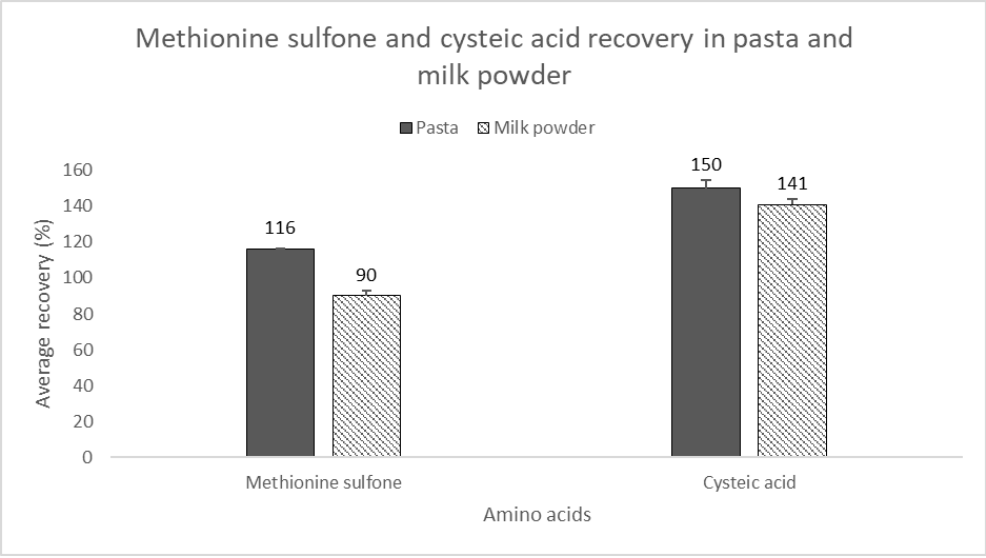


Figure 4.19. The recovery of methionine sulfone and cysteic acid in pasta (black) and milk powder (striped) reference materials following acid hydrolysis at 110 ºC for 22 h. Error bars represent the standard deviation of repeat measurements (n = 2).

Considering that cysteic acid could not be reliably quantified with the Waters method, a different oxidation and hydrolysis procedure was adopted from the AOAC Official Method 985.28. The method describes performic acid oxidation at 0-5 ºC for 16 h, as opposed to 50 ºC for 1 h that was previously applied. The method also recommends adding 3 mL HBr after the oxidation step to quench the oxidation reaction. However, when HBr was added, test samples were digested by the acid such that there was no sample left in the hydrolysis tubes upon visual inspection. The addition of HBr was set aside and instead the hydrolysis conditions were changed from 110 ºC for 24 h, to 145 ºC for 4 h as previously recommended (Gehrke et al., 1987). Test samples were oxidised and hydrolysed using the recommended parameters, and the resulting sulphur-containing amino acids were quantified. The recovery of methionine sulfone in pasta was determined to be 100%, and cysteic acid recovery was 126% (Figure 4.20). The results showed that modifications of the hydrolysis parameters to a shorter hydrolysis time at an increased temperature were suitable for methionine sulfone analysis. However, the recovery of cysteic was outside the 80-120% recovery range, and thus the method required further development.

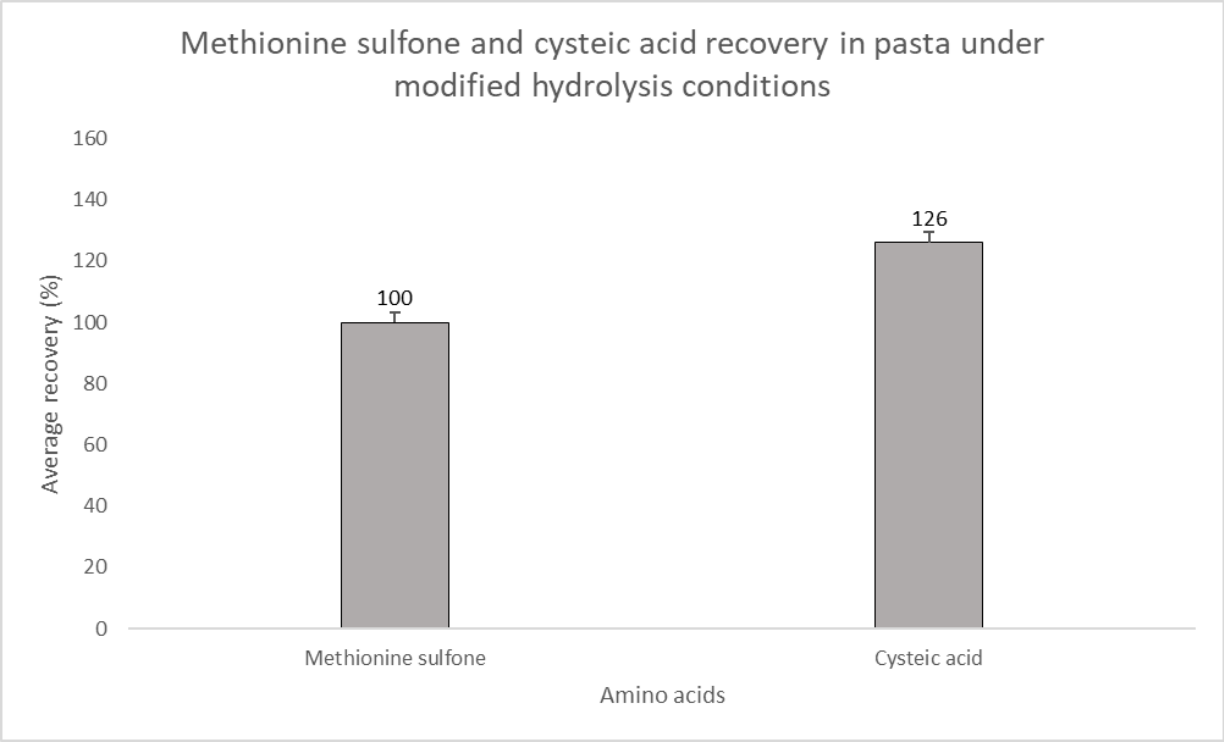


Figure 4.20. The recovery of methionine sulfone and cysteic acid in pasta following performic acid oxidation at 0-5 ºC for 16 h, and hydrolysis with 6 M HCl at 145 ºC for 4 h. Methionine sulfone recovery fell within the 80-120% recovery tolerance, but cysteic acid recovery was higher than the recovery limit. Error bars represent the standard deviation of independent measurements (n = 4).

Modifications to Official Method 985.28 produced excellent results for methionine sulfone in pasta. Cysteic acid results were also improved at 126% compared to the recovery of 150% with the previous method. The subsequent method parameter investigated to further optimise the method was the volume of HBr added following performic acid oxidation. A 3 mL volume was previously added, and it led to undesired sample digestion. The HBr volume was reduced to 1 mL with the expectation that a lesser quantity of the acid will minimise sample digestion. Visual inspection of the hydrolysis tubes showed the decreased volume did not lead to losses. Official Method 985.28 describes evaporating performic acid/HBr at 37 ºC in a vacuum rotary evaporator before acid hydrolysis can be performed. The rotary evaporator available in the laboratory could only evaporate one sample at a time, which would not be time efficient. Instead of using a rotary evaporator, drying was performed at 37 ºC overnight on a Reacti-Therm™ heating block, under a stream of nitrogen. Visual inspection of the hydrolysis tubes showed that although performic acid was sufficiently evaporated following incubation, the drying temperature was unable to adequately evaporate HBr. The temperature was increased from 37 ºC to 65 ºC, and the higher temperature successfully evaporated HBr.

Pasta samples were oxidised using performic acid as described, and 1 mL HBr was added. Following the evaporation of HBr at 65 ºC, acid hydrolysis was performed at 145 ºC for 4 h. The revised method produced a recovery of 102% for methionine sulfone, and a cysteic acid recovery of 110% (Figure 4.21). The data provided evidence that methionine and cysteine can be reliably quantified as their oxidised derivatives using the method developed in this study.

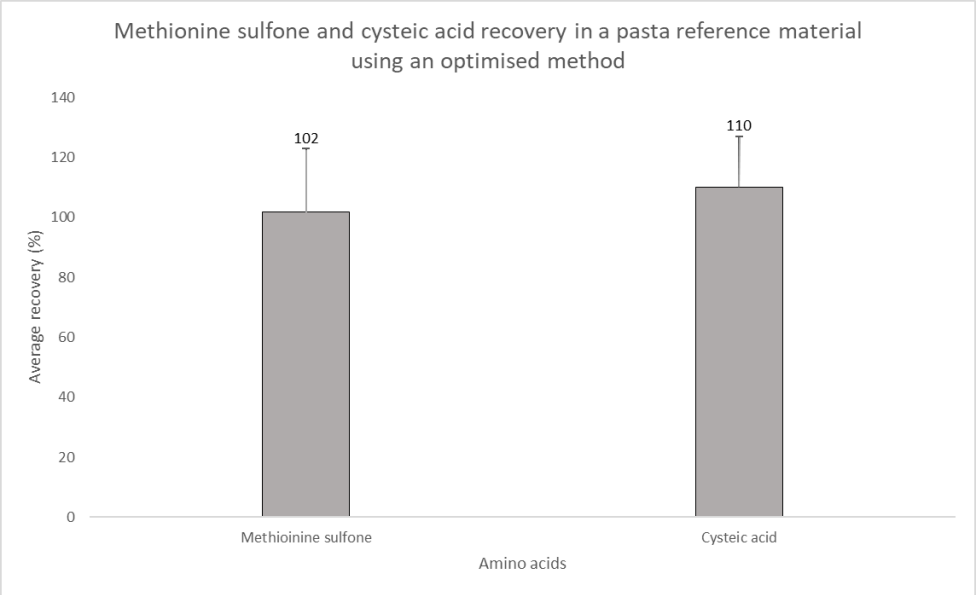


Figure 4.21. The recovery of methionine sulfone and cysteic acid following performic acid oxidation at 0-5 ºC for 16 h, followed by the addition of 1 mL HBr, and lastly hydrolysis with 6 M HCl at 145 ºC for 4 h. Amino acid analysis was performed on three different days (n = 4, n = 2 on day 1). Error bars represent the standard deviation of 10 independent measurements.

The method developed for the quantification of methionine and cysteine was implemented to evaluate the impact of coelution on methionine sulfone recovery. Amino acids have thus far been separated by gradient elution, which means the concentration of the organic mobile phase was increased over the course of a chromatographic run (9.50 min in this study). Two experiments were conducted to possibly resolve coelution and determine whether the variation in recovery was significant. Since methionine sulfone elution occurred between 0.54 min and 5.74 min, the first experiment shortened the elution time to 0.54-5.00 min, meaning the organic mobile phase increased rapidly, and in the second experiment the elution time was prolonged to 0.54-6.30 min, indicating a slower increase of the organic phase. The chromatograms obtained from each analysis are shown in Figure 4.22 (A and B). When the elution time was shortened, the two peaks closely coeluted, with a retention time of 3.93 min for the methionine sulfone peak. When the elution time was lengthened, the retention time increased to 4.43 min, and although coelution was observed, the peak resolution was relatively improved.

