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Extraction and Optimization of Natural Protein (Keratin) from Waste Chicken Feather for the Development of Anti-Ageing Cream

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This is to certify that the thesis prepared by Tewodros Abebe Lebenu entitled: **Extraction and Optimization of Natural Protein (Keratin) from Waste Chicken Feather for the Development of Anti-Ageing Cream** and submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemical Engineering complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations and Acronyms

β	-	Beta
DNA	-	deoxyribonucleic acid
UV-Vis	-	Ultraviolet–visible spectroscopy
FTIR	-	Fourier-transform infrared spectroscopy
XRD	-	X-ray diffraction
H-NMR	-	Hydrogen nuclear magnetic resonance
TGA	-	Thermo gravimetric analysis
N	-	Normality
BSA	-	Bovine Serum Albumin
ANOVA	-	Analysis of variance
3D	-	Three Dimensional

Abstract

Treatment and conversion of by-products into value added products would help not only to strengthen the economy of a country but also to protect the environment from pollution and to improve the socio-economic status of the people by creating employment. Keratin is abundantly available as a byproduct from poultry, slaughterhouse, tanning and fur processing industry. Chicken feathers, cattle and buffalo horns, tannery hair are the abundantly available sources of keratin which could be successfully converted into high value products on a large scale. In the present investigation has been made to extract valuable protein from chicken feathers and to study change in temperature, time and chemical concentration during thermo-chemical treatment thereby optimize the extraction conditions for the development of anti-ageing cream. Sodium hydroxide is used to digest the raw chicken feathers. Once the feathers are dissolved, hydrochloric acid solution is added to the solution for the precipitation of protein. The precipitated protein is washed with water several times and the protein was subjected to separation by centrifugation and freeze-drying. The effect of different parameters, such as extraction temperature, time, and NaOH concentration was studied in relation to extraction yield of keratin protein. The percentage keratin yield was found to be 23 to 82% in different extraction conditions. The extracted keratin protein was further analyzed by biuret test, FT-IR, XRD and TGA. Biuret test, FT-IR studies have been done to confirm the protein nature. XRD and TGA were used to know physical characteristics of the regenerated protein from chicken feather. A general factorial design was applied to both extraction processes using ***Design Expert*** *software and linear regression model was obtained growing the individual effect of extraction temperature, time, and NaOH concentration and their interaction in the entire extraction process. The optimum condition for the extraction of keratin protein from chicken feathers was found to be 5g feather, 0.75 N NaOH and 45 minutes reaction time at 60°C temperature. The keratin extraction developed by the optimized thermo-chemical conditions was used to produce anti-aging cream by using ingredients which include emulsifier, emollient, preservatives and surfactant.*

Keywords*: - Chicken feathers; Keratin; Extraction; Optimization; Anti-ageing cream*

Chapter 1

1. Introduction

1.1 Background

Processing poultry feather biomass into useful products presents interesting opportunities of recycling agricultural waste material (Martelli *et al*., 2006). The disposal process for chicken feather is expensive because of its important physical property(difficulty in hydrolyzing it) and also be difficult because the chicken feather is burning up with the incinerator plant, buried in the soils and also recycled as a low quality poultry feed. These processes mostly give the bad effects to the environment, especially the burning of chicken feather which will release the greenhouse gases in the air. There are several alternatives for chicken feather application, but still it is going as the waste globally because of its lack of understanding to use this biomass waste.

Post forecasts that chicken meat production in 2017 will climb slightly from the previous year to 53,000 metric tons in Ethiopia (USDA Staff, 2017). According to the information obtained from the Ministry of Agriculture and Ministry of Industry, Ethiopia, the number of poultry and meat processing industry increasing exponentially from time to time and there is no processing plants currently which can covert feather waste into low-nutritive animal feed in Ethiopia. The consumption of poultry meat grows year by year, which results in an increase of its production in poultry and poultry meat processing industry (Korniłłowicz-Kowalska, T. and Bohacz, J). This poultry and poultry meat processing industry produces a great amount of waste feathers each year which causes an environmentally difficult disposal problem. Therefore, from both an economic and environmental point of view, it is quite desirable to develop effective and profitable process to use these resources.

Several attempts have been made to degrade the keratin to ensure appropriate utilization of the protein for different industrial purposes. It is possible to influence and change the keratin structure through hydrothermal treatment, oxidation, Reduction and by microbial treatment (Eggum, 1970, Williams *et al*., 1990 and Issei and kamimura, 1983). These reactions unfold and modify the main polypeptide configuration of the protein. This transformation leads to the formation of soluble peptide products and sometimes when the reaction conditions are vigorous

the resulting protein will lose its structure and even all the proteins will go into amino acids level or sometimes even some of the essential amino acids may also lost.

Keratin is a natural protein extracted from the chicken feather. In the developed country, the usage of keratin in the personal care products is widely used. The personal care product produced from the keratin protein is conditioning shampoo, anti-aging cream, facial cleanser and others. There are some differences between the personal care products already produced by the other developed countries because the raw material used to extract the protein is sheep wool, while in this research, the extraction of keratin protein is from chicken feathers. The keratin extracted from chicken feathers is categorized as beta (β) keratin (Sawyer *et al* 2000). Keratin is being used in the production of personal care products to impart several functions: improving skin firmness; stimulate the production of new cell, anti-inflammatory and anti-oxidant properties.

Collagen synthesis plays a big role in the ageing process. Collagen synthesis in skin is inhibited because incomplete degradation of collagen by UV leads it to accumulate as partial fragments in the skin. One of collagen degradation product is keratin based anti-aging cream. As the collagen degradation products increased, the less or no collagen will produce. There are four keys in the ageing process. The processes are oxidative stress, inflammation, glycation and deoxyribonucleic acid (DNA) damage (Gupta *et al*, 2014).

The present research work aim was regarding extraction and optimization of process parameters of natural keratin protein from chicken feathers waste for the development of anti-aging cream. The optimum extraction conditions will help in decreasing the stability of keratin fibers in the solid form found in feathers and this will break down disulphide bonds, hydrogen bonds and salt linkages of the keratin fibers in order to dissolve it into protein solution.

1.2 Problem Statement

Chicken feathers are the wastes generated from the poultry industries; create a serious solid waste problem (Menandro, 2010). It is estimated that 400 million of chickens are processed every week worldwide. Chicken feathers constitute about 10% of the total weight of chicken (Grazziotin *et al*., 2006) and roughly each bird has up to 125 grams of feather, the weekly worldwide production of feather waste is about 3000 tons (Santos, 1996).The annual production of feather waste in Ethiopia at the end of 2017 is 53,000 metric tons because Post forecasts that chicken meat production in 2017 will climb slightly from the previous year to 53,000 metric tons in Ethiopia (USDA, 2017).

In Ethiopia Slaughter houses are usually located in urban or peri-urban locations, where the transport cost to markets is minimized and where there is abundant labor supply. This situation increases the risk of environmental impacts. This is because most of slaughterhouses in the underdeveloped countries often lack the land required to set-up waste-management facilities. The pollutants/byproducts that are discharged from the poultry meat processing plants create serious disposal problems and the neighboring communities are directly affected by serious diseases by the contamination of surface and ground water.

Among the solid wastes, chicken feather plays an important role because of difficulty in hydrolysing it. The disulfide bond plays an important role in safeguarding the keratin against its degradation. The current practices of collection and disposal of feather wastes are highly dependent upon the socio-economic conditions of the respective countries. Due to lack of infrastructural facilities for collection and handling, optimum utilization of these waste become difficult and considerable quantities are wasted. Improper collection and treatment of feather waste is not only economic loss but also damages the atmosphere. Thus in the present investigation, an attempt has been made to conduct studies for the extraction of valuable protein from chicken feathers for its application in anti-aging cream.

1.3 Objectives

1.3.1 General objective

The general objective of this study was to extract and optimize the extraction conditions to improve the yield and convert the waste chicken feather in to valuable product called keratin protein for industrial applications.

1.3.2 Specific objectives

The specific objectives of this research work include:

- To extract keratin protein from waste chicken feather.
- To study the effect of temperature, time and alkali concentration on the final yield of keratin and quality.
- To optimize processing parameters of the reaction.
- Development of anti-Aging cream by using extracted keratin.

1.4 Significance of the Study

This research has been done to extract natural protein from chicken feather, optimize processing parameter and develop Anti-Ageing cream. Chicken feathers are resistance to degradation and these characteristics contributes to environmental effects. Extraction of natural protein called keratin will be one of a solution in managing the wastage of chicken feathers. The research will be a base for the further study on development of keratin based personal care products. Keratin protein has a wide range of use in cosmetic and biomedical products such as conditioning shampoo, and anti-aging cream. The beta keratin protein will be used to develop new product instead of using alpha keratin protein because the natural protein extracted from chicken feathers is beta keratin protein and the amino acid composition of these alpha and beta keratin are both different.

1.5. Conceptual framework of the study

The design of experiments carried out on this study, is indicated in Figure 1.1.

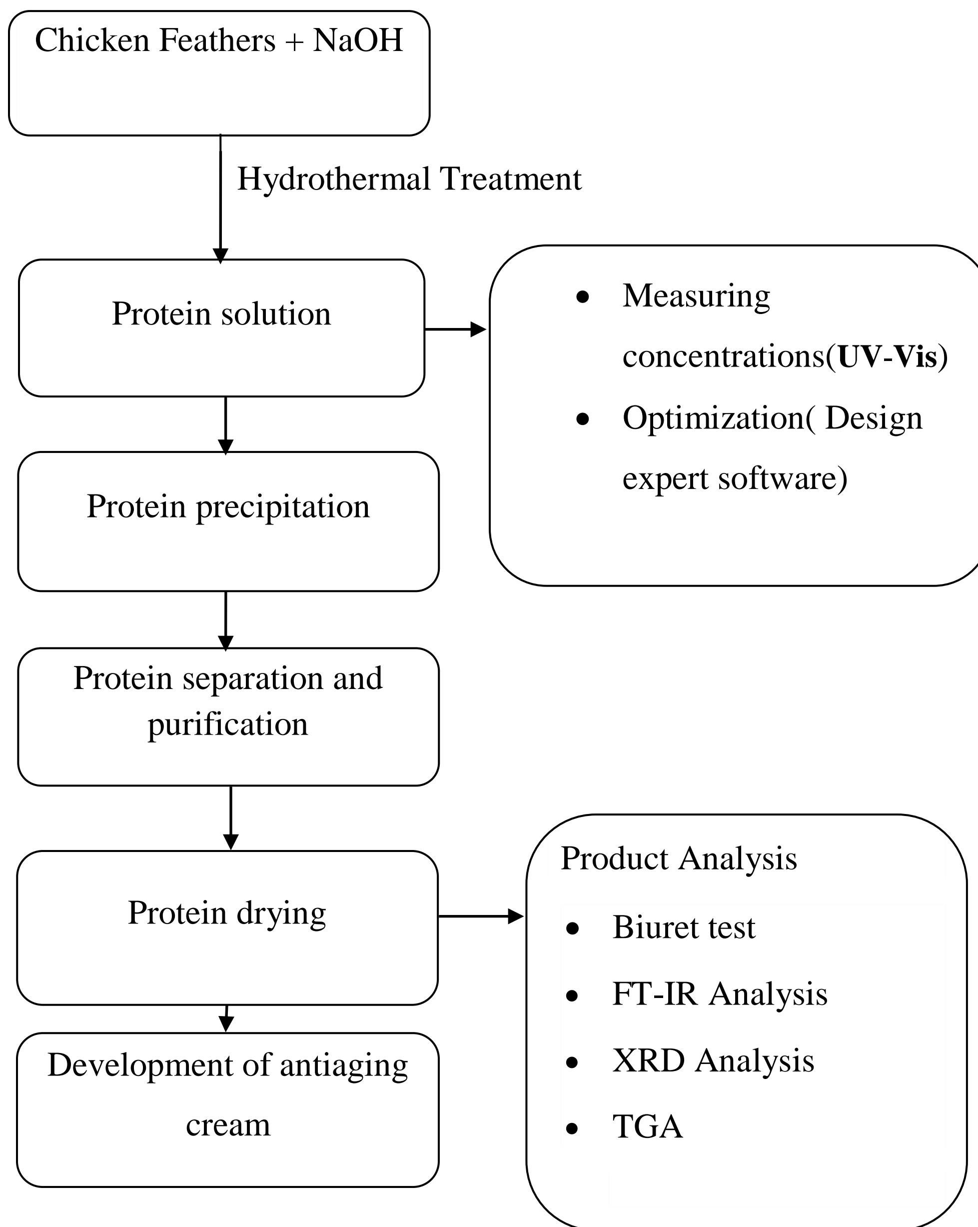

Figure 1.1 Flow chart for experiments on extraction of keratin protein from waste chicken feathers

Chapter 2

2. Review of Literature

2.1 Keratinous wastes

Increasing population, industrialization, agricultural and commercial activities have brought many troubles some changes in the environment due to the disposal of huge amount of solid wastes (Tchobanoglous *et al*., 1993). Keratin is abundantly available as a by-product from poultry, slaughterhouse, tanning and fur processing industries. Keratins are insoluble fibrous proteins found in hair, wool, feather, nail, horns, hooves, bristles and other epithelial covering. Keratins are difficult to degrade by the common proteolytic enzymes and their disposal leads to environmental problems. Among the solid wastes, keratin plays an important role because of difficulty in hydrolyzing it. The disulfide bond plays an important role in safeguarding the keratin against its degradation. The current practices of collection and disposal of keratinous wastes are highly dependent upon the socio-economic conditions of the respective countries. Due to lack of infrastructural facilities for collection and handling, optimum utilization of these waste become difficult and considerable quantities are wasted. Improper collection and treatment of keratin containing waste is not only economic loss but also damages the atmosphere. Research is ongoing to determine the innovative routes for the conversion of keratin containing solid waste into value added products.

2.2 Sources of keratin

Chicken feathers from poultry processing industries, animal hairs from tanneries, horns and hooves from slaughterhouses are the important and abundantly available sources of keratin which could be successfully converted into value added products on a large scale.

2.2.1 Poultry Chicken Feather

Chicken feather is the most abundant keratinous waste in the world. During processing of chicken for human consumption, poultry feather accumulates as a waste and it has become a burden to dispose of. Feathers constitute up to 10% of total chicken weight (Grazziotin *et al*.,

2006) and it is estimated that several million tons of chicken feathers are generated every year by the poultry processing plants world-wide (Santos, 1996). For example, the US poultry industry produces about 4 billion pounds of waste feather each year (Parkinson, 1998). Currently, the feathers are autoclaved into a low nutritional value animal feed. In some locations, regulatory prohibitions do not allow the feather waste to be used as feed. In these cases, feather disposal is by burial. The generation of this waste in developing countries, particularly in Ethiopia is not concentrated at processing plants alone like in other well developed parts of the world. Thus both generation and waste disposal in Ethiopia are different from that of developed countries.

2.2.2 Tannery Hair

Bovine and ovine hair is obtained as a by-product from the tanneries during hair-shaving process and it is estimated that about 5% of dry hair is recovered based on the raw hide weight (Scroggie 1982, Cranston et al 1986). Wool shares 15-20% of the body weight of sheep depending on the breed and climatic conditions. But still most of the tanneries are following hair-burning process, which destroy the hair completely and contribute high amount of COD, BOD, TDS etc., to the effluent (Taylor et al., 1987, Marsal et al., 1999, Thanikaivelan et al., 2002). Microbial proteases offer potential solution to remove the hair completely from the raw skins (Rao et al., 1998; Paul et al. 2001; Gehring et al., 2002; Foroughi et al., 2006).

2.2.3 Horns and Hooves from Slaughterhouse

Horns and hooves of cattle and buffaloes make up a large amount of waste products of the slaughterhouses. Traditionally, horns and hooves are used for the manufacture of buttons, combs, knife handles, and other decorative articles. Horns and hooves, particularly from buffaloes are used for the manufacture of handicrafts like buttons, cutlery handles and used as a material in tools, furniture etc. However, only selected horns and hooves are used for this purpose.

2.3 Chemistry of keratin

Based on physical characteristics, histology and chemical composition, keratins are divided into two categories, the soft keratin and hard keratin. The outer layer of epidermis of the skins is an example of soft keratins. Horn, hoof and nails are classified under the hard keratins. Hair, wool and feather though classified as hard keratins also have some histological characteristics

common with soft keratin. Primarily the distinction is based on the immediate sensation of hardness or softness, and the fact that soft keratins (epidermis) desquamate while the hard keratin persists. Chemically the difference between the soft and hard keratins is known by the percentage of sulfur content which is as high as 5% in the case of hard keratin (Block and Bolling, 1951) and as low as 1% in soft keratins. Wool and feather keratins constitute about 3% of sulfur content. The distribution of sulfur content of various keratins is given in Table 2.1 (Lindley, 1948). The properties of hard and soft keratins are summarized in Table 2.2.

Table 2.1 Sulfur content of various keratins

Source	Sulfur Content in % on dry weight of keratin
Duck feathers	3.26
Calf hair	3.97
Cow tail hair	3.93
Horse hair	3.71
Camel hair	3.28
Dog hair	5.30
Rabbit hair	4.35
Cow horn	3.77
Sheep horn	2.08

Table 2.2 Properties of hard and soft keratins

Hard Keratin	Soft Keratin
Tough and Hard	Soft and Pliable
Permanent, non-desquamating	Desquamating
Low lipid content	Higher lipid content
Higher sulfur content	Lower sulfur content
Higher thermal stability	Lower thermal stability
Better oriented	Less perfect ordering

Keratins belonging to the sclera peptides group are compounds that are extremely resistant to the action of physical, chemical and biological agents. One of the main characteristics of keratin is that they have high mechanical stability and resistance to proteolytic degradation, which depends on the disulfide and hydrogen bonds, salt linkages and other crosslinking (Korkmaz et al., 2004). Therefore, keratinous material is insoluble in water, weak acids and bases, as well as in organic solvents and extremely resistant to degradation by common proteolytic enzymes such as trypsin, papain and pepsin (Gradisar, 2005). On the other hand, keratin is very reactive, as cystine can easily be reduced, oxidised, and hydrolysed (Akahane *et al*., 1977; Thannhauser *et al*., 1984).

