EXTRACTION OF PROTEINS FROM NATURAL RUBBER LATEX WASTEWATER FOR METAL REMOVAL AND ITS ANTIMICROBIAL PROPERTIES

HAIRANI BINTI TAHIR

FACULTY OF SCIENCES
UNIVERSITI MALAYA
KUALA LUMPUR

2022

EXTRACTION OF PROTEINS FROM NATURAL RUBBER LATEX WASTEWATER FOR METAL REMOVAL AND ITS ANTIMICROBIAL PROPERTIES

HAIRANI BINTI TAHIR

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF SCIENCE

DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2022

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **HAIRANI BINTI TAHIR**

Matric No: **SHC 110034**

Name of Degree: **DOCTOR OF PHILOSOPHY**

Title of Thesis ("this Work"):

EXTRACTION OF PROTEINS FROM NATURAL RUBBER LATEX WASTE FOR METAL REMOVAL AND ITS ANTIMICROBIAL PROPERTIES

Field of Study: **PHYSICAL CHEMISTRY**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date: 20 Feb 2022

Subscribed and solemnly declared before,

Witness's Signature Date: 22 Feb 2022

Name:

Designation:

EXTRACTION OF PROTEINS FROM NATURAL RUBBER LATEX WASTEWATER FOR METAL REMOVAL AND ITS ANTIMICROBIAL PROPERTIES

ABSTRACT

Natural rubber latex plant waste from a centrifuge process contains *hev* protein. However, these proteins are mostly allergenic. The precipitation process carried out to purified protein from the waste bulk at different pH conditions, namely, from pH 3 to pH 8. The purpose of varying pH condition was to capture specific proteins. Amount of protein collected at different pH measured and it found that greater amounts collected at pH 4 and pH 7. Precipitation also carried out without the use of ammonium sulphate compare the amount of protein collected. Overall, the amount of protein collected from the salt-free condition was lesser by 25 %. The purified protein was then isolated using polyarcryl amide gel *Biorad* P-60 gel via open column chromatography. Each isolate containing protein introduced to SDS PAGE electrophoresis using benchmark protein marker as a standard to identify the isolated protein. Amongst the proteins isolated, seven types found to match the molecular weight of 20 kDa *hevea* protein to 40 kDa. Isolated protein was characterized using FTIR to countercheck the existence of protein for the particular isolate and using UV at 280 nm and 595 nm to estimate the concentration of protein in each number of isolates. Each isolate containing protein freeze-dried for storage purposes. Powder samples were more convenient to carry about and occupied smaller space compared to liquid samples, which were more susceptible to degradation or putrefaction, especially at warmer temperatures. The identified proteins were cross-matched with the hevea protein databank proposed earlier and used according to its specific function. At first, *Hevein, hev b6*, one of the isolated proteins, intended for antifungal study and mangan superoxide dismutase, *Hev b10* for metal extraction study, but the limited amount of proteins collected meant that the overall study of precipitated

protein undertaken, instead. Freeze-dried precipitated proteins analyzed for traces of metal and it was found that by using AAS, about 2 ppm of copper and 4 ppm of lead were recorded. However, many samples was saved by using ICP and additionally, 254 ppm zinc, 0.013 ppm aluminum, 0.129 ppm of selenium, 0.001 ppm nickel, 0.749 of ferum and 19.7 ppm of magnesium were detected. Proteins collected from different purification techniques exposed to 2 ppm, 5 ppm, 10 ppm, and 20 ppm sets of metal ion concentration to study their binding capability. Results showed that as the concentration of protein was increased, the metal removal intensity was also increased. It also observed that the binding capability differed between metals.

Keywords: NRL waste, MC extracted, SP extracted, metal removal.

PENGEKSTRAKAN PROTIN DARI SISA AIR GETAH ASLI CECAIR BAGI APLIKASI NYAH-LOGAM DAN CIRI-CIRI ANTIMIKROB

ABSTRAK

Sisa air kilang getah asli dari proses emparan mengandungi protin *hev.* Walaubagaimanapun kebanyakan protin ini adalah alergen. Proses presipitasi dijalankan untuk menulenkan protin sisa air pada keadaan pH berbeza iaitu dari pH 3 hingga pH 8. Tujuan menjalankan perbezaan pH adalah untuk mendapatkan protin tertentu. Jumlah protin yang di kumpul pada pH berbeza diukur dan jumlah yang tertinggi ialah pada pH 4 dan pH 7. Proses penulenan juga dijalankan tanpa dan denganmenggunakan ammoniam sulfat dan dibandingkan melalui hasil protin yang dikumpul. Secara keseluruhan jumlah protin yang dikumpul melalui proses emparan berulang adalah kurang dua puluh lima peratus daripada yang menggunakan ammoniam sulfat. Protin yang ditulenkan kemudian diuraikan kepada komponen lebih kecil meggunakan gel P-60 *Biorad* menggunakan kaedah kromatografi kolum terbuka. Setiap pecahan komponen mengandungi protin di analisa menggunakan SDS PAGE (kaedah elektroforesis) dengan penanda aras protin *(benchmark)* digunakan sebagai rujukan untuk mengenalpasti protin yang telah diasingkan. Dari semua protin yang diasingkan , tujuh jenis dikenalpasti sepadan dengan protin yang mempunyai berat molekul 20 kDa hingga 40 kDa. Protin yang di asingkan di perincikan menggunakan FTIR untuk menguji silang kewujudan protin untuk setiap komponen yang telah diasingkan dan UV pada 280 nm dan 595 nm digunakan untuk menganggarkan kepekatan protin dalam setiap. Setiap pecahan mengandungi protin dikeringkan menggunakan kaedah beku kering untuk tujuan penyimpanan. Sampel serbuk lebih mudah disimpan dan memerlukan ruang yang lebih kecil berbanding sampel protin cecair yang lebih mudah rosak terutama pada suhu tinggi. Protin yang telah dikenalpasti juga dibandingkan dengan maklumat yang dicadangkan pada awal dan digunakan mengikut ciri khas protin tersebut. Pada mulanya *hev b6* salah satu protin yang

berjaya ditulenkan hendak digunakan sebagai bahan anti-kulat dan *hev b10* untuk proses pengekstrakan logam, tetapi oleh kerana jumlah protin yag dikumpulkan terlalu kecil, perkara itu tidak dapat dijalankan dan hanya protin yang ditulenkan sekali sahaja diuji untuk keupayaan mengekstrak logam. Dengan menggunakan kaedah penyerapan atom (AAS) hanya 2 ppm kuprum dan 4 ppm plumbum terdapat dalam sisa air getah asli cecair (GCR). Penggunaan AAS memerlukan sampel protin yang banyak, oleh itu kaedah Plasma induktif berganda (ICP) digunakan dan sebanyak 254 ppm zink, 0.013 ppm aluminium, 0.129 ppm selenium, 0.001 ppm nikel, 0.749 ferum dan 19.7 ppm magnesium dikesan berada di dalam Sisa air GCR. Protin yang dikumpul melalui pelbagai kaedah penulenan dicampurkan dengan 2 ppm, 5 ppm 10 ppm dan 20 ppm kepekatan campuran logam untuk mengkaji keboleupayaan mengikat. Hasil yang diperolehi menunjukkan yang pertambahan kepekatan protin memberi kesan yang sama kepada keupayaan penyingkiran logam dan kebolehupayaan mengikat berbeza diantara logam logam.

Keywords: Sisa air getah asli cecair (GAC), Kaedah emparan berulang (KE), Kaedah pengekstrakan piawai (PP), nyah-logam.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Dr. Misni bin Misran for the continuous support of my Ph.D. study, for his patience, motivation and immense knowledge.

This work would not have been possible without the financial support of PPP UMRG. My special gratitude also goes to all lecturers and staffs in the Department of Chemistry Universiti Malaya for their support. Without their precious support, it would not be possible to conduct this research

I am grateful to all of those with whom I have had the pleasure to work during this project, my fellow lab mates, for the sleepless nights we were working together. Also, I thank my colleagues in the Faculty of Applied Sciences UiTM Shah Alam for enlightening me the first glance of research.

Nobody has been more important to me in the pursuit of the project than the members of my family. I would like to thank my parents, Hj Tahir and Hjh Rosnah and father in law Hj Ismail, not forgotten, late Hjh Hamsiah and all family members, whose love and guidance are with me in whatever I pursue. They are the ultimate role models. Most importantly, I wish to thank my loving and supporting husband, Mr. Johary and my two wonderful children, Ghazaly and Nur Nafeesa who provide unending inspiration.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ζ	:	Zeta potential
AAS	:	Atomic absorption spectroscopy
BOD	:	Biological Oxygen Demand
COD	:	Chemical Oxygen Demand
EP	:	Extractable protein
FTIR	:	Fourier Transform Infrared
ICP	:	Inductively coupled plasma
IEP	:	Isoelectric point
k Da	:	Kilo Dalton
Mw	:	Molecular weight
MWCO	:	Molecular weight cut-off
MC	:	Multiple centrifuge extraction
NA	:	Nutrient agar
NR	:	Natural rubber
NRL	:	Natural rubber latex
PAGE	:	Polyacrylamide Gel Electrophoresis
PDA	:	Photometric Dispersion Analyser
SDS	:	Sodium dodecyl sulphate
SEC	:	Size exclusion chromatography
SP	:	Standard precipitation
TEM	:	Transmission Electron Microscopy
UV	:	Ultra-violet

CHAPTER 1: INTRODUCTION

1.1 Latex and Serum

Latex of *Hevea brasiliensis* is the cytoplasm of specialized cells known as laticifers (Jekel *et al.*, 2003). Latex is a two-phase system, consisting of a dispersed phase with small particles and polymeric in nature. The aqueous dispersion medium is also known as a continuous or external phase and the term serum is also used. A complete characterization of dispersion medium involves all dissolved and suspended constituents, together with their concentrations, such as knowledge of the acidity or alkalinity of the aqueous phase, as well as the concentration of colloidal electrolytes, simple electrolytes, and water-soluble hydrocolloids. The pH is also an important variable, although this parameter is related to acidity and alkalinity. Two important types of surface phase are present in latex. They are an air-aqueous interface and an aggregate of polymer-aqueous interface. In this work, the later phase is of concern. The nature of the interfacial region that separates the polymer and the aqueous phase determines several partially interrelated latex characteristics, such as the electric charge carried by the particles, and also the speed and direction in which the particles migrate in an electric field.

Fresh, preserved, and prevulcanized natural rubber latex is all milky-white fluids. Preserved latex may sometimes darken on standing because of the action of enzymes in the latex. When kept in iron containers for a long time, the action of hydrogen sulfide (which is often present in minute quantity in preserved latex) on the metal may also cause the latex to darken. Prevulcanized latex, on the other hand, tends to develop a slight pinkish color when left undisturbed for some time. This is attributed to the residual zinc dithiocarbamate accelerator forming colored complexes with certain non-rubber substances in the latex. Fresh latex from rubber trees has a smell similar to that of milk. It soon develops an unpleasant, rancid smell if left unpreserved because putrefaction occurs. Most preserved latex used today has a very strong ammonia odor because

ammonia is added as a preservative. The density of preserved latex is between 950 and 980 kg/m^3. It is the resultant of two factors: the density of the rubber particles, and the density of the serum in which they are suspended. The former is between 900 and 930 kg/m^3. Meanwhile, the density of the serum depends on the number of dissolved materials. It is slightly greater than that of water. As rubber particles are lighter than serum, it is, therefore, expected that they will gradually tend to rise to the surface of the latex if the latex is undisturbed for some time.

The pH of latex varies from 6.5 to 7.0. The acidity of fresh latex increases during the first few hours after tapping. Eventually, the latex coagulates unless ammonia is added. Normal ammonia-preserved latex has a pH value between 10.2 and 10.5. The viscosity of ammonia-preserved latex is considerably lower than that of fresh field latex. This is because viscosity depends on the pH of the latex, and the minimum viscosity reached a pH value of about 10. This is the condition that exists in ammonia-preserved latex. A study for the effect of ammonia content and storage period on the viscosity of concentrated natural rubber (NR) showed an increment in viscosity due to gel formation in concentrated NR latex through crosslinking formation by hydrogen bonding and chemical crosslink, hence supporting the idea that hydrogen bonding via proteins and chemical crosslinks play a predominant role in the viscosity (Santipanusopon & Riyajan, 2009).

The number of rubber particles present in even a small volume of latex is very large. Langland (1936) determined that in 35 % latex, 1 mL of the latex contains about 6.4 × 10^{12} rubber particles (Kemp, 1937). Those particles, which are sufficiently large to be visible through a microscope, are seen to exhibit the characteristics of Brownian movement. Like particle shape, there are many opinions regarding the structure of the latex particles, and there are several opposing views. Nevertheless, certain features of the

particle structure are generally agreed upon. The inner core of the particle consists of rubber hydrocarbon. On the surface of the core of the particle is an adsorbed layer of phospholipids, sterols, sterol esters, fats, and waxes. Outer adsorbed non-rubber substances detected in fresh latex are distributed and some of the non-rubber substances, which are of particular significance, are further considered.

The transcriptome sequencing and comparative analysis by Wei *et al*. revealed a long-term flowing mechanism in *H. brasiliensis* latex, and the authors also discussed the importance of increasing the rubber yield of rubber trees so that high-quality material could be produced (Wei *et al.*, 2015). Longer flow time is a phenomenon in latex flowing due to intensive tapping. The transcriptome and expression profiling data for long-term flowing latex are needed as an important resource to identify genes and to better understand the biological mechanism of latex flow in rubber trees (Wei *et al.*, 2015). Upon bark tapping, the laticifer vessels are opened and their latex is expelled due to the turgor pressure of the laticifer vessels, and the latex flow rate and duration of flow are the first intrinsic factors known to limit rubber yield (d'Auzac *et al.*, 1989). This work shows the importance of protein study in the NR because almost all rubber production in the tree from biosynthesis to the last part of production involves protein. Centrifuging is the most favored method, and centrifuged latex is used for the majority of latex applications. Various batches of field latex are blended together to ensure a uniform product, and the process gives 60 % latex concentrate and skim latex with 5 to 8 wt. %.

There are more than 250 types of proteins in the latex with a total amount between 1 and 1.8 wt. % (Danwanichakul *et al.*, 2014). The skim latex is removed and subsequently converted into dry skim rubber (S Chaiprapat & Sdoodee, 2007).Natural rubber protein, which is also known as *hevea* protein, has been studied since as early as 1927. The extraction of the protein is mainly achieved by the freeze-dried method. Most of the

studies conducted focused on identifying the allergen and the characteristic of the protein, and suggested a method to control the existence of the allergen. The matter of interest is the allergy, which causes skin irritation when rubber gloves are worn for more than 8 h. The use of protein diversifies. Research from around the world classifies the use and the main role of protein, such as an enzyme, as a carrier in the reaction in our human system. *Hevea* protein can be isolated from the seed, leaves, or bark of *H. brasiliensis*. The seed gives the highest content of protein than other parts. A review on *hevea* protein from 1927 to 2020 shows that the research area is on the determination of 15 protein allergens, covering the function of some allergens, how to extract protein allergens, how to control the blooming of allergens to the surface of latex products, and characterization of roles and functions of allergens in the natural rubber latex (NRL) system and genomic studies.

The allergen content of disposable rubber gloves varies with different types of gloves produced, with gloves and toy balloons appear to be more important sources of rubber allergens (Yunginger *et al.*, 1994). Allergen is not only confined to latex-related products, where researchers found the presence of latex allergens in passenger cars and truck tire tread, in debris deposited from the atmosphere near a freeway, and also in airborne particulate matter (Miguel *et al.*, 1996). Natural rubber latex, a starting material in glove production, contains latex proteins that are carried over to the finished products due to inadequate manufacturing practice, which may pose a risk of allergen to medical workers or patients (Yip & Cacioli, 2002). Thus, it leads to the implementation of various steps in the manufacturing process to remove or immobilize the proteins.

In the manufacturing process of latex goods, various methods have been introduced, including the alternative starting material using low-protein latex (LOPROL) with a reduced amount of protein in the NRL, or in the manufacturing line where the extractable protein (EP) could be removed by the process called leaching, which comprises a few

passes of washing with warm water. The average rubber tree may exude 100 mL of latex (33% rubber content) at each tapping of the tree, and approximately 2 g of protein per tapping or about 330 g of protein per year (165 tapping days per year) (Perrella & Gaspari, 2002). The protein will flow to the waste stream and pool in the treatment pond. The focus of this research is that instead of flowing to the waste stream, the *hevea* protein is collected, isolated, characterized, identified, and searched for any suitable application. Natural rubber latex waste from the latex concentrate factory contains *hevea* protein. This *hevea* protein consists of several types of common proteins, which are subject to investigation in terms of characteristics and applications.

The use of protein as a metal binder has been widely discussed, but *hev* proteins are not a typical example of the application. The production of latex products requires the NRL in a concentrated form via centrifugation, and this step produces a secondary product (i.e., skim rubber). Direct coagulation of skim latex to produce skim rubber is not easy because the colloid stability is enhanced by the high content of ammonia and protein substances. The usual method to coagulate skim latex is by coagulation with sulfuric acid. This acid coagulation method leads to the generation of highly acidic effluent that necessitates additional treatment before being discharged to the environment in order to avoid pollution (Kongkaew *et al.*, 2012). A study on wastewater includes the reuse of the wastewater as sorbent and other potential management practices. Most of the research using the wastewater is intended to investigate the use of wastewater, including exploring the valuable properties it possesses.

Table 1.1: *Hev* proteins and amino acids

Allergen	Major Amino Acids	Properties
Hev b1	11% Alanine, 10.8% Valine	Non-polar hydrophobic amino acid
Hev b2	10.7% Serine 9.4% Leucine	Non-polar hydrophobic amino acid
Hev b3	17.1% Valine 9.3% Alanine	Non-polar hydrophobic amino acid
Hev b4	10.4%Leucine 7.3% Alanine	Non-polar hydrophobic amino acid
Hev b5	30.5% Glutamate 18.5% Alanine	Negatively charged (Acidic amino acids)
Hev b6	14% Cysteine 12% Glycine	Uncharged polar amino acid
Hev b7	12%Leucine 9% Isoleucine	Non-polar hydrophobic amino acid
Hev b8	13% Glycine 8.4%Isoleucine	Uncharged polar amino acid
Hev b9	10% Alanine 9.9% Glycine	Non-polar hydrophobic amino acid
Hev b10	12.8% Leucine 9.4% Alanine	Non-polar hydrophobic amino acid
Hev b11	15% Glycine 9% Alanine	Uncharged polar amino acid
Hev b12	13.8% Alanine 12%Threonine	Non-polar hydrophobic amino acid
Hev b13	7.9% Alanine 7.9% P-alanine	Non-polar hydrophobic amino acid
Hev b14	10.2% Glycine 10.2% Leucine	Uncharged polar amino acid

Discussion on the biological roles of *hev* protein has identified the function of each established *hev* protein. *Hev b1*, which involves in biosynthesis, is a regulatory protein. *Hev b2*, a type of defense protein, is used in the defense against fungal pathogens. *Hev b3* involves in biosynthesis, whereas *Hev b4* has no specific application. *Hev* b5 is a structural protein that provides mechanical support. *Hev b6* is an antifungal protein, and both *Hev b7* and *Hev b8* are regulatory proteins. *Hev b9* is a structural protein and a housekeeping enzyme. *Hev b10* is a superoxide dismutase, an enzyme that causes radical destruction. *Hev b11* class I endochitinase is a defense-related protein, while *Hev b12*, which is a non-specific lipid transfer protein 1, is also a defense-related protein. *Hev b13* and *Hev b14* are esterase and hevamine, respectively, which are also defense-related proteins, whereas *Hev b15*, which is a serine-protease inhibitor, is an allergen (Berthelot, Lecomte, Estevez, & Peruch, 2014). This skim rubber waste contains a fresh and of higher

concentration of *hev* proteins compared to other waste streams. As long as the production of concentrate NRL exists, the amount of waste-containing proteins also increases, leading to abundant proteins that can be purified and used. As of now, research has been conducted to treat skim serum wastewater with gamma radiation and water to irrigate plants. Many industries are still in search of the most effective method for handling their waste, including metal waste, in order to purify proteins from the industrial line. It is hoped that some of the heavy metal waste could be treated with *hev* proteins.

Table 1.2: Allergen, domain, and cross-reference from the Swiss Protein Database

Allergen	Binding/Molecular Function	Domain	Cross-Reference PDB
Hev b1			
Hev b2	Glycosidase Hydrolase, cation binding,	IghsA00 Glycosidases	3D structures of two plant beta glucan endohydrolases
Hev b3			Small rubber particle protein, patatin like
Hev b4	Lipid metabolic process, acting on ester bonds		
Hev b5			
Hev b6	Chitin binding, disulfide bond	ln10A01 Barwin-like endoglucanases (53%) Chitin binding type 1	Crystal structure of php p1 a major timothy grass pollen allergen
Hev b7	Lipid metabolic process, hydrolase activity	10xwA00 Cytosolic phospholipase A2 catalytic domain (99%)	Crystal structure of semet patatin
Hev b8	Actin cytoskeleton organization	Ig5uA00 Dynein light chain 2a, cytoplasmic (100%)	Latex profilin hev b8-pollen allergen
Hev b9	Glycolysis, Phosphopyruvate hydratase complex		Crystal structure of putative mandelate racemase from bondetella bronchiseptica rb 50
Hev b11	Cell wall molecule catabolic process,	Chitin binding type 1 domain	The refined crystal structure of an endochitinase
Hev b12	Lipid transport, Lipid binding,	1fk5A00 plant lipid – transfer	Structural basis of non-specific lipid binding
Hev b13	Defense response, lipid metabolic process, lgE binding,		
Hev b14	Carbohydrate metabolic process, Chitin catabolic process cation binding, hydrolase activity		

Source: Protein database bank (PDB)

1.2 Problem Statement

Studies on *hevea* protein address the issue of latex allergens by suggesting methods to overcome problems related to the allergen. Most of the works focused on the isolation and characterization of 13 types of latex allergens, from *Hev* b1 to *Hev* b13. The methods for reducing or removing protein allergens have been extensively studied. Instead of appreciating the existence of such proteins in natural rubber latex, the roles in the systems are always deprived due to interest in the allergen and related consequences. It is a necessity to put some values to unwanted or rejected proteins by detailed works on the roles of each protein in the colloidal system itself and outside the system. The protein will flow to the waste stream and pool in the treatment pond. To date, there is no discussion on collecting and using these proteins. Thus, instead of flowing to the waste stream, the *hevea* protein is collected, isolated, characterized, identified, and searched for any suitable application.

Most of the research focused on the isolation, identification, and characterization of allergens with the aim of altering the genetic code in the rubber tree or using any method that can eliminate or tie the allergen. This research aims to utilize unwanted proteins.

1.3 Significance of the Study

In general, wastewater from industries needs to be treated before discharge to water bodies. Various treatment steps can be avoided by re-using wastewater, especially in bulk. Simultaneously, the extraction process generates useful materials for other purposes, such as metal removal and antimicrobial agents.

Untreated wastewater also causes pollution, especially when the wastewater is discharged directly to the river and flows to the sea. As a consequence, aquatic life is always in danger, and sometimes, human life is also affected indirectly due to the biological enhancement of our food source. Reducing the amount of wastewater and

generating proteins give a great impact on a sustainable environment for future generations.

It is also important to encourage the reuse of waste not only for the environmental factors and problems caused by the mistreated waste, but the cost from starting materials to the final process of extraction is also comparatively lower with promising benefits. Hence, the approach is economically viable.

1.4 Objectives of the Study

This study pursues to suggest the reuse of wastewater from natural rubber latex waste and to convert those collectibles to useful forms. The objectives of the study are:

1. To investigate protein extraction techniques from an NRL concentrate processing plant, specifically skim rubber serum based on the extracted amount and their characteristics.
2. To examine physical properties and identify extracted proteins.
3. To study the interaction of proteins with various types of metals.
4. To investigate the antifungal and antibacterial properties of the extracted proteins.

The extraction process begins with the selection of most protein-rich and structurally stable from skim rubber serum, which can be used directly as bio-sorbents. The purification process involves physical techniques and a chemical agent. The extracted proteins can be stored in solid powder or liquid aqueous form. A series of characterizations are carried out to understand the physical and chemical properties of the extracted proteins so that the application of the proteins can be made viable.

1.5 Scope and Limitations of the Work

The protein source wastewater, which was collected directly from the waste serum of skim rubber production, is a by-product of the latex concentrate processing factory of

MARDEC Industrial Latex Sdn. Bhd., Tapah, Perak, Malaysia. NRL waste is normally collected a day or two after the coagulation process of skim serum. This period is critical to ensure the serum sample still has a reasonable amount of protein and there is no microorganism activity that has occurred. Several simple characterizations were performed to ensure the sample fulfilled certain criteria, including pH, zeta potential, and particle size. The waste was centrifuged, purified, or filtrated and fractionated. The extraction from the source was carried out first, followed by fractionation based on the molecular weight. The fractionation process was performed through open-column chromatography using size-exclusion chromatography with a column bed (Polyacrylamide P-60 Gel). SDS-PAGE was performed for molecular weight determination using a standard protein marker as a benchmark. Particle size and zeta potential were determined using a static light-scattering instrument (Malvern Zetasizer Nano ZS). A photometric dispersion analyzer (PDA) and an ultraviolet (UV) visible spectrophotometer were used to construct a chromatographic profile.

Bactericidal and fungicidal studies were performed using disc diffusion assay or well diffusion assay method. Extracted and fractionated proteins were spread on two different bacteria and fungi to evaluate their antimicrobial activities. Atomic absorption spectroscopy (AAS) and inductively coupled plasma (ICP) were used for the metal-binding study. The amount of protein varies at various concentrations and metal elements used. The percentage of metal removed was then calculated. In this research, proteins were extracted and purified from the wastewater body originating from the production line of skim rubber. The properties of proteins depend largely on the starting material, which is natural rubber latex. NRL constituents largely depend on weather and soil, especially mineral content, which is one of the parameters considered in this study. The variations in some properties are due to the mineral content of NRL, which limits the consistency in some of the data.

CHAPTER 2: LITERATURE REVIEW

This chapter covers latex products containing proteins that cause skin allergy, wastewater that contains proteins, waste management technology, and the study on *hev* proteins. A common problem associated with the presence of *hev* proteins is also addressed. Various purification techniques and also characterization or identification methods are listed and discussed. Studies involving proteins are also considered for discussions later on. Other related aspects are also covered briefly.

2.1 Natural Rubber Latex Concentrate Processes and Properties

Centrifuged and creamed latexes are normally preserved using ammonia, where the level is adjusted to 0.70 % to 0.75 % based on latex weight (high-ammonia (HA) latex). For low-ammonia latex, a secondary preservative is used. In all cases, latex is bulked at various stages in the production process to ensure consistency in the final product. At every stage of storage, samples are taken for quality control testing. Latex is handled in conditions of high cleanliness to avoid bacterial infection. Tanks, pipes, filters, and pumps are all disinfected daily, and specific disinfectants are used in rotation to prevent the formation of resistant bacteria. In concentrated latex production, ammonia is added to the arriving field latex for preservation, while various chemicals are added for latex conditioning.

Table 2.1: Particle size and zeta potential of cream, skim, fresh, and centrifuged latex

Properties	Cream Rubber	Skim Rubber	Fresh Latex	Centrifuged Latex
Particle Size, d (nm)	467	297	636	655
Zeta Potential, ζ (mV)	-70	-64	-45.7	-39.3

Ref: (Rippel *et al.*, 2003), (Sansatsadeekul *et al.*, 2011)

This pretreated latex is subjected to rubber centrifugation to remove liquid in order to concentrate the latex from 30% to 60 % dry rubber content. The rejected liquid from centrifugation is called skim latex, which still contains 4 % to 8 % dry rubber content. Particle size distribution depends on the time and condition of centrifugation. Table 2.1 shows the size and zeta potential of skim and cream rubber measured in 10^{-3} mol L^{-1} KCl. The size and zeta potential of fresh and centrifuged latex are measured at pH 9 of 0.015 M NaCl.

2.2 Protein Definition

In the 1800s, Johannes Mulder came up with the name protein, a term derived from a Greek word, which means "of first importance". Proteins are a very important class of food molecules because they provide an organism not only with carbon and hydrogen, but also nitrogen and sulfur (Denniston *et al.*, 2013). Protein carries out most of the work in a cell, such as protection of the body from infection, mechanical support and strength, and catalysis of metabolic reaction.

Protein has many biological functions. Enzymes (e.g., pepsin and trypsin) break down proteins in our diet so that the subunits can be absorbed for use by our cells. Defense protein includes antibodies (also called immunoglobulins), which are specific protein molecules produced by a specialized cell of the immune system in response to antigens. Transport protein carries materials from one place to another in the body. The protein called transferrin transports iron from the liver to the bone marrow. Other specific functions of proteins include regulatory proteins, structural proteins, movement proteins, and nutrient proteins.

Proteins have many biological functions, including enzymes as biological catalysts, where the majority of enzymes that have been studied are proteins. Defense protein includes antibodies, also known as immunoglobulin, which are specific protein molecules

produced by specialized cells of the immune system in response to a foreign antigen. Transport protein carries materials from one place to another in the body. For instance, hemoglobin and myoglobin are responsible for the transport and storage of oxygen in higher organisms. Regulatory protein controls many aspects of cell functions, structural protein provides mechanical support to large animals and provides them with their outer covering, and movement protein is necessary for all forms of movement. The most important muscle, which is the heart, contracts and expands through the interaction of actin and myosin proteins. Moreover, sperm can swim due to the long flagella made up of proteins.

Nutrient protein serves as a source of amino acids for embryos or infants, where egg albumin and casein in milk are examples of nutrient-storage proteins (Denniston *et al.*, 2013). All functions or biological roles in proteins are carried out by each and every type of protein investigated in this study.

2.2.1 Protein Structure

Proteins are the functional forms of polypeptides. Protein structures are defined as much by the environment as by the chemical properties of polypeptide chains. Thus, there are distinctive categories of proteins: water-soluble globular proteins, water-insoluble fibrous proteins, and proteins that are associated with the hydrophobic environment of membrane bilayers. Each protein is characterized by distinct amino acid compositions and sequences, but all the structures can be described using an identical set of basic principles (Van Holde *et al.*, 2006).

Molecules formed by linking two or more amino acids are called peptides. Each amino acid in a peptide is called a residue. Molecules with many amino acid linkages are polypeptides, and those with long polypeptide chains are proteins. Proteins are made up of some combinations of 20 different subunits called α-amino acids. The carboxyl group

of one amino acid is linked to the amino group of another amino acid in a linear polymer. The peptide bond is an amide bond formed between the –COO- group of one amino acid and α-NH_3^+ group of another amino acid. The primary structure is the amino acid sequence of the protein chain, which results from the peptide bonds. It is a translation of information contained in genes. Each protein has different primary structures with different amino acids in different places along the chain. The primary structure will determine its biologically active form. The interactions among R groups of the amino acid in the protein chain depend on the location of those R groups along the chain. This interaction will govern how the protein chains fold, which in turn dictates its final three-dimensional structure and its biological function.

The secondary structure is the result of hydrogen bonding between the amide hydrogen and carbonyl oxygen of the peptide bonds. Different regions of protein chains may have different types of secondary structure. Some regions of a protein chain may have a random or non-regular structure; however, the most common types of secondary structure are the α-helix and the β-pleated sheet because they maximize hydrogen bonding in the backbone. The helical shape is maintained by hydrogen bonds between neighboring residues. The β-pleated sheet structure is found in fibers like silk. Fibrous protein is a structural protein arranged in fibers or sheets with only one type of secondary structure. The three-dimensional structure of a protein is formed due to the association of different amino acid residues far away from each other in the primary sequence involving long-range interactions. Most fibrous proteins, such as silk, collagen, and α-keratins are insoluble in water. However, the majority of cellular proteins are soluble in the cell cytoplasm. Soluble proteins are usually globular proteins, which have a three-dimensional structure called tertiary structure. Protein may also have a quaternary structure, which describes how polypeptide chains stack together in a multi-chain protein (Denniston *et al.*, 2013).

Protein gelation is important in food products, and the factors affecting gelation will help in the knowledge of protein structure. Heat-induced protein gels are known to form in a two-step process: unfolding of proteins that may expose the protein active sites, resulting in the aggregation of protein molecules. This aggregation continues with the association of protein particles, and a gel network may form (Zhou *et al.*, 2015). The non-covalent interaction that maintains the three-dimensional structure of a protein is weak, and it can be disrupted easily. The unfolding of a protein is called denaturation. The reduction of disulfide bonds leads to an even more extensive unraveling of the tertiary structure. Protein can be denatured in several ways. One of them is heat, where an increase in temperature favors vibrations within the molecule; consequently, the energy of vibrations within the molecule can become great enough to disrupt the tertiary structure. At either high or low extremes of pH, at least some of the charges on the protein are missing; hence, the electrostatic interactions that would normally stabilize the native protein are drastically reduced (Campbell, 2009).

The primary structure conveys all the information necessary to produce the correct tertiary structure, but the folding process in vivo can be a bit trickier. In the protein-dense environment of the cell, proteins may fold incorrectly, as they are produced or they may begin to associate with other proteins before completing their folding process. Correctly folded proteins are usually soluble in the aqueous cell environment or they are correctly attached to membranes. However, when proteins do not fold correctly, they may interact with other proteins and form aggregates. To help avoid the folding problem, special proteins call chaperons aid in the correct and timely folding of many proteins (Campbell, 2009). In 2003, there were about 498 common folds that made up the structures in the protein database (over 21,000 protein structures). The folds are considered the basic building blocks of all tertiary structures and may be structural or functional (Van Holde *et al.*, 2006).

2.3 Manufacturing Process in the Latex Industry and Wastewater

The manufacturing process in the latex industry begins from the production of latex concentrate to rubber or latex production. In each case, the wastewater produced varies in protein compositions, which are influenced by the processing steps involved. Latex concentrate produced from centrifugation contains proteins, which are still structurally stable and can be further used by utilizing the role or biological functions of those proteins. The wastewater or effluent can be collected from both bowl washing of centrifuge or skim serum, which is separated from the cream that undergoes a secondary process to produce skim rubber sheets. This skim waste is acidified serum and technically, only a few proteins can survive this condition. However, in terms of stability, this acidified serum is better and less susceptible to microorganism attacks. On the other hand, latex products mean any dipped, cast, and foamed products, and also other manufacturing techniques to produce rubber articles. Dipped rubber products are commonly used in gloves, condoms, pacifiers, and catheters. Cast rubber products are more prevalent in the mask-making industry, whereas foamed rubber products are used to produce pillows, mattresses, and other foamy articles.

In all latex product manufacturing, the concern is on removing excess proteins, which may cause an allergic reaction to consumers. Non-rubber components in concentrated NRL, such as proteins, long-chain fatty acids, soaps, and polypeptides are presumed to distribute in the serum fraction. The rubber particle surface protein on the surface of NRL gloves is considered as the allergen, and several methods have been developed to reduce the protein content. One of the methods is the use of dialdehyde sodium alginate to immobilize the protein, which confers a potential approach to tackle such a problem (Gong *et al.*, 2013). Proteins in NR latex and some rubber products may cause an increase in the incidence of sensitization, adversely allergic reactions, and even death through anaphylactic shock. Furthermore, proteins have an effect on the stability, quality, and

durability of some rubber products, especially latex gloves because they contact with the skin more than other rubber products (Bang-iam *et al.*, 2013). In all product manufacturing, the effluent contains proteins, but most of them are already denatured or changed form due to special treatments to remove those proteins.

2.4 Wastewater Reuse and Management

A study of fungi associated with the waste of a rubber-processing factory managed to isolate three fungal species: *Mucor racemosus, Mucor* sp., and *Aspergillus niger*. During latex coagulation, a large aqueous fraction called natural rubber waste serum is obtained as a by-product. Although this serum is presently being used as a commercial source of nitrogen and potassium fertilizer, the waste management technology is still poorly developed (Atagana *et al.*, 1999). In the study of the characterization and recycling of wastewater from guayule latex extraction, the researchers found that the wastewater is suitable for irrigation based on the sodium absorption ratio, whereas the waste is unsuitable for irrigation but may be suitable for recycling and latex extraction (Coffelt & Williams, 2009). Chaiprapat and Sdodee (2007) studied the effect of wastewater recycled from natural rubber smoked sheet production on the economic crop in southern Thailand by investigating the potential use of wastewater for agricultural purposes (S Chaiprapat & Sdoodee, 2007). The wastewater used did not meet the discharge standard, and Chinese green mustard and cucumber were used as the test plants, whereas paddy and rubber trees were used as the economic crops. The results showed that plants irrigated with effluent mixtures had a higher yield than control, and for economic crops, the latex production of rubber trees irrigated with effluent was significantly higher. However, empty grain occurred in the paddy field experiment. This works promotes an alternative application of wastewater, which can be used directly from the waste of NR. There is a slight difference compared to our study because the waste collected is preconditioned and purified to isolate proteins from the waste and use it later. Sulfuric acid, which is used to

coagulate skim latex in the production of skim rubber, produces H_2S gas in the wastewater. Therefore, an attempt was made to use hydroxypropyl methylcellulose polymer as a sulfuric acid substitute, and this work offers an integrated approach in waste management in the latex concentrate industry (Sumate Chaiprapat *et al.*, 2015).

The effluent from a latex concentrate factory is the most contaminated wastewater from rubber processing factories. A common treatment method requires a large land area, high-energy consumption, and high operating and maintenance costs. These factors drive the research on membrane technology application, which was initiated by concentrating natural rubber serum using reverse osmosis (Lau, 1994). Skim latex serum was treated using ultrafiltration with spraying (Harunsyah and Sulaiman, 2002) and natural rubber field latex was concentrated using a tubular cross-flow ultrafiltration system (Veerasamy *et al.,* 2003). In-situ ultrasonication was used to reduce fouling during the recovery of natural rubber skim latex by ultrafiltration, and the approach managed to achieve the ideal preservation level. The in-situ ultrasonication improved the permeate flux and simultaneously maintained the feed stability (D Veerasamy *et al.*, 2009), whereas the use of a membrane bioreactor managed to obtain BOD and COD removal efficiencies of 96.7 % and 96.99 %, respectively (Sulaiman *et al.*, 2009).

In the investigation of foulant characteristics and cleaning protocols, the rehabilitation of fouled membrane from natural skim latex through membrane autopsy and ultrasonication enhanced the membrane cleaning procedures, where the skim latex still contained 4%–5% dry rubber and sulfuric acid. The parameter measured was flux recovery using deionized water after chemical cleaning, and the cleaning was very good even without ultrasonication (Devaraj Veerasamy & Ismail, 2012).In the microbial desulfurization of waste latex rubber using *Alicyclobacillus sp.*, Yao *et al.* (2013) utilized a microbe with the desulfurizing capability to recycle waste latex rubber (WLR) (Yao *et*

al., 2013). The growth characteristics of the microorganism and the technical conditions of co-culture desulfurization were studied, and the mechanism for the microbial desulfurization of WLR was explored. The results of XPS and Fourier transform infrared (FTIR) revealed that *Alicyclobacillus sp.* could break the crosslinked sulfur bonds and oxidize them to sulfone groups. This work provides information on other applications of NR waste and useful microorganism species as a basis for our work. In the consideration of a specific type of *hev* protein, lectin is one of the heterogeneous proteins that are physiologically important. A number of roles have been proposed, for example in wall extension, stimulation of plant cell proliferation and growth, carbohydrate transport, protein storage protein, and plant defense (Wittsuwannakul *et al.*, 1998).