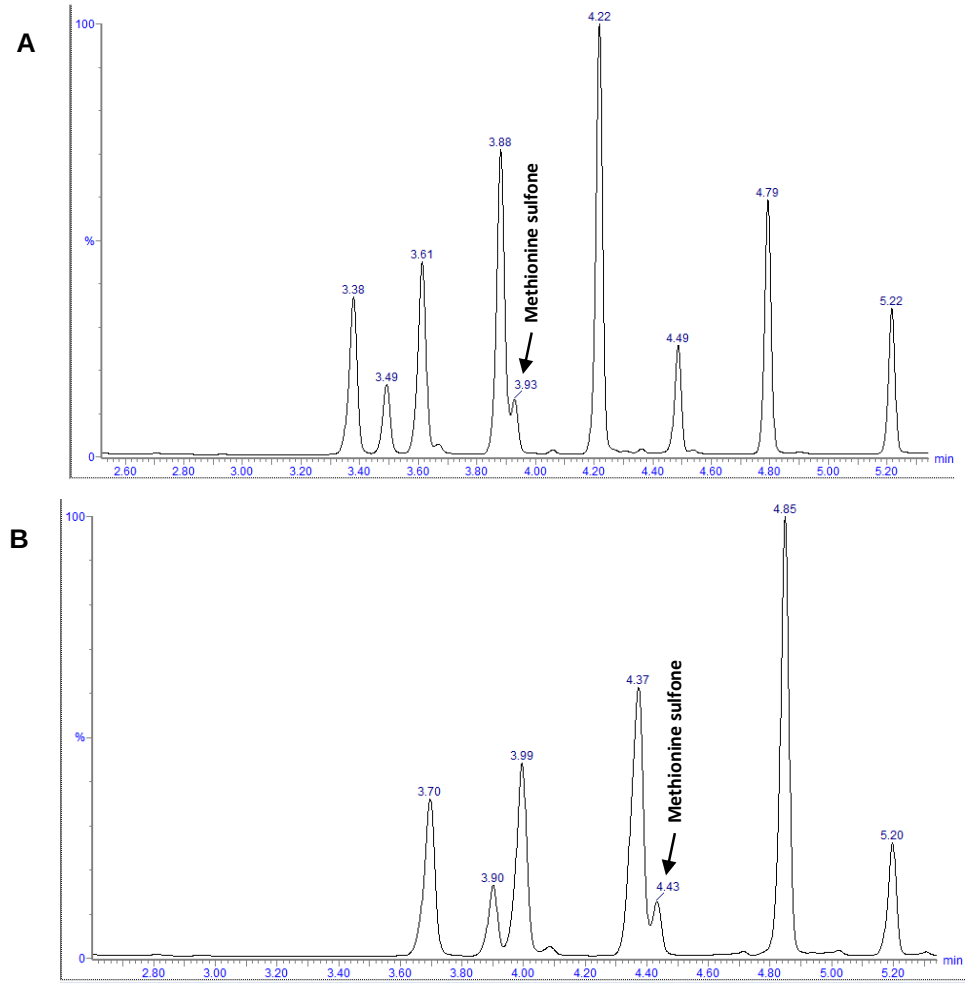


Figure 4.22. Chromatograms obtained from UHPLC-UV analysis of a pasta sample to resolve methionine sulfone coelution. (A) The effect of shortening the elution time on coelution and (B) The effect of prolonging the elution time. A relatively good peak resolution was observed when the elution time was increased. On-column concentration range: 100-3000 ng/g.

An additional parameter was studied to determine whether methionine sulfone coelution could be further resolved. The parameter was the percentage volume of the organic mobile phase used during separation. The liquid chromatography method separated analytes at a flow rate of 0.7 mL/min and 0.1% of the flow rate was the organic mobile phase. The percentage of the organic mobile phase was increased to 1.0%, and although coelution was observed, the peak resolution showed a relative improvement following the increased amount of the mobile phase (Figure 4.23: A and B).

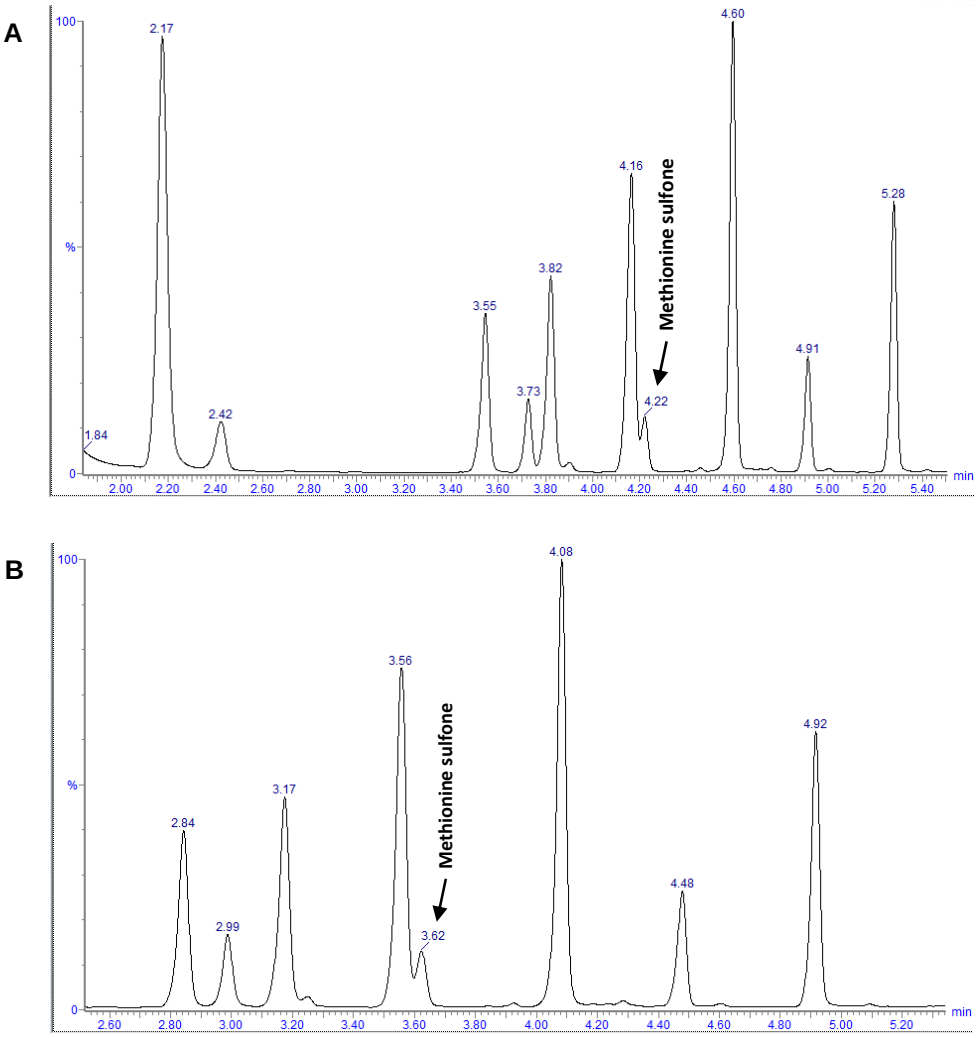


Figure 4.23. Chromatograms obtained from UHPLC-UV analysis of pasta to resolve methionine sulfone coelution at a flow rate of 0.7 mL/min. (A) The chromatogram obtained when the percentage volume of the organic mobile phase was 0.1%. (B) The chromatogram obtained at an increased percentage of 1.0%. A relatively good peak resolution was observed when the amount of the mobile phase was increased. On-column concentration range: 100-3000 ng/g.

The impact of coelution was determined by calculating methionine sulfone recovery under the different chromatography conditions. A recovery of 87% was obtained when the elution time was shortened, and 81% when the elution time was increased (Figure 4.24: A). A paired t-test was conducted on the data set and it indicated the recovery was not significantly different

(p > 0.05). The recovery obtained at the conventional flow rate percentage of 0.1% organic mobile phase was 86%, and the recovery was 82% when the percentage was increased to 1.0% (Figure 4.24: B). The difference in recovery was also not statistically significant (p > 0.05). Attempts to resolve methionine sulfone coelution were therefore unsuccessful. However, since the QC results fell within the 80%-120% recovery tolerance, the results for methionine sulfone were accepted as accurate.

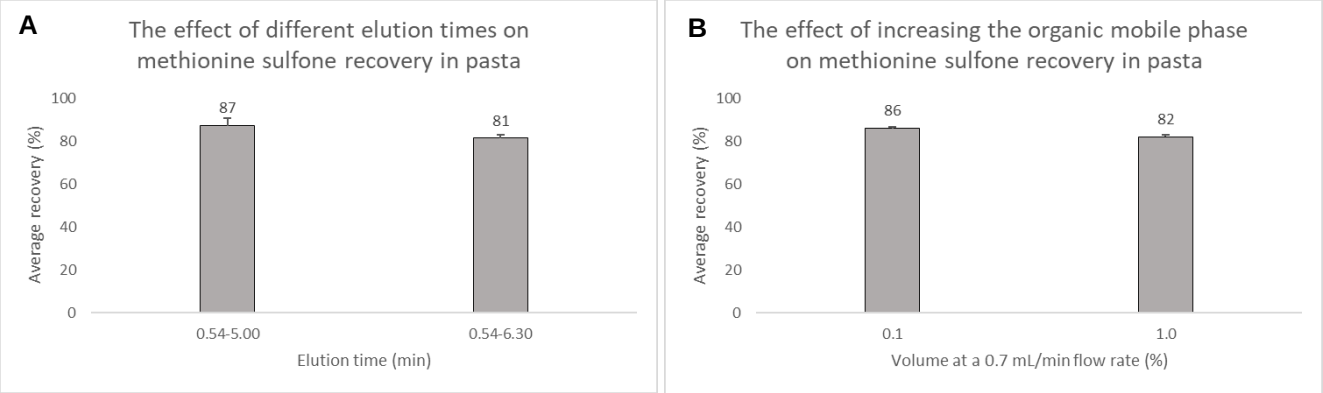


Figure 4.24. The recovery of methionine sulfone under different chromatography conditions. (A) The recovery obtained when the elution time was shortened and prolonged (n = 2). (B) The recovery obtained when the percentage volume of the organic mobile phase was increased (n = 2).

4.3.4. Validation of the optimised method for methionine and cysteine analysis

The method developed for the quantification of methionine sulfone and cysteic acid was validated through analysis of a pasta reference material from BIPEA. The validation criteria shown in Table 4.10 were based on method performance parameters as recommended by the Eurachem Guide (Magnusson and Örnemark, 2014). Method validation was conducted to provide confirmation that the optimized method satisfied requirements for use in amino acid analysis of staple food samples.

Table 4.10. Parameters evaluated for validating the optimised method based on analysis of a pasta reference material from BIPEA (Magnusson and Örnemark, 2014).

Parameter	Description
Confirmation of identity (selectivity)	%RSD ≤ 5% between samples and calibrant retention times
Accuracy	80-120% amino acid recovery
Repeatability (%RSD_r)	%RSD ≤15% for measurement results obtained on a single day
Reproducibility (%RSD_R)	%RSD ≤15% for measurement results obtained on different days

4.3.4.1. Confirmation of identity

The identity of methionine sulfone and cysteic acid was confirmed through comparison of the retention time %RSD of the calibrant from Sigma-Aldrich, the pasta QC, and the five staple food samples. %RSD ≤ 5 in retention times between all samples and the calibrator served as the parameter to confirm amino acid identity. Retention times are shown in Table 4.11 and all %RSD values were below 5% which confirmed identity of the analytes.

Table 4.11. Retention time %RSD to confirm amino acid identity (n = 2).

	Retention time (min)	
Samples	Cysteic acid	Methionine sulfone
Calibrant*	2.66	4.30
Pasta	2.65	4.26
Corn-soya	2.63	4.40
Teff	2.63	4.39
Cassava	2.64	4.27
Sorghum	2.63	4.38
Maize	2.63	4.26
Mean	2.64	4.32
Standard deviation	0.01	0.06
%RSD	0.42	1.49

*Cysteic acid and methionine sulfone calibrants from Sigma-Aldrich.

4.3.4.2. Accuracy

To evaluate accuracy of the proposed method, four replicates of the pasta reference material were analysed on three different days (n = 2 on day 1). Recoveries of methionine sulfone and cysteic acid were averaged and they are shown in Figure 4.25. The recovery for methionine sulfone was 102%, and cysteic acid had a recovery of 110%. Recovery of the two amino acids fell within the 80-120% tolerance parameters, and thus requirements for accuracy were satisfied.

