ANALYTICAL QUANTIFICATION METHOD OF PROTEIN DENATURATION IN SPRAY DRYING

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BORANG PENGESAHAN STATUS TESIS♦

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Signature :……

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Date : 12 JANUARY 2012

ANALYTICAL QUANTIFICATION OF PROTEIN DENATURATION IN SPRAY DRYING

SURIA BINTI MAHMUD

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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STUDENT'S DECLARATION

I declare that this thesis entitled *"Analytical quantification of protein denaturation in spray drying"* is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Name : SURIA BINTI MAHMUD

Date : 12 JANUARY 2012

DEDICATION

To my beloved father, Mahmud bin Abdul Lahak, families, and friends, who gave me everlasting inspiration, never ending encouragements and priceless support towards the success of this research.

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بسم الله الرحمن الرحيم

In the name of Allah SWT, most Grateful and most Merciful

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ABSTRACT

Protein is the most valuable constituent of bovine milk, due to its high nutritional quality, unique physicochemical and functional properties, which were exploited to manufacture diverse range of dairy products. This research is to identify the protein denaturation by heat in lab scale spray dryer. The Lab-Plant SD06AG Laboratory Scale Spray Dryer is used to produce protein powder from a pure whey protein powder. This work is to establish a method for preserving protein content in whey protein during the spray drying which is by employing the microencapsulation technique and also by controlling the spray drying operating parameters. Protein denaturation was reduced by adding of microencapsulation by using Maltodextrin. The moisture content analysis was determined via MS-07 Moisture Content Analyzer. Then, the morphology and particle size distribution of milk powders are determined by using Malvern Mastersizer. The quality of protein that undergo spray drying process which is before and after it analyzed by using high-performance liquid chromatography (HPLC). The analysis suggested a severe protein denaturation (60% and 40%) at higher spray drying temperature (180 °C and 190°C) but can be reduced up to 29% and 20% when microencapsulation technique is employed.

ABSTRAK

Protein adalah konstituen susu lembu yang paling berharga, kerana kadar nutrisi yang tinggi, fizikokimia yang unik dan sifat berfungsi, yang dieksploitasi untuk mengeluarkan pelbagai produk tenusu. Cadangan ini adalah untuk mengenal pasti penyahaslian protein dengan haba pengering semburan skala di dalam makmal. Lab-Plant SD-06AG Makmal Skala Sembur pengering digunakan untuk menghasilkan serbuk protein dari serbuk protein whey tulen. Kerja ini adalah untuk membentuk satu kaedah untuk memelihara kandungan protein dalam protein whey semasa pengeringan semburan yang menggunakan teknik microencapsulasi dan juga dengan mengawal parameter semburan pengeringan operasi. Penyahaslian protein telah dikurangkan dengan menambah mikroencapsulasi dengan menggunakan Maltodextrin. Analisis kandungan lembapan telah ditentukan melalui MS-07 Kandungan Lembapan Analyzer. Kemudian, morfologi dan taburan saiz zarah susu tepung ditentukan dengan menggunakan Malvern Mastersizer. Kualiti protein yang sebelum dan selepas menjalani proses semburan kering dianalisis dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC). Analisis telah mencadangkan penyahaslian protein (60% dan 40%) pada semburan suhu pengeringan yang lebih tinggi (180 ° C dan 190 ° C), tetapi boleh dikurangkan sehingga 29% dan 20% apabila teknik mikroencapsulasi diaplikasikan.

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LIST OF ABBREVIATIONS & SYMBOLS

%	-	Percentage
wt%	-	Weight percent
v/v%	-	Volume per volume percent
WPI	-	Whey protein isolate
α-lac	-	Alpha lactalbumin
β-lg	-	Beta lactoglobulin
IgG	-	Immmunoglobulin
BSA	-	Bovine Serum Albumin
g	-	Gram
rpm	-	Revolution per minute
K	-	Kelvin
m	-	Meter
ml	-	Milliliter
µl	-	Microliter
ACN	-	Acetonitrile
H_2O	-	Water
TFA	-	Triflouroacetic Acid
^oC	-	Degree Celsius
MPa	-	Mega Pascal
V	-	Volume
HPLC	-	High Liquid Performance Chromatography

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Drying is the oldest method of preserving food, and dried products which have useful attributes in terms of good storage stability, economical way of transport and unique structural qualities. Drying also is one of the most important unit operations and has probably the widest applications in the food and pharmaceutical industries. There are three common drying methods used to create particles from solutions which namely as freeze-drying, spray-drying and drum drying.

Based on Masters (1991), spray-drying is one of the well-established methods for producing dry powders and it is the direct opposite to the spray-freeze-drying technique, i.e. an atomized spray is contacted with hot gas which is used as the drying medium. Then, evaporation takes place to yield dried particles, which are subsequently separated from the gas stream by a variety of methods. Currently, spray drying is the preferred method for producing whey proteins in powder form. Normally, it comes at the end-point of the processing line, as it is an important step to control the final product quality (Anandharamakrishnan *et al.*, 2008). Recently, many techniques have been developed to microencapsulate food ingredients, spray-drying is the most common technology used in food industry

due to low cost and available equipment. Microencapsulation by spray-drying has been successfully used in the food industry for several decades (Gouin, 2004) and this process is one of the oldest encapsulation methods used since the 1930s to prepare the first encapsulated flavors using gum acacia as wall material (Shahidi and Han, 1993).

1.2 Problem Statement

Spray drying is a preferred method to protect milk powder. During spray drying protein will be denatured. Protein denaturation ranged from 30 to 40% in a pilot scale spray dryer when the outlet temperature was set at 120 °C (Anandharamakrishnan *et al.,* 2008). Lower protein content can be harmful, 50 babies died in China in 2004 when the substandard baby milk with as little as one-sixth the required amount of protein was consumed (Watts, 2004). So, one method to minimize protein denaturation is by employing low spray drying temperature (Anandharamakrishnan *et al.,* 2007) but result in lower powder yield. Another method is by using microencapsulation method which has been applied for encapsulating food ingredients such as flavors, lipids, and carotenoids (Gharsallaoui, 2007). Microencapsulation is a relatively new technology that is used for protection, stabilization, and slow release of food ingredients. The encapsulating or wall materials used generally consist of starch, starch derivatives, proteins, gums, lipids, or any combination of them. Methods of encapsulation of food ingredients include spray-drying, freeze-drying, fluidized bed-coating, extrusion, cocrystallization, molecular inclusion, and coacervation (Shahidi *et al.,* 1993). This work is to establish a method for preserving protein content in fresh milk during operation and also to develop a reliable analytical method for quantifying the protein in milk powder.

1.3 Research Objective

The objectives of this work are to establish a method for preserving protein content in whey protein isolate (WPI) during operation and also to develop a reliable analytical method for quantifying the protein in whey protein powder.

1.4 Scope of Research

The following are the scopes of this research to support the above mentioned objectives:

- To perform spray drying of protein using the lab scale spray dryer
- To minimize the denaturation of protein during spray drying process by changing the operating parameters such as inlet temperature and via microencapsulation of maltodextrin
- To examine the particle size distribution of whey powder using Malvern Mastersizer
- To determine the moisture content of spray drying whey powders by using MS-07 moisture content analyzer
- To analyze the quality of protein that undergoes spray drying process which is before and after spray drying by using HPLC
- To establish relationship between spray drying condition and protein denaturation by using HPLC

1.5 Rationale and Significance

This study will provide a better understanding of protein denaturation in lab scale spray dryer hence establishing optimum operating conditions for spray drying to minimize protein denaturation which is expected to be achieved upon the completion of this study.

1.6 Thesis outline

In this research, it will divide into five chapters. Firstly, Chapter 1 is an overview about this research. It consists of the introduction on spray dryer which gives a brief idea on what spray-drying process and the effect to the protein. The problem statement, objective and the scope of the study also are included in this chapter.

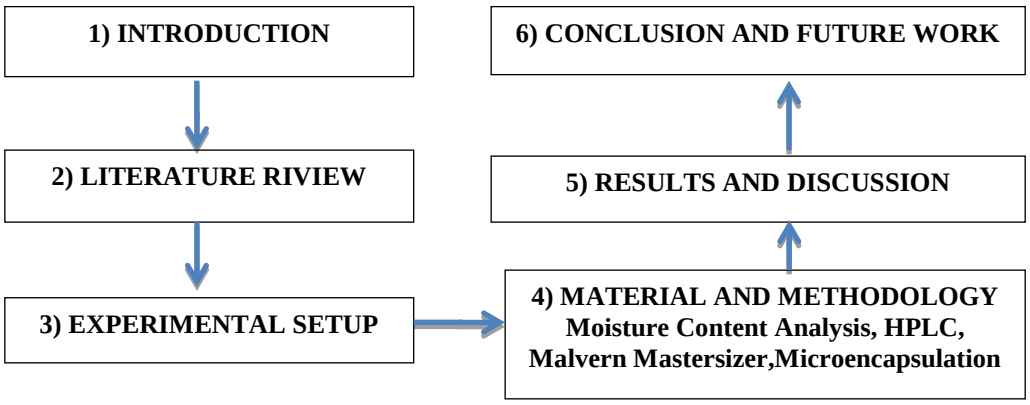

Figure 1.1: The road map for thesis

Chapter 2 is about literature review on protein denaturation during spray drying process and includes all parameters taken to minimize protein denaturation during spray drying in the lab scale dryer. In this chapter, all the relevant technical paper, journals, and books taken from those researches will be studied and discussed.

Then, **Chapter 3** will be covered the parts of experimental set up and will be explained more details on methodology and operating procedures. The techniques and the algorithms that will be used in performing this study will be applied. The methods and techniques used for this system are described in detail. In addition, in this chapter also explained the material used in this experiment and the method used to analysis the data.

Chapter 4 will be covered on the results and discussion of the research during the operation process. All the experimental result and data will be discussed in details which are including the effects of microencapsulation and effect of controlling parameters of lab scale spray dryer to whey proteins content. The detailed report on the product quality analysis was evaluated. Implementation of process that is involved during development of this analysis is explained in detail in this chapter.

Chapter 5 will be discussed on the conclusion can be made for the study and some recommendations can be taken.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

According to Anandharamakrishnan *et al.* (2008), spray drying is the process of transforming a feed (solution or suspension) from a fluid into a dried particulate form by spraying the feed into a hot drying medium. Spray drying is a widely used in industrial process for the continuous production of dry powders. Then, spray drying is widely used in the food and pharmaceutical industries for producing high quality powders with low moisture content (Charm, 1971; Masters, 1991). Spray drying also is used for a wide range of products such as proteins, vitamins, bacteria, enzymes, yeast, flavors, milk, eggs, soups, beverages, fruit juices, vegetables and encapsulated products. Several studies and patents on spray drying have been summarized by Masters (1991).

2.2 Spray Drying

Spray-drying is the most common and cheapest technique to produce microencapsulated food materials. Equipment is readily available and production costs are lower than most other methods. Compared to freeze-drying, the cost of spray-drying method is 30–50 times cheaper (Desobry *et al.,* 1997). Spray-drying has been considered as a solution for conventional drying problems because the process has usually proved not only efficient but also economic (Masters, 1968).

2.3 Principles of Spray Drying

According to Gharsallaoui (2007), spray-drying is a unit operation by which a liquid product is atomized in a hot gas current to instantaneously obtain a powder. The gas generally used is air or more rarely an inert gas as nitrogen. The initial liquid feeding the spray dryer can be a solution, an emulsion or a suspension. Spray-drying produces, depending on the starting feed material and operating conditions, a very fine powder (10–50 lm) or large size particles (2–3 mm). Water removal by spray-drying solutions is a common engineering practice. By decreasing water content and water activity, spray-drying is generally used in food industry to ensure a microbiological stability of products, avoid the risk of chemical and/or biological degradations, reduce the storage and transport costs, and finally obtain a product with specific properties like instantaneous solubility for example. The spray-drying process has been developed in connection with the manufacture of dried milk.

However, based on Gharsallaoui (2008), when milk is spray-dried, the process can be considered as a microencapsulation one which is milk fat is being the core material that is protected against oxidation by a wall material consisting of a mix of lactose and milk proteins. In this mix, the carbohydrates provide structure through glass formation whereas the proteins provide emulsification and film forming properties.

2.4 Applications of Spray drying

The application of spray-drying process in microencapsulation involves three basic steps (Dziezak, 1988). Firstly is by preparing of the dispersion or emulsion to be processed. Secondly is by homogenizing of the dispersion and lastly is by atomizing of the mass into the drying chamber. However, Shahidi and Han (1993), suggested that the microencapsulation by spray-drying involves four stages which are preparation of the dispersion or emulsion, homogenization of the dispersion, atomization of the in feed emulsion and dehydration of the atomized particles. The first stage is the formation of a fine and stable emulsion

of the core material in the wall solution. The mixture to be atomized is prepared by dispersing the core material, which is usually of hydrophobic nature, into a solution of the coating agent with which it is immiscible. The dispersion must be heated and homogenized, with or without the addition of an emulsifier depending on the emulsifying properties of the coating materials because some of them have themselves interfacial activities. In the spray-drying process, the initial emulsion droplets are in the order of diameter 1–100 lm.

Before the spray-drying step, the formed emulsion must be stable over a certain period of time (Liu *et al.,* 2001), oil droplets should be rather small and viscosity should be low enough to prevent air inclusion in the particle (Drusch, 2006). So, emulsion viscosity and particle size distribution have significant effects on microencapsulation by spray-drying. High viscosities interfere with the atomization process and lead to the formation of elongated and large droplets that adversely affect the drying rate (Rosenberg *et al.,* 1990). As the sprayed particles fall through the gaseous medium, a spherical shape with the oil encased in the aqueous phase will form (Dziezak, 1988).

2.5 Advantages and Disadvantages of Spray Drying

Gohel (2009), reported that by using spray drying can gives lots of advantages likes the actual spray drying process is very rapid, with the major portion of evaporation taking place in less than a few seconds, wide ranges of spray dryer designs are available to meet various product specifications and offers high precision control over particle size, bulk density, degree of crystallinity, organic volatile impurities and residual solvents.

Other than that, powder quality remains constant during the entire run of the dryer and nearly spherical particles can be produced, uniform in size and frequently hollow, thus reducing the bulk density of the product.

There are some disadvantages by using spray drying such as the equipment is very bulky and with the ancillary equipment is expensive and the overall thermal

efficiency is low, as the large volumes of heated air pass through the chamber without contacting a particle, thus not contributing directly to the drying.

2.6 Issues on Spray Drying Of Heat Sensitive Material

According to Liu *et al.* (2004), the main factors in spray-drying that must be optimized are feed temperature, air inlet temperature, and air outlet temperature. However, Zakarian and King (1982) suggested that a high air inlet temperature causes an excessive evaporation and results in cracks in the membrane inducing subsequent premature release and a degradation of encapsulated ingredient or also a loss of volatiles. While, the air inlet temperature is usually determined by two factors which are the temperature which can safely be used without damaging the product or creating operating hazards and the comparative cost of heat sources (Fogler and Kleinschmidt, 1938).

The best spray-drying conditions are a compromise between high air temperature, high solid concentration of the solution, easy pulverization, drying without expansion and cracks of final particles (Bimbenet *et al.*, 2002). Reineccius (1988) reported that the greatest loss of the volatiles during microencapsulation by spray-drying takes place at early stages of drying, prior to the formation of a dry crust at the surface of the drying particles. It was expected also that heat denaturation of whey proteins influences emulsification characteristics and thus microencapsulating properties (Rosenberg and Sheu, 1996).

2.7 Protein

Sander *et al.* (1997) implies that proteins can be derived from plants, e.g. wheat gluten, soy and pea protein, or from the milk or skin and hides of animals, such as casein and whey protein, and gelatin respectively. Currently a number of industrial applications are based on industrial proteins (Skeist, 1990; Mulder, 1997). Proteins can be processed in the presence of a high amount of water (e.g. coatings, adhesives, surfactants), or under low-moisture conditions (extrusion).

Generally, protein based coatings and adhesives are produced by dissolving the protein in water at high or low pH and using denaturants such as urea (Skeist, 1990; Somanathan *et al.*, 1992; Gennadios *et al.*, 1994; McHugh *et al.*, 1994).

2.8 Structure of Proteins

Kuntz *et al.* (1974) suggested that protein molecules in solution are surrounded by a hydration shell which is composed of water molecules attached to the protein surface mainly by hydrogen bonds and is indispensable for supporting the native protein conformation. If an organic solvent is present in solution, its molecules tend to displace water from the hydration shell thus distorting the finely balanced interactions responsible for maintaining the native conformation of the protein molecule. According to a generally accepted notion, the destruction of the hydration shell is one of the main reasons of protein denaturation by organic solvents.

2.9 Denaturation of Proteins

According to Goetz and Koehler (2003), denaturation is due to the ability to form and stabilize gels, foams, emulsions and fibrillary structures, proteins are able to highly influence the texture of food. Thermal processing of protein-containing food systems and its components is used in order to increase the shelf life (pasteurization, sterilization) and to modify selectively the texture of the food. On a molecular level, heat treatment causes denaturation of proteins including unfolding and aggregation of the molecules. The changes depend on protein concentration, process temperature, duration, pH-value, concentration of salt, sugar, and hydrocolloids. Depending on the composition of the system, heat treatment is applied to increase the viscosity (thickening), to form gel structures or to stabilize the structure.

Denaturation is the modification of the native structure of a protein without cleavage of peptide bonds within the amino acid sequence (Cheftel *et al.*, 1985).

Depending on the conditions, partial or complete as well as reversible or irreversible denaturation can be observed (McKenzie and Ralston, 1971).

2.10 Whey Protein

Whey proteins are the proteins that appearing in the supernatant of milk after precipitation or in other words whey proteins are the proteins that remain in solution after removal of casein. These kind of proteins are more water soluble than caseins and are subject to heat denaturation. Native whey proteins plays an extremely important role in the food industry and have good gelling and emulsification properties. The beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin (BSA), and immunoglobulins (Ig) are the principle fractions in whey proteins. Whey proteins are commonly used in food and formulated pharmaceutical products (Anandharamakrishnan *et al.*, 2005; Anema *et al.*, 2005; Schokker *et al.*, 2000)

Table 2.1: Distribution of milk proteins

Component	% Total	% Whey
Whey	17	
Beta-lactoglobulin	10	58
Alpha-lactalbumin	2	13
Immunoglobulins	2	12
Serum Albumin	1	6
Minor proteins	2	12

2.10.1 Beta-Lactoglobulin

In whey protein, ß-lactoglobulin gives the highest fraction of protein. It comprises 10% of the total milk protein or about 58% of the whey protein. It contains 162 amino acids with a molecular weight of about 18,300. There are two genetic variants, A and B, which differ in the substitution of a glycine in Variant B for an aspartic and in Variant A. The molecule contains two disulfide and 1 free sulfhydryl groups and no phosphorus.

β-lactoglobulin is manufactured specifically in the mammary gland for inclusion in milk where its role is unknown. All ruminant milk contains ß-lactoglobulin while the milk of almost all non-ruminants does not. The molecule has a very hydrophobic area that is quite effective in binding retinol. Some speculate that the binding of Vitamin A may have a regulatory role in the mammary gland. Because of its prevalence in bovine milk, to a large extent the properties of whey protein concentrates are in effect, the properties of ß-lactoglobulin.

The secondary structure of β-lactoglobulin is homologous to that of retinol-binding proteins. The lone α-helix is located on the surface of the molecule. The center of the barrle is hydrophobic and can be involed in the binding of hydrophobic molecules. The three deminsional structure of β-lactoglobulin is presented in Figure 2.1.

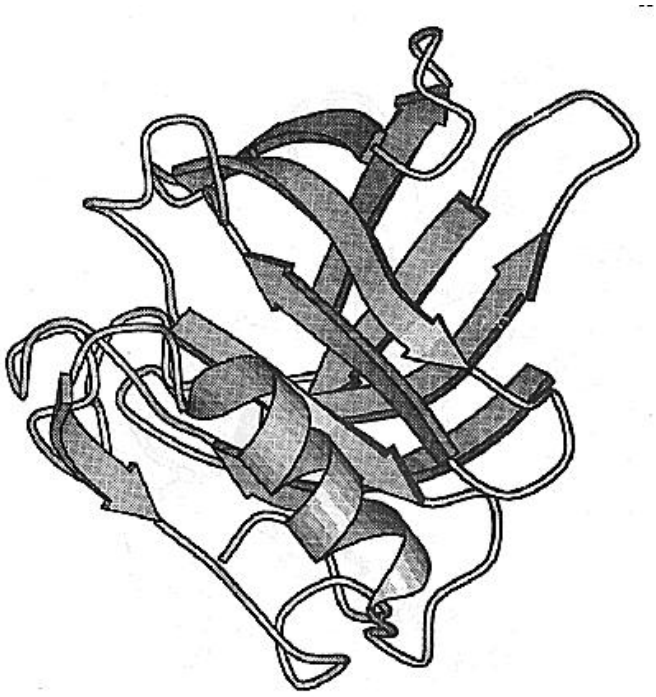

Figure 2.1: The three-dimensional structure of Bovine β-lactoglobulin.

Source: Original structure by Sawyer, reproduced from Swaisgood (1996)

2.10.2 Alpha-Lactalbumin

The second most prevalent protein in whey is α-lactalbumin which comprises about 2% of the total milk protein which is about 13% of the total whey protein. The molecule consists of 123 amino acids and has a molecular weight of 14,146. The molecule contains 4 disulfide linkages and no phosphate groups.

Alpha-lactalbumin has been shown to modify the activity of the enzyme galactosyl transferase. In the absence of α-lactalbumin, this enzyme adds UDP-galactose to N-acetyl glucosomine groups that are attached to proteins. It can transfer the UDP galactose to glucose, but the Km for glucose is 1400mM and thus, the reaction proceeds slowly, if at all. Alpha-lactalbumin serves to lower the Km for glucose to 5mM and the enzyme complex now will add UDP-galactose to glucose to produce lactose and UDP. Thus, the milk of all mammals that contain lactose also contains α-lactalbumin. The α-lactalbumin of any species isolated so far will serve to modify bovine galactosyl transferase activity.

When the sequences of α-lactalbumin and lysozyme are compared, about 40% of the residues are found to be the same, including all the cysteine residues. Another 20% of the residues have similar structures. This information coupled with the fact that α-lactalbumin helps to synthesize the same linkage that lysozyme cleaves, suggests that the molecules are closely related. In fact, knowledge of the three-dimensional structure of lysozyme has been utilized to predict the three-dimensional structure of α-lactalbumin.