Keratins contain a high percentage of sulfur-containing amino acids, largely cystine, which may vary between 2-18% (Fraser et al., 1972). The disulphide bridges formed between two cysteine molecules resulting in a fairly rigid structure (Arai, 1983). Keratin contains more than 22 amino acids especially in the helical regions of their structure. Of the amino acids present in the keratin, important amino acids are cystine, arginine, serine and glycine. In the amino acid analysis the presence of high portion of cysteine disulphide linkages are noticed. Nearly all the known amino acids are present in significant amounts although there is very little histidine, tryptophane and methionine. A group of 95 amino acid sequence in keratin has been identified in multiple avian species. About 40% of these amino acids in keratin are hydrophilic and the remaining 60% are hydrophobic (Shih, 1993). Some researchers claim this to be 50:50 (Barone and Schmidt, 2005). The amino acid sequence of chicken feather is very similar to that of other feathers. The sequence is largely composed of cystine, glycine, proline, and serine, and contains almost no histidine, lysine, or methonine (Schmidt and Jayasundrera, 2003). The amino acid composition of chicken feather, cattle horn, sheep wool and human hair (Ward and Lundgren, 1954) is presented in Table 1.3.

Table 2.3 Amino acid composition of chicken feather, cattle horn, sheep wool and human hair

Amino acid g/100g of dry keratin	Chicken Feather	Cattle Horn	Sheep Wool	Human Hair
Glycine	7.2	9.6	5.2-6.5	4.1-4.2
Alanine	5.4	2.5	3.4-4.4	2.8
Valine	8.3-8.8	5.3-5.5	5.0-5.9	5.5-5.9
Leucine	7.4-8.0	7.6-8.3	7.6-8.1	6.4-8.3
Isoleucine	5.3-6.0	4.3-4.8	3.1-4.5	4.7-4.8
Phenylalanine	4.7-5.3	3.2-4.0	3.4-4.0	2.4-3.6
Proline	8.8-10.0	8.2	5.3-8.1	4.3-9.6
Serine	10.2-14.0	6.8	7.2-9.5	7.4-10.6
Threonine	4.4-4.8	6.1	6.6-6.7	7.0-8.5
Tyrosine	2.0-2.2	3.7-5.6	4.0-6.4	2.2-3.0
Aspartic acid	5.8-7.5	7.7-7.9	6.4-7.3	3.9-7.7
Glutamic acid	9.0-9.7	13.8	13.1-16.0	13.6-14.2
Arginine	6.5-7.5	6.8-10.7	9.2-10.6	8.9-10.8
Lysine	1.0-1.7	2.4-3.6	2.8-3.3	1.9-3.1
Hydroxyl lysine			0.2	
Histidine	0.3-0.7	0.6-1.0	0.7-1.1	0.6-1.2
Tryptophan	0.7	0.7-1.4	1.8-2.1	0.4-1.3
Cystine	6.8-8.2	10.5-15.7	11.0-13.7	16.6-18.0
Methionine	0.4-0.5	0.5-2.2	0.5-0.7	0.7-1.0
Cysteine	0.4	0.8-1.6	0.4	0.5-0.8

2.4 Structure of keratins

There are two main forms of keratin, alpha-keratin and beta-keratin. Alpha-keratin is found in the hair, wool, horn, hoof, nail of mammals, while beta-keratin is present in the scales and claws of reptiles, and in the feathers, beaks, and claws of birds. Beta-keratin is harder than alpha-

keratin. Structurally alpha-keratin has alpha-helical coiled structure while beta-keratin has twisted beta sheet structure. X-ray diffraction and electron microscope studies of hard keratins such as horn, feather (Filshie and Rogers, 1962) have shown that they all have a filamentous texture but the molecular structure of the filaments in mammalian keratins is quite different from that in avian keratins. Parallel X-ray diffraction studies have revealed that the framework of the filaments in mammalian keratin consists of two-strand coiled-coils of α-helices whereas the framework in avian keratins is composed of β sheets (Rudall, 1947). The X-ray diffraction pattern of reptilian hard keratin is very similar to that obtained from avian hard keratins leading to the supposition that the framework of the filaments is also composed of β sheets (Fraser and Parry, 1996).

Alpha keratin fiber is a hierarchically structured material that shows a fibrous organization from the micrometer to the nanometer scale (Zahn *et al*., 1980). The main part of the fiber, called the cortex, is composed of spindle-shaped cells of ~100µm long and 3 µm wide. These cells contain macro fibrils of 0.3-µm diameter which are glued together by an intermacro fibrillar matrix. A schematic of a wool fiber which is similar to a human hair is shown in Figure 2.1.

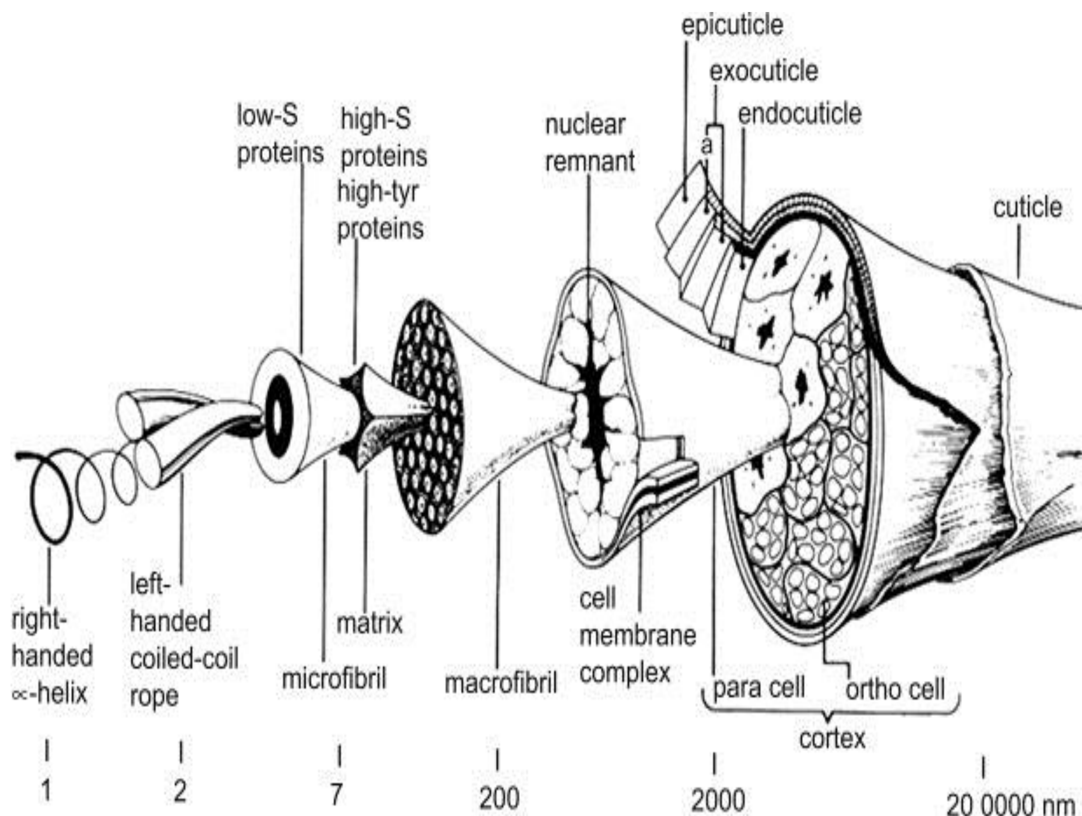

Figure 2.1 Structure of wool fiber

2.5 Approaches for the degradation of keratin

Several attempts have been made to degrade the keratin to ensure appropriate utilization of the protein for different industrial purposes. It is possible to influence and change the keratin structure through hydrolysis, oxidation and reduction. These reactions unfold and modify the main polypeptide configuration of the protein. This transformation leads to the formation of soluble peptide products possessing a number of reactive groups on their chains.

2.5.1 Hydrothermal Treatment

Hydrothermal process usually employs high steam pressure (10-15 psi) and/or high temperature (100-140°C) in the presence of acid (HCl, H_2SO_4, HCOOH etc.) (Eggum, 1970) or alkali (NaOH, KOH, Na_2CO_3, K_2CO_3, etc.)(Gousterova *et al*., 2003). Treatment with acid or alkali at the boiling temperature for over 2-3 h opens disulfide linkages of keratin and yields water soluble polypeptides, oligopeptides or even amino acids (Barret 1985, Wang and Parsons 1997;

Pernaud *et al*., 1999). The effect of change in pressure, temperature, chemicals and pH during thermo-chemical treatment has been studied to analyze the nutritional value of feather meal (Steiner *et al*., 1983; Papadopoulos *et al*., 1986, Moritz and Latshaw, 2001). Main drawback in hydrothermal process is that hydrolysis may result in partial or even complete destruction of amino acids, which contain peptides with varying molecular weight and nutritional improvement. This method leads to losses of essential amino acids (lysine, methionine and tryptophan) and causes the formation of non-nutritive amino acids (lysinoalanine, lanthionine, etc) (Papadopoulos 1989; Latshaw *et al*., 1994). Merits and demerits of alkali versus acid hydrolysis (Asquith, 1977) have been reported.

2.5.2 Microbial Treatment

Microbial conversion of keratin wastes is a potential technique for degradation and utilization of keratin as a hydrolysate in terms of cost-effective and environmentally benign processing (Williams et al., 1990, Shih 1993). Presence of disulfide bonds in keratins hinders their degradation by proteolytic enzymes (trypsin, pepsin and papain), (Parry *et al* 1977; Papadopoulos, 1986; Cohlberg, 1993; Steinert 1993) which can be efficiently degraded by soil microorganisms (Kaul and Sumabali 1997, Lucas *et al*., 2003), actinomycetes, bacteria and fungi by synthesis of keratinolytic proteases-keratinases (Dozie *et al*., 1994; Hirchsman *et al*., 1994; Lin *et al*., 1996; Sangali and Brandelli, 2000; Ichida *et al*., 2001; Kim *et al*., 2001; Manczinger *et al*., 2003). Keratinases are robust enzymes with a wide temperature and pH activity range and are largely serine or metallo proteases (Onifade *et al*., 1998). Keratinases attack keratin residues (feather, wool, steam hydrolyzed horn powder etc.) (Szabo *et al*., 2000; Gousterova *et al*., 2005) and convert them into degradative and cost effective products. Application of keratinase producing microorganisms is being explored in feed, fertilizer, detergent, leather and pharmaceutical industries where there is great need for materials derived from alternative raw materials specifically animal wastes derived from meat processing plants, poultry units, marine and slaughter houses.

2.5.3 Reaction of Keratins with oxidizing Agents

Oxidation of keratin is an important reaction particularly for hair and wool. For example, hydrogen peroxide is used as a bleaching agent. To prepare soluble derivatives performic and

peracetic acids are used. A number of oxidising agents are used for the oxidation of protein groups such as thiol, disulphide, thioether, imidazole, phenolic, and indole which are susceptible to oxidation attack.

Oxidation of wool with solutions of potassium permanganate at pH 9.0-2.0 and sodium hypochlorite at pH 10 led to the formation of lanthionine from the reactive cystine fraction. Acidic solutions of potassium permanganate, which also react with only 30% of the total cystine, gave cysteic acid and sulphate (Alexander et al., 1951). The reaction of wool keratin with hydrogen peroxide is relatively slow, expect under alkaline conditions. When wool is immersed in hydrogen peroxide solutions having pH in the range of 2.5-9.0, some of the reagent is initially absorbed on the amino and imino groups (Alexander 1950). The absorbed peroxide seems to be remarkably stable and is removed by washing.

A study has been made of the reaction of different oxidising agents with the tyrosine in wool, horn and solubilised keratin. Chlorine and hypochlorous acid oxidise all the tyrosine in wool, whereas acid permanganate and alkaline hypochlorite oxidize only 30% of the tyrosine present. The tyrosine in wool may therefore be divided into two fractions, one of which is oxidised by the latter two reagents and the other not. Peracetic acid does not oxidise the tyrosine in any of the keratinous materials studied (Alexander et al., 1951a).

2.5.4 Reaction of Keratins with Reducing Agents

Actions of reducing agents on keratin fibres have been almost totally confined to the behavior of the disulphide bond not only of its use for the preparation of soluble derivatives but also because it is the basis of some setting methods. For example keratin hydrolysate is prepared by conducting the reduction of keratin with a reducing agent such as a mercaptan or a sulfide under alkaline conditions, by which disulfide linkages of keratin are severed to produce mercapto groups, and then subjecting the resulting reduction product to enzymatic hydrolysis, by which the peptide linkages are severed to lower the molecular weight (Issei and Kamimura, 1983).

2.6 Options available for the utilization of keratins

Keratins find applications in food, pharmaceutical, cosmetic and fertilizer industry. Research is being done globally to utilize these wastes. The resulting products have several potential

applications in various industries such as retanning and finish agent in leather industry, as a fertilizer in the agricultural industry, as additives in the building industry, as an eco-adhesive in the wood industry, as a binder to partially substitute casein in the paper industry.

Chicken feather is used as animal feedstuff in the form of feather meal (Steiner et al., 1983, Papadopoulos *et al*.; 1986, Kelly *et al*.; 2002; Kelly *et al*., 2006). Acid, alkali or enzymes hydrolyze keratin and hydrolysates have number of applications (Naito and Nemoto, 1986; Fleischner, 1989; Edens *et al*., 2005; Gousterova *et al*., 2005; Grazziotin, 2006). Cosmetics based on keratin preparations have been reported for the treatment of human hair and skin (Kim *et al*., 1990; Wiegmann *et al*., 1990). Keratinous materials are used as additive in the preparation of concrete and ceramics (Kawashima, 1993; Kawashima, 1994). Sulfur bound amino acid solution is used to prepare organic fertilizer, which enhances plant metabolism (Chikura et al., 1994). Firefighting composition is prepared from a solution of organic colloid derived from the hydrolysis of horns and hooves (Datta, 1993). Oxidization of keratinous materials cleaves and oxidises some of the disulfide linkages to form water-soluble peptides and this material is used as a wound healing agent (Van Dyke *et al*., 2001). Keratin derived from hair is converted into scaffold for use in biomedical implant, tissue engineering and wound dressing applications (Timmons *et al*., 2000). Keratin hydrogel formed from clean, washed hair by partially oxidising a significant percentage of disulfide linkages to form cysteic acid groups can be used as a wound dressing and cell scaffolding (Blanchard *et al*., 1999). Peptides derived from human hair or sheep wool keratins by breaking the disulfide linkage with oxidants are used for the preparation of compositions for pharmaceutical or topical administration or for use in cosmetic preparations (Cowsar, 2003).

Formulation containing keratin hydrolysate helps to deposit on the scale faces of the hair surface which increases the hydrophilicity of the hair fiber (Wiegmann et al., 1990) and it is also reported to cure the damaged hair. Composition for both cleansing and moisturisation of skin comprise a combination of surfactant, an emollient, a cationic material, a film former and a polyamino condensate has been reported (Baker and Zucker, 1993).

Jet printing inks containing water soluble keratin proteins have the ability to give prints with good coloured and water resistance properties (Masahiro *et al*., 1996). Keratin proteins added to porous ceramic products offer uniform and evenly distributed gas bubbles in the ceramic materials (Kawashima, 1994). Biocompatibility of rubber is enhanced when keratin proteins are dissolved during its preparation (Sakaki and Nakade, 1994). The finishing of silk or wool fabrics are treated with aqueous solution containing keratin proteins and cross linking agents for improving dyeing or printing color yield (Nomura 1994) and pleat retention properties. Cotton fabrics treated with an aqueous solution containing sheep keratin hydrolysate and NaCl at 50°C have the ability to improve heat retention.

Chicken feathers can be used as a low cost adsorbent to remove heavy metals from waste water. Natural and chemically treated chicken feathers were tested for their ability as adsorbents to remove copper and zinc from wastewater (Al-Asheh *et al*., 2003). Dyes can also be adsorbed by chicken feathers (Gupta *et al*., 2006, Mittal, 2006).

2.7 Development of Anti-Ageing Cream from Chicken Feathers

Human skin cell is containing keratin and it is the first protection line in defending diseases. The integumentary system plays as natural sunscreen, provide spaces for fatty tissue storage and regulating the body temperature by providing the sensory input to brain (Colbert *et al*.,2009). As a multifunctional layer, skin is exposed to variety defect factors that lead to skin ageing. Skin ageing happens in several ways. It may looks as wrinkles, dullness and pigmentation occurs in skin (Kato,E. and Tsuzuki,T., 2011). As human beings grow older, the maximal functioning and reverse capacity is decreasing and this phenomenon called an ageing process (Yaar, M. and Gilchrest, B.A., 2007). Collagen synthesis plays a big role in the ageing process. Collagen synthesis in skin is inhibited because incomplete degradation of collagen by UV leads it to accumulate as partial fragments in the skin. As the collagen degradation products increased, the less or no collagen will produce. There are four keys in the ageing process. The processes are oxidative stress, inflammation, glycation and deoxyribonucleic acid (DNA) damage. The formulations of anti-ageing creams were done based on the ratios (Table 2.4).

Table 2.4:- Ingredients for Cream formulation of anti-ageing cream

Ingredients	K1-L0 (g)	K2-L0 (g)	K1-L1 (g)	K2-L1 (g)
Cetostearyl alcohol	3.0	3.0	3.0	3.0
Palm Oil	3.0	3.0	3.0	3.0
Glycerin	4.0	4.0	4.0	4.0
Zinc oxide	2.0	2.0	2.0	2.0
Citric acid	1.0	1.0	1.0	1.0
Keratin	2.0	4.0	2.0	4.0
Distilled Water	q.s.	q.s.	q.s.	q.s.
Cremophor	2.0	2.0	2.0	2.0
Lecithin	-	-	1.0	1.0

Chapter 3

3. Materials and Methods

The experiments were carried out in the School of Chemical and Bio Engineering laboratory, Addis Ababa Institute of Technology, and in the Department of Chemistry, Natural Science campus, Addis Ababa University, Addis Ababa, Ethiopia.

3.1 Materials

Materials and chemicals used during extraction and characterization of keratin protein from chicken feather were: Test tubes and Beakers, Electronic weighing balance , Water bath, measuring cylinder, Round bottom flask, Electric heater, Distilled water, sodium hydroxide, BSA, dichloromethane, toluene, Biuret reagent, XRD, TGA, centrifugal separator, FT-IR,UV visible spectroscope ,oven, Cuvet, micro pipet.

3.2 Experimental methods

3.2.1 Extraction of keratin protein

3.2.1.1 Soxhlet Extraction

Chicken feathers were collected from ELFORA poultry processing industry Debre Zeyit (Bishoftu) is located 47.9 kilometers south east of Addis Ababa, along its route highway. Initially by using soap water the feathers were washed, and dried. Further in order to remove the stains, oil and grease compounds. Soxhlet extraction system was used in order to eliminate lipid and residual compounds found in chicken feather (Figure 3.1). Organic solvents such as dichloromethane and toluene were used to conduct the extraction process. Time of the extraction was 6 hours. After the completion of extraction process, the chicken feathers were dried. The dried feathers were dissolved in aqueous solution of NaOH at different concentration level in order to break “Disulfide Bridge” that confer additional strength and rigidity.

