The Evaluation and Laboratory Screening of Selected Commercially Available Protein Supplement Products

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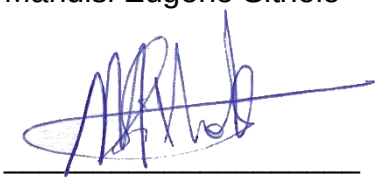
Declaration

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

Mandisi Eugene Sithole



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Abstract

Protein supplements have grown in popularity in recent years since they are utilised by both physically active people and athletes. However, the regulation of supplement products by the relevant authorities within the sector has been inadequate. As a result, unscrupulous manufacturers of these products take advantage of this by mislabelling, adding inadequate protein, and incorporating adulterants to enhance the apparent protein content. This is a widespread practice throughout the world, including South Africa. Unsuspecting customers may be misled by incorrectly labelled products. According to research, adding adulterants such as melamine to these products may cause kidney disease. The purpose of this study was to evaluate the label information of selected protein supplements sold in South Africa, quantify the protein content, and determine the prevalence of melamine, cyanuric acid, and uric acid in these products. Predetermined labelling information categories for protein supplement products (n = 21) were assessed, including claims, warnings, disclaimers, and product pricing. Label claims were typically observed to be vague and unsupported by scientific literature/evidence. The actual protein content of the selected protein supplements was determined using a multi-protein assay approach. In this study BCA, Bradford, and Lowry assays were used for the protein assessment. There was a statistically significant difference (p<0.05) between analysed protein values and the label reported values, in 86% products analysed by BCA, and 67% by Lowry assay. A simple and rapid reverse phase high performance liquid chromatography (HPLC-UV) method was developed and validated to determine presence of adulterants simultaneously in protein supplements. All of the supplements tested positive for one or more adulterants using this approach. The mean concentrations estimate for the adulterants were 12.5 $\mu g.ml^{-1}$ for melamine, 120 $\mu g.ml^{-1}$ for cyanuric acid and 57.8 $\mu g.ml^{-1}$ for uric acid. The findings of this study demonstrate that protein supplement manufacturers take advantage of the lack of effective governance through unethical practices. This research is significant as it supports the need for an improved regulatory framework within the supplement sector.

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Mandisi Eugene Sithole

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List of Congresses Attended

1. Sithole M, and Gabriels G. *The Evaluation of Commercially Available Protein Supplement Products' Labelling Information*. South African Sports Medicine Association (SASMA) and the BRICS Council of Exercise and Sports Science (BRICSESS) Congress 2019. Century City Conference Centre, Cape Town, South Africa, 10th – 13th October 2019 (Poster presentation).
2. Sithole M, and Gabriels G. *The Quantification of Actual Protein-Content of Locally Available Protein Supplement Products*. Wits University School of Therapeutic Sciences Biennial Research Day, Parktown, South Africa, 15 October 2020 (Poster presentation).
3. Sithole M, and Gabriels G. *Assessment of Label Information and Product Cost of Commercially Available Protein Supplements in Johannesburg*. Wits University School of Therapeutic Sciences Biennial Research Day, Parktown, South Africa, 14 September 2021 (Poster presentation).
4. Sithole M, and Gabriels G. *The Detection of Adulterants in Protein Supplements Commercially Available in South Africa*. Virtual 7th All Africa Congress on Basic and Clinical Pharmacology, Nairobi, Kenya, 15th – 17th September 2021 (Virtual poster presentation).
5. Sithole M, and Gabriels G. *Protein Supplement Products Contain Lower Actual Protein-Content than Declared on Labels*. 36th World Congress of Sports Medicine, Athens, Greece, 23rd – 26th September 2021 (Virtual poster/oral presentation).

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List of Abbreviations

%	Percentage
% v/v	Percentage volume
Abs	Absorbance
ACN	Acetonitrile
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
^{o}C	Celsius
CYA	Cyanuric acid
DAD	Diode-Array Detection
DOH	Department of Health
e.g.	Example
EFSA	European Food Safety Authority
g	Grams
GC-MS	Gas chromatography-mass spectrometry
HPLC	High Performance Liquid Chromatography
ICH	International conference on Harmonisation
K_2HPO_4	Dipotassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen phosphate
$LC\text{-}MS^2$	Liquid Chromatography-Triple-Quadruple Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
mAU	Milli-Absorbance Unit
MeOH	Methanol
Mel	Melamine
Min	Minutes
ml	Millilitre
ml/min	Millilitre per Minute
Mm	Millimetres
mM	Millimolar
ND	Not Detected
Nm	Nanometres

pKa	Dissociation constant of acid
ppb	Parts per billion
ppm	Parts per million
RP-HPLC	Reverse Phase - High Performance Liquid Chromatography
SAHPRA	South African Health Products Regulatory Authority
SD	Standard deviation
TDI	Tolerable Daily Intake
UA	Uric acid
$\mu g.ml^{-1}$	Micrograms per Millilitre
µl	Microlitre
µm	Micrometre
USA	United States of America
U.S. FDA	United States Food and Drug Administration
UV-Vis	Ultraviolet-Visible Spectroscopy
WHO	World Health Organization

CHAPTER ONE - INTRODUCTION

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1.1. Nutritional supplements

Nutritional supplements can be described as any product that can be taken in addition to a regular diet to promote extra health-promoting nutrients (Hassan *et al*., 2020). There are many types of these products on the market, including botanicals, minerals, vitamins, amino acids, proteins, carbohydrates, and others (U.S. FDA, 2017). The formulations consist of powder or shakes, tablets, capsules, liquids, gels, and energy bars (Dlugaszewska *et al*., 2019; Gabriels *et al*., 2018). The global industry's estimated market value in 2018 was US$115 billion, and the use of nutritional supplements spans all ages, from infants to the elderly (Grand View Research, 2020). According to a research report, the annual growth rate of the nutritional supplement industry in South Africa was 13.5% on average between 2014 and 2016 (Schönfeldt *et al*., 2019). Based on these trends, studies speculate that the supplements industry should be expected to grow exponentially in the coming years due to strong demand (Schönfeldt *et al*., 2019).

The purpose of nutritional supplements is to help people obtain adequate amounts of essential nutrients without eating a variety of nutritious foods (NIH, 2020). It should be noted that supplements cannot replace the various foods that are essential for a healthy diet (U.S. FDA, 2017). Nutritional supplements are used for a variety of reasons and are often designed to meet an individual's specific nutritional needs. Some people, for example, take iron supplements to treat iron deficiency or supplements to lower their risk of developing a medical condition such as anaemia and hypertension (Alfolayan and Wintola, 2014). Supplements are usually unnecessary when a person consumes a well-balanced diet and exercise regularly, unless a specific deficiency is identified by a healthcare professional (Numata, 2020). Protein supplements are commonly considered to be safer than other types of supplements (Maughan, 2013). However, the safety of many other nutritional supplements is unclear, as more research is needed to determine their value (da Costa *et al.*, 2021). As a result, the proper use of supplements can help an individual avoid the side effects and toxicities associated with overuse of these products (NIH, 2020). Many supplements, according to the National Institute of Health (NIH), contain

active ingredients that have a strong effect on the body (NIH, 2020). Most nutritional supplements are generally regarded as safe if the user follows the product's instructions (NIH, 2020). On the contrary, high doses of certain nutrients can have negative effects, and even an overdose of certain supplements can cause serious damage and possibly death (U.S. FDA, 2017).

1.2. Protein Supplements

Protein supplements are one of the most popular nutritional supplements that can be obtained from both animals and plants (Ali *et al*., 2019). The increased use of these supplements by athletes, gym-goers, and health-conscious people has contributed to their popularity (Naidoo *et al.*, 2018a; Sanchez-Oliver *et al.*, 2018). The primary goal of using these products is to increase muscle mass and strength, improve exercise recovery, reduce injury during training, improve endurance and performance, and live a healthy lifestyle (Ali *et al*., 2019; Arensberg *et al*., 2014; Kreider *et al*., 2010; Lieberman *et al.*, 2015). According to a recent survey of gym users in South Africa (Fig 1.1), 84% of those who use supplements prefer protein-based supplements as part of their supplement use with exercise (Coopoo *et al*., 2020).

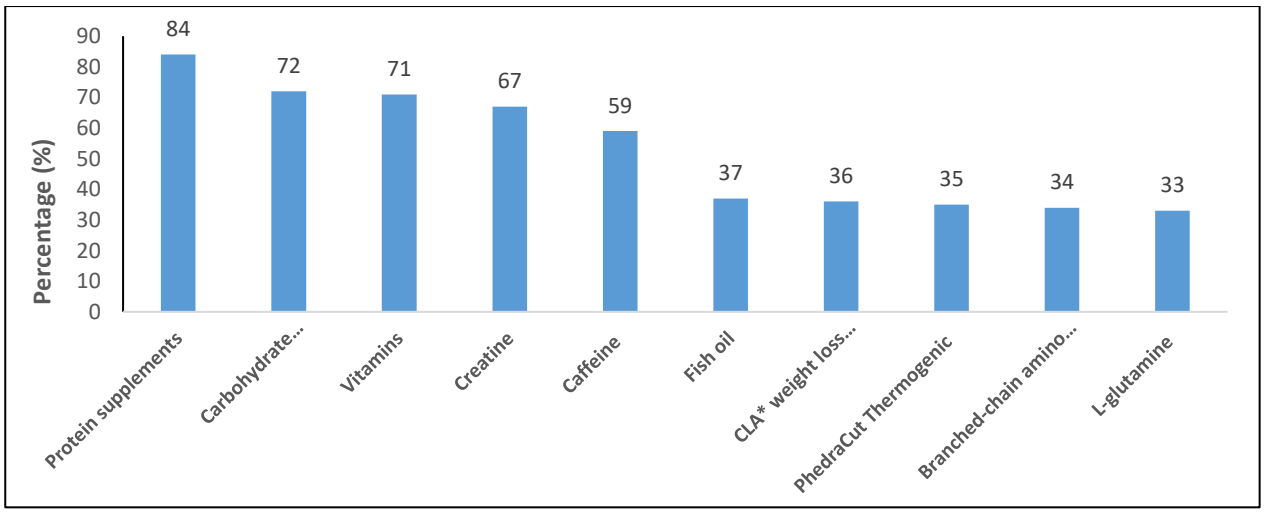

Figure 1.1: Consumption of nutritional supplement by gym users (n=364) in Johannesburg, South Africa (Coopoo *et al*., 2020)

The increased demand for supplements for recreational purposes by demographic groups other than elite athletes, such as gym goers, is said to play a significant role in the industry's growth (Gabriels *et al*., 2015). Globally, the annual growth rate was

6.8% in 2007, while in South Africa, the rate was 7.7% (Naidoo *et al*., 2018a). This expansion can be attributed to manufacturers' aggressive marketing rather than the development of more effective nutritional supplements (Gabriels *et al.*, 2011).

1.3. Protein as a nutrient

To fully understand why protein supplements are among the most popular nutritional supplements, one must first comprehend the importance of protein as a nutrient. Protein is an essential macronutrient for humans in maintaining normal bodily functions (Sá *et al.*, 2020). Proteins, according to Marieb and Hoehn (2016), make up more than half of the body's organic matter and serve the most diverse organic functions. Kårlund *et al.* (2019) emphasised the important role proteins play in tissue growth, maintenance, and repair, as they are a component of all cells, tissues, bones, tissues, and muscles.

Proteins are classified into two types: structural proteins and functional proteins (Marieb and Hoehn, 2016). Structural proteins, such as myosin, actin, haemoglobin, and collagen, play a role in the formation of cell shapes, movement, and the support of major structures such as bones and muscles (Numata, 2020). Functional proteins are involved in a variety of activities in the human body, such as hormones and coordinated activities of different body systems, such as insulin, whereas other proteins, such as immunoglobulins, are responsible for defense against foreign pathogens as part of the immune system (Rose, 2019).

Proteins are made up of amino acids that are linked together by peptide bonds (Numata, 2020). Animals and plants contain twenty different amino acids. There are two types of amino acids: essential amino acids and non-essential amino acids (Table 1.1). Human bodies cannot produce essential amino acids and thus they are obtained from diet (Gavelle *et al*., 2017). Non-essential amino acids can be produced by human bodies, and therefore do not need to come from diet (Boye *et al.,* 2012).

Table 1.1: Essential vs non-essential amino acids (Marieb and Hoehn, 2016)

Essential amino acids	Non-essential amino acids
Histidine	Alanine
Isoleucine	Arginine
Leucine	Asparagine
Lysine	Cysteine
Methionine	Glutamate
Phenylalanine	Glutamine
Threonine	Glycine
Tryptophan	Proline
Valine	Serine
	Taurine
	Tyrosine

A protein is typically composed of 300 or more amino acids (Numata, 2020; Sá *et al.,* 2020). Each protein has a distinct number and sequence of amino acids that can be arranged in a variety of ways (Berg *et al*., 2002). The number and sequence of amino acids determines the shape of the resulting protein. A protein's shape is critical because it influences the protein's function (Alberts *et al*., 2002).

1.3.1. Protein sources and the daily recommended daily intake

Since proteins are constantly used for the growth and maintenance of cells and tissues, our bodies require a constant supply of amino acids for protein synthesis (Berg *et al*., 2002; Marieb and Hoehn, 2016). Adequate protein intake is especially important in infants, childhood, adolescence, pregnancy, lactating women, and with exercise sessions (Sá *et al*., 2020). Humans receive their protein from a variety of sources. Food derived from animals and plants, for example, contain protein and amino acids that are required for the growth and maintenance of our cells and tissues (EFSA, 2012).

Plant-based protein foods like soy, whole grains, and beans are thought to account for 60% of the world's protein supply per capita (Steinke *et al*., 1991). Meat, dairy products, and fish are animal-derived protein foods that account for 70% of protein consumption in the United States alone (Young and Pellet, 1994). A similar trend can be seen in South Africa, where poultry, beef, dairy, and sheep are the main sources of protein for South Africans, according to research (Vorster *et al*., 2013).

The World Health Organization (WHO, 2007) recommends 0.8 g of protein per Kg of body weight to maintain normal bodily processes, which corresponds to approximately 56 g of protein per day for an average male and 46 g of protein per day for females. Protein supplements are not recommended for people who eat a well-balanced diet (Young and Pellet, 1994).

1.4. Source and types of protein supplements

Protein supplements are concentrated protein sources derived from animal and plant foods such as dairy, rice, peas, and rice (Tinsley, 2018;Sanchez-Oliver, 2018). Protein supplements, like other nutritional supplements, come in a variety of forms. However, powder-based protein supplement products are the most popular because they are easier and faster to consume (Coopoo *et al*., 2020; Gabriels *et al*., 2015). There are numerous protein powders available that contain all nine essential amino acids as well as branched-chain amino acids. This type of supplementation can be used based on an individual's nutritional needs or preferences (Almeida *et al*., 2015).

Whey protein powder is one of the most effective ways to introduce protein to daily diet since it is easily absorbed at physiological levels (Schönfeldt *et al*., 2015). This form of protein is produced when the liquid residue of milk is processed into cheese or yoghurt (Sanchez-Oliver, 2018). The liquid is heat-treated by means of evaporation or ultrafiltration, and protein separation and concentration are accomplished through enzymatic or chemical processes (Sanchez-Oliver, 2018). The final stage of the process involves drying to produce whey powder rich in essential proteins and branched chain amino acids (Hoffman *et al*., 2015).

1.4.1. The benefits of protein supplements

Protein supplements have been suggested to stimulate muscle protein synthesis in physically active people, particularly those who do resistance training, such as working out in the gym or athletes (Phillips, 2012; Kuswari *et al*., 2021). Physical activity is said to promote metabolic processes; consequently, when a person is active, the body utilises protein supplements more efficiently than with inactive people (Børsheim *et al*., 2002; Kuswari *et al*., 2021).

According to Tipton and Wolfe (2004), athletes and individuals undergoing muscle-building training have a much higher need for protein in order to support maximum muscle growth. Some athletes and individuals, however, believe that dietary protein is insufficient to meet their protein requirements (Børsheim *et al*., 2002). They regularly use protein supplements in addition to their training and diet regimens to achieve their goal (Phillips, 2012). Proteins, in general, can only be delivered effectively to meet individual needs if all other nutrients required for normal cell function are met (WHO, 2007). As a result, before relying on protein supplements, one must ensure that they are getting enough protein from their diet. This can be accomplished by consuming high-quality foods with adequate protein during meals for muscle protein synthesis, muscle mass maintenance, and muscle function (Symons *et al*., 2009).

Apart from the use of protein supplements in sports and exercise, there are several other advantages reported, such as weight loss and lower cholesterol levels in overweight people (Frestedt *et al*., 2008). Although the evidence is inconclusive, protein supplements have been shown to have anti-cancer properties as well (Frestedt *et al*., 2008; Pal *et al.*, 2010).

1.4.2. Risks associated with supplements

Many countries, including South Africa, do not classify nutritional supplements as medicine, therefore they are not subject to the same stringent regulations as pharmaceutical drugs (Naidoo *et al*., 2018a). The Food and Drug Administration of the United States (U.S. FDA) does not play a role in determining whether nutritional supplements are effective before they are commercially available on the market (U.S. FDA, 2017). The same is true in South Africa, where there is currently no authority in charge of nutritional supplement quality control and regulation (Schönfeldt *et al.,* 2019; Naidoo *et al.,* 2018a). Manufacturers are often responsible for ensuring the safety and efficacy of their products; however, they are not required to demonstrate this (Garthe and Maughan, 2018).

The lack of proper regulations in the nutritional supplement industry has created a gap in the market, resulting in poor manufacturing practices (da Costa *et al.*, 2021). Unscrupulous supplement manufacturers have been reported in recent years to (i) falsely advertise or represent product contents by mislabelling, (ii) adulterate product

by omitting or adding substances that may make the product to appear to be of good quality, and (iii) add prohibited substances in sports and potentially harmful substances that are not listed on labels, which may have health implications (Gabriels *et al.,* 2015; Schönfeldt *et al.,* 2019; Naidoo *et al.,* 2018b). These practices have gone unchecked for a long time, which has alarmed proponents of this industry and prompted focused research on supplement products. Consequently, researchers have scrutinized each of these supplement products' areas of concern, and each component will be discussed in this section.

1.5. Legislation of supplements

For many years, the regulation of nutritional supplements has been a contentious issue because they are not classified as medicines, but rather as food (Hassan *et al*., 2020). Furthermore, supplements are subject to less stringent regulations than over the counter or prescription drugs (NIH, 2020). Due to a lack of regulation in the nutritional supplement industry, unethical manufacturers have mislabelled and added harmful substances to their products (Naidoo *et al.,* 2018b). Supplements are regulated at the national level rather than globally, and regulations differ from one country to the next (Garthe and Maughan, 2018). Nutritional supplements are regulated and acted upon by different types of legislation around the world, including in South Africa, in stark contrast to those that regulate and are acted upon for medicine (Gabriels, 2013). As an example, this section will look at the laws and regulations governing the nutritional in South Africa.

The legislation in South Africa does not clearly define the regulation of nutritional supplements (Naidoo *et al*., 2018a). The unregulated supplement industry provides an opportunistic environment for false nutrition claims and the adulteration of undeclared substances, both of which can be harmful to consumers (Gabriels *et al*., 2015; Schönfeldt *et al*., 2019). The SA Institute for Drug-Free Sport (SAIDS) has expressed concern over consumers' lack of awareness about the safety and governance of these products, implying that demand for these products remains high and manufacturers are unwilling to change the status quo (Gabriels *et al*., 2011). The lack of consumer awareness of the supplement industry creates the false impression that products are regulated as medicine (Gibson and Taylor, 2010). This provides supplement users with an unfounded sense of security that label claims and efficacy

compliance have been met prior to marketing checks (Carvey *et al*., 2012; Naidoo *et al*., 2018b). South African supplement consumers, according to Gabriels *et al.* (2011), are at risk due to a lack of industry control.

For years, SA's regulation of supplements has been obscure (Naidoo *et al.,* 2018b). However, sections of various Acts in the South African Constitution deal with specific aspects of nutritional supplements (Gabriels *et al*., 2011). The following Acts are associated with the governance of nutritional supplements in South Africa: (i)The Consumer Protection Act (CPA), (ii) The National Health Act, (iii) The Medicines and Related Substances and Amendment Act, (iv) The SA Institute for Drug-Free Sport and Amendment Act, and (v) The Medical Research Council Act (Gabriels *et al*., 2011).

Despite the fact that these laws do not directly regulate supplements, they are intended to promote supplement-related regulatory enforcement, consumer protection, and education (Gabriels *et al*., 2011; Naidoo *et al*., 2018b). For example, the CPA aims to protect consumers from product hazards and maintain safety, but it is limited in terms of liability claims jurisdiction (Gabriels *et al*., 2011; Gabriels *et al*., 2012). SAIDS is concerned with testing for prohibited substances used by athletes, which can result in a positive drug test with the use of adulterated or contaminated nutritional supplements (Gabriels *et al*., 2011). As a result, none of these Acts or institutions are directly involved in nutritional supplement regulation.

Some changes have been implemented by the newly formed South African Health Products Regulatory Authority (SAHPRA). Products that make medicinal claims, for example (e.g., testosterone boosters, fat loss agents), are now classified as complementary medicines and fall under the scope of the "Medicines and Related Substances Act 101 of 1965" (Naidoo *et al*., 2018a; Schönfeldt *et al*., 2015). SAHPRA issued guidelines on complementary medicine regulations in June 2020. SAHPRA defines health supplements as any substance, extract, or mixture as determined by the Authority, sold in dosage forms, or purported to be used in restoring, correcting, or modifying any physical or mental state by – (a) complementing health; (b) supplementing the diet; or (c) a nutritional effect (SAHPRA, 2020). This demonstrates that products in these categories are now strictly regulated and controlled by the government before they are commercially

available. However, for a variety of reasons, these regulations are still insufficient to regulate the South African nutritional supplement industry. For example, in terms of protein source, the SAHPRA guidelines state that if there is no statement that the product made or inferred, then the product may be supplied with claims that comply with Foodstuff legislation (SAHPRA, 2020). Furthermore, products that are not in the form of doses, tablets, soft gels, or capsules are governed by the Department of Health (DOH) R429 draft regulations governing food labelling and advertising (DOH, 2011). As a result, there is a void in the supplement industry, and the regulation of products such as whey protein and other protein powder blends is in a predicament.