2.5 Protein and Allergen

It is well known that NR latex contains proteins recognized as allergenic proteins. A small amount of these proteins can give rise to latex allergy. Upon centrifugation, rubber latex is divided into a layer of rubber particles, the cytosol and lutoid body fraction, and organelles of vacuolar origin. Proteins are the condensation polymerization products of amino acids, which are produced through the removal of water and formed peptide links. For example, for the polymerization of glycine (or α-amino acetic acid), which is the first member of amino acids for the proteins in natural rubber latex, there is a point at which both $–NH_2$ and $–CO_2H$ species are at maximum ionization. The net charge is the difference between positive and negative species known as zeta potential (ζ), and at this point, it is obviously zero. This zero net charge point occurs at around pH 5, which is called the isoelectric point, and the ranges of the isoelectric point for *Hev* proteins are from pH 4.5 to pH 8.0. Proteins that can be found in the bottom fraction are also called the lutoid body (B-serum) that consists of essentially a gelatinous lump bounded by a semi-permeable membrane containing proteins and nitrogenous compounds, rubber and carotenoids, lipids, and metal ions suspended in colloidal dispersion in a cationic

environment (acidic pH 5.4). The semi-permeable membrane is polarized, where the outside is of negative polarity, but the inside is positively charged. C-serum is essentially aqueous and contains dissolved materials, including carbohydrates and inositols, protein derivatives and nitrogenous compounds, nucleic acid and nucleosides, and also inorganic ions and metal ions. The protein is probably the most important non-rubber substance present in NR latex. Together with phospholipids, they confer colloidal stability to the freshly tapped latex when they are adsorbed onto the rubber particles. They may also act as vulcanization activators during dry rubber vulcanization. Tata (1971) established that the total protein content in fresh latex is 0.95 %. From this value, 27.2 % is in the rubber phase, 47.5 % is in the serum phase, and 25.3 % is in the bottom fraction. Two major anionic proteins, α-globulin and hevein, have been isolated and studied in some detail (Tata, 1971). α -globulin is made up of 17 constituent amino acids, one of which is cystine that contains a high sulfur content. These two proteins have similar isoelectric points. Although α-globulin is readily adsorbed at a water-oil interface, resulting in the decrease of interfacial tension, hevein has very little surface activity.

The total protein content in fresh latex is approximately 1 % to 1.5 %, in which about 20 % is adsorbed on the rubber particles, and a similar proportion is associated with the bottom fraction. The adsorbed proteins, together with adsorbed lipids, impart colloidal stability to the latex and remain associated with the rubber phase when the latex is coagulated by acid during the manufacturing of dry rubber. About half of the serum and bottom fraction protein coagulated and remain associated with the rubber in the process. In 1942, it was shown that the serum from unpreserved latex contained seven electrophoretically distinct protein components. Ammonia preservation treatment carried out for several months reduced the number of electrophoretically resolvable components from seven to two. However, in the 1960s, the use of starch gel electrophoresis demonstrated the presence of 22 protein components in the serum phase. At least eight

proteins were present in the bottom fraction of fresh latex, and some of these components might be identical to the ones occurring in the serum phase.The isoelectric points of the protein are in the range from approximately pH 3 to greater than pH 9. The proteins adsorbed on the rubber particles have not been studied in much detail due to the difficulties in removing them from the particle surface. However, particle electrophoresis shows that fresh latex particles have isoelectric points ranging from pH 4.0 to pH 4.6, depending on the clone. This variation in isoelectric points indicates that more than one protein is adsorbed on the rubber particle, and the relative proportion of the adsorbed proteins is clonal characteristics. Hevein and α-globulin are two proteins present in a relatively high concentration in latex, which have been separated and purified, and their properties have been studied in some detail. α-globulin is the protein present in the highest concentration in fresh latex serum. It is soluble in salt solutions at pH values away from its isoelectric point (pH 4.55), coagulated by heat, and readily adsorbed at hydrocarbon-water interfaces.

The sulfur content is low and there is little phosphorous present. It precipitates from solution at approximately the same pH at which fresh latex is coagulated, suggesting that it may be one of the proteins adsorbed on the surface of the rubber particles, and thus partly responsible for the colloidal stability of the latex. Approximately 20 % of the dry matter in the bottom fraction of latex from mature trees is water-soluble protein, of which about 70 % is hevein. Its isoelectric point is 4.7 and it contains an abnormally high amount of sulfur (about 5 %), essentially all of which is present as disulfide groups in cystine. Hevein is readily soluble in water over a wide range of pH, including its isoelectric point, not coagulated by heat, and has a molecular weight of about 5 kDa. These properties indicate that sheet or crepe rubber should contain very little hevein. A protein with very similar properties as hevein, called pseudo-hevein, has also been isolated. It is slightly less anionic than hevein and is present in a much smaller amount. Several basic proteins

with high isoelectric points (pH 8.6) are detected in the fresh latex bottom fraction. The main basic component, which forms about 17 % of the total protein in the bottom fraction, has an isoelectric point in the region of pH 10. It is soluble in water or diluted solutions over wide pH values. Although this protein has a high isoelectric point, no precipitation is observed with the anionic proteins isolated from latex. These basic proteins may have an important influence on the colloidal stability of fresh latex due to the positive electric charge they carry at neutral or slightly alkaline pH values.

2.6 Proteins from NRL Concentrate Processing Waste

Based on the literature survey, skim rubber contains a very low amount of rubber particles, and other ingredients are ammonia, water, and non-rubber components. It is proven that the protein content in the skim fraction of natural rubber latex is excessive. A previous study characterized the enzyme deproteinized skim rubber and proposed that skim natural rubber latex is a protein-rich by-product obtained during the centrifugal concentration of natural rubber latex (George *et al.*, 2009). The skim has very low dry rubber content but a higher proportion of non-rubber solids, which are mostly proteinaceous in nature. The characterization using gel permeation chromatography and particle size analysis showed no significant changes in particle size, but the reduction in the quantity of fatty acids reflected the improvement of aging characteristics. The excessive amount of protein was also supported by the study of the effluent of natural rubber factories, and from the study, it was found that the effluent was enriched in the antifungal protein hevein (U. M. Soedjanaatmadja *et al.*, 1995).

NRL protein acts as a surfactant in the colloid system for the first few hours after tapping to ensure stability (Perrella & Gaspari, 2002). Besides, proteins also have other roles in the NRL, such as biosynthesis to produce the latex in the laticifer and coagulation of the tapping cut of the rubber tree. Another protein in NRL, known as *Hevea* latex lectin

(HLL) protein, is suggested to function as a mediator in rubber particle aggregation and latex coagulation. Wititsuwannakul studied this lectin or carbohydrate-binding protein (Wititsuwannakul, Pasitkul, Kanokwiroon*, et al.*, 2008). The HLL was purified by chitin-binding separation, followed by DEAE-Sepharose chromatography. Its molecular weight obtained by SDS-PAGE was 17 kDa, with a *pI* value of 7.2. The hemagglutinin activity of HLL was enhanced by Ca^{2+}, and most interestingly, the HLL strongly induced the aggregation of the *Hevea* latex rubber particles. This strong rubber particle aggregation leads to latex coagulation, indicating the possibility that it is involved in the formation of coagulum that plugs the latex vessel ends and stops the flow of latex upon tapping (Wititsuwannakul, Pasitkul, Jewtragoon*, et al.*, 2008).

The colloidal properties of skim and cream natural rubber particles were investigated by Rippel *et al.* (2003). The work studied the topology and charge properties in film formation using atomic force microscopy and scanning electric potential microscopy. The elemental distribution maps of the particles were obtained using electron energy-loss imaging in low-energy transmission electron microscopy. The cream fraction was found to be stable, whereas rapid coagulation occurred in the skim fraction. Several factors had been proposed to explain the spontaneous destabilization: i) the development of acidic environment due to bacterial activity, ii) the interaction of adsorbed volatile fatty acid anions with cations, and iii) the aggregation by cationic protein and attack by enzymes from the lutoid serum (Rippel *et al.*, 2003). The finding was the rubber particles from the cream rubber particles contained a higher amount of adsorbed protein-phospholipid material compared to those in the "self-cleared" concentration fraction. Natural rubber is synthesized by over 2,000 species of plants, but *Hevea brasiliensis* is the most important species that produced commercially viable natural rubber compared with other rubber-producing plants (Xiang *et al.*, 2012).

Berthelot (2014) studied *H. brasiliensis* REF (*Hevb1*) and SRPP (*Hevb3)* through an overview of rubber particle proteins and reviewed the knowledge of two important proteins associated with *H. brasiliensis* rubber particles. They covered three decades of research on these two proteins and their homologous in plants, and particularly emphasized on different possible properties and functions of various plant proteins (Berthelot, Lecomte, Estevez, Coulary-Salin*, et al.*, 2014; Berthelot, Lecomte, Estevez, & Peruch, 2014). The major constituents of NRL are cis-1,4-polyisoprene, which is about 94 %, and the remaining 6 % is called non-rubber components, constituting 1.5 % to 3 % lipids, 2 % proteins and polypeptides, 0.4 % carbohydrates, and 0.2 % minerals (Rolere *et al.*, 2015).

2.7 Roles of *Hevea* Proteins in Natural Rubber

NR particles are made up of a hydrophobic core of NR molecules surrounded by a mixed layer of proteins and phospholipids as the shell (Nawamawat *et al.*, 2011). The proteins and phospholipids are associated with the terminal ends of the rubber chains. The carboxylic groups of the proteins and the phosphate groups of the phospholipids contributed to the negative charges of the latex particles. Enhanced ionization of proteins and phospholipids at alkaline pH caused an incremental increase in the negative charges of the latex particles and contributed to increased stabilization of latex (Sakdapipanich *et al.*, 2015). The organic-organic nanomatrix structure was discovered upon observation of the morphology of natural rubber. Natural rubber particles with an average diameter of about 1 μm are well-dispersed in a nanomatrix of non-rubber components, which has a thickness of about 15 nm (proteins and phospholipids). Removing the proteins will destroy the nanomatrix structure and significantly reduce the mechanical properties. For example, the storage modulus value without protein is one-tenth of that of natural rubber. Hence, organic-organic nanomatrix plays a crucial role in the outstanding properties of natural rubber (Kawahara *et al.*, 2014).

Berthelot *et al.* discussed the proteins involved in the latex coagulation in their work on homologous *H. Brasiliensis* (*Hevb1*) and SRPP (*Hevb3*) present auto assembling (Berthelot, Lecomte, Estevez, Coulary-Salin*, et al.*, 2014). Their function was unclear but they discovered that REF had amyloid properties, which could be of particular interest during coagulation. In this work, aggregation, auto-assembling, yeast and erythrocyte agglutination, and co-interactions by various biochemical (PAGE, spectroscopy, microscopy), biophysical (DLS, ellipsometry), and structural (TEM, ATR-FTIR, PM-IRRAS) approaches were used to investigate various aspects of protein interactions. They found that the interaction of proteins with membranes could help in the colloidal stability of latex, whereas the protein-protein interaction would contribute to the coagulation process by bringing rubber particles together or eventually disrupting the particle nano-membrane. This work could help in the identification of our extracted protein.

2.8 *Hevea* Protein Extraction and Fractionation

Proteins and amino acids in natural rubber have been thought to be physically held by the polymer, and the presence of residual amino groups is reported to be spotted in highly purified *hevea* rubber (Eng *et al.*, 1992). Separation by precipitation requires large solubility differences between the analyte and potential interferences. The theoretical feasibility of this type of separation can be determined by solubility calculations (Skoog, 2004). The accelerating growth of the market and growing interest in proteins result in the demand for faster, cost-efficient, and easily understood purification of proteins (Kröner *et al.*, 2013). The purification of native REF and SRPP from *hevea* requires a large number of detergents, which is a problem for a structural study, and the purification of gloves can be achieved using only ammonium salt precipitation combined with hydrophobic chromatography (Berthelot, Lecomte, Estevez, & Peruch, 2014). Proteins can be concentrated from the source by removing impurities and a series of centrifugation to further purify the proteins. At every centrifuge cycle, the serum-containing proteins are

taken from the middle layer to ensure the floating materials and sediments are removed. Each purified protein is assessed physically to obtain a clear picture of the properties of concentrated proteins. Other common methods used in purifying proteins involve tedious steps of salt and solvent preparation, including standard ammonium sulfate precipitation and acetone purification, which are claimed to be cleaner purification methods.

2.9 Salt-Induced Precipitation of Proteins

A common way to separate proteins is by adding a high concentration of salt. This process is termed salting out. The solubility of protein molecules shows a complex dependence on pH, temperature, the nature of the protein, and the concentration of the salt used. The salting-in effect is explained by the Debye-Huckel theory. The counter ions of the salt surround the protein, and the screening results in a decrease in the electrostatic attraction of protein molecules. Proteins are commonly least soluble at their isoelectric point. Hence, a combination of high salt concentration and pH control is used to achieve salting out. A study of the effect of salts and pH on protein flavor binding was conducted through fundamental investigations on molecular forces between proteins and flavors (Kun Wang & Susan D.Arntfield, 2015).

2.9.1 Fractionation via Open Column Chromatography

Freeze-dried protein was re-dissolved before being subjected to open column chromatography using Bio-gel P-60 as a stationary phase and Tris buffer as a mobile phase. P-60 gel was used to suit the size exclusion limit from 1.8 to 400 kDa, where *hevea* protein was predicted to have molecular weight with a maximum around 200 kDa and a minimum of 5 kDa. Mixed bed chromatography is a method suggested to be packed in a single column to reduce the dynamic protein concentration when used in large overloading (Boschetti & Righetti, 2011).

2.9.2 Characterization and Identification of *Hevea* Proteins

One of the most important tasks of organic chemistry is the determination of organic structure. When an interesting compound is isolated from a natural source, its structure must be completely determined before the synthesis begins. In many cases, a compound is identified by chemical means. Analytical techniques that work with tiny samples and do not destroy the samples are needed. Spectroscopic techniques often meet these requirements, unlike chemical tests, where most spectroscopic techniques are non-destructive (Wade, 2003).

A study on molecular cloning, expression profiles, and characterization of a novel translationally controlled tumor protein (TCTP) in *H. brasiliensis* by Li *et al.* (2013) discussed the isolation of a new TCTP gene designated as HbTCTP1. Real-time reverse-transcription PCR analyses showed that HbTCTP1 was expressed throughout different tissues and development stages of leaves. The recombinant HbTCTP1 fusion protein was shown to protect supercoiled plasmid DNA from damages induced by metal that catalyzed the generation of reactive oxygen species (Li *et al.*, 2013). Hence, this study is useful in the characterization study of our work.

The identification of laticifer-specific genes and their promoter regions from a natural rubber-producing plant of *H. brasiliensis* was conducted by Aoki *et al.* (2014), which discussed on enhancing natural rubber production through gene expression. Quantitative analysis revealed that the genes highly and predominantly expressed in laticifers were rubber elongation factor (REF), small rubber particle protein (SRPP), and putative protease inhibitor protein (Aoki *et al.*, 2014). A study on the identification and characterization of the 14-3-3 gene family in *H. brasiliensis* by Yang *et al.* (2014) investigated the 14-3-3 gene, which is a phospho-specific binding protein gene. The tissue-specific expression profile showed that this protein participated in numerous

cellular processes and suggested the target HbGF14c protein was related to SRPP, a major rubber particle protein involved in rubber biosynthesis (Yang *et al.*, 2014). The non-isoprene compound is normally studied by chemical analysis. Protein is generally quantified using a simple nitrogen titration Kjeldahl method by assuming that all nitrogen atoms are brought about by peptide bonds. Another method used to characterize protein content is the Lowry method, but it is limited to extractable protein only. Nevertheless, protein link to cis-1,4-polyisoprene is cannot be estimated (Rolere *et al.*, 2015). In the characterization and identification of latex allergen by two-dimensional electrophoresis and protein microsequencing research, it was reported that 2 DE map was very complex and exhibited about 200 distinct polypeptides, and the proteins eluted consisted primarily of two groups of acidic proteins located in the 8–14 kDa and 22 to 24 kDa regions (Posch *et al.*, 1997). The characterization of associated proteins and phospholipids in natural rubber latex by Sansatsadeeekul *et al.* suggested that the presence of phospholipid and protein layers enhanced the colloidal stability of NR latex, and the observation using scanning electron microscopy clearly displayed a gray ring near the particle surface that corresponded to the protein-lipid membrane layer (Sansatsadeekul *et al.*, 2011).

2.9.3 FTIR Study of *Hevea* Proteins

Fourier transform infrared spectroscopy is well suited to detect relative changes in protein secondary structure due to external factors by analyzing the amide I band of proteins between 1700 and 1600 cm^{-1}. This band is influenced by hydrogen bonds, which are mainly affected during conformational changes in the protein secondary structure (Ulrichs *et al.*, 2015). The measured vibrational bands of FTIR are assigned according to the literature. NR specific bands are attributed to some non-isoprene compounds. Five major bands are highlighted: amine (3283 cm^{-1}), ester (1748 to1738 cm^{-1}), carboxyl (1711 cm^{-1}), amide I (1630 cm^{-1}), and amide II (1541 cm^{-1}). Amide I and amide II are specific to peptide bonds and linked to NR protein content (Rolere *et al.*, 2015). Other peaks that

are not associated with isoprene are an intense band between 3200 and 3500 cm^{-1}, indicating the presence of hydroxyl groups. An amide (N-H) peak at 3280 cm^{-1} (more apparent in cream rubber), a carbonyl peak (C-O) peak at 1737 cm^{-1}, a small amide (N-H) peak at 1548 cm^{-1} (both are more apparent in cream rubber), and a series of peaks between 1130 and 1010 cm^{-1} indicate oxygenated compounds. The peak at around 1080 cm^{-1} is assigned to the (C-O) groups (Rippel *et al.*, 2003). The FTIR spectrum of natural rubber shows the characteristics bands of attached nitrogenous compounds at 3280 and 1540 cm^{-1}. These bands diminish if the fresh field latex is treated with enzymes, but the band at 3320 cm^{-1} remains after treatment, suggesting the presence of residual amino acids bonded to the rubber molecules (Eng *et al.*, 1992).

2.9.4 Zeta Potential and Particle Size of *Hevea* Proteins

Proteins adsorbed on NR particles are composed of many types of amino acids resulting in electrophoretic separation; thus, proteins from latex can be separated and identified based on their electrophoresis mobility (Sansatsadeekul *et al.*, 2011). It should be noted that the latex particles are amphoteric in nature and the global negative charge of the latex (pI 3.0 to 5.0) with a negative zeta potential value is due to the presence of negatively-charged proteins and lipids at the surface (Berthelot, Lecomte, Estevez, & Peruch, 2014). A wide range of particle size could be observed in all the latex samples, confirming the polydispersity nature of the NR latex particles, and a decrease in the zeta potential values indicated the loss of natural stabilizers (i.e., proteins and phospholipids) (Sakdapipanich *et al.*, 2015).

2.9.5 pH of Proteins

At physiological pH, most proteins are above their isoelectric points and have a net negative charge. A pH lower than the isoelectric point causes protonation of acidic groups and a net positive charge on the protein. In each case, the like-charges formed repel each

other, reducing the likelihood of protein aggregation (Agilent Application Note). Protein comprises amino acids, some of which have polar side chains or contain acidic or basic groups. At the isoelectric point, the protein is uncharged and charge repulsion of the similar functional group will be at a minimum, allowing aggregation to take place. Many proteins precipitate under these conditions (Agilent Application Note).

The zeta potential values of all latexes at pH 9 in the presence of 0.015 M NaCl clearly showed a significant drop after deproteinization and saponification because both processes removed proteins and phospholipids, both of which carried negative charges at alkaline pH (Sakdapipanich *et al.*, 2015).

2.9.6 SDS-PAGE of Extracted and Fractionated Proteins

SDS-PAGE is one of the tools used to identify the approximate molecular weight of proteins or fractions of the proteins isolated. As seen on a polyacrylamide gel electrophoresis stained by Coomassie blue, latex contains many proteins (Berthelot, Lecomte, Estevez, & Peruch, 2014). This method involves a very minute sample and a list of steps to ensure the reduced proteins can be located and align with the selected references. The stain used depends on the concentration of protein. Coomassie blue is the common stain but for lower concentrations, silver staining is recommended (U. M. Soedjanaatmadja *et al.*, 1995). The total protein content of fresh latex is approximately 1 % to 1.5 %, and it is believed that 20 % is to be adsorbed on the rubber particles, and the remaining dissolved in the serum phase. The adsorbed proteins are composed of many types of amino acids, including neutral amino acids, acidic amino acids, and basic amino acids, resulting in electrophoretic separation, so that the proteins can be separated and identified based on their electrophoresis mobility (Sansatsadeekul *et al.*, 2011).

2.9.7 Atomic Spectroscopy of Metal Proteins

The research by Gomez-Ariza *et al.* (2004) used mass spectrometry techniques for the characterization of metal bound to proteins (metallomics). In a biological system, a new generation of analytical tools substitutes traditional atomic detectors based on the use of photons for ion characterization: atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectroscopy (ICP-AES), mass spectrometry (MS), and inductively coupled plasma-mass spectrometry (ICP-MS). Many cases related to biological molecules involving proteins and multiprotein systems in which metals frequently participate have been described (Gómez-Ariza *et al.*, 2004). This work will help in measurement techniques and understanding metal-protein binding. A study conducted by Xiang *et al*. (2012) revealed 22 gene products from 53 spots using 2D-DIGE combined with MALDI-TOF/TOF. This study analyzed the specific types of proteins of large and small rubber particles to understand the biosynthesis activity, where seven were downregulated from small rubber particles, including SRPP and HMGSC, and about seven were upregulated from large rubber particles, including REF 19.6 kDa, β-1,3-glucanase. Nevertheless, the biosynthesis and coagulation activity still remains unexplained (Xiang *et al.*, 2012).

N.M Mubarak *et al.* (2011) produced carbon nanotubes (CNTs) using two-stage chemical vapor deposition and their potential use in protein purification was studied using skim latex serum as a starting material. The sample was first adjusted to five using acetic acid to promote the coagulation of small rubber particles. Centrifugation was performed at 10,000 rpm for the separation of cell debris, clear serum, and coagulated latex. The clear purified serum was submitted to analysis with 10 kDa MWCO against a buffer solution to remove low molecular weight solutes. The purification process was conducted using CNT as a column material (Mubarak *et al.*, 2011). This work uses the same material and method but a different column material with a different target. Our work is only

interested in using the isolated protein of different molecular weights and investigating the potential use of the extracted proteins.

A study on the design and fabrication of an artificial neural network-digital image-based colorimeter (DIC) for protein assay in NRL and medical gloves was conducted by Bang-Iam *et al.* (2013). They discussed the development of the DIC using a complementary metal oxide semiconductor (CMOS) camera as a detector, coupled with an artificial neural network (ANN). This method was based on the red, green, and blue (RGB) values of different color intensities from the reaction of protein complexes with the modified Lowry reagent (Bang-iam *et al.*, 2013). The proposed method was successfully applied for the determination of extractable proteins in NR latex and medical latex gloves and proved to be convenient and inexpensive. This method is not be considered in this work for now, but the concept might be useful as an option in determining the concentration of protein extracted and also in application study.

2.10 Antifungal Study of Proteins

A plant produces a number of cysteine-rich proteins of antifungal peptide classes, including *α-defensins*, lipid-transfer proteins, *thionins*, *hevein*, and knottin-type peptides (De Lucca *et al.*, 2005). An antifungal protein GAFP-1, also called *gasrodianin*, was purified from *Gastrodia elata* (*Orchidaceae*), a parasitic plant that can inhibit the hyphal growth of some pathogenic fungi (Xu *et al.*, 1998) An antifungal protein was isolated from the intercellular fluid of bitter gourd leaves during screening for potent antimicrobial proteins from plants (Zhang *et al.*, 2015). Plant immune responses involve a wide diversity of physiological reactions induced by a recognition of pathogens, such as hypersensitivity responses, cell wall modification, and synthesis of antimicrobial molecules, including antimicrobial peptides (de Souza Cândido *et al.*, 2014). Two proteins similar to peroxide and trypsin inhibitor and other defense-related proteins were

identified on *H. brasiliensis* rubber particles, suggesting the involvement of the particles in response to environmental stimuli and against pathogens (Dai *et al.*, 2013). Hevein, a lectin-like protein, has the ability to bind N-acetyl-D-glucosamine, the monomeric unit of polymer chitin present in the cell walls of fungi (Archer, 1960). Parjis *et al.* (1990) studied hevein, a cysteine-rich protein with a molecular weight of about 4.7 kDa, and established that hevein is an antifungal protein (Van Parijs *et al.*, 1991).

The isolation method used was based on hevein binding affinity for chitin from the bottom fraction. It was found that hevein showed strong antifungal activity against several fungi in vitro. As hevein is localized in the vacuole-derived lutoid bodies of NRL, it might be an effective system of self-protection or defense mechanism. An antimicrobial activity study of proteins purified from the latex of *H. brasiliensis* on oral microorganisms revealed that hevein showed strong antimicrobial activity, especially against *Candida spp*. (Kanokwiroon *et al.*, 2008). Latex and C- or B-serum contain components with antimicrobial or antifungal activities, in which hevein and β-1,3-glucanase are clearly involved in such processes; however, the antifungal or antimicrobial activities of REF and SRPP have never been clearly reported (Berthelot, Lecomte, Estevez, & Peruch, 2014).

2.11 Protein-Metal Binding Application Studies

Immobilized metal ion chromatography (IMAC) was used to purify proteins using the metal-binding concept (Ueda *et al.,* 2003). The binding of proteins (or peptides) to metal ions is based on the interaction between an electron-donating group present on a protein surface and a metal ion presenting one or more accessible coordination sites. IMAC uses a sorbent or a matrix, in which metal-chelating groups are covalently attached. When metal ion was added (loaded), the multidentate chelators and metal ion formed complexes, in which the metal ions were secured for subsequent interaction with the

compound to be resolved (Ueda *et al.*, 2003). A comprehensive platform to investigate protein-metal ion interactions by affinity capillary electrophoresis was studied by Alhazmi *et al.* (2015), and they discussed the behavior of several metal ions with different globular proteins (Alhazmi *et al.*, 2015). The methods used have been applied for the interaction study of metal and semi-metal groups, and the calculated results have been used to detect interactions and estimate further coordination of bound metal ions.

Abraham *et al.* (2009) conducted the electrochemical treatment of skim serum effluent from natural rubber latex centrifuging units using metal electrodes and compared the efficiency of different electrodes in removing pollutants. Various metal electrodes like aluminum, cast iron, steel, and mild steel were compared in removing the pollutants. The aluminum anode was the best material. The aluminum ions released from the sacrificial electrodes neutralized the electrostatic charges on the colloidal/suspended solids, enabling the formation of flocks (Abraham *et al.*, 2009). Guo *et al*. (2014) studied the binding between lead ions and the high-abundance serum proteins and discussed the interaction between three of the most abundant bovine serum proteins (i.e., serum albumin, transferrin, and IgG) with Pb^{2+} using electrochemistry (Guo *et al.*, 2014).

The binding constant (β) of Pb^{2+} to the individual proteins and a mixture of proteins was measured according to the non-ideal state theoretical equation, as well as the McGhee-Von Hippel equation for the ideal state. Protein-protein interactions and micro-environmental influences affected the binding between Pb^{2+} and serum protein. This work provides useful information on theoretical and experimental work regarding heavy metal binding interactions. A study was conducted for the influence of mixed layer of proteins and phospholipids on the unique film formation behavior of *hevea* natural rubber latex to obtain more insight into the role of the mixed layer of proteins and phospholipids on latex film formation, covering a comparative study of fresh NR, deproteinized NR, and

saponified NR using AFM. The film formation of NR latex was investigated by monitoring the change in surface morphology and roughness for the topmost layer of the latex film surface. Based on the finding, it can be concluded that the film formation can be divided into three stages: 1) Water evaporation stage: As water is removed, the particles are crowded into a decreasing volume. 2) Particle deformation stage: Irregularly arranged neighboring particles are forced into contact and deformed concurrently to fill up the void. However, the mixed layer of proteins and phospholipids surrounding the latex particles resisted the deformation of the particles. 3. Coalescence stage: When the capillary forces of deformation exceeded the resistance of the mixed layer of proteins and phospholipids, it ruptured and allowed direct mixing of the rubber molecules. Therefore, it can be confirmed that a mixed layer of proteins and phospholipids caused the stabilization of latex particles pertaining to the charges they carry (Sakdapipanich *et al.*, 2015).

A study on metal-binding proteins and peptides in the bioremediation and phytoremediation of heavy metals by Mejare and Bulow (2001) focused on the role of microorganisms in enhancing the heavy metal accumulation, and the scope was restricted for different peptides and proteins towards cadmium (Cd). Microorganisms could be used to clean up metal contamination by removing metals from contaminated water and waste stream, sequestering metals from soil and sediments, or solubilizing metals to facilitate their extraction; however, bacteria and microorganisms have developed resistance mechanisms to toxic metals, which make them innocuous (Mejáre & Bülow, 2001).. The above study concerns only Cd, whereas our work covers other metals, such as Pb, Cu, Al, and Zn, but the mechanism suggested is a good reference to better understand our work.

2.12 Metal Decontamination Studies

Metal removal or decontamination from aqueous solutions is a process of treating the contaminants containing metal with the use of physical, chemical, and biological techniques and technologies. Conventional methods for removing toxic metal ions from aqueous solutions have been recommended, such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technology, floatation, adsorption on activated carbon, evaporation, photocatalysis, and magnetic nanoparticles. These methods have been investigated for their potential to remove metal ions; however, biosorption of heavy metal ions has become a popular environmentally-driven research topic (Gautam *et al.*, 2014). Bioremediation methods include bioaccumulation, biosorption, and phytoremediation. These methods are environmentally-benign, free of secondary pollution, and have low cost; therefore, they have gained significant attention to remediate industrial wastewater (Vijayaraghavan & Balasubramanian, 2015). One of the methods used to treat metal is biosorption, which is a relatively new process for the removal of contaminants.

Biosorbent from agricultural waste proved to be effective for the removal of hexavalent chromium from aqueous solutions. Lignocellulose is a biosorbent containing biomolecules, similar to polysaccharides and proteins with specific functional groups that are mainly responsible for metal ion biosorption. The mechanisms involved in metal biosorption include chemisorption, ion exchange, complexation, adsorption-complexation on surface and pores, microprecipitation, heavy metal hydroxide condensation onto the surface, and surface adsorption (Rangabhashiyam & Selvaraju, 2015). Vijayaraghavan and Balasubramaniam (2015) studied the suitability of biosorption for the decontamination of metal-bearing wastewater. The study critically reviewed the state-of-the-art biosorption processes and future directions for a number of biomass with good biosorption capacity, but large-scale commercial application has not been employed

yet. The key factors affecting the growth of biosorbents for the decontamination of wastewater are not determined due to a lack of important data of biosorption and incomplete understanding of the physical characteristics of biomass (Vijayaraghavan & Balasubramanian, 2015). The review focused on identifying the practical limitations of biosorption and providing future research directions. This work helps in finding the gap in the decontamination of metals in the wastewater.

Jamil Anwar *et al.* (2010) studied the removal of Pb (II) and Cd (II) from water by adsorption on banana peels to remove metal ions by adsorption. Langmuir, Freundlich, and Temkin isotherms were employed to describe adsorption equilibrium. The study concluded that banana peels have a good potential as an adsorbent to remove toxic metals (Anwar *et al.*, 2010). This work also used AAS for metal estimation, thus helping in understanding our work in certain aspects.A study on biomass-derived biosorbents for metal ion sequestration was done by Gautam *et al.* (2014) involving adsorbent modification and activation methods. The review provides a comprehensive appraisal of the equilibrium modeling of a number of biosorption processes, as well as structural, chemical, and morphological modifications and activation of biosorbents (Gautam *et al.*, 2014). This work is used as a reference for the metal removal mechanism.

2.13 TEM Study of NRL Particles and Proteins

TEM was used to compare SRPP, a membrane protein suggested as a key protein closely related to rubber biosynthesis in different plants. The microscopic distribution of SRPP on the rubber particles during the washing process was investigated by TEM-immunogold labeling (Wang *et al.*, 2016). The structural study of REF and SRPP was investigated using TEM to demonstrate that both proteins auto-assemble into different aggregative states and both proteins are able to interact together (Berthelot, Lecomte, Estevez, Coulary-Salin*, et al.*, 2014).

CHAPTER 3: METHODOLOGY

Factory waste of latex concentrate processing plant contains proteins. Those proteins flow to the waste stream in the washing of concentrator. Waste collected from MARDEC Industrial Latex Tapah, Perak Malaysia factory analyzed for pH, particle size and zeta potential. Wastewater chilled at 4 to 11° C and filter prior to centrifugation for 15 minutes to remove rubber and other impurities. From this, only 25 ml of middle layer taken out and proceed to extraction. Materials used and experimental described in detailed further on.

3.1 Chemical

Ammonium sulphate granule of Qrec brand purchased from TRP Technologies Malaysia. 3.5 kDa molecular weight cut off (MWCO) snakeskin dialyzing tube (regenerated cellulose) was purchased from Research Instrument. Standard metal solution Cd $(NO_3)_2$ in HNO_3, Cu $(NO_3)_2$ in HNO_3, Pb $(NO_3)_2$ in HNO_3, Zn $(NO_3)_2$ in HNO_3 and Al $(NO_3)_3$ in HNO_3 were purchased from Merck Millipore Malaysia. Disodium phosphate *Systerm Chemar* brand and Citric acid from MERCK used for pH buffer purchased from a local supplier. Bradford reagent, Biorad P-60 gel, (Polyacrylamide gel) and Invitrogen Silver staining kit *novex* by life technologies also purchased from local supplier. All reagents were of analytical grade.

3.2 Latex concentrate manufacturing plant layout

The field latex collected from various plantations was blended in a tank and stored in a storage tank. In the storage tank, ammonia (preservative) was added to ensure that the field latex would not destabilize when it was subjected to high rotational speed during centrifugation.

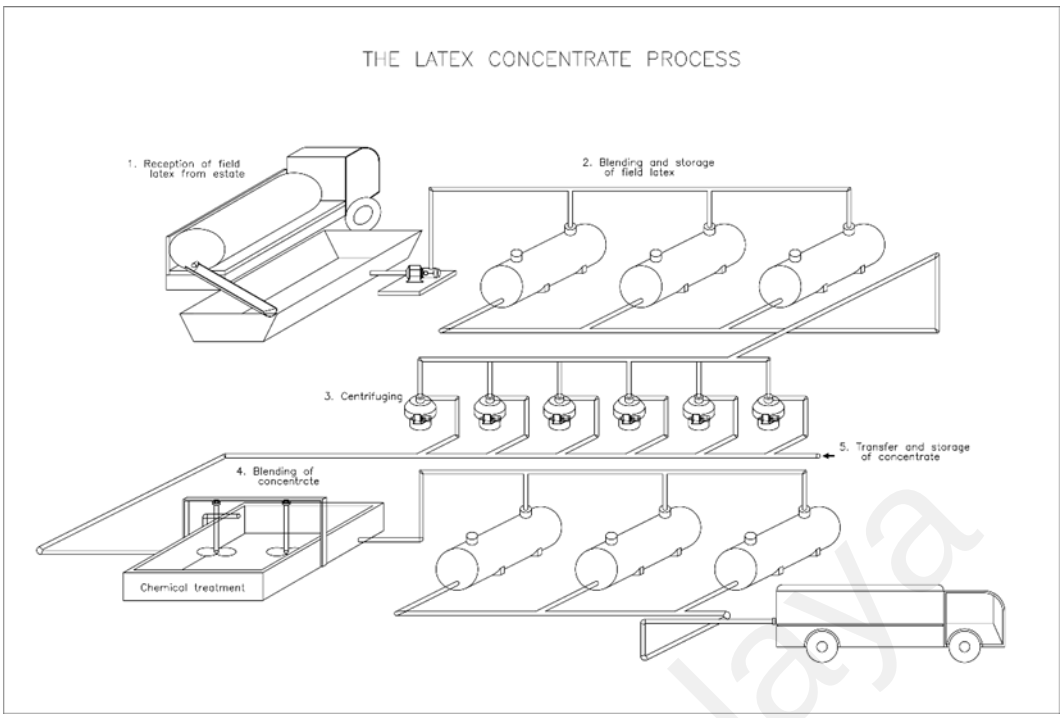


Figure 3.1: Latex concentrate manufacturing plant layout

From the blending tank, latex will flow to the centrifuge by gravity (Figure 3.1). Hence, a latex concentrate plant is placed in a hilly area where sufficient gradient will cause the fresh latex to flow to the centrifuge and later to the blending trough for chemical addition before it moves to the storage tank for trading. The final storage tank will cater to the request from buyers on the grades or if any chemical addition is needed.

3.2.1 Latex concentrate process and starting material collection

The fresh field latex from the collection station was transferred to the reception tank for blending purposes. The blended field latex was tested for its total solid content (TSC), dry rubber content (DRC) alkalinity, and volatile fatty acid (VFA) number to ensure that only good sample proceeds to the manufacturing line. Centrifugation was carried out where the cream and skim fractions were separated into their specific pipelines. Centrifugation was stopped after one batch to clean the centrifuge. Cream latex was collected in the blending tank for chemical addition as required by the customer before being transferred to the storage tank. Meanwhile, skim latex was transferred to the skim rubber production line. Skim rubber normally contains a high ammonia level; hence, it is

difficult to be coagulated. To obtain a low level of acid, the ammonia level must be reduced using strong acids, such as sulfuric acid. The sample for protein purification was withdrawn from the skim serum wastewater of skim rubber production. In general, skim latex is residual latex available as a by-product of the concentration process (Kongkaew *et al.*, 2012). If the sample from bowl washing of the centrifugation unit is used, greater attention is needed to ensure that the sample is free from contamination by microorganisms. Sampling is normally done on the second day immediately after the coagulation of skim latex. The longer the serum is in the coagulation trough, the higher the possibility of contamination by microorganisms. It is strongly recommended that when collecting NRL waste, the sample is kept in an icebox throughout the journey to the laboratory. The cool temperature will inhibit the growth of microorganisms and ensure that the waste sample can be used for at least a month to complete the extraction process. This is due to the difficulty in maintaining the stability of wastewater for a long period. The protein content in the wastewater is a good food supply for the proliferation of microorganisms and the loss of protein content in this current work is because the concentration of proteins in the wastewater will keep decreasing. It is common to ensure the low temperature of the sample when dealing with microorganisms, especially if the sample is a fresh latex, but in this case, it is wastewater without proper treatment. Therefore, the collection must be done a day after the coagulation process of skim rubber. Otherwise, the wastewater will flow to the treatment pond and will be mixed with other waste streams.

3.2.2 Latex concentrate manufacturing plant wastewater (effluent)

Wastewater from a latex concentrate plant can be from several places until the final collection at the treatment ponds. Each effluent line (Figure 3.2) was screened for protein content and a decision was made to focus on effluent B due to the protein level and stability of the sample. Effluent from bowl washing only consisted of a diluted protein.

The stability against microorganisms is subjected to management practice because effluent A will flow directly to the drain and without good housekeeping, the drain is the most suitable place for every species of microorganism proliferation.