3.2.1.2 Size reduction

After drying, the feathers were cut into small segments using scissors for size of around 1mm and kept in a sealed plastic bag.

3.2.1.3 Keratin extraction

Chicken feathers (5 g) were mixed with, 0.5, 0.75 and 1 Normality (N) NaOH. The reaction was conducted in a water bath under dynamic conditions at 40, 60 and 80 ^{o}C for 30, 45 and 60 minutes (Figure 3.2). By varying the reaction time, temperature and NaOH concentration, different experiments were conducted and the absorbance of every sample (extracted keratin) was measured using UV-visible spectrophotometry. With the help of mechanical stirrer, the chicken feathers were mixed and dissolved under the basic condition. The solution was then filtered and centrifuged at 10,000 rpm for 5 minutes. The supernatant liquid was carefully collected and then filtered using filter paper in order to make the extraction particle free. The absorbance change to concentration was measured using standard curve (See Equation 3.1). Finally, the percentage yield of protein from each experiment was calculated by using equation 3.2 and the results were summarized in Table 4.1.

3.2.1.4 Estimation of Protein by Biuret Method

Reagents Required:

a. **Biuret Reagent**: Dissolve 3 g of copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium potassium tartrate in 500 ml of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodium hydroxide (www.ruf.rice.edu/biuretreagent).

b. **Protein Standard**: 5 mg BSA/ml (www.ruf.rice.edu/proteinstandard).

Procedure:

a. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working protein standard in to the series of labeled test tubes.
b. Pipette out 1 ml of the given samples in to the series of labeled other test tubes.
c. Make up the volume of working standard to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
d. Now add 3 ml of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
e. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10 min.

f. Now cool the contents to room temperature and record the absorbance at 540 nm against blank.
g. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.
h. Then from this standard curve calculate the concentration of protein in the given samples by using equation 3.1.

Table 3.1 Standard protein for determination of protein in unknown sample

<table>
<tr><th>Volume of standard BAS(ml)</th><th>Volume of distilled water(ml)</th><th>Concentration of protein(mg)</th><th>Volume of biuret reagents(ml)</th><th></th><th>A_{540}</th></tr>
<tr><td>0.0</td><td>1</td><td>0.0</td><td>3</td><td rowspan="6">Incubate at 37°C for 10 min & cool</td><td>0.00</td></tr>
<tr><td>0.2</td><td>0.8</td><td>1</td><td>3</td><td>0.041</td></tr>
<tr><td>0.4</td><td>0.6</td><td>2</td><td>3</td><td>0.095</td></tr>
<tr><td>0.6</td><td>0.4</td><td>3</td><td>3</td><td>0.149</td></tr>
<tr><td>0.8</td><td>0.2</td><td>4</td><td>3</td><td>0.203</td></tr>
<tr><td>1</td><td>0.0</td><td>5</td><td>3</td><td>0.257</td></tr>
</table>

3.2.1.5 Determination of percentage of keratin extracted using UV spectrophotometry

The mass of soluble protein was determined from that standard curve using the following equation and expressed as mg per ml of test sample.

$$y = mx \qquad (3.1)$$

Where,

y = absorbance at 540 nm

x = protein concentration in mg/ml

m= slope of the standard curve

The percentage of extraction yield was calculated using the formula below. Since chicken feather have protein content about 90% the yield (Swati,S. and Arun,G., 2016).

$$\text{Percentage protein yield} = \frac{\text{mass of protein}}{0.9\text{x mass of the sample}} x100\% \qquad (3.2)$$

3.2.1.6 Protein precipitation

After the protein extraction process, the pH of keratin solution was adjusted to 4.2 by using diluted HCl in order to precipitate keratin (Fig 3.3). Then the solution was centrifuged at 10,000 rpm for 5 minutes and the solids particles were carefully collected. The supernatant liquid was also collected separately and step 2 and 3 were repeated (Arun, *et al*., 2012).

3.2.1.7 Protein purification

The collected solid particles were added into 100ml deionized water and stirred (washing). The solution was centrifuged at 10,000 rpm for 5 minutes and the solids were collected. The collected solid particles were then dissolved in 100ml of 2M sodium hydroxide solution (Fig 3.4). The precipitating, washing and dissolving steps are repeated 3 times (Arun, *et al*., 2012). Finally the product was grounded with mortar and pestle and sited with stainless steel mesh (pore size 30 μm) in order to acquire fine powder keratin (Fig3.4).

3.2.2. Analysis of keratin protein

3.2.2.1 Biuret test

1% copper sulphate solution and 1% potassium hydroxide solution were prepared. The 5ml of the solution collected was mixed with potassium hydroxide solution with 1:1 ratio. Three drops of copper sulphate solution were added to the mixture solution. Changes in the solution observed and recorded (Arun, *et al*., 2012).

3.2.2.2 FTIR

Fourier transform of infrared (FTIR) used to understand all details of structure of keratin. The characteristic bands and signals from these spectra were analyzed in order to confirm the protein nature of the extracted keratin (Azila, *et al*., 2014).

3.2.2.3 XRD

It is a non-destructive analytical technique which reveals the information about the crystal structure, chemical composition and physical properties of materials and thin films. These techniques are based on observing the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy(Azila, *et al*., 2014).

3.2.2.4 TGA

Thermo gravimetric analysis or thermal gravimetric analysis (TGA) is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature with constant heating rate, or as a function of time with constant temperature and/or constant mass loss. Keratin heated up from $25^{o}C$ to $700^{o}C$ under N_2 atmosphere with a heating rate $10^{o}C/min$ (Azila, *et al*., 2014).

3.3 Design of the Experiment

3.3.1General factorial Design

Data analysis has performed by design expert software using General factorial Design Method. For hydrolysis extraction we had three factors temperature, time and NaOH concentration with three levels for all three factors. This design of the experiment helps us to differentiate the significance of the main and the interaction factors. This program software also used to develop the mathematical model that will describe the effects of the main and interaction factors on the response.

3.4 Preparation of anti-ageing cream

In the formulation of anti-ageing creams, water bath was used to mix all the ingredients in the beaker at 60°C because the heat will be distributed more evenly in the beaker when the water bath is used. Oil and water phase was prepared separately. Cetostearyl alcohol was mixed into glycerin until clear solution obtained. Then, palm oil was added in the clear solution and the oil phase was done. Water phase solution was prepared by dissolving citric acid in the distilled water. Water and oil phase were mixed. Then, camphor and zinc oxide were added. Finally, the prepared cream mixture was cooled with continuously stirring. Finally, keratin was added to the

cold solution and homogenized (Gupta *et al*, 2014). The recipe for the formulation of anti-ageing cream is presented in Table 3.2.

Table 3.2 Ingredients for Cream formulation of anti-ageing cream

Ingredients	**Amount (g)**
Cetostearyl alcohol	3.0
Palm Oil	3.0
Glycerin	4.0
Zinc oxide	2.0
Citric acid	1.0
Keratin	4
Distilled Water	Small quantity
Camphor	2.0
Lecithin	1

Chapter 4

4. Results and Discussion

4.1 Construction of standard curve

For determination of keratin protein concentration by biuret method, a standard curve with BSA (Bovine Serum Albumin) was prepared. A range of standard solutions of BSA was prepared according to the procedure described in section 3.2.1.4. Standard curve for estimation of protein in unknown samples was constructed and showed in Figure 4.1.The best fit linear equation intercepting zero is derived. The equation y =0.0521x-0.0062 was used for estimation of protein in unknown sample where 'y ' is the absorbance at 540 nm and ' x' is the concentration of protein in unknown sample in mg/ml

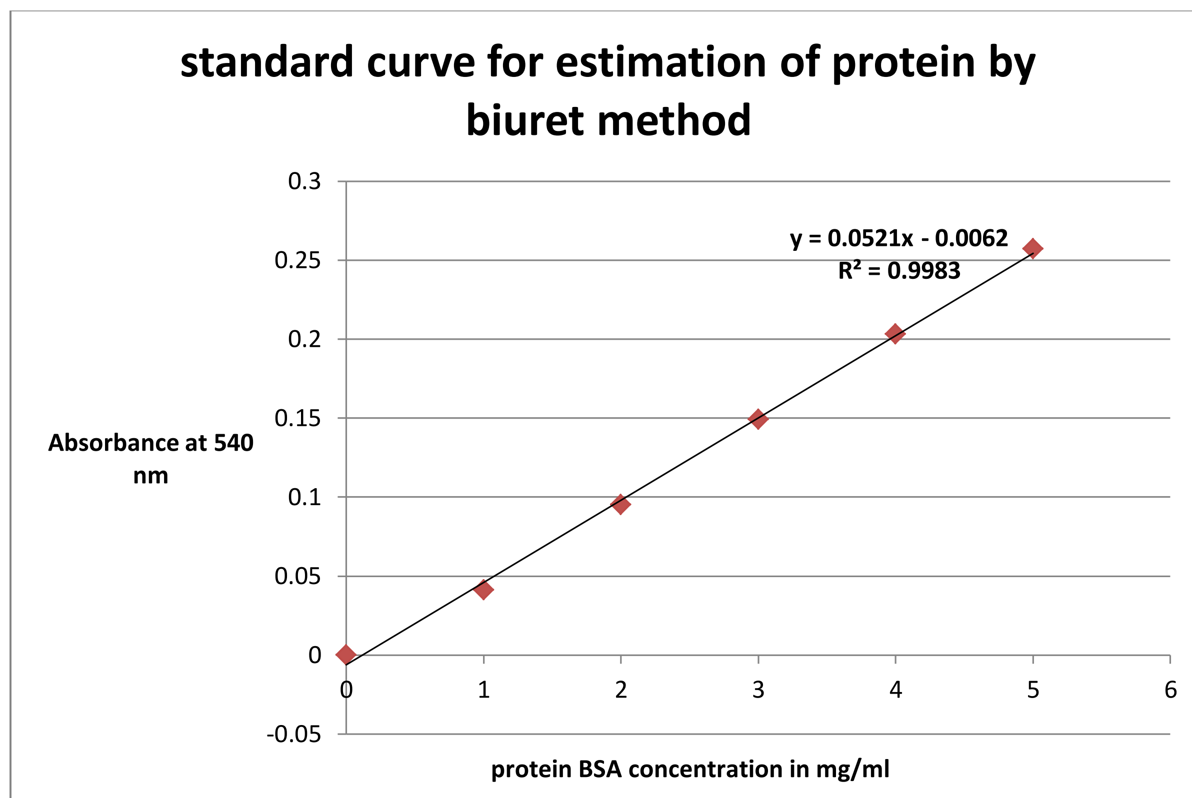

Figure 4.1 Standard curves for estimation of protein concentration

The strong linear relationship (**R^2>0.99**) between the absorbance of protein and concentration demonstrates exceptional reliability in estimating protein content of unknown samples.

4.2 Extraction of Keratin from Chicken Feather

The schematic of breakage of disulphide of bond during the application of NaOH was presented in Figure 4.2. After disulfide bond breaking, keratin was precipitated with Diluted HCl.

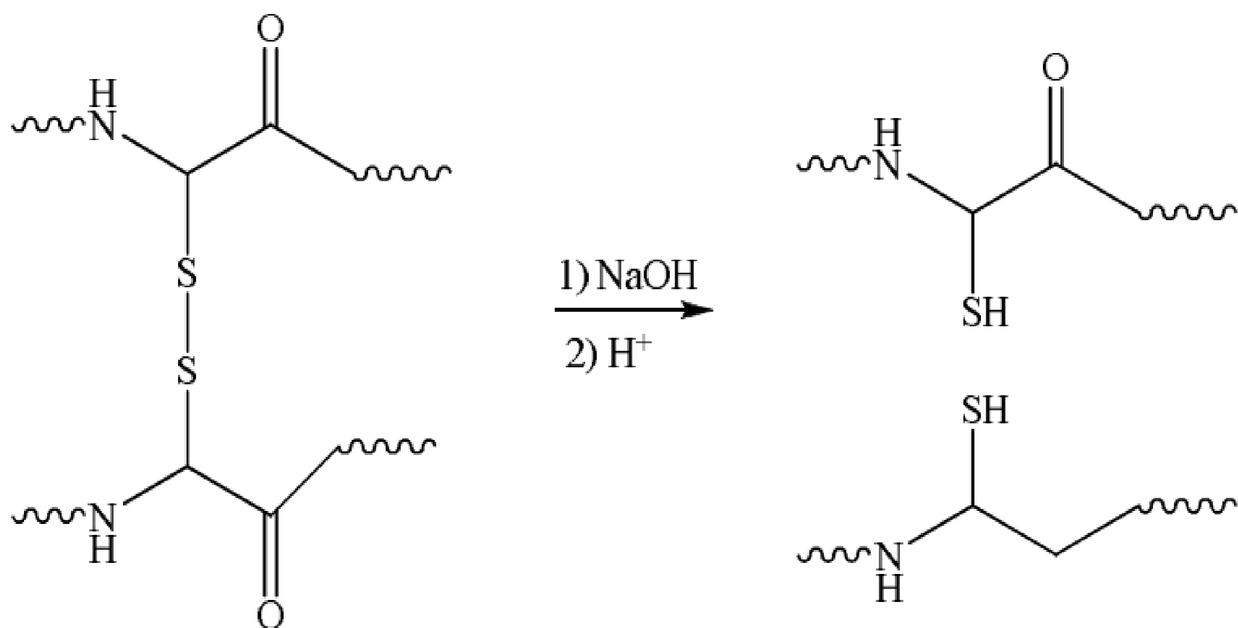

Figure 4.2 The process of disulfide bond break using NaOH solution

Feather hydrothermal experiment performed on various parameters of the process has shown the variability of output of the process. The content of keratin yield was in the range 23 - 82%. The lowest content of keratin yield (23%) was obtained for the parameters of process: temperature 40°C, duration of process 60 minutes , solution concentration 0.5 Normality. The highest yield (82 %) reported for the test conducted at the Temperature of 60°C and for 45 minute and 0.75 Normality of NaOH solution. Temperature and NaOH concentration mainly affect the yield of final keratin. This is due to its content in the final product after the reaction because NaOH remains in the system and enters into the composition of the product. The greater concentration of NaOH makes the lower content of total protein yield. When temperature

increase from 40 to 60 °C reaction can provide much energy to accelerate physical and chemical change of feather keratin and thus promote the dissolution of feathers (the yield increase) but when the temperature increase from 60 to 80 °C the yield become constant because of when the reaction conditions are vigorous the resulting protein will lose its structure and even all the proteins will go into amino acids level. The amount of kerain yield in the final product depends on the degree of hydrolysis of the feather. The environment of the process (Temperature, Time and NaOH concentration) influences the degree of degradation of that biopolymer. An increasing of keratin yield in the bases is caused by peptide and disulfide bonds cleavage. The solubility is dependent on the concentration of base in the, temperature, NaOH concenteration and process time. Tests performed during the waste feathers conversion into keratin showed that the yield of protein depends on many parameters of the process.

The parameters of the process and results of the product's analysis are summarized in Table 4.1. The highest yield of keratin was found to be 82% by the 9th run. The optimum extraction conditions are 5 g keratin, 60°C, 45 minute and 0.75 NaOH mole/l.

Table 4.1 Total percentage yields of keratin protein for different condition

Run	Factor 1 A:Temperature (^{o}C)	Factor 2 B:Time (Minute)	Factor 3 C:Concenteration (Normality)	Absorbance (average)	Response Keratin Yield (%)
1	80.00	60.00	1.00	0.189905	81
2	60.00	45.00	1.00	0.18756	80
3	60.00	60.00	0.50	0.171852	73.3
4	80.00	45.00	1.00	0.189905	81
5	80.00	30.00	0.50	0.107847	46
6	80.00	30.00	1.00	0.143015	61
7	60.00	60.00	1.00	0.188029	80.2
8	40.00	60.00	0.75	0.113943	48.6

9	60.00	45.00	0.75	0.192249	82
10	40.00	45.00	0.50	0.058613	25
11	60.00	30.00	0.75	0.128948	55
12	40.00	30.00	0.75	0.105503	45
13	40.00	60.00	1.00	0.117225	50
14	60.00	30.00	1.00	0.14067	60
15	40.00	30.00	0.50	0.04689	20
16	60.00	60.00	0.75	0.185216	79
17	40.00	45.00	0.75	0.114881	49
18	40.00	60.00	0.50	0.053924	23
19	80.00	45.00	0.75	0.189905	81
20	80.00	60.00	0.50	0.168804	72
21	40.00	45.00	1.00	0.14067	60
22	80.00	60.00	0.75	0.168804	72
23	80.00	45.00	0.50	0.164115	70
24	80.00	30.00	0.75	0.168804	72
25	60.00	45.00	0.50	0.171149	73
26	60.00	30.00	0.50	0.109254	46.6
27	40.00	30.00	1.00	0.112536	48

4.3 Effect of process parameters in percentage yield of keratin protein from waste chicken feather

In general there are many parameters that will influence final yield of keratin during thermo-chemical treatment. But in our study, we focused on three important reaction conditions (i) reaction temperature, (ii) reaction time and (iii) NaOH concentration in order to understand the effectiveness of different conditions on the quality and quantity the of final yield.

4.3.1 Effect of extraction temperature on percent yield of keratin protein.

Temperature is an important factor affecting the yield of keratin, and the rate of dissolution of feather keratin at various temperatures (40-80^{0}C) under different time and

NaOH concentration is plotted in the form of graph and presented in Figure 4.3. From the Figure, it is clear that the yield increased obviously with the raise of temperature from 40°C to 60°C and approached a flat stage from 60°C to 80°C. It was because higher reaction temperature can provide much energy to accelerate physical and chemical change of feather keratin and thus promote the dissolution of feathers. However, when the temperature was beyond 60°C, the yield was constant. Peptide bond cleavage of feather keratin occurred at the higher temperature but when we use high temperature during thermos-chemical treatment, there may be chances for the loss of protein/amino acid structure. Therefore, 60°C is selected as the optimal reaction temperature for the development of anti-aging cream.