Despite their similarity, they do not work on the same substrates and are not related antigenetically. The site of synthesis of α-lactalbumin like beta-lactoglobulin is the mammary gland. Alpha-lactalbumin is unusual in that the molecule is more stable to heat in the presence rather than the absence of calcium. Most proteins show increased heat sensitivity in the presence of calcium. This is probably due to the ability of calcium to promote the formation of ionic intermolecular cross links with most proteins. These crosslinks hold the molecules in proximity and increase the likelihood of aggregation upon heating. Alpha-lactalbumin, on the other hand, uses calcium to form intramolecular ionic bonds that tend to make the molecule resistant to thermal unfolding. Under favorable conditions of calcium and pH, α-lactalbumin can remain soluble after exposure to 100C. The structure of α-lactalbumin is presented in figure 2.2.

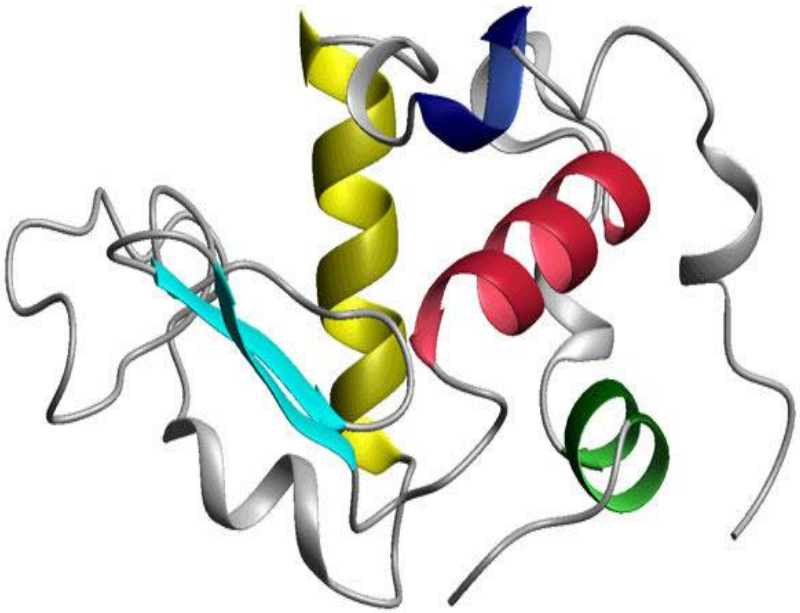

Figure 2.2: Three-dimensional structure of α-lactalbumin, from *human alpha-lactlabumin stabilized with human milk-specific fatty acids.*

2.10.3 Bovine Serum Albumin

The Bovine Serum Albumin (BSA), isolated from milk, is identical to the blood serum molecule. Thus, BSA is not synthesized in the mammary gland, but rather into the milk through passive leakage from the blood streams. The protein has a molecular weight of 69,000. It contains no phosphorus, 17 disulfides and 1 free sulfhydryl group. In blood plasma albumin is a carrier of free fatty acids. The molecule has specific binding sites for hydrophobic molecules and may bind them in milk as well. The rasmol version of the human enzyme with attached myristic acid. To see the fatty acids set the color scheme to chain. The albumin will be in blue and the myristic acid in red.

2.10.4 Immunoglobulins

The immunoglobulins comprise at least 2% of the total milk protein. There are four classes of immunoglobulins found in milk: 1gG1, lgG2, lgA and lgM. All of these molecules have a similar basic structure being composed of 2 light

chains with molecular weights of 20,000 - 25,000 and two heavy chains, having molecular weights of 50,000 - 70,000.

These molecules are not synthesized in the mammary gland and thus must first enter into the gland and then be transported through it to be able to enter the milk. In the case of at least one class of antibodies, lgG1, a specific receptor site has been located on the membrane of the cells of the mammary gland that facilitates the entry of this protein into the gland. The immunoglobulins supply passive immunity to the calf when supplied in the colostrum. This protection lasts until the animal is old enough to begin synthesis of its own antibodies.

2.10.5 Proteose-Peptones

This fraction of milk has been defined as those proteins that remain in solution after milk has been heated at 95C for 20 minutes and then acidified to pH 4.7. These proteins are precipitated by 12% trichloroacetic acid.

This fraction can be divided into 4 major components while other minor components are recognized. Proteose peptone component 3 is found only in whey and is not associated with casein. This protein contains over 17% carbohydrate and has a molecular weight of 20,000. Antibody to proteose peptone component 3 will cross react with fat globule membrane and it has been suggested that this component is of membrane origin.

Proteose peptone component 5 has a molecular weight of 13,000 and is associated with both the whey and casein fractions of milk. The molecule contains phosphorus and has been shown to consist of the N-terminal 107 amino acids of β-casein that arrive from the proteolytic cleavage that yields the g-caseins.

In a like manner, proteose peptone component 8 fast with a molecular weight of 3,900 represents the N terminal 28 amino acids released from the cleavage of β-casein. The other major proteose peptone component, 8 slow, has not yet been shown to have been derived by the proteolysis of any milk proteins. In time,

however, this will probably occur. The protein has a molecular weight of 9,900. As a group, the proteose peptones are by definition resistant to heating. They are also very surface active due in part to their low molecular weights and also to the carbohydrate associated with component three. About 1.1% of the total milk proteins consist of proteose peptone. As some of these molecules are derived from the proteolysis of beta-caseins, their concentration in any given milk can be expected to increase with time.

2.11 High Performance Liquid Chromatography (HPLC)

Scientists involved in pharmaceutical process research and development (PR&D) rely heavily on analytical data obtained using chromatographic techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC). Through critical evaluation of these data, scientists assess the progress of processes, formation of reaction byproducts, and the quality of synthesized materials. HPLC, in particular, is widely accepted in pharmaceutical laboratories for the following reasons: sample preparation time is usually short, qualitative and quantitative data can be recorded for samples of widely varying polarity in a single run, various forms of detection can increase the specificity and information content of assays, and regulatory agencies have accepted HPLC as a mature and reliable technique. Within the past 15 years, HPLC has very likely impacted the development of every drug candidate within the pharmaceutical industry (Karcher, 2005).

2.12 Principle of HPLC

According to Karcher (2005), a unique high-performance liquid chromatographic (HPLC) workflow specifically designed for the rigors of process development has been developed. A key feature of the workflow is the creation of an HPLC software–hardware platform designed to automatically and systematically screen samples using a matrix of columns and eluents to aggressively search for impurities. The screening conditions are complementary to each other, and are useful to assess the complexity of a sample and to

chromatographically resolve impurities that may coelute using any single method. The workflow has been designed to support several different modes of HPLC, and can be used with absorption detection, photodiode array spectrometers, evaporative light scattering (ELS) devices, and mass spectrometric (MS) detection. The custom software interface contains a data-viewing feature to simplify analysis of results. The platform is designed to be used by process scientists, and the same simple user-interface is used to control analytical HPLC, LC–MS, and preparative HPLC.

2.13 Particle Size Distribution (PSD)

The particle size distribution (PSD) of a powder, or granular material, or particles dispersed in fluid, is a list of values or a mathematical function that defines the relative amounts of particles present, sorted according to size. The particles in a colloidal suspension or emulsion are seldom all of the same size and they often have varying shapes. Describing the size and shape is therefore a significant problem. Emulsion droplets can usually be assumed to be spherical (Colloidal Dynamics, 1999).

Based on article in Malvern Instruments Ltd (2011), particle size analysis and measurement is an important parameter across many industries. The stability, chemical reactivity, opacity, flowability and material strength of many materials are affected by the size and characteristics of the particles. Getting things right at every stage of production, from R & D through to manufacture and quality control of the finished product is vital. Malvern has a range of particle size analysis solutions from sub-nanometer to millimeters in size.

2.14 Application of Particle Size Distribution

As particle size decreases, surface area increases as a function of total volume. In the colloidal size range there is much interest in particle-particle interactions. Most colloidal commercial products are designed to remain in a stable condition for a defined shelf life. Milk is an example where homogenization is used to reduce droplet size to delay the onset of phase separation (i.e., creaming with the fat rising to the surface). Commercial suspensions may be formulated to keep particles in suspension without sedimenting to the bottom. Examples of phase separation mechanisms are shown below. The following forces play an important role in the interaction of colloid particles which are van der Waals forces. This is due to interaction between two dipoles that are either permanent or induced. Then, electrostatic interaction, colloidal particles often carries an electrical charge and therefore attracts or repels each other. Lastly are steric repulsive forces between polymer-covered surfaces arising due to osmotic repulsion and volume restriction (Horiba Scientific, 2011).

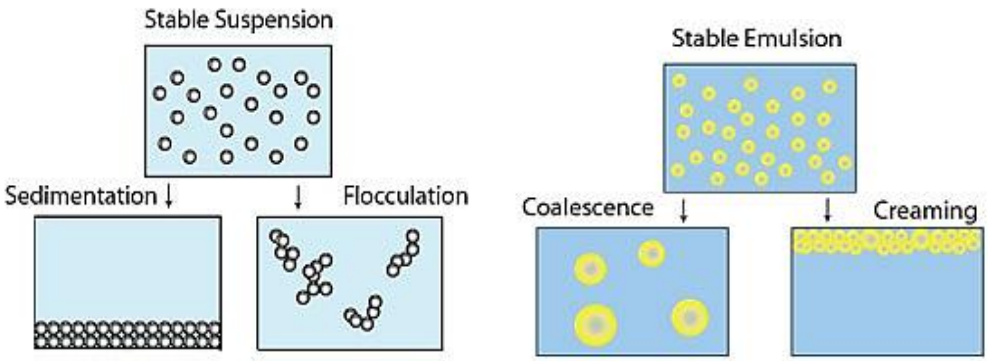

Figure 2.3: Examples of phase separation mechanisms

Source: Horiba Scientific (2011)

2.15 Microencapsulation

2.15.1 Introduction

Shahidi and Han (1993), proposed five reasons for applying microencapsulation in food industry which are to reduce the core reactivity with environmental factors, to decrease the transfer rate of the core material to the outside environment, to promote easier handling, to control the release of the core material, to mask the core taste and finally to dilute the core material when it should be used in only very small amounts.

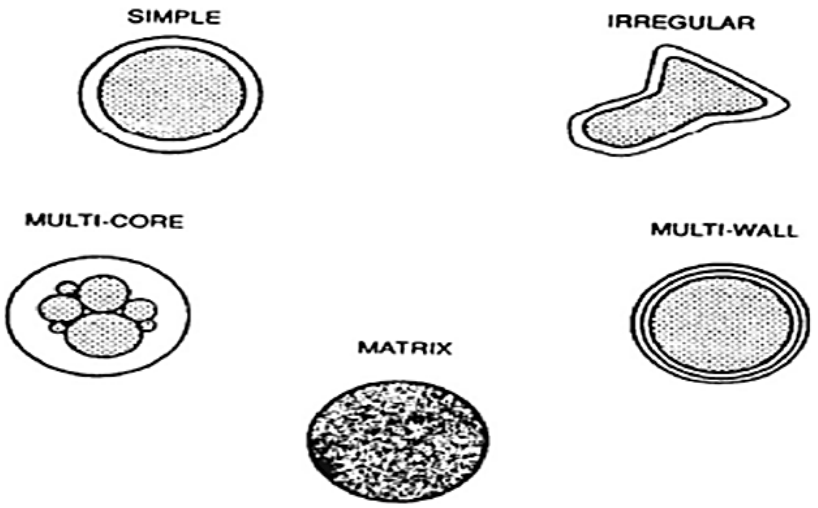

Figure 2.4: Morphology of different types of microcapsules

Source: Gibbs *et al.* (1999)

The different types of microcapsules and microspheres are produced from a wide range of wall materials (monomers and/or polymers) and by a large number of different microencapsulation processes such as: spray-drying, spray-cooling, spray-chilling, air suspension coating, extrusion, centrifugal extrusion, freeze-drying, coacervation, rotational suspension separation, co-crystallization, liposome entrapment, interfacial polymerization, molecular inclusion and so on (Desai and Park, 2005; Gibbs *et al.*, 1999; Gouin, 2004; King, 1995; Shahidi and Han, 1993).

Although most often considered as a dehydration process, spray-drying can be used to encapsulate active material within a protective matrix formed from a polymer or melt (Dziezak, 1988).

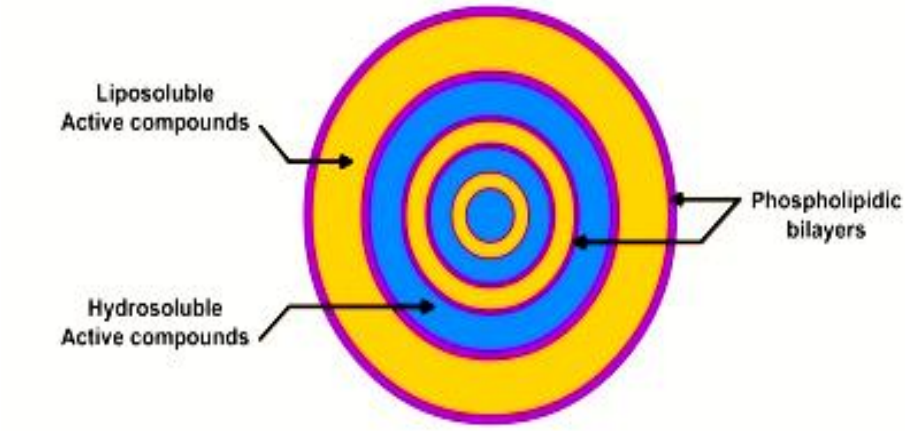

Figure 2.5: Very tiny droplets or particles of liquid or solid material are Surrounded or coated with a continuous film of polymeric material

Source: Gate2tech (2011)

Microencapsulation is described as a process of enclosing micron sized particles of solids or droplets of liquids or gasses in an inert shell, which in turn isolates and protects them from the external environment (Ghosh, 2009). The impact of microencapsulation on drying and storage of dried probiotic microorganisms prior to application in food systems is barely investigated, since efforts with respect to increase in survival are mainly focused on the application of protective substances (Carvalho *et al.,* 2002). While capsules from dried hydrogels generally remain water insoluble after rehydration, microcapsules prepared from proteins by spray drying are water soluble in most cases (Carvalho *et al.,* 2007).

2.15.2 Microencapsulation Technique

Table 2.2: Different techniques used for microencapsulation

Chemical processes	Physico-chemical processes	Physico-Mechanical process
Interfacial polymerization In situ polymerization Poly condensation	Coacervation and phase separation Sol-gel encapsulation Supercritical CO2 assisted microencapsulation	Spray drying and congealing Fluid bed coating Pan coating Solvent evaporation

Source: Ghosh (2009)

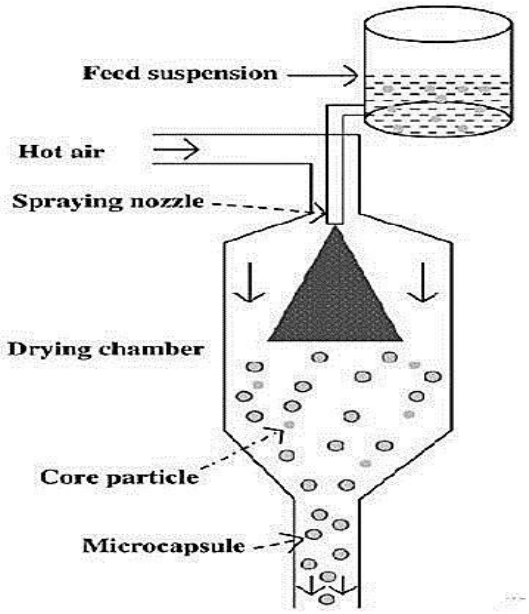

Figure 2.6: Schematic illustrating the process of micro-encapsulation by spray-drying

Source: Ghosh (2009)

The above mentioned techniques are widely used for microencapsulation of several pharmaceuticals. Among these techniques fluidized bed or air suspension method, coacervation and phase separation, spray drying and spray congealing, pan coating, solvent evaporation methods are widely used. Depending on the physical nature of the core substance to be encapsulated the technique used will be varied (Ghosh, 2009).

Table 2.3: Microencapsulation processes and their applicabilities

Microencapsulation process	Nature of the Core material	Approximate particle size(µm)
Air suspension	Solids	35-5000*
Coacervation and phase separation	Solids and Liquids	2-5000*
Multi orifice centrifugation	Solids and Liquids	1-5000*
Pan coating	Solids	600-5000*
Spray drying and congealing	Solids and Liquids	600
Solvent evaporation	Solids and Liquids	5-5000*

*The 5000µm size is not a particle size limitation. The methods are also applicable for macrocoating (Bakan *et al.*, 2009)

The micro-capsules have a number of benefits such as converting liquids to solids, separating reactive compounds, providing environmental protection, improved material handling properties (Gate2tech, 2011). Active materials are then encapsulated in micron-sized capsules of barrier polymers such as gelatin, plastic and wax.

Jyothi *et al.* (2009) proposed that micro particles or microcapsules consist of two components namely core material and coat or shell material. Core material contains active ingredient while coat or shell material covers or protects the core material. Different types of materials like active pharmaceutical ingredients, proteins, peptides, volatile oils, food materials, pigments, dyes, monomers, catalysts, pesticides etc. can be encapsulated with different types of coat or shell materials like ethyl cellulose, hydroxyl propyl methyl cellulose, sodium carboxy methyl cellulose, sodium alginate, PLGA, gelatine, polyesters, chitosans and so on.

2.15.3 Wall Material for Microencapsulation

The wall system is designed to protect core material from factors that may cause its deterioration, to prevent a premature interaction between the core material and other ingredients, to limit volatile losses, and also to allow controlled or sustained release under desired conditions (Shahidi and Han, 1993). Depending on the core material and the characteristics desired in the final product, wall materials can be selected from a wide variety of natural and synthetic polymers. According to Gouin (2004), since almost all spray-drying processes in the food industry are carried out from aqueous feed formulation, the wall material must be soluble in water at an acceptable level. In addition Reineccius (1988) suggested that a wall material for microencapsulation by spray-drying should possess good properties of emulsification, film forming, and drying and the wall concentrated solutions should have low viscosity to its high solubility.

Based on Dziezak (1988), many available wall materials possess these properties but the number of materials approved for food uses is limited. Many biopolymers have been used in microencapsulation of various food ingredients by spray-drying. The microencapsulation of food ingredients is often achieved with biopolymers of various sources, such as natural gums (gum arabic, alginates, carragenans, etc.), proteins (milk or whey proteins, gelatin, etc.), maltodextrins with different dextrose equivalence, waxes and their blends.

However, typical wall materials for microencapsulation by spray-drying are low molecular weight carbohydrates, milk or soy proteins, gelatin and hydrocolloids like acacia gum (Reineccius *et al.,* 1995; Thevenet, 1995) and more recently local materials, such as mesquite gum (Beristain and Carter, 1994; Beristain *et al.,* 2001) have been used to overcome the expensive cost of some commonly used materials. Among these methods, a process of electrostatic layer-by-layer deposition was successfully developed to prepare spray-dried microcapsules containing tuna oil (Klinkesorn *et al.,* 2006). These microcapsules were reported to be unaffected by air inlet temperature probably because of the robustness of the wall barrier.

Table 2.4: Experimental conditions recently optimized for the encapsulation of some different food ingredients by spray drying

Encapsulated ingredient	Wall material	Feed temperature (°C)	Air inlet temperature (°C)	Air outlet temperature (°C)	References
Anhydrous milk fat	Whey proteins/lactose	50	160	80	Young et al. (1993)
Ethyl butyrate ethyl caprylate	Whey proteins/lactose	5	160	80	Rosenberg and Sheu (1996)
Oregano, citronella and marjoram flavors	Whey proteins/milk proteins	NR	185–195	85–95	Baranauskiené et al. (2006)
Soya oil	Sodium caseinate/carbohydrates	NR	180	95	Hogan et al. (2001)
Calcium citrate calcium lactate	Cellulose derivatives/ polymethacrylic acid	NR	120–170	91–95	Oneda and Ré (2003)
Lycopene	Gelatin/sucrose	55	190	52	Shu et al. (2006)
Fish oil	Starch derivatives/glucose syrup	NR	170	70	Drusch et al. (2006)
Cardamom essential oil	Mesquite gum	Room T	195–205	105–115	Beristain et al. (2001)
Arachidonyl L-ascorbate	Maltodextrin/gum arabic/soybean polysaccharides	NR	200	100–110	Watanabe et al. (2004)
Cardamom oleoresin	Gum arabic/modified starch/ maltodextrin	NR	176–180	115–125	Krishnan et al. (2005)
Bixin	Gum arabic/maltodextrin/sucrose	Room T	180	130	Barbosa et al. (2005)
D-Limonene	Gum arabic/maltodextrin/modified starch	NR	200	100–120	Soottitantawat et al. (2005a)
L-Menthol	Gum arabic/modified starch	NR	180	95–105	Soottitantawat et al. (2005b)
Black pepper oleoresin	Gum arabic/modified starch	NR	176–180	105–115	Shaikh et al. (2006)
Cumin oleoresin	Gum arabic/maltodextrin/modified starch	NR	158–162	115–125	Kanakdande et al. (2007)
Fish oil	Sugar beet pectin/glucose syrup	NR	170	70	Drusch (2006)
Caraway essential oil	Milk proteins/whey proteins/ maltodextrin	NR	175–185	85–95	Bylaité et al. (2001)
Short chain fatty acid	Maltodextrin/gum arabic	NR	180	90	Teixeira et al. (2004)

NR: not reported.

2.15.4 Application of Microencapsulation

The encapsulation efficiency of the microparticle or microcapsule or microsphere will be affected by different parameters (Jyothi *et al.,* 2009). Fig.2.7.5 illustrates the factors influencing encapsulation efficiency.