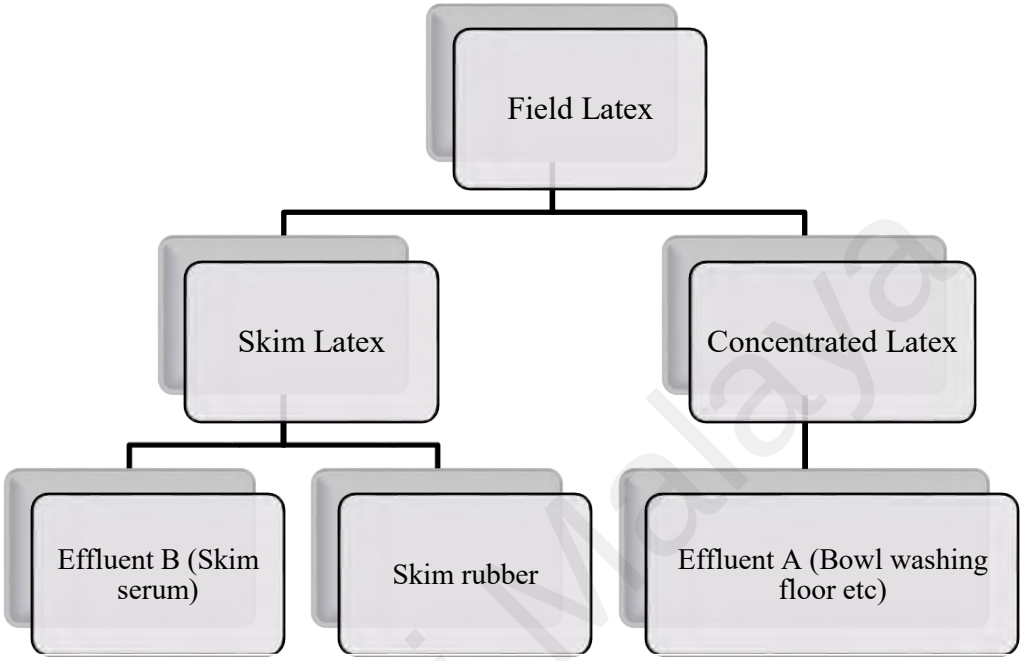


Figure 3.2: Wastewater (effluent) from latex concentrates manufacturing plant

On the other hand, effluent B was collected at the coagulation trough where hygiene is one of the main concerns in factory housekeeping practice. For the suitability of the waste chosen as the starting material, the waste from SMR 10 and SMR 20 processing plants and the DPNR manufacturing plant was collected and tested for protein content and the result was negative. SMR 10 and 20 processing plants have very contaminated wastewater because the starting material is a lower grade rubber. On the other hand, DPNR that is expected to contain more proteins has an unsuitable value of protein level that may be due to the chemical reactions that occurred during deproteinization, which destroyed and denatured proteins. Moreover, the DPNR process uses enzymes to remove proteins attached to rubber particles, where the presence of enzyme must be at a significant amount in the wastewater of the plant to affect protein concentration. Besides wastewater, other protein sources, such as seeds and leaves of NR tree also contain the

proteins of interest. However, these sources are the last option because they are not waste. Similarly, the wastewater from a leaching tank in the manufacturing of rubber gloves is expected to have proteins due to the step taken in the manufacturing line to remove proteins, but the investigation proved differently. As for now, the best starting material is the skim serum of a latex concentrate manufacturing plant that must be taken a day after the coagulation of skim latex. The serum is still in the coagulation trough and it must be taken before the lump is washed so that it is pure acidified serum. The good serum pH for this current work is around pH 4. pH 3 to 4 is good but theoretically, it may cause disturbance in protein activity.

3.3 Protein extraction

Purification is a first step taken when a cell containing protein being disrupted to separate protein from its original positions in the plant for example; the organelle or subcellular which will be removed later. Various purification methods designed for specific protein types and degree of purity intended. The common technique used for protein purification is salt precipitation and acetone purification. Both techniques have pros and cons such as cleaner protein but the supernatant containing high salt content and solvents. This is in contrast to the multiple centrifuge method, which introduced as an attempt to limit the use of ammonium salt and solvent such as acetone in protein purifications. Equally important this technique also reduced certain steps such as salt preparation and save on time and material cost too. Other than that current work involved the use of waste as a starting material, the use of chemicals and high cost material will not support the side goals of the works which is it will be economically viable. The use of ammonium salt for example produce more waste of supernatant and even the stripping may overcome the situation extra cost incurred.

3.3.1 Ammonium sulphate extraction (SP)

About 40 mL of natural rubber latex (NRL) waste serum was centrifuged at 14,000 rpm for 15 min. The centrifuged waste was left for 30 min to allow rubber particles to rise to the surface because rubber is less dense than its surrounding fluid. 25 mL of the middle layer centrifuged waste was poured into a conical flask. 100 mL of liquid ammonium sulfate was added dropwise to the waste and stirred using a magnetic stirrer. 40 mL of the mixture was withdrawn from the conical flask and recentrifuged at the same rpm and duration as the first centrifuge run. The supernatant was removed and the centrifuge tube was placed upside down to dry the precipitates (i.e., extracted proteins). The precipitates were redissolved using 5 mL of the pH buffer (Table 3.1) accordingly or only deionized water (DI) for each centrifuge tube. A 3.5 kDa MWCO SnakeSkin dialyzing tube was used to remove ammonium sulfate for at least 24 h in distilled water or DI, preferably DI. Ammonium salt precipitation of protein involved drop-by-drop addition of ammonium sulfate to the centrifuged waste. The middle layer of the centrifuged serum was removed from the tube. Ammonium sulfate was dissolved to 4.1 M. The saturated ammonium sulfate was added slowly and stirred using a magnetic stirrer. The mixture was then recentrifuged at 14,000 rpm for 15 min to precipitate the proteins. After that, the supernatant was removed, leaving the precipitates behind. The precipitates or protein pallets were redissolved in DI and kept in a chiller for 4 days at 4–11 °C. The dialyzed precipitate was collected in a 250 mL Schott bottle and frozen at -80 °C prior to freeze-drying. The freeze-dried protein was weighed before the isolation of individual proteins.

3.3.2 Multiple Centrifugations (MC)

Salt-free purification only used filter paper to remove large natural rubber (NR) lump from wastewater. The material was centrifuged at 14,000 rpm for 15 min to separate small rubber particles and other non-protein constituents. Small rubber particles normally

pooled at the top while heavier particulates and non-rubber constituents settled at the bottom. Only the middle layer was used for the second-round centrifuge. After each centrifugation, a disposable pipette was used to collect about 10 mL of the original volume to prepare for the next centrifugation step. After four cycles of centrifugation, the middle layer was dialyzed using a 3.5 kDa MWCO SnakeSkin dialyzing tube with similar procedures for standard purification for at least 4 days in DI and kept in a refrigerator to promote conditions to slow down microorganism growth. The dialyzed proteins were then freeze-dried for at least a week. After that, the freeze-dried proteins were weighed and recorded. Table 3.1 shows the amount of phosphate and acid conjugate used to prepare the pH buffer in order to create a condition that might favor specific *hevea* proteins.

Table 3.1: Preparation of the buffer solution in pH range 3-8

Volume of 0.2 M Disodium phosphate ($Na_2HPO_4.2H_2O$) (ml)	Volume of 0.1 M Citric Acid ($H_3C_6H_5O_7.H_2O$) (ml)	pH
20.55	79.45	3±0.5
38.55	61.45	4±0.5
51.50	48.50	5±0.5
63.15	36.85	6±0.5
82.35	17.65	7±0.5
97.25	2.75	8±0.5

The buffer was used in the beginning where the wastewater and extracted proteins were exposed to specific pH before the fractionation process. The pH buffer was used in the first part of the current work to have an overview of the behavior of the targeted *hev* proteins at a specific condition.

3.3.3 Preparing Bio-Gel P-60 gel for fractionation

Dry Bio-Gel P-60 gradually added to buffer in a beaker. Buffer use was twice as the expected packed bed volume. P 60 gel requires 12 hours at 20°C to hydrate. For column packing, the funnel affixed to the top of the column, column exit closed, and enough

buffers added to fill about 20% of the column. Even slurry poured into the column in a single, smooth movement. Avoid splashing the slurry to ensure even packing and to avoid air bubbles trapped. The packed column is only suitable for pH 3–8; otherwise, it may swell and affect the protein fractionation process. Table 3.2 shows the molarity and pH of Tris buffer used as a mobile phase. It is recommended to check the pH to avoid column swelling each time the Tris buffer is prepared.

Table 3.2: Tris buffer preparation

Molarity	pH by dilution
0.01	9.88
0.005	9.52
0.001	9.49
0.0005	8.69

The packed column only suitable for pH from 3 to pH 8 otherwise it may swell and affect the protein fractionation process. Table 3.2 showed the molarity and pH of tris buffer used as a mobile phase. Each time when preparing tris buffer it is recommended to check for pH to avoid column swelling.

3.3.4 Freeze drying-technique of *hevea* proteins

The sample was frozen at -80 °C before being transferred to a freeze-drying unit. To prepare a 60 mL sample, a week or more is required for drying. In contrast to preparing a 25 mL sample, approximately 24 h is required, depending on sample purity.

3.4 Characterization of *Hevea* Protein Fractions

Each isolate was characterized using FTIR and SDS PAGE for its pH, particle size, and ζ potential. The pH of the isolate was measured directly after collecting a 25 mL sample. The FTIR results were obtained the next day or after a few days to find out whether the fractions contain protein or not. Particle size and ζ potential were also

measured after isolation to investigate the relationship of particle size, ζ potential, and molecular weight of protein isolates.

3.4.1 Yield of precipitated *hevea* proteins

The yield of *hevea* proteins was measured after precipitation using the weight of the centrifuge tube (*w1*) and the weight after removing the supernatant (*w2*). At this stage, NR protein was still mixed with ammonium sulfate. The pure yield was measured after freeze-drying by weighing the initial dialyzed sample (*w1*), followed by the weight of the freeze-dried protein (*w2*), where the calculated percentage gives the final yield. The wet yield was used as the initial estimation of *hevea* proteins when a new batch of wastewater was collected, especially for wastewater with different seasons and freshness.

3.4.2 Fourier Transform Infrared (FTIR) Perkin Elmer Spectrum 400 ATR

An FTIR instrument contains no dispersing element and all wavelengths are detected and measured simultaneously. IR radiation is not sufficiently energetic to cause electronic transition, but it can induce transition in the vibrational and rotational states associated with the ground electronic state of the molecule (Skoog *et al*., 2004). The frequency of the stretching vibration depends on the masses of the atoms and the stiffness of the bond. Heavier atoms vibrate more slowly than lighter ones. In a group of bonds with similar bond energies, the frequency decreases with increasing atomic weight. Stronger bonds are generally stiffer, requiring more force to stretch or compress them. Thus, stronger bonds usually vibrate faster than weaker bonds. Triple bonds are stronger than double bonds; thus, triple bonds vibrate at a higher frequency than double bonds. In a group of bonds having atoms of similar masses, the frequency increases with bond energy (Wade, 2003).Molecules undergo three different types of quantized transitions when excited by ultraviolet, visible, and infrared radiation. For ultraviolet and visible radiation, excitation involves the promotion of an electron residing in a low energy molecular or atomic orbital

to a higher energy orbital. In addition to electronic transition, molecules also exhibit two types of radiation-induced transition: vibrational transition and rotational transition (Skoog, 2004).

The infrared (from the Latin, "*infra*" meaning "below") region of the spectrum corresponds to frequencies from just below the visible frequencies to just above the highest microwave and radar frequencies, with the wavelength range of 8×10^{-5} to 1×10^{-2} cm. A common infrared spectrometer operates in the middle of this region, at a wavelength between 2.5×10^{-4} and 25×10^{-4} cm, corresponding to energies of about 1.1 to 11.0 kcal/mol (4.6 to 46.0 kJ/mol). The position of an infrared band is specified by its wavelength (λ) and measured in microns (μm). A micron (or micrometer) corresponds to one-millionth (10^{-6}) of a meter or 10^{-4} cm. A more common unit, however, is the wavenumber ($\tilde{\nu}$), which corresponds to the number of cycles (wavelength) of the wave in centimeters. The wavenumber is the reciprocal of the wavelength (in cm). As 1 cm is equal to 10,000 μm, the wavenumber is calculated by dividing 10,000 by the wavelength in microns. Wavenumber has become the most common method for specifying IR absorption. The wavenumber is proportional to the frequency (v) of the wave, and it is also proportional to the energy of a photon of this frequency ($E = hv$) (Wade, 2003).

Rolere *et al.* (2015) investigated the NR composition with FTIR spectroscopy. It is a rapid and non-destructive method to determine both protein and lipid contents simultaneously. They stated that specific vibrational bands attributed to non-isoprene compounds naturally present in NR composition (Rolere *et al.*, 2015). Specific functional groups in lipids (ester and carboxyl groups) and proteins (amides) were quantified using the calibration curves developed. This work is related to our work on the identification of a specific band for proteins as one of the control elements in predicting the existence of protein in each sample produced.

A drop of the sample was placed on the attenuated total reflectance (ATR) diamond (Perkin-Elmer) and the spectrum was obtained from the frequency of 400 to 4000 cm^{-1}. After each treatment to the sample, FTIR was used to analyze the area under the peak from 990 to 1200 cm^{-1} and from 1500 to 1800 cm^{-1}. The precipitate sample and each isolate were also analyzed using FTIR to measure the area under the peak, especially to screen the isolates containing protein. The ATR-FTIR technique was used to detect the presence of protein and variation in the concentration of specific isolates. The area under the graph from 990 to 1200 cm^{-1} was calculated to estimate the concentration of protein for comparison with the ultraviolet (UV) absorbance data.

3.4.3 pH measurement of NRL waste, extracted and fractionated proteins

pH is used as a measure to control raw NRL serum properties, particularly in determining the effect when a series of treatments is exposed to the NRL serum and deciding whether the sample is suitable for further processing. Buffer from pH 3 to 8 was prepared using disodium phosphate and citric acid and was then used as a medium prior to purification. The pH of every sample and fraction was measured to observe any specific outline during treatment and fractionation. pH is the simplest method to have a knowledge of a sample, yet it gives reliable information about other physical properties of certain materials.

3.5 Fractionation of extracted proteins

Protein fractionation was conducted after ammonium sulfate purification. The purified protein was re-dissolved before introducing into the column. The buffer solution was added to the column as the fractionation process was completed. The fractions were withdrawn by time or by the volume collected. Next, each fraction was characterized and analyzed for the concentration of protein and other physical characteristics, such as pH, particle size, and ζ potential. Proteins and amino acids in NR have been thought to be

physically held by polymers and the presence of residual amino groups was observed in highly purified *hevea* rubber (Eng *et al.*, 1992). Separation by precipitation requires large solubility differences between the analyte and potential interferences. The accelerating growth of the market and growing interest in proteins result in demand for a faster, cost-efficient, and easily understood process for the purification of proteins (Kröner *et al.*, 2013). The purification of native REF and SRPP from *hevea* requires a large number of detergents, which is a problem for structural study. The purification of gloves was achieved using ammonium salt precipitation combined with hydrophobic chromatography (Berthelot, Lecomte, Estevez, & Peruch, 2014).

3.5.1 Fractionation via open column chromatography

Freeze-dried protein was redissolved before it was transferred to open column chromatography using Bio-Gel P-60 as a stationary phase and Tris buffer as a mobile phase. P-60 gel was used to suit the size exclusion limit from 1.8 to 400 kDa. *Hevea* protein was predicted to have a molecular weight with a maximum of 200 kDa and a minimum of 5 kDa. Mixed-bed chromatography is a method suggested in a single column to reduce the dynamic protein concentration when used in large overloading conditions (Boschetti & Righetti, 2011). Size exclusion chromatography (SEC) is used as a powerful technique for the qualitative and quantitative evaluation of aggregates (Fekete *et al.*, 2014).

3.5.2 Column chromatography for *hevea* protein fractionation

The word "chromatography" comes from the Greek words "*chroma*", which means color, and "*graphein*", which means to write. The technique was first used in the 20th century to separate plant pigments with easily visible colors. Chromatography is based on the fact that different compounds can distribute themselves to varying extents between different phases, or separable portions of matter (Campbell, 2009). Many

chromatography techniques used for the research of proteins are forms of column chromatography, in which the material that makes up the stationary phase is packed in a column. The sample is a small volume of concentrated solutions applied to the top of the column, and the mobile phase called the eluent passed through the column. Three different types of chromatography use different modes in isolating proteins. They are SEC, affinity chromatography, and ion-exchange chromatography. This research used SEC, which is also known as gel filtration chromatography. This size exclusion technique separates protein based on size. The column unit consists of the stationary phase of cross-linked gel particles. The gel particles are usually in bead form and consist of one or two kinds of polymers. The first is a carbohydrate polymer, such as dextran or agarose. These two polymers are often referred to by the trade name Sephadex and Sepharose, respectively. The second is based on polyacrylamide, which is sold under the trade name Bio-Gel. It is produced by the polymerization of acrylamide and methylene bisacrylamide, stable in aqueous buffers at pH 1 to 10, and its molecular size exclusion limit is 1.8 to 400 kDa (Campbell, 2009).

The cross-linked structure of these polymers produces pores in the material. The extent of cross-linking is controlled to select the desired pore size. When a sample is introduced into the column, smaller molecules, which can enter pores, tend to delay their progress down the column, unlike the larger molecules. As a result, the larger molecules elute first, followed by the smaller ones, after escaping from the pores (Campbell, 2009). In this study, the extracted proteins were fractionated using open column chromatography. BioRad polyacrylamide P-60 gel was used as the stationary phase or column packing, while Tris buffer of pH 8 was used as the mobile phase. The isolate was taken every 10, 15, or 25 mL. The maximum volume of the column was 230 mL, with a diameter of 2.5 cm and a length of 50 cm. P-60 gel was used due to the size of the targeted proteins to be fractionated. P-60 gel is recommended by the manufacturer for the protein ranges between

5 and 50 kDa. The amount of gel dissolved and buffer added can be chosen as required. For example, for 4 g gel, about 44 mL of buffer was added, while for 20 g gel, about 220 mL of buffer was added.

3.5.3 Ultraviolet and Visible Spectroscopy of Protein Fractions

Spectroscopy uses the interactions of radiation with matter to obtain information about a sample. The sample is usually stimulated in some way by applying energy in the form of heat, electrical energy, light, particles, or a chemical reaction. Prior to applying the stimulus, the analyte is predominantly in its lowest energy, the ground state. The stimulus then causes some of the analyte to undergo a transition to a higher energy or excited state (Skoog, 2004). Beer's law describes the absorption behavior of only dilute solutions, and in this case, is the limiting law. At concentrations exceeding 0.01 M, the average distances between ions or molecules of the absorbing species diminished to the point where each particle affects the charge distribution, and thus the extent of absorption of its neighbors. The chemical deviation occurs when the absorbing species undergoes association, dissociation, or reaction with the solvent to give products that absorb differently from the analyte. The extent of such departures is predicted from the molar absorptivity of the absorbing species and the equilibrium constant for the equilibrium involved. A UV spectrophotometer was used to detect the presence of proteins and the concentration of specific fractions.

3.5.4 Determination of Particle Size and Zeta Potential

A Zetasizer (Malvern Nano Series Zetasizer, Malvern UK) was used to determine the particle size and ζ potential of the filtered NRL waste, purified proteins, and fractions. Each purified protein and fraction was introduced to the instrument to determine the stability of purified proteins, while the size will indicate if a specific range of size is successfully fractionated at a specific time. The principle behind the technique is light

scattering that is captured by particles, which provides the information of aggregations that correlate with the stability of sample solutions. Chilled raw serum from a factory was filtered before the ζ potential and particle size were determined. If the results satisfy the early set required, then further steps are taken. In the extraction of proteins, ζ potential and particle size are also used as quality control checks for proteins. This is because the stability of proteins is estimated from ζ potential, while particle size determines whether rubber particles are still present in the extract or not. Furthermore, the ζ potential of fractionated proteins also serves as the rough estimation for the presence, concentration, and stability of proteins in specific fractionated samples.

3.5.5 Transmission Electron Microscope (TEM) Microscopy

An energy-filtered transmission electron microscope (EFTEM) model LIBRA 120 equipped with an Olympus SIS-iTEM (ver. 5) was employed to investigate the microstructure of NRL waste, purified proteins, protein fractions, and metal-bound proteins. The TEM image gives information on the size, purity, and contamination at a micro-molecular level. The extracted proteins were viewed to determine if contaminants or rubber particles are still present together with protein fractions and to measure the particle size or cluster of particles. Finally, metal-bound proteins were observed to confirm the presence of metals in the metal-bound protein sample. The morphological study of raw serum was conducted to locate the presence of proteins besides rubber particles. The extracted and dialyzed proteins were also subjected to the surface morphology test to understand the overall structure of extracted proteins. Meanwhile, the metal-binding protein sample was assessed to understand the reaction of metals with protein particles.

3.5.6 Dispersion Analysis

For a photometric dispersion analyzer (PDA 2000), transparent flexible tubing fits in a Perspex block, which houses two precisely aligned fiber-optic probes. The optical fibers carry the incident and transmitted light. The light source is a high-intensity light-emitting diode and the transmitted light is continuously monitored by a sensitive photodiode. The output from the photodiode is converted to a voltage, which consists of a large DC component, together with a small fluctuating AC component. The DC component is simply a measure of the average transmitted light intensity and is dependent on the turbidity of the suspension.

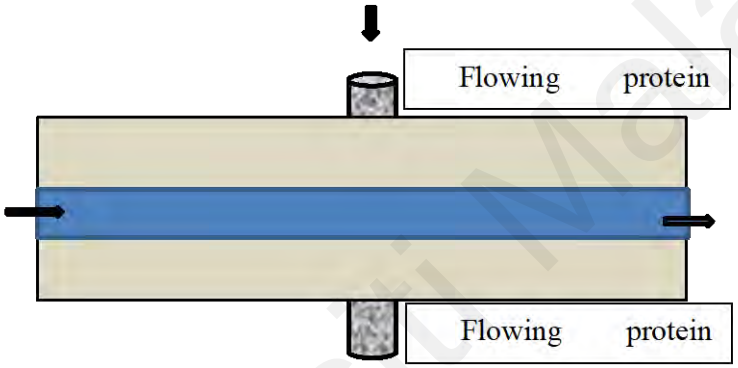


Figure 3.3: Photometric dispersion analyzer instrument

3.6 Characterization of *Hevea* Protein Fractions

Each individual fractions were characterized for pH, FTIR, particle size and zeta potential and SDS PAGE. A pH of fractions measured directly after collecting the 25ml sample. FTIR obtained the next day or after a few days to know whether the fractions contain protein. Particle size and zeta potential measured also after extraction to investigate the relationship of particle size, zeta potential and molecular weight of protein fractions. Meanwhile, SDS PAGE provide the information on specific *hev*s protein of interest especially after extraction.

3.6.1 Gel casting for electrophoresis of *hevea* protein

The gel used for SDS PAGE can be prepared using different ingredients based on the percentage required. The gel is also sold by various manufacturers of SDS PAGE in cassettes at a single percentage or a gradient percentage, depending on the application and degree of softness. The prepared gel will be slotted into the SDS PAGE. The cast gel can be prepared or bought, depending on the requirement. The casting procedure started by mixing acryl amide with buffer and other ingredients, but Ammonium Persulfate (APS) and Tetramethylethylenediamine(TEMED) were added last for only half a minute. The mixture was poured into a glass plate set up earlier. DI or 50:50 butanol:water was added to remove bubbles. APS and TEMED should be prepared fresh and the cast gel must be stored in liquid. The gel is normally stored in the running buffer to avoid contamination and drying prior to use. 10 % cast gel or 4 % to 12 % polyacrylamide gel was used with 10 or 12 lanes. About 10 to 12 samples can be loaded for each run.

It is recommended that for each gel, one lane is reserved for the protein marker. Several selections of protein markers are available and used according to the nature of the protein sample and the treatment or characteristics preferred. Pre-stained and benchmark markers are among common markers used in protein identification. Once completed, the gel cassette was removed from the electrophoresis unit and washed, followed by carefully removing the gel from the cassette. Proper steps should be followed to ensure that the appearance of the band is captured at the right time. The gel was removed from the cassette using a special knife to avoid gel tearing. Each end of the gel cassette was loosened first before the front cover was pulled out. It is advisable to slide the gel into DI while pulling so that the gel will not be stuck on the cassette.

3.6.2 SDS PAGE unit for proteins Mw determination

The SDS PAGE unit is used to identify proteins using protein markers based on the molecular weight of proteins. This unit consists of a power supply and an electrophoresis unit where a gel cassette is put in the unit and an electric current will separate the proteins electrophoretically. To begin electrophoresis, the mixture of the sample (3 µL), buffer (3.5 µL), and reducer (1 µL) was prepared and made up to 10 µL with DI. The mixture was then heated at 70 °C for 10 min in a water bath and upon completion, 5 µL of the mixture was pipetted into the gel lane. The total formulation can be half of the total mixture to 10 µL if no duplication is needed. If a commercial cast gel is used, the comb that covers the lane is removed and it is filled with the sample.

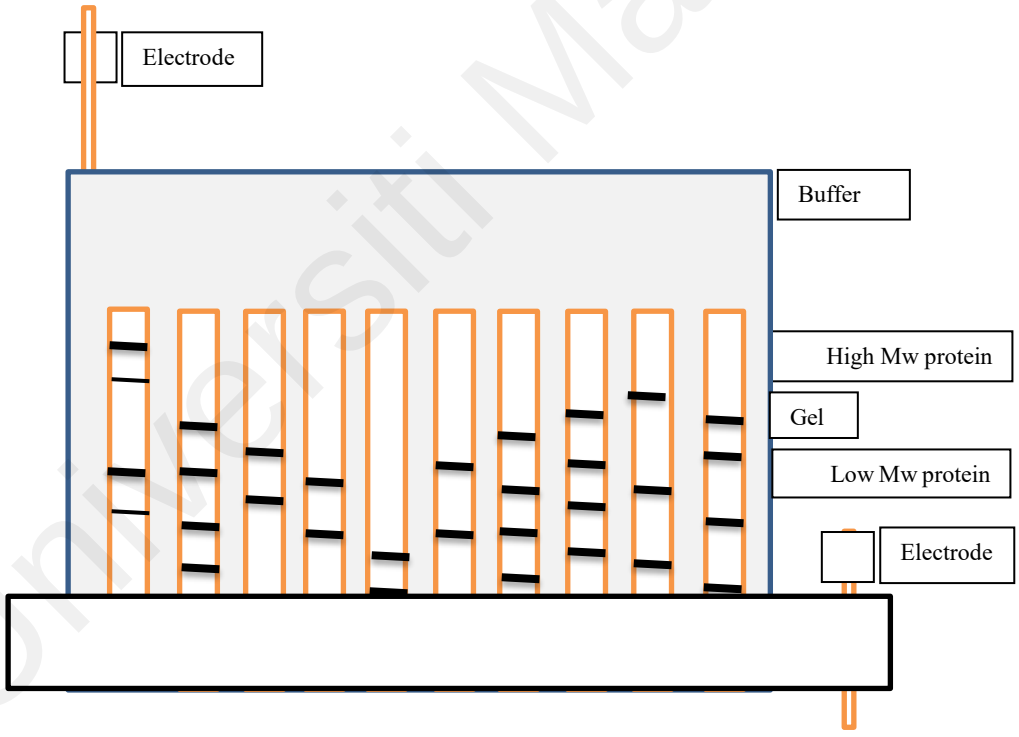


Figure 3.4: SDS PAGE unit for extracted and fractionated protein Mw determination

An antioxidant was added to the unit prior to electrophoresis to ensure that the heat build-up during the process would not affect the band formation or denature the protein fractions. The power supply was maintained at 102 mA, 200 V, and 20 W for 35 min. Electrophoresis time depends on the separation of proteins of interest. If a longer time is

needed to separate the proteins, the process can be prolonged for up to a few hours. The process is considered complete when the entire sample in the lane had migrated to the whole gel. Other indication that can dictate complete migration is the blue lines have reached the bottom of gel. Staining procedure is the next step in SDS PAGE and is better to conduct as soon as electrophoresis complete or else fixation might be necessary in order to preserve the bands formed.

3.6.3 Staining procedure of SDS PAGE gel

The chemicals and apparatus used for staining procedure preparation include ultrapure water, a staining tray, a rotary shaker, Teflon coated stir bars, disposable pipettes, graduated cylinders, 30 % ethanol made of ultrapure water, 100% ethanol, fixative (40 % ethanol), and 10 % acetic acid made with ultrapure water. The reagent provided in the kit was used to prepare the staining solution.

Table 3.3: Steps in staining and duration

Steps	Duration	Remarks
Fixative	20 min	To fix the protein
Ethanol wash	10	Wash
Sensitizer	10	identification of mw
Ethanol wash	10	Wash
Ultrapure	10	Wash
Staining	15	Band formation agent
Ultrapure	20-60 s	Wash
Developer	4-8	Band start to form
Stopper	10	To inhibit band formed smear
Ultrapure	10	Wash

The first step was sensitizing the solution using ethanol (30 mL) and a sensitizer (10 mL) with the addition of ultrapure water to 100 mL, followed by staining the solution using 1 mL of stainer with the addition of ultrapure water to 100 mL. The next step was to develop the marker using the developing solution with 10 mL of developer and one

drop of developer enhancer with the addition of ultrapure water to 100 mL. Table 3.3 lists the steps taken to stain the protein and the time suggested. The time taken for the band to develop varies for different proteins, as it may take at least 1 min. This is the only step that needs attention to avoid the thickening of the band if left unattended. The gel used was removed from the cassette and washed twice using DI to remove excess SDS solution. A fixative was used to fix the gel for at least 20 min and used overnight before any staining could be done. In all steps, the gel in the solution was swirled continuously using a shaker.

The crucial step was developing the bands. Once the bands appeared, they could be clearly observed prior to adding a stopper so that the bands could be compared with the reference bands. If there is no smearing, then the exact molecular weight could be perfectly identified. Staining was conducted the following day if the gel was fixed. The preparation of all solutions followed basic staining protocols provided by the silver staining kit of Silver Quest Novex by Life Technologies. The gel was then transferred to a gel imager to obtain better images of all the bands.

3.7 Application study of *hevea* proteins

Purified and fractionated proteins were used to study the metal binding and antifungal properties because *hevein* and *hevb6* have antifungal characteristics. The metal binding was aimed for *hevb10*, mangan superoxide dismutase. However, as the fractionated protein collected was very little in amount, a mixture of *hevs* was used instead. Meanwhile, the fractionation process needs to be optimized so that a greater amount of fractions is collected in the column. Also, the purification conditions were varied to determine specific proteins of interest.

3.7.1 Antifungal and antibacterial activities of *hevea* proteins

The antifungal and antibacterial activities of individual proteins or a mixture of proteins were studied using the Kirby-Bauer method or disc diffusion assay. The sample was weighed and dissolved in DI before 20 µL of the sample was dropped on a disc and applied onto agar. The agar containing the sample and the specific fungus was then incubated in an oven at 27 °C for at least 2 days. The purified protein was weighed and 5 mL of sterile DI was added to the purified protein. The mixture was filtered using a PES syringe filter and the fungi or bacteria were swabbed on the agar plate (potato for fungi and nutrient agar (NA) for bacteria) using either a disc or well to drop the proteins prepared earlier. The Petri dish was left in the oven at 37 °C for bacteria and 27 °C for fungi. The growth of fungi or bacteria was monitored every 24 h up to 2 days for antibacterial study and 3 days for antifungal study. The analysis was carried out when the growth of bacteria or fungi was sufficient to cover the area investigated. Different types of agar were used for antifungal and antibacterial studies. Nutrient agar was used for the antibacterial study while potato dextrose agar was used for the antifungal study. The preparation steps are similar but the dilution factor is different. The broth of the same agar was used to grow the fungi prior to casting.

3.7.2 Preparation of Nutrient Agar (NA) and Potato Dextrose Agar

About 10 g of NA (MERCK/Difco) was dissolved in 500 mL of distilled water in a Schott bottle, microwaved for 4 min at 100 °C, and autoclaved for 15 min at 121 °C. The agar was cooled in a laminar airflow chamber with only a lamp and exhaust fans were switched on for at least 10 min. After that, the agar was poured onto a 9 cm plastic Petri dish to about half-full and waited until cool. The agar was then exposed to a UV source for 30 min and cooled again for 10 min to make sure that there is no vapor on the Petri dish cover. The same procedures were used for the potato dextrose agar, except for the formulation (i.e., 39 g in 1 L solution).

3.7.3 Disc Diffusion Assay using Kirby Bauer Method

The freeze-dried sample was weighed and diluted in 5 mL of autoclaved DI. Next, the sample was filtered using a 0.45 µL syringe filter. The agar was labeled into four sections, with the middle as control, either negative or positive. If a disc was used, the sample was dropped onto the disc and the disc was placed on the agar as a label. The sample was incubated at 37 °C for antibacterial study and 27 °C for antifungal study. De Lucca *et al.* (2005) studied the antimicrobial activity of specific plant-derived antifungal proteins and peptides. They found that *hevein*, *hevein*-like peptides, and chitin-binding peptides inhibited the hyphal growth of fungi by binding to chitin. *Hevein*-like peptides and small chitin-binding peptides (43 residues) inhibited the growth of *Alternaria brassicicola*, *Ascochyta pisi*, and *Fusarium culmorum* at low doses (De Lucca *et al.*, 2005). In our work, *hevein* (*hev b6*) is one of the target proteins to be isolated; hence, the application study was used to counter-check our fractionated protein. Disc diffusion assay was employed for investigating the effect of a *Hevea* proteinase inhibitor (Kanokwiroon *et al.*, 2008).

3.7.4 Metal Extraction Study of NRL Waste and Extracted *Hevea* Proteins

Atomic spectroscopic methods are employed for qualitative and quantitative measurement of elements. This method can detect elements in parts-per-million to parts-per-billion level. Spectroscopic determination of atomic species is performed on a gaseous medium in which the individual atoms or elementary ions are well separated from one another. The first step in all atomic spectroscopic procedures is atomization. Several methods have been used to atomize samples for atomic spectroscopic studies, including inductively coupled plasmas and flames, as well as electrothermal atomizers. In absorption spectroscopy, an external source of radiation impinges on the analyte vapor. If the external source radiation is of the appropriate frequency (wavelength), it can be absorbed by the analyte atoms and promoted to excited states (Skoog, 2004).

These spectroscopic techniques are complementary and most powerful when used together. In many cases, an unknown compound cannot be completely identified from one spectrum without additional information, yet the structure can be determined with confidence using two or more different types of spectra (Wade, 2003). Approximately 30% of all proteins are metalloproteins, in which metals are important for their function (Swart, 2013). The protein properties (e.g., net charge, dipole moment, donating and accepting electrons, and the number of potential ligands for metal ions inside a binding site) are the major factors affecting the interaction with metal ions. Therefore, the selectivity of each protein for specific metal ions is of particular interest (Dudev & Lim, 2013). The metal-binding activity can be studied using atomic absorption spectroscopy (AAS) and inductively coupled plasma (ICP). AAS uses different lamps for different metals; thus, this method requires many samples. Analytical techniques are often categorized according to the atomization/ionization source (flame, furnace, or plasma) or the type of spectroscopy used (absorption, emission, fluorescence, or mass). Combinations of these techniques produce numerous analytical techniques with different performance characteristics (Laursen *et al.*, 2014). At present, AAS, inductively coupled plasma-optical emission spectrometry (ICP-OES), and inductively coupled plasma-mass spectrometry (ICP-MS) are the most frequently used techniques for multi-element analysis. Furthermore, limited amounts of proteins purified make ICP the best method to measure metal-binding activity.

3.7.5 Inductively coupled plasma (ICP) and atomic absorption (AAS) for metal-removal study of extracted proteins

An ICP source offers the greatest advantage in terms of sensitivity and freedom from interferences. It consists of three concentric quartz tubes through which streams of argon flow at a total rate of 11 to 17 L/min. The diameter of the largest tube is about 2.5 cm. Surrounding the top of this tube is a water-cooled induction coil powered by a radio-

frequency generator capable of producing about 2 kW energy at either 27 or 40 MHz. Ionization of the flowing argon is initiated by a spark from a Tesla coil. Samples are introduced into the ICP by argon flowing at about 1 L/min through the central quartz tube. The sample can be an aerosol, a thermally-generated vapor, or a fine powder. The sample is transported to the tip by the Bernoulli effect. This transport process is called aspiration. The high-velocity gas breaks up the liquid into fine droplets of various sizes, which are then carried into the plasma. In on-line solid-phase extraction, Cd from protein fractions of serum was studied using oxidized carbon nanotubes coupled to electrothermal atomization atomic absorption spectrometry (ETAAS) to determine Cd in biological samples as it requires minimal sample handling. The procedure joins the simplicity of protein separation by cellulose acetate electrophoresis (CAE), the pre-concentration of solid-phase extraction using ox-CNT, and the sensitivity attainable by ETAAS (Acosta *et al.*, 2013).

A review on specific metal ion binding sites in unstructured regions of proteins by Kozlowski *et al*. (2013) summarizes the most recent observations on some highly effective binding sites (e.g., poly-His, poly-Cys, or Met-containing sequences). Metal ions often coordinate to binding sites located in loops or unstructured regions of those proteins. Metal ion binding usually exerts a distinct impact on the binding pocket structure due to the secondary or tertiary structure donors from the residue being often very far away in the peptide sequence. Several metalloproteins have disordered structures (e.g., α-nuclein, prions, or β-amyloid) and some specific metal chaperons consist of long poly-His sequences, which are very effective binders of metals that do not show any specific secondary structure. This work provides a binding site, an example of some metal-binding types and regions where binding normally occurs. AAS was used to analyze metal-binding proteins. A 0.5 g sample was weighed and topped up to 100 mL in a volumetric flask. Standard metal solutions were prepared at different concentrations. The sample was

mixed with a standard metal solution of a specific concentration. The sample with the standard solution was left for about 2 h and then centrifuged. Standard metal solutions were used at different concentrations to investigate the binding activity of protein precipitates. AAS was carried out by preparing the standard solution and a standard calibration curve was constructed. The instrument measured the absorption and the same set of standard solutions was reacted with a specific amount of precipitate solution. The data obtained were analyzed based on the difference between the initial and final concentrations and also the percentage of metal removed from the protein-metal mixture. From the literature, AAS was used to describe the quantification of Al in immunobiological (Mishra *et al*., 2007).

3.7.6 Protein-Metal Solution preparation

Metal solutions were prepared to the desired concentrations from a standard solution of 1,000 ppm. The prepared solutions were reacted with 0.5 g of *hevea* proteins. Four sets of mixtures were prepared (MeX 2:10 contains 2 ppm of mixed metal solution with 10 mL of *hevea* proteins and MeX 5:20 contains 5 ppm of mixed metal solution with 20 mL of *hevea* proteins). Two different designs were used as follows:

1. Fixed metal concentration with varied NRL waste protein.
2. Fixed NRL waste protein with varied metal concentrations.

Table 3.4: Variation in protein amount for metal removal; Pb, Cu, Cd, Al, Zn

Method 1: Metal conc. (ppm)	Protein (ml)	Method 2: Metal conc.(ppm)	Protein (ml)
2,5,10,15,20	0	1	20
2,5,10,15,20	10	2	20
2,5,10,15,20	20	3	20
2,5,10,15,20	30	5	20
2,5,10,15,20	40	10	20

Table 3.4 shows the metal concentration and protein sample used. The first method requires a large number of proteins, which is time-consuming and costly. Furthermore, a clear picture of a single sweep cannot be easily obtained by this method. The second method is mostly used because the knowledge on maximum metal concentration can be easily obtained and the use of multiple elements for a mixture provides greater information on protein-metal removal. The metal concentrations were prepared from 2 ppm to 20 ppm, where the solutions consist of five different metals. For example, a 2 ppm metal solution will have 100 µL of Pb, Zn, Al, Mg, and Se and then topped up to a 50 mL solution. This solution was then mixed with 0, 10, 20, 30, or 40 mL of *hevea* proteins.

3.8 Graphical representation of extraction set-up and process flow

The graphical representation of the whole process was constructed to show the process flow of extracting *hevea* proteins, characterization, and application study. The parameters used are also stated as a guide. Figure 3.5 shows the experimental setup for extraction and fractionation of proteins and Figure 3.6 on application study.

