ASSESSMENT OF ANALYTICAL METHODS FOR VALIDATING THE CLAIMS OF MILK PROTEIN BASED HEALTH SUPPLEMENTS

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY BY

SADHNA MAURYA

B.Tech. (Dairy Technology)

DAIRY CHEMISTRY DIVISION

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

KARNAL-132001(HARYANA), INDIA

2020

Regn. No. 18-M-DC-07

ASSESSMENT OF ANALYTICAL METHODS FOR VALIDATING THE CLAIMS OF MILK PROTEIN BASED HEALTH SUPPLEMENTS

BY

SADHNA MAURYA
(18- M-DC-07)
B.Tech. (Dairy Technology)

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY

Approved by

(Dr.Madhav R Patil)
EXTERNAL EXAMINER

02.09.2020
(Dr.Rajan Sharma)
Major Advisor & Chairman

MEMBER OF ADVISORY COMMITTEE

1. **Dr.Bimlesh Mann**
   Principal Scientist, Dairy Chemistry
2. **Dr. Kamal Gandhi**
   Scientist, Dairy Chemistry
3. **Dr.Yogesh Khetra**
   Senior Scientist, Dairy Technology
4. **Dr.Naresh Goel**
   Pricipal Scientist, Dairy Microbiology
   (Jt. Director Nominee)

3/9/2020

Gandhi 3/9/2020

2/9/2020

2/9/2020

Dairy Chemistry Division
ICAR- National Dairy Research Institute
Karnal-132001, Haryana

Dr. Rajan Sharma
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled, "**ASSESSMENT OF ANALYTICAL METHODS FOR VALIDATING THE CLAIMS IN MILK PROTEIN BASED HEALTH SUPPLEMENTS**" submitted by **SADHNA MAURYA** in partial fulfilment of the requirement for the award of the degree of **MASTER OF TECHNOLOGY** in **DAIRY CHEMISTRY** of the **ICAR-National Dairy Research Institute,** (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

(Dr. Rajan Sharma)
Major Advisor

Dated: 30.07.2020

Dedicated

To

My Beloved

Family

ACKNOWLEDGEMENT

It is a pleasant task to express my thanks to all those people who made this thesis possible and an unforgettable experience for me.

First of all I would like to thank God for being my strength, knowledge, ability and opportunity to undertake this research study and complete it satisfactorily. Without his blessings, this achievement would not have been possible.

This thesis is prepared under the supervision and guidance of Dr. Rajan Sharma, Principal Scientist, Dairy Chemistry division, National Dairy Research Institute. I am deeply indebted and sincerely grateful to him for his inspiring guidance, constant encouragement, intellectual supervision and all kind of help extended to me that led to the successful completion of my research. It was a great privilege and honour to work under his guidance.

I would like to extend my heartiest thanks to the member of my advisory committee Dr. Bimlesh Mann (Principal scientist, DC Division), Dr. Kamal Gandhi (Scientist, DC Division), Dr. Yogesh Khetra (Scientist, DT Division) and Dr. Naresh Goel (Principal scientist, DM Division) for their valuable suggestions and insightful comments.

My sincere thanks are humbly submitted to Dr. M.S. Chauhan, Director and Vice Chancellor, ICAR-NDRI, Karnal, Dr. R.R.B Singh, Joint Director (Academics), Dr. Dheer Singh, Acting Joint Director (Research), Dr. Bimlesh Mann, Head, DC Division for providing necessary facilities to carry out the present research.

I am extremely grateful to Dr. Bimlesh Mann for her unconditional support, guidance and suggestions during my whole research work.

I would like to extend my gratitude to all the Scientists of Dairy Chemistry Division for their guidance, help and cordial assistance that helped me sort out my research work problems.

I am wholeheartedly thankful to my most favourite seniors Nishi mam and Neelima mam for their never ending support and for extending helping hands throughout my research work.

I would like to extend my thanks to my seniors and lab mates Mitul sir, Yashwant sir, Gautam sir, Monika mam, Anamikia mam, Preeti mam and Reetika mam for their sincere cooperation and support. I am thankful to the trainees of my laboratory Upasna &Pragati and also to the Bittu bhaiya for their help during my bench work.

I would also like to say thanks to my friends Mitul, Amila, Srinidhi, Akash and Rishabh for their long discussions, help and fun filled environment which made this journey unforgettable.

And finally my greatest regards to my father Mr. Shrikrishna Maurya, other family members, Mr. Vijay Kumar and Mr. Deepak Jendre for their tremendous support, encouragement and understanding in tough times. Without their support and love, it would not have been possible to complete this work successfully.

Date: 29/07/2019 **Sadhna Maurya**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1.	EAAs	Essential amino acids
2.	BCAAs	Branched chain amino acids
3.	PDCAAS	Protein digestibility-corrected amino acid score
4.	BV	Biological value
5.	WPI	Whey protein isolate
6.	WPC	Whey protein concentrate
7.	WPH	Whey protein hydrolysate
8.	FAO	Food and agriculture organization
9.	BIS	Bureau of Indian Standard
10.	FDA	Food and Drug Administration
11.	USFDA	United states Food and Drug Administartion
12.	FSSAI	Food Safety and Standard Authority of India
13.	RP-HPLC	Reversed phase high pressure liquid chromatography
14.	IDF	International Dairy Federation
15.	ISO	International organization for standardization
16.	UV	Ultraviolet
17.	FT-NIR	Fourier Transform near Infrared
18.	Flame-AAS	Flame Atomic absorption spectroscopy
19.	MIR	Mid Infrared
20.	NIR	Near Infrared
21.	ppm	Parts per million
22.	DMAB	Dimethyl amino benzaldehyde
23.	LC-MS	Liquid chromatography mass spectroscopy
24.	ELISA	Enzyme linked immune sorbent assay
25.	NMR	Nuclear magnetic resonance
26.	NDRI	National Dairy Research Institute
27.	%	Percentage
28.	<	Less than
29.	>	More than
30.	℃	Degree Celsius
31.	µl	Microliter
32.	µg	Microgram
32.	GMP	Glycomacropeptide

33.	CAGR	Compound Annual Growth Rate
34.	CAPD	Continuous Ambulatory Peritoneal Dialysis
35.	PDE-5	Phosphodiesterase type-5
36.	WADA	World Anti-Doping Agency
37.	ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
38.	α-la	α- lactalbumin
39.	β-lg	β- lactoglobulin
40.	Na	Sodium
41.	Ca	Calcium
42.	mg	Milligram
43.	ml	Millilitre
44.	mg/l	Milligram per litre
45.	ml/min	Millilitre per minute

Abstract

Milk protein has a wide range of potential health benefits and functional properties. Milk protein based health supplements are quite popular in India and all over the world especially among the people aware about their health and wellness. These products are marketed at premium rates, but quality of these products is rarely checked. Even FSSAI has not suggested any analytical tools or methods to assess the quality of health supplements. Thus, the present study was undertaken to evaluate the quality of milk protein based health supplements and also to check the suitability of existing methods to assess the quality of these product using raw ingredients like Whey Protein Concentrate (WPC), Micellar Casein and Milk Protein Concentrate (MPC). For protein estimation, 3 methods have been used i.e. Kjeldahl, bicinchoninic acid (BCA) and Lowry assay and found that Lowry assay is better method for calculating the true protein in milk based ingredients as estimated protein values well correlated with reference method i.e. Kjeldahl method. For validating the same, blends of different raw ingredients (WPC, MPC, Micellar casein) and urea equivalent to the 1% protein have been made and found that spectrophotometric methods are more authenticated as these are based on biuret reaction and measure only true protein and not the non-protein nitrogen. Overall composition of raw ingredients like moisture, ash, minerals etc. was also estimated and compared to the health supplements. Total 12 samples of whey protein based health supplements were procured from local market and analysed. It was found that the protein content in 59% samples, fat content in 35% was varied from their claimed values. Individual proteins (α-lactalbumin, β- lactoglobulin) were also estimated using HPLC method and results indicated that in 2 samples, the ratio of α-lactalbumin and β-lactoglobulin was not 1:3 indicating alteration in the proteins used for manufacturing such blends. Lactose content varied from 2.63 to 59.97% and one samples also contained carbohydrate other than lactose. Moisture content and ash content varied from 1.9 to 5.73% and 2.5 to 5.79%, respectively. Although it was not mentioned on the label, all the samples were tested positive for presence of urea and caffeine indicating fraudulent use of urea for increasing Kjeldahl nitrogen and caffeine as a stimulant. Urea and caffeine contents in the samples ranged from 0.043 - 0.93% and 1.03 – 8.02 ppm respectively, which is much higher than the raw ingredients. Thus, it was concluded that overall 25% samples significantly varied from their claimed values and none of the samples followed the FSSAI guideline that the list of ingredients should be given in descending order.

सारांश

दूध प्रोटीन सहित संभावित स्वास्थ्य लाभ और कार्यात्मक गुणों की एक विस्तृत श्रंखला है। प्रोटीन युक्त दूध स्वास्थ्य के लिए लाभदायक है, इसलिए भारत सहित विश्व में काफी लोकप्रिय है। दूध निर्मित उत्पाद स्वास्थ्य और कल्याण के बारे में लोगों को जागरूक करते हैं। इन उत्पादों का विपणन प्रीमियम दरों पर किया जाता है, लेकिन इन उत्पादों की गुणवत्ता की जाँच शायद ही कभी की जाती है। यहां तक कि एफएसएसएआई ने स्वास्थ्य की खुराक की गुणवत्ता का आंकलन करने के लिए विश्लेषणात्मक उपकरणों या तरीकों का सुझाव नहीं दिया है। हालांकि, वर्तमान में मट्ठा प्रोटीन कॉन्सेंट्रट (डब्ल्यूपीसी), माइस्लर कैसीन और मिल्क प्रोटीन कॉन्सेंट्रेट (एमपीसी) जैसे अवयवों का उपयोग करके दूध प्रोटीन आधारित स्वास्थ्य की खुराक की गुणवत्ता का मूल्यांकन करने की व्यवस्था की गई है। प्रोटीन के आंकलन के लिए तीन विधियों का उपयोग किया गया है। प्रथम विधि जेल्डहाल, द्वितीय बिसिनकोनिक एसिड (बीसीए) व तीसरी लोरी परख विधि है। इनमें से लोरी परख विधि दूध आधारित अवयवों में वास्तविक प्रोटीन की जांच करने के लिए सबसे ज्यादा उपयुक्त है। अनुमानित प्रोटीन की जांच के लिए संदर्भ विधि यानी जेल्डहाल विधि सही है। इस विधि के तहत 1 प्रतिशत पोटीन के समतुल्य अपरिष्कृत कच्चे माल (डब्ल्यूपीसी, एमपीसी, माइक्रेलियर कैसिइन) का मिश्रण बनाया गया, और पाया गया कि स्पेक्ट्रोफोटोमेट्रिक तरीके अधिक प्रमाणिक हैं। जो कि मूत्रवर्धक प्रतिक्रिया पर आधारित हैं, और केवल सही प्रोटीन को मापते हैं। इस विधि में यह भी पाया गया कि नमी, राख, खनिज आदि कच्चे माल की औसत सरंचना भी स्वास्थ्य की खुराक की तुलना में अनुमानित थी। वहीं स्थानीय बाजार से मट्ठा प्रोटीन आधारित हेल्थसुप्पल के कुल 12 नमूने खरीदे गए और उनका विश्लेषण किया गया। जिसमें पाया कि 59 प्रतिशत नमूनों में प्रोटीन सामग्री , 35 प्रतिशत वसा सामग्री किए गए दावों से भिन्न थी। एचपीएलसी विधि का उपयोग करते हुए इंडिंबिजुवल प्रोटीनस (α-lactalbumin और β-lactoglobulin) का भी अनुमान लगाया गया था। 2 नमूनों में α-lactalbumin और β-lactoglobulin का अनुपात 1:3 प्रोटीन में मिश्रणों के निर्माण के लिए इस्तेमाल किए गए प्रोटीन में परिवर्तन था। लैक्टोज की मात्रा 2.63 से 59.97% थी और एक नमूने में लैक्टोज के अलावा अन्य कार्बोहाइड्रेट भी थे। नमी सामग्री व राख की मात्रा 1.9 से 5.73% और 2.5 से 5.79% तक भिन्न थी। हालांकि, लेबल पर इसका उल्लेख नहीं किया गया था, सभी नमूनों का परीक्षण किया गया, जिसमें कैफीन एक उत्तेजक के रूप में और नाइट्रोजन बढ़ाने के लिए यूरिया के कपटपूर्ण उपयोग का संकेत है। नमूनों में यूरिया और कैफीन की मात्रा क्रमशः 0.043 से 0.93% और 1.03% से 8.02 पीपीएम थी, जो कच्चे माल की तुलना में बहुत अधिक है। इस प्रकार यह निष्कर्ष निकाला गया कि कुल मिलाकर 25 प्रतिशत नमूने दावा किए गए मूल्यों से भिन्न थे। सामग्री की सूची को अवरोही क्रम में दिया जाना चाहिए , जबकि इन नमूनों में पाया गया कि एफएसएसएआई के दिशा निर्देशों का पालन नहीं किया गया है।

Introduction

1. INTRODUCTION

Mammary secretion habitually referred as milk, which is produced by milking of healthy milch animals without either addition thereto or extraction therefrom. The protein content in bovine milk is 3.5%, fractioned into two fragments i.e. casein and whey protein and their proportion is 80:20 (Patterson *et al*., 2019). Milk protein is considered as the model ingredient for manufacturing the foods intended for certain nutritional purpose as it comprises great amounts of bioavailable amino acids. In dairy industries different type of milk protein products are being manufactured including caseins, caseinates, milk protein isolates (MPI), milk protein concentrates (MPC), whey powders, whey protein concentrates (WPC: 50-80%) and whey protein isolates (WPI: ~90%) (McGregor and Poppit, 2013).

Whey protein is considered as complete protein as it comprises all the 20 amino acids as well as essential amino acids and their amount is in the proportion as required in the human body. The protein digestibility corrected amino acid score (PDCAAS) and biological value (BV) of whey protein are 1.00 and 104, respectively (Patterson *et al*., 2019). Whey protein is reflected as “fast” protein as it digests rapidly and thus increases amino acid concentration in blood at a faster rate than casein, while casein is reflected as “slow” protein, because it evacuates gradually from the stomach and thus leading to a prolonged and slow appearance of amino acids in the blood (Patterson *et al*., 2019). Indian market of whey protein is expected to develop at a CAGR of about 20% from 2017 to 2022 (researchandmarket.com). The casein can be fractionated as - αs_1, αs_2, ß, and k-CN. Generally the amount of protein in high protein milk powders ranges from of 50–85% and are generally denoted as milk protein concentrates (MPCs) and these are usually characterised based on the protein content like MPC56, MPC70 and MPC85 containing protein of 56, 70 and 85 %, respectively (Singh *et al*., 2007).

Health supplements defined as the foods, specifically processed or manufactured to fulfil the individual dietary requirements. These requirements are either due to any biological or physical condition or due to any disease and disorders. These supplements are served as it is. The composition of supplements

must be different from the normal foods having nature comparable to it. Health supplements also referred as food or dietary supplements. Powder, tablets and capsules etc. are the common form of dietary supplements.

According to FSSAI,

“Health supplement’ is a dietary substance(s) which is used by human beings in their diet for supplementing the diet. It may include ingredients like:

(a) Plant or the powder, extract or concentrate of their parts
(b) Protein, minerals or vitamins, amino acids, enzymes or
(c) Constituents of animal sources.

And these supplements cannot be used as conventional food”.

Health supplements are different from the foods for special medical purposes or foods for special dietary uses or normal foods as they are meant for different purpose. The first regulation in India for Health supplements and Neutraciticals has been executed from 1st January 2018. A collaborative initiative has been established by “International Alliance of Dietary Supplements Associations” (IADSA) and “Confederation of Indian Industries” (CII) under the “Resource Centre on Health Supplements and Neutraciticals” (ReCHaN).

There are 3 types of Milk Protein based Health supplements are available in market:

1. Whey Protein based Health Supplements
2. Casein based Health Supplements
3. Milk Protein based Health Supplement

Whey protein isolate (WPI), whey protein concentrate (WPC), glycomacropeptide, whey protein hydrolysate (WPH) are the base ingredients being used for the production of whey protein based health supplements. Milk Protein based supplements are principally manufactured by combining milk protein and some non-dairy constituents like cocoa based products, flavour, caffeine, artificial sweeteners etc. They should not be marketed as the supplements for diagnosing, treating or curing any disease which means that the manufacturers cannot make claims like “treats heart diseases” or “lowers high cholesterol” for these products (ReCHaN).

These products are gaining popularity among health conscious individuals because products of milk protein are beneficial for the health. Such products are aggressively marketed in health fitness centres across India. Due to increase in the awareness about the health and wellness, consumers are demanding more value in these supplements. Consumption of milk protein is increasing consistently in India as well as in the world. Different varieties of these products are abundantly available in market. Although a wide range of supplements are accessible in market, the products containing protein are constantly most popular among all these supplements. Thus, adulteration in these products is more common with substitute products such as inexpensive proteins having lower biological value (Andrade *et al*., 2019).

There is no well-matched regulation for milk protein based health supplements in most of the countries. Lack of a specific regulation and better observance in the manufacturing process of health supplements may result in irreconcilabilities between the content and label. There are so many risks allied with the use of unregulated health supplements including absence of active ingredients, presence of unsafe materials (like foreign objects and microbiological agents), toxic agents and also the occurrence of potentially dangerous drugs (Lukacs *et al*., 2018). There are so many cases of athletes or sports persons failing the doping test because they were using the dietary supplements. Due to the use of dietary supplements, there are also evidences of health problems and of serious adverse happenings, also small number of fatalities (Danezis *et al*., 2016). Generally these products are marketed at superior rates, but quality of these products is not checked regularly being available in unregulated environment. No specific quality parameters are available for marketing of such products. Regulatory bodies of India like FSSAI also has not proposed any analytical tools or methods for the assessment of the superiority of milk protein based health supplements. Numerous reports in the literature has indicated the adulteration of such products with different components like maltodextrin, melamine, plant proteins, other nitrogenous compounds (urea, ammonium sulphate) with low biological values etc. As the major component in such products is protein, the adulteration of such produces with high nitrogen-based substances is very common (Finete *et al*., 2013).

As already been mentioned that these products are often mixed with caffeine, cocoa powder, non-nutritive or nutritive sweeteners and other nitrogenous sources, so it may be a challenge to estimate the true protein content as Kjeldahl method is susceptible to manipulation being targeting the nitrogen compounds. Kjeldahl method is not an effective method for detecting the adulteration as apart from determining the total nitrogen of protein, it also determines other nitrogenous compounds like ammonium sulphate, melamine, and urea added to the samples. At laboratory level, there is no method available that can detect each and every substance declared on the label of health supplements, thus the complexity of supplements is also a great challenge (Moore *et al*., 2012). So, we require a method to determine the true protein content as well as the milk protein content in these products. It is also required to develop the methods for determining the composition, various components or adulterants, mineral profile for developing the analytical tools to evaluate the quality of health supplements (Maughan *et al*., 2013).

Hence, to address the above-mentioned problems, the present research work entitled as **"Assessment of analytical methods for validating the claims of Milk protein based health supplements"** has been proposed with the following objectives:

1. To check the suitability of existing methods to assess the quality of milk protein based health supplements.

2. To analyse composition of various milk protein based health supplements.

Review of Literature

2. REVIEW OF LITERATURE

Milk proteins are nutritionally important and provide a varied range of functional properties. For the production of milk proteins, several methods have been developed. Different milk protein products have been designed for particular applications including whey powders, whey protein isolates and concentrates, caseins and caseinates, and milk protein isolates and concentrates.

2.1 Health supplements

Health supplements are foods which are specially processed to satisfy particular dietary requirements. The purpose of these supplements is for balancing the diet, compensating the lack of nutrients, maintenance of health, improving the appearance and enhancement of sports as well as sexual performances. These supplements are very popular throughout the world with the major market is being shared by the U.S. and European countries. The value for sports supplements in global market is projected to approximately double in the 2015-2021 period, summiting at $13.6 billion. While the protein supplements accounted for 4905.9 million USD in global market in 2017 which is expected to touch 9785.8 million USD by 2026 rising at a compound annual growth rate (CAGR) of 8.0% (Persistence Market Research, 2016). While the market of sports nutrition in India is worth Rs. 1376 crore and estimated to grow at a CAGR of 22.8% by 2023 and there is 65% growth in products related to sports nutrition product introduced in India in 2018 as compared to the data of 2015 to 2017 as per Mintel Global data (Financial Express, 2019). Dietary supplements market in India is estimated to grow at a CAGR of 11.60% during the period of 2016 to 2021. Milk protein based health supplements accounts for substantial market of dietary supplements. Fig. 2.1 depicts the share of different types of supplements in the market (Research and Market.com, 2018).