Policymakers were expected to shift in favour of stricter regulations, according to industry advocates. The new SAHPRA guidelines, on the other hand, have had little impact on how the South African nutritional supplement industry is regulated. Because there is no authority that directly enforces quality controls prior to entering the market, manufacturers can continue to make unsubstantiated claims at will and fraudulently add hazardous substances to products. As a result, interested parties are now responsible for investigating and conducting studies on these market supplement products in order to provide the government and relevant authorities with substantial evidence demonstrating that some of these products are not suitable for consumption and may have adverse health effects. This is expected to encourage the further development of nutritional supplement policies while also ensuring consumer safety.

1.6. Legislation relating to labelling information on supplements

Food label information is important not only because it is required by law, but also because it allows consumers to better understand the nutritional value of the food (Wahab, 2018). As a result, the primary function of food labelling is to inform consumers and sell products (Koen *et al*., 2016). The purpose of food labelling has become increasingly complex in recent years as a result of the influence of food legislation, food companies, public authorities, and consumers (Cheftel, 2005).

In South Africa, a label is defined as "any tag, brand, mark, pictorial, graphic, or other descriptive matter, which is written, printed, stencilled, marked, embossed, impressed upon, or permanently attached to a container of a foodstuff, and includes labelling for

the purpose of promoting its sale or disposal" (DOH, 2011). According to the World Health Organization, food labels are "any written, printed, or graphic matter that is present on the label, accompanies the food, or is displayed near the food, including that for the purpose of promoting its sale or disposal" (Hawkes, 2004).
This information is typically reported on nutritional supplements as a Supplemental Facts label, which lists the active ingredients, serving size, and other ingredients such as fillers, binders, and flavours (NIH, 2020). Certain health-related claims may also appear on the label of nutritional supplements. In the United States, for example, supplement manufacturers may claim that their products can promote or support bodily functions such as immunity (NIH, 2020). According to the U.S. FDA, these claims must be followed by the words "This statement has not been evaluated by the Food and Drug Administration." The purpose of this product is not to diagnose, treat, cure, or prevent any disease" (U.S. FDA, 2017).

Nutritional supplements, as previously stated, are classified as food. As a result, supplement products sold on the South African market for consumption must still adhere to the guidelines outlined in South African Department of Health Article R.146 (labelling and advertising of foodstuffs) when displaying information on the label such as date marking, nutritional information, statements/claims, and allergens (DOH, 2011).

Similarly, supplement manufacturers in the United States must adhere to certain labelling guidelines, such as the DSHEA of 1994 and the Nutrition Labelling and Education Act of 1990, which are enforced by the U.S. FDA (U.S. FDA, 2005). These guidelines limit what can be said about a product, not its alleged benefits. Manufacturers, however, continue to claim that their product can "enhance the immune system" or "treat" specific conditions, even though there is insufficient scientific evidence to support such claims (U.S. FDA, 2017).

Several studies have found that manufacturers in the nutritional supplement industry frequently evade these labelling requirements due to poor product regulation. As a result, manufacturers' claims on product labels are frequently unsubstantiated by peer review or scientific evidence (Lambert, 2007). Furthermore, consumers frequently believe these products without question, resulting in adverse effects that

may be attributed to adulterants or contaminants that are not specified on the product label (Gabriels *et al*., 2011; van der Merwe and Grobbelaar, 2005; van der Merwe and Kruger, 1992). In general, nutritional supplement labels do not always accurately reflect the content, and consumers are unable to differentiate between true and false claims on these labels (Lambert, 2006; Prins, 2008).

1.7. Adulteration in supplements

Food adulteration is the intentional addition of another substance to food in order to increase the quantity of raw or prepared food, which may result in a reduction in the actual quality of the food (Manoharan *et al*., 2021). Nutritional supplements, as previously stated, fall under the food category in South Africa. In addition to the supplement industry's lack of regulations, studies have shown that unethical manufacturers may add harmful substances to their products in order to increase the quantity of their products, thereby adulterating supplements (Rocha *et al*., 2016). Several studies have discovered a variety of undeclared substances that are not listed on nutritional supplement labels. Products such as antidepressants, nifedipine, sildenafil, anabolic-androgenic steroids, and prohormones are added due to their biological effects, while contaminants/adulterants such melamine, heavy metals, and pesticides have all been found in these products (Gabriels *et al*., 2018; Gabriels *et al*., 2015; Geyer *et al*., 2004; van der Merwe *et al*., 2004; Chen, 2020).

According to Brown (2017), pharmaceutical drugs are the most common adulterants in nutritional supplements. When ingested in large quantities, some of these substances can have disastrous health consequences, and consumers consume these products unaware of the dangers lurking within (Gibson and Taylor, 2005; Gabriels *et al*., 2011; Da Justa Neves and Caldas, 2015; Naidoo *et al*., 2018b). Stroke, acute liver injury, kidney failure, and, in some cases, death are all serious side effects of adulterated supplements (Czepielewska *et al*., 2018).

Melamine and its derivatives have received attention in recent years as a result of their widespread use as an adulterant (Sun *et al*., 2010a). Because melamine and cyanuric acid are structurally similar to protein and have properties (Table 1.2) that are appealing for adulteration in products such as dairy products and protein supplements (Fig 1.2). (Byungchul, 2009; WHO, 2013). Since Kjeldahl and Dumas methods measure nitrogen from the entire sample for protein quantification, these

compounds evade detection. As a result, unethical manufacturers employ these techniques to conceal the presence of these compounds, giving the product the appearance of being high in protein. However, because there have been fewer studies on these compounds in relation to protein supplements, melamine, cyanuric acid, and uric acid have emerged as compounds of interest (Kuswari *et al*., 2021; Schönfeldt *et al*., 2019). Due to the lack of regulations, studies have suggested that these compounds may be prevalent in protein supplement products (Montesano *et al.,* 2013; Gabriels *et al.,*2015).

Table 1.2: Properties of the selected adulterants (Pubchem, 2021)

	Melamine	**Cyanuric Acid**	**Uric Acid**
Chemical name (IUPAC name)	1,3,5 – triazine-2,4,6-triamine	1,3,5-triazine-2,4,6-triol	7,9- dihydro-1H-purine-2,6,8-trione
Chemical formula	$C_3H_6N_6$	$C_3H_3N_3O_3$	$C_5H_4N_4O_4$
Molecular weight (g/mol)	126.12	129.07	168.11
Solubility (mg/L)	3240	2000	6000
pKa	5.0	6.9	5.6
Pharmacology	Absorbed in the gastrointestinal tract (GIT) in humans (Cruywagen *et al.,* 2011) Plasma half-life of 3hrs (OECD, 1998) Eliminated through urine within 24hrs (Mast *et al.,* 1983)	Rapidly absorbed in the GIT (Bischoff, 2017). 98% eliminated through urine unaltered after 24hrs (Allen *et al*., 1982).	By-product of purine metabolism in humans (Villegas *et al*., 2012). Distribution includes blood, plasma, and traces in various organs (Pubchem, 2021). Excreted through urine (Pubchem, 2021)

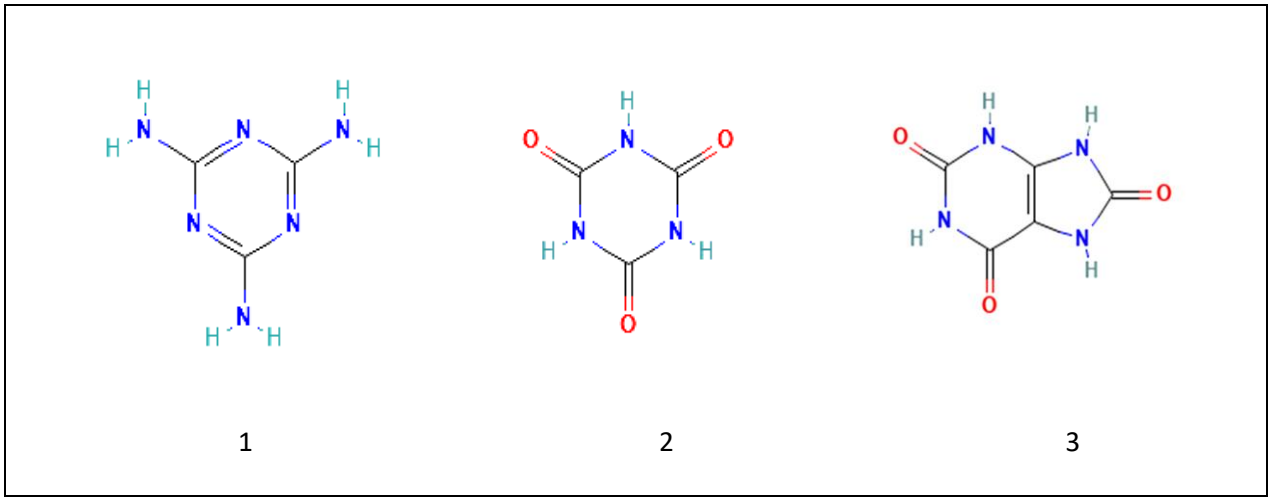

Figure 1.1: Structural formulas of the adulterants. 1. Melamine, 2. Cyanuric acid, and 3. Uric acid (Pubchem, 2021)

These added materials are typically not declared and are done to reduce costs and/or increase the quantity of a given food product (Teen Teh and Dykes, 2014). Adulteration is a worldwide phenomenon that has existed since ancient times and affects nearly all foods (Banerjee *et al.,* 2017). Adulteration can occur for a variety of reasons. This includes (i) an increase in population, which results in increased food demand, and food producers take advantage of this by adding cheaper materials to adulterate food and increase profits; (ii) practiced by those who do not have a proper understanding of its dangers, due to a lack of awareness and proper knowledge; and (iii) the government lacks measures to control it (Zhang *et al.,* 2021). These practices are similar to those observed in the supplement industry (Naidoo *et al.,* 2018a; Schönfeldt *et al*., 2019).

1.7.1. Adulteration incidences of melamine and its derivatives

In March 2007, the U.S. FDA received numerous reports of cat and dog illness and death in the United States (Byungchul, 2009). This incident prompted an U.S. FDA investigation, which revealed that these animals had consumed melamine-contaminated pet food (U.S. FDA, 2009). As a result of this incident, hundreds of dog and cat deaths and kidney diseases have been reported (U.S. FDA, 2009). The U.S. FDA responded by recalling all of the affected pet food products, making it the largest recall in the agency's history (Dobson *et al*., 2008). Furthermore, studies have revealed the presence of melamine and other structurally related compounds such as cyanuric acid, ammelide, and ammeline in contaminated pet food (Cohen, 2008).

According to the WHO (2009), the pet food manufacturers of the implicated products deliberately added melamine to increase the apparent protein content and reduce costs. Melamine contamination in pet food has also been reported in Taiwan and South Korea in 2004, as well as in Italy in 2008. (Brown *et al*., 2007; Cocchi *et a*l., 2009). The 2007 outbreak coincided with the same clinical, histological, and toxicological findings in both cases (Byungchul, 2009).

China reported in September 2008 that over 50,000 infants had been admitted to hospitals with renal failure and kidney stones (Sun *et al*., 2010a). This incident resulted in six deaths, which research determined were caused by infants who consumed melamine-contaminated baby formula (Chan *et al*., 2008; Kuehn, 2009). Frequent consumption of tainted infant formula can aggravate the situation by not allowing enough time between meals to eliminate melamine, resulting in a gradual increase in body contaminants (Gabriels *et al*., 2015; Yan *et al*., 2012). Despite Chinese authorities' efforts to contain the situation, the United Nations (UN) proceeded to warn countries about importing dairy products from China, and the European Union followed by banning the imports of Chinese baby food (Perry 2008, Byungchul, 2009).

These melamine food adulteration incidents in pets and humans have elicited a wide range of reactions and piqued the interest of researchers worldwide in investigating other potential sources of melamine contamination (da Costa *et al.*, 2021). According to studies, the general population can be exposed to melamine through the use of everyday products such as melamine-containing tableware. Melamine migration to food and liquids as a result of high temperatures can exacerbate the situation (Chien *et al*., 2011; Sathyanarayana *et al*., 2019). According to Zhu and Kannan (2019), the sources of melamine and its derivatives are food packaging and animal feed. Lin *et al*. (2008) and Baek *et al*. (2014) postulated that the specific route of exposure could be due to ingestion of melamine-contaminated food.

Studies have also revealed the presence of melamine in nutritional supplement products (Montesano *et al*., 2013; Gabriels *et al*., 2015). There is no doubt that the presence of melamine in these products is due to a lack of adequate industry regulations.

These events culminated in concerns about the safety and quality of these food products. To protect public health, organizations such as the World Health Organization (WHO) and the United States Food and Drug Administration (U.S. FDA) established the Tolerable Daily Intake (TDI) of melamine and its derivatives in food, as summarized in Table 1.3. (Filazi *et al*., 2012). In several countries, the most common TDI for melamine is 2.5 ppm in foods and dairy products, and 1 ppm in infant formula (Sun *et al*., 2010a). According to the WHO (2008), the TDI for cyanuric acid in foodstuffs is 1.5 mg/kg because it appears to be more toxic when combined with melamine.

Table 1.3: Tolerable daily intake (TDI) for melamine (Gabriels *et al*., 2015)

Organisation	TDI maximum dose
CAC	Infant formula – 1mg/kg Food and animal feed – 2.5mg/kg
EFSA	0.2mg/kg
U.S. FDA	2.5ppm Infant formula – 1ppm
SA	2.5ppm
WHO	0.2mg/kg Infant formula – 1ppm

1.7.2. Toxicity of melamine, cyanuric acid, and uric acid

Early melamine research presumed that melamine alone could cause kidney failure due to crystals found in the kidneys (Byungchul, 2009). Recent research has demonstrated that melamine forms hydrogen bonds with cyanuric acid in the kidneys to form an insoluble crystalline complex known as melamine-cyanurate (Fig 1.3). (Dobson *et al*., 2008; Cohen, 2008; Sun *et al*., 2010a). According to research, when these two compounds combine to form a crystalline complex, the crystals precipitate in the kidneys, resulting in kidney failure (WHO, 2009).

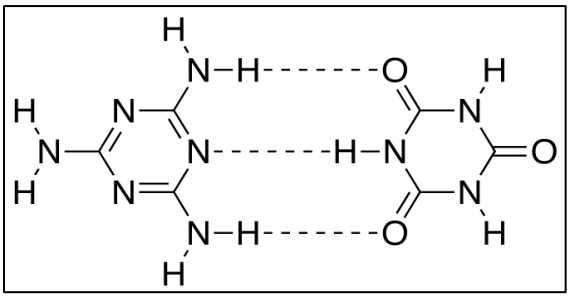

Figure 1.2: Chemical structure of melamine-cyanurate with hydrogen bonds between the two compounds (Sun *et al*.,2010a)

Melamine and cyanuric acid have low toxicity to humans and animals when consumed separately, but their toxicity increases when consumed together (Chao *et al*., 2011). Puschner *et al*. (2007) confirmed this, by demonstrating that only melamine or cyanuric acid exposure in the diet does not cause renal failure in cats. However, when melamine and cyanuric acid were combined in high doses in cat food, cats developed kidney failure and had to be euthanized after 48 hours (Puschner *et al*., 2007). Another study discovered that when rats were fed a melamine and cyanuric acid mixture, the nephrons formed crystals, causing renal damage (Stine *et al.,* 2014).

To maintain a normal level of uric acid in the serum, a balance must be maintained between uric acid synthesis and degradation (Wijemanne *et al*., 2018). Abnormal uric acid levels in serum and urine are linked to a lack of purine-metabolizing enzymes and have been linked to kidney disease, cardiovascular disease, type 2 diabetes, gouty arthritis, obesity, and hypertension (Wei *et al*., 2009; Jinnnah *et al*., 2013; Maiuolo *et al*., 2016; Wijemanne *et al*., 2018). Zhang *et al*. (2008) demonstrated that uric acid plays a role in melamine-related kidney injury caused by crystal formation in humans. According to the study's findings, melamine and uric acid form a crystal

complex in the kidney in the absence of cyanuric acid or other melamine analogues (WHO, 2008; Sun *et al*., 2010b; Rai and Banerjee, 2017).

Melamine and its derivatives have been extensively studied in terms of their effects on human kidney function. A study conducted in the United States found concentrations of melamine and cyanuric acid in children's urine that were higher than those reported by other countries (Sathyanarayana *et al*., 2019). They discovered elevated levels of kidney injury molecule 1 (KIM1) in the cohort, as well as high levels of cyanuric acid. This implies that exposure to these compounds raises the risk of kidney damage and/or failure. Melamine exposure was found to be directly related to high levels of N-acetyl b-D-glucosaminidase (NAG), a urine marker of early renal tubular damage, in urine samples from adults in Taiwan (Liu *et al*.,2017).

According to the literature, melamine and its derivatives may be linked to other health effects such as reproductive, endocrine, and neurological function (Bolden *et al*., 2017). Melamine has been shown to be toxic to reproductive systems in rodents, piglets, and humans (Stine *et al*., 2014; Wang *et al*., 2013; Chang *et al*., 2014). Melamine has been shown to pass through the placenta to the foetus in pregnant rats, affecting neonates (Chu *et al*., 2017). This is a major public health concern, indicating that exposure to this toxic compound may harm unborn babies. Currently, little is known about the likely adulteration of melamine and its derivatives in protein supplements, as well as the impact these chemicals may have on persons who use supplements on a regular basis, such as athletes.

1.8. Analytical methods

Melamine and its derivatives have been linked to a variety of health problems in both humans and animals, according to research. The adulteration of foods using these compounds is economically motivated and is extremely concerning because it not only lowers food quality but also exposes consumers to serious health risks (Schieber, 2018; Bansal *et al*., 2017). As a result, detecting food adulteration or contamination is a top priority in order to ensure food safety and protect consumers from fraudulent activities. The development of rapid, efficient, and dependable methods for detecting food adulteration is on the rise (Banerjee *et al*., 2017). Spectroscopy, chromatography, stable isotope analysis, proteomics, metabolomics,

enzymatic methods, and DNA-based technologies are among these methods (Schieber, 2018).

Various studies have documented melamine exposure in great detail using the abovementioned techniques in different food sources. However, research on protein supplements remains lacking (Gabriels *et al*., 2015). Furthermore, as the supplement industry expands, consumers are becoming more vulnerable to health issues such as renal failure. There is a growing need to rigorously evaluate these products and collect enough data as evidence to persuade legislators to enact strong policies to regulate the supplement industry, particularly in South Africa.

As a result, in order to achieve this goal, appropriate methods for conducting in-depth research on protein supplements must be used. Many methods for analysing melamine and its derivatives in food and pet foodstuffs have been proposed in the scientific literature since the melamine incidents in 2007 and 2008, which drew international attention (WHO, 2008; Montesano *et al*., 2013). This section will focus on various methods and determine which ones are best for detecting adulterants and quantifying the protein content of protein supplement products.

1.8.1. Protein quantification methods

Studies have shown that there are some discrepancies between the content on the label and the actual content of protein supplements (da Costa *et al.*, 2021). According to the findings of these studies, the manufacturers of these products use non-protein substances such as melamine to increase the apparent protein content (Gabriels *et al*., 2015; Kuswari *et al*., 2021). Consequently, it is crucial to test commercially available protein supplement products in a South African setting. Part of this research looked at whether the protein content measured in the laboratory correlates with the protein percentage stated on the product label.

Johnson (2012) demonstrated that protein quantification is pivotal to protein research in a variety of research topics. Multiple methods for measuring proteins have been developed, ranging from a single type to a more complex mixture of proteins (Jain *et al*., 2020; Johnson, 2012). These methods' fundamental principles include determining nitrogen, peptide bonds, aromatic amino acids, dye binding capacity, light scattering properties, and ultraviolet absorption (Jain *et al*., 2020). The Bradford

assay, protein absorbance at 280 nm, the Lowry, and the Bicinchoninic acid (BCA) assays are the most commonly used methods for quantifying proteins. Other methods, such as Western blot, mass spectrometry, and Enzyme-Linked Immunosorbent Assay (ELISA), are frequently used in research and diagnostics to quantify individual proteins (Johnson, 2012).

When selecting a protein quantification method, it is essential to first understand the nature of the protein sample in order to determine the best method (ThermoFisher Scientific, 2018; Jain *et al*., 2020). Protein in food is complex, and so is its analysis (Jain *et al*, 2020). There are several methods for quantifying proteins in food (Mæhre *et al*., 2018). The Dumas and Kjeldahl methods have been used in recent studies on protein supplements (Schonfeldt *et al*., 2019; Kuswari *et al*., 2021). One limitation of these methods is that they do not consider foods that may contain nitrogen from other compounds (such as creatine, choline, and free amino acids) (FAO, 2003). According to the literature, some protein supplement products may contain nitrogen-rich compounds like melamine, which can cause errors in total protein concentration (Gabriels *et al*., 2015).

Gabriels *et al.* (2015) recommend using multi-protein detection methods such as BCA, Lowry, and Bradford assays. The use of these methods, selected for their peptide and amino acid specificity, can assist in avoiding the detection of adulterants in protein samples, which traditional methods fail to do. (Johnson, 2012, Field and Field, 2010). Finally, a study should be conducted in conjunction with these assays to determine whether adulterants such as melamine, cyanuric acid, and uric acid can interfere with protein detection and the extent to which these compounds affect protein quantification in the samples.

1.8.2. Methods for detecting adulterants

Following the scandal of melamine being discovered in food products, much attention was given to research focusing on melamine detection in recent years. So far, several methods for this purpose have been published, including CE, LC-MS, HPLC, GC-MS, NMR spectroscopy, LTP, and DAPCI, as summarized in Table 1.4 below (Sun *et al*.,2010a). Colorimetric sensors based on polydiacetylene (PDA) liposomes, surface-enhanced Raman spectroscopy (SERS), and quantum dots-based fluorescence are other techniques used to analyse melamine (WHO, 2013). Each of

these methods is chosen based on its own set of advantages and disadvantages. These methods are the most sensitive and accurate, but they are often too costly and time consuming to use for routine analysis (Rai and Banerjee, 2017).