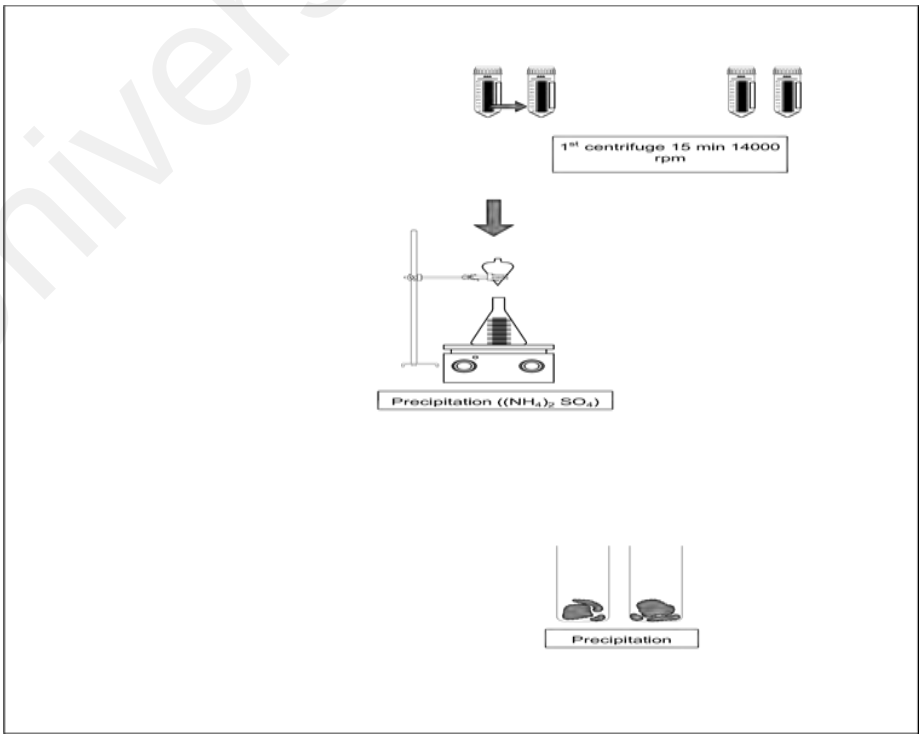
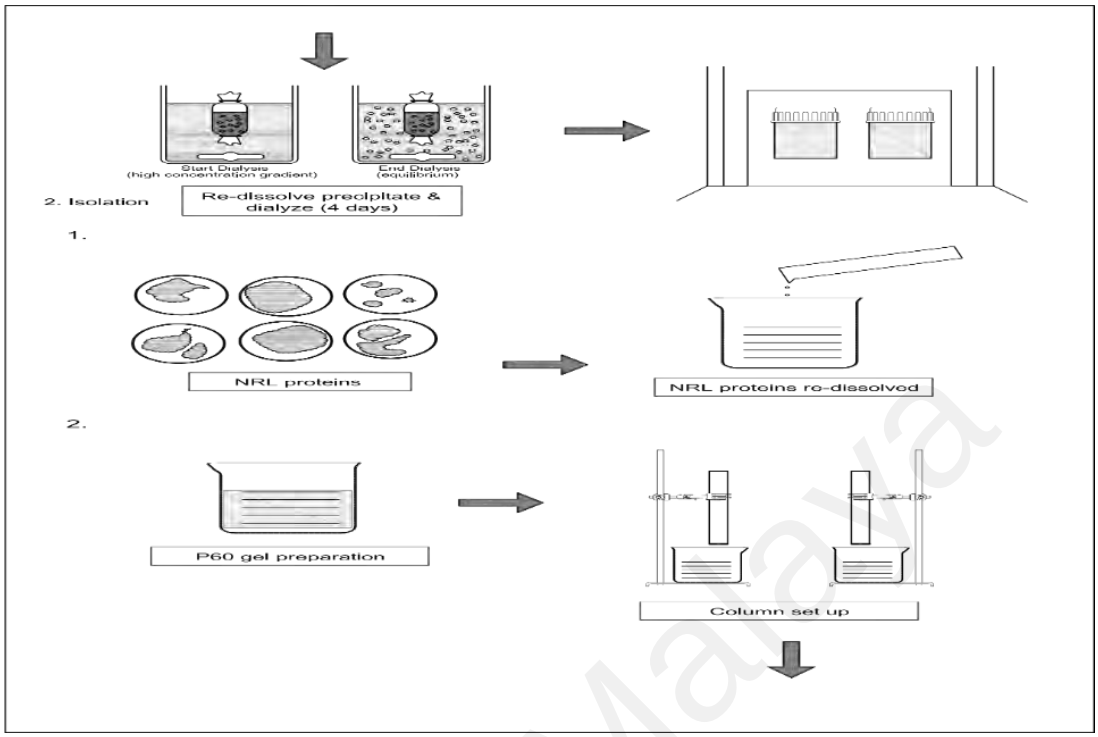
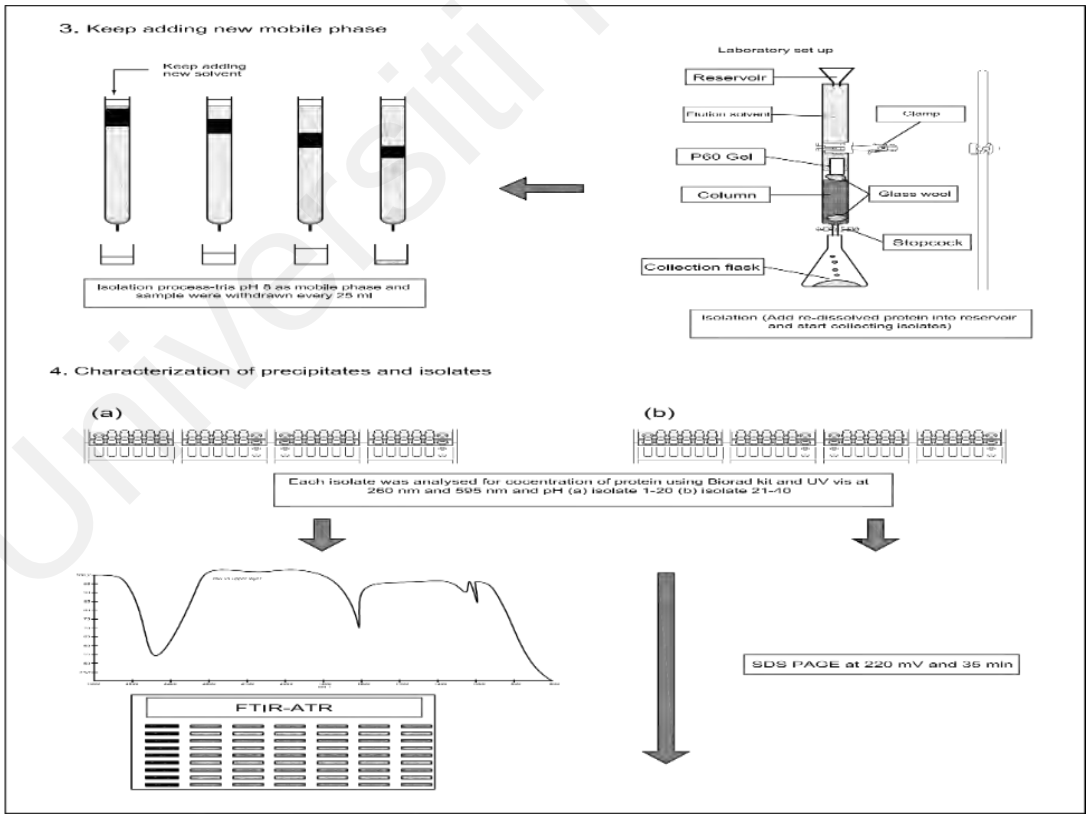


Figure 3.5: Experimental set-up of a) *hevea* protein extraction



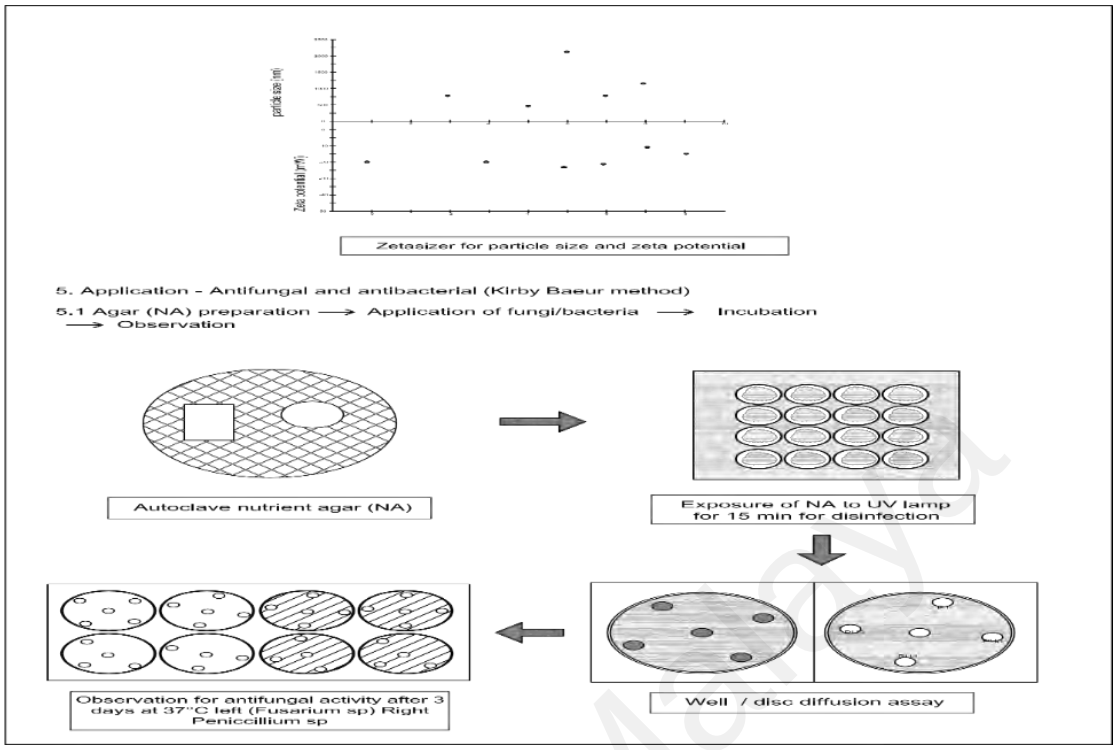
b)



c)

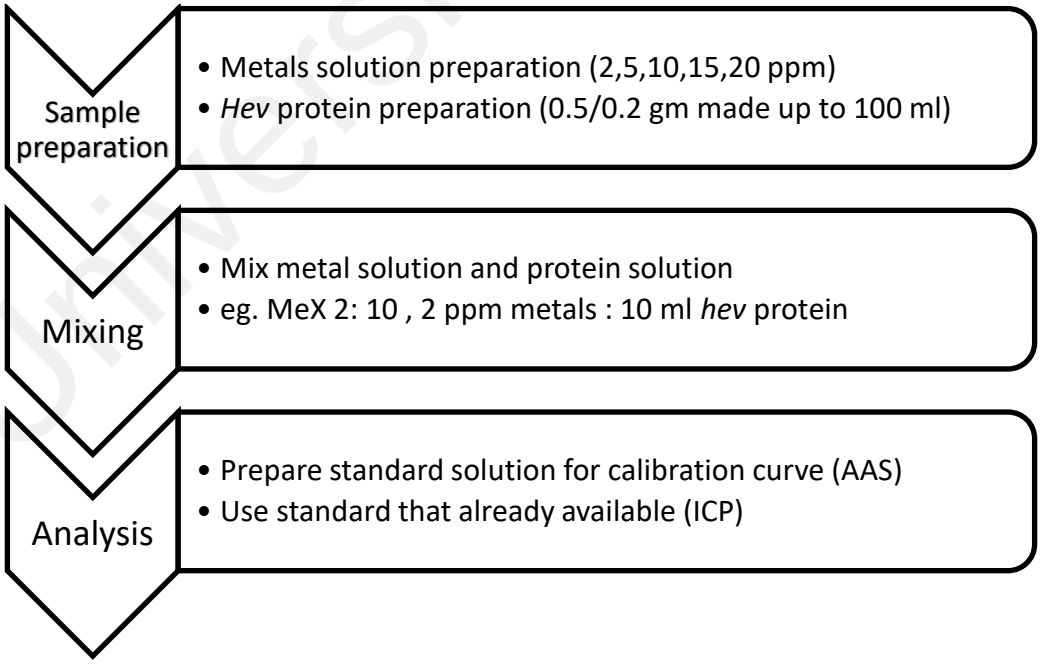
Figure 3.5, continued

b) Fractionation column preparation and set up c) Fractionation process and characterization



a)

Figure 3.6: Application study a) Antimicrobial study of NRL *hevea* protein and b) metal removal process flow



b)

Figure 3.6, continued

CHAPTER 4: RESULT AND DISCUSSION

The natural rubber latex (NRL) waste effluent containing proteins collected from the skim rubber production line was analyzed for its particle size and zeta (ζ) potential. Various techniques have been used to remove rubber particles and purify *hevea* proteins. The NRL waste effluent was subjected to centrifugation, filtration, and conditioning with buffer pH to understand the behavior of the waste when treated with the above-mentioned processes.

Table 4.1: Particle size and zeta potential of NRL waste effluent

Sample	Particle size	Zeta potential
Cw,rs,wash,filter	8088	-0.671
Cw,48f3t,filter	215.6	-0.07
Cw,24f3t,filter	197.6	1.99
Skim wash, centrifuge	372.1	-0.0935
Cw, centrifuge	41.55, 6110	0.8474
Cw pH 3.06	430.7	-0.0239
Cw, bottom fraction	5461	-0.0351
Skim bottom fraction	4663	-3.81
Sw, centrifuge, pcp	6315	0.00075
Sw, centrifuge	678	-6.64
Sw pH 6.3	5065,405	-21.7
Cw,centrifuge,pcp, dialyzed	931.9	-9.32, -4.19
Cw,centrifuge,fr,rs,wash,pcp	5531	-4.09
Cw,centrifuge,pcp,centrifuge,dialyzed3d	969.3	-8.74
Cw,centrifuge,pcp,dialyzed 7d	931.9	-9.32
Cw,centrifuge,rs,wash1,	3000	0.832
Cw,centrifuge,rs,wash2	6165	-0.387
Cw,pH 7	1276	-7.74
Cw,pH 8	4689	-13
Cw, pH 6	1613	-8.87
Cw,pH 5	1073	-4.05
Cw,pH 4	1523	-0.69
Cw,centrifuge,pcp	0.00046,4308	-5.09,5.78
Cw,filter,pcp,dialyzed	1522	-23.6

Cw- coagulation waste, pcp-precipitate, fr- freeze, rs-rubber solvent, 24f3t- 24hours freeze, 3 hours thaw, sw-skim wash

The data in Table 4.1 show that the coagulation waste is more stable than the skim wash. Thus, it was decided to focus on the skim serum based on the protein content after precipitation. Subsequently, the coagulation waste at acidic pH produced a higher yield. The choice of waste-containing proteins was coagulation of waste or skim serum. The

wastewater was collected immediately after the skim coagulation bowl washing or skim washing, which was taken from the rubber trap before the treatment pond. Both waste samples were centrifuged, filtered, and conditioned with buffer pH prior to purification. The ζ potential data are used as a basis in choosing the starting material due to the stability of serum, which can also be correlated with the protein to be purified. From the table, only precipitated protein showed good ζ potential compared to the freshly collected serum, indicating that all the serum samples require proper storage, preferably a chiller, and need to be purified as soon as possible to prevent the loss of protein in the starting material.

4.1 Effect of various purification steps on the amount of protein collected

NRL centrifuge plant wastewater contains protein, upon centrifugation, rubber latex is divided into a layer of rubber particles, C serum and bottom fraction containing protein (U. Soedjanaatmadja *et al.*, 1995). The skim serum from centrifugation still containing 4-8 % DRC, the wastewater from the skimming process is highly acidic pH 2.0 – 4.5 (Sumate Chaiprapat *et al.*, 2015). Skim natural rubber latex is residual latex available as a by-product of the concentration process of the NR latex by centrifugation. Direct coagulation of the skim latex is not easy to achieve because the suspension stability is enhanced by the high content of ammonia and proteinaceous substances (Kongkaew *et al.*, 2012).

Table 4.2: Effect of dialyzing tube pore size on the weight of proteins precipitated

Tube MWCO	Weight 1(g)	Weight 2(g)	Weight 3(g)	Average (g)
3.5	0.2847	0.2993	0.3712	0.3184
20	0.1696	0.1773	0.1671	0.1713
40	0.1354	0.1597	0.1159	0.137

Table 4.2 shows effect of dialyzing tube pore size on final weight of proteins. However, in working out for the best dialyzing tube size, the smaller size the better due

to *hev* protein ranges from about 10 to 200 k Da, that's the answer why the use of 40 k Da tube reduced the amount of protein collected by more than 50%. This proteinaceous substance precipitated out from skim wastewater via various methods. Effect of tube size carried out to measure the amount of protein collected when different size used at the same starting volume and other parameter kept constant. The result showed for 20 k MWCO the average amount of protein collected reduced by 46% and for 40k MWCO further reduced to 57 %, which was more than half. This lead to the decision of using 3.5 k MWCO. The reason for this is the range of size of *hev* proteins. Theoretically, NRL serum contains proteins ranges from 5 k Da to 50 K Da in molecular weight.

Table 4.3: Effect of pH on weight of proteins precipitated

Pre-conditioned	Weight 1 (g)	Weight 2 (g)	Weight 3 (g)	Average (g)
DI water	0.2467	0.2454	0.2047	0.2322
pH4	0.2885	0.2003	0.2844	0.2577
pH5	0.1931	0.1719	0.1651	0.1767
pH6	0.1905	0.2687	0.2992	0.2528
pH7	0.223	0.211	0.3	0.2446
pH8	0.1665	0.2610	0.3076	0.2450

Table 4.3 provides the data when NRL waste was pre-conditioned at different pHs. This experiment was conducted to investigate if there are any significant changes in the amount of protein collected when NRL waste was exposed to different pHs because the identified 15 *hev* proteins have an isoelectric point (IEP) range of 3.5 to 9.5. It is expected that the active center of a specific *hev* protein will be its surface. However, from the data obtained, a slight reduction in the collected amount was observed only at pH 5. Hence, it is insignificant to conclude the focus on specific pH. The pH of NRL effluent waste was measured and it was found that the coagulation trough recorded the lowest pH of 3.1 to 4.8, followed by bowl washing (pH 4.8 to 5.2) and the drain before the effluent was pooled at the rubber trap, where the pH was higher than 5.5. The initial pH of wastewater

provides guidelines for purification. A higher pH indicates that microbial activity has taken place, which affects the protein content. Usually, microbe activities are limited at acidic pH due to the unsuitable surrounding, and a certain type of protein is also affected and only a few types that can resist acidic conditions (e.g., *hevein*) remain at a moderately high concentration (U. M. Soedjanaatmadja *et al.*, 1995).

The exposure of precipitates to 10.01 pH Tris buffer resulted in the sedimentation of proteins. The raw sample exposed to a certain pH resulted in the precipitation of specific proteins and not all proteins precipitated. However, these proteins can be excluded via fractionation by column chromatography. The resulting precipitates from different pH exposure can be directly dialyzed and characterized for particle size, ζ potential, and molecular weight. The limitations of this precipitation process are some of the protein might not belong to any pH used; thus, the protein will not be captured. Using other types of salts to prepare buffer may interfere with the precipitation because it will not be compatible with Tris buffer. Therefore, it is necessary to use another salt for another pH buffer from the beginning. In other words, the buffer should be added to the raw material and not after precipitation. The fractionation step is not possible unless the same buffer is used and provided that the buffer is compatible with the SDS PAGE column. The use of multiple centrifugation methods is to purify the *hev* proteins introduced as an attempt to replace salts and to find any irregular properties.

4.2 Physical characteristics of purified NRL protein

The solid precipitated using a standard method was dried, weighed, and used to estimate the protein collected before the dialyzing steps. Latex yield is influenced by tree clone, age, seasons and climate, and soil conditions (Coulen *et al.*, 2017). In this study, the protein yield was investigated by conducting various measurements steps during precipitation. The NRL waste serum was exposed to different pHs prior to precipitation

with the goal of obtaining different types of proteins, which have different chemical active sites. From Figure 4.1, the exposure of the NRL waste serum at even pH showed an unexplained profile. The NRL waste from different batches might be the answer to the inconsistency in the yield profile. From the plot, it was obvious that to achieve a high yield, it is worth omitting the pre-conditioned waste unless it is proven that specific types of proteins of interest are identified at specific pH conditions. Therefore, the fractionation process was carried out only for the deionized water (DI) precipitated protein. The fraction term is used when the mixture of proteins is in one fraction and the isolate term is used if only a single protein species is available in that fraction.

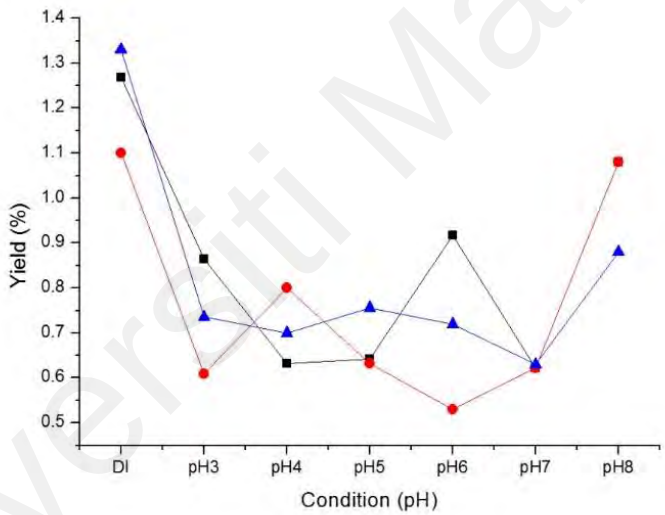


Figure 4.1: Extracted *hevea* protein yield (%) Effect of pre-conditioned on NRL protein yield for first batch (■), second batch (●), and third batch (▲)

Statistical analysis

Variable	N	N*	Mean	SE Mean	StDev	Variance	Minimum	Median	Maximum
DI	3	0	1.2330	0.0688	0.1192	0.0142	1.1000	1.2690	1.3300
pH3	3	0	0.7370	0.0736	0.1275	0.0163	0.6100	0.7360	0.8650
pH4	3	0	0.7110	0.0485	0.0840	0.0071	0.6330	0.7000	0.8000
pH5	3	0	0.6771	0.0396	0.0685	0.0047	0.6330	0.6422	0.7560
pH6	3	0	0.722	0.112	0.194	0.037	0.530	0.720	0.917
pH7	3	0	0.62500	0.00252	0.00436	0.00002	0.62200	0.62300	0.63000
pH8	3	0	1.0133	0.0667	0.1155	0.0133	0.8800	1.0800	1.0800

NRL waste serum exposed to different pH prior to precipitation with the goal to a captured different type of proteins, which has a different chemical active site. The

presence of different percentage of amino acids in the specifics *hevs* is expect to favor specific pH conditions selected. From Figure 4.1 exposure of NRL at even pH showed somehow unexplained profile. The increasing and decreasing trend in each batch from DI to pH 8 shown by different batch is due to seasonal factor. The constituent of rubber and non-rubber constituents slightly effected by the local season such as dry and rainy season experience throughout the year. The NRL waste was of the different batch that might be the answer of inconsistency in yield profile. From the plot, it was obvious that to develop with high yield it is worth to omit the preconditioned unless proved that specific type of protein of interest been captured at specific pH condition. For that reason, the fractionation process carried out only for the DI precipitated protein. Fraction term is used when mixture of proteins are in one fraction and isolates used if only single protein species are in that fraction.

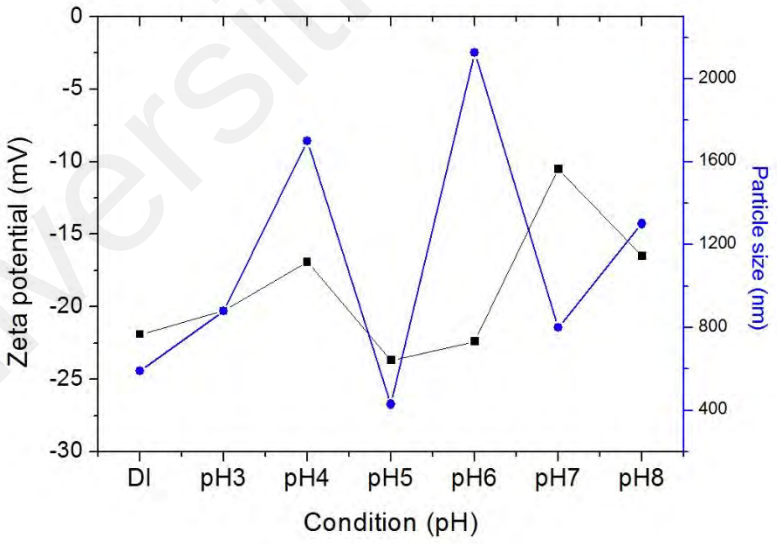


Figure 4.2: ζ potential and particle size of extracted *hevea* proteins. Effect of pre-conditioned on ζ potential and particle size of NRL protein on particle size (●) and ζ potential (■)

Statistical analysis of zeta potential and particle size

N	N*	Mean	SE Mean	StDev	Variance	Minimum	Median	Maximum
7	0	-18.89	1.74	4.59	21.09	-23.70	-20.30	-10.50
7	0	1118	234	619	382882	430	879	2126

Figure 4.2 shows the ζ potential of precipitates. The highest ζ potential was observed at pH 5, and the least stable ζ potential occurred at pH 7 with -10 mV and pH 8 with -16 mV. Meanwhile, the lowest particle size was observed at pH 5. The overall profile in ζ potential showed an almost identical pattern with the particle size from DI to pH 5 but a large variation in size was displayed. It is suggested that at pH 5, the protein is at the best state where the stability and size represent the targeted proteins. This pH is also close to that of *pI* for most of the listed *hev* proteins in the range of 4.5–8 and supports the zero net charge that occurred at pH 5 (Refer section 2.4).

4.3 *Hevea* protein Chromatography

In this work, dialyzed or freeze-dried NR protein was introduced to the column and characterized for its pH, particle size, and ζ potential using UV-vis spectroscopy, FTIR, and SDS PAGE. The first part of this work used precipitates of different pHs and analyzed the characteristic profile. In this part, the pH condition was varied while precipitating the protein, where a photometric dispersion analyzer (PDA) was used online with a chromatography column to construct a chromatogram. Meanwhile, the second part only focused on DI precipitates but at different loadings. UV absorbance at 280 nm was employed as a tool to construct a chromatogram.

4.3.1 Photometric Dispersion Analyzer (PDA) of fractionated proteins

PDA is a sensitive monitor for flowing suspensions and emulsions. The DC component is simply a measure of the average transmitted light intensity and is dependent on the turbidity of the suspension. At least three peaks appeared in Figure 4.3(a) indicating about three different strong identities of protein, and the higher concentration is from the second peak from 200 to 300 min. Other protein fractions displayed very weak separation or no separation at all. The load amount was fixed at 0.1 g and the mobile phase was set at pH 8. However, for other pH from 4 to 7, an almost flat plot was viewed, indicating the

absence or very low amount of protein fractionated. This might be because the starting sample has a very low protein concentration; thus, fractionation is not possible and the use of pH alone to condition the serum could not precipitate those proteins.

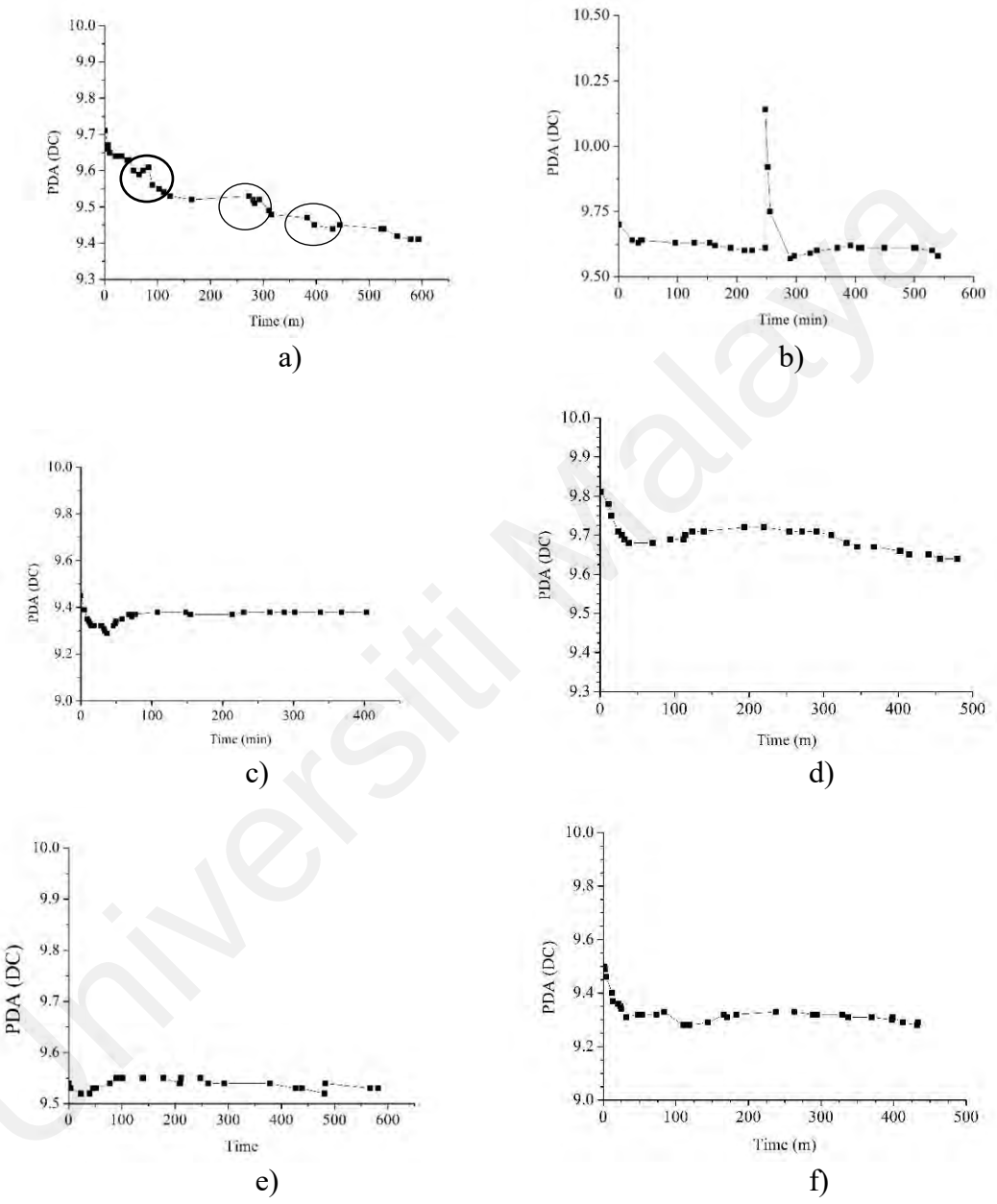


Figure 4.3: PDA of pH varies proteins plotted against time. PDA data for a) DI-purified, b) pH 3, c) pH 4, d) pH 5, e) pH 6, and f) pH 7 fractions from recorded as time versus DC reading fraction number 1 to fraction number 14

Previous work suggested the use of mixed chromatography method with modifications involving ligand libraries to target all proteins present in the sample. They proposed that

the modification of this principle is done to enhance proteins of very low concentrations and also to enable the use of different chemical agents (Boschetti & Righetti, 2011).

4.3.2 Effect of pH of NRL Waste, Extracted Proteins, and Fractions

The pH of the precipitates was measured to investigate if the protein with different pHs still had the same pH after precipitation. Meanwhile, the pH of the fractions was measured to observe if there was any significant pH change for each fraction containing different types of proteins. The overall data suggested that different fractions have different pHs if the fractions contain proteins of the same type or a mixture of different molecular weight proteins. When the pH of a protein solution is above the IEP, all the protein molecules will have a net negative surface charge. On the other hand, the protein molecules will have a net positive charge below the IEP. In either case, these like-charged molecules repel one another, and this repulsion helps keep these very large molecules in solution. At the IEP, the protein molecules no longer have a net surface charge. As a result, they no longer strongly repel one another and are at their least soluble. Under these conditions, there is a tendency for a particle to clump together and precipitate out of the solution. In this case, protein may coagulate even though they are not denatured.

By comparing the PDA plot of DI with the pH plot, it is consistent that at a higher concentration of protein, the pH is the lowest and for each set of isolates, the increase in pH is a signal of a complete set of isolation, which means that all proteins have eluted. The specific trend observed with protein fractions are discussed in section 4.3.7 to evaluate separation performance and the effect of it on different proteins fractions. The implications of the increased pH of the latex are the increase of the density of bound electric charge and the electrokinetics (ζ potential) at the interface between the rubber, particles, and the aqueous phase, thereby increasing the colloid stability of the latex. In the methodology, the variants were established for the largest interaction coverage by

modifying either the initial conditions of pH or the ionic strength, as well as the chemical modification of the column (Boschetti & Righetti, 2011). The *hevea* rubber particles are usually 0.02 to 3.0 µm in diameter and mature rubber particles are spherical to pear-shaped when observed under a microscope (Xiang *et al.*, 2012). For purification, it is expected that no more rubber particles should be depicted.

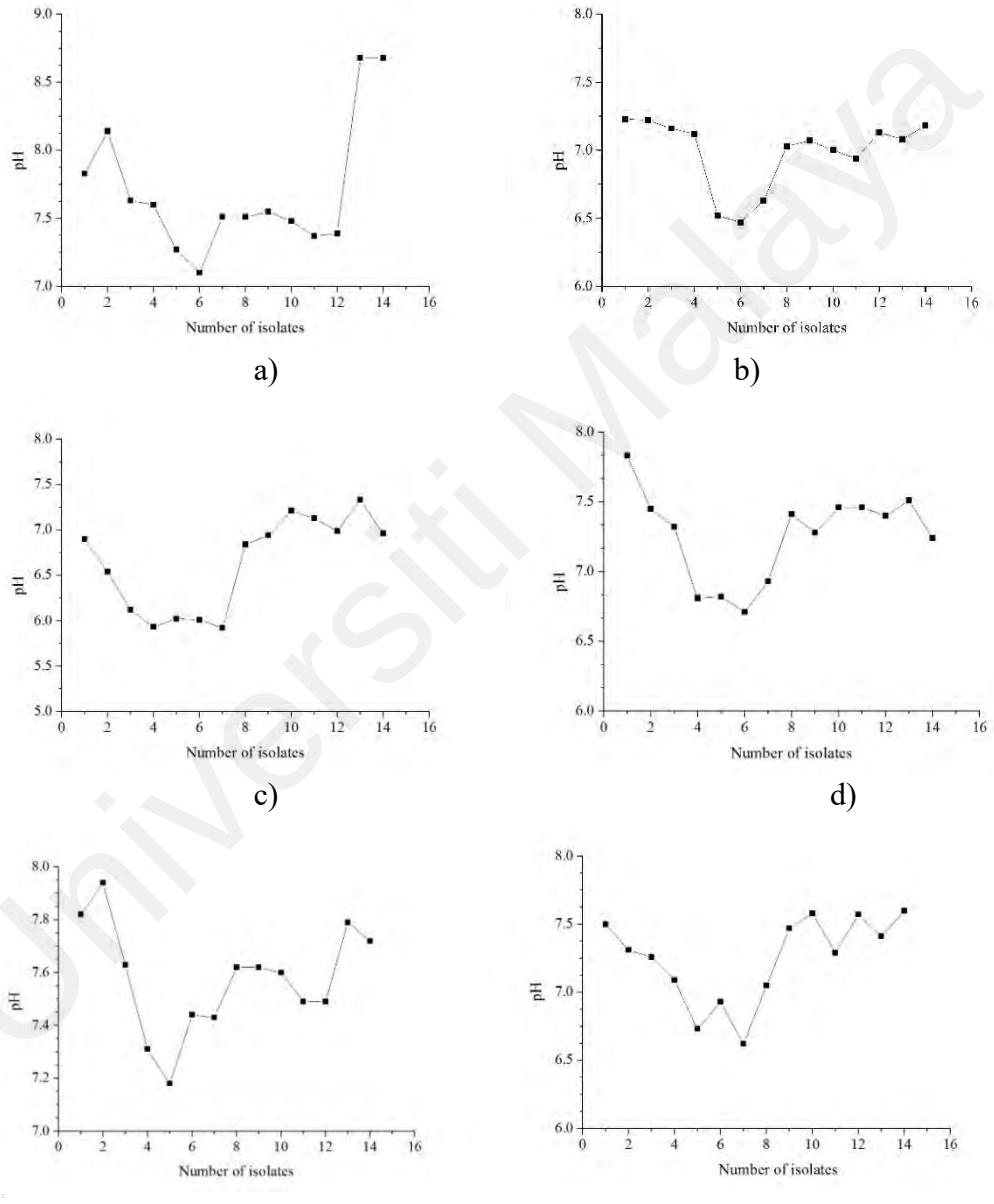


Figure 4.4: pH of each protein fractions. pH profile of a) DI, b) pH 3, c) pH 5, d) pH 6, e) pH 7 and pH 8 fractions

From each plot, the pH dropped at the first point of fractions containing proteins. This indicates that the properties of a collected protein are mostly acidic; thus, the pH decreases

as soon as the protein is added. From this pH profile, no specific profile is used as a guide in predicting the protein characteristics or properties in the fractions besides pH. This pH data might be useful in constructing a database of protein fractions or properties summary.

4.3.3 Particle size of Fractions from various pH Conditions

Theoretically, the particle size of NRL and its serum considers how the latex is dispersed. The concept of the equivalent spherical particle arises in connection with particular properties of the colloidal systems, such as light scattering propensity, specific surface area, and behavior in a centrifugal field. Supposedly the particle size distribution of all suspensions is polydisperse to a lesser or greater extent. In protein isolation using SEC, it should be expected that folded protein will come out last due to its small size, and unfolded protein will come out first. The Mark-Houwink equation is used in estimating the relationship between particle size and molecular weight. Ammonium sulfate provides partial stabilization, but also tends to crystallize and increase in particle size. Thus, by removing ammonium sulfate using a dialyzing tube, the size of protein particles is almost definite. The particle size distribution of pH 3 isolates showed a different profile from the DI sample, which is expected because the activity of protein largely depends on pH. However, the overall profile is still as expected. It is perceived that fraction number 7 has the highest particle size, which may contain unfolded protein. However, the folded protein may be fraction number 9 that eluted, which is too close to fraction number 7, hence influencing their characteristics (Figure 4.5).

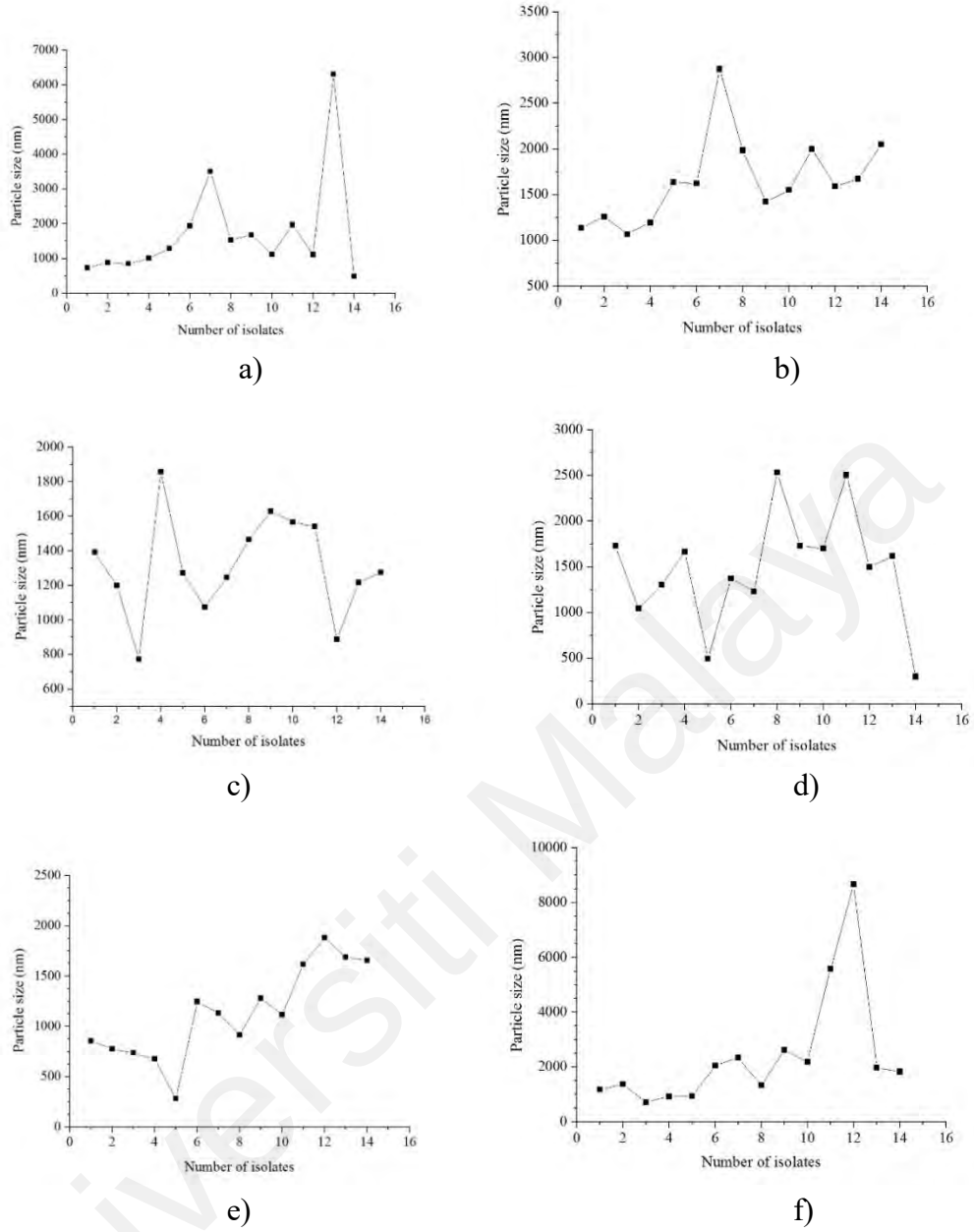


Figure 4.5: Particle size of pH varied fractions a) DI, b) pH 3, c) pH 5, d) pH 6, e) pH 7 and f) pH 8 fractions

The particle size of pH 3, 5 and 6 is almost identical without any specific profile; this shows the distribution of size with that pH is unpredictable. The particle size of proteins for pH 7 and pH 8 fractions shows the same profile but most of small particle size protein eluted out first and the large particle size protein retains longer in the column. Means that

in a basic condition, the set of proteins behave in the opposite manner of in acidic condition.