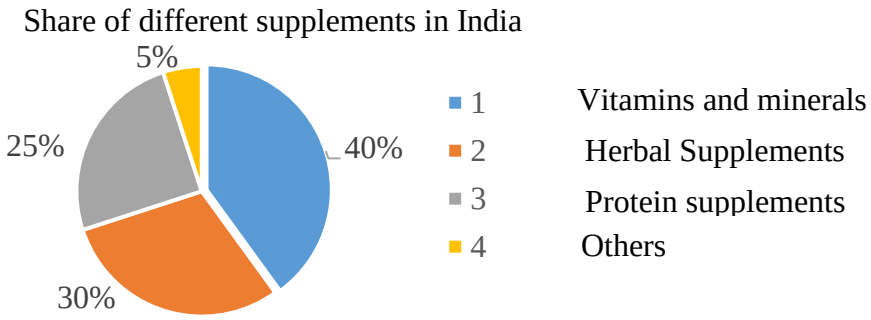


Fig. 2.1 Share of different types of supplements in Indian market

2.2 Milk protein – A quality protein

Nowadays milk protein based health supplements are very popular among health conscious public due to growing awareness about the health benefits of milk protein. Milk is a source of complete and balanced diet and consists of several valuable constituents. Significant nutritional advantages of milk are mainly because of the nutritive value and biological properties of milk proteins. Proteins are the complex molecules and their structural basic units are amino acids. Protein is important for various nutritional, physiological and functional activities. Milk is a well-known source of high-grade proteins which supplies major and important amino acids which in turn contribute in absorption of various trace elements and nutrients (Sharma *et al*., 2011). Milk protein is a source of various bioactive peptides which imparts various biological functions (Nagpal *et al*., 2011). Milk proteins, primarily from whey are acknowledged as an important functional food (Smithers, 2008). Nutritionally whey proteins have been recognised superior over casein in various aspects. More than 300 amino acids have been found in nature, out of which only 20 contributes in building of proteins (Wu, 2009). Whey protein is recognised as complete protein because it consists all the 20 amino acids including 9 essential amino acids in the same proportion as required for human body. Out of 9 essential amino acids, 3 are branched chain amino acids (BCAA) having unique characteristics (Sanz *et al*., 2017). Milk is the richest source of BCAA like leucine, valine and isoleucine as compared t other sources of protein. BCAAs, especially leucine, prevent muscle wasting and also stimulates muscle protein synthesis even in high protein breakdown condition. Whey proteins being rich source of BCAAs, are highly soluble under acidic condition of stomach causing their fast digestion and thus are also called “fast protein” or “fast-digested

proteins" (Cynober *et al*., 2003). While casein are reflected as "slow protein" or "slow-digested protein" as it gets clotted under the acidic condition of stomach and released slowly in small intestine (Bos *et al*., 2003). Patterson *et al*., 2019, also reported that the digestion as well as absorption of casein occurs slowly as compared to the whey proteins. Milk proteins have various health benefits as they contain higher amount of sulphur containing amino acids like methionine and cysteine which are precursor amino acids of glutathione. Glutathione is a tripeptide having antioxidant, immune-stimulatory and anti-carcinogenic properties. Whey proteins also helps in enhancing the serotonin activity which helps in sound sleep, stress reduction, supports immune system, helps to build lean physique by decreasing extra fat from the body, increases energy level and higher metabolic rate (Hoffman and Falvo, 2004).

Different benefits of milk protein specifically whey proteins are discussed below:

2.2.1 High nutritional value

Nutritive value is the measure of contribution of any food to the nutrient content of the diet. Nutritive value depends upon absorbed and digested quantity of food and also on the essential nutrients (protein, carbohydrate, fat) amount. As whey protein has good digestibility and also contains substantial amount of EAAs, thus it is considered as protein containing higher biological value and nutritive value (Smithers, 2008).

2.2.2 Essential amino acids

An essential amino acid is an amino acid which has to be taken from outer sources because it cannot be synthesized de novo by living beings. There are 9 essential amino acids e.g. histidine, lysine, leucine, threonine, isoleucine, valine, tryptophan, methionine and phenylalanine. Milk protein mainly whey protein is an excellent source of essential amino acids as compare to the other protein sources. All the essential amino acids which are necessary for growth of human body are present in sufficient quantity in whey (Gürsel, 2015). The critical factor for repairing and growth is leucine. It is needed for promoting the muscle protein synthesis. Some amino acids are also supplied by leucine in greater amount, which are necessary for stimulating the muscle growth (Pennings et al., 2012).

2.2.3 High biological value

The amount of absorbed protein from a food which is retained by the body to do the physiological functions is called biological value. Biological value also signifies that how readily and efficiently consumed protein can be utilised by human body. Whey protein is considered as the ideal protein for body building, athletes, sports person and fitness as they have quick digestibility and also due to their high biological value.

Table 2.1 Biological values of different protein sources

Protein source	Biological value	References
Whey protein	104	Hoffman and Falvo (2004)
Casein	77	Hoffman and Falvo (2004)
Soybean	94.5	Cahill et al. (1944)
Egg	95-100	Sumner (1938)

2.2.4 Sulphur containing amino acids

Out of different milk proteins, casein contains less sulphur containing amino acids like methionine and cysteine. α-s_1 and β-casein does not contain cysteine or cystine but k-casein and α-s_2 contains 2 cysteine residues per mole. The principal and rich source of sulphur containing amino acid is whey protein (Fox and McSweeney, 2013). These sulphur containing amino acids acts as the precursors of glutathione (act as antioxidant, anticarcinogen) which act as an antioxidant and also helps in metabolism (Shoveller *et al*., 2005).

2.2.5 Branched chain amino acids

The essential amino acids having protein anabolic properties are called branched chain amino acids. Leucine, valine and isoleucine are the examples of BCAAs as they don't have linear carbon bonds. All these three BCAA differs in hydrophobicity, shape and size but they have similar structure (Brosnan and Brosnan, 2006). Different biological functions of these amino acids include insulin secretion, muscle protein synthesis and brain amino acids uptake. In skeletal muscle, these AAs can be oxidized easily hence these are related to the energy metabolism in muscles. Literature has also indicated the BCAAs have benefits in sports nutrition also. These amino acids helps in controlling weight and act as

metabolic regulators for lipid metabolism, glucose and protein homoeostasis (Tipton and Wolfe, 2004).

Numerous health benefits of BCAAs are given below:

(1) They stimulate the synthesis of muscle protein

(2) Prevents the breakdown of muscle protein

(3) They also act as a muscles fuel during exercise

(4) Reduces the fatigue feelings,

(5) These AAs also speeds up the recovery after a intense exercise

These AAs are quickly utilised in muscles as a source of energy and thus used by people before and after doing the exercise (Sowers, 2009).

A study among the fatty young men revealed that taking extra protein during exercise and dieting can help in losing more fat and helps in gaining more muscles than those not taking extra protein. It has also been revealed that consuming whey protein shake before meal helps in improving the blood sugar as well as insulin response in both men and women suffering from type-2 diabetes. It has been shown by the 22 clinical studies that the individuals who have received the additional amount of protein either in the form of supplements or foods containing high protein, experienced significant elevation in muscle mass and strength (Consumerlab.com).

2.3 Importance of health supplements

Use of health supplements is very common among general population. It is due to the belief that health benefits on consumption of these supplements is above and beyond the health benefits that can be attained by the normal food (Maughan, 2013).

The common reason for using these supplements as reported by Maughan in 2013 are given below:

i. They improve health.
ii. They provide more energy.
iii. They enhance the performance.
iv. They increase the muscle strength.

According to a survey done in the above study, the proportion of using dietary supplements for different reason is given below:-

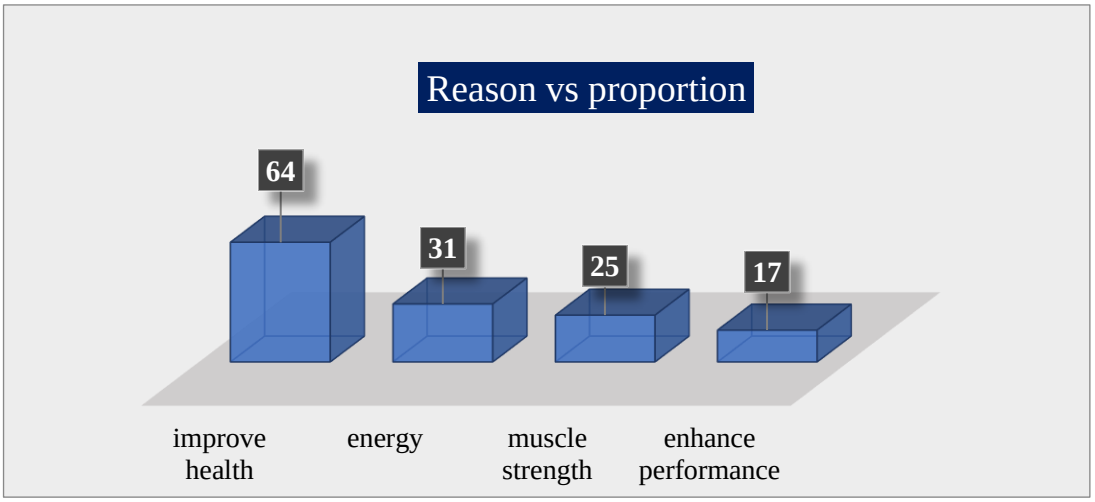


Fig 2.2 Proportion of different factors for the consumption of health supplements

A study was done by Kang and his team in 2019 to check the effect of whey protein supplementation and resistance exercise on frail and pre frail older people for 3 months and found that whey protein based health supplements are beneficial for stimulating the synthesis of muscles and also improved frailty.

Lensu and his team, 2019 studied about the interactive effects of milk protein supplements and the running and concluded that whey protein supplements can develop metabolic strength with or very less running. Milk protein can also reduce the metabolic disorders. Also protein supplementation can improve the phosphorylation of acetyl-CoA-carboxylase (Lensu *et al*., 2019). In another study it was also found that supplementing the whey protein along with resistance training can increase LST and Intracellular water (ICW) and also can reduce the ratio of Extracellular water (ECW) to the Intracellular water.

The study to check the clinical efficacy of whey protein supplements for treating the malnutrition in continuous ambulatory peritoneal dialysis (CAPD) was done by Sahathaven and his team in 2018 and found that the whey protein works with the a particular micronutrient to improve the Body mass index, weight, skin fold measures nPCR level and serum urea (Sahathaven *et al*., 2018).

Whey proteins also have antioxidant properties as it can increase, the endogenous anti oxidative enzyme system's activity and also improve the availability of glutathione in reduced form (Nabuco *et al*., 2019).

2.4 Quality issues in milk protein based health supplements

There are wide range of supplements but protein-based supplements are most popular mainly among the resistance training, military and sports personnel (Lun *et al*., 2012).

The use of unregulated health supplements may cause following risks (Maughan, 2013)

i. Absence or lack of active ingredients.
ii. Presence of harmful substances.
iii. Presence of toxic agents.
iv. Presence of pharmaceuticals which can be potentially dangerous.

The customer expects that the supplements should contain only the mentioned ingredients on the label in the specified amount and not any other ingredient. But the reality is not the same and there are various evidences about the fraudulent practices and poor quality as well (Maughan, 2005).

FDA has reported about the issues with supplements like presence of contaminants, foreign objects, food allergens which were not declared on label. The main cause of use of milk powder at the place of protein powders because these two have close resemblance (Lukacs *et al*., 2018).There are products containing little or no active ingredient in the supplements which are expensive (Green *et al*., 2001). It has also been observed by the FDA investigators that the rodents were running in the blending room, there was a dead rodent on the motor , the area used for storing finished product was contaminated by the dead rodent encircled by excreta and there were gnawed bags containing raw material covered with rodent's faeces and urine (Maughan, 2013). They also indicated the addition of melamine which is less expensive than the protein (ConsumerReports.org. 2012). In some products, the amount of adulterants is not significant thus it can be assumed that it may due to cross contamination during processing and packaging. The other reason of contamination could also be the poor quality

control. But most of time, adulteration is being done by manufacturers intentionally.

According to a study in U.S., it was estimated that each year over $ 20 billion is being spent by the customers on health supplements. The main purpose of adulteration of these products is the assumption that if the customers couldn't get any early effects, then they will abandon such products. So unscrupulous manufacturers add different components to get the quicker effect (Rocha *et al*., 2015).

Rocha and his team, 2015 reported different type of adulterants in different health supplements-

i. **Supplements for muscle building and athletic performance enhancement –** Common adulterants are anabolic steroids (methandienone and testosterone esters), prohormones, diuretics and designer drugs (drugs which don't have approval from any governmental regulatory body for therapeutic use in human), stimulants like subutramine (furazepam and 2 benzodiazepines diazepam which help in reducing the anxiety and also work as stimulants due to added anorexics). It has been studied that 18 out of 416 public notification issued by FDA were concerned about steroids presence in muscle building supplements.
ii. **Supplements for weight loss -** common adulterants are appetite suppressors, stimulants, antidepressants, diuretics etc.
iii. **Supplements for sexual performance enhancement-** Usually adulterated with pharmaceutical drugs like Phosphodiesterase-5 (PDE-5).

These adulterants can also be classified according to the purpose for which they are being used in health supplements (Andrade *et al.,* 2019) as given below:

i. **Stimulant adulterants-** The purpose of these adulterants is to stimulate the central nervous system of users e.g. Caffeine.

ii. **Economic adulterants**- These are the adulterants which are being used to mint more money. Manufacturer mainly use cheap products at the place of main ingredients e.g. melamine, plant protein, urea.

iii. **Effectual adulterants-** These adulterants can increase the initial effect of dietary supplements to attract more customer, as consumers will

discontinue the use of supplements if there are no really effects of these supplements e.g. anabolic steroids, prohormones (Rocha *et al*., 2015).

Some manufacturers also add pharmaceuticals to attain the stated aim. Some products contain higher amount of these pharmaceuticals which is not just sufficient for higher efficiency but can also raise the potential health hazards (Geyer *et al*., 2008). There are also reports about the supplements adulterated with the numerous agents which are banned by the regulations of anti-doping out of which some were also hazardous to health (Geyer *et al*., 2004). There are so many cases of innocent athletes which have been registered for positive doping test due to the presence of one or more banned substances in health supplements which have been consumed by those athletes (Watson *et al*., 2009). In the last few years, a large number of health supplements have been identified which contains undeclared doping substances (Danezis *et al*., 2016). There are reports about dietary supplements containing dimethylamylamine as many military personnel got positive test for the amphetamines in the programs of military screening (Geyer *et al*., 2008). Dimethylamylamine has various names like methylhexanamine, dimethylpentylamine, pentylamine, forthane etc. but according to the list of World anti-doping agency (WADA) (2011), only the first two name have been prohibited making the identification of these stimulants as complicated (Danezis *et al*., 2016).

Recently hydrogen collagen protein is also being marketed highly among the strength training people. But it is not a high quality protein because it lacks in tryptophan, so it will not stimulate the synthesis of protein by itself (Maughan, 2013). There are also not proper methods for the assessment of adulterants due to which the measurement of protein cannot be done properly and thus there are chances for adulteration like in the recent example of adulteration of milk powder by melamine, wherein children of Republic of China were hospitalized after its consumption (Lukacs *et al*., 2018).

There are also other adulterants found in protein based supplements like thermogenic substances, amino acid derivatives and carbohydrates. The adulteration of whey protein concentrate with spray dried milk whey powder has also been reported in the literature, as whey powder contains 70% lactose giving the economical profit to the manufacturers and is less effective to the consumers

(Andrade *et al*., 2019). Some supplements have also been adulterated with the maltodextrin which is the degraded product of starch. Creatine is also used as a supplement but in some cases it is also used as adulterant without claiming it on the label because it helps in the recycle of ATP in the muscles thereby improving the physical performance (Maughan, 2013). Free amino acids like glycine have also been used as adulterants as they are cheaper than whey protein isolate and whey protein concentrate. Trainers and physicians don't have any agreement about the health benefits of dietary supplements because there are so many contaminated and ineffective supplements in the market which may cause serious health hazard and false positive doping test to the athletes (Garrido *et al*., 2016). In some cases, it has been found that level of contaminants is not enough to attain any performance or health effect but it is more than enough to give the false positive results to the athletes who have been evaluated for the doping test (Danezis *et al*., 2016).

There are frequently cases of presence of stimulants like analogues and ephedrine whose natural source is Ma Huang or ephedra sinica. As customers are not aware about the natural name of ephedrine thus unscrupulous manufacturers mention these names over the label instead of the name of active ingredients. In some cases, they may contain sibutramine, which is not declared directly on the label but can give the information that it contains 'pure herbal ingredients'. Health supplements are also adulterated with the significant amount i.e. more than 1mg/g of anabolic steroids which is either not declared on the label or declared with the fancy or non-approved names. There are also evidences about the presence of androgenic steroids primarily prohormones and almost 15% of non-hormonal health supplements like proteins, vitamins and minerals which were not mentioned on the label.

Some designer steroids also found as an adulterant which are complicated to be identified as these are not listed as the ingredient of any medication and prohibited list of WADA also not contain their names. In the period from 2009 to 2010, it was found that supplements contained the prohibited growth hormones which releases the peptide-2 (GHRP-2) which is prohibited in the WADA's list (Danezis *et al*., 2016).

Sometimes manufacturers also give the written declaration to the athletes that their product do not contain any prohibited substances but still the doping test

of those athletes were found to be positive. Sealed package of health supplements was examined and it was found that it contained banned ingredients (Danezis *et al*., 2016).

Thus to regulate the composition of protein and other constituents of these protein supplements, there must be the implementation of quality control programme over milk protein products. In addition to that, the precautions should be taken while choosing the type of health supplements as it has been seen that these products are adulterated with other undesired type of protein and some other adulterants (Garrido *et al*., 2016).

2.5 Reported Incidences about quality issues

There are many incidences that have been reported to the mislabelling and adulteration of milk protein based health supplements. Some of these are given in Table-2.2.

Table 2.2 Incidences of adulteration in health supplements reported in literature

S. No	Title	Findings	Reference
1.	Caffeine contents of Dietary Supplements commonly purchased in the US	✓ Analysed 53 products with HPLC method ✓ Average caffeine ranged from 1 to 829 mg caffeine /daily dose	Andrews *et al.* *(*2007)
2.	Quality Assurance issues in the use of dietary Supplements, with reference to Protein Supplements	✓ Out of 24, 31% products failed in quality assurance test ✓ Lesser protein content ✓ Presence of heavy metals (lead- 6 to 18 µg in daily dose) ✓ Stimulants were also present ✓ 4 g of extra sugar/ serving ✓ out of 58 , 25% contained low levels of steroid contaminants and 11% were contaminated with stimulants	Maughan, (2013)
3.	Quality Assurance issues in the use of dietary Supplements, with reference to Protein Supplements	✓ In New York, 15 products were tested for heavy metals like Ar, Cd, Pb and Hg. ✓ 3 products contained heavy metals higher than the safe level.	Maughan, (2013)

4.	Proteomics in quality control: whey protein-based supplements	✓ 16 samples of whey protein based health supplements were tested. ✓ Only 10 samples contained proteins from bovine milk and out of which, 7 samples had the different profile expected for whey protein i.e. the contribution of β-lactoglobulin was < 20 %. ✓ 523 unique protein were identified out of which 162 were assigned to Bos Taurus, 19 to triticum aestivum, 25 to glycine, 1 for each of oryza sativa, solanumm tuberosum and zea mays and 2 to Gallus gallus.	Garrido *et al*., (2016).
5.	Drug adulteration of food Supplements: A threat to public health in European Union	✓ In Germany, Netherlands, and Sweden too much caffeine content was present ✓ In many countries, stimulants and pharmaceuticals were also present in various products	Czepielewska *et al* ., (2018)
6.	Quality Assurance issues in the use of dietary Supplements, with reference to protein supplements	✓ 634 non hormonal nutritional supplements from 13 different countries were analysed. ✓ Prohormones were presented in 94 samples. ✓ Prohormones of testosterone and nandrrolone were presented in 23 samples ✓ 64 samples contained only prohormones for testosterone ✓ While supplements contained only the prohormones for nadrolone ✓ Could not obtain reliable data for 66 samples	Maughan, (2013)
7.	Adulteration of Dietary Supplements by the Illegal Addition of Synthetic Drugs	✓ Pharmaceutical adulterants include appetite suppressors, stimulants, antidepressants, anxiolytics, diuretics, PDE-5 and laxatives in weight-loss protein food supplements	Rocha *et al*., (2015)

8.	The FDA and Adulterated Supplements— Dereliction of Duty	✓ FDA identified that between 2004 to 2012, 332 brands and between 2007 and 2016, 746 brands of supplements adulterated with pharmaceutical agents	Pieter and Cohen, (2018)
9.	FSSAI orders probe into health supplements, claims by FBOs	✓ FBOs were not following labelling regulations ✓ Marked with green dot, while it contained animal origin ingredients	FSSAI, (2019)
10.	Careful with food supplements	✓ 20% health supplements contained unapproved pharmaceuticals	The New Indian Express, (2019)
11.	Illegal Health Supplements manufacturing unit raided	✓ Ashraf Ahmed was running illegal unit ✓ Food safety officer found plastic jars containing supplements and sacks full of gluten and cereals and drugs also	The Times of India, (2019)
12.	Adulteration and safety issues in nutraceuticals and dietary supplements: innocent or risky?	✓ 10 fatalities were happened due to the consumption of sexual enhancement dietary supplements adulterated with sildenafil and glibenclamide up to 40 times more than the therapeutic daily dose (25-20 mg) used for treating the maturity onset diabetes mellitus.	Orhan *et al*., 2016
13.	Quality Assurance issues in the use of dietary Supplements, with reference to Protein Supplements	✓ In U.S., 58 samples of health supplements were analysed. ✓ 25% contained steroids at low level. ✓ 11% contained stimulants	Maughan, (2013)

Dietary supplements are also commonly adulterated with phosphodiesterase type-5 (PDE-5) like sildenafil, tadalafil and vardeanfil etc. These are the drugs for treating erectile dysfunction. These analogues of PDE-5 can also cause drop in blood pressure. Some of the incidents are shown in the Table 2.3.

Table 2.3 Incidences of adulteration of dietary supplements by Phosphodiesterase analogues and other synthetic drugs

S. No	Title	Findings	Reference
1.	Adulteration and safety issues in nutraceuticals and dietary upplements: innocent or risky?	✓ 13 slimming dietary supplements were tested using High Performance Thin Layer Chromatography (HPTLC-UV) densitometry method and found that 69% samples containeds ibutramine per se or its derivative	Orhan *et al*., 2016
2.	Detection, identification and quantification by 1H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance	✓ Out of 150 samples tested of dietary supplements using Hydrogen-1 Nuclear Magnetic Resosnance (^{1}H NMR) and Mass Spectrometry (MS) techniques, 61% contained sildenafil, tadalafil and vardenafil, >64% contained any one of the PDE-5 inhibitors, 36% contained at least 2 synthetic analogues	Gilard *et al*., 2015
3.	Determination of flibanserin and tadalafil in supplements for women's sexual desire enhancement using high-performance liquid chromatography with tandem mass spectrometer, diode array detector, and charged aerosol detector	✓ In China 88 samples of Dietary Supplements were analysed using UPHPLC-Q-Orbitrap HR-MS and found that 8 samples contained sildenafil, 1 sample contained tadalafil and norautildenafil both and 2 samples contained flibanserin and other synthetic drugs	Poplawaska *et al*., 2014
4.	Determination of nonopioid analgesics in adulterated food and dietary supplements by LC-MS/ MS	✓ Using (Liquid Chromatography Mass Spectrometry/ Mass Spectrometry) LCMS/MS, 214 dietary supplements were tested for the presence of nonsteroidal anti-inflammatory drugs, steroids and analgesics and found that 53 samples contained acetaminophen, diclofenac, ibuprofen and indometasine etc. also the most used adulterant was ibuprofen in these supplements	Kim *et al*., 2014

These products are used very frequently but adulteration or mislabelling can cause the terrible results. In 2009, 23 cases were reported of very serious health issues like jaundice, elevation in liver enzymes and also damage of liver. It caused one death due to the liver damage because of the consumption of health supplement containing hydroxycitric acid. Also, there was an incident of hepatitis developments among 20 Iranian bodybuilders because of taking the cocktail of different health supplements (Maughan, 2013).