Table 1.4: A summary of published methods for determination of melamine

Method	Matrix	Sensitivity (LOD/LOQ)	Reference
LC-MS^2	Catfish & Trout, serum	0.8µg/ml	Stine *et al.* (2012)
HPLC-UV	Milk powder	0.006mg/kg, 0.019mg/kg	Londoño *et al.* (2017)
LC-MS^2	Infant formula powder	4ng/g	Tittlemier *et al.* (2010)
HPLC-DAD	Liquid milk	18mg/kg, 60mg/kg	Sun *et al.* (2010b)
NMR	Milk product	0.69mg/kg	Lachenmeier *et al.* (2009)
LC-MS	Nutritional supplements	19ng/g	Gabriels *et al.* (2015)
GC-MS	Muscle, egg & milk	10mg/kg	Zhu *et al.* (2009)
CE-DAD	Milk	0.047mg/ml	Chen *et al.* (2009)
LTP- MS^2	Milk powder	6ppb	Huang *et al.* (2009)
DAPCI-MS	Milk	0.8mg/kg	Yang *et al.* (2009)
NIR/FTIR	Infant formula powder	1ppm	He *et al.* (2008)

LC-MS^2, Liquid chromatography-triple-quadruple tandem mass spectrometry; HPLC-UV, High-performance liquid chromatography tandem ultra-violet detector; HPLC-DAD, High-performance liquid chromatography tandem diode-array detection; NMR, Nuclear magnetic resonance spectroscopy; LC-MS, Liquid chromatography tandem mass spectrometry; GC-MS, Gas chromatography-mass spectrometry; CE-DAD, Capillary electrophoresis and diode-array detection; LTP- MS^2, Low-temperature plasma probe combined with tandem mass spectrometry; DAPCI-MS, Desorption atmospheric pressure chemical ionization mass spectrometry; NIR/FTIR, Near-infrared/mid-infrared spectroscopy

HPLC is a widely used method for detecting melamine and its analogues in a variety of matrices simultaneously (He *et al*., 2008; Kim *et al*., 2008). This instrumental method has been combined with a range of different selective detection techniques. According to WHO (2008), the highest degree of sensitivity and selectivity is provided by liquid-chromatography triple-quadruple tandem mass spectrometry (MS^2), followed by single-stage mass spectrometry (MS), diode array detection (DAD), and finally ultraviolet (UV) absorption. As previously stated, some laboratories have limited resources but are required to perform routine monitoring for contaminated foods. These laboratories cannot use the most sensitive detectors but can choose inexpensive and commonly used detectors such as DAD and UV-Vis.

Melamine was detected in pet food using LC-MS^2 with LOD of 0.001 mg/kg (Varelis and Jeskelis, 2008; Taylor *et al*., 2008). Gabriels *et al*. (2015) demonstrated high sensitivity for detecting melamine in nutritional supplements using LC-MS^2, with a lower limit of quantification (LLOQ) of 1.92 ng/ml. Montesano *et al*. (2013) detected melamine in protein supplements using HPLC-DAD and achieved an LLOQ of 0.05 mg/kg.

Several studies have shown that HPLC-UV can detect melamine at sensitive levels. Filazi et al. (2012), for example, used HPLC-UV to detect melamine in dairy products with a LOD of 35 *µ*g/kg. Maleki *et al.* (2018) observed that detection of melamine in infant formula was as sensitive, with a LOD of 30 *µ*g/kg. Using HPLC-UV, simultaneous detection of melamine and its analogues, ammelide, ammeline, and cyanuric acid in cereal flour allowed for detection levels (LOD) ranging from 5 µg/g to 90 *µ*g/g (Ehling *et al*.,2007). As a result, the findings of these studies using HPLC-UV are comparable to those of more sensitive detectors. However, due to its low sensitivity, few studies have used the HPLC-UV method to determine the prevalence of melamine and its analogues in more complex matrices (Sun *et al*., 2010a).

Lastly, the HPLC-UV method was used in this study to confirm the presence of adulterants (melamine, cyanuric acid, and uric acid) in protein supplements. A multi-protein approach was used to quantify the protein content of protein supplement products, utilising BCA, Lowry, and Bradford assays as recommended in the literature.

1.9. Aim and objectives

The health of unsuspecting consumers is threatened due to an increase in adulteration and inaccurate labelling practices of protein supplement products. According to research, unethical manufacturers omit key nutrients such as protein to reduce costs and falsify nutritional quantities on the label. Protein supplement products must be thoroughly evaluated and scrutinized from the inside out to ensure the safety of individuals who use these products. The aim of this study was to evaluate the labelling information on commercially available protein supplement products, perform laboratory quantification for actual protein content, and determine the prevalence of adulterants in these products.

To achieve this aim, the objectives of this study were:

- To evaluate the product label information for selected protein supplement products that are commercially available in South Africa.
- To use a multi-protein assay approach to determine the actual protein content in the selected protein supplement products.
- To establish an in-house laboratory HPLC validated method for the simultaneous determination of melamine, cyanuric acid, and uric acid.
- To use the established HPLC method to assess and quantify for melamine, cyanuric acid, and uric acid in the selected protein supplement products.

Chapter Two – Methods & Materials

CHAPTER 2 - METHODS & MATERIALS

2.1. Materials used

Protein supplements products were purchased from Dischem Retail Pharmacy in Rosebank Mall and Chrome Supplements at The Zone Shopping Centre in Rosebank in May 2019. The selection of protein supplement product was based on the retailer's recommendations and the best-selling products. A total of twenty-one (n = 21) commercially available products were obtained, which was informed by the available research budget. Nineteen of the twenty-one supplements were manufactured by different companies, while two were manufactured by the same company. These supplements were marketed to both active people who take them for health reasons and athletes.

The highest quality reagents and chemicals used in this study were obtained from various suppliers, as shown below (Table 2.1). They were handled and stored in accordance with the supplier's instructions or supplied manuals.

Table 2.1: List of chemicals and reagents used in this study

Chemicals/Materials/Reagents	Supplier
Melamine (analytical grade, 99%)	Sigma-Aldrich (USA)
Cyanuric acid (analytical grade, 98%)	Sigma-Aldrich (USA)
Uric acid (analytical grade, ≥99%)	Sigma-Aldrich (USA)
Methanol (HPLC gradient grade)	Sigma-Aldrich (Germany)
Acetonitrile (HPLC gradient grade)	Sigma-Aldrich (France)
Acetic acid	Rochelle Chemicals (SA)
Formic acid	Sigma-Aldrich (UK)
Ammonium acetate	Fischer Scientific (UK)
Dipotassium hydrogen orthophosphate	Sigma-Aldrich (Germany)
Potassium dihydrogen phosphate	Labchem (SA)
Sodium 1-heptanesulfonate	Sigma-Aldrich (Germany)
Trichloroacetic acid	Ace Chemicals (SA)

2.2. Product label information study

In Chapter 1 the importance of specific knowledge and the understating of claims and labelling on protein supplements was highlighted. This is part of a practical intervention to ensure the maintenance of quality products and to ensure the establishment of knowledge and awareness. In this section, product label information and statements are evaluated using predetermined categories for the selected 21 protein supplement products (Gabriels *et al.*, 2012). For the purposes of this research, the categories investigated were (i) general labelling information, (ii) advertising/ consumer public relations, (iii) claims, (iv) disclaimers, (v) quality assurance, and (iv) scientific pledge warnings. In addition, the price of each product was evaluated to determine the cost per serving and the cost of protein per gram of the product (Equation 2.1-2.2). at the time of purchases (May 2019).

$$\text{Cost per serving} = \frac{\text{Product price}}{\text{No. of servings per product}}$$

Equation 2. 1: The formula to calculate the cost of product per serving

$$\text{Cost per gram of protein} = \frac{\text{Cost per serving}}{\text{No. of servings per grams of protein per serving}}$$

Equation 2. 2: The formula to calculate the cost of protein per gram of product

2.2.1. Data analysis

The data from each protein supplement label were captured and the information was transcribed into an Excel spreadsheet (Microsoft® Office Excel, 2016). These statements were captured as “yes” or “no” statements. Following that, these statements were transcribed as "1" or "0" to allow the statistics program to read them and quantify them. Excel file was imported to Statistica software (version 13.3) for the statistical analysis.

2.3. Protein content quantification study

2.3.1. Sample preparation and stock solutions

The preparation of the protein sample was performed in duplicate for each product respectively. This was done by accurately weighing 20.0 mg of the protein supplement powder on an analytical balance (Radwag, AS 310.R2, Poland). The samples were then dissolved in 50 ml polypropylene tube with 10 ml double-distilled water. The protein samples were stored in 4^{O}C until required for the protein assays. Stock solutions of melamine (1000 µg.ml^{-1}), cyanuric acid (1000 µg.ml^{-1}) and uric acid (100 µg.ml^{-1}) were prepared in deionised water. The stock solutions were stored in -18 ^{O}C and had integrity of up to a month, after which they started degrading.

2.3.2. Working standards

Working standard solutions were prepared daily, by diluting the stock solutions with deionised water as previously described to final concentrations of 50,100, and 250 µg.ml^{-1} for melamine and cyanuric acid 50,100 and 250 µg.ml^{-1} and uric acid 25, 50 and 100 ug.ml^{-1}, for respective experiments. All the protein assays were performed using a Thermo MULTISKAN GO Microplate spectrometer (ThermoFisher Scientific, USA).

2.3.3. BCA assay

The BCA assay was performed using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, catalog number 23227). This assay is based on the reduction of Cu^{2+} to Cu^{1+} (cuprous cation) by protein under alkaline conditions, a process which is known as the Biuret reaction (Fig 2.1.a.). The Biuret reaction is affected by several amino acid residues, such as cysteine, cystine, tyrosine, tryptophan, and the peptide backbone (Johnson, 2012). The second step consists of the highly selective and sensitive colorimetric detection of the copper cation using the BCA reagent (ThermoFisher Scientific, 2018). The result of this reaction is a purple colour product formed by the chelation of two BCA-protein molecules with one cuprous cation (ThermoFisher Scientific, 2018). The reaction can be measured using a spectrometer at 562 nm, and

the absorption of the reduced copper ion is directly proportional to the concentration of proteins in the solution (Smith *et al.,* 1985).

For each test sample, 25 µl was added to 96-well microplate. The BCA reagent A (containing sodium carbonate, sodium bicarbonate, Pierce™ BCA detection reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA reagent B (with 4% cupric sulphate) were mixed in a ratio 50:1 in a 50 ml polypropylene tube. Approximately 200µl of this mixture was added to each well with test samples in the microplate to bring final volume to 225 µl. The microplates with test samples were incubated at 37^{o}C for 30 minutes (Fig 2.2.a). The samples were analysed by spectrometer to determine their absorbance at 562 nm. Standard curves were prepared using bovine serum albumin (BSA) as a control for all experiments. The BSA concentration range of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg.ml^{-1} was prepared. Then a standard curve of the concentrations versus the absorbance at 562 nm was plotted using an Excel spreadsheet. From this, the protein concentration of the test supplement samples was determined.

2.3.4. Lowry assay

A modified Lowry protein assay kit (ThermoFisher Scientific, catalog number 23240) was used to perform this assay (ThermoFisher Scientific, 2018). As with BCA assay, the first step is the Biuret reaction, in which Cu^{2+} is reduced to Cu^{1+} under alkaline conditions, and then a complex is formed by the cuprous cation and peptide bond (Fig 2.1.b.). The second step involves a reduction in the Folin-Ciocalteu reagent to form Cu^{1+}-peptide bond complex, which causes the colour of the solution to turn blue, which can be detected at 750 nm using a spectrometer (Olson and Markwell, 2007). The amount of protein present in the solution is estimated using standard curves of standard solutions of selected proteins, such as BSA (ThermoFisher Scientific, 2018)

A volume of 40 µl BSA protein standard or test sample was added to a 96-well microplate (Fig 2.2.a). Followed by addition of 200 µl of Modified Lowry Reagent (containing cupric sulphate, potassium iodide, and sodium tartrate in an alkaline sodium carbonate buffer) to each well, mixed and incubated at room temperature for 10

minutes. After the incubation, 20 µl of 1X Folin-Ciocalteu reagent was added to the samples in the microplate and incubated at room temperature for 30 minutes. Absorbance reading was done at 750 nm using a spectrometer, and a BSA standard curve was plotted using concentrations versus absorbance readings.

2.3.5. Bradford assay

The Bradford assay is one of the most widely used methods for determining protein concentration and was created by Dr. Marion Bradford in 1976 (Johnson, 2012). The assay uses Coomassie Brilliant blue G250 dye, which forms a complex with proteins in an acidic solution. As a result, the colour is observed to change from brown/green to blue, and the colorimetric response can be measured at 595 nm with a spectrometer (Fig 2.1.c) (ThermoFisher Scientific, 2018). The Coomassie dye mostly binds to arginine, tryptophan, tyrosine, histidine, and phenylalanine residues (Olson and Markwell, 2007). For Bradford assay, protein concentration is determined by amount of dye in the blue ionic form measured using spectrometer (Kruger, 1994).

The Bradford assay was performed by mixing 10 µl of the protein samples or BSA protein with 300 µl of Coomassie dye reagent (Thermo Scientific, catalog number 23246) in a 96-well microplate (Fig 2.2.a). This was mixed for 30 seconds and then incubated at room temperature for 10minutes. Absorbance of the samples was read at 592 nm using a spectrometer. A standard calibration curve was plotted for the absorbance of BSA protein against the concentration range.

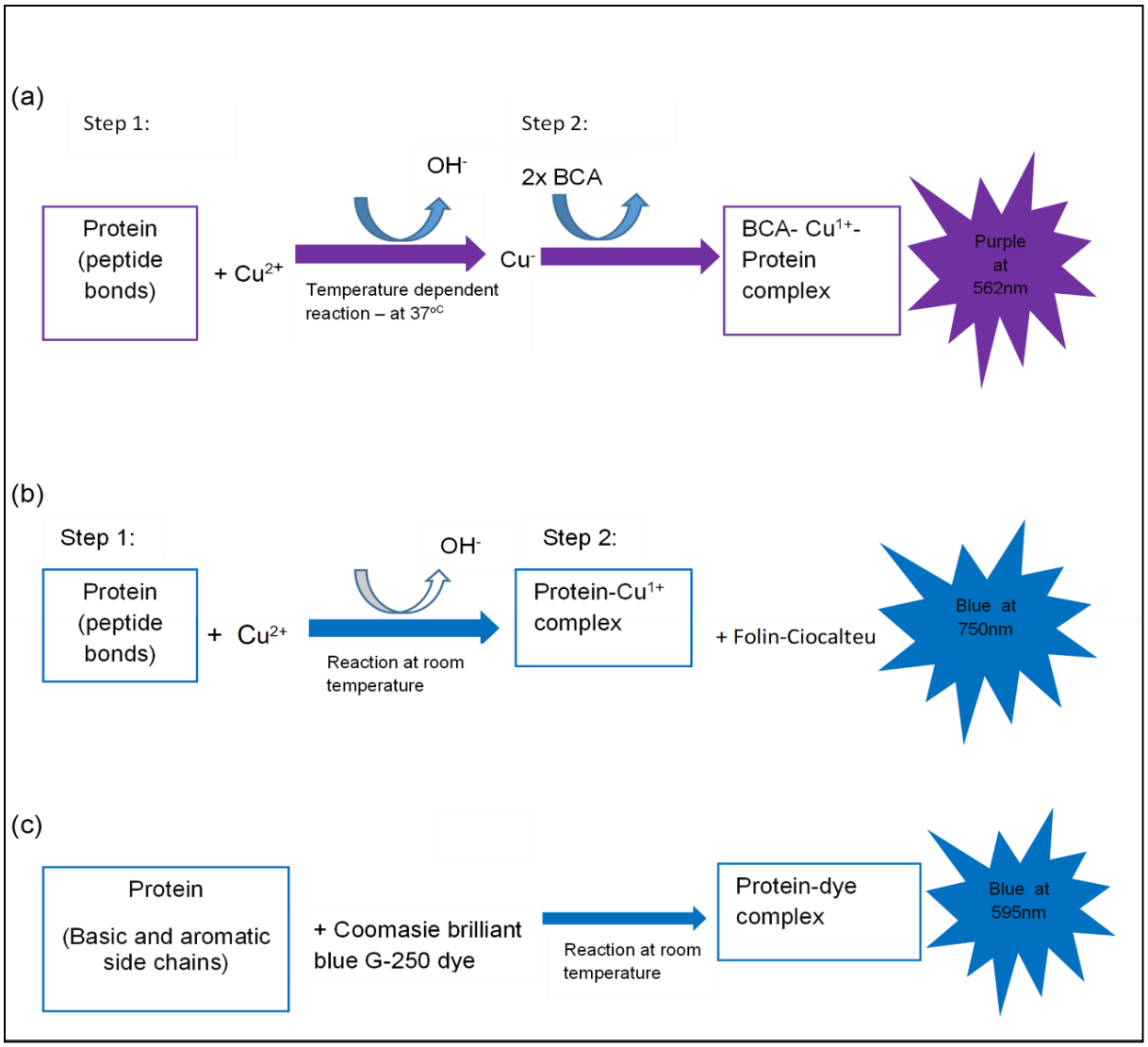

Figure 2.1: Schematic representation of multi-protein assay reactions. (a) BCA assay, (b) Lowry assay and (c) Bradford assay chemical reaction (Johnson, 2012)

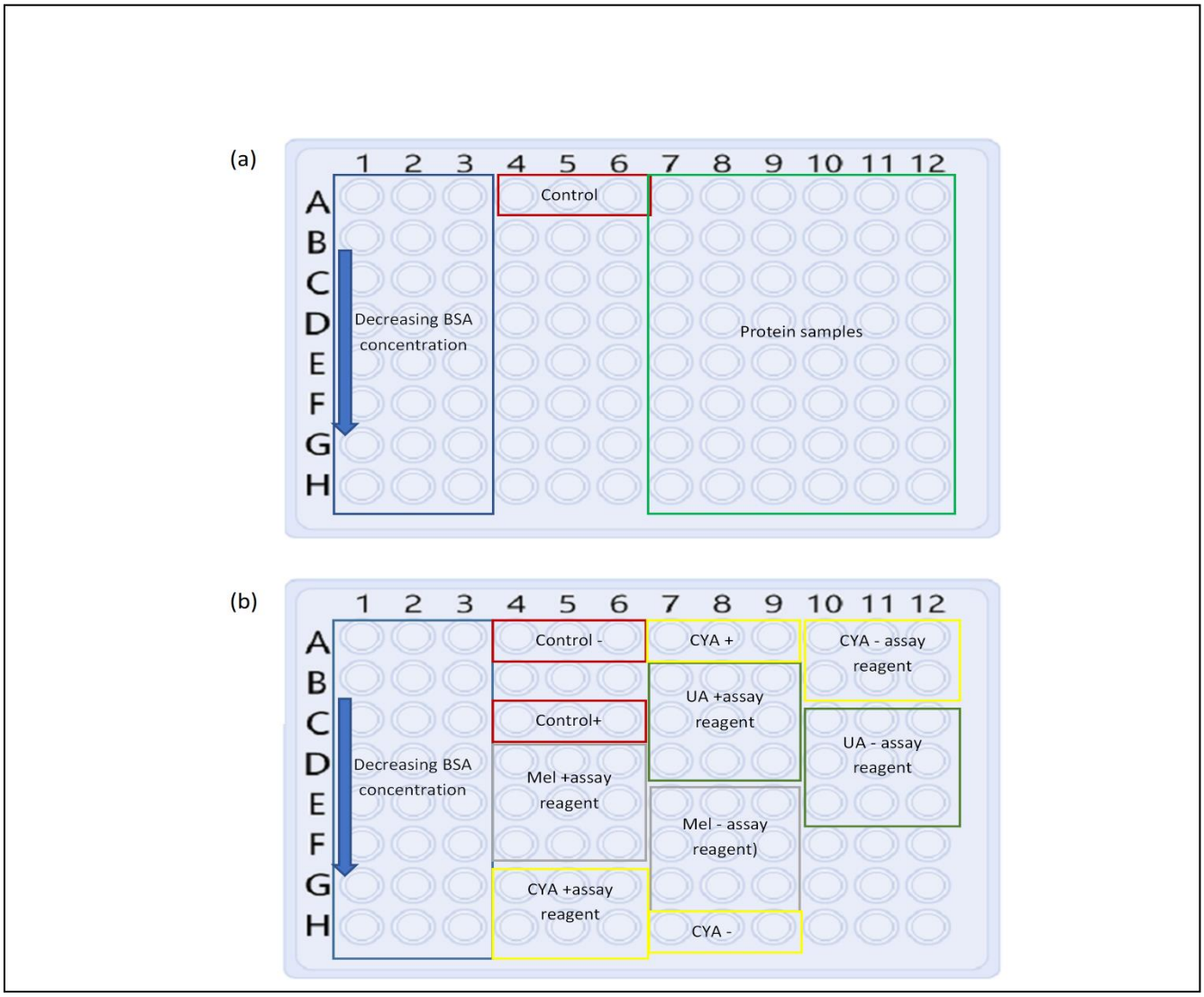

Figure 2.2: Schematic representation of the 96-well microlitre plates used in a) the multi-protein assays and, b) interference study of the assays with melamine, cyanuric acid, and uric acid respectively, with the appropriate BSA concentrations and controls

2.3.6. Interference study

An interference is a substance, other than the test sample, that can be measured by the selected analytical method (Deepachandi *et al*., 2020). Interference can lead to erroneous analysis of the results. Therefore, in this study we investigated interfering substances in each of the three assays described above using melamine (50, 100, 250 ug.ml^{-1}), cyanuric acid (50, 100, 250 ug.ml^{-1}) and uric acid (25, 50 and 100 ug.ml^{-1}) as test samples in the assay. Control samples of these compounds were prepared

without the use of assay reagents. Instead, deionised water was used to obtain the same volume as the test samples. The test samples and control were arranged on 96-well microplates as depicted on figure 2.2 (b) and absorbance read at 562 nm, 595 nm and 750 nm for BCA, Bradford, and Lowry assays, respectively.