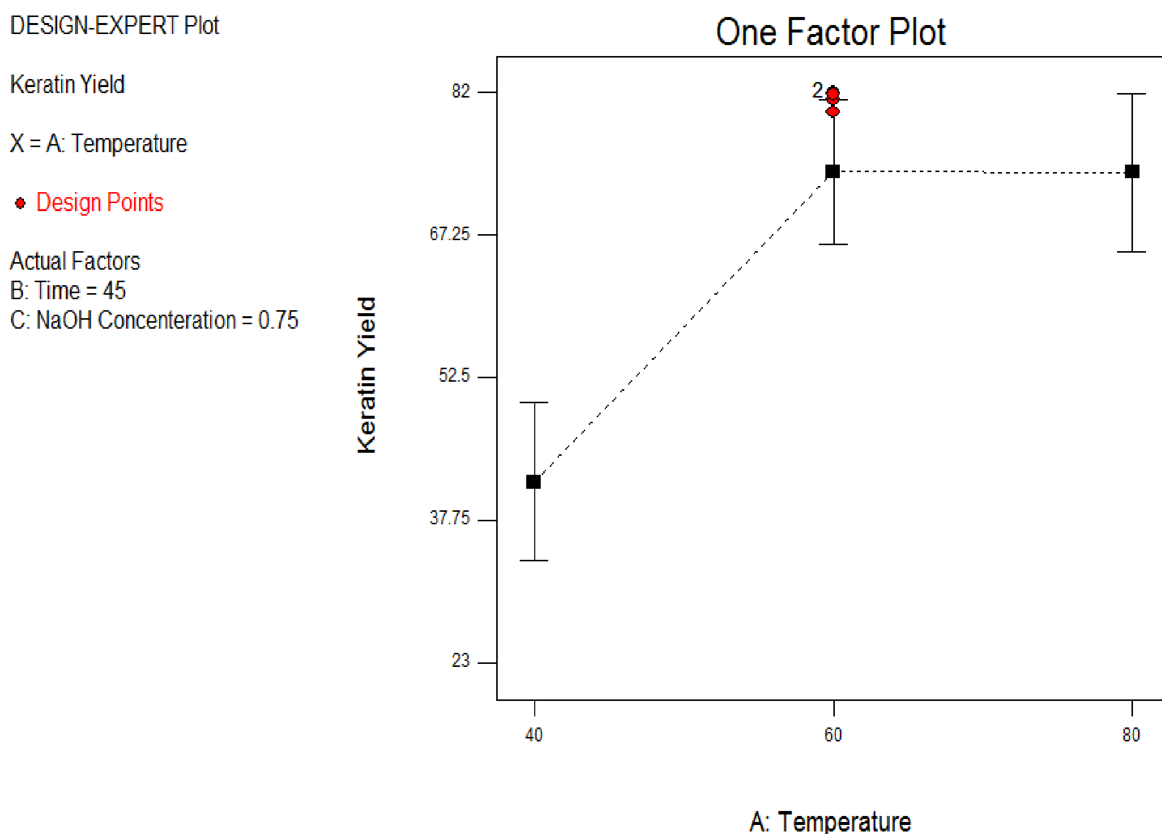

Figure 4.3 Effect of extraction Temperature on percent yield of keratin protein.

4.3.2 Effect of extraction time on percent yield of keratin protein

Figure 4.4 shows the influence of extraction time (30-60 min) on the final yield of keratin at 60°C temperature and 0.75 N NaOH concentration . From the Figure, it is observed that most of the keratin was extracted during 30 to 45 minutes reaction time and the maximum yield, 82% was obtained at 45 min reaction time. Further increase of extraction time results in no significant improvement on the yield. In general, the rate of extraction was high at the beginning of the reaction but get gradually constant at the end. This reveals that no significant improvement on the keratin yield when extending the extraction time.

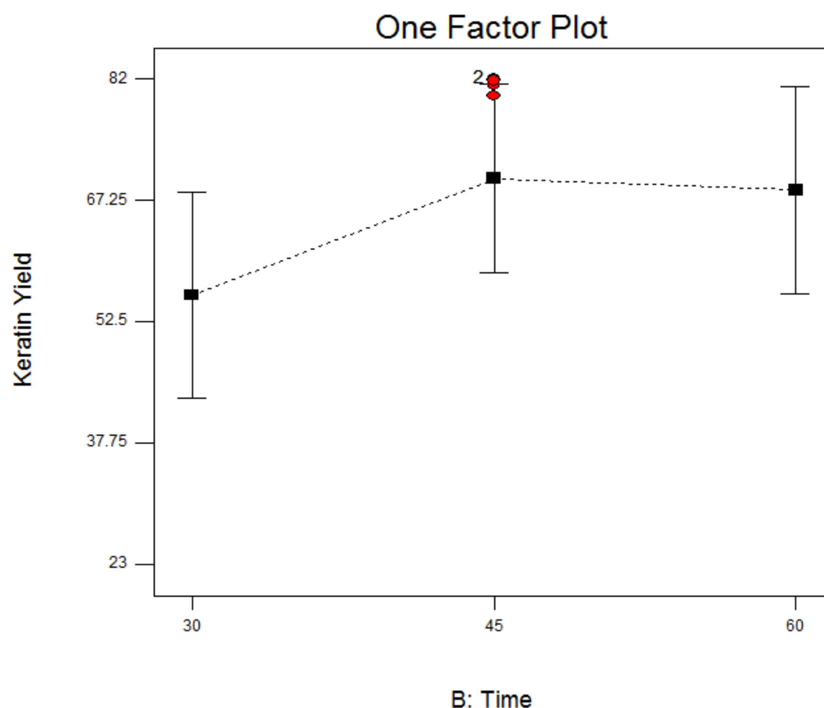

Figure 4.4 Effect of extraction time on percent yield of keratin protein

4.3.3 Effect of NaOH concentration on percent yield of keratin protein

From the Figure 4.5, it is observed that a gradual increase of the yield with the increase of concentration of NaOH solution from 0.5-0.75N after that it becomes constant. Treatment with 0.75N NaOH increases the yield when compare to 0.5N alkali. The yield became constant when using 1N NaOH solution.

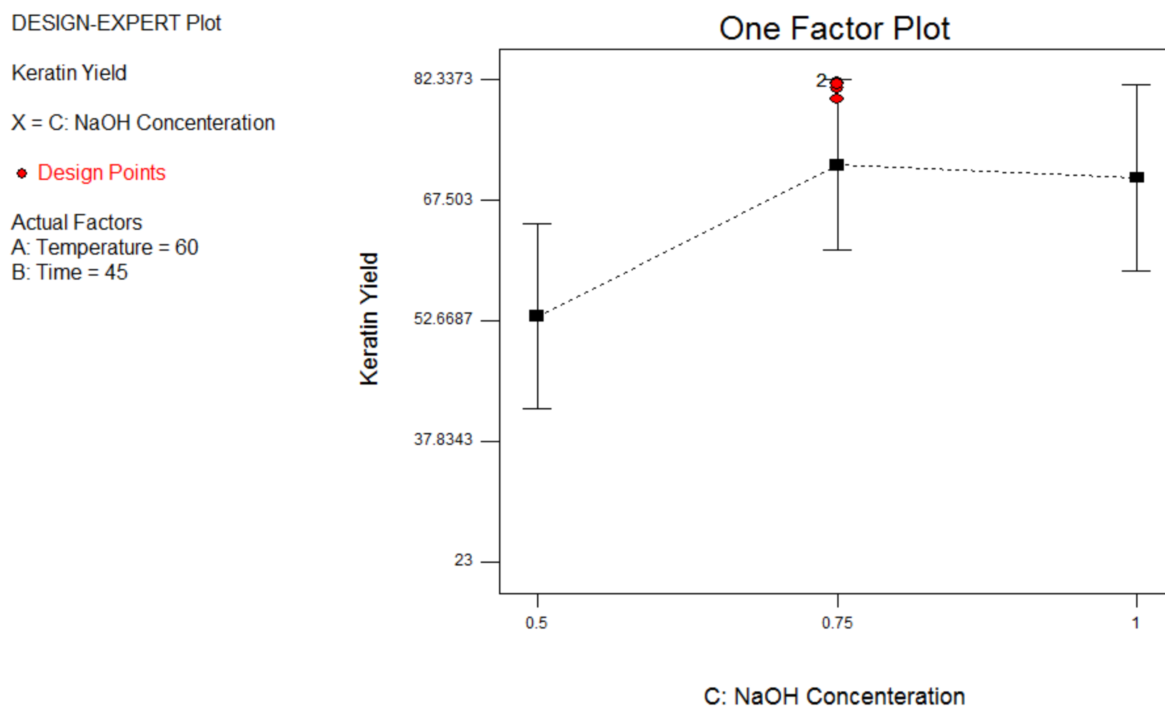

Figure 4.5 Effect of extraction NaOH concentration on percent yield of keratin protein

4.4. Data analysis using Design expert 7.0.0 software

Three basic factors were selected for design expert to determine the total experiment runs. Total of three factors and three levels were selected to give a total experimental runs of 27 with a factorial design of 3^3 for all experiment. The results were replicated two times to improve reliability of the data and that resulted to perform 54 experiments. A planning matrix was set up to take account of the factors that could influence with responses, such as measurement of solution concentration, extraction time and temperature at different levels. Table 4.2 shows the factors and levels chosen for the planning matrix. The experiments were performed in batches under constant stirring (200 rpm) and using constant amount of chicken feather (5g)

Table 4.2 Factors and levels for the extraction experiments

Factors	Level	Level value	Unit
Temperature	1	40	^oC
	2	60	^oC
	3	80	^oC
Time	1	30	Minute
	2	45	Minute
	3	1	Minute
NaOH concentration	1	0.5	Normality
	2	0.75	Normality
	3	1	Normality

The aim of applying a factorial design analysis was to identify the most significant factors affecting the yield percentages after batch extraction experiments.

Table 4.3 Analysis of variance (ANOVA) Influence of the factors studied in batch extraction experiments

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	Prob> F
Model	8495.55	9	943.95	24.06	< 0.0001
A	3972.38	1	3972.38	101.25	< 0.0001
B	875.01	1	875.01	22.30	0.0002
C	1288.63	1	1288.63	32.85	< 0.0001
A^2	1190.98	1	1190.98	30.36	< 0.0001
B^2	530.79	1	530.79	13.53	0.0019
C^2	253.93	1	253.93	6.47	0.0210
AB	116.56	1	116.56	2.97	0.1029
AC	252.08	1	252.08	6.43	0.0214
BC	15.19	1	15.19	24.06	0.5421

A = Temperature, **B** = Time, **C** = solvent (NaOH) Concentration

The Model generated for keratin extraction was significant that ANOVA analysis shown in table 4.3 proves the results discussed above at a confidence level of 92%. As shown from table 4.3 values of P less than 0.05 indicate model terms are significant. Results showed that the effect of temperature, time and NaOH concentration are of major importance for extraction of keratin from chicken feather. Combination of A*C (temperature and NaOHconcentration), were significant model terms for extraction process. As shown from the ANOVA results, temperature and NaOH concentration was the dominant factor that affects extraction process. This result confirmed from many literatures that temperature and NaOH concentration are the main factor influences the extraction process. Extraction time showed insignificant; meaning itscontribution

was very low. Interaction of temperature and time,time and NaOH concentration did not influences extraction process. Modeling equations show the yield of keratin in relation to different factor levels.

Final Equation in Terms of Coded Factors and actual factors:

Final Equation in Terms of Coded Factors:

Keratin Yield = +80.51+ (14.86 * A) + (6.97 * B) + (8.46 * C) – (14.09 * A2) – (9.41* B2) – (6.51* C) + (3.12* A * B) – (4.58* A * C) – (1.13* B * C)

Final Equation in Terms of Actual Factors:

Keratin Yield = -303.68426 + 5.18944* Temperature + 3.82870 * Time + 258.47778* NaOH Concentration - 0.035222 * Temperature2-0.041802 * Time^2 104.08889 * NaOH Concenteration^2 + 0.010389 * Temperature * Time-0.91667 * Temperature * NaOH Concenteration-0.30000 * Time * NaOH Concentration

4.4.1. Model adequacy check

The adequacy of model was tested by analysis of variance (ANOVA). The regression model was found to be significant with the correlation coefficients of determination of R-Squared, adjusted R-Squared and predicted R-Squared having a value of 0.9972, 0.8887 and 0.8279 receptively. The value of R squared for the developed correlation is 0.9972. It implies that experimental variables studied attributed for the extraction process is 99.72% shows that the regression model equations provided a description of the experimental data, in which all the points are close to the line of perfect fit. This result indicates that the agreement between the experimental and the predicted values was good. In other ways it indicates that there is a linearity relationship between the extraction process and the three factors considered. The figure 4.6 show how the data generated from developed model equation is close to the actual data obtained.

Table 4.4 The R-squared values for extraction process

Std. Dev.	6.26	R-Squared	0.9272
Mean	60.51	Adj R-Squared	0.8887
C.V.	10.35	Pred R-Squared	0.8279
PRESS	1577.32	Adeq Precision	17.009

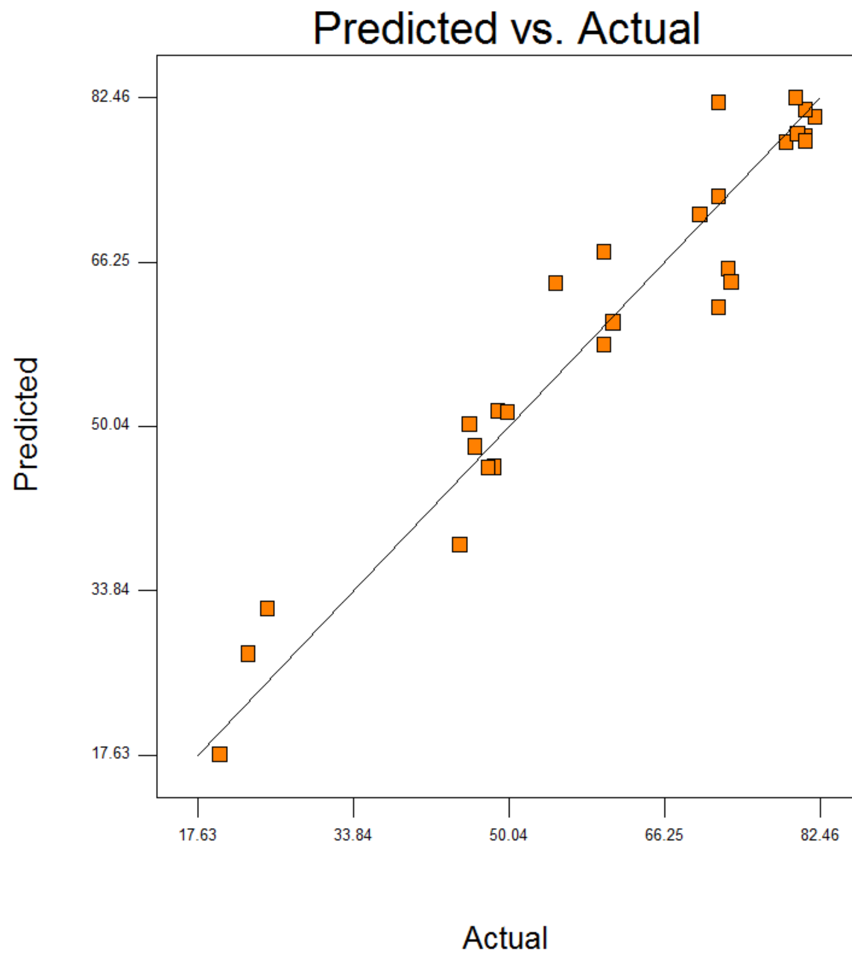

Figure 4.6 Predicted Vs actual experimental value for keratin extraction

4.8.2. Interaction Effects

The keratin yield was plotted as a function of the interactions of two of the factors by holding the other variable at average value. There are three interaction factors analyzed by the model equation. These are:

i. AB (Temperature and Time)

ii. AC (Temperature and NaOH concentration)

iii. BC (Time and NaOH concentration)

The three-dimensional response surfaces, plots are shown in Figures 4.7, 4.8, and 4.9 as a function of the interactions of two of the factors.

i. AB (Temperature and Time)

The effect of temperature and extraction time on keratin yield is shown form 3D plot of Figure 4.7. As it can be observed from the keratin yield plot, as both temperature and time increased the keratin yield also increased and it started to become constant after reaching its maximum. The maximum yield was found at the moderate values of both factors. On the other hand, the higher keratin yield observed at middle and higher value of both temperature and time. This confirmed to the fact that maximum keratin yield occurred at temperature (60°C) and time (45minute)

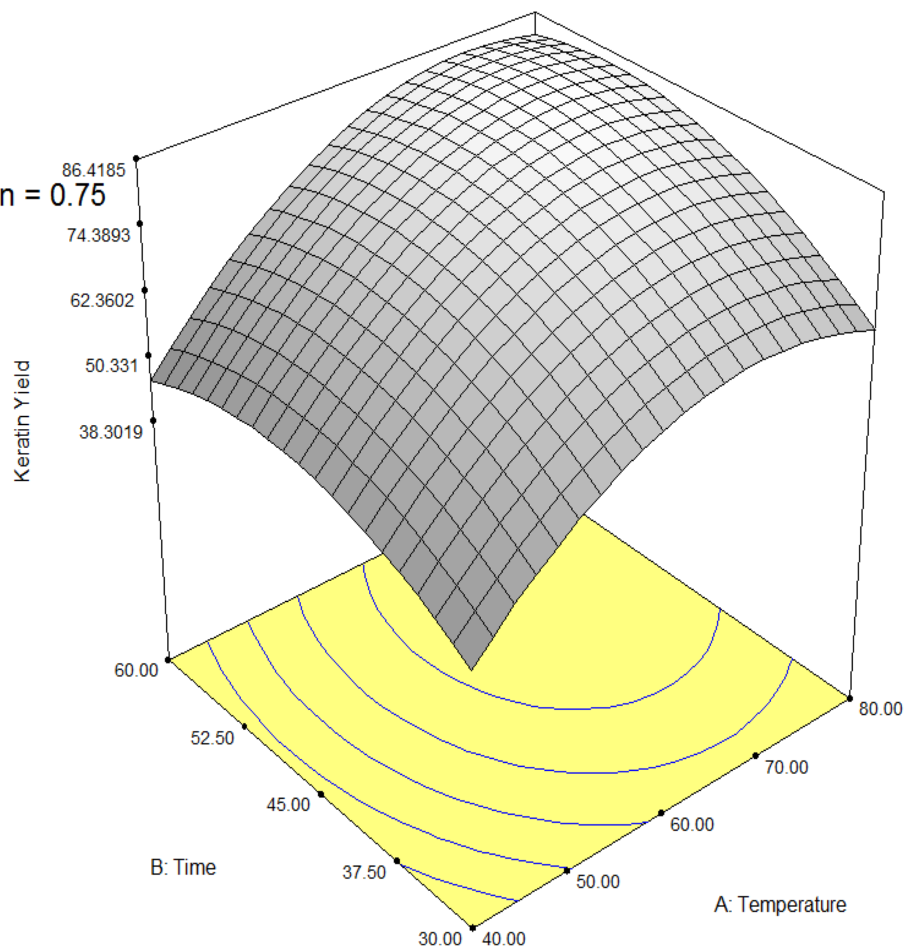

Figure 4.7 3D plot for keratin yield showing the interaction effect of temperature and time

ii. AC (Temperature and NaOH concentration)