2.6 Analytical Methods

There are some conventional method used for protein measurements like-

1. Reversed Phase-High Performance Liquid Chromatography
2. Mid Infrared Spectroscopy
3. Near Infrared Spectroscopy
4. Dumas Method
5. Kjeldahl Method

The results of protein in whey protein powders by dumas method and Near-infrared Spectroscopy were compared and it was found that dumas method can be replaced by NIR spectroscopy for measuring the protein as it takes less time i.e. 1min per sample and this method is also very cost effective (Ingle *et al*., 2016).

Some recently developed analytical methods to check the adulteration in milk protein based health supplements have been discussed below-

2.6.1 Fourier transform infrared spectrometry-Attenuated total reflectance (FTIR-ATR)

FTIR is an extremely rapid method which also have high through put. This method can analyse wide array of samples in industries within a short period of time. The principle of this method is that when a sample comes under the beam of infrared radiations, then these radiations will be absorbed by the functional group of the sample and vibrate in different recognized ways and these absorptions/vibrations can directly be correlated with the bio or chemical species (Andrade *et al*., 2019). Some adulterants identified by FTIR are given in Table 2.4.

Table 2.4 List of some adulterants in health supplements using Fourier transform infrared spectrometry

S. No.	Sample Preparation	Operation	Adulterants	Reference
1.	Ground to homogeneity in a mortar and pestle	FTIR-ATR in MIR (4000-650 cm^{-1}) with TGS detector Spectrum collected at 4cm-1 resolution Assure ID software used for spectral comparison	Melamine, Soy protein, drugs	Champagne and Emmel, 2011
2.	250 mg homogenized sample Hard press treatment Tablet formation of 10 mm dia. and 1mm thickness	FTIR-ATR in MIR (4000-400cm^{-1}) with chemometrics approach is done using Sprint rapid analyser Spectrum collected at 4cm^{-1} resolution	Milk Whey Powder in place of Whey Protein Concentrate	Andrade *et al*., 2019

2.6.2 Liquid Chromatography

It has been proven that the HPLC-MS/MS is a promising approach for analysing the trace levels of analytes in different complex matrixes because it has high selectivity and this method can also be used for the determination of different adulterants like vardenafil, tadalafil, and sildenafil in different dietary supplements (Venkatasami *et al*., 2010).

Although it is said that mass spectrometry is better in precision and selectivity than HPLC but the latter one is more economical. HPLC is also being used for detection as well as authentication of different adulterants in dairy protein based products and ingredients (Moore *et al*., 2012). The list of some adulterants analysed by liquid chromatography are given in Table 2.5.

Table 2.5 List of some adulterants in health supplements liquid chromatography

S.No.	Sample Preparation	Operation	Adulterants	Reference
1. Shotgun mass spectrometry				
• 1g sample, dissolved in 30ml of solution containing urea, ammonium borate, SDS • Vortex and centrifuged @14,000g/ 40 min • Washing and centrifugation @ 12,000g/10 min • Collect filtrate and store at -20℃		Protein quantification using 2D Quant kit Protein digestion by using trypsin nanoUPLC analysis coupled to a hybrid quadrupole/ion mobility mass spectrometry /orthogonal acceleration time-of-flight (Q-IMMS-oaTOF) MS geometry UNIPROT Protein databank is used	Soy protein, wheat protein, amino acid like glycine	Garrido *et al*., 2016
2. HPLC				
20 mg sample in 2 ml water Centrifugation @ 1.5x g/ 5 min Solid phase extraction is carried out then samples were evaporated under nitrogen flow at 45℃		LC effluent pumped to Q. exactive Orbitrap HRMS Data obtained were processed using Qual browser and Formula calculator Comparision between theoretical and experimental molecular mass was evaluated	Banned substances like Andarine	Roiffe *et al*., 2019
Samples were homogenized and 1 g sample was diluted in 15ml water and boil for 3 to 5 min and then cooled and made up volume up to 100 ml and then filtered through 2V filter paper		HPLC conditions: Column: ODS-3 Mobile phase A- 0.1% Phosphoric acid in water Mobile phase B- 100% ACN Flow rate- 1ml / min UV absorbance- 272 nm	Average caffeine ranging from 1 to 829 mg caffeine /daily dose	Andrews *et al*., 2007

2.6.3 Near Infrared Spectroscopy

There was no study available about the comparison of NIR spectroscopy and dumas method for detecting the common adulterants like melamine, urea and also amino acids in the milk protein based health supplements but the use of multivariate curve resolution with near-infrared spectroscopy has been proven as a promising method for detection as well as quantification of three different type of adulterant in milk powder i.e. whey, urea and starch (Forchetti and poppi, 2016).

Lukacs and his team in 2018 investigated about the suitability of the NIR spectroscopy to detect as well as to quantify the multiple adulterants present in the protein powder. They also checked the potential of same method as an alternative method for determination of protein. They used the NIR spectroscopy data to develop quantitative models to quickly and easily determine different adulterants in whey protein concentrate. They found that NIR spectroscopy is a reliable method for determining the protein content in whey protein powder even in the presence of different nitrogen based adulterants. They used 20 mg sample and applied NIR spectroscopy at 12500 to 3600 cm^{-1} coupled with chemometrics and collected the spectrum at 32 cm^{-1} resolution. They were able to find 3 adulterants i.e. urea, L-histidine, L-taurine in protein powder. (Lukacs *et al*., 2018).

2.6.4 Inductively Couples Plasma- Mass Spectrometry (ICP-MS)

For human being, some are essential metals while others can also affect health even if their level is low. There are mainly 4 elements in dietary supplements which have toxicological effect viz. Hg, Cd, Pb and As and the permitted daily dose suggested by FAO/WHO is 15, 5, 5 and 15 µg/day.

A study done by Pinto et al, (2019) for analysing 26 trace elements in 49 whey protein based health supplements using iCAP^{TM} inductively coupled plasma –mass spectrometry. They found different toxic elements viz. Rb, Pb, Be, Ag, Ce, Sn, Sb, Ti and U and the most abundant elements was Rb (5.7 ± 2.2 µg/g). Out of 49, 69% samples contained Hg and one sample contained higher Hg (11.4 ± 8.7 µg/g). Two samples contained high amount of As (13.4 ± 5.2 µg/g and 14.7 ± 5.1 µg/g) and the less abundant toxic element was Pb (0.004 ± 0.0055 µg/g). They also found significantly higher amount of Fe, Zn, Cu, Mn, Cr, and Co in chocolate

flavour of whey protein based health supplements, Fe, Zn, Mn, Cu, and Co in strawberry flavoured whey protein based health supplements and Se in coffee flavoured whey protein health supplements (Pinto *et al*., 2019).

2.7 Challenges

Authenticity of any food product is the major concern of producers, consumers and regulators since a long time (Danezis *et al*., 2016). There are practices of adding compound having high nitrogen content in place of the milk protein which can mask the actual protein content while measuring with common methods used for measuring the protein. Cheaper amino acids such as glycine also used as a nitrogenous source in place of protein, it is difficult to identify these adulterants by cited methods as these methods may find the other nitrogenous components but not the amino acids (Garrido *et al*., 2016). During the protein quantification, the inaccuracy and variations may occur and observed reasons are:

i. The amount of nitrogen recovered during analysis may vary (Simonne and others 1997).
ii. In food containing high protein, both protein nitrogen and the substance containing organic and inorganic nitrogen are also present.
iii. The nitrogen content as well as the conversion factor from nitrogen to protein may also change even for the same protein source (Moore *et al*., 2012).

As milk protein base health supplements is a complicated matrix of different components, it is difficult to obtain a reliable data. In most of the cases, limited panel of different substances has been tested and also the test applied had lower sensitivity making it difficult to find present foreign material. Hence, for the nutritional supplements like protein powder which are being used widely, there is a requirement of highly sensitive test as compare to the supplements taken in form of pills and capsules (Maughan *et al*., 2012).

The present analytical method to determine the total protein content is based on the total amount of nitrogen present in that product and cannot distinguish between the protein based and non-protein based nitrogen. Thus, the lack of appropriate method directly leads towards the addition of melamine and other non-protein nitrogen compounds in these supplements as an adulterant. In the high protein containing commodities which are marketed in huge volume, the

measurement of protein is done by either the rapid method or possibly by the infrared spectroscopy methods. As these method do not measure the protein or nitrogen directly but are calibrated against the methods which are nitrogen based, these are not accurate (Moore *et al*., 2012).

Many studies have reported that the methods for assessment of total protein which are based on the total nitrogen content have limited accuracy. The reason for inaccuracy could be due to the inaccurate weighing of sample, use of contaminated glassware or reagents, use of wrong reagent and due to the lower recovery of nitrogen (Moore *et al*., 2012). It has been also observed that glycosylation also interferes with the measurement of protein by the common used method for total protein estimation.

Protein measurement can also be influenced by the processing like heat processing which may affect the solubility of milk or due to the denaturation of protein (Koppelman *et al*., 2004)

Different methods for measuring the protein content suffers from one or more limitations which are discussed below**:-**

2.7.1 Kjeldahl method

Generally, Kjeldahl method is quite specific for measuring the total organic nitrogen (Simmone *et al*., 1997) but it has been reported that during analysis, the inorganic nitrogen may be covered partially or fully. Therefore, this method is not specific for measuring the protein nitrogen as well as this method also lacks the selectivity of high degree for organic nitrogen. Thus, the results of this method are unacceptable for samples containing different source of non-protein nitrogen as adulterants and thus adulteration of protein with melamine, ammonium compounds and urea cannot be prevented (Moore *et al*., 2012). In addition to that, other factors can also be responsible for introducing the error in the protein measurement by Kjeldahl method like accuracy in weighing of samples, contaminated glassware, contaminated reagents, less nitrogen recovery during digestion, accuracy of boric acid and the alkali used for titration (Lynch and Barbano, 1999).

2.7.2 Lowry method

The selectivity of Lowry method is somewhat better than the Kjeldahl method but this method is susceptible when authentic protein will be substituted

by the non-authentic protein which has either equal or greater response factor than the authentic protein (Moore *et al*., 2012).

2.7.3 Bicinchoninic Acid (BCA) Method

This method measures any substance that can reduce the Cu^{2+} to Cu^{1+}, can be indicate as the BCA positive compound like free amino acids, di- or tri peptides, reducing sugars etc. Some compounds like EDTA can chelate the copper thus restrict its reactivity during BCA (Krohn, 2005). The phenols give positive response to BCA have greater response factor than the actual protein e.g. the response factor for per unit pyrocatechol was 106 times more than the protein. As the potential number of BCA-positive substances is high, this method is more vulnerable to adulteration (Moore *et al*., 2012).

2.7.4 Dye Binding method

Various food components like starch, calcium chloride can make a complex with the azo dyes and can give false positive results (Kotakowski, 2001). Protein hydrolysis can also interfere in results as hydrolysis will increase the number of amino groups with free terminal group and they will bind to the anionic dyes and thus give the false positive results (Owusu-Apenten, 2002).

2.7.5 Bradford Method

The method is selective to different protein thus sensitivity of detecting adulteration with the Bradford positive compound will be increased (Owusu-Apenten 2002). This method gives proper result only for the polypeptide protein having molecular weight more than 3000 Da, which can be soluble under acidic condition (Krohn 2002).

2.7.6 Mid Infrared Transmittance

This method is susceptible to the adulterants that absorb at 6.465 μm (1550 cm^{-1}). The compounds like carboxylic acids and lipolysis product can give false positive result as they can absorb in the protein region (Ribadeau-Dumas and Grappin, 1989).

2.7.7 Near Infrared Spectroscopy

For protein determination, wavenumbers of 2180, 2100 and 2055 cm^{-1} are commonly used. But for protein determination by this method is not specific as other compounds like starch can also absorb at these wavelength (Moore *et al*., 2012).

2.7.8 High Performance Liquid chromatography

Although this method is good at different perspective but it also has some limitations like it takes high time for analysis and also identifying the specific protein from food matrices is also a great challenge and the cost of equipment is also high (Moore *et al*., 2012).

2.7.9 Mass Spectrometry

In this modern era, this method is very popular but it also has some limitations, including less solubility of some proteins (Leonil *et al*., 2000). The accuracy and mass resolution is lower for the compound with molecular weight more than 30 KDa and equipment cost is also high (Moore *et al*., 2012).

The detection of different adulterants having biological value lower than the protein is challenging from methodical point of view as manufacturers add other protein source but don't mention on label thus consumers imagine that they are purchasing the product with 100% bovine milk protein but actually it is not the same (Moore *et al*., 2012). The accuracy and precision of the method for protein measurement can also be influenced by the complex food matrices. While using fluorescence methods for detecting, there would be the interaction between the caffeine or other constitutes of food matrix and the fluorophore and so change the intensity of the fluorescence measured. Some activator agents also can manipulate the results as they will increase the intensity of fluorescence due to different substances will interact with them (Brandao *et al*., 2017). Thus there is a need to develop alternative methods which can detect the target analyst selectively and with high sensitivity. The developed method should also have high accuracy and precision while analysing any food matrix (Moore *et al*., 2012).

2.8 Work done at NDRI

Quality assessment of whey protein based health supplements

The related study has been done in National Dairy Research Institute (Suthar, 2019). In which 14 different samples of various whey protein based health supplements were taken from the market and were analysed for different components .The study revealed that both the estimated total protein and true protein content were less than the claimed values in 84% of the estimated samples. Out of 14 samples, 6 samples of health supplements contained significantly higher fat content than the labelled amount of fat. In the analysed

samples, the lactose content ranged from 4 to 42.9%. Calcium, sodium and iron were present in all the samples. The ash content ranged from 1.88 to 5.28%. All the samples were found to be positive for caffeine and the amount varied from 1.57 to 19.77 ppm. Some of the supplements were also adulterated by the other nitrogen sources like urea and its level varied from 200 to 8700 ppm. Thus, the study indicated that there are mislabelling and adulteration in the whey protein based supplements available in the market.

Material and Methods

3. MATERIALS AND METHODS

The present study was carried out for the assessment of analytical methods for validating the claims in milk protein based health supplements. In this work, proximate composition (protein, moisture, fat, ash, minerals etc.) of milk protein based health supplements was determined. Apart from compositional analysis, presence of adulterants like urea, caffeine, maltodextrin etc. were also checked. The present chapter comprises of materials and methods used for carrying out the above mentioned study.

3.1 Materials

3.1.1 Whey protein based health supplements

Different whey protein based health supplements were procured from market**.** These products contained varied amount of protein content and also different composition.

3.2 Apparatus and glasswares

All volumetric flasks, pipettes and burettes were class “A” type. Burette (50 ml), funnels (small and large), graduated centrifuge tubes (2, 15, and 50 ml), measuring cylinder (5, 10, 25, 50, 100, 250, 500 and 1000 ml), silica crucible, volumetric flasks (5, 10, 25, 50, 100, 250, 500 and 1000 ml), mojonnier flasks were purchased from Borosil India Ltd., Mumbai, India; Whatman Filter Papers (Whatman no. 1, 4 and 42) were procured from Whatman International Ltd., Kent, England. Various syringe filters (0.22 µm and 0.45 µm) and disc filter were procured from Millipore India Pvt. Ltd., Bengaluru, India.

3.3 Equipment

1. Weighing balance (Sartorius India Pvt. Ltd., Mumbai, India)

2. Water purifier (Sartorius atrium pro, Sartorius India Pvt. Ltd., Mumbai, India)

3. Magnetic stirrer (SPINOTMC 02, Tarsons Products Pvt. Ltd., Kolkata, India)

4. High speed centrifuge (Sigma 1-15K, UK)

5. High precision water bath (LABCO 3612, Timber market Ambala Cantt, India.)

6. Auto pipettes

- 100-1000µl (BRAND Transferpette, Germany)
- 20-200 µl (LabQuest Borosil, Pune, India)
- 10-100 µl (LabQuest Borosil, Pune, India)

7. Hot plate (Advanced Technocracy Inc., Ambala, India)

8. Hot air oven (Tempo Instruments and Equipment (I) Pvt. Ltd., Mumbai, India)

9. Syringe driven filter unit (33 mm, 0.22 µm pore size) (Millex, Millipore, Billerica, Massachusetts, USA)

10. Muffle furnace (Metrex scientific Instruments Pvt. Ltd., New Delhi, India)

11. Agilent microliter syringe (250 µl capacity, for manual injections) (Hamilton Company, Nevada, USA)

12. Vacuum filtration assembly (1000 ml) (Schott Duran, Riviera, Wertheim, Germany)

13. Refrigerated centrifuge (Eppendorf centrifuge 5810 R, New Delhi, India)

14. Ultrasonicator (SB-50D, Biochem life sciences Pvt. Ltd., New Delhi, India). Throughout this study, deionized water having conductivity less than 0.055 µS/cm prepared in water purifier (Cascada IX water, Labwater technology, UK) was used.

15. HPLC system and accessories: Agilent reverse phase high performance liquid chromatography (Model: 1260 Infinity, Agilent, US). It consisted of pump control module II with two Agilent 1260 Infinity pumps; manual injector, temperature-controlled column compartment and photodiode array detector and refractive index detector, sample loop (20 µl). Chromatograms were analysed using Open lab software.

16. Atomic Absorption Spectrophotometer (AA-7000,Shimadzu, Tokyo, Japan) flame mode, air-acetylene flame (temperature 2300°C with flow rate 1.5 L/min)

17. Spectrophotometer (UV-2700 230V, Shimadzu Corporation. Kyoto, Japan)

18. Automated ELISA plate reader (Infinite M200 PRO, Nano quant, Tecan, Switzerland)

3.4 Methodology

3.4.1 Objective 1: To check the suitability of existing methods to assess the quality of milk protein-based health supplements.

3.4.1.1 Activity 1: To collect the different raw ingredients.

Different raw ingredients were collected. Whey Protein Concentrate was gifted by Devisco Food International, Milk Protein Concentrate was collected from Dindigul Farm Product Private limited, Tamil Nadu and Micellar casein was purchased from A. M. Neutratech Private Limited, Delhi.

3.4.1.2 Activity 2: To check the suitability of Kjeldahl method and other spectrophotometric methods for protein analysis.

To find the suitable method for analyzing the protein in milk protein based ingredients, the protein content was estimated in different raw ingredients (WPC, Micellar casein and MPC) by three different methods viz. Kjeldahl method, Lowry assay and Bicinconinic acid assay. And the results of all three methods were compared to get the most preferred method for protein analysis. .

3.4.1.2.1 Estimation of protein by Kjeldahl method

Total nitrogen was estimated using Kjeldahl method (ISO 8968-1:2014) and the protein content in each sample was determined by multiplying the estimated total nitrogen with the factor of 6.38.

Reagents

Concentrated sulphuric acid, potassium sulphate, copper sulphate solution (5%), mixed indicator (methyl red- saturated alcoholic methyl red dye, methylene blue- 0.2% solution in ethanol), saturated boric acid (4%) and sodium hydroxide (40%), hydrochloric acid (0.1 N).

Procedure

1. Test portion and pre-treatment

To the clean and dry digestion tubes, 15 g of potassium sulphate and 1 ml of copper sulphate solution was added. About 0.15 g ± 0.05 g of the sample was

accurately weighed and was added into digestion tube. Then, 25 ml concentrated sulphuric acid was poured along the sides of the digestion flask to wash down any copper sulphate solution, potassium sulphate or test portion left on the neck of the flask and contents were mixed gently and tube was left at room temperature for 10 min.

B. Digestion

1. Digestion tubes were fixed in the digestion block and the main switch of instrument was turned on. Digestion block was set at a low initial temperature to control foaming (at approximately between 180°C and 230°C).
2. Test portion was digested for 30 min or until white fumes develop. Then temperature of digestion block increased to between 410°C and 430°C. Test portion was digested until clear and free of undigested remnant was obtained.
3. The digestion process was completed in 2-2.5 hours.
4. The digestion tube was removed from the block with the exhaust manifold in place and allowed to cool to room temperature for approximately 25 min.

C. Distillation

1. Turned on the condenser water for the distillation apparatus. Digestion tube containing digested test portion was attached to distillation unit and automatic programme was started in distillation unit.
2. Conical flask placed under the outlet of condenser where 50 ml of boric acid solution was delivered automatically as set in method.
3. In distillation unit, method was set to dispense 85 ml of 40% sodium hydroxide solution in the digestion tube.
4. Liberated ammonia was collected in the conical flask containing excess boric acid solution with indicator.
5. Conical flask was removed for further titration.

D. Titration

1. 0.1 N Hydrochloric acid was filled in burette of 25 ml with the help of funnel.
2. Then the content of the conical flask was titrated against 0.1 N Hydrochloric acid till the appearance of pink colour.
3. The amount of 0.1N HCL used was measured.

Blank test

Blank test was carried out by following the same procedure as described above, taking 5 ml of water and about 0.85 g of sucrose instead of test portion.