2.3.7. Data analysis

The mean (n=6) analysed values for protein content, including standard deviations and standard mean error, were determined using an Excel spreadsheet (Microsoft® Office Excel, 2016). Statistical probability (*p*-value) of difference between the analysed values and the reported amount on labels was analysed using a two-unequal variance (heteroscedastic) *t*-test, with two-tailed distribution using STATA (STATA® 16.1 Statistics/Data analysis Special Edition, 2019). Additionally, compliance was determined by calculating the percentage difference between the mean of the analysed values and the reported value on the label product as previously indicated.

Protein concentration in microplate = Protein concentration with assay x dilution factor

Equation 2. 3: The formula to calculate protein concentration in microplate after absorbance reading

$$\text{Initial protein concentration} = \left(\frac{\text{Protein concentration in microplate}}{\text{Dilution factor}} \text{x final volume}\right) 1000$$

Equation 2. 4: The formula to calculate the protein concentration from the initial sample

$$\text{Actual protein \%} = \frac{\text{Initial protein concentration}}{\text{Protein mass in initial sample}} \text{x } 100$$

Equation 2. 5: The formula to calculate the actual protein percentage from the initial protein sample

2.4. HPLC method validation and determination of adulterants

2.4.1. Instrumentation

The selection of the HPLC-UV method was informed by the availability of the HPLC instrument at the Department of Pharmacy and Pharmacology, University of the Witwatersrand. The analysis was performed using the Perkin Elmer Flexer HPLC system, which included a Flexer LC binary pump auto-sampler and an UV-Vis detector (U.S.A) (Fig 2.3). The column consists of Kinetex C18 reverse phase (RP) column (150 x 4.6, 5 µm particle size) and C18 column guard (4.6 mm, 3 µm particle size) (Phenomenex, U.S.A).

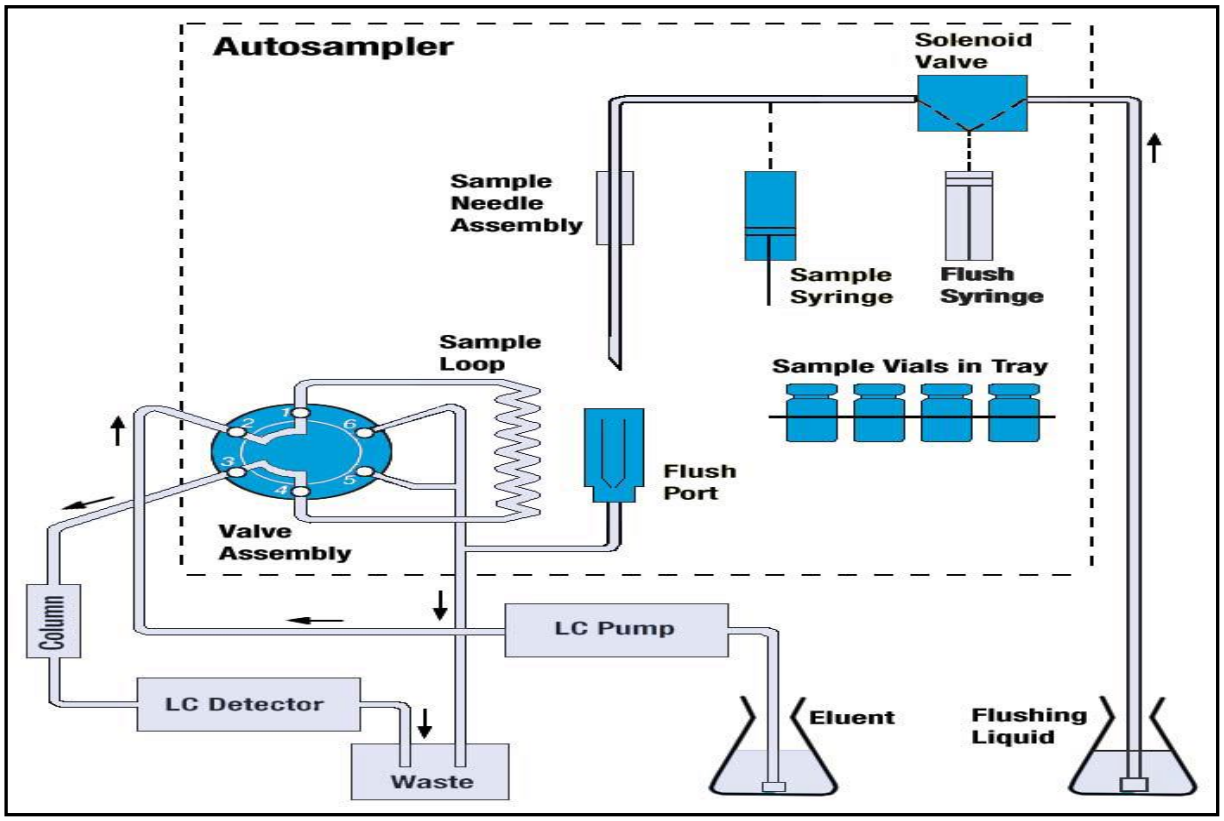

Figure 2.3: Schematic diagram of the Perkin Elmer Flexar HPLC system (Source - https://www.labmakelaar.com/fjc_documents/171.pdf)

2.4.2. Preparation of standard stock solutions

The stock solutions of melamine (100 ug.ml^{-1}) and cyanuric acid (1000 ug.ml^{-1}) were prepared in methanol: water (50:50, % v/v). While uric acid (100 ug.ml^{-1}) was prepared in methanol: water (50:50, % v/v) and a few drops of 0.5 M sodium hydroxide were added to help dissolve the crystals. All stock solutions were stored at -18^{o}C and filtered through 0.45 µm before use.

2.4.3. HPLC method development

To find a suitable method to investigate adulterants in protein supplements by HPLC, various mobile phases and conditions were explored from the literature. This includes 0.1% formic acid: acetonitrile (1:1, v/v) (Gabriels *et al*., 2015), 0.01 M acetate buffer: methanol (80:20, % v/v) (Montesano *et al.,* 2013) and 20 mM phosphate buffer: acetonitrile (99:1, % v/v) (Wijemanne *et al*., 2018). These buffers were selected based on their use for detecting melamine, cyanuric acid in their respective studies. The buffers were tested with the RP-column described above. Optimal conditions for each buffer were investigated by testing suitable temperature, adjusting the pH and the organic solvent ratio to achieve the best separations for melamine, cyanuric acid, and uric acid.

2.4.4. Method optimization and robustness

The proposed HPLC method was optimised by analysing samples in various conditions by making minor changes in the mobile phase composition (pH 2.9 – 8), in the flow rate (0.4 to 1 ml/min), in the temperature of the column (25 – 40^{o}C), and by changing the wavelength (203 – 240 nm).

2.4.5. Preparation of mobile phase

A 20 mM potassium phosphate buffer was prepared by accurately weighing 2.98 g of K_2HPO_4 and 936 mg of KH_2PO_4 in 800 ml deionised water. To this solution, 560 mg of sodium 1-heptanesulfonate (2.5 mM) as an ion-exchanger was added. The pH of the solution was adjusted to 7.25 with phosphoric acid, and then buffer made up to 1L with deionised water. The buffer was filtered through a 0.45 µm nylon membrane

filter prior HPLC analysis. The final mobile phase solution consisted of a 99:1 (% v/v) mixture of potassium phosphate buffer to acetonitrile. The buffer was stored at 4^{o}C.

2.4.6. Chromatographic conditions

Optimal HPLC analysis was achieved by using an isocratic elution, RP C18 column, with a mobile phase 20 mM potassium phosphate buffer: ACN (99:1, % v/v; pH 7.25) which was pumped at a flowrate of 0.5 ml/min. The column temperature was set to 30^{o}C, the sample injection volume was 20 µl, and all analytes were monitored at a wavelength of 203 nm.

2.4.7. Test samples preparation

The extraction method performed for the 21 protein supplements in this study was a modified approach to that of Londoño *et al.* (2017). From each protein product respectively, 0.5 g powder was weighed into a 15 ml polypropylene tube. A 10 ml mixture of 1% TCA and ACN (75:25, % v/v) was added to each tube to precipitate the proteins and then vortexed for 1 minute. The samples were sonicated for 30 minutes, and then placed in a shaker for 10 minutes at 420rpm. The samples were then centrifuged at 4000 rpm for 15 minutes. The resulting supernatant was transferred to new tubes and filled with 1% TCA to a volume of 10 ml. The samples were then centrifuged at 3600 rpm for 15 minutes. The extracted protein sample was filtered through a 0.45 µm nylon membrane filter. For HPLC analysis, the extracted protein samples were diluted accordingly, with the mobile phase, in a vial to a total volume of 2 ml. The remainder of the protein extract was aliquoted and stored at -18^{o}C until required for further, subsequent analysis.

2.4.8. Linearity

Standard working solutions of melamine, cyanuric acid, and uric acid were prepared fresh by serially diluting stock solutions with the mobile phase. Final concentrations of 0.4, 1.56, 3.12, 12.5, 25 and 50 µg/ml of melamine; 0.78,1.56,3.12, 25, 50 and 100µg/ml of cyanuric acid and, 0.78,1.56,3.12, 12.5 25, and 50 µg/ml of uric acid were prepared to obtain calibration curves. During the analysis, for each analyte, each concentration was injected in 3 times (n=3) into the column. The mean peak values were plotted against concentrations. The linearity was assessed by a linear

regression analysis, which was considered by the least squared regression method using an Excel spreadsheet (Microsoft® Office Excel, 2016).

2.4.9. Specificity

The specificity for this method was determined by evaluating if a specific peak would be generated at the same retention time for melamine, cyanuric acid, and uric acid respectively. An injection of a blank (mobile phase) was done during each chromatographic run to ensure that there was no interference.

2.4.10. Stability

Stability is determined by comparing the response and impurity profile from standards or samples to that of freshly prepared standard/sample and to its own response from earlier time points (Bliesner, 2006). The stability of the standards (melamine, cyanuric acid, and uric acid) in this study was assessed for three days under ambient conditions and refrigerator (4^{o}C). Each solution was analysed with the selected HPLC method, and the percentage recovery was calculated daily.

2.4.11. Precision

The precision of the proposed HPLC method was assessed by repeatability and reproducibility. Precision of an analytical method is the degree of agreement between individual test results when a method is repeatedly applied to multiple samples (Kayesh *et al.,* 2013). The repeatability for this method development was assessed at 3 different concentrations for each analyte in this study (3.12, 12.5, and 25 µg.ml^{-1} for melamine and uric acid; 3.12, 25, and 50 µg.ml^{-1} for cyanuric acid). The proposed HPLC method was used to repeat six injections on the column, continuously analysing the different concentrations of each analyte. In addition, intra- and inter-day repeatability of responses after replicate injections for the different concentrations of each analyte was assessed by comparing results from 5 different days (n=5). This was expressed as RSD% among the responses using the formula from Equation 2.6.

$$\text{RSD\%} = \frac{\text{Standard deviation}}{\text{Mean}} \text{ x } 100\%$$

Equation 2. 6: Percentage relative standard deviation (RSD %) used for precision of method

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2.4.12. Accuracy

The accuracy of an analytical method represents the proximity between the expected value and the value obtained by the method (Kayesh *et* al., 2013; Bhavyasri *et al.,* 2013). In this study, a selected protein sample was spiked with known amounts of the stock solutions of analytes to result three different concentrations (similar concentrations used in precision). These solutions were injected in triplicate and the assay was repeated 4 times consecutively using the proposed method. The percentage recovery (%R) of the response factor (area and concentration) was calculated using Equation 2.7.

$$\%\text{Recovery} = \frac{\text{Recovered concentration}}{\text{Injected concentration}} \text{x } 100$$

Equation 2. 7: The percentage recovery of analytes used to determine accuracy of method

Table 2.2: Acceptance criteria for parameters used in method validation (Bliesner, 2006)

Parameter	Criteria
%RSD (interday & intraday)	≤ 2%
%Recovery (accuracy)	85% - 104%
%Recovery (stability)	± 1.5%

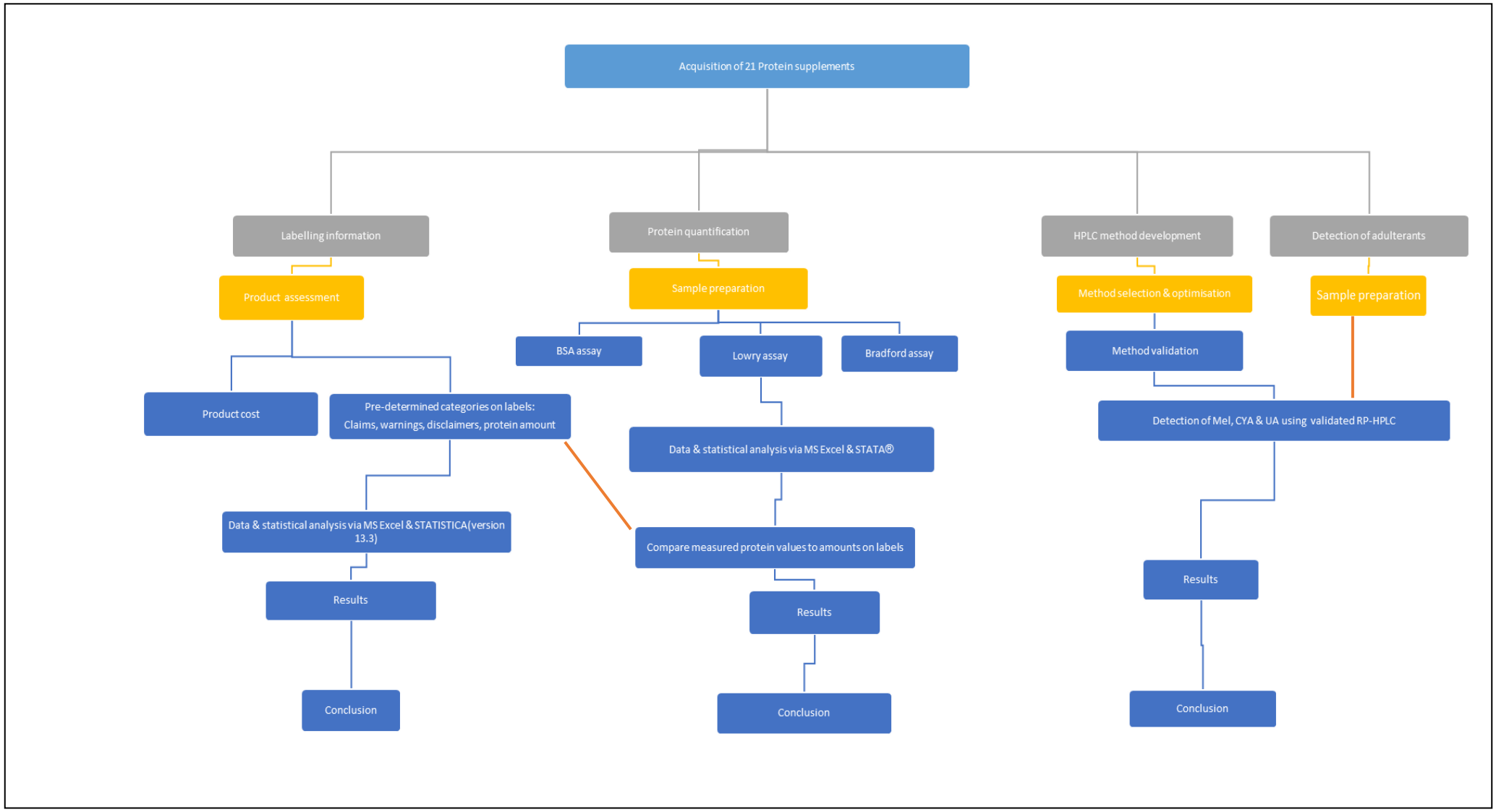


Figure 2.4: Action plan to accomplish objectives for the investigation of selected protein supplements (n = 21)

CHAPTER THREE – RESULTS

CHAPTER 3 – RESULTS

3.1. Label information study

3.1.1. General information on labels

Twenty-one protein supplements (n = 21) were selected for analysis, of which 14 (67%) were locally manufactured and 7 (33%) were imported. Ninety percent (n = 19) of the products were packaged in plastic containers, while 5% of the products were packaged in a vertical pouch, and 5% packaged in a metal container. The average number of colours on products was 5. The average number of ingredients ranged between 6 and 19 per product and the overall average for the products was 12. All the supplement products had one or more flavours. All products provided information with direction for use and 100% of the products emphasized compliance with storage details. The most common flavour among the products was chocolate with 38%, followed by vanilla flavour with 33%, as shown in Figure 3.1. The 'best before' or the expiration date statements were also present on all the products. All the products had a batch number visible on the container.

The average protein per serving (scoop) was 27.80 g. The average protein per 100 g/

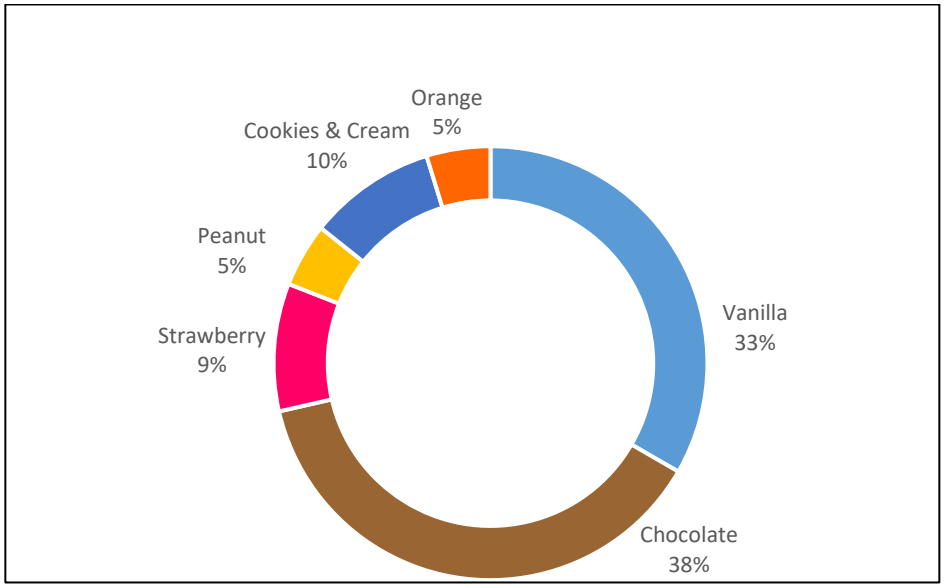


Figure 3.1: Flavour profile of selected protein supplement products (n=21)

percentage reported on the labels was 74.10% for the supplement products as shown in Table 3.1.

Table 3.1: Protein information from product labels (n = 21)

Product code	Weight (g)	Servings	Protein per serving (g)	Protein % per 100g
1	908	28	22.30	69.70
2	920	31	22.00	73.33
3	908	28	22.00	68.80
4	420	12	20.00	57.14
5	1000	33	22.00	73.33
6	925	33	20.20	72.14
7	908	28	25.00	75.76
8	908	30	23.50	77.30
9	908	30	20.60	68.70
10	1000	40	17.80	71.20
11	500	20	20.00	80.00
12	1000	25	32.00	80.00
13	1000	33	22.22	74.00
14	907	27	25.20	76.42
15	600	15	33.00	82.50
16	1000	31	23.00	71.88
17	1002	30	24.00	72.73
18	896	28	25.00	78.13
19	502	20	20.00	79.73
20	900	30	21.00	70.00
21	750	30	21.00	84.00

In this study, 33% of the products had social media accounts where consumers could find more details or information about the product. Social media platforms included Facebook, Instagram, Snapchat, Twitter, and YouTube. Many of these social networks also serve as an advertising platform for these products. Eighty-one percent of the protein supplement labels referred to a website or some type of advertising with a QR code that consumers could use to get more promotional information about the products.

Figure 3.2 shows that a blend of whey-protein types constitutes the majority (67%) of the protein types among the supplement products, followed by concentrate and isolate proteins which constitute 14% each. Locally produced supplements contain more blend

proteins (48%) compared to imported products (Figure 3.2). Bone-broth protein represents only 5% (n = 1) of the products.

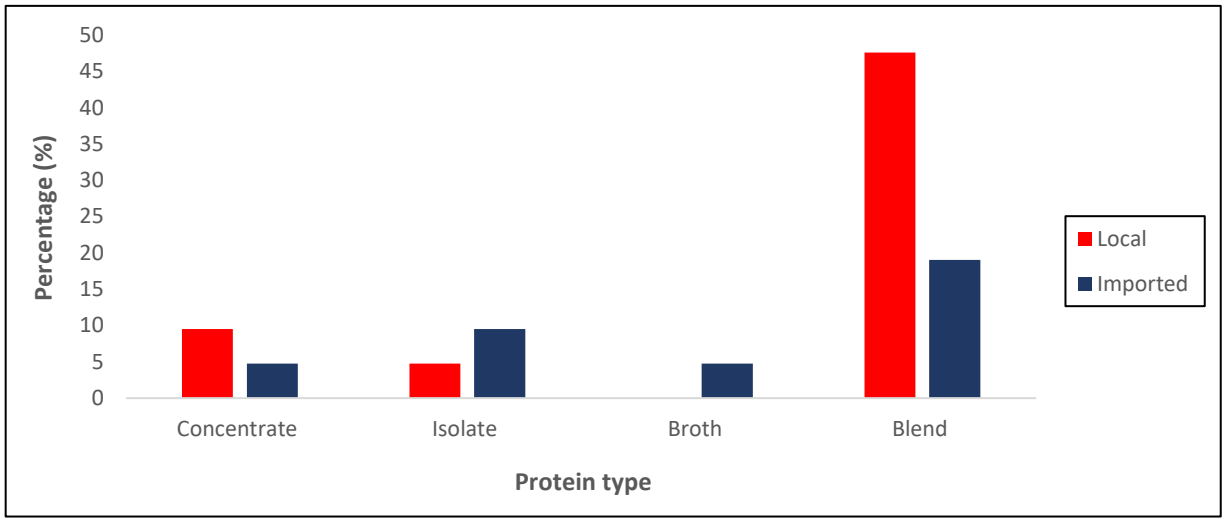


Figure 3.2: Distribution of protein types within the selected protein supplements

This study found that 5% of the supplement products are intended for muscle recovery, 14% provide muscle growth benefits, while 5% reported no muscle benefits. However, 76% of the protein supplements offered a combination of muscle benefits per product, as highlighted in Table 3.2. The most popular combination among the products (52%) was muscle growth and recovery, and the least common was lean muscle growth and maintenance with 5% (Table 3.2).