The effect of temperature and NaOH concentration on keratin yield is shown form 3D plot of Figure 4.8. As it can be observed from the keratin yield plot, as both temperature and NaOH concentration increased the keratin yield also increased and it started to become constant after reaching its maximum. The maximum yield was found at the moderate values of both factors. On the other hand, the higher keratin yield observed at middle and higher value of both temperature and NaOH concentration. This confirmed to the fact that maximum keratin yield occurred at temperature (60°C) and NaOH concentration (0.75N)

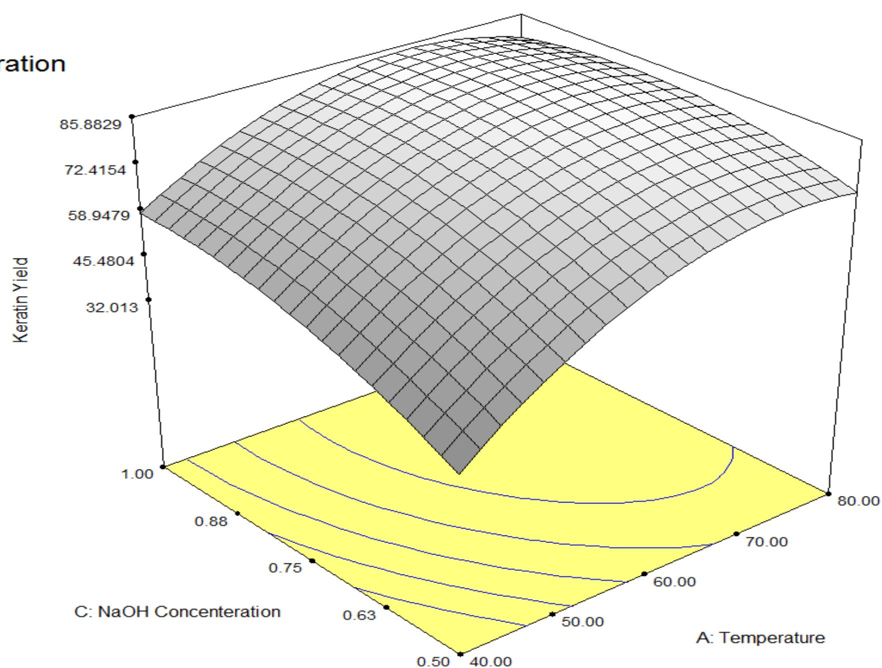

Figure 4.8 3D plot for keratin yield showing the interaction effect of temperature and NaOH concentration

iii. BC (Time and NaOH concentration)

The effect of time and NaOH concentration on keratin yield is shown form 3D plot of Figure 4.9. As it can be observed from the keratin yield plot, as both time and NaOH concentration increased the keratin yield also increased and it started to become constant after reaching its maximum. The maximum yield was found at the moderate values of both factors. On the other hand, the higher keratin yield observed at middle and higher value of both time and NaOH concentration. This confirmed to the fact that maximum keratin yield occurred at time (45 minute) and NaOH concentration (0.75N)

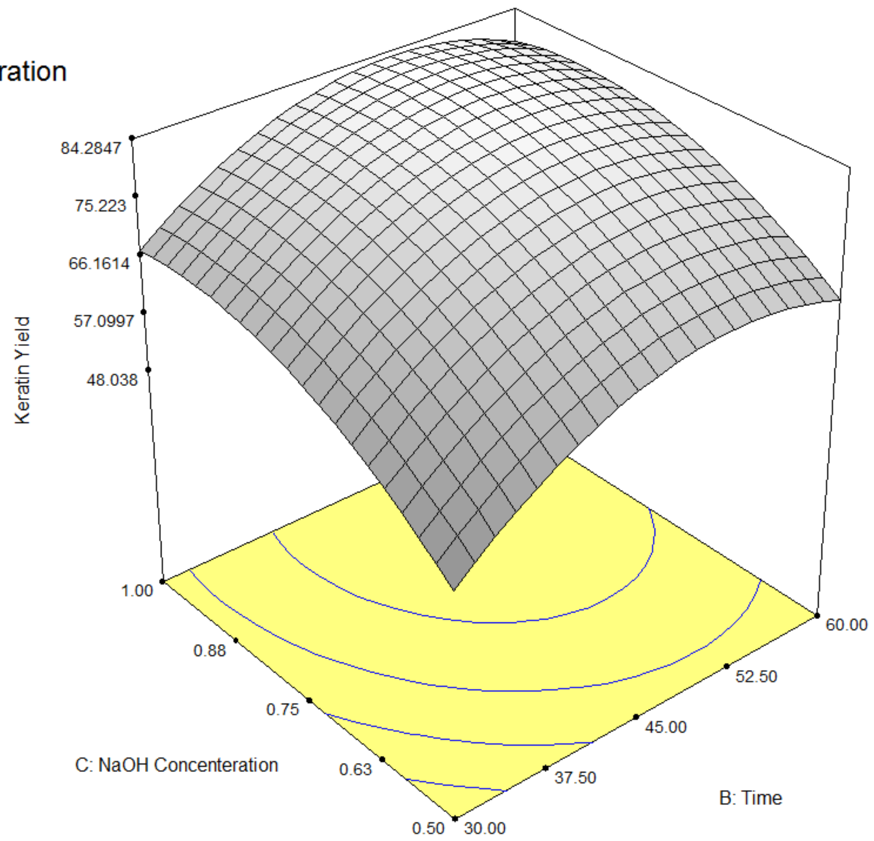

Figure 4.9 3D plot for keratin yield showing the interaction effect of time and NaOH concentration.

4.4 Analysis methods of keratin

4.4.1 Biuret test

The protein solution turned into purple colour after the Biuret reagent is added and this is possible only if the peptide bonds are present in extraction. The more peptide bonds in the keratin extraction, the higher is the intensity of the purple color as shown in Figure 4.10. Also the difference between the purple color for the different experimental conditions is clearly visible in the Figure. In the figure, the sample towards left hand side shows darker in color than the right one, this is because the left sample is extracted at low temperature (45°C) and the right one is at the highest temperature (80°C). From Biuret analysis it is confirmed that the protein will lose its structure (lose of peptide bond) when the extraction temperature is very high (~100°C).

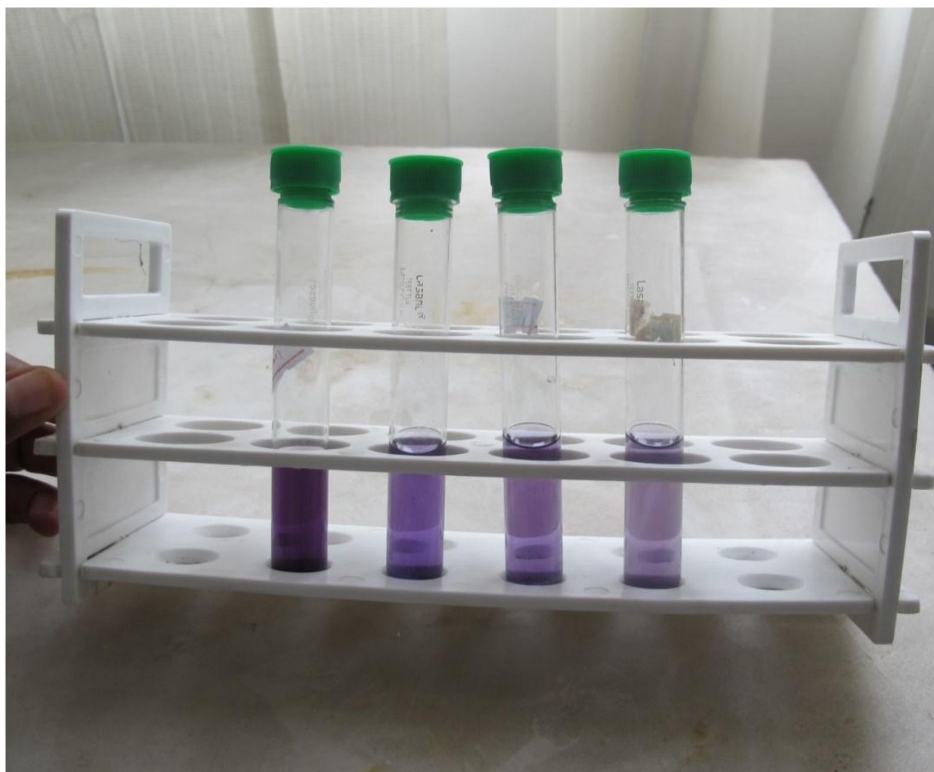

Figure 4.10 Biuret test for extracted protein from chicken feather

4.4.2 Fourier Transform Infrared Spectroscopy

Structure of keratin is a type of natural polymer and it has so complicated structure. Therefore, Fourier transform of infrared (FTIR) used to understand all details of structure of keratin. The

characteristic bands and signals from these spectra was analyzed in order to confirm the products as keratin.

Keratin proteins give rise to several characteristic absorption bands known as amide A, amide B, amide I, amide II and amide III. Amide A and amide B bands are connected with the frequency of stretching vibrations of located N-H bonds. According to the FTIR spectrum of keratin, amide A absorption band was seen at 3287 cm-1 and amide B was seen at 3072 cm-1 (Aluigi et al., 2008; Wojciechowska et al., 2004).

In our data (Figure 4.11- 4.13), amide A absorption band was seen 3316 cm-1 ,3317 cm-1 and 3306 cm-1 at sample 1,2 and 3 respectively. In addition, amide B was seen 2928 cm-1, 2926 cm-1 and 3074 cm-1 at sample 1, 2 and 3 respectively.

The amide I mode is the peptide carbonyl stretching vibration of the CONH unit. According to literature, β-sheets have a strong absorption band at 1610-1640 cm-1 and a weaker band at 1680-1690 cm-1. The α-helix and random coil structure are located at 1640-1650 cm-1 and 1650-1660 cm-1, respectively (Akhtar et al., 1997; Zoccola et al., 2008b Wojciechowska et al., 2004.,). In the FTIR spectrum of keratin that shown at Figure 4.11- 4.13, amide I absorption band was observed at 1660cm1, 1660cm-1 and 1666 cm-1 indicating the α-structure of keratin.

The amide II mode is N-H in plane bending plus C-N stretching vibrations with a contribution from C-C streching (Akhtar et al., 1997). Amide II around 1547 cm-1 suggests the presence of β-sheet type keratin, whereas the amide II around 1515 cm-1 indicates the presence of α-helix structure in the keratin chain (Wojciechowska et al., 2004). As a result, the amide II mode of keratin was attributed to the vibration at 1547cm-1,1550 cm-1 and 1558cm-1 which indicated the β-sheet type keratin (Figure 4.11- 4.13).

The amide III mode is the in-plane combination of N-H in-plane bending and C-N stretching, with contributions from the C-C stretch and C-O in-plane bend. The NH in-plane bending mode is a significant component of a number of modes in the 1400- 1200 cm-1 region. The vibration at around 1240 cm_{-1}, 1271 cm-1 and 1247 cm_{-1} was therefore attributed to amide III absorption band of keratin (Figure 4.11- 4.13).