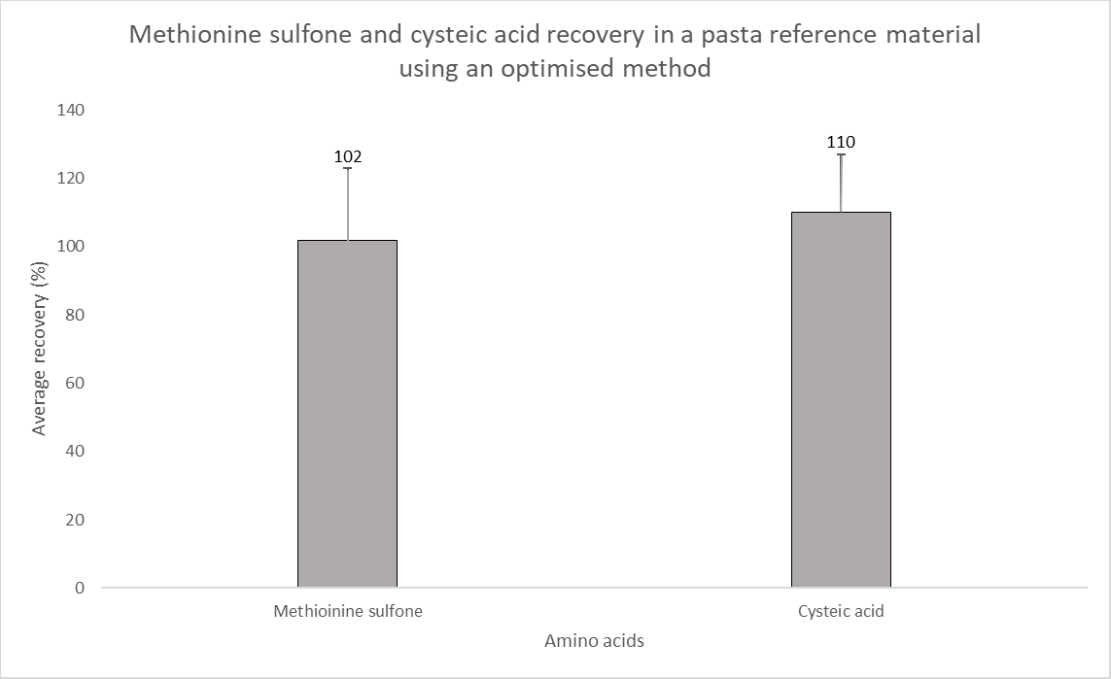


Figure 4.25. The average recovery of methionine sulfone and cysteic acid in pasta analysed on three different days (n = 4, n = 2 on day 1). The recovery fell within the 80-120% range for both amino acids. Error bars represent the standard deviation of 10 independent measurements.

4.3.4.3. Repeatability

Repeatability measures the closeness of results under identical experimental conditions. To determine repeatability of the method, four replicates of the pasta sample were analysed on the same day. The method validation criteria outline the parameter for repeatability to be $\%RSD_r \leq 15\%$. Table 4.12 shows the data following analysis of a pasta standard, and both analytes presented $\%RSD_r$ values below 15%, with the highest variation observed in methionine sulfone with a $\%RSD_r$ of 11.61%. Methionine sulfone and cysteic acid thus satisfied the criteria for repeatability.

Table 4.12. $\%RSD_r$ from four replicates of a pasta sample to demonstrate repeatability of the analysis method developed in this study.

	Concentration (g/100 g)	
Samples	Cysteic acid	Methionine sulfone
Sample 1	0.196	0.274
Sample 2	0.191	0.255
Sample 3	0.190	0.281
Sample 4	0.214	0.332
Mean	0.198	0.286
Standard deviation	0.011	0.033
%RSD	5.48	11.61

4.3.4.4. Reproducibility

Reproducibility refers to repeatability of the method on different days. Two replicates of the pasta sample were analysed on two different days. The method validation criteria outline the parameter for reproducibility to be $\%RSD_R \leq 15\%$. Table 4.13 shows the repeatability results following analysis of the pasta standard. $\%RSD_R$ was below 15% for both amino acids, indicating that the method satisfied requirements for reproducibility.

Table 4.13. $\%RSD_R$ from analysis of pasta on two different days to demonstrate reproducibility of the method developed in this study (n = 2).

	Concentration (g/100 g)	
Day of analysis	Cysteic acid	Methionine sulfone
Day 1	0.198	0.198
Day 2	0.237	0.201
Mean	0.217	0.199
Standard deviation	0.028	0.002
%RSD	12.84	1.04

4.3.5. Method development for the quantification of tryptophan

Classic acid hydrolysis with 6 M HCl is not suitable for tryptophan analysis as it is highly destructive, resulting in complete tryptophan losses. An alternative alkaline hydrolysis method using a base, typically NaOH, can be applied. An alkaline hydrolysis method for tryptophan analysis was adapted from AOAC Official Method 988.15. The protocol describes adding 4.2 M NaOH to test samples, followed by hydrolysis at a constant temperature of 110 ºC for 22 h. As part of initial implementation of the method, dual experiments were conducted using 1.2 mL and 10 mL NaOH to determine the most suitable volume for hydrolysis. Amino acids in test samples and calibration blends were derivatised and analysed by liquid chromatography. A chromatograph for the calibration blend with the highest concentration (3000 ng/g) in the calibration series showed good peak separation for tryptophan (Figure 4.26).

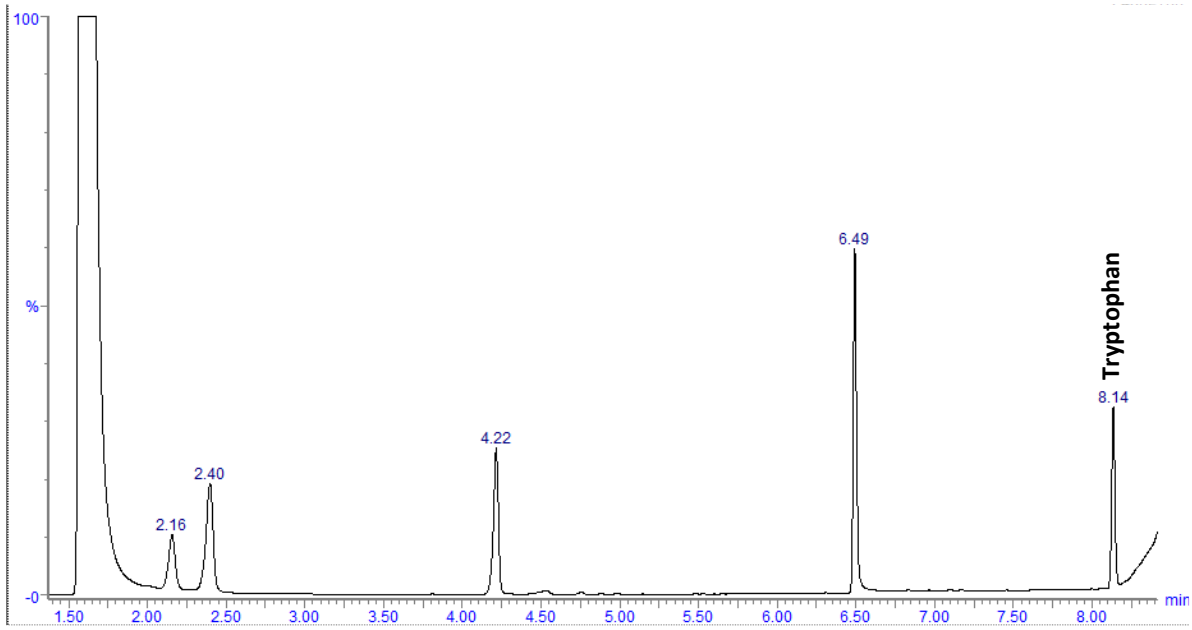


Figure 4.26. A chromatogram for a tryptophan calibration standard from Sigma-Aldrich prepared in 0.1 M HCl (concentration 3000 ng/g). The prepared calibration standards were derivatized using the AccQ·Tag™ Ultra Derivatization kit and the amino acids were separated by gradient elution using UHPLC-UV.

Peak areas from chromatograms of the calibration blends were plotted against the gravimetrically prepared concentrations to construct a calibration curve for tryptophan quantification (Figure 4.27). The calibration curve showed good linearity with an r^2-value of 0.9967, meeting the tolerance for linearity ($r^2 \geq 0.9900$).

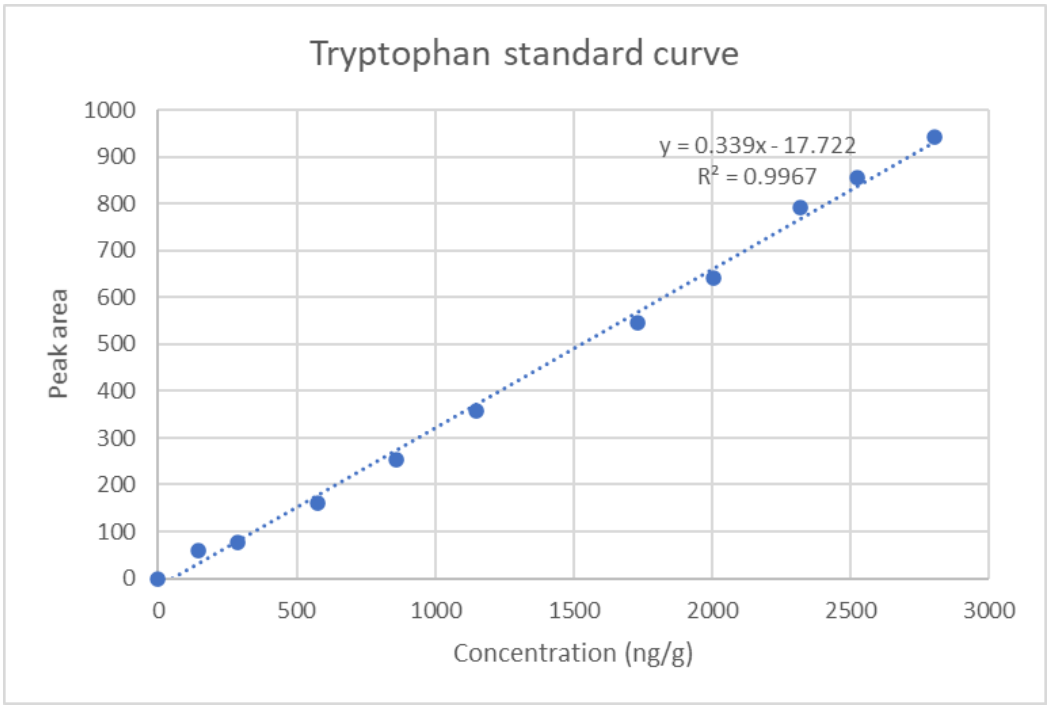


Figure 4.27. A 10-point calibration curve for a tryptophan calibrant prepared in 0.1 M HCl used to determine the quantity of tryptophan in test samples (concentration range 100 – 3000 ng/g).

A chromatogram was also obtained for the pasta reference material which showed good peak resolution for tryptophan (Figure 4.28). The peak area data was used to determine the concentration of tryptophan using the linear equation. Thereafter, the concentration was multiplied by the dilution factor to obtain the final concentration.

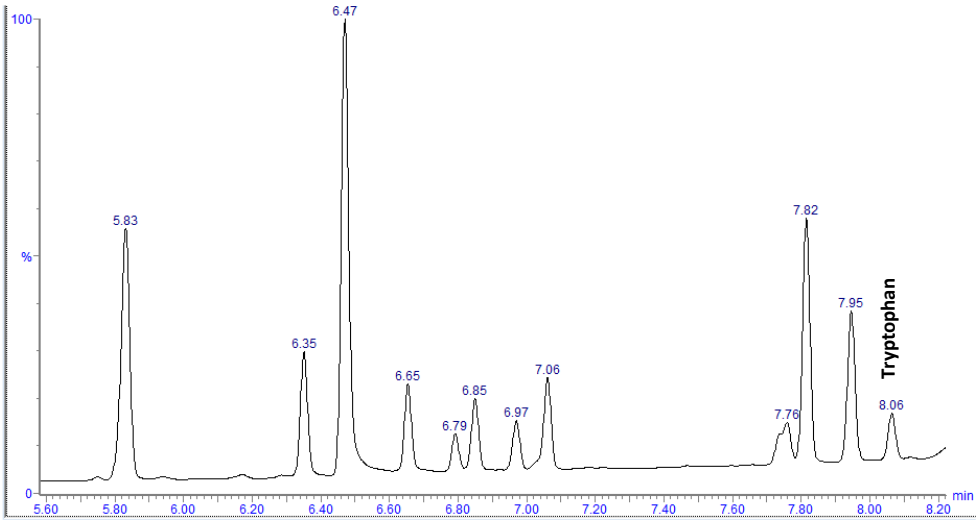


Figure 4.28. A chromatogram for tryptophan in a pasta reference material hydrolysed with 1.2 mL NaOH (4.2 M) at 110 ºC for 22 h. Hydrolysed amino acids were derivatized using the AccQ·Tag™ Ultra Derivatization kit, and thereafter separated by gradient elution on a UHPLC-UV instrument. On-column concentration range: 100-3000 ng/g.

The final concentration was substituted into the formula for recovery, and results for tryptophan recovery are presented in Figure 4.29. Pasta samples hydrolysed with 1.2 mL NaOH had an average recovery of 125%, and samples hydrolysed with 10 mL NaOH performed poorly with a recovery of 40%. The 10 mL volume may have been too high resulting in highly diluted samples. The 1.2 mL volume produced a comparatively good recovery, making the volume better suited for further method optimisation.