Table 3.2:Physiological benefits declared on protein supplement products

Benefit	Percentage (%)
Lean muscle gain/support & maintenance	5
Lean muscle gain & muscle growth/building	14
Muscle growth/building & maintenance	19
Lean muscle gain/support & recovery	19
Muscle maintenance & recovery	24
Muscle growth/building & recovery	52

3.1.2. Product cost

The product cost calculations were based on the supplements' product purchase price and the weight values stated on the labels. At the time of purchase of the supplement products (April 2019), the average cost price of all the products was R 420.80 (Table 3.3). The results of this study showed that locally produced products cost R290.60, while imported products cost an average of R681.10. The overall average cost of protein per gram was 70 cents. For imported supplements, the average cost of protein per gram was R1.11. In terms of the cost of the type of protein, this study found that the bone-broth product was a more costly type of protein per gram at R2.31 (Table 3.3), followed by isolate protein at 83 cents.

Table 3.3: Summary of the cost of protein the consumer will spend per protein product

Product code	Price of product* (Rands)	Cost per serving (Rand)	Cost per gram of protein (Rand)
1	319.95	11.28	0.51
2	499.95	16.30	0.74
3	299.95	10.57	0.48
4	199.95	16.66	0.83
5	279.00	8.37	0.38
6	299.00	9.05	0.45
7	269.95	9.81	0.39
8	699.95	23.43	1.00
9	289.95	9.58	0.47
10	334.95	8.37	0.47
11	219.95	11.00	0.55
12	329.00	13.16	0.41
13	299.00	8.97	0.40
14	699.00	25.43	1.01
15	289.00	19.27	0.58
16	299.00	9.57	0.42
17	699.95	23.05	0.96
18	899.00	32.11	1.28
19	934.95	46.75	2.34
20	289.95	9.67	0.46
21	384.95	12.83	0.61

*****Note: Prices are inclusive of VAT and mark-up from retailers i.e., cost to consumer

3.1.3. Claims

All of the supplement products (100%) had some form of claim or statement that overstated the product's quality (Table 3.4). Only 5% of the supplements claimed that their product may be used as a meal replacement and as a weight-loss management supplement. Only one product (5%) cited claims as being tested by a reputable laboratory, however there was no further information provided to support this. One supplement product (5%) included a health claim stating that the product improves the immune system. Physiological benefits of the products evaluated included metabolism support (10%), increased strength (19%), improved recovery (62%), and muscle mass changes (86%).

Table 3.4: Description specific claims from product labels (n=21)

Claims description	Total	Total as %
Serves as a weight loss/management product	1	5
Give support to immune system	1	5
Research supporting evidence	1	5
Serves as a meal replacement	1	5
Supports general metabolism	2	10
Provides increased strength	4	19
Provides increased energy	5	24
Improves recovery	13	62
Muscle mass changes	18	86
Use of pseudo-science terms	21	100
Quality of product emphasized	21	100
Outrageous claims	21	100

3.1.4. Disclaimers

Notably, not all products had a disclaimer on the labels. Disclaimers that were found on the products primarily focused on warning users that the products are not intended for medical purpose and 57% of the products declared that the products have not been tested by the relevant authorities (Table 3.5). The results show that 29% of the products declared on the labels do not contaminates. This indicates that 71% of product labels did not contain contaminants. Six of the products (29%) indicated on labels that the supplement should not replace medication.

Table 3.5: Description of disclaimer from product labels (n= 21)

Disclaimer	Total	Total as %
Product is free of stimulants, contaminants, colourants, flavourings and/or preservatives	6	29
This product should not replace medication	6	29
Product does not cure illness	9	43
Product or claim made by product has not been evaluated by U.S. FDA/MCC/SAHPRA	12	57

3.1.5. Warnings

The number of warnings varied with an averaging of 4 per product (Table 3.6). However, sometimes the warnings were not immediately visible because the font was very small. Forty-three percent of the supplements highlighted discontinuation of use in the event of side effects and highlighted that their products are not intended to treat any disease. All the products underscored the importance of compliance with storage instructions. The second most common warning on the product labels was the "presence of food items associated with allergies/intolerance" (95%).

Table 3.6: Description of warnings from product labels (n =21)

Warning	Total	Total as %
Lower dose when experiencing adverse effects	3	14
Time restriction consideration when consuming product	4	19
Maximum dose "dangers"	4	19
No presence of banned substance in product	5	24
Exclusion of use, and not cure of disease states	9	43
Exceeding recommended dose	9	43
Age categories for consumption	11	52
Supplement should not replace dietary requirements	11	52
Medical conditions and use of supplements	15	71
Keep product out of reach of children	16	76
Consultation with health care professionals	16	76
Presence of food items associated with allergies/intolerance	20	95
Adherence to storage details	21	100

3.2. Protein quantification – multi-protein assay approach

Protein supplements (n = 21) were prepared in duplicate as described in Chapter 2 and analysed six times (n = 6) in the BCA, Lowry, and Bradford assay. Standard calibration curves were plotted using BSA as the known protein using mean absorbance and BSA concentrations (Fig 3.3 a-c). Only data showing a strong correlation coefficient (R^2 of >0.95) was accepted for each assay. The BSA standard curves generated for the three assays were linear, allowing the concentrations of the protein supplement samples to be quantified. The absorbance values measured of the supplement products were in the range of the established standard calibration curves. The mean of all the absorbance measurements per protein sample in each assay was calculated. From this, the unknown concentrations of the supplement products were calculated from the standard curves using the equation $y = mx+c$. These concentrations allowed the calculation of the initial amounts of protein in the supplement products using Equations 2.3 to 2.5. Figure 3.4. illustrates the colour change observed in the microplates during the assay analysis. The colour change for BCA and Lowry assays, purple and blue, respectively, was as expected, indicating the presence of protein in the samples (Fig. 3.4. a-b). However, it

was observed that protein samples in Bradford assay showed poor colour development (green instead of blue), indicating low protein detection with this method (Fig 3.4.c).

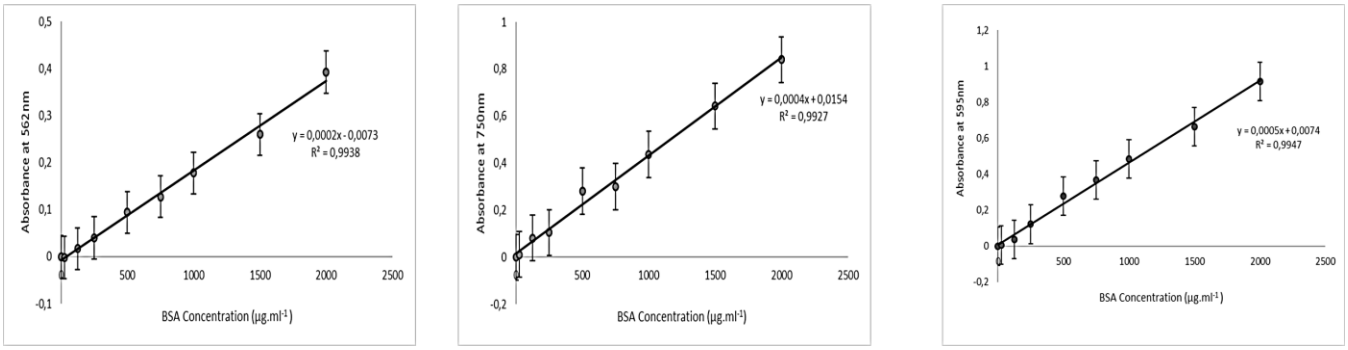


Figure 3.3: Standard calibration curves of BSA protein using linear regression in multi-protein assay. (a) BCA assay, (b) Lowry assay and (c) Bradford assay

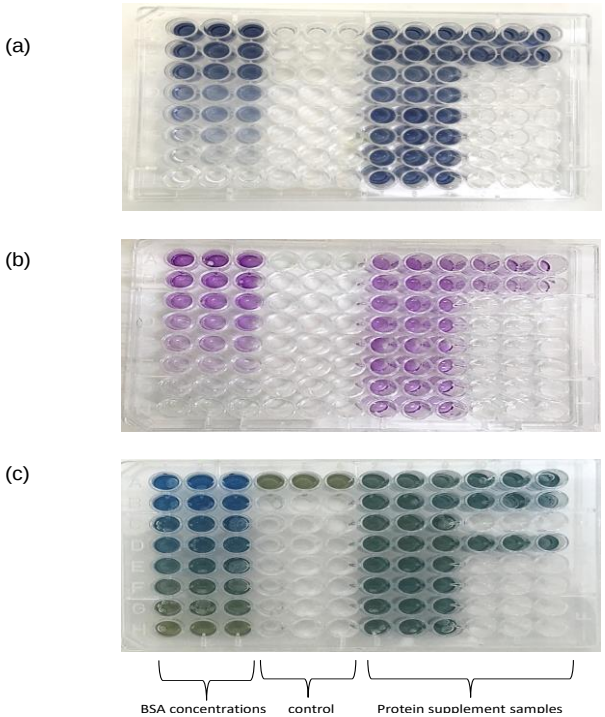


Figure 3.4: Examples from the protein assays after incubation period. (a) Lowry assay (b) BCA assay and (c) Bradford assay

3.2.1. BCA assay

Table 3.7 summarizes the data collated for the BCA assay in this study. The mean standard deviation of 5.8 indicates a small distribution of the mean values, and average standard mean error of 2.37 indicates how close the means are to the values declared on the labels. The mean protein values analysed by this assay differed statistically significantly (p<0.05) from the reported values in 18 (86%) of the products. Only 3 of the supplement products had a p>0.05 suggesting that they were not statistically significantly different from the declared values. The percentage difference between the mean protein content reported and analysed in the BCA assay ranged from 3.5% to 46.7%, with an average variance of 17% for all samples (n = 21). In this study we found that three products had over-reported protein content by more than 25% as highlighted (in red) in Table 3.7.

Table 3.7: Comparison between protein amounts declared on labels and analysed protein in BCA assay for protein supplements products (n =21)

Product code	Protein content declared on label per 100g	Analysed Protein per 100g (n=6)	*SD	**SEM	***% difference	*P-value*
1	69.7	64.3	5.6	2.7	5.4	0.0622
2	73.3	59.1	4.7	1.9	14.3	0.0007
3	68.8	55.0	6.1	2.5	13.7	0.0026
4	57.1	48.3	8.9	3.7	8.8	0.0618
5	73.3	39.2	5.7	2.3	**34.1**	<0.0001
6	72.1	63.9	7.0	2.9	8.3	0.0351
7	75.8	55.0	9.7	3.9	20.7	0.0034
8	77.3	61.2	6.1	2.5	16.1	0.0013
9	68.7	65.2	5.7	2.3	3.5	0.1913
10	71.2	54.6	5.4	2.2	16.6	0.0006
11	80.0	58.0	5.2	2.1	21.9	0.0001
12	80.0	60.3	5.6	2.3	19.7	0.0004
13	74.0	58.2	5.3	2.2	15.8	0.0007
14	76.4	63.4	5.8	2.4	13.0	0.0028
15	82.5	61.7	4.3	1.8	20.8	0.0001
16	71.9	46.8	2.9	1.2	**25.1**	<0.0001
17	72.7	62.6	3.9	1.6	10.1	0.0015
18	78.1	63.0	7.6	3.1	15.1	0.0046
19	79.7	33.0	1.7	0.7	**46.7**	<0.0001
20	70.0	60.5	5.1	2.1	9.5	0.0061
21	84.0	63.5	9.3	3.8	20.5	0.0030

*SD= standard deviation
**SEM= standard error of the mean
***% difference = percentage difference
% Difference highlighted in red are values over 25% difference between analysed and reported values on labels

3.2.2. Lowry assay

In the Lowry assay, the average percentage difference analysed was 13% (Table 3.8). The average standard deviation and the mean standard error were 7.3 and 3 respectively for this assay. Compared to the BCA assay, these Lowry method values were slightly higher, with the exception of the average percentage variance value of 13%. Table 3.8 shows that 14 (67%) of the products had a p <0.05 suggesting a statistically significant difference between the analysed mean values and the reported values on the product label. Only 33% (n = 7) of the products analysed by the Lowry assay had analysed means values that were not statistically significant (p>0.05). The range of percentage variation for the Lowry assay in this study was from 1.77% to 31.5% (Table 3.8). The results of this assay also showed that three of the products had over-reported protein content by more than 25%.

Table 3.8: **C**omparison between protein amounts declared on labels and analysed protein in Lowry assay for protein supplements products (n = 21)

Product code	Protein content declared on label per 100g	Analysed Protein per 100g (n=6)	*SD	**SEM	***% difference	*P-value*
1	69.7	66.1	4.2	1.7	3.7	0.085
2	73.3	62.5	5.1	2.1	10.9	0.0038
3	68.8	53.7	6.8	2.8	15.1	0.0030
4	57.1	54.3	9.5	3.9	2.8	0.5025
5	73.3	53.3	6.8	2.8	20.1	0.0008
6	72.1	60.2	7.7	3.1	11.9	0.012
7	75.8	50.2	6.3	2.6	**25.6**	0.0002
8	77.3	67.8	9.0	3.7	9.5	0.0486
9	68.7	63.6	9.0	3.7	5.0	0.2273
10	71.2	66.8	4.9	1.9	4.4	0.0774
11	80.0	56.9	6.7	2.7	23.0	0.0004
12	80.0	64.3	9.1	3.7	15.7	0.0083
13	74.0	64.6	6.9	2.8	9.4	0.0203
14	76.4	66.2	8.1	3.6	10.2	0.0351
15	82.5	56.6	8.9	3.6	**25.9**	0.0009
16	71.9	51.8	4.2	1.7	20.1	0.0001
17	72.7	66.9	7.2	2.9	5.8	0.1275
18	78.1	66.5	4.5	1.8	11.6	0.0014
19	79.7	48.1	8.9	3.7	**35.6**	0.0003
20	70.0	68.2	7.9	3.2	1.8	0.6063
21	84.0	78.7	10.6	4.3	5.3	0.2713

*SD= standard deviation
**SEM= standard error of the mean
***% difference = percentage difference
% Difference highlighted in red are values over 25% difference between analysed and reported values on labels

3.2.3. Bradford assay

With an average of 54%, the Bradford assay showed the largest percentage difference between the analysed protein values and the reported values on the labels (Table 3.9). In addition, it was found that the *p*-values for 20 (95%) out of the 21 samples (Table 3.9) were statistically significantly different ($p<0.05$) from the protein values reported on the label. The discrepancy is further underlined by the marginal colour change in the reaction between the Coomassie-dye and the protein samples, as seen in Fig. 3.4. (c). In a Bradford assay, a deep blue colour indicates a presence of protein; however, the protein samples in this study remained mostly green, indicating little protein-dye interaction during the reaction.

Table 3.9: Comparison between protein amounts declared on labels and analysed protein in Bradford assay for protein supplements products (n = 21)

Product code	Protein content declared on label per 100g	Analysed Protein per 100g (n=6)	*SD	**SEM	***% difference	*P-value*
1	69.7	13.0	6.4	3.7	**56.7**	0.0043
2	73.3	11.8	5.5	3.2	**61.5**	0.0027
3	68.8	9.7	5.2	3.0	**59.1**	0.0025
4	57.1	19.8	9.6	5.5	**37.3**	0.0212
5	73.3	10.2	4.5	2.6	**63.1**	0.0017
6	72.1	19.8	5.6	3.3	**52.4**	0.0039
7	75.8	8.2	4.9	2.8	**67.6**	0.0017
8	77.3	36.9	12.1	7.0	**40.4**	0.0287
9	68.7	17.8	8.7	5.0	**50.9**	0.0097
10	71.2	19.7	7.9	4.6	**51.5**	0.0079
11	80.0	31.6	18.6	10.8	**48.4**	0.0461
12	80.0	26.9	5.5	3.2	**53.1**	0.0035
13	74.0	42.3	17.5	10.1	**31.7**	0.0881
14	76.4	22.3	3.7	2.1	**54.1**	0.0016
15	82.5	15.6	3.2	1.8	**66.9**	0.0007
16	71.9	28.4	2.9	1.7	**43.5**	0.0016
17	72.7	27.7	4.9	2.8	**45.0**	0.0040
18	78.1	16.9	3.5	2.0	**61.3**	0.0011
19	79.7	3.9	2.1	1.2	**75.8**	0.0002
20	70.0	13.3	2.9	1.7	**56.7**	0.0009
21	84.0	26.9	5.7	3.3	**57.1**	0.0032

*SD= standard deviation
**SEM= standard error of the mean
***% difference = percentage difference
% Difference highlighted in red are values over 25% difference between analysed and reported values on labels

3.2.4. Method competency

Of the three methods used in this study, only two were considered acceptable enough for protein quantification in supplements products and for further analysis. Table 3.10 shows the statistical analysis of the difference between the means of the analysed protein and the calculated *p*-values of BCA and Lowry assays. There was no significant discrepancy between the analysed protein content of these two methods, the lowest being 1.5% and the highest 15%. Fifteen of the products had a $p > 0.05$, which indicates that 71% had significant statistical difference between the label values and the analysed protein content. This confirms that both the BCA and Lowry assays are competent in the assessing of actual protein content in supplement products.

Table 3.10: Statistical variance between BCA and Lowry assays

Product code	Difference between analysed protein content	*P-value*
1	1.76	0.5490
2	3.41	0.2563
3	-1.34	0.7277
4	5.98	0.2872
5	14.02	0.0031
6	-3.67	0.4097
7	-4.83	0.3306
8	6.55	0.1721
9	-1.53	0.7309
10	12.17	0.0021
11	-1.06	0.7703
12	3.94	0.3887
13	6.36	0.1022
14	2.78	0.5314
15	-5.07	0.2379
16	5.03	0.0360
17	4.28	0.2310
18	3.48	0.3563
19	15.12	0.0023
20	7.77	0.0710
21	15.13	0.0255

3.2.5. Interference study

Melamine, cyanuric acid, and uric acid were tested at various concentrations in each of the three assays in this study to determine if they interfere with protein quantification. The results (Fig. 3.5. a-b) show that melamine and cyanuric acid could be detected in negligible amounts for the BCA and Lowry assays. However, some slight activity was observed with uric acid when analysed in BCA and Lowry assays as highlighted by the upward shift in graphs (Fig. 3.6.1(c) & Fig. 3.6.2 (c)). This was observed particularly at higher concentrations of uric acid (50 and 100 µg.ml^{-1}), where a pale purple and blue colour change was observed in the microplates (Fig. 3.5 a-b). At lower concentrations, uric acid did not show colour change with the Lowry assay (Fig. 3.5.a), indicating there was no detectable reaction with the assay reagent. This result suggests that the reagent contains a component that interacts with uric acid at high concentrations. The BCA assay showed some activity for all three compounds of interest, as highlighted by the purple colour reactions in Figure 3.5(b). Melamine showed the highest activity for all the assay reagents (Fig. 3.6.). The 3 compounds of interest showed little activity in the Bradford assay (Fig. 3.6.3.a-c).