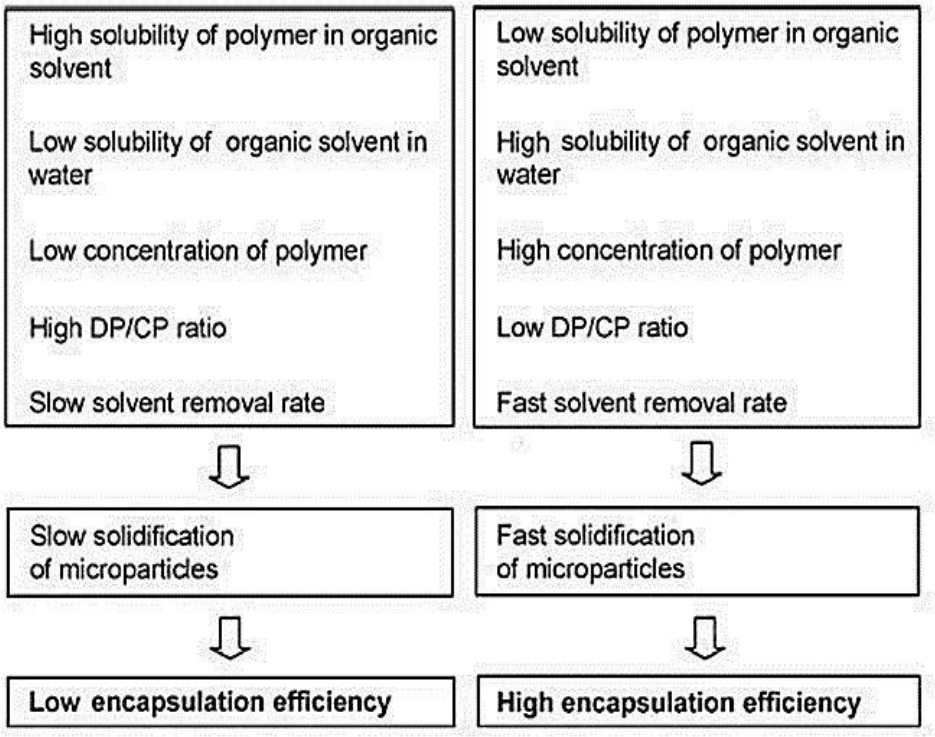

Figure 2.7: Factors influencing encapsulation efficiency

According to Jimenezet et *al.*(2004) ; Re (1998) ; Huezo *et al.* (2004) ; Schierle *et al.* (1997) in food processing field, microencapsulation technique has been widely used to protect food ingredients against deterioration, volatile losses, or premature interaction with other ingredients. The protective mechanism therein is to form a membrane (wall system) to enclose droplets or particles of the encapsulated material (core). So far, various kinds of microencapsulation techniques such as solvent dispersion/evaporation, phase separation (coacervation), corystallization, interfacial polymerization etc., have been developed, among which, spray-drying is the most commonly used one in the food industry due to its continuous production and easiness of industrialization.

2.16 Summary

Spray drying process requires high temperature which can cause protein denaturation. Many research concluded that the whey protein started to denature at outlet temperature, 60°C until 70°C such as Adhikari *et al*. (2009), Anandharamakrishnan *et al.* (2008), Madueireira *et al*. (2007) and Elgar (2000). In most cases, spray drying process studies without include microencapsulation protein, its only focusing on the wall material of microencapsulation like Gharsallaoui *et al*.(2007), Loksuwan (2007) and Sheu and Rosenberg (1995).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Introduction

This chapter describes the materials use and the methodology applies during this research. The structure of this chapter is shown in figure 3.1. The analytical methods are divided into two categories related which are product characteristics (particle size analysis and moisture content) and product quality (protein denaturation and composition of milk).

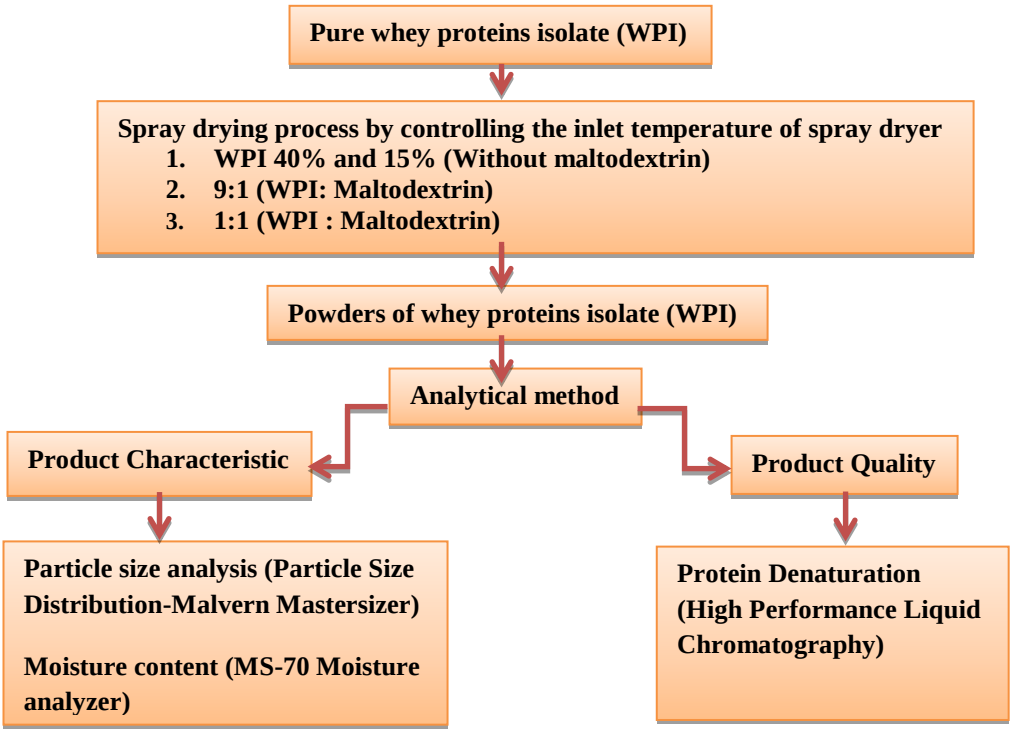

Figure 3.1: Structure of Chapter 3

3.2 Equipment / Apparatus

There are several types of apparatuses and equipment's are required during completing this study. There are analytical balance, hot plate, magnetic stirrer, frezeer, fume hood, vacuum pump, sonicator, centrifuge tubes, vortex, dropper, micropipettes, Scott bottles, sample bottles to store the WPI powder after done the spray drying process, beakers, measuring cylinder, spatula and weighing boat. Then, syringe, needles and syringe filter are used to filter the sample before analyze it. Moisture analyzer was used for measure moisture content in the sample. SD-06A Laboratory Scale Spray Dryer to spray the whey powder, High-performance liquid chromatography (HPLC) and Particle Size Distribution (Malvern Mastersizer) are used for analysis powder morphology and size.

3.3 Materials Used

There are several materials and chemicals used in this research. Some of them are: Ultra-Pure Water, maltodextrin, Whey Protein Isolate (WPI) powder is obtained from Ultimate Nutrition (Fleetwood, Lancashire, UK) and manufacturer claims that 99% of the whey proteins are undenatured and Protein Standard (α-lactalbumin and β-latoglobulin) with 85% and 80% purity respectively to determine the protein content, Tri-fluoroacetic acid (TFA) and Acetonitrile (ACN) which is HPLC Grade used for UPLC analysis purchased from Merck.

Table 3.1: WPI powder composition

Mass per 100g of dry powder	
Protein	92.0 g
Carbohydrate	3.0 g
sugars	3.0 g
Fat	3.0 g
Fiber	1.0 g
Sodium	Nil
Calcium Content	200mg
Lecithin (E322)	400mg

3.4 Preparation of Spray Drying

3.4.1 Whey Protein Without Maltodextrin Solution Preparation

The solutions of whey proteins with concentration 15% and 40% (w/v) prepared as follows: The 15% (w/v) whey protein solution was prepared at room temperature by dissolving 15g of powder in 100 ml of distilled water in beaker. This mixture was gently stirred in a laboratory hot plate without heating effect for 10 min to dissolve all the whey proteins in water. The mixture was kept for a consistent period before spray drying for the protein to hydrate. The same procedure was followed for 40% (w/v) concentrations, using 40 g WPI powder respectively.

3.4.2 Whey Protein With Maltodextrin Solution Preparation

The solutions of whey proteins added with maltodextrin with concentration 15% and 40% (w/v) prepared as follows: For 15% (w/v) concentration whey protein solution with ratio 90% WPI and 10% of maltodextrin was prepared at room temperature by dissolving 13.5g of WPI and 1.5g of maltodextrin in 100 ml of distilled water in beaker. Then, for 15% (w/v) concentration whey protein solution with ratio 50% WPI and 50% of maltodextrin was prepared at room temperature by dissolving 7.5g of WPI and 7.5g of maltodextrin in 100 ml of distilled water in beaker. These mixtures were gently stirred in a laboratory hot plate without heating effect for 10 min to dissolve all the whey proteins in water. The mixtures were kept for a consistent period before spray drying for the protein to hydrate. The same procedure was followed for 40% (w/v) concentrations by using 36g WPI: 4 g maltodextrin and 20g WPI: 20 g maltodextrin respectively.

Figure 3.2: Preparation of whey protein solution

3.5 Spray Drying Experimental Set Up

The spray dryer operates co-currently and has a spray-nozzle which is 1 mm in diameter. The spray drying equipment was set up for spray drying process. The spray drying process initiated by passing hot air through the chamber for 15 minutes to warm up the chamber. After that, all the parameters involved were set up as shown in figure 3.4 and 3.5. Then, after the system was ready to be operated and the inlet temperature reached, the solution of whey protein was fed into drying chamber. The feed concentration of solution will be spray dried over different range of gas inlet and outlet temperatures. Inlet gas temperature was varying started from 189 ºC until 192 ºC for 15% concentration of whey protein isolate and 181 ºC until 183 ºC for 40% concentration of whey protein isolate. Outlet gas temperature was kept between 88 ºC and 89 ºC for both concentrations. Liquid feed to the dryer was 8 rpm as shown in Figure 3.4.

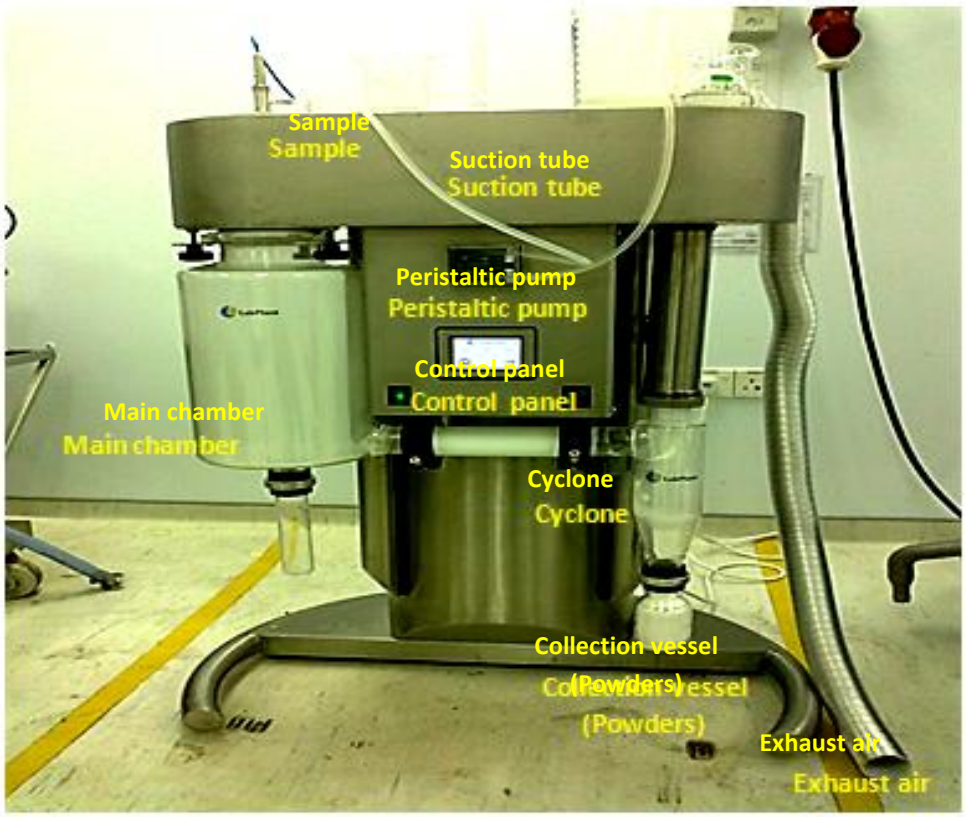

Figure 3.3: SD06AG spray dyer

Figure 3.4: Setting of the parameters (fan, pump and deblocker) at control panel

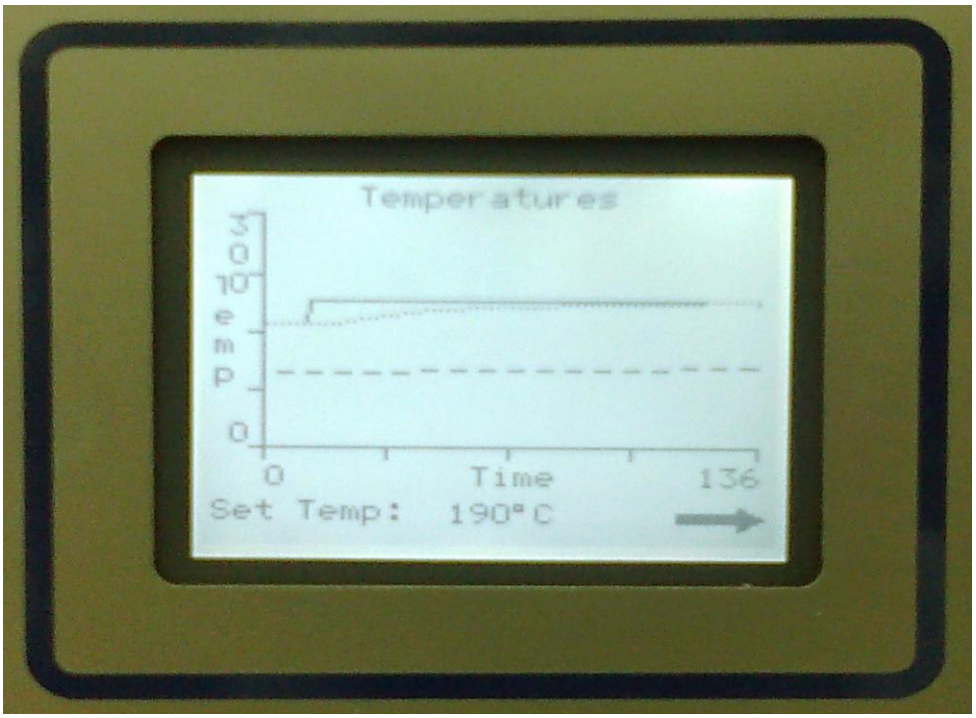

Figure 3.5: Temperature rise up until reached setting temperature

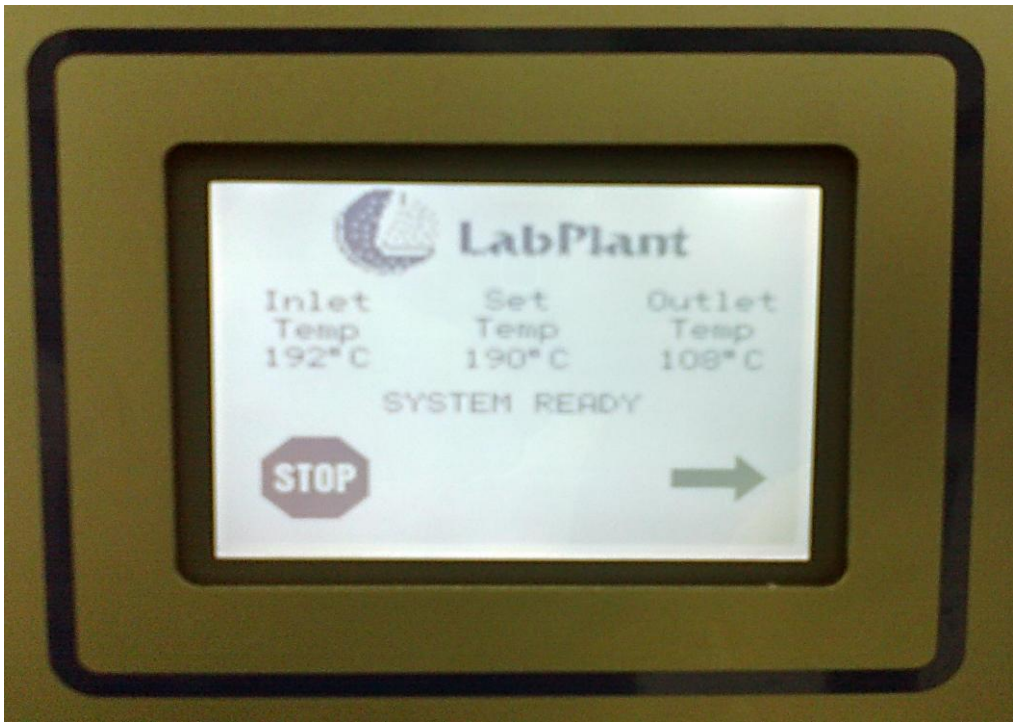

Figure 3.6: Temperature reached to the setting temperature, system is ready to operate.

Figure 3.7: Whey isolate powder after spray drying

The spray dried products of whey protein isolate were collected in a cyclone at the collection vessel after 10 to 15 minutes run the spray drying process. It kept in sample bottles as showed in Figure 3.7 and was stored in closed containers within a dessicator containing silica gel at room temperature until analysis.

3.6 Moisture Content Analysis

The moisture contents analysis were analyzed by using MS-70 Moisture Analyzer from A&D Company from Tokyo, Japan. WPI samples after spray drying may contain varies amounts of moisture and it may increase when exposed to air. The analysis were carried out by setting the temperature at 140°C and putting 2 g of sample on the plate (standard temperature and weight for dairy products) and the value of moisture was calculated by MS-70 analyzer. All the steps for analysis were shown in figure 3.8, 3.9 and 3.10.

Figure 3.8: Preparation before analyze the moisture content using MS-70 analyzer

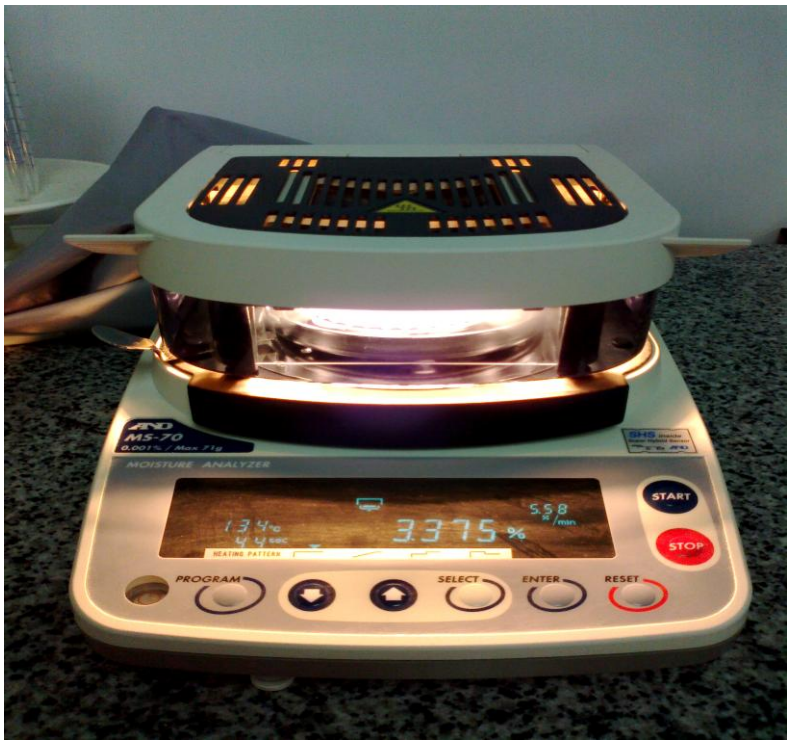

Figure 3.9: During the analysis process using MS-70 analyzer

Figure 3.10: After analysis process using MS-70 analyzer

3.7 High Performance Liquid Chromatography

3.7.1 Preparation of Whey Protein Solution

The sample was weighted based on the calculation made from moisture contents from moisture content analyzer. The target was to make sure the total solid contains was the same with the whey protein isolate pure. The weighted sample then placed to the 10 ml centrifuge tube that contains appropriate volume of Ultra-Pure Water according to the concentration from moisture content analysis at appendix. Then, the vortex was used to make sure the powder was mixed gently. The sample was sonicated around 3-4 minutes to dissolve the solutions and also bust the bubble if present. Then, the mixture solution was transferred into the syringe.

Prior to HPLC analysis, all samples were filtered through 0.22µm nylon syringe filters and buffers were filtered through 0.22µm Durapore membrane filters (Millipore, Bedford MA, USA) and degassed. From this sample solution, only 10 uL was injected into HPLC system.

3.7.2 HPLC Analytical Procedure

High Performance Liquid Chromatography (HPLC) of whey protein isolate was using C4 Jupiter Column (300 A, 250 x 4.6 mm id., 5µm particles size; Phenomenex, Cheshire, UK).The injection volume is 10µL.

Then, the operating conditions were as follows (Enne *et al*., 2005):

Flow rate 1 ml/min: Solvent A, 0.1% (wt/vol) triflouroacetic acid (TFA) in double distilled water; Solvent B, 0.1% (wt/vol) triflouroacetic acid (TFA) in HPLC-grade acetonitrile (ACN)
The column was equilibrated with 20% solvent B
Absorbance was recorded at 205 nm

The elution was performed as follows:

0 to 1 min, 35% solvent B

1 to 8 min, 35 to 38 % solvent B

8 to 16 min, 38 to 42 % solvent B

16 to 22 min, 42% to 46% solvent B

22 to 24 min, 46% to 90% solvent B

24 to 25 min, 90% solvent B

25-30 min, 90% to 35% solvent B

30-35 min, 35% solvent B

Figure 3.11: WPI analysis by using Ultra Performance Liquid Chromatography (UPLC) with column HPLC

3.7.3 Protein Standard

The protein standard which are α-lactalbumin (product L5385, approximately 85% purity as per manufacturer), β-lactoglobulin (product L3048, approximately 80% purity as per manufacturer) and Bovine serum albumin (product L3048)

were obtained from the Sigma-Aldrich (Malaysia) and were used as proteins standard for HPLC analysis.

3.7.4 Protein Standard Calibrations

The whey protein were identified by means of retention time and peak were quantified by comparing peak areas with the results of calibration series with pure native standards (supplied by Sigma-Aldrich) for α-lactalbumin, β-lactoglobulin, Bovine Serum Albumin and Immunoglobulin. α-lactalbumin, β-lactoglobulin and Bovine Serum Albumin protein calibration standard were prepared by serial dilution on the day of use just like in table 3.2, 3,3 and 3.4 respectively using ultra-pure water.