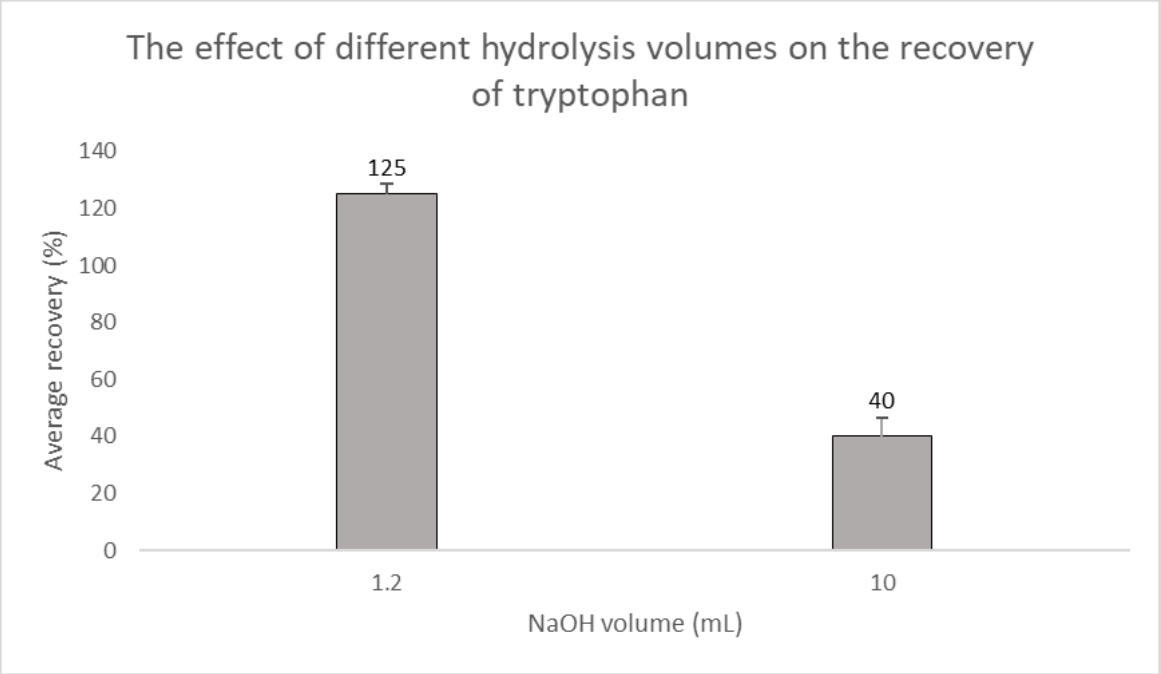


Figure 4.29. The recovery of tryptophan in pasta following alkaline hydrolysis (4.2 M NaOH) at 110 ºC for 22 h using hydrolysis volumes of 1.2 mL and 10 mL. The 1.2 mL volume produced an over recovery of 125%, and the 10 mL volume led to tryptophan under recovery at 40%. Error bars represent the standard deviation of independent measurements (n = 2).

The hydrolysis experiment was repeated using the 1.2 mL volume to verify reproducibility of the analysis method. Pasta samples were prepared and following alkaline hydrolysis, the samples had congealed into a gel-like substance. The gel material complicated aliquoting 1 mL hydrolysates for downstream processes because most of the 1.2 mL NaOH volume was retained in the gel. The samples were nonetheless analysed by liquid chromatography, and thereafter recovery was calculated. Tryptophan recovery in pasta was found to be 308% as shown in Figure 4.30, which was significantly higher than the 125% recovery obtained in the previous analysis (t-test, $p < 0.05$). The inconsistent recovery between the two analyses indicated the 1.2 mL hydrolysis volume was not reproducible.

The hydrolysis volume was further interrogated to establish a reproducible method for tryptophan analysis. To this end, a volume of 5 mL NaOH was implemented for hydrolysis. The hydrolysed samples were analysed, and tryptophan recovery in pasta was determined to be 83%, which fell within the recovery tolerance of 80-120% (Figure 4.30). Although the recovery was within the tolerance limit, the analysis was repeated to ensure the results were not a product of chance or random events. The repeat experiment using 5 mL NaOH produced a significantly poor recovery of 47% (t-test, $p < 0.05$) as shown in Figure 4.30. The significant difference in recovery from 83% to 47% indicated modification of the hydrolysis volume was unable to produce reliable, reproducible measurement results.

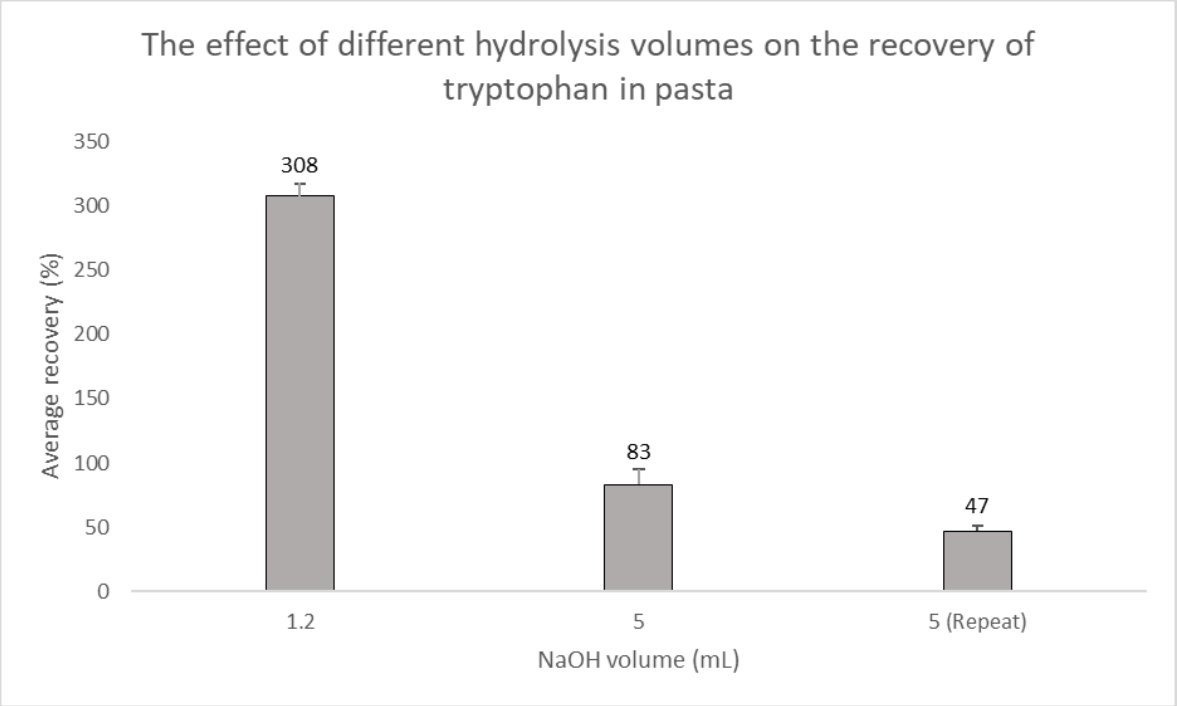


Figure 4.30. The recovery of tryptophan in pasta following alkaline hydrolysis using 4.2 M NaOH at a volume of 1.2 mL (n = 2) and 5 mL (n = 3) at 110 ºC for 22 h. Error bars represent the standard deviation of independent measurements.

Unusually low tryptophan recoveries obtained from numerous analyses raised concerns about tryptophan stability over the course of a hydrolysis experiment. A time-course study was therefore designed with hydrolysis conducted at 110 ºC for 8 h, 16 h, 24 h, and 48 h. The study was performed to assess whether the time implemented for hydrolysis was suitable for tryptophan analysis and did not lead to losses. Pasta samples were prepared and hydrolysed with 5 mL NaOH. The samples were analysed, and tryptophan recovery was calculated across the time intervals. Results of the time-course study are shown in Figure 4.31. Tryptophan recovery was 10% following an 8-h hydrolysis time, and it increased to 14% after a 16-h hydrolysis period. The recovery remained at 14% after 24 h and increased to 17% following a 48-h hydrolysis period. A linear regression analysis of the data set showed the increase in recovery was not significant ($p > 0.05$), thereby demonstrating the hydrolysis time did not contribute significantly towards the poor tryptophan recoveries that were obtained.

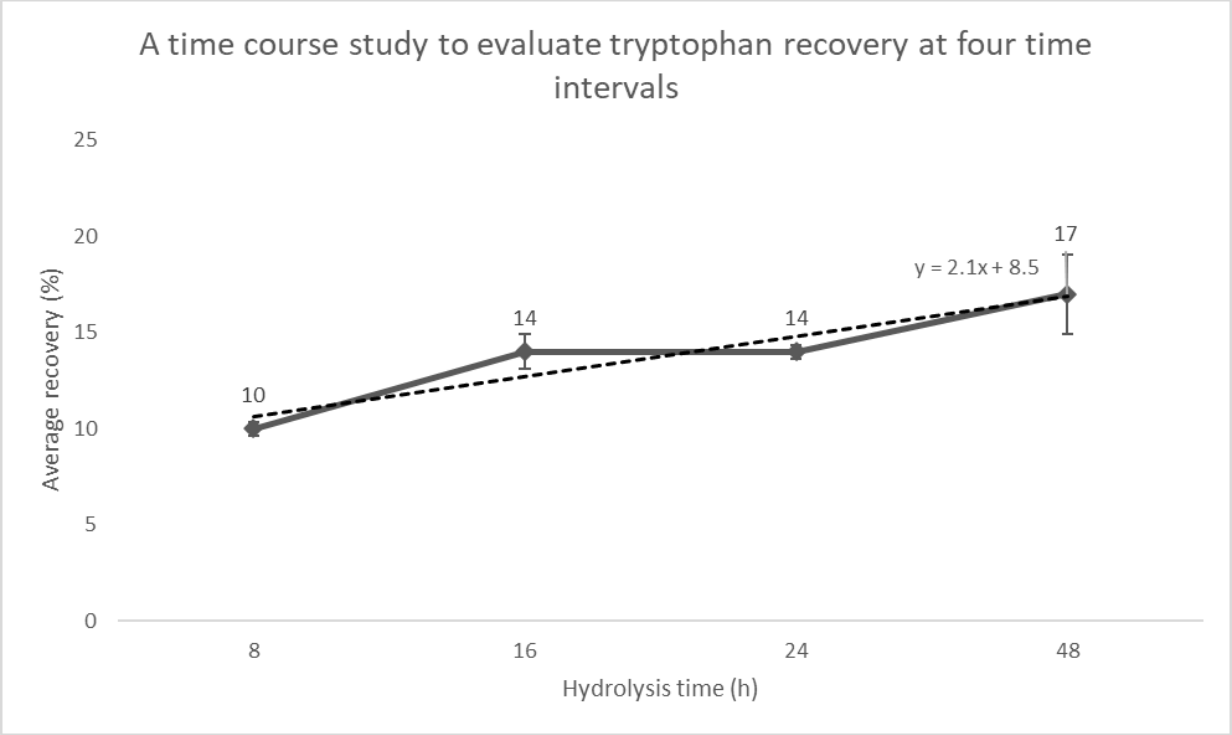


Figure 4.31. A time-course study evaluating the recovery of tryptophan in pasta following alkaline hydrolysis using 4.2 M NaOH at 110 ºC for 8 h, 16 h, 24 h, and 48 h. A trend analysis of the data showed the hydrolysis time did not contribute significantly ($p > 0.05$) to the low tryptophan recoveries. Error bars represent the standard deviation of independent measurements ($n = 2$).

The different parameters tested to quantify tryptophan have thus far been unsuccessful in producing repeatable recovery results. Available literature has suggested tryptophan might be susceptible to degradation owing to the presence of trace oxygen during hydrolysis (Wu and Tanoue, 2001). The addition of antioxidants has been demonstrated to be effective in overcoming tryptophan losses (Wu and Tanoue, 2001; la Cour et al., 2019), and thus this study investigated the effect of ascorbic acid as an antioxidant for reliable tryptophan determination. Two different masses of ascorbic acid (50 mg and 100 mg) were tested to determine a suitable amount that would produce good, reproducible recovery results. Following liquid chromatography analysis, tryptophan recovery was determined. The addition of 50 mg ascorbic acid produced a poor recovery of 45%, and 100 mg also produced a poor recovery of 51% (Figure 4.32). Ascorbic acid was therefore unable to improve recovery to satisfy the criteria for accuracy, and thus a suitable method for tryptophan determination could not be established in the current study.