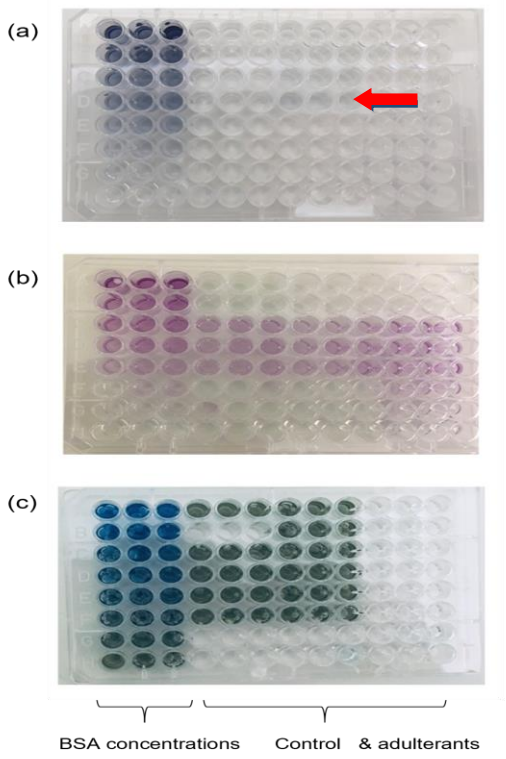
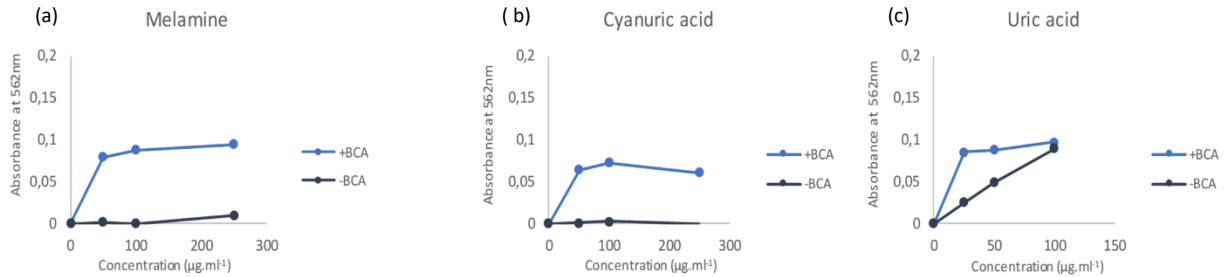


Figure 3.5: Interference study of adulterants in a multi-protein assay. (a) Lowry assay (b) BCA assay and (c) Bradford assay

1



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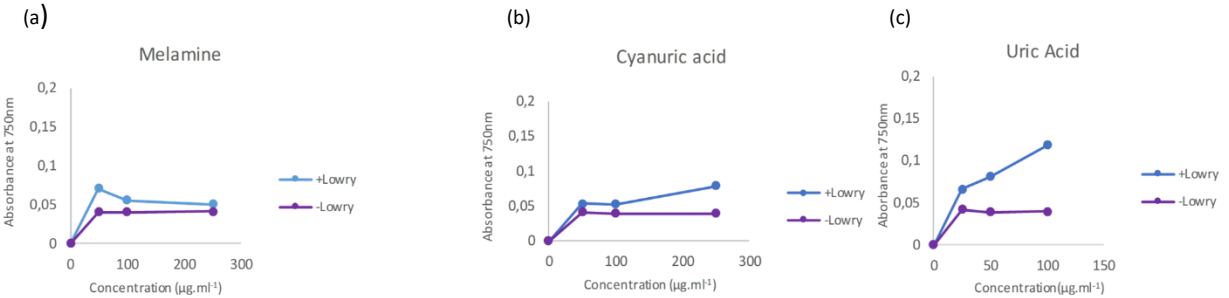

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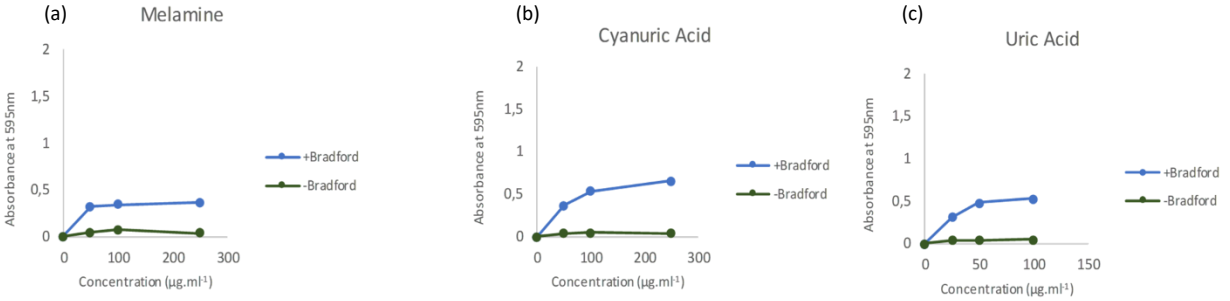

Figure 3.6: Interference study of multi-protein assays. The bottom line in the graph representing compound without the assay reagent (control) 1. Lowry assay with a) Melamine (50-250µg.ml^{-1}), b) Cyanuric acid (50-250µg.ml^{-1}), c) Uric acid (25-100µg.ml^{-1}); 2. BCA assay with a) Melamine (50-250µg.ml^{-1}), b) Cyanuric acid (50-250µg.ml^{-1}), c) Uric acid (25-100µg.ml^{-1}) and 3. Bradford assay with a) Melamine (50-250µg.ml^{-1}), b) Cyanuric acid (50-250µg.ml^{-1}), c) Uric acid (25-100µg.ml^{-1})

3.3. HPLC method development

3.3.1. Selection of mobile phase

Four mobile phases were tested for detection of the analytes of interest based on similar studies from the literature. The results of the retention times from this study are summarised in Table 3.11. The mobile phases Formic acid: ACN, Acetate buffer:ACN and Phosphate buffer:MeOH initially tested, did not provide sufficient resolution for the analytes. This was caused by analytes eluting close to the other compounds such as cyanuric acid eluting close to melamine with Formic acid: ACN, and uric acid eluting close to cyanuric acid with Acetate buffer:ACN and Phosphate buffer:MeOH as mobile phases (Table 3.11).The peaks for cyanuric acid had troughs, indicating negative peaks were forming for when Formic acid:ACN and Acetate buffer:ACN where used (Appendix A, Figure A1).

Table 3.11: Summary of retention times for the mobile phases tested for method development

Analyte	Retention time (min)			
	Formic acid:ACN	**Acetate buffer:ACN**	**Phosphate buffer:MeOH**	**Phosphate buffer: ACN**
Melamine	1.28	1.59	3.59	3.37
Cyanuric acid	1.34	1.63	3.01	2.60
Uric acid	1.94	1.71	2.98	2.18

However, the addition of an ion-exchanger, sodium 1-heptanesulfonate at 2.5 mM to Phosphate buffer: ACN as the mobile phase provided better resolution for the analytes (Table 3.11). HPLC analysis using this mobile phase at 203 nm provided good symmetry, separation, and resolution for the peaks of the three analytes. Melamine eluted at 3.37 min, cyanuric acid at 2.60 min and uric acid at 2.18 min (Fig. 3.7 a-c). This mobile phase was selected for further studying.

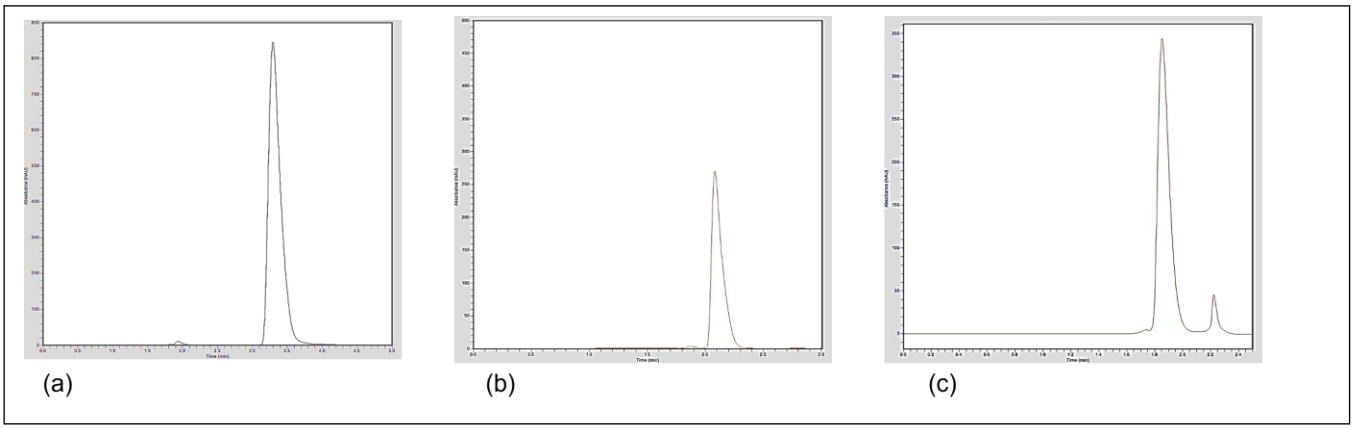

Figure 3.7: Chromatographs of (a) 25 µg.ml^{-1} Melamine, (b) 25 µg.ml^{-1} Cyanuric acid and (c) 12.5 µg.ml^{-1} Uric acid in 20 mM Phosphate buffer with ion-exchanger (pH 7.25): Acetonitrile (99:1, % v/v)

3.3.2. Method optimization

3.3.2.1. Effect of mobile phase ratio

The results show that the retention times of melamine, cyanuric acid and uric acid changed when the organic solvent of the mobile phase was increased up to 10% (Table 3.12). All analytes were detected in less than 3 minutes. Although the increase in organic solvent shows faster elution with good peak symmetry, the resolution becomes poor as the analytes elute closer together. However, as the organic solvent percentage was decreased, the retention times of the analytes increased and in the case of 99:1 ratio, good retention and resolution was observed.

Table 3.12: Elution of analytes with 20 mM Phosphate buffer with ion-exchanger (pH 7.25): Acetonitrile at different ratios

Analyte at 50 µg.ml^{-1}	**Retention time (min)**			
	Mobile phase ratio 90:10	**Mobile phase ratio 92:8**	**Mobile phase ratio 95:5**	**Mobile phase ratio 99:1**
Melamine	2.74	2.84	3.09	3.47
Cyanuric acid	2.29	2.29	2.55	2.62
Uric acid	2.11	2.16	2.36	2.36

3.3.2.2. Effect of temperature

After confirming the appropriate ratio of the mobile phase, the column temperature was observed at different levels. At room temperature (25^0C), the analytes showed poor symmetry and a peak front. At 40^0C, the peaks and retention times of the analytes were similar to those at 30^0C. Therefore, 30^0C was selected as the column temperature because it provided the best peak resolution and symmetry.

3.3.3. Chromatographic conditions

Following confirmation of the mobile phase ratio and column temperature, chromatographic conditions such as the pH, injection volume, and the flow rate remained consistent with the literature, as they provided the best results for the analysis of the three compounds of interest. The optimal chromatographic conditions for the analysis of melamine, cyanuric acid, and uric acid are summarised in Table 3.13. This optimised method was further validated and used for the detection of adulterants in protein supplements.

Table 3.13: Summary of the optimal chromatographic conditions for the proposed HPLC method in this study

Parameter	Conditions
Colum	Kinetex C18 (150x 4.6 mm, 5 µm) with guard column C18 (4.6 mm, 3 µm)
Column temperature	30ºC
Pump mode	Isocratic
Flow rate	0.5 ml/min
Injection volume	20 µl
Run time	4 min
pH	7.25
Mobile phase	20 mM phosphate buffer: ACN, plus ion exchanger (2.5 mM sodium 1-heptanesulfonate)

3.4. Method validation

3.4.1. Linearity and range

Calibration curves for melamine, cyanuric acid and uric acid were plotted against the peak responses (peak area from the absorbance) using these concentrations. The experiments were repeated three times for each analyte, and the average peak areas were calculated to plot the standard curves shown in Fig. 3.8. The regression coefficient (R^2) for each analyte was found to be greater than 0.999 as shown in Table 3.14, confirming the linear relationship between the concentration and the peak area for the three analytes.

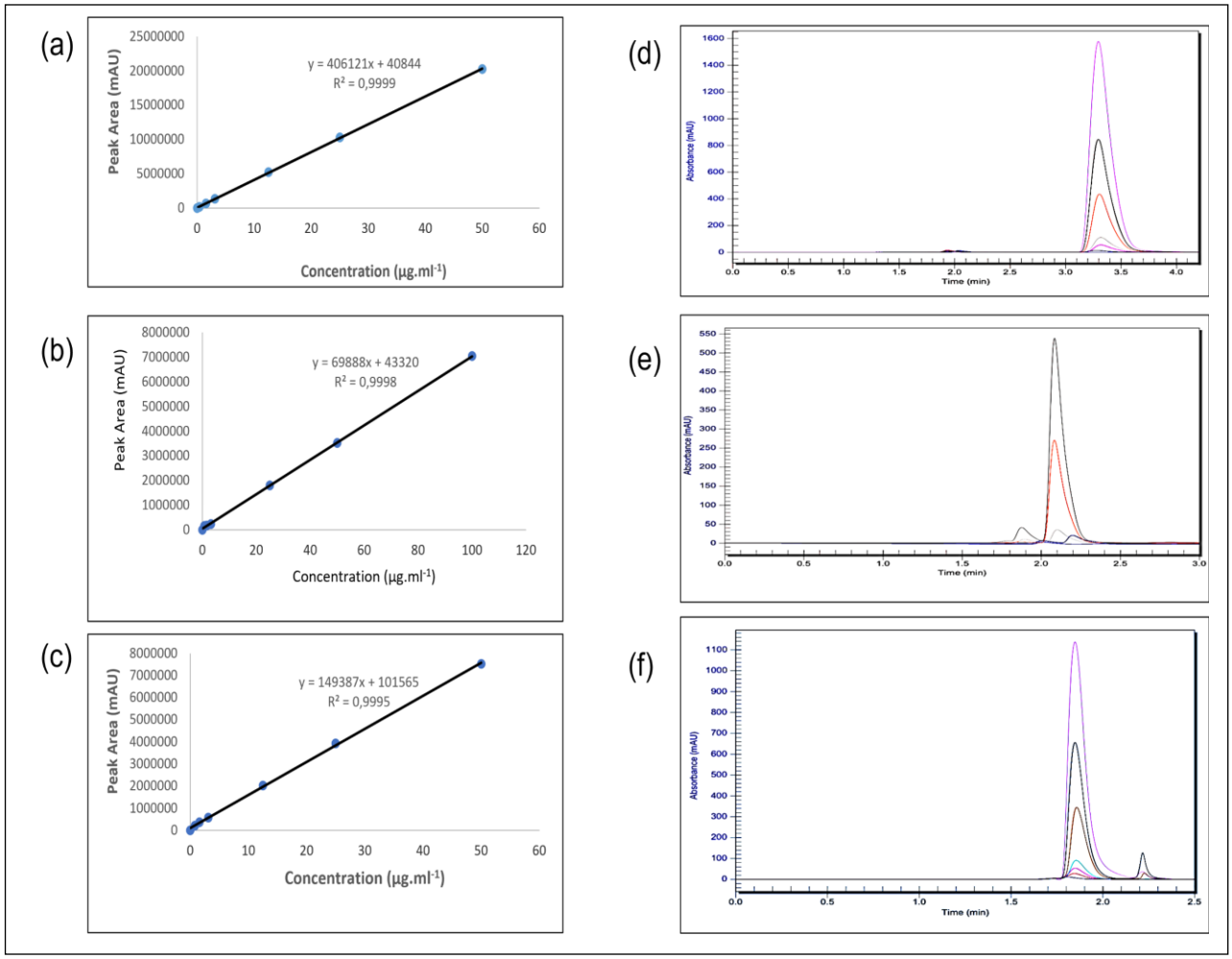

Figure 3.8: The standard calibration curves and chromatographs of analytes from HPLC analysis using the proposed method. Standard calibration curve of: (a) melamine, (b) cyanuric acid and (c) uric acid. Overlaid chromatographs of (d) melamine standard concentrations (0.39 – 50 µg.ml^{-1}), (e) cyanuric acid standard concentrations (0.78 – 100 µg.ml^{-1}) and (f) uric acid standard concentrations (0.78 – 50 µg.ml^{-1})

Table 3.14: Summary of regression analysis including the LOD and LOQ of the analytes from standard calibration curves

Analyte	Retention time (min)	Regression equation	Regression coefficient (R^2)	Range ($\mu g.ml^{-1}$)	LOD ($\mu g.ml^{-1}$)	LOQ ($\mu g.ml^{-1}$)
Melamine	3.30 ±0.013	y = 406121x+ 40844	0.9999	0.39 - 50	0.81	2.43
Cyanuric acid	2.10 ±0.007	y = 69888x + 43320	0.9998	0.78 - 100	2.17	6.67
Uric acid	1.85±0.006	y = 149387x + 101565	0.9995	0.78 - 50	1.92	5.82

3.4.2. Specificity

The peaks of each analyte were found to be uniform, symmetrical and with good resolution. Figure 3.8. d-f illustrate the excellent chromatographic separation of the analytes. The retention times were distinct and the average retention times were 1.85 min, 2.10 min, and 3.30 min for melamine, cyanuric acid, and uric acid, respectively (Table 3.14).

3.4.3. Accuracy

The accuracy of the method was determined by recovery studies and expressed as percentage recoveries for each analyte. The percentage recovery for melamine ranged from 96.8% to 100.8%, from 99.3% to 102.9% for cyanuric acid, and from 99.6% to 104.9% for uric acid (Table 3.15). All the recoveries of the analytes were within the acceptable criteria for accuracy.

Table 3.15: Determination of method accuracy

Amount of analyte added (µg.ml^{-1})	Peak area (mAU)	Slope (*m*)	Intercept (*c*)	Mean recovered amount (n=4) (µg.ml^{-1})	% Recovery (n=4)
	Melamine				
3.12	1316064 1327876 1314671 1305984	406121	40844	3.14±0.022	100.7
12.5	4954908 4949872 4954099 4965102			12.10±0.016	96.8
25	10283216 10276414 10357668 10218977			25.22±0.14	100.8
	Cyanuric acid				
3.12	261371 260978 260437 262490	69888	43320	3.12±0.012	99.9
25	1840140 1840201 1840030 1841440			25.71±0.0095	102.9
50	3512560 3504789 3520984 3511478			49.64±0.095	99.3
	Uric acid				
3.12	590061 590165 591689 589958	149387	101565	3.27±0.0055	104.9
12.5	1961433 1957465 1960507 1963482			12.45±0.017	99.6
25	3837734 3830834 3842689 3839806			25.01±0.034	100.0

3.4.4. Precision

The precision of the method was assessed in terms of interday, intraday and repeatability studies. Three standard solutions of melamine, cyanuric acid, and uric acid from the standard curves were analysed five times on the same day to determine intraday precision and on five consecutive days to determine interday precision. Tables 3.16 and 3.17 summarise the interday and intraday precision studies for this method. The %RSD values obtained for all analytes were within the limits of ≤2% as shown in Tables 3.16 and 3.17.

A repeatability study was carried out with the standard stock solutions of melamine, cyanuric acid, and uric acid was performed by injecting the same concentrations of each analyte six times into the column using the proposed method. Table 3.18 represents the data from this study. The results show that the %RSD values for the three compounds were below 2% and within the acceptable limits.

Table 3.16: Determination of method precision - interday

Amount of analyte added ($\mu g.ml^{-1}$)	Mean recovered amount (n=5) ($\mu g.ml^{-1}$)	%RSD (n=5)	%Recovery (n=5)
Melamine			
3.12	3.14±0.020	0.63	100.8
12.5	11.78±0.010	0.087	94.3
25	25.43±0.32	1.25	101.7
Cyanuric acid			
3.12	3.10±0.015	0.48	99.5
25	24.82±0.11	0.46	99.3
50	50.03±0.28	0.55	100.0
Uric acid			
3.12	3.18±0.011	0.36	101.8
12.5	12.77±0.053	0.41	102.1
25	25.01±0.030	0.12	100.1

Table 3.17: Determination of method precision – intraday

Amount of analyte added ($\mu g.ml^{-1}$)	Mean recovered amount (n=5) ($\mu g.ml^{-1}$)	%RSD (n=5)	%Recovery (n=5)
Melamine			
3.12	3.10±0.0022	0.070	99.4
12.5	11.78±0.020	0.17	94.3
25	25.43±0.17	0.67	101.7
Cyanuric acid			
3.12	3.10±0.022	0.71	99.6
25	25.55±0.12	0.46	102.2
50	49.75±0.13	0.26	99.5
Uric acid			
3.12	3.20±0.0046	0.15	102.6
12.5	12.64±0.024	0.19	101.1
25	24.99±0.069	0.28	99.9

Table 3.18: Repeatability study

Repeat	Recovery		
	Melamine (25 $\mu g.ml^{-1}$)	Cyanuric acid (25 $\mu g.ml^{-1}$)	Uric acid (25 $\mu g.ml^{-1}$)
1	24.95	25.07	24.11
2	25.06	25.38	24.24
3	25.10	25.16	24.61
4	25.17	24.86	24.46
5	24.93	25.43	24.38
6	25.27	25.30	24.59
mean	25.08	25.20	24.40
SD	0.13	0.21	0.20
%RSD	0.52	0.85	0.80

3.4.5. Stability study

The stability of melamine, cyanuric acid and uric acid was determined at room temperature and 4^{o}C by assay analysis at 24-hour intervals for three consecutive days. In this study, standard solutions of 50 $\mu g.ml^{-1}$ were used for melamine and cyanuric acid and 25 $\mu g.ml^{-1}$ for uric acid. The solutions were analysed from day zero, when they were freshly prepared until the last day (day 3), without protection from light. The recoveries of each analyte were calculated for all solutions for each day. The solutions were found to be stable for up to 72-hours (Table 3.19). The assay values attained after 72-hours were statistically identical with the initial value (day zero) without any significant considerable difference from the initial samples.

Table 3.19: Evaluation data of solution stability study

Time intervals	Absolute difference in assay for standard solution, %					
	Melamine (50 µg.ml^{-1})		Cyanuric acid (50 µg.ml^{-1})		Uric acid (25 µg.ml^{-1})	
	At 4ºC	At room temperature	At 4ºC	At room temperature	At 4ºC	At room temperature
24 hrs	1.31	0.11	0.04	0.04	0.14	0.14
48 hrs	1.34	0.17	0.61	0.60	0.18	0.17
72 hrs	1.85	0.18	0.90	0.90	0.24	0.23

3.5. Detection of adulterants in protein supplements

Twenty-one protein supplement products were analysed by the validated HPLC method to determine the presence of melamine, cyanuric acid, and uric acid. All the products tested positive for one or more of the adulterants. Table 3.20 shows the results of this study. Melamine was detected in 86% of the products with an average of 12.5 µg.ml^{-1}, cyanuric acid was present in 90% of the products with an average of 119.5 µg.ml^{-1}, and uric acid was detected in 76% of the products with an average of 57.8 µg.ml^{-1}.