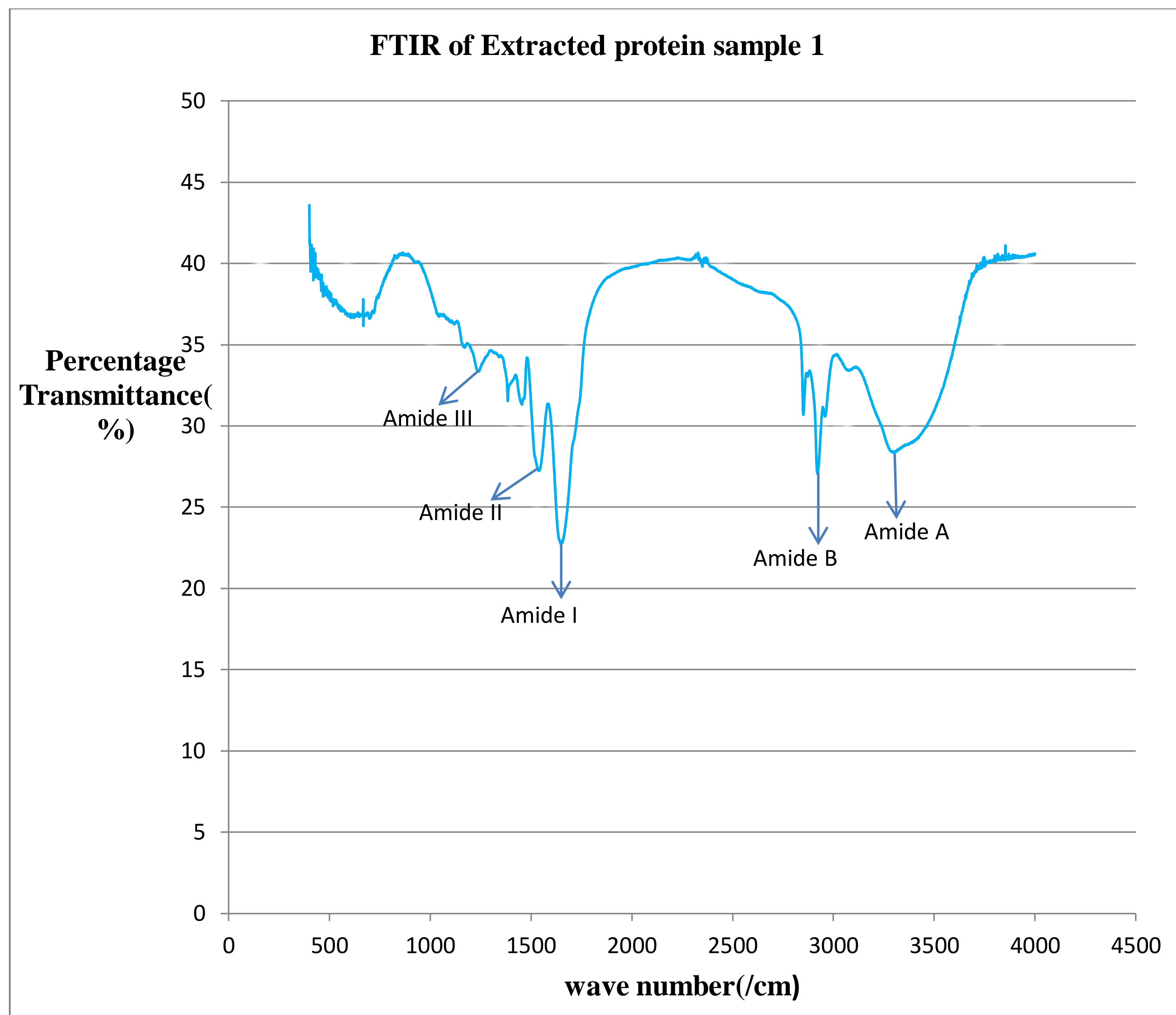

Figure 4.11 FT-IR of Extracted Protein Sample 1

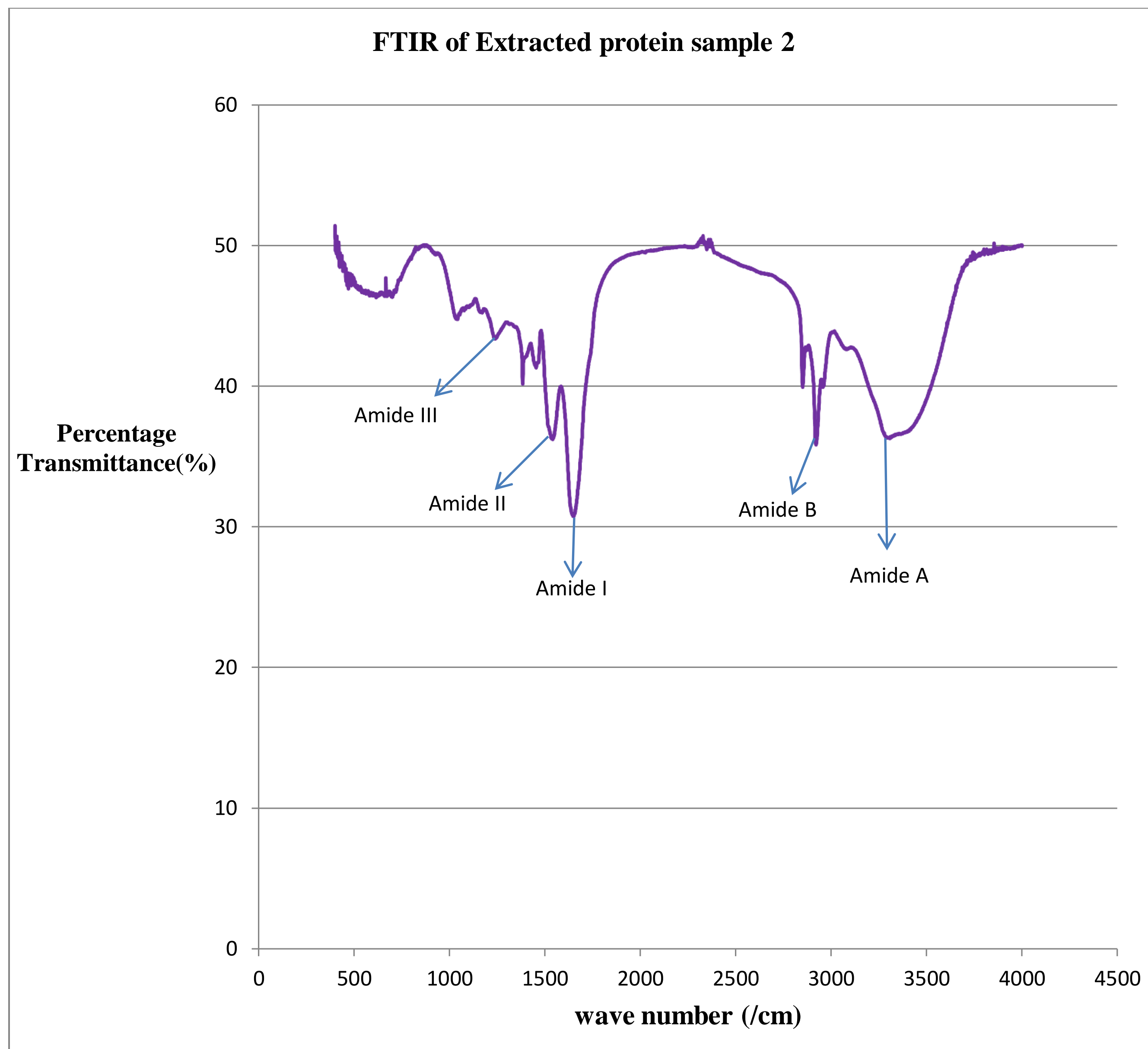

Figure 4.12 FT-IR of Extracted Protein Sample 2

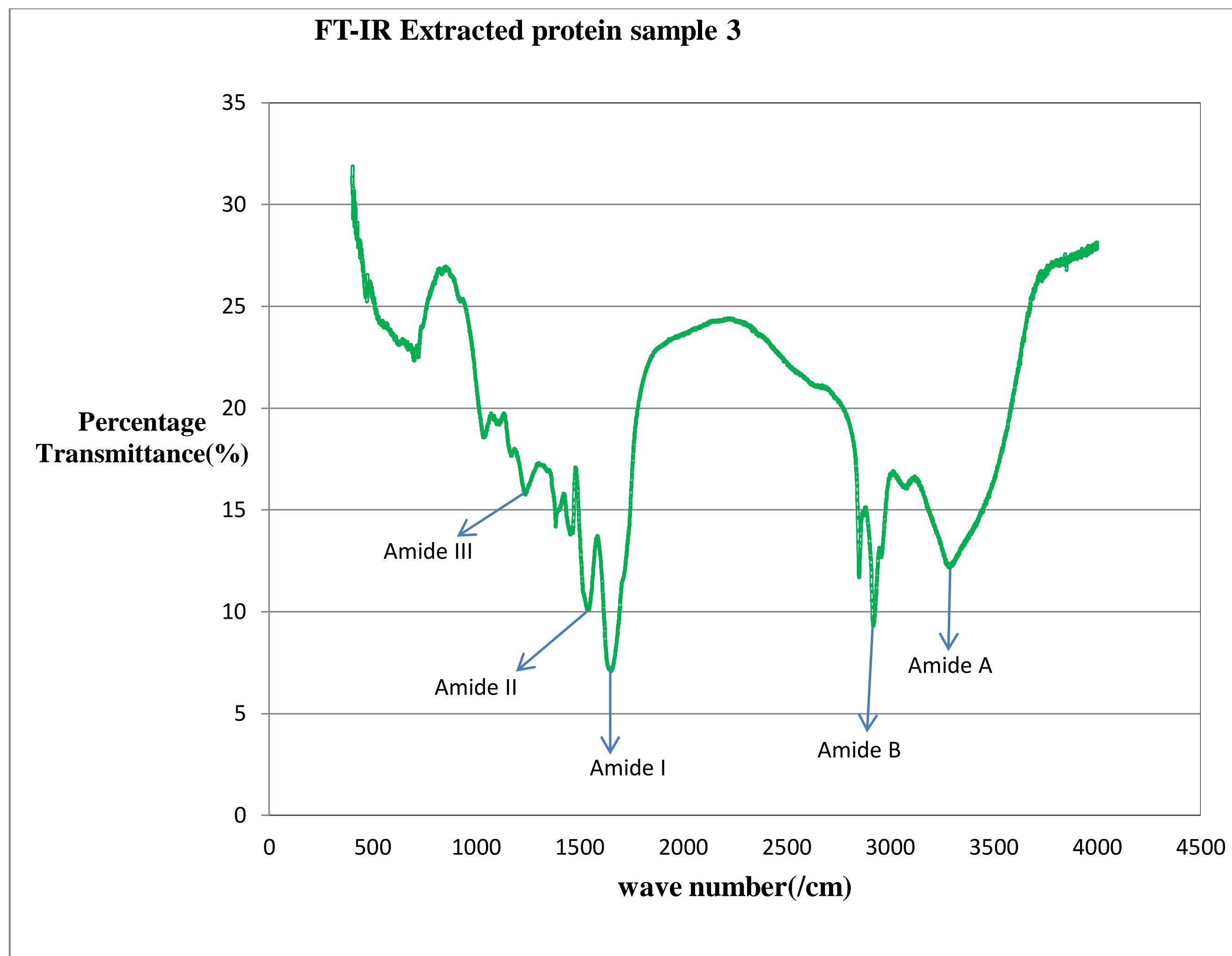

Figure 4.13 FT-IR of Extracted Protein Sample 3

4.4.3 XRD

The powdered sample of the regenerated feather keratin as well as raw chicken feather was characterized by XRD to study the crystallinity of the materials, and the results are shown in Figure 4.15. Both of them shows the characteristics of the α-helix appearing and β-sheet structures at 2θ = 9° (0.98 nm) (Meredith 1956, Rao et al 1986). The peak at about 17.8° (0.51 nm) corresponds to the diffraction pattern of the α-helix whereas the peak at about 19° (0.47 nm) is typical of the β-sheet structure (Meredith 1956, Rao et al 1986). However, due to the overlapping signals at about 17.8° and 19° from the α-helix and β-sheet, both are unable to be unambiguously assigned. It can be seen from Fig. 4.15 that the regenerated keratin exhibits similar diffraction patterns, indicating regeneration of the crystallinity of the original keratin.

However, the peaks at about 9° and 19° are both significantly stronger in the regenerated material, suggesting a greater content of the β-sheet structure.

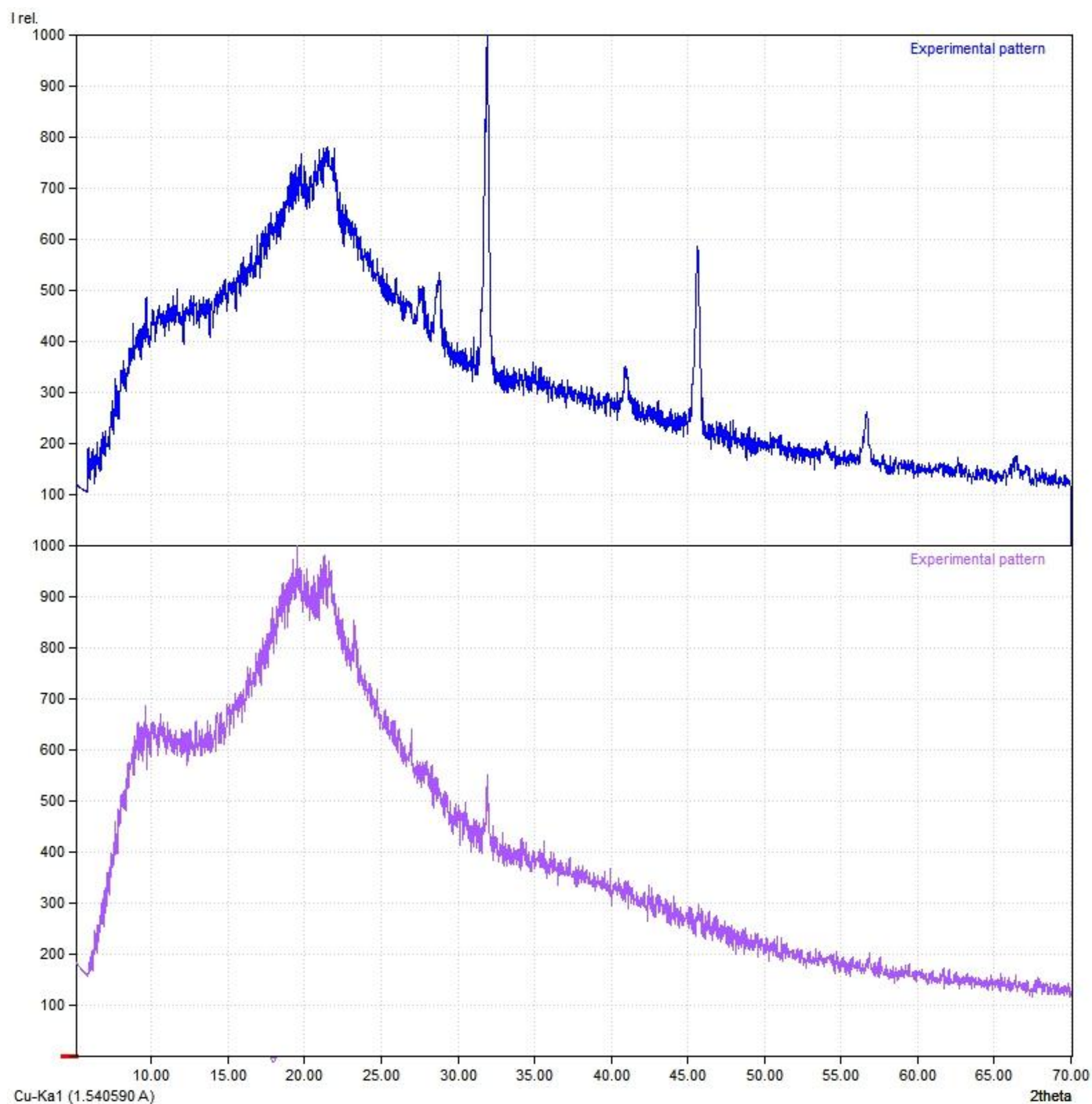

Figure 4.14 XRD analysis of extracted protein (blue) and raw feather (pink)

4.4.4 Thermo Gravimetric Analysis of Extracted Keartin

TGA analysis has been done for raw chicken feathers as well as the extracted keratin. The TGA curve of the raw chicken feather shows stability up to 200°C. In contrast, the regenerated feather keratins degrade at lower decomposition temperatures. This shows that the stability of the raw feather is influenced by the presence of strong cross-linking (disulphide bond) between the keratin fibers, whereas in the regenerated keratin the cross-linking networks are disrupted somewhat, leading to lower stability. The TGA of these materials show two steps of mass loss. The first step occurs close to 100 °C corresponding to the evaporation of water bound to the material. The second step involves the keratin degradation that is understood to be associated with the rupture of the helical conformation and disulfide bond breakage (Ullah et al 2011, Davies et al 2000).

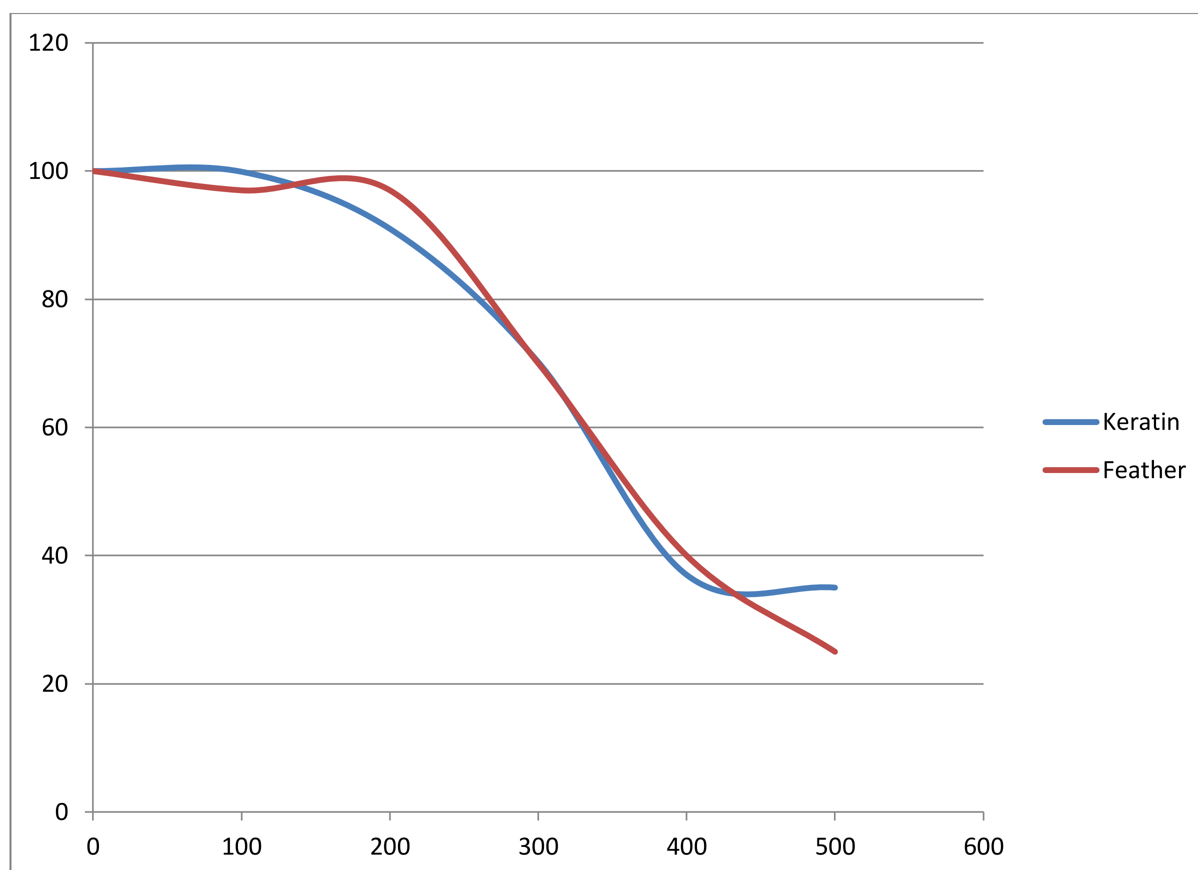

Figure 4.15 TGA analysis of extracted protein

Chapter 5

5. Conclusions and Recommendation

5.1 Conclusions

- This research work was intended to study the influence of different factors (Extraction time, temperature and solvent concentrations) on the quality and quantity of extraction of keratin protein from chicken feathers that can be used for the preparation of anti-aging cream. Variability of these operating conditions is the pre-dominant factor which determine the quantity of the protein. There are different methods of keratin extraction from chicken feathers. In this thesis, hydrothermal extraction (thermos-chemical) by using NaOH was employed based on the facility available.

- The optimum condition for the extraction of keratin protein from chicken feathers was found to be 5g feather, 0.75 N NaOH and 45 minutes reaction time at 60°C temperature. The yield of keratin was 82 %.

- The FT-IR spectroscopy of extracted keratin samples indicated the presence of different functional groups (amide A, B, i, ii, and iii) which reveals that the optimum extraction conditions retain the protein and aminoacid structure which is most essential for the preparation of anti-aging cream. Also it is conclude that optimum use of NaOH /hydrothermal conditions do not destruct the aminoacid structure.

- XRD analysis of extracted keratin revealed the presence of α-helix and β-sheet peaks and confirms the crystallinity of the regenerated keratin.

- In conclusion, the study reveals that by employing optimum thermo-chemical conditions, the protein structure/quality can be maintained even after the extraction and can be effectively utilized for the development of value added products, for example anti-aging creams.

5.2 Recommendations

Based on the results obtained from the study, the following suggestions have been made for the future work.

- Further researches have to be carried out to increase the yield of keratin protein from chicken feather by using other extraction methods for example by microbial degradation and by using reducing agents.

- Optimization of thermo-chemical extraction process is carried out in this study. But further optimization of the down-streaming processes recommended to maximizing the yield of keratin protein.

- The anti-aging cream was formulated using safe ingredients that include competitively price materials which are easily available but it needs characterization, optimization of ingredients and further investigation.

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Appendices

Appendix A

Laboratory equipment and experimental photos

Figure 3.1 Setup of the Soxhlet Apparatus

Figure 3.2 Experimental setup for extraction of keratin

Figure 3.3 Protein solution PH adjustments (left) and precipitated protein (right)

Figure 3.4 Centrifugal separation (Left) and separated keratin protein inside test tube (right)

Figure 3.5 powder keratin protein after drying and grinding

Figure 3.6 Formulated anti-aging cream

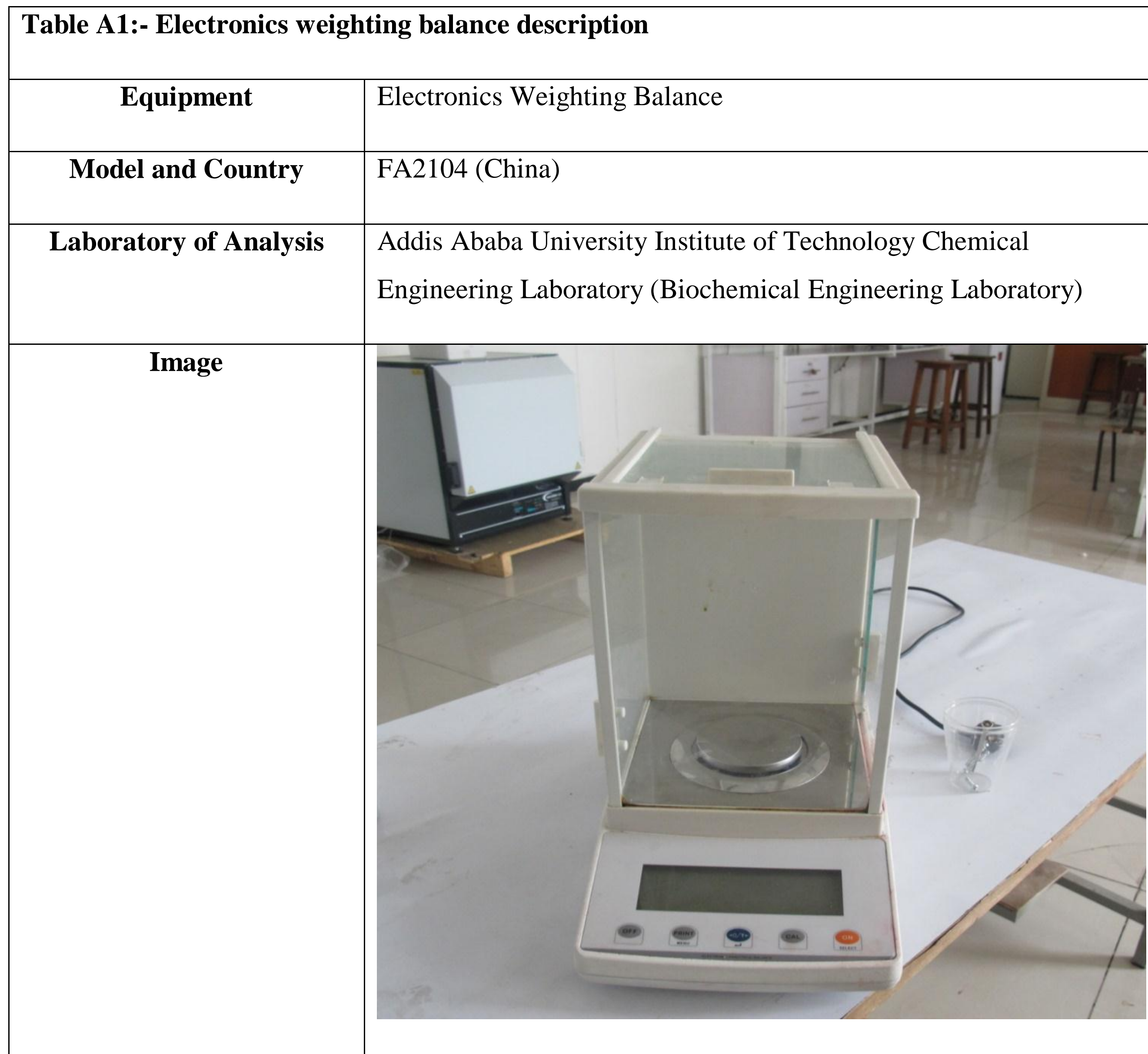
Table A1:- Electronics weighting balance description	
Equipment	Electronics Weighting Balance
Model and Country	FA2104 (China)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