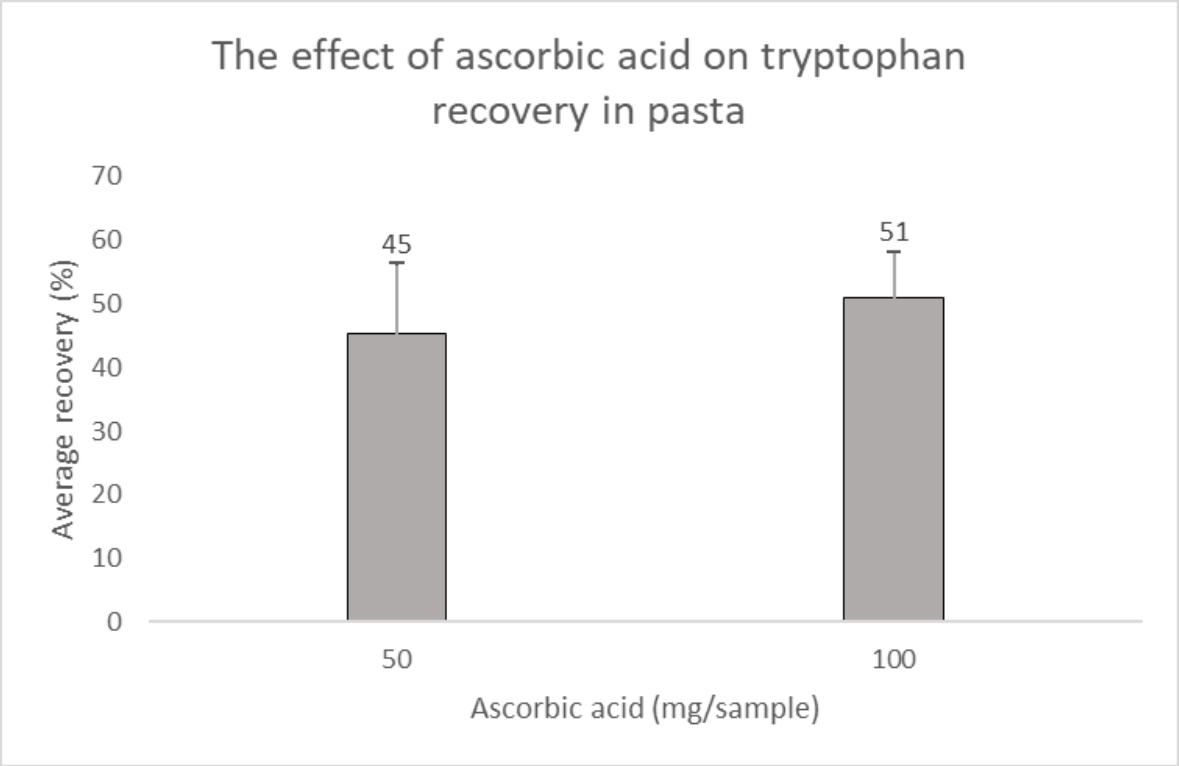


Figure 4.32. Tryptophan recovery in pasta following the addition of 50 mg and 100 mg ascorbic acid as an antioxidant to prevent oxidative degradation. Samples were hydrolysed with 4.2 M NaOH at 110 ºC for 22 h. The antioxidant was unable to produce recovery values within the 80-120% tolerance. Error bars represent the standard deviation of independent measurements (n = 2).

4.3.6. The amino acid composition of staple foods

An analysis method was developed and validated for use in quantifying 15 acid-stable amino acids in staple foods. The optimized method involved adding 10 mL 6 M HCl to 100 mg finely milled food samples, followed by acid hydrolysis at 110 ºC for 72 h. A separate hydrolysis method was also developed to quantify methionine and cysteine in food samples. The method included performic acid oxidation conducted at 0-5 ºC for 16 h, followed by the addition of 1 mL HBr and the subsequent evaporation of performic acid/HBr at 65 ºC. Samples were hydrolysed in 6 M HCl at 145 ºC for 4 h. Hydrolysed amino acids were derivatised and analysed using liquid chromatography. The final concentrations were corrected for recovery to reflect accurate amino acid compositions of the staple foods being investigated.

Amino acid concentrations, as determined by amino acid analysis, and the protein content determined using the Dumas method are summarised in Table 4.14. Corn-soya was the first staple analysed and it revealed glutamic acid to be the most abundant amino acid in its constitution with a concentration of 2.38 g/100 g, followed by leucine and aspartic acid with comparable concentrations of 1.25 g/100 g and 1.24 g/100 g, respectively. The lowest amino acid concentrations were observed in methionine with a value of 0.226 g/100 g, cysteine with

0.242 g/100 g, and histidine with 0.336 g/100 g. Corn-soya had the highest sum of amino acids with a content of 12.6 g/100 g, which matched the protein content of 12.8 g/100 g obtained by the Dumas method in Chapter 3.

Sorghum showed the highest concentration of glutamic acid out of all the staple foods, with a concentration of 2.47 g/100 g. Leucine and alanine were also detected in high concentrations with a content of 1.58 g/100 g and 1.08 g/100 g, respectively, followed by proline with 0.937 g/100 g and aspartic acid (0.815 g/100 g). Methionine was the least abundant amino acid with a concentration of 0.179 g/100 g, followed by a comparable cysteine content of 0.182 g/100 g. Lysine was the third least abundant amino acid with a content of 0.259 g/100 g. Sorghum showed the second highest sum of amino acids with a value of 11.5 g/100 g, matching the protein content of 11.3 g/100 g obtained by the Dumas method.

The most abundant amino acids in teff were glutamic acid with a content of 2.18 g/100 g, leucine with 0.740 g/100 g, and aspartic acid with 0.640 g/100 g. Amino acids with the least concentrations were cysteine with a content of 0.221 g/100 g, histidine with 0.232 g/100 g, and lysine with 0.325 g/100 g. Teff showed the third highest sum of amino acids with a value of 9.29 g/100 g, which compared with the Dumas value of 9.53 g/100 g.

The amino acid composition of maize revealed relatively high concentrations of glutamic acid with a content of 1.54 g/100 g, leucine with 0.962 g/100 g, and proline with 0.714 g/100 g. Amino acids with the lowest concentrations were cysteine and methionine with comparable concentrations of 0.182 g/100 g and 0.183 g/100 g, respectively, histidine with 0.246 g/100 g, and lysine with a content of 0.276 g/100 g. The total amino acid concentration of maize was the fourth highest with a value of 8.16 g/100 g, consistent with the protein concentration of 8.88 g/100 g obtained by the Dumas method

Cassava presented poor concentrations of all amino acids being studied, with the highest concentration observed in glutamic acid with a content of only 0.105 g/100 g, followed by aspartic acid with 0.069 g/100 g, and leucine with 0.050 g/100 g. Concentrations of the least abundant amino acids were 0.011 g/100 g for methionine, 0.013 g/100 g for cysteine, and 0.020 g/100 g for histidine. The sum of amino acids in cassava was 0.660 g/100 g which was lower than the protein content of 1.13 g/100 g determined by the Dumas method. However, the variation between the two methods was not significant since the protein and amino acid concentrations fell within limits of the estimated uncertainty budget.

Table 4.14. Amino acid composition and the protein content of five staple foods. Amino acid concentrations are reported as the mean ± standard deviation (n = 4). The sum of amino acids is compared to the protein content shown as the mean ± expanded uncertainty at a 95% confidence level (n = 23).

	Concentration (g/100 g)									
Amino acids	Corn-soya	σ/U	Sorghum	σ/U	Teff	σ/U	Maize	σ/U	Cassava	σ/U
Histidine	0.336	0.010	0.262	0.004	0.232	0.005	0.246	0.013	0.020	0.003
Serine	0.640	0.029	0.524	0.036	0.449	0.021	0.402	0.013	0.033	0.003
Arginine	0.798	0.029	0.404	0.010	0.430	0.018	0.425	0.021	0.031	0.002
Glycine	0.538	0.015	0.358	0.018	0.385	0.018	0.361	0.044	0.036	0.003
Aspartic acid	1.24	0.025	0.815	0.031	0.640	0.031	0.560	0.027	0.069	0.005
Glutamic acid	2.38	0.103	2.47	0.148	2.18	0.075	1.54	0.040	0.105	0.005
Threonine	0.505	0.028	0.390	0.032	0.405	0.034	0.311	0.014	0.029	0.002
Alanine	0.737	0.029	1.08	0.072	0.553	0.026	0.614	0.018	0.058	0.005
Proline	0.864	0.029	0.937	0.061	0.510	0.031	0.714	0.015	0.037	0.003
Lysine	0.637	0.011	0.259	0.014	0.325	0.013	0.276	0.012	0.045	0.003
Tyrosine	0.410	0.012	0.415	0.025	0.355	0.014	0.292	0.007	0.025	0.002
Valine	0.623	0.017	0.591	0.025	0.510	0.025	0.407	0.031	0.040	0.003
Isoleucine	0.528	0.020	0.477	0.015	0.400	0.044	0.284	0.025	0.029	0.001
Leucine	1.25	0.046	1.58	0.106	0.740	0.042	0.962	0.027	0.050	0.003
Phenylalanine	0.643	0.015	0.610	0.039	0.500	0.024	0.400	0.014	0.031	0.002
Methionine	0.226	0.037	0.179	0.029	0.461	0.016	0.183	0.026	0.011	0.006
Cysteine	0.242	0.014	0.182	0.015	0.221	0.002	0.182	0.021	0.013	0.003
Total	12.6	0.51	11.5	0.59	9.29	0.44	8.16	0.34	0.660	0.02
Protein (N x 6.25)	12.8	5.92	11.3	5.93	9.53	5.93	8.88	5.92	1.13	5.93

σ = Standard deviation. U = Expanded uncertainty.

A key parameter to determine the nutritional value of staple foods is the composition of essential amino acids. The distribution of 8 of the 9 **essential amino acids** in the five staple foods being investigated is presented in Figure 4.33. Histidine showed the highest concentration in corn-soya with a value of 0.336 g/100 g, followed by sorghum with a content of 0.262 g/100 g. Maize showed the third most abundant histidine content of 0.246 g/100 g, followed by teff with 0.232 g/100 g and lastly, cassava showed a significantly lower histidine content of 0.020 g/100 g. Corn-soya also showed the highest concentration of threonine with a value of 0.505 g/100 g, followed by teff with 0.405 g/100 g, and sorghum with 0.390 g/100 g. Maize had a lower threonine concentration of 0.311 g/100 g, and cassava had the lowest concentration at 0.029 g/100 g.

Lysine showed the highest concentration in corn-soya with a content of 0.637 g/100 g, and the second highest content was observed in teff with 0.325 g/100 g. Maize and sorghum revealed comparable lysine concentrations of 0.276 g/100 g and 0.259 g/100 g, respectively. Cassava had a poor lysine content with a value of only 0.045 g/100 g. Valine concentrations were highest

in corn-soya at 0.623 g/100 g, followed by sorghum with a value of 0.591 g/100 g. Teff showed a valine content of 0.510 g/100 g, and maize showed a comparatively lower content of 0.407 g/100 g. Cassava had a significantly poor valine concentration of 0.040 g/100 g.

Corn-soya had the highest isoleucine concentration at 0.528 g/100 g, followed by sorghum with 0.477 g/100 g and teff with 0.400 g/100 g. Maize showed a significantly lower concentration of 0.284 g/100 g, and cassava had the lowest isoleucine concentration of 0.029 g/100 g. Leucine was the most abundant essential amino acid in all five staple foods, with the highest concentration observed in sorghum with a content of 1.58 g/100 g, followed by corn-soya with 1.25 g/100 g. Maize revealed a leucine content of 0.962 g/100 g, and teff revealed a lower content of 0.740 g/100 g. Although leucine was the most abundant essential amino acid in cassava, the staple food showed an overall poor concentration with a value of 0.050 g/100 g.

Phenylalanine had the highest abundance in corn-soya with a concentration of 0.643 g/100 g, followed by sorghum with 0.610 g/100 g and teff with 0.500 g/100 g. Maize had a phenylalanine content of 0.400 g/100 g and cassava had the lowest content with 0.031 g/100 g. Methionine showed the highest concentration in teff with a value of 0.461 g/100 g, followed by corn-soya with a significantly lower content of 0.226 g/100 g. Maize and sorghum revealed similar methionine concentrations of 0.183 g/100 g and 0.179 g/100 g, respectively. Cassava showed an exceptionally low methionine concentration of 0.011 g/100 g.