Table 3.2: Standard curve for α-lactalbumin

Concentration (mg/ml)	Area
0.01265	12933.456
0.01625	12293.724
0.25	2635899.785
0.5	3374651.137
0.8	6559655.026
1	8052639.47
2	13372738.34
4	24597385.23

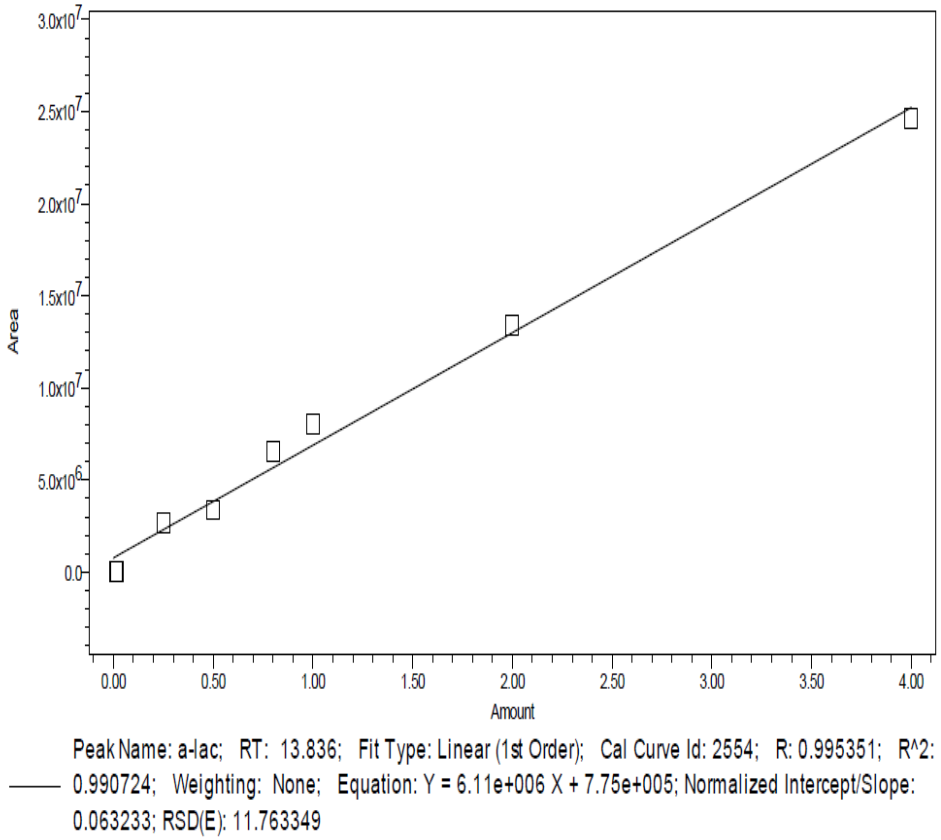

Figure 3.12: Graph of calibration curve for α-lactalbumin

Table 3.3: Standard curve for β-lactoglobulin 1

Concentration (mg/ml)	Area
0.0625	65880.267
0.125	184533.625
0.25	1016683.576
0.5	2012701.687
0.8	3295982.426
1	4374851.835
2	8674645.401
4	17044425.615

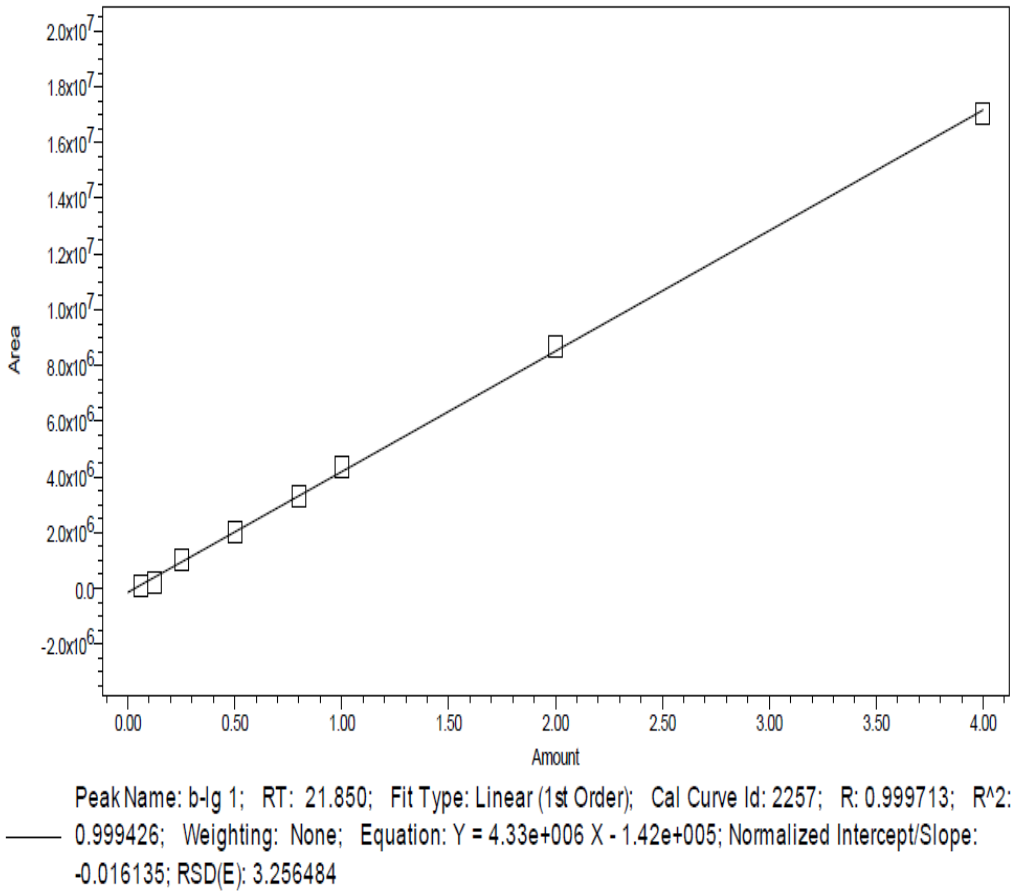

Figure 3.13: Graph of calibration curve for β-lactoglobulin

Table 3.4: Standard curve for β-lactoglobulin 2

Concentration (mg/ml)	Area
0.0625	65880.267
0.125	184533.625
0.25	1016683.576
0.5	2012701.687
0.8	3295982.426
1	4374851.835
2	8674645.401
4	17044425.615

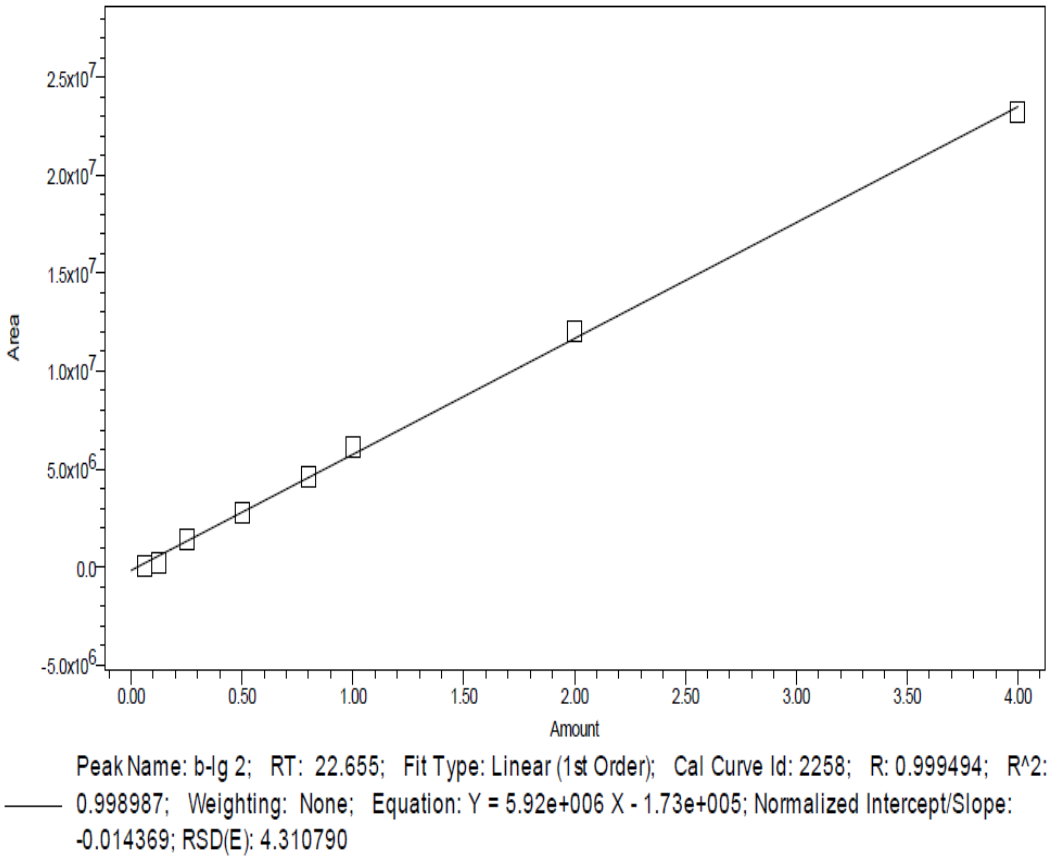

Figure 3.14: Graph of calibration curve for β-lactoglobulin 2

Table 3.5: Standard curve for BSA

Concentration (mg/ml)	Area
0.125	1101806.621
0.25	2893564.025
0.5	5960192.422
0.8	11546341.069
1	15588853.193
2	35142627.129

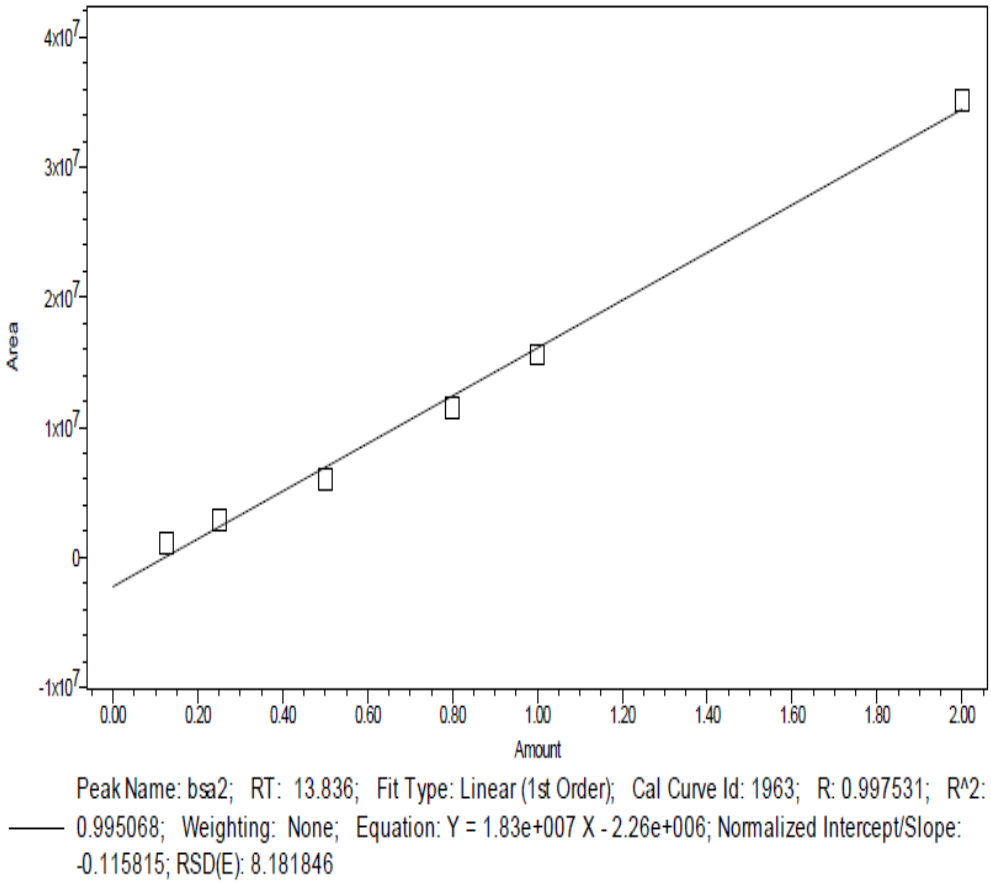

Figure 3.15: Graph of calibration curve for BSA

Figure 3.16 shows the chromatographic pattern of bovine serum albumin, α-lactalbumin and β-lactoglobulin. The proteins were resolved with retention time a) ~15min one peak for bovine serum albumin; b) ~ 16 min one peak for α-lactalbumin and c) ~ 21 min and 22 min β-lactoglobulin1 and β-lactoglobulin 2. The same trend of separation was observed in other reports (e.g. Elgar *et al*., 2000 & Enne e*t al*., 2005)

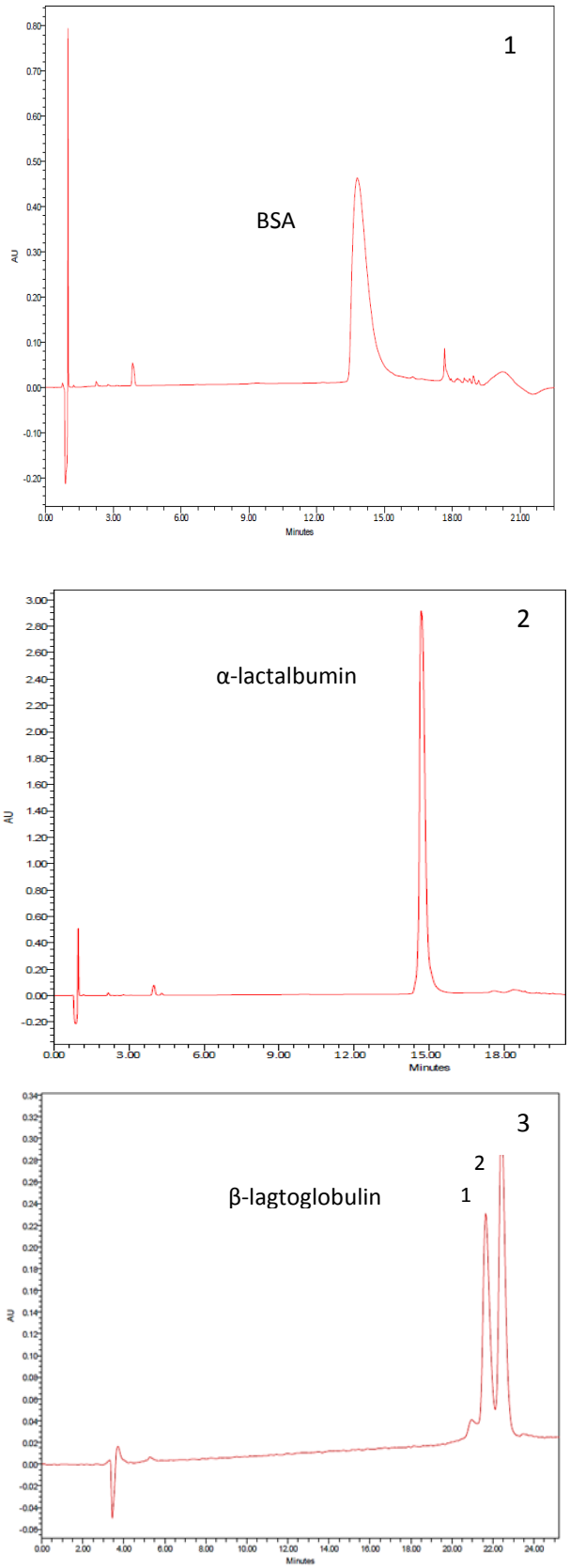

Figure 3.16: Chromatogram pattern of protein standards 1) Bovine serum albumin, 2) α-lactalbumin and 3) β-lactoglobulin 1 and β-lactoglobulin 2

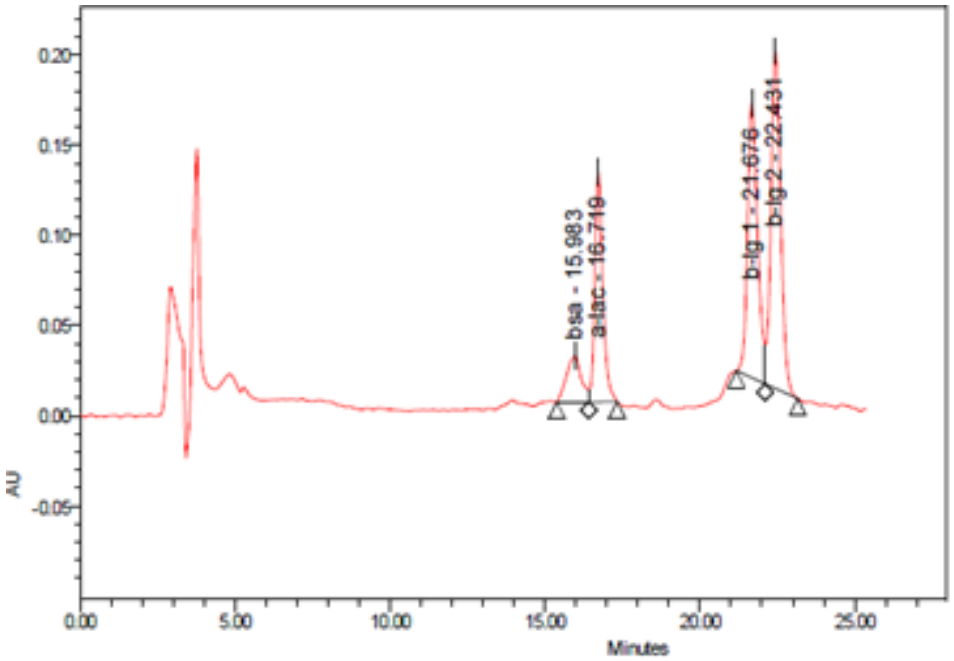

Figure 3.17: Peak of chromatogram of spray dried whey protein

3.8 Particle Size Distribution Analysis

The particle size distributions of the spray dried powders was measured at 25 ºC by laser light scattering using MasterSizer instrument (Malvern Instrument, UK) are showed in Figure 3.15. The instrument uses an approximation of Mie-Scattering theory, which utilizes the refractive index of dispersed phase and its absorption. A relative refractive index n_{oil}/n_{water}= 1.095 and absorption value of 0.1 were used. The result was recorded as volume distributions.

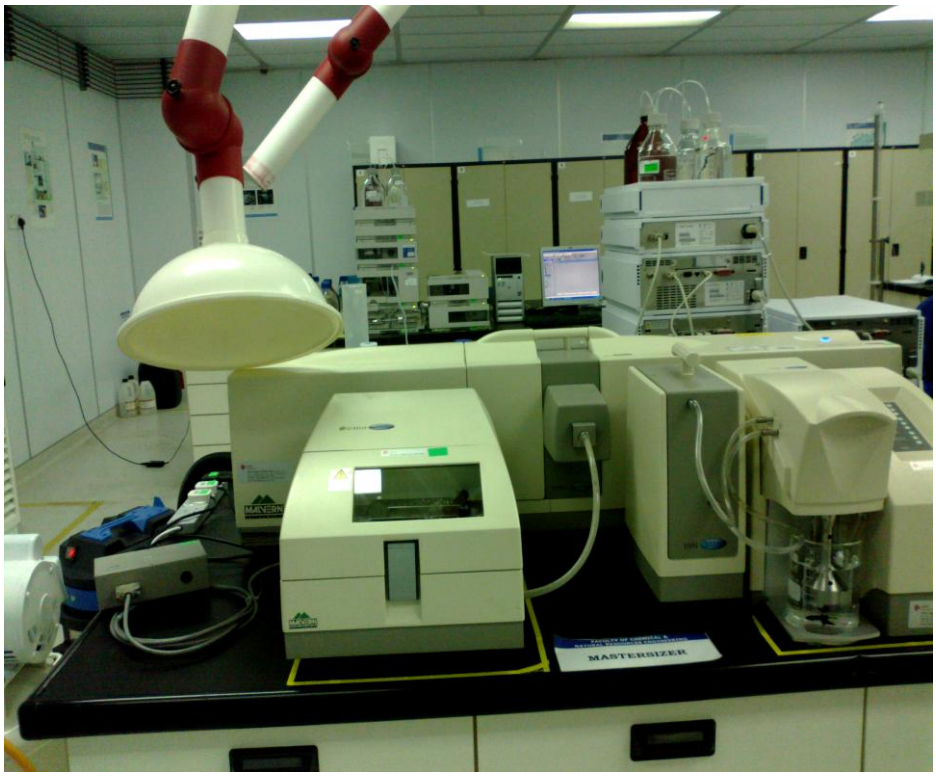

Figure 3.18: Particle Size Distribution

Figure 3.19: Tested sample loaded into analyzer

3.9 Summary

The experiment was conducted start from spray drying of whey protein isolate until form a powder then do the further analysis. Next step is the preparation of the powder milk to analysis by using Particle size distribution, Moisture analyzer and HPLC. All the analysis has a great contribution for this research. During spray drying, the parameter was use to controlling the spray dryer were inlet temperature which using variable temperature (180ºC and 190ºC) and via microencapsulation technique.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter is about the analysis of the experiment. The HPLC is a direct method for measure the loss of solubility of protein content in whey protein isolate (WPI). The high inlet temperature for spray drying process has affected the protein denaturation in whey protein composition. The higher inlet temperature of spray dryer used is increased; the protein denaturation for less solubility of the protein also will be increased. The counter-current operation the dry powder exiting the chamber will closely approach the inlet gas temperature (Master, 1991). The analysis such as HPLC, Moisture Content Analyzer and Particle size Analysis has been used for this study based on the study by Enne (2005), Anandharamakrishnan *et al.* (2008), Ye *et al*. (2006) and Elgar *et al.* (2000).

4.2 Spray Drying

The main operating variables in the spray drying that must be optimized were the feed rate, the atomization pressure, and the inlet and outlet temperatures (Li *et al*., 2004). The full sets of experimental operating conditions are presented in table 4.1 and table 4.2.