4.3.4 Zeta Potential of Extracted Proteins and Fractions

In the 1940s, Derjaguin, Verway, Landau, and Overbeek developed a theory regarding colloidal stability, in which the stability of a colloidal system is determined by the sum of electrical double layer repulsive force and van der Waals attractive force that the particles experience as they approach one another.

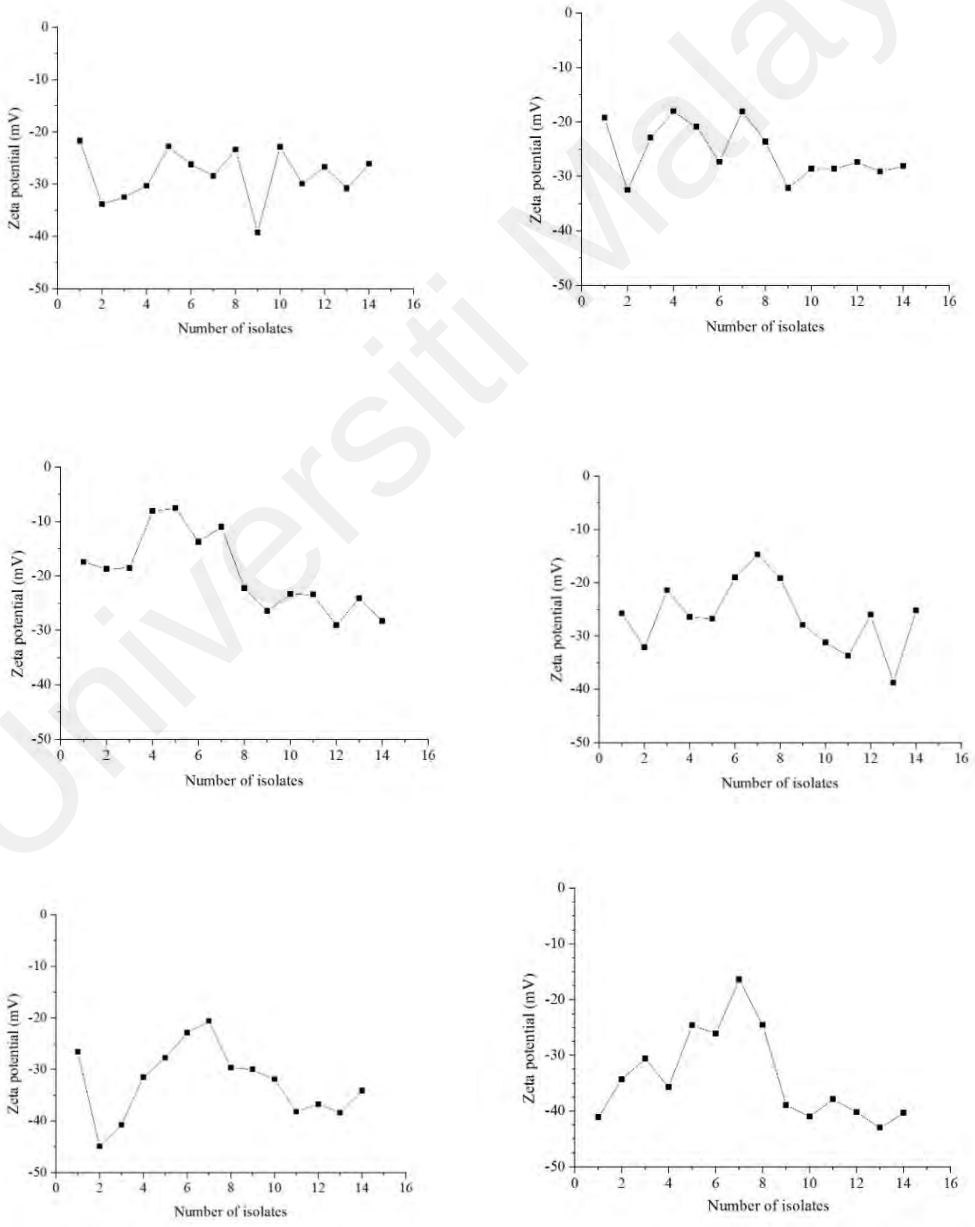


Figure 4.6: ζ potential of pH varied fractions. Zeta potential of a) DI, b) pH 3, c) pH 5, d) pH 6 , e) pH 7 and f) pH 8 fractions

. The theory proposed that an energy barrier resulting from the repulsive force prevents two particles from approaching one another and adhering together. Nevertheless, if the particles collide with sufficient energy to overcome the barrier, the attractive force will pull them into contact. ζ potential is well-known for the characterization of electrochemical surface properties. The application includes determining the material properties in the membrane separation, mineral processing, water treatment, and protein separation and purification (Salgın *et al.*, 2012). The ζ potential of DI and pH 3 fractions (Figure 4.6) showed an almost identical pattern. However, the ζ potential of each fraction did not show any specific trend. On the other hand, the ζ potential of pH 5, 6, 7, and 8 showed a specific trend, where most of the ζ potential is low at higher protein concentrations and increased again when most of the protein have eluted. This profile might be due to the ζ potential concept, where at higher protein concentrations, the ζ potential is low due to the agglomeration of particles. As the concentration of protein fractions decreased, the ζ potential is high because the distance between particles increased. Particle charge is measured by modifying the suspending liquid. The modification includes changing the liquid's pH or changing the ionic species of solution. An IEP is the pH of the protein solution in which the net charge or ζ potential is zero. At the IEP of protein, its structure is more hydrophobic, more compact, and less stable due to the absence of inter-particle repulsive forces. Hence, protein can easily aggregate and precipitate at their IEPs.

The difference in the IEP values of biomolecules is caused by different ionic environments, such as ionic strength, pH, and ion type. The experimental method used can change the IEP value; therefore, there are several different values of IEPs for the same protein in the literature (Salgın *et al.*, 2012). Nevertheless, the use of ζ potential in the determination of colloidal stability is reliable because the system involves a wide range of particles with different sizes and different physicochemical properties

Each step conducted in this work considers the ζ potential and size to ensure that a precise sample is extracted. Additionally, the sample is temperature and pH-sensitive. Thus, the best way to ensure that the sample is still in a good condition is to use a countermeasure, such as a tracking system in terms of characteristics or property checking that will be necessary as a complement to other data. In summary, the ζ potential of all fractions (-10mV to -40 mV) is acceptable for continuing the next step of the research work, and only the fractionated amount limits the application study. The pH of the surrounding is suitable for the sample of *hevea* proteins but the exact pH for fractionation remains inconclusive with this profile.

4.3.5 Investigation on Column Optimum Loading and Resulting Fractions

In the first part of the work, the sample was loaded into the column without freeze-drying and immediately after dialyzed. The concentration of the protein extracted is unknown and there is a possibility of the presence of ammonium salt. Therefore, it is difficult to determine the exact amount of protein, but a rough estimation is possible when the fractions are freeze-dried. These chromatography systems managed to produce a sample that inhibits bacterial growth. It is recommended that for the antimicrobial activity study, different pH fractionations are used instead of a normal pH sample. By comparing the overall data plot from a PDA, only the pH 3 sample (Figure 4.6(b)) showed the same outcome using UV absorbance at 280 nm. Other samples showed very weak signals for the isolation. In the second part, only DI precipitates were used and the variable employed was the weight of NRL protein after freeze-dried. The chromatographic profile was constructed using the data of UV absorbance at 280 nm for each fraction. Different weights were loaded onto five columns and the same Tris buffer pH 8 was used. The fractionated species and the concentration of protein for specific fractions were obtained from the chromatographic profile.

4.3.6 UV Absorbance of Purified Proteins and Fractions

The UV absorbance at 280 nm assay is fast and convenient as no additional reagent is required. The principle is the protein in the solution absorbs UV light with absorbance at 280 nm and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm and peptide bonds are responsible for the peak at 200 nm. Research on *Hevea* latex lectin binding using chromatographic separation with Sepharose also obtained the chromatographic profile using UV absorbance at 280 nm by varying the packed column used and heat treatment was subjected to their protein to obtain baseline resolution (Wititsuwannakul, Pasitkul, Jewtragoon*, et al.*, 2008).

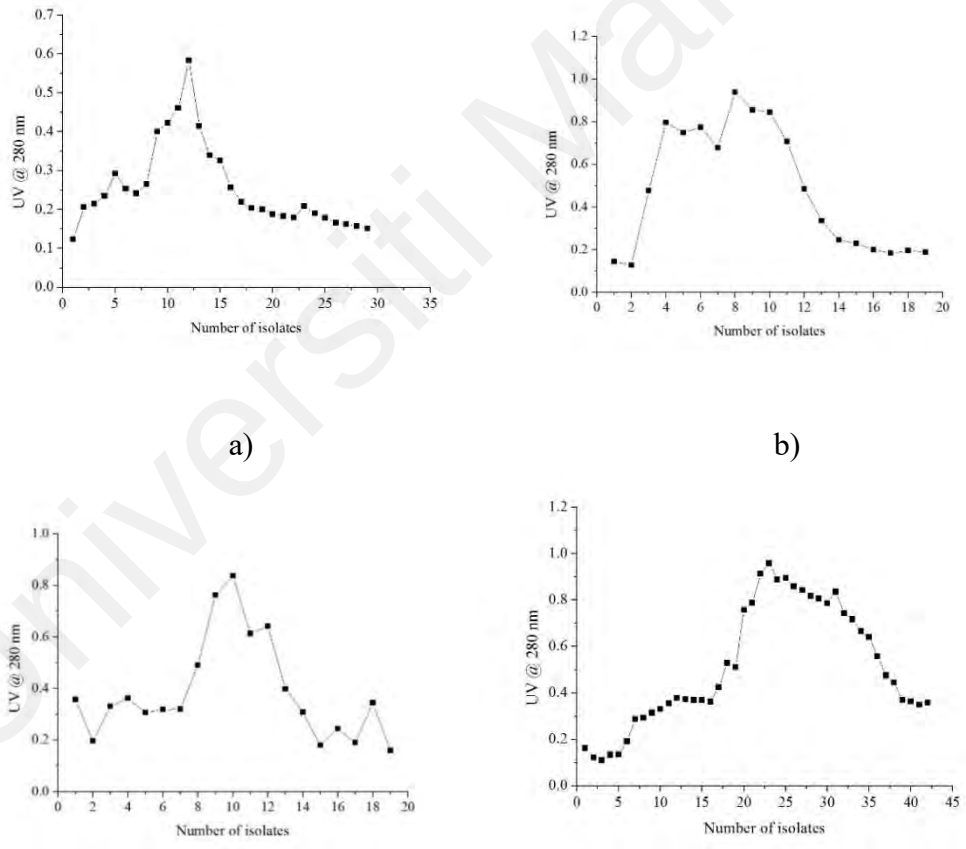
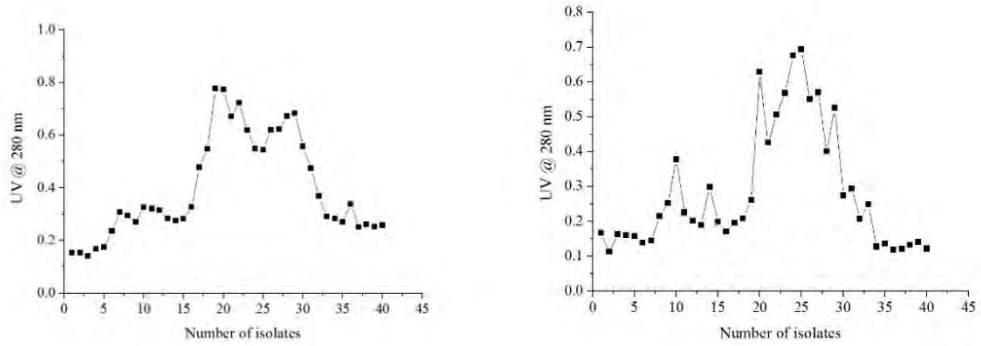


Figure 4.7: UV visible plot of each fraction. UV at 280 nm of purified proteins and fractions,a) 1.0 g in 30 ml TRIS and b) 0.96 g in 30 ml TRIS, c) 0.874 g in 35 ml TRIS and d) 0.5801 g in 250 ml TRIS e) 0.3501 g in 30 ml TRIS f) Sample 0.2538 g in 30 ml deionized water



e) f)

Figure 4.7, continued

Chromatogram (a) and (b) Figure 4.7 show that a very dilute sample does not affect the isolation process in terms of the number of species. Meanwhile, chromatogram (e) and (f) show that the correct ratio or dilution factor may help in attaining baseline resolution. From chromatogram (c) and (d), it is clear that loading affects the isolation process. The higher the loading, the smaller the number of the fractionated species. The lowest loading recorded higher species when Tris buffer was used as the diluent. In contrast, when DI was used as the diluent, the number of fractionated species decreased. The possible reason for this would be due to the pH of the initial sample, as pH plays a very important role in protein activity. Considering the information gathered, it is predicted that different chromatogram profiles are produced by varying the pH of buffer. Although the method is rapid and relatively easy to use, spectroscopic methods depend on light absorbance/transmittance and may be compromised by opaque or colored solutions (Guo *et al.*, 2014).

4.3.7 pH Profile of Various Load Amount Fractions

The pH profile of fractions was found consistent with the load amount of protein in each fraction. The higher the load amount of protein, the lower the pH of the fractions. These profiles are useful in estimating a complete fractionation process for a specific load.

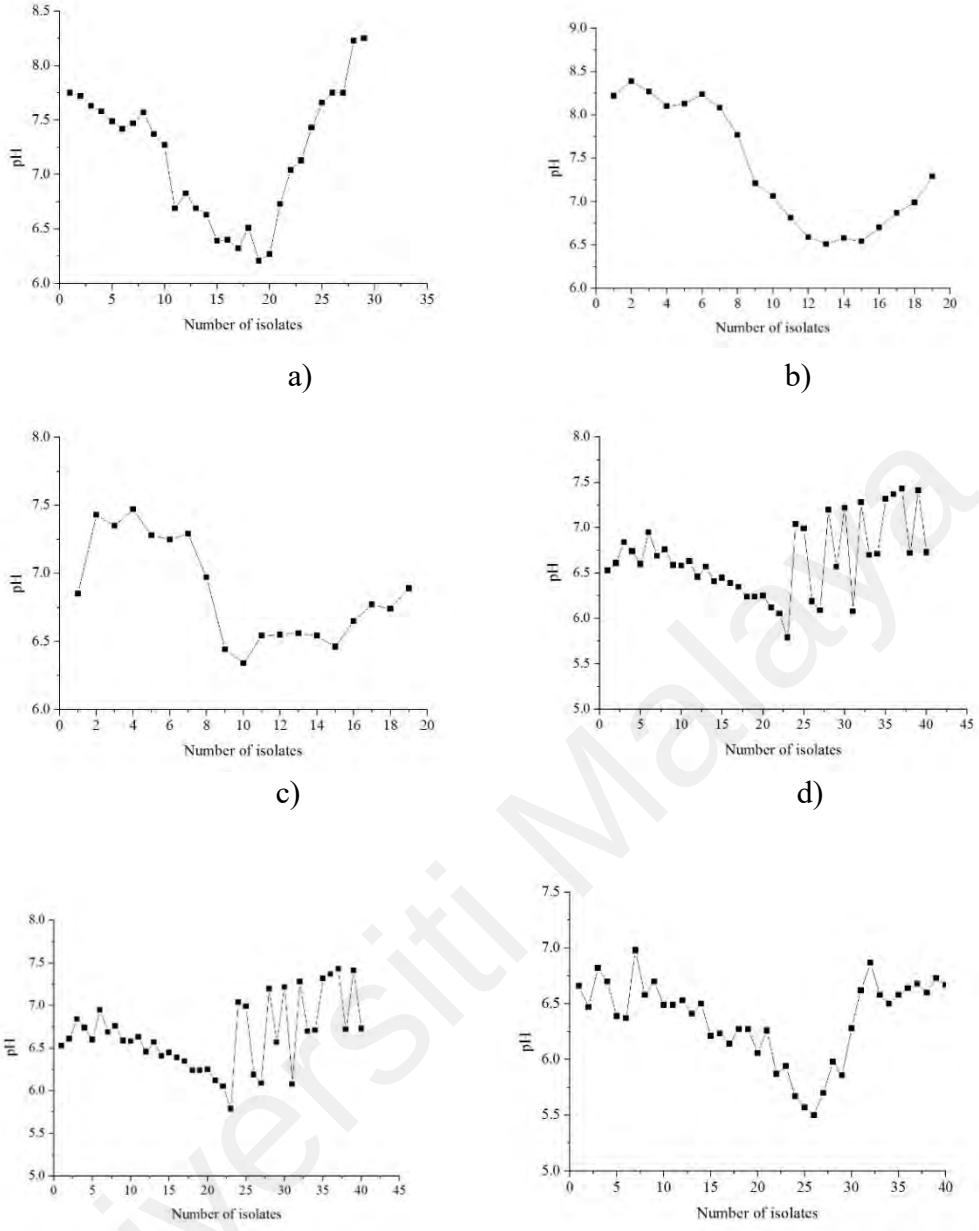


Figure 4.8: pH profile of load amount varied fractions. a) 1.0 g in 30 ml TRIS and b) 0.96 g in 30 ml TRIS, c) 0.87 g in 35 ml TRIS and d) 0.58 g in 30 ml TRIS, e) 0.35 g in 30 ml TRIS and f) 0.26 g in 30 ml TRIS, n) 0.25 g in 30 ml Deionized water

A two-bed volume is normally used for estimating all the proteins that eluted, and it can be confirmed with UV absorbance at 280 nm or by assay using kits, such as BCA or Bradford assays. This obtained profile provides a basic knowledge of specific fractions and is useful in identifying the exact physical properties of specific fractions.

4.3.8 ζ Potential of Fractions

The ζ potential of each load amount was varied to investigate all fractions and to evaluate column performance. From the ζ potential profile of the fractions, the consistent profile was viewed from the highest amount of protein load until the fewest, except for the fewest load amount.

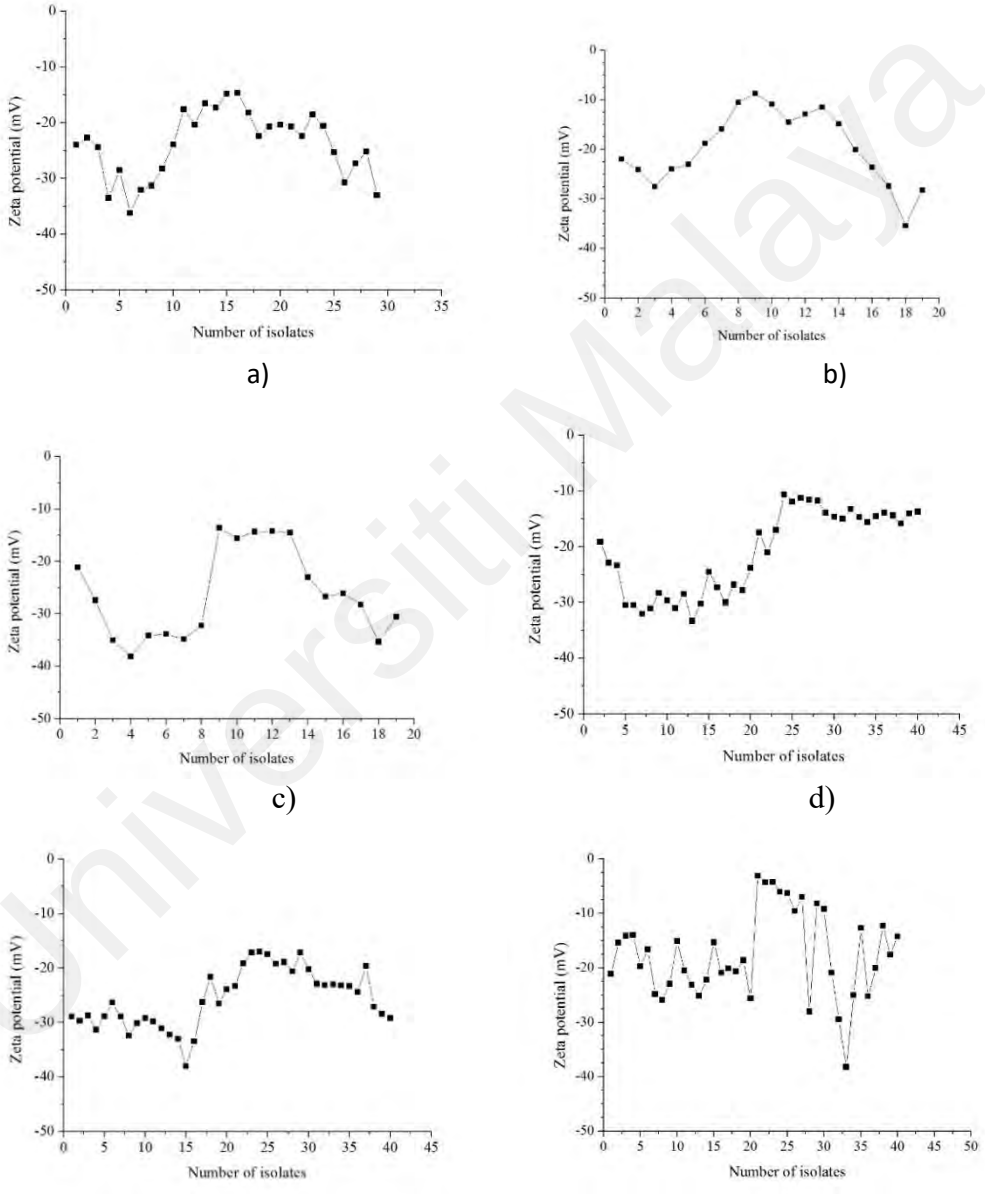


Figure 4.9: ζ potential of load amount varied fractions. a) 1.0 g in 30 ml TRIS and b) 0.96 g in 30 ml TRIS, c) 0.87 g in 35 ml TRIS and d) 0.58 g in 250 ml TRIS, e) 0.35 g in 100 ml TRIS and f) 0.26 g in 30 ml TRIS, f) 0.25 g in 30 ml TRIS

Figure 4.9 (f) is similar to the profile of pH, where the initial and final readings are exactly or almost the same. It is predicted that the fraction containing proteins at a

physiological state will have higher ζ potential compared to the fraction with very little or without proteins at liquid state. However, for the higher load amount in Figures 4.9 (a) and (b), the ζ potential is approximately -10 mV, which is less stable than the lower load amount. The reason for this profile might be due to the separation of individual *hevs* that is restricted at a higher load amount compared to a lower load amount. Another reason is a low concentration gives more space and provides free movement of a particle.

4.3.9 Particle Size of Fractions

The particle size of fractions was measured concurrently with ζ potential to have a better picture of the size of individual *hev* proteins. Other than that particle size, the size of specific fractions was also used as an estimation of specific *hev* protein size. Each fraction containing protein usually displays a smaller size compared to a fraction without proteins. The size of 400 to 600 nm seems to be ideal for fractions containing proteins. From Figure 4.10, the highest load amount showed acceptable particle size when measured. However, at a lower load amount, the particle size did not represent the protein. This makes sense because the concentration of protein in the fractions is very low; thus, it will restrict the measurement and the instrument will only measure the mobile phase. In addition, particle size will be useful if the load amount is greater than 0.3 g; otherwise, the size measured may not be useful. From Figure 4.10, by comparing all profiles of DI, pH 5, 6, 7, and 8 fractions, it shows that the profiles are identical. The profile shows that the lower pH belongs to fractions with a high concentration of protein.

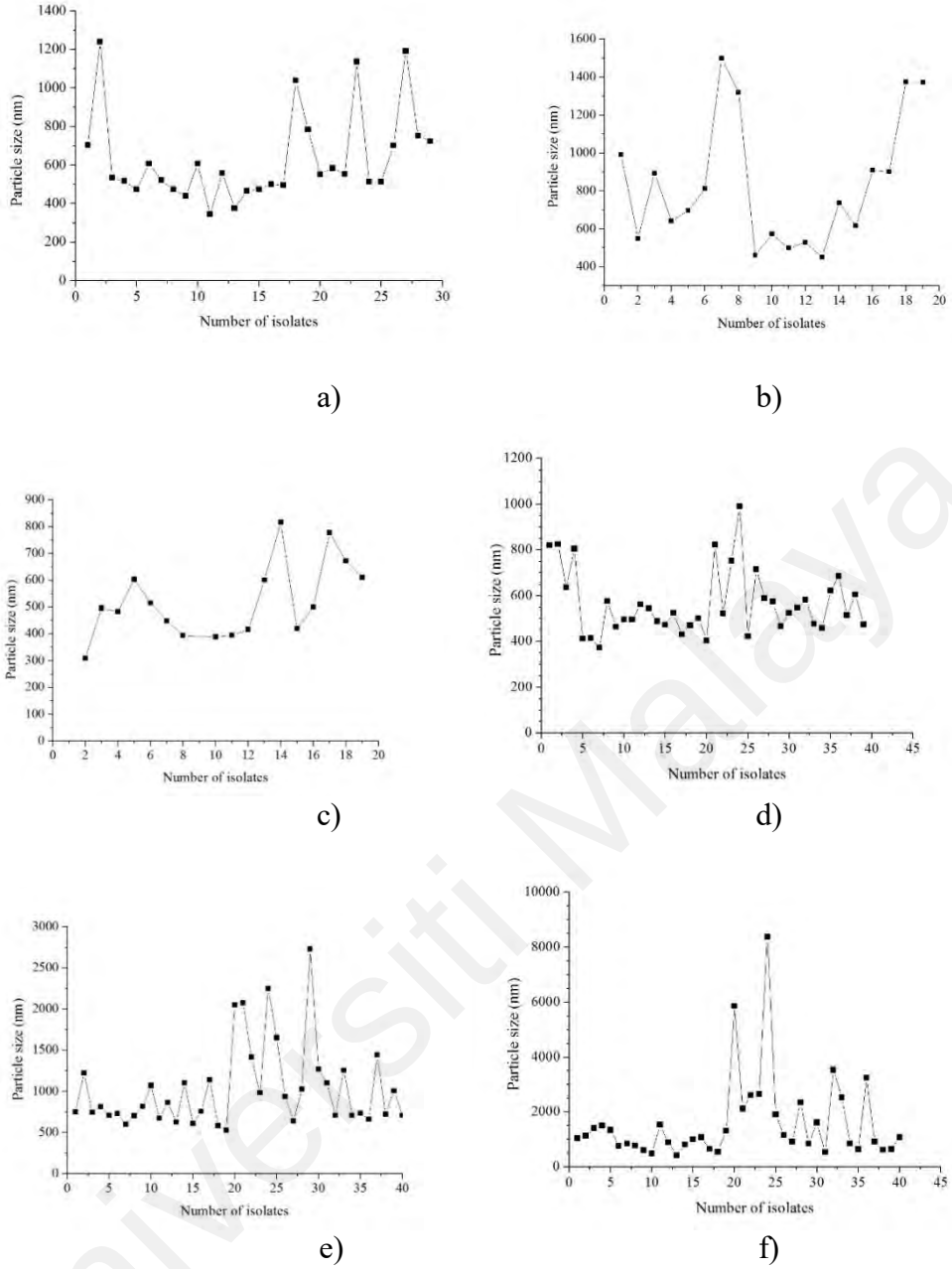


Figure 4.10: Particle size of fractions. Particle size distribution of a) 1.0 g fractions and b) 0.96 g fraction 1 to 14, iii) particle size distribution of c) 0.87 g and d) 0.58 g fractions, e) 0.35 g and f) 0.25 g fractions

4.3.10 Assay or concentration of proteins in each fractions by;

Besides direct plot measurement, secondary data were calculated from the area under the FTIR peaks in the region where the most significant changes occurred in each sample.

i. Area under the peak for region 990 to 1100 wavelength

a) b)

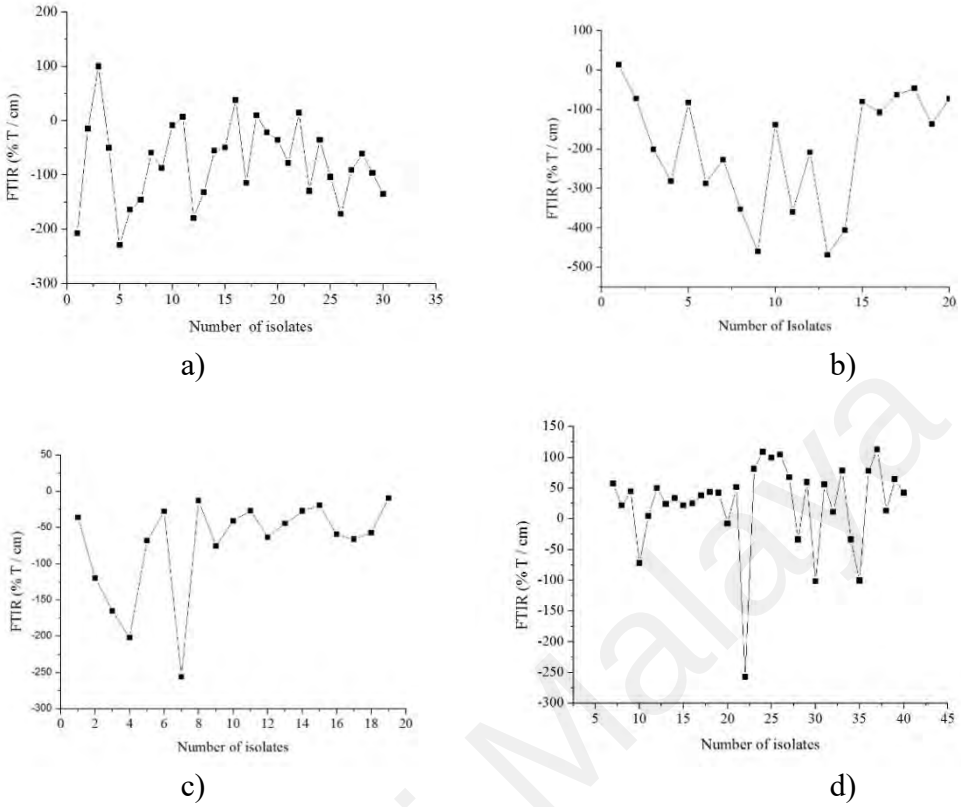


Figure 4.11: Area under the graph plot of load amount varied protein fractions. Estimation of concentration by the area under the peak of FT-IR. For a) 1.0 g and b) 0.96 g fractions, for c) 0.874 g and d) 0.2627 g fractions

From the UV data, the concentration of proteins was calculated at 280 nm and 595 nm using the reagent and the calibration curve of estimated concentration.

ii. Assay plot calculated from UV at 595 nm

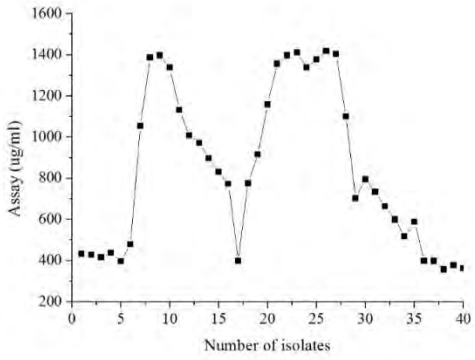
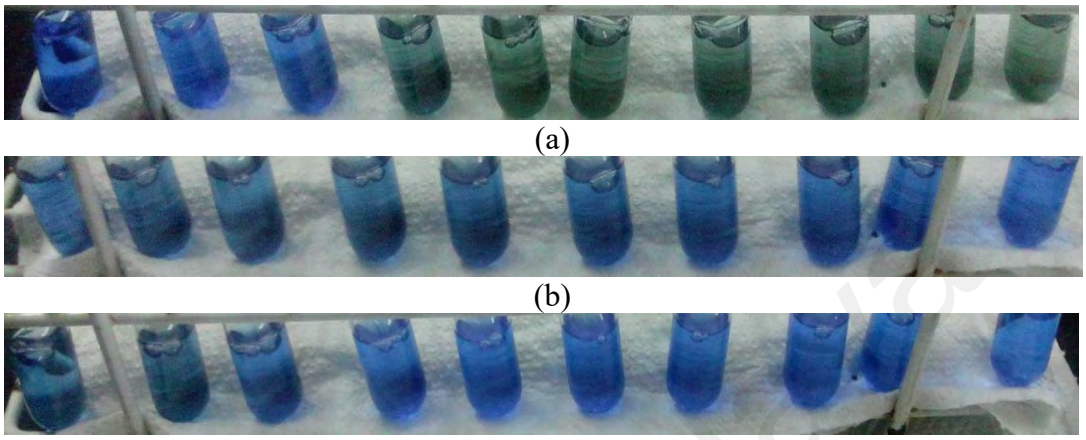


Figure 4.12: Assay plot using Bradford reagent kit for UV at 595 nm using PS cuvette. Assay for 0.2627 g from fractions number 1 to 40

This assay plot were used in investigating protein concentration from calculation using reagent and two major peaks constructed will provide information for final selection of fraction as below sample.



(a)

(b)

(c)

Figure 4.13: Colour indication of fraction containing proteins. Selection of fraction for freeze-drying. Using colour indication of Bradford reagent for UV@ 595 for assay. (a) Sample : 0.26 g in 30 ml TRIS fr 1 to 10 from right, (b) Sample 0.26 g in 30 ml TRIS fr 11 to fr 20 from first right , and (c) Sample 0.26g in 30 ml TRIS fr 21 to fr 30 from right

Figure 4.13 shows the color changes when each isolate interacted with the Bradford reagent. Blue color indicates the presence of protein at a specific fraction. For the above set of fractions, the protein first appeared at isolate 7 (a) and the last protein appeared at isolate 27 (c). The Bradford reagent is used to measure the protein concentration and it is useful when a particular fraction needs to be freeze-dried or further investigated. Solid blue color represents a higher protein concentration and slight purple to grey color represents a very low concentration of protein present in that particular fraction. Besides protein concentration, this step can also be used as a guideline on the time to start collecting fractions and the endpoint of fractionation. Thus, this means that the proteins only come out in fraction number 7 and complete fractionation occurs when the fraction color stays purple or grey.

4.4 Physical properties of fractions

In order to have a better bird's eye view of all the proteins, a summary of the physical properties of fractionated proteins is tabulated. It is also used to identify if any consistency or profile has the potential to exploit further works. The general profile displayed at present will easily locate the highest amount of specific proteins at a specific fraction. For the proteins of interest, the specific fraction could either be used directly or further scaled up to increase the dried amount. The summary includes the concentration of protein from UV absorbance at 595 nm using the Bradford reagent, the ζ potential of fractions, and the area under the graph calculated from the FTIR spectra, pH of each fraction, particle size, elution time, and SDS PAGE. The concentration and area under the graph were used as an initial prediction of protein concentration. Meanwhile, ζ potential was used to predict protein stability at a specific fraction. Particle size provides the information on the size of particles for specific fractions containing proteins and lastly, SDS PAGE displays the molecular weight of specific proteins at a specific fraction when compared with the protein marker. SDS PAGE of fractions was used as a tool to investigate the molecular weight of proteins in specific fractions, which can be predicted from the column selection and the mode of separation can either be via size exclusion or selectivity.

Table 4.4: Prediction of *hevea* protein order of elution base on Mw and IEP

Proteins	Mw	IEP pI
Hev b4	53-55	4.5
Hev b9	47.7	5.6
Hev b13	43	5.0
Hev b7	42.9	4.8
Hev b2	35.1	9.5
Hev b14	33.7	8.7
Hev b11	33	5.1
Hev b10	26	6.3
Hev b3	22.4	4.8
Hev b6	21	5.6
Hev b5	16	3.5
Hev b8	15	4.9
Hev b1	14.7	5.0
Hev b12	9.3	10.8

At physiological pH, most proteins are above their IEPs and have a net negative charge. A pH lower than the IEP causes protonation of acidic groups and a net positive charge on the protein. In each circumstance, the like-charged molecules repel each other, reducing the likelihood of protein aggregation (Agilent application Note). Protein consists of amino acids, some of which have polar side chains or contain acidic or basic groups. At the IEP, the protein is uncharged and the charge repulsion of similar functional groups will be at a minimum, allowing aggregation to take place. Many proteins precipitate under these conditions (Agilent Note). The pH of the running buffer is maintained at 8.

Tables 4.4 and 4.5 present the type of proteins eluted first based on the IEP and molecular weight. It is predicted that a higher molecular weight protein elutes first based on the principle of SEC. The bigger the size, the shorter the retention time in the column. Meanwhile, the smaller the size, the longer the retention time in the column. Both theories are used as the basis in predicting the order of elution of *hev* protein.

Table 4.5: Prediction of *hev b* proteins order of elution base on IEP, *pI*

Proteins	IEP *pI*	Mw
Hev b14	8.7	30/33.7
Hev b2	9.5	35.1
Hev b10	6.3	22.9/26
Hev b9	5.6	47.7
Hev b6	5.6	21
hev b12	10.8	9.3
Hev b11	5.1	33
Hev b13	5.0	42.9
Hev b1	5.0	14.7
Hev b8	4.9	13.9/15
Hev b7	4.8	42.9
Hev b3	4.8	22.4
Hev b4	4.5	53-55
Hev b5	3.5	16

Tables of fraction characteristics was constructed to compile the physical properties in a single table. Each table comprises the load amount and each fraction has its physical properties in line with the estimated elution time. The fractions containing proteins based on the initial prediction using the Bradford reagent and UV-vis spectroscopy were freeze-dried for the application study.