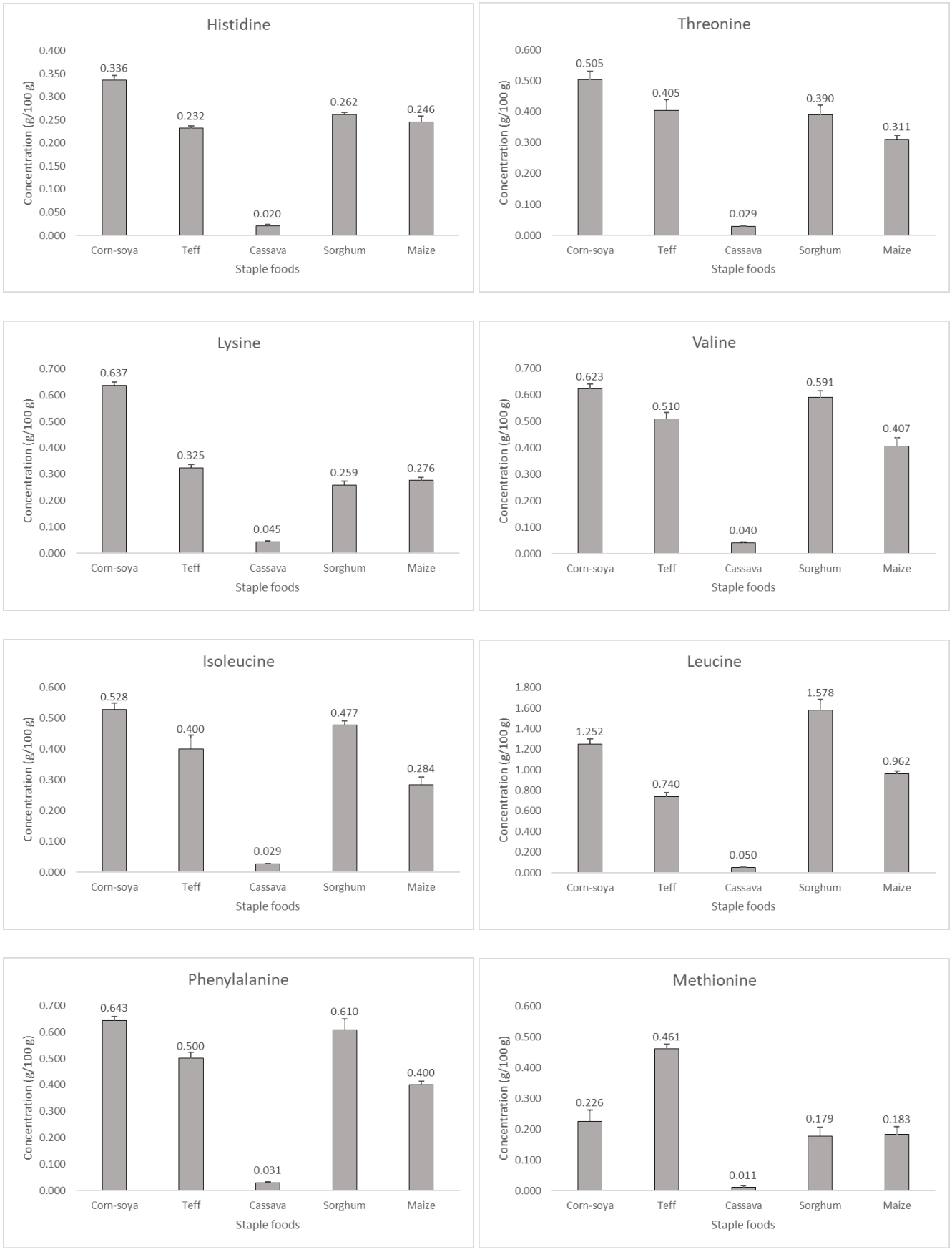


Figure 4.33. The distribution of essential amino acids in five African staple foods: Corn-soya, teff, cassava, sorghum, and maize. Error bars represent the standard deviation of independent measurements obtained on four different days (n = 4).

4.4. Discussion

The current work documents the characterisation of proteins in African staple foods based on amino acid analysis. Accurate quantitative analysis of amino acids is essential to describe the nutritional and economic value of food material. Furthermore, outlining methods for determining the composition of proteins and amino acids serves as conception for the development of reference materials.

4.4.1. Method development

4.4.1.1. Acid-stable amino acids

The development of a suitable method to quantify amino acids was based on analysis of a pasta sample, which provided a source of plant proteins that would better represent the chemical properties of the plant-based test samples during analysis. Initial testing performed on the pasta sample showed that recoveries for the essential amino acids valine and isoleucine were below the lower limit for recovery. Pasta samples were therefore milled to improve recovery of these two amino acids. Teff was also subjected to the milling process because the sample was received as whole grain seeds. The recovery of valine and isoleucine in pasta was not significantly improved by the milling process. However, teff seeds showed a significant improvement in the recovery of both analytes. These results suggested that although pasta samples were granular before the milling process, the granules were not significantly larger in size compared to particles obtained after milling and it is for this reason that a significant improvement in recovery was not observed.

In contrast, whole teff seeds are typically ~1 mm in size and following the milling process, particle sizes were half the size of the 0.25 µm sieve (Herak, 2016). That is, milled teff seeds were 0.125 µm (0.000125 mm) in size. Comparison of the results from whole teff seeds and milled seeds showed that milling the samples significantly improved the recovery of valine and isoleucine. This observation can be linked to the inverse relationship between particle size and surface area (Dubois et al., 2010). Milling decreased the grain particle size, which resulted in the inverse increase of the surface area. Therefore, a larger surface area was available for interactions between milled teff seeds and the hydrolysis agent to liberate amino acids more efficiently from the protein substrate. This was particularly important in the case of valine and isoleucine as they are notoriously difficult to hydrolyse (Albin et al., 2000; Lamp et al., 2018; Mustățea et al., 2019). The recovery of the remaining amino acids in both pasta and teff was not significantly improved by milling, and this may be because these amino acids were already sufficiently accessible to the acid hydrolysis reagent before the milling process. Although the analysis method had not yet been optimized, observations in milled teff seeds highlighted the

importance of adequately processing foods to improve amino acid analysis. If teff seeds had not been milled, the valine and isoleucine compositions would have been underestimated leading to undervaluing of the nutritional and economic value of the grain.

To improve valine and isoleucine recovery, the hydrolysis period was increased, which was effective in improving recovery to fall within the set tolerance of 80-120%. As previously described, proteins containing valine and isoleucine are well-catalogued to be difficult to hydrolyse, and this present report corroborates literature findings (Albin et al., 2000; Lamp et al., 2018). The two amino acids are hydrophobic residues which are sequestered in hydrophobic regions of the protein (Tsugita and Scheffler, 1982; Lamp et al., 2018). Furthermore, they contain aliphatic side chains which cause steric hindrances preventing protein unfolding (Kathuria et al., 2016). As a result, both amino acids are only slowly released during acid hydrolysis because peptide bonds between valine-valine, valine-isoleucine and isoleucine-isoleucine are not easily accessible to the hydrolysis agent. Thus, a longer hydrolysis period is often necessary to liberate valine and isoleucine.

Although pasta was used as the relevant quality control sample, a milk powder reference material was analysed in parallel. Complications with valine and isoleucine recovery were not encountered in milk powder as was the case in pasta. Hydrolysis of milk powder with the conventional hydrolysis time of 24 h produced excellent recovery for all acid-stable amino acids, including valine and isoleucine. A possible explanation for the observed results may be linked to different types of proteins present in the two samples. It is possible the proteins present in milk powder contain far less valine-valine, valine-isoleucine, and isoleucine-isoleucine bonds and thus the amino acids are liberated more efficiently. An alternative theory for the different results may be that milk powder readily dissolves in the HCl reagent, thus ensuring maximum exposure to the acid from the onset of the hydrolysis reaction, and as such less energy is required to liberate amino acids. Modification of the analysis method to yield optimal results for different quality control samples emphasizes the importance of conducting experiments with fit-for-purpose reference materials. Had this project relied on the milk powder sample for quantification, it is possible the amino acid content of staple foods would have been misrepresented. The provision of relevant reference materials is therefore one of the core motivations for this study to ensure the accuracy of testing practices on local food commodities.

The results from amino acid analysis showed that leucine, glutamic acid, and aspartic acid were consistently among the most abundant amino acids in food samples. Leucine was the most abundant essential amino acid in all food samples under study, which matches previous reports about the leucine content of plant-based food sources (Bong et al., 2010; Mofokeng

and Shimelis, 2018; Zhang et al., 2016). These findings suggest that plant proteins are generally excellent sources of dietary leucine.

The observations for glutamic acid and aspartic acid can be linked to amino acid modifications during acid hydrolysis. Glutamine and asparagine undergo deamidation which converts the residues to glutamic acid and aspartic acid, respectively (Riggs et al., 2019; Delmar et al., 2019). Therefore, it is not possible to quantify individual concentrations of glutamine, glutamic acid, asparagine, and aspartic acid using chemical hydrolysis methods. They can only be quantified as a summed determination of glutamic acid + glutamine (collectively denoted as glutamic acid), and aspartic acid + asparagine (denoted as aspartic acid). It is on this basis that determination of the two amino acids consistently revealed high concentrations. A summed determination of the four amino acids is an important problem in areas such as protein and peptide delineation and resolving molecular structures. However, quantifying glutamine and asparagine as a sum of their acidic analogues is widely accepted in food testing practices because it does not significantly impact total protein quantification (Kambhampati et al., 2019).

4.4.1.2. Methionine and cysteine

Sulfhydryl groups present on methionine and cysteine are unstable in the presence of HCl, which leads to amino acid losses. One of the most commonly applied solutions to overcome stability problems is the use of performic acid to oxidise the amino acids prior to acid hydrolysis. The oxidative function of performic acid serves to cleave disulphide bonds in cysteine resulting in the formation of cysteic acid (Thera et al., 2018). Methionine is similarly oxidised to methionine sulfone. These oxidised derivatives contain sulphur in a more stable state, facilitating accurate quantification of methionine and cysteine. The current study therefore implemented performic acid oxidisation followed by acid hydrolysis to analyse the content of methionine and cysteine in staple food samples.

Initial implementation of a performic acid oxidation/acid hydrolysis method in pasta produced good recoveries for methionine sulfone and an over-recovery for cysteic acid. Not convinced of the validity of the results, the experiment was repeated with inclusion of a milk powder reference sample. The repeat experiment produced excellent recoveries for methionine sulfone in both samples, and an over-recovery of cysteic acid was observed. The results demonstrated that methionine sulfone can be reliably determined in two separate reference standards using the analysis method. Reasons for the cysteic acid over-recovery are currently unclear, but matrix effects are suspected to have contributed to the observed results. Complex food samples contain ingredients such as salts and carbohydrates that may interfere with essential method parameters including accuracy (Song et al., 2015). Internal standards can be

used to overcome matrix effects, but this is not a cost-effective approach. A different oxidation-hydrolysis method was considered to obtain acceptable recoveries for the two analytes. The new method involved performic acid oxidation, followed by the addition of HBr and thereafter acid hydrolysis. HBr or sodium metabisulfite are normally added to the oxidation reaction to reduce performic acid to formic acid, thereby quenching the reaction (Mustățea et al., 2019). The resultant formic acid/HBr should be evaporated before acid hydrolysis can be conducted. A number of research laboratories use a vacuum rotary evaporator to dry samples. For this work, a cost-effective heating block set to 65 ºC was used to successfully achieve a similar objective of evaporating HBr.

Test samples were hydrolysed, and the recovery of methionine sulfone fell within the recovery range. However, the recovery of cysteic acid was greater than the acceptable recovery range, similar to previous analyses. The analysis method was presently not optimal for cysteic acid analysis; and as a result, the impact of the HBr volume was investigated with the aim of improving cysteic acid recovery. The HBr volume was reduced from 3 mL to 1 mL, and under these conditions, methionine sulfone and cysteic acid recovery fell within the 80-120% recovery range. The results suggest a higher volume of HBr added to quench the oxidation reaction may negatively interfere with cysteic acid analysis. The mechanism thereof is not immediately clear, perhaps a higher volume of HBr present in test tubes led to inadequate evaporation of HBr which influenced the recovery of cysteic acid. Nonetheless, a working protocol for methionine and cysteine analysis had been developed.