Table 4.1: Spray dryer operating parameter for 15% concentration of solid

Sample	Temp setting (°C)	Temp inlet gas (°C)	Temp outlet gas (°C)	Pump (rpm)	Fan (rpm)
WPI (Without malto)	190	Avg: 190 Max: 191 Min: 189	Avg: 89.3 Max: 90 Min: 89	8 rpm	50
9:1 (Malto added)	190	Avg: 192 Max: 193 Min: 191	Avg: 88.3 Max: 89 Min: 88	8 rpm	50
1:1 (Malto added)	190	Avg: 191 Max: 192 Min: 190	Avg: 88.3 Max: 89 Min: 88	8 rpm	50

Table 4.2: Spray dryer operating parameter for 40% solid concentration

Sample	Temp setting (°C)	Temp inlet gas (°C)	Temp outlet gas (°C)	Pump (rpm)	Fan (rpm)
WPI (Without malto)	180	Avg: 134 Max: 181 Min: 98	Avg: 89.3 Max: 90 Min: 89	8 rpm	50
9: 1 (Malto added)	180	Avg: 176 Max: 183 Min: 98	Avg: 88.3 Max: 89 Min: 88	8 rpm	50
1: 1 (Malto added)	180	Avg: 192 Max: 182 Min: 179	Avg: 88.3 Max: 89 Min: 88	8 rpm	50

4.3 Moisture Content Analysis

WPI samples after spray drying may contain varying amounts of moisture and it may increase when exposed to air. The moisture contents of the sample were analyzed by using MS-70 Moisture Analyzer from A&D Company from Tokyo, Japan. The percentages of moisture content for all samples were shown in Table 4.5. The purpose of finding samples moisture content is for wet basis of each samples powder. Based on published reports from Etzel *et al*. (1996) and Samborska *et al*. (2005), the moisture content reduces based on increasing the

outlet and inlet gas temperature because water in the samples will evaporate at high temperature.

Table 4.3: Results of moisture contents for WPI 15% solid concentration

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
WPI Pure	7.06	2	6.20	140
WPI	7.46	2	6.45	140
9:1	9.10	2	7.7	140
1:1	8.52	2	8.75	140

From the above table, the moisture content for the WPI Pure is 7.06% while the percentage of moisture content of WPI 15% after done spray drying is 7.46%. Then, the moisture content for WPI added with 10% maltodextrin is 9.10% while the moisture content for WPI added with 50% maltodextrin is 8.52 %. The driest product was WPI added with 10% maltodextrin which is 9.10%.

Table 4.4: Results of moisture contents for WPI 40% solid concentration

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
WPI Pure	7.71	2	6.73	140
WPI	8.61	2	6.80	140
9:1	9.17	2	7.3	140
1:1	7.46	2	7.9	140

From the table above, the moisture content for the WPI Pure is 7.71% while the percentage of moisture content of WPI 40% after done spray drying is 8.61%. Then, the moisture content for WPI added with 10% maltodextrin is 9.17% while the moisture content for WPI added with 50% maltodextrin is 7.46 %. The driest product was WPI added with 10% maltodextrin which is 9.17%.

Table 4.5: Preparation of WPI 15% solid concentration samples' for analysis by HPLC

Sample preparation	Concentration of each sample
Preparation of WPI sample	
WPI Pure	**1 mg/ml**
WPI	**1 mg/ml**
Preparation of WPI sample + maltodextrin	
9:1	**1.1355 mg/ml**
1:1	**2.0217 mg/ml**

The Table 4.5 shows the appropriate amount of concentration preparations for each sample. The concentrations of each sample were obtained by applying some formula and all data of moisture contents analysis were used in this calculation. The calculation can be referred at Appendix A. The results obtained from table 4.5 shows that WPI sample added with maltodextrin has the highest concentration compared to the other samples. WPI that added with 50% of maltodextrin shows the highest amount of concentration.

Table 4.6: Preparation of WPI 40% solid concentration samples' for analysis

Sample preparation	Concentration of each sample
Preparation of WPI sample	
WPI Pure	**1 mg/ml**
WPI	**1.01 mg/ml**
Preparation of WPI sample + maltodextrin	
9:1	**1.12 mg/ml**
1:1	**1.98 mg/ml**

The Table 4.6 shows the appropriate amount of concentration preparations for each sample. The concentrations of each sample were obtained by applying some formula and all data of moisture contents analysis were used in this calculation. The calculation can be referred at Appendix A. The results obtained from table 4.6 shows that WPI sample added with maltodextrin has the highest concentration compared to the other samples. WPI that added with 50% of maltodextrin shows the highest amount of concentration. It was shown that an increased maltodextrin coverage, it will improve the wettability and same goes case study by Faldt *et al*. (1996).

4.4 High Performance Liquid Chromatography Analysis

There are HPLC peak of chromatogram to clarify whey protein isolate composition in each samples are shown in figures 4.1 and 4.2. The major peaks, corresponding to the main whey proteins which is β-lactoglobulin (β-lg) and α-lactalbumin (α-lac) appeared at elution times within 22-24 min and 17-18 min, respectively. The peaks of Bovine Serum Albumin (BSA) appeared between the major peaks at times within 15-16 min, respectively. From the chromatogram obtained at figures 4.1 and 4.2, WPI pure shows the highest peak followed by 9:1, 1:1 and WPI 15%. These showed that, maltodextrin coverage more important to reduced protein denaturation (Faldt *et al*., 1996)

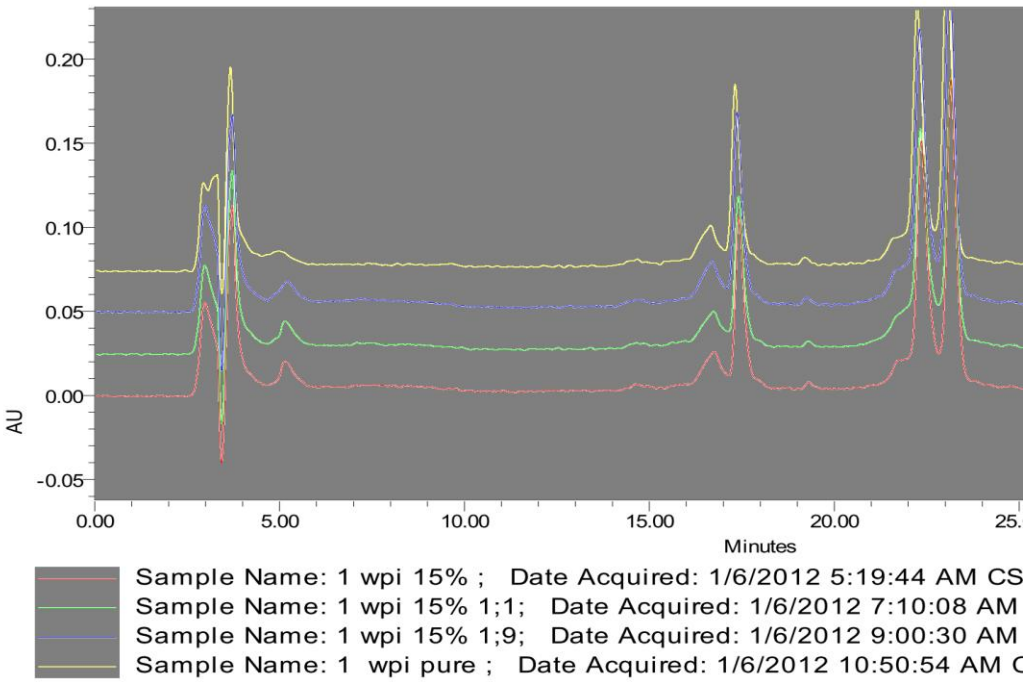

Figure 4.1: HPLC peak of chromatogram of spray dried whey protein isolate (WPI) powder without maltodextrin and with maltodextrin for WPI 15% solid concentration

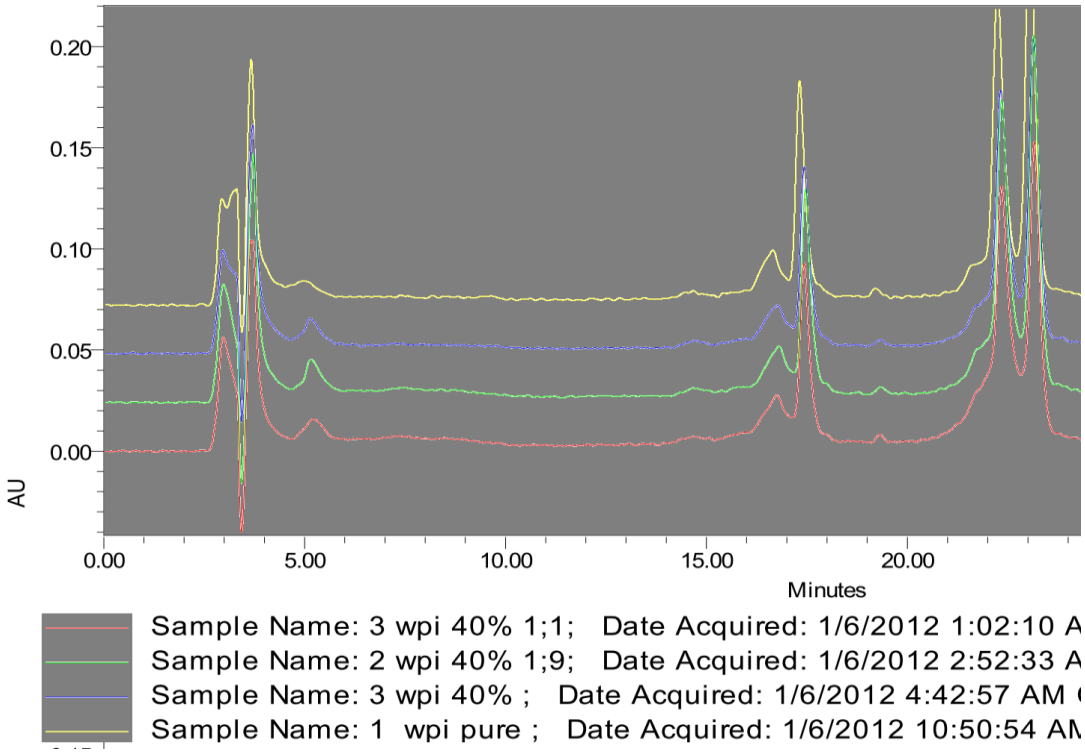

Figure 4.2: HPLC peak of chromatogram of spray dried whey protein isolate (WPI) powder without maltodextrin and with maltodextrin for WPI 40% solid concentration

Table 4.7: Raw data of peak area from HPLC analysis for WPI 15% solid concentration

Type of sample/ Area	1st trial				2nd trial				3rd trial			
	wpi pure	**wpi**	**9:1**	**1:1**	**wpi pure**	**wpi**	**9:1**	**1:1**	**wpi pure**	**wpi**	**9:1**	**1:1**
α-lac	2173738	1113840	1823014	1378784	1645713	1449941	1265769	1483856	2173738	1529934	1823014	1760845
β-lg 1	3228079	1712198	2789305	2298326	2675730	2465986	2142992	2332253	3228079	2417220	2789305	2739939
β-lg 2	3898912	2062998	3306177	2754486	3161634	2975203	2549988	2754486	3898912	2919597	3306177	3235372
BSA	756366	403972	555944	318251	554125	277565	283355	318251	756366	448494	555944	533039

Table 4.8: Raw data of peak area from HPLC analysis for WPI 40% solid concentration

Type of sample/Area	1st trial				2nd trial				3rd trial			
	wpi pure	wpi	9:1	1:1	wpi pure	wpi	9:1	1:1	wpi pure	wpi	9:1	1:1
α-lac	1822892	1366289	1617280	2264775	2173738	1328611	1739644	1579324	1822892	1551841	1739644	1579324
β-lg 1	3619417	3057765	3522581	4247369	3228079	2928992	3612580	3536504	3619417	328008	3612580	3536504
β-lg 2	3576925	2656720	30690701	5332520	3898912	2564123	3235793	2842942	3576925	2831796	3235793	2842942
BSA	640940	491964	642816	1003013	756366	478933	706497	683780	640940	632347	706497	683780

Table 4.9: Concentration for WPI 15% concentration solid samples'

Trial/ Conc	wpi pure				wpi				9:1				1:1			
	α-lac	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**
1	0.229	0.778	0.688	0.165	0.055	0.428	0.378	0.146	0.172	0.677	0.588	0.154	0.099	0.564	0.495	0.141
2	0.143	0.651	0.563	0.154	0.110	0.602	0.532	0.139	0.080	0.528	0.460	0.139	0.116	0.571	0.495	0.141
3	0.229	0.778	0.688	0.165	0.124	0.591	0.522	0.148	0.172	0.677	0.588	0.154	0.161	0.666	0.576	0.153
Avg	**0.200**	**0.736**	**0.646**	**0.161**	**0.096**	**0.541**	**0.477**	**0.144**	**0.141**	**0.627**	**0.545**	**0.149**	**0.125**	**0.600**	**0.522**	**0.145**

Table 4.10: Concentration for WPI 40% concentration solid samples'

Trial/ Conc	wpi pure				wpi				9: 1				1: 1			
	α-lac	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**	**α-lac**	**β-lg 1**	**β-lg 2**	**BSA**
1	0.229	0.778	0.688	0.165	0.055	0.428	0.378	0.146	0.172	0.677	0.588	0.154	0.099	0.564	0.495	0.141
2	0.143	0.651	0.563	0.154	0.110	0.602	0.532	0.139	0.080	0.528	0.460	0.139	0.116	0.571	0.495	0.141
3	0.229	0.778	0.688	0.165	0.124	0.591	0.522	0.148	0.172	0.677	0.588	0.154	0.161	0.666	0.576	0.153
Avg	**0.200**	**0.736**	**0.646**	**0.161**	**0.096**	**0.541**	**0.477**	**0.144**	**0.141**	**0.627**	**0.545**	**0.149**	**0.125**	**0.600**	**0.522**	**0.145**

The Figure 4.9 and 4.10 showed the solid concentration for 15% WPI and 40% WPI. All the concentration was obtained by referring to the proteins calibration curve at Figure 3.12, 3.13 and 3.14 respectively.

The Table 4.11 and Table 4.12 showed the percentage of denaturation of protein with and without microencapsulation of maltodextrin. Table 4.12 showed the protein denaturation was up to 46% at 15% WPI concentration at 190°C temperature. Besides, from Figure 4.12 it showed that the protein denaturation was up to 60% at 40% WPI concentration solid at 180°C temperature. Both temperatures typically were at very high inlet temperatures but just slightly difference. Then, 15% WPI concentration solid gave much lower denaturation compared to 40% WPI. This is because the amounts of protein contents were lower compared to 40% WPI which much higher. So, undoubtedly 40% WPI will denatured more compared to 15% WPI.

Table 4.11: Percentage of 15% WPI solid concentration denaturation

Sample	**Denaturation (%)**		
	α-lac	β-lg	BSA
wpi pure	-	-	-
without maltodextrin	46.03	24.06	10.21
with maltodextrin (9:1)	25.07	13.74	6.64
with maltodextrin (1:1)	29.52	15.33	7.4

Table 4.12: Percentage of 40% WPI solid concentration denaturation

Sample	Denaturation (%)		
	α-lac	β-lg	BSA
wpi pure	-	-	-
without maltodextrin	60.42	20.09	9.2
with maltodextrin (9:1)	31.03	1.58	1.65
with maltodextrin (1:1)	42.5	7.31	2.41

The degree of each WPI samples denaturation was calculated according to the equation below (Fureby *et al*., 2001):

$$\text{Degree of denaturation protein} = \left(\frac{1-\text{soluble protein}}{\text{soluble protein in untreated sample}}\right) \text{x } 100$$

Figures 4.3 showed the comparison of denaturation without maltodextrin between WPI 15% and WPI 40% concentration solid. The results showed α-lactalbumin for 40% showed the highest denaturation, 60.42% compared to 15% WPI, 46.03%. Otherwise, the denaturation of BSA and β-lactoglobulin of 15% WPI concentration solid (10.21% and 24.06%) were little bit higher compared to 40% WPI concentration solid (9.20% and 20.09%). Supposedly, when the concentration solid is increased, the percentage of loss solubility also will be increased (Anandharamakrishnan *et al.*, 2007).

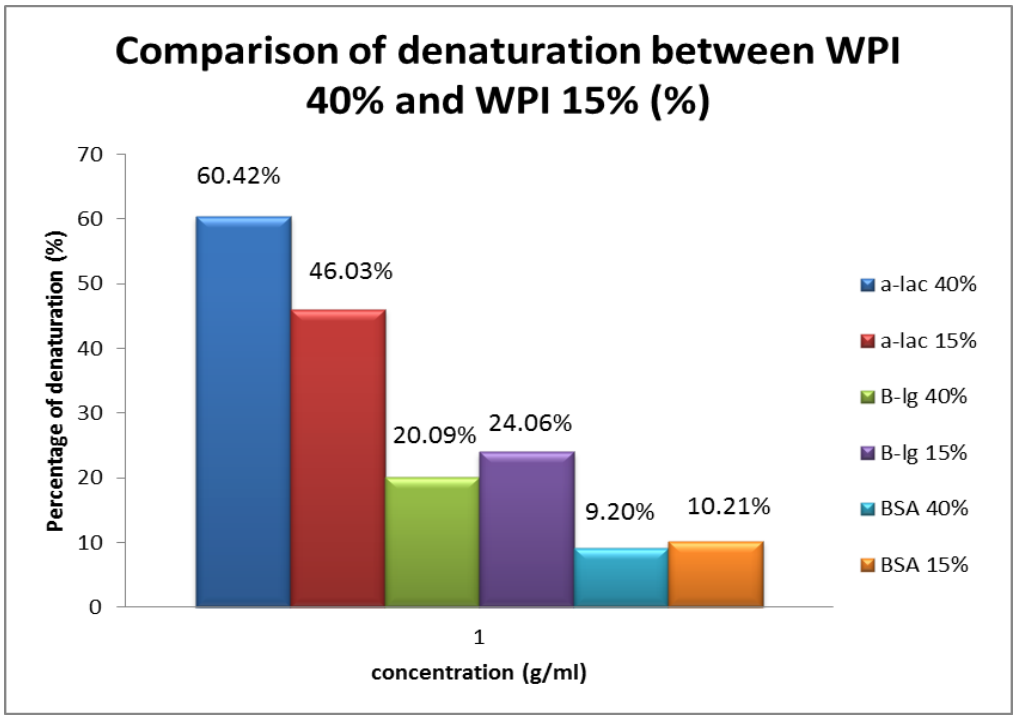

Figure 4.3: Comparison of denaturation between WPI 40% and WPI15%

Figures 4.4 showed the comparison of denaturation with ratio 90% WPI powders to 10% maltodextrin (9:1) between WPI 15% and WPI 40% concentration. The results showed α- lactalbumin for 40% showed the highest denaturation, 31.04% compared to 15% WPI, 25.07%. Otherwise, the denaturation of BSA and β-lactoglobulin of 15% WPI concentration solid (6.64% and 13.74%) were little bit higher compared to 40% WPI concentration solid (1.65% and 1.58%). So, as a conclusion, BSA and β-lactoglobulin were less denatured in WPI 40% compared to WPI 15%.

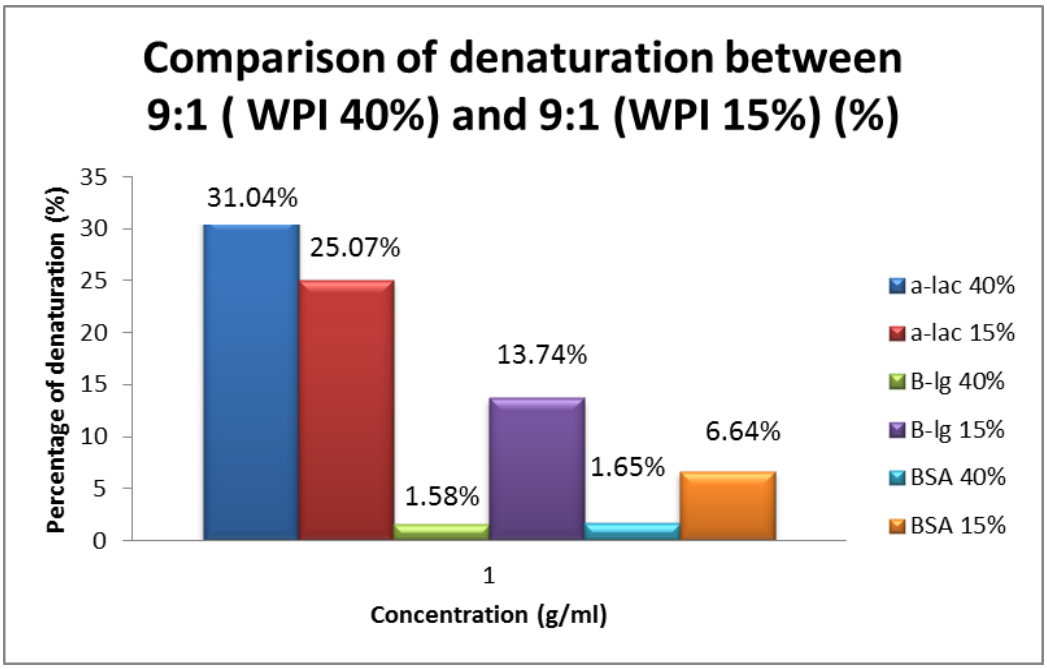

Figure 4.4: Comparison of denaturation between 9:1 (WPI 40%) and 9:1 (WPI15%)

Figure 4.5 showed the comparison of denaturation with ratio 50% WPI powders to 50% maltodextrin (1:1) between WPI 15% and WPI 40% concentration. The results showed α- lactalbumin for 40% showed the highest denaturation, 42.50% compared to 15% WPI, 29.52%. Otherwise, the denaturation of BSA and β-lactoglobulin of 15% WPI concentration solid (7.40% and 15.33%) were little bit higher compared to 40% WPI concentration solid (2.41% and 7.31%). So, BSA and β-lactoglobulin were less denatured in WPI 40% compared to WPI 15%.