Calculations

Nitrogen content was calculated by following equation

$$Wn\ (\%\ Nitrogen\ content) = \frac{1.4007 * (Vb - Vs) * N}{W}$$

Where,

W_n = nitrogen content of the sample, expressed as percentage by mass,

V_s = millilitre of standard hydrochloric acid used for milk sample

V_b = millilitre of standard hydrochloric acid used for blank sample

Calculate the crude protein content W_p using the following equation:

$$Wp = Wn * 6.38$$

3.4.1.2.2 Estimation of protein by Lowry method

Reagents

a) **Copper sulphate solution 1% (w/v):** Dissolve 1 g of copper sulphate in distilled water and make up the volume 100 ml.
b) **Sodium potassium tartrate 2% (w/v):** Dissolve 2 g of sodium potassium tartrate in distilled water and make up the volume 100 ml.
c) **Sodium carbonate 2% (w/v) in 0.1N NaOH:** Dissolve 21.2 g of sodium carbonate and 4 g sodium hydroxide in distilled water and make up the volume 1000 ml.
d) **Alkaline reagent:** Mix 0.5 ml of reagent (a), 0.5 ml of reagent (b) and 100 ml of reagent (c) immediately before use.

e) **Folin's reagent:** Dilute Folin's reagent (2N, commercially available) with the equal amount of distilled water immediately before use.

f) **Standard protein:** Bovine serum albumin (BSA)

I. Stock solution (1 mg/ml): Weigh 50 mg BSA in 50 ml of 20 mM phosphate buffer of pH 7.

II. Working solution: Dilute stock solution 1:1, to make the final concentration of 0.5 mg/ml.

Procedure

Sample preparation

I. Stock solution: 100 mg of different blends were dissolved in phosphate buffer and made up the volume to 100 ml.

II. Working solution: Dilution of stock solution was done to 1:5 using phosphate buffer.

Protocol

1. 0, 25, 50, 100, 150, 200, 250 and 300 µl of standard protein solution was dispensed in different labelled test tubes and volume was adjusted to 500 µl to prepare the dilution of 0, 25, 50, 100, 150, 200, 250 and 300 µg, respectively for preparing standard curve. 500 µl sample was dispensed in separate labelled test tubes.
2. 5 ml of alkaline reagent was added to each of the tubes of the standards and samples and tubes were vortexed immediately to develop optimum colour.
3. Tubes were left undisturbed at room temperature for 10 min.
4. 0.5 ml of Folin's reagent was added and vortexed immediately.
5. Incubation for 30 min was done at room temperature.
6. Absorbance was taken at the wavelength 660 nm using spectrophotometer, zeroed against blank.
7. Standard curve was plotted by graphing the absorbance values of each standard against protein concentration in µg/ml.
8. Protein concentration was determined by interpolating from the standard curve.

Calculation

$$Total\ protein\ (\mu g/ml) = Y * 5$$

Where,

Y= concentration of protein from standard curve in µg/ml

3.4.1.2.3 Estimation of protein by Bicinchoninic acid (BCA) method

Reagents

(a) Reagent A:

Bicinchoninic acid: 1g

Sodium carbonate: 2 g Na_2CO_3. H_2O

Di sodium tartarate: 0.16 g

Sodium hydroxide: 0.4 g

Sodium bicarbonate: 0.95 g

Dissolved in 100 mL of distilled water and pH of the solution was adjusted to 11.25 if necessary.

b) Reagent B (4 % copper sulphate): Dissolve 4 g $CuSO_4$ (SRL) in distilled water and volume was made up to 100 ml.

c) BCA Working solution: Mixed reagent A and B in the ratio of 50:1.

d) Standard protein: Bovine serum albumin

III. Stock solution (1 mg/ml): Weigh 50 mg BSA in 50 ml of 20 mM phosphate buffer of pH 7.
IV. Working solution: Dilute stock solution 1:1, to make the final concentration of 0.5 mg/ml.

Sample preparation

I. Stock solution: 100mg of different blends were dissolved in phosphate buffer and made up the volume to 100ml.
II. Working solution: Dilution of stock solution was done to 1:1 using carbonate buffer (0.1M, pH 10).

Procedure

Protein content in the fractions was estimated by the method of Smith *et al.* (1985) using bicinchoninic acid (Sigma-Aldrich). Fifty microliter of sample was mixed with 950 µl of carbonate buffer and 1 ml of BCA working reagent (prepared by mixing 50 ml of reagent A and 1 ml of reagent B) in glass tubes and the contents were vortexed. The contents were incubated at 60ºC for 30 min followed by cooling to room temperature. Then the reaction mixture was incubated at 37°C for 30 min and the absorbance was taken at 570 nm using automated ELISA plate reader.

Calculation

$$Total\ protein\ (\%) = Y * 2$$

Where,

Y= concentration of protein from standard curve in µg/ml

3.4.1.2.4 Validation for the suitable methods for protein estimation among above.

For validating the thing that spectrophotometric methods only calculates true protein, the raw ingredients (WPC, MPC, Micellar casein) were adulterated with urea (NPN) equivalent to the 1% protein. Followed by the protein estimation was done using methods used in Section 3.2.1.2.

3.4.1.3 Activity 3- Compositional analysis of raw ingredients using different analytical methods.

3.4.1.3.1 Estimation of protein content

Protein was estimated using all the three methods, given above in Activity 3.2.1.2.

3.4.1.3.2 Estimation of ash content

Ash content of samples was estimated by using the method of Bureau of Indian Standards (IS 14433 (Part 1), 2005).

Apparatus:

Flat-bottom dish of silica, Muffle furnace-maintained at 550 ± 20℃, desiccator.

Procedure:

About 3 g of the sample was weighed in the dish, previously dried in an air-oven and weighed. Heated the dish gently on a flame at first and then strongly in a muffle furnace till grey ash was obtained. Cooled the dish in a desiccator and weighed. Heated the dish again for 30 min in the muffle furnace. Cooled the dish in a desiccator and weighed. Repeated this process of heating for 30 min, cooling and weighing until the difference between two successive weighing was less than 1 mg was obtained. Recorded the lowest mass.

Calculation

The ash content, expressed as percentage by mass is equal to

$$ASh\ (\%\ by\ mass) = \frac{W3 - W1}{W2 - W1} * 100$$

Where,

W_1 is the mass in grams of the dish

W_2 is the mass in grams of the dish and test portion.

W_3 is the mass in grams of the dish and dried test portion.

3.4.1.3.3 Estimation of minerals (Ca and Na)

3.4.1.3.3.1 Estimation of calcium content:

Calcium content of samples was estimated by method proposed by International Organization for Standardization AOAC 2005 using Atomic Absorption Spectrophotometer (AAS).

Cleaning of glass wares

All the glasswares used for AAS analysis were washed properly by nitric acid and then washed with deionized water.

Reagents

Nitric acid solution (volume fraction of 25%), Lanthanum trichloride solution (27%), calcium standard solution purchased from Sigma-Aldrich.

Preparation of standard:

Stock solution (50 ppm): Accurately 2.5 ml of calcium standard solution was taken in 50 ml volumetric flask and volume was made up to 50 ml with ultrapure water. This stock solution was gently mixed and diluted to get standards of desired concentration: 2.0, 4.0, 6.0, 8.0 and 10 ppm calcium.

Preparation of sample

The ash of the sample was prepared as procedure discussed earlier in the section 3.4.1.3.2. The obtained ash was dissolved in 1 ml of nitric acid solution. The crucible contents were then quantitatively transferred into a 50 ml volumetric flask. The contents were diluted to 50 ml mark with water. Further, diluted to 100 times by taking 0.5 ml of prepared sample in 50 ml volumetric flask, volume fraction of 10% lanthanum chloride (5 ml) was also added and volume made up to 50 ml in a volumetric flask. Further, estimation of calcium was carried out using AAS at wavelength λ_{max} of 422.7 nm. Blank test was performed in the similar way as for the test portion except that it was devoid of the sample.

3.4.1.3.3.2 Estimation of sodium content

Sodium content of sample was estimated by method proposed by International Organization for Standardization AOAC 2005b using Atomic Absorption Spectrophotometer (AAS).

Cleaning of glass ware

All glassware used for AAS analysis were washed properly by nitric acid and then with deionized water.

Reagents

Nitric acid solution (volume fraction of 25%), sodium standard solution purchased from Sigma-Aldrich.

Preparation of standard:

Stock solution (10 ppm): Accurately 0.5 ml of sodium standard solution was taken in 50 ml volumetric flask and volume was made up to 50 ml with deionized water. This stock solution was gently mixed and diluted to get standards of desired

concentration: 0.125, 0.25, 0.50 and 1 ppm and 0.1, 0.2, 0.5, 1.00, 1.50, 2 ppm for sodium and iron respectively.

Preparation of sample

The ash content of sample was prepared as discussed earlier in the section 3.4.1.3.2. The obtained ash content was dissolved in 1 ml of nitric acid solution. Transferred quantitatively the crucible content into a 50 ml volumetric flask. Diluted to the 50 ml mark with water. Mixed thoroughly and diluted the sample 100 times by taking 0.5 ml of prepared sample and volume made up to 50 ml volumetric flask. Further, estimation of sodium was carried out using AAS at wavelength λ_{max} 589 nm. Blank test was carried out by using the same procedure and the same amount of each reagent added in parallel with the procedure of the test portion.

3.4.1.3.4 Estimation of urea using p-dimethyl amino benzaldehyde (DMAB) method

Urea in whey protein based health supplements was estimated by DMAB method of BIS (IS: 1479 (Part-1), 2016).

Reagents:

p-Dimethyl amino benzaldehyde (DMAB) solution (1.6% m/v), Phosphate buffer pH 7.0, Trichloroacetic acid (24%), Diluting reagent (equal volume of 24% TCA and phosphate buffer), Urea standard solution (1 mg/ml)

Preparation of standard curve

Standard solutions of urea were prepared by taking 20, 40, 80, 120, 160, 200 and 300 µl of standard solution (1 mg/ml) in 2 ml microcentrifuge tubes and volume made to 1 ml by adding 980, 960, 920, 880, 840, 800 and 700 µl diluting reagent. The concentration of each solution would be 20, 40, 80, 120, 160, 200 and 300 µg/ml. One millilitre of DMAB solution was added to each micro centrifuge tube to develop yellow colour. Reagent blank containing 1 ml diluting reagent and 1 ml DMAB solution was also prepared. The contents of the tube were mixed by shaking the tubes vigorously. Tubes were allowed to stand for 10 min and optical density was measured at 425 nm. Graph of optical density along Y-axis against concentration of urea along X-axis was plotted.

Procedure:

Accurately 3.5% protein solution of milk protein based health supplements was prepared and vortexed for 5 minutes. Five millilitres of sample solution was taken and mixed with 5 ml of trichloroacetic acid to precipitate the protein and then filtered. One millilitre of filtrate was treated with 1 ml of DMAB reagent to develop colour. The optical density of the solution was measured at 425 nm.

Calculations:

$$Urea\ content(mg/100\ ml\ of\ sample\ solution) = \frac{Y}{5}$$

Where,

Y = concentration of sample from the standard curve for urea (mg)

3.4.2 Objective 2: - To analyse composition of various milk protein based health supplements

3.4.2.1 Activity 1- Collection of different milk protein based health supplements

Particular types of milk protein based health supplements i.e. whey protein based health supplements were purchased from market. The analysis of 12 different brands of whey protein based health supplements was done.

3.4.2.2 Activity 2- Compositional analysis of collected samples

3.4.2.2.1 Estimation of components as per method standardized in objective 1

Some components (total protein, ash, minerals, and urea) in different types of health supplements were estimated using methods standardized in objective 1 of this study, given in Table 3.1.

Table 3.1: Parameters estimated by the methods standardized in objective 1

S. No.	Particular	Reference
1.	Total Protein	Lowry *et al*., 1951 and Smith *et al*., 1985
2.	Ash	IS 14433 (Part 1), 2005
3.	Calcium	AOAC, 2005
4.	Sodium	AOAC, 2005
5.	Urea	IS:1479 (Part 1), 2016

3.4.2.2.2 Estimation of moisture content

Moisture content was determined using method proposed by Bureau of Indian Standards (IS: 11623 – 1986 (Reaffirmed)).

Apparatus:

Flat-bottom aluminium moisture dishes with cover having approximately 50 mm diameter and 25 mm depth was used, Drying oven – a well-ventilated air oven, thermostatically controlled at 102 ± 2°C, Desiccator – containing an efficient desiccant.

Procedure:

Approximately 1.0 g of sample was weighed in a moisture dish, covered the disc with lid and weight was recorded. The dish was uncovered and placed along with its lid in the oven at 102 ± 2°C for 2 h. After drying, replaced the lid and transferred the covered dish to the desiccator. Allowed it to cool to room temperature and accurately weighed the dish. The process was repeated until successive weightings do not differ by more than 0.5 mg.

Calculation:

$$Moisture\ (\%\ by\ mass) = \frac{M1 - M2}{M1 - M} * 100$$

Where,

M_1 – initial mass in g of the dish and lid with the material taken for analysis

M_2 - final mass in g of the dish and lid with the material after drying

M – Mass in g of the empty dish

3.4.2.2.3 Estimation of fat content

Fat content was determined using method proposed by International Dairy Federation (IDF: 127A (1998)).

Apparatus

Analytical balance, drying oven, boiling water bath, Mojonnier fat extraction flask, fat collecting vessel (Flat bottom flask of capacity 125 to 250 ml), measuring cylinders (5 and 25 ml).

Reagents:

Ammonia solution (25% m/m), ethyl alcohol (95%), diethyl ether (free from peroxide), petroleum ether (boiling range 40-60°C).

Procedure:

1. Accurately 1.5 g sample was weighed and transferred into Mojonnier flask.
2. Ten millilitres of warm (65 ± 5°C) distilled water was added to dissolve the powder. Two millilitres of ammonia solution was added and contents were mixed.
3. Mojonnier flask was gently heated at 65 ± 5°C for 15-20 minutes with occasional shaking and then cooled to laboratory temperature.
4. Ten millilitres of ethanol was added and mixed gently but thoroughly by allowing the contents of the flask to flow backward and forward between the two bulbs.
5. Twenty five millilitres of diethyl ether was added, closed the flask with pre wetted cork and mixed the content properly. Twenty five millilitres of the petroleum ether was added and again repeated the previous step.
6. Allowed the content to stand for 30-40 minutes at room temperature (30°C) under undisturbed condition till the upper layer is clear.

7. Carefully removed the cork (rinse it inside of the neck of the flask with mixed solvent so that rinsing runs into the flask) and decanted the ether solution from Mojonnier flask into the pre weighed fat collective vessel (100 ml conical flask).
8. Outside of the neck of extraction flask was rinsed with a little of the mixed solvent and collected the rinsing in the fat collecting vessel.
9. Solvent was then evaporated by keeping the fat collecting vessel over boiling water bath.
10. Five millilitres of ethanol was added to the contents of the Mojonnier flask as described in step 4.
11. Second extraction was carried out by repeating the operations as described in step 5 to 10.
12. Third extraction was carried out as described in step 5 to 10 but using 15 ml of diethyl ether and 15 ml of petroleum ether.
13. After extraction, the solvent in the fat collecting vessel was evaporated in hot air oven at 102 ± 2°C for 1 h.
14. After the completion of time, the fat collecting vessel was removed from oven, cooled and weighed.
15. Blank test was carried out simultaneously as described in the step from 2 to 15, while the test portion was replaced with 10 ml of water.

Calculation:

The fat content, expressed as a percentage by mass, is equal to

$$\frac{(M1 - M2) - (M3 - M4)}{M0} * 100$$

Where,

M_0 = mass in g of the test portion.

M_1 = mass in g of the fat-collecting vessel along with the extracted matter.

M_2 = mass in g of the empty fat-collecting vessel.

M_3 = mass in g of the fat-collecting vessel used in the blank test and any extracted matter determined.

M_4 = mass in g of the empty fat-collecting vessel used in blank test.

3.4.2.2.4 Estimation of whey proteins (α-lactalbumin and β-lactoglobulin) by RP-HPLC

Reverse phase HPLC method suggested by Yuskel and Erdem (2010) with slight modification in sample preparation was used to determine the elution profile of the whey proteins.

Reagents

Solvent A: Acetonitrile, water and TFA in a ratio 100:900:1 (v/v/v)

Solvent B: Acetonitrile, water and TFA in a ratio 900:100:1 (v/v/v).

Apparatus

The reverse phase HPLC system, Millipore filtration assembly with 0.22 µm filters for filtering all the reagents and water.

Standard preparation

Standards (α-lactalbumin and β-lactoglobulin) stock solutions of 25 mg/ml was prepared individually by dissolving in the mixture containing solvent A and solvent B in the ratio of 70:30. This stock solution was gently mixed and diluted to get desired concentration: 0.5, 1, 2, 4, 6, 8 and 12.5 mg/ml. The individual standards of α-lactalbumin and β-lactoglobulin were mixed together in 1:1 ratio followed by filtration through 0.22 µm syringe filter and then injected into the column.

Sample preparation

Accurately 1.0% protein solution of each sample was prepared in the solvent mixture (solvent A and B in 70:30 ratio) by heating at 40-50°C and vortexed for 5 minutes. The contents were centrifuged at 12000 x g for 30 min followed by filtration through 0.22 µm syringe filter.

HPLC conditions

Sample of 20 µl was injected into manual injector through a 20 µl loop. Gradient solvent delivery was achieved using binary gradient pump at a flow rate 1 ml/min. The separation of whey proteins was achieved by column (Zorbax 300 SB-C_8 4.6 X 150 mm, 5 µm) with linear gradient elution sequence as described in Table 3.2. The column temperature was maintained at 40°C in column heater chamber. The absorbance of the elute was monitored at 220 nm using photodiode array detector.

Table 3.2 Linear gradient elution sequence for whey proteins

Time (min)	Solvent A (%)	Solvent B (%)
0	80	20
45	40	60
47.4	80	20

3.4.2.2.5 Estimation of lactose by HPLC

Reverse–Phase HPLC:

Reverse-phase HPLC method of Sharma *et al*., 2009 was followed to determine lactose with slight modifications in sample preparation.

Reagents:

Carrez I solution (500 mM aqueous potassium ferrocyanide), Carrez II (500 mM aqueous zinc acetate), acetonitrile, ultrapure water, α-lactose monohydrate (sigma Aldrich).

Apparatus:

The reverse phase HPLC system, Millipore filtration assembly with 0.22 µm filters for filtering all the solvents and water.

Standard preparation:

Stock solution of lactose standard (25 mg/ml) was prepared in ultrapure water. This stock solution was gently mixed and diluted to get standards of desired

concentration: 0.5, 1, 2.5, 5 and 10 and 25 mg/ml. The individual standards were filtered through 0.22 µm syringe filter and then injected into column. Standard curve of 0.5-25 mg/ml concentration of lactose was prepared.

Sample preparation

Sample was appropriately diluted in deionized water to obtain final solution containing 3.5% protein and was vortexed for 5 min. 1.5 mL of ultrapure water was added in two millilitre of reconstituted sample and diluted content was incubated at 60°C for 10 min. Then, 0.25 ml Carrez I solution (500 mM aqueous potassium ferrocyanide), 0.25 ml Carrez II (500 mM aqueous zinc acetate) and 1 ml acetonitrile were added. The contents were gently mixed and then kept undisturbed for 1 h at room temperature. The precipitate obtained was removed by centrifugation (10000xg, 8 min and 20°C). The remaining supernatant containing the extracted lactose was then filtered through a 0.22 µm nylon membrane and 20 µl of this final clarified extract was injected into the HPLC system for analysis.

HPLC conditions

Isocratic solvent used for separation of lactose was acetonitrile and water in the ratio of 70:30. Sample (20 µl) was injected into manual injector with 20 µl loop. The RI detector was used to detect lactose content in the samples. The column and RI detector cell temperatures were maintained at 40°C.

3.4.2.2.6 Estimation of caffeine content of whey protein based-health supplements

Andrews and his team (2007) suggested the RP-HPLC method, was followed to estimate caffeine content and its elution time with slight modifications in sample preparation.

Reagents: Methanol (HPLC grade), water (HPLC grade)

Apparatus: The reverse phase HPLC system, Millipore filtration assembly with 0.22µm filters for filtering all the solvents and water.

Standard preparation

Caffeine stock solution of 100 ppm was prepared by accurately weighing 10 mg of pure caffeine (Sigma-Aldrich) in a beaker. The content was mixed with deionized water and transferred to 100 ml volumetric flask and volume made up to 100 ml by deionized water. Working standards of 2, 4, 6, 8 and 10 ppm were prepared by serial dilution of the stock solution with deionized water.

Sample preparation

Accurately 3 g of the sample was weighed in 250 ml conical flasks. Then 200 ml of ultrapure water was added and placed over water bath (100°C). Extraction was done for half an hour. Then the solution was cooled, volume maintained to 250 ml and filtered through Whatmann number 1 filter paper. One milliliter of the filtrate was pipetted into clean 10 ml volumetric flasks and volume was made to the mark with HPLC water. The prepared sample was then filtered through micro filter (0.22 µm) and filled into HPLC vials for analysis.

HPLC conditions

Isocratic solvent used for estimation of caffeine consisted of methanol and water in the ratio of 40:60. 20 µl sample was injected into the HPLC. Isocratic solvent delivery was achieved using binary gradient pump at a flow rate of 1 ml/min. The UV detector was used to detect caffeine content in the samples. The column and UV detector cell temperatures were maintained at 40°C.

3.4.2.2.7 Detection of maltodextrin by maltodextrin detection strip

Maltodextrin in milk protein based health supplements was estimated by maltodextrin detection strip (Delmos Pvt. Ltd.)

Procedure:

Accurately 3.5% protein solution of whey protein based health supplements was prepared and vortexed for 5 minutes. A drop of samples was placed on the test strip and the colour was compare with the colour chart.

3.4.2.2.8 Detection of glucose by glucose detection strip

Glucose in milk protein based health supplements was estimated by Glucose detection strip (Delmos Pvt. Ltd.)

Procedure:

Accurately 3.5% protein solution of whey protein based health supplements was prepared and vortexed for 5 minutes. A drop of samples was placed on the test strip and the colour was compared with the colour chart.

Result and Discussion

4. Result and Discussion

This chapter includes the results of the study, “Assessment of analytical methods for validating the claims of milk protein based health supplements”. In this study three methods, modified in the laboratory were used for the protein estimation and results were compared to get more suitable method for estimating the protein in whey protein based health supplements. These methods were validated by adulterating the raw ingredients (WPC, MPC, Micellar casein) with a non-protein nitrogen source viz., urea. Compositional analysis of raw ingredients was also done. The compositional analysis of whey protein based supplements was also estimated e.g. protein was estimated using spectrophotometric methods, for individual protein and lactose estimation, HPLC methods were used. As these products are very popular among young people and are priced at premium rates, the adulteration of these products is suspected. Thus to check the adulteration, the urea and caffeine were estimated and maltodextrin was estimated qualitatively. The results obtained for raw ingredients were also compared with the composition of whey protein based health supplements and the estimated values were compared with the claimed values on the label of health supplements to check the authenticity of these products.