4.4.1.3. Tryptophan

Despite the proven nutritional importance of tryptophan, it is not uncommon for quantitative studies to omit measurement results for this amino acid as it is an entirely independent measurement, contributing results for only a single amino acid to the complete amino acid profile (Dahl-Lassen et al., 2018; Bals et al., 2007; Gorissen et al., 2018; Rozan et al., 2001). Tryptophan analysis is complicated by the oxidative destruction of the indole side chain under acidic conditions, making analysis of the amino acid by traditional acid hydrolysis impossible (Friedman, 2018). Alkaline hydrolysis with NaOH has been proposed and successfully implemented in other studies to overcome degradation issues, and this hydrolysis technique was applied in the current study (Zhang et al., 2009; Yust et al., 2004).

In the initial implementation of an alkaline (4.2 M NaOH) hydrolysis method, 1.2 mL and 10 mL hydrolysis volumes were tested to determine the optimal volume for hydrolysis. Following hydrolysis and quantification, the 10 mL volume produced a poor recovery which suggested the higher volume led to overly diluted samples. The 1.2 mL volume produced an over-recovery

of tryptophan, indicating the volume may have been too low. However, 1.2 mL NaOH produced better recoveries, closer to the acceptable recovery range and therefore the volume was implemented for subsequent method optimisation. A repeat experiment with the 1.2 mL volume produced a gel-like substance which may be a result of the hydrolysis volume being too low to completely suspend samples in solution. Similar issues involving gel-formation following alkaline hydrolysis have been reported (la Cour et al., 2019). In the cited study, NaOH was abandoned in favour of lithium hydroxide (LiOH). However, this project proceeded with evaluating the NaOH volume since LiOH was unavailable.

To establish a suitable volume for tryptophan hydrolysis, a 5 mL hydrolysis volume was tested, and it initially produced excellent recoveries. However, repeat analyses using the same volume resulted in significantly poor recoveries. A clear scientific basis for the observed results could not be identified due to the large variability and poor reproducibility of the data generated.

Hydrolysis times of 8 h, 16 h, 24 h, and 48 h were subsequently investigated to determine whether the time implemented for hydrolysis may be leading to tryptophan losses. Following hydrolysis and quantification, the recovery of tryptophan at progressively longer hydrolysis times did not significantly improve suggesting the hydrolysis period did not influence the recovery of tryptophan. Studies on tryptophan analysis at the University of Copenhagen arrived at similar conclusions regarding the minimal influence of the hydrolysis time on tryptophan recovery (la Cour et al., 2019).

This study has demonstrated that the hydrolysis volume and the time applied for hydrolysis did not contribute significantly towards the observed tryptophan recoveries. A possible explanation for the poor recoveries could be due to oxidative degradation of tryptophan from residual oxygen since the indole side-chain is highly susceptible to oxidation (Bellmaine et al., 2020). The addition of antioxidants provides a probable solution to overcome oxidative losses. Ascorbic acid was included as the antioxidant, but only half of the tryptophan was successfully recovered. Previous reports combining ascorbic acid with LiOH were successful in obtaining desired recoveries (la Cour et al., 2019), thus LiOH may be a more suitable hydrolysis reagent. The recovery results obtained herein did not meet specifications for accuracy, and thus the method could not be implemented to quantify tryptophan in staple foods.

4.4.2. The amino acid composition of staple foods

The human body does not have natural amino acid reserves, and thus requires a continuous supply of amino acids during the synthesis and degradation of proteins and other nitrogenous metabolic products. Humans are unable to produce sufficient quantities of the 9 essential amino acids, and thus emphasis is placed on these indispensable nutritional elements because

they can only be obtained from the diet. The composition of essential amino acids is therefore crucial when determining the value of staple foods.

Corn-soya is provided by supplementary feeding programmes to curb the onset of acute malnutrition and the subsequent progression to mortality (Karakochuk et al., 2012). A review of official communication from global authorities has shown that a minimum amino acid content for corn-soya is not specified (USDA, 2017). Guidelines for corn-soya production only provide specifications for the protein content as determined by nitrogen analysis. The current investigation found that essential amino acids made up a significant proportion of the protein content, representing a desirable protein quality. Lysine is ordinarily the first limiting nutrient in diets predominantly consisting of maize (Kato et al., 2019). The high lysine content in corn-soya shows that maize-soybean combinations can compensate for nutritional limitations in maize. However, the methionine concentration in corn-soya was not significantly improved from the content found in conventional white maize. Soybeans and the maize endosperm have limiting concentrations of the sulphur-containing amino acid, which would explain the low content in the fortified preparation (Liu et al., 2020; Guo et al., 2020). A Malawian study demonstrated the recovery of children diagnosed with moderate wasting disease using corn-soya (Matilsky et al., 2009). However, RUTFs showed improved efficacy when compared to corn-soya, and the poor composition of methionine may be one of the contributing factors for the reduced efficacy.

Amino acid analysis of a representative sorghum sample showed that essential amino acids including leucine, phenylalanine, isoleucine, and valine were present in high concentrations. Lysine and histidine were determined to be the least abundant essential amino acids, which compares with previously reported quantities (Mohapatra et al., 2019). In addition, the methionine composition of sorghum was among the lowest, suggesting lysine, histidine, and methionine may be limiting nutrients in a diet comprising primarily of sorghum. Apart from these three amino acids, sorghum otherwise presents a good source of quality proteins. However, this observation does not consider protein digestibility, which refers to susceptibility of a protein substrate to proteolysis, leading to increased bioavailability of free amino acids (Joye, 2019). Proteins with a high digestibility index are consequently good quality proteins because they are easily digested into their constituent amino acids for use in essential metabolic pathways. The digestibility of sorghum proteins markedly decreases following wet cooking and preparation methods (Mokrane et al., 2010; Liu et al., 2019). Protein digestibility is therefore a salient research subject to address limitations of sorghum nutrition.

Teff is another staple food that was analysed in this work, and the findings show teff to be a relatively good source of several essential amino acids compared to other grains. The results obtained in the current analysis support previous reports as they demonstrate a grain with a balanced composition of essential amino acids including leucine, valine, phenylalanine, and isoleucine (Abebe et al., 2015). Additionally, teff results show a relatively high lysine content which is in agreement with previous findings (Gebru et al., 2020; do Nascimento et al., 2018). The methionine concentration in teff was determined to be significantly higher compared to corn-soya, maize, sorghum, and cassava. Studies into teff seed storage proteins (SSPs) attribute the high methionine content to a group of SSPs called prolamins which are abundant in teff (Zhang et al., 2016; Adebowale et al., 2011). These results provide merit to the increased global interest in the teff grain. Teff is currently an underexploited food crop, thus continued scientific considerations into the crop will contribute towards food security and generating income for small-scale producers.

Maize is one of the most important principal source of energy and nutrients in sub-Saharan Africa, which contributes significantly towards household food security. This study determined the composition of maize to comprise some of the lowest concentrations of essential amino acids including lysine, histidine, isoleucine, and methionine. These amino acid deficiencies, particularly the low lysine concentrations, are consistent with previous reports (Erdal et al., 2019; Abiose and Ikujenlola, 2014). Maize may therefore not be a suitable primary source of nutrition for the provision of some nutritionally important amino acids. Maize has been a subject of extensive studies that have produced genetic mutants with an improved protein quality (Hossain et al., 2019; Jilo, 2021). However, the improved maize cultivars are not widely adopted by food producers because they are associated with reduced yields, and a soft endosperm making the crop more susceptible to diseases and pest infestations (Toro et al., 2003; Sofi et al., 2009). Further research still needs to be conducted to ensure the economic viability of modified maize cultivars with improved nutritional qualities.

The amino acid composition of the cassava variety analysed in this study ranged from 0.011 g/100 g to 0.105 g/100 g, with a sum of amino acids that is only equal to 0.660 g/100 g, representing a significantly poor source of all essential amino acids. These results further support previous reports that cassava cannot meet dietary demands for essential amino acids (Ceballos et al., 2006; Gomes and Nassar, 2013; Morgan and Choct, 2016). Unsurprisingly, cassava-based diets have led to health complications. Congolese women and children sustaining on a diet of processed cassava roots were reported to have a high incidence of *konzo* – a severe, but largely neglected disease condition characterised by irreversible paralysis which is commonly observed in cases of insufficient dietary amino acid intake

(Diasolua-Ngudi et al., 2002). Cassava serving as the major source of nutrition thus poses significant health risks as it cannot meet demands for nutritionally important amino acids.

4.5. Chapter conclusions

This study demonstrated that valine and isoleucine require a prolonged hydrolysis time to obtain acceptable recoveries owing to difficulties in cleaving peptide bonds between the two residues. Methionine showed evidence of higher stability compared to cysteine, because it could be accurately quantified by several, fundamentally different analysis protocols. Cysteine results were initially more unpredictable, which could be correlated to instability of the side chain sulfhydryl group. In the end, analysis methods were developed for the quantification of acid-stable and sulphur-containing amino acids in staple foods. The methods were shown through statistical appraisal to be selective, accurate, repeatable, and reproducible and thus were implemented to characterise the amino acid composition of staple foods.

Corn-soya was found to be an excellent source of essential amino acids including lysine, which is commonly a limiting nutrient in cereal grains. Sorghum revealed high concentrations of most essential amino acids, except for lysine, methionine, and histidine. Teff showed a good composition of lysine and methionine likely due to a high composition of the prolamin group of seed storage proteins. Cassava roots are a poor source of all essential amino acids which may have health implications. Maize was determined to have low quantities of nutritionally important amino acids such as lysine, methionine and histidine, supporting previous reports on the poor amino acid quality of the crop.

Alkaline hydrolysis of tryptophan led to tryptophan losses which had a significant impact on accuracy of the method. Different method parameters including the hydrolysis time, hydrolysis volume, dilution volume and the addition of an antioxidant were investigated but conclusions could not be drawn based on the highly variable and irreproducible measurement results obtained, and as such, tryptophan quantification could not be performed in the current study. This showed that methods for amino acid analysis need to be modified for every sample type since optimised methods from literature may not be applicable for different samples.

Chapter 5

Conclusion

This study successfully developed and implemented analysis methods to determine the moisture content, particle size distribution, and the protein and amino acid composition of major African staple foods. Moisture determination by Karl Fischer titration showed that corn-soya, sorghum, teff and maize have a moisture content below maximum limits outlined for safe, long-term storage. These results therefore lead towards acceptance of the formulated hypothesis stating that staple foods have a moisture content below recommended limits to minimise storability challenges. However, the results for cassava revealed it has a moisture content above the maximum limit. The hypothesis is therefore rejected with regard to cassava. The staple food has a higher potential to support the growth and proliferation of fungal species that would produce harmful mycotoxins.

Particle size distribution measurements by laser diffraction revealed that corn-soya and teff had coarse particles distributed within a uniform size fraction. These findings lead towards rejection of the formulated hypothesis stating that particle sizes in milled food samples are uniformly sized fine particles. Although the food samples showed uniformly sized particles, the particle sizes were coarser than hypothesised. The particle size distribution of maize, sorghum and cassava comprised fine particles distributed between multiple size fractions. Therefore, the hypothesis is also rejected with regard to these three staple foods. All five staple foods that were investigated in this work did not comprise uniformly sized fine particles.

Two quantitative methods were successfully developed to hydrolyse food proteins, followed by analysis using UHPLC coupled to UV detection. The first method involves a prolonged 72-h hydrolysis time to determine acid-stable amino acids. The second method includes performic acid oxidation, followed by quenching the reaction with HBr, and lastly acid hydrolysis at 145 ºC for 4 h to determine the sulphur-containing amino acids methionine and cysteine. The methods were validated for accuracy, repeatability and reproducibility and thus are applicable for various plant-derived food sources. The proposed methods produced good amino acid recoveries between 87% and 117%, and thus were implemented to determine the amino acid composition of five staple foods. The findings from amino acid analysis were supported by protein analysis results obtained in this study using an independent Dumas combustion method.

Corn-soya showed a high amino acid composition with a balanced distribution of most essential amino acids, excluding methionine. Sorghum had the second highest amino acid composition

rich in valine, leucine and phenylalanine, but poor in lysine and methionine. Teff revealed the third most abundant amino acid composition comprising relatively high lysine, methionine and threonine concentrations. The formulated hypothesis stating that plant-derived food sources have a high protein and amino acid composition is therefore accepted with regard to these three staple foods. Maize showed a lower overall amino acid quantity revealing low concentrations of histidine, lysine, isoleucine and methionine. Cassava showed exceptionally poor concentrations of all amino acids. Therefore, this work rejects the stated hypothesis with regard to maize and cassava – these two staple foods have a poor protein and amino acid composition.