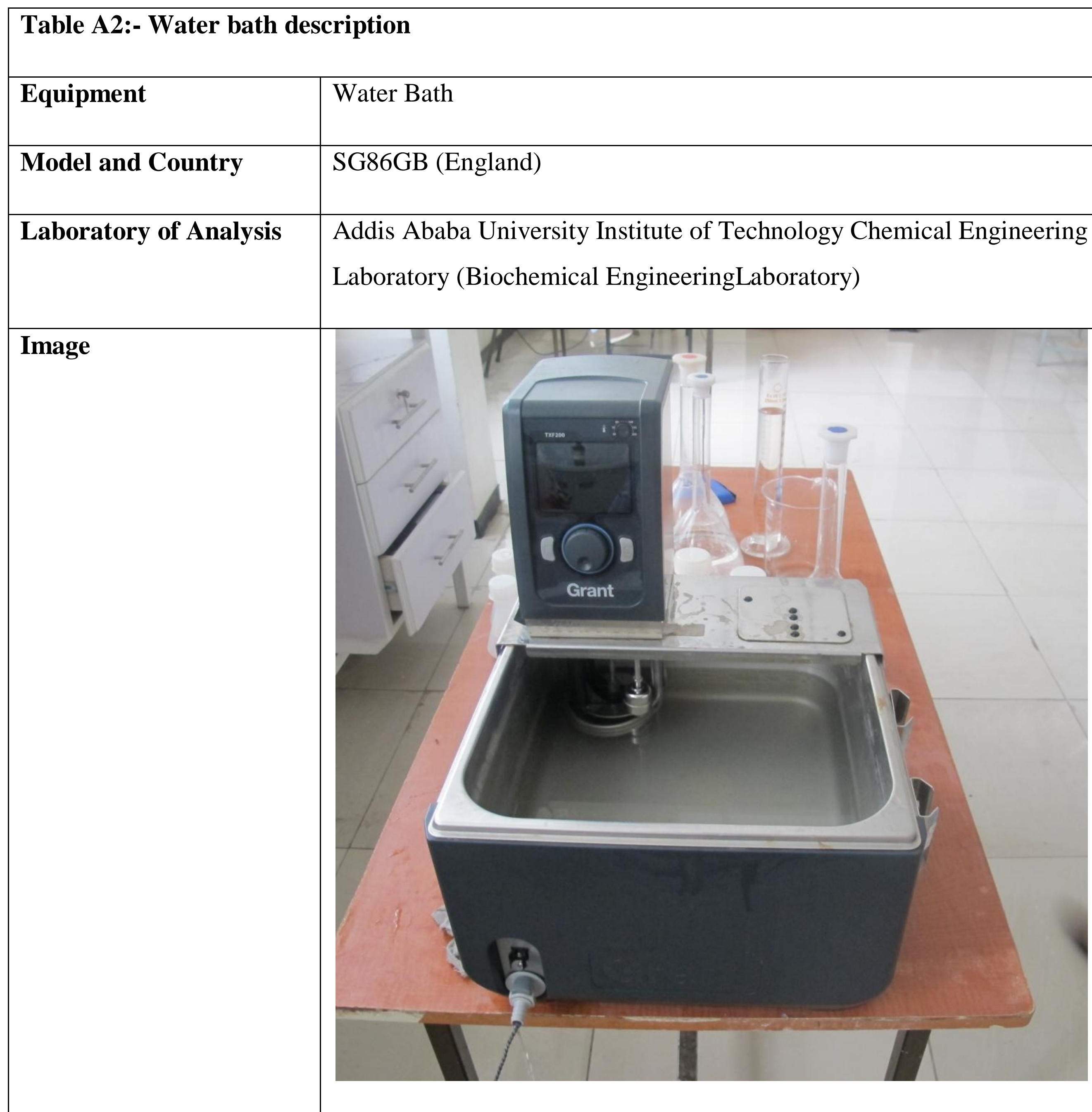
Table A2:- Water bath description	
Equipment	Water Bath
Model and Country	SG86GB (England)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical EngineeringLaboratory)
Image	

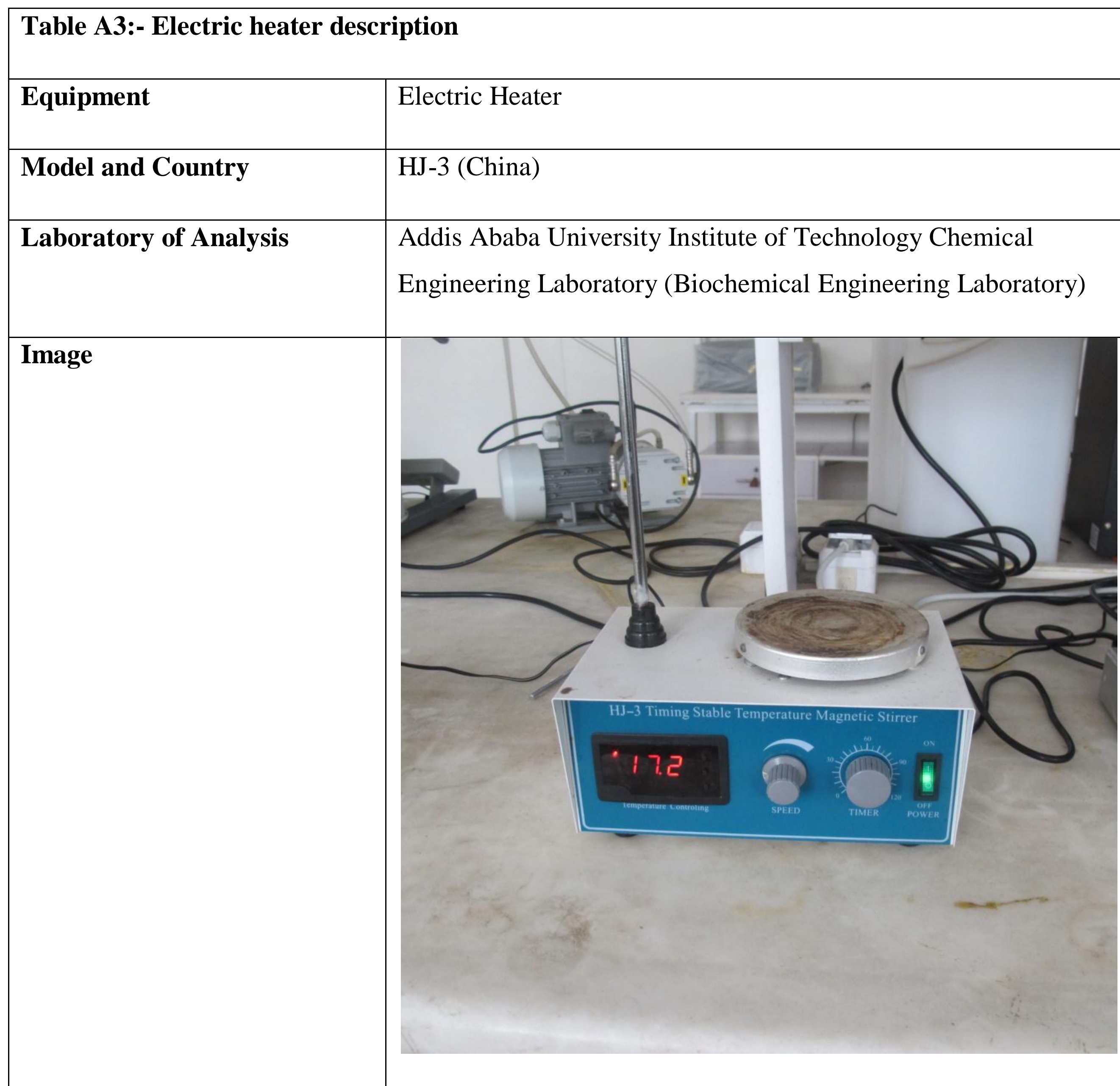
Table A3:- Electric heater description	
Equipment	Electric Heater
Model and Country	HJ-3 (China)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

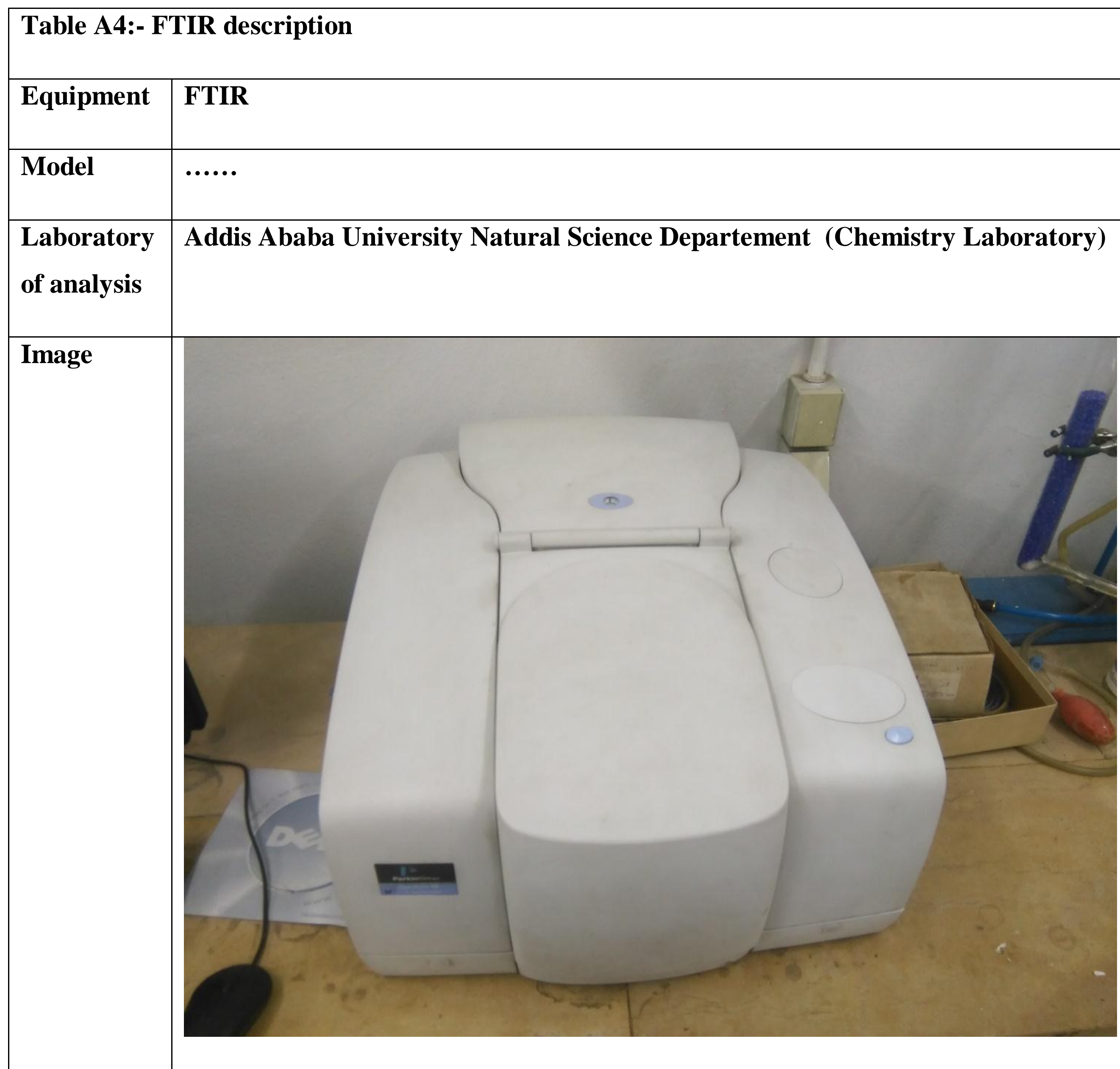
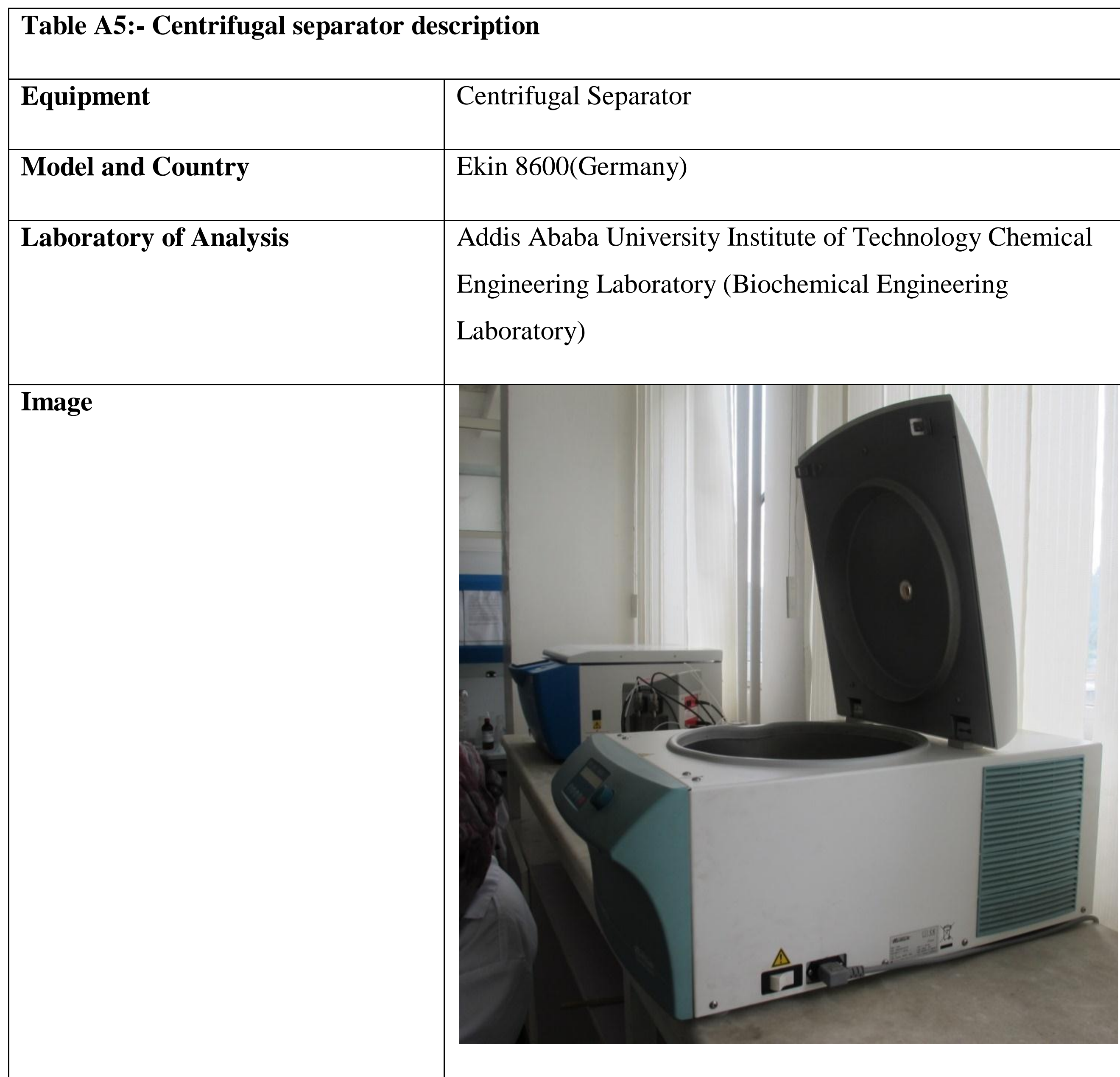
Table A4:- FTIR description	
Equipment	**FTIR**
Model	**……**
Laboratory of analysis	**Addis Ababa University Natural Science Departement (Chemistry Laboratory)**
Image	

Table A5:- Centrifugal separator description	
Equipment	Centrifugal Separator
Model and Country	Ekin 8600(Germany)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

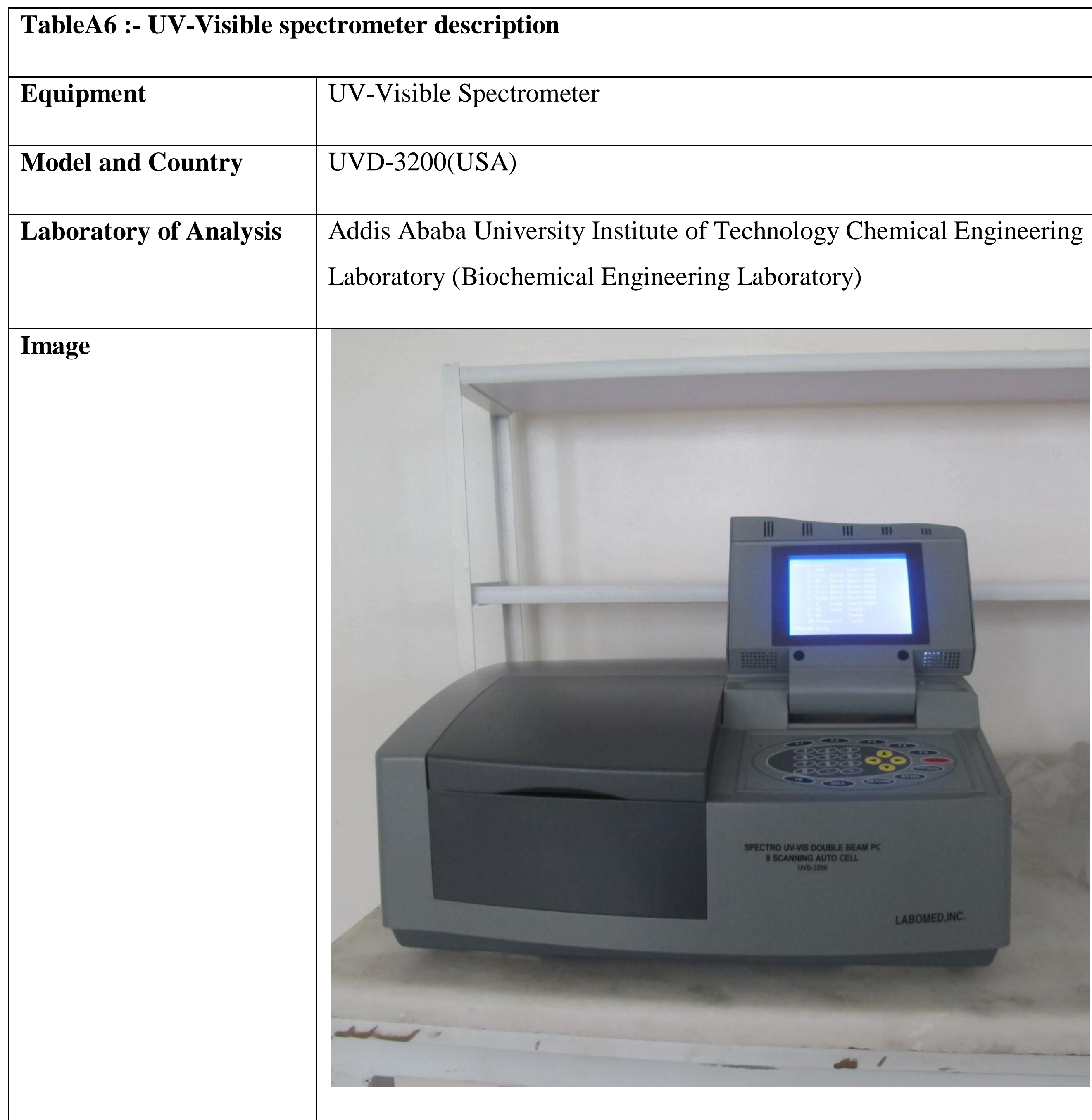
TableA6 :- UV-Visible spectrometer description	
Equipment	UV-Visible Spectrometer
Model and Country	UVD-3200(USA)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