4.1 Objective 1: To check the suitability of existing methods to assess the quality of milk protein-based health supplements

4.1.1 Checking the suitability of Kjeldahl method and other spectrophotometric methods for protein analysis

To check the suitable method for protein estimation, three methods were applied viz. Kjeldahl method and spectrophotometric methods like Lowry assay and bicinchoninic acid assay (BCA). Both spectrophotometric methods are based on biuret reaction. The standard curve for both Lowry and BCA assay is prepared using bovine serum albumin (BSA) and same is presented in the Figure 4.1 and 4.2, respectively. All the three methods were used to analyse the protein in WPC, MPC, Micellar Casein and the results were compared to analyse which method was more reliable for protein estimation in different constituents. The colour of final solution of BCA assay was violet-purple and for Lowry assay it was bluish-green

as shown in Figure 4.3a and 4.3b. The protein content proportionally increased with the intensity of the colour. And the results for these are given in Table 4.1 and graphical presentation is given in Figure 4.4.

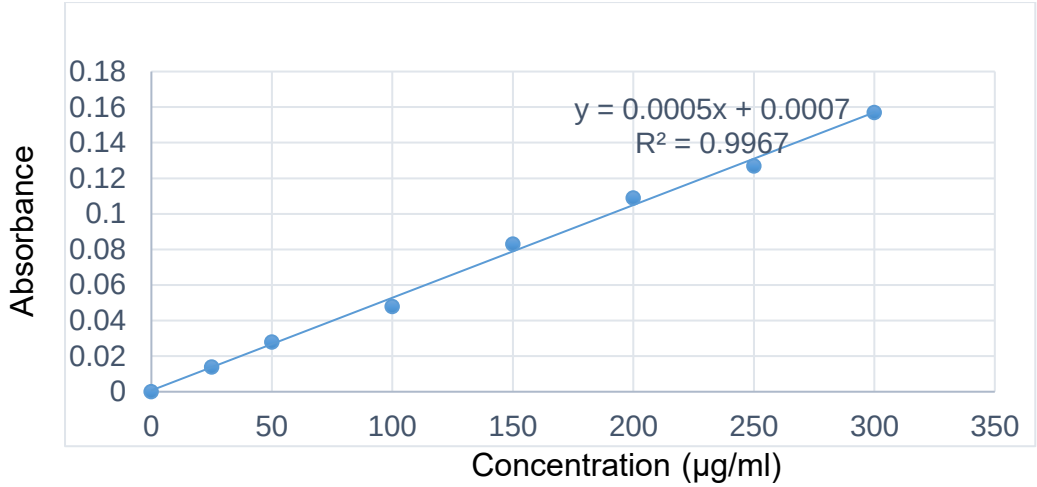


Fig 4.1 Standard curve for Lowry method

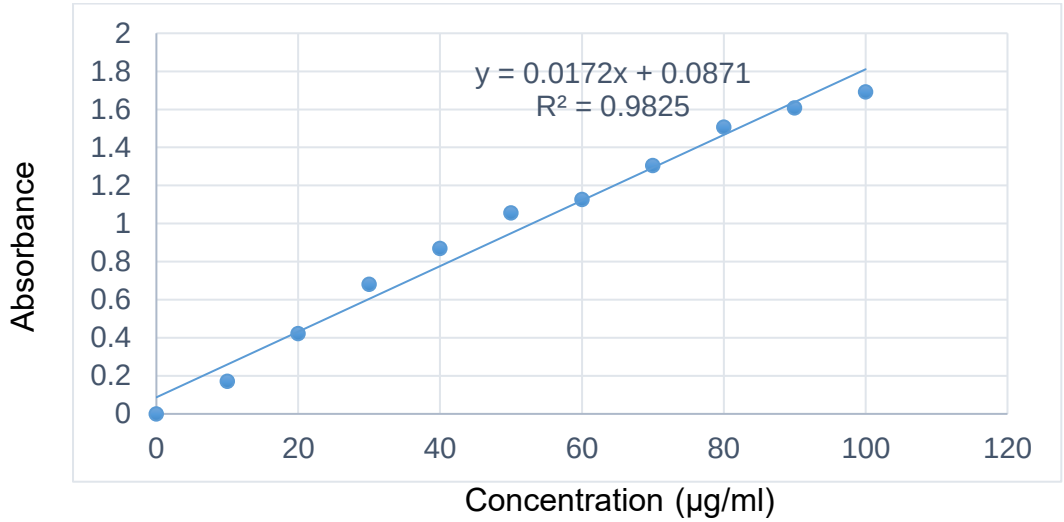


Fig 4.2 Standard curve for bicinchoninic acid assay (BCA) method

4.3a: ELISA plate for BCA

4.3b: Test tube for Lowry method

FIG4.3 Colour of final solution in bicinchoninic acid assay (BCA) and Lowry method

Table 4.1 Protein content in different ingredients by different methods

S. No.	Ingredients	Protein content (%) estimated by various method		
		Lowry method	Kjeldahl method	Bicinchoninic acid assay
1.	Whey Protein Concentrate	69.47 ± 0.85	69.83 ± 0.5	69.97 ± 1.65
2.	Micellar Casen	81.88 ± 1.22	82.05 ± 0.94	83.65 ± 1.50
3.	Milk Protein Concentrate	77.9 ± 0.45	78.2 ± 0.03	79.13 ± 1.34

Values are expressed as Mean ± Standard Error of Mean, n=3.

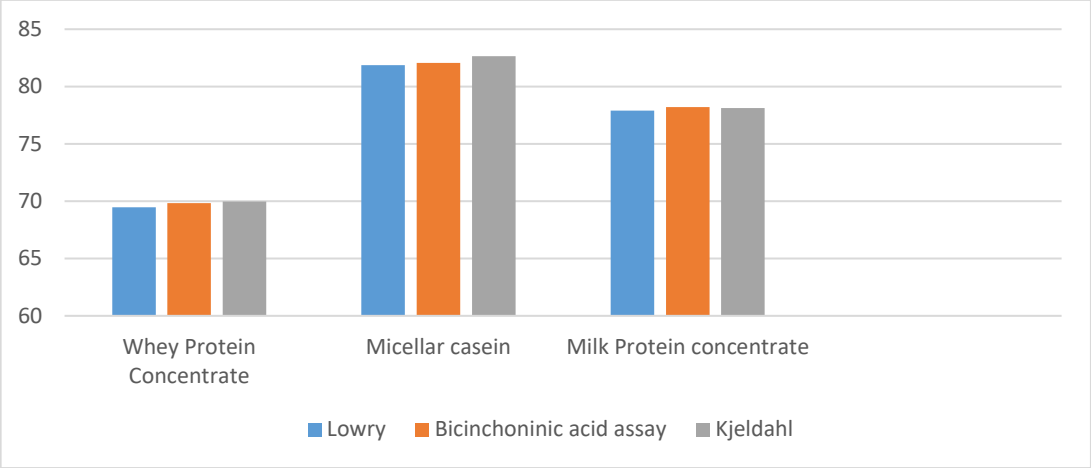


Figure 4.4 Graphical demonstration of protein content by different methods

As the spectrophotometric methods are based on the biuret reaction, such methods only calculate the true protein but Kjeldahl method calculate the total nitrogen thus it is vulnerable to over-estimation due to the presence of other non-protein nitrogen substances present in the sample. So, we can say that spectrophotometric methods are more suitable for estimating the protein in milk constituents. The paired t-test was applied to compare the results of Lowry and BCA methods, by taking the null hypothesis that there is no significant difference between the protein content estimated by Lowry and BCA methods. It was found that the p-value was greater than 0.05. So, we cannot reject the null hypothesis. Thus we can say that there is no significant difference between the protein values of these two methods, means both the methods are applicable for protein

estimation in milk protein products. But the most preferable method would be Lowry method as it takes less time and also the reagents are less costly compared to BCA method.

4.1.2 Validation of the suitable methods for protein estimation among above

To validate the same thing that spectrophotometric methods only measure the true protein, the analysis of raw ingredients was done after adulterating it with the other nitrogen source i.e. urea equivalent to the 1% of protein and the results are given in Table 4.2.

Table 4.2 Protein content before and after adulterating with urea

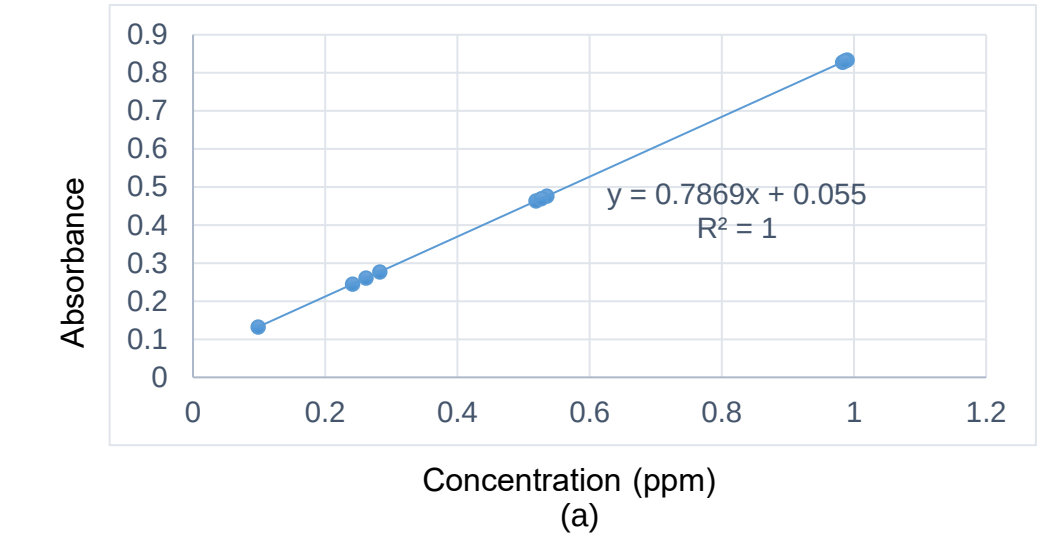
S. No.	Ingredients	Protein content (%) estimated by various method	
		Lowry method	Bicinchoninic acid assay
1.	Whey Protein Concentrate without urea	69.47 ± 0.85	69.97 ± 1.65
2.	Whey Protein Concentrate with urea (~ 1% protein)	68.03 ± 0.83	68.78 ± 0.45
3.	Micellar Casein without urea	81.88 ± 1.22	83.65 ± 1.50
4.	Micellar casein with urea (~ 1% protein)	80.04 ± 0.91	82.05 ± 0.45
5.	Milk Protein Concentrate without urea	77.9 ± 0.45	79.97 ± 1.34
6.	Milk Protein Concentrate with urea (~ 1% protein)	76.09 ± 0.21	77.97 ± 0.84

Values are expressed as Mean ± Standard Error of Mean, n=3.

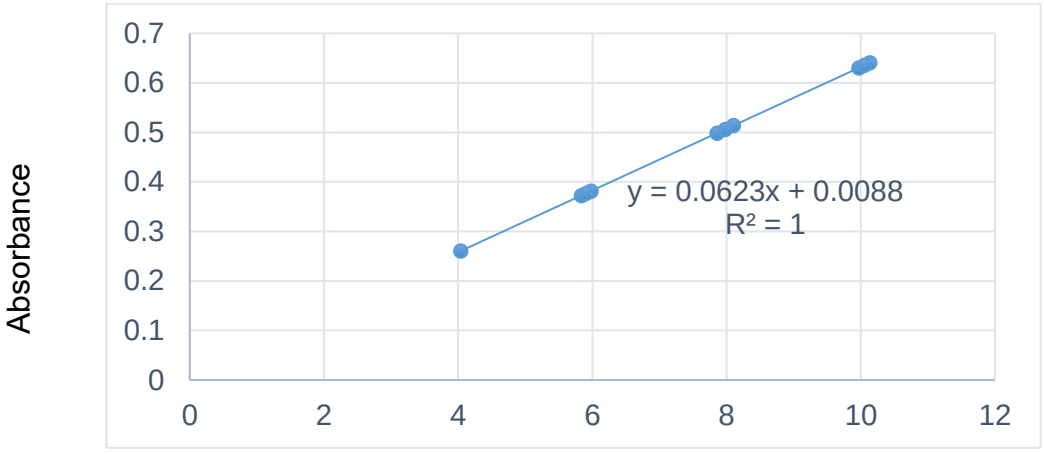
In Table 4.2, there are two types of results are given, first is without urea and other is with urea. We can see after adding urea equivalent to 1% of protein, the protein content is decreased approximately 1% when analysed by spectrophotometric methods. It means that spectrophotometric methods gives colour only for true protein and not for other nitrogen sources. Thus, these methods are more authentic for protein estimation in milk protein products.

4.1.3 Compositional analysis of raw ingredients using different analytical methods

Different parameters like protein, ash, minerals (sodium, calcium) and urea were estimated and compared with the estimated values of health supplements in second objective. For estimating the ash content, IDF method was used, while minerals were estimated by AOAC methods using Flame- Atomic Absorption Spectrophotometer (AAS) and for urea estimation p-dimethyl amino benzaldehyde (DMAB) method was applied. Protein is already estimated in section 4.1.1. Standard curve for minerals analysis and urea content are given below in Figure 4.5a and 4.5b and 4.6, respectively. The final colour in urea analysis is yellow and the intensity of yellow colour increased proportionally with the urea content. The results for the same are given below in Table 4.3.



(a)



(b)

Fig 4.5 Standard curve for mineral analysis (a) Sodium (b) Calcium

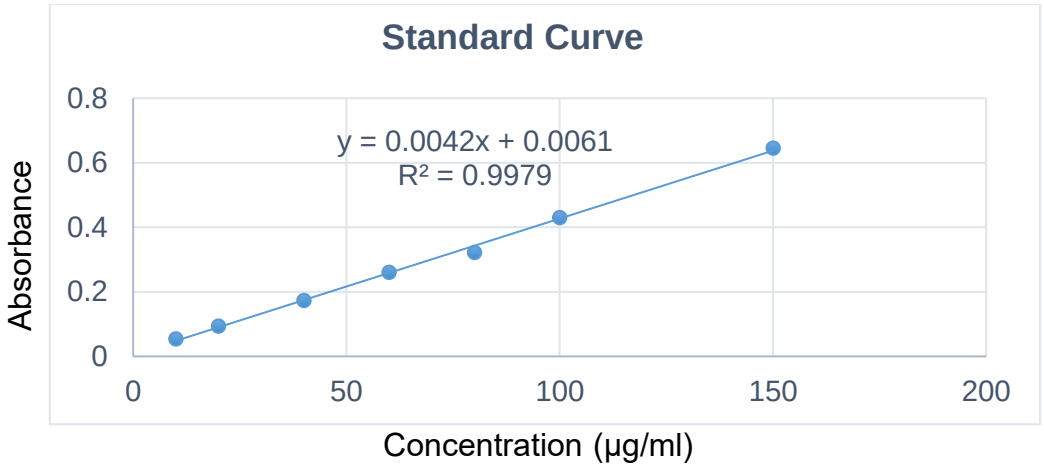


Fig 4.6 Standard curve for urea estimation

Table 4.3 Compositional analysis of raw ingredients

S. No.	Ingredients	Ash (%)	Sodium (mg/100 g)	Calcium (mg/100 g)	Urea (%)
1..	Whey Protein Concentrate	3.01 ± 0.09	130.55 ± 0.6	799.47 ± 0.13	0.029± .86
2.	Micellar Casein	6.0 ± 0.32	233.3 ± 1.0	850.93 ± 0.40	0.024± .94
3.	Milk Protein Concentrate	5.9 ± 0.72	144.86 ± 0.31	5300.07± .73	0.012± .27

Values are expressed as Mean ± Standard Error of Mean, n=3.

4.2 Objective 2: To analyse the composition of various milk protein based health supplements

4.2.1 Compositional analysis of collected samples

4.2.1.1 Protein estimation

For estimating the protein in whey protein based health supplements, the spectrophotometric methods standardized in section 4.1.1 were used, as we have already seen that spectrophotometric methods are better for milk protein based products. The results for the same are given in Table 4.4. In table we can see that the protein content in 33% is not matched with the claimed values especially in S-5, S-7 and S-8 as the claimed values varied significantly from the estimated values also 33% sample contained little higher amount of protein than the claimed value. A study done by Proteste organization in 2014 demonstrated that 53% out

of 28 samples has less protein content than the claimed values while in this study 59% samples are varied from their claimed values (Almeida *et al*., 2016). The graphical presentation for the same given in Figure 4.7.

Table 4.4 Protein content in health supplements

S. No.	Samples	Claimed value (%)	Estimated protein content (%) in supplements	
			Lowry method	Bicinchoninic acid assay
1.	S-1	80	78.06 ± 0.83	80.09 ± 0.65
2.	S-2	77.8	77 ± 0.11	79.53 ± 0.23
3.	S-3	61	63.3 ± 0.73	63.87 ± 0.04
4.	S-4	68	72.53 ± 0.80	71.89 ± 0.33
5.	**S-5**	**78**	**71.3 ± 0.093**	**74.86 ± 0.12**
6.	S-6	83.3	81.3 ± 0.07	82.31 ± 0.26
7.	**S-7**	**34.48**	**23.3 ± 0.55**	**23.06 ± 0.09**
8.	**S-8**	**22.46**	**4.3 ± 0.67**	**6.51 ± 0.48**
9,	S-9	68	66.8 ± 0.81	67 ± 0.83
10.	S-10	84	79 ± 0.52	83.7 ± 0.49
11.	S-11	78.98	81.39 ± 0.56	82.87 ± 0.08
12.	S-12	66.6	68.86 ± 0.91	69.7 ± 0.73

Values are expressed as Mean ± Standard Error of Mean, n=3.

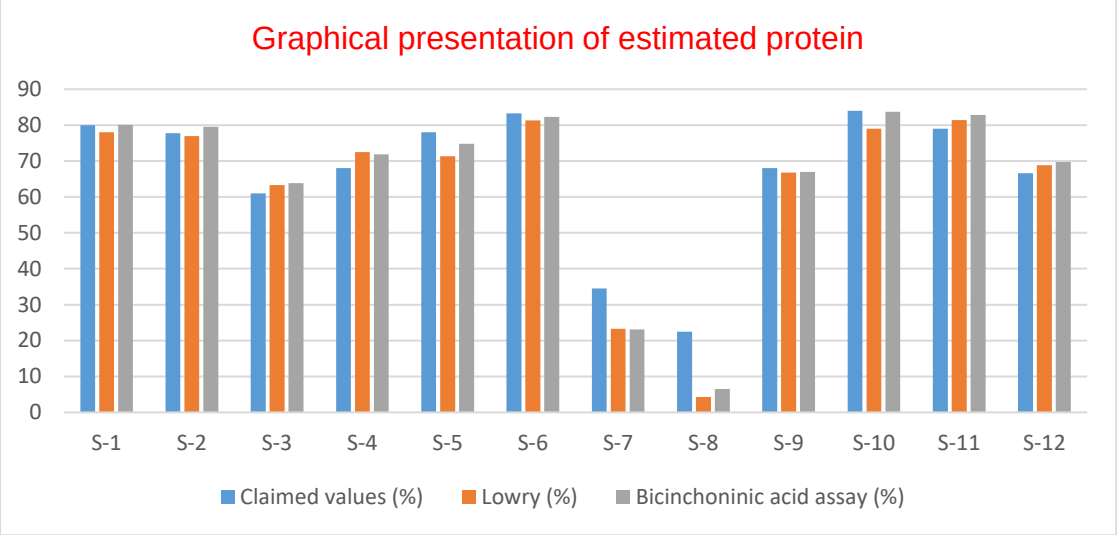


Fig 4.7 Graphical presentation of protein content in health supplements

In this graph we can see that the estimated protein values in three supplements i.e. S-5, S-7 and S-8 are significantly varied from their claimed values.

4.2.1.2 Moisture, fat and ash analysis

For all moisture, fat and ash estimation, gravimetric method were used. The results for the same are given in Table 4.5. The given table shows that out of 12, the fat content in 41% samples was deviated from the claimed value, mainly in S-7 and S-8 the fat content is much higher than the claimed value thus, these products will provide more energy rather than quality protein. The moisture content of two samples (16%) i.e. (S-11 and S-12) is more than 5%, which is not suitable for dried products, as it may cause problems during storage of the health supplements. The ash content in WPC generally varies from 3 to 6% but the ash content in WPC was 3.01%, measured in section 4.1.3. After comparing the estimated ash content in health supplements and whey protein concentrate, it was found that the ash content in 41% of samples is higher than the estimated value but theoretically it was within the expected range.

Table 4.5 Estimated values of moisture, fat and ash content

S. No.	Samples	Fat(%)		Moisture (%)	Ash (%)
		Claimed values	Estimated values	Estimated values	Estimated values
1.	S-1	5.0	6.8 ± 0.5	1.9 ± 0.32	2.5 ± 0.1
2.	S-2	6.6	5.3 ± 0.2	2.95 ± 0.4	2.9 ± 0.04
3.	S-3	6.9	7.0 ± 0.03	2.46 ± 0.8	3.1 ± 0.3
4.	S-4	9.0	7.22 ± 0.28	2.16 ± 0.09	3.4 ± 0.05
5.	S-5	7.2	6.8 ± 0.1	2.38 ± 0.3	3.2 ± 0.15
6.	S-6	3.33	3.63 ± 0.7	2.50 ± 0.52	3.8 ± 0.26
7.	**S-7**	**6.8**	**10.09 ± 0.91**	2.9 ± 0.67	3.01 ± 0.05
8.	**S-8**	**0.53**	**8.9 ± 1.09**	6.0 ± 0.05	5.79 ± 0.37
9.	S-9	4.11	4.5 ± 0.57	3.73 ± 0.63	3.87 ± 0.3
10.	S-10	7.6	7.3 ± 0.7	2.47 ± 0.1	4.99 ± 0.49
11.	S-11	3.2	3.0 ± 0.39	**5.73 ± 0.05**	3.0 ± 0.97
12.	S-12	5.0	5.92 ± 0.7	**5.09 ± 0.3**	4.07 ± 0.71

Values are expressed as Mean ± Standard Error of Mean, n=3

4.2.1.3 Estimation of mineral content

Mineral content in whey protein based health supplements was also estimated as minerals also shows the nutritional status of these products. We estimated specifically two minerals (Ca and Na), as in most of the samples these two minerals were mentioned. AOAC method was used employing Flame-AAS. The standard curves for the same are given in section 4.1.3. The results for minerals content are given in Table 4.6. The sodium content varied from 187.93 to 573.09 mg/100 g and calcium content varied from 501.03 to 1475.68 mg/100 g. The given table shows that all the samples contained significant amount of these minerals, but it was not mentioned on the labels of some health supplements like S-1, S-8 and S-10. Furthermore, some samples like S-4, S-11, and S-12 contained significantly higher amount of calcium than their claimed values i.e. $p<0.05$.