Table 4.6: Fractions characteristics, sample: 1.0 g in 30 ml tris

Fr	Conc. (UV595)	ζ	Area FTIR %T cm^{-1}	pH	Size Nm	Time (Min)	SDS PAGE Mw (KDa)
1.0g	29.42		-46.49				80,60,50,45,40, 30,25,10
1	0.3792	-24	-206.84	7.32	704.6	13	60
2	0.649	-22.7	-14.6	7.72	1242	27	50
3	4.235	-24.4	-100.24	7.63	535.1	38	50
4	24.51	-33.5	-49.8	7.58	516.6	50	50
5	25.63	-28.5	-228.40	7.49	472.9	63	50
6	25.56	-36.2	-164.09	7.42	607.8	73	50
7	23.53	-32	-145.36	7.47	521.4	83	50
8	23.12	-31.3	-58.96	7.57	472.9	93	50
9	24.14	-28.2	-87.67	7.37	440	106	-
10	36.30	-23.9	-8.80	7.27	608.3	116	25, 50
11	44.44	-17.6	6.85	6.69	344.2	126	-
12	38.21	-20.3	-179.15	6.83	557.9	138	10, 40
13	38.79	-16.5	-131.94	6.69	376.6	150	10, 25, 40
14	31.77	-17.3	-55.29	6.63	467.2	161	40
15	23.77	-14.8	-49.11	6.39	474	171	-
16	20.24	-14.6	38.49	6.40	500.2	186	40
17	15.25	-18.2	-114.06	6.32	496.1	198	-
18	11.27	-22.4	9.54	6.51	1040	211	10,25, 40
19	10.24	-20.7	-21.54	6.21	785	223	10
20	6.44	-20.3	-35.06	6.27	550.3	235	10, 25, 40
21	4.8	-20.7	-78.08	6.73	584	248	-
22	5.35	-22.4	-14.96	7.04	553.6	261	-
23	3.02	-18.5	-129.56	7.13	1138	277	-
24	3.10	-20.6	-35.36	7.43	513.5	289	-
25	3.23	-25.3	-103.58	7.66	513.7	309	-
26	2.94	-30.7	-171.81	7.75	701.9	321	-
27	1.81	-27.3	-91	7.75	1193	337	-
28	1.65	-25.2	-60.41	8.23	752.6	351	-
29	0.73	-33	-95.90	8.25	722.8	368	10
30	1.89	-33	-135.5	8.34	704.6	381	10, 40

Table 4.6 shows a summary of the characteristics of 1.0 g isolates and protein identification. The highest protein concentration occurred at fraction number 11, but the SDS page did not show any band. This indicates that either the concentration of the measured protein is not in the selected reference band or there might be a mistake in the procedure. The highest ζ potential value was recorded for fraction number 6 and the highest protein concentration determined from the FTIR band was for fraction number 5. Other data, such as pH, volume, and time were used for the estimation of specific fraction characteristics at a specific time and volume. By observing the common profile of pH, lower pH at 5 and 6 can be used to indicate the presence of proteins in those fractions. A complete fractionation process is also significant when the pH value started to approach the buffer pH. Other common rules used are doubling the packed volume of the mobile phase when fractionating and an approach to check if the proteins are still in the column

is by suing fraction characteristics. Variation in load amount and dilution factor is part of the trial run in optimizing the column. By reducing the load amount to about 70 %, the number of fractionated proteins increased from 3 to 12 (Table 4.10). The number of protein fractions was affected by the load amount, but specific changes in protein species were not observed when diluted protein samples were introduced.

Tables 4.6 to 4.11 were constructed for easier identification of the exact fraction and their physical properties. The constructed profile for each physical property was used earlier to estimate the number of fractionated proteins but it was quite difficult to read the value from the plot. The tables also provide information on the fraction containing proteins and the total running time for chromatography. These tables are important when the fractions are used in applications, such as antimicrobial activity, where the backward operation is used to identify the process that produces such fractions and for which applications.

Table 4.7: Fractions characteristics sample 0.87 in 30 ml tris

Fraction	Conc g/l	ζ	Area %T/cm^{-1}	pH	Size nm	Time	SDS PAGE Mw KDa
0.87	555.6		-43.10			-	6,5,4,35,3,2,10
1	-	-21.1	-36	6.85	1124	12	-
2	-	-27.4	-119.6	7.43	309.4	24	-
3	8.49	-35	-165.16	7.35	495.8	40	60
4	69.12	-38.1	-201.70	7.47	481.9	53	60
5	35.34	-34.1	-68.10	7.28	603.5	68	60
6	8.49	-33.9	-27.37	7.25	515.2	83	60
7	5.41	-34.8	-12.83	7.29	448.3	94	-
8	98.66	-32.2	-256.12	6.97	392.8	109	40,35,30,20
9	253.3	-13.6	-75.35	6.44	389	124	40,30,20
10	112.7	-15.6	-40.53	6.34	395	139	60,40,30,20
11	-	-14.3	-26.3	6.54	416.1	151	-
12	-	-14.2	-63	6.55	601.4	166	60,10
13	-	-14.5	-44.45	6.56	816.8	179	-
14	-	-23	-27.06	6.54	419.9	191	-
15	-	-26.7	19.2	6.46	499.8	204	-
16	-	-26.1	-59.19	6.65	777.9	218	-
17	-	-28.2	-65.87	6.77	673.2	235	40,20,10
18	-	-35.3	-57.01	6.74	610.4	249	-
19	-	-30.6	-9.09	6.89	1124	275	-

Table 4.7 summarizes the characteristics of each fraction for the initial load amount. From the table, the highest protein concentration was recorded for fraction number 8 to10. Referring to Figure 4.5(b) the highest peak was recorded for fraction number 7 to 14. By comparing the SDS PAGE and the chromatographic profile in Figure 4.7(b), the number of proteins at specific fractions based on molecular weight matched. Figures 4.7(b) and 4.11(b) are the plots for each physical characteristic listed above. The elution time and SDS PAGE added in the table of physical properties are used for the mapping process from fractionation to characterization. Elution time is useful for predicting the size of proteins commonly eluted first and last. SDS PAGE also provides the estimation of the molecular weight of proteins separated by electrophoresis, which is used to compare the fractionated *hevea* proteins.

By observing the particle size from fraction number 1 to 19, a larger size provides information on the absence of protein at that particular fraction. However, at some fractions, proteins with smaller particle sizes were detected. Referring to Tables 4.7 and 4.8, only 8 out 19 fractions contained proteins, while at 1 g loading, the fraction still contained protein and was distributed evenly. Reduction in the area under the peak of the FTIR spectra also indicates reduced protein concentration. Based on the pH of fractions, complete looping was displayed when the fractionation took place, where the initial pH was also recorded toward the end of the process. The starting load amount of the sample from Table 4.8 was 0.58 g and 250 mL of buffer.

Table 4.8: Fraction characteristics Sample 0.58 g in 250 ml tris

Fraction	Conc µg/l	ζ	Area %T/cm	pH	Size nm	Time min
1	0.554	-19.1	18.56	6.53	820.8	8
2	0.535	-22.9	-38.13	6.61	824.8	17
3	0.534	-23.4	14.88	6.84	637.2	23
4	0.557	-30.5	-22.15	6.74	805.5	32
5	0.740	-30.5	-22.68	6.60	412.3	40
6	1.319	-32	-13.08	6.95	414.2	48
7	1.622	-31.1	-67.77	6.69	373.9	56
8	1.646	-28.3	5.23	6.76	576.8	64
9	1.629	-29.6	-10.81	6.59	464.2	73
10	1.641	-31	-11.03	6.58	496.1	80
11	1.649	-28.5	-21.32	6.63	495.2	90
12	1.621	-33.3	-16.71	6.46	561.7	98
13	1.605	-30.2	1.81	6.57	544.4	105
14	1.601	-24.5	-14.42	6.41	488.2	111
15	1.614	-27.3	12.48	6.45	473.5	118
16	1.638	-30	-2.39	6.39	524.2	126
17	1.711	-26.8	-79.67	6.35	431.8	135
18	1.704	-27.8	-25.66	6.24	470.5	141
19	1.753	-23.8	-34.17	6.24	501.3	149
20	1.753	-17.5	-27.96	6.25	403.9	158
21	1.773	-21	-4.92	6.12	823.1	165
22	1.766	-17	2.91	6.05	521.8	172
23	2.381	-10.6	-	5.79	752.5	179
24	1.757	-11.9	-	7.04	991.8	185
25	1.696	-11.2	-	6.99	422.8	192
26	1.689	-11.6	-	6.19	715	197
27	1.667	-11.7	-	6.09	588.4	204
28	1.673	-13.9	-	7.20	574.3	210
29	1.633	-14.6	-	6.57	466.1	215
30	1.614	-15	-	7.22	525.4	227
31	1.583	-13.2	-	6.08	547.8	235
32	1.583	-13.2	-	7.28	581.3	242
33	1.588	-14.7	-	6.70	476.2	250
34	1.506	-15.6	-	6.71	458.4	256
35	1.381	-14.5	-	7.32	622.2	263
36	1.2706	-13.9	-	7.37	685.4	270
37	1.21085	-14.4	-	7.43	515.4	276
38	1.05859	-15.8	-	6.72	605.4	286
39	1.03008	-14	-	7.41	474	294
40	1.07902	-13.7	-	6.73	-	301

A very dilute sample was used to investigate the effect of sample dilution on the characteristics of the fractions. As the sample was diluted, the fraction number increased as predicted, and the first protein fraction that eluted was fraction number 6 or 7. Other characteristics displayed an almost identical profile as a higher load amount and a thicker sample were used.

Table 4.9: Fraction characteristics 0.35 g in 100 ml tris

Fraction	Conc g/l	ζ	Area %T cm^{-1}	pH	Size Nm	Time (Min)
1	2.76	-28.9	-	7.37	750	8
2	3.42	-29.7	-	7.26	1223	13
3	6.58	-28.7	-	6	744.1	26
4	9.68	-31.3	-	6.96	811.1	30
5	40.97	-28.8	-	7.14	707.2	39
6	78.43	-26.3	-	7.27	728.3	57
7	98.35	-28.9	-68.85	7.38	599.3	71
8	94.28	-32.4	37.9	7.45	701.2	89
9	80.99	-30.1	-11.43	7.32	815.6	98
10	75.49	-29.2	-132.41	7.37	1070	108
11	80.76	-29.8	-97.47	7.38	672.2	120
12	79.9	-31.1	-41.48	7.35	865.2	126
13	77.82	-32.2	-34.61	7.34	623.2	135
14	77.36	-33	-75.11	7.35	1104	146
15	39.86	-38	-15.85	6.73	609.9	156
16	80.6	-33.4	-21.3	7.19	753.7	171
17	102.47	-26.2	-82.15	5.82	1141	181
18	103.94	-21.6	-11.38	6.45	584.3	192
19	102.695	-26.5	-13.43	5.15	524.2	203
20	106.07	-23.9	-1.41	5.76	2050	214
21	102.32	-23.3	-	5.48	2076	227
22	100.09	-19.1	-	4.56	1417	241
23	100.22	-17.1	-	5.03	983.5	251
24	84.36	-17	-	5.56	2250	260
25	81.7	-17.5	-	4.96	1653	271
26	84.42	-19.2	-	6.53	939	285
27	90.21	-18.9	-	4.81	641.5	299
28	96.62	-20.6	-	5.56	1026	315
29	97.94	-17.1	-	4.68	2730	331
30	83.83	-20.2	-39.69	4.91	1272	345
31	59	-22.9	-10.8	6.6	1103	362
32	42.39	-23.1	-73.2	6.31	706.4	376
33	40.72	-23	-172.06	6.74	1254	387
34	24.06	-23.2	-32.27	6.12	709.2	397
35	19.62	-23.3	-41.52	6.45	734	411
36	19.32	-24.4	-109.57	6.61	659.6	422
37	15.78	-19.6	-33.15	6.13	1444	437
38	16.44	-27.1	-6.39	6.62	721.3	456
39	17.36	-28.4	-6.39	6.76	1006	470
40	18.22	-29.2	-22.46	6.81	709.6	491

. Table 4.9 tabulates the characteristics of the fraction containing proteins with 0.35 g of load amount and was diluted three times. Based on Tables 4.9 and 4.8, the concentration of proteins could not be calculated for UV absorbance at 595 nm as the sample was too dilute. This may be due to the limitation of the reagent used. It is recommended that the maximum dilution should be up to 300 % if the same reagent is used. Moreover, SDS PAGE could not be conducted at this dilution due to the limit of

silver staining used for comparing the proteins with the standard, which is below the recommended concentration limit of 20 ng. The load amount of 0.26 g was the smallest weight introduced to the column and the total number of fractionated proteins was 11 out of 15 known *hev* proteins. The fraction containing known proteins started to elute at fraction number 8 to 23. This dilution method works well in separating proteins into more different types, but the method also offers a smaller number of fractions, which limits its application. Both antimicrobial and metal removal studies require at least 0.1 g of protein, but with dilution, approximately 0.01 g is the greatest amount collected for each type. Scaling up the sample requires a bigger column and is also time-consuming. Other modifications suggested in previous works might be useful, but they will make this wastewater project less viable. Therefore, the separation of individual proteins will not be considered for the application study. The main interest of modern SEC is to improve the analysis throughput by reducing the analysis time (Fekete *et al.*, 2014).

For the 0.26 g sample fraction, the smaller molecular weight band and the higher molecular weight came out in the first fraction containing protein. The smaller proteins were discarded based on the SEC principle that the smaller size is retained longer in the column. Thus, the band may be due to the bead trapped in the protein from the previous sample and not from the 0.26 g sample. By comparing 0.58 g and 0.2 g of load amount, the diluted sample with a high amount of protein does not have a better resolution. Hence, dilution does not influence the baseline resolution but the load amount must be very low for the proteins to be well separated in a gravitational flow chromatographic column, where a bigger column may help to tolerate high protein concentrations.

Table 4.10: Fraction characteristics sample 0.26 g in 30 ml tris

Fraction	Conc. g/l	ζ	FTIR %T cm^{-1}	pH	Size nm	Time (Min)	SDS PAGE Mw (k Da)
1	432.17	-19.3	-	6.83	624.5	9	-
2	427.18	-22.8	-	6.9	1008	19	-
3	416.57	-19.8	-	7.01	497.2	26	-
4	437.05	-22.7	-	7	965.9	33	-
5	396.48	-22.7	-	7.05	1219	45	-
6	478.80	-28.2	-	7.06	1168	52	-
7	1054.9	-33	57.4	7.5	580	61	-
8	1386	-38.4	21.8	7.1	693.3	68	10, 120
9	1398	-34.4	44.4	6.99	657.7	76	15, 120
10	1338.6	-35.7	-72.44	7.43	590.9	82	10,15,25
11	1131.5	-35.1	4.73	7.13	646.8	90	15,25
12	1009.4	-33.7	50.01	7.06	711.6	99	15, 25
13	971.5	-29.8	24.45	6.85	1718	106	10,15,25,40,
14	897.04	-32.9	33.49	6.89	762.4	114	25,40,50,
15	830.44	-31.9	21.87	6.9	598.8	119	15,50,60
16	772.15	-22.5	25.4	6.64	876.7	129	20
17	398.15	-24.9	38.35	6.52	588.3	143	20
18	775.02	-18.5	44.09	6.53	1355	151	20,50
19	915.09	-25.8	42.43	6.5	753.3	160	20,50
20	1159.6	-20	-7.6	6.41	998	167	20,30,40,50
21	1356.7	-7.79	51.58	6.44	3951	175	20,30,40,50
22	1398.0	-7.98	-257.56	6.18	1944	181	20,30,40,50
23	1411.1	-15.8	81.59	6.15	1520	189	20,30,40,50
24	1338.0	-13.3	109.11	6.13	2004	198	-
25	1376.8	-10.7	99.97	6.26	3996	207	-
26	1417.5	-11.2	104.54	6.29	1356	213	-
27	1404.2	-10.2	68.06	6.3	1455	223	-
28	1101.0	-12.9	-33.69	6.33	1351	232	-
29	701.79	-10.9	59.64	6.32	1596	242	-
30	794.04	-18.7	-101.62	6.45	796.6	250	-
31	734.89	-20.7	56.37	6.54	493.9	260	-
32	663.56	-23.5	11.33	6.88	481.7	271	-
33	599.18	-30.5	78.99	6.95	545	281	-
34	517.27	-36.4	-33.64	7.08	526.3	290	-
35	587.69	-34.2	-100.59	7.1	566	299	-
36	398.56	-34.2	77.89	6.7	770.8	309	-
37	397.65	-29.3	113.08	6.77	1049	319	-
38	356.59	-37.7	13.21	7.01	636.4	332	-
39	377.62	-30.4	64.82	6.66	824.7	342	-
40	361.93	-30.1	42.47	6.87	1084	355	-
41	-	-30.5	-	6.76	1498	366	-
42	-	-29.8	-	6.95	862	376	-
43	-	-30 .5	-	6.78	737.8	388	-
44	-	-32.4	-	6.98	857.5	398	-
45	-	-32.1	-	6.92	1452	407	-

0.25 g of load amount was used to check whether the load amount results in better resolution of protein fraction. It was confirmed that for this 2.5 cm column inner diameter, only 0.25 g or less proteins tolerated in terms of protein species. SEC separates biomolecules according to their hydrodynamic radius. SEC is an entropically controlled

separation process in which molecules are separated based on molecular size differences rather than by their chemical properties (Fekete *et al.*, 2014).

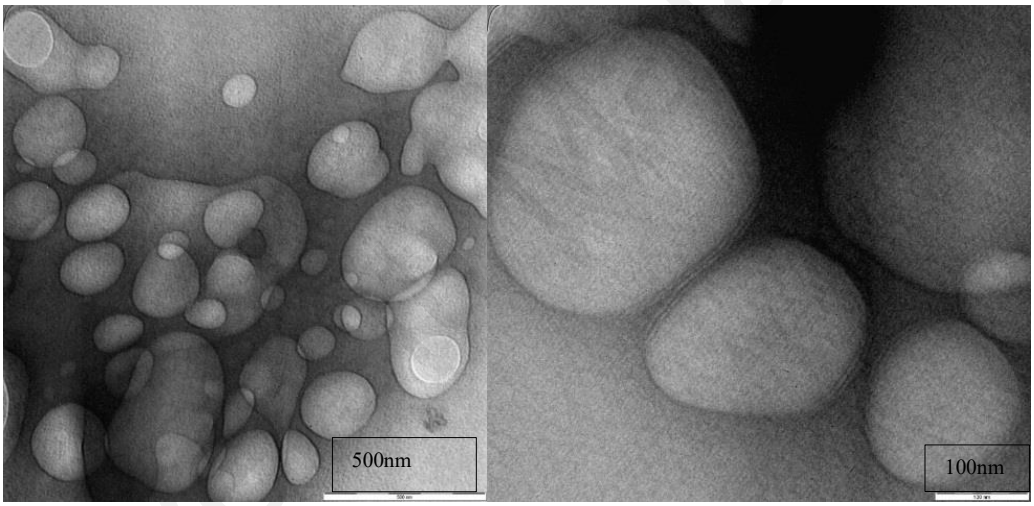
Table 4.11: Fraction characteristics 0.25 g in 30 ml DI

Fraction	ζ	pH	Size nm	Time (Min)
0.25				
1	-21.1	6.66	1047	11
2	-15.4	6.47	1132	20
3	-14.1	6.82	1405	30
4	-14	6.7	1508	38
5	-19.7	6.39	1337	44
6	-16.6	6.37	755.8	52
7	-24.8	6.98	840.7	62
8	-25.9	6.58	782	70
9	-22.9	6.7	605.8	77
10	-15.1	6.49	481.1	88
11	-20.5	6.49	1530	96
12	-23.1	6.53	899.5	106
13	-25.1	6.41	412.3	116
14	-22.2	6.5	816.2	125
15	-15.3	6.21	1007	135
16	-20.9	6.23	1080	145
17	-20.1	6.14	650.8	152
18	-20.6	6.27	551.7	160
19	-18.6	6.27	1317	168
20	-25.6	6.06	5860	175
21	-3.07	6.26	2117	185
22	-4.32	5.87	2611	193
23	-4.18	5.94	2656	201
24	-5.99	5.67	1801	212
25	-6.29	5.57	1908	224
26	-9.55	5.5	1161	234
27	-6.98	5.7	914.6	246
28	-28	5.98	2353	261
29	-8.19	5.86	849.1	270
30	-9.18	6.28	1612	284
31	-20.9	6.62	536.4	296
32	-29.4	6.87	3536	309
33	-38.2	6.58	2533	319
34	-25	6.5	852.9	333
35	-12.7	6.58	645.3	347
36	-25.2	6.64	3248	358
37	-20	6.68	914.5	368
38	-12.2	6.6	619.3	376
39	-17.6	6.73	647.7	384
40	-14.2	6.67	1077	394

From Table 4.11, the pH of the first and last fractions indicated a complete fractionation process but the pH profile was slightly acidic compared to the 0.26 g fraction. A complicated particle size profile was observed at a lower load amount, which

might be due to very large size variation. Furthermore, the measurement or the packed column used is not suitable for specific proteins fractionated at this specific load even though 11 species out of 15 targeted proteins managed to be fractionated. Previous work on latex lectin binding used acetone to precipitate proteins and chromatographic separation using Sepharose 6B column managed to obtain 14 and 24 kDa proteins using UV absorbance for constructing the chromatographic profile (Wititsuwannakul, Rukseree*, et al.*, 2008).

4.5 TEM images- NRL waste, precipitate, dialyzed and freeze-dried NRL waste protein

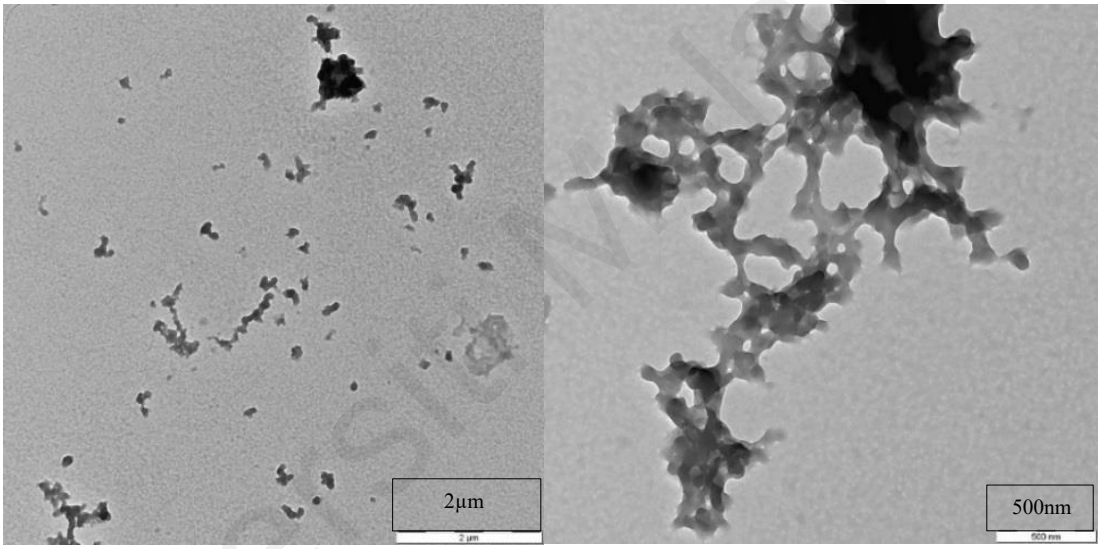


(a) **(**b)

Figure 4.14: NRL waste effluent at (a) 500 nm and (b) 100 nm 10k and 40k magnification

Figure 4.14 shows the transmission electron microscopy (TEM) image of NRL waste to investigate the particle size of rubber and other solids that may exist in the serum phase. The image size matched the average NR particle size range of 20–400 nm or a mean diameter of approximately 636 nm (Sansatsadeekul *et al.*, 2011). The smallest particle was around 100 nm and the largest particle in this image (Figure 4.14(b)) was approximately 200 nm. After ultracentrifugation or washing, spherical rubber particles isolated from the cream layer represent a mean diameter of approximately 170 nm (Wang

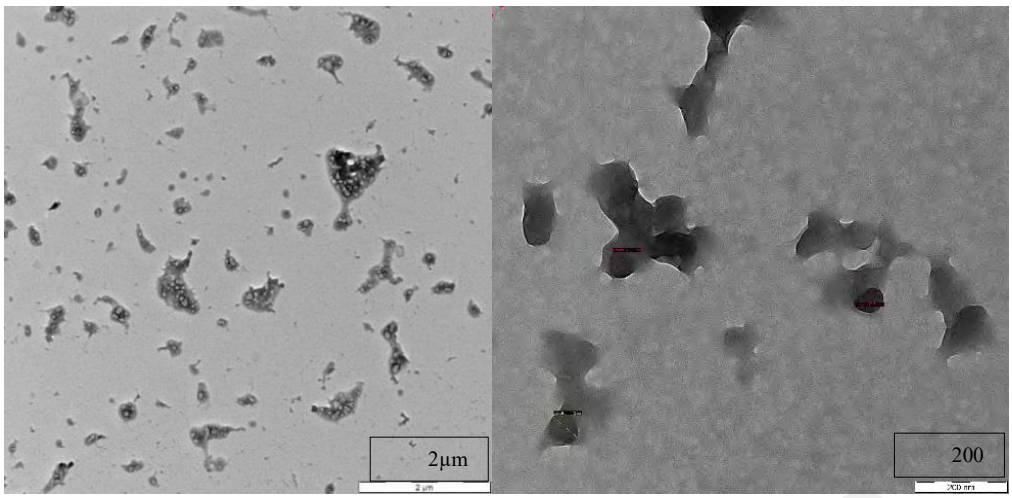
et al., 2016). For the image taken from the NRL wastewater, the surrounding water and the fused image (arrow) are common for a cluster of rubber particles, which are easily attracted to each other due to polarity. At a stable state, water acts as a hydration or bound layer and causes repulsion between rubber particles. However, at an unstable state, due to the absence of electrical repulsion, rubber particles agglomerate and cause coagulation. These particles were filtered out using filter paper and the free protein layer was collected for extraction purposes. The extracted proteins were freeze-dried and redissolved before being observed using TEM.



(a) (b)

Figure 4.15: Dialyzed protein unstained at 2 µm or 4k (a) and 500 nm or 10k (b) magnification

Figure 4.15 shows the captured image of clustered particles, showing protein particles or aggregates of proteins. Dialyzed proteins at 500 nm showed clear linkages as looks alike ribbon or helical structure constructed from the amino acids sequence.



(a) (b)

Figure 4.16: DI pcp fractionated protein at (a) 2 µm bar (fraction 7 sample 220314) (b) 200 nm

Figure 4.16 presents the extracted proteins at 2 µm. The distribution of particles or clusters of particles contains white spots in each big cluster (Figure 4.16(a)). The dark spots in Figure 4.16(b) were measured. The dark particles in the cluster were measured individually, where the sizes were found to be 68 nm, 78 nm, and 70.5 nm. By comparing these values with the values in Tables 4.6 and 4.7 where the particle size for the fraction containing proteins was measured using a Zetasizer, the value tripled to approximately 600 nm. This might probably be due to the measurement of clusters and does not represent individual particles. As proposed by Guidelli (2011), the diameter obtained by the dynamic light scattering technique is of the same order of magnitude as those of agglomerates, as indicated by TEM micrographs, which suggests that the DLS result is associated with a large state of aggregation of particles (Guidelli *et al.*, 2011).

4.6 Study of Multiple Centrifugation against Ammonium Sulphate Extraction

For the investigation of other alternative methods besides salt precipitation, physical methods such as centrifugation and filtration were applied with some of the data below. Saturated ammonium sulfate solution was used to remove the surrounding water layer from protein particles, causing the particles to aggregate and precipitated out. The salt

that overtakes the surrounding water layer was removed using a 3.5 k MWCO dialyzing tube due to the molecular weight of *hevea* protein of 5 to 200 kDa. The middle layer of NRL waste was withdrawn after each centrifugation and recentrifuged to concentrate protein particles. The protein particles were still surrounded by a water layer and the rubber particles limited the formation of protein aggregates. The rubber particles of *H. brasiliensis* were observed to have a bimodal size distribution and could be classified into large rubber particles (LRPs, 0.20–0.35 μm) and small rubber particles (SRPs, 0.08 to 0.19 μm) (Wang *et al.*, 2016).

Table 4.12: Effect of number of centrifugation on particle size, ζ, and yield

Process	Size nm	ζ	Appearance	Yield
1st pH 4.3	272.9	-7.92	Sticky	
2nd pH 4.3	223.4	-8.5	Less sticky	
3rd pH 4.3	168.9	-8.68	Less sticky	
4th pH 4.3	159.2	-8.01	Not sticky	0.4325 g

The sticky appearance of the freeze-dried centrifuged waste is because the rubber particles still exist. After repeated filtration using a 0.2 μm nylon filter, the sticky portion decreased but the filtration resulted in additional cost. Only the final yield was measured, which amounted to 0.4325 g. A previous study using polyethylene glycol (PEG) as a surfactant to extract proteins from skim serum found that the extractable protein (EP) from the serum increased between the extraction in centrifugation and a continuous process (Danwanichakul *et al.*, 2014). This said work used the same method and instrument. However, the use of PEG is beyond our scope and interest because it is proven that *hevea* proteins could be extracted by using centrifuge alone, but the rubber particles still exist and caused sticky appearance. Thus, weighing and storage are the issues to be solved.

4.7 Fourier Transform Infrared Spectroscopy of NRL waste and Extracted proteins

A Fourier transform infrared spectrometer uses an interferometer to measure a spectrum. An infrared spectrum does not provide enough information to identify a structure conclusively unless an authentic spectrum is available to compare "fingerprints". Recent studies used attenuated total reflectance (ATR) configuration to characterize NR. ATR allows FTIR characterization and avoids material destruction compared to the classic transmission that requires pre-dissolution or pressing the sample at a rather high temperature (Rolere *et al.*, 2015).

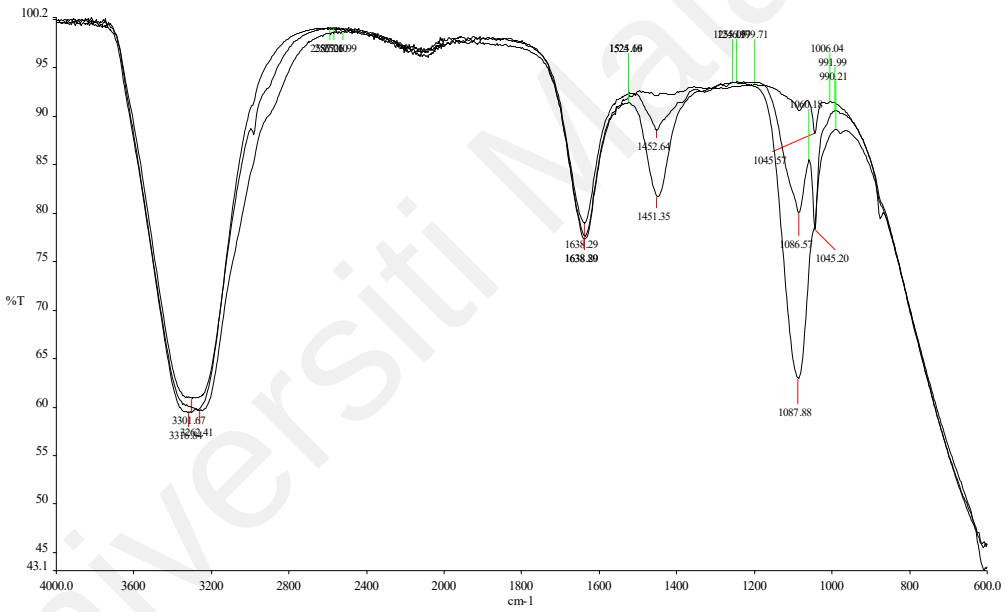
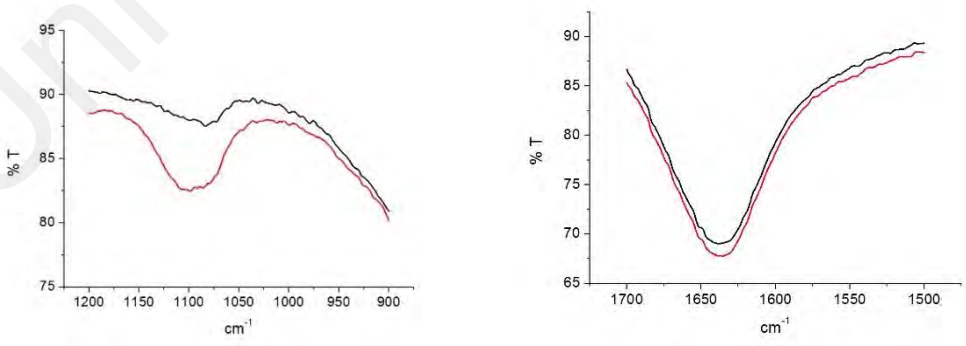


Figure 4.17: FTIR spectrum of various protein extraction methods. NRL waste (--) MC extracted proteins (--) and SP extracted proteins (--)

The purpose of using FTIR spectroscopy is to identify any specific protein peak or rubber phase peak in order to distinguish between proteins and rubber particles. Significant changes were observed when physical treatment methods of extraction were implemented (e.g., centrifugation and salt precipitation). In Figure 4.17, the arrow pointed at the region where the significant differences of three peaks were observed. The peak is at 1045 cm^{-1}. From Figure 4.17, peak number two, which is multiple centrifugations (MC) purified protein, gives a broader peak that indicates a higher protein concentration.

Meanwhile, peak number three, which is ammonium sulfate purification (SP) purified protein, gives a sharp but thin peak that indicates a lower protein concentration. Compared to the use of the Bradford reagent for protein concentration measurement, MC contains a higher amount of protein.

A previous study on protein using FTIR listed that amide I peak occurred at frequency 1630 cm^{-1}, amide II at 1541 cm^{-1}, and N-H stretching at 3283 cm^{-1} (Rolere *et al.*, 2015). From Figure 4.17, significant changes in the peak transmittance intensity could be observed at 1060 cm^{-1} (NR), 1086.57 cm^{-1} (MC), and 1087.88 cm^{-1} (SP), and at frequency 1451 cm^{-1} for MC and SP. Meanwhile, at 1638 cm^{-1}, only 2% difference in transmittance was determined. The peak at 1090 cm^{-1} was assigned to twisting $–CH_2-$ and $–CH_2-$ deformation at 1446 cm^{-1}. Only one sample of the purified protein showed the existence of amide II. However, a broad peak at 1638 cm^{-1} confirmed the existence of protein but there was a little variation in intensity, which could be seen from the amide I peak (stretching $–(C=O)$ - $NH-R_2$). An intense broad band between 3200 and 3500 cm^{-1} indicated the presence of hydroxyl groups (Rippel *et al.*, 2003). The N-H stretching peak at 3283 cm^{-1} overlapped with the hydroxyl peak because this protein dissolved in water.



a) b)

Figure 4.18: Comparison of FTIR of MC and SP purified protein at (a) 900 - 1200 frequency and (b) 1500-1700 frequency, where MC purified (---) and SP purified proteins (---)

Furthermore, the peak at 3280 cm^{-1} was more apparent in cream rubber, but our sample is skim rubber. Thus, this region is not taken into consideration for this study. By comparing the FTIR spectra of purified proteins at 1000–1200, 1500–1600, and 1600–1700 cm^{-1} (Figures 4.18(a) and (b)), it can be seen that there was only a small variation in the profile for 1600–1700 cm^{-1} and 1500–1600 cm^{-1} regions, which are amide I and amide II. The region of 900–1300 cm^{-1} showed a huge variation in the peak obtained. The consistent profile leads to the investigation on the metal extraction sample and the relationship with the region of 900–1200 cm^{-1}.

4.7.1 Effect of pH on Amount of MC- and SP-extracted proteins

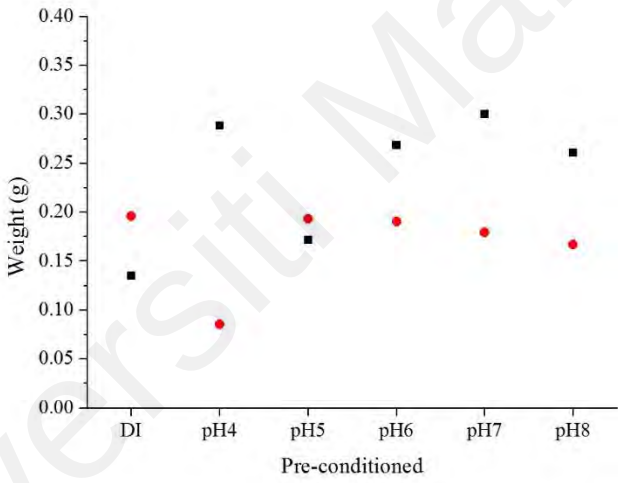
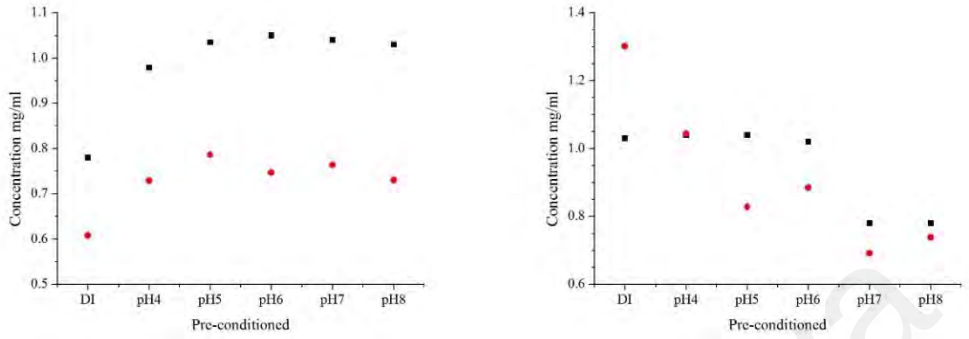


Figure 4.19: Effect of pH on purified amount using SP and MC methods. SP: Standard ammonium sulphate (■) and MC: Multiple centrifuge (●)

NRL waste was conditioned prior to extraction to evaluate the extracted amount of proteins. From Figure 4.19, it was found that both acidic and basic pH of SP gave the highest extracted amount. The lowest amount was recorded for pH 4 of MC. This profile showed that pH 4 is the turning point from a low to high amount of extracted proteins in the extraction process, but this profile is still affected by the season or batch of the sample. The concentrations of both extracts were measured using UV spectroscopy at different

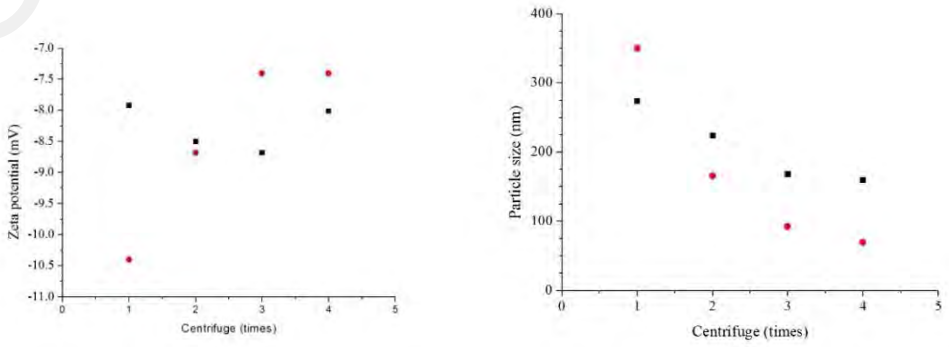
wavenumbers. The results showed a better profile for SP but an inconsistent profile for MC.



a) SP b) MC

Figure 4.20: Effect of pre-conditioned on concentration of purified proteins in mg/ml estimated from UV at 280 (●) and 595 nm (■), SP – Standard Ammonium sulphate, MC – Multiple centrifugation

Figure 4.20 shows the concentration of isolated proteins from SP and MC. From the plot, it is clear that SP gives higher concentrations in most pH conditions but at the alkaline condition, the concentration decreased. For MC, the concentration increased at the acidic condition and dropped slightly at the alkaline condition. Proteins are commonly least soluble at their IEPs. Hence, a combination of high salt concentration and pH control is used to achieve salting out (Skoog, 2004). Protein mixtures are separated by a stepwise increase in ionic strength. It is important to give extra consideration for some proteins because ammonium sulfate can denature proteins.



a) b)

Figure 4.21: Effect of times of centrifugation on (i) ζ potential and (ii) particle size of MC extracted

Figure 4.21 shows the effect of MC on ζ potential and particle size. The particle size showed a significant reduction, whereas the ζ potential recorded increased values in centrifugation for both MC and SP. The increase in ζ potential suggests that the destabilization process occurred due to the interaction of protein particles that become smaller, and smaller particle size suggests that most of the non-protein substances are removed.