Table 3.20: Detection of adulterants using the proposed HPLC method in this study

Product code	Detected amounts (µg.ml^{-1})		
	Melamine	Cyanuric acid	Uric acid
1	3.24	18.51	122.47
2	4.20	11.60	5.88
3	ND*	3.58	ND*
4	4.41	0.23	97.12
5	0.36	2.08	ND*
6	ND*	6.29	ND*
7	1.15	6.96	275.05
8	134.79	14.27	1.51
9	3.16	1196.39	13.10
10	0.61	1146.90	8.79
11	63.81	14.20	353.60
12	0.32	10.38	10.79
13	0.05	ND*	ND*
14	9.04	10.77	97.51
15	0.05	14.38	115.38
16	0.27	0.21	6.33
17	35.64	30.26	13.21
18	0.02	1.59	56.33
19	2.30	20.19	20.81
20	0.04	0.72	ND*
21	ND*	ND*	15.18

ND*= not detected

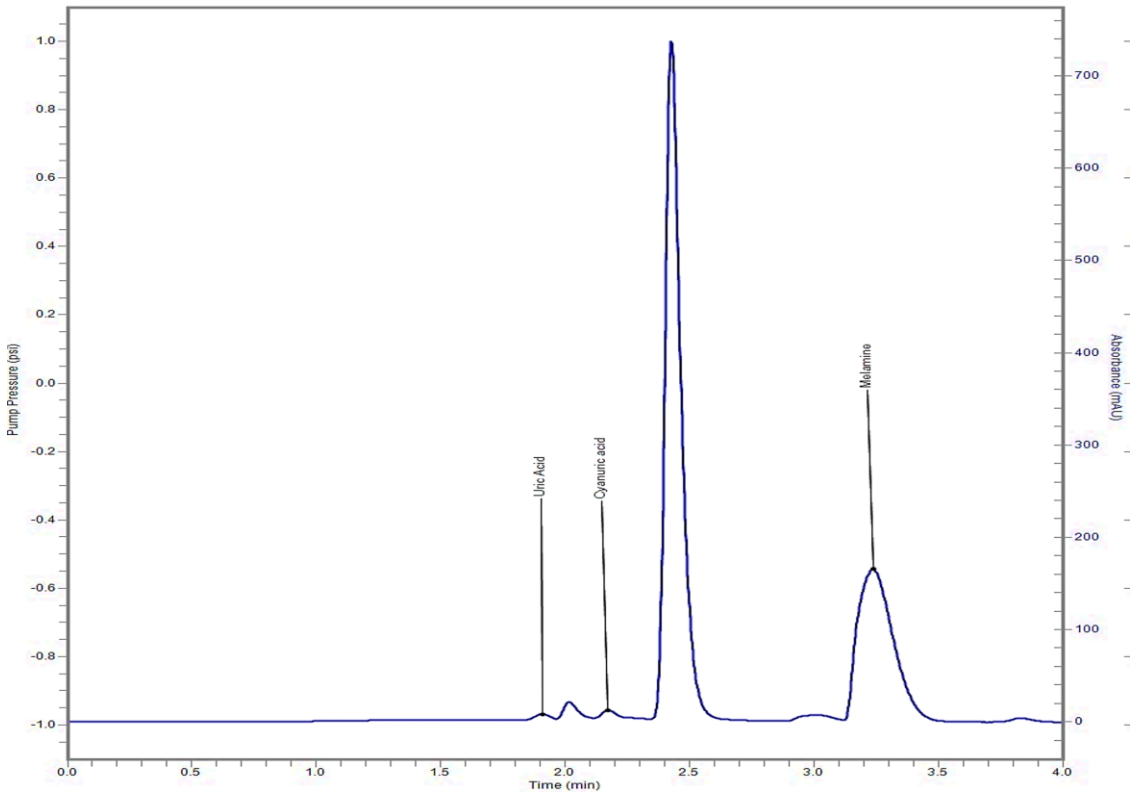

Figure 3.9: Chromatograph of test sample, product 8 (1:4 dilutions) with melamine, cyanuric acid and uric acid detected using proposed HPLC method

Table 3.21 contains illustrative examples of this study on the required amount to achieve the TDI limit as established by the respective authorities. The data represented here shows that locally produced protein supplements contain the highest levels of melamine of 319 mg for consumption per day. While imported products have the highest amounts of cyanuric acid and uric acid at 1837.7 mg and 1768 mg respectively, for consumed product per day.

Table 3.21: Illustrative examples of adulterants contamination levels and consumption relative to accepted TDI

	Melamine				Cyanuric acid		Uric Acid		
Description	**High**	**Medium**	**Low**	**High**	**Medium**	**Low**	**High**	**Medium**	**Low**
Concentration ($\mu g.ml^{-1}$)	134.82	0.61	0.016	1196.42	10.41	0.21	353.62	13.14	1.51
Product consumption quantity per day based on consumed and dose information (grams)	0.319	0.0009	0.000021	1.84	0.01	0.00016	1.77	0.015	0.0012
Indication of adulterant content on label (Y/N)	No	No	No	No	No	No	No	No	No
Local/International product purchase South Africa	Local	Local	Local	International	International	International	International	International	Local
Total adulterant consumption per day (mg)	319	0.91	0.021	1837.71	10.14	0.16	1768	15.18	1.21

CHAPTER FOUR – DISCUSSION

CHAPTER 4 – DISCUSSION

Protein supplements, particularly whey protein products, are widely used in the fitness and wellness sectors around the world. The physiological benefits mentioned in Chapter 1 add to the appeal of these products. However, due to poor regulation in the supplement industry, these products have recently prompted the interest of researchers. Several studies have reported the presence of prohibited substances and compounds that are not listed on nutritional supplement labels. As a result, the safety and reliability of these products have been called into question, as has the accuracy of the information on the labels regarding the reported protein content and the absence of prohibited substances. The current study found that the protein content of all 21 protein supplement products was lower than indicated on labels, indicating that the information on the labels is incorrect. In addition, according to the label information study, most product labels made no mention of the possibility of contamination. In contrast, melamine, cyanuric acid, and uric acid, were found in all of the products, proving that this was incorrect. The findings of the various studies conducted as part of this protein supplements research are further discussed below.

4.1. Label information study

Twenty-four percent of the labels on protein supplement products indicated that they did not contain prohibited substances in sports and potentially harmful substances (Table 3.6). About 76% of the product labels did not provide evidence or indication of any contamination. These results can be interpreted as either an expression of confidence that the quality of the products is good and therefore there are no concerns about possible contamination, or as an indication that the manufacturer is unaware of possible contamination in the products or that they simply omitted to make any claims (Gabriels *et al.,* 2012). These results are consistent with the findings of Gabriels (2013), who investigated various formulations of nutritional supplements and discovered that 71% of the product labels did not contain indications of products being “contaminant-free” or have any banned substances. This suggests that there is a common trend and consistency in such practices among supplement manufacturers. Furthermore, the

findings of Gabriels and Lambert (2013), who conducted a survey of users of nutritional supplement, revealed that 70% of the respondents were strongly influenced by the labels on the packaging, which indicated that the nutritional supplement was free of banned substances. However, upon closer inspection of the 21 protein supplements in this study, some of the most popular products are among the 76% that do not report the presence of contaminants. This could imply that consumers disregard this information about these well-known brand-name products, placing false trust in them, or that consumers are unaware of the potential dangers.

The average cost price of all the supplement products is R 420.8 (Table 3.1). This finding is in line with a recent survey in South Africa, which discovered that majority of consumers spend around R500 on nutritional supplements (Coopoo *et al.,* 2020). Table 4.1 summarizes the four major types of protein found among the 21 protein supplements in this study. As illustrated in Figure 3.2, imported products contain more expensive protein. This could be due to the presence of more costly proteins imported products, such as whey isolate and hydrolysate (Table 4.1). Shipping fees and taxes levied on the imported supplement products are two other factors that can affect the prices. Suppliers may also purposefully inflate imported pricing to entice consumers who may assume that higher-priced items are superior. As part of this ongoing study, it would be interesting to assess the preferences and perceptions of users of protein supplements; whether one type of whey protein is preferred over the other and whether imported products are of higher quality than locally manufactured products. It is worth noting that the average cost per serving is determined by the type of protein used (as shown in Table 4.1). The higher the cost of protein per serving, the more expensive the protein type. One of the objectives in this study was to investigate the actual protein content quantity within the product, to assess whether the indicated protein quantities are correct. Furthermore, based on the findings, this information will allow us to determine whether product pricing is justified.

Table 4.1: Protein powder types found in the products of this study (Tinsley, 2018)

Protein	Description
Concentrate	Less processed whey protein that consists of 80% protein and 20% carbohydrates and fats. Generally cheaper.
Isolate	More processed form of whey protein that contains less fats and carbohydrates and more protein (≥90%). More costly
Hydrolysate	Pre-digested form of whey protein has no fats or carbohydrates. Easily digested because it has undergone hydrolysis. Most expensive
Blend	Contains a mixture of whey proteins which can be concentrate, isolate and hydrolysate
Broth	Made from animal bones, cooked to form a concentrated form of bone-broth protein

The physiological benefits that products promise is one of the main reasons people use them. Most protein supplements (>52%) in this study are intended for muscle growth (Table 3.2), which is consistent with the findings of Coopoo *et al.* (2020), who found that most gym users mostly consume protein supplements for the purpose of gaining muscle mass. As a result, most of the supplement products on the market make this muscle gain benefit claim in order to attract more customers.

Whey protein, according to studies, is superior to other types of protein such as soy or casein protein, for muscle growth (Hartman *et al.,* 2007, Tang *et al.,* 2009, Pennings *et al.,* 2011). Despite the slight nutritional difference, there is no evidence that whey isolate is more beneficial than whey concentrate (Tinsley, 2018). In fact, studies suggest that taking these two whey proteins in the same dosages would provide the same desired physiological benefits (Tinsley, 2018). Naclerio and Larumbe-Zabala (2016) discovered that whey protein supplements improved muscle strength. This was supported by Fardi and Welis (2018), who found that protein supplements not only promote muscle growth and strength, but also accelerate recovery during and after exercise. Therefore, based on available scientific research, this would support claims about protein supplements products related to muscle building and recovery. However, no such scientific evidence was found to support the claims of almost all the protein supplements products in this study. In fact, only 5% of

the products had stated that claims are based on scientific research, but no evidence or citation was given on the label (Table 3.4). It should be noted that while the short-term physiological benefits of protein supplements have been thoroughly researched, the long-term effects are still unknown (Fardi and Welis, 2018).

All the supplements in this study made exaggerated claims about product quality and were often unclear and devoid of substantial scientific evidence (Table 3.4). On the product labels, outrageous claims, and pseudoscientific terminology such as “Time Released Formula,” “Fast Digesting,” and “Instant Gratification” were observed. Ambiguous and outrageous claims on nutritional supplement labels may promise physiological benefits to the customer, but these frequently unproven claims do not have to be true because they have been scientifically validated (Gabriels, 2013). Furthermore, upon closer inspection, there was no evidence to support the claims made on the product label by the manufacturers. The main concerns about the content of the labels include the lack of supporting scientific data on safety and efficacy, as well as a lack of ethical and clinical content. This type of label information is critical because it ensures that policy makers, healthcare professionals, consumers, and the general public have sufficient information to make informed decisions (Gabriels, 2013; Joshi *et al*., 2010). Earlier studies support this study's findings, stating that advertisements involving claims, often health or illness claims, are misleading or scientifically unsubstantiated, despite restrictions barring such statements (Gabriels and Lambert, 2013; Lachenmeier *et al*., 2013; Petroczi *et al*.,2011).

A study to determine the characteristics that draw customers' attention to nutritional information indicated that display size, colour theme, labelling ease of use, logo, and location on the front of the pack were important determinants (Bialkova and van Trijp, 2010). This study found that a better understanding of nutritional information and paying attention to label content influenced the selection of nutritional supplements (Bialkova and van Trijp, 2010). In this present study, all of the above-mentioned claims and statements were frequently in a bold and clear font in all of the products. On the contrary, warnings and disclaimers were presented in small print and were often difficult to see and read. This appears to be intentional on the part of the manufacturers, who employ this type of marketing to bring consumers' attention to the promised benefits. While crucial information such as the existence of allergens

might be overlooked by an individual who is allergic to a specific substance that may be present in the product, ingesting such a product can result in an adverse reaction. This is in violation of DOH regulation R.146, which regulates supplement product labelling. Warnings on labels must be in clear and visible font for the customer, according to the regulations (DOH, 2011). This finding emphasizes need for proper labelling guidelines for these products (Gabriels *et al.,* 2012).

All the supplement products (n = 21) underscored the importance of following storage instructions (Table 3.6). Compliance with storage instructions is essential, as studies have shown if nutritional supplement products are stored at the incorrect temperature, they can convert inactive compounds into completely harmful active chemicals or unidentified compounds (Gabriels, 2013; van der Merwe and Grobbelaar, 2005). Consumption of such nutritional supplements may result in disastrous side effects (Gabriels *et al.,* 2012).

In terms of claims, disclaimers, and warnings, data from this study revealed no significant variations in information between imported and locally produced supplements. Lastly, this research indicates that SAHPRA needs to improve its oversight and develop industry-specific guidelines that must be followed by the supplement industry and enforced by the mandated authority with regards to labelling information on protein supplements in particular.

4.2. Quantification of protein content

Recent research on protein supplements has revealed that the protein content of these products may be deliberately manipulated (da Costa *et al.*, 2021). To exacerbate the situation, the Dumas and Kjeldahl methods currently used to quantify protein content in protein supplement products are considered inadequate (Field and Field, 2010; Mæhre *et al.*,2018). This limitation stems from the fact that these methods also measure the nitrogen content of the entire protein sample, including non-protein compounds (FAO, 2003). According to the literature, nutritional supplements can contain nitrogen-rich compounds such as melamine, which can interfere with the quantification of protein content (Gabriels *et al*., 2015). In order to address this issue, a multi-protein assay approach was used in this study to determine the protein content of 21 protein supplement products. The results of this study are discussed further below.

4.2.1. Multi-protein assay

The Bradford assay results (Table 3.9) in this study show that there was a significant discrepancy between the values measured in the laboratory and the values reported on the label of protein supplements. Given that 95% of the products are statistically significantly different (p<0.05) from the values reported by the label, as well as the poor colour development in the Bradford assay (Fig.3.5. b), this indicates a poor reaction between the assay-reagent and protein samples. One plausible explanation is that the Bradford assay has a major limitation in that Coomassie-dye does not bind to low molecular weight peptides (<3000 Da), as a result, samples containing proteins of varying sizes cannot be accurately measured (Kruger, 1994). A study confirmed this by demonstrating that the molecular weight distribution of whey protein ranged from 300 to 1400 Da, with most whey peptides being less than 1000 Da (Li-Jun *et al.,* 2008). As a result, the Bradford assay was deemed insufficient for quantifying protein content in protein supplements for the purposes of this study.

The results of the BCA and Lowry assay in Tables 3.7 and 3.8, respectively, revealed that the protein content analysed in the 21 protein supplements was lower than indicated on the product labels. The percentage difference between the measured protein values and the protein values declared on the labels ranged from 5.4% to 46.7% for BCA and from 1.8% to 35.6% for the Lowry assay. A statistical comparison of these two protein assays revealed that no significant difference (p>0.05) between the protein content values determined by the BCA and Lowry assays (Table 3.10). These findings are comparable to those of Schönfeldt *et al.* (2019) who used the Dumas method and Kuswari *et al.* (2021) who used the Kjeldahl method to quantify the protein content in protein supplements. Both the Kjeldahl and Dumas methods rely on a nitrogen conversion factor (6.26) to determine the protein content in a sample, which can result in an overestimation of the protein due to nitrogen from non-protein sources in the sample (Mæhre *et al.,*2018). The protein content determined by the protein assays in this study is entirely derived from the amino acids and proteins in the products. In the work of Schönfeldt *et al.* (2019), approximately 10% of the 70 products analysed by the Dumas method had higher measured protein content than the declared values on the label by 0.5 to 13.7%.

These protein content results from traditional methods contradict the findings of this study, which found that all the protein content analysed was lower than the declared values. This confirms that the Kjeldahl and Dumas methods incorrectly measure nitrogen from non-protein compounds, giving the impression that protein supplements have a higher protein content.

For compliance purposes, the guidelines of the regulations relating to the Labelling and Advertising of Foodstuff (The DOH Guidelines R. 146 of March 2010) stipulate that a 25% tolerance for protein nutrient claim is allowed if a product has more than 10g of protein per 100g, and a difference of ±2 g is acceptable if product has less than 10g of protein per 100 g (Schönfeldt and Pretorius, 2011). In the current study, three products were found to have over-reported protein content by more than 25% (highlighted in red) in both the BCA and Lowry assays (Table 3.7-3.8) This implies that product manufacturers are not complying with the government regulations, and that product labels should not make claims like “high in protein” when they are not (Schönfeldt *et al.*, 2019).

4.2.2. Factors affecting protein content and quality

In terms of protein content being lower than that declared in protein supplements, the current study’s findings are consistent with those of Schönfeldt *et al.* (2019) and Kuswari *et al.* (2021). The consistent discrepancy between the protein content analysed and the protein content reported on labels across the studies confirms that manufacturers of protein supplements falsely overestimate the protein content (Naidoo *et al*., 2018a). Manufacturers use less expensive multi-amine compounds such as melamine, as a non-protein material, incorrectly increasing the protein content in the product simply by increasing the presence of nitrogen in the product without adding amino acids (Gabriels *et al.,* 2015; Schönfeldt *et al.,* 2019; Kuswari *et al.,* 2021). Methods such as Dumas and Kjeldahl, for example, cannot differentiate between the nitrogen from protein and the non-protein melamine when measuring protein content, as a result, manufacturers maintain a high retail value for their products (Naidoo *et al*., 2018a). Protein supplement manufacturers’ blatant practice seriously infringes on unsuspecting consumers who buy these products based on their claims of high in quality protein and a desire for optimal physiological benefits. In the BCA and Lowry assays, for example, the protein content of Product 19 was

inflated by 46.7 % and 31.5%, respectively. This product had the greatest percentage difference, in both methods, implying that the manufacturers were disingenuous in reporting the protein content on the label when both methods discovered a significant discrepancy in the same product. Interestingly, the same product had the highest protein cost per gram, as shown in Table 3.3, at R2.34. This suggests that manufacturers may be manipulating their products by labelling them as high quality and high in protein when, in fact, this is not the case, according to this study.

Other factors that contribute to the difference in protein content between laboratory results from what is indicated on the protein supplement label include the manufacturing process, storage conditions, and inconsistency of the product (Kuswari *et al.,* 2021). The final stage of the manufacturing process for whey protein products such as isolate and concentrate (Table 4.1), involves drying by heating at temperatures ranging from 170 ^{o}C to 260 ^{o}C (Bernard *et al.,* 2011; Kuswari *et al.,* 2021). As a result, the protein can be denatured by 30% to 47%, causing undesirable reactions and influencing the type of nutrients in the product (Rufián-Henares *et al*., 2004; Bernard *et al.,* 2011). As stated in Section 4.1 above, it is essential to maintain the correct storage conditions for supplement products. According to Le *et al.* (2011), when exposed to varying temperatures (30 - 45 ^{o}C) and relative humidity (44 - 85%), whey protein activates a non-enzymatic browning reaction, which has a negative impact on the nutritional properties of the product (van Boekel, 1998). Additionally, when protein supplements are exposed to conditions that exceed the recommended limits, lactose blocks the amino group of lysine to form lactulosyllysine, which can alter the bioavailability and, through chelation, affect the value and quality of the protein (Tamanna and Mahmood, 2015; Kuswari *et al.,*2021). Lastly, product inconsistency can be caused by the changes in the composition of the product components over time, primarily because manufacturers change certain ingredients or use different methods to make the same product (Kårlund *et al*., 2019).

4.2.3. Interference of adulterants in protein assays

According to Shen (2019), during a biuret reaction, Cu^{2+} interacts with 4 nitrogen atoms of a peptide to form a copper (I) complex. When melamine and cyanuric acid are combined with copper salts, they form a complex (Wiles *et al.,* 2005; Dante *et al.,*

2016). Cyanuric acid reacts with readily available Cu^{2+} ions to form copper (II) cyanurate at high concentrations above 100 ppm (Dante *et al.,* 2016). Similarly, uric acid has been reported to react to form copper urate (Bernardi *et al.*, 2009). In this study, the red arrow in Figure 3.5a shows a slight colour change in the Lowry assay with 100µg.ml^{-1} uric acid, confirming that a reaction is occurring. This finding suggests that the reagent contains a component that interacts with uric acid in high concentrations, which is consistent with the findings of Dante *et al.* (2016) and Bernardi *et al.* (2009). Melamine, cyanuric acid, and uric acid are likely to react with the Cu^{2+} as nitrogen-rich compounds in the biuret reactions of the BCA and Lowry assays, and by coordinating with the amine groups of these compounds, the dye of the assay binds, resulting in a false-protein positive assay as observed in this study. From this, it can be concluded that melamine, cyanuric acid, and uric acid have only minor interference with the protein assays. This result is consistent with the findings of Field and Field (2010), who conducted a similar study with Bradford assay and discovered that melamine and cyanuric acid had no effect on the assay.

4.2.4. Application of biuret methods

The accurate assessment of food protein content is fundamental because it affects the economic value of the food product and can affect the economic feasibility of new protein producing sectors (Hayes, 2020). The present techniques for measuring protein concentration in protein supplements, such as Kjeldahl and Dumas, are insufficient and costly (Field and Field, 2010; Gabriels et al., 2015; Hayes, 2020). Moreover, the results of this study demonstrated that none of the product labels disclosed which method was utilised to measure the protein content claimed on the labels. The International Serum Industry Association, a trade organization for serum providers such as BSA protein, has laws that require the use of biuret methods to determine protein content in serum products (Johnson, 2012). This is a good example of how similar regulations can be implemented in the protein supplement industry to control product quality and regulatory adherence. Based on the findings of this study the biuret methods (BCA and Lowry assays) should be considered as potential methods for determining protein content in protein supplements. These spectrometric methods are reliable, inexpensive, and less prone to fraud, making

them a viable alternative for analysing protein content (Chang *et al.*, 2017; da Costa *et al.*, 2021).

The findings of the present study provide practical evidence of current supplement industry practices and could be used as a guide by authorities such as SAHPRA to implement specific and stringent regulations for these products.

4.3. HPLC method for detection of adulterants

Gabriels *et al* (2015) who discovered the presence of melamine in nutritional supplements, made the recommendation to evaluate for the presence of other melamine derivatives. Following that, the present study sought to investigate the prevalence of melamine, cyanuric acid, and uric acid in protein supplements. Several HPLC methods for detecting melamine and its derivatives in food products have been proposed in the literature, as discussed in Chapter 1. At the start of this study, no HPLC method was found that could detect melamine, cyanuric acid, or uric acid simultaneously. As a result, several methods from the literature were evaluated to determine which would be best applicable for the simultaneous detection of the compounds interest in this study, namely melamine, cyanuric acid, and uric acid, using RP-HPLC (Montesano *et al.,* 2013; Gabriels *et al*., 2015; Wijemanne *et al*., 2018).

4.3.1. Method development and optimization

A trend among the analytes was observed after a series of tests with different mobile phases used in this study. Uric acid eluted closely to either melamine or cyanuric acid using the test mobile phases. Even after adjusting the pH of the buffers and the organic solvent ratio, uric acid would elute with the other analytes. One possible explanation is that uric acid's structure is similar to melamine and cyanuric acid (Figure 2.1, in Chapter 2). During analysis, uric acid at a given pH is very likely to have physiochemical properties similar to cyanuric acid or melamine, causing it to elute closely to these compounds. These findings are consistent with those of Wiljemanne *et al.* (2018) who showed that uric acid retention time varied with changes in pH. Uric acid made up of highly reactive carboxyl and amine groups that, depending on the pH value of the buffer, either donate or accept protons (PubChem, 2021).