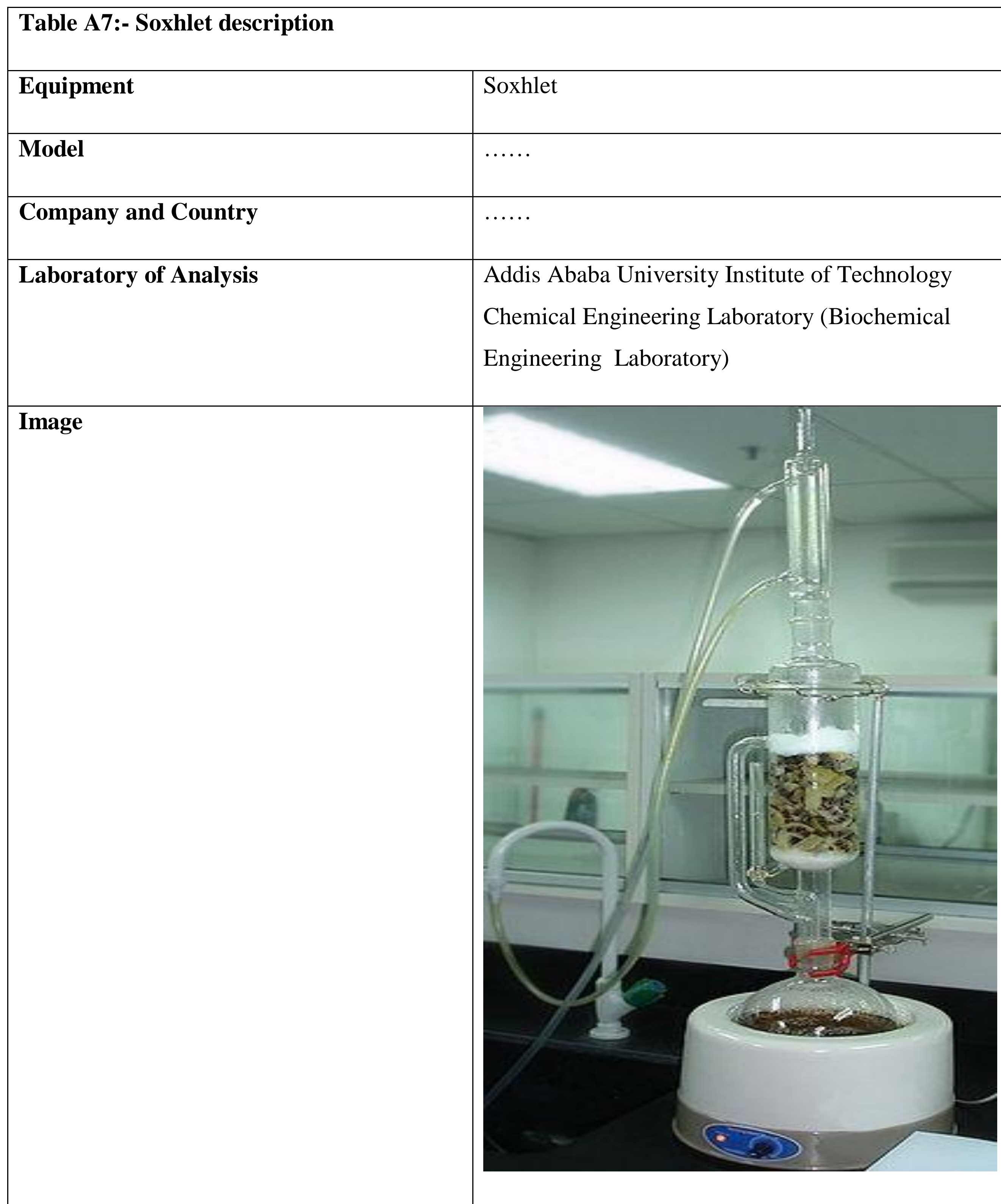
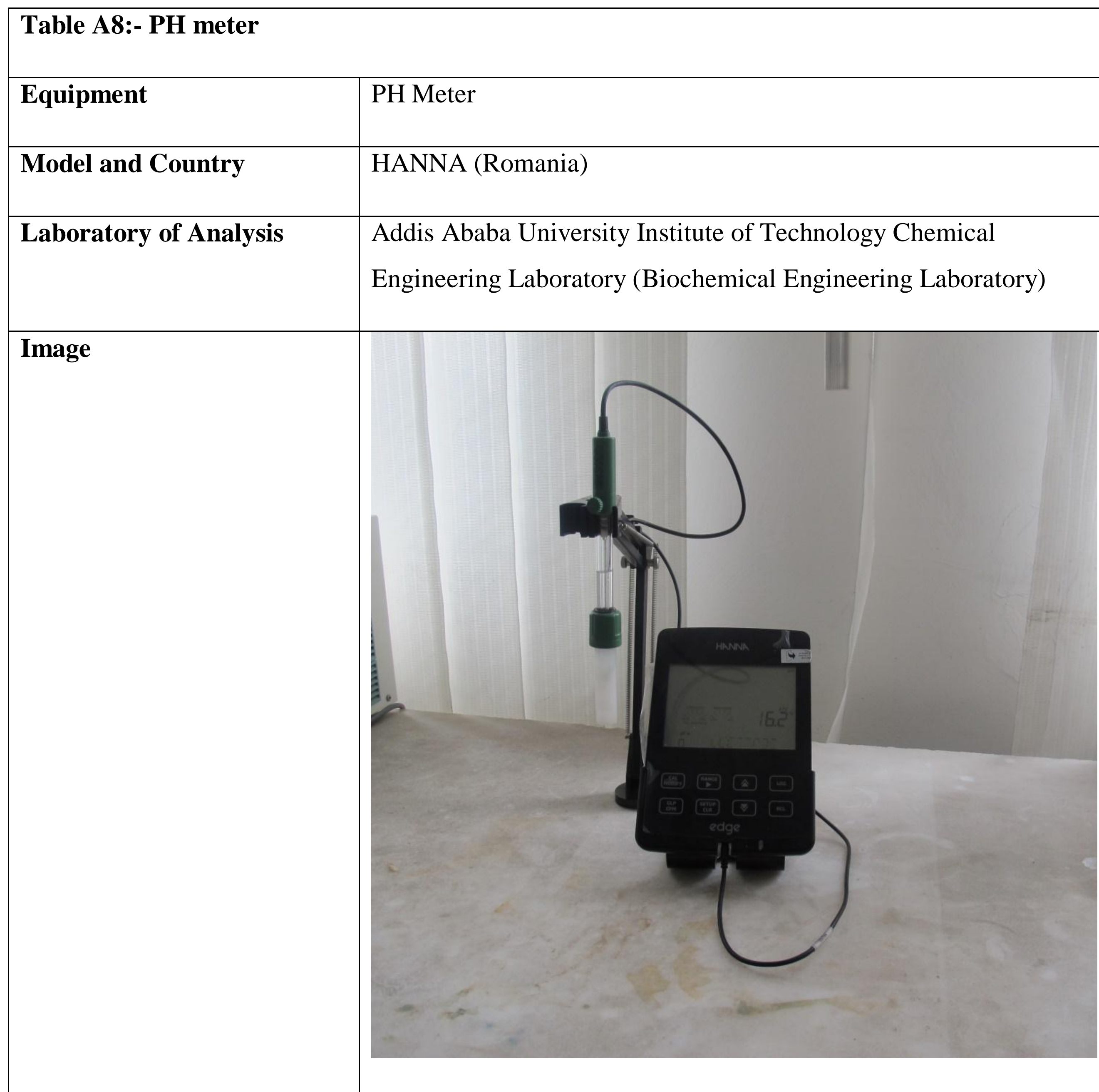
Table A7:- Soxhlet description	
Equipment	Soxhlet
Model	……
Company and Country	……
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

Table A8:- PH meter	
Equipment	PH Meter
Model and Country	HANNA (Romania)
Laboratory of Analysis	Addis Ababa University Institute of Technology Chemical Engineering Laboratory (Biochemical Engineering Laboratory)
Image	

Appendix B

Extracted Data Analysis by design expert

Table B1 Analysis of experimental (actual) data of keratin protein from chicken feather

Run	Block	Factor 1 A:Temperature oC	Factor 2 B:Time Minute	Factor 3 C:NaOH Concentra Normality	Response 1 Keratin yield %
1	Block 1	80.00	30.00	0.75	72
2	Block 1	80.00	45.00	0.50	70
3	Block 1	60.00	45.00	1.00	80
4	Block 1	80.00	60.00	0.50	72
5	Block 1	80.00	60.00	1.00	81
6	Block 1	60.00	60.00	0.75	79
7	Block 1	40.00	30.00	1.00	48
8	Block 1	40.00	60.00	1.00	50
9	Block 1	60.00	45.00	0.75	82
10	Block 1	40.00	45.00	0.75	49
11	Block 1	40.00	60.00	0.75	48.6
12	Block 1	40.00	30.00	0.75	45
13	Block 1	80.00	30.00	0.50	46
14	Block 1	40.00	45.00	1.00	60
15	Block 1	80.00	45.00	1.00	81
16	Block 1	40.00	45.00	0.50	25
17	Block 1	40.00	30.00	0.50	20
18	Block 1	60.00	60.00	0.50	73.3
19	Block 1	60.00	30.00	0.75	55
20	Block 1	60.00	60.00	1.00	80.2
21	Block 1	60.00	30.00	1.00	60
22	Block 1	80.00	30.00	1.00	61
23	Block 1	60.00	30.00	0.50	46.6
24	Block 1	80.00	60.00	0.75	72
25	Block 1	40.00	60.00	0.50	23
26	Block 1	60.00	45.00	0.50	73
27	Block 1	80.00	45.00	0.75	81

Table B2 Values for reasonable agreements

Std. Dev.	6.26	R-Squared	0.9272
Mean	60.51	Adj R-Squared	0.8887
C.V.	10.35	Pred R-Squared	0.8279
PRESS	1577.32	Adeq Precision	17.009

The "Pred R-Squared" of 0.8279 is in reasonable agreement with the "Adj R-Squared" of 0.8887."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.009 indicates an adequate signal.This model can be used to navigate the design space.

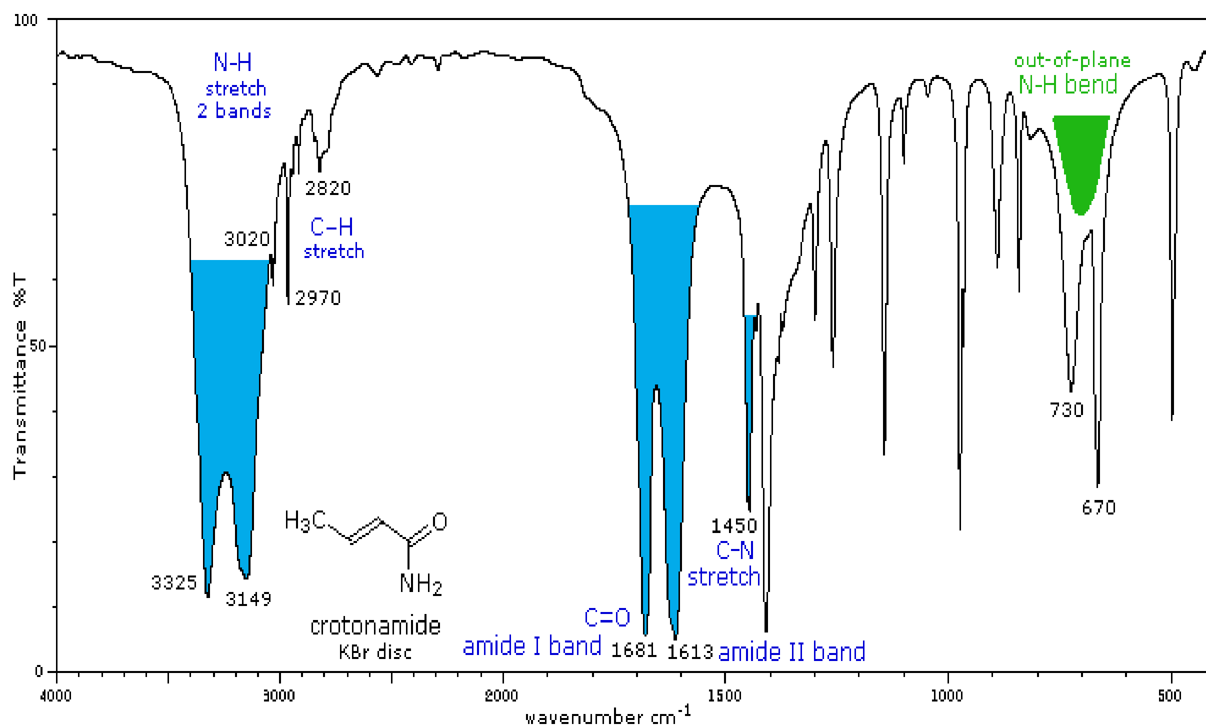
The high F value (F model = 24.6) with low probability value ($P < 0.0001$) indicates the significance of the fitted model. The low value of the coefficient of variation (CV = 10.35%) indicates that results of the fitted model are reliable. The quality of the model fit was evaluated by the coefficient of determination (R^2), this value being calculated to be 0.92 for the response, indicating that the developed model equation successfully captured the correlation between the process parameters to the yield of bottom product. The adjusted coefficient of determination (R^2Adj.) value reconstructs the expression with all the significant terms included. The value of the adjusted coefficient of determination (R^2 Adj. = 0.88) is also very high, supporting the significance of the model. As the fitted model provides a good approximation to the experimental condition, the model was employed to find the values of the process variables for optimum yield of keratin protein.

Appendix C

IR-Spectroscopy: Functional Group Identification

Infrared spectroscopy (IR spectroscopy or Vibrational Spectroscopy) is the spectroscopy that deals with the infrared region of the electromagnetic spectrum that is light with a longer wavelength and lower frequency than visible light. It covers a range of techniques, mostly based on absorption spectroscopy (www.wikipedia.com).

The exact frequency at which a given vibration occurs is determined by the strengths of the bonds involved and the mass of the component atoms. For a more detailed discussion of these factors. In practice, infrared spectra do not normally display separate absorption signals for each of the 3n-6 fundamental vibrational modes of a molecule. The number of observed absorptions may be increased by additive and subtractive interactions leading to combination tones and overtones of the fundamental vibrations, in much the same way that sound vibrations from a musical instrument interact. Furthermore, the number of observed absorptions may be decreased by molecular symmetry, spectrometer limitations, and spectroscopic selection rules. One selection rule that influences the intensity of infrared absorptions, is that a change in dipole moment should occur for a vibration to absorb infrared energy. Absorption bands associated with C=O bond stretching are usually very strong because a large change in the dipole takes place in that mode.



Some General Trends:

i. Stretching frequencies are higher than corresponding bending frequencies. (It is easier to bend a bond than to stretch or compress it.)

ii. Bonds to hydrogen have higher stretching frequencies than those to heavier atoms.

iii. Triple bonds have higher stretching frequencies than corresponding double bonds, which in turn have higher frequencies than single bonds. (Except for bonds to hydrogen).

Table C1 Characteristic IR absorption frequencies of organic functional groups

Functional Group Names	Absorption Ranges(cm_{-1})	Type of Vibration causing IR absorption
Alkanes	3000-2800	H-C-H Asymmetric & Symmetric Stretch
	1500-1440	H-C-H Bend
Alkenes	3100-3000	C=C-H Asymmetric Stretch
	1675-1600	C-C=C Symmetric Stretch
Alkynes	3300-3200	C H Stretch
	2200-2100	C C Stretch

Aromatic Rings	3100-3000 1500-1450	C=C Stretch -H Asymmetric
	1600-1580	C-C=C Symmetric Stretch
	1500-1450	C-C=C Asymmetric Stretch
Phenols & Alcohols	3600-3100	Hydrogen Stretch -bonded O-H
	1730-1650	C=O Stretch
Ketones	1750-1625	C=O Stretch
	1750-1625	C=O Stretch
Aldehydes	2850-2800	C-H Stretch off C=O
Esters	1755-1650	C=O Stretch
	(1300-1000)	C-O Stretch
Ethers	1300-1000	C-O Stretch
Amines—Primary	3500-3100	(TWO PEAKS!) N-H Stretch
	1640-1560	N-H Bend
Amines—Secondary	3500-3100	(ONE PEAK!) N-H Stretch
	1550-1450	N-H Bend
Nitriles	2300-2200	C N Stretch
Nitro Groups	1600-1500	N=O Stretch
	1400-1300	N=O Bend
Amides	3500-3100	N-H Stretch (similar to amines)
	1670-1600	C=O Stretch
	1640-1550	N-H Bend
Aldehydes	2750-2700	C-H Stretch off C=O