4.8 Application Study of NRL Waste and Extracted Proteins

4.8.1 Antibacterial and Antifungal Activities

Latex contains numerous non-rubber constituents, including proteins that are presently discarded as waste. Purified non-rubber constituents from latex contain biologically active molecules, such as β-1,3-glucanase isozyme and polyphenol oxidases (Kanokwiroon *et al.*, 2008). *Hevea* proteins contain an antifungal protein *hevein*, *hev b6*. The SDS PAGE of extracted proteins confirmed its existence. Even though the concentration has not been established, it is good to study microorganism activity.

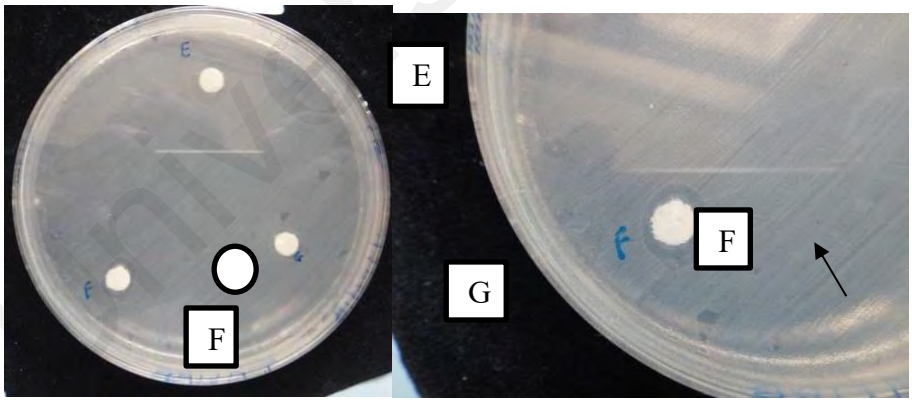


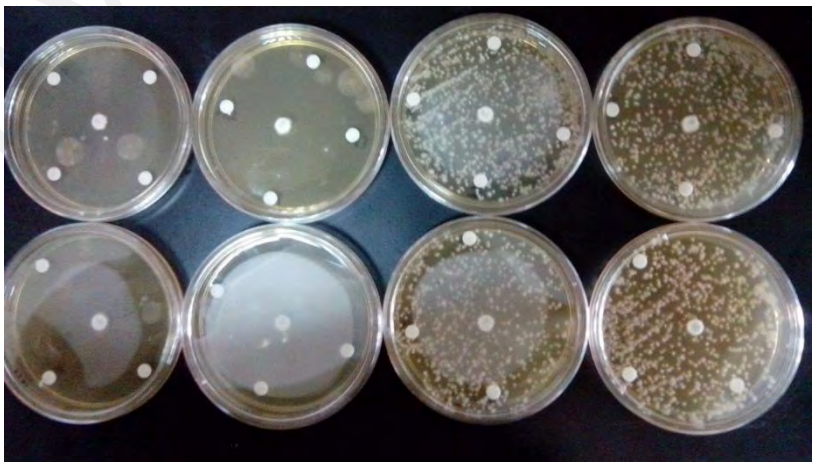
Figure 4.22: Antibacterial study on the extracted protein of pH varied fractions. Fraction 6 pH 3, (E), fraction 6 pH 3 (F) and (G) pH 3 extract against *Escherichia E coli*

The extracted proteins were exposed to two different species of fungi and bacteria. The studies focused on the NRL protein antibacterial activity using *Staphylococcus aureus* and *Escherichia coli*, whereas *Penicillium sp.* and *Fusarium sp.* were used for fungicidal

studies. The zone inhibition activity of the extracted proteins was only observed for sample F, which used the freeze-dried fraction number 6 of pH 3 extracted *hevea* protein. For the fractionation of this pH 3 protein, the sample showed a sharp peak (Figure 4.4(b) Section 4.3.2 and Figure 4.5(b) Section 4.3.3 shows that fraction number 6 is the onset of the big peak. From Figure 4.4(b), the pH is the lowest compared to other fractions, which is around 7.2, and the ζ potential is approximately -28 mV. This sample offers limited information on the protein species at that fractions and repeating the extraction process does not give the same result of antibacterial activity. An investigation is still ongoing for this particular section. On the other hand, the antifungal study was also conducted to assess the behavior of *hevea* proteins toward some fungi. The findings might be useful in paint industries, but the limited amount of extracted proteins collected restricts the entire study of this section.

4.8.2 Fungicidal study

Fusarium sp. and *Penicillium sp.* were applied on potato dextrose agar and a drop of *hevea* proteins was introduced onto the disc. If the concentration of *hevea* proteins used is very low, the disc may affect the result due to some losses that might occur.



Fusarium sp *Penicillium sp*

Figure 4.23: Disc diffusion assay of extracted protein. 4 left petri dish (*Fusarium sp*) 4 right Petri dishes (*Penicillium sp*) after 24 hours

The growth of fungi confirmed no inhibition zone was exhibited by *hevea* proteins. From Figure 4.23, the spots on four left Petri dishes showed the colonization of *Fusarium sp.* and the dots on four right Petri dishes showed the colonization of *Penicillium sp*. The concentration of *hevea* proteins introduced in this study is not the only issue of unsuccessful inhibition. The species and purity also need to be established first to ensure a correct and sufficient sample is used. It has been suggested that a rubber tree must be well equipped with antimicrobial compounds to protect itself from any invaders, and another report showed that another substance with antifungal activity was *hevein* (Kanokwiroon *et al.*, 2008).

4.8.3 Metal Extraction Analysis of *hevea* proteins

In metal extraction analysis, the NRL waste protein was mixed with standard metal solution and the atomic absorption spectroscopy data were recorded (Figure 4.24). Visual observation showed that the NRL waste protein formed a complex with metal ions at a high metal concentration above 2 ppm. Below 2 ppm, the NRL waste protein showed a cloudy solution without a specific form of agglomerates.

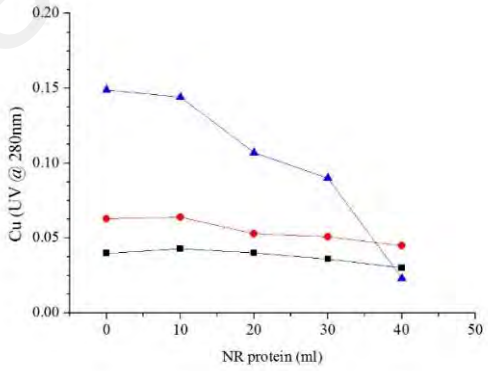


Figure 4.24: AAS of NRL protein (SP) bind with copper. Various metal concentration with NRL protein from 10 ml to 40 ml. Cu 1 ppm (■), 2 ppm (●), and 5 ppm (▲)

Histidine is the amino acid with the strongest affinity for metal ions in protein metal binding, as indicated by several studies. It is widely accepted that histidine, tryptophan, and cysteine residue are vital in the binding of proteins due to strong interactions with

metal ions (Ueda *et al.*, 2003). Although the retention of proteins or peptides is primarily due to metal affinities of their individual amino acids, other factors also contribute toward their metal affinity, including amino acid sequences, folding, and surface properties (Mejáre & Bülow, 2001). There are two ways in which amino acids could contribute to apparent metal affinity, which are by direct or indirect binding of the metal. There is evidence indicating that the effect is indirect, in which nearby aromatic side chains enhanced Cu(II)-histidyl (Arnold, 1991).

4.8.4 Traces of Metal in NRL Waste

The NRL waste serum was filtered using filter paper several times and then analyzed (Sumate Chaiprapat *et al.*, 2015) for traces of metal using inductively coupled plasma (ICP). It was found that zinc (Zn), aluminum (Al), selenium (Se), nickel (Ni), and magnesium (Mg) recorded 254, 0.013, 0.129, 0.001, 0.749, and 19.7 ppm, respectively. Meanwhile, lead (Pb), cadmium (Cd), copper (Cu), and aurum (Au) showed negative traces in this waste effluent. The traces of elements are important in predicting metal selectivity and capability for protein-metal interaction. Zinc is one of the metals that usually exist as it is normally added as a secondary accelerator in the manufacturing of latex concentrate. Other metals normally come from the soil absorbed by a tree during biosynthesis.

4.8.5 Metal Removal Analysis for NRL Waste

From Figure 4.25, it can be seen that the metal removal capability was different between each metal but the protein still showed very good interaction with Pb, followed by Cd, 50% reduction with Cu, and 50% reduction with Al. The overall performance of those five metals at 2 ppm concentration is very good. Between 2 ppm and 5 ppm metal solutions, Pb achieved good interaction with the NRL protein but at lower extraction

capabilities of 40 %, followed by Cd (20 %), Cu (50 %), and Al (30 %). In each sample, the

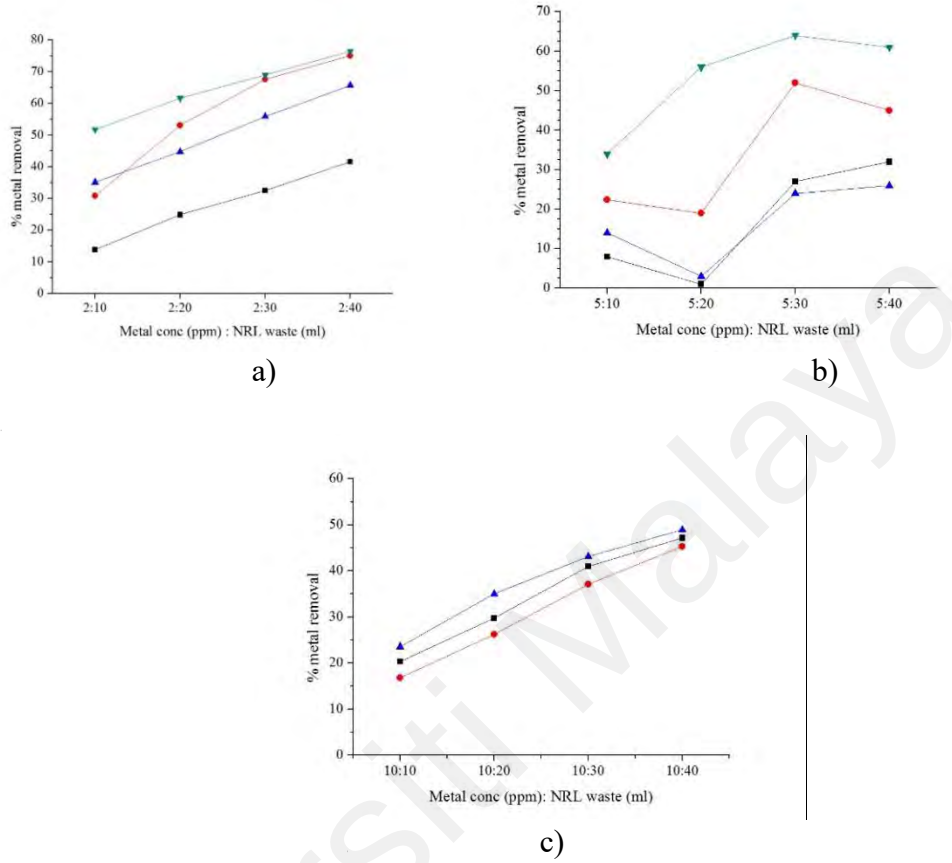


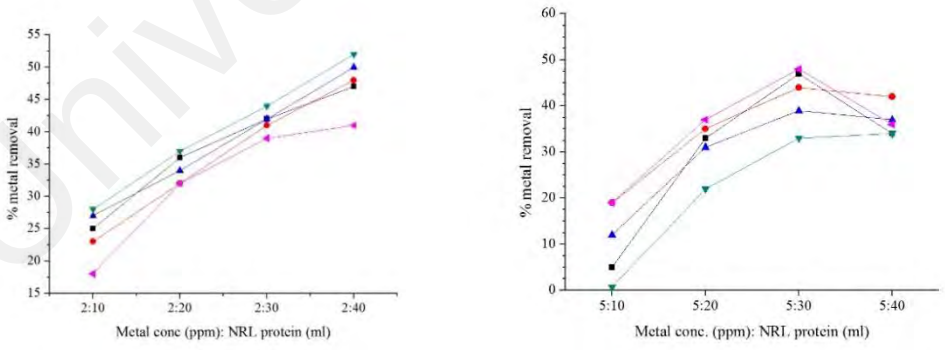
Figure 4.25: Effect of NRL waste concentration on metal removal a) 2 ppm metal, b) 5 ppm and at c) 10 ppm metal solution concentration Al (■), Cd (●), Cu (▲), and Pb (▼)

NRL waste was filtered using filter paper and mixed with 5 ppm metal solution. It can be seen from the graph that Pb and Cd showed good binding while only 30 % Cu and Al managed to bind at the maximum protein concentration. The interaction of the metals with the NRL protein at 10-ppm metal concentration was observed, where only three types of metals could be treated. It was significant that Pb contributed to the highest interaction with a 30 % reduction in extraction capability compared to 5-ppm concentration. In addition, Cd experienced the same decreased interaction with 13 % reduction, followed by Cu with 12 % reduction.

The overall data suggest that NRL waste can be used to treat metals added to the waste effluent. However, the limitation is that as the metal concentration increased, the extraction capability reduced by about 12 % to 30 % and metal species extraction is limited to Pb, Cu, and Cd. The skim serum (NRL waste) contains a significant amount of non-rubber components, including proteins, sugar, lipids, carotenoids, and organic and inorganic salts originating from latex and a small amount of uncoagulated latex (Abraham *et al.*, 2009). This suggests the weak performance exhibited by NRL waste proteins. The investigation was carried out with purified NRL protein using SP.

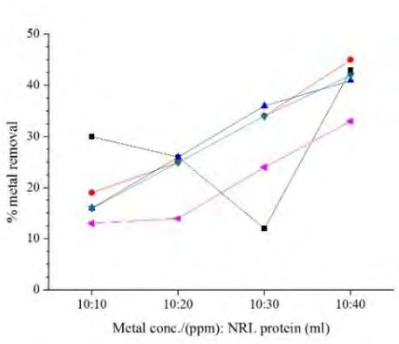
4.8.6 Metal Removal of Ammonium Sulphate Extracted (SP) proteins

Standard metal solutions of various concentrations diluted from 1,000 ppm were mixed with SP proteins to investigate the metal removal capability of SP-extracted proteins and compared with MC-extracted proteins and also the direct use of NRL waste. Ammonium sulfate-extracted NRL or SP-extracted proteins were also added to the metal solution to investigate the metal-protein interaction. It is expected that there will be higher removal than the other two protein precursors.

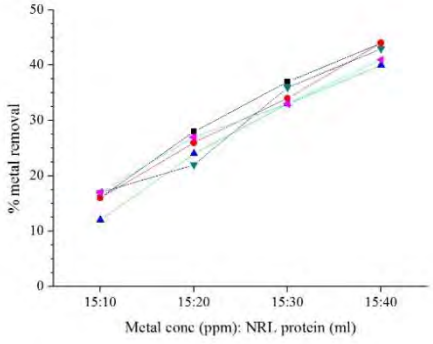


a) b)

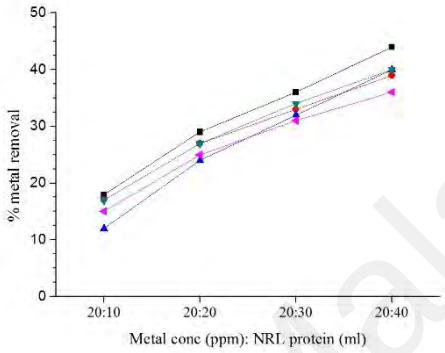
Figure 4.26: Effect of concentration of (SP) protein on metal removal a) 2 ppm b) 5 ppm c) 10 ppm d) 15 ppm and e) 20 ppm metal solution concentration for Al (■),Cd (●), Cu (▲), Pb (▼), and Zn (◀). pH for 15:10, 15:20 15:30, 15:40 were 1.52, 1.62, 1.68, 1.76 accordingly. pH for 20:10, 20:20, 20:30, 20:40 were 1.74, 1.76, 1.82, 1.84 accordingly



c)



d)



e)

Figure 4.26, continued

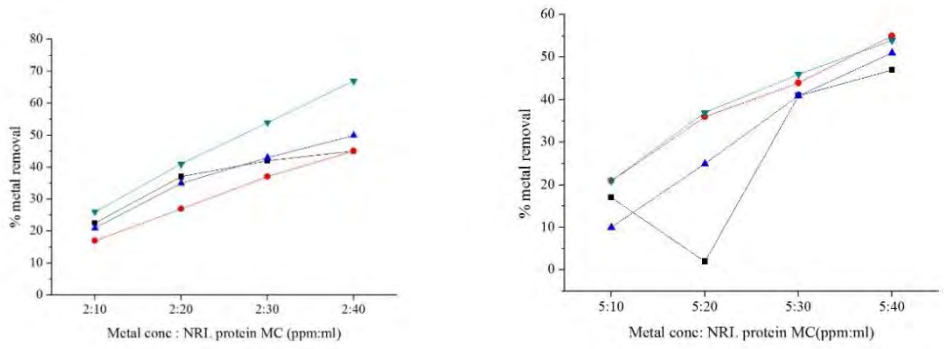
From Figures 4.26 (a) and (b), Pb exhibited the highest binding or removal, followed by Cu, Al, Cd, and Zn for 2 ppm metal concentration. A general trend was consistent using NRL waste. The interaction was more consistent with each metal and the difference was only around 10 % between each metal. Zn interaction was slightly lower with about 30% difference from Cd interaction.

Zn still showed poor interaction due to the presence of Zn in the waste, where only a certain amount was reduced after the purification process. From Figure 4.26(c), it can be seen that Zn achieved the highest binding, followed by Cd, while Pb contributed to the lowest binding. Figure 4.26(d) shows metal removal at 15 ppm metal solution. The interaction of Cd and Al was the highest, followed by Pb, Zn, and Cu. Figure 4.26(e) shows the metal removal of SP-extracted proteins at 20 ppm metal solution, where good binding was achieved with Al, followed by Cu, Pb, Cd, and Zn. From the overall plot, it can be determined that as the concentration of metal solution increased, the order of

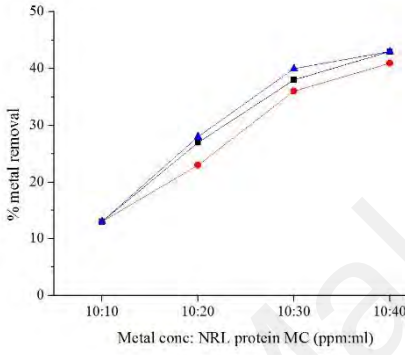
binding with specific metal changed. At 2 ppm metal concentration, Pb showed the highest binding capability or metal removal, whereas at 5, 10, and 15 ppm, Cd showed the highest binding or metal removal. Meanwhile, at 20 ppm, NRL is composed of rubber particles that are mainly cis-1,4 polyisoprene, proteins, phospholipids, carbohydrates, and inorganic substances, such as potassium (K), Mg, Zn, Cu, and iron (Fe) (Danwanichakul *et al.*, 2014). Thus, the presence of these elements influences metal removal.

4.8.7 Metal Removal of Multiple Centrifuge Extracted Proteins

About 0.2 g of MC-extracted proteins was redissolved using 100 mL of DI. 2, 5, and 10 ppm metal solutions were prepared, and 10, 20, 30, and 40 mL of redissolved MC-extracted proteins were mixed with the metal solution. The mixture was analyzed using ICP and the initial reading for an unmixed metal solution was used as blank. The data for each mixture with blank were used to estimate the percentage of protein that bound or reacted with a metal solution. MC-purified protein is a salt-free purified protein. The highest binding was achieved by Pb, followed by Cu, Cd, and Al, while Zn was not considered because the initial concentration used as blank is much lower than the sample, hence there is no removal of Zn in this case. Figure 4.27(b) shows the metal removal of MC-purified proteins at 5 ppm metal solution. The highest binding was achieved by Cd, followed by Pb, Cu, and Al. The trend for Pb, Cd, and Cu is consistent and increased continuously, while Al showed a significant reduction for 20 mL protein solution. More data points are needed to understand the relationship of Al with 20 mL protein solution. An investigation on a higher metal concentration showed that the metal species removed by *hevea* proteins were only Pb, Cu, and Cd. This suggests that at a lower metal concentration, MC-purified proteins can tolerate a higher amount of Al in the NRL waste, but as the metal concentration increased, the NRL waste proteins could not tolerate the metal strength, thus could not bind to any specific metal.



a) b)



c)

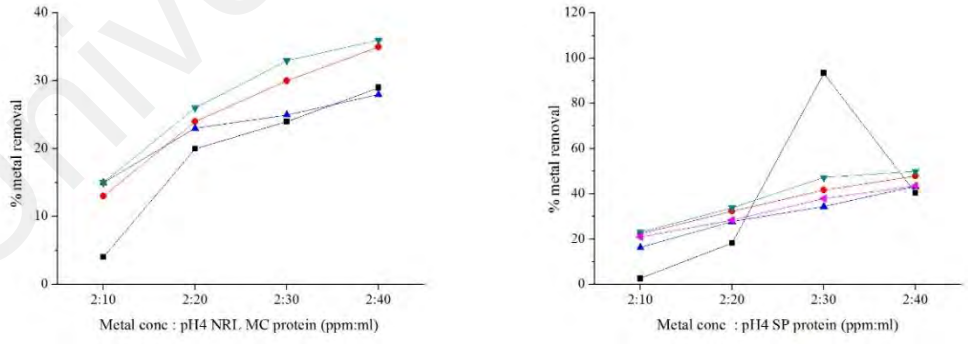
Figure 4.27: Effect of concentration of (MC) protein on metal removal 2 ppm (a) 5 ppm (b) and 10 ppm (c) metal solution concentration pH for 2:10, 2:20, 2:30, and 2:40 were 2.63, 2.73, 2.78, 2.86 accordingly. pH for 5:10,5:20,5:30 and5:40 were 2.20, 2.26, 2.36, 2.46 accordingly. Al (■), Cd (●), Cu (▲), and Pb (▼)

Figure 4.27(c) shows the metal removal of MC-purified proteins at 10 ppm metal solution. From the graph, MC-purified proteins only reacted with three types of metals for 10 ppm metal solution. The maximum removal was achieved for Pb, followed by Cd and Cu. From Figures 4.27(a) and (b), the binding capability of MC-purified proteins is the highest at a lower metal concentration, but as the metal concentration increased, the number of metal species removed decreased; moreover, the binding capability also weakened. For 10 ppm metal solution, the extracted proteins could not bind to Al and the removal percentage dropped insignificantly. By comparing the results from the lowest to the highest metal solution concentrations on pH, the magnitude decreased as the concentration increased. This might be due to a higher metal concentration that is highly acidic in the surrounding, which limits protein activities. For the removal of protein by Pb, the removal was reduced by 20 % for 2 to 5 ppm metal concentration and the removal

was further reduced by 20 % as the metal concentration increased to 10 ppm. A similar profile was observed for MC-purified proteins and NRL waste. Hence, the overall performance of MC-extracted proteins is lower than that of SP-extracted proteins. However, it is not appropriate to conclude that the lower purity of MC-extracted proteins accounts for its capability in metal removal. The total extraction process from the initial sample to the freeze-dried sample is similar, except that no ammonium salt is used. Nevertheless, the final protein amount collected was lower than 60 % due to the difficulty in scraping "wet" proteins. Similar performance may be achieved if the same amount of protein extract is used.

4.8.8 Metal Removal of Different pH MC-extracted proteins against SP-extracted protein

NRL waste protein was purified at different pHs to study the amount of proteins that can be isolated and to study their metal removal capabilities. The fractionated proteins of various pHs were mixed with 2 ppm Al, Cd, Zn, Pb, and Cu solutions, and the removal percentage was calculated from the ICP data.



a) b)

Figure 4.28: Effect of concentration of pH varies extracted proteins on metal removal a) pH 4(MC) and b) pH 4(SP) protein on metal removal at 2 ppm metal solution concentration of c) pH 5 (MC) protein and d) pH 5 (SP) on metal removal at 2 ppm metal solution concentration e) pH 6 (MC) and f) pH 6 (SP) protein on metal removal at 2 ppm metal solution concentration g) pH 8 MC and h) pH 8 SP protein on metal removal at 2 ppm metal solution concentration.
Al (■), Cd (●), Cu (▲),Zn (◀)and Pb (▼)

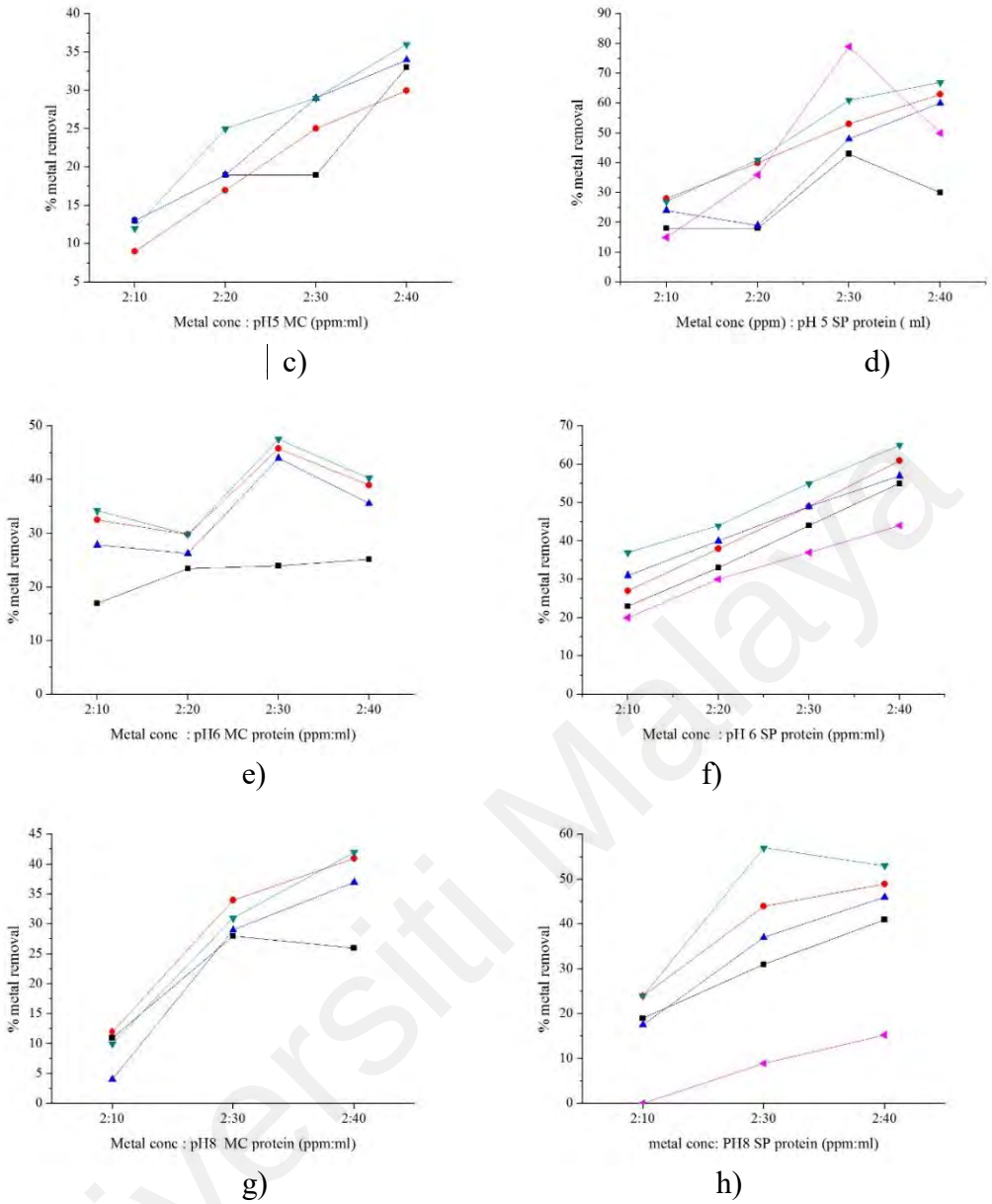


Figure 4.28, continued

From Figure 4.28, Pb achieved the highest binding capability, followed by Cu, Al, and Cd. From Figure 4.28(b), Zn achieved the highest binding capability at a 2:30 ratio but dropped at a 2:40 ratio. A similar trend was observed for Al, except that Al showed the lowest binding capability. Other metals demonstrated a standard trend with Pb maintaining its good binding capability. From Figures 4.28(e) and (f), the order of binding capability is as follows: Pb > Cd > Cu > Al > Zn.

4.8.9 The Behavior of Several Preparation Methods of Protein towards Different Types of Metals

Two methods were used in *hevea* protein extraction, and both methods have been proven suitable for metal removal. However, the metal removed showed different degrees of selectivity corresponding to metal concentration and protein concentration. This is expected because different extraction methods result in different protein concentrations, hence affecting the capability to remove metals. Based on the overall performance of individual metals, Pb and Cd showed a good and consistent profile for their removal. Pb is among the hazardous contaminant that can seriously deteriorate the environment because it is readily enriched in soil and water, and it is also difficult to remove or degrade naturally. Pb interferes with the physiology and metabolism of the plants by binding to the sulfhydryl groups of various proteins, leading to structure disruption or activity inhibition (Liu *et al.*, 2009).

Environmental pollution of Cd is mainly from mining and smelting, dispersal of sewage sludge, and the use of phosphate fertilizers. Cd is readily taken up by many crops, including cereals, potatoes, vegetables (leafy and root), and fruits (Mejáre & Bülow, 2001). Organisms respond to heavy metal stress using different defense systems, such as exclusion, compartmentalization, making complexes, and the synthesis of binding proteins, such as metallothioneins (MTs) or phytochelatins (PCs). Naturally occurring Cd-binding proteins and peptides, such as MTs and PCs are very rich in cysteine residues. This explains why Cd and Pb could perform better in *hevea* protein-metal binding, which is due to the presence of *hevein* in the *hevea* protein. *Hevein* is a cysteine-rich protein. By comparing Pb and Cd removal from the same batch and technique, the difference is only on metal concentration. Hence, there is a possibility that the influence or competition between metals affects metal removal.

i. Lead Removal by *Hevea* Proteins

A good performance was exhibited for Pb removal by metal-*hev* protein from NRL waste to SP-extracted proteins. Pb can cause oxidative damage by stimulating the formation of free radicals and reactive oxygen species, resulting in oxidative stress (Kozlowski *et al.*, 2013). Thus, it is good that proteins are attracted to Pb.

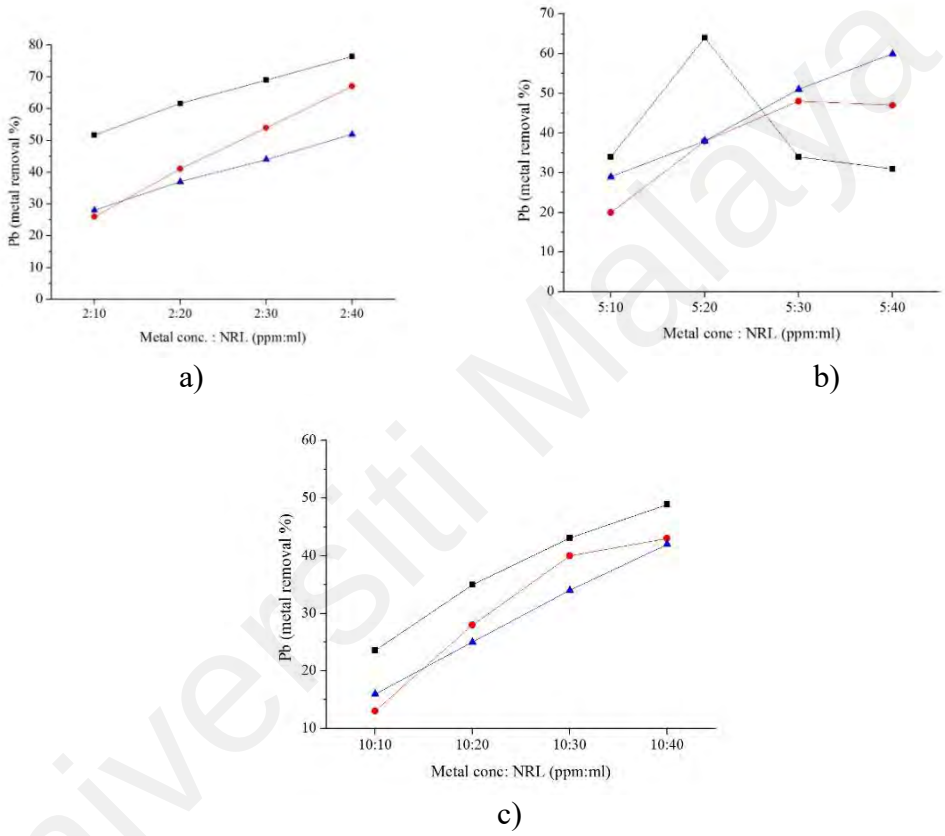


Figure 4.29: Effect of protein extraction method on lead removal 2 ppm,5 ppm and 10 ppm metal solution concentration NRL waste (■), MC protein (●), and SP protein (▲)

Figure 4.29 shows the influence of different extraction methods on the metal removal capability toward Pb. The NRL waste was filtered using filter paper and the waste was mixed with metal solutions of 2, 5, and 10 ppm, which was then subjected to ICP. Initially, the NRL waste showed good binding with Pb, and the highest removal was achieved at a 5:20 ratio. However, it started to decrease at a 5:30 ratio and further decreased at a 5:40 ratio. Meanwhile, for MC-extracted proteins, the proteins could not bind to Pb at a 5:20 ratio, increased at a 5:30 ratio, and started to decrease at a 5:40 ratio. From the graph,

only SP displayed a straight line. The reason for such a curve is because NRL waste is unpurified proteins and still contains impurities, which may disrupt metal-protein binding. Furthermore, the binding process is probably interrupted by the non-protein constituents in the waste.

ii. Cadmium Removal by *Hevea* Proteins

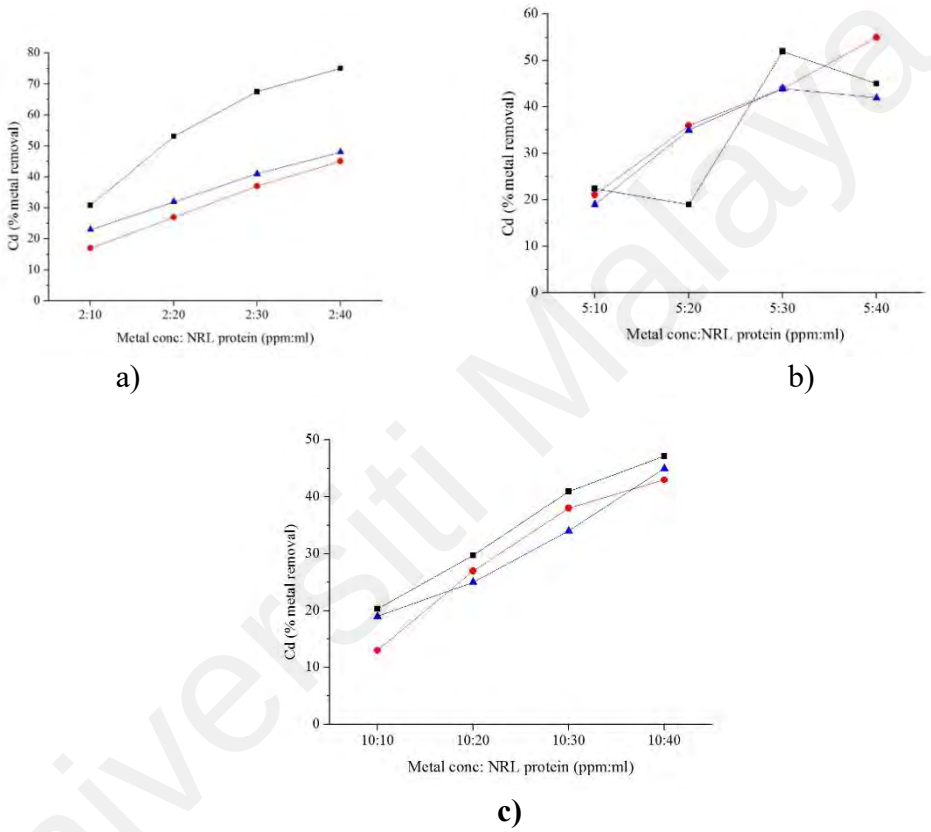


Figure 4.30: Effect of protein extraction method on cadmium removal at 2ppm, 5ppm and 10 ppm cadmium solution concentration. NRL waste (■), MC protein (●), and SP protein (▲)

MC-extracted proteins were produced using purification, which involved a physical or repeated concentration process. The protein in the NRL waste was concentrated using centrifugation. It is assumed that the NRL protein is partially purified for MC. SP is the standard purification method where ammonium sulfate is used to precipitate specific or targeted proteins. As the concentration of ammonium sulfate increased, more protein precipitated. In this case, SP-extracted proteins are considered to be 50 % more purified

than the NRL waste. Thus, it gives a consistent profile in metal binding. Previous studies suggested that selectivity is provided by the pre-organized structure of the donor site itself in the protein. The metal ion binding usually exerts a distinct impact on the binding pocket structure due to the secondary or tertiary structure donors from the residues being often very far away in the peptide sequence (Kozlowski *et al.*, 2013). Further investigation on a single metal at different metal concentrations can be suggested for metal selectivity study.

4.8.10 Metal Selectivity of NRL Proteins

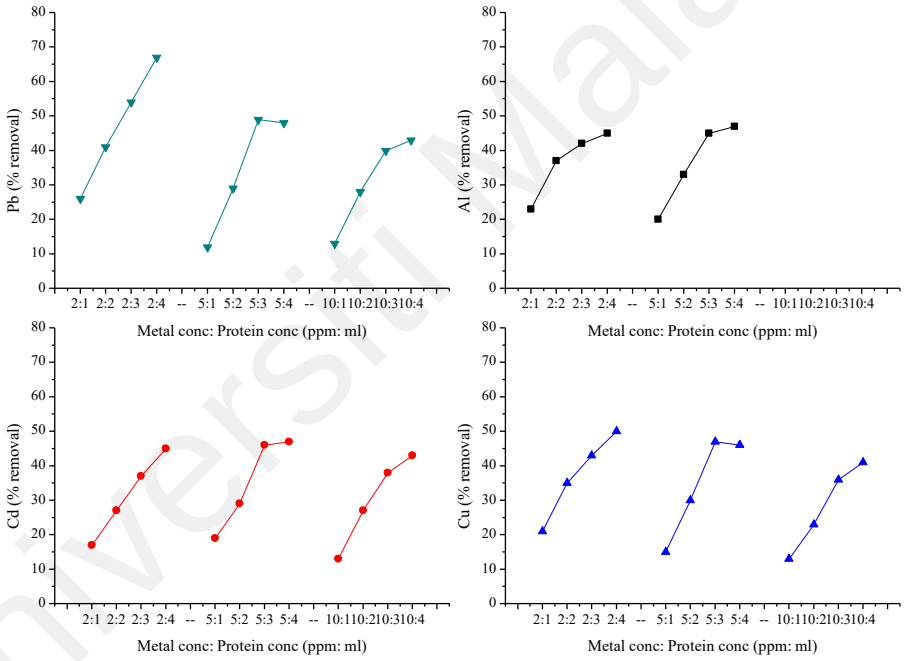


Figure 4.31: Metal selectivity of NRL MC Protein

From Figure 4.31, the order of metal uptake and strength of metal ions that can be tolerated by MC-extracted proteins was evaluated. The metal extraction of NRL waste, MC-purified proteins, and SP-purified proteins was conducted using 5 ppm metal solution. Figure 4.32 shows the metal removal for 5 ppm metal solution of mixed Pb, Cu, Cd, Al, and Zn. From Figure 4.32(a), a broad range of removal can be seen for different metals, with Pb showing the highest removal at all concentrations while Cu recorded the

lowest removal. Figure 4.32(b) shows the removal by MC-purified proteins and a very close gap was identified. Only metal species were removed and Zn still could not be removed. However, Figure 4.32(c) shows a significant gap for Pb, Cu, and Al, whereas Cd and Zn demonstrated an almost identical profile, especially at higher protein loadings. The metal concentration of the samples was tested using FTIR spectroscopy.