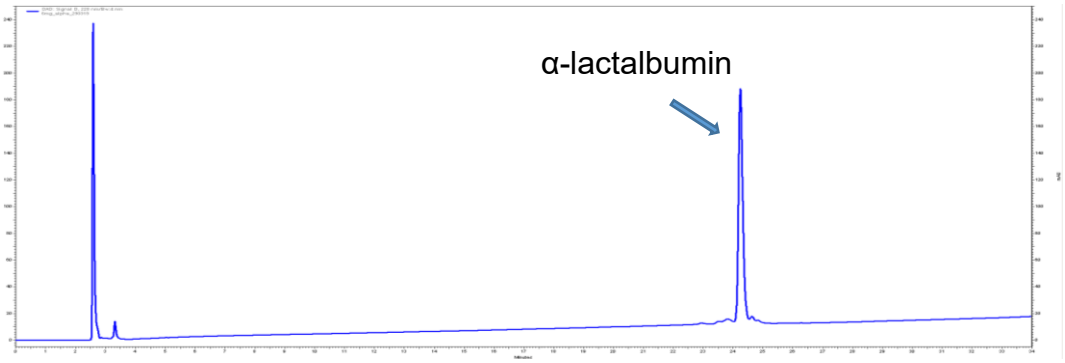
Table 4.6 Estimated values of minerals (Na and Ca)

S. No.	Samples	Sodium (mg/100 g)		Calcium (mg/100 g)	
		Claimed values	Estimated values	Claimed values	Estimated values
1.	**S-1**	-	**195.26 ± 0.15**	**-**	**966.85 ± 0.16**
2.	S-2	170	266.57 ± 0.88	-	850.50 ± 0.69
3.	S-3	-	313.32 ± 1.6	330	733.29 ± 0.59
4.	S-4	318	292.55 ± 0.53	**477**	**1066.22 ± 0.34**
5.	S-5	185	245.7 ± 0.17	365	807.27 ± 0.18
6.	S-6	175	279.04 ± 0.99	290	660.15 ± 0.16
7.	S-7	218.39	193.95 ± 0.81	270.11	773.01 ± 0.97
8.	**S-8**	**-**	**421.03 ± 1.09**	**-**	**1475.68 ± 0.72**
9.	S-9	275.37	393.71 ± 0.5	-	634.81 ± 0.54
10.	S-10	**-**	**187.93 ± 0.7**	**-**	**501.03 ± 0.73**
11.	**S-11**	427.63	573.09 ± 0.86	**463.81**	**1095.52 ± 1.67**
12.	**S-12**	338.83	375.93 ± 0.03	**491.67**	**1285.87 ± 0.79**

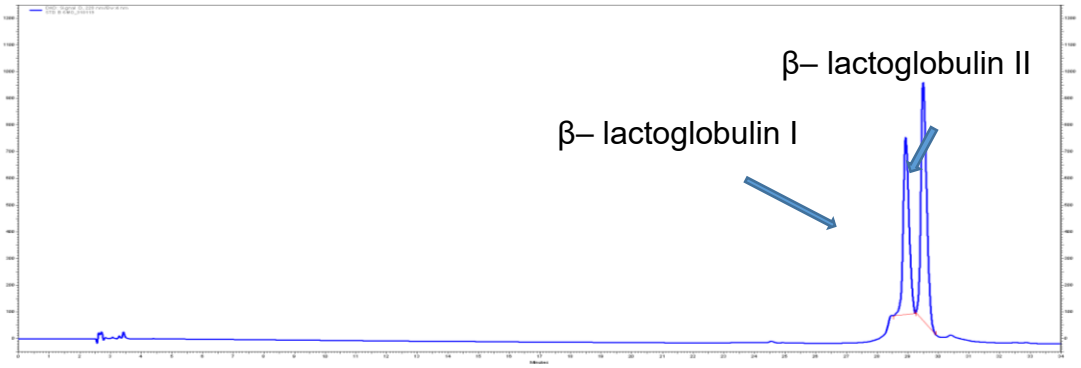
Values are expressed as Mean ± Standard Error of Mean, n=3.

4.2.1.4 Estimation of individual whey proteins (α-lactalbumin and β-lactoglobulin)

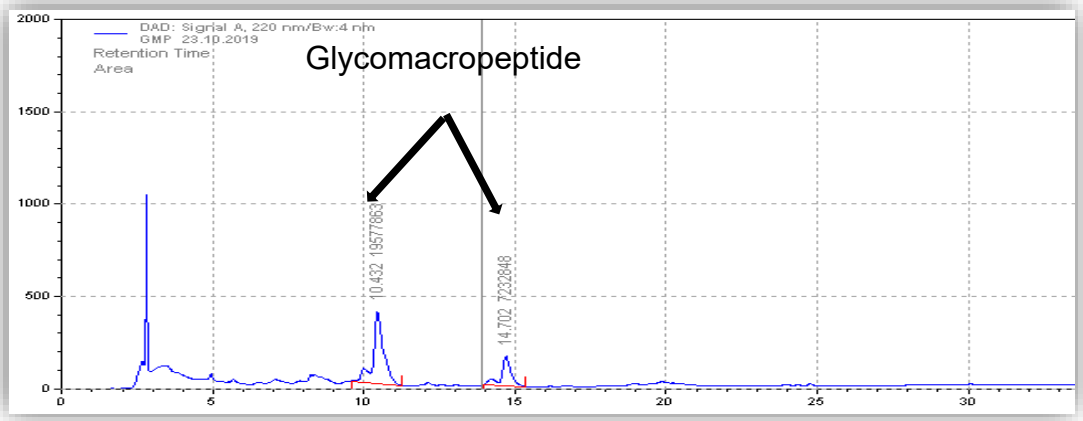
For estimating individual whey proteins, RP-HPLC method was used with C8 column and UV deterctor at 220 nm. In all the samples it was claimed that, they contains only whey proteins. Thus to check the authenticity of these products invidual proteins were estimated. The chromatograms for standards of both proteins (α-la, β-lg-1 and β-lg-2) and glycomacropeptide are given in Figure 4.8a, 4.8b and 4.8c and the elution time was **24.5, 29.3**,**29.9** and **10.42, 14.7** minutes, respectively. HPLC profile of some samples are given in Figure 4.9. The standard curves for these proteins are shown in Figure 4.10a, 4.10b and 4.10c. The protein value of major proteins (α-lactalbumin and β-lactoglobulin) was calculated using these standard curves and the results are given in the Table 4.7.



(a)



(b)



(C)

Figure 4.8 Chromatograms for standard protein (a) Chromatogram of α-la (b) Chromatograms of β-lg-1 and β-lg-2 (c) Chromatogram of glycomacropeptide

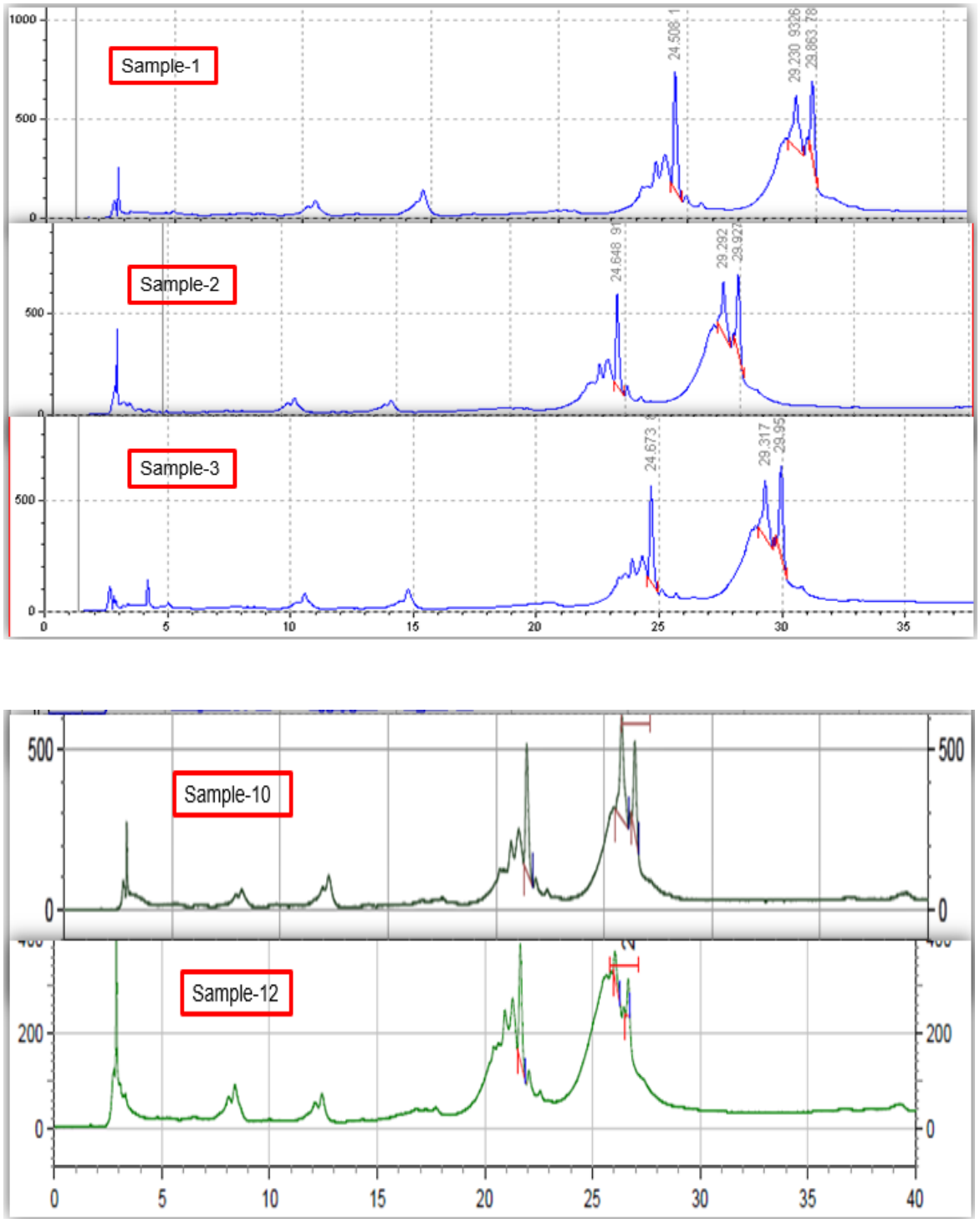
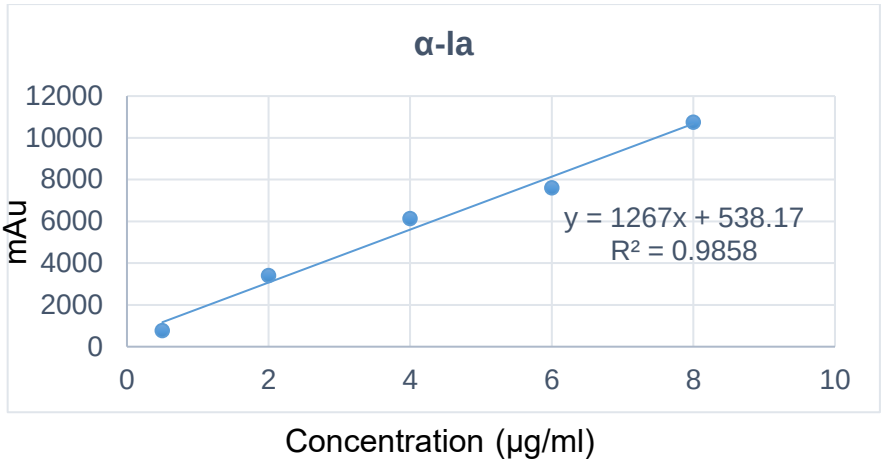
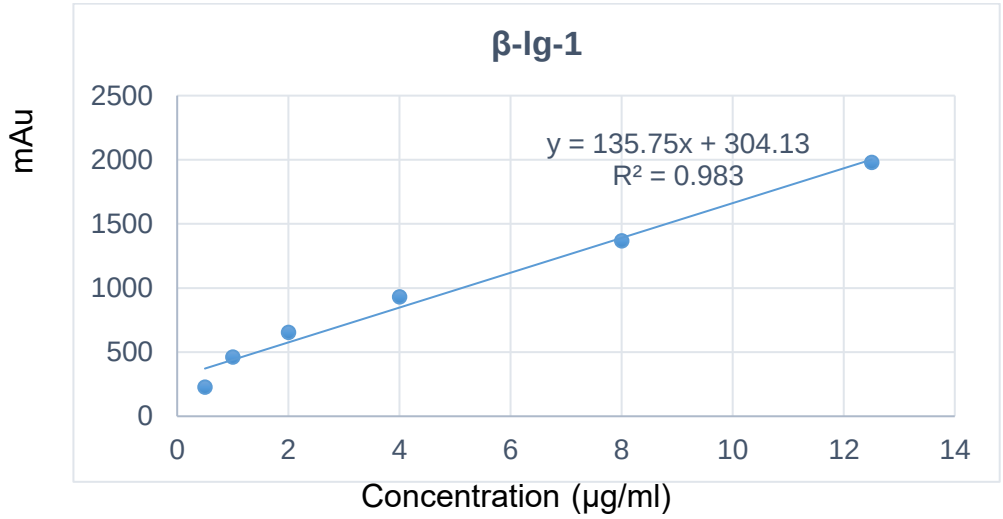


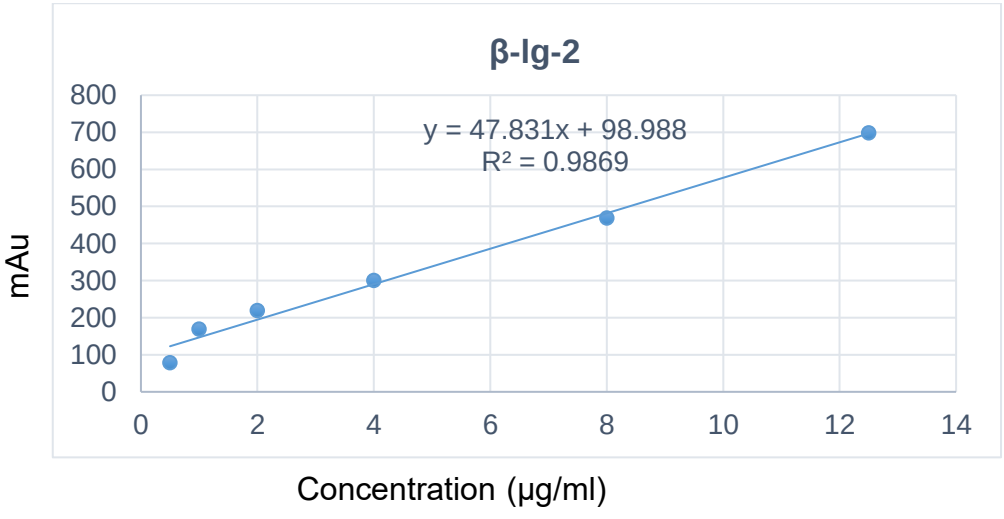
Figure 4.9 Chromatograms of whey protein based health supplements



(a)



(b)



(c)

Figure 4.10 Standard curves of standard proteins (a) Standard curves of α-la (b) Standard curve of β-lg-1 and (c) Standard curve of β-lg-2

Table 4.7 Estimated values of individual proteins (α-lactalbumin and β-lactoglobulin)

Samples	α-lactalbumin (%)	β-lactoglobulin (%)	Total whey protein (%)	Estimated protein values (%)
S-1	19.35	57.03	76.38	78.06 ± 0.83
S-2	18.54	52.94	71.48	77 ± 0.11
S-3	12.09	39.09	51.18	63.3 ± 0.73
S-4	15.47	47.02	62.49	72.53 ± 0.80
S-5	16.03	53.57	69.61	71.3 ± 0.093
S-6	19.83	58.93	78.76	81.3 ± 0.07
S-7	4.02	13.73	17.75	**23.3 ± 0.55**
S-8	-	-	-	**4.3 ± 0.67**
S-9	13.09	51.54	64.63	66.8 ± 0.81
S-10	18.56	60.4	79.6	79 ± 0.52
S-11	**25.87**	**51.68**	**77.55**	81.39 ± 0.56
S-12	**45.22**	**19.83**	**65.05**	68.86 ± 0.91

In the table we can see that in some of the samples the calculated protein by HPLC is less than the total estimated values by Lowry method because we have only calculated the major proteins i. e. α-la, β-lg and in the chromatograms for sample it can be observed that there are two more peaks at the retention time of 10 and 15 minutes. By running the standards of glycomacropeptide (GMP), we came to know that these peaks are of GMP. So, these two points may be the reason for less estimated values of protein by using HPLC. In whey protein the amount of β– lactoglobulin is about 58% (Fenelon et al., 2019) and α-lactalbumin is about 20% (Sharma, 2019). Thus the ratio of β– lactoglobulin is approximately 3 times of α-lactalbumin. But this ratio is not followed in 2 sample (S-11, S-12). In S-11 the amount of β– lactoglobulin is approx. twice of α-lactalbumin and in S-12 the amount of α-lactalbumin is more than the β– lactoglobulin. It is also shown in the chromatogram of S-12 that the peak area of β– lactoglobulin is less than the α-lactalbumin. So, we can say, there may be the alteration in the proteins used in manufacturing of these whey protein based health supplements.

4.2.1.5 Carbohydrate estimation in whey protein based health supplements

For carbohydrate estimation in whey protein based health supplements, RP-HPLC method with amino column and RI detector was used. In this methods Carrez solutions and acetonitrile are used to make the solution free from protein and other interfering substances. The chromatograms of standard of different sugars are given in figure 4.11a, 4.11b 4.11c and 4.11d. The retention time for glucose, galactose, sucrose and lactose are **5.00, 5.23, 5.71 and 6.50 minutes** respectively. The HPLC profile of some samples is given in Figure 4.12.

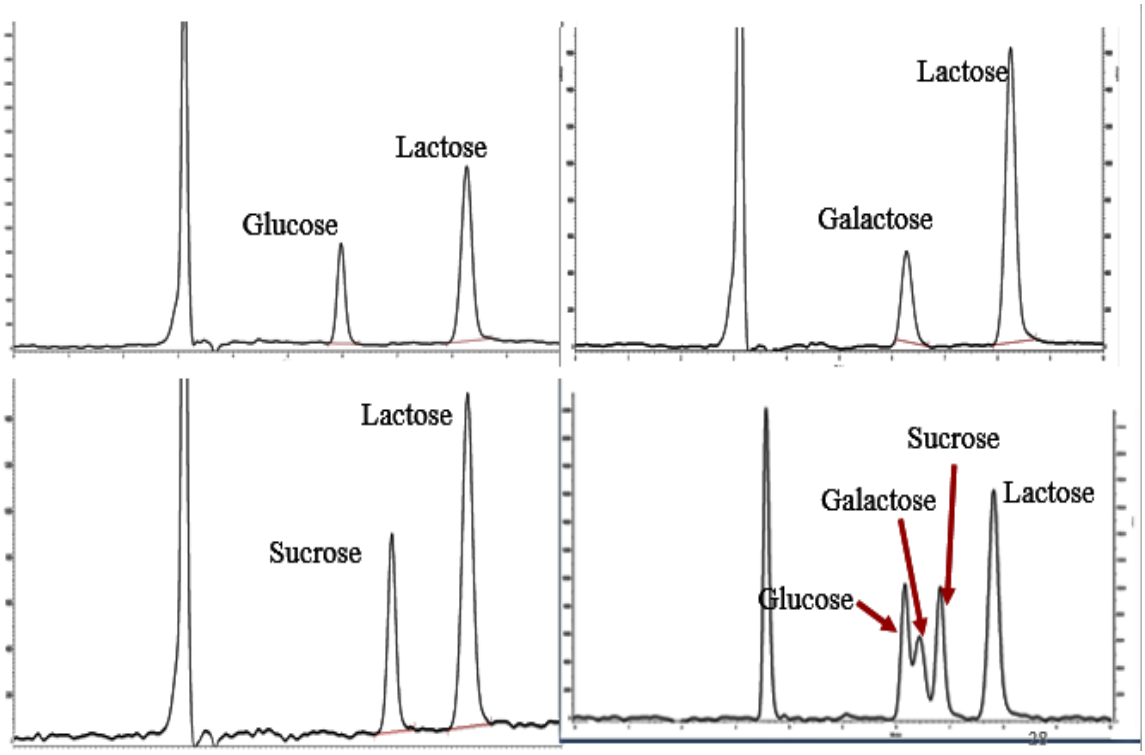


Figure 4.11 Chromatograms for standard of different sugars (a) Chromatogram of glucose and lactose (b) Chromatogram of galactose and lactose (c) Chromatogram of sucrose and lactose (d) Combined chromatogram of all the sugars

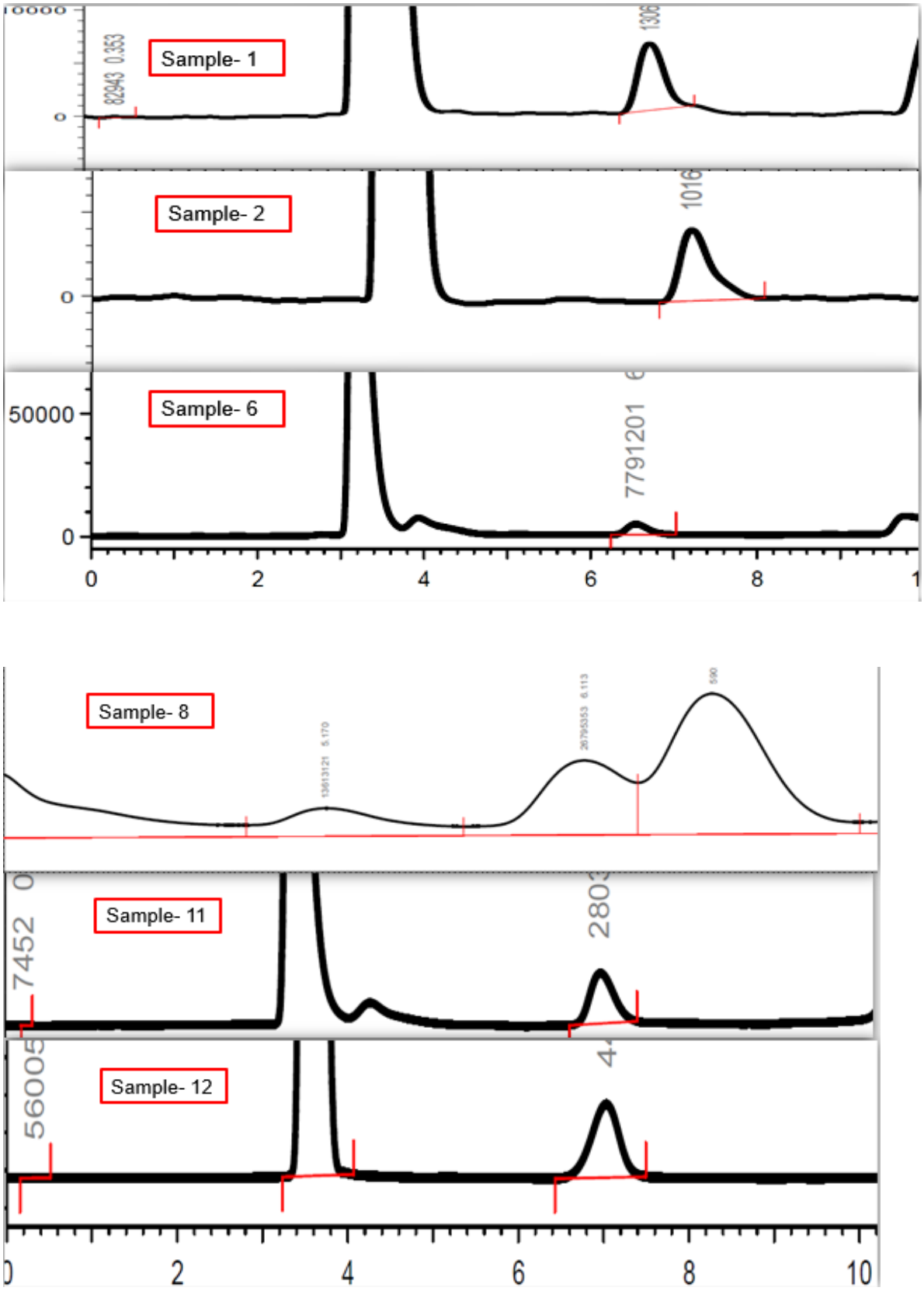


Figure 4.12 HPLC profile of some of whey protein based health Supplements

As we can see that in most of the samples only lactose is present as carbohydrate. So, only the quantity of lactose was estimated in all the samples. But one sample (S-8) also contained other sugar i.e. sucrose. The standard curve for lactose estimation is given in Figure 4.13. The estimated values of lactose in all the samples are given in Table 4.8. In the table we can see that some of the samples are significantly deviated from their claimed values especially **S-1, S-4, S-7 and S-8**. Furthermore S-8 also contained carbohydrate other than lactose as shown in Fig. 4.12, but it was not mentioned on the label that is contained additional sugar.