The two protocols established for protein hydrolysis were not suitable to reliably determine tryptophan. Future work would therefore seek to establish a reproducible method for this amino acid. The investigations would interrogate different hydrolysis agents such as lithium hydroxide or barium hydroxide (Lucas and Sotelo, 1980). Alternatively, a different antioxidant such as tryptamine can be trialled to overcome oxidative degradation of tryptophan during hydrolysis (la Cour et al., 2019). Future investigations would also consider comparative studies between different cultivars such as bitter and sweet cassava, and brown and white teff to highlight varieties with a superior nutritional constitution.

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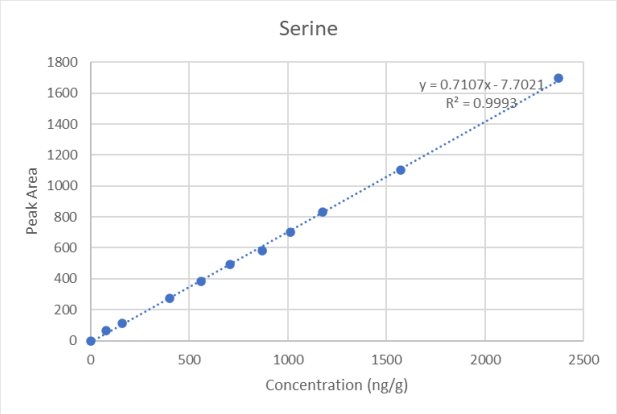
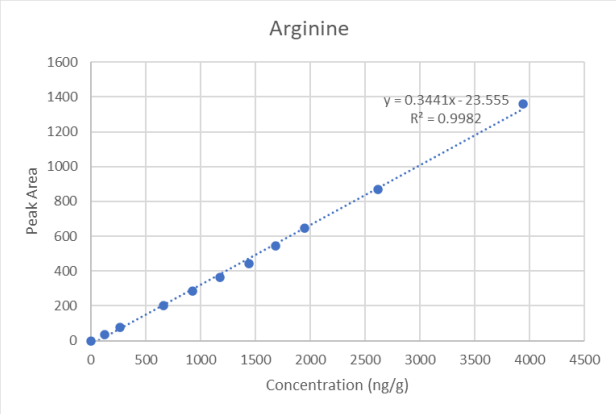
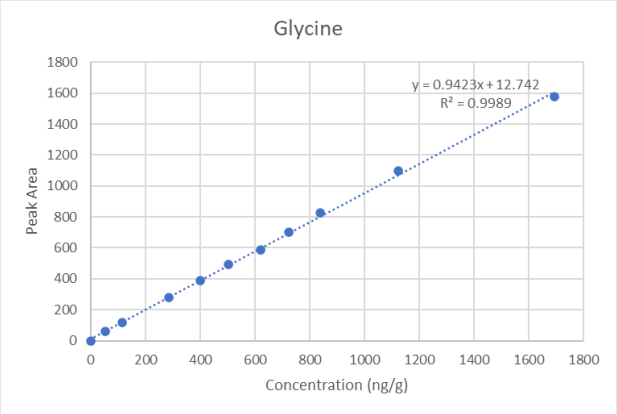
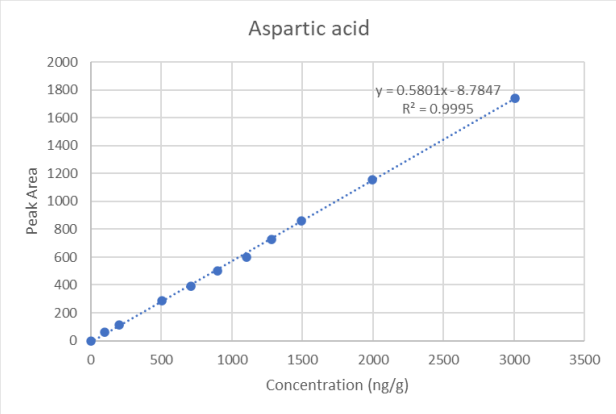
Appendix

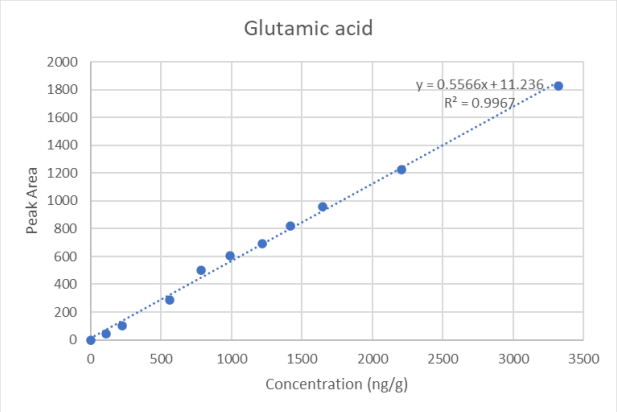
Amino acid calibration curves

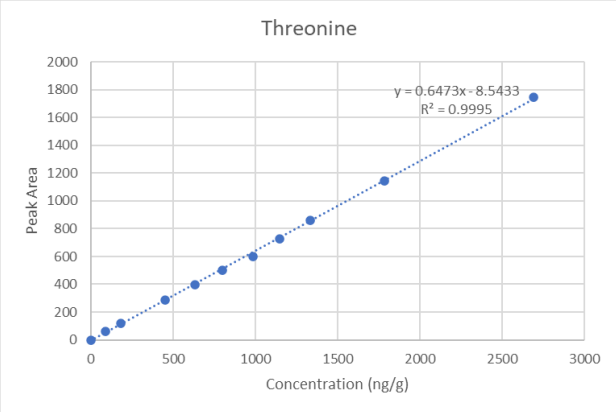


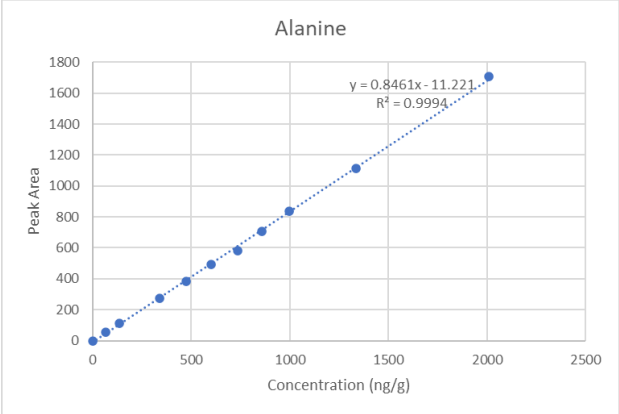


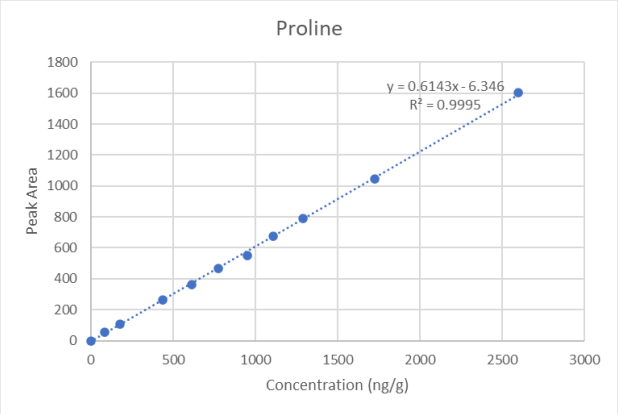


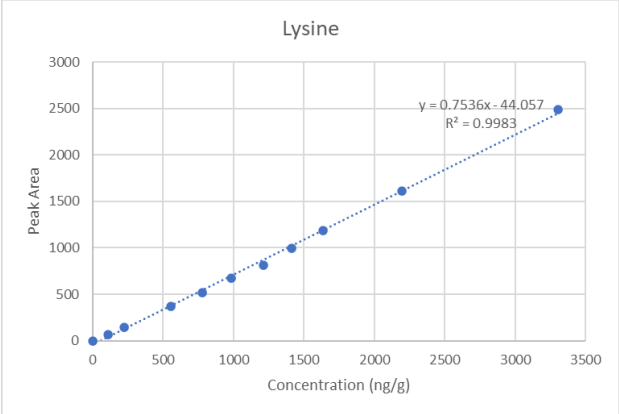


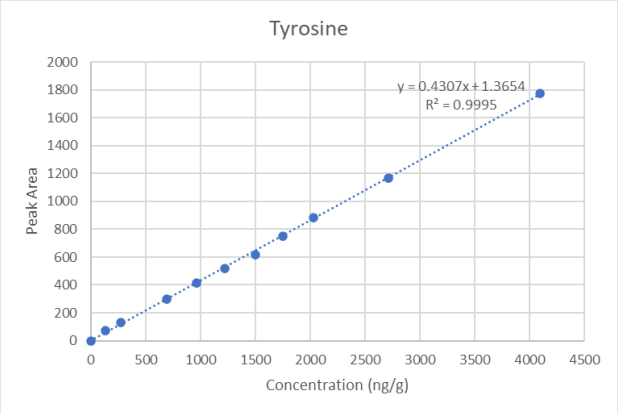


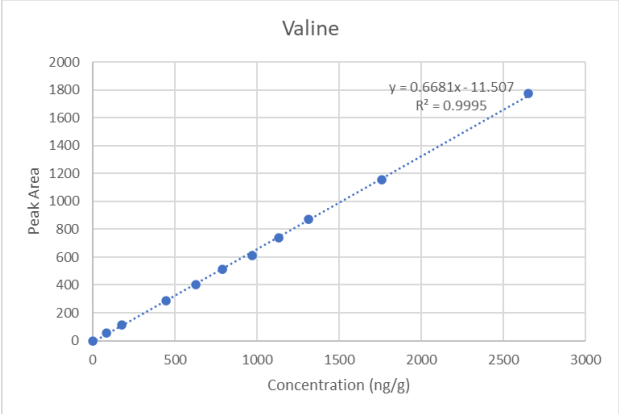


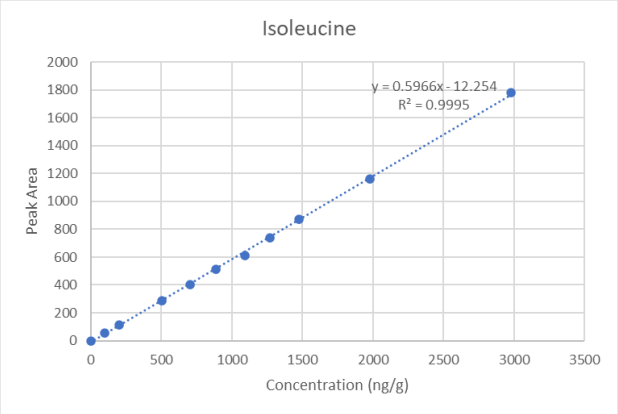


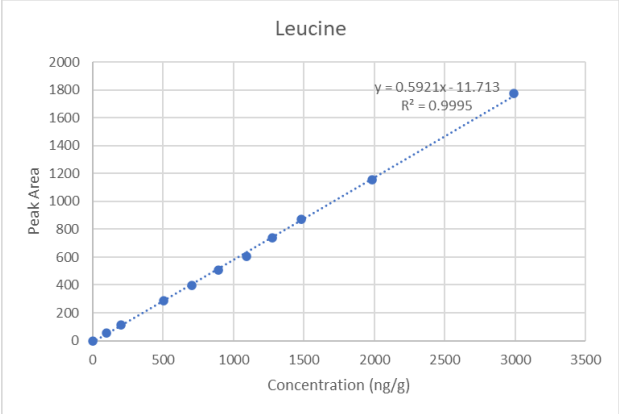


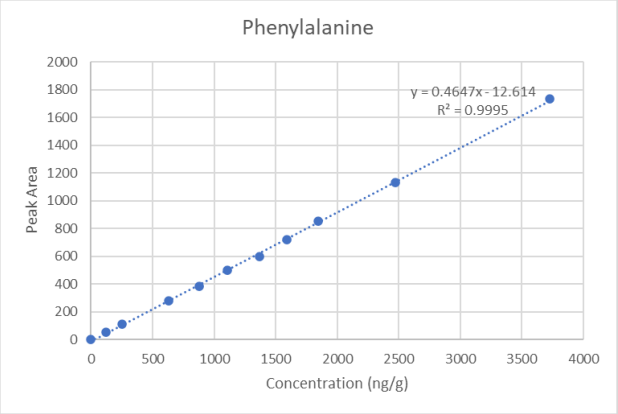












Appendix A. Calibration curves used to extrapolate the concentration of acid-stable amino acids in plant-derived staple food samples.