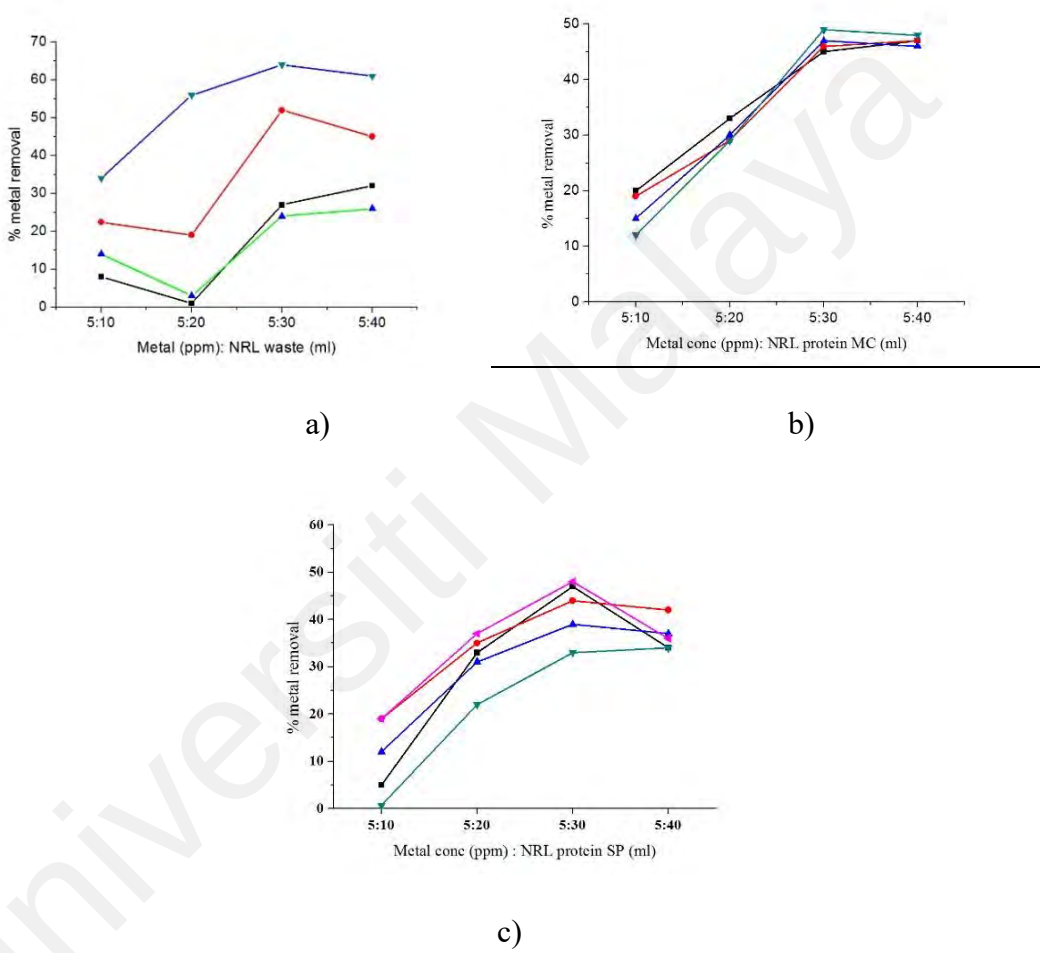
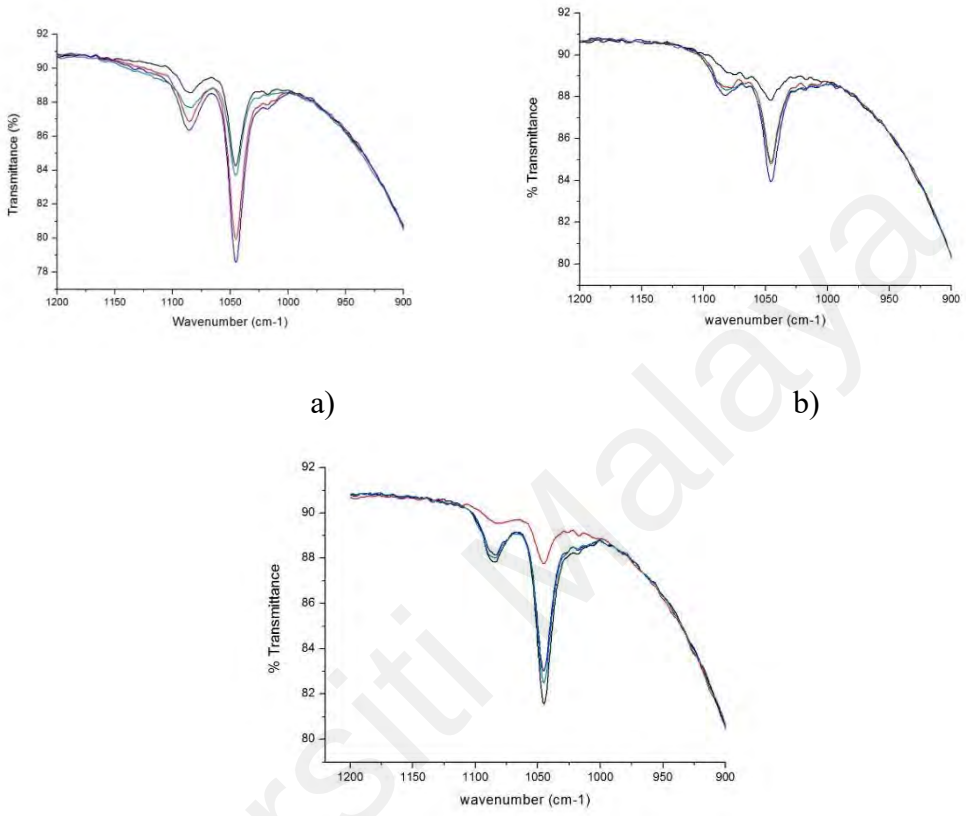


Figure 4.32: Metal removal of extracted protein and waste
a) NRL waste, b) MC purified the protein and c) SP purified protein at 5 ppm metal concentration - Pb - Cu - Cd - Al - Zn

The highest removal of Pb is achieved by the NRL waste compared to highly purified proteins because there might be a high concentration of *hevein* in the waste. Furthermore, extraction will remove other *heveas* and only some amount of *hevein* remains. The selection of proteins to react with metals is based on individual metal performance.

4.8.11 FTIR Spectrum of Metal Extracted Solution

The FTIR-ATR spectra obtained were used to compare the preparation methods toward metal removal.



a) b)

c)

Figure 4.33: FTIR spectrum of metal bind protein (a) NRL waste, (b) MC protein and (c) SP protein. 5:10 (—),5:20 (—), 5:30 (—), and 5:40 (—)

The profile in the spectra is quite consistent with the concentration of proteins measured using ICP. For Figure 4.33(a), the maximum intensity in transmittance was achieved for 5 ppm metal solution with 40 mL protein solution, followed by the 5:20, 5:30, and 5:10 peaks. The 5:30 peak may be due to impurities present in the filtered NRL waste. Figure 4.33(b) shows MC-purified proteins, where the 5:40 peak showed the highest intensity, followed by the 5:20 and 5:30 peaks at the same position, and the lowest intensity was recorded for the 5:10 peak. This shows the profile where the concentration increases as the intensity increases. Figure 4.33(c) represents SP-purified proteins, which also gave an unexpected profile. The 5:20 peak showed very weak intensity compared to

the 5:10 peak, but in terms of total intensity, different SP-purified proteins performed better than other protein specimens.

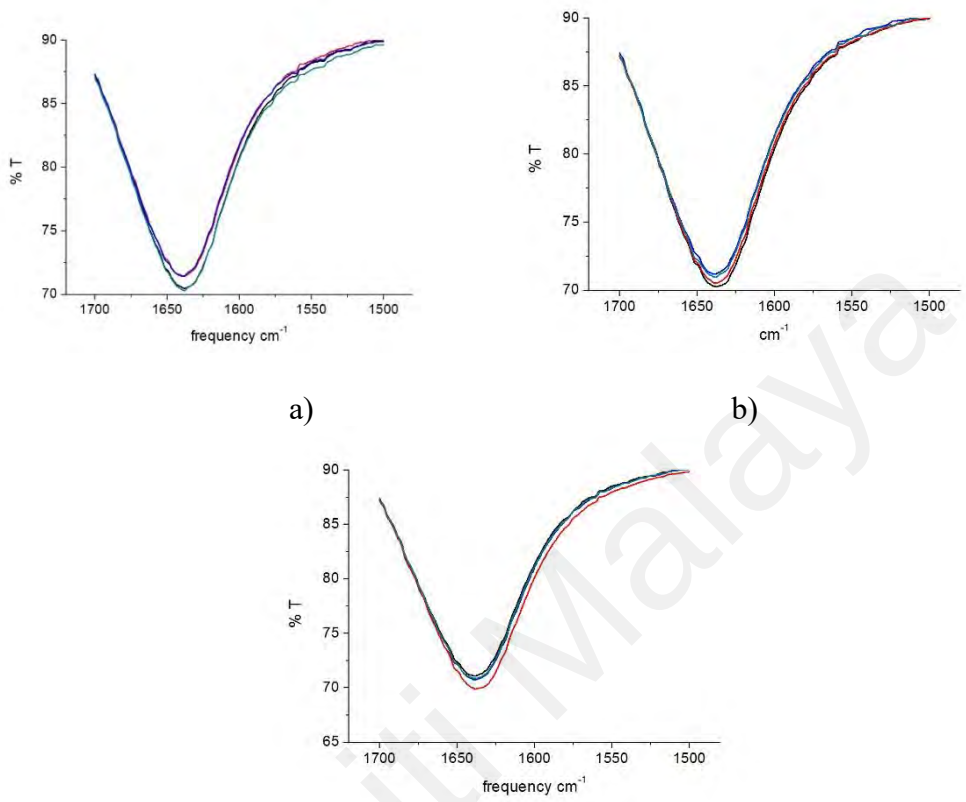
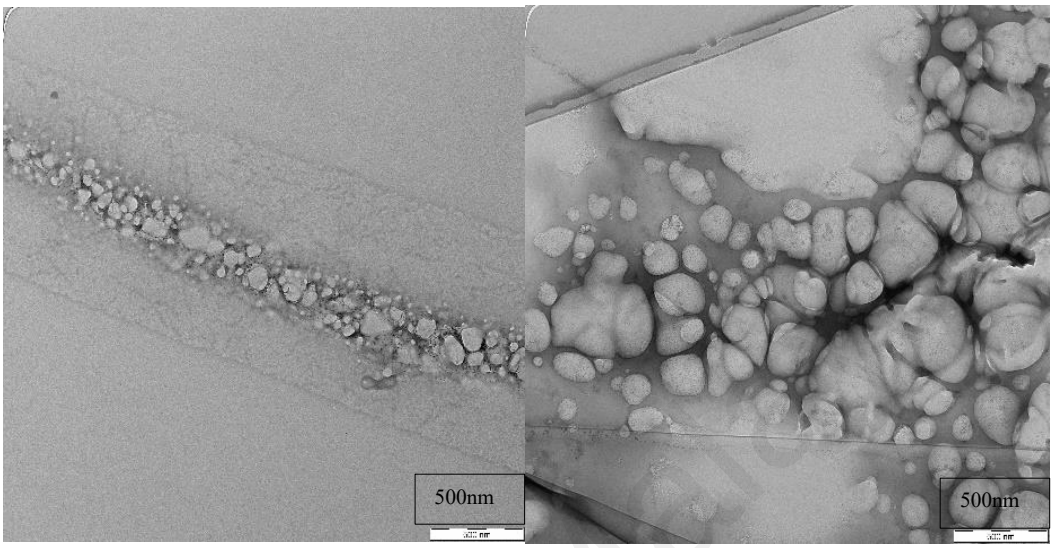


Figure 4.34: FTIR spectrum of NRL waste, MC and SP-5ppm metal a) Metal-NRL waste 5 ppm: 10 ml,20 ml,30 ml,40 ml FTIR spectrum b) metal-MC protein 5 ppm:10 ml, 20 ml, 30 ml,40 ml c) metal-SP for amide I and amide II

The spectroscopic method is rapid and relatively easy to use but it depends on light absorbance transmittance and may be compromised by opaque or colored solutions (Guo *et al.*, 2014). By comparing Figures 4.33 and 4.34, the purified protein region did not display much difference. However, a significant difference could be observed at the region of 900–1200 cm^{-1}. The region of 900 to 1200 cm^{-1} was assigned to $–CH_2$- based on the IR spectrum atlas, where the region of 1040 to 1060 cm^{-1} was assigned to S-O: a) R_2S^+-O^- and b) R_2 S = O (1120 to 1160 cm^{-1}). The significant changes in the intensity at 900 to 1200 cm^{-1} are most probably due to S or cysteine residue, which is abundant in NRL waste or more specifically, *hevea* proteins (J Van Parijs, 1991; U. M. Soedjanaatmadja *et al.*, 1995).

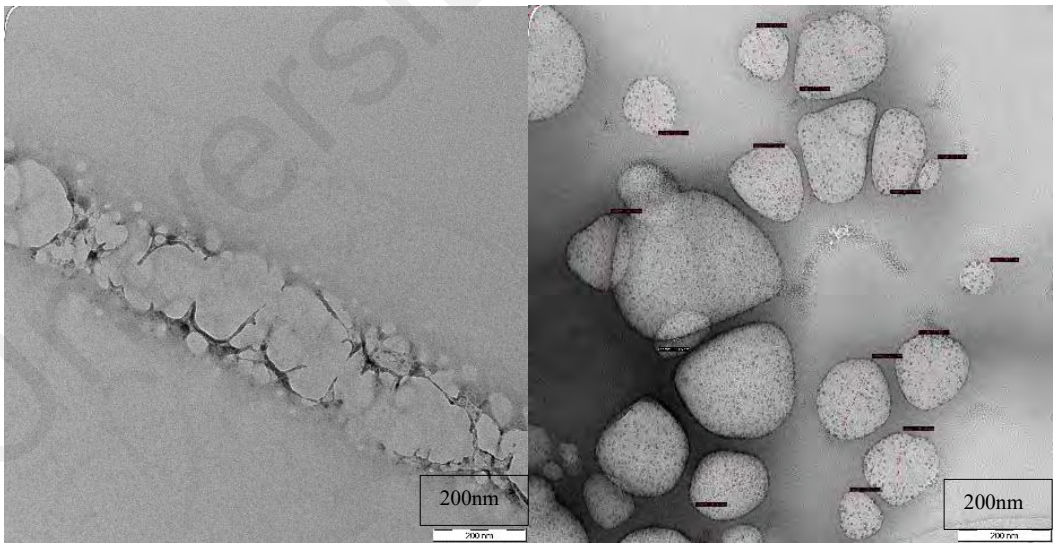
4.8.12 Transmission Electron Microscopy for Metal Removal Proteins

TEM was carried out to investigate the size and microstructure of *hevea* proteins. A clear image was captured when the metals were bound to the proteins.



a) b)

Figure 4.35: a) and b) TEM micrograph of metal-protein at 500 nm magnification



c) d)

Figure 4.35, continued c) and d) TEM micrograph of metal bound proteins at and 200 nm magnification

TEM micrograph of metal bound protein showed black dots on the particle signifying the attachment of metal with protein particles. The size of particles ranged from 71 nm

for the smallest particles to 230 nm for the largest particles, as shown in Figure 4.35(d). This suggests the reduction of metal concentration in the solution (from metal removal calculation) and the formation of cloud or complex in the sample solution. The binding mechanism could not be suggested unless more assessment is conducted to support the findings. The TEM of REF and SRPP at 200 nm is quite cohesive with rubber particles. Due to their very hydrophobic content, they are available in lipids, and acidification may occur with the lysis of lutoids and proteases released in the serum. Oxidation occurs at the air interface. Hence, various processes can lead to the destruction of proteolipid membrane and the generation of protein aggregation (Berthelot, Lecomte, Estevez, Coulary-Salin*, et al.*, 2014). Previous works on the synthesis of silver nanoparticles in skim latex showed the image of metal and nanoparticles that looked like *hevea* proteins in clusters with white and black particles in nano size (Suwatthanarak *et al.*, 2016).

CHAPTER 5: CONCLUSION

5.1 Conclusion

In this work, natural rubber latex (NRL) waste effluent was used as a starting material to extract *hevea* proteins. *Hevea* proteins are well known as an allergen, especially in glove and other latex-related products. The allergen causes rashes and spina bifida to a patient allergic to those proteins. Many studies have been conducted to remove *hev* proteins, thus contributing to the presence of these compounds in the waste effluent of the industry. This leads to the direction of this work, which is to extract *hev* proteins through several approaches and to use these proteins as proteins with common roles in a living organism, such as defense proteins and metalloproteins. The conclusion part of this work provides the findings and suggestions that can be attempted to overcome current issues.

From the data collected, it was observed that the lower the pH of the NRL waste serum, the better properties achieved in terms of yield, odor, and stability. The yellowish color of the serum indicates that the pH is lower. The preferable serum pH is below 4.5. Above 4.5, the serum is pale white and a bad odor is present around pH 5, which is an indication of microorganism activity. Thus, low serum pH provides information on the safety margin for processing and storage. Acidic pH is good for preventing microbial attack but some of the proteins might lose their activity due to acidic pH. Nevertheless, the proteins are still useful structurally. The balance between the initial and final pH may affect the amount of proteins extracted. A measure was taken to keep the sample in cold condition, such as in a chiller (4–11 °C) from the industry and the sample was freeze-dried to ensure minimum contamination by microorganisms. The protein was extracted using saturated liquid ammonium sulfate of 4.1 M, where the yield varied with the incoming material properties. The pH below 4.5 is stable for storage for at least a few months in a fridge (4–11 °C), which means that the amount of precipitate collected will not decrease

significantly. At pH below 4.5, the yield is normally 0.2 g of freeze-dried protein for every 50 mL of NR waste. The salt used is good in preserving the precipitates but an issue arises regarding the amount of wasted salt, and there is an urge to find an alternative method to purify proteins. The multiple centrifugations (MC) method, also known as the pre-concentration method, was introduced to overcome the issue of removing the salt, which resulted in additional salt waste. The procedure used is almost similar to salt extraction. The NRL serum was centrifuged at least four times and the middle layer of the serum was withdrawn after each centrifuge. For the optimization of the amount of SP- and MC-extracted proteins, the NRL waste was conditioned at various pH from pH 3 to 8. The concentration of the resulting proteins was measured using ultraviolet (UV) absorbance at 280 nm and 595 nm. The absorbance at both wavenumbers provided different concentrations of proteins. Other physical properties of the extracted proteins were also measured. Based on the overall performance of SP and MC for the extracted protein amount and physical properties, SP is more consistent and easier to handle. The amount of extracted protein is higher for SP but some measurement says otherwise. SP-extracted proteins, which are found to be powdery at a physical state, are suitable for further purification or isolation. MC-extracted proteins, which are quite sticky, can be applied to avoid column problems, such as clogging.

This work focused on two parts. In the first part, the pH of precipitates was varied and the precipitates were fractionated using pH 8 Tris buffer. The pH profile, zeta (ζ) potential, and size of the fractions/isolates were plotted and compared with the second part of the work, where the loading amount in the column was varied and the same Tris buffer was still used, except for the freeze-dried natural rubber (NR) protein that was redissolved in the buffer itself. In the second part of the work, a chromatogram was plotted using the data from UV absorbance at 280 nm. The plot gives several fractionated species. The pH profile, ζ potential, and particle size did not show any specific profile but the

profiles are still useful in understanding each fraction. SDS PAGE provides the information on the number of *hev* proteins managed to be fractionated or isolated; hence, the degree of column resolution was estimated based on the information. The particle size of each fraction containing proteins is in the range from the lowest to the highest depending on the type and fractionated protein species. Meanwhile, the ζ potential varied from -5 mV to -40 mV, indicating the presence of different protein species and stability. Protein concentration was measured using the area under the graph of the Fourier transform infrared peak, and UV absorbance at 280 nm and 595 nm was used as an estimation of the total protein concentration. Single protein isolates for any specific fraction are found to be acceptable. Furthermore, the pH of fractions seems to be higher at the beginning of fractions, decreased as the protein reached the maximum separation, and increased to its original pH at the end of fractions. The complete loop is useful to ensure complete fractionation and also the time to end the process. The prediction for the order of elution of proteins was made based on the isoelectric point (IEP) and the predicted molecular weight was close to the actual molecular weight, except for certain samples, which might be due to the limitation of certain criteria, such as overloading or the pH condition is too strict for certain protein types.

The application study of this work evaluated the activity of NRL protein with certain bacteria and fungi. Two types of bacteria were used (*Staphylococcus aureus* and *Escherichia coli*) to investigate the inhibition of NR protein. Meanwhile, three types of fungi and one yeast were used to study the fungicidal activity (*Fusarium sp.*, *Penicillium sp.*, *Aspergillus sp.*, and *Candida albicans*). The study does not give any consistent result, which may be due to the practice or the sample itself. For the bactericidal study, there was positive inhibition for the fractionates but not the extracted sample. The sample used was from the first part of the study, which used different pHs to precipitate the sample. Meanwhile, the sample from the second part of the study used deionized water for

precipitation, and no inhibition was observed. The third objective aims to investigate metal removal by *hev* proteins upon exposure to certain metal solutions. The objective was evaluated by mixing NRL waste, SP-purified proteins, and MC-purified proteins from the waste. Several metals were used to investigate the binding characteristics of proteins. Cu, Pb, Cd, Al, and Zn were used at different concentrations of 2, 5, 10, 15, and 25 ppm. It is clear that at a lower metal concentration, there was no formation of cloud complex in the metal-protein mixture, but at a higher concentration, the cloud complex could be seen suspended in the mixture proportionate to the amount of protein added. The metal removal percentage calculated from the initial and final concentrations proved that the extracted proteins managed to remove metals at varying degrees. The SP-extracted proteins showed a consistent profile, followed by MC-extracted proteins, and NRL waste that managed to remove some metals up to 10 ppm metal concentration.

5.2 Future works

A specific study on the starting materials should be performed so that only a good sample will be subjected to precipitation. For chromatographic analysis, the most important thing is to have different pH precipitates and to fractionate at different pH conditions instead of only using pH 8 Tris buffer. This is because one of the objectives of this work is to use proteins that precipitate at different pHs, which will give a positive result in bactericidal activity. For metal extraction, other types of heavy metals can be used to study any specific pattern or any limitation in the concentration of proteins or metals.

REFERENCES

Abraham, Nair, & Madhu. (2009). Electrochemical treatment of skim serum effluent from natural rubber latex centrifuging units. *Journal of Hazardous Materials, 167*(1), 494-499.

Acosta, Savio, Talio, Ferramola, Gil, & Martinez. (2013). On-line solid phase extraction of Cd from protein fractions of serum using oxidized carbon nanotubes coupled to electrothermal atomization atomic absorption spectrometry. *Microchemical Journal, 110*, 94-98.

Alhazmi, Nachbar, Albishri, El-Hady, Redweik, El Deeb, & Wätzig. (2015). A comprehensive platform to investigate protein–metal ion interactions by affinity capillary electrophoresis. *Journal of Pharmaceutical and Biomedical Analysis, 107*, 311-317.

Anwar, Shafique, Salman, Dar, & Anwar. (2010). Removal of Pb (II) and Cd (II) from water by adsorption on peels of banana. *Bioresource Technology, 101*(6), 1752-1755.

Aoki, Takahashi, Takayama, Ogata, Sakurai, Suzuki, Asawatreratanakul, et al. (2014). Identification of laticifer-specific genes and their promoter regions from a natural rubber producing plant Hevea brasiliensis. *Plant Science, 225*, 1-8.

Archer. (1960). The proteins of Hevea brasiliensis latex. 4. Isolation and characterization of crystalline hevein. *Biochemical Journal, 75*(2), 236-240.

Arnold. (1991). Metal-affinity separations: a new dimension in protein processing. *Nature Biotechnology, 9*(2), 151-156.

Atagana, Ejechi, & Ayilumo. (1999). Fungi associated with degradation of wastes from rubber processing industry. *Environmental Monitoring and Assessment, 55*(3), 401-408.

Bang-iam, Udnan, & Masawat. (2013). Design and fabrication of artificial neural network-digital image-based colorimeter for protein assay in natural rubber latex and medical latex gloves. *Microchemical Journal, 106*, 270-275.

Berthelot, Lecomte, Estevez, Coulary-Salin, & Peruch. (2014). Homologous Hevea brasiliensis REF *(Hevb1)* and SRPP *(Hevb3)* present different auto-assembling. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1844*(2), 473-485.

Berthelot, Lecomte, Estevez, & Peruch. (2014). Hevea brasiliensis REF (Hev b 1) and SRPP (Hev b 3): An overview on rubber particle proteins. *Biochimie, 106*, 1-9.

Boschetti, & Righetti. (2011). Mixed-bed chromatography as a way to resolve peculiar protein fractionation situations. *Journal of Chromatography B, 879*(13), 827-835.

Campbell. (2009). K. & Farrell, S. *Biochemistry, 6th edition.* Thomson Brooks, Belmont, CA ; Mexico.

Chaiprapat, & Sdoodee. (2007). Effects of wastewater recycling from natural rubber smoked sheet production on economic crops in southern Thailand. *Resources, Conservation and Recycling, 51*(3), 577-590.

Chaiprapat, Wongchana, Loykulnant, Kongkaew, & Charnnok. (2015). Evaluating sulfuric acid reduction, substitution, and recovery to improve environmental performance and biogas productivity in rubber latex industry. *Process Safety and Environmental Protection, 94*, 420-429.

Coffelt, & Williams. (2009). Characterization and recycling of waste water from guayule latex extraction. *Industrial Crops and Products, 29*(2), 648-653.

Coulen, Sanders, & Bruins. (2017). Valorisation of proteins from rubber tree. *Waste and Biomass Valorization, 8*(4), 1027-1041.

d'Auzac, Jacob, & Chrestin. (1989). *Physiology of Rubber Tree Latex. The Laticiferous Cell and Latex-A Model of Cytoplasm*:, Boca Raton , Florida US: CRC Press Inc.

Dai, Kang, Li, Nie, Duan, & Zeng. (2013). In-depth proteome analysis of the rubber particle of Hevea brasiliensis (para rubber tree). *Plant Molecular Biology, 82*(1-2), 155-168.

Danwanichakul, Rattanaphan, Srisatjang, & Danwanichakul. (2014). Extraction of protein from skim natural rubber latex using PEG as a surfactant via low speed centrifugation and continuous flow. *Journal of Applied Polymer Science, 131*(4).

De Lucca, Cleveland, & Wedge. (2005). Plant-derived antifungal proteins and peptides. *Canadian Journal of Microbiology, 51*(12), 1001-1014.

de Souza Cândido, Sousa, Viana, de Oliveira-Júnior, Miranda, & Franco. (2014). The use of versatile plant antimicrobial peptides in agribusiness and human health. *Peptides, 55*, 65-78.

Denniston, Topping, Woodrum, & Caret. (2013). *General, Organic, and Biochemistry*: New York : McGraw-Hill Higher Education.

Dudev, & Lim. (2013). Competition among metal ions for protein binding sites: determinants of metal ion selectivity in proteins. *Chemical Reviews, 114*(1), 538-556.

Eng, Tanaka, & Gan. (1992). FTIR studies on amino groups in purified Hevea rubber. *Journal of Natural Rubber Research, 7*(2), 152-155.

Fekete, Beck, Veuthey, & Guillarme. (2014). Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *Journal of Pharmaceutical and Biomedical Analysis, 101*, 161-173.

Gautam, Mudhoo, Lofrano, & Chattopadhyaya. (2014). Biomass-derived biosorbents for metal ions sequestration: Adsorbent modification and activation methods and adsorbent regeneration. *Journal of Environmental Chemical Engineering, 2*(1), 239-259.

George, Alex, Joseph, & Thomas. (2009). Characterization of enzyme-deproteinized skim rubber. *Journal of Applied Polymer Science, 114*(5), 3319-3324.

Gómez-Ariza, Garcıa-Barrera, Lorenzo, Bernal, Villegas, & Oliveira. (2004). Use of mass spectrometry techniques for the characterization of metal bound to proteins (metallomics) in biological systems. *Analytica Chimica Acta, 524*(1), 15-22.

Gong, Liu, Peng, Su, & Chen. (2013). Immobilization of the proteins in the natural rubber with dialdehyde sodium alginate. *Carbohydrate Polymers, 98*(2), 1360-1365.

Guidelli, Ramos, Zaniquelli, & Baffa. (2011). Green synthesis of colloidal silver nanoparticles using natural rubber latex extracted from Hevea brasiliensis. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 82*(1), 140-145.

Guo, He, Strong, & Wang. (2014). Binding between lead ions and the high-abundance serum proteins. *Chemosphere, 112*, 472-480.

J Van Parijs. (1991). Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. *Planta*. 183(2), 258-264.

Jekel, Hofsteenge, & Beintema. (2003). The patatin-like protein from the latex of Hevea brasiliensis (Hev b 7) is not a vacuolar protein. *Phytochemistry, 63*(5), 517-522.

Kanokwiroon, Teanpaisan, Wititsuwannakul, Hooper, & Wititsuwannakul. (2008). Antimicrobial activity of a protein purified from the latex of Hevea brasiliensis on oral microorganisms. *Mycoses, 51*(4), 301-307.

Kawahara, Yusof, Noguchi, Kosugi, & Yamamoto. (2014). Organic–inorganic nanomatrix structure and properties of related naturally occurring rubbery macromolecules. *Polymer, 55*(20), 5024-5027.

Kemp. (1937). Purified Rubber for Electrical Insulation. *Rubber Chemistry and Technology, 10*(3), 474-491.

Kongkaew, Dokkhan, Pattanawanidchai, Chaikumpollert, & Loykulnant. (2012). Factors affecting creaming efficiency of bio-based polymers, vulcanization and mechanical properties of creamed skim rubber. *Biomass and Bioenergy, 46*, 233-241.

Kozlowski, Potocki, Remelli, Rowinska-Zyrek, & Valensin. (2013). Specific metal ion binding sites in unstructured regions of proteins. *Coordination Chemistry Reviews, 257*(19), 2625-2638.

Kröner, Hanke, Nfor, Pinkse, Verhaert, Ottens, & Hubbuch. (2013). Analytical characterization of complex, biotechnological feedstocks by pH gradient ion exchange chromatography for purification process development. *Journal of Chromatography A, 1311*, 55-64.

Laursen, Schjørring, Kelly, & Husted. (2014). Authentication of organically grown plants–advantages and limitations of atomic spectroscopy for multi-element and stable isotope analysis. *TrAC Trends in Analytical Chemistry, 59*, 73-82.

Li, Deng, Liu, & Qin. (2013). Molecular cloning, expression profiles and characterization of a novel translationally controlled tumor protein in rubber tree (Hevea brasiliensis). *Journal of Plant Physiology, 170*(5), 497-504.

Liu, Liu, Guan, Ma, Chen, Gu, & Qu. (2009). Transcriptional profiling of Arabidopsis seedlings in response to heavy metal lead (Pb). *Environmental and Experimental Botany, 67*(2), 377-386.

Maznah, Baharin, Hanafi, Azhar, & Hakim. (2008). Effect of acid treatment on extractable protein content, crosslink density and tensile properties of natural rubber latex films. *Polymer Testing, 27*(7), 823-826.

Mejáre, & Bülow. (2001). Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. *TRENDS in Biotechnology, 19*(2), 67-73.

Miguel, Cass, Weiss, & Glovsky. (1996). Latex allergens in tire dust and airborne particles. *Environmental Health Perspectives, 104*(11), 1180.

Mubarak, Yusof, & Alkhatib. (2011). The production of carbon nanotubes using two-stage chemical vapor deposition and their potential use in protein purification. *Chemical Engineering Journal, 168*(1), 461-469.

Nawamawat, Sakdapipanich, Ho, Ma, Song, & Vancso. (2011). Surface nanostructure of Hevea brasiliensis natural rubber latex particles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 390*(1), 157-166.

Perrella, & Gaspari. (2002). Natural rubber latex protein reduction with an emphasis on enzyme treatment. *Methods, 27*(1), 77-86.

Posch, Chen, Wheeler, Dunn, Raulf-Heimsoth, & Baur. (1997). Characterization and identification of latex allergens by two-dimensional electrophoresis and protein microsequencing. *Journal of Allergy and Clinical Immunology, 99*(3), 385-395.

Rangabhashiyam, & Selvaraju. (2015). Evaluation of the biosorption potential of a novel Caryota urens inflorescence waste biomass for the removal of hexavalent chromium from aqueous solutions. *Journal of the Taiwan Institute of Chemical Engineers, 47*, 59-70.

Rippel, Lee, Leite, & Galembeck. (2003). Skim and cream natural rubber particles: colloidal properties, coalescence and film formation. *Journal of Colloid and Interface Science, 268*(2), 330-340.

Rolere, Liengprayoon, Vaysse, Sainte-Beuve, & Bonfils. (2015). Investigating natural rubber composition with Fourier Transform Infrared (FT-IR) spectroscopy: A rapid and non-destructive method to determine both protein and lipid contents simultaneously. *Polymer Testing, 43*, 83-93.

Sakdapipanich, Kalah, Nimpaiboon, & Ho. (2015). Influence of mixed layer of proteins and phospholipids on the unique film formation behavior of Hevea natural rubber latex. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 466*, 100-106.

Salgın, Salgın, & Bahadır. (2012). Zeta potentials and isoelectric points of biomolecules: the effects of ion types and ionic strengths. *International Journal Electrochemistry Science, 7*, 12404-12414.

Sansatsadeekul, Sakdapipanich, & Rojruthai. (2011). Characterization of associated proteins and phospholipids in natural rubber latex. *Journal of Bioscience and Bioengineering, 111*(6), 628-634.

Santipanusopon, & Riyajan. (2009). Effect of field natural rubber latex with different ammonia contents and storage period on physical properties of latex concentrate, stability of skim latex and dipped film. *Physics Procedia, 2*(1), 127-134.

Skoog, D. A., (2004). *Fundamentals of Analytical Chemistry*. Belmont, CA: Thomson-Brooks/Cole.

Soedjanaatmadja, Subroto, & Beintema. (1995). The effluent of natural rubber factories is enriched in the antifungal protein hevein. *Bioresource Technology, 53*(1), 39-41.

Soedjanaatmadja, Subroto, & Beintema. (1995). Processed products of the hevein precursor in the latex of the rubber tree (Hevea brasiliensis). *FEBS Letters, 363*(3), 211-213.

Sulaiman, Ibrahim, & Abdullah. (2009). Membrane Bioreactor for the treatment of natural rubber wastewater. *International Journal of Environmental Engineering, 2*(1-3), 92-109.

Suwatthanarak, Than-ardna, Danwanichakul, & Danwanichakul. (2016). Synthesis of silver nanoparticles in skim natural rubber latex at room temperature. *Materials Letters, 168*, 31-35.

Swart. (2013). Metrology for metalloproteins—where are we now, where are we heading? *Analytical and Bioanalytical Chemistry, 405*(17), 5697-5723.

Tata. (1971). New methods for the location of proteins after electrophoresis in starch gel and on paper. *Analytical Biochemistry, 42*(2), 470-475.

Ueda, Gout, & Morganti. (2003). Current and prospective applications of metal ion–protein binding. *Journal of Chromatography A, 988*(1), 1-23.

Ulrichs, Drotleff, & Ternes. (2015). Determination of heat-induced changes in the protein secondary structure of reconstituted livetins (water-soluble proteins from hen's egg yolk) by FTIR. *Food Chemistry, 172*, 909-920.

Van Holde, Johnson, & Ho. (2006*). Principles of Physical Biochemistry*. Upper Sadle River N.J. United States :Pearson Prentice Hall

Van Parijs, Broekaert, Goldstein, & Peumans. (1991). Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. *Planta, 183*(2), 258-264.

Veerasamy, & Ismail. (2012). Rehabilitation of fouled membrane from natural rubber skim latex concentration through membrane autopsy and ultrasonication enhanced membrane cleaning procedure. *Desalination, 286*, 235-241.

Veerasamy, Supurmaniam, & Nor. (2009). Evaluating the use of in-situ ultrasonication to reduce fouling during natural rubber skim latex (waste latex) recovery by ultrafiltration. *Desalination, 236*(1-3), 202-207.

Vijayaraghavan, & Balasubramanian. (2015). Is biosorption suitable for decontamination of metal-bearing wastewaters? A critical review on the state-of-the-art of biosorption processes and future directions. *Journal of Environmental Management, 160*, 283-296.

Wade L. G., (2003). *Organic Chemistry (5th edition),* Upper Sadle River N.J United States : Prentice Hall.

Wang, Liu, Wu, You, He, Zhang, Zhang, et al. (2016). Micromorphological characterization and label-free quantitation of small rubber particle protein in natural rubber latex. *Analytical Biochemistry, 499*, 34-42.

Wei, Luo, Zheng, Qiu, Yang, Wu, & Xiao. (2015). Transcriptome sequencing and comparative analysis reveal long-term flowing mechanisms in Hevea brasiliensis latex. *Gene, 556*(2), 153-162.

Wititsuwannakul, Pasitkul, Jewtragoon, & Wititsuwannakul. (2008). Hevea latex lectin binding protein in C-serum as an anti-latex coagulating factor and its role in a proposed new model for latex coagulation. *Phytochemistry, 69*(3), 656-662.

Wititsuwannakul, Pasitkul, Kanokwiroon, & Wititsuwannakul. (2008). A role for a Hevea latex lectin-like protein in mediating rubber particle aggregation and latex coagulation. *Phytochemistry, 69*(2), 339-347.

Wititsuwannakul, Rukseree, Kanokwiroon, & Wititsuwannakul. (2008). A rubber particle protein specific for Hevea latex lectin binding involved in latex coagulation. *Phytochemistry, 69*(5), 1111-1118.

Wittsuwannakul, Wititsuwannakul, & Sakulborirug. (1998). A lectin from the bark of the rubber tree (Hevea brasiliensis). *Phytochemistry, 47*(2), 183-187.

Xiang, Xia, Dai, Kang, Li, Nie, Duan, et al. (2012). Proteome analysis of the large and the small rubber particles of Hevea brasiliensis using 2D-DIGE. *Plant Physiology and Biochemistry, 60*, 207-213.

Xu, Liu, Wang, Gu, & Chen. (1998). Purification and characterization of a novel anti-fungal protein from Gastrodia elata. *Plant Physiology and Biochemistry, 36*(12), 899-905.

Yang, Li, Guo, Tang, & Peng. (2014). Identification and characterization of the 14-3-3 gene family in Hevea brasiliensis. *Plant Physiology and Biochemistry, 80*, 121-127.

Yao, Zhao, Wang, Wang, Wei, & Hu. (2013). Microbial desulfurization of waste latex rubber with Alicyclobacillus sp. *Polymer Degradation and Stability, 98*(9), 1724-1730.

Yip, & Cacioli. (2002). The manufacture of gloves from natural rubber latex. *Journal of Allergy and Clinical immunology, 110*(2), S3-S14.

Yunginger, Jones, Fransway, Kelso, Warner, & Hunt. (1994). Extractable latex allergens and proteins in disposable medical gloves and other rubber products. *Journal of Allergy and Clinical Immunology, 93*(5), 836-842.

Zhang, Xie, Wei, Li, & Yang. (2015). Purification and characterisation of an antifungal protein, MCha-Pr, from the intercellular fluid of bitter gourd (Momordica charantia) leaves. *Protein Expression and Purification, 107*, 43-49.

Zhou, Zhang, Gao, Wang, & Qian. (2015). Influence of pH and ionic strength on heat-induced formation and rheological properties of cottonseed protein gels. *Food and Bioproducts Processing, 96*, 27-34.