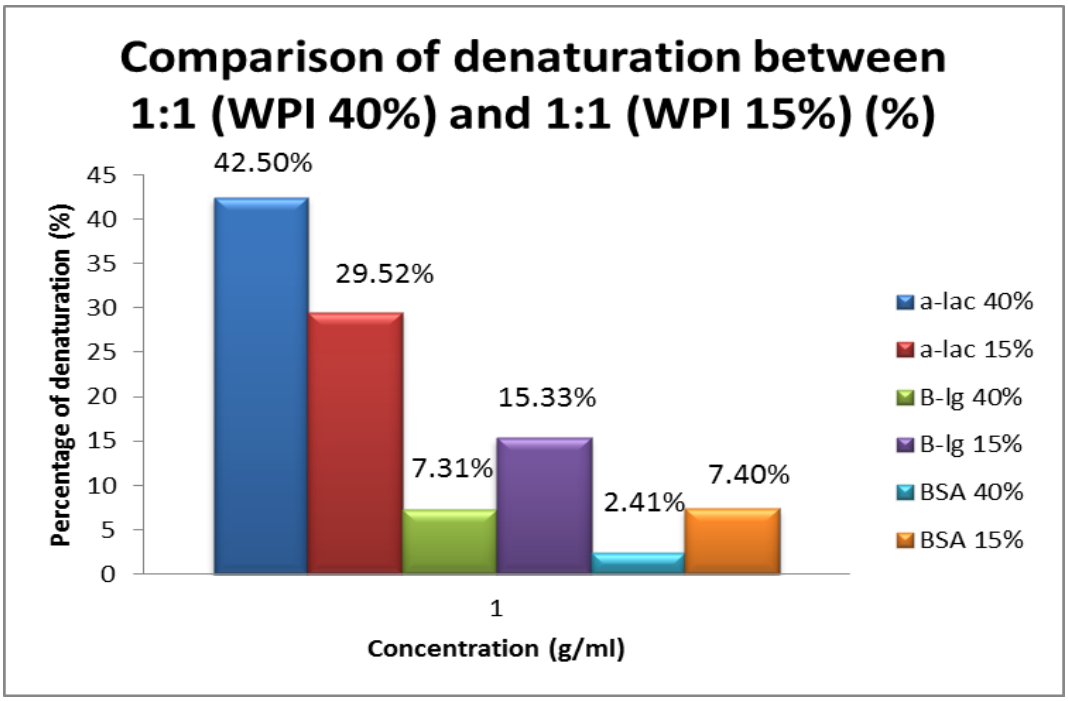

Figure 4.5: Comparison of denaturation between 1:1 (WPI 40%) and 1:1 (WPI15%)

Figures 4.6, 4.7 and 4.8 were discussed about the effects of encapsulation ratio in WPI solutions. From the results obtained, all samples without maltodextrin showed the highest denaturation. Then, followed by applying 50% maltodextrin which gave the second highest of protein denaturation. Lastly, by adding only 10% of maltodextrin, it gave least percentage of denaturation for both concentrations. So, the 90% WPI powder to 10% maltodextrin (9:1) was the best ratio for microencapsulation.

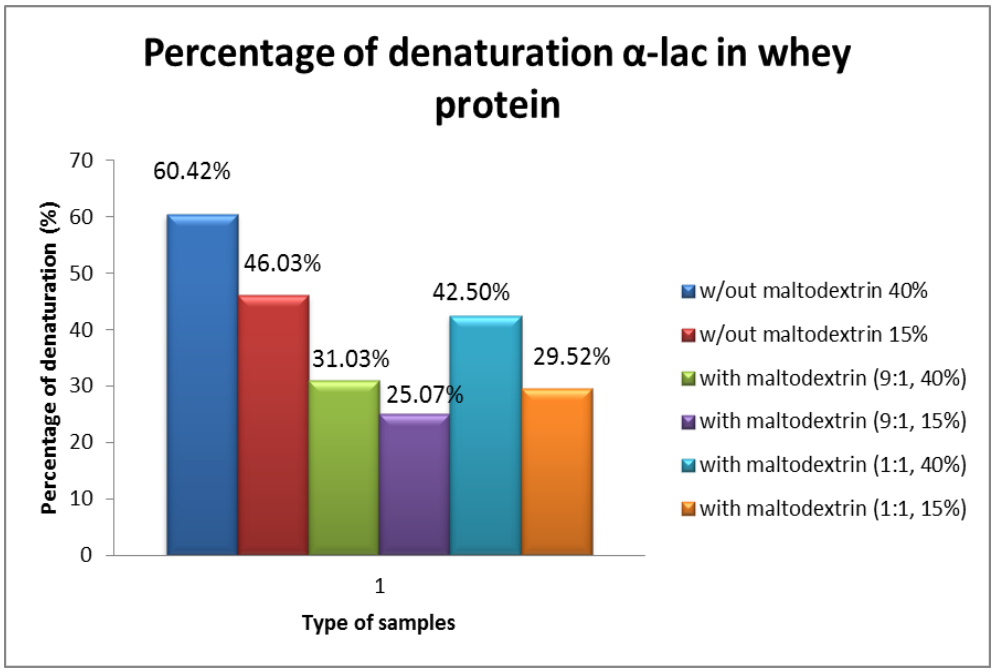

Figure 4.6: Percentage of denaturation α-lac in whey protein

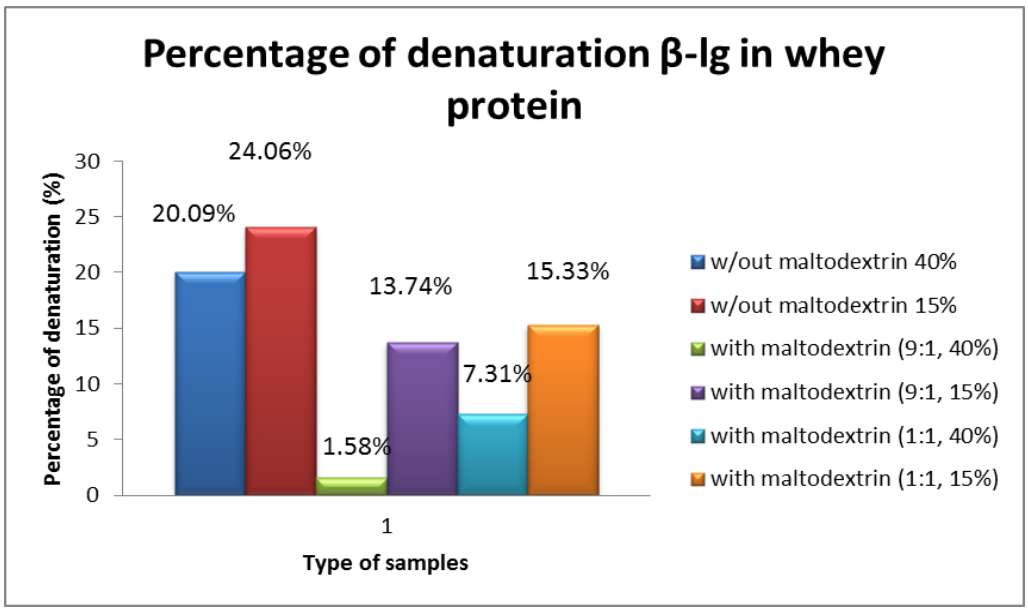

Figure 4.7: Percentage of denaturation β-lg in whey protein

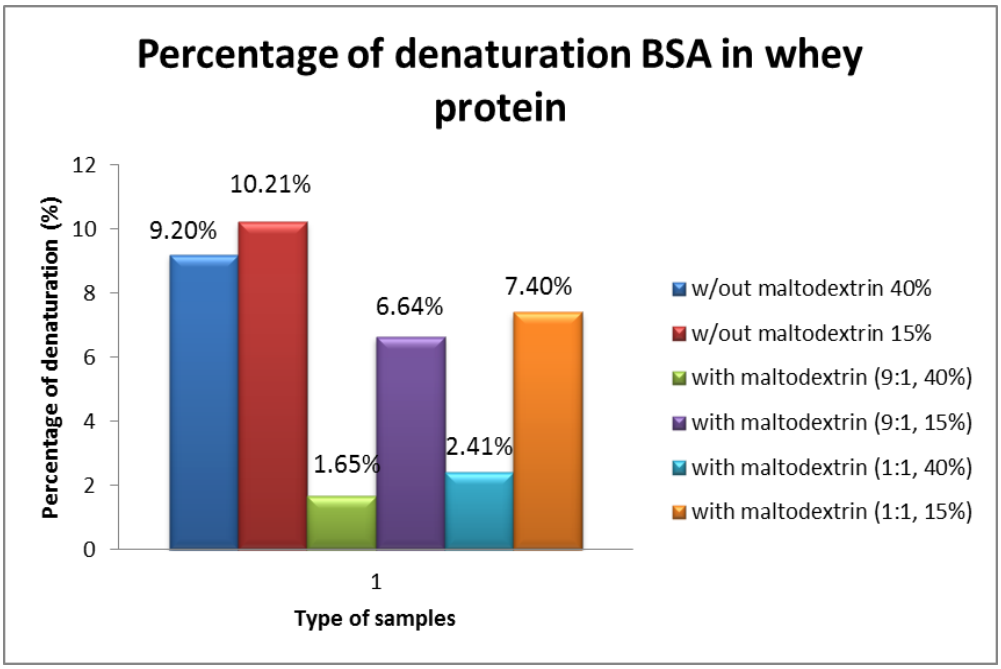

Figure 4.8: Percentage of denaturation BSA in whey protein

As a result, about 21% reductions in protein denaturation for the whey protein isolate powder with microencapsulation for using WPI 15% concentration solid. Otherwise the reduction in protein denaturation with microencapsulation for WPI 40% concentration solid was up to 29%. The WPI 40% concentration solid give the highest percentage reduction of protein denaturation. All the data were shown in figures 4.9 and 4.10.

Table 4.13: Percentage of reduction of denaturation for WPI 15% solid concentration

Sample	**Percentage reduction of denaturation for WPI 15% (%)**		
	α-lac	β-lg	BSA
Without maltodextrin	46.03	24.06	10.21
With maltodextrin (9:1)	20.95	20.53	3.56
With maltodextrin (1:1)	16.51	17.33	2.8

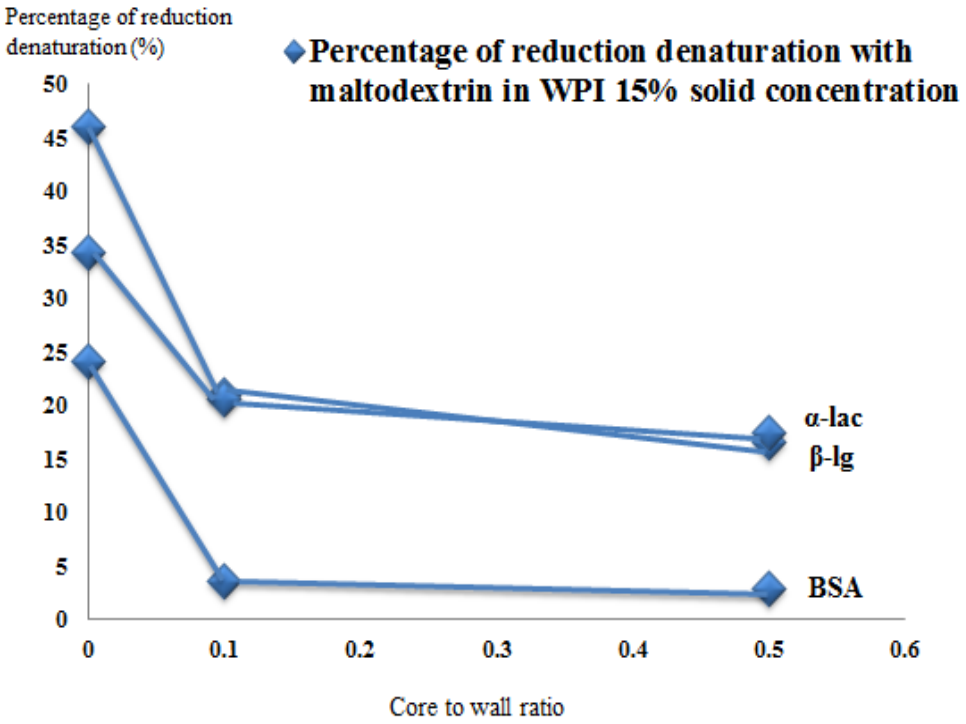

Figure 4.9: Percentage of reduction denaturation with maltodextrin for WPI 15%

Table 4.14: Percentage of reduction of denaturation for WPI 40% (%)

Sample	Percentage of reduction of denaturation for WPI 40% (%)		
	α-lac	β-lg	BSA
Without maltodextrin	60.42	20.09	9.2
With maltodextrin (9:1)	29.39	18.51	6.79
With maltodextrin (1:1)	17.929	12.78	7.54

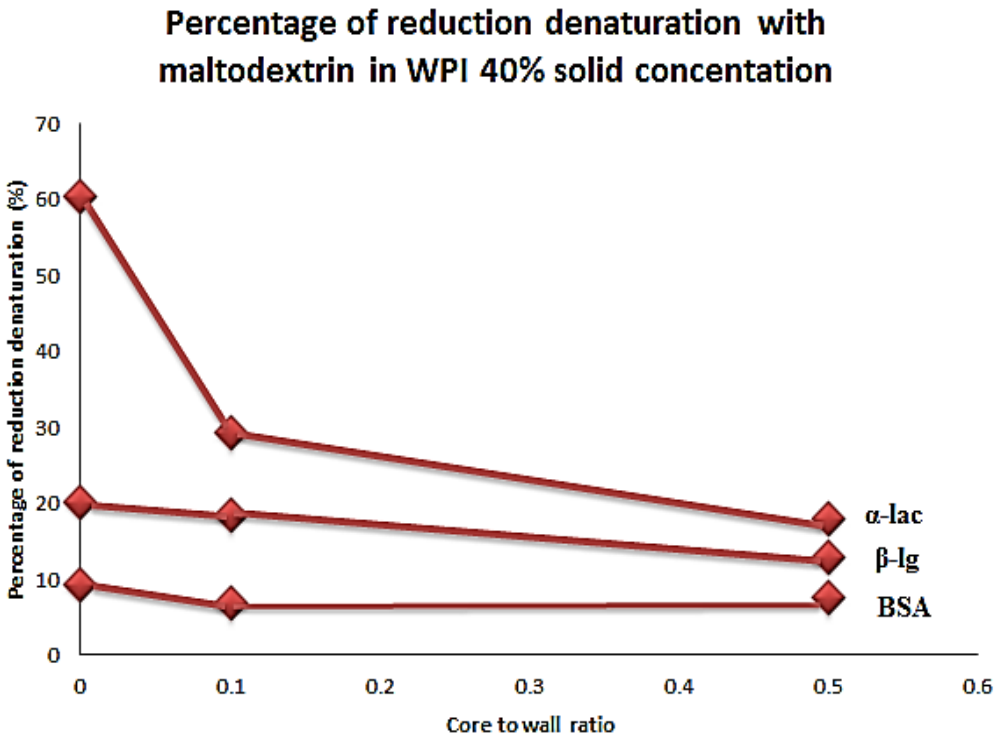

Figure 4.10: Percentage of reduction denaturation with maltodextrin for WPI 40%

4.6 Particle Size Distribution

Particle size at higher inlet temperature is large due to more elastic properties of the encapsulation material which sustain the ballooning effect during droplet drying (Sheu et al., 1998). Particle size distribution were varies from each samples. Based on table 4.15, when increasing the amount of maltodextrin, the particle size distribution will be increased too.

The droplet size of milk powder sample was measured after spray drying. The size distribution profile for without maltodextrin, 9:1 and 1:1 were shown in Figure 4.11, 4.12 and 4.13. .The most predominant diameters was without maltodextrin for whey protein powders 35.113 μm, for 9:1, 59.339 μm and for 1:1, 353.484 μm.

The most severely denatured powder provides bimodal size distribution. The larger powder particle sizes of maltodextrin can be explained by agglomeration or

caking powder particles, which were observed in this sample (Loksuwan, 2007). Agglomeration has reported to be partly related to the presence of a high level of surface free fat (Vega , 2006). The tailing into the larger droplet sizes increased with increasing protein denaturation (Fureby *et al.*, 2001).

The measurement of particle size is dependent on convention involved in particles size definition and upon the physical principle employed in determination process (Herdan, 1990).When different physical principles are used in particle size determination; it cannot be assumed that they should give identical result. So, it recommended that characteristic particle size be selected to be measured according to the property or the process under study.

Table 4.15: Particle size distribution without microencapsulation and with microencapsulation

Samples	Particle size distribution ,d(0.5) µm
without microencapsulation	35.113
with microencapsulation (9:1)	59.339
with microencapsulation (1:1)	353.484

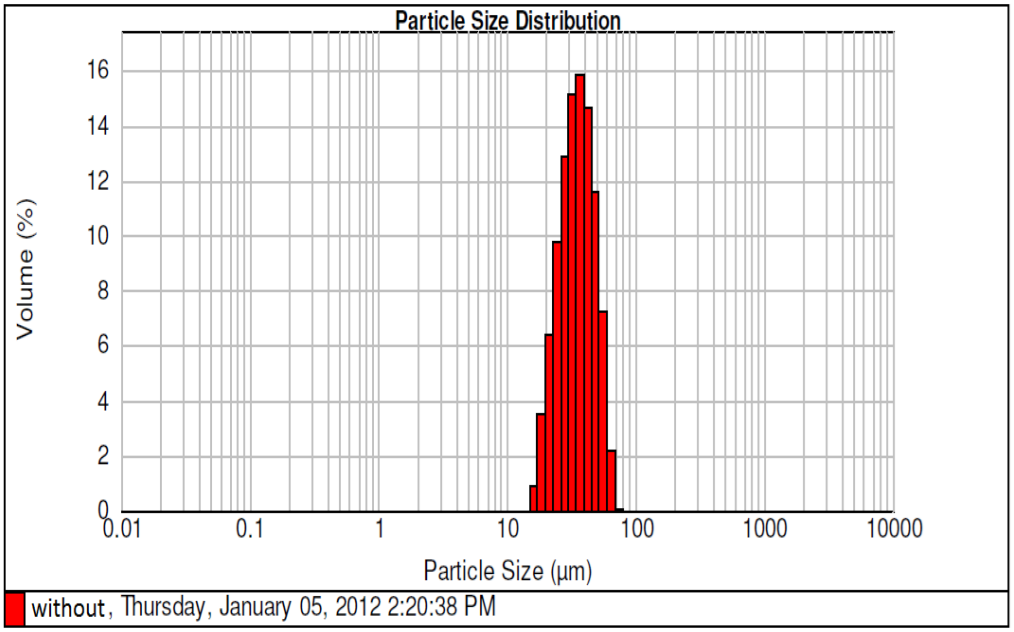

Figure 4.11: Particle size distribution without microencapsultion

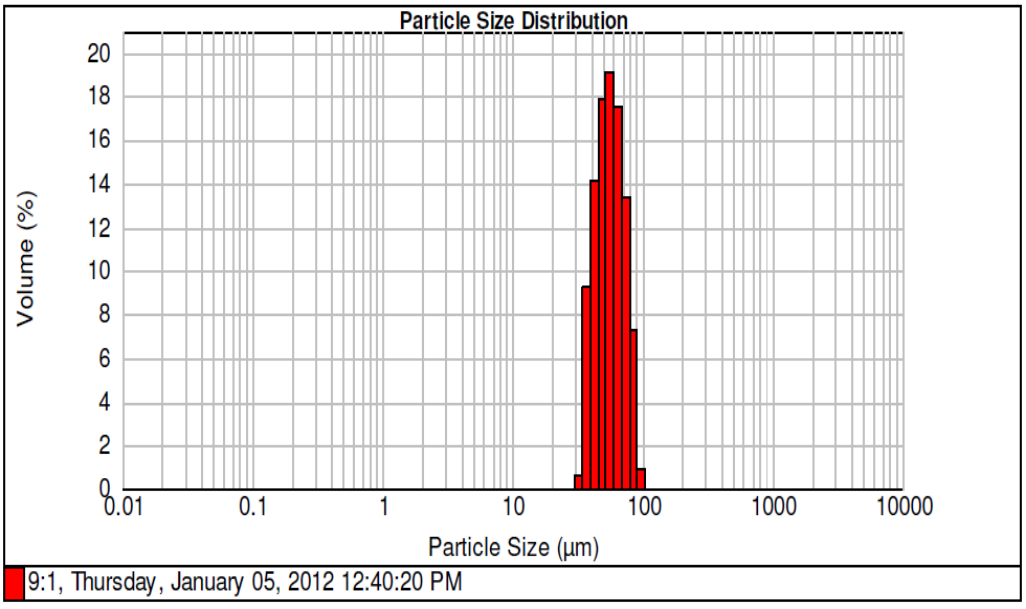

Figure 4.12: Particle size distribution with microencapsulation (9:1)

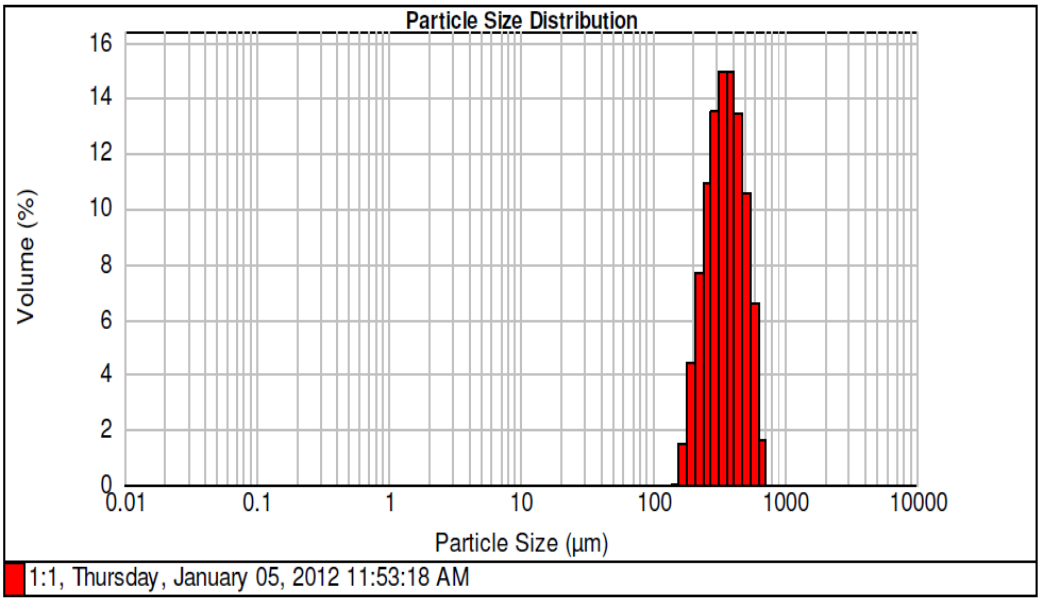

Figure 4.13: Particle size distribution with microencapsulation (1:1)

4.5 Summary

To sum up, this study shows High Performance Liquid Chromatography (HPLC) is a method for determining the protein content in powder milk. It supported by other analysis of Moisture Content Analysis and Particle size distribution. At higher inlet temperature the protein denaturation whey protein isolate for spray dryer process will increase. But by using microencapsulation technique it can significantly reduce the protein denaturation by up to 29% for WPI 40% concentration solid and 21% for WPI 15% concentration solid compared to the ones without microencapsulation.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

To sum up, the study of "Analytical Quantification of Protein Denaturation in Spray Drying" has done successfully. Based on the results obtained, the objectives of this study which are to establish a method for preserving protein content in whey protein isolate (WPI) during operation and also to develop a reliable analytical method for quantifying the protein in whey protein powder were achieved.