The first two experimental methods in this study used high percentage organic solvents (50% and 20%, respectively). The results from these methods showed that all three analytes eluted in less than 2 minutes. However, when the organic solvent ratio in the mobile phase was reduced to 5% and 1% for the third and final methods, respectively, the retention times for all the analytes increased to more than 2 minutes. This is consistent with the findings of Schuster *et al.* (2013) who found that in reverse-phase HPLC, the retention times of the compounds increase as the organic solvent is reduced.

The decision to add an ion exchanger to the buffer was made after considering the physical and chemical properties of melamine, cyanuric acid, and uric acid, as well as information from the literature and method development results. The use of an ion exchanger was justified because it was discovered to aid in the separation of analytes that eluted in close proximity (Small, 1989). An ion exchanger works on the basis of an ion-pair reagent with a high ionic charge that interacts with the analyte of interest. The ion-pair reagent is made up of a charged region that interacts with the analyte and a hydrophobic region that interacts with the column's stationary phase (Small, 1989; Weiss, 1995).

In this study, sodium 1-heptanesulfonate was used as an ion exchanger in the buffer. Sodium 1-heptanesulfonate is a cation with a high positive charge (Sigma-Aldrich, 2020). To successfully elute melamine, cyanuric acid, and uric acid, the buffer pH was adjusted to 7.25, which is above the pKa for all the analytes (Table 2.1, in Chapter 2). All the analytes are deprotonated at pH 7.25, resulting in an overall negative charge for the analytes that allows interaction with the positive charge of the sodium 1-heptanesulfonate. While the hydrophobic region of the ion exchanger interacts with the neutral C18 stationary phase. Because of these ionic interactions, analytes can elute at a specific retention time, which is unique for each compound (Weiss, 1995).

The results of the buffer with ion exchanger in the buffer demonstrated adequate separation of melamine, cyanuric acid, and uric acid, with distinct retention times of the for three analytes (Figure 3.7). In a similar study, Londono *et al.* (2018) used sodium 1-heptanesulfonate in the mobile phase to detect melamine levels in milk.

The chromatographic conditions for this study were optimised, and a satisfactory analytical method was obtained (Table 3.13).

4.3.2. Method validation

The proposed RP-HPLC method was validated using the standard procedures outlined in the U.S. FDA and ICH guidelines for linearity, range, specificity, LOD, LOQ, accuracy, precision, and stability (ICH, 2005; U.S. FDA, 2015). According to the guidelines, all of the parameters were within the acceptable limits (Table 2.2) (ICH, 2005; Bliesner, 2006; U.S. FDA, 2015). As shown in Table 3.14, all three analytes had a strong linear relationship between the standard concentrations and the peak, with a regression coefficient (r^2) of more than 0.999. Melamine, cyanuric acid, and uric acid chromatographs demonstrated satisfactory elution, separation, and good resolution of compounds at 3.30 min, 2,10 min and 1.85 min, respectively (Figs. 3.8. d-f). When compared to previous HPLC studies on these compounds, these results show an improvement in the retention times of melamine and its derivatives. Previous HPLC methods eluted melamine and its derivatives after 5 minutes, whereas elution for all three compounds was achieved in less than 4 minutes in this study (Montesano *et al*., 2013; Sun *et al.,* 2010b). This method also revealed that melamine had the lowest LOD of 0.81 $\mu g.ml^{-1}$ and cyanuric acid with the highest LOD of 2.17 $\mu g.ml^{-1}$. Uric acid showed had midrange values between the analytes for LOD and LOQ (Table 3.14). Filigenzi et *al.* (2008) used HPLC-MS^2 to achieve LOD of 1 $\mu g.ml^{-1}$ for melamine, whereas Ding et *al.* (2008) used HPLC-DAD to investigate melamine residue in protein powders of plant origin and found a LOD of 10 $\mu g.ml^{-1}$. As a result, the current study's findings show that the proposed HPLC method has good sensitivity for melamine and its derivatives when compared to other existing methods with more sensitive detectors. The percentage Relative Standard Deviation (%RSD) for repeatability ranged from 0.52 to 0.85 for the three analytes (Table 3.18). This indicated that the protocol was reliable and repeatable (Bliesner, 2006).

4.3.3. Detection of adulterants using proposed HPLC method

Using the validated RP-HPLC method, the current study demonstrated the presence of melamine, cyanuric acid, and uric acid in 21 protein supplement products (Table

3.20). Interestingly, none of the products that tested positive for any of the adulterants had information on the label indicating that either melamine, cyanuric acid, or uric acid could be present as a contaminant in the product (Table 3.6). This is consistent with the findings of Gabriels et al. (2015), who discovered that 47% of the 138 products tested positive for melamine and that there was no melamine mentioned on the labels of nutritional supplements that tested positive. Food manufacturers are required by Department of Health regulation R.146 (labelling and advertising of foodstuffs) to list all ingredients used in food products on their labels, including potential contaminants or allergens (DOH, 2011). Therefore, the results of this study could be interpreted as supplement manufacturers falsely advertise their products and deliberately omitting crucial information on the label about the potential presence of these adulterants. These compounds have been shown to have serious health effects and are not approved for use in nutritional supplements (Venter *et al.*, 2011, Meltzer *et al.,* 2004; Gabriels *et al.,* 2015).

Approximately 15 (71%) of the products tested positive for all three adulterants investigated in this study (Table 3.20). Although the concentrations of the adulterants were generally low, the presence of all three adulterants in these products suggests that the melamine chemical complex is more likely to precipitate (Ching *et al.,* 2013; Sprando *et al*., 2012). The combination of these adulterants is said to be more toxic, and when consumed through these products over time, it can be fatal for the unwary consumer (WHO, 2008). This is concerning because, as discussed in Chapter 1, no regulatory authority or agency is currently dedicated to testing for safety and effectiveness of these supplement products.

The daily intake doses of cyanuric acid and uric acid in the highest concentrations were found to be 1837.7 mg and 1768 mg, respectively, in this study (Table 3.21). These amounts are significant in comparison to the TDIs established by institutions around the world (Table 1.5, in Chapter 1). Melamine, cyanuric acid, and uric acid individually have low toxicity at low concentrations (Ching *et al*., 2013), however the co-occurrence of these adulterants is said to be more toxic and likely to cause potentially adverse health effects (Chen *et al.,* 2014; Hua *et al.,* 2012). Furthermore, as discussed in Chapter 1, daily consumption of protein supplement products with detectable uric acid levels, as demonstrated in this study, would increase the likelihood of developing diseases such as gout.

In this study, 90% of the protein supplements contained cyanuric acid, which was higher than 86% of melamine-containing products (Table 3.20). The concentration of cyanuric acid was approximately ten times higher than that of melamine. This finding is consistent with previous research on human and pet urine, which found higher levels of cyanuric acid than melamine in the samples of up to five times higher (Zhu and Kannan, 2019; Karthikraj *et al.,* 2018; Sathyanarayana *et al.,* 2018). This could be due to the manufacturers adding more cyanuric acid than melamine to the supplement products, or it could be due to the adulterated melamine converting to cyanuric acid (WHO, 2008). These findings add to existing evidence that the prevalence of cyanuric acid as an adulterant has increased, implying that more attention should be paid to this compound because its presence in a sample with melamine is said to be more toxic (Jacob *et al.,* 2012; Puschner *et al.,*2007; Zhu and Kannan, 2019).

As discussed earlier in this study, supplement manufacturers add adulterants to the products for a variety of reasons. This includes adding weight to the product, manipulating strength or quality, making the product appear better than it is, or omitting a valuable ingredient to reduce costs (Bejar, 2018; Newmaster *et al.,* 2013; Rocha *et al.,* 2016). The presence of melamine, cyanuric acid, and uric acid in these supplements may indicate that these adulterants were added to the products to increase the apparent protein content. These findings are consistent with the findings of the protein quantification study of the same supplement products (Section 4.2) using a multi-protein assay approach, which showed that protein content was lower than the amounts indicated on the labels. This would imply that manufacturers only add certain amounts of protein and replace the rest with melamine, cyanuric acid and/or uric acid. These compounds are nephrotoxic and in no case should they be added to foods or edible materials (Montesano *et al*., 2013).

The findings of this study highlight the importance for adequate regulations in the nutritional supplement industry to monitor adulteration/contaminants and implement a robust quality control programme. This is to ensure consistent, high-quality products and boost consumer confidence.

4.4. Limitations of study

Due to research funding constraints, only 21 protein supplements were investigated. However, a broader range of protein supplements (>100 products) would provide a better insight of the supplement industry practices. Another limitation of this work is the use of a UV-Vis detector in the HPLC, which may be insensitive to detect adulterants/contaminants at lower concentrations. Methods such as LC-MC^2 and GC-MC are more sensitive and have been shown in the literature to detect melamine and its derivatives at minimal levels. With a few modifications, future research could employ the suggested approach in this work to detect melamine and its compounds at sensitive levels.

4.5. Future prospects

Several pharmacological substances, including fluoxetine, have been found in nutritional supplements (Gabriels *et al.,* 2018; Hassan *et al.,* 2020; Brown, 2017). Elite athletes who consume these protein supplements may not only experience adverse effects, but they may also test positive for doping (da Costa *et al.*, 2021). This could jeopardise the athletes' career, so research into the presence of pharmacological compounds in protein supplements is needed to prevent this.

Protein supplements are typically marketed as complete proteins that contain all nine essential amino acids (Hoffman and Falvo, 2004). For that reason, a study of these commercial protein supplements is required to determine whether they contain all nine essential amino acids and other amino acids as claimed on labels. This information could be compared to the protein content as determined by the analysis and the amounts declared on the label.

As shown in Figure 3.9, a number of unidentified peaks were observed. These peaks were not identified due to time constraints in this project. These peaks could be melamine derivatives such as melamine-cyanurate, ammelide, ammeline, and cyromazine (pesticide). In the future, a similar HPLC method could be designed with similar parameters to identify these peaks. This could be accomplished by running protein supplement samples through HPLC then use a fraction collector to verify the identity of these peaks.

CHAPTER FIVE - CONCLUSION

CHAPTER 5 – CONCLUSION

Protein supplements are considered as the prime source of protein for many athletes and active individuals striving to improve their fitness and performance. This has driven the growth of the supplement industry in the recent years into a multi-billion dollar industry today. Reports from the literature about incompatibilities between what is on the label and the actual content of nutritional supplements are concerning, owing to high production demand, combined with poor manufacturing practices, and the lack of specific regulations (da Costa *et al.*, 2021; Fibigr *et al*., 2018). This study focused on 21 protein supplements commercially available in South Africa.

Ambiguous and outrageous claims on protein supplement labels may promise a physiological benefit to the consumer, but these frequently untested statements may not necessarily be true because they have not been scientifically validated. Manufacturers can currently (i) make unsubstantiated claims, such as promising muscle changes without scientific evidence, (ii) mislead consumers through advertising practices, (iii) practice non-disclosure of unfavourable health consequences and (iv) fail to declare the presence of adulterants and/or banned substances in the product, on labels. The current study's findings indicate that regulation for protein supplement products needs to be improved and made more scientifically specific. Manufacturers must explicitly state what health conditions are likely to be triggered when using their products so that consumers can make more informed decisions. As a result, SAHPRA must evaluate the claims and disclaimers made on the labels, as well as test the contents of these products. Such statements on product labels should be based on standardised laboratory screen methods. On all products, warning statements such as 'adherence to storage details' and 'keep out of reach of children' should be maintained and enforced.

The actual protein content of the 21 protein supplements was successfully quantified in this study using the BCA and Lowry assays. The results revealed that all supplement products had less protein content than reported on the label when measured by both methods. These findings contradict previous studies that used commonly used Dumas and Kjeldahl methods, which revealed that some of the products had higher protein content than stated on the labels. This confirms that the traditional methods which measure nitrogen from other non-protein compounds in the

samples as protein content, are inaccurate and not reliable. In addition, five of the products were found not to be non-compliant with the threshold of the regulation R. 146 (>25%) and had over-reported the protein content. The inconsistent levels of accuracy between analysed protein content and the amounts reported on labels, is not unique to South Africa. The same discrepancies with protein content in protein supplements have been reported in studies conducted in the United States, Brazil, and Indonesia (Kuswari *et al.,* 2021). These findings highlight the producers' poor manufacturing practices, who intentionally report false protein content on the labels. Other factors that contribute to the protein content disparity include manufacturing processes that cause protein denaturation, inaccurate protein measuring methods, and improper storage conditions. As a result of the reduced quantity, consumers of these products may not receive the desired maximum physiological benefits as communicated on the product label. Consequently, consumers spend more on these products while receiving less value for their money. This study emphasizes the need of stricter quality control measures and appropriate methods to ensure that what is reported on product label is essentially what is in the product. SAHPRA's recently implemented regulatory framework on complementary supplements, is, however, inadequate and does not specifically address the gaps in the market that manufacturers exploit.

This study developed a novel RP-HPLC method that is simple, accurate and rapid for the simultaneous detection of melamine, cyanuric acid, and uric acid. All three adulterants were successfully separated and detected simultaneously in the 21 protein supplements using this validated method. Despite the fact that product labels made no mention of contaminations or the possibility of harmful substances, adulterants were discovered in protein supplements. Seventy-one percent of the protein supplements contained all three adulterants, implying that the toxic combination of these compounds could have serious health consequences overtime for the consumer. The presence of cyanuric acid and uric acid was significant, indicating a net adulteration of protein supplement products on the market. The amount of the adulterants detected in these products is a cause of concern in terms of both quality and safety. This implies that when monitoring for adulteration, these compounds should be given more attention than just melamine.

In conclusion, this study found that protein supplements in South Africa are not as high in protein and as safe as they are claimed to be. Despite claims of high quality and protein content, this study revealed that consumers may not receive maximum physiological benefits due to inadequate protein. The methods used in this study are an excellent illustration of supplement industry methods that should be standardised and consistent. The application of precise analytic procedures would ensure supplement product validity and quality. In addition, the presence of adulterants in protein supplements endangers users' health. This emphasizes the need of educating the public on alternate ways to meet protein requirements through diet. These findings provide a more refined insight into current practices in the protein supplement industry. When implementing reforms in the nutritional supplement sector, policy response must be more vigorous, and the data from these findings provides specific areas of concern that can be used for this purpose. Furthermore, the study emphasizes the importance of appropriate controls, enforcement, and well-trained officials capable of identifying problems or potential problems in the sector.

CHAPTER SIX - RECOMMENDATIONS

CHAPTER 6 – RECOMMENDATIONS

- For manufacturers:
    - Supplement-specific regulations on what claims/statements can be made by manufacturers of protein supplements.
    - Clear font for warnings, expiration dates, batch numbers, and disclaimers
    - Using standardised methods to determine protein content in protein supplement products, such as the BCA and Lowry.
    - All protein supplement product labels must specify the method used to measure protein content.
- For regulatory bodies:
    - SAHPRA, the new regulatory body, must establish clear labelling guidelines, particularly for supplements. Minimum requirements are simply insufficient; product labels must meet specific requirements in order to be available on the market.
    - For regulatory agencies such as WHO, SAHPRA, and the Department of Health to: (i) include uric acid as one of the substances added to supplements and establish an appropriate TDI for it; and (ii) lower the current TDI values for cyanuric acid and melamine. (iii) be more stringent in terms of quality-control of supplement products by conducting more frequent tests for adulterants/contaminants to ensure the product's quality and safety for consumers; and (iv) prohibit the use of certain substances in supplements that can have fatal health implications.
    - South African regulatory authorities, such as SAHPRA, must conduct ongoing random tests on protein supplement products to ensure adherence to improved guidelines.
- For consumers:
    - Educating the public about nutritional information, such as how to interpret label information and what to look for in order to make more informed decisions.

- Future work:
    - To better understand or postulate the mechanism of action of melamine complexes such as melamine-cyanurate, the pharmacokinetics and pharmacodynamics of these complexes would need to be studied.
    - Conduct a cohort study of urine samples from individuals who take these supplements on a regular basis to determine (i) the presence and concentrations of adulterants (melamine, cyanuric acid, and uric acid), and (ii) the presence of biomarkers of early kidney damage.

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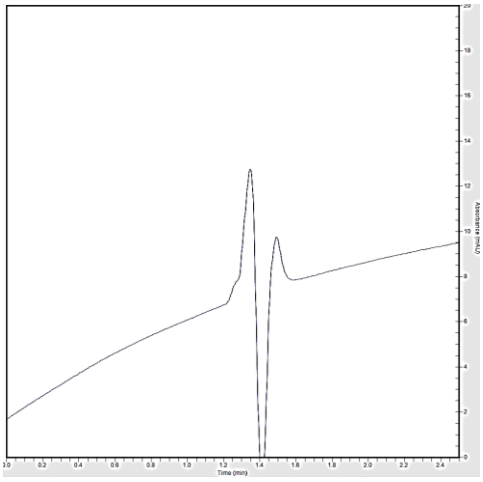
APPENDICES

Appendix A

Table A 1: Description of selected categories found on product labels (Gabriels, 2013)

Category	Definition	Examples from product labels
Claim	An assertion/declaration that something is the case, without providing evidence or proof	(i) Supports lean muscle gain. (ii) Source of high-quality proteins
Disclaimer	A statement intended to specify/delimit the scope of rights & obligations that may be exercised & enforced by parties in a legally recognised relationship	(i) This product is not intended to cure or prevent any illness or disease & should not replace any medication. (ii) These statements have not been evaluated by SAHPRA/FDA/MCC
Warning	A statement that serves as a cautionary advice on the risks associated with the product	(i) Store in a cool, dry place. (ii) keep out of reach of children
Outrageous claims	Highly unusual/unconventional/ extravagant assertations made without proof or evidence	(i)Instant muscle gratification (ii)The "last word" in ultra-enhancing sports supplements
Pseudo-science	A collection of beliefs or assumptions mistakenly regarded as being based on scientific method	(i)Triggers anabolism and builds muscle (ii) Ignites intense workouts

1.



2.

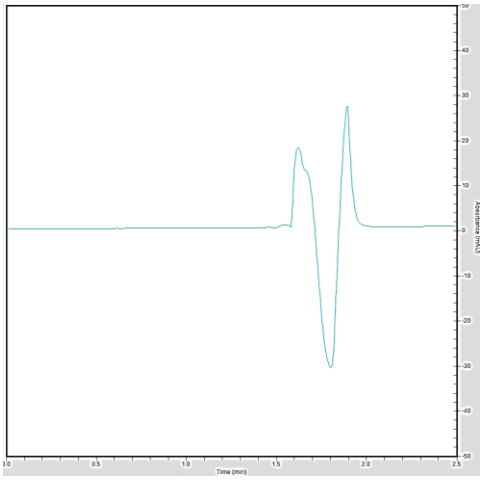


Figure A 1: Detection of cyanuric acid with different mobile phases showing a negative peak. 1. 50 µg.ml^{-1} Cyanuric acid with 0.1% formic acid: ACN mobile phase and 2. 50 µg.ml^{-1} Cyanuric acid with Acetate buffer mobile phase

Table A 2: The percentage relative standard deviation values for the analytes in accuracy study

Amount of analyte added ($\mu g.ml^{-1}$)	**Mean recovered amount (n=4) ($\mu g.ml^{-1}$)**	**SD**	**%RSD**
Melamine			
3.12	3.14	0.022	0.71
12.5	12.10	0.016	0.13
25	25.22	0.140	0.56
Cyanuric acid			
3.12	3.12	0.012	0.40
25	25.71	0.0095	0.04
50	49.64	0.095	0.19
Uric acid			
3.12	3.27	0.0055	0.17
12.5	12.45	0.017	0.13
25	25.01	0.034	0.14

Table A 3: Summary of peak area values used for the intraday study

Amount of analyte added (µg.ml^{-1})	Peak area	Slope (m)	Intercept (c)	Mean recovered amount (n=5) (µg.ml-1)
Melamine 3.12	1300185 1299053 1300653 1298672 1300465	406121	40844	3.10
12.5	4824949 4830061 4822019 4840140 4820105			11.78
25	10356317 10467451 10290573 10322671			25.43
Cyanuric acid 3.12	260043 261207 261859 257960 261256	69888	43320	3.10
25	1826163 1828278 1817592 1834258 1839341			25.55
50	3520248 3505945 3521517 3522509 3530952			49.75
Uric acid 3.12	579603 578355 580176 579865 579635	149387	101565	3.20
12.5	1989817 1993579 1983524 1989853 1988609			12.64
25	3834746 3846345 3837653 3833678 3817835			24.99

Table A4: Summary of peak area values used for the interday study

Amount of analyte added (µg.ml^{-1})	Peak area	Slope (m)	Intercept (c)	Mean recovered amount (n=5) (µg.ml-1)
Melamine 3.12	1316551 1307561 1320089 1314523 1329589	406121	40844	3.14
12.5	4824949 4830578 4826917 4831095 4821032			11.78
25	10368501 10178181 10358724 10539886 10404628			25.43
Cyanuric acid 3.12	259973 260107 261859 258967 260256	69888	43320	3.10
25	1777940 1776798 1784276 1765874 1786401			24.82
50	3539817 3518672 3523782 3565792 3549964			50.03
Uric acid 3.12	576616 573364 577104 576543 577809			3.18
12.5	2009237 2010987 2006895 1997894 2019763	149387	101565	12.77
25	3837734 3844768 3832985 3835698 3839965			25.01

Appendix B

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

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

23/12/2021

Ref: W-CBP-211223-02

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Mr M Sithole
Student No. (if appropriate): 05 0725 8D
Staff No. (if appropriate):

Supervisor: Dr G Gabriels

School: Therapeutic Sciences
Department: Pharmacy and Pharmacology
Pharmacology
Medical School
University

Project title: *The evaluation and laboratory screening of selected commercially-available protein supplement products*

Reason: In vitro laboratory study
No human participants will be involved in the study

Dr CB Penny
Chairperson: Human Research Ethics Committee (Medical)

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