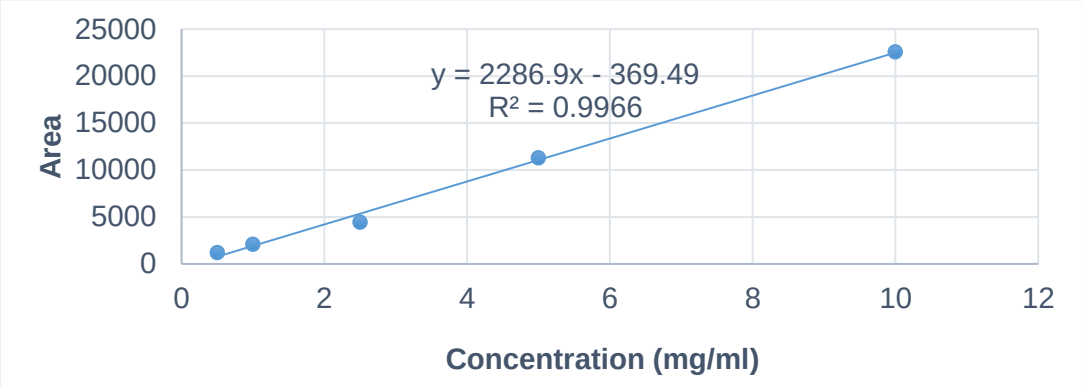


Figure 4.13 Standard curve for lactose estimation

Table 4.8 Estimated values of lactose in whey protein based health supplements

S. No.	Samples	Lactose content (%)	
		Claimed values	Estimated values
1.	**S-1**	**7.0**	**2.63 ± 0.61**
2.	S-2	6.7	4.14 ± 0.05
3.	S-3	19.0	9.38 ± 1.4
4.	**S-4**	**9.0**	**2.57 ± 0.17**
5.	S-5	6.8	7.73 ± 0.07
6.	S-6	6.0	3.20 ± 0.31
7.	**S-7**	**45.9**	**24.65 ± 1.03**
8.	**S-8**	**70.39**	**59.97 ± 1.5**
9.	S-9	15.4	12.09 ± 0.95
10.	S-10	4.0	5.41 ± 0.83
11.	S-11	9.8	9.0 ± 0.05
12.	S-12	15	17 ± 0.7

Values are expressed as Mean ± Standard Error of Mean, n=3.

4.2.2 Checking the adulterants in whey protein based health supplements

As whey protein based health supplements are very popular among health conscious people and the person goes to training centre and wants to build muscles, also the cost of these products is very high. So, adulteration in these products is expected either to increase its quantity or to increase its efficiency without changing its price (Lukacs *et al*., 2018). Like some manufacturers use cheaper source of nitrogen, instead of true milk protein to increase the nitrogen content e.g. urea, soy protein etc. (Champagne and Emmel, 2011). They also use some bulking agents and some stimulating agents to increase its efficiency but they sell these products at their original price (Garrido *et al*., 2016). So, in this study some parameters like caffeine, urea, maltodextrin and glucose are also estimated to check the adulteration in whey protein based health supplements.

4.2.2.1 Caffeine estimation

As we know that the caffeine is a stimulating agent, some manufacturers used it to increase the efficiency of health supplements. In earlier studies number of supplements adulterated with such ingredients (caffeine, drugs) has been noticed. Thus caffeine is also estimated in present study by using HPLC method with C18 column and UV detector. The HPLC profile of standard of caffeine is given in figure 4.14. The elution (retention) time for caffeine is **3.093 minutes**. After running the samples, it was found that all the 12 samples contained varied amount of caffeine though it was not mentioned on the label of such whey protein based health supplements. The chromatograms of some samples are given in Figure 4.15. The standard curve for caffeine estimation is given in Figure 4.16 and the estimated values of caffeine in all the samples are given in Table 4.9.

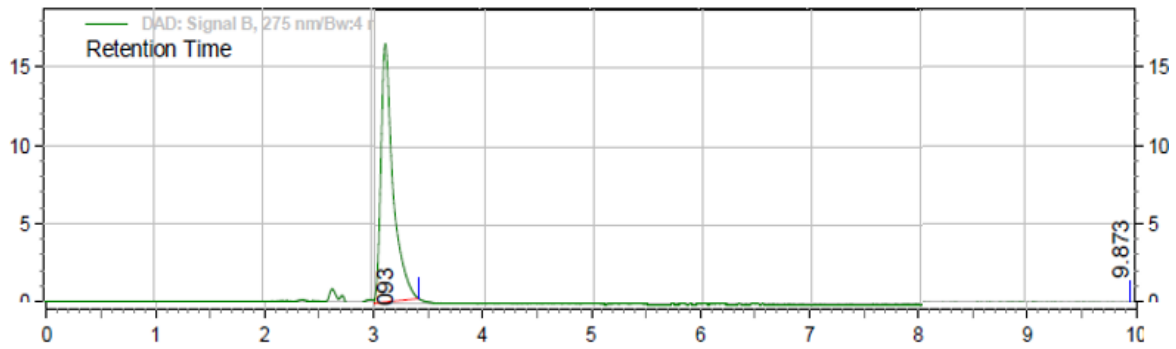


Figure 4.14 Chromatogram of standard caffeine

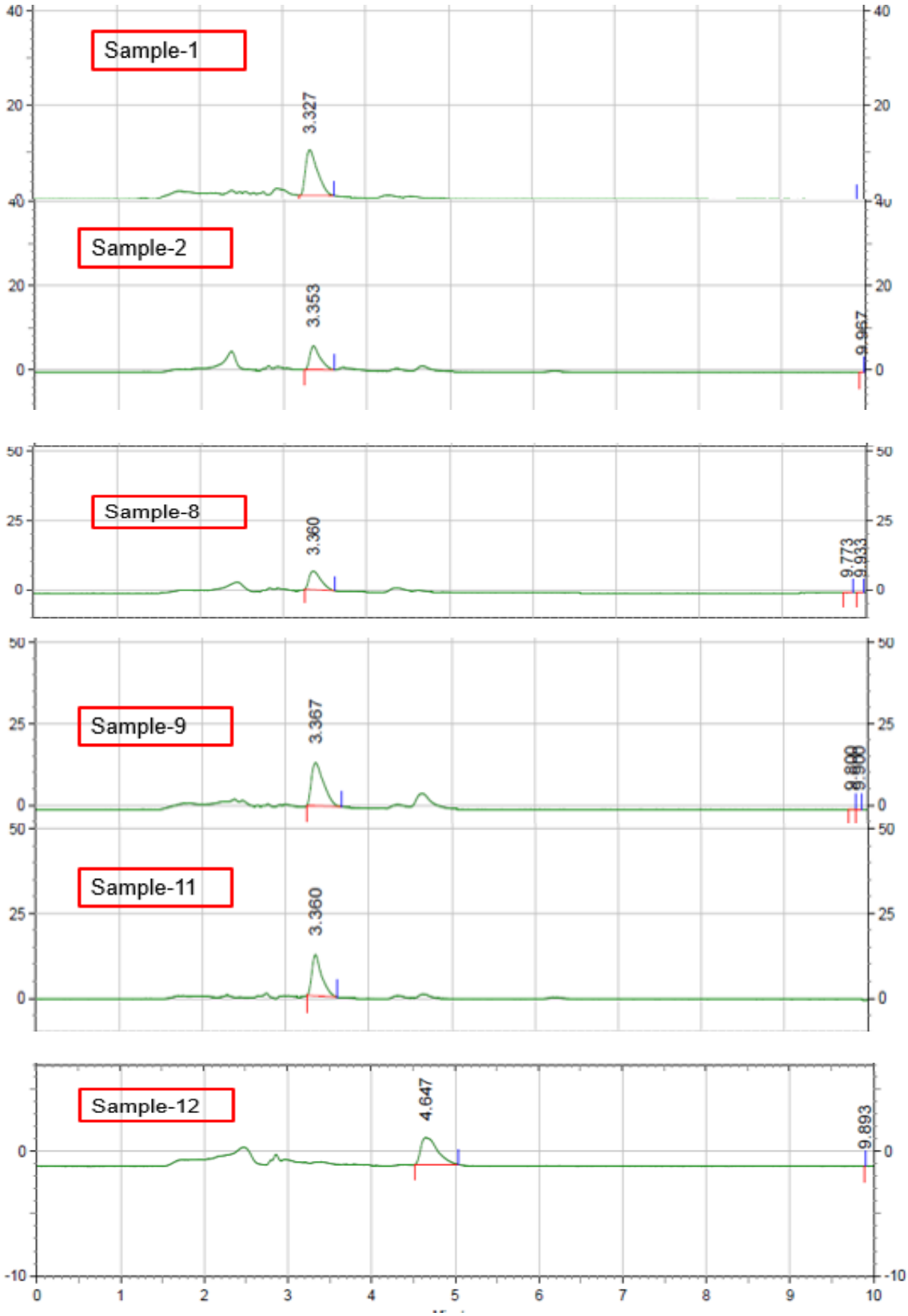


Figure 4.15 HPLC profile of whey protein based health supplements

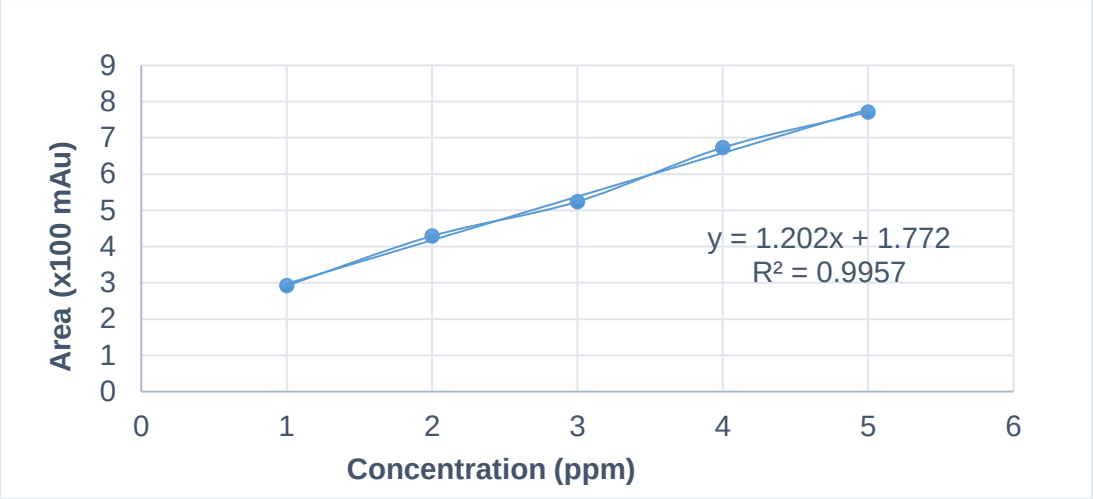


Figure 4.16 Standard curve for caffeine estimation

Table 4.9 Estimated values for caffeine in whey protein based health supplements

S. No.	Samples	Caffeine (ppm)
1.	S-1	3.13 ± 0.15
2.	S-2	1.57 ± 0.08
3.	S-3	4.28 ± 0.14
4.	S-4	3.29 ± 0.09
5.	S-5	5.93 ± 0.32
6.	S-6	8.02 ± 0.44
7.	S-7	3.91 ± 0.12
8.	S-8	2.84 ± 0.04
9.	S-9	4.11 ± 0.63
10.	S-10	1.03 ± 0.12
11.	S-11	4.36 ± 0.93
12.	S-12	4.97 ± 0.05

In the given table, it is showed that all the samples contained caffeine, varied from **1.03 to 8.02 ppm** and the **S-6** contained the highest amount of

caffeine among all but there was no statutory warning on the label. So, we can say manufacturers either add the caffeine as stimulant to increase the efficiency or it came from chocolate in the chocolate flavour whey protein based health supplements.

4.2.2.2 Urea estimation

As urea is the cheaper source of nitrogen, so manufacturers use this to increase the nitrogen as protein but marketing price of these products is same as original products. The final colour in urea estimation is yellow colour and showed in Figure 4.17. The results of estimated urea are given in Table 4.10 and the graphical presentation is given in the Figure 4.18. In the table we can see that all the samples contained higher amount of urea varied from 0.043 to 0.093% especially **S-5, S-8 and S-12** contained much more amount of urea. In the Figure 4.18, the comparison between urea content of WPC and health supplements has been done. In this graph we can see that the urea content in WPC was 0.025% but all the supplements contained urea more than the raw WPC and the visible can be seen in S-8 and S-12. So, it can be concluded that additional urea was present in all the whey protein based health supplements and this much urea on daily basis could also affect the health of consumers.

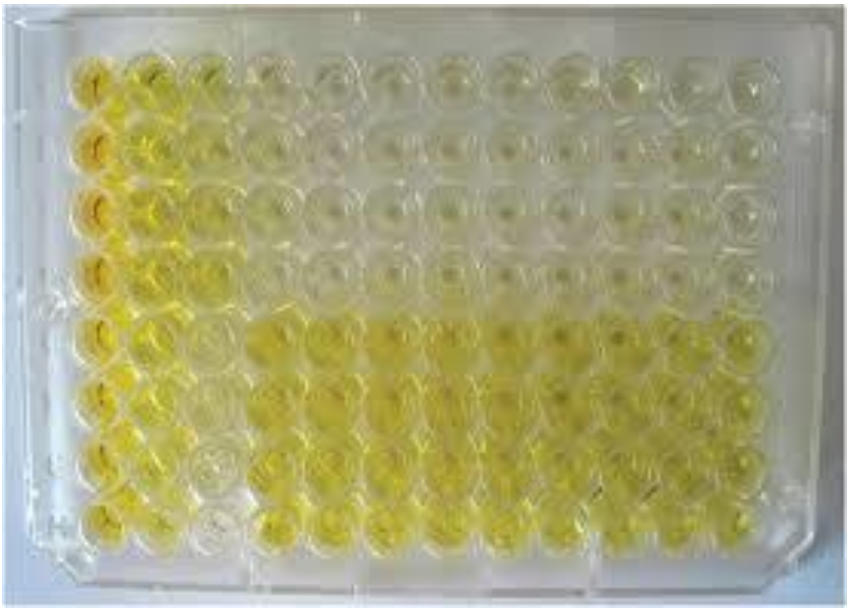


Figure 4.17 ELISA plate of urea analysis

Table 4.10 Estimated value of urea content in whey protein based health supplements

S. No.	Samples	Urea (%)
1.	S-1	0.045 ± 0.43
2.	S-2	0.046 ± 0.97
3.	S-3	0.097 ± 0.81
4.	S-4	0.049 ± 0.19
5.	**S-5**	**0.15 ± 0.27**
6.	S-6	0.052 ± 0.77
7.	S-7	0.045 ± 0.41
8.	**S-8**	**0.93 ± 0.37**
9.	S-9	0.072 ± 0.39
10.	S-10	0.043 ± 0.41
11.	S-11	0.091 ± 0.79
12.	**S-12**	**0.73 ± 0.57**

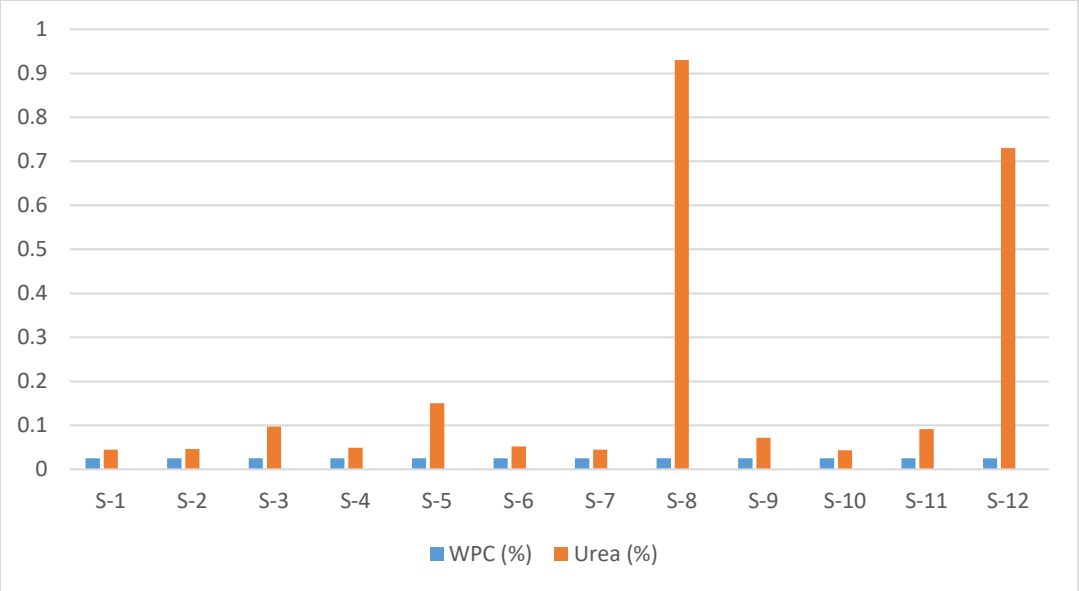


Figure 4.18 Graphical presentation for comparing urea content in raw whey protein concentrate and whey protein based health supplements

4.2.2.3 Maltodextrin detection

Manufacturers also use maltodextrin for adulterating the whey protein based health supplements, since maltodextrin is the cheaper source of carbohydrate and also used as a bulking agent. So, to detect the maltodextrin, strip based method developed at NDRI was used. For positive result strips give yellow to brown colour and for negative it remains white. The intensity of colour is directly proportional to the amount of maltodextrin. The strips of this test is shown in Figure 4.19. It was found that out of 12, 41% of whey protein based supplements contained maltodextrin but there was no warning on the label of these 5 supplements. In figure we can see that S-7 and S-8 contained very high amount of maltodextrin as strip turned into dark brown colour.

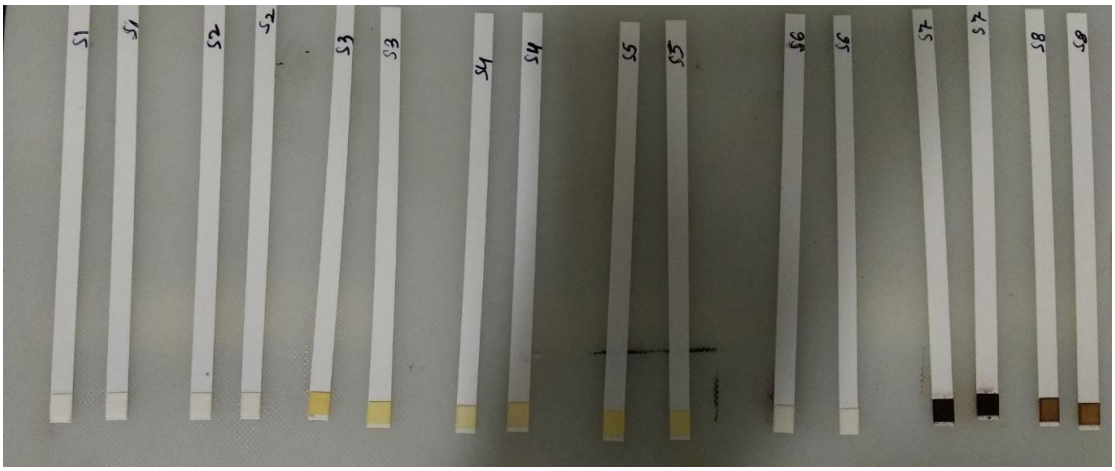


Figure 4.19 Tested strips for maltodextrin

4.2.2.4 Glucose detection

As maltodextrin strips can also give positive results for glucose. So, for verifying the results of maltodextrin, glucose was also estimated using strip based method. These glucose strips show light green to dark green colour for substances containing glucose but give light yellow colour for negative substances. The results for glucose estimation are given in Figure 4.20. In the figure it is showed that 3 samples contained glucose. Here we can see that S-8 contained more glucose than S-7 but it contained less maltodextrin than the S-8. So, we can say that S-7 had much higher amount of maltodextrin.

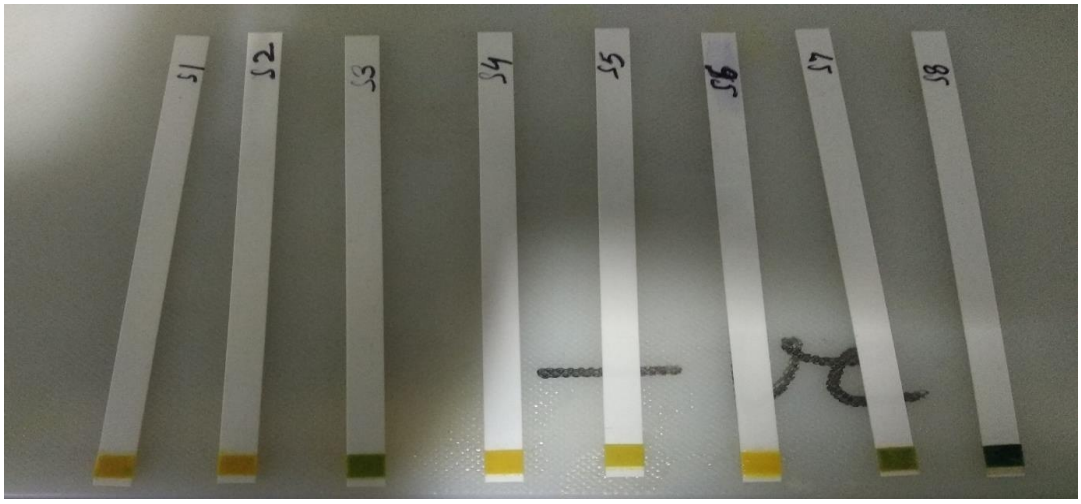


Figure 4.20 Strip results for glucose estimation

So, by comparing both the figures (Figure 4.19 and figure 4.20), it was noticed that S-3, S-7 and S-8 contained both Maltodextrin and glucose but S-4 and S-5 contained only maltodextrin.

Summary and Conclusion

5. Summary and Conclusion

The present study was done to check the suitability of existing methods to assess the quality of milk protein based health supplements and also to check the authenticity of these supplements. For checking suitable method for protein estimation three different methods (Kjeldahl method, Lowry method and Bicinchononic acid assay) were applied to estimate the protein in raw ingredients (WPC, MPC, Micellar casein) and their results were compared and found that spectrophotometric methods are more reliable than the Kjeldahl method as it only measured true protein content but most preferred method was Lowry method as it also takes less time and reagents cost for this method is also very low as compare to BCA. For validating the fact that spectrophotometric methods estimate only true protein, the protein in raw ingredients was estimated after adulterating it with non-protein nitrogen (urea) equivalent to the 1% protein and results showed approximately 1% less protein in all the ingredients. Also other composition like ash, minerals and urea in raw ingredients were also estimated. The ash and urea content was varied from 3-6% and less than 0.029% respectively.

The compositional analysis like protein, carbohydrates, individual proteins, moisture, ash, fat, minerals (Na, and Ca), in 12 whey protein based health supplements collected from the market was also done. Initially total protein was estimated and that 66% samples contained less protein while 25% samples were contained significantly less protein than their claimed values. The major individual whey protein (α-lactalbumin and β-lactoglobulin) were also analysed using HPLC method and the calculated protein was somewhat less than the estimated total protein. As the ratio of β-lactoglobulin to the α-lactalbumin is approximately 3:1 but in one sample this ratio is approx. 2:1 and in one sample the amount of α-lactalbumin was more than the β-lactoglobulin showed the alteration in the protein used to manufacture these supplements.

Moisture content in all the health supplements varied from 1.9 to 5.73% and 2 samples contained moisture more than 5% and may cause issues in storage. Ash content varied from 2.5 to 5.79% which is under the value of ash content in WPC. The sodium and calcium content varied from 187.93 to 573.09 mg/100 g and 501.03 to 1475.68 mg/ 100 g respectively. All the samples contained both the

minerals but it was not mentioned in 5 samples. Furthermore, all the samples contained significantly higher mineral content than the claimed values.

Fat content in 25% samples was more than the claimed values while 2 samples contained significantly high and low fat content each.

Carbohydrates were estimated using HPLC method and found that all the samples contained only lactose as carbohydrate except one sample, contained sucrose also. The lactose content in samples varied from 2.63 to 59.97% and 33% samples contained less carbohydrates than the claimed values.

To check the adulteration in whey protein based health supplements some parameters like caffeine, urea and maltodextrin were also analysed. Caffeine is used as a stimulant, estimated using HPLC method and found that all the samples contained caffeine and varied from 1.03 to 8.02 ppm though it was not mentioned on the labels of health supplements.

As urea is a cheaper source of nitrogen, analysed by DMAB method and urea content varied from 0.045 to 0.93%. After comparing these results with the urea content in raw ingredients, found that all the samples contained additional urea especially 25% samples contained much higher urea content, which can also affect the health of consumers.

Maltodextrin is used as a bulking agent. Out of 12, 42% samples were found positive for maltodextrin in strip based detection method. As maltodextrin strips also gives positive result for glucose. So, the glucose was also estimated using strip based method and found that 25% samples contained both maltodextrin and glucose while 17% samples contained only maltodextrin.

As per FSSAI, 2011 guidelines all the ingredients should be labelled in the descending order based on the amount of it present in the products. But none of the supplements followed the same.

Overall it was concluded that 25% samples were significantly varied from their claimed values. All the samples contained caffeine, urea and 5 samples also contained maltodextrin though it was not mentioned on the labels of the whey protein based health supplements. At present no analytical methods and quality standards are prescribed by FSSAI to analyse these products. So, the legal

standards and analytical methods are required to check the authenticity of milk protein based health supplements.

Bibliography

BIBLIOGRAPHY

Almeida, C. C., Alvares, T. S., Costa, M. P., & Conte-Junior, C. A. (2016). Protein and amino acid profiles of different whey protein supplements. *Journal of Dietary Supplements*, *13*(3), 313-323.

Andrade, J., Pereira, C. G., de Almeida Junior, J. C., Viana, C. C. R., de Oliveira Neves, L. N., da Silva, P. H. F., Jose, M., & dos Anjos, V. D. C. (2019). FTIR-ATR determination of protein content to evaluate whey protein concentrate adulteration. *LWT*, *99*, 166-172

Andrews, K. W., Schweitzer, A., Zhao, C., Holden, J. M., Roseland, J. M., Brandt, M., Dwyer, J. T., Picciano, M. F., Saldanha, L. G., Fisher, K. D., Yetley, E., Betz, J. M., & Douglass, L. (2007). The caffeine contents of dietary supplements commonly purchased in the US: analysis of 53 products with caffeine-containing ingredients. *Analytical and Bioanalytical Chemistry*, *389*(1), 231-239.

AOAC, 2005. Method for estimation of Calcium content, Association of Analytical Communities, Maryland.

AOAC, 2005.Method for estimation of Sodium Content, Association of Analytical Communities, Maryland.

Bos, C., Metges, C. C., Gaudichon, C., Petzke, K. J., Pueyo, M. E., Morens, C., & Tome, D. (2003). Postprandial kinetics of dietary amino acids are the main determinant of their metabolism after soy or milk protein ingestion in humans. *The Journal of Nutrition*, *133*(5), 1308-1315.

Brosnan, J.T., Brosnan, M.E. (2006). Branched-chain amino acids: enzyme and substrate regulation. *Journal of Nutrition*, 136 (1 Suppl.), 207-217.

Brandao, M. P., dos Anjos, V. D. C., & Bell, M. J. V. (2017). Time resolved fluorescence of milk powders–A pilot study. *International Dairy Journal*, *64*, 31-36.

Cahill, W. M., Schroeder, L. J., & Smith, A. H. (1944). Digestibility and biological value of soybean protein in whole soybeans, soybean floor, and soybean milk. *Journal of Nutrition*, *28*, 209-218.

Champagne, A. B., & Emmel, K. V. (2011). Rapid screening test for adulteration in raw materials of dietary supplements. *Vibrational Spectroscopy*, *55*(2), 216-223. Consumer Lab.

Protein powders and drinks review—for body building, sports & dieting.2014. Accessed Jun. 21, 2014. Available at https://www.consumerlab.com/reviews/ProteinPowders Shakes Drinks Sports %20Meal Diet/NutritionDrink.

Cohen, P. A. (2018). The FDA and Adulterated Supplements—Dereliction of Duty. *JAMA Network Open*, *1*(6), e183329-e183329.

Cynober, L. A. (Ed.). (2003). *Metabolic & therapeutic aspects of amino acids in clinical nutrition*. CRC Press.

Czepielewska, E., Makarewicz-Wujec, M., Różewski, F., Wojtasik, E., & Kozłowska-Wojciechowska, M. (2018). Drug adulteration of food supplements: A threat to public health in the European Union?. *Regulatory Toxicology and Pharmacology*, *97*, 98-102.

ConsumerReports.org. Protein drinks [cited 2012 Nov 26]. Available From: http://www.consumerreports.org/cro/2012/04/protein-drinks/index.htm.f.

Danezis, G. P., Tsagkaris, A. S., Camin, F., Brusic, V., & Georgiou, C. A. (2016). Food authentication: Techniques, trends & emerging approaches. *TrAC Trends in Analytical Chemistry*, *85*, 123-132.

Fenelon, M. A., Hickey, R. M., Buggy, A., McCarthy, N., & Murphy, E. G. (2019). Whey proteins in infant formula. In *Whey Proteins* (pp. 439-494). Academic Press.

Finete, V. D. L. M., Gouvêa, M. M., de Carvalho Marques, F. F., & Netto, A. D. P. (2013). Is it possible to screen for milk or whey protein adulteration with melamine, urea and ammonium sulphate, combining Kjeldahl and classical spectrophotometric methods?. *Food chemistry*, *141*(4), 3649-3655.

Forchetti, D. A. P., & Poppi, R. J. (2016). Use of NIR hyperspectral imaging and multivariate curve resolution (MCR) for detection and quantification of adulterants in milk powder. Lebensmittel-Wissenschaft und -Technologie-Food Science and Technology, 1–7.

Fox, P. F., & McSweeney, P. L. (Eds.). (2013). *Advanced Dairy Chemistry: Volume 1: Proteins, Parts A&B*. Springer.

FnBnews.com (2019). FSSAI orders probe into health supplements, claims by FBOs.

Geyer, H., Parr, M. K., Koehler, K., Mareck, U., Schänzer, W., & Thevis, M. (2008). Nutritional supplements cross-contaminated and faked with doping substances. *Journal of mass spectrometry*, *43*(7), 892-902.

Garrido, B. C., Souza, G. H., Lourenço, D. C., & Fasciotti, M. (2016). Proteomics in quality control: Whey protein-based supplements. *Journal of proteomics*, *147*, 48-55.

Gilard, V., Balayssac, S., Tinaugus, A., Martins, N., Martino, R., & Malet-Martino, M. (2015). Detection, identification and quantification by 1H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *Journal of pharmaceutical and biomedical analysis*, *102*, 476-493.

Green, G. A., Catlin, D. H., & Starcevic, B. (2001). Analysis of over-the-counter dietary supplements. *Clinical Journal of Sport Medicine*, *11*(4), 254-259.

Gürsel, A. (2015). Whey Technology. *Ankara University Publications*, (451). Ha, E., & Zemel, M. B. (2003). Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people. *The Journal of Nutritional Biochemistry*, *14*(5), 251-258.

Singh, H. (2009). Protein interactions and functionality of milk protein products. In *Dairy-Derived Ingredients* (pp. 644-674). Woodhead Publishing.

Harris, E. S., Cao, S., Littlefield, B. A., Craycroft, J. A., Scholten, R., Kaptchuk, T., ... & Zhao, Z. (2011). Heavy metal and pesticide content in commonly prescribed individual raw Chinese Herbal Medicines. *Science of the Total*

Environment, *409*(20), 4297-4305. Hoffman, J. R., & Falvo, M. J. (2004). Protein–which is best? *Journal of Sports Science & Medicine*, *3*(3), 118.

IDF, 009:2008. Method of estimation of Fat by Mojonnier method, International dairy Federation, Belgium.

IDF, 026:2004. Method of estimation of Moisture by Gravimetric method, International dairy Federation, Belgium.

IDF, 459:2012. Method for estimation of Protein by Kjeldahl, International dairy Federation, Belgium.

Ingle, P. D., Christian, R., Purohit, P., Zarraga, V., Handley, E., Freel, K., et al. (2016). Determination of protein content by NIR spectroscopy in protein powder mix products. Journal of AOAC International, 99(2), 360–363.

IS:14433-1 (Reaffirmed 2005) Infant milk substitutes, Part 1: Milk Protein Based, Bureau of Indian Standards, New Delhi.

Kim, H. J., Lee, J. H., Park, H. J., Kim, J. Y., Cho, S., & Kim, W. S. (2014). Determination of non-opioid analgesics in adulterated food and dietary supplements by LC-MS/MS. *Food Additives & Contaminants: Part A*, *31*(6), 973-978.

Koppelman, S. J., Lakemond, C. M., Vlooswijk, R., & Hefle, S. L. (2004). Detection of soy proteins in processed foods: literature overview and new experimental work. *Journal of AOAC International*, *87*(6), 1398-1407.

Krohn, R. I. (2002). The colorimetric detection and quantitation of total protein. *Current Protocols in Cell Biology*, *15*(1), A-3H.

Kolakowski, E. (2001). Protein determination and analysis in food systems. *Chemical & Functional Properties of Food Proteins. Technomic Publishing Company, Lancaster, Pennsylvania*, 57-112.

Léonil, J., Gagnaire, V., Mollé, D., Pezennec, S., & Bouhallab, S. (2000). Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides. *Journal of Chromatography A*, *881*(1-2), 1-21.

Lensu, S., Pekkala, S. P., Mäkinen, A., Karstunen, N., Turpeinen, A. T., Hulmi, J. J., & Koch, L. G. (2019). Beneficial effects of running and milk protein

supplements on Sirtuins and risk factors of metabolic disorders in rats with low aerobic capacity. *Metabolism open*, *4*, 100019.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*, 265-275.

Lukacs, M., Bazar, G., Pollner, B., Henn, R., Kirchler, C. G., Huck, C. W., & Kovacs, Z. (2018). Near infrared spectroscopy as an alternative quick method for simultaneous detection of multiple adulterants in whey proteinbased sports supplement. *Food Control*, *94*, 331-340. Lun, V., Erdman, K. A., Fung, T. S., & Reimer, R. A. (2012). Dietary supplementation practices in Canadian high-performance athletes. *International journal of sport nutrition and exercise metabolism*, *22*(1), 31-37.

Lynch, J. M., & Barbano, D. M. (1999). Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. *Journal of AOAC international*, *82*(6), 1389-1398.

McGregor, R. A., & Poppitt, S. D. (2013). Milk protein for improved metabolic health: a review of the evidence. *Nutrition & Metabolism*, *10*(1), 46.

Maughan RJ. 2005. Contamination of dietary supplements and positive drug tests in sport. *J Sport Sci* **23**: 883– 9.

Moore, J. C., DeVries, J. W., Lipp, M., Griffiths, J. C., & Abernethy, D. R. (2010). Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety*, *9*(4), 330-357.

Maughan, R. J. (2013). Quality assurance issues in the use of dietary supplements, with special reference to protein supplements. *The Journal of nutrition*, *143*(11), 1843S-1847S.

Nabuco, H. C., Tomeleri, C. M., Junior, P. S., Fernandes, R. R., Cavalcante, E. F., Dos Santos, L., Analiza, M. S., Luis, B. S., & Cyrino, E. S. (2019). Effect of whey protein supplementation combined with resistance training on cellular health in pre-conditioned older women: A randomized, double-blind,

placebo-controlled trial. *Archives of gerontology and geriatrics*, *82*, 232-237.

Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Morotta, F., Jain, S., & Yadav, H. (2011). Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & Function*, *2*(1), 18-27.

Owusu-Apenten, R. (2002). *Food protein analysis: quantitative effects on processing* (Vol. 118). CRC press.

Orhan, I. E., Senol, F. S., Skalicka-Wozniak, K., Georgiev, M., & Sener, B. (2016). Adulteration and safety issues in nutraceuticals and dietary supplements: Innocent or risky. *Nutraceuticals, Nanotechnology in the Agri-Food Industry; Grumezescu, AM, Ed*, 153-182.

Owusu-Apenten, R.K. (2002). The Bradford method—Principles. In: Owusu-Apenten RK, editor. Food protein analysis: quantitative effects on processing. New York, N.Y.: Marcel Dekker. p 169–94.

Patterson, S. D., Waldron, M., & Jeffries, O. (2019). Proteins and Amino Acids and Physical Exercise. In *Nutrition and Skeletal Muscle* (pp. 183-196). Academic Press.

Persistence Market Research (2016). Global market study on sports Supplements: Propelled by increasing consumer awareness and augmenting number of health & fitness centers.

Pennings, B., Groen, B., de Lange, A., Gijsen, A. P., Zorenc, A. H., Senden, J. M., & Van Loon, L. J. (2012). Amino acid absorption and subsequent muscle protein accretion following graded intakes of whey protein in elderly men. *American Journal of Physiology-Endocrinology and Metabolism*, *302*(8), E992-E999.

Pinto, E., Ferreira, I. M., & Almeida, A. (2020). Essential and non-essential/toxic trace elements in whey protein supplements. *Journal of Food Composition and Analysis*, *86*, 103383.

Poplawska, M., Blazewicz, A., Zolek, P., Fijalek, Z., 2014. Determination of flibanserin and tadalafil in supplements for women's sexual desire

enhancement using high-performance liquid chromatography with tandem mass spectrometer, diode array detector, and charged aerosol detector. J. Pharm. Biomed. Anal. 94, 45–53.

Rebiere H, Guinot P, Civade C, Bonnet PA, Nicolas A. 2012. Detection of hazardous weight-loss substances in adulterated slimming formulations using ultra-high-pressure liquid chromatography with diode-array detection. *Food Addit Contam A* **29**: 161– 71.

Research and Market.com (2018). The Indian Market for Dietary Supplements 2018-2023 - CAGR of 20% is expected for 2015-2023

Ribadeau-Dumas, B., & Grappin, R. (1989). Milk protein analysis. *Le Lait*, *69*(5), 357-416.

Rocha, T., Amaral, J. S., & Oliveira, M. B. P. (2016). Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. *Comprehensive reviews in food science and food safety*, *15*(1), 43-62.

Roiffé, R. R., Sardela, V. F., Lima, A. L. D. S., Oliveira, D. S., Aquino Neto, F. R. D., Lima, K. D. S. C., & Cruz, M. N. D. S. D. (2019). Determination of adulterants in whey protein food supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. *Brazilian Journal of Food Technology*, *22*.

Sanz, J. M. M., Norte, A., García, E. S., & Sospedra, I. (2017). Branched Chain Amino Acids and Sports Nutrition and Energy Homeostasis. In *Sustained Energy for Enhanced Human Functions and Activity* (pp. 351-362). Academic Press.

Sahathevan, S., Se, C. H., Ng, S., Khor, B. H., Chinna, K., Goh, B. L., & Karupaiah, T. (2018). Clinical efficacy and feasibility of whey protein isolates supplementation in malnourished peritoneal dialysis patients: A multicenter, parallel, open-label randomized controlled trial. *Clinical nutrition ESPEN*, *25*, 68-77

Sharma, S., Singh, R., & Rana, S. (2011). Bioactive peptides: a review. *International Journal Bioautomation*, *15*(4), 223-250.

Shoveller, A. K., Stoll, B., Ball, R. O., & Burrin, D. G. (2005). Nutritional and functional importance of intestinal sulfur amino acid metabolism. *The Journal of Nutrition*, *135*(7), 1609-1612.

Simonne, A. H., Simonne, E. H., Eitenmiller, R. R., Mills, H. A., & Cresman III, C. P. (1997). Could the Dumas method replace the Kjeldahl digestion for nitrogen and crude protein determinations in foods?. *Journal of the Science of Food and Agriculture*, *73*(1), 39-45.

Sharma, R., Rajput, Y. S., Dogra, G., & Tomar, S. K. (2009). Estimation of sugars in milk by HPLC and its application in detection of adulteration of milk with soymilk. *International Journal of Dairy Technology*, *62*(4), 514-519.

Smith, P. E., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M., ... & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical biochemistry*, *150*(1), 76-85.

Sharma, R. (2019). Whey proteins in functional foods. In *Whey Proteins* (pp. 637-663). Academic Press.

Smithers, G. W. (2008). Whey and whey proteins—from 'gutter-togold'. *International Dairy Journal*, *18*(7), 695-704.

Sowers, S. (2009). A primer on branched chain amino acids. *Huntington College of Health Sciences*, *5*, 1-6.

Sumner, E. E. (1938). The Biological Value of Milk and Egg Protein in Young and Mature Rats. *Journal of Nutrition*, *16*, 129-39.

The New Indian Express, (2019). *Careful with food supplements.*

The Times of India, (2019). *Illegal Health Supplements manufacturing unit raided.*

Tipton, K. D., & Wolfe, R. R. (1982). Protein and amino acids for athletes. *Journal of Sports Sciences*, *22*(1), 65-79.

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids*, *37*(1), 1-17.

Watson, P., Judkins, C., Houghton, E. D., Russell, C., & Maughan, R. (2008). Supplement contamination: Detection of nandrolone metabolites in urine

after administration of small doses of a nandrolone precursor. *Proceedings of the Nutrition Society*, *67*(OCE8).

Yüksel, Z., & Erdem, Y. K. (2010). Detection of the milk proteins by RP HPLC. *GIDA-Journal of Food*, *35*(1), 5-11.