Analysis of the protein denaturation during pilot scale spray drying process has demonstrated that at higher operating inlet temperature resulted in more protein denaturation up to 60% at inlet temperature of 180ºC with 40% solid concentration of whey protein isolate (WPI) and about 46% of protein denaturation for 15% solid concentration of whey protein isolate (WPI) at inlet temperature of 190ºC.

The study was showed that microencapsulation technique can significantly reduce the protein denaturation by up to 29% for 40% solid concentration of whey protein isolate (WPI) and 21% for 15% solid concentration of whey protein isolate (WPI) compared to the ones without microencapsulation.

Others, the investigations demonstrated that there are difference in size and composition of whey protein powders by the conditions of spray dried process such as temperature, effect of concentration and microencapsulation that analyze by Malvern Mastersizer. It shows that by increasing the amount of maltodextrin, the size will increase too. This is because of the balloon effect by microencapsulant.

Because of the above conclusion, the researcher can conclude that finding from this work may useful for production of high quality milk powder, due to the economic and safety of microencapsulation process. The described method may have wide application in the routine analysis of sample milk powder produced by dairy industries. Further development of the method to enable analysis of minor basic whey protein component in milk powder is currently ongoing.

5.2 Recommendations

There are some recommendations that researcher had found during the study. Firstly, analysis should be performed as soon as possible to prevent contamination of the product. Then, while doing the analysis, the samples should be placed in desiccators because it can absorb the moisture content in whey powder. So, the samples can remain dry and still in powder form although in long term period of study.

Other than that, this research may useful for dairy products and food industry especially in Malaysia. This is because this work may be useful for production of high quality milk powder by using microencapsulation technique. It can protect the composition of protein from being denatured. In addition, microencapsulation techniques is suitable for nutraceutical and pharmaceutical too because it involves preservation of bioreactive compound.

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APPENDIX

The following items are including in this Appendix.

- Appendix A : Calculation for moisture content
- Appendix B : Calculation of dilution for calibration curve
- Appendix C : Particle Size Distribution

Appendix A: Calculation for moisture content

Calculation for moisture content WPI 15%:

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
WPI Pure	7.218	2	6.7	140
	6.901	2	5.7	140
Average	**7.060**	**2**	**6.2**	**140**

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
SD WPI 15 %	7.494	2	6.3	140
	7.418	2	6.6	140
Average	**7.456**	**2**	**6.45**	**140**

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
9:1	9.707	2	7.5	140
	8.493	2	7.9	140
Average	**9.1**	**2**	**7.7**	**140**

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
1:1	8.628	2	7.6	140
	8.412	2	9.9	140
Average	**8.52**	**2**	**8.75**	**140**

Average of moisture content:

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
WPI Pure	7.06	2	6.20	140
SD WPI 15 %	7.46	2	6.45	140
9:1	9.10	2	7.7	140
1:1	8.52	2	8.75	140

Basis: $\dot{m}_1 = 1mg/ml$ = concentration of WPI Pure
So;
$a = 0.93\ x\ \dot{m}_1$
$a = 0.93\ x\ 1mg/ml$
$a = 0.93\ mg/ml$
$a = 0.93\dot{m}_2$
$\dot{m}_2 = 1\ mg/ml$
$a = 0.9\ x\ 0.91\ x\ \dot{m}_3$
$\dot{m}_3 = 1.1355\ mg/ml$
$a = 0.5\ x\ 0.92\ x\ \dot{m}_4$
$\dot{m}_4 = 2.0217\ mg/ml$

Lastly;

$\dot{m}_1 = 1mg/ml$ = concentration of WPI Pure
$\dot{m}_2 = 1mg/ml$ = concentration of SD WPI 15%
$\dot{m}_3 = 1.1355\ mg/ml$ = concentration of 1:9

$\dot{m}_4 = 2.0217\ mg/ml$ = concentration of 1:1

Suitable concentration for sample preparation

Sample preparation	Concentration of each sample
Preparation of WPI sample	
WPI Pure	**1 mg/ml**
SD WPI 15%	**1 mg/ml**
Preparation of WPI sample + Maltodextrin	
9:1	**1.14 mg/ml**
1:1	**2.02 mg/ml**

Calculation for moisture content WPI 40%:

Average of moisture content:

Sample	Percentage of moisture (%)	Weight (g)	Time to dry (min)	Temperature (°C)
WPI Pure	7.7053	2	6.73	140
SD WPI 15 %	8.613	2	6.80	140
9:1	9.169	2	7.3	140
1:1	7.459	2	7.9	140

Basis: $\dot{m}_1 = 1mg/ml$ = concentration of WPI Pure
So;
$a = 0.92\ x\ \dot{m}_1$
$a = 0.92\ x\ 1mg/ml$
$a = 0.92\ mg/ml$
$a = 0.91\dot{m}_2$
$\dot{m}_2 = 1.011\ mg/ml$

$a = 0.9\ x\ 0.91\ x\ \dot{m}_3$
$\dot{m}_3 = 1.1233\ mg/ml$
$a = 0.5\ x\ 0.93\ x\ \dot{m}_4$
$\dot{m}_4 = 1.9785\ mg/ml$
Lastly;
$\dot{m}_1 = 1mg/ml$ = concentration of WPI Pure
$\dot{m}_2 = 1.011mg/ml$ = concentration of SD WPI 15%
$\dot{m}_3 = 1.1233\ mg/ml$ = concentration of 9:1
$\dot{m}_4 = 1.9785\ mg/ml$ = concentration of 1:1

Suitable concentration for sample preparation

Sample preparation	Concentration of each sample
Preparation of WPI sample	
WPI Pure	**1 mg/ml**
SD WPI 15%	**1.011 mg/ml**
Preparation of WPI sample + maltodextrin	
9:1	**1.12 mg/ml**
1:1	**1.98 mg/ml**

Appendix B: Calculation of dilution for calibration curve

1) Dilution for α-lactalbumin and β-lactoglobulin

   a) 4 mg/ml
   b) 2 mg/ml
   c) 1 mg/ml
   d) 0.8 mg/ml
   e) 0.5 mg/ml
   f) 0.25 mg/ml
   g) 0.125 mg/ml
   h) 0.0625 mg/ml

Then, calculate it by using equation $\mathbf{M_1V_1 = M_2V_2}$

Stock solution = 4 mg/ml

So,

1) For concentration 2 mg/ml;

$$\left(\frac{\mathbf{4mg}}{\mathbf{ml}}\right)(\boldsymbol{V_1}) = (\frac{\mathbf{2mg}}{\mathbf{ml}})(\mathbf{2ml})$$

$$\boldsymbol{V_1} = \mathbf{1\ ml} + \mathbf{1\ ml}\ \text{Of}\ \boldsymbol{H_2O}$$

2) For concentration 1mg/ml;

$$\left(\frac{\mathbf{4mg}}{\mathbf{ml}}\right)(\boldsymbol{V_1}) = (\frac{\mathbf{1mg}}{\mathbf{ml}})(\mathbf{2ml})$$

$$\boldsymbol{V_1} = \mathbf{0.5\ ml} + \mathbf{1.5\ ml}\ \text{Of}\ \boldsymbol{H_2O}$$

3) For concentration 0.8 mg/ml;

$$\left(\frac{4\text{mg}}{\text{ml}}\right)(V_1) = \left(\frac{0.8\text{mg}}{\text{ml}}\right)(2\text{ml})$$

$$V_1 = 0.4\ \text{ml} + 1.6\ \text{ml}\ \text{Of}\ H_2O$$

4) For concentration 0.5 mg/ml;

$$\left(\frac{4\text{mg}}{\text{ml}}\right)(V_1) = \left(\frac{0.5\text{mg}}{\text{ml}}\right)(2\text{ml})$$

$$V_1 = 0.25\ \text{ml} + 1.75\ \text{ml}\ \text{Of}\ H_2O$$

5) For concentration 0.25 mg/ml;

$$\left(\frac{4\text{mg}}{\text{ml}}\right)(V_1) = \left(\frac{0.25\text{mg}}{\text{ml}}\right)(2\text{ml})$$

$$V_1 = 0.125\ \text{ml} + 1.875\ \text{ml}\ \text{Of}\ H_2O$$

6) For concentration 0.125 mg/ml;

$$\left(\frac{4\text{mg}}{\text{ml}}\right)(V_1) = \left(\frac{0.125\text{mg}}{\text{ml}}\right)(2\text{ml})$$

$$V_1 = 0.0625\ \text{ml} + 1.9375\ \text{ml}\ \text{Of}\ H_2O$$

7) For concentration 0.0625 mg/ml;

$$\left(\frac{4\text{mg}}{\text{ml}}\right)(V_1) = \left(\frac{0.0625\text{mg}}{\text{ml}}\right)(2\text{ml})$$

$$V_1 = 0.0313\ \text{ml} + 1.9687\ \text{ml}\ \text{Of}\ H_2O$$

2) Dilution for Bovine Serum Albumin (BSA)

1. 2 mg/ml
2. 1 mg/ml
3. 0.8 mg/ml
4. 0.5 mg/ml
5. 0.25 mg/ml
6. 0.125 mg/ml

Then, calculate it by using equation $\mathbf{M_1V_1 = M_2V_2}$

Stock solution = 2 mg/ml

So,

1) For concentration 1 mg/ml;

$$\left(\frac{\mathbf{2mg}}{\mathbf{ml}}\right)(\boldsymbol{V_1}) = (\frac{\mathbf{1mg}}{\mathbf{ml}})(\mathbf{2ml})$$

$$\boldsymbol{V_1} = \mathbf{1\ ml} + \mathbf{1\ ml}\ \text{Of}\ \boldsymbol{H_2O}$$

2) For concentration 0.8 mg/ml;

$$\left(\frac{\mathbf{2mg}}{\mathbf{ml}}\right)(\boldsymbol{V_1}) = (\frac{\mathbf{0.8\ mg}}{\mathbf{ml}})(\mathbf{2ml})$$

$$\boldsymbol{V_1} = \mathbf{0.8\ ml} + \mathbf{1.2\ ml}\ \text{Of}\ \boldsymbol{H_2O}$$

3) For concentration 0.5 mg/ml;

$$\left(\frac{\mathbf{2mg}}{\mathbf{ml}}\right)(\boldsymbol{V_1}) = (\frac{\mathbf{0.5mg}}{\mathbf{ml}})(\mathbf{2ml})$$

$$\boldsymbol{V_1} = \mathbf{0.5\ ml} + \mathbf{1.5\ ml}\ \text{Of}\ \boldsymbol{H_2O}$$

4) For concentration 0.25 mg/ml;

$$\left(\frac{2\text{mg}}{\text{ml}}\right)(V_1) = (\frac{0.25\text{mg}}{\text{ml}})(2\text{ml})$$

$$V_1 = 0.25\ \text{ml} + 1.75\ \text{ml}\ \text{Of}\ H_2O$$

5) For concentration 0.125 mg/ml;

$$\left(\frac{2\text{mg}}{\text{ml}}\right)(V_1) = (\frac{0.125\text{mg}}{\text{ml}})(2\text{ml})$$

$$V_1 = 0.125\ \text{ml} + 1.875\ \text{ml}\ \text{Of}\ H_2O$$

$$V_1 = 0.0313\ \text{ml} + 1.9687\ \text{ml}\ \text{Of}\ H_2O$$

LC Calibration Report

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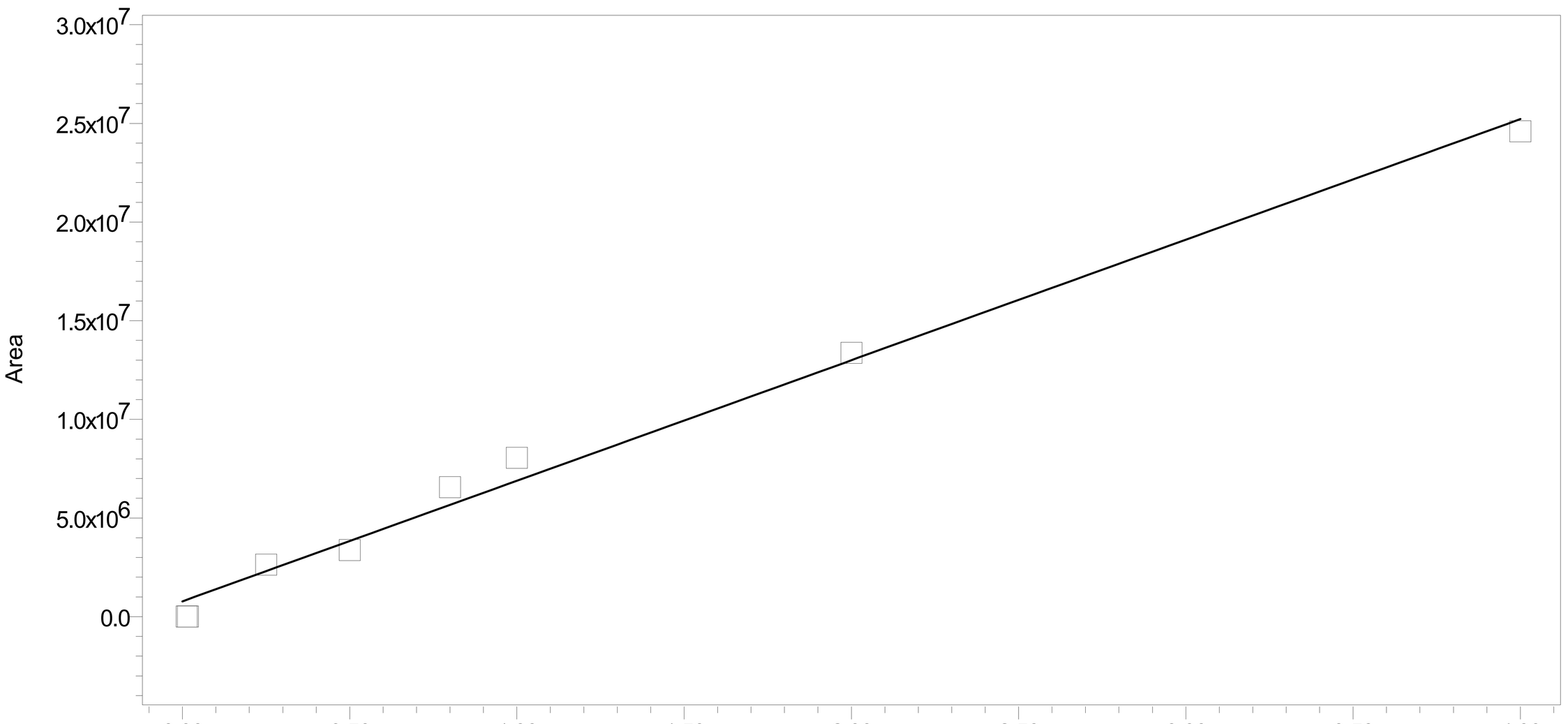

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Peak: a-lac

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1	a-lac 0.01265 2	2563	a-lac		0.013	12933.456	-0.125	-1086.16	No
2	a-lac 0.01625 1	2564	a-lac		0.016	12293.724	-0.125	-868.29	No

Peak: a-lac

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual
3	a-lac-0.25	2565	a-lac		0.250	2635899.785	0.305	21.82	No
4	a-lac - 0.5	2566	a-lac		0.500	3374651.137	0.425	-14.91	No
5	a-lac- 0.8	2567	a-lac		0.800	6559655.026	0.947	18.34	No
6	a-lac-1	2568	a-lac		1.000	8052639.470	1.191	19.11	No
7	a-lac-2	2569	a-lac		2.000	13372738.343	2.062	3.09	No
8	a-lac-4	2570	a-lac		4.000	24597385.230	3.899	-2.53	No

Peak: a-lac

	Ignore
1	No
2	No
3	No
4	No
5	No
6	No
7	No
8	No

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Calibration Plot group contains no data.

LC Calibration Report

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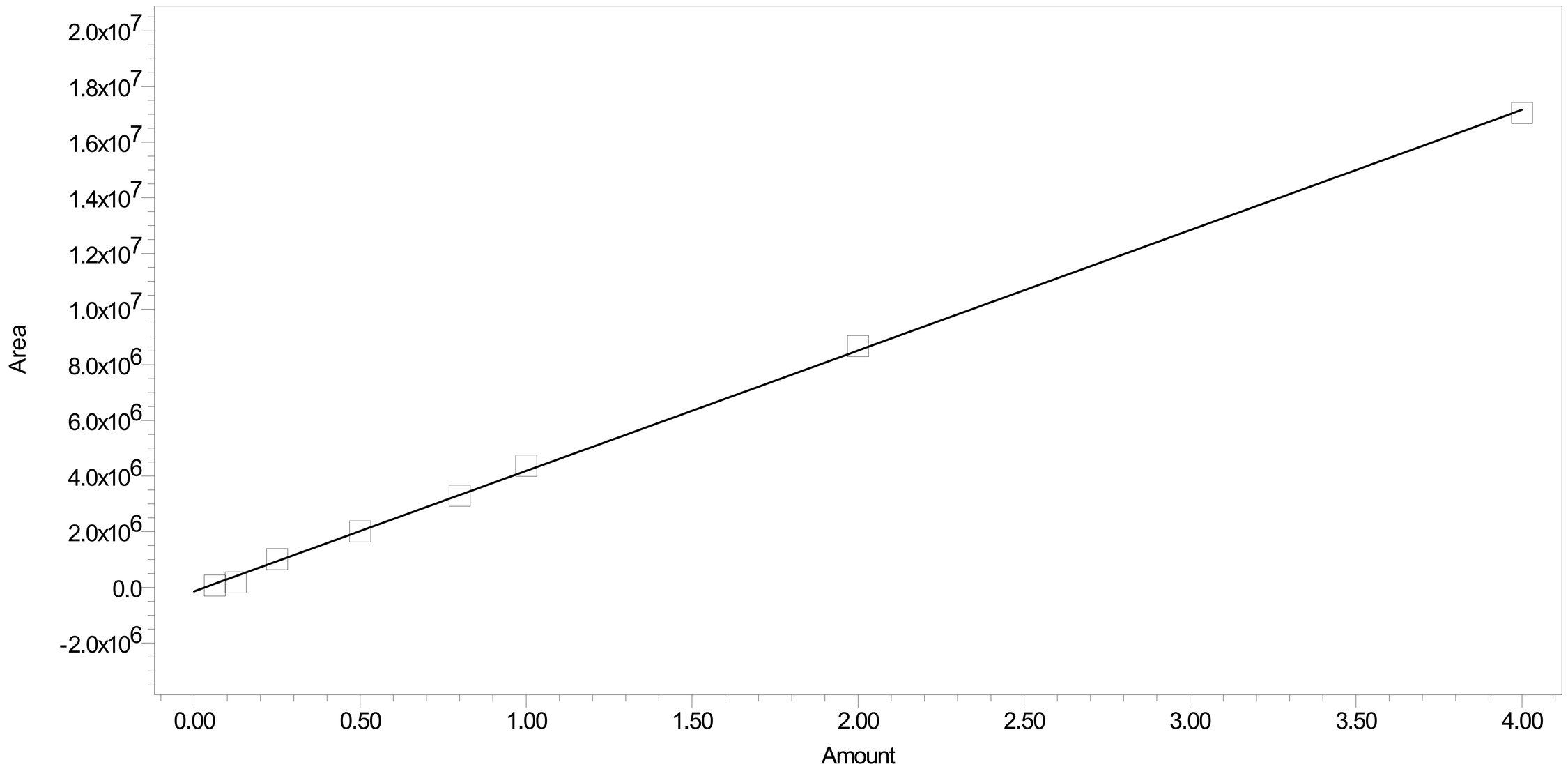

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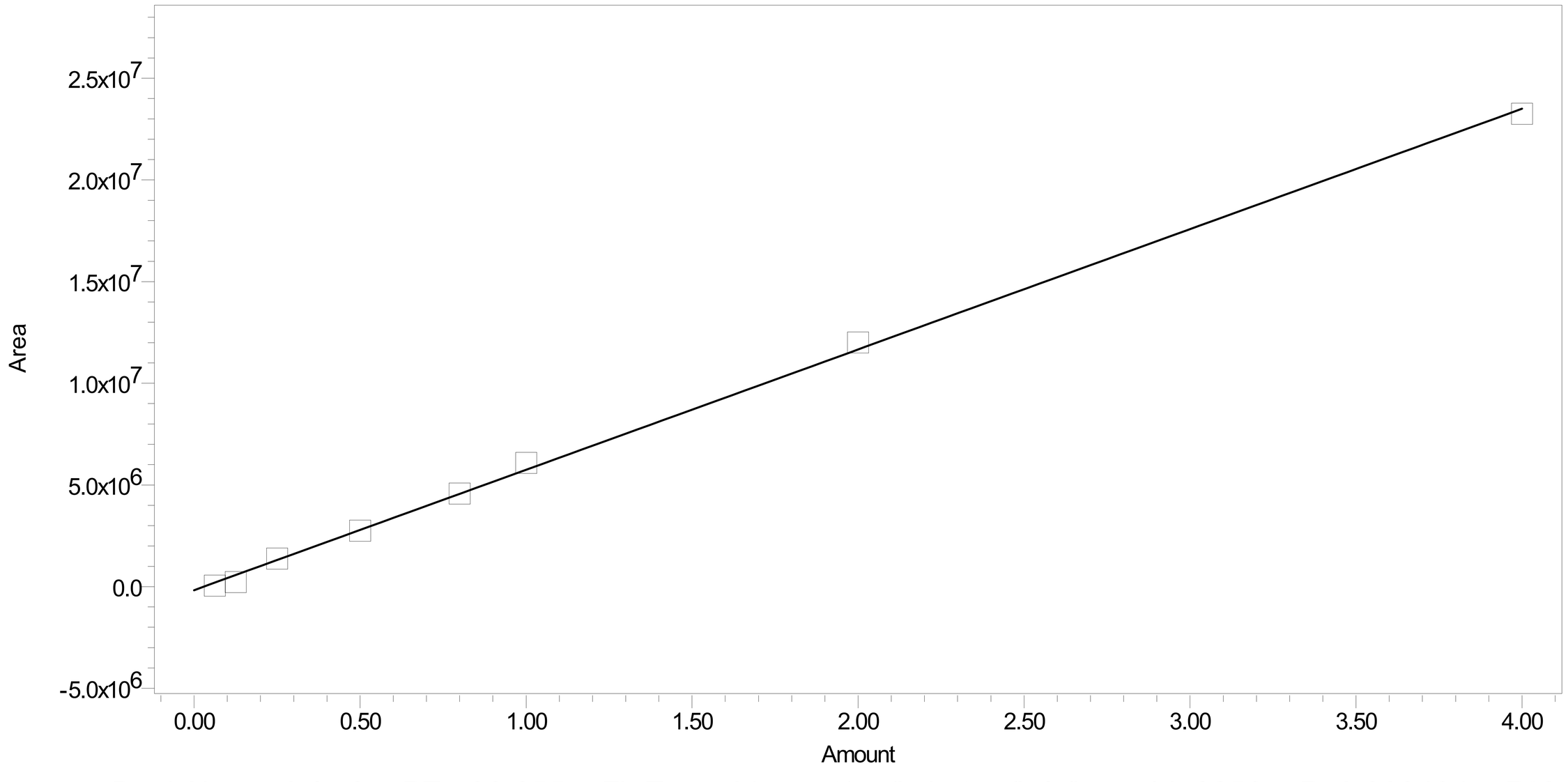
Peak: b-lg 1

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual
1	b-lg0.0625	2288	b-lg 1		0.063	65880.267	0.048	-23.20	No
2	b-lg0.125	2289	b-lg 1		0.125	184533.621	0.075	-39.66	No
3	b-lg-0.25	2290	b-lg 1		0.250	1016683.516	0.268	7.10	No
4	b-lg-0.5	2291	b-lg 1		0.500	2012701.687	0.498	-0.41	No
5	b-lg-0.8	2292	b-lg 1		0.800	3295982.426	0.795	-0.68	No
6	b-lg-1	2293	b-lg 1		1.000	4374851.835	1.044	4.39	No
7	b-lg-2	2294	b-lg 1		2.000	8674645.401	2.038	1.89	No
8	b-lg 4	2295	b-lg 1		4.000	17044425.615	3.972	-0.70	No

Peak: b-lg 1

	Ignore
1	No
2	No
3	No
4	No
5	No
6	No
7	No
8	No



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Peak: b-lg 2

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual
1	b-lg0.0625	2288	b-lg 2		0.063	46524.858	0.037	-40.72	No
2	b-lg0.125	2289	b-lg 2		0.125	224019.646	0.067	-46.37	No
3	b-lg-0.25	2290	b-lg 2		0.250	1376342.254	0.262	4.70	No

Peak: b-lg 2

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual
4	b-lg-0.5	2291	b-lg 2		0.500	2755396.089	0.495	-1.05	No
5	b-lg-0.8	2292	b-lg 2		0.800	4581475.208	0.803	0.41	No
6	b-lg-1	2293	b-lg 2		1.000	6086800.289	1.058	5.77	No
7	b-lg-2	2294	b-lg 2		2.000	12005961.800	2.058	2.89	No
8	b-lg 4	2295	b-lg 2		4.000	23252906.320	3.958	-1.05	No

Peak: b-lg 2

	Ignore
1	No
2	No
3	No
4	No
5	No
6	No
7	No
8	No

LC Calibration Report

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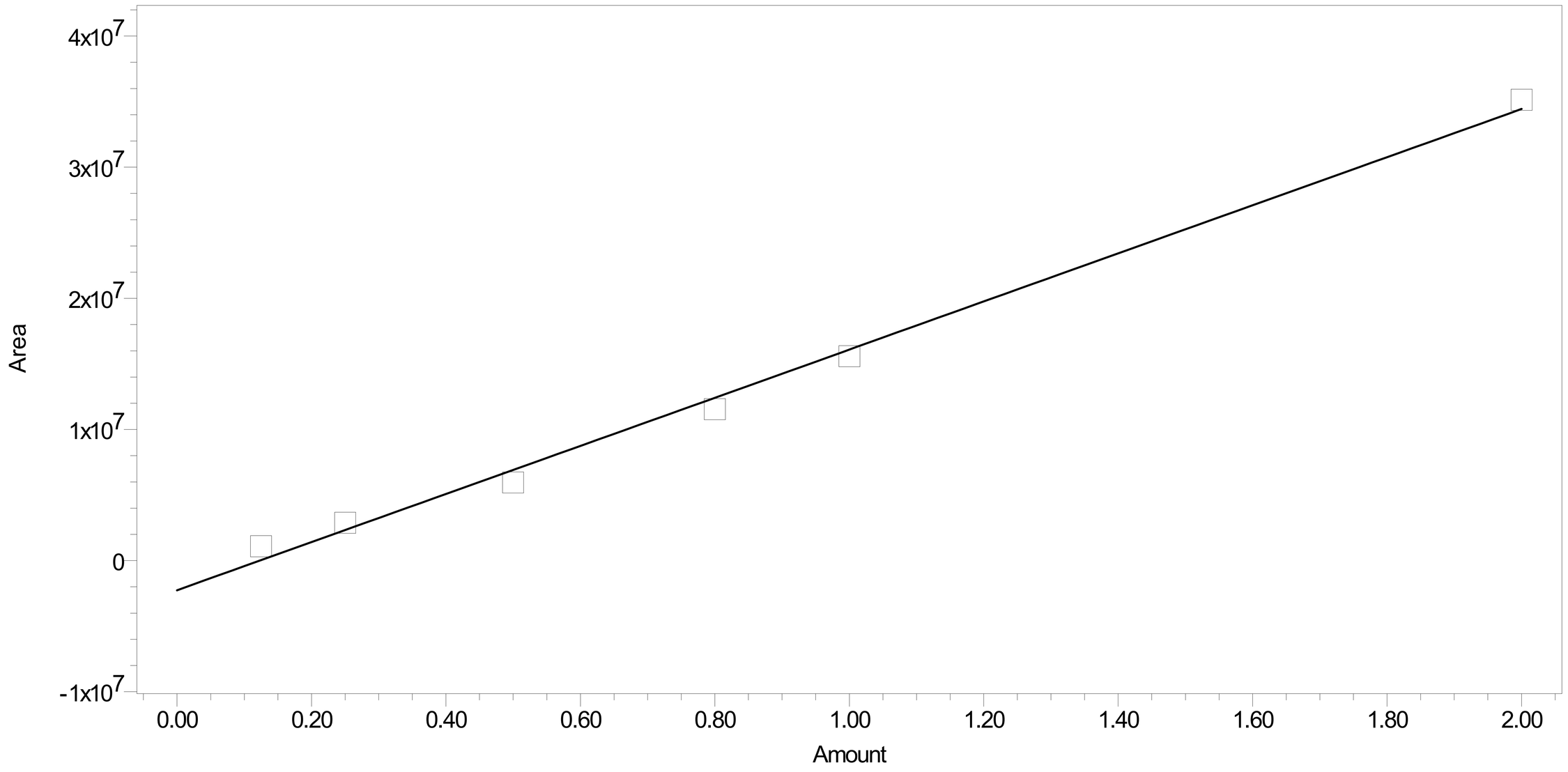
Calibration ID:	1962	Proc. Chnl. Descr.:	PDA Ch1 205nm@4.8nm
Date Calibrated:	12/26/2011 4:55:25 PM CST		



Peak Name: bsa2; RT: 13.836; Fit Type: Linear (1st Order); Cal Curve Id: 1963; R: 0.997531; R^2: 0.995068; Weighting: None; Equation: Y = 1.83e+007 X - 2.26e+006; Normalized Intercept/Slope: -0.115815; RSD(E): 8.181846

Peak: bsa2

	Sample Name	Result Id	Peak Name	Level	X Value	Response	Calc. Value	% Deviation	Manual
1	bsa 0.125	1982	bsa2		0.125	1101806.621	0.183	46.48	No
2	bsa 0.25	1983	bsa2		0.250	2893564.025	0.281	12.30	No
3	bsa 0.5	1984	bsa2		0.500	5960192.422	0.448	-10.42	No
4	bsa 0.8	1985	bsa2		0.800	11546341.069	0.752	-5.96	No
5	bsa 1	1986	bsa2		1.000	15588853.193	0.973	-2.74	No
6	bsa 2	1987	bsa2		2.000	35142627.129	2.038	1.92	No

Peak: bsa2

	Ignore
1	No
2	No
3	No
4	No
5	No
6	No

MASTERSIZER 2000

Result: Sieve BS 410 : 1986 / (ISO 565 : 1990) Report

Sample Name:
wpi 15%

SOP Name:
whey1

Measured:
Thursday, January 05, 2012 2:20:38 PM

Sample Source & type:
Factory = Paris

Measured by:
Malvern

Analysed:
Thursday, January 05, 2012 2:20:40 PM

Sample bulk lot ref:
123-ABC

Result Source:
Measurement

Particle Name:
milk

Accessory Name:
Scirocco 2000 (A)

Analysis model:
General purpose

Sensitivity:
Enhanced

Particle RI:
1.500

Absorption:
0

Size range:
0.020 to 2000.000 um

Obscuration:
0.00 %

Dispersant Name:

Dispersant RI:
1.000

Weighted Residual:
34.939 %

Result Emulation:
Off

Concentration:
0.0000 %Vol

Span :
0.839

Uniformity:
0.26

Result units:
Volume

Specific Surface Area:
0.182 m²/g

Surface Weighted Mean D[3,2]:
32.991 um

Vol. Weighted Mean D[4,3]:
36.369 um

Density:
1.000 g/cm³

d(0.1): 22.608 um **d(0.5):** 35.113 um **d(0.9):** 52.078 um

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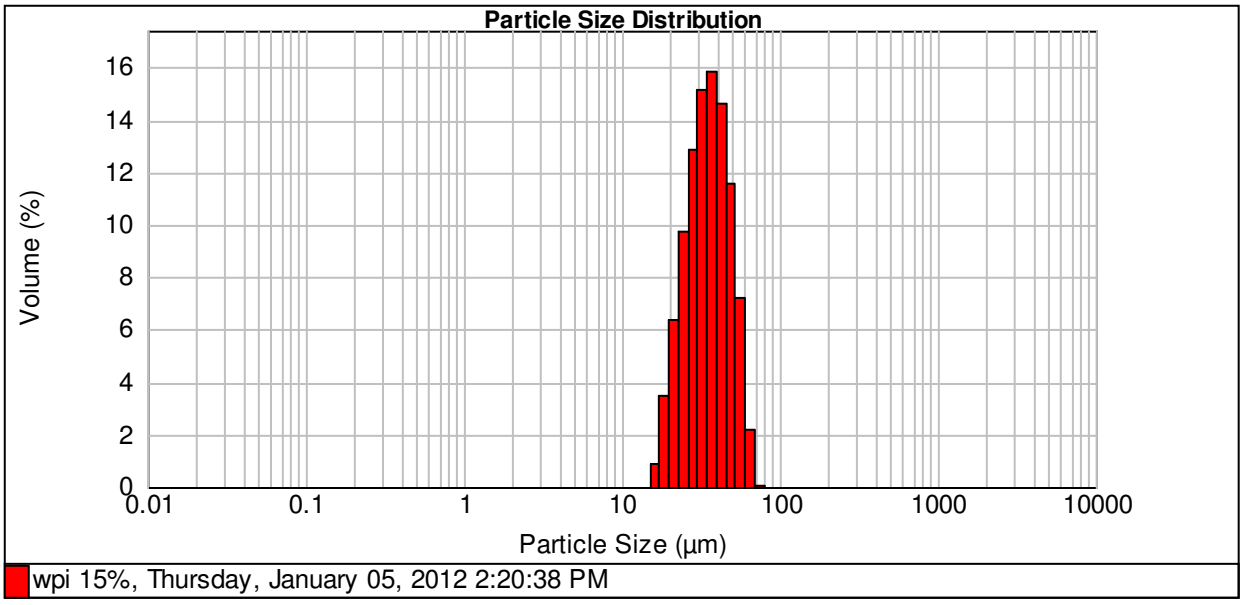
Mesh No	Aperture µm	Volume In %	Vol Below %
10	2000	0.00	100.00
12	1700	0.00	100.00
14	1400	0.00	100.00
16	1180	0.00	100.00
18	1000	0.00	100.00
20	850	0.00	100.00
25	710	0.00	100.00
30	600	0.00	100.00
35	500		100.00

Mesh No	Aperture µm	Volume In %	Vol Below %
35	500	0.00	100.00
40	425	0.00	100.00
45	355	0.00	100.00
50	300	0.00	100.00
60	250	0.00	100.00
70	212	0.00	100.00
80	180	0.00	100.00
100	150	0.00	100.00
120	125		100.00

Mesh No	Aperture µm	Volume In %	Vol Below %
120	125	0.00	100.00
140	106	0.00	100.00
170	90	0.00	100.00
200	75	1.01	100.00
230	63	7.76	98.99
270	53	13.76	91.23
325	45	18.37	77.46
400	38		59.09



Operator notes:

MASTERSIZER 2000

Result: Sieve BS 410 : 1986 / (ISO 565 : 1990) Report

Sample Name: 9:1
SOP Name: whey1
Measured: Thursday, January 05, 2012 12:46:21 PM
Sample Source & type: Factory = Paris
Measured by: Malvern
Analysed: Thursday, January 05, 2012 12:46:25 PM
Sample bulk lot ref: 123-ABC
Result Source: Measurement

Particle Name: milk
Accessory Name: Scirocco 2000 (A)
Analysis model: General purpose
Sensitivity: Enhanced
Particle RI: 1.500
Absorption: 0
Size range: 0.020 to 2000.000 um
Obscuration: 0.00 %
Dispersant Name:
Dispersant RI: 1.000
Weighted Residual: 33.049 %
Result Emulation: Off

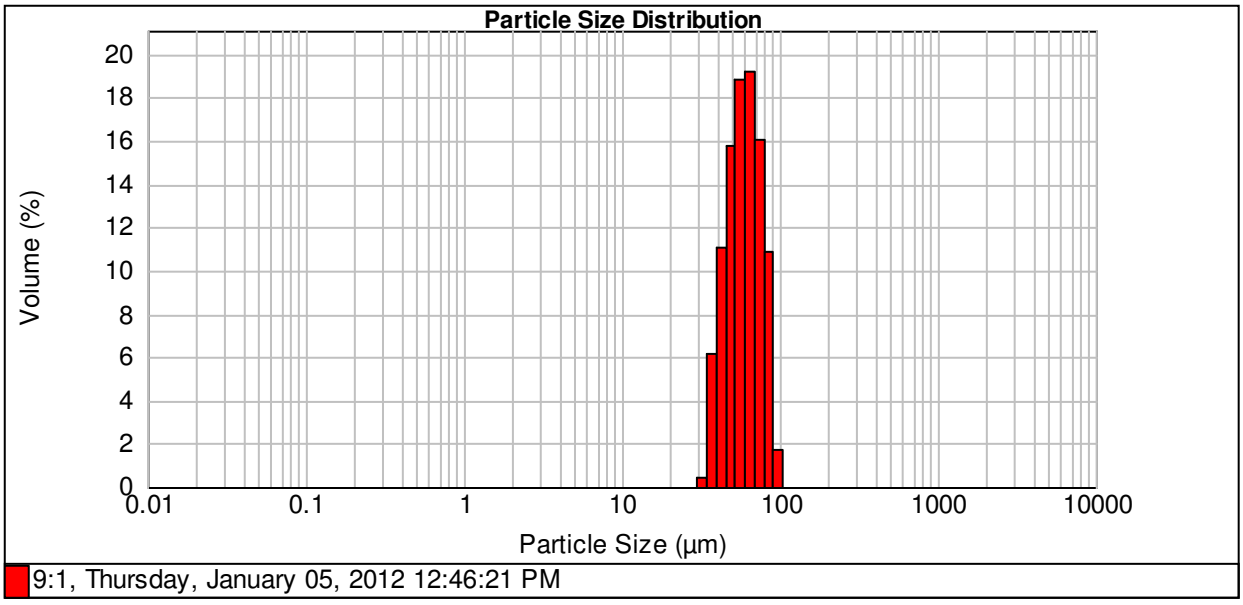
Concentration: 0.0000 %Vol
Span : 0.670
Uniformity: 0.208
Result units: Volume
Specific Surface Area: 0.105 m²/g
Surface Weighted Mean D[3,2]: 57.073 um
Vol. Weighted Mean D[4,3]: 60.648 um
Density: 1.000 g/cm³

d(0.1): 41.867 um **d(0.5):** 59.339 um **d(0.9):** 81.599 um

Mesh No	Aperture µm	Volume In %	Vol Below %
10	2000	0.00	100.00
12	1700	0.00	100.00
14	1400	0.00	100.00
16	1180	0.00	100.00
18	1000	0.00	100.00
20	850	0.00	100.00
25	710	0.00	100.00
30	600	0.00	100.00
35	500		100.00

Mesh No	Aperture µm	Volume In %	Vol Below %
35	500	0.00	100.00
40	425	0.00	100.00
45	355	0.00	100.00
50	300	0.00	100.00
60	250	0.00	100.00
70	212	0.00	100.00
80	180	0.00	100.00
100	150	0.00	100.00
120	125		100.00

Mesh No	Aperture µm	Volume In %	Vol Below %
120	125	0.00	100.00
140	106	2.38	100.00
170	90	16.35	97.62
200	75	22.81	81.27
230	63	23.86	58.46
270	53	18.55	34.61
325	45	12.25	16.06
400	38		3.81



Operator notes:

MASTERSIZER 2000

Result: Sieve BS 410 : 1986 / (ISO 565 : 1990) Report

Sample Name:
1:1

SOP Name:
whey1

Measured:
Thursday, January 05, 2012 11:53:18 AM

Sample Source & type:
Factory = Paris

Measured by:
Malvern

Analysed:
Thursday, January 05, 2012 11:53:19 AM

Sample bulk lot ref:
123-ABC

Result Source:
Measurement

Particle Name:
milk

Accessory Name:
Scirocco 2000 (A)

Analysis model:
General purpose

Sensitivity:
Enhanced

Particle RI:
1.500

Absorption:
0

Size range:
0.020 to 2000.000 um

Obscuration:
0.00 %

Dispersant Name:

Dispersant RI:
1.000

Weighted Residual:
54.800 %

Result Emulation:
Off

Concentration:
0.0000 %Vol

Span :
0.872

Uniformity:
0.267

Result units:
Volume

Specific Surface Area:
0.018 m²/g

Surface Weighted Mean D[3,2]:
332.418 um

Vol. Weighted Mean D[4,3]:
368.302 um

Density:
1.000 g/cm³

d(0.1): 226.221 um **d(0.5):** 353.484 um **d(0.9):** 534.507 um

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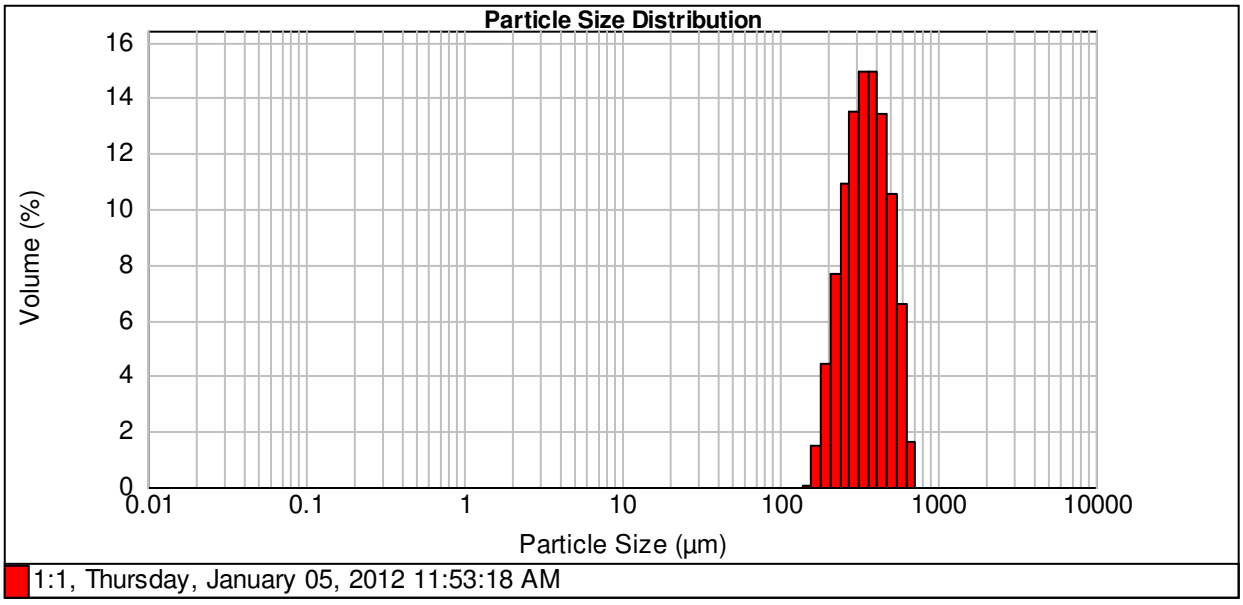
Mesh No	Aperture µm	Volume In %	Vol Below %
10	2000	0.00	100.00
12	1700	0.00	100.00
14	1400	0.00	100.00
16	1180	0.00	100.00
18	1000	0.00	100.00
20	850	0.00	100.00
25	710	3.50	100.00
30	600	11.48	96.50
35	500		85.01

Mesh No	Aperture µm	Volume In %	Vol Below %
35	500	15.16	85.01
40	425	19.39	69.85
45	355	17.81	50.47
50	300	16.05	32.66
60	250	9.99	16.61
70	212	5.26	6.63
80	180	1.37	1.37
100	150	0.00	0.00
120	125		0.00

Mesh No	Aperture µm	Volume In %	Vol Below %
120	125	0.00	0.00
140	106	0.00	0.00
170	90	0.00	0.00
200	75	0.00	0.00
230	63	0.00	0.00
270	53	0.00	0.00
325	45	0.00	0.00
400	38		0.00



Operator notes: