THE RECOVERY OF FUNCTIONAL PROTEIN FROM FISH WASTE

by

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A Doctoral thesis submitted in
partial fulfilment of the
requirements for the award of
Doctor of Philosophy
of the Loughborough University
of Technology.

October 1979.

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Summary

This research project is concerned with the recovery of "Functional Fish Protein" from fish filleting waste by an extraction method utilising sodium chloride solutions.

It was found that the greatest amount of extracted protein could be obtained after one hour of extraction time using 4% NaCl as a solvent. The optimum solid to liquid ratio was 1:8 and the best particle size was found to be 0.04 cm. diameter. The Silverson homogenizer was found to give a higher protein yield than other extraction apparatuses which utilise orbital or rotary motions.

It was found that minced fish waste samples stored at $-30^{\circ}C$ for 60 days did not result in a noticeable deterioration in protein extractability values. Those values decreased by about 27% and 60% after 70 days storage at $-15^{\circ}C$ and $0^{\circ}C$ respectively.

The protein-saline extract was desalted and concentrated by means of an ultrafiltration process. The effect of protein concentration, temperature and pH of the feed stream on the flux rate were studied. It was found that the flux rate; i) decreased as protein concentration rose; ii) increased with increasing temperature of solution; iii) did not change at basic pH, but decreased at acidic pH.

The functional properties of the freeze-dried product were assessed. The characteristics of solubility, coagulation, foam and emulsification showed that "Fish Protein Concentrate" was suitable for use as a functional agent in food products.

Acknowledgments

I wish to express my gratitude to the Iraqi Ministry for Higher Education and Scientific research who offered me the financial support to do this research project.

I would like to express my sincere thanks to my supervisor Professor J. Mann for his helpful, encouragement and invaluable suggestions during this project.

I wish to express my great thanks to my external supervisor Dr. M. N. Clifford previously of the Grimsby College of Technology, and presently of the University of Surrey for his advice, help and guidance throughout this work. His help in obtaining fish samples, too, is appreciated.

Thanks are due to Professor D. C. Freshwater, Head of the Department of Chemical Engineering for extending the use of the departmental facilities.

My thanks are also due to Dr. J. D. Selman for his advice in writing up this manuscript.

Thanks are due to the technical staff in Department of Chemical Engineering and in the Grimsby College of Technology for their cooperation.

Thanks are due to Professor R. A. Lawrie of the Department of Applied Biochemistry and Nutrition, University of Nottingham (Sutton Bonington), for allowing me to conduct some experiments at his department. Thanks are due also to Mr. Philip Glover of the same Department for drying fish protein extract, and to Mr. David Bozon of the same Department for analysing the amino acid content of FPC.

I wish to express my special thanks to my parents for their sacrifices and encouragement and to my wife for her patience and understanding and encouragement throughout the period of this work.

I would like to thank Mrs. C. Sharpe for typing this thesis.

Contents

List of Figures

List of Tables

Chapter One

The Recovery of Functional Protein From Fish Waste

Chapter One

Introduction

Most proteins obtained from animal sources traditionally were utilized to meet the nutritional needs of man and also for their contribution in up grading the nutritional value of foods of plant origin. For example, fish protein powders have been incorporated with wheat flour to improve the nutritive value of bread (Ismail et al., 1968; Hale, 1972; Howell, 1978).

At the present time, proteins are also in demand not only for their nutritional capabilities, but also more importantly, in economic terms, for their functional properties.

The functional capacity may be defined as the ability of protein to perform physical functions in a food system. For example, protein solubility (in beverages) can be important, as can the ability to emulsify (in sausages and luncheon meat formulations) (Kinsella, 1977).

The by-products and fish wastes (FW) from fish processing plants, which are useless for direct human consumption and are usually converted into fish meal or fertilizer, can be more efficiently utilized to produce functional protein, which is

valuable for direct application in many food industries.

One such product is fish protein concentrate (FPC). This is defined as that product which contains fish protein in a more concentrated and stable form than natural fish and is suitable for human consumption (Finch, 1977). The common methods which are employed for processing fish and fish wastes into FPC depend on using organic solvents such as 2-propanol to remove fat and water simultaneously, leaving a residual high protein material which is then steam-stripped and dried to remove the residual solvent. This material is then ground to the desired particle size to yield a greyish powder (FPC), which is ideally odourless and tasteless. The product made in this way has high nutritional value, but lacks many functional properties (Spinelli et al., 1971). It is known however that native proteins possess potential functional capacity, but during the different stages of processing, especially when concentrated acids or bases, high temperature and organic solvents are employed, protein denaturation takes place (Hermansson, 1973; Kinsella, 1977). Protein denaturation in this context refers to the loss of solubility over a range of pH and ionic strength. This has been taken by many workers as a simple and practical method to measure protein functionality (Kinsella, 1976), but clearly it is not the only means by which denaturation could be indirectly measured.

It would therefore be expected that by using mild extraction conditions for the recovery of fish protein from fish waste proteins would be preserved in their native state as much as possible, and thus could avoid considerable loss in protein functionality.

In this investigation,an aqueous saline solution has been used as the solvent material.

Sodium chloride was chosen as a salt for the extraction process because of the well known ability of dissolved salts to solubilize muscle proteins (King et al., 1974). Although a variety of salts (KCl and NaCl) have been used for many years in the biological investigations of fish and mammal muscle, they have never been employed in pilot scale extraction of muscle proteins.

The functional properties and the approximate price of some protein sources which are commonly used as functional agents are shown in Table 1.1 as follows:

Material	% Protein	£/tonne	Binding capacity	Emulsification capacity	Water absorption
Soya flour	50%	250-350	Poor	Fairly good	Good
Caseinate	≈100%	800-1000	Poor	Good	Fair
Soya concentrate	60-70%	450-600	Fairly good	Good	Good
Blood plasma	≈100%	1800-2000	V. good	V. good	Fair
Soya isolate	. 90%	1000-1300	Good	Good	V.good
Egg albumin	≈100%	2500-3000	V. good	V. good	Fair

Table 1.1

The functional properties and the approximate prices of some protein sources. (The data were kindly supplied by Dr. Cordell of Unilever Ltd.).

If the suggested processing method can recover fish protein that is able to perform two or three of the above-mentioned functional tasks described in Table 1.1, the product would be likely to be sold at value ranging between £1000 - 3000 per tonne. This means that the product would have a great economic value and therefore would be more able to support the expected additional processing costs.

The production method envisaged consists of the following steps:

i) Extracting the soluble protein from minced fish waste by using mild saline solution as a solvent, leaving the remaining fish constituent in insoluble form. This can then be removed by means of a centrifuge. The factors which affect the extraction process will be further explained in the following chapters.

ii) The soluble protein-saline extract can then be concentrated and desalted by means of a membrane separation process, in this case the ultrafiltration technique, which both increases protein concentration and reduces the salt content, whilst not subjecting the solution to high temperatures.

The effect of protein concentration, pH and temperature of fish protein extract on the flux rate and the performance of the concentration process, have been studied.

iii) The drying of the product is envisaged by any mild form of drying such as spray drying or by using the freeze-drying technique.

The ultimate aim of this research project was to elaborate procedures necessary for the recovery of functional FPC, which will maximise the retention of protein functionality, in the course of which it was necessary to evaluate the functionality of the product.

Chapter Two

Chapter Two

Literature Survey:

2.1 Historical Background

The idea of processing fish protein concentrate (FPC) for human consumption is not new. It was possibly the Romans who produced FPC, since a vase unearthed in the ruins of Pompeii, Italy, has the inscription *"Liquamen optimum siccatum ex officino umbrici agathopi"*. This means, the best liquamen (fermented and dried fish products) in the world are from the factories of Agathopus (Pariser, 1967).

A Roman cookery book of the first century A.D. has described in detail the utilization of otherwise useless and unsaleable fish for FPC manufacture.

The processing method comprises:

i) salting the fish;
ii) leaving it to dry in the sun with frequent shaking of the mixture;
iii) removing the remaining liquid by means of a fine meshed basket.

This product can be considered as a prototype FPC. The Roman recognized that this food was of economic and dietary importance and of therapeutic value.

The art of making FPC was lost in post - Roman times. Probably because of the development of agriculture, and the increasing consumption of meat, and preference for fish in its natural form, such processing of fish was displaced from economic activity.

The fish industry was of great importance to Asian peoples. It has been established that the oldest known form of money in India and Ceylon was the fish hook. It was also known that the manufacture of FPC from fish, in the form of fish sauces and pastes was developed very early all over the far east.

However, neither Europe nor Asia saw much development in fish processing until the introduction of canning by Appert in 1810 in France, some 2000 years later (Kreuzer, 1974).

The development of fish meal and fish flour production occurred because of the development in animal husbandry (Chapman, 1967). However, since the second world war, there have been great efforts by FAO and UNICEF of the

United Nations, towards utilizing the fish for direct human consumption where aesthetically and economically feasible, rather than converting to fish flour for poultry and animal feed (Heen and Kreuzer, 1962).

The term FPC was adopted by FAO in 1961 in preference to earlier name "Fish Flour" to avoid confusion with cereal flours which it resembles in general physical form but not in chemical properties (Anon, 1962).

Only in the last twenty years has FPC had full recognition and world wide serious interest, as a valuable source of high quality protein.

Numerous processes for the manufacture of FPC have been described in a comprehensive bibliography (Library of congress - 1970).

Patents in the United Kingdom, Canada, USA and other countries have been granted for many of these processes which ranged from simple methods of solvent extraction of fish meal to more sophisticated methods.

2.2

Recovery of Protein from Unconventional Sources

In order to help meet the demand for food for both nutritional and functional purposes, man had to search for new sources of protein, in the main protein from unconventional sources. Some of these sources were considered as waste materials, animal feeding stuffs and fertilizers.

The waste materials are mainly derived from agricultural sources and food industry by-products, such as those from meat, poultry and fish processing plants, leaf protein, single cell protein (SCP), dairy waste and oil seed residues (Young et al., 1976; Rolfe, 1976).

Biochemical engineering techniques have been applied in processing the new proteins, such as in the use of fermentation to produce SCP, or the use of ultrafiltration in recovery of protein from food factory effluents (Lacey, 1972; Humphrey, 1974).

In addition to the functional and nutritional rich protein materials which can be obtained from the wastes, the utilization of such materials can help in preserving the environment and in avoidance of pollution.

Some of the unconventional sources of proteins are as described below:

2.2.1. Protein from Meat, Poultry and Fish Processing Plants

Effluents from meat, poultry and fish processing plants contain large amounts of protein and fat and usually have high BOD values. Such effluents are highly polluting and can impose heavy loads on public sewerage treatment works.

A process has been developed by Grant (1976) which allows the separate recovery of fat and protein from effluents, accompanied by a large reduction in BOD value. The protein recovery consisted of two stages, the first stage being a flocculation pretreatment whereby a proportion of soluble protein is rendered insoluble and is recovered by air flotation or sedimentation, followed by dewatering and drying. The second stage consisted of extraction of residual soluble protein from the effluent by passage through a bed of ion exchange resin. If the effluent contained a large amount of fat, this is recovered prior to protein recovery by air flotation.

The recovered protein was evaluated nutrionally by amino acid analysis and by feeding trials to pigs and poultry which resulted in good growth rates without evidence of toxic effects.

Thus it was possible by such treatment to produce a colourless effluent with a reduction of 90% of the original BOD value.

Another method for recovery of fish waste has been developed by Mackie (1974), whereby the fish wastes, mainly cod, are solubilized by proteolytic enzymes. This process described in section 2.4.2 can help in recovering a soluble fish protein as well as minimizing the wastes in the fish processing plants.

2.2.2. Single Cell Proteins

Single cell proteins (SCP) in a general term for protein produced through utilization or fermentation of organic materials (such as hydrocarbons, including petroleum derivatives, cellulose, starch and sugar) by organisms such as bacteria, yeast and fungi (Tannenbaum and Wang, 1975).

The production of SCP from wastes of agricultural industries has received attention in recent years with two main objectives in view:

i) upgrading the feed value of solid wastes;
ii) reducing the BOD from liquid wastes, with a bonus in the form of high protein animal supplement (Imrie & Righelato, 1976).

Any organism used for production of SCP from waste and/or organic materials must have a number of special properties:

i) it must be capable of growth on a wide range of carbon sources, preferably simultaneously and have high growth rates to minimise the size of the fermentation system;

ii) it must have a high efficiency of conversion of the substance to biomass

The organisms that grow on wastes in nature may fulfil these conditions and could be used for SCP production (Imrie and Righelato, 1976). Popular organisms for food protein application are yeasts, mainly Saccharomyces and Candida species, which have been used in food for a long time as vitamin rich additives and flavouring agents.

However, bacteria have some advantages over other organisms, particularly for animal feed, because they grow more rapidly and have higher protein contents with higher sulphur - containing amino acid contents, than other single cell proteins (Tannenbaum, 1977). Until recently the higher fungi have received little attention in industrial scale projects but the possibility exists for successful growth in continuous culture on very inexpensive waste carbohydrate sources. Fungi may be of considerable economic interest due to their inherent enzymic capacities and their ease of harvesting (Young et al., 1976).

The nutritional value of SCP sources for human consumption has been reviewed by Waslein (1975). In summary, the protein nutritional value and digestibility of SCP is good and could be made to meet the total protein need of adults. However, there are some contra-indications for using SCP for human consumption:

i) high RNA content;
ii) the possible presence of toxic materials such as aromatic compounds which are found in SCP processed from petroleum origin (Young et al., 1976).

The first point may be overcome by additonal processing to reduce the RNA to acceptable levels. Whereas the toxic compounds can be removed by purification processes prior to the fermentation itself, but costs are raised (Young R.H., 1975). The use of SCP for human consumption has been reviewed by Scrimshaw (1975), who stated that a variety of safe palatable, nutritious and economically feasible SCP can be developed for human consumption.

SCP have a potential for use as a functional agents where nutritive value is of secondary value (Rha, 1975). Preliminary tests showed that SCP has a potential as a surface active agent. It was also found that the functional properties of the dried yeast such as solubility, performance in bread dough and baking quality are quite inferior compared

to those of more conventional protein sources such as milk solids.

2.2.3. Leaf Protein Concentrates (LPC)

The leaves of plants contain useful quantities of protein for supplying the requirements of protein for both monogastric animals and humans. The green leaves are the primary, and the largest, producers of protein in the world, supplying protein to other plant tissues including the crop seeds which nourish humans and animals (Young et al., 1976).

The factor limiting the use of LPC for human consumption is the high levels of structural materials such as celluloses, hemicelluloses and lignins, which are largely indigestible by man (Pirie, 1971). The processing of LPC has seen no radical innovations since its inception, and there are no valid patents covering any essential part of the extraction process. However, the various methods depend upon the extraction of juice from the leaf, followed by protein recovery in several categories of purity as follows:

i) whole green leaf protein;
ii) decolourised whole green leaf protein;
iii) protein fractionated to yield a white fraction and green fraction.

iv) protein fractionated to yield pure crystalline protein plus other protein fractions (Pirie, 1971).

The nutritional value of LPC has been found to be well balanced and generally to contain adequate levels of essential amino acids, with the limiting amino acid being methionine in some products (Bickoff et al., 1975).

LPC prepared from alfalfa leaves was shown to possess functional properties. The solubility value of 23% (Nitrogen solubilized) was obtained with water adjusted to pH 7.3 (Betschart, 1974). This value is affected by concentration of LPC, ionic strength and pH of solutions. LPC had an emulsifying capacity comparable to meat and soya proteins (Wang & Kinsella, 1976), and also formed gel upon heating in the presence of calcium ions at pH 12 (Lu and Kinsella, 1972).

2.2.4. Oil-Seed Proteins

Oil seeds traditionally have been utilized as a source of edible oil, but now they are also offering considerable potentials as a source of protein for nutritional purposes (Richard-Jansen, 1972) and as functional agents in processed food (Kinsella, 1976).

Oil seeds have several features which make them desirable

for human food-protein production. When the oil and crude fibre is removed, the content of protein is approximately 50% by weight and is of a quality which is generally superior to that of cereal protein. However, soy bean is low in sulphur - containing amino acids, and cottonseed, peanut and sesame flour are deficient mainly in lysine (Young et al., 1976). Cereal proteins are often low in lysine and in some instances they lack methionine, tryptophan, and occasionally threonine (Anglemier & Montgomery, 1976). This indicates that oil seed proteins and soy protein, in particular, can be used effectively in combination with most cereal grains to improve the overall quality of total protein intake, Thus, combinations of soy protein, high in lysine, with a cereal containing adequate S-amino acids results in a complementation of the two protein sources and an increase in the protein quality of the mixture above that for either protein alone. (Young et al., 1976).

The net protein utilization (NPU) is defined as a percentage of dietary protein which is converted into body protein (Gaman & Sherrington, 1977).

Table 2.1 shows the NPU value of some protein sources.

No	Protein type	NPU
1	Egg protein	100
2	Mother milk protein	100
3	Fish protein	83
4	Meat protein	80
5	Soya bean protein	56
6	Maize protein	55
7	Wheat (white flour) protein	52
8	Ground nut protein	48
9	Yeast	59

Values 1 - 8 were taken from King et al., (1972), value 9 was taken from Tannenbaum (1977).

Table 2.1 - The NPU of some protein sources.

Soy proteins preformed the following functional properties:

i) Soya protein has traditionally been added to wheat flour to aid maturation and bleaching. In addition, small amounts of soy proteins are being increasingly added to bakery products for several claimed functions although soy protein has also been known to be deleterious in some applications (Wolf, 1970).

ii) A dispersion of soy bean globulins formed a gel as demonstrated by Circle & Smith (1972), who stated that gel formation is affected by protein concentration, duration and temperature of heating, and pH.

iii) Soy protein preparations have been recommended by food processors as suitable, if not superior, replacements for the salt-soluble myofibrillar meat proteins in emulsified meat products such as frankfuters, bologna, sausages and salami (Rakosky, 1970; Wolf, 1970; Smith & Circle, 1972).

iv) Whipping or foaming agents have prepared from soy proteins by peptic hydrolysis. These hydrolysates were used in confections, particularly creams, nougats, kisses and fudge and also used by the baking industry for icings and meringues (Burnett & Gunther, 1949; Sair & Rathman, 1950).

v) Soy proteins have also showed the ability to coagulate and form soy-curd, which is essentially calcium coagulated protein gels, and the chemical basis of their formation has been reviewed by Watanabe et al., (1974).

The other oil seed proteins such as cotton seed, sunflower and coconut have also showed the following functional properties:

i) Carefully prepared oil seeds (coconut, sunflower and cotton seed) were soluble in different salt solutions. The solubility profile of each protein is affected by type of protein, concentration of salt solution and pH (Mattil, 1971).

ii) The effects of various oil seed flours on bread quality have been studied by Matthews et al., (1970) and Rooney et al., (1972) who found that the heated cotton seed, peanut, sunflower and sesame seed protein (at 17%) were less deleterious than the non heated proteins, though the bread crumb was very coarse. Generally, at replacement levels above 10% protein, loaf volume is decreased and crumb grain, colour and texture are inferior.

iii) Oil seed proteins are being used as foaming agents in whipped toppings and frozen desserts. They are also used in confections, such as nougats and marshmallows, to partially replace egg whites in cake formations, and to impart certain desirable characteristics to cake and cookie icings (Johnson, 1970; Wolf, 1970).

2.3. The Functional Properties of Proteins:

The term"functional properties" has different meaning for different workers, depending on their interests. Generally for the food technologist, the functional properties of proteins refer to the overall physico-chemical properties of the protein exhibited in food system, and reflects the interactions that are influenced by protein composition, (structural conformation), intermolecular association(s) of protein with other food ingredients (water, carbohydrate and lipid), and the environment in which these properties are measured (Kinsella, 1977).

2.3.1. Classification of the Functional Properties:

Kinsella (1976) broadly categorized the functional properties of protein which are important in food applications as shown in Table 2.2.

General Property	Specific functional term*
Organoleptic	Color, flavor,odor,texture,mouth-feel
Kinesthetic	smoothness,grittiness,turbidity,etc.
Hydration	Solubility,dispersibility,wettability, water absorption,swelling,thickening, gelling,rheological,water holding capacity, syneresis,viscosity,dough formation, etc.

Surface	Emulsification, foaming, aeration, whipping, protein/lipid film formation, lipid binding, flavor binding, stabilization, etc.
Structural	Elasticity, grittiness, cohesion, chewiness,
Textural	viscosity, adhesion, network cross-binding,
Rheological	aggregation, stickiness, gelation, dough formation, texturizability, fiber formation,
Other	Compatability with additives, enzymatic, inertness, modification properties

* These functions vary with pH, temperature, protein concentration, protein species or source prior treatement, ionic strength, and dielectric constant of the medium. They are also affected by other treatments, i.e. mac romolecules (carbohydrates, lipids) in the medium, and by processing treatment i.e. modification, etc.

Table 2.2. General Classes of Functional Properties of Proteins Important in Food Applications.

Some protein sources are utilized for their unique functional properties, for example, the foaming capacity of egg-white in cake, the visco-elastic properties of wheat glutein in bread and the curdling of milk in cheese processing. However, different protein sources showed several additional functional capacities in food and these must be determined and manipulated to enhance their use in food systems (Anglemier & Montgomery, 1976; Howell, 1978). Wolf (1970) and Johnson (1970) catalogued the range of functional properties which are important in several products. For example, solubility and viscosity are desired in beverages.

compatability with gluten and dough formation is desired in bread, and several functional properties such as emulsification, water holding capacity and gelation are required in sausage and meat products.

The functional properties of FPC which were examined in this project are defined below as follows:

i) Hydration (solubility, swelling, rehydration), which reflects the ability of protein to absorb water (Paul & Palmer 1972).

ii) Surface-active capacities (emulsifying and foaming), the emulsifying capacity being the volume of oil (ml.) that can be emulsified by protein (g) before phase inversion or collapse of emulsion occurs. Emulsion stability refers to the ability of a protein to form an emulsion that remains unchanged for a particular duration, under specific conditions (Kinsella, 1976). The foaming capacity is the ability of protein to form a film at the gas-liquid surface, the film strength depending upon the magnitude of protein-protein interaction and protein-water interaction (Hermansson & Skjoldebrand, 1971).

iii) Heat coagulation which is defined as the ability of a protein suspension to form a coagulum upon heating through the thermal denaturation which causes unfolding of protein chains and the formation of a network, as new linkages

between hydrogen bonds and other reactive groups present on proteins occurs (Jensen et al., 1950).

iv) Dough formation, which is confined to cereal proteins, specifically wheat gluten (and rye and barley, to a lesser extent). Kneading of wheat proteins with water causes hydration of the poly-peptides folded in the aleurone granules and permits their partial uncoiling (particularly the glutenins), and facilitates extensive intra-and inter-molecular association of these polypeptides via labile disulfide bonds, hydrogen bonding and hydrophobic associations, with the resultant formation of gluten dough (Kinsella, 1976).

2.3.2. Protein Structure and Functional Properties

The conformation of protein molecules is an important factor determining the functional properties in food systems since the native conformation of globular protein in which the polar amino acids are exposed to the aqueous phase, favours solubility. This protein conformation predisposes the molecules to be useful as emulsifying or foaming agents, while an unfolded elongated conformation is more indicative in a protein of ability to gel, to stabilise foams, and to be desirable in fibre spinning. The ability of protein to change conformation under certain conditions is an important character in functional applications (Franks, 1975). The contention that the primary structure of proteins (amino acid sequence) dictates the conformation of a

protein, and the possibility that the tertiary and quaternary structures may be related to the behaviour of several proteins in food, is probable (Kinsella, 1976).

There have been some theoretical studies to predict the changes in protein conformation by estimating the type and magnitude of all forces and constraints acting upon a poly peptide chain of a given amino acid sequence in solution (Nagano, 1974). But such an approach is not reliable and needs further investigation. There has also been some data concerning the interrelationships between composition, structure, and functional properties of proteins, which have been obtained preponderantly from biochemical studies concerned with explaining enzyme mechanisms, protein-protein associations, and lingand-protein binding. These studies provide food chemists with techniques and basic interrelations that could be applicable to research designed to elucidate the physicochemical phenomena underlying protein functions in foods. However, food systems are generally too heterogenous and complex to apply classical physicochemical techniques in studying the molecular changes, i.e., chemical and/or conformational, particularly the latter, which are occuring a technical process. Usually, the particular molecules in question are studied before and after the treatment. Some researchers contend that it is futile to attempt to derive thermodynamic data for proteins in food systems. Simple analytical model systems are required, and the data obtained from these simple systems should be comparable to those observed in the complex system (Buckingham, 1970; Hermansson, 1971;

Graham & Phillips, 1975; Hermansson & Akesson, 1975; Franks, 1975).

2.3.3. Protein Denaturation and Functional Properties

The term "denaturation" has different meaning for different workers. Usually, to both chemists and biologists, denaturation is undesirable, because it results in altered physicochemical properties, particularly a loss in solubility and functionality (Hermansson, 1973).

There has been much controversy concerning the definition of denaturation. The process of denaturation usually involves an alteration in the ordered structure of the native protein, i.e. a change in conformation of polypeptide chains from the native state, without the rupture of any primary covalent bonds. Native protein can undergo subtle changes in conformation, and a number of proteins demonstrate major, but reversible changes in conformation, usually via disulfide bond scission; whether or not these can be included in denaturation is debatable. Generally, denaturation is an "all or none" reaction, reflecting the cooperative nature of transition from the native conformation to a less structure state (Tanford, 1970).

Denaturation may initially involve unfolding of polypeptides, without any apparent loss in solubility. This step is usually followed by aggregation. This sequence, and the relative rates of each, may vary with protein source and the nature of denaturant. These factors should be studied and evaluated in

in each case of food proteins (Kinsella, 1976).

However, denaturation is an integral part of several technical processes in the preparation and texturization of foods; boiling an egg and curdling of milk are traditional examples. Unfolding of globular proteins occurs during emulsion or foam formation, (Bull, 1947; Swift & Sulzbacher, 1963) and in meat emulsions, thermal gelation of these proteins is essential. The formation of stable foams requires the concurrent denaturation of the functional proteins (Eldridge et al., 1963). Coagulation of milk proteins is an initial step in cheese making, and the preparation of alkaline dope for the spinning of protein fibres is needed to cause unfolding of polypeptides chains (Huang & Rha, 1974). Thus, while solubility is critical, information concerning the environmental conditions causing coagulation, aggregation, or gelation of food protein is also of practical significance.

2.3.3.1. Effect of Physical and Chemical Treatments on Protein Denaturation

Denaturation, i.e., disruption of noncovalent bonds, is caused by various physical treatments, if of sufficient intensity and duration, i.e., heating, freezing, radiation, extreme dilution, exposure to air-water or oil-water interfaces, and sonication. A wide variety of chemical denaturants exist. These may act by disrupting the specific types of secondary

forces which stabilize the primary conformation of native proteins; for example, urea, guandine hydrochloride, and some surfactants disrupt hydrophobic interactions, by promoting the solubilizion of hydrophobic residues in the aqueous phase (Gordon & Warren, 1968).

The presence of detergents may minimise hydrophobic interactions and may actually form chemical bridges between hydrophobic and hydrophilic portions of polypeptides, thereby facilitating denaturation. However, certain anionic detergents may also stabilize proteins agains thermal aggregation (Hegg & Lofquist, 1974).

The effect of heat denaturation on gluten protein was studied by Pence et al.(1953), who showed a close parallelis between baking performance and protein solubility, and concluded that solubility could be used for determining the extent of heat denaturation and predicting the performance of gluten in bread making. The effect of heat and chemical denaturations of plant protein with regards to their functionality and food applications were reviewed by Wu & Inglett (1974) where they discussed the correlation between denaturation, loss in solubility, and the associated deterioration in functionality of several proteins from soy, wheat, peanut, cotton seed, rye and rice.

The processing method plays a significant role in preserving or destroying the functional properties of proteins, since high temperatures, strong acids, basis and/or organic solvents can result in considerable alteration to protein structure and inferior functional properties. Mattil (1971) for example concluded from the solubility profile of FPC made by 2-propanol extraction of Mullet (Mugil spp.), that the protein has been substantially denatured.

2.4 FPC Processing Methods

Many countries and organisations have been involved for the last two decades in processing and manufacturing FPC on a commercial scale (Finch, 1970; Meade, 1971). FPC is not a single product, but a family of products manufactured by different processes. Depending on the process that is used, the member of the family or the product will have different characteristics and can be used either for nutritional purposes or as a functional ingredient (Stillings & Knobl, 1971; Cobb & Hyder, 1972).

The processing methods of FPC can be classified as follows:

i) Chemical

ii) Biological

iii) Physical (Knobl, 1967; Knobl et al., 1971 and Hale, 1972).

The main objective of the various methods is to release the protein from fish flesh in a stable form, which can later be converted to a stable powder with good odour and taste. These processing methods are described here as follows:

2.4.1. The Chemical Methods

These widely used methods have been developed to the point of commercialisation. The processes employ organic solvent to remove water and fat from fish and the remaining protein and minerals can then be dried and ground (Moorjani & Lahiry, 1962; Library of Congress, 1970, and Stillings & Knobl, 1971). To date three methods have been developed to produce FPC commercially. These are described below:

2.4.1.1. Viobin Method.

The comminuted fish is dehydrated by an azeotropic distillation of water with dicloroethane. The dehydrated fish is then treated with more dichloroethane to remove the lipids, and then vacuum dried to remove residual solvents (Levin, 1959).

According to Morrison and his co-worker (1965), dichloroethane destroys cystein, histidine and methionine which results in inferior nutritional value of the product.

2.4.1.2. 'Canadian', or 2-Propanol (Iso-Propanol) Extraction Method:

This method was first conducted in Canada (Guttmann & Vandenheuvel, 1957) and further developed in the USA by the Bureau of Commercial Fisheries (1966).

This process employs a series of extractions with azeotropic 2-propanol moving countercurrent to the product, to both dehydrate and defat comminuted fish. Residual solvent in then removed by either vacuum drying, or drying at atmospheric pressure (Spinelli et al., 1971).

2.4.1.3. Astra Method:

This process has been developed in Sweden and depends on decolouring the fish under mildly alkaline conditions at a pH value of not greater than 9.5, by using a mixture of alkali and hydrogen peroxide at 30 - 70°C.

The decolourized fish is then treated with 2-propanol to remove the lipids and the residual protein can be dried (Carpenter et al., 1975).

The processor claimed that the product has high nutritional value and an acceptable white colour. Although most of the above processes are capable of producing high nutritional

quality FPC, there are many handicaps or disadvantages still to be overcome, among which are:

i) the difficulty of removing the solvent traces which may be toxic, even after many leaching steps or subjection to reduced pressure. Also, these additional steps increase the production costs (Hyder et al., 1972; Chu and Pigott, 1973).

ii) the products have poor functional properties, which limit the use of FPC as functional agents (Knobl et al., 1971; Bhumiratana et al., 1977).

iii) the loss of some amino acids which is caused by the use of some solvents, such as in the case of the Viobin process.

2.4.2. The Biological Methods:

These methods use either enzymes or microorganisms to convert fish tissues into stable concentrates with desirable properties. The fish tissues can be broken down by the introduction of either living proteolytic microorganisms, such as bacteria, moulds or yeasts, or by the use of isolated enzymes from plant or animal origins. The resulting hydrolyzates can be separated from the undigested sludges by means of filtration or centrifugation (Library of Congress, 1970; Hale, 1972)

Numerous methods have been reported for processing FPC by biological methods. In Japan, Tomiyama (1968) has prepared FPC by autolysis of sardines under acidic conditions. The process involved comminution of fish, mixing with water and pH adjustment. Chloratetracycline was then added as a preservative, and then the digestion process was carried out at 50°C, until a clear autolysate was obtained, which was free from fat and indiestable residue.

In France, Ploqum and his co-workers (Hale, 1972) have carried out concentrated efforts to develop a fish hydrolysis process, in which papain and relatively high temperature and short digestion times were used. The final product was highly soluble and was said to possess good functional properties.

In the United Kingdom, Mackie (1974) has developed an enzymic process for FPC processing from whole fish or filleting wastes. The process involves comminution of fish, enzymic digestion with trypsin, pronase, papain, bromelain, pancreatic proteinase and bacterial proteinase for about 30 minutes at 50 - 60°C. The bones and skin are then removed by sieving, and the hydrolysate freeze-dried. The final product tends to be off-white to brown in colour. It disperses easily in water and does not have a gritty texture.

In the USA, Hale (1974) has carried out an extensive research project on processing FPC by biological methods, where the effects of various conditions and commercially available

proteolytic enzymes on the yield were studied. The soluble hydrolysates were prepared from red hake (Urophycis chuss) using a variety of enzymes and digestion conditions. It was found that the concentrations of tryptophan and histidine in the products were critical nutritionally and varied with the pH value of the hydrolysis, acidic conditions destroying the tryptophane, and the alkali causing racemization of most amino acids. However, a soluble FPC having a protein efficiency ratio (PER) equal to that of casein was prepared with alkaline bacterial enzymes at a pH value of 8.5.

The final product was highly soluble in water but off-white in colour, which made it useful in food industries where the solubility is desirable but a bland colour is not a limiting factor.

In general the biological methods had the following characteristics:

i) the product possessed functional properties, but poor nutritional value;
ii) low production costs;
iii) the processes required simple plant and services and in principle could be used easily in remote areas or on board ship. However, this method has not advanced beyond the laboratory or small pilot scale.

2.4.3. The Physical Methods:

These methods employ physical means such as pressure or electric current to break down the fish tissues and release their contents. Water and fat can then be removed easily (Knobl, 1967).

These methods are not used for FPC production on a commercial scale; all the investigated processes were performed on a bench scale. A German engineer, Doevenspeck, applied an electric current through raw fish slurry, followed by centrifugal separation of the components. One likely explanation of phenomenon underlying this method is that the ruptured cells will release their contents. Another method has been designed to remove moisture from fish. Here the raw fish is finely divided and then dispersed in a non-volatile liquid, preferably immiscible with water, which acts as a heat transfer medium. The dispersion is heated in a vessel that cna be evacuated. When pressure is suddenly reduced over the surface of the dispersion containing the heated fish particles, the moisture present is removed by evaporation while the non-volatile heat transfer medium retains the dehydrated fish particles The dehydrated fish particles are eventually separated from the heat transfer liquid by filtration or centrifugation, and the particles can then be defatted with the desired solvent (Knobl, 1971), as described in Section 2.4.1.

2.5 Extraction of Protein from Fish Muscle:

2.5.1. Historical Background

The earliest work on fish muscle protein consisted of the extraction of muscle of Atlantic haddock with solutions of sodium chloride and potassium phosphate (Logan, 1930). The water-soluble and water-insoluble fractions were distinguished. Reay (1933, 1935) and Reay and Kuchel (1937) found that haddock muscle fractions were similar to those in rabbit (which consisted of a water soluble fraction, myogen, an albumin type protein and a salt soluble fraction, myosin, and globulin). About 85% of the protein could be extracted with 7% lithium chbride and about 95 - 97% with successive extractions with 0.013 N. HCl. These workers found that soluble protein content was reduced on frozen storage. Subba Rao (1948) and Dyer et al., (1950) showed that this was due to a change in the globulin fraction, while the water-soluble fraction apparently remained unaffected.

Dyer and his co-workers (1950) found that 95% of cod and haddock could be extracted with 3-5% NaCl at pH between 7 - 9. The muscle protein consisted of about 75 - 80% myosin; 20% globulin X, myogen and myoalbumin; and 3% stroma, collagen, elastin. Hamoir (1951) isolated and fully characterized the myofibrillar proteins of carp muscle which were tropomyosin and nucleo tropomyosin.

The components most nearly resembled rabbit actomyosin.

Hamoir (1955) reviewed the modern methods of analysis, including electrophoresis and ultracentrifugation, which have been applied to the study of fish muscle components.

2.5.2. The Composition of Fish Muscle.

The classification of fish muscle proteins according to the extraction with various solutions was reviewed by Palmer & Bowers (1972) as follows:

i) the sarcoplasmic proteins which can be extracted with water or a weak salt solution and comprise about 16-22% of the total protein.

ii) the myofibrillar proteins which can be extracted with electrolyte solutions of ionic strengths greater than 0.5. Three principle myofibrillar proteins are myosin, actin and tropomyosin.

Myosin, in fish as well as mammals in the most abundant fraction and makes up about 40% of the total protein of cod. Actin formed about 15-20%, and tropomyosin is the smallest fraction and is estimated to be about 1.8 - 2.6%.

iii) the stroma is that protein which remains insoluble after extraction with salt solutions and is made up largely of myocommata (connective tissues), vascular tissue and probably fibre membrane or sarcolemma.

Fish collagen contains more hydroxy groups than does bovine collagen. The quantity of collagen in fish muscle varied from about 3% in godoids to about 10% in elasmobranchs, as compared with 17% for mammals. The low amount of connective tissues in fish muscle probably accounts for the flakiness and tenderness of the muscle.

2.5.3. The Extractability of Fish Protein

The solubility or extractability of fish protein in 5% NaCl has been taken as a criterion for measuring the rate of protein alteration or denaturation which occurred during froxen storage of fish (Dyer, 1951). The main factors which can alter protein extractability are described below:

3.5.3.1 Composition of Fish

The composition of fish is extremely varied, because they can be caught in different sizes and nutritional states. The tissue constituents of a catch of a single species of fish can not only vary in composition depending on maturity, but also can vary depending on where and when the fish are caught (Gould & Peters, 1971; Spinelli et al., 1972).

2.5.3.2. Handling of Fish

It must be appreciated that the condition of fish can vary considerably at the time they appear on deck, both within a single catch and between catches. These differences arise in part from the factors mentioned in section 2.5.3.1. out in part from differences in how the fish were caught e.g. in traps, nets etc. and whether the fish had just been caught or whether it had been caught for some time (perhaps 12-24 hours in the core of traps) (Paul & Bowers, 1972). The handling of fish on board ship i.e. whether gutted and/or filletted, whether iced or frozen and any delays prior to any of these treatment can modify the post mortem. (rigor mortis) changes (Amlacher, 1961). These in turn can modify the extractability of protein (Tomlinson, et al., 1965). Delays should be minimal, consistent with the avoidance of gaping and thaw rigor in the case of fish that are frozen (Love & Haq, 1970b), but under commercial conditions, the ideal can not always be achieved.

The literature is extensive but it would be seen that the greater damage to the protein occurs when very low pH values are attained post morten, (Tomlinson, et al., 1965) particularly when these are associated with high temperature, the latter is usually arising from exposure to the sun (Love & Haq, 1970 a).

2.5.3.3. Method of Extraction:

The method of extraction itself can have a major influence on the final product quality. According to Cowie & Mackie,(1968); and Ravesi & Anderson, (1969), the degree of muscle comminution the ionic strength and pH of the extracting solution, the duration

of the extraction process and the type of extraction apparatus can affect the soluble protein yield. This would suggest that low protein extractability is not always an inidcation of protein denaturation.

2.5.3.4. Storage Temperature and Protein Denaturation:

Freezing is one of the best methods which is being applied to preserve and extend the shelf life of fish both on shore and on board fishing vessels. However, after prolonged storage at temperatures as low as $-20^{o}C$, marked and undesirable sensory changes in the product occur. In fatty fish, the deterioration is mainly reflected by the development of oxidative rancidity, while the quality of lean fish predominantly suffers from protein denaturation, which is reflected by significant deterioration in texture, solubility and functional properties, especially when frozen in a minced state. (Sikorski et al., 1976).

The deterioration of frozen fish muscle has been reviewed and studied by Dyer & Dingle, 1961; Connell, 1964, 1968; Love, 1966; Powrie, 1973; Sikorski et al., 1976.

Some aspects of protein denaturation during frozen storage are discussed below:

i) The Loss of Protein Solubility and Formation of Aggregates

The decline in protein solubility is mainly due to the alteration of the actomyosin - myosin system, while the sarcoplasmic protein does not undergo significant changes (Dyer & Dingle, 1961; Connell, 1968).

Anderson & Ravesi (1970a) found that the amount of readily extractable sarcoplasmic proteins in cod stored for 32 weeks at -12°C, decreased by about 40% of its original value, and they attributed this result to the increased resistance of muscle to homogenization.

Most of the alteration in the protein system is caused by aggregation of myosin- actomyosin.

Lowey & Halzer (1959) demonstrated that the spontaneous aggregation of rabbit myosin in 0.6 M - KCl is a step-wise process in which the molecules attach to themselves almost exclusively side to side to form at least octamers. The rate of aggregation is increased with the rise of storage temperature and was found to be about ten times greater at 25°C than at 4°C.

Connell (1962) found that the molecules of cod myosin in solutions aggregate upon standing especially rapidly when frozen. Buttkus (1970) found that aggregated trout myosin tends to resist solubilization in 1M - NaCl, 8 M urea and 6 M guanidine hydrochloride or detergents at pH 8.

The literature reported the existence of different types of inter-molecular cross linkages in myofibrillar protein of frozen fish. Most of these linkages are of the secondary type, i.e. hydrogen bond or hydrophobic adherences, and these are sensitive to 1% sodium dodecyl sulphate (SDS) solution. (Connell, 1965; Iwata & Okado, 1971).

The results of ultracentrifugal separations carried out by King (1966) showed that during frozen storage of cod there was an initially rapid decline in the extractability of the F-actomyosin fraction, accompanied by a fairly large increase in the concentration of a fraction consisting of myosin and G-actomyosin, and a subsequent gradual decline of the extractability of all the investigated fractions of myofibrillar proteins. On the basis of this evidence, King suggested that during frozen storage of cod there was a "rapid dissociation of F-actomyosin into G-actomyosin followed by a slower rate of dissociation of G-actomyosin into components that then aggregate to form inextractable protein". He also considered the possibility of direct aggregation of F-actomyosin macromolecules to form insoluble polymers. Anderson & Ravesi (1970 a) also indicated aggregation that increased with the time of frozen storage of fish. Childs (1973) found that in Pacific true cod the suspensibility of whole myofibrils decreased during storage at -40^{o}C at a higher rate than the extractability of myofibrillar proteins.

Jarenback & Liljemark (1975) discovered that the electron microscope image of KCl-phosphate buffer extracts of cod myofibrillar proteins was changed after frozen storage of the fish. In solutions obtained from unfrozen cod, they found large numbers of actomyosin filaments, i.e. F-actin, with attached myosin molecules at regular interals in the form of arrowheads.

However, in samples extracted from fish stored for different periods of time at $-20^{o}C$, the number of regular filaments gradually decreased. After 38 weeks, large aggregates formed by actomyosin appeared as well as others suspected of being actomyosin filaments bound to fragments of Z-discs. In extracts obtained from cod stored at $-10^{o}C$, similar changes were noticeable earlier. These results made Jarenback & Liljemark (1975) to conclude that the myosin - myofilaments can be almost completely extracted from fresh muscle but are resistant to extraction in fish stored for long perid of time in the frozen state.

The state of the frozen fish played a significant role in molecular interaction between the different constituents. Mincing the fish enhanced chemical changes induced by cryoconcentration because it caused disintegration of many membrane systems including lysozomes and brings together the enzymes and substrates of many reactions according to Sorenson (1976) and Sikorski et al., (1976).

ii. The Effect of Rancidity and Free Fatty Acids (FFA)

Rancidity in food can be defined as the development of off-flavours which make the product unfit for human consumption, resulting from oxygen reaction with unsaturated fatty acids which may or may not be part of a fat (triglyceride) or phospholipid (Labuza, 1971).

The major problem in frozen storage of fish is toughening which was suspected to be caused by FFA. The effect of FFA on the texture and protein extractability of frozen fish was investigated by several workers as follows:

Dyer (1951) and Dyer & Dingle (1961) presented a hypothesis on the contribution of unesterified fatty acids and the presence of lipids in the tissues to the protein changes in frozen fish. They observed that FFA formation preceded the loss of extractability of myofibrillar proteins. They hinted at a possibility of a stabilizing effect of lipids bound to the actomyosin in lipo-protein and subsequent lack of stabilization resulting from the hydrolysis of the lipid. As an alternative they suggested a sensitizing effect of the products of hydrolysis on the protein. They also considered the surface activity of both lipids and fatty acids as a basis for such effects.

Olley & Lovern (1960); Nishimoto (1962); and Olley and her co-workers (1962) showed that the release of FFA occurred concurrently with protein denaturation during frozen storage. However, Olley & Lovern (1960) pointed out that similar reaction rates at various temperatures are not, in themselves proof that one effect is causing the other and suggested caution in

drawing any simple relationship between lipid hydrolysis and protein denaturation.

King & his co-workers (1962) suggested that the protective effect of natural fish lipids is caused by partial removal of FFA from reaction sites due to their dispersion in the lipid fraction.

Olley and her co-workers (1960, 1962) found that most of the FFA in many fish originated from phospholipids, which probably concentrated in the lipoprotein of the cell membrane.

A thorough study was carried out by Olley and her co-workers (1962) to explain the phenomenon of FFA liberation and the decline of protein extractability in cod, lemon sole, halibut and dogfish. They indicated that there is no simple cause and effect relationship. They also studied the protecting effect of lipid on protein extractability and found that protein denaturation rapidly occurred in both lean and fatty fish. For the lean fish no neutral lipids are present to counteract the FFA, whereas in the fatty fish the lipids are not available. The slightly fatty fish are the most stable since enough lipids are available.

Anderson and her co-workers (1965) indicated that the ionic interaction of fatty acid is the driving force for protein insolubilization.

Ackman (1967) found that during the frozen storage of lean fish such as cod and haddock, the hydrolysis of phospholipids gave FFA capable of interacting with protein to produce texture deterioration. He also mentioned that the rancidity is more commonly associated with lipids in fatty fish.

Anderson & Ravesi (1970 b) discovered that the interaction of FFA with myofibrillar proteins produced a network of cross-links which increased the resistances of muscle fibres to fragmentation and reduced the protein solubility.

Jarenback & Liljemark (1975) found large aggregations in electron microscope images of mixtures of cod myofibrillar proteins and emulsified linoleic acid. Micelles of fatty acids could be seen adhering to surface of actomyosin filaments. This interaction of the acid with myofibrils (pH 7.6 and $2^{o}C$) resulted in significant reduction of protein extractability, aggregation of the extracted proteins, and an increase in the volume of myofibrillar residue after extraction. Although the residue contained more protein, most thick myosin myofilaments were dissolved. This result made Sikorski and his co-workers (1976) suggest that interaction with fatty acids did not significantly impair the solubility of myosin.

The fatty acids which were liberated from phospholipids as a result of hydrolysis may as a result of their energy of translation and concentration gradient, diffuse into the non-frozen liquid solution and interact with the solutes. Originally being part of the lipo-protein structures of the cells, they were not able to expose their reactivity towards distant substrates. As free fatty acids or salts, they can attach themselves to appropriate binding sites of either neutral lipid droplets or hydrophobic polar or ionized fragments of the polypeptide chains. When they interact with neutral lipids, they become unavailable for interaction with protein.

When they bind to polypeptide side chains of polar groups, they may decrease the protein solubility due to the formation of intermolecular hydrophobic-hydrophilic or hydrophobic-ionic linkages, especially at appropraite concentrations of inorganic ions. Shenouda & Pigott (1975) found that the presence of Ca^{++} as well as higher ionic strength solutions favours polymerisation of actin and binding of neutral and polar lipids. The rate and equilibrium of lipid hydrolysis should obviously be related to the interactions of the reaction products with protein and the temperature of storage. The interaction of FFA with protein should enhance the hydrolysis, according to the general law of mass action, while at lower temperatures, due to increased viscosity of the remaining solution and more frequent gaps between pools of unfrozen lipids, the migration of liberated acids should be hindered, and the hydrolysis

stopped, at higher concentrations of intact lipids. This reasoning could furnish an interpretation for the rates and equilibria of lipid hydrolysis in frozen fish as found by Olley and her co-workers (1969) and later confirmed by Anderson & Ravesi (1970b).

The other factors which affect protein denaturation during frozen storage are cited in the review of Sikorski et al (1976), concerning "Protein changes in frozen fish".

2.5.4. Effect of Frozen Storage on Protein Functionality

Prolonged storage, especially at temperatures around $-18^{o}C$, brings about significant deterioration of the texture of frozen fish described as increased toughness, chewiness and rubberiness or stringiness (Connell, 1964; Sikorski, et al., 1976). Connell (1962) stated that the development of toughness and the loss of water-holding capacity is caused by formation of additional linkages and by the higher strength of existing linkages, between the myofibrillar proteins.

Grabowska & Sikorski (1974) found that after 7 weeks storage at $-18^{o}C$ up to 70% of the initial fat emulsifying capacity of cod myofibrillar protein was lost, while that of the sarcoplasmic part underwent only minor changes. They also found that frozen storage of Baltic cod in minced state at $-18^{o}C$ resulted in significant deterioration of the gel forming

capacity of myofibrillar protein.

The storage condition significantly affects the functional properties of protein. According to Childs (1974), the flesh of several Atlantic fish species, stored as fillets and packed in evacuated moisture-vapour proof pouches for 2 - 12 months at -40°C, did not lose its emulsifying capacity.

2.6 The Functional Properties of FPC and Fish Muscle

FPC prepared by the solvent methods described previously in Section 2.4.1. lacks most of the functional properties. However, the literature reports some data in which FPC showed functional behaviour.

Hermansson et al.(1971) found that FPC had low solubility values ranging between 4 - 35% within a broad pH range. Only the soluble fraction of the FPC (1 - 2%) was found to be responsible for foam formation. The insoluble fraction acted as a foam stabilizer. The foam volumes obtained from the soluble fraction were equivalent to those obtained using egg white. The product also showed wetting and swelling properties.

Baldwin & Sinthalavais (1974) reported that different FPC preparations showed varying foam capacity.

Best foams (volume and stability) were obtained with FPC concentrations of 10% at pH 10. Sodium chloride exerted little effect on foaming. The alkaline hydrolysis dramatically improved foaming capacity of FPC, probably by increasing the quantity of soluble peptides. This result is in agreement with the Hermansson et al. (1971) finding.

Spinelli et al. (1975) prepared functional protein isolates and derivatives from fish muscle by enzymic modification. The isolates were coagulated, but the derivatives were heat stable. Both preparations had foaming and emulsifying properties.

Grabowski & Sikorski (1974) found the myofibrillar proteins of cod fish far superior to other muscle proteins. Frozen storage decreased the emulsifying capacity and this was correlated with the decrease in protein solubility. Minimum emulsification occurred at pH 4.5.

Inklaar & Fortuin (1969) reported that FPC was successfully used in sausage meat. They also stated that the emulsifying capacity is related to the amount of soluble protein and factors affecting solubility of the myofibrillar proteins.

2.7. Membrane Separation and Concentration Processes:

Membrane separation processes are gaining widespread interest as an important process for separation and concentration.

of biological and industrial liquids. Two facts explain this importance. Firstly, the process allows separation of dissolved materials from each other, or from a solvent with no phase change (Mac Bean & Smith, 1977). Secondly, the process does not have high energy consumption. Thus, it is cheaper than other separation methods such as evaporation or crystallization (Lacey, 1972).

There are many different types of membrane processes such as ultrafiltration (UF), pervaporation and electrodialysis. However, all the processes have common characteristics. Firstly, when a liquid containing two or more components is in contact with one side of a membrane that is more permeable to one component (or a group of like components) than to the other components, this is a 'selective membrane'. The other side of the membrane is in contact with a fluid that receives the components transferred through the membrane. Secondly, to cause the transfer of components through the membrane, there must be a driving force of some kind. Such a force may be transmembrane differences in concentration, as in dialysis and osmosis; electrical potential as in electrodialysis; or hydrostatic pressure such as in Reverse Osmosis (R.O.), Ultrafiltration (U.F.) and microfiltration (Lacey, 1972).

The heart of the separation process is the membrane, which is usually constructed from polymeric materials. Polymeric membranes have the ability to discriminate between

molecules on the basis of molecular size and conformation and/or chemical composition (Fenton-May et al., 1971).

However UF and RO membranes in regular use are anistropic diffusive membranes, which consist of a highly consolidated but very thin skin (0.1 - 10μ) supported by a relatively thick (20μ - 1mm) porous substructure as explained in Figure 2.1 (Michaels, 1968).

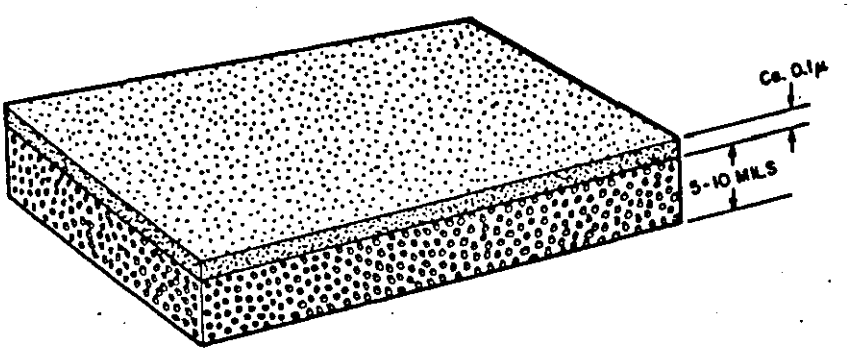


Figure 2.1 Schematic of an anisotropic, diffusive membrane

The thin layer (or the active layer) determines the retention ability of the membrane, which is usually controlled during membrane fabrication (Loeb & Sourirajan, 1960; Bank & Sharples, 1966).

Because the consolidated layer is so thin, such membranes display high flux rates, coupled with the ability to block the passage of some solute molecules. These membranes can withstand high hydraulic pressure, and they do not readily become plugged or fouled by the retained solutes. Although membranes are designed to have as far as possible a uniform pore size, in practice a distribution of pore sizes will exist, resulting in an imprecise molecular 'cut-off' even for spherical molecules (Melling, 1974).

2.7.1. Ultrafiltration and Reverse Osmosis (UF & RO)

Both ultrafiltration and Reverse Osmosis are membrane processes for separation and concentration of homogenous liquid solutions, which are based on the ability of polymeric membranes to discrimate between molecules on the basis of molecular size, shape or/and chemical composition (Fenton-May 1970; Dejemek, 1975). These terms are used interchangeably, not only by the lay public but some of the experts in the field as well. Others distinguish between the two processes on the basis of the molecular mass of the separated solutes (Madsen, 1974), applied pressure, or the mechanism of the separation (Sourirajan & Matsuura, 1973).

In macroscopic descriptions, the two processes are identical, a solution under pressure is brought into contact with a thin layer of a solid (the membrane), the properties of the membrane being such that the components of the solution pass through the membrane with different velocities, resulting in a difference between the composition of the original solution and the liquid that passed through the membrane, the permeate.

Both processes are explained schematically in Figure 2.2

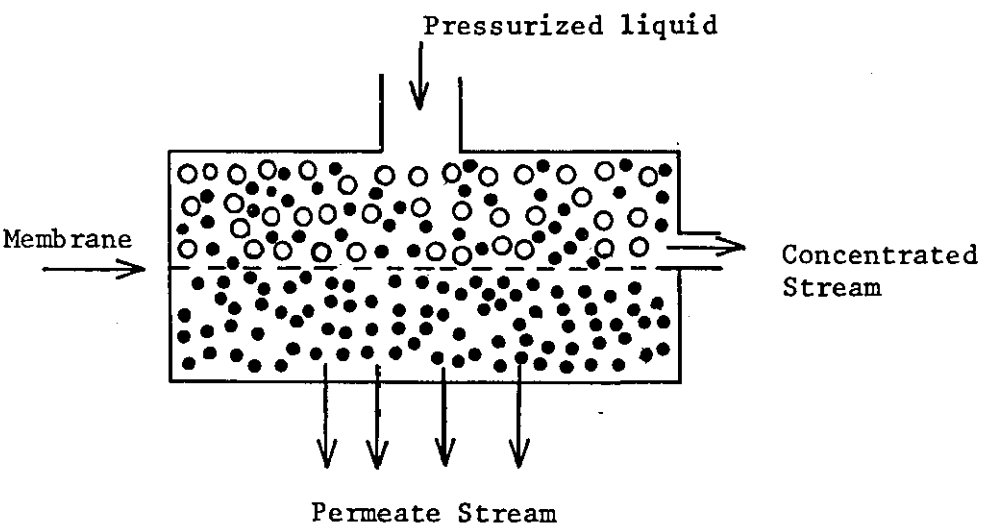


Figure 2.2. Schematic diagram of membrane ultrafiltration and Reverse osmosis processes.

In Reverse Osmosis, all species except water are rejected by the membrane. The osmotic pressure of the feed liquid in such a process will often be quite hight. Consequently,in order to achieve satisfactory water flux rates through the membrane, it is necessary to apply a hydrostatic pressure of 60 bar (880 psi) or more.

Ultrafiltration is described as that membrane separation process in which the membrane is permeable to solvent and relatively low molecular mass solute, but impermeable to higher molecular mass species. The osmotic pressure difference across the membrane in such a system will generally be very low, thus allowing the process to be operated at low hydrostatic pressure, such as 1 - 3 bar (15-44 psi).

2.7.2 Diafiltration

Addition of further solvent to a solution from which solvent and microspecies have ultrafiltered is called diafiltration. This process effectively washes the micro species from the solution, purifying the retained species. In contrast with conventional dialysis, the rate of micro-species removal is independent of their concentration but is simply a function of the rate relative to solution volume.

Diafiltration also extends itself to efficient salt exchange or washing in of a micro-species, changing the environment of the retained material. Solutions may be diafiltered by repeated dilution and concentration, or continuously keeping process volume constant. Reference is made to this technique in the Amicon brochure "Amicon ultrafiltration No.427".

2.7.3. Solute and Solvent Transport Mechanism:

According to Michaels and his co-workers (1965), and Michaels (1968), there are two basic classes of mass-transport mechanisms which take place in RO/UF diffusive membranes. "Tight" or RO membrane i.e. those capable of retaining solutes whose molecular diameters are about 10 $\mathring{A}$ or less, function as diffusive transport barriers. In these membranes, both solute and solvent species migrate by molecular diffusion within the polymer driven by applied pressure difference. "Loose" or UF membrane, those retaining particles larger than 10 $\mathring{A}$, appear to function as molecular screens. Small molecules or micro-solute can transfer through the membrane readily. However, this passage can be hindered by intermolecular interaction in the boundary layer, which is formed as a result of concentration polarization (see section 2.7.4).

Solvent flux through an "RO" membrane can be described as follows:

$$J_1 = \frac{K_1}{t}(\Delta P - \Delta\pi)$$

$$= \frac{\bar{C}_1 D_1 \bar{V}_1}{t\,RT}(\Delta P - \Delta\pi) \quad \text{.....2.1}$$

where J_1 = Solvent flux ($cm^3/cm^2.sec$).

t = Membrane thickness (cm)

K_1 = Membrane permeability constant

ΔP = Hydrostatic pressure difference ($dynes/cm^2$)

$\Delta\pi$ = Solute osmotic pressure difference between upstream and downstream solutions ($dynes/cm^2$)

$\bar{C}_1$ = Mean concentration of solvent in membrane (cm^3/cm^3)

D_1 = Diffusivity of solvent in membrane (cm^2/sec)

V_1 = Partial molar volume of water in solution ($cm^3/mole$)

R = Gas constant.

T = Absolute temperature

Solute flux through an "RO" membrane can be described as follows:

$$J_2 = \frac{k_2 D_2}{t} (C_i - C_o) \quad \text{.....2.2}$$

where J_2 = Solute flux ($gm/cm^2.sec$)

k_2 = Solute distribution coefficient between membrane and solution

D_2 = Solute diffusivity in membrane

C_i = Upstream solute concentration (g/cm^3)

C_o = Downstream solute concentration (g/cm^3)

Solvent flux through UF membrane can be described as follows:

$$J_1 = \frac{K_2 \Delta P}{\mu t} \quad \text{.....2.3}$$

where K_2 = membrane permeability content $\frac{ER^2}{30}$

μ = Solvent viscosity

E = Porosity of membrane

R = Hydraulic mean pore radius

Donnelly & Delaney (1974) applied Michaels equations to examine the performance of UF plant in the concentration of cheese whey. They examined the effects of temperature, pressure and flow rate on the rate of flux. They found that flux rate was linearly related to temperature, which would affect the viscosity of the liquid (equation 2.3) whereas for RO plant, the temperature would effect all three variables in the numerator of the membrane permeability constant (equation 2.1) (Donnelly & Dunkley, 1971). They also found that flux rate would be linearly related to the pressure up to a point, and then become independent of pressure. The point at which pressure independence occurred varied with flow rate and feed concentration. At low flow rate and high feed concentration it occurred quite rapidly. Pressure independence occurs because of the concentration polarization effect. With RO it may arise due to a combination of microsolute and macrosolute polarization, while in UF micro-solute polarization

is negligible and pressure independance arises solely from macro-solute. These results are in agreement with data of Fenton-May et al.(1971), who studied the use of UF and RO for concentrating and fractionating skim milk. They also found that the flux rate was inversely proportional to the concentration of protein in the concentrated stream.

This result is in agreement with Michaels equation (2.3). When pressure independance occurred and protein molecules built up at the membrane wall and formed a gel layer, Blatt et al, (1970) proposed the following relationship to describe the flux rate under these conditions.

$$J = K_s \ln \frac{C_g}{\bar{C}} \quad \text{.....2.4}$$

where K_s = mass transfer coefficient

C_g = solute concentration at which gel formation occurred.

$\bar{C}$ = bulk solute concentration

Using the above equation, Donnelly & Delaney (1974) found that the flux rate was inversely proportional to the logarithm of protein concentration in the concentrated stream during the UF of cheese whey.

2.7.4 Concentration Polarization

As the membrane separation process is carried out, one or more of the macro-solutes will be rejected at the membrane surface. Solvent and microsolute which pass through the membrane are supplied to the membrane boundary by bulk flow of the feed solution normal to the membrane. The rejected solute will be carried along in this convective flow. If, however, steady state operation is to be maintained, with all or part of the solute molecules being rejected at the membrane surface, the excess of the rejected solute which builds up there must diffuse back into the bulk feed stream. Thus a gradient in the concentration of the rejected molecules. is established in the fluid near the membrane surface such that the net diffusive flux of the rejected molecules away from the membrane is equal to the convective flux of that material to the wall.

The existence of this concentration profile is referred to as "concentration polarization" Figure 2.3 (Porter & Michaels, 1971).

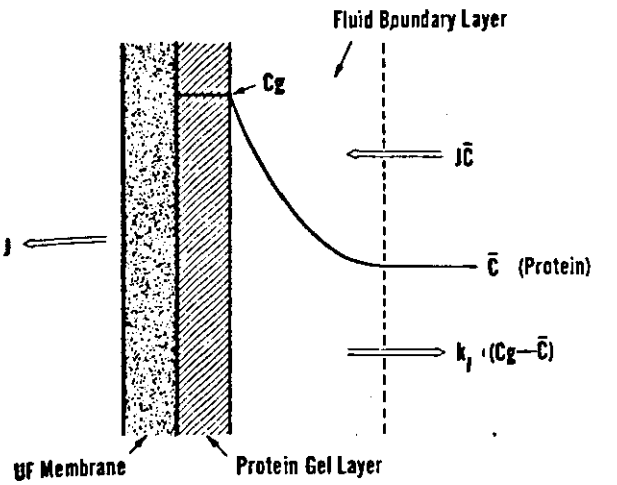


Figure 2.3

"Concentration polarization"

J = Solvent flux

Cg= Solute concentration at membrane

$\bar{C}$ = Solute concentration in solution

$J\bar{c}$= Solute transport toward the membrane

k_j =Solute diffuse back from protein gel layer

Under such conditions, Porter & Michaels (1971) proposed the following relationship to describe the flux rate,

$$J = \frac{\Delta P}{R_G + R_M} \quad \text{.....2.5}$$

where R_G = flow resistance due to gel layer

R_M = flow resistance due to membrane

Concentration polarization is extremely important because this increased concentration at the membrane boundary (equation 2.5) may have a number of adverse affects on mass transfer rates and therefore on the economics of membrane separation processes.

In RO, concentration polarization can markedly increase the apparent osmotic pressure of the feed. As equation (2.1) shows, this increase will raise the operating pressure required to produce a given flux and hence increase the operating costs.

In the UF of protein and other high molecular mass solute solutions, concentration polarization may cause the viscosity of the feed stream adjacent to the membrane to increase, thereby causing non-Newtonian effects or intermolecular interactions of such magnitude that mass transport from the bulk feed stream to the low pressure side of the membrane may actually be controlled by the rate of diffusion of solvent and micro-solute molecules through the macro-solute boundary layer.

Concentration polarization also increases the possibility of precipitation of marginally soluble constituents in the feed. Such precipitation can clog the membrane and reduce the flux rate of the feed solution.

2.7.5. Applications of RO/UF in Food Industry

RO and UF show promise as new techniques in separation and concentration of numerous food solutions in many food industries, such as the concentration of fruit juices (orange and apple), protein recovery from milk whey, protein recovery from soy whey and concentration of egg white (Lacey, 1972). These processes can be used wherever there is a need to concentrate or fractionate aqueous solutions with out loss of flavour or nutritional value or functional properties.

These concentration processes have decided advantages over alternative dehydration processes, such as vacuum dyring and spray or freeze drying, in that protein and other heat-sensitive materials are preserved. Taste change due to thermal alteration of components are avoided, and equipment and operating costs are reduced (Michaels, 1968).

UF processes have been performed on purification of raw beet sugar juice prior to evaporation and crystallization. Filtration through sucrose - permeable, macro solute retentive membranes yield an ultrafiltrate essentially free of colloid, polysaccharides, colour bodies, and other objectionable components which are normally removed by chemical and sedimentative processes. UF can perform these functions with considerable cost saving for the sugar refinery (Rickles, 1967).

The processing of dairy products is one of the most important applications of UF in food industries. Salt- and sugar-free milk protein concentrate can easily be processed by ultra-filtering whole or skim milk through casein-lactalbumin retentive membranes. Cheese whey can be deacidified, desalted, desugared and concentrated similarly to obtain edible protein concentrate.

The permeate from such processing can be refiltered through a lactose-retentive membranes to yield a purified lactose concentrate from which sugar can be readily crystallized and recovered (Michaels, 1968; Fenton-May, 1971). Another application of a UF system was the recovery of protein from fish waste, in which the waste materials were solubilized by proteolytic enzymes to polypeptides which can pass through a UF membrane. Eventually the unsolubilized fish waste and enzymes are retained by the membrane, thus obtaining the most favourable kinetics for the proteolysis reaction (Cheftel et al., 1971).

Chapter Three

Chapter Three

Materials and Methods

3.1. The Raw Materials

The cod filleting waste (FW) used in this project was supplied by fish merchants from Grimsby. The FW is the residue remaining after the removal of fillets from gutted whole fish, that is, it constist of head, backbone and adhering flesh.

The FW was washed with potable water and then passed through a mincer (Hobart model E4522), fitted with a 0.33 cm sieve plate. The minced FW was stored in polyethylene bags at $-15^{o}C$ until required.

3.2. The Investigation of the Extraction Process

3.2.1. The Time of Extraction:

This test was carried out to study the extractability of minced FW by distilled water, and to determine the optimum extraction time required.

A minced FW sample of 25 gram was suspended in 250 cm^3. of distilled water to give a mixing ratio of solid : liquid (S : L) of 1 : 10.

This ratio was arbitrarily chosen.

The FW particle size nominally did not exceed 0.33 cm diameter. In practice some larger particles may have been forced through the aperture in the sieve.

The suspended FW samples were shaken at room temperature ($25^{o}C$) for various times. The solvent volume was 250 cm^3 shaken at a speed of 170 rpm in an orbital shaker. After shaking, the suspension was clarified by centrifugation for five minutes at 1000 x g. The clear supernatant liquor was decanted and the extracted protein was determined by the micro-Kjeldahl method (see section 3.6.2).

In the presentation of experimental data, the term 'protein' will be treated as Nx 6.25. The term 'extracted protein' represents the percentage of total crude nitrogen x 6.25 present in the supernatant. The term 'true extracted protein' represents the percentage of total crude nitrogen minus non-protein nitrogen compounds x 6.25 present in the supernatant.

3.2.2. The Saline Extraction

For this investigation the procedure mentioned in section 3.2.1. was followed, except that the extraction time was for 1 hour, and different concentrations of NaCl were used as solvents (see section 3.6.1.1.). The concentrations of 1-10%

NaCl w/v were examined to determine the optimum saline concentration to be used in further processes.

3.2.3. The Mixing Ratio

For this test the extraction procedure outlined in section 3.2.1. was used, except that the extraction time was 1 hour, using 4% NaCl as a solvent, and different mixing ratios of S : L were examined to determine the optimum mixing ratio for the extraction process.
Table 3.1 shows the weights and volumes of minced FW and saline solution used to prepare the mixing ratios

FW(g)	4 NaCl% (cm^3)	mixing ratio
20	400	1 : 20
15	225	1 : 15
12	144	1 : 12
10	100	1 : 10
8	64	1 : 8
5	25	1 : 5

Table 3.1 The mixing ratios

3.2.4. The Temperature of Extraction

For this test the extraction procedure outlined in section 3.2.1. was followed, except that 4% NaCl was used as a solvent. The extraction time was 1 hour and the extraction was carried out in a water bath controlled by thermostat at a temperature which ranged between 0 and 60°C.

The test was carried out to examine the effect of extraction temperature on the protein yield from FW.

3.2.5. The Particle Size of FW

This experiment was carried out to examine the effect of nominal maximum particle size of FW on the extracted protein yield.

For this test the extraction procedure described in section 3.2.1 was used, except that the extraction time was one hour, using 4% NaCl as a solvent, 1 : 8 S : L ratio, and different nominal maximum particle sizes in the range 0.04 to 0.97 cm were examined.

3.2.6. Using another Type of Salt:

Calcium chloride was used as an alternative salt to examine the resultant yield of extracted protein from FW.

In this test 0 - 9% calcium chloride solutions (see section 3.6.1.2) were used as solvents, at a mixing ratio of 1 : 8 (S : L), and an extraction time of one hour. The other extraction conditions were as described in section 3.2.1.

3.2.6.1 A Comparison Between Distilled Water, 4% NaCl and 4% $CaCl_2$:

This test was carried out to compare the protein yield which could be obtained from FW when different solvents were used.

The salt solutions were prepared as described in sections 3.6.1.1. and 3.6.1.2. The extraction time was 1 hour, using a mixing ratio of 1 : 8 (S : L). The other extraction conditions were as described in section 3.2.1.

3.2.7. The Number of Extraction Stages

The test was carried out to determine the number of extraction stages which were required to completely extract the soluble protein from FW. The first extraction stage was performed in the manner described in section 3.2.1.
The second stage was performed by extracting the residual FW from the first stage with a fresh quantity of 4% NaCl. The same procedure was repeated for the third stage. The extracts were bulked.

Each of the extraction stages was for one hour using 4% NaCl as a solvent and S:L ratio of 1:10. Figure 3.1 explains the extraction stages schematically.

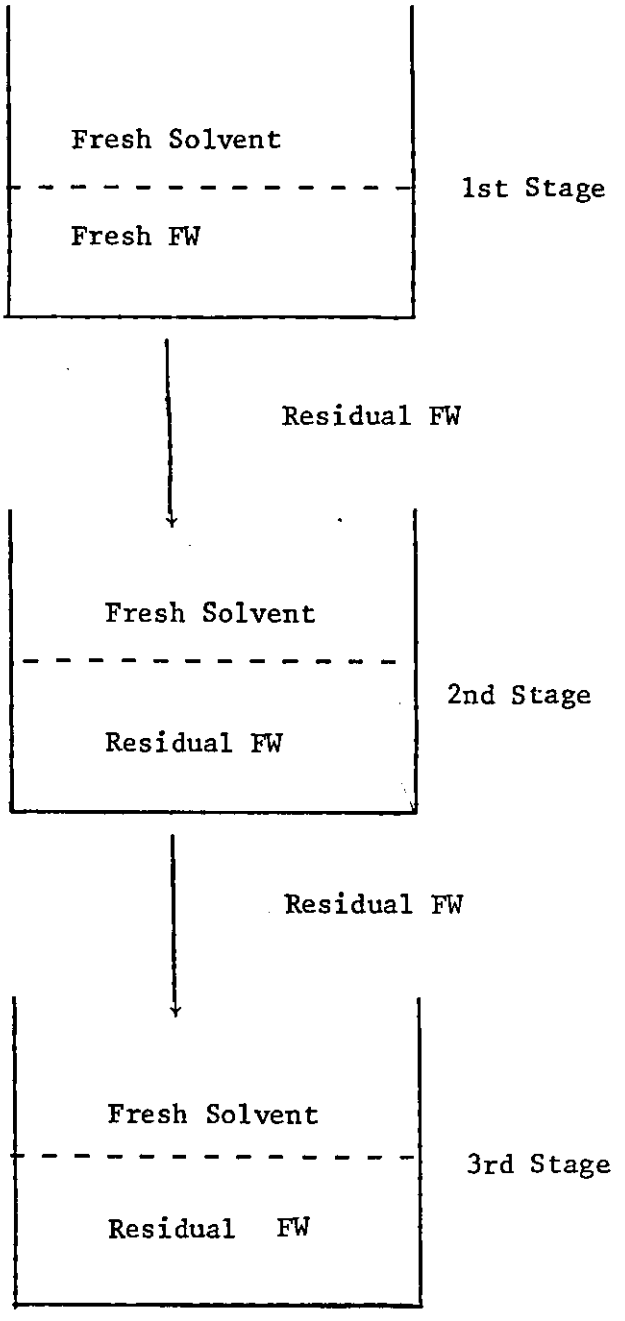


Figure 3.1 - The extraction stages

3.2.8. Effect of Shaking Speed on the Protein Yield

The test was carried out to examine the effect of using different shaking speeds on the amount of protein extracted from FW. In this experiment an orbital incubator (supplied by Gallenkamp & Co. Ltd - London - England) fitted with speed control was used to provide regular and constant shaking speed during the extraction process.

Speeds in the range 100 to 300 rpm were investigated. The following extraction conditions were held constant during the test: 4% NaCl used as a solvent, the S : L ratio was 1 : 8 and the volume of extraction medium was 250 cm^3.

3.2.9. Effect of Shaking motion on Protein Yield

Two types of extraction apparatus were employed. The first was the orbital type (mentioned in section 3.2.8), and the second was the rotary type which consisted of a stirrer driven by an electric motor.

The following extraction conditions were kept through out the test: S : L ratio of 1 : 8, 4% NaCl as a solvent, one hour was the extraction time at 200 rpm and the solvent volume was 250 cm^3.

The test was performed to examine the effect of different extraction apparatus on the yield of soluble fish protein from FW.

3.2.9.1. Effect of Homogenizing the Minced FW on the Yield

The Silverson homogenizer, which is supplied by Silverson Machines Ltd., Waterside, Chesham, England was employed to examine the effect of breaking down the minced FW on the protein yield.

The extraction conditions mentioned in section 3.2.9 were kept throughout the test.

3.2.10. The Optimum Extraction Conditions:

The following are the optimum extraction conditions and were used in all further work:-

i) the extraction time was one hour using 4% NaCl as a solvent;
ii) the S : L ratio of 1 : 8 was used;
iii) the nominal maximum particle size of minced FW was 0.04 cm diameter
iv) the rotary shaking speed was 200 rpm and 250 cm^3 of extraction solvent were used;
v) the centrifugation of suspended FW was carried out at 1000 x g for 5 minutes.

The tests which were mentioned in sections 3.2.1. - 3.2.9 were performed at least in duplicate and two samples from each run were analysed to obtain at least four observations. If higher volumes of fish protein extract were required, the weight of FW, volume of solvent and the shaking speed were multiplied by the ratio of increased volume to original volume.

3.2.11. Effect of Temperature and Storage Time of Minced FW on Protein Extractability

The cod filleting wastes were cleaned with potable water to remove any dirt or residual blood and minced as described in section 3.2.1. The minced FW was mixed thoroughly to provide uniformity in the samples. The samples were then divided into lots, of approximately 70g, placed in polyethylene bags and kept in plastic containers for storage at $0^{o}C$, $-15^{o}C$ and $-30^{o}C$.

The first test was performed as soon as the FW samples were delivered, and then repeated at 10 day intervals. When the test was performed, samples of 50 g of frozen FW were taken from each lot and separately suspended in 400 cm^3 of NaCl and extracted at a shaking speed of 320 rpm.

The other extraction conditions and the anlysis were as mentioned in section 3.2.10.

The following analysis was carried out:

i) Total soluble protein ;
ii) NPN content of minced FW;
iii) NPN content of soluble protein extract;
iv) the free fatty acid value of minced FW. The analytical methods are described in sections 3.6.2 3.6.4.

3.2.12. Effect on the Yield of the Amount of Fish Flesh in FW

The test was carried out to examine the effects of the amount of fish meat remaining on the skeleton after the fillet removal on the soluble yields from FW.

Fresh iced cod fillets were purchased from local retail sale outlets in Loughborough and minced as described in section 3.2.10. This minced material replaced 0, 5, 10 and 15% of the FW and the mixtures were extracted using the orbital shaker and conditions specified in section 3.2.10.

3.3. The Concentration and Desalination of Fish Protein Extract

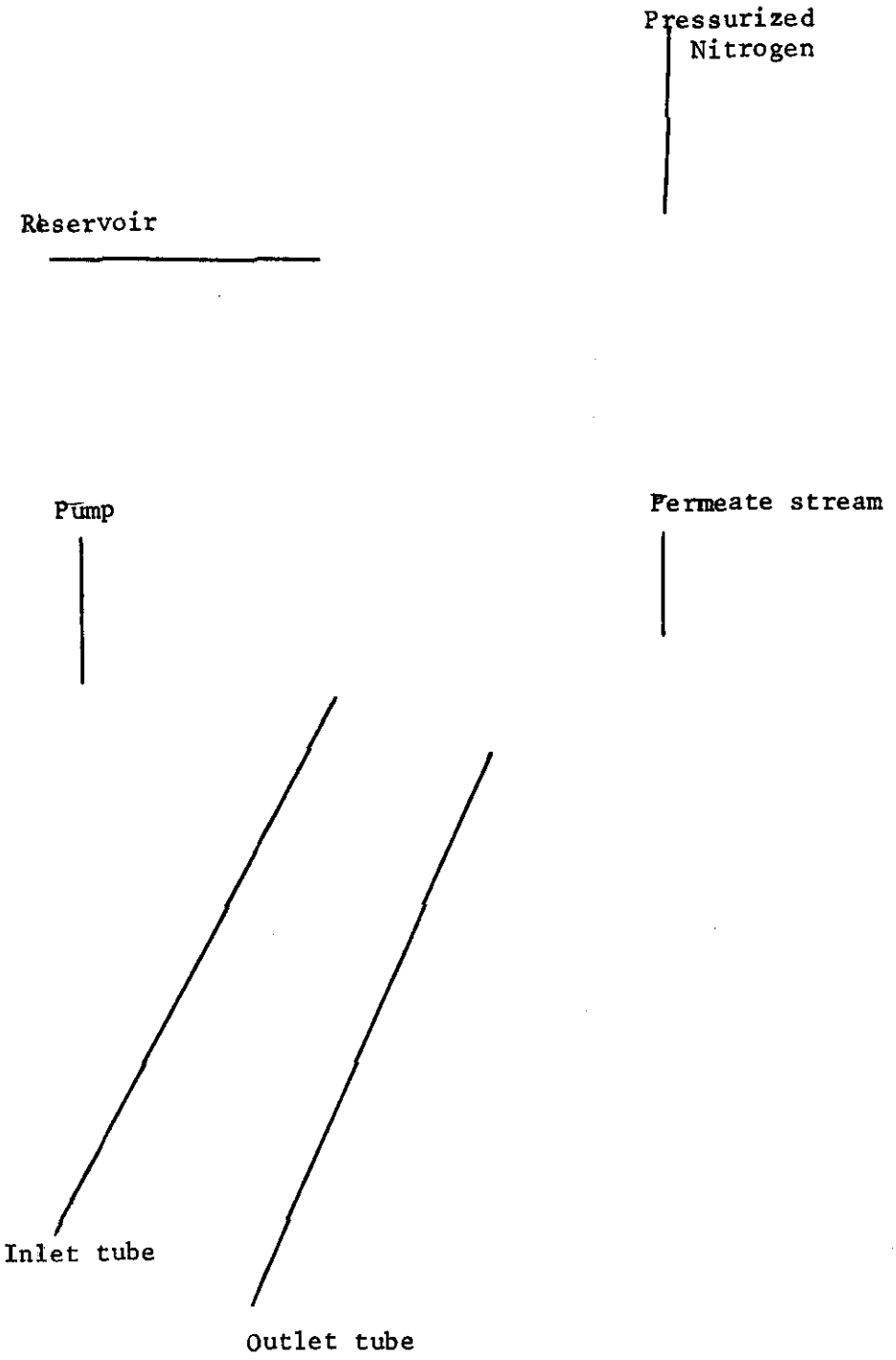
3.3.1. Bench Scale Concentration Process

The soluble protein extract was prepared as described in section 3.2.10.

The concentration process was performed by using Amicon ultrafiltration unit TCF-10 which supplied by Amicon, Lexington, Massachusetts, USA.

The unit is supplied by a 1 litre reservoir and a membrane diameter of 90 mm is used. The unit is fitted with a Sartorius pump SM 16896 supplied by Sartorius - Membrane-filter GMBH - Postrach 142 - Federal Republic of Germany. The pump had a flow rate of 1.5 litre/minute.

Figure 3.2 illustrates this unit.


Pressurized
Nitrogen
Reservoir
Pump
Permeate stream
Inlet tube
Outlet tube

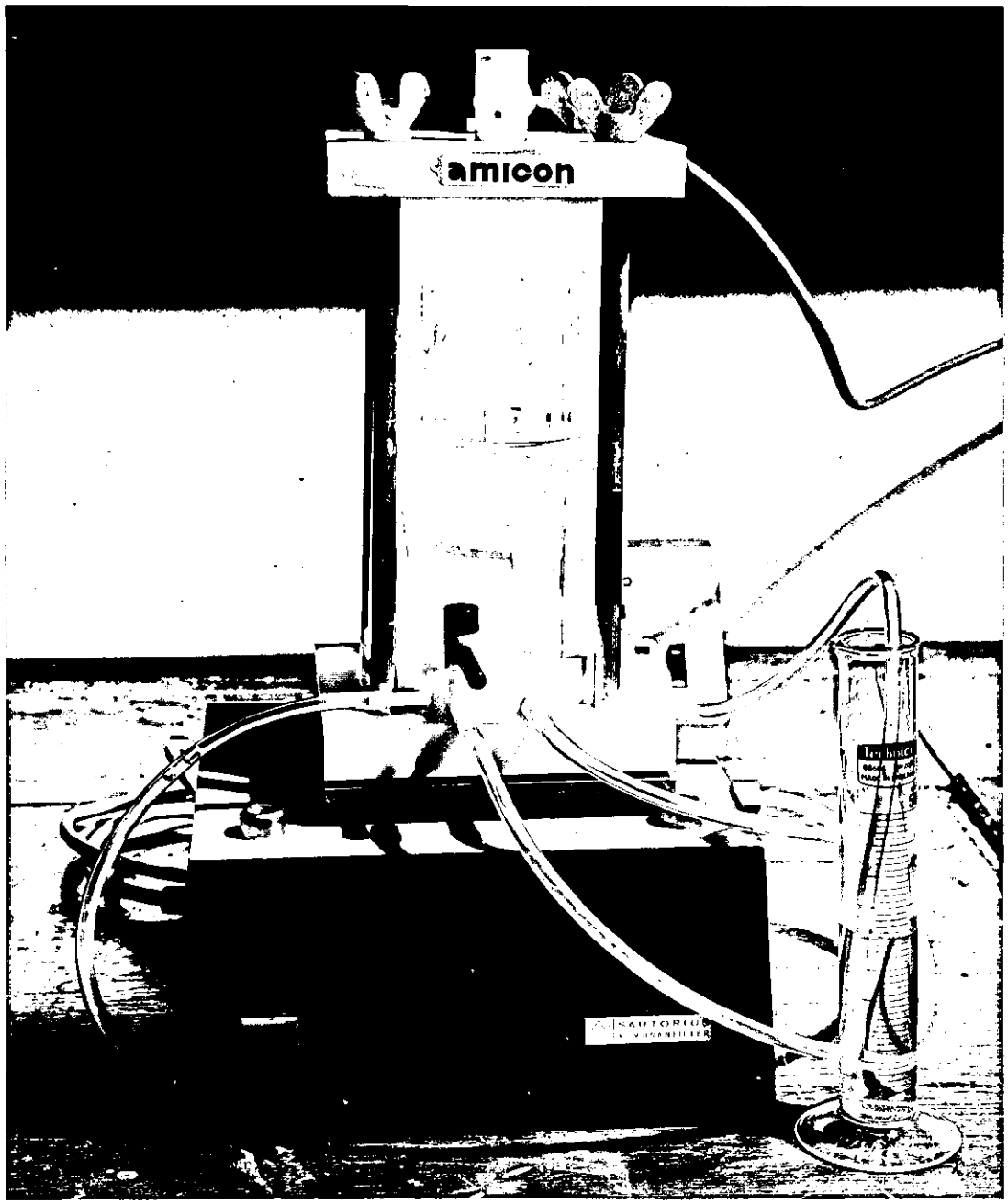


Figure 3.2 Amicon TCF-1 0 Ultrafiltration Unit.

3.3.2. The Desalination Process:

Desalination was performed by using the apparatus specified in section 3.3.1.

The soluble and concentrated fish protein extract was desalted by diafiltering with volumes of distilled water, which can be calculated according to the following relationship:

NaCl% in FPC x volume of FPE = NaCl% required x final volume of extract.

FPE = Fish protein extract

The final volume of extract = volume of FPE + volume of H_2O

3.3.3. Effect of Membrane Cut-Off on the Flux

The procedure mentioned in section 3.2.10 was used to prepare two litres of fish protein extract which contained 0.43% protein. The extract was divided into four lots. Two different membranes were used, each ultrafiltering two lots. The membranes PM-30 and UM-10 which had molecular mass cut-offs of 30,000 and 10,000 respectively.

The UF unit mentioned in section 3.3.1 was used.

3.3.4 Effect of pH on the Flux Rate

The procedure mentioned in section 3.2.10 was used to prepare four litres of fish protein extract which contained 0.52% protein.

The tested pH values were 3.5, 4.5, 6.0, 6.8 (normal) 8.0, 9.0, 10.0 for each pH value, 450 cm^3 was taken from the original fish extract and 0.5 N-NaOH or 0.5 N-H_2SO_4 were used to adjust the required pH. The final volume was made 500 cm^3 to give the same protein concentration in all tested samples.

Each lot was separately ultrafiltered through a PM-30 membrane using the UF unit described in section 3.3.1.

It was not possible to run duplicate samples in this test because of the difficulty in preserving the product over a period of one week.

However, second experiments were performed with different protein solution, the results of which agreed with the results obtained from the test described above.

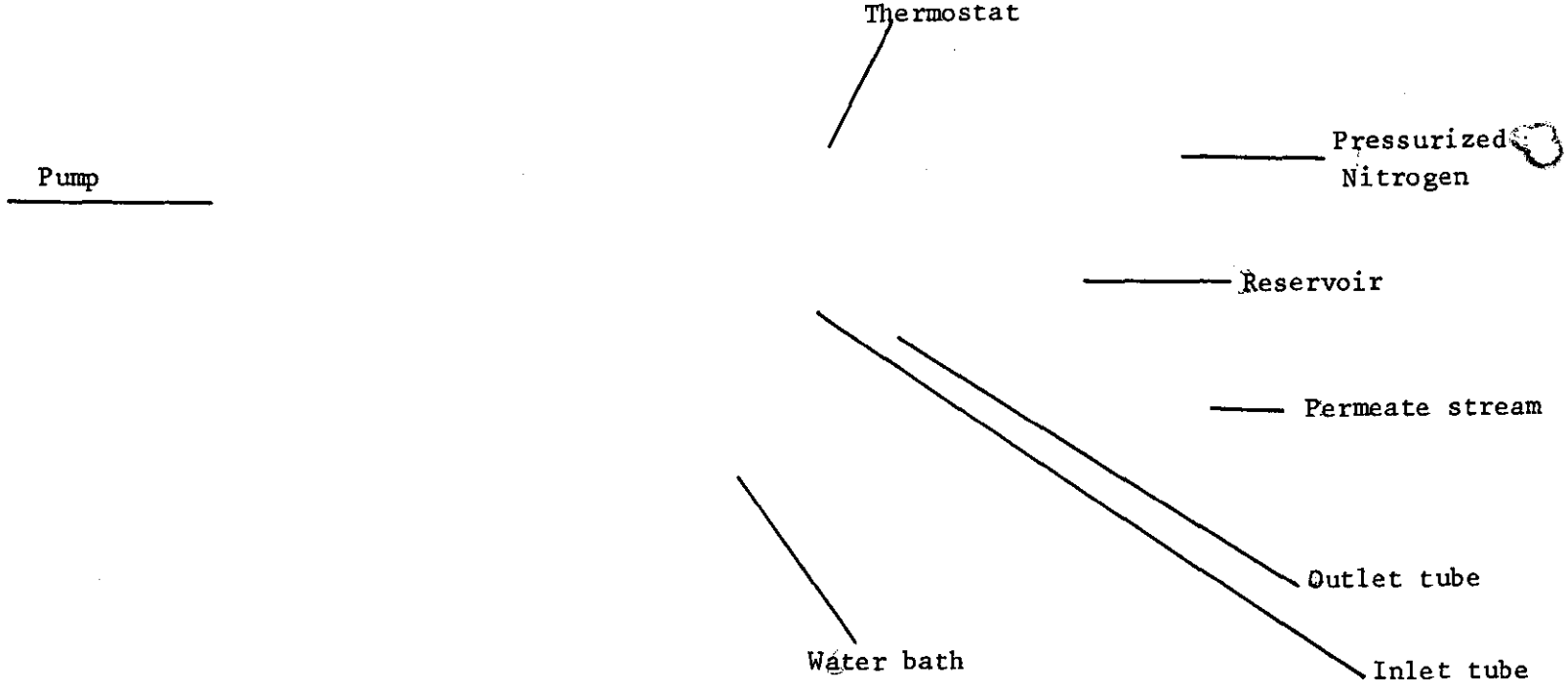
3.3.5. Effect of Temperature on Flux Rate

The UF unit described in section 3.3.1. was fitted with coiled stainless steel and rubber tubes between the pump and the reservoir. A thermometer was also fitted on the inlet tube to the UF unit as shown in Figure 3.3.

The coiled and rubber tubes were placed in a water bath fitted with a thermostat.

Some 1.5 litres of fish protein extract was prepared as mentioned in section 2.2.10 and then divided into six lots of 500 cm^3.

Each lot was separately introduced to the UF unit. The liquid was firstly pumped around the equipment circuit for 10 minutes to adjust to the required temperature and then the UF process commenced.


Thermostat
Pressurized
Nitrogen
Pump
Reservoir
Permeate stream
Outlet tube
Water bath
Inlet tube

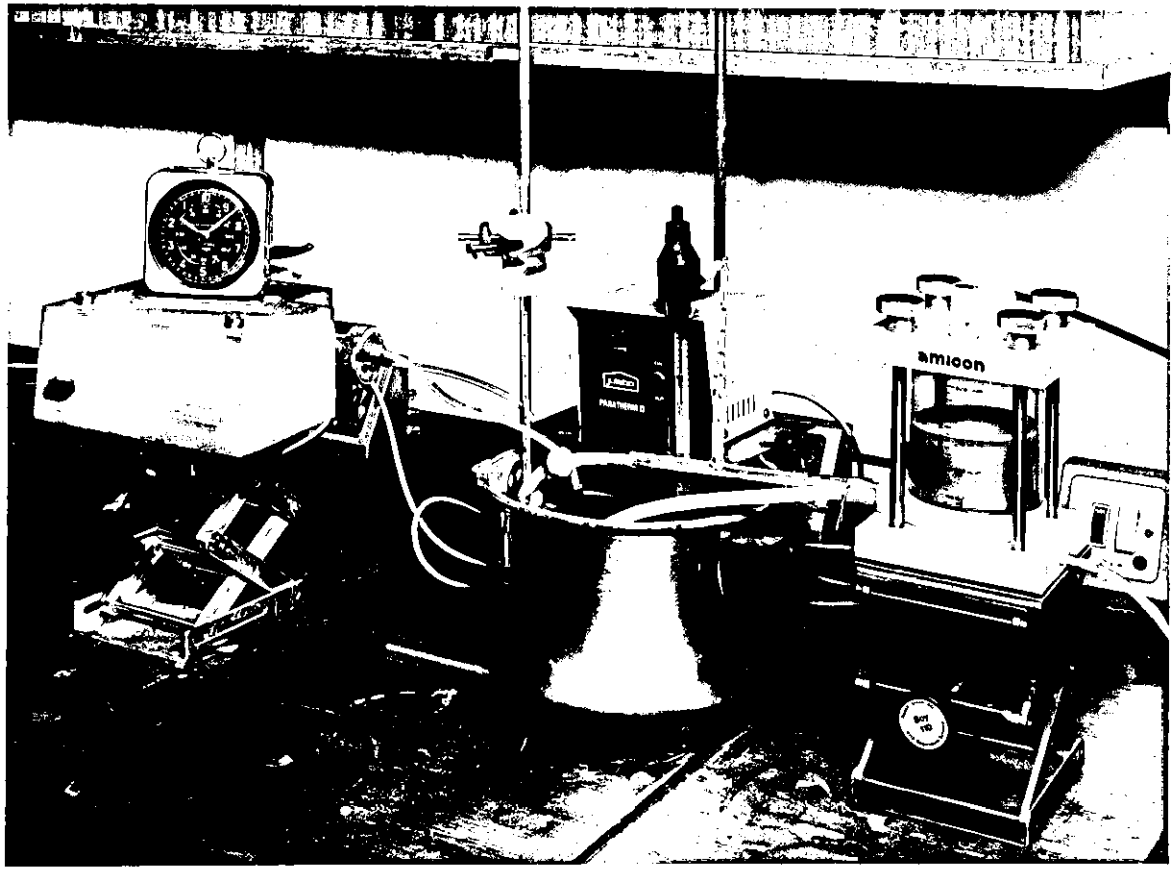


Figure 3.3 The Modified TCF-10 Ultrafiltration Unit.

3.3.6. Preparation of FPC on Bench Scale

i) Some 340 cm^3 of concentrated fish protein extract, which contained 1.63% protein and 3.65% NaCl was prepared as previously described in section 3.3.1.

ii) The drying process was performed in a rotary evaporator apparatus at 40°C for five hours.

iii) The approximate contents of the dried product on a free water basis were as follows:

Protein	29 - 35 %
Fat	4.95 - 5.62 %
Ash	55.87 - 61.27%

iv) The analytical methods used to determine protein, fat and ash are described in sections 3.6.2 and 3.6.6.

3.3.7. Preparation of FPC on Pilot Scale

3.3.7.1. Preparation of soluble fish protein extract

i) Some 12.5 kg of freshly minced FW was suspended in 100 dm^3 of 4% NaCl.

ii) The extraction procedure was carried out in a 600 litre stainless steel vessel fitted with a mechanical stirrer driven by electric motor. The extraction process was continued for one hour.

iii) The unextracted FW was left to settle for 15 minutes, and the soluble protein extract was drawn by means of a pump and centrifuged by using a continuous centrifuge to remove remaining suspended particles present in the extract. If necessary the extract was re-centrifuged.

iv) The soluble extract is now ready for the UF process.

3.3.7.2. The Concentration and Desalination Processes

The test was carried on a "PC1" pilot scale ultrafiltration unit supplied by Paterson Candy International Ltd, Whitchurch Hampshire - England.

The unit was modular in design, with each module containing 18 tubular membranes each 1.2 cm diameter mounted in series (Figure 3.4)

The modules were surrounded by a tubular plastic shroud and there was a permeate outlet in the module shroud. The end caps contained feed inlet and outlet to individual membrane tubes. and also fulfilled the function of connecting the 18 tubes in series.

Two modules were used. Each individual membrane measured 1.2 cm in diameter and 2440 cm in length. The total area of the membranes was 3.4 square metres. The membranes were of cellulose acetate and had a cut-off of a molecular mass of

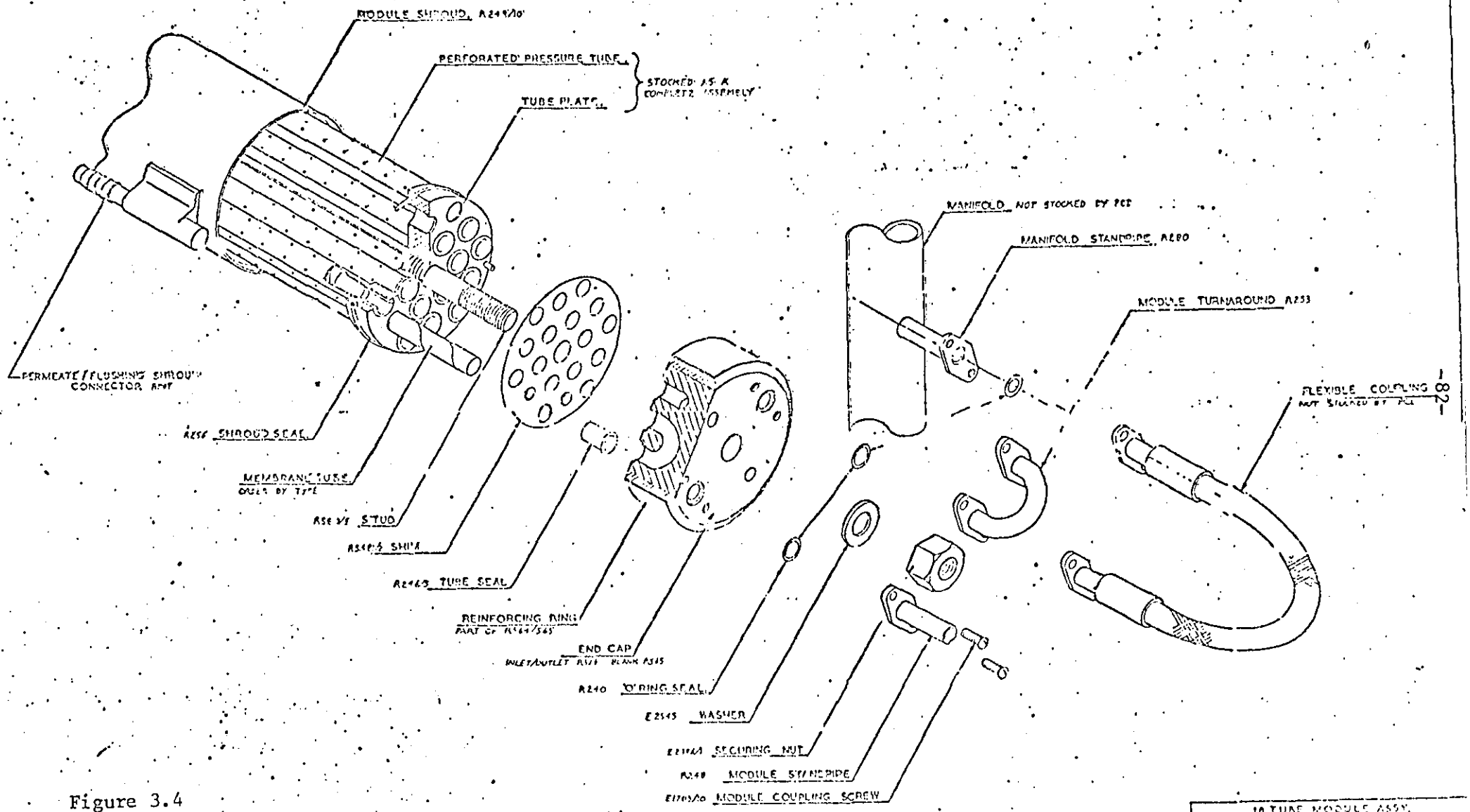


Figure 3.4

Exploded View of a PCI Ultrafiltration Module
(this Figure is taken from PCI brochure)

approximately 20,000 (Type T5A).

In this test, the soluble fish protein extract was fed from a container through a pump via the heat exchanger to the modules. The unit was run at $30^{o}C$, a flow rate of 13.5 litre/min and 14.2 kg/cm^2(130 psi).

2.3.7.3 The Drying Process

The concentrated soluble fish protein extract was dried by means of an accelerated freeze dryer, which was manufactured by Vickers-Armstrong Ltd - South Marston Works - Swindon - Wiltshire - England.

The dryer was operated at $-30^{o}C$ and a vacuum of 10^{-1} Torr.

3.4 Estimation of the Functional Properties of FPC

3.4.1. The Solubility Test:

The test comprised the following steps:

i) determine the total protein content (P_1) of FPC (N x 6.25) by Kjeldahl method as described in section 3.6.2;

ii) suspend 1 gram of FPC in 100 cm^3 of distilled water (or NaCl solution or buffer solution). The preparation of these solutions is mentioned in section 3.6.1.;

iii) agitate the suspension by means of a mechanical stirrer for 45 minutes in a water bath at 30°C;

iv) centrifuge at 1000 x g for 5 minutes;

v) determine the protein content (P_2) of the soluble extract by the Kjeldahl method;

vi) calculate the solubility according to the following equation:

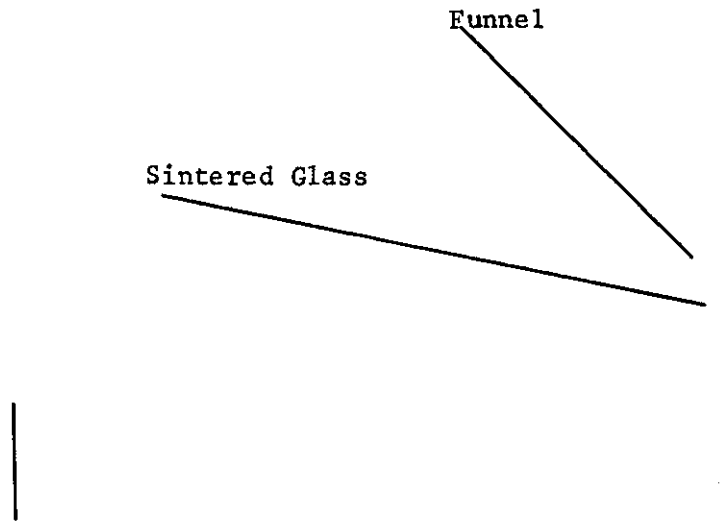
$$\text{Solubility}(\%) = \frac{P_2}{P_1} \times 100$$

The test was performed in duplicate

3.4.2. The Swelling Test:

The test was carried out to measure the spontaneous uptake of water by FPC. The apparatus used in this test was a modification of that used by Hermansson (1972) and consisted of a funnel fitted with a sintered glass support for the filter paper on to which the samples were introduced.

The funnel was connected to a graduated glass capillary tube. Figure 3.5 shows the apparatus.


Funnel
Sintered Glass
Capillary tube

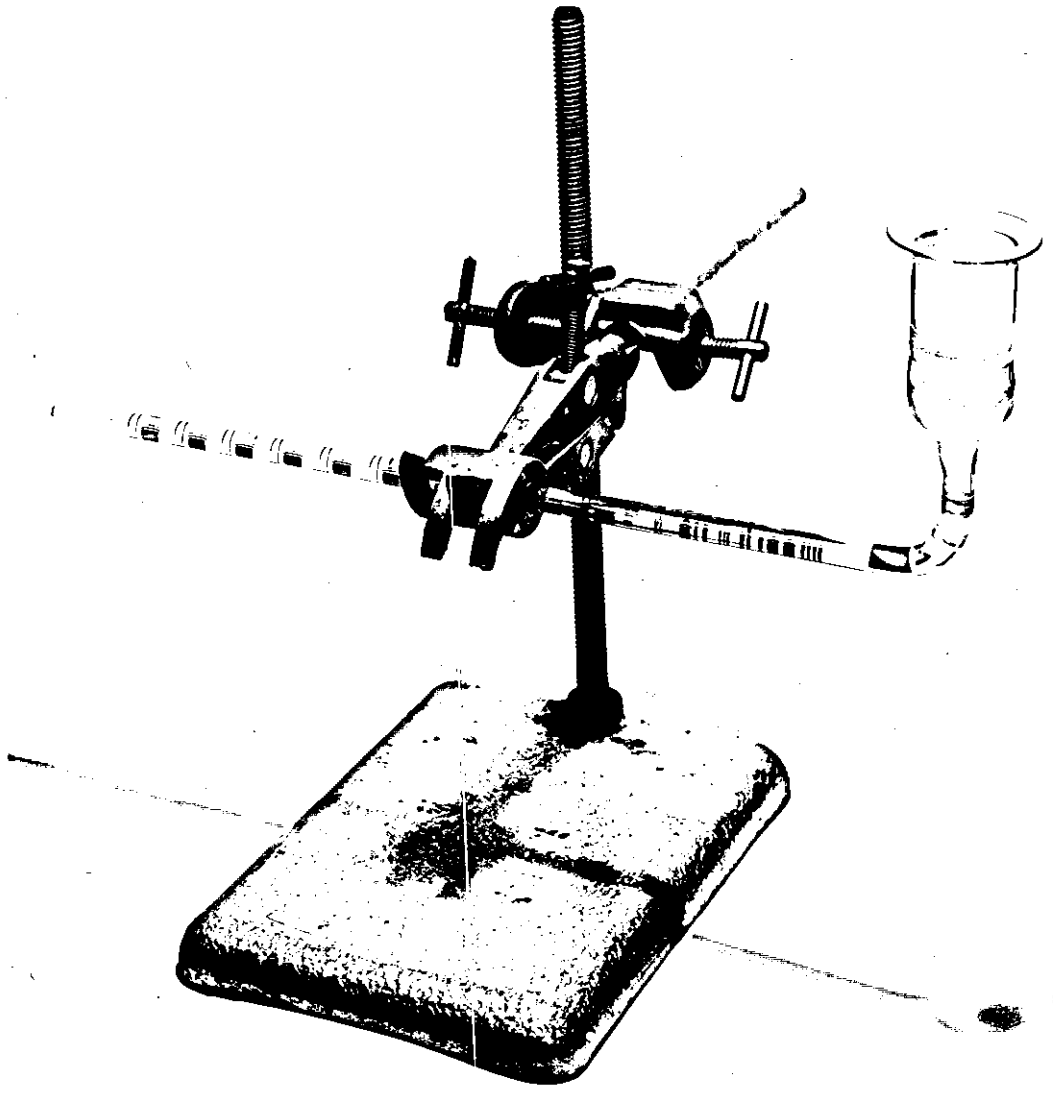


Figure 3.5

The Apparatus For Measuring The Swelling Capacity.

Method:

i) fill the apparatus with distilled water and remove any air bubbles;

ii) place the filter paper (Whatman No.4) in the funnel;

iii) introduce the sample as evenly as possible;

iv) cover the funnel with a watch glass to reduce evaporation;

v) record the volume of absorbed water after 30 minutes.

It had been observed that no further uptake occurred between 30 minutes and 5 hours;

The tested weights of FPC were 50, 100, 150, 200, 250 mg, and a minimum of 10 readings were obtained (at 25°C) on each sample.

3.4.3. The Heat Coagulation Test:

The percent of coagulated protein is defined as the percent of protein from the total soluble protein that will coagulate upon heating to 100°C.

The tests described below are modifications of a method developed by Kwee (1970) which depends on measuring the optical density of the purple colour which developed as the Biuret reagent (see section 3.6.1.) reacted with the peptides in the protein solution.

The method consists of the following steps:

i) prepare the soluble fish protein extract as mentioned in section 3.4.1.

ii) determine the protein content in the soluble extract by Kjeldahl method;

iii) pipette 15 cm^3 of the soluble extract into a test tube, heat for 30 minutes in a 100°C water bath, cool to about 25°C and centrifuge at 1000 x g for 15 minutes. Pipette 2 cm^3 of the supernatant liquor to each test tube and add 8 cm^3 of Biuret reagent to each test tube. Keep in the dark for 30 minutes. The test was done in duplicate.

(In some experiment different proportions of soluble protein extract and Biuret reagent were used as will be mentioned in the text).

iv) pipette 2 cm^3 of soluble extract into each test tube, add 8 cm^3 of Biuret reagent and keep in the dark for 30 minutes.

v) measure the optical density (O.D.) or absorbance at wave length 540 mm for solutions mentioned in steps iii, iv by using SP6-400 UV spectrophotometer.

vi) calculate the coagulation percent according to the following equation:

$$\text{Coagulation}(\%) = \frac{\text{O.D. before heating} - \text{O.D. after heating}}{\text{O.D. before heating}} \times 100$$

Samples of blood albumin were also examined for comparison

3.4.4. The Emulsion Test:

Part One

i) Some FPC samples ranging between 1 and 5 gram were separately blended for 2 minutes with 50 cm^3 of distilled water and 10 cm^3 of corn oil.
Two layers were formed. The upper was the foam layer and the lower was the cream layer.
When the cream layer separated two new layers were formed, namely the emulsion and water layers. Figure 3.6 explains these layers schematically.

ii) The mixtures were separately placed in 100 cm^3 graduated cylinders and the volumes of foam, emulsion and water layers were measured at intervals.

Samples of soy bean concentrate and egg albumen flakes (BDH Chemicals Ltd.- Poole - Dorset - U.K.) were also examined in the same manner for comparison.

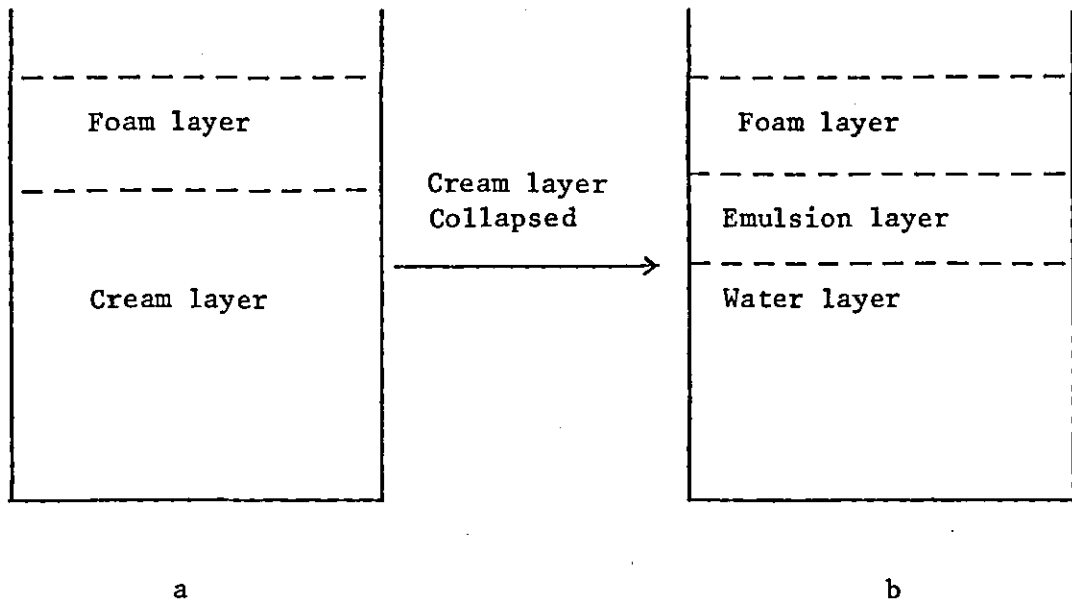


Figure 3.6 Layers formed after blending FPC, oil and water

a) After blending

b) Collapse of cream layer

Part Two:

The experiments described above were repeated using different volumes of corn oil with 5 grams FPC and 50 cm^3 of distilled water.

All experiments were carried out at temperatures of 25°C.

3.4.5. Prepration of Mayonnaise

Mayonnaise is an example of an oil in water emulsion. This experiment was carried out to test the performance of FPC as a partial replacement for egg yolk in mayonnaise preparation.

The basic recipe for mayonnaise preparation was as follows, (Patten, 1972).

2 egg yolks (35 grams)
½ - 1 teaspoon English or French mustard
1/4 - ½ teaspoon salt
good shake pepper
pinch sugar.
142 cm^3 corn oil
1-2 tablespoons vinegar
1 tablespoon water

Two batches of mayonnaise were prepared, the first as described above, the second containing 10 grams of FPC in place of 10 grams of egg yolk.

Method

i) Put the egg yolks, seasoning and sugar into the mixing bowl of a domestic food mixer. Whisk well and add the corn

oil gradually drop by drop, beating all the time.

ii) When the oil has been incorporated, whisk in the vinegar, then add the boiled water gradually to give a very light creamy texture.

In the mayonnaises containing FPC, the same method was followed except that the FPC was added after all the oil was incorporated. Mayonnaises were stored at 5°C, and examined for character and stability at intervals of approximately 7 days, for six months.

The approximate protein and fat content of the above mentioned recipes are calculated and shown in Table 3.2 as follows:

	Fat (g)			Protein (g)		
	Yolk	FPC	Total	Yolk	FPC	Total
Mayonnaise	10.85	0	10.85	6	-	6
Mayonnaise + FPC	7.75	0.03	7.78	4.25	6	10.25

Protein and fat are present in egg yolk at 17% and 31% respectively (Gaman & Sherrington, 1977).

Protein and fat are present in FPC at 60% and 5% respectively.

Table 3.2 The approximate fat and protein content of Mayonnaise and Mayonnaise - FPC recipes.

3.4.6. Preparation of Meringues:

Meringues are an example of a solid foam, normally stabilised by denatured egg white proteins or egg white substitute. In this investigation egg white was partially replaced by FPC to test the foam stability properties of the FPC.

The basic recipe for meringues preparation is as follows; (Beeton, 1963):

2 egg whites (60 g)
Pinch of salt
113 g of castor sugar

Method

i) Place egg whites and salt in a bowl, whisk until whites stand up in points
ii) Beat in 2-3 teaspoons of sugar, then lightly fold in remainder with continuous beating.
iii) Place the meringues on a baking tray, and bake in a very cool oven (about 130^{o}C) until dry and crisp.

In the meringues containing FPC, the same method was employed except that a quantity (5 gram) of FPC replaced the same weight of egg whites. The FPC was added after the sugar

had been incorporated into the mixture.

The approximate protein content of the above mentioned recipes is calculated and shown in Table 3.3.

Samples	Protein (g)		
	Egg albumin	FPC	Total
Meringues	6.0	-	6.0
Meringues + FPC	5.5	3	8.5

Protein present in egg white at 10% (Gaman & Sherrington, 1977).

Protein present in FPC at 60%

Table 3.3. The approximate protein content of meringue and meringues - FPC recipes

3.4.7. The Baking Test:

The test was carried out to examine the effect of FPC on bread quality. The basic recipe was as follows (Kent-Jones and Amos, 1967).

Hard commercial flour	1120 gram
Dried yeast	21 "
Sugar	5 "
Soft oil	7.5 "
Hard oil	0.5 "
Salt	21 "
Potassium bromate	25 milligram
Ascorbic Acid	84 "
Water	672 cm^3

Work in at 3.18 watt hour/kg (7 watt hour/lb) = 29.61 watts

Method

i) Dissolve the yeast and sugar in about 500 cm^3 of water at 43°C and hold at 38°C for 15 minutes.

ii) Place the flour in the dough mixer (Morton Machine Co. Ltd-Wishaw-Scotland). Add the yeast suspension and the salt, ascorbic acid and bromate dissolved in the residual water.

iii) Close the lid and mix for 30 seconds on the slow speed. Open, scrape down, pour the melted fat over the mix, close the lid and mix at high speed until the required work input has been attained.

iv) Open the mixer, tilt the mixing bowl and scale off 2 x 500 gram dough pieces into greased loaf tins. Proof for 45 minutes at 30°C.

v) Place the loaf tins in Simon rotary test baking oven (Henry Simon Ltd - Cheadle Heath-Stockport-England) and bake at 232°C for 25 minutes.

vi) Leave the loaf to cool down and measure loaf height and crust by means of a vernier caliper, and the cross-sectional girth by measuring tape, as explained schematically in figure 3.7. Also, assess the crub colour and texture by sense.

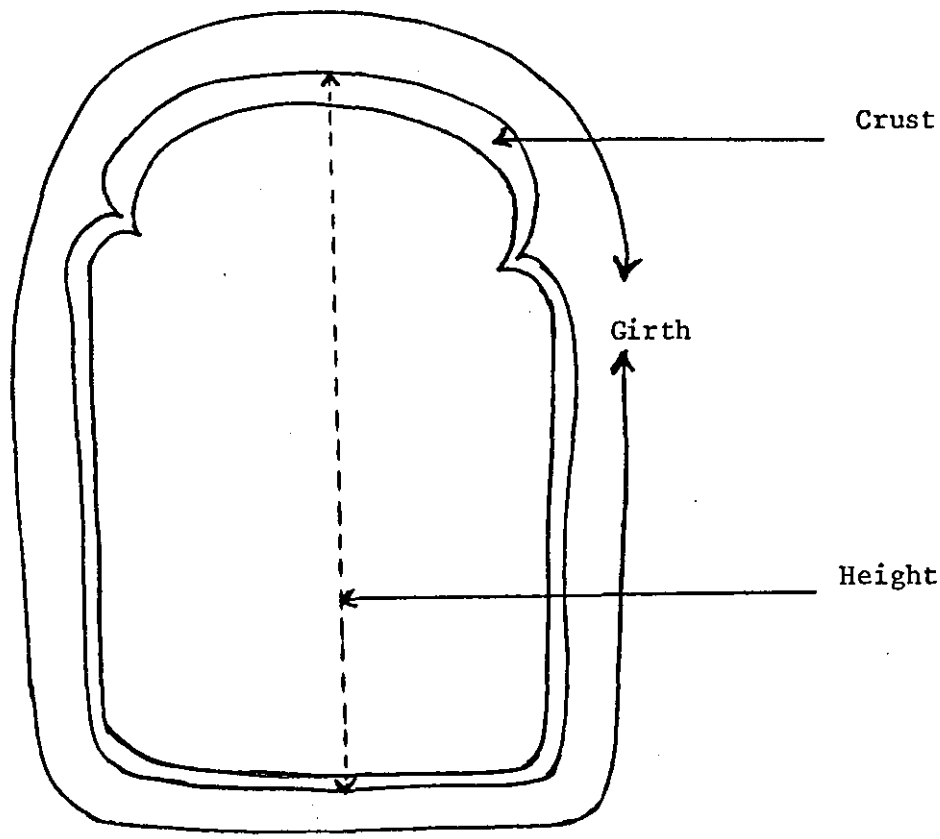


Figure 3.7 The loaf dimensions.

Four doughs were prepared, the first one as described above, while in the second, third and fourth doughs, 10, 20, 30 gram of flour respectively were replaced by similar weights of FPC.

The approximate protein and carbohydrate contents of the above four doughs are calculated and shown in Table 3.4

Sample No.	Weight of flour (g)	Weight of FPC (g)	Protein (g)			Carbohydrate (g)		
			Flour	FPC	Total	Flour	FPC	Total
1	1120	0	136.64	0	136.64	806	0	806
2	1110	10	135.42	6	141.42	799	0	799
3	1100	20	135.20	12	147.20	792	0	792
4	1090	30	132.98	18	150.98	785	0	785

Protein and carbohydrate are present in wheat flour at 12.2% and 72% respectively (Lillevik, 1970; Gaman and Sherrington, 1977).

Protein present in FPC at 60%

Table 3.4 The approximate protein and carbohydrate present in dough recipes.

3.4.7.1. The Extensibility of Doughs:

The extensibility and resistance of the doughs described in section 3.4.7 were tested by a Research Extensometer (Henry Simon Ltd-Cheadle Heath-Stockport-England) as follows:

i) Weigh out 2 x 100 gram of proven dough from each of the four samples described above.

ii) Introduce each dough separately to mixer shaper unit (Henry Simon Ltd-Cheadle Heath-Stockport-England) to obtain a uniform shape.

iii) Adjust the research extensometer and introduce each dough to measure the resistance and extensibility.

3.5 The Viscosity of Fish Protein Extract

An Ostwald viscometer was used to determine the viscosity of fish protein extract (FPE) as follows:

i) Adjust the water bath at $25^{o}C$.

ii) Fill a clean viscometer (size B) with 25 cm^3 of distilled water, place the viscometer in the water bath for 5 minutes and then determine the flow time of the water through the capillary tube taking at least three readings and find the mean t_1 .

iii) Take the specific gravity (ρ_1) and viscosity (μ_1) values of distilled water from Muller (1973).

iv) Dry the viscometer thoroughly

v) Determine the specific gravity (ρ_2) of FPE by using specific gravity bottle. Apply the following equation:-

$$\rho_2 = \frac{\text{weight of the bottle filled with FPE}}{\text{weight of the bottle filled with } H_2O}$$

vi) Repeat step ii but use FPE instead of water to find t_2

The viscosity of FPE (μ_2) can be determined by the following equation:

$$\frac{\mu_2}{\mu_1} = \frac{\rho_2 t_2}{\rho_1 t_1}$$

3.6 Analytical Methods:

3.6.1. Preparation of Solutions:-

3.6.1.1. Sodium Chloride Solution:

Table 3.5 shows the accurate weights of analytical reagent Sodium chloride used to prepare NaCl solutions.

Each weight was separately dissolved in distilled water and made up to 100 cm^3.

g NaCl	Concentrations	
	g NaCl in 100 cm^3 distilled water or NaCl%	Molar Concentration
1	1	0.17
2	2	0.34
3	3	0.52
4	4	0.69
5	5	0.86
6	6	1.03
7	7	1.21
8	8	1.38
9	9	1.55
10	10	1.72

Table 3.5 Preparation of NaCl Solutions

3.6.1.2. Calcium Chloride Solutions:

Table 3.6 shows the accurate weights of analytical grade calcium chloride used to prepare $CaCl_2$ soloutions. Each weight was separately dissolved in distilled water and made up to 100 cm^3.

g. $CaCl_2$	Concentrations	
	g $CaCl_2$/100 cm^3 H_2O or $CaCl_2$ %	Molar Concentrations
1	1%	0.13
2	2%	0.26
3	3%	0.40
4	4%	0.53
5	5%	0.66
6	6%	0.79
7	7%	0.93
8	8%	1.06
9	9%	1.19

Table 3.6 Preparation of $CaCl_2$ solutions

3.6.1.3 Buffer Solutions

Phosphate-citrate buffer solutions were prepared according to Vogel (1961) as follows:

i) Dissolve a mixture of 6.008 g of analytical grade citric acid, 3.893 g of analytical grade potassium dihydrogen phosphate, 1.769 g of analytical grade boric acid and 5.266 g of pure diethylbarbituric acid in distilled water and make up to 1 dm^3.

ii) Mix 100 cm^3 of the above solution with various volumes (x) of 0.02 N-NaOH (free from carbonate) to obtain the pH values tabulated below:-

pH	X(cm^3)
4.0	15.5
4.4	19.9
5.0	27.1
5.6	34.2
6.0	38.9
7.0	50.6
8.0	63.7

iii) Check the pH values with a pH meter and correct any deviation with 0.05 N-H_2SO_4 or 0.05 N-NaOH.

3.6.1.4 Biuret Reagent:

Dissolve 9 g of sodium potassium tartrate in 400 cm^3 of 0.2 N-NaOH. Add 3 g of ground hydrated copper sulphate, followed by 5 g of potassium iodide. Make up to 1 dm^3 with 0.2 N-NaOH (free of CO_2) (Diamond and Denman, 1966).

3.6.2 Protein and Non-Protein Nitrogen (NPN) Determinations:

Protein determinations were carried out by semi-micro Kjeldahl method which is described by Pearson (1970). The term protein refers to N x 6.25. The NPN determination involves protein precipitation by 10% Tri-chloro-acetic acid (TCA), e.g 1 cm^3 FPE or 1 g FW in 9 cm^3 TCA solution, and Kjeldahl determination on filtrate.

Apparatus:

i) Markham or Parnas and Wagner semi-micro Kjeldahl unit.

ii) Kjeldahl digesting racks.

Preparation of Reagents:

i) 10% TCA: Dissolve 10g TCA in 100 cm^3 of distilled water.

ii) Digestion catalyst:

25 g K_2SO_4 + 2g $CuSO_4$ + 1g SeO_2.

iii) 40% NaOH. Dissolve 400 g of sodium hydroxide in 1 dm^3 of distilled water.

iv) 2% Boric acid: Dissolve 20 g of A.R. boric acid in 1 dm^3 of distilled water.

v) Screened Methyl Red: 1 part of 0.2% methyl red + 3 parts of 0.1% bromo cresol green.

iv) 0.02 N - H_2SO_4 and 0.1 N - H_2SO_4:

Prepared from a ready made concentrated sulphuric acid supplied by BDH Ltd.

Procedure

i) The digestion: Transfer 1.00 cm^3 of FPE or (0.10 g FW) into a 30 ml Kjeldahl flask. Add 2.00 cm^3 of nitrogen-free concentrated sulphuric acid (5.00 cm^3 to the FW), and 0.8 g of catalyst mixture. Mix the flask contents carefully.

Initially warm slowly and carefully to minimize frothing, then increase the heat and boil until the solution clears, and continue boiling for a further 30 mintes.

ii) The Distillation

Transfer the digest into the steamed-out Markham or Parnas and Wagner unit, rinse the flask carefully to ensure complete transfer of the digest.

Make alkaline with 40% NaOH (about 15 cm^3), steam out the ammonia completely into 10 cm^3 of 2% boric acid containg 3 drops of screened methyl red. When the distillation is complete, lower the flask, distil for a further 2 minutes and rinse the condenser tip.

iii) The Titration:

Titrate the distillate with 0.02 N-H_2SO_4 (or 0.10 N-H_2SO_4 for FW) and calculate the protein content as follows

1.00 cm^3 of 0.10 N - H_2SO_4 = 0.0014 g Nitrogen

gN x 6.25 = g Protein

iv) Carry out a blank determination and subtract any Nitrogen present from the result obtained above.

v) Determine the efficiency of protein recovery using a protein standard and correct the results according to the value obtained.

3.6.3 Chloride Determination

The determination of chloride ion was carried out by titration with mercuric nitrate solution as explained in the following equation.

$$2\,Cl^- + Hg^{++} \longrightarrow \underset{\downarrow \text{ ppt.}}{HgCl_2}$$

The end point was detected by diphenyl carbazone which forms blue-violet compound with excess mercuric ion (Vogel, 1961).

Reagents:

i) Mercuric nitrate solution 0.02N: Dissolve 3.4 g of recrystallized mercuric nitrate $Hg(NO_3)_2.H_2O$ accurately weighed, in 800 cm^3 of distilled water to which 20 cm^3 of 2N-HNO_3 has been added. Dilute to 1 dm^3.

ii) Sodium chloride solution 0.1%: Dissolve 0.10 g of dry analytical grade NaCl in 100 cm^3 of distilled water.

iii) Diphenyl carbazone: Dissolve 0.10 g of pure diphenyl carbazone in 100 cm^3 of analytical grade ethanol.

iv) Nitric Acid 2N: Dilute 12.8 cm^3 of analytical grade nitric acid (relative density 1.42) to 1 dm^3 with distilled water.

Procedure

Take 5.00 cm^3 of 0.1% NaCl, add 3 drops of diphenyl carbazone and titrate with 0.02 N - $Hg(NO_3)_2$ until the first prominent blue-purple tinge.

Take the average reading of quadruplicate titres and work out the relationship between mercuric nitrate and sodium chloride which was found to be as follows:

1.00 cm^3 of 0.02 N-$Hg(NO_3)_2$ = 0.00127 g NaCl.

Notes:

In order to obtain clear and sharp end points as well as reproducable results, it is necessary to precipitate the FPE with 10% sodium tungstate and $\frac{N}{12}$ - H_2SO_4, then determine the chloride ion in the supernatent liquor. 1.00 cm^3 of sodium tungstate and 8.00 cm^3 of $\frac{N}{12}$ .H_2SO_4 were used to precipitate the soluble fish protein in 1.00 cm^3 of extract.

3.6.4. The Determination of Free Fatty Acids (Acid value)*

The method used was that described by Lees (1975) which consists of the following steps:

i) Weigh out approximately 1.00 g of minced FW.

ii) Disperse the sample in 50 cm^3 of hot neutralized ethanol.

iii) Titrate the mixture using 0.01 N-NaOH with phenol-phthalein as indicator, shake well during the titration and keep the solution warm.

iv) Calculate the content of free fatty acids as oleic acid by the following equation:

$$\text{Acid value} = \frac{0.561 \times \text{Volume of titre 0.01 N-NaOH}}{\text{weight of sample in grams}}$$

The test was performed at least in triplicate.

× This method may not be suitable for minced FW.

3.6.5 Estimation of the Number of Colonies

The methods described by the Society of American Bacteriologists (1957) and Hall (1975) were followed.

The test consists of the following steps:

i) Preparation of Plate Count Agar

Tryptone	5 g
Yeast extract	2.5g
Glucose	1g
Agar	15 g
distilled water	1 dm^3

Bring the above ingredients to the boil, adjust the pH to 7.0 and sterilize at 121°C for 15 minutes.

ii) Preparation of Dilutions:

A sterilized 1/4 strength Ringer solution was used to prepare fish protein extract dilutions, where 1 cm^3 of FPE was added to 9 cm^3 of 1/4 strength Ringer solution to prepare 10^{-1} dilution.

Further dilutions were prepared in the same manner up to 10^{-8}. Each dilution was prepared in duplicate.

iii) Inoculations:

The petri dishes were innoculated with 0.1 cm^3 of the appropriate dilutions. Samples were examined in quadruplicate.

iv) Incubation and Counting:

The plates were incubated at 15 and 30°C for 48 hours. Then the colonies in each of the appropriate dilutions were counted (i.e. those having between 30 - 300 colonies) The means of counts were multiplied by the dilutionfactor to obtain the number of colonies per cm^3 of solution. Aseptic techniques were employed throughout the test.

3.6.6. Determination of Calcium,Sodium & Magnesium:

The method employed for determination of calcium, magnesium and sodium was atomic absorption spectrophotometry (Robins, 1966).

3.6.6.1. Calcium

The soluble protein and phosphate in the extract were precipitated with 0.5% $LaCl_3$ in 10% TCA which had been prepared by dissolving 0.5 g of Lanthanum chloride in 100 cm^3 of 10% w/w TCA.

Lanthanum chloride was added to remove the effect of phosphate which tends to interfere with calcium ions and reduces the accuracy of the test.

Preparation of the Sample:

1.00 cm^3 of FPE was precipitated with 9.0 cm^3 of the above mentioned $LaCl_3$-TCA solution to give 1 : 10 dilution of calcium ions which fall within the range detected by the apparatus. The supernatant liquor was then ready for analysis. The same sample was also used for magnesium determination.

Preparation of Calcium Standards

A standard solution of calcium ions supplied by BDH, was used to prepare standard solutions which ranged between 0.01-0.1 mg % Ca^{++}. TCA-$LaCl_3$ solution was used to dilute the original stock solution.

3.6.6.2 Magnesium

Preparation of Standards

A standard solution of magnesium ions supplied by BDH was used to prepare the standard solutions which ranged between 0.01 - 0.1 mg % Mg^{++} $LaCl_3$-TCA solution was used to dilute the original stock solution.

3.6.6.3 Sodium

Preparation of the Sample

1.0 cm^3 of FPE was precipitated with 9.0 cm^3 of 10% TCA. If necessary a further ten-fold dilution was prepared.

Preparation of the Standards:

A standard solution of sodium ions supplied by BDH was used to prepare the standard solutions which ranged between 0.001 - 0.005 % NA^+.

A solution of 10% TCA was used to dilute the standard solution.

3.6.6.4. Atomic Absorption Apparatus and Method:

EEL - 140 - Atomic absorption spectrophotometer manufactured by Evans Electroselenium Ltd - Halstead - Essex-England.

Method

i) Set the atomic absorption spectrophotometer for each tested ion according to the manufacturer's instructions.
ii) Introduce the tested ion standards into the appartus and plot a calibration curve of ion concentration against the absorption.

iii) Introduce samples, record the absorption and refer to the calibration curve, then multiply by the concentration factor to determine the ion concentration in the sample.

iv) The tests were performed in duplicate, and the apparatus needs recalibration every day.

3.6.7. Determination of FPC Composition:

3.6.7.1. Moisture

The method which is outlined in Lees (1975) was used as follows:

i) Weigh out accurately 5 g of finely grounded FPC into a stainless steel dish.

ii) Place the sample in a vacuum oven and dry for four hours at 70°C with the pressure reduced to 30 mm Hg.

iii) Remove the sample, allow to cool in a desiccator and weigh.

iv) Repeat the drying procedure for a further thirty minutes, weigh and continue to dry to constant weight.

v) Calculate the moisture content from the weight loss from the sample. Six samples were examined for each determination.

3.6.7.2 Protein

The Kjeldahl method, as outlined in section 3.6.2, was used to determine the protein content of moisture-free FPC which had been prepared as described above in section 3.6.6.1

Six samples were analysed for each determination.

3.6.7.3 Ash

The ash content of moisture-free FPC was determined according to the method outlined in Lees (1975) as follows:

i) Weigh out accurately 2 g of sample into a porcelain crucible and char over a low bunsen flame.
ii) Ash at 550^{o}C until a white ash was obtained. Allow the sample to cool in a desiccator and then weigh.
iii) Ash for a further 15 minutes and reweigh after cooling Repeat if a significant decrease in weight is noticed. Six samples were analysed for each determination.

3.6.7.4. Fat:

The fat content of moisture-free FPC was determined according to the method outline by Lees (1975) as follows:

i) Weigh out accurately 5 g of powder sample directly into a fat-free extraction thimble and plug the end of the thimble with fat-free cotton wool. Place the thimble and contents into the central syphon portion of Soxhlet apparatus.
ii) Weigh a dried 250 cm^{3} glass joint flask and add 100 cm^{3} of chloroform.
iii) Connect the flask ot th Soxhlet unit and condenser. Reflux for five hours.
iv) Distil off the chloroform and place the flask and contents in an oven at 105^{o}C, dry for about three hours, cool the flask in a dessicator and weigh.

v) Reflux for a further thirty minutes and repeat step iv to ensure complete extraction of fat.

vi) The fat content can be determined from the weight of material held in the receiver flask. Six samples were analysed for each determination.

3.6.8. The Composition of FPC

The composition of FPC prepared on the pilot scale (section 3.3.7) were as shown below:

	Range	$\bar{x} \pm s$
a) Moisture	2.79 - 3.18	2.92 ± 0.15

b) On the above basis of free moisture content, the composition of FPC was found to be:

	Range	$\bar{\bar{x}} \pm s$
Protein	48.50 - 54.44	52.34 ± 3.01
Fat	2.19 - 7.33	5.59 ± 2.45
Ash	32.22 - 33.13	32.74 ± 0.40
NPN	0	

Chapter Four

Chapter Four

The Extraction of Fish Protein

4.1 Introduction

The aim of the work described in this chapter was to examine the extractability of Fish Waste (FW) proteins in salt solutions and to elucidate the role of the different factors which affect the extraction process.

In order to explain the mechanism of the protein extraction process, the composition and the charactersistics of fish muscle must be studied (see section 2.5.3.1.) Based upon this data, some 75% of the muscle protein remaining on the skeleton should theoretically be recoverable by an extraction technique using salt solutions of ionic strengths greater than 0.5.

The basic approach to the extraction process includes three steps:

i) Comminution of the fish waste.

ii) Solubilization of muscle protein by saline solution.

iii) Removal of the undissolved fish residue, e.g. by means of centrifugation and the recovery of soluble fish protein as supernatant.

The factors which affect the extraction process have been examined and are as follows:

4.2 Extraction with Water (see 3.2.1.)

Figure 4.1 shows the protein concentration in the supernatant (mean value and range) achieved by aqueous extraction. The maximum yield was obtained after 1 hour and this time of extraction was adopted for subsequent investigations. The decrease in yield beyond 4 hours presumably reflects the insolubilization of extracted protein.

4.3 Extraction with Saline Solutions (see 3.2.2)

The yield of soluble protein rose when sodium chloride was added to the extraction medium.

The best yield was obtained at a level of 4% NaCl as shown in Figure 4.2 and was about 46% higher than that obtained with water.

The protein yield extracted with water is also 16% less than that previously obtained in section 4.2 As both tests were conducted under the same operating conditions, this difference must have occurred as a result of batch variations in the cod FW (see section 4.10).

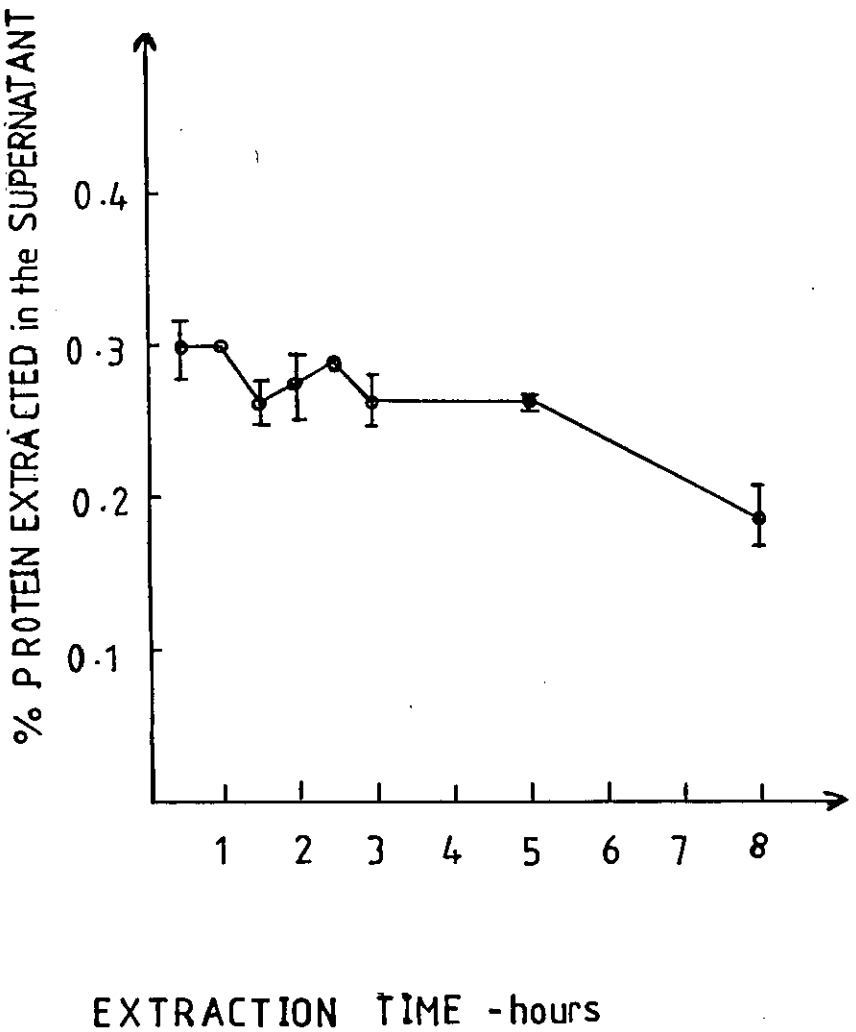


FIGURE 4.1 PROTEIN EXTRACTED FROM FW WITH WATER

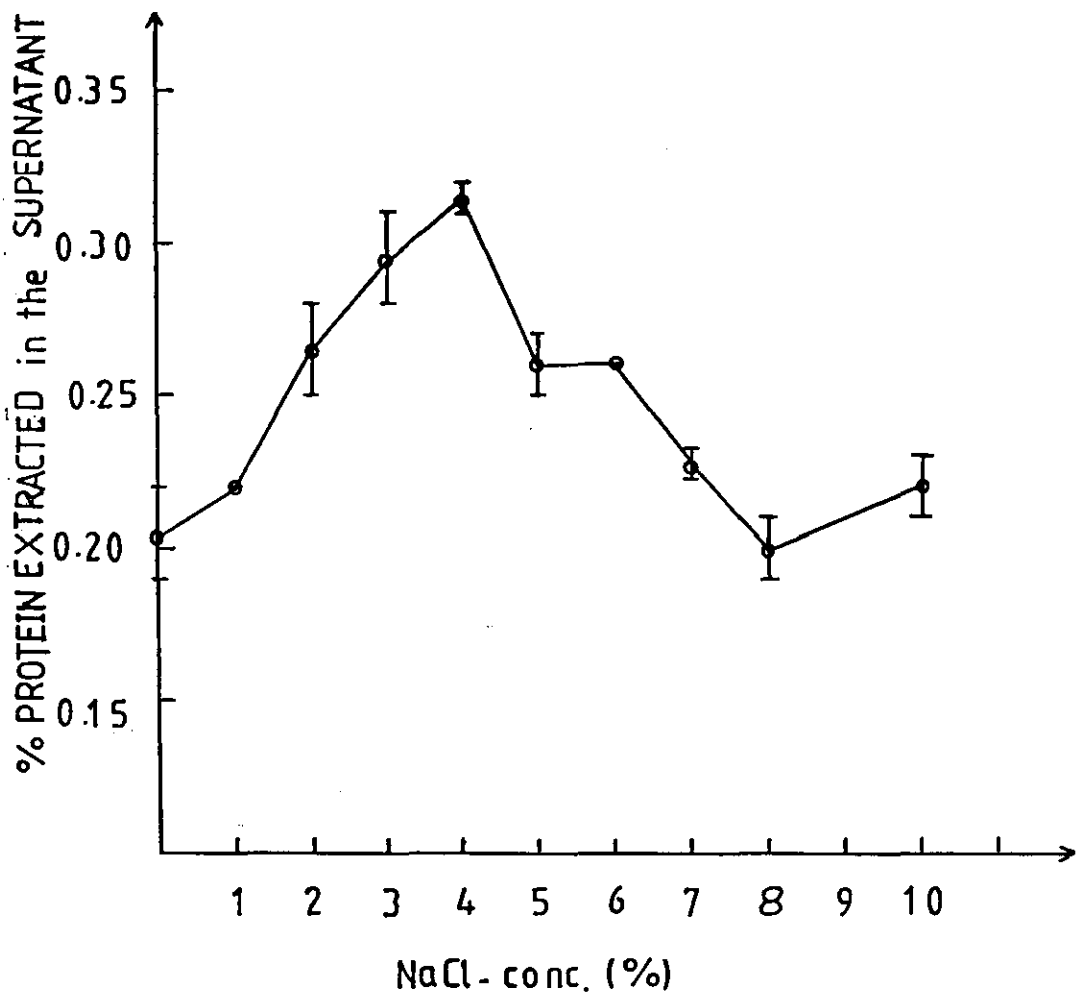


Fig. 4.2 Protein Extracted From FW With Saline Solution After 1 Hour

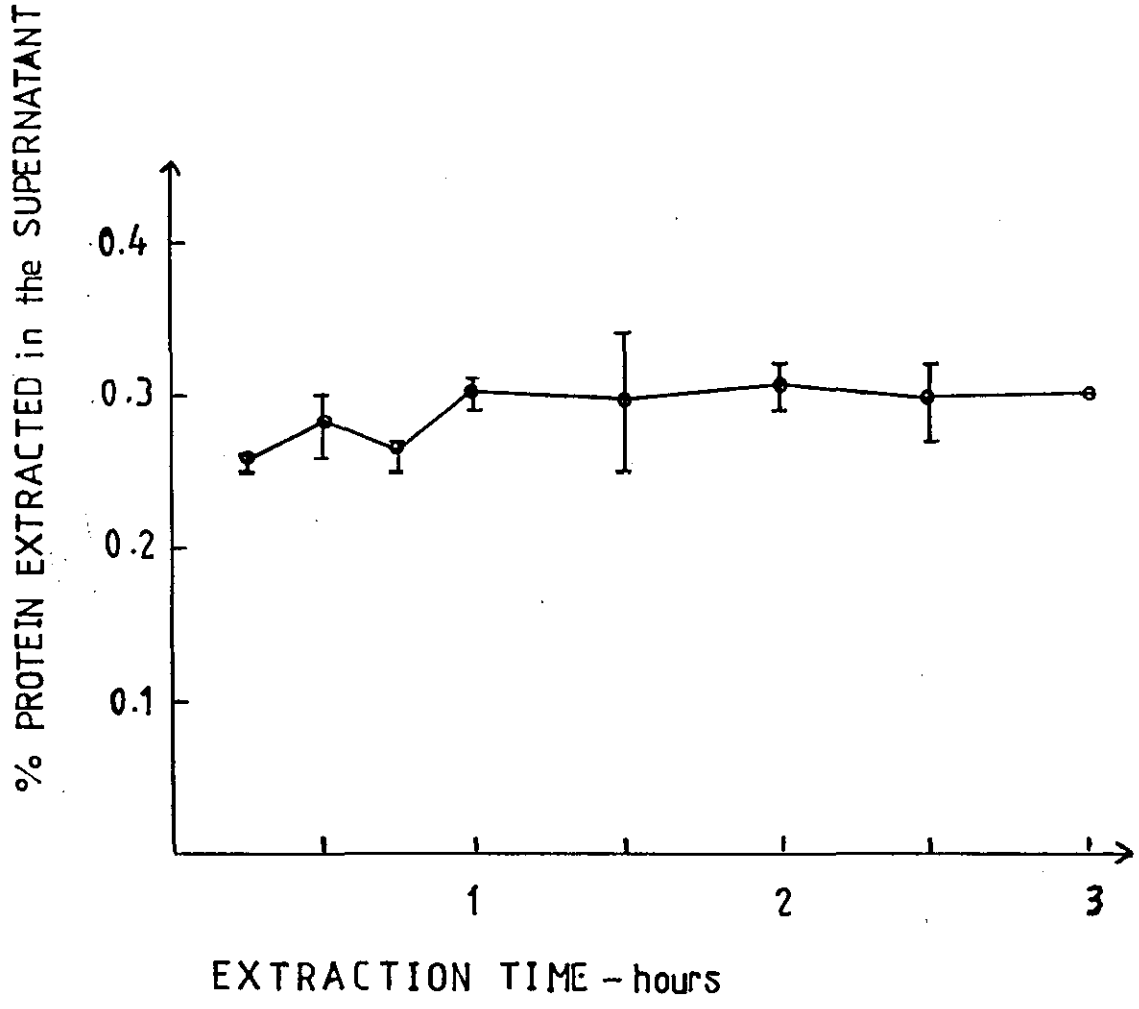


Fig. 4.3 Effect Of Extraction Time On Protein Yield From FW

The optimum extraction time for 4% NaCl solution was found to be 1 hour as shown in Figure 4.3.

In an attempt to improve the yield of soluble protein, multistage extractions were examined (see section 3.2.7). Of the total soluble protein extracted in these stages, 93% was extracted in the first stage, 5% in the second and 2% in the third stage. This is shown in Table 4.1. The result indicates that little extra protein was obtained by a second or third stage and it was therefore concluded that such additional stages would probably not be economic to operate.

Stage	Extracted protein (%) in Supernatant		Total Protein (%)
	Range	$\bar{X} \pm S$	
1st	0.25 - 0.27	0.26 ± 0.01	90
2nd	0 - 0.03	0.02 ± 0.2	7
3rd	0 - 0.01	0.01 ± 0.01	3

$\bar{X}$ = mean ; S = standard deviation

Table 4.1 Effect of number of extraction stages on protein yield from FW

4.4 Effect of Solid to Liquid Ratio on Protein Yield (see 3.2.3).

The results presented in Table 4.2 show that the optimum solid to liquid ratio to obtain the greatest yield of soluble protein from FW, was 1 : 8.

The 't' test (see appendix 1.1) showed that the 1 : 8 ratio is significantly higher in the yield than the other tested ratios, with 99% confidence (t = 5.81, under 4 degrees of freedom).

Solid:Liquid ratio	g Protein extracted from 100 g FW	
	Range	$\bar{X} \pm S$
1 : 20	2.8 - 3.00	2.90 ± 0.12
1 : 15	3.00 - 3.10	3.05 ± 0.07
1 : 12	3.08	3.08 ± 0
1 : 10	2.90	2.90 ± 0
1 : 8	3.25 - 3.13	3.21 ± 0.07
1 : 5	2.80 - 3.00	2.87 ± 0.12

Table 4.2 Effect of Solid : Liquid ratio on protein yield

4.5 The Extraction Temperature (see 3.2.4)

The soluble protein extracted in iced water at a temperature in the range 0^{o} to $10^{o}C$ was 0.23% as shown in Figure 4.4. At $10^{o}C$, the yield decreased to 0.19%. Above that temperature, the yield increased progressively, particularly in the range between 20^{o} and $30^{o}C$.

4.6 The Particle Size of Minced FW (see 3.2.5)

The effect of particle size of minced FW (as defined by the mincing mesh used) is shown in Table 4.3, in which the greatest yield was obtained from the smallest particle, the yield being about 40% greater than that obtained with the largest particle.

Sieve plate Aperture (cm)	Extracted protein (%) in the Supernatant	
	Range	$\bar{X} \pm S$
0.97	0.43 - 0.52	0.48 ± 0.06
0.44	0.54	0.54 ± 0
0.33	0.49 - 0.57	0.54 ± 0.04
0.24	0.73 - 0.77	0.75 ± 0.03
0.09	0.75	0.75 ± 0
0.04	0.81 - 0.82	0.82 ± 0.01

Table 4.3 Effect of the particle size of minced FW on the yield

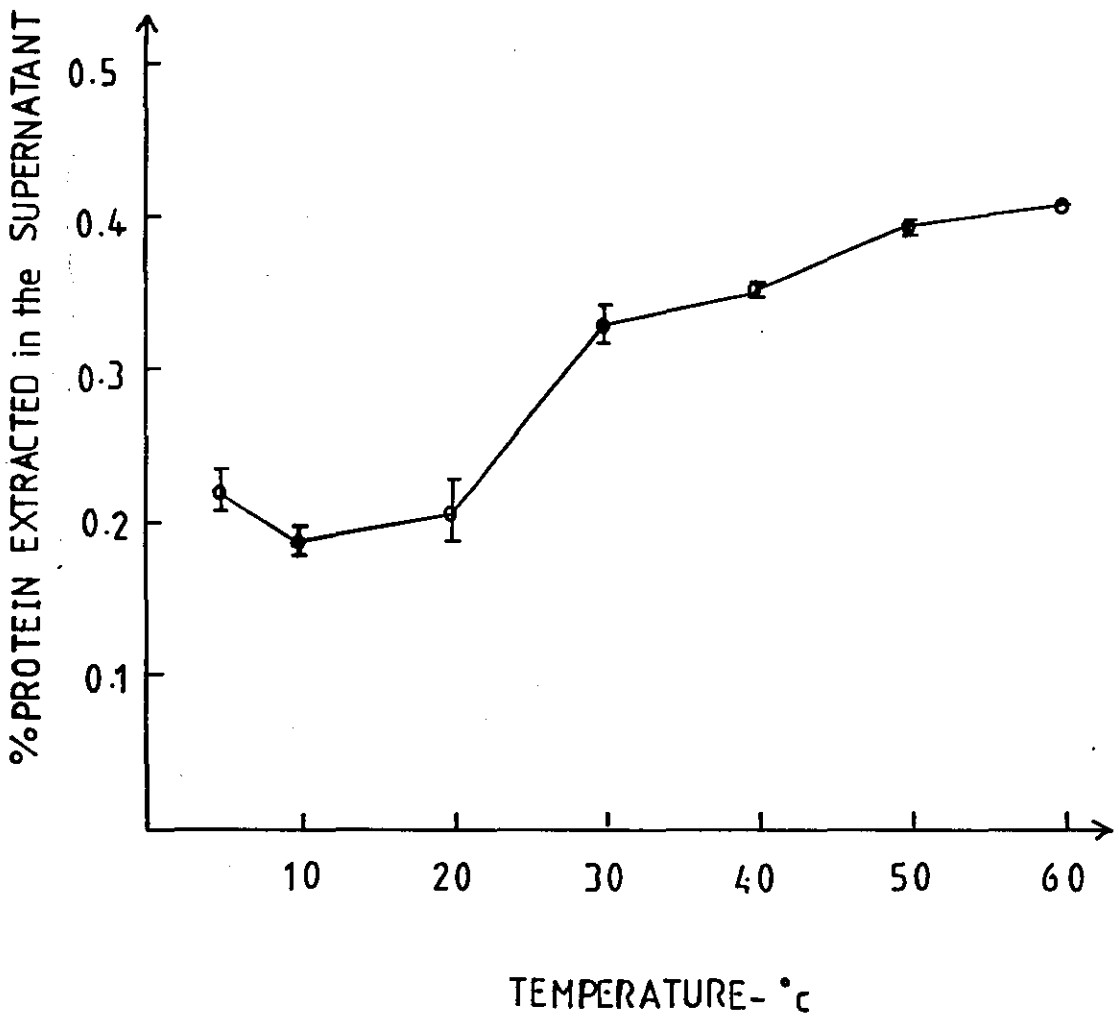


Fig. 44 Effect Of Extraction Temperature On Protein Yield From FW After 1 Hour.

4.6.1. Effect of Mincing on the Surface Area of FW:

It is assumed that the particle of FW produced by mincing is spherical in shape, and of uniform size.

Let the total mass of FW minced be M.

Then $M = n. \rho. \frac{4}{3} \pi r^3$ .....4.1.

where n = number of particles produced.

ρ = density of FW.

r = radius of particles.

or $n = M/\rho. \frac{4}{3} \pi r^3$ 4.2

and the total surface area is given by:

$S.A. = n. 4 \pi r^2$ 4.3

Substituting for n from equation 4.2:

$$S.A. = \frac{M. 4 \pi r^2}{\frac{4}{3} \rho r^3} \quad \text{.....4.4}$$

i.e. $S.A. = \frac{M}{\rho} \frac{3}{r}$

Hence the surface area is proportional to $\frac{1}{r}$

One would therefore expect, as a first approximation, that the amount of protein extracted from a given mass of FW would also be proportional to $\frac{1}{r}$. This relationship has been plotted in Figure 4.5, together with the data obtained above in section 4.6 (see appendix 1.2).

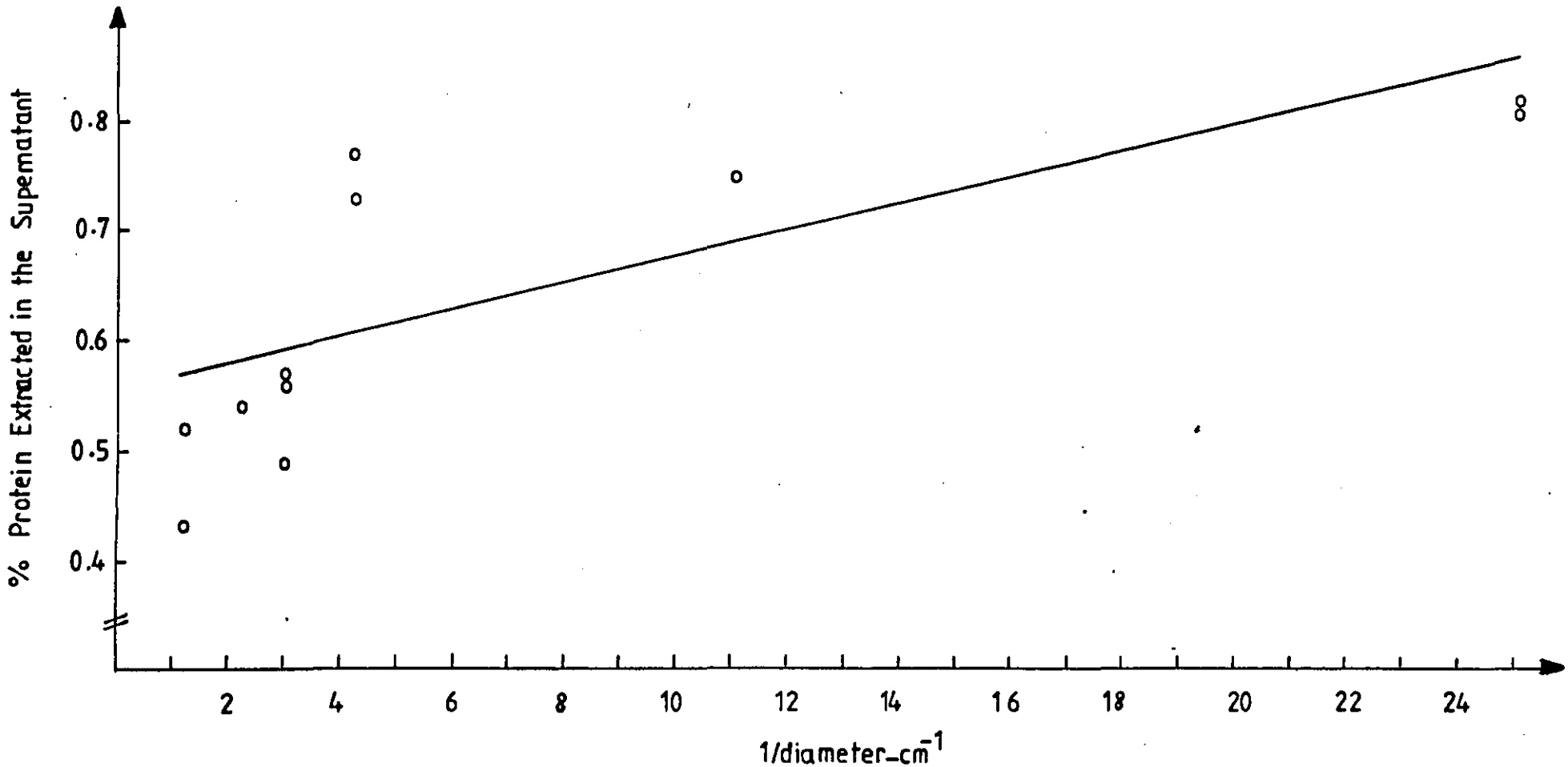


Figure 45 Effect of the Particle Diameter of FW on Protein Yield After 1 Hour

Whilst the trend of the experimental data does not contradict the above assumptions, little else can be said.

4.7 Effect of Extraction Apparatus and Shaking Speed on The Protein Yield:

4.7.1. Shaking Speed (see 3.2.6)

Figure 4.6 shows that the yield of soluble protein gradually increased with the shaking speed until it reached a maximum of 250 RPM in the orbital shaker. Above that speed froth was formed and apparently caused a slight reduction in protein recovery.

4.7.2 Extraction Apparatus and Protein Yield (see 3.2.9)

Table 4.4 shows that the amount of extracted protein from FW depended upon the form of agitation applied in the process.

The data showed a difference of 13% between the two techniques - The t-test (see appendix 1.3) was applied on the data and revealed that with 99.0% confidence, a difference exists between the two extraction methods ($t = 4.38$ under 6 degrees of freedom).

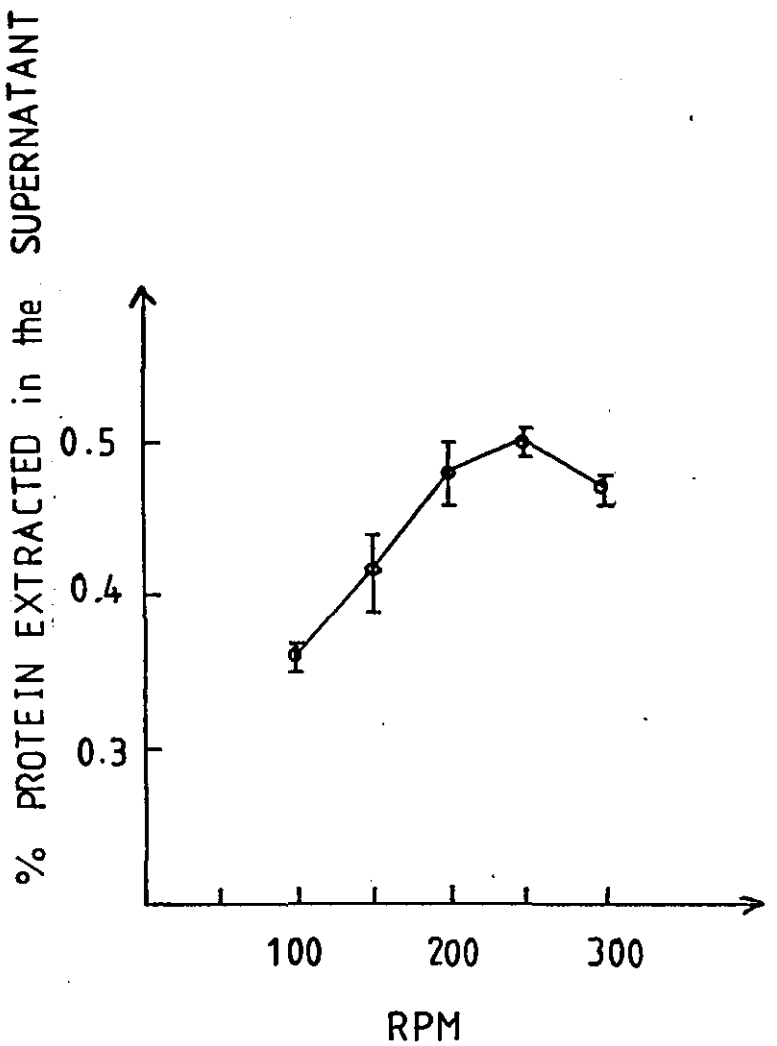


Fig. 46 Effect Of Shaking Speed On Protein Yield From FW After 1 Hour

Run	Extracted protein (%) in Supernatant	
	Rotary motion	Orbital motion
1	0.51, 0.53	0.43, 0.44
2	0.58, 0.56	0.44, 0.45
3	0.53, 0.55	0.53, 0.49
4	0.53, 0.53	0.49, 0.52
Mean $\bar{+}$ S	0.54 $\bar{+}$ 0.02	0.47 $\bar{+}$ 0.04

Table 4.4 Effect of extraction apparatus on protein yield from F.W.

In an attempt to improve the yield, a Silverson homogeniser was employed to extract fish protein. The results which are presented in Table 4.5 indicate an increase in the soluble yield. This increase was about 15% above the yield obtained using the previous extraction technique which had employed a rotary motion.

Run	Extracted protein (%) in the Supernatant	
	Rotary motion	The Silverson homogeniser
1	0.42, 0.43	0.46, 0.48
2	0.39, 0.41	0.47, 0.47
Mean $\bar{+}$ S	0.41 $\bar{+}$ 0.02	0.47 $\bar{+}$ 0.01

Table 4.5 Effect of using a homogeniser on protein yield from FW

4.8 Effect on the Yield of the Amount of Fish Flesh in FW (see 3.2.12).

Table 4.6 indicates that the addition of fish flesh to minced FW increased the soluble protein yield. The recovered protein progressively increased with the amount of fish flesh incorporated with FW up to 10%. Above that, the soluble protein yield apparently did not increase.

This presumably occurred because the extraction condition employed were only optimum to extract the soluble protein presents in FW.

Amount of fish flesh added (%)	Extracted protein (%) in Supernatant	
	Range	$\bar{X} \pm S$
0	0.37 - 0.40	0.39 ± 0.02
5	0.44 - 0.51	0.46 ± 0.03
10	0.46 - 0.50	0.48 ± 0.02
15	0.41 - 0.47	0.44 ± 0.02

Table 4.6 Effect on the Yield of the Amount of Fish Flesh added to FW.

4.9 Effect on the Yield of Using an Alternative Salt (see 3.2.6)

Figure 4.7 shows that Calcium Chloride solutions are efficacious in solubilizing fish protein. The greatest yield was obtained with 3% $CaCl_2$ solution.

4.9.1. A comparison Between Water, NaCl and $CaCl_2$ solutions (see 3.2.6.1.)

As can be seen from Figure 4.7 there was no marked difference in the soluble protein values obtained with calcium chloride solutions between 3% (0.4M) and 8% (1.06M).

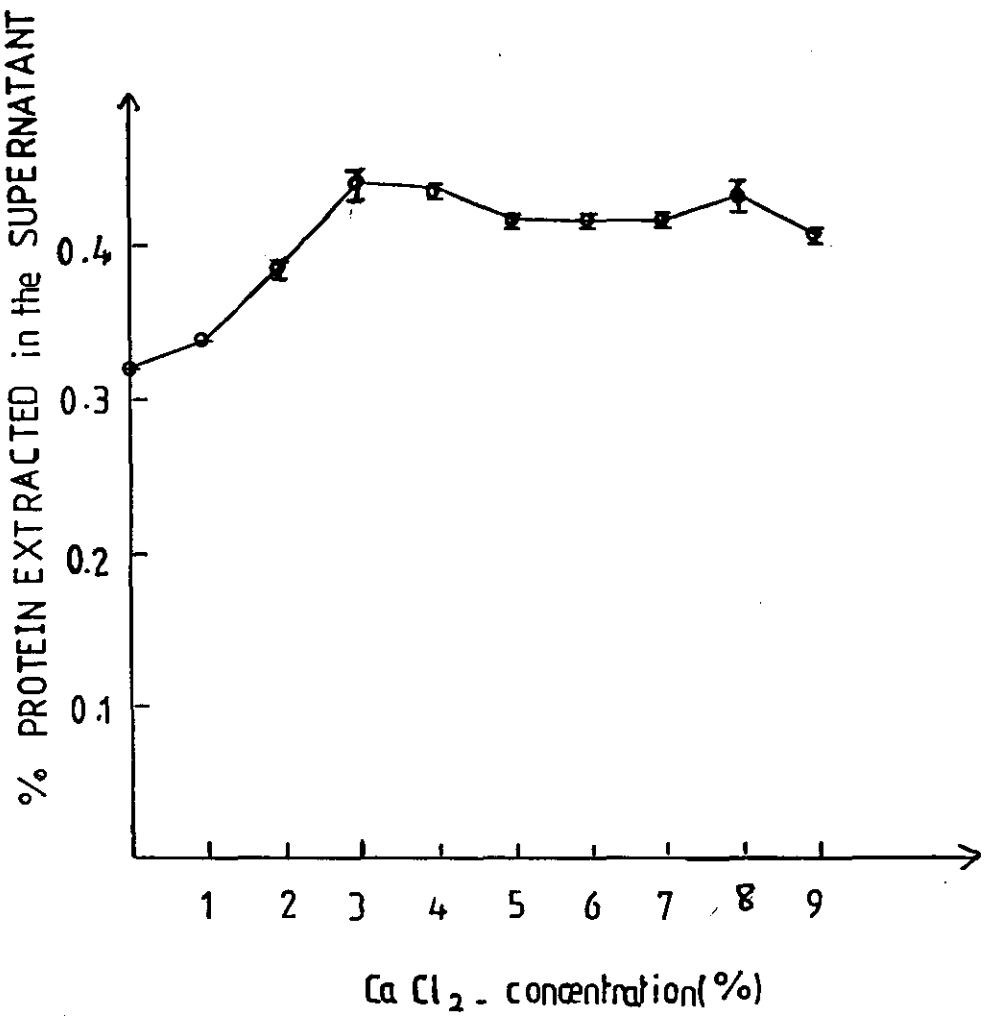


Fig. 4.7 Protein Extracted From FW With $CaCl_2$

After 1 Hour

In a further experiment, a comparison between $CaCl_2$ and NaCl solutions based upon salt weight was made. Table 4.7 shows that Calcium Chloride solution increased the amount of extracted fish protein by 7% over the yield obtained with Sodium Chloride solution.

Solvent	Extracted protein (%) in the Supernatant	
	Range	$\bar{X} \pm S$
Distilled water	0.52 - 0.53	0.52 ± 0.01
4% NaCl (0.7M)	0.68 - 0.70	0.69 ± 0.01
4% $CaCl_2$(0.52M)	0.76 - 0.77	0.76 ± 0.01

Table 4.7 Effect of different solvents on protein yield extracted from FW.

4.10 Discussion

Using water as a solvent (Figure 4.1) it was found that the greatest amount of soluble protein could be extracted from FW through the first hour of the extraction process. The extracted material is probably sarcoplasmic protein according to Connell (1964). This fraction forms about 16 - 22% of fish muscle and can be extracted with water or weak salt solution according to Dyer & Dingle (1961). Sodium chloride solution upto 4% (or 0.7M) was found in the present work to increase the soluble protein yield (Figure 4.2), when it is used as an extraction solvent.

This increase occurred because of the characteristic ability of dissolved salts to solubilize muscle proteins (King et al., 1974). The protein solubility increased gradually with the ionic strength of NaCl solutions until it reached the maximum at the 4% level. This can be considered as the point of maximum "salting-in". After that the protein solubility gradually declined with the higher salt concentrations which results in the "salting-out"effect. It can therefore be seen that fish proteins show the typical salting-in and salting-out phenomena described by Anglemier & Montgomery (1976).

According to Dyer & Dingle (1961), the increased protein yield obtained by substituting NaCl solutions for water is produced by the extraction of myofibrillar proteins which form about 75% of fish muscle.

It was found that one hour was the optimal extraction time with saline solutiosn (Figure 4.3). It was also found that one extraction stage would be adequate to deplete the soluble protein from FW, since more than 90% of the total yield obtained from three extraction stages was extracted in the first stage. The extra yield would probably not justify running the process for more than one stage since it would only increase the production cost without a substantial increase in yield.

In assessing the efficiency of the extraction procedure

one should take the initial true protein content of the FW and the yield of true protein. However, these figures are not easily obtained, and the efficiency is estimated here from the initial crude protein content e.g. 14.32 g/ 100 g FW (NX 6.25)found in one particular sample, which includes some inherently insoluble protein and some NPN (Meinke, et al., 1972). The effect of the NPN is slight and less than the sample to sample variation and the yield of 23% [$(\frac{3.5}{14.32} \times 100)$ where 3.5 g of soluble protein was obtained from 100 g FW] can thus be accepted as a reasonable estimate of the yield of true protein. However no correction can be made for the protein that is inherently insoluble but it can be assumed that the true efficiency is greater than 23% and equal to:

$$\frac{3.5}{14.32 - \text{insoluble protein}} \times 100$$

However it should also be borne in mind that the content of soluble protein in the FW will vary due to oneor more of the following factors:

i) biological variations in the fish composition, e.g. seasonal variation, maturity (Spinelli, et al., 1972);

ii) variation in the FW arising from filleting technique can result in considerable differences in the yield, as demonstrated in section 4.8;

iii) the history of how the fish was handled affects the

protein solubility significantly. The long storage period at high temperatures (around $0^{o}C$ higher) might cause considerable denaturation, which is reflected mainly by a marked decrease in protein solubility (Ravesi & Anderson, 1969; Sikorski, et al., 1976).

It was found that the optimum ratio of weight of FW to the volume of solvent (S : L) was 1 : 8. This was approximately 10% greater than the yield obtained from a S : L ratio of 1 : 5, the lowest yield found in these experiments.

The importance of S : L ratio in the extraction process arises from ensuring optimum conditions for FW to disperse freely and easily in the solvent. This will provide an efficient extraction process and subsequently higher yield. The higher L ratio may meet these conditions, but the extra volumes which are unnecessary for FW solubilization will form an additional load on effluent treatment and pollution control, and on concentration costs. On the other hand, the low L ratio resulted in low protein recovery which according to Meinke, et al., (1972) is caused by the problem of increased viscosity and gelation.

It is therefore seen that optimization of the S : L ratio will avoid these problems and ensure high protein recovery.

The soluble protein yield was found to be proportionally increased with increasing temperature in the range 10°C to 60°C. There are however a number of factors which would account for this:

i) The solubility of protein is generally increased when temperature increases between 0° and 40°C (Anglemier & Montgomery, 1976).

ii) There could be greater microbial or autolytic activity at higher temperature, resulting in a higher yield of soluble nitrogenous components (Hale, 1972).

iii) The rate of protein denaturation is also expected to increase with raising temperature, particularly above 50°C (White, et al., 1978). While, it is commonly accepted that denaturation would decrease solubility, the increase could have been partly due to the denaturation of low solubility nitrogenous materials to more soluble forms.

These hypothese are not mutually exclusive. Clearly, to elucidate the precise mechanisms involved, more detailed studies would be necessary. The above results are not in agreement with those found by Dyer and his co-workers (1950), who detected no difference in the amount of soluble protein extracted from fresh cod muscle at 5°C and 25°C. However, after being frozen and stored for one week at -18°C, they found that the protein extracted at 25°C decreased by 32% of the original value compared to 17% with that extracted at 5°C. Dyer et al were however working with relatively undenatured

(freshly killed) fish than in the present work.

The smallest partical size of FW produced the highest protein yield, since the fine partical divisions as explained in section 4.6.1. must have a greater surface area, and therefore increased capacity for releasing protein into solution. This result is in agreement with the findings of Dyer et al (1950).

It was found that the protein yield increased with increased shaking speed in an orbital shaker up to 250 RPM (Figure 4.6). Above this speed froth was formed and apparently caused a slight reduction in protein recovery. However, a good shaking speed ensures that the local bulk concentration is equal to the mean bulk concentration; in other words, it means that there are no local higher concentrations to impede the overall extraction process.

The type of extraction apparatus (Tables 4.4 and 4.5) was shown to affect the protein yiled considerably. The experimental results showed a difference of about 15% between the three techniques employed.. It was suspected that the type of motion created during the extraction process may have affected the degree of cell subdivision resulting in a difference in the yield.

More simply, the different shaking may have resulted in a different level of turbulence in the extraction solution. This eventually could improve solute-solvent mixing and increase the soluble yield.

These results are in agreement with the work of Cowie & Mackie (1968), who found that the protein extractability curves for cod muscle stored at different temperatures were higher in an Ultra-Turrax homogeniser than in the Marsh Snow homogeniser. A greater degree of subdivision of cells was obtained with the Ultra-Turrax homogeniser, which would allow a greater solubilization of proteins.

Calcium chloride was tested as an alternative salt for the extraction process. As a divalent salt, it may have greater ability to solubilize fish muscle protein than Sodium Chloride. The experimental results revealed that 4% $CaCl_2$ solution increased the soluble yield by 7% over that obtained by 4% NaCl extraction. However, $CaCl_2$ was eliminated from subsequent work because of the disproportional increase in the cost. Dyer and his co-worker (1950) however found that sodium chloride was superior to calcium chloride by 4% when extracting fish muscle protein.

4.11 Conclusions

The greatest amount of extracted protein that can be obtained from fish waste was found to be after one hour of extraction.

4% Na Cl was found to be optimal as a solvent, with S : L ratio of 1 : 8 and mean nominal particle size of 0.04cm. diameter. It was also found that more than one extraction stage would not substantially increase the total soluble protein yield.

The optimum shaking speed was found to be 250 RPM for 250 cm^3 of extracting medium using an orbital extraction apparatus. The Silverson homogeniser was found to give higher protein yield than both orbital and rotary apparatuses. In addition, the yield was found to be proportional to the extraction temperature in the range 10^oC to 60^oC.

Calcium chloride was found to be efficacious in extracting fish protein and indeed gave a marginally higher yield over that obtained with sodium chloride, but $CaCl_2$ was omitted from further tests due to the disproportional increase in its cost.

Chapter Five

Chapter Five

Frozen Storage and Protein Extractability of Fish Waste

5.1 Introduction

Freezing is one of the most effective methods which has been applied to preserve and extend the storage life of fish and other food products.

The literature indicates however (see section 2.5.3.4) that, after prolonged storage even at temperatures as low as -20°C, substantial sensory and textural deterioration of fish and fish products still takes place. In fatty fish, this change is mainly reflected by the development of oxidative rancidity, while the quality of lean fish is affected by protein denaturation which resulsts in significant deterioration of the texture and functional properties of fish particularly when frozen in the minced state (Sikorski, et al, 1976).
The work described in this chapter was carried out to examine:

i) The extent of change in true protein extractability of minced FW stored at 0°, -15° and -30°C.

ii) a) The relationship between true protein extractability and non-protein nitrogen compounts (NPN) which developed during frozen storage of minced FW.

b) The relationship between true protein extractability and NPN extracted from minced FW samples which stored at $0^{o}C$,$-15^{o}C$ and $-30^{o}C$.

iii) The effect of free fatty acids (FFA) liberated from FW during frozen storage at different temperatures on the extractability of true protein. The term 'true protein' used in this chapter represents the total crude nitrogen minus the NPN compounds multiplied by 6.25 as determined by 10% TCA method which described in section 3.6.2.

5.2 Effect of Temperature and Length of Storage of Minced FW on Protein Extractability (see 3.2.11).

Figure 5.1 shows the solubility profile of minced FW stored at different temperatures. The true protein extractability of samples stored in iced water at $0^{o}C$ rapidly declined from 0.45% to 0.14% after 12 days storage. The development of unpleasant odours and some rubbery texture were also observed. After that period of time, no further deterioration in the extractability appeared to take place.

There was a gradual decline in the true protein extractability during the 70 days storage period of minced FW at $-15^{o}C$. The final value of protein extractability was only approximately 27% of the original value. A slight off-flavour and rubbery texture was observed after 50 days of storage.

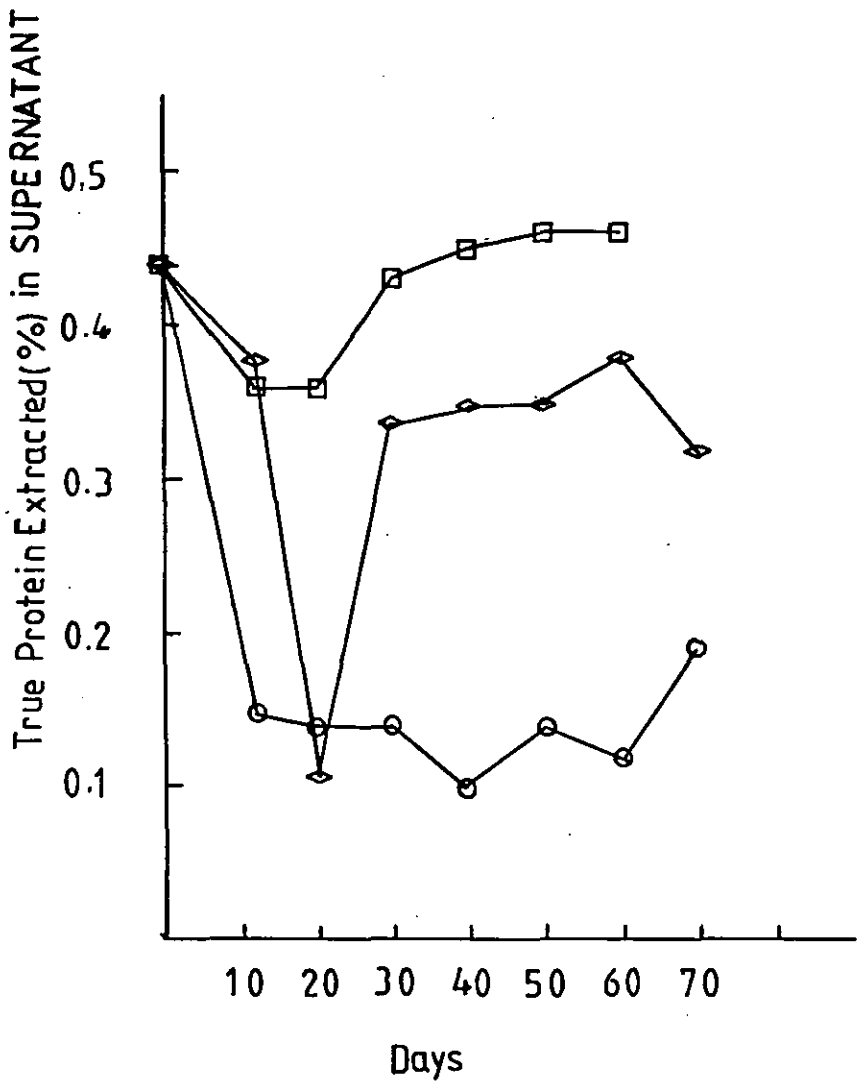


Fig. 5.1

Effect of temperature & storage period on protein extractability of minced 'FW'.

○ Samples stored at 0 °C

◇ 〃 〃 〃 −15 °C

□ 〃 〃 〃 −30 °C

There was no apparent change in the true protein extractability of samples which were stored at -30°C for up to 60 days. The lower values obtained after 12 and 20 days of storage were probably due to sample variations. Also, there was no noticeable deterioration in the smell and texture of stored samples.

5.3 Effect of NPN on Extractability of True Protein:

5.3.1. Effect of NPN of FW on Extractability of True Protein:

Table 5.1 shows the changes in NPN values of minced FW stored at different temperatures.

Storage Period (Days)	NPN in minced FW (%N)					
	Storage Temperature					
	0°C		-15°C		-30°C	
	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$
0	←		0.12-0.17	0.14±0.02	→	
12	0.25-0.27	0.26±0.01	0.26	0.26±0	0.26	0.26±0
20	0.42-0.79	0.67±0.17	0.44-0.65	0.55±0.12	0.40-0.66	0.48±0.12
30	0.68-0.73	0.70±0.02	0.84-0.86	0.85±0.01	0.73-0.79	0.75±0.03
40	1.03-1.19	1.14±0.08	1.09-1.22	1.15±0.07	0.97-1.18	1.09±0.11
50	0.64-0.69	0.67±0.02	0.69-0.74	0.71±0.02	0.61-0.66	0.64±0.02
60	0.61-0.65	0.62±0.02	0.72-0.75	0.74±0.02	0.59-0.66	0.63±0.03
70	0.36-0.42	0.39±0.03	0.37-0.41	0.39±0.02	-	-

Table 5.1 The changes in NPN content of minced FW samples stored at 0°C, -15°C and -30°C.

Table 5.1 shows that the NPN content of minced FW samples stored at 0, -15 and -30°C progressively increased with the storage time upto 40 days. After that the values gradually declined. The reason for this is not known. It is also apparent that the storage temperature had no marked effect on the level of NPN developed during the test. The relationship between true protein extractability values (Figure 5.1) and NPN content of minced FW (Table 5.1) (See appendices 2.1, 2.2 and 2.3) were statistically analysed by the correlation coefficient test (r) which was separately applied on each of the true protein values against their NPN counterpart. The results are as explained in Table 5.2

Storage temperature °C	r between extracted true protein and NPN
0	r =-0.77 Significant to 95% at 6 degrees of freedom
-15	r =-0.16 No correlation could be established even at 90% confidence
-30	r = +0.37 No correlation could be established even at 90% confidence

Table 5.2

The correlation coefficient for true protein extractability against NPN values of minced FW stored at 0°C, -15°C and -30°C.

It is therefore seen that the true protein values are inversely related to NPN values for samples stored at $0^{o}C$, whereas no such relationship could be established for samples stored at $-15^{o}C$ and $-30^{o}C$.

5.3.2 Effect of Extracted NPN on True Protein Extractability:

Storage Period (Days)	NPN Extracted from Minced F.W. by Saline Solution(%N)					
	Storage Temperature					
	$0^{o}C$		$-15^{o}C$		$-30^{o}C$	
	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$
0	←		0.01-0.02	0.02 ± 0.01	→	
12	0.03	0.03 ± 0	0.02	0.02 ± 0	0.02	0.02 ± 0
20	0.03-0.04	0.04 ± 0.01	0.06-0.07	0.07 ± 0.01	0.04-0.06	0.05 ± 0.01
30	0.04	0.04 ± 0	0.02-0.03	0.03 ± 0.01	0.01-0.06	0.04 ± 0.03
40	0.03-0.06	0.05 ± 0.02	0.01-0.03	0.02 ± 0.01	0.03	0.03 ± 0.01
50	0.02-0.04	0.03 ± 0.02	0.02-0.03	0.02 ± 0.01	0.01-0.02	0.02 ± 0.01
60	0.01	0.01 ± 0	0.01-0.03	0.02 ± 0.01	0.01-0.02	0.01 ± 0.02
70	0.01-0.04	0.03 ± 0.02	0.01-0.04	0.02 ± 0.01	-	-

Table 5.3 The changes in NPN extracted by saline solution from minced FW stored at $0^{o}C$, $-15^{o}C$ and $-30^{o}C$

Table 5.3 shows that the NPN compounds extracted from minced FW fluctuated during the frozen storage at 0, -15 and $-30^{o}C$. These values and true protein extractability values (Figures 5.1) corresponding to each storage temperature were separately analysed by r-test (see appendices 2.4, 2.5 and 2.6).

The results which are shown in Table 5.4 suggest no relationship could be established.

Storage temperature oC	r between extracted NPN and true protein extractability
0	r = 0
-15	r = 0
-30	r = 0

Table 5.4 The correlation coefficient for true protein extractability values against their extractable NPN values for samples stored at $0^{o}C$, $-15^{o}C$ and $-30^{o}C$

On comparing the data in Tables 5.1 and 5.3, the trend of NPN changes in both FW and soluble extract was only consistent in samples stored at $0^{o}C$. This result, however is not beyond question as NPN compounds are present in the insoluble form in fish muscle and bone (Meinke, et al, 1972) and it is therefore expected that NPN compounds cannot be fully extracted from FW, that is, the extracted NPN does not necessarily follow the same pattern of changes which occurred in FW.

5.4 Effect of FFA on True Protein Extractability

Table 5.5 shows that the FFA values of minced FW stored at 0, -15 and $-30^{o}C$ fluctuated during frozen storage. These FFA values cannot clearly explain the loss in protein extractability of FW.

However it is possible that these values do not truely reflect the FFA content because of the difficulty involved in testing unhomogenized samples such as FW. The apparent lack of a storage temperature effect on the FFA value may arise for a similar reason.

Storage Period(Days)	Storage temperature					
	0°C		-15°C		-30°C	
	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range	$\bar{x} \pm s$
0	←		2.72-2.78	2.75 ± 0.04	→	
12	2.90-3.31	3.11 ± 0.30	2.50-2.90	2.70 ± 0.03	2.64-2.69	2.67 ± 0.04
20	2.89-4.49	3.69 ± 1.13	3.28-4.63	3.96 ± 0.95	2.27-2.78	2.53 ± 0.40
30	3.00-3.37	3.12 ± 0.21	1.82-2.89	2.49 ± 0.58	2.05-2.47	2.19 ± 0.24
40	2.10-2.66	2.41 ± 0.28	2.10-2.66	2.40 ± 0.28	2.13-2.58	2.38 ± 0.23
50	2.67-3.39	3.06 ± 0.40	2.89-3.74	3.30 ± 0.43	2.56-2.66	2.61 ± 0.05
60	3.03-3.79	3.40 ± 0.38	2.21-2.51	2.40 ± 0.17	2.80-3.07	2.96 ± 0.14
70	2.73-3.19	2.90 ± 0.25	2.03-2.32	2.13 ± 0.16	-	-

Table 5.5 The changes in FFA value of minced FW stored at 0°C, -15°C and -30°C. Triplicate samples were examined.

The relationship between true protein extractability values which are shown in Figure 5.1 and FFA values (Table 5.5) were statistically analysed for correlation (see appendices 2.7, 2.8, 2.9). The results are as shown in Table 5.6

Storage temperature ^{o}C	r between FFA and true protein extractability
0	r =-0.18 No correlation could be established even at 90% confidence
-15	r =-0.61 No correlation could be established even at 90% confidence
-30	r = 0

Table 5.6 The correlation coefficient for true protein extractability against FFA values of minced FW stored at 0, -15 and $-30^{o}C$.

It is therefore confirmed that no definite correlation could be established between true protein extractability and FFA level in minced FW.

5.5 Discussion

5.5.1. Effect of Temperature and Length of Storage on Protein Changes:

The experimental results revealed that protein denaturation in minced FW does take place during frozen storage. This is mainly evidenced by a sharp decline in protein solubility profile, particularly when fish is stored at high temperature, i.e. in iced water.

The decline in protein solubility according to Dyer and Dingle (1961) and Connell (1968) is caused by alteration of myofibrillar protein while the sarcoplasmic protein does not undergo significant changes. The alteration in myofibrillar protein is due to aggregation of myosin protein in which different types of intermolecular cross linkages i.e. H...bonds or hydrophobic adherence, take place. These ionic interactions are concluded by Connell (1965); Anderson & Ravesi (1970 a,b) and Iwata & Okoda (1971) to cause insolubilization of fish protein during frozen storage.

The rate of myosin aggregation increases with a rise in storage temperature (Connel, 1968). This may explain the rapid decline in protein extractability of minced FW samples stored in iced water in which the protein extractability decreased by about 60% after 70 days storage compared with 27% in samples stored at $-15^{o}C$. However, samples stored at $-30^{o}C$ for 60 days did not show deterioration of quality or noticeable

alteration in protein extractability.

The development of off-flavour and rubbery texture which occurred after 12 and 50 days storage at $0^{o}C$ and $-15^{o}C$ respectively cannot only explain the decline in protein extractability according to Cowie and Little (1966, 1967), who found that the protein extractability of cod fillets stored at $-7^{o}C$, $-14^{o}C$ and $-29^{o}C$ for 34 weeks, 34 weeks and 82 months respectively had no correlation with the development of muscle toughness. They also stated that muscle toughness is mainly dependent on the muscle pH and not protein extractability. At higher storage temperatures toughness due to protein denaturation becomes more significant.

An important point to consider is that biological variation in fish may significantly affect their susceptibility to changes induced by freezing and frozen storage (Love, 1966). This would suggest that some if not all these biological variations function through modifying the susceptibility of muscle protein to denaturation prior to freezing, variations in muscle pH value being particularly important. Thus, a sample with 100% denaturation before freezing could show no changes after freezing. Another point to consider is the variation in FW samples which can result in marked differences in protein extractability values, as demonstrated in section 4.8. This may explain the unusual values obtained after 20 days storage at $-15^{o}C$ and after 12, 20 days storage at $-30^{o}C$ (see Figure 5.1).

5.5.2. Inter relationship Between Yields of NPN and True Protein

The zero relationship obtained in Table 5.4 between true protein and NPN extracted by 4% NaCl suggests that the mechanism of protein insolubilization is possibly not caused by the latter factor. It would also appear that the small and constant amount of extracted NPN was a surplus quantity originating from NPN compounds present in FW. On integrating the data in Tables 5.1 and 5.3 it would appear that at $0^{o}C$ NPN is produced at the expense of the saline extractable true protein. During storage at $-15^{o}C$ and $-30^{o}C$ there is no evidence of such a simple relationship.

It is expected that the microbial and enzymic activities are contributing to NPN production at $0^{o}C$ as reflected by the significant correlation obtained above. It was surprising that NPN was also produced at $-15^{o}C$ and $-30^{o}C$ at which temperatures the microbial and enzymic activities are expected to slow down. The reason for this is not clear. It is apparent that NPN was generated by an unknown chemical mechanism and there were some factor(s) which reduced the yield of true protein independently of producing NPN. Insolubilization of extractable true protein could occur through rancid product precipitating protein as will be explained in section 5.5.3. and/or through formaldehyde precipitating protein.

The 10% TCA method used to determine the NPN compounds (see section 3.6.2) will include nitrogenous compound such as

trimethylamine oxide (TMAO) and dimethylamine (DMA).

The literature, however reported (Sikorski, et al., 1976) that some of the differences in the rate of freezing deterioration of proteins in various fish species can be explained on the basis of formaldehyde (FA) in tissues and its effect on proteins. FA and DMA are both formed from TMAO decomposition, which is catalyzed by an enzyme system present in the sarcoplasmic protein of fish (Tokunaga, 1964; Yamade, et al., 1969 and Kostuch, et al., 1975), and bacteria Psedomonas aminovorans (Large, 1971) and Bacillus P.M.6. (Kostuch, et al., 1975).

The above experimental results for FW samples stored at $0^{o}C$ are in agreement with those of Tokunaga (1964) who found that FA formation in frozen stored Alaska pollack was accompanied by loss of extractability of myofibrillar proteins. He also found that regardless of storage conditions the increase of DMA content was always followed by a decrease of protein extractability. The correlation coefficient for DMA and protein extractability ranged between -0.77 and -0.84. A similar result was obtained by Sorenson (1976) who found that FA formation in minced cod stored at $-24^{o}C$ was inversely related to the content of salt soluble protein ($r = o.89$, significant to 99.9% level at 10 degrees of freedom).

A different result was obtained by Connell (1975) who carried out an experiment in which samples of cod, either fresh or after lyear of frozen storage at -15°C, were blended with water or different concentrations of FA, held from 1-28 days at 0°C and analyzed for protein solubility in 1% SDS (at ionic strength (μ) = 0.05 and pH 7.5). In severely frozen denatured flesh only a small fraction of protein was rendered in extractable in SDS, while in samples treated with FA, extensive covalent-cross-linking had taken place, enhanced by the concentration of FA and the time of reaction. Thus, he concluded that apparently FA does not participate in the freezing deterioration of protein in situ by inducing methylene cross-links, which does not exclude other mechanisms of contribution to freezing changes.

5.5.3. Effect of FFA on True Protein Extractability:

Rancidity may be considered as a factor decreasing protein extractability. It should be expected to obtain an inverse relationship between the latter and FFA values. Such a relationship appeared to exist for samples stored at 0°C and -15°C, but it was not found to be statistically significant.

However, an important point to consider is whether the measured amount of FFA as determined by the method described in Section 3.6.4, reflects the actual content in stored FW samples.

The apparent fluctuations with time in FFA values obtained were unexpected according to Anderson & Ravesi (1968) who found a 200% increase in FFA over 32 days at 0°C when working on cod fish, and using chromatographic methods.

The reason for this may be related to:

i) The unsuitability of the method used to determine FFA (see section 3.6.4) e.g. it was difficult to determine the end point which resulted in a marked difference in FFA values as reflected by high standard deviation in some tested samples, as shown in Table 5.5

ii) When FFA oxidized to hydroxy perioxide and radicals, some of these products could combine with actomyosin to form a very insoluble polymer according to Castell and Smith (1970), who suggested that oxideized fatty acids are more closely bound to protein than unoxidized FFA. The former is unextractable by solvent. This mechanism may provide a possible explanation of the role of FFA on protein insolubilization. Thus, it is likely that some FFA of FW was liberated and bound to protein during the different stages of handling and processing and this may also explain why FFA did not apparently rise during the storage period.

There is disagreement in the literature concerning the role of FFA on the extractability of fish protein during frozen storage. For example Olley & Lovern (1960); Nishimoto (1962); and Olley, et al, (1962) indicated there was no simple casual

relationship between FFA liberation and the loss of protein extractability. They also stated that caution must be exercised in drawing any conclusion betweenthe two factors. However, Anderson & Ravesi (1970b) and Jarenback & Liljemark (1975) showed that production of FFA significantly impaired the extractability of myofibrillar protein. The role of FFA on protein extractability was fully discussed in section 2.5.3.4

5.6 Conclusion

Protein denaturation as reflected by loss of extractability occurred during frozen storage of minced FW. The deterioration in protein extractability and texture took place rapidly at high storage temperature, i.e. in iced water. Using low storage temperature e.g. -30°C, was found to be effective in slowing down the rate of denaturation and extending the storage life of the raw material.

The true protein extractability could not be correlated with the production of FFA during the storage period.

The NPN level in the soluble extract had no relationship with true protein yield. While, the NPN level in FW was inversely related to true protein extractability values for samples stored in iced water. No relationship could be established for samples stored at -15 and -30°C. However, as mentioned in section 2.5.3.4, mincing the fish favours many reactions that occur during storage.

Therefore it is advisable to avoid frozen storage of minced FW where possible, or if unavoidable to use low storage temperature such as $-30^{o}C$ or below which would permit upto 60 days storage with little change.

Chapter Six

Chapter Six

The Concentration and Desalination of Fish Protein Extract

6.1. Introduction:

Ultrafiltration (UF) has been recognized as a successful unit operation for concentrating and the separation of numerous liquid solutions in food processing operations, such as the concentration of fruit juice, protein recovery from whey and the concentration of egg white.

As previously mentioned in section 2.7, the ultrafiltration process can be used either for concentration or for fractionation of many food solutions, and these are being particularly important when the concentrated liquid contains a heat labile solute. One reason for increasing importance is because the separation does not involve a phase change, and thus conserves energy. Further, the method permits the retention of product quality, since volatiles are retained to a greater extent. The purpose of the work described in this chapter was to examine the performance and feasibility of using the UF process for concentration and desalination of soluble protein extract, and also to examine:

i) the effect of protein concentration, pH and temperature of the feed stream on membrane permeation rate, or 'flux rate'.

ii) the ionic transfer across the UF membrane. All the bench scale processes were performed with an Amicon TCF-10 unit, supplied by a 1-litre reservoir.

6.2 Primary Examination of UF Performance (Section 3.3.1.)

Figures 6.1 and 6.2 show the changes in protein and salt concentration of two different extracts of soluble fish protein with increasing volume of permeate stream during the concentration process.

The protein content was progressively increased as the volume of permeate removal increased. Simultaneously the salt content decreased in absolute terms. This indicated that some of the salt was removed with the permeate stream. As Figures 6.1, 6.2 indicate, protein contents were respectively increased by 4.6 and 4.8 fold while the salt levels were reduced by the same ratio.

These tests were performed primarily to examine the performance of the UF process and no duplicate samples were tested. Comparting Figures 6.1, 6.2, it would appear that both protein and salt concentrations are increasing and decreasing respectively in the same manner. Thus, it is seen that the UF techniques can be successfully applied to desalting and concentrating fish protein extract.

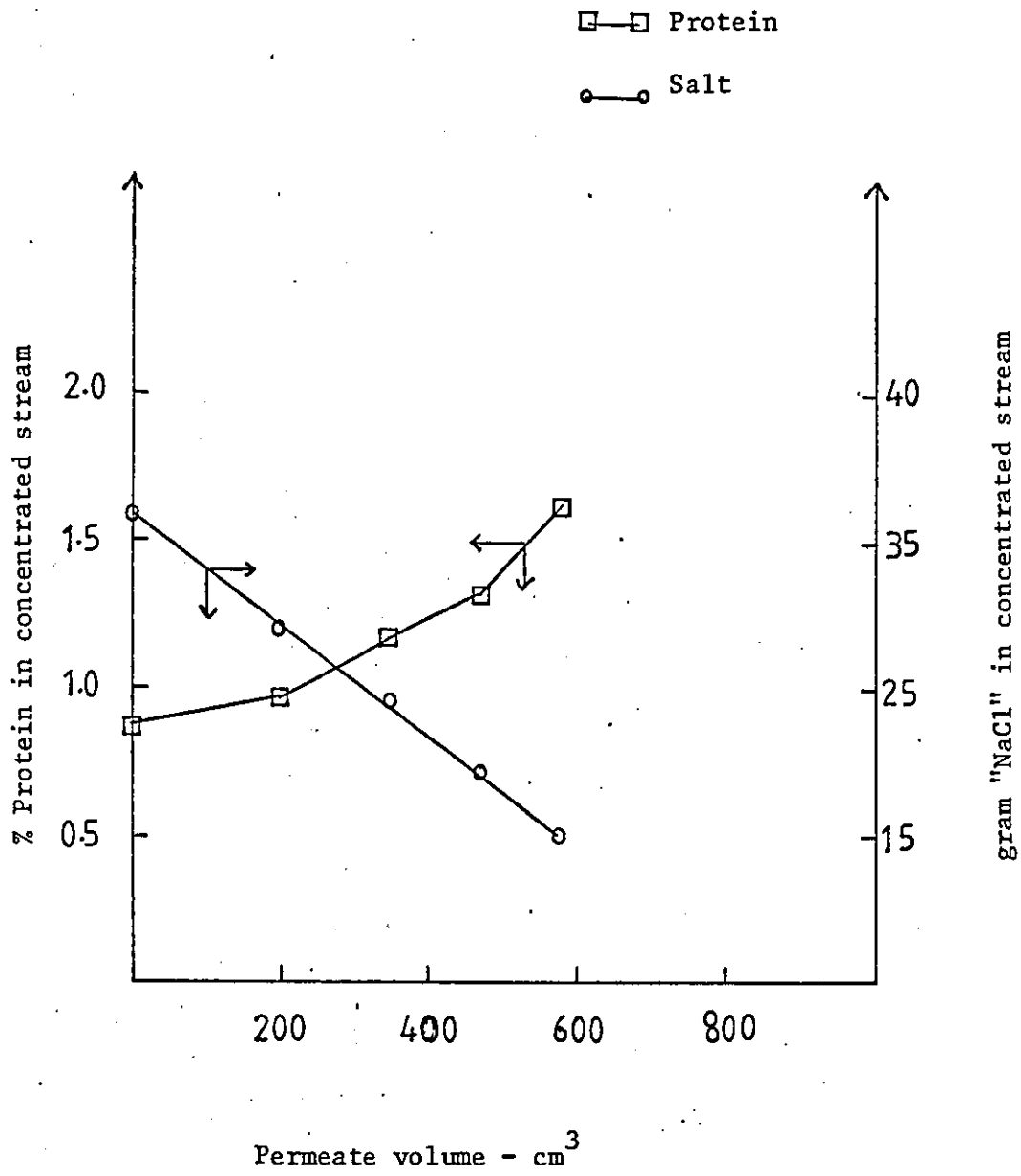


Figure 6.1 The Changes in protein and salt concentration of fish protein extract during U.F. process

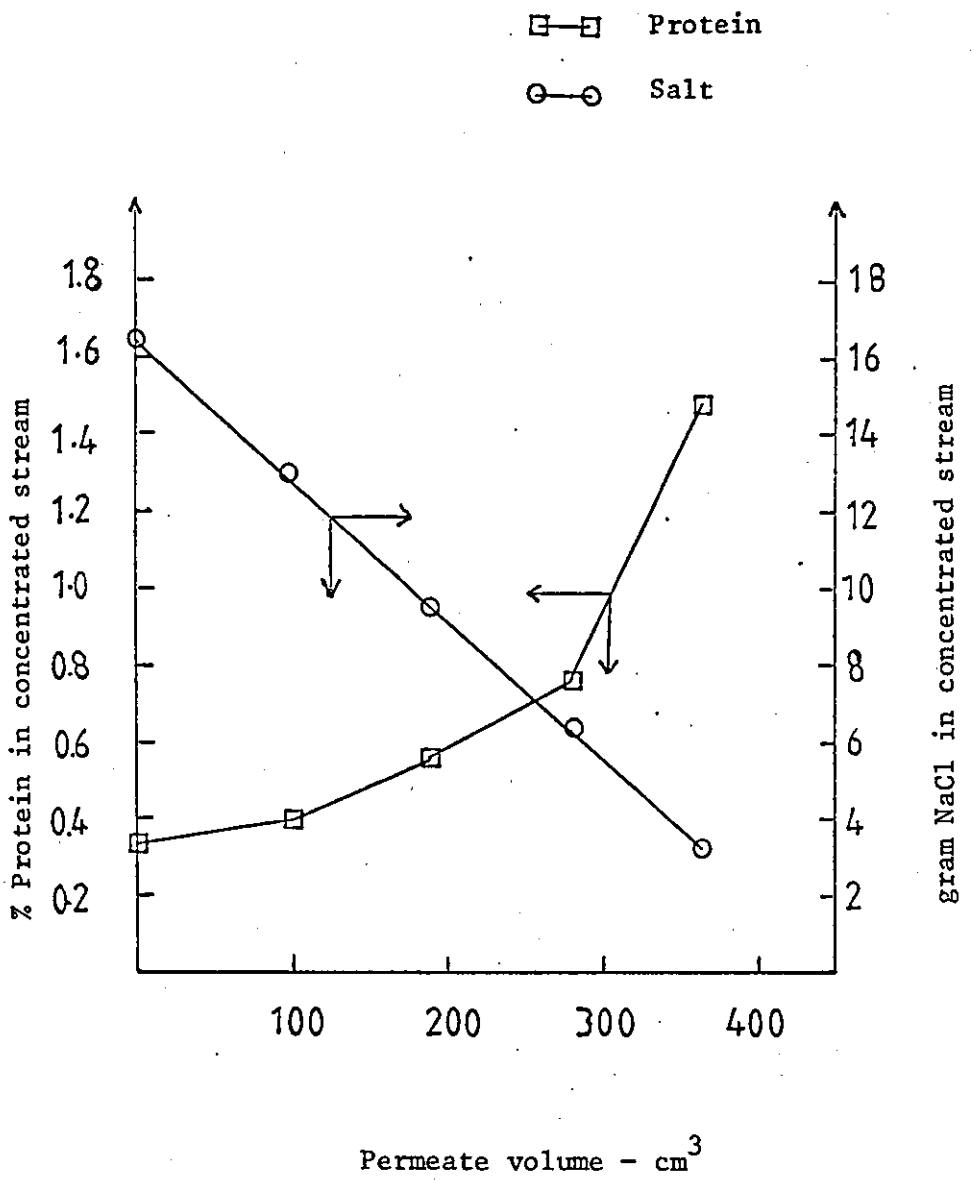


Figure 6.2 The change in protein and salt concentration of fish protein extract during U.F. process.

6.3. Desalination of Fish Protein Extract (see 3.3.2)

Results in section 6.2 showed that the UF process reduced the quantity of NaCl in soluble fish extract, but in terms of percentage (g NaCl/100 cm^3 solution) the concentrated stream has the same salt percentage the content as the original extract which is also equal to NaCl% in the permeate stream (Refer to the ionic concentrations in Table 6.1). However, in order to achieve further reduction in the salt level, it was necessary to diafilter the fish protein with distilled water. This technique is explained in section 2.7.2 and basically involves addition of further solvent to a solution from which solvent and micro-species have ultrafiltered. Figure 6.3 shows the change in salt content after each diafiltration process.

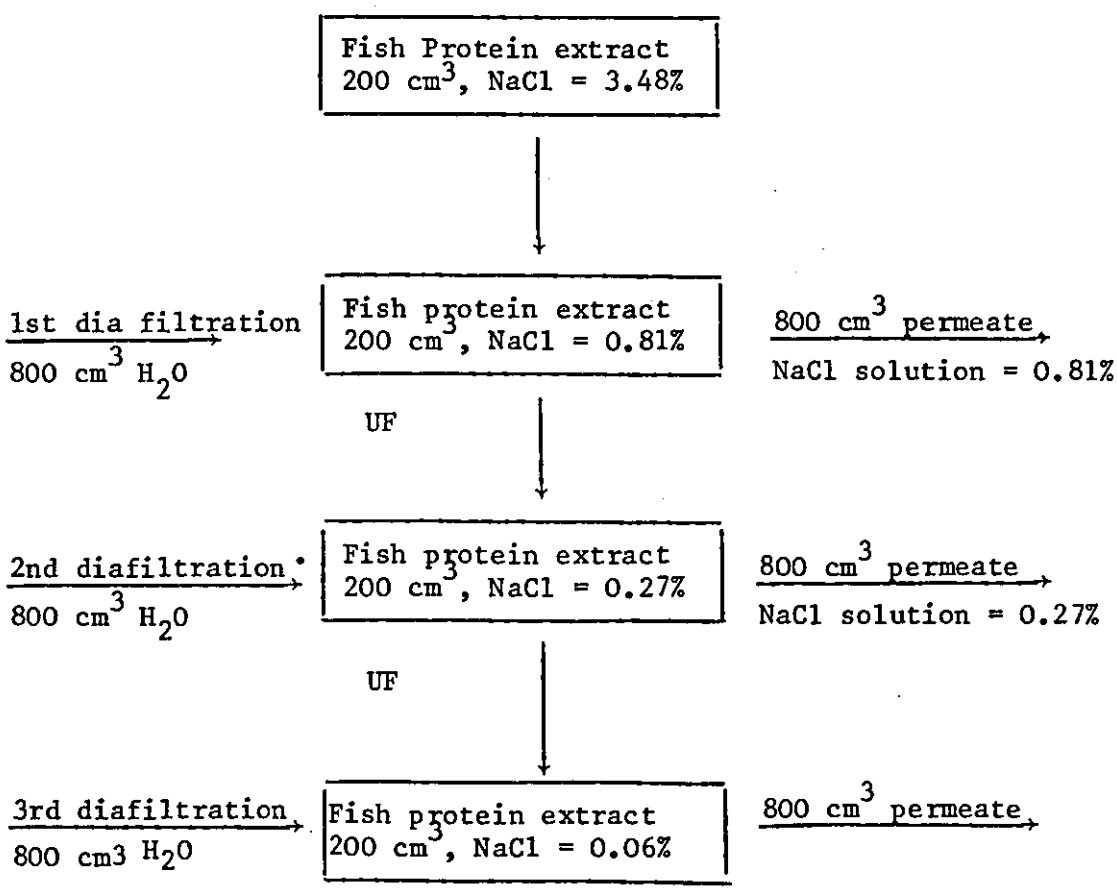


Figure 6.3 Desalination of fish protein extract

The above results show that the salt content of fish protein could be reduced to the level which is naturally present in fish tissue (see Table 6.1, part 3).

The volume of distilled water required to desalt the fish protein extract is dependent on the concentration of salt in the extract and can be calculated by the relationship mentioned in section 3.3.2.

The test confirms that FPE can be effectively desalted by UF process.

6.4. Membrane cut-off and Flux rate (section 3.3.3.)

Figures 6.4 and 6.5 show the effect of membrane cut-off on the flux rate of fish protein extract through two different membranes PM-30 and UM-10 which had molecular cut-offs of 30,000 and 10,000 respectively.

Comparing Figures 6.4 and 6.5 clearly shows that a higher flux rate was obtained with PM-30, for example, at 0.6% protein, 2 and 4.8 flux units were obtained with UM-10 and PM-30 respectively.

The above result was as expected according to equation 2.3 (section 2.7.2) in which the membranes with larger pore sizes produced a higher flux rate than those with smaller pore sizes.

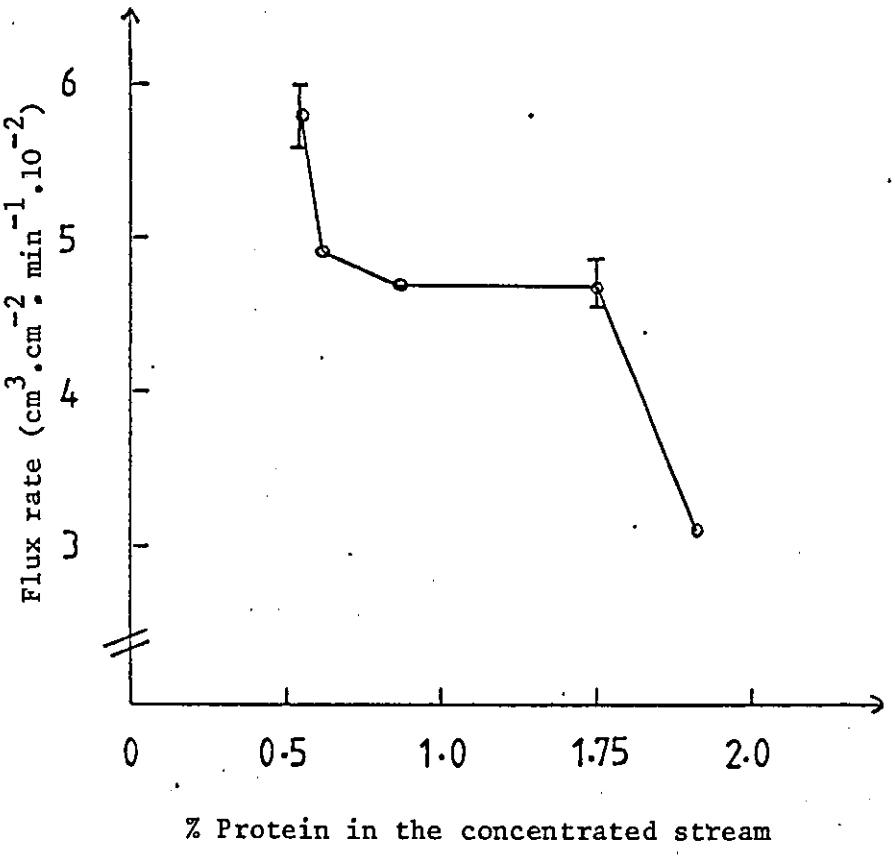


Figure 6.4

The effect of membrane cut-off (PM-30)
on the flux rate of fish protein extract at 2.12 Kg/cm^2
and flow rate of 1.5 litre/min.

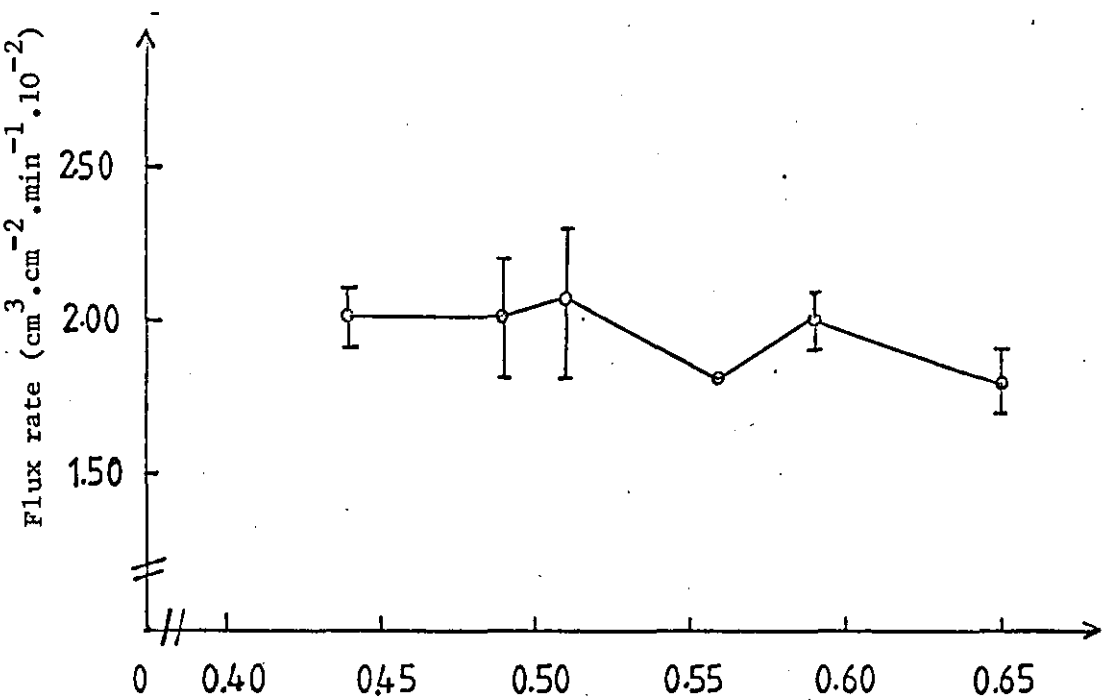


Figure 6.5

The effect of membrane cut-off (UM10)
on the flux rate of fish protein extract at 2.12 Kg/cm^2
and flow rate of 1.5 litre/min.

6.5. Protein Concentration and Flux Rate:

Figure 6.6 shows the effect of protein concentration on flux rate of fish protein extract through an UF membrane (PM-30) which had a molecular mass cut-off of 30,000. The experimental results revealed that as the protein concentration of the concentrated stream increased, the flux rate decreased accordingly. The flux rate decreased by 36% of the original value as protein level in the concentrated stream increased from 1.30% upto 6%.

6.6. Protein Concentration and Viscosity (section 3.5)

The dynamic viscosities (μ - cp) of concentrated fish protein extracts were determined from five different concentration experiments. The straight line equation was fitted to the data as shown in Figure 6.7 (see appendix 3). The correlation coefficient test (r) was applied to the data and was found to be 0.97, which is significant to 99% at 4 degrees of freedom.

According to the straight line, the relationship between protein concentration and viscosity was found to be:

$$\mu = 0.79 + 0.24\ P \qquad \text{.....6.1}$$

where μ = viscosity (cp)

P = protein concentration (%)

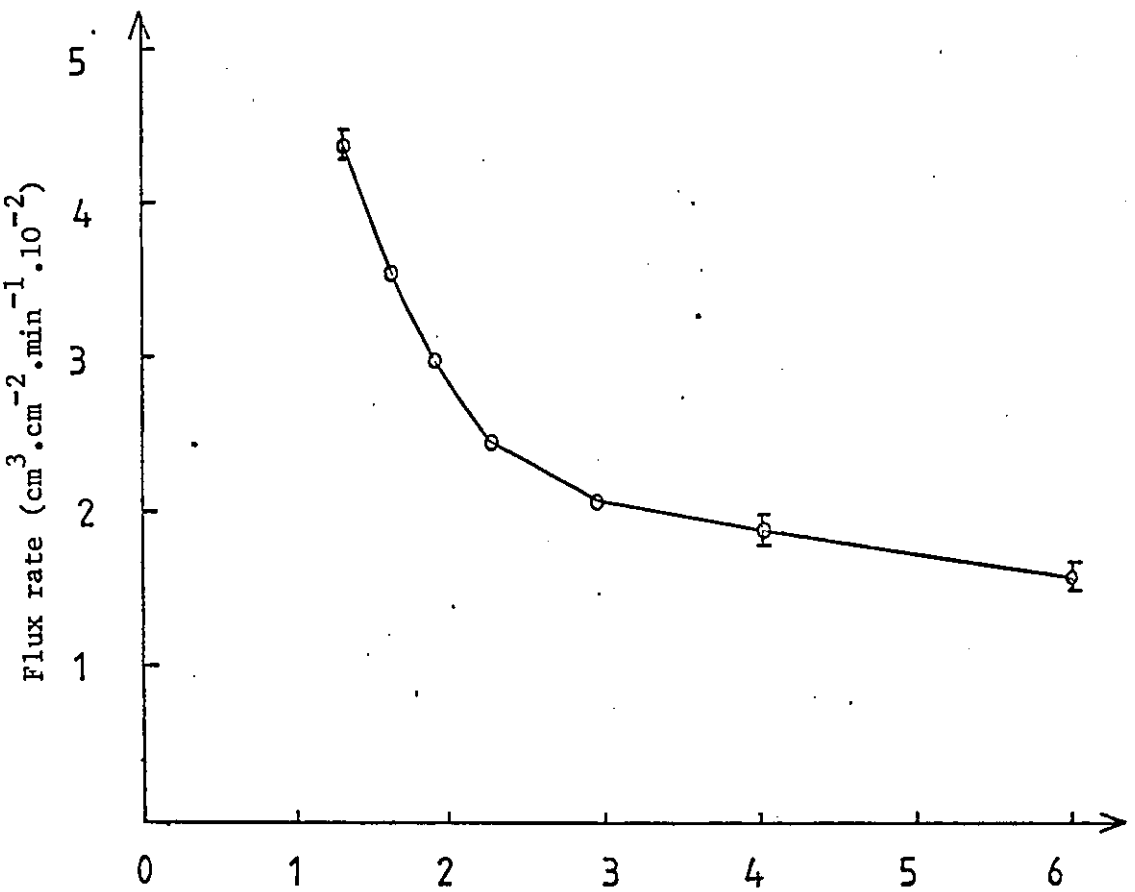


Figure 6.6.

The effect of concentration of fish protein extract on the flux rate through an PM-30 UF membrane at pressure 2.12 Kg/cm^2 (30 psi) and flow rate of 1.5 litre/min.

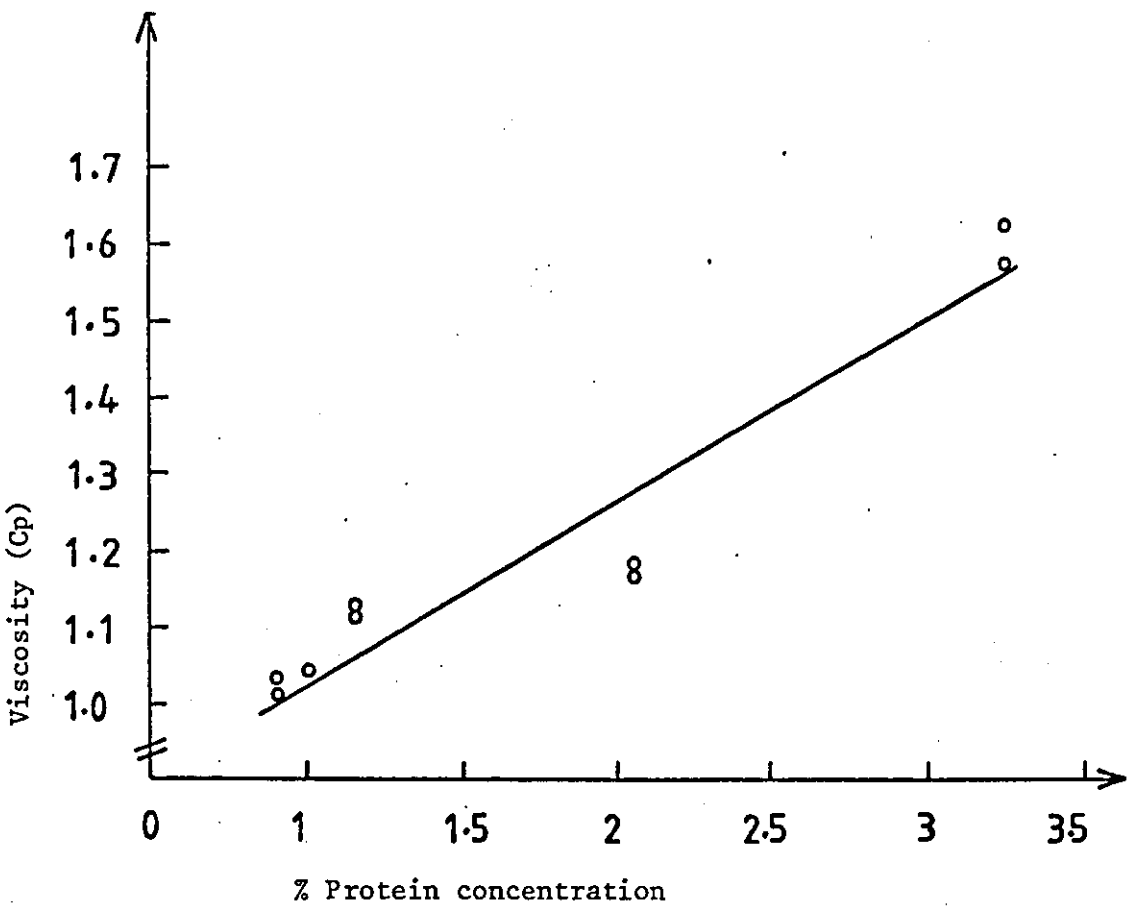


Figure 6.7

Effect of protein concentration of fish protein extract on the dynamic viscosity

6.6.1. The Relationship between Viscosity, Protein Concentration and Flux Rate

Equation 6.1 is plotted in Figure 6.6 together with the experimental data and shows good agreement as explained in Figure 6.8. When the logarithm values of these data are plotted, it would be expected to obtain a straight line according to equation 2.3. Figure 6.9 shows that no such relationship resulted, and therefore the simple equation (2.3) is not sufficient to describe the flux in this case.

6.7 Effect of pH on the Flux Rate (section 3.3.4)

Figures 6.10 and 6.11 show the effect of the pH of fish protein extract on the flux rate. It can be seen that there were no marked changes in the flux rate around the basic pH between 7-10 (Figure 6.10). On the other hand, the flux rate generally declined on the acidic side (Figure 6.11) and particularly so around pH 4.5 which protein precipitation occurred. The white precipitate was deposited on membrane and apparently hindered the flux rate.

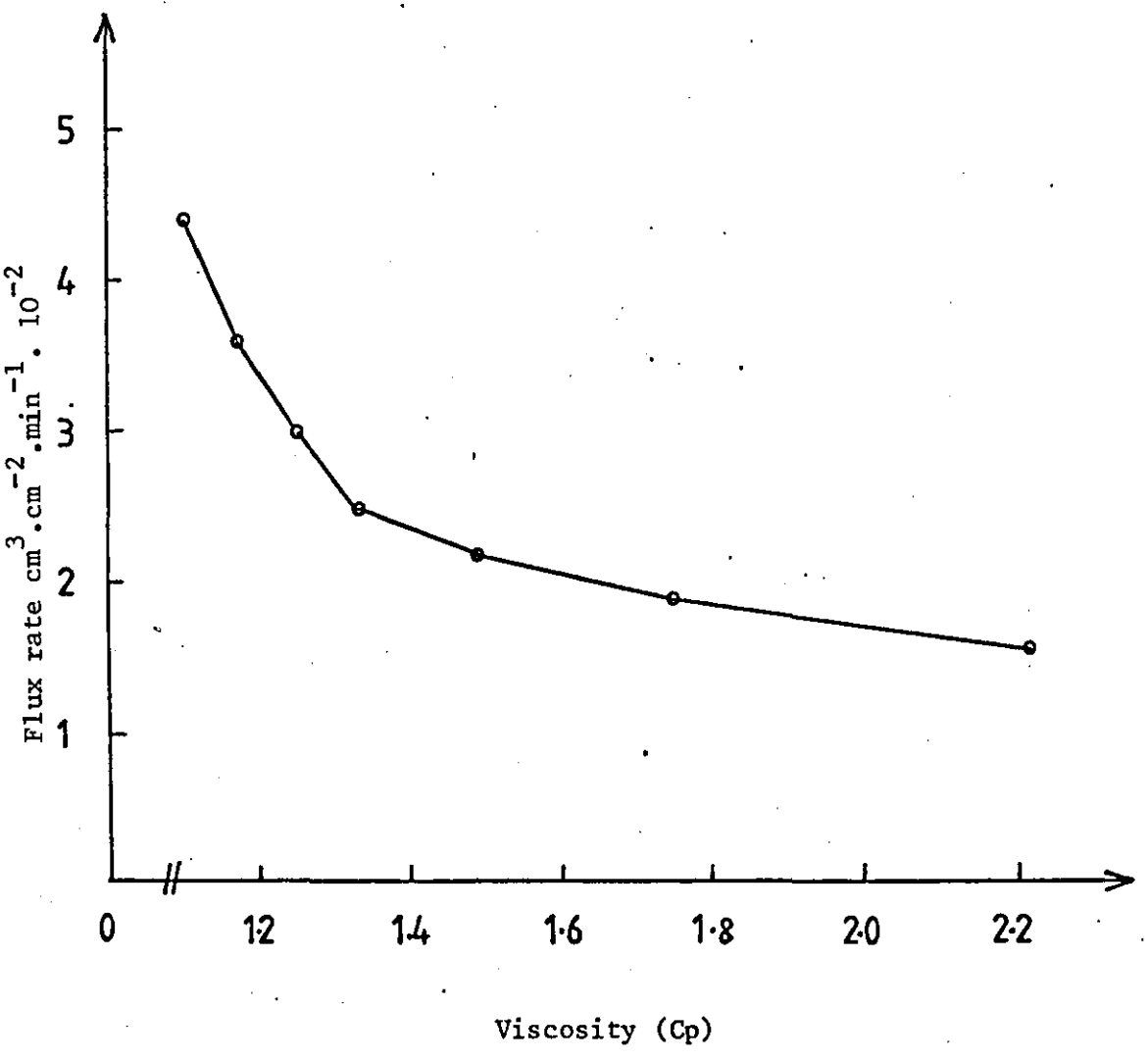


Figure 6.8

Effect of dynamic viscosity on flux rate during the UF of fish protein extract.

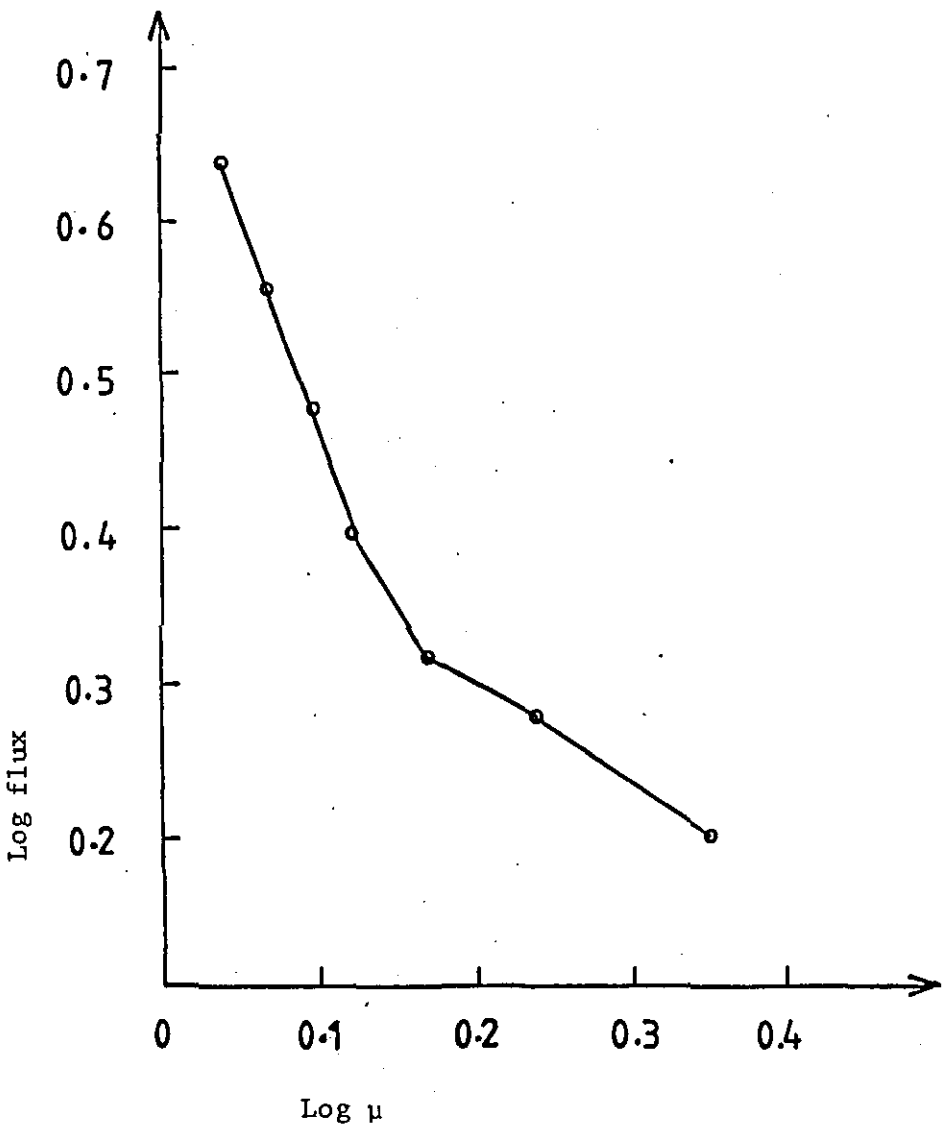


Figure 6.9

The log-log relationship between flux rate and dynamic viscosity of fish protein extract.

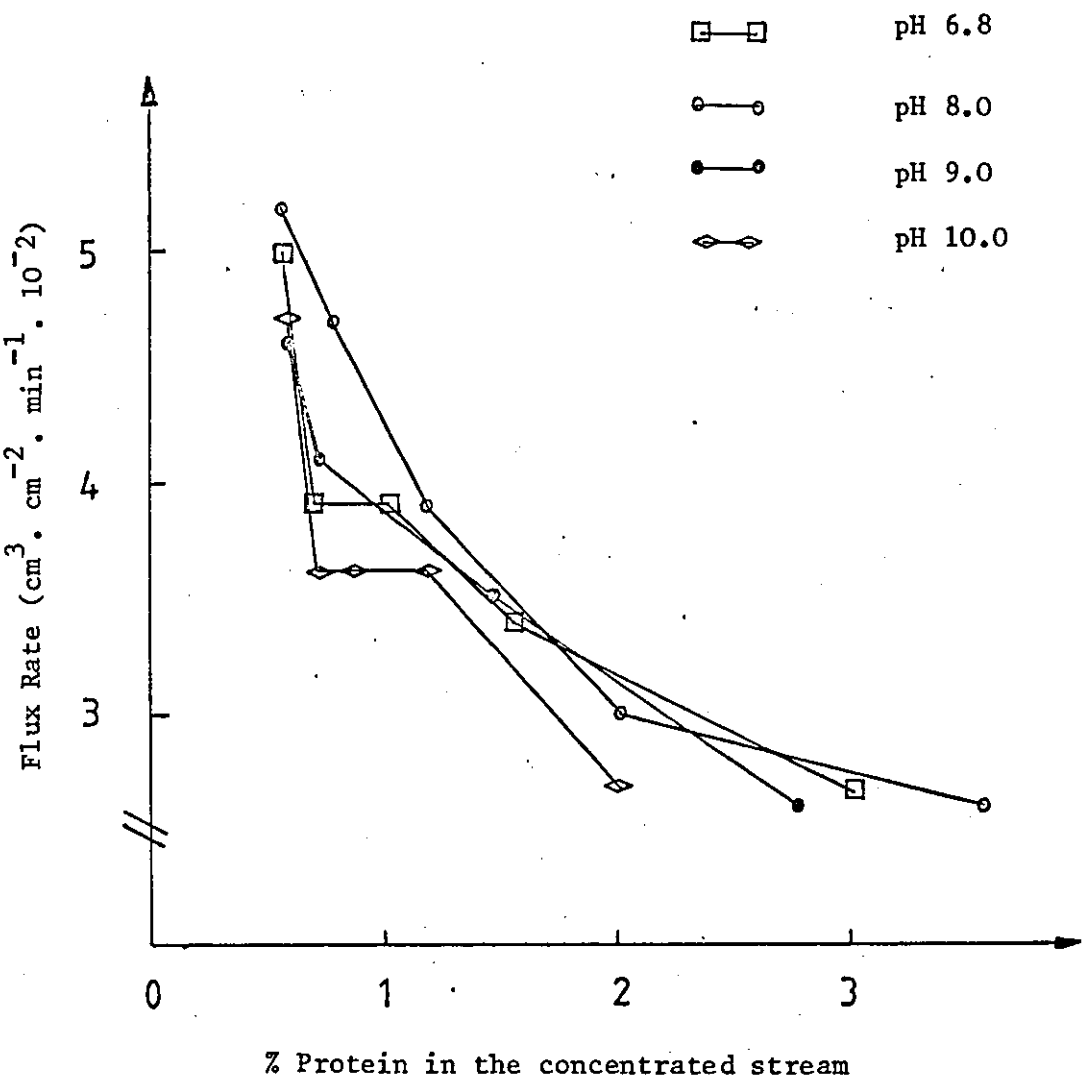


Figure 6.10

The effect of basic pH of fish protein extract on the flux rates through an PM 30 at 2.12 Kg/cm^2 and flow rate of 1.5 litre/min.

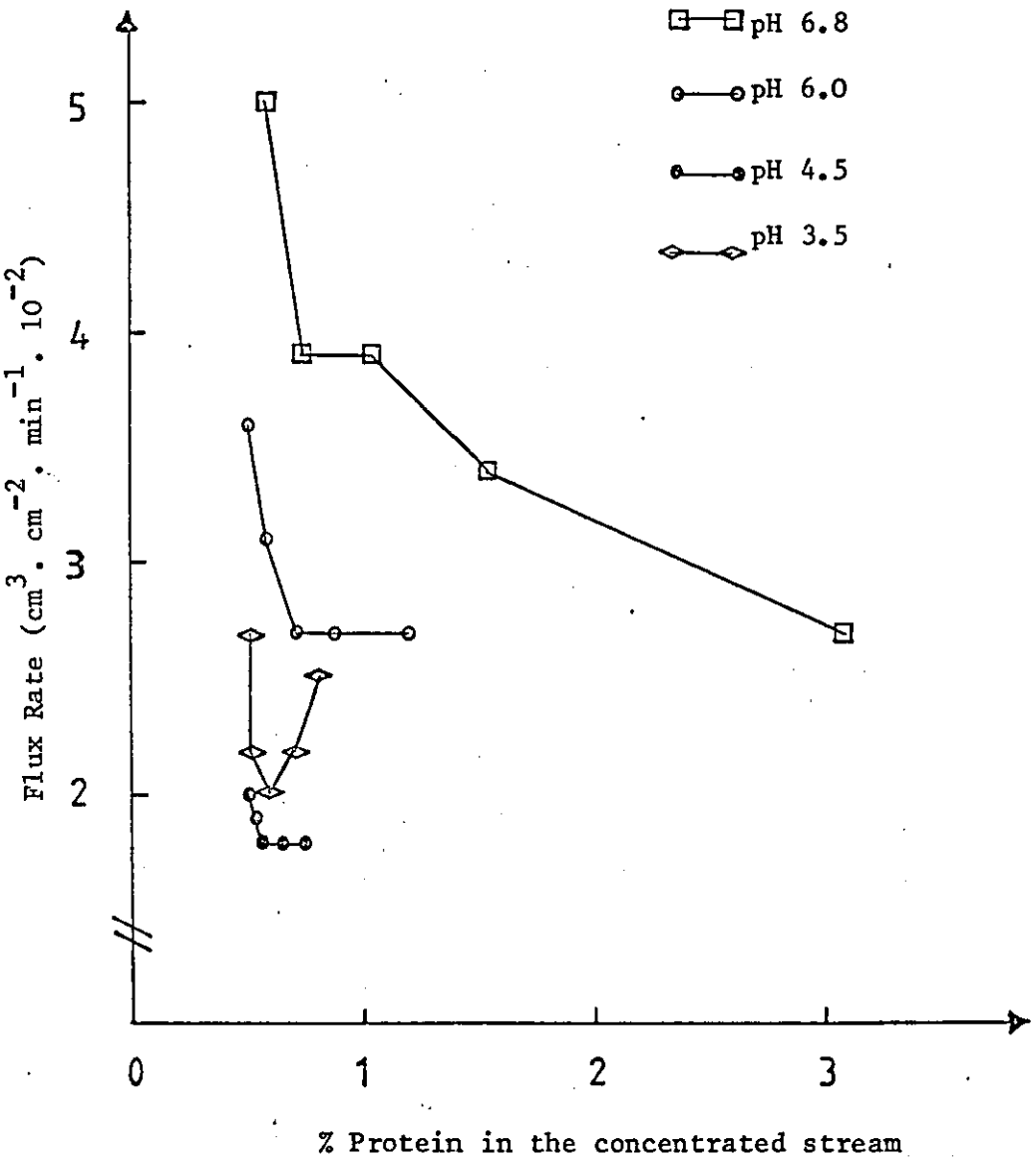


Figure 6.11

The Effect of acidic pH of fish protein extract on the flux rate through a PM-30 at 2.12 Kg/cm^2 and flow rate of 1.5 litre/min

6.8 Effect of Temperature on Flux Rate (section 3.3.5)

Figure 6.12 shows the effect of feed temperatures on flux rate, in which the flux rate is proportionally increased with the rise in temperature. The result was expected and agreed with equation 2.3. mentioned in section 2.7.3
The above data was obtained at 30 minute intervals over the process time of 3 hours. It was not possible to run the ultrafiltration equipment for more than 2½ hours at 40°C due to excessive pump overheating.

6.9 Ionic Transfer Through UF Membranes

Table 6.1 shows the concentrations and distributions of ions in feed, concentrate and permeate streams after the UF of fish protein extract.

Examination of the three streams after removal of 50% of the initial volume of feed stream showed that there were no differences in their ionic concentration within the analytical error.

6.10 Pilot Scale Concentration and Desalination Processes (Section 3.3.7.2)

The PCI ultrafiltration unit fitted with a membrane with cut-off of 20,000 (T5/A) was used to examine the performance

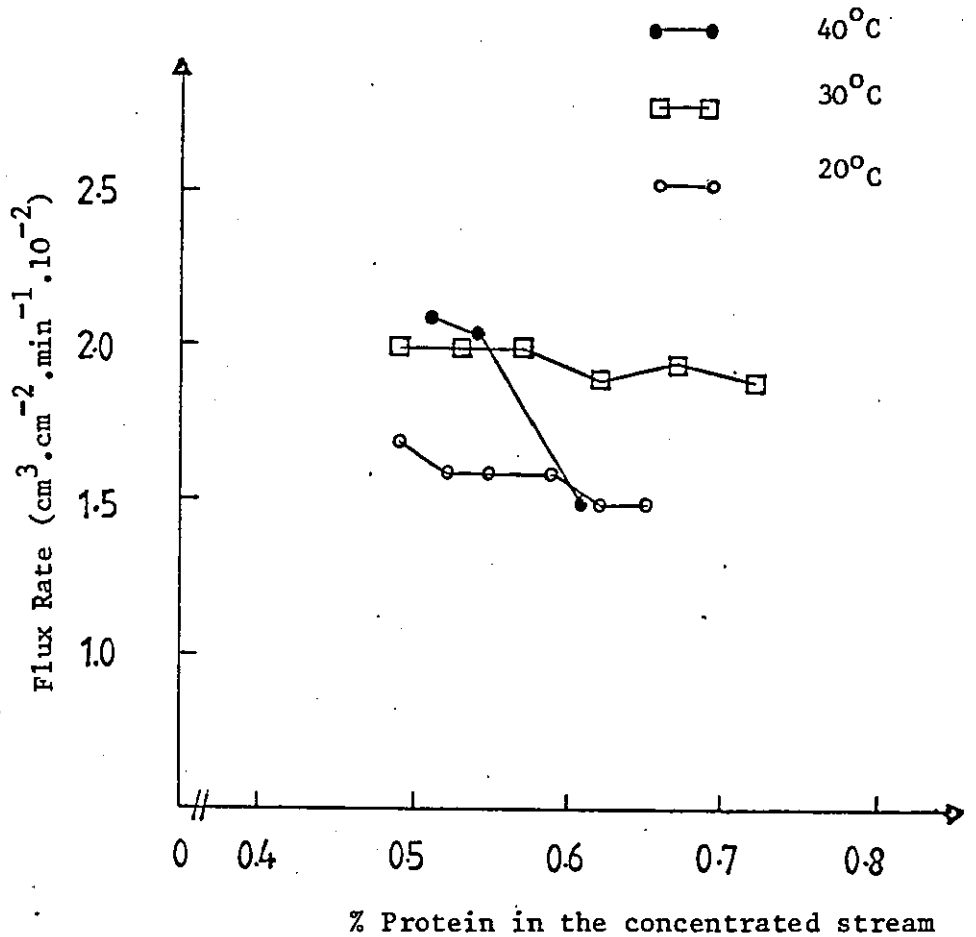


Figure 6.12

Effect of temperature on the flux rate through an UM-10 membrane at pressure of 2.12 Kg/cm^2 and flow rate of 1.5 litre/min.

Run One:

Fish Protein was extracted with 4% NaCl, the run performed in triplicate, at least 3 samples from each lot was analysed.

Run Two:

Fish protein was extracted with 4% NaCl, the run performed in duplicate, two samples from each lot were examined.

Run Three:

Fish protein was extracted with distilled water, the run performed in duplicate, two samples from each lot were examined.

All the processes were performed by using XM-50 membrane (50,000 molecular cut-off)

$$\text{Removal or Retention \%} = \frac{\text{Ion concentration in concentrate or permeate} \times 100}{\text{Ion concentration in Feed.}}$$

	Protein (%) N x 6.25	Volume of liquid cm^3	Cl^- g(%)	removal or retention (%)	Na^+ g (%)	removal or retention (%)	Ca^{+2} mg (%)	removal or retention (%)	Mg^{+2} mg (%)	removal or retention(%)	
Feed	0.46	500	2.39 ± 0.05	-	2.60	—	3.85 ± 0.9	—	0.59 ± 0.07	—	RUN ONE
Concentrate	0.77	250	2.33 ± 0.03	97.40	2.60	100	3.97 ± 0.3	103.12	0.63 ± 0.05	107.11	
Permeate	0.13	250	2.34 ± 0.04	97.74	2.60	100	3.85 ± 0.8	100	0.64 ± 0.03	107.6	
Feed	0.38	500	2.38 ± 0.05	-	1.46	-	2.5	-	0.25	-	RUN TWO
Concentrate	0.61	250	2.37 ± 0.02	99.58	1.46	100	2.5	100	0.24	96	
Permeate	0.08	250	2.38 ± 0.03	100	1.46	100	2.5	100	0.25 ± 0.003	100	
Feed	0.30	500	0.06 ± 0.002	-	0.03	-	2.84 ± 0.02	-	0.33 ± 0.03	-	RUN THREE
Concentrate	0.47	250	0.06 ± 0.003	100	0.03 ± 0.002	100	2.59 ± 0.03	91.97	0.31 ± 0.03	93.94	
Permeate	0.09	250	0.06 ± 0.002	100	0.03 ± 0.003	100	2.55 ± 0.03	89.79	0.30 ± 0.03	90.91	

Table 6.1. Concentration of Protein, Na^+, Ca++, Mg^{++}, Cl^- ions in the three streams after UF process

of pilot scale UF operations. The feasbility of applying this technique for concentrating and desalting soluble fish protein extracts has been examined. Some 85 dm^3 of soluble fish protein extract which contained 0.5% protein and 3.15% NaCl was introduced into the above units and 125 dm^3 of distilled water was added at various intervals to maintain the high flux rate before the development of a protein layer on the membrane surface.

After removal of 125 dm^3 of solvent, a mixture of distilled and saline water with the permeation stream, the concentration process proceeded until the minimum volume of liquid required to run the unit was reached.

Table 6.2 and 6.3 show the change in flux rate, salt level and protein content during the concentration and desalination processes.

Time min	Flux $cm^3.min^{-1}.cm^{-2}$	NaCl (%)
0	0.0253	1.84
30	0.0288	1.81
60	0.0247	1.43
90	0.0244	1.23
120	0.0240	0.61

Table 6.2

The change in flux rate and salt content of permeation stream as 125 dm^3 of distilled water added continuously during the desalinatio of fish protein extract in PC1 unit, flow rate 13.5 litre/min, 14.2 Kg/cm^2 (130 psi) and 30°C.

Time min	Flux rate $cm^3 . min^{-1} . cm^{-2}$	Protein (%) N x 6.25
0*	0.0240	0.33
30	0.0232	0.43
90	0.0215	0.53
120	0.0206	0.85
135	0.0200	0.95

Table 6.3

The change in flux rate and protein content of fish protein extract during the concentration process in a PCl unit. The flow rate was 13.5 litre/min, at $30^{o}C$ and 14.2 Kg/cm^2(130 psi)

The above experimental results showed that the salt content of soluble fish protein extract was reduced as distilled water was added continuously to the feed stream. The results are also in agreement with those obtained from the bench scale UF process.

6.11 Discussions and Conclusions:

6.11.1 Concentration of Protein and Flux Rate

The experimental results revealed that the UF technique is a successful means for concentrating and desalting fish protein extract, in which both simultaneous increases in protein concentration and reductions in the salt content can be

achieved.

The flux rate is significantly affected by protein concentration of feed stream. Figure 6.6 shows that the flux rate decreased by 36% of the original value as protein concentration rose from 1.3% to 6%. This decline in flux rate can be related to the following facts:

i) the build up of a concentration polarization layer will seriously reduce the flux rate, where firstly it will form an additional hydrodynamic resistance (R_G in equation 2.5, section 2.7.4) for mass transport of liquid and permeable ionic species, and secondly it will result in a localized increase in osmotic pressure of the concentrated stream, thus reducing the driving force available to induce flow of permeate.

ii) Michael's et al.(1965) equation (2.3 in section 2.7.3) suggested that the flux rate is inversely proportional to the dynamic viscosity of the liquid. As found in sections 6.5 and 6.6 the general trend of flux is decreasing with increasing viscosity or protein concentration of fish protein extract, but not in the manner described in equation 2.3 which should be linear when the logarithm values of the above data are plotted.

The literature has reported different data concerning the effect of protein concentration on the flux rate. For example, the results obtained by Fenton-May, et al,(1971) concerning the fractionation of whey protein by RO/UF are in agreement with Michael's et al, (1965) equations.

On the other hand Donnelly & Delaney (1974) found that the flux rate was linearly decreasing with the logarithic values of protein concentration according to the equation of Blatt, et al., 1970 (equation 2.4 in section 2.7.3).

Blatt et al, (1970) proposed this equation to describe the flux rate under concentration polarization conditions, when pressure indepenance occurred. Blatt's treatment of the equation assumes that Cg is constant throughout the experiment, but even if this were true, the increasing thickness of the gel layer next to the membrane would invalidate his conclusions.

Thus it would seem that the Donnelly & Delaney (1974) approach was unsuccessful in establishing such a relationship between flux rate and protein concentration.

Examining Figure 6.9 reveals that flux rate decreased apparently linearly up to a protein conentration of 2.25% (see Figure 6.6) and then diverted away. The reason for this is not clear. It may possibly reflect the effect of the concentration polarization layer, which as described above can reduce the flux rate in a manner which varies from that applicable before the development of the concentration polarization layer. However, no experimental data are available to confirm this explanation.

6.11.2 The pH and Flux Rate

The pH of the solution affects the solubility and

the stability of protein dispersions, thus any changes in the pH may involve an alteration in protein molecular conformation (size, shape and charges). The arrangement, aggregation or dissociation of protein sub units will change according to the new environment. All these changes will greatly affect the viscosity of protein solution. (Paul and Palmer, 1972).

Therefore, the viscosity will change toward the resultant ionic atmosphere. For example, if the pH changed from the ios-electric point in either direction one should expect an increase in viscosity as the repulsive force increased and the protein chains unfolded (Angelmier and Montgomery, 1976). The contrary would be expected if the pH approached the iso-electric point, when one would expect the viscosity to drop.

The normal pH of soluble fish protein extract was found to be 6.8. The experimental results showed that there was no marked change in the flux rate around the basic pH between 7-10, while the flux rate decreased around the acidic pH as protein tends to precipitate and clog the membrane.

In membrane separation processes, it is necessary to keep the pH of the solution within the limits recommended by the membrane manufacturer, for example (2 and 8 in case of PCl membranes at 15^{o}C, whereas Amicon membrane had a wider pH range between 2 and 12 at temperatures between 50^{o}C - 70^{o}C), that is to maintain the ionic stability of the membrane in order to keep membrane performance as high as possible and membrane life as long as possible. According to the above results obtained,

it is not recommended to alter the pH of fish protein extract, because

i) no significant improvement in the flux rate results, and

ii) the protein denaturation which might be caused by pH alteration, which will possibly cause some deterioration in the functional properties.

6.11.3. The Temperature and Flux:

The experimental results showed that the flux rate is proportionally increased with the operating temperature of the UF unit. The viscosity is reduced by an increase in temperature. (West 1963). These results are in agreement with equation 2.3 (section 2.7.3) which suggests a strong dependence of flux on the viscosity of the liquid. Thus it is seen that the flux rate is strongly affected by the operating temperature of UF system.

It was found that the flux rate increased by 18.75% as liquid temperature rose from 20 to 30^{o}C and by 16% in the range 30-40^{o}C subsequently.

The most desirable operating temperature of the UF unit is determined by two factors:

i) from the engineering point of view, it is favourable to run the system at the highest possible temperature recommended by membrane manufacturer, to achieve maximum flux rate.

ii) from the microbiological and sanitary point of view it was found that the average of total microbial counts of fish protein extract incubated at 15°C, 30°C were 1 x 10^5 and 25 x 10^7 colonies cm^3 respectively. This indicates that the microbial activity can increase with operating temperature.

It is also know however that at 50°C the microbial growth will be reduced (Stainier et al., 1971), but such high temperatures coupled with long operating times, would impair some of the functional properties of protein through thermal denaturation. It is therefore necessary to compromise between the high flux, high microbial activity and protein denaturation by operating the system at moderate temperature (around 25°C), where the microbial growth will be acceptable from the sanitary point of view, and the flux rate is reasonable.

6.11.4 Membrane cut-off and Flux Rate

The membrane cut-off is determined by the hydraulic mean of pore radius and membrane porosity (R,E. respectively in equation 2.3 section 2.7). These values are greater in the membranes with large pore sizes than in the membranes with smaller pore sizes, thus RM-30 loose membranes gave higher flux rates, as shown in Figures 6.4 and 6.5.

It is therefore recommended to use the higher molecular membrane cut-off, provided it is able to retain the concentrated species. The molecular mass of salt-soluble fish protein is

around 500,000. (Paul and Palmer 1972), so even the PM 30 membrane is a conservative choice.

6.11.5 Ionic Transfer Through UF Membrane:

As previously mentioned in section 2.7, UF membranes act as molecular screens, where any species smaller than the membrane cut-off is able to pass with the permeation stream.

The UF membrane cut-offs ranged between 10 and 10^3 Å. The radius of ions of interest to this study (Na^+, Ca^{+2}, Mg^{+2}, Cl^-) ranged between 0.65 and 1.81 Å. (Daniels & Alberty,1961). One should therefore expect these ions to pass easily through the UF membranes used. The statistical analysis of ionic concentrations (Table 6.1) showed that the difference between the three streams was less than, or within, the range of standard deviation of each sample. This would suggest that the concentration of ions in the three streams are identical, or that the ions were equally transferred through the UF membrane. It is therefore likely that the above ions are present in unbonded form.

The above ion behaviour is clearly restricted to the test conditions, so that care should be exercised before assuming that this would be true under different experimental conditions or in another biological liquid. In fact it is thought that the subject needs further investigation.

The following point may be relevant in this regard:

i) The ions in the liquid system may be free or bonded to the protein molecules.

ii) The presences of free ions as indicated in the tests, necessitates the consideration of the development of the concentration polarization layer, especially during the concentration of high protein content solutions. It is thought such layers will be greatly affected by the ready access of these ions and permeable molecules to the membrane, where some of the ions will be trapped or bonded to the concentration polarization layer, which contains functional groups ready to react or accept some ions.

iii) If the ions are bonded to the protein molecules then they will be retained with the concentrated stream, and it will be very easy to detect and measure the ionic concentration in each stream of the UF process.

iv) The permeate stream may contain some proteins which can bind some ions. This possibility will affect the ionic transfer through the UF membrane.

v) An important fact is that the nature and composition of the protein system will play a significant role in the ionic transfer through the UF membrane. For example, Terepka and co-worker (1970) found in their study of Na^+,K^+,Cl^- and water distribution in human serum, a difference between the composition of concentrate, permeate and feed streams after prolonged ultrafiltration. They proposed that the original serum contains non-diffusible and freely diffusible components. The latter only is passed with the

permeation stream and this explained the difference in the compositions of the three streams after prolonged ultrafiltration.

Chapter Seven

Chapter Seven

Examination of the Functional Properties of FPC

The work described in this chapter was carried out to examine and assess the functional properties of FPC prepared on pilot scale equipment from Fish Waste (FW) by the saline extraction process.

The dried product was firstly examined in solutions such as water and oil and then incorporated in some food recipes to demonstrate its functionality these systems.

The experimental results are as discussed below:

7.1. Solubility in Water (section 3.3.6, 3.4.1)

In the early work performed on this project, the solubility test conducted on FPC prepared on bench scale resulted in a solubility value of 79% in distilled water at 25°C. This is significantly different from subsequent results described below, and is discussed further in Section 7.9.1.

7.2 Solubility in Saline Solutions (section 3.4.1)

Figure 7.1 shows the solubility profile of FPC suspended in sodium chloride solutions at pH7, in which the solubility value of 42% was obtained with distilled water. The solubility values increased by 10% with solutions containing upto 0.51 M-NaCl. Above that the solubility declined and then levelled at a value of 46% with 0.85 M-NaCl.

7.3 Solubility in Buffer Solutions (section 3.4.1.):

Figure 7.2 shows that the solubility of FPC in different

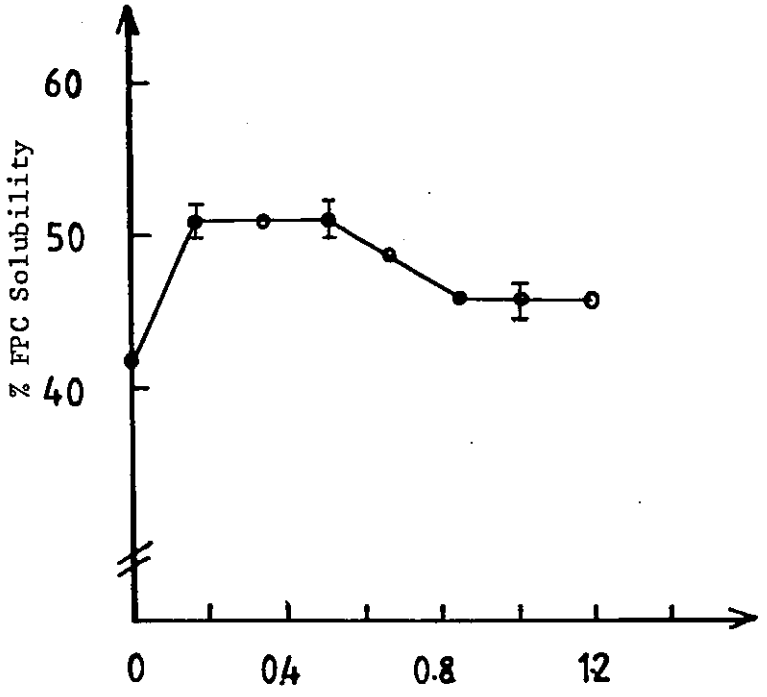


Figure 7.1

The solubility of FPC as a function of NaCl solutions strength at pH 7 and 25°C.

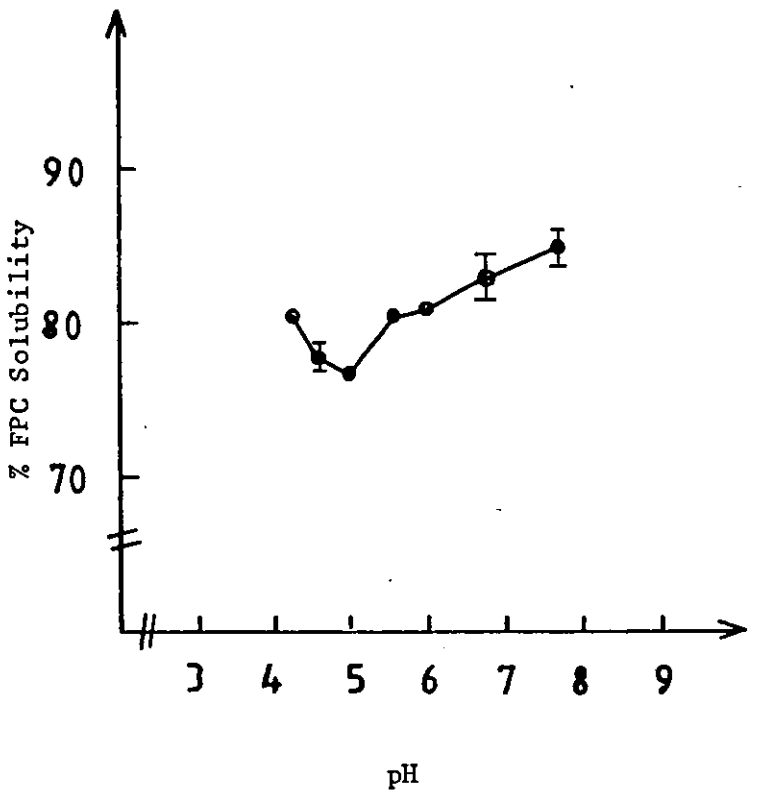


Figure 7.2

The Solubility of FPC as a function of pH and 25°C

phosphate buffers between pH 4.2 - 7.8 was lowest at pH 5 and increased as pH changed in both the acidic and basic directions.

7.4. Water Holding (Swelling) Capacity (Section 3.4.2):

Water Holding Capacity (Swelling) was defined in section 2.3.1. Figure 7.3 shows that the volume of absorbed water progressively increased with the weight of FPC.

It was therefore confirmed that the protein wetted readily throughout its bulk. Note however that the approximate straight-line relationship was only obtained by taking ten samples at each quantity of FPC. Only in this way could the variability be reduced to enable the straight line relationship to be obtained.

The correlation coefficient analysis (r) was applied to the date and found to be 0.98 which is significant to 99% confidence at 8 degrees of freedom (see appendix 4.1).

7.5 The HeatCoagulation Test (section 3.4.3)

The soluble fish protein extract at pH 6.8 did not coagulate upon heating for 15, 30 and 60 minutes in boiled water bath.

The absorbance (A) or optical density (O.D.) at 540 nm of heated and unheated extracts are as shown in Table 7.1

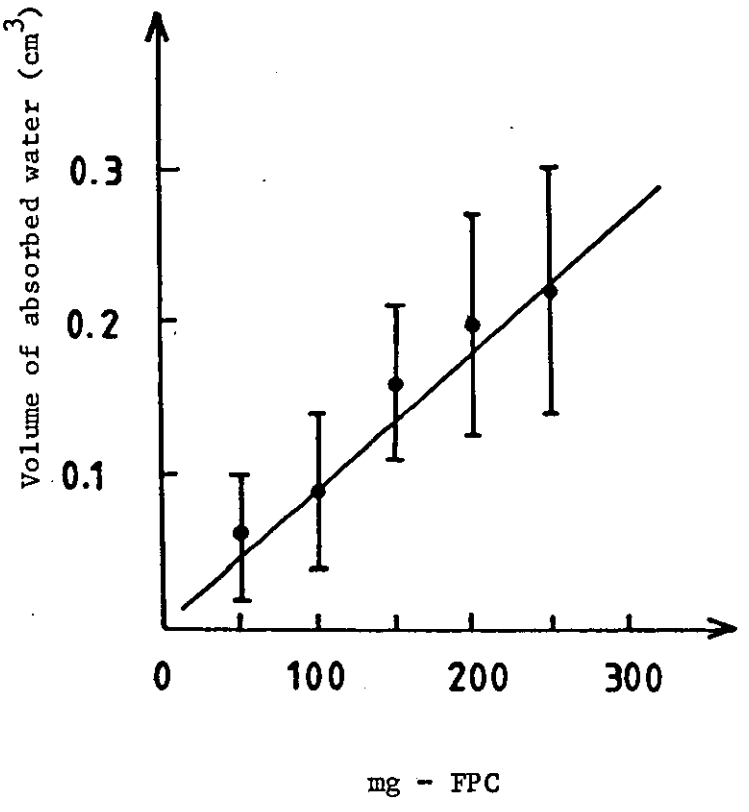


Figure 7.3

The Swelling Capacity of FPC

Heating time min	% P N x 6.25	A unheated	A heated
15	0.10	0.13	0.21
30	0.10	0.13	0.20
60	0.10	0.13	0.21

Table 7.1

The change in the absorbance (A) at 540 nm of heated and unheated fish protein extracts to which Biuret reagent was added.

The heated protein solutions became turbid as reflected by higher absorbance values.

The results suggest that the ionic balance of fish protein extract at pH 6.8 renders protein solutions heat resistant which eventually prevent coagulation.

It is apparent that the ionic balance of fish protein must be changed to allow coagulation.

This was done by suspending FPC in different phosphate-citrate buffers.

The protein extracts were heated for 30 minutes in a boiling water bath and measuring the absorbance at 540 nm as shown in Table 7.2

pH	% Protein N x 6.25	A - unheated		A - Heated		% * Coagulation
		Range	x	Range	x	
4.3	0.81	0.22-0.26	0.24	0.01-0.05	0.03	x
4.6	0.78	0.22-0.24	0.22	0.01-0.04	0.03	x
5.0	0.77	0.22-0.26	0.23	0.03-0.07	0.06	74
5.6	0.80	0.17-0.20	0.19	0.88-1.04	0.96	nil
6.0	0.81	0.14-0.17	0.16	0.72-0.80	0.77	nil
6.8	0.83	0.15-0.17	0.16	0.56-0.59	0.58	nil
7.8	0.81	0.16-0.17	0.17	0.40-0.45	0.44	nil

*% Coagulation $= \frac{\text{A before heating - A after heating}}{\text{A before heating}} \times 100$

x Protein precipitation occurred at this pH before heating

o Equal volumes of Biuret reagent and fish protein extract were tested. The test was performed in duplicate.

Table 7.2

Effect of pH on the change in the absorbance at 540 nm of heated and unheated fish protein extracts to which Biuret reagent o was added.

When fish protein extract at different pH was heated, white elastic coagulum was only formed at pH 5, which suggested that this ionic balance enhanced or favoured the coagulation of protein. This pH was used during the subsequent test. Figure 7.4 shows the coagulation (%) of different concentrations of fish protein extract between 0.10 - 1.40% adjusted to pH 5.0.

Samples of suspended blood albumin in distilled water prepared so that the protein concentration was between 0.10% and 0.50% at pH (5 - 5.1) were also examined for comparison. Both protein coagulation percentages were found to progressively increased as the protein concentration increased. Significantly higher values were obtained with blood albumin.

The r-test was separately applied to fish protein and blood albumin data and showed:

i) for fish protein, r = 0.97 which is significant to 99.0% confidence at 10 degrees of freedom.

ii) for blood albumin, r = 0.94 which is significant to 99.0% confidence at 8 degrees of freedom (see appendices 4.2 and 4.3).

7.6 Surfactant Capacity (Section 3.4.4)

7.6.1. Emulsion Stability as a function of FPC Weight

The emulsion stability of FPC in distilled water and corn oil as a function of sample weight is shown in Figures 7.5 and 7.9 inclusive. Samples of egg albumen flakes and soy bean isolate were also examined for comparison as shown in Figure 7.10 and 7.11 respectively. In all these Figures, the emulsion layer before collapse represents the volume of cream layer.

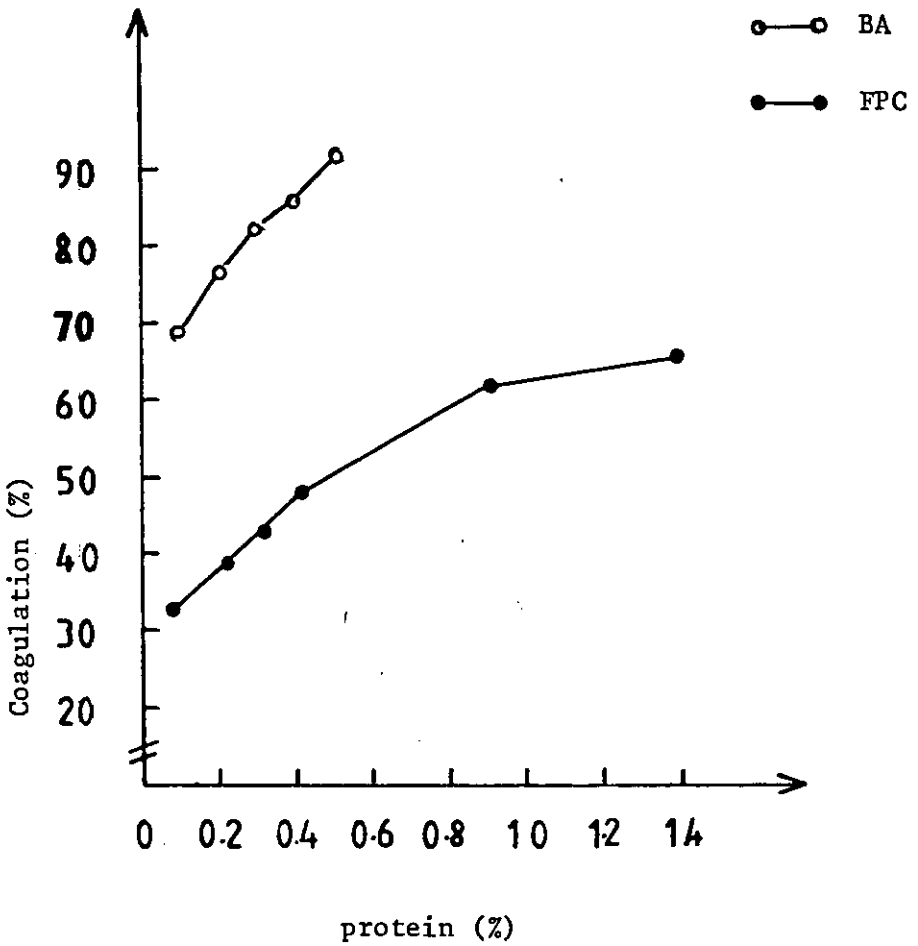


Figure 7.4

The coagulation test of FPC and Blood albumin (BA)

The experimental results showed the following observations:

i) The initial volume of cream layer was progressively increased with increase of FPC up to 3 g, above which no further significant increase occurred.

ii) The collapse time of the cream layer of 1 g FPC was 30 min (Figure 7.5), which increased to 5 h with 2 g FPC (Figure 7.6) and reached about 24 hours with 3,4, and 5g FPC (Figures 7.7, 7.8,7.9).

iii) As can be seen from Figures 7.5 - 7.9, the final volume of stable emulsion after 120 hours was as shown below in Table 7.3

Weight of FPC (g)	Volume of emulsion (cm^3)
1	22
2	21
3	20
4	27
5	23

Table 7.3

The volume of stable emulsion layer of FPC,50 cm^3 of water and 10cm^3 of oil after 120 hours.

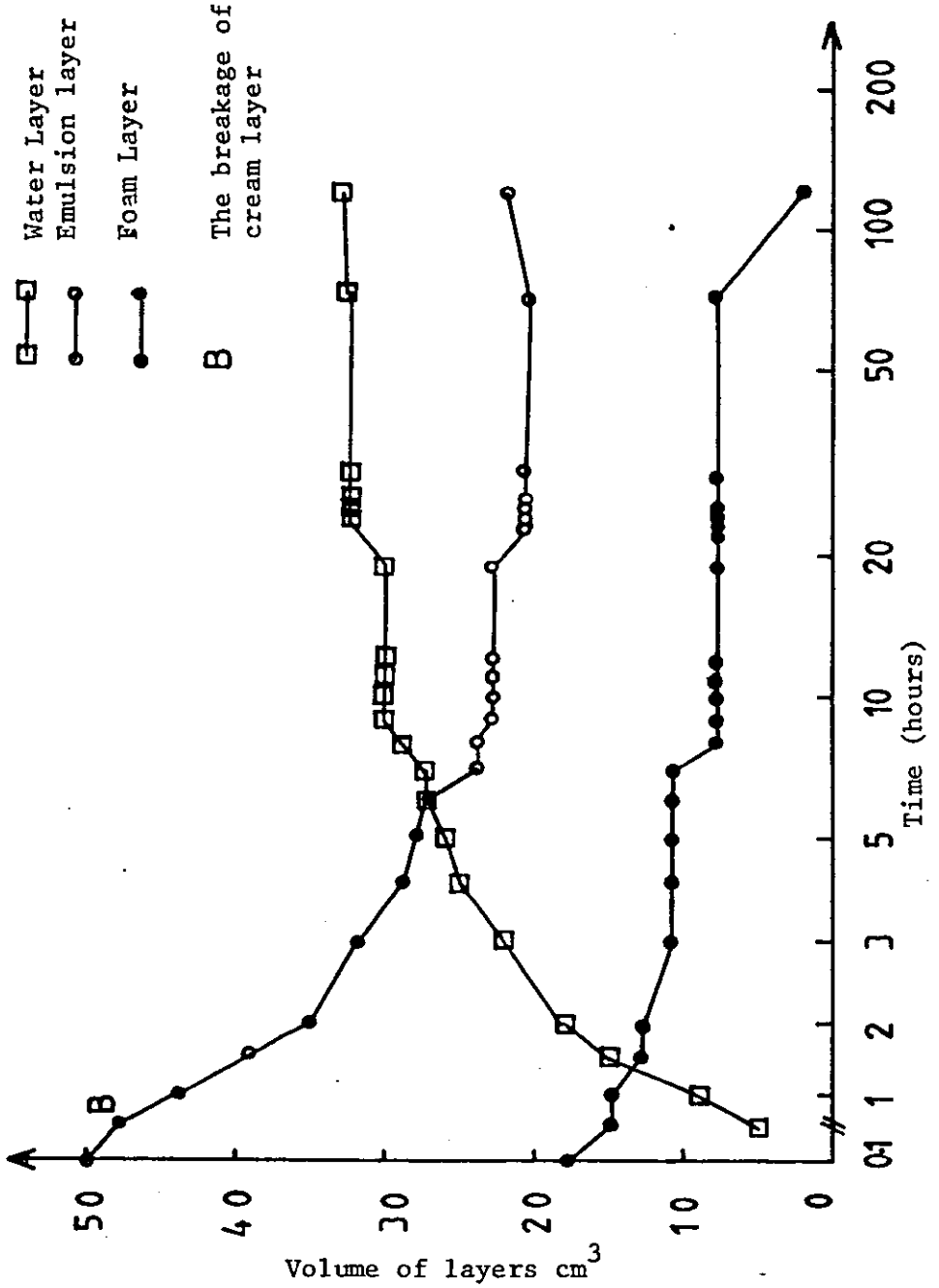


Figure 7.5 The emulsion stability of 1 g FPC + 10 cm^3 corn oil + 50 cm^3 distilled water

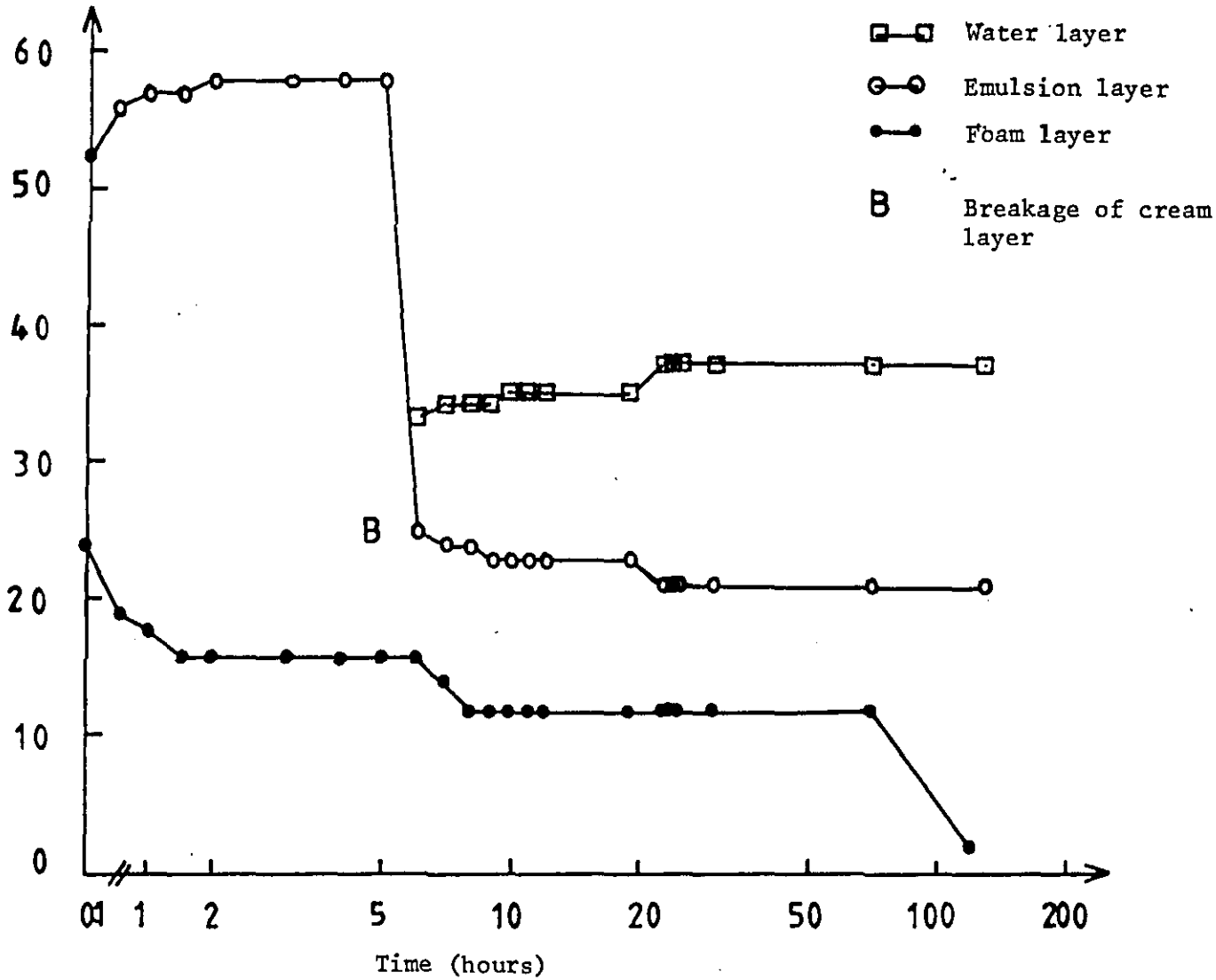


Figure 7.6 The emulsion stability of 2g FPC + 10 cm^3 corn oil + 50 cm^3 distilled water.

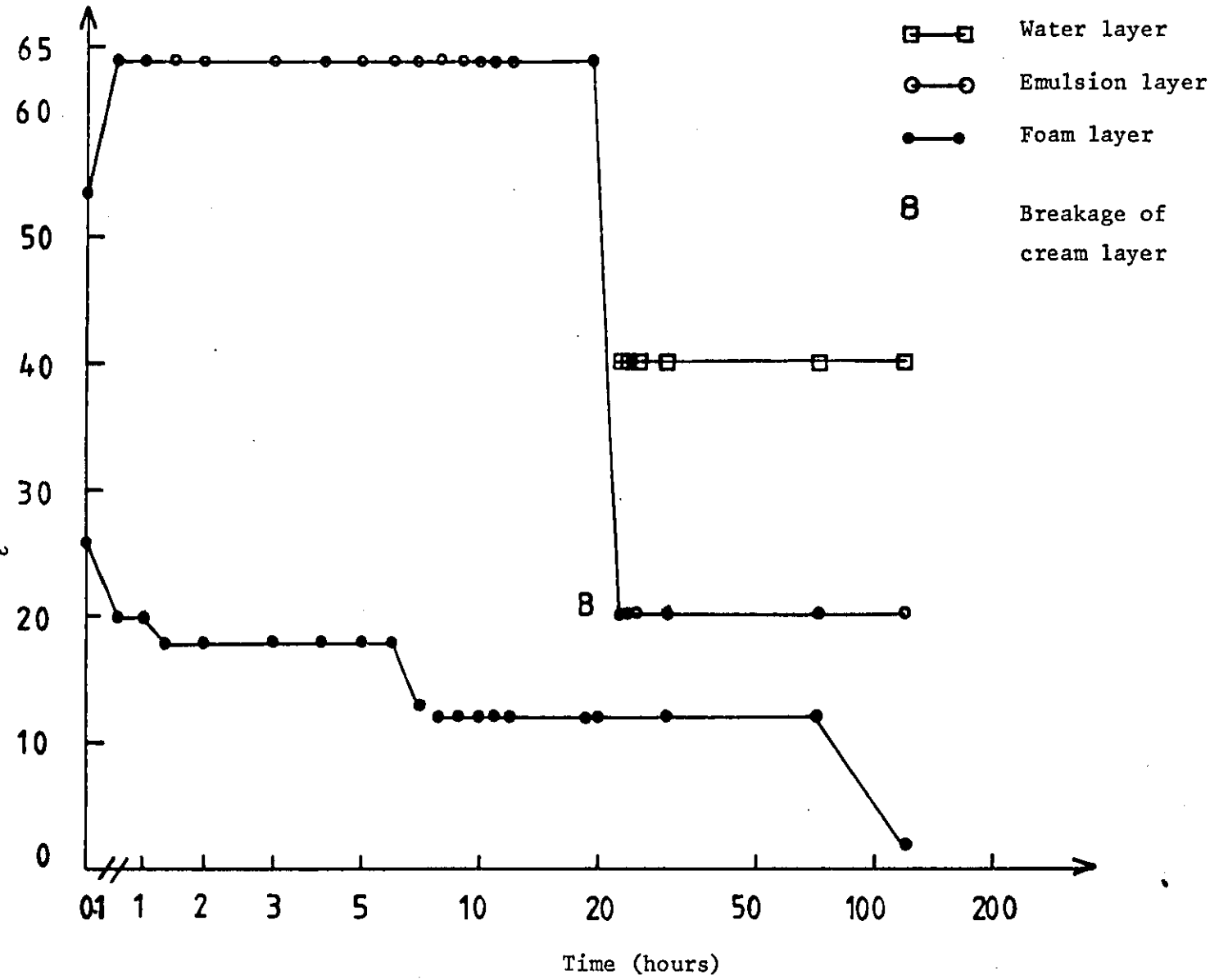


Figure 7.7. The emulsion stability of 3g FPC + 10 cc oil + 50 cc distilled water.

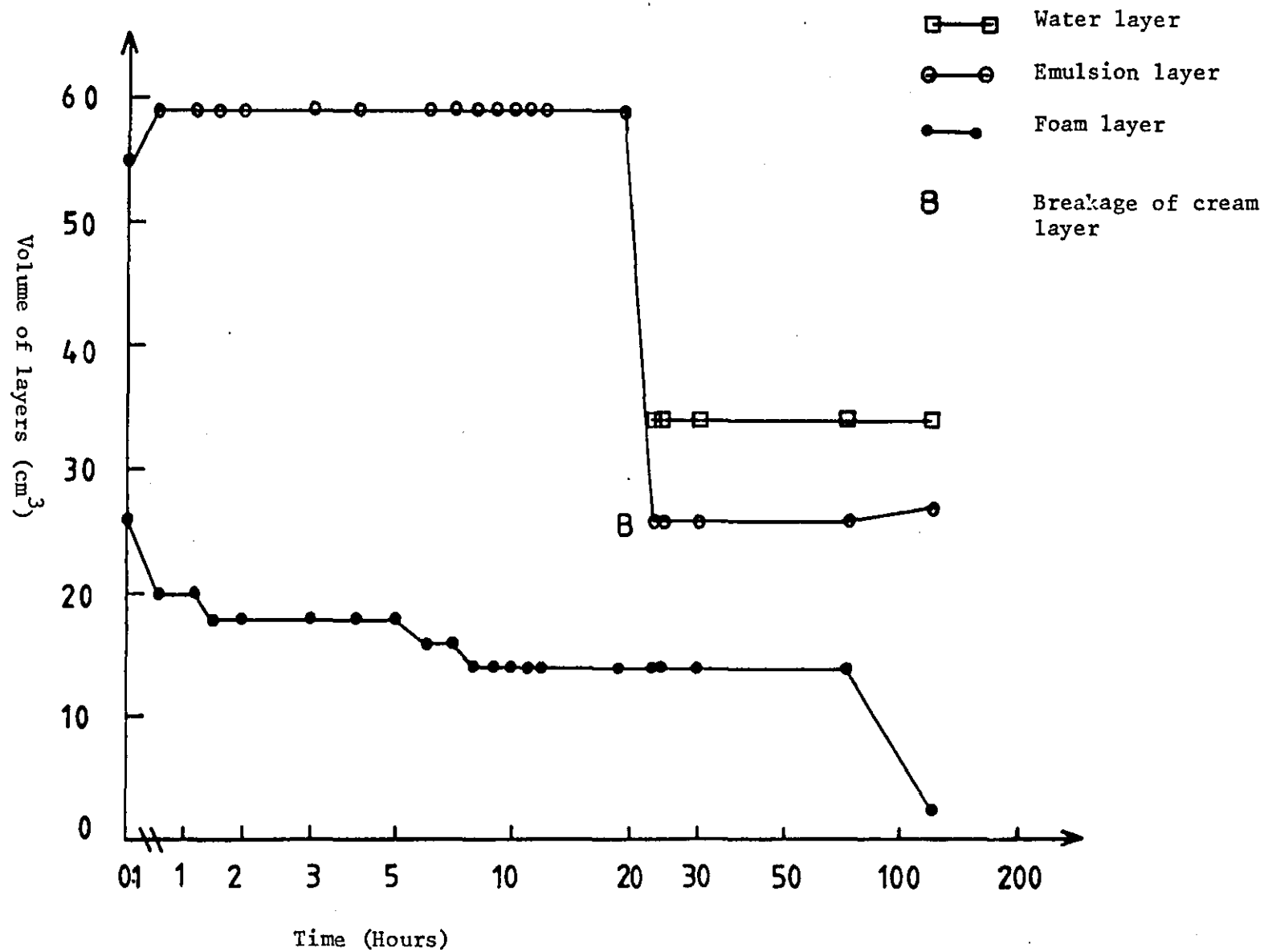


Figure 7.8 The emulsion stability of 4g FPC + 10 cm^3 oil + 50 cm^3 distilled water.

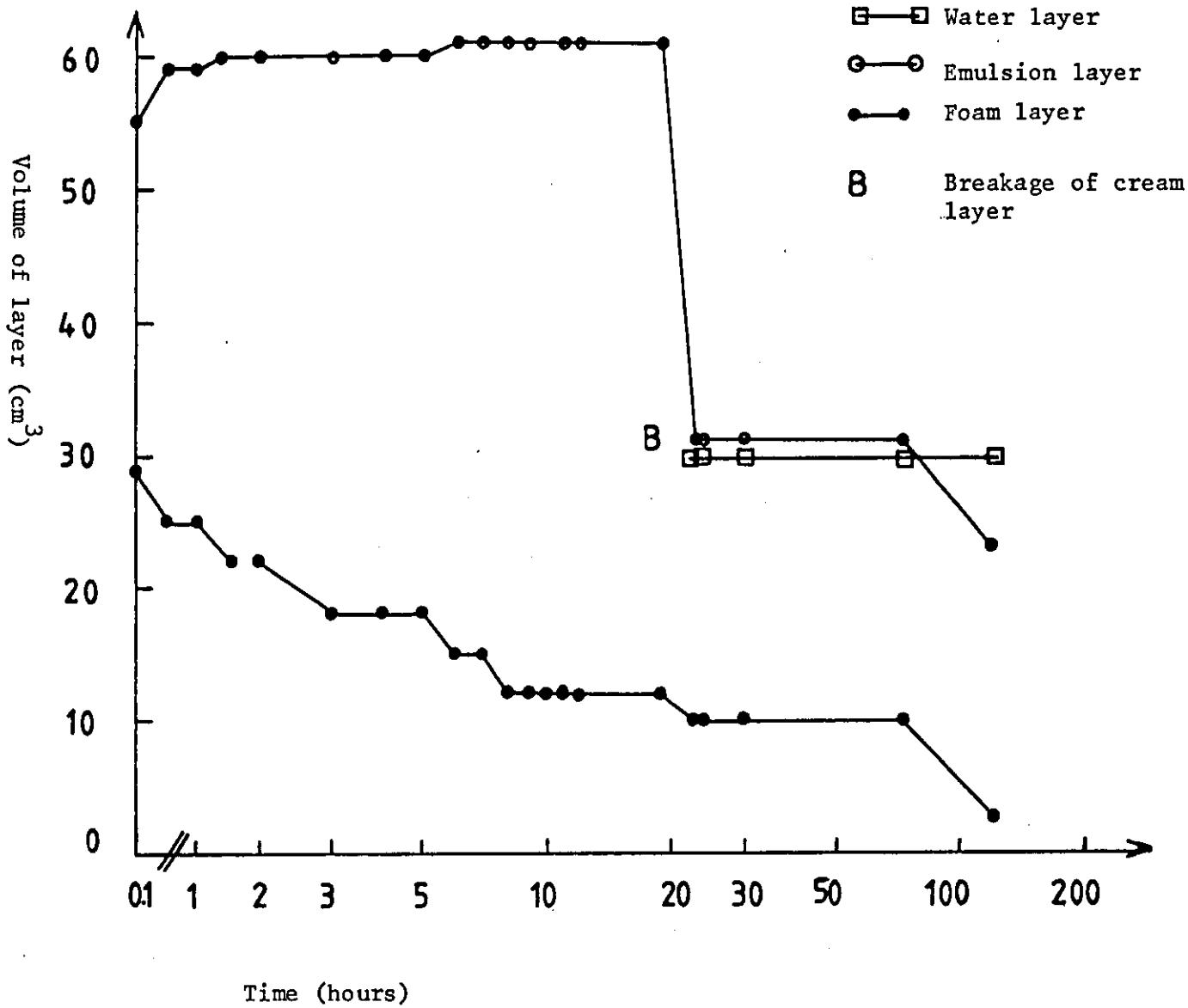


Figure 7.9 The emulsion stability of FPC (5g) + 10 cm^3 oil + 50 cm^3 distilled water.

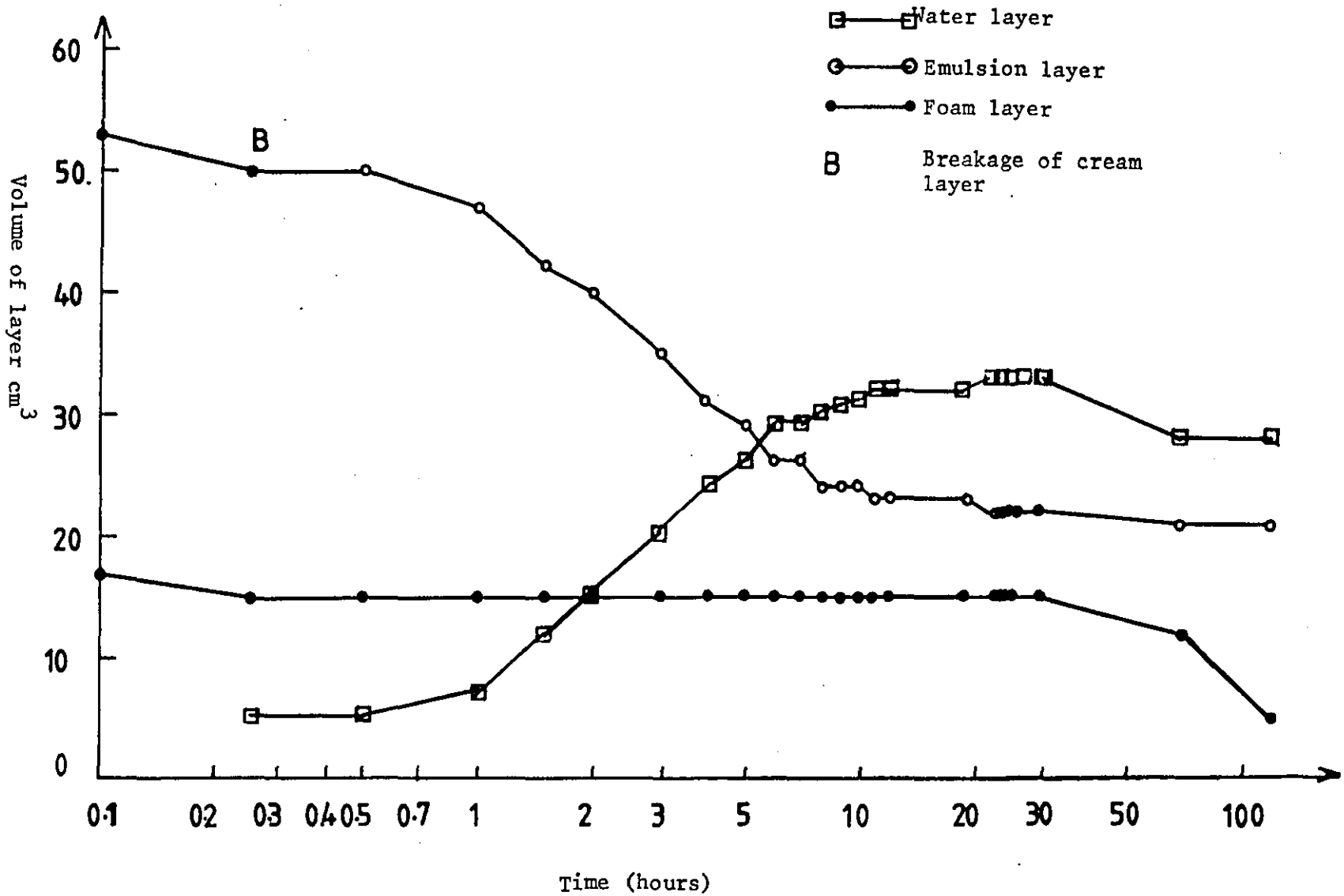


Figure 7.10 The emulsion stability of 5g of egg albumen flakes + 50 cm^3 distilled water + 10 cm^3 Oil

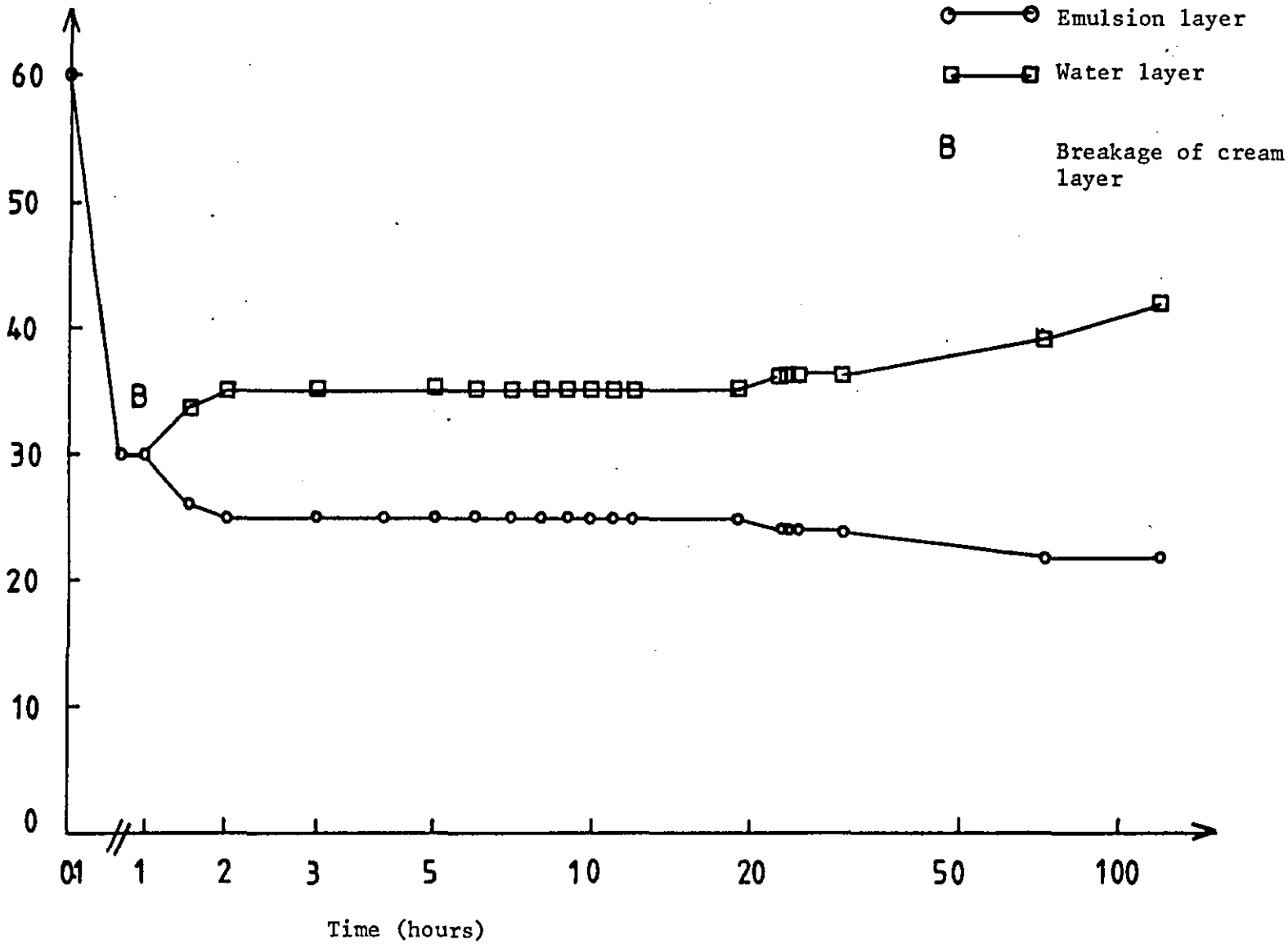


Figure 7.11 The emulsion stability of 5g soy bean + 10 cm^3 oil + 50 cm^3 distilled water.

When FPC samples were emulsified with 10 cm^3 of corn oil and 10 - 17 cm^3 of distilled water, the increased weight of FPC did not result in higher emulsion volumes. Therefore it seems that when 1 g FPC was emulsified in 10 cm^3 of oil and water, the excess weight of FPC appeared to stabilizie the emulsion as reflected by the longer collapse time.

iv) The cream layer of 5 g egg albumen (Figure 7.10) collapsed after 15 min. The emulsion layer gradually declined until 24 h, after which it levelled out at 21 cm^3. Although the volume of final stable emulsion was similar to that obtained with FPC samples, the short collapse time indicates poor stability.

v). The cream layer of the emulsion of 5 g soy bean, 10 cm^3 corn oil and 50 cm^3 water (Figure 7.11) collapsed after 2 min. The emulsion layer declined during 2 h after which it levelled out at 25 cm^3 and then stabilized at 22 cm^3 after 70 h.

Therefore it can be seen that 10 cm^3 of corn oil and each 5 g of egg albumen and soy bean separately emulsified with 11 cm^3 and 12 cm^3 of distilled water respectively.

vi) The volume of the foam layer of FPC samples was progressively increased with increasing weight of the sample.

It gradually declined until 70 h after which it further declined and had a volume of 2 cm^3 after 120 h. Although less foam volume was obtained with egg albumen (Figure 7.10) compared with FPC samples, better stability was obtained as

foams stood for 30 h without noticeable deterioration. After this time they gradually declined.

7.6.2. Emulsion Stability of FPC as a Function of Oil Volume

The emulsion stability of FPC, distilled water and corn oil as a function of oil volumes is shown in Figures 7.12 - 7.15 which indicate the following observations:

i) The initial volumes of cream layers were progressively increased with the volume of added oil.

ii) The cream layer collapsed after 26 h in all tested samples.

iii) As can be seen from Figures 7.12 - 7.15 the final emulsion layers after 360 h - were as shown in Table 7.4.

Oil added (cm^3)	Volume of emulsion (cm^3)
10	15
20	28
30	46
40	62

Table 7.4 Effect of oil volume on the volume of emulsion layer of 5g of FPC,50 cm^3 water and oil after 360 hours.

The above results show that FPC and 10, 20, 30 and 40 cm^3 of oil respectively emulsified 15, 18, 16 and 22 cm^3 of distilled water, indicating that FPC tends to bind oil rather than water.

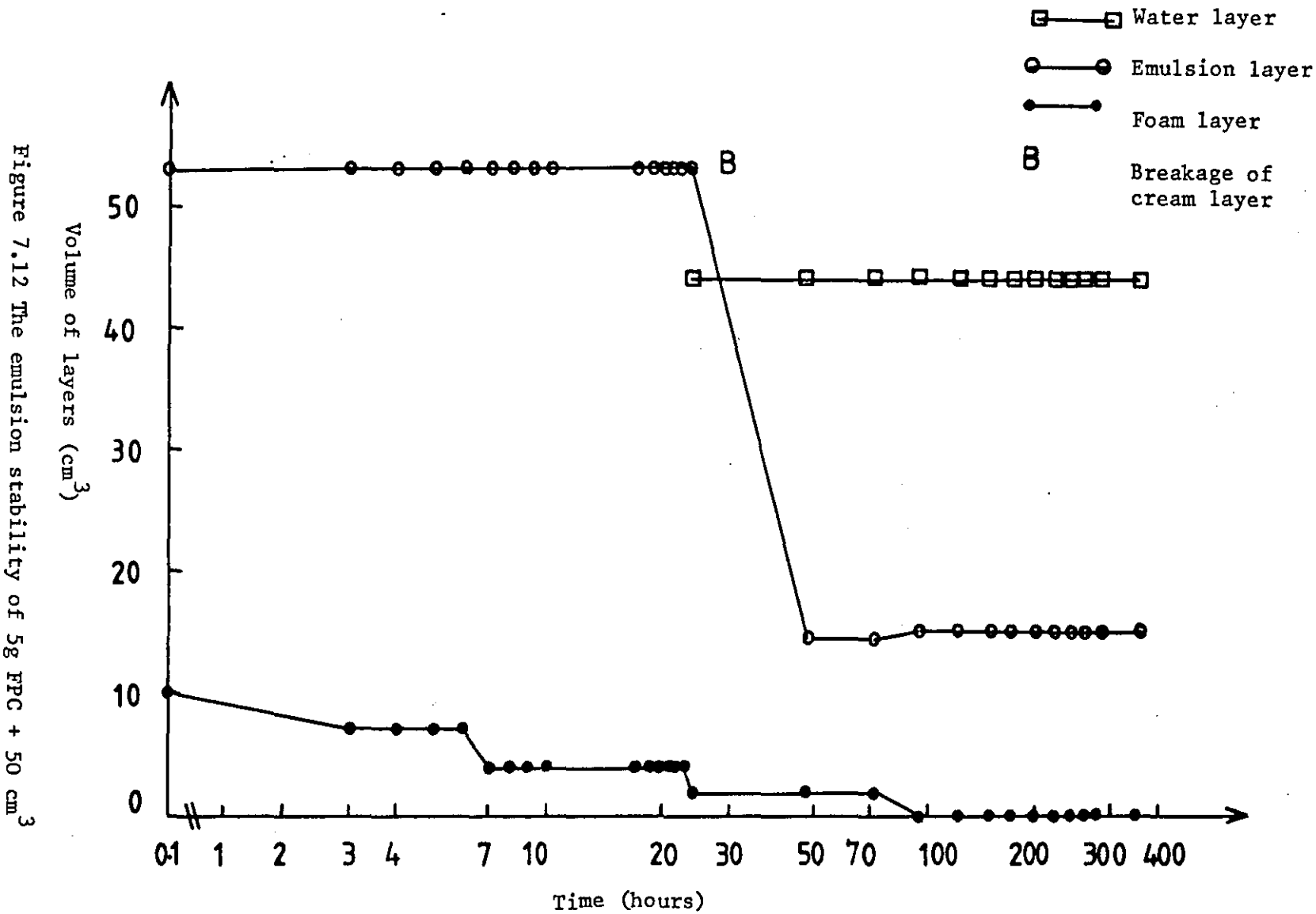


Figure 7.12 The emulsion stability of 5g FFC + 50 cm^3 distilled water + 10 cm^3 oil

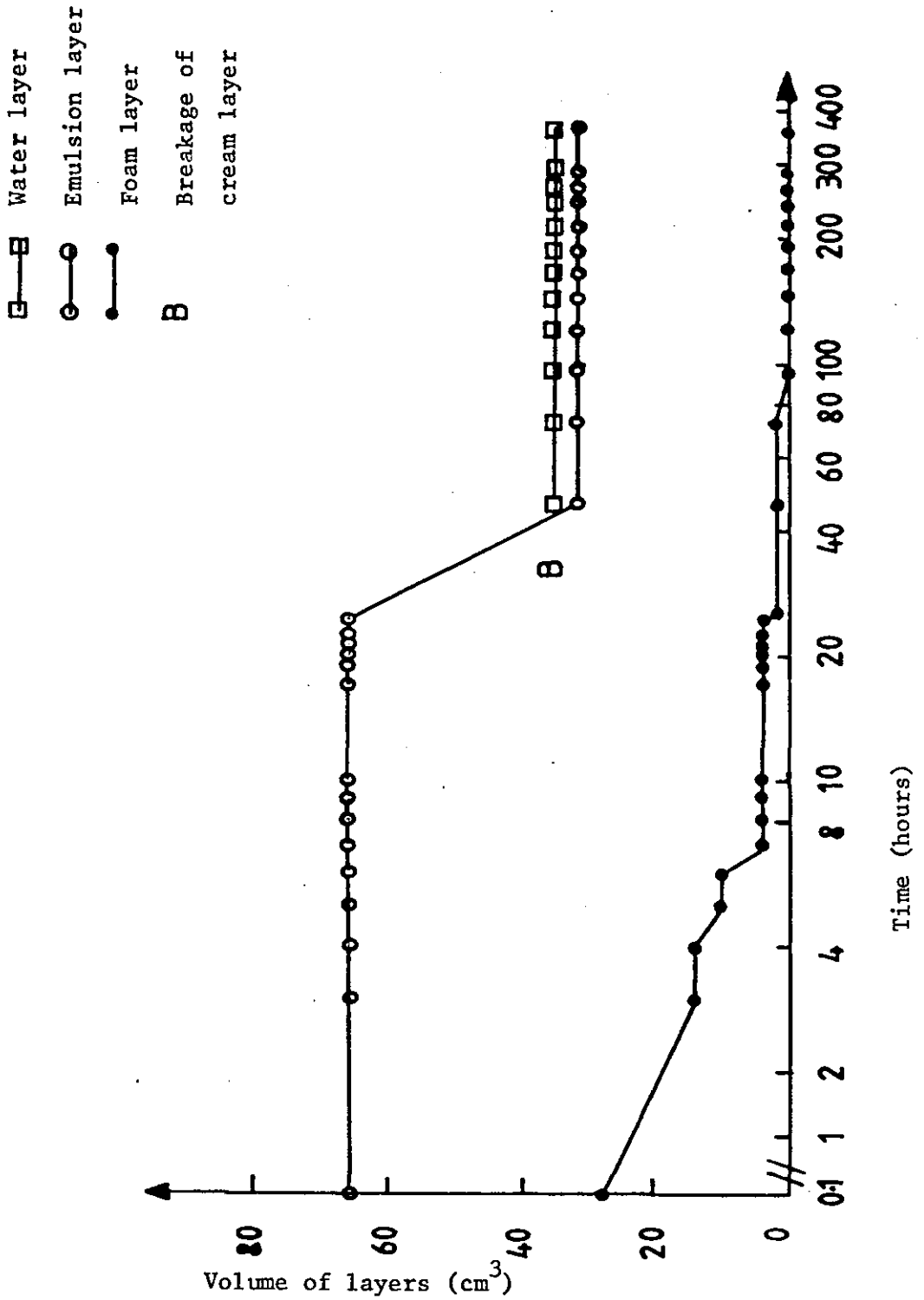


Figure 7.13 The emulsion stability of 5g FPC + 50 cm^3 distilled water + 20 cm^3 oil.

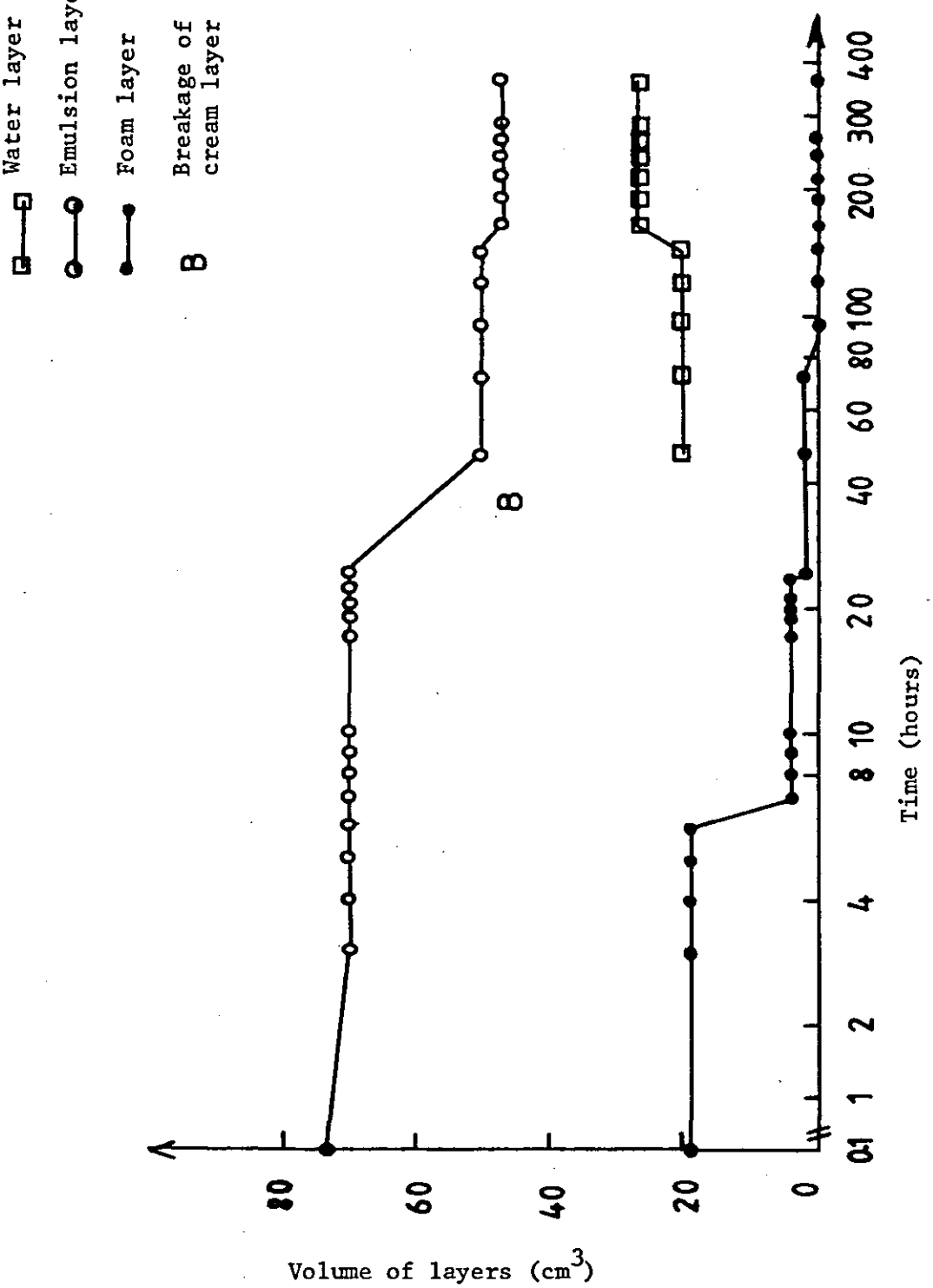


Figure 7.14 The emulsion stability of 5g FPC + 50 cm^3 distilled water + 30 cm^3 oil

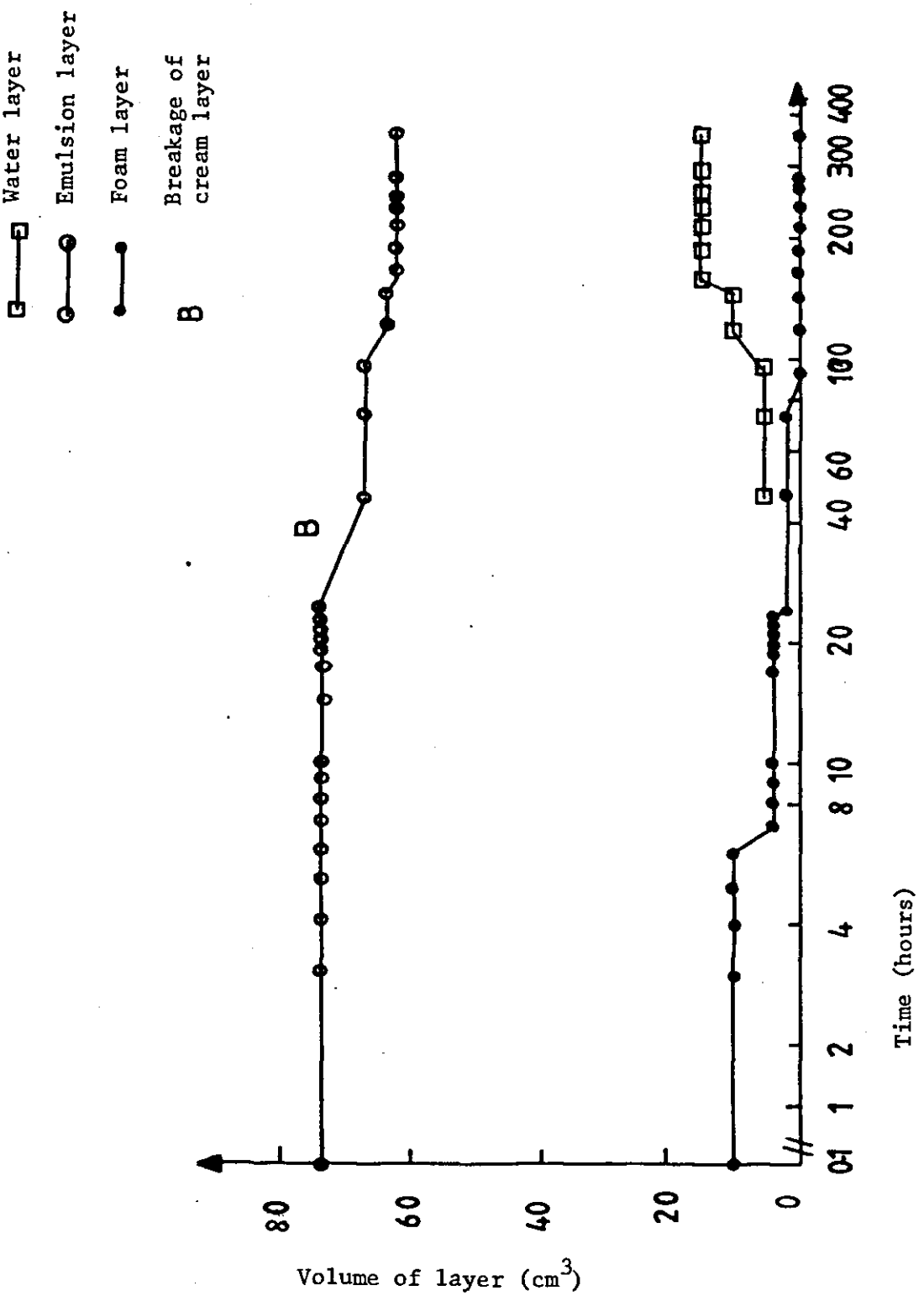


Figure 7.15 The emulsion stability of 5g FPC + 50 cm^3 distilled water + 40 cm^3 oil

iv) The initial foam volumes increased with the amount of added oil up to 20 cm^3 (Figure 7.12, 7.13). Above that, the volume gradually declined as shown in Figures 7.14 and 7.15 to that of the lowest oil content. All the samples reached 4 cm^3 after 7 h and then collapsed gradually to zero after 96 h.

7.6.3. Mayonnaise preparation

Two mayonnaise samples were prepared, the first from egg yolk and other ingredients (as previously described in section 3.4.5). In the second sample, FPC partly replaced the egg yolk by 15%.

The results showed that FPC successfully functioned in the recipe without any change in the texture or smell, although the colour was pale-greyish compared with pale yellow in the conventional sample.

The two samples were stored for 6 months at 5°C without any noticeable change in their appearance.

7.7 Meringue Preparation (section 3.4.6)

It was found that FPC spoiled or collapsed the meringue structure when added to replace 12% of the egg white content. But, it was also noticed that the meringue structure stood until about half of the FPC was added, which implies that

FPC could successfully replace egg white at about the 6% level.

Another FPC-meringue sample (at 12% replacement) was made in a different way, i.e. FPC was added at the beginning of beating with egg white, but no improvement was resulted.

In both cases, the final product can be described as a thick sugar solution, which could not be shaped in the baking tray. Meringues were also made from all white egg for comparison. Both samples were baked in a cool oven at about 130°C.

The all white egg samples resulted in a firm shaped structure, whereas the other sample was off-white, unshapted with an easily breakable structure. Figure 7.16 shows the two products after baking.

7.8 The Baking Test (section 3.4.7)

Table 7.5 shows the effect of FPC incorporation with wheat flour on the dimensions of baked bread loaves.

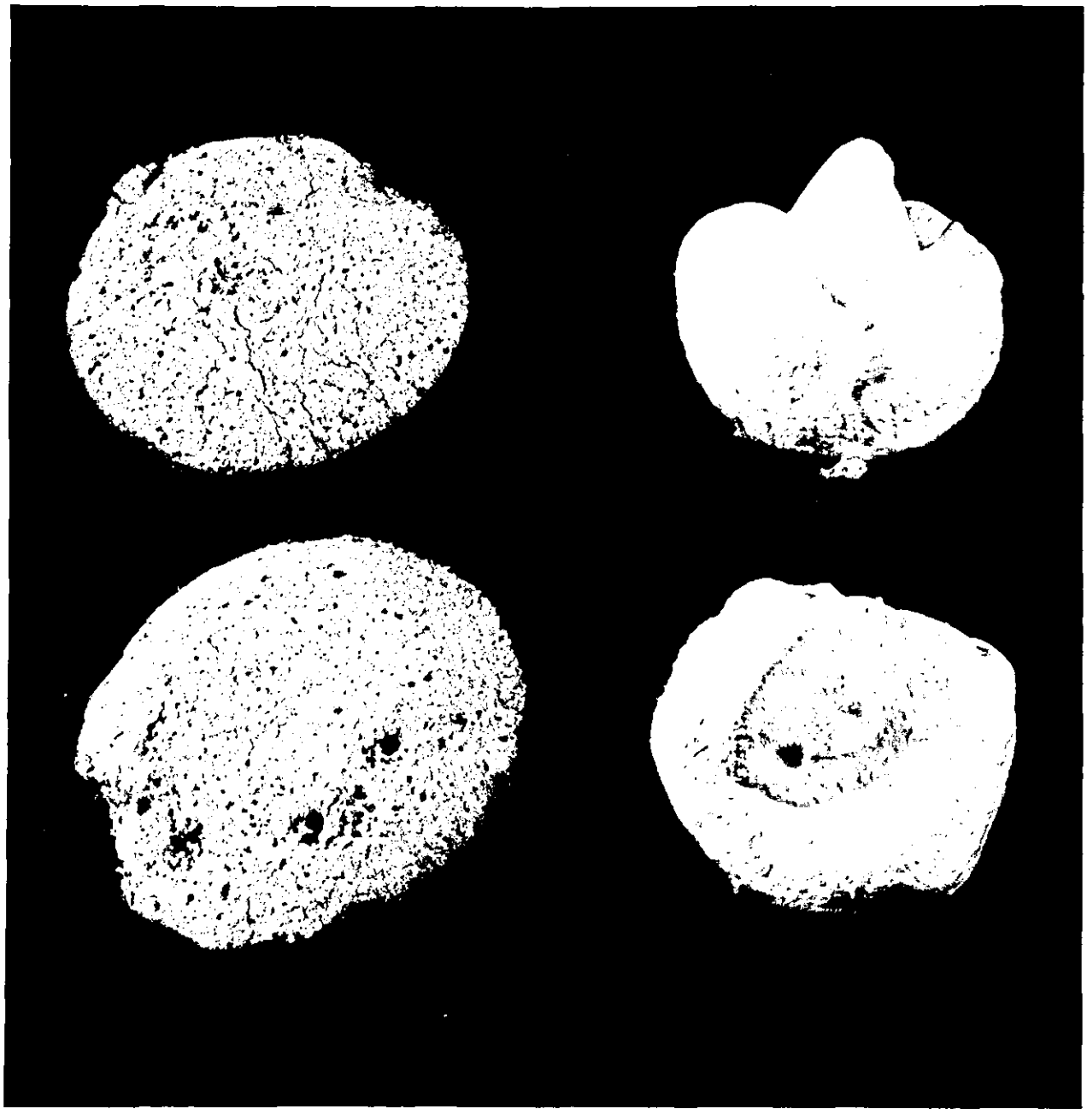


Figure 7.16

Effect of Incorporating FPC in Meringue Recipe.

Left: FPC and Egg White.

Right : Egg White Only.

Sample No.	Weight of FPC (g)+	Loaf Dimensions					
		Girth (cm)		Height (cm)		Crust Thickness (cm)	
		Values	$\bar{x}$	Values	$\bar{x}$	Values	$\bar{x}$
1	0	44.0,39.0	41.50	11.180,11.600	11.390	0.450,0.458	0.454
2	10	34.0,36.4	35.20	10.766,9.974	10.370	0.518,0.352	0.435
3	20	37.8,37.5	37.65	10.774,10.910	10.842	0.446,0.432	0.439
4	30	34.8,36.1	35.45	10.312,9.820	10.066	0.452,0.422	0.437

+ The weight of FPC which replaced the same weight of flour.

o Duplicate samples $\bar{x}$ The average

Table 7.5 Effect of FPC on bread loaf dimensions

FPC reduced all the dimensions of loaves. All the loaves had the same external colour, texture and appearance. The inside colour became slightly darker in loaf 4. No traces of fishy smell were detected in the tested loaves. Figure 7.17 shows the extensometer curves of the above four dough samples mentioned in Table 7.5, where the highest resistance value (950 unit) obtained with the standard sample (No.1) decreased sharply to 250 units with sample 2 and then rose again as the level of incorporated FPC increased. No change was noticed in the average extensibility of the four samples examined.

7.8.1. The Amino Acid Content of FPC:

The amino acid contents of FPC are as shown in Table 7.6 The amino acids that are of most importance in bread making are cysteine and cystine which have an important role during dough development (Kinsella, 1971).

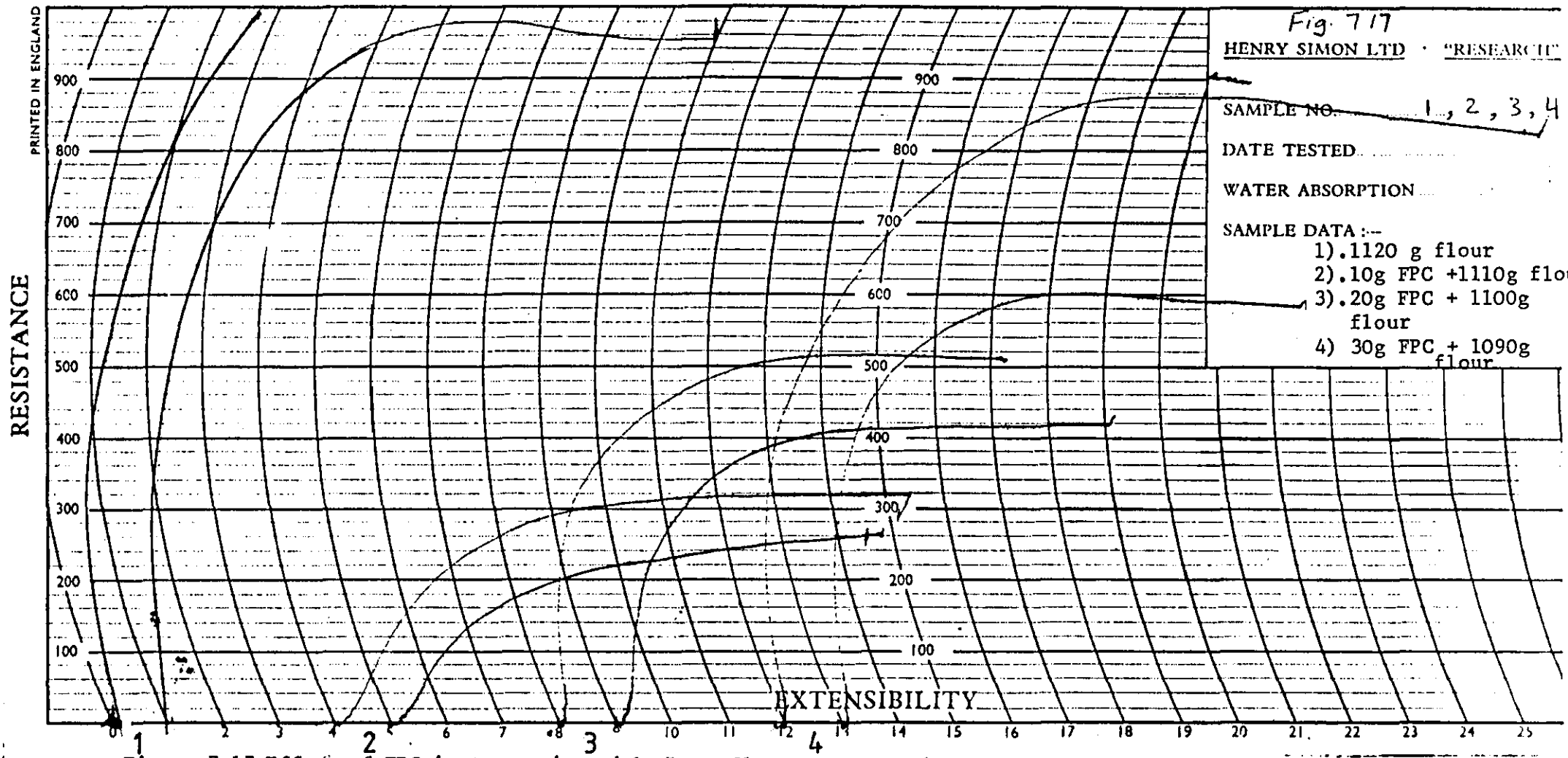


Figure 7.17 Effect of FPC incorporation with wheat flour on the resistance and extensibility of doughs.

Amino acid (AA)	g AA/100 g FPC
Aspartic acid	6.17
Threonine	3.61
Serine	3.39
Glutamic acid	8.28
Proline	2.60
Glycine	6.25
Alanine	4.33
Valine	3.14
Cysteine	0.68
Methionine	1.16
Iso-Leucine	2.47
Leucine	4.52
Tyrosine	2.11
Phenyl-alanine	2.86
Lysine	4.46
Histidine	1.49
Arginine	4.20

Total polar amino acid = 43.24%

Total nonpolar amino acid = 18.48%

Table 7.6 The amino acid content of FPC

The level of cysteine in FPC and flour in the above mentioned samples in section 7.8 are calculated below in Table 7.7

Sample No	g Cysteine in flour	g cysteine in FPC	g- Total
1	3.72	0	3.72
2	3.68	0.07	3.75
3	3.65	0.14	3.79
4	3.62	0.21	3.83

1). Cysteine value in flour was taken as 0.33 g/100g flour (Lillevik, 1970).

2) The weight of protein (N x 6.25) in FPC and flour were calculated, Cysteine value was calculated as g cysteine/100g protein and then referred to the weight of flour and FPC used.

3) Protein contents were 60% and 12.2% for FPC and flour respectively.

Table 7.7 Cysteine level in flour and FPC samples

Incorporation of FPC in bread recipes slightly increased the total level of cysteine. This means enough cysteine was present in the samples and the reduction in loaf dimensions can therefore not be related to the decrease in sulphur amino acids as FPC was added to the flour.

However, it is not know whether the cysteine was unavailable for reaction or whether there were other reactions occurring between FPC and flour which caused this effect.

7.9 Discussion

7.9.1. Solubility

As previously mentioned in chapter 1, the solubility value of proteins was considered as a criterion for measuring the functional properties, i.e. high solubility means good functional properties (Kinsella, 1977). The solubility of FPC in NaCl solutions at pH 7 and ionic strength (0_0.2) ranged between 40 - 51% as shown in Figure 7.1. These values represent a great improvement in this characteristic compared with those obtained with FPC prepared by the Astra method (see section 2.4.1.3) which only had a solubility value ranging between 0.7- 2% at the ionic strength of (0-1.00) (Hermansson, et al, 1971).

The improvement in the solubility values of FPC prepared by the method used in this project, suggests that the product retains good functional properties. This result was expected, as the mild extraction and processing method had a predominant role on the retention of the functional properties of protein. In contrast, the organic solvent and high temperatures which are employed during FPC processing by the Astra method caused a great reduction in the functional properties, as reflected by low solubility values.

The increasing solubility of FPC with increasing salt concentration in the region of low salt concentration (Figure 7.1) is to be expected because of the salting-in effect. As salt concentration increased however, the solubility values declined, due to the salting-out effect (Paul & Palmer, 1972).

The early tests performed on FPC which had been prepared on the bench scale, showed a solubility value of 79% in distilled water at 25°C, as mentioned in section 7.1. The above-mentioned solubility values between 42 - 51% was lower than expected and possibly occurred because the drying process lasted for 21days due to a technical fault in the freeze dryer. Drying by this means would normally take less than one day. It is possible that the lower solubilities resulted from ionic interactions occurring between protein molecules, particularly ionic interactions of the nature suggested by Hamm (1970) and Holdsworth (1971) to take place during freeze dyring of flesh foods.

When FPC was suspended in phosphate-citrate buffers in the range pH 4 - 8 (Figure 7.2), the solubility values were increased to 80-85% which indicates the efficacious effect of the buffer solutions in solubilizing or breaking the ionic interactions which were resistant to the NaCl solutions.
The effectiveness of the buffer solution arose from the insolubilizing divalent ions being removed by the buffer components thus increasing protein solubility (White et al, 1978).

The solubility values between 80-85% represent a distinct improvement in this characteristic compared with FPC prepared by the Astra method, which had solubility values between 4-35% at pH 2-12. (Hermansson, et al, 1971).

The solubility profile of FPC (Figure 7.2) agreed with the general property of proteins in having lower solubility around their iso-electric point. (pH 4.5 for FPC), as the charge difference then becomes very small. At a pH other than the iso-electric point, proteins possess like charges and repel each other, thus allowing protein molecules to disperse easily in solution and thus increase their solubility. (Anglemier & Montgomery, 1976).

The solubility values of some commerically available proteins were tested by Hermansson (1973), who found that soya-bean protein isolate, sodium caseinate and whey protein had respectively the solubility values of 55-25%, 90-5%, and 90-75% between the ionic strength (M-NaCl) of 0-4 at pH 7. These figures may suggest that FPC processed in this project had high solubility values which would enable the product to be comparable with if not superior to some protein sources.

7.9.2. Water Holding Capacity

FPC was shown to possess water holding capacity as shown in Figure 7.3. The average volume of entrained water per g FPC calculated from 15 mg was found to be 1.13 cm^3. This value represents a very good capacity for FPC which contains

about 60% protein (N x 6.25) when compared with the values of pure protein, which was found by Berlin, et al (1973) to be 1 g per 2 cm^3 water.

On the other hand, Hermansson (1973) presented higher values of the water holding capacity of some protein sources. She found that each 20 mg. of soya-bean isolate, sodium caseinate and whey protein concentrate had respectively swelled to entrain 0.12, 0.06 and 0.04 cm^3 water after 15 min. at 19°C. Re-calculating these values on the basis of 1g protein resulted in 6, 3 and 2 cm^3 water/g protein respectively.

This is an example of the difficulty involved in making comparisons between results obtained from different protein sources using investigations by empirical tests, in which great variance may be inherent in the tested materials, equipment and methods. Caution must be used in explaining, and comparing different experimental results. The water holding capacity of different proteins must therefore be determined in a simple model system to facilitate adjustments in food formulations when using or testing new protein sources (Kinsella, 1976).

7.9.3. The Heat Coagulation Test:

The suspension of FPC in distilled water at pH 6.8 did not coagulate when heated in a boiling water bath for periods as long as one hour.

To explain this phenomenon, there are two possible reaction mechanisms responsible for aggregate formation.

i) Protein molecules are opened or unfolded upon heating. Such molecular changes will enhance the formation of aggregates or coagulum through new linkages between hydrogen bonds and other functional groups present on protein molecules (Mirsky & Pouling, 1936; Jensen, et al, 1950; and Haurowitz, 1963).

ii) Sulph hydryl-disulphide interchange SH - SS, where in protein aggregation occurs under denaturation conditions. For example, heating a small amount of sulphhydryl groups initiates a chain reaction with disulphide linkages, at the same time generating a new sulphhydryl group capable of repeating the process to form a coagulum. (Jensen, 1959).

Coagulation did not apparently take place in the fish protein extract at pH 6.8. This can be related to two factors;

i) The sulphur amino acids did not function in the SH-SS interchange mechanism. As reported by Jenson (1959) this was caused by the blockage or unavailability of -SH groups for reactions.

ii) The ionic repulsive force of fish protein extract at pH 6.8 appeared to be high to an extent which did not allow protein chains to lie closely together to form the coagulum structure. Instead it is apparent that a partial chain conjunction occurred as reflected by high absorbance values (Table 7.1) of the heated solutions, because protein chains partially joined and formed a physical barrier to the light.

However, as pH changed toward the iso-electric point, the repulsive forces decreased, encouraging the new linkages of heated protein to react and form a coagulum at pH 5. This pH was then maintained in all the subsequent experiments performed in the coagulation test.

The pH at which coagulation occurred varied according to the composition of the buffer used in the test or with the level of salt ions present in the product, because the protein configuration is affected by the presence of salt ions (Paul & Palmer, 1972). It would therefore be advisable to run a simple test to determine the coagulation environments before using the product in a food system.

Kirchmeier (1962) presented data that suggested that the high cysteine content (-SH) favours coagulation at low temperature, whearas high proline discourages coagulation.

Thus a protein with high proline and low sulphur amino acids might coagulate, but not easily i.e. it might require very specific conditions. For example casein which contains 13% proline and 0.3% cysteine coagulates at temperatures between 160-200^{o}C. (Kirchmeier, 1962).

Based upon the Kirchmeier (1962) concept, coagulation temperatures were plotted against their proline/cysteine ratio as shown in Figure 7.18 This shows the result in which a straight line can be drawn through the points. FPC data is also plotted in the Figure and shows a behaviour much as Kirchmeier suggested.

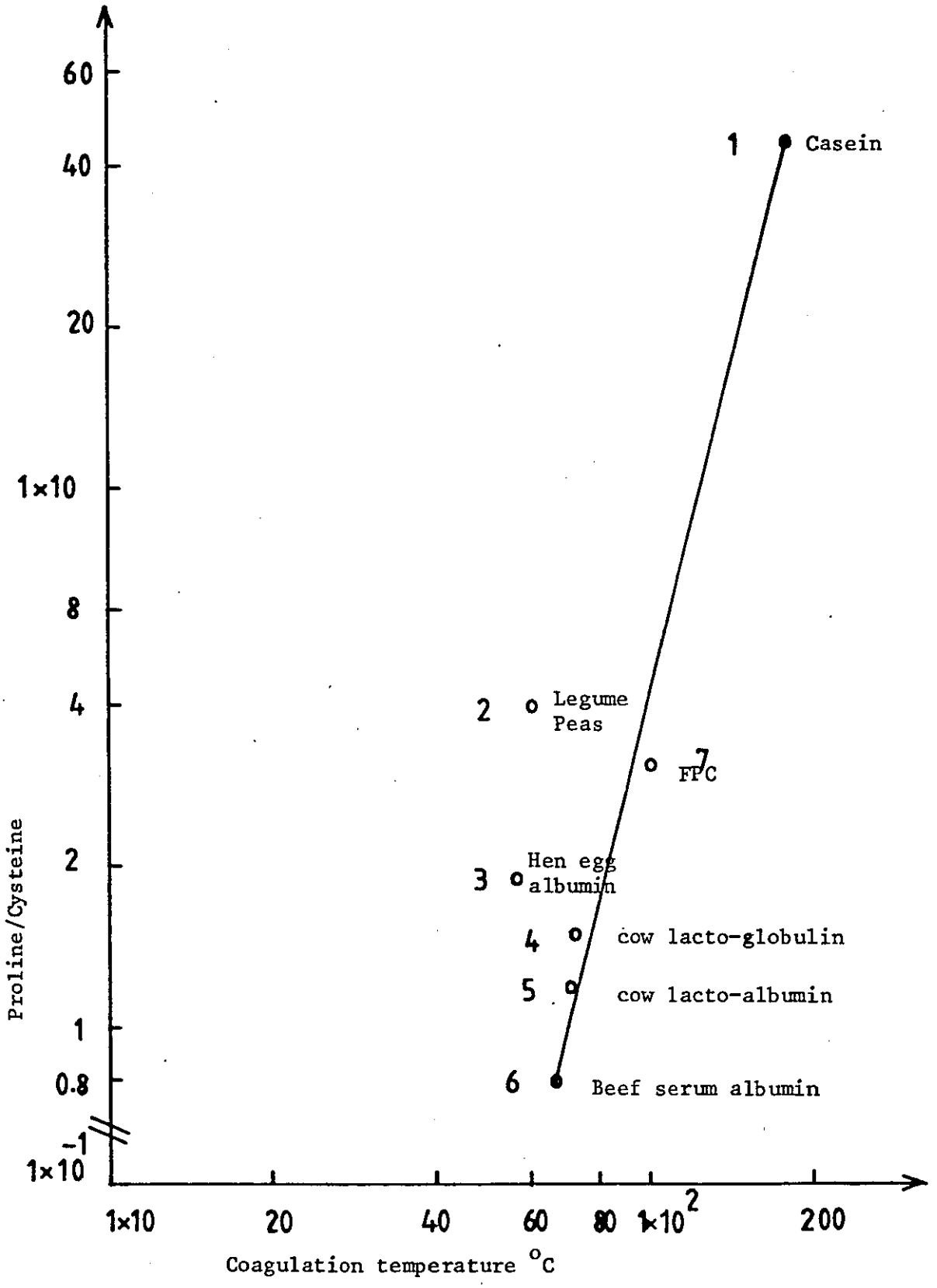


Figure 7.18. The relationship between coagulation temperature and Proline/Cysteine content of some protein sources.
Protein values for source, 1,2,3,4,6 were taken from Lillevik (1970). Protein value for source 5 was taken from Altman and Dittmer (1968). Coagulation temperature was taken from Kirchmeier (1962).

7.9.4. The Surfactant Properties:

7.9.4.1. Emulsifying Capacity:

According to the definitions of emulsifying capacity and emulsifying stability mentioned previously in section 2.3.1, FPC showed a substantial ability to emulsify the different volumes of water and oil.

In the first test mentioned in section 7.6.1, the final volume of stabilized emulsion layer (Figures 7.5 - 7.9) showed a stability period ranging between 60-95 h, while egg white flakes and soy-bean isolate (Figures 7.10, 7.11) were stable for 60 and 50 h respectively. This would suggest FPC to have a superior stability over soybean and egg white flakes. In the second test (section 7.6.2) the emulsion stability was studied as a function of oil volumes. FPC showed the ability to emulsify the added volume of oil as recorded in Figures 7.12 - 7.15. The solutions were examined for a longer period than that taken in first test. The stability periods were ranged between 180 - 312 h.

The high affinity of FPC to oil rather than water is unexpected in view of the high proportion of polar or hydrophilic amino acids (43.24% compared to 18.48% for non-polar amino acids, see Table 7.6). Theoretically such a protein would be expected to have a high affinity for binding with water, but the hydrophilic/hydrophobic properties of proteins are difficult to predict due to their complex ionic nature which is affected by pH, the presence of salt ions and the temperature of solutions

(Paul, 1972).

The ionic nature of protein is a disadvantage for their use as emulsifiers in food emulsions, because they can react with various ions present in food systems to form complexes which may have a reduced emulsifying capacity and low solubility in both types of liquid phase (Powrie & Tung, 1976). For this reason, non-ionic emulsifers are extensively used in food industry.

7.9.4.2. Foam Capacity

FPC showed the ability to form a stable foam when emulsified with water and oil. The foam volume was progressively increased with the weight of FPC as recorded in Figures 7.5 - 7.9 Egg-white flakes (Figures 7.10) produced less foam volume compared with FPC samples, but they showed superior stability over FPC, while soybean isolate failed to produce any foam.

FPC at 6% replacement of egg white did not spoil the meringue structure. Above that level however, the meringue structure collapsed. The failutre of FPC to function in meringue recipes at 12% replacement of egg white suggested that in order to maintain foam stability, the proteins must exhibit a critical balance between their ability to engage in the limited inter-molecular cohesion required to form a stable elastic membrane,

and the tendency to self-associate excessively, which would result in aggregation and breakdown of foam (Kinsella, 1976). It was felt that the ionic balance in the meringue recipe (egg white, sugar and salt) did not favour the former mechanism. An experiment was conducted to test this factor. The pH of meringue was 9 at which unfolding of fish protein was expected to occur, resulting in a retarding of foam formation as the repulsive charges would be so high. Then the pH of the meringue mixture was reduced to 5 by adding lemon juice, and then the FPC was added to the mixture. The meringue structure collapsed, suggesting that there are other factors influencing this effect, which may be related to unknown ionic interactions between FPC, egg white, sugar and salt and which acts to prevent the formation of meringue when FPC is added.

The foam stability and capacity of FPC prepared by Astra (2.4.1.3) was found to be comparable with egg white according to Hermansson et al, (1971).

7.9.5. The Baking Test

The unique properties of wheat gluten in forming an elastic-cohesive mass when wetted and mixed with water is a well known function in the formation and development of dough (Anglemier & Montgomery, 1976).

The literature is lacking in sufficient data discussing the function of FPC in the mechanism of dough formation.

The experimental results revealed that incorporation of FPC with wheat flour reduced the dimensions of baked loaves. FPC previously showed the ability to absorb water, but this is not enough to ensure that FPC can perform elastic-cohesive functions or supplement wheat gluten in the formation of dough.

It is very difficult to explain the inhibiting role of FPC in dough formation, in the light of this baking test, although such a test is considered as a criterion by which protein functionality is assessed in bread making. Indeed the subject is extensive and needs further work which is outside the scope of this project.

It is difficult to say whether the FPC functioned by the mechanism of thiol-disulphide interaction (Kinsella, 1971), or was involved with wheat gluten in ionic interaction which inhibited its role in dough formation, or alternatively FPC may contain a loaf-depressing component similar to the one which is present in milk powders and which known to reduce loaf dimensions (Kinsella 1971).

The point which can be understood from this test is the ratio of $\frac{SS}{SH}$ $\left(\frac{\text{cystine}}{\text{cysteine}}\right)$ was probably not optimum in FPC-flour mixtures to produce the larges loaf volume. According to Belderok (1966), the maximum loaf volumes were obtained when the $\frac{SS}{SH}$ ratio of $\frac{15}{19}$ was present in the wheat flour. Higher or lower ratios reduced the volume of baked loaves.

Examination of table 7.7 shows an increase in the cysteine level as FPC was added progressively to flour. This would suggest the optimum ratio of $\frac{SS}{SH}$ with regard to the standard sample (all flour) was changed and thus reduced the loaf volumes.

The other point arising from the extensibility test (Figure 7.17), is that incorporation of FPC in the flour mixture reduced the resistance without significant change in the extensibility. The resistance of dough is an important characterist: in biscuit making, where flour should have low resistance and more extensibility (Kent, 1966). Therefore, providing a flour possesses high extensibility and resistance, a small weight of FPC can be added to flour to reduce the resistance without noticeably affecting the smell or colour of the dough of baked product.

This result suggested that the functional properties of FPC could be useful in biscuit making, and FPC addition by raising the NPU could also be valuable.

7.10. Conclusion

The experimental results revealed that FPC possesses functional properties such as solubility, coagulation and emulsification ability, and can also be used in biscuit making. The high solubility is an important characteristic, enabling the FPC to be used in beverage and semi-solid food products, such as gel and meat analogues (Hermansson et al, 1971).

The fishy smell which is caused by the presence of lipids in the product must be considered before incorporating FPC in food systems. The water holding capacity suggest incorporating the FPC in viscous foods - soups, meat roles, sausages, comminuted meats and cheeses (Hermansson, 1972). The coagulation of FPC is an important functional property which can be useful as a structural and binding agent in processing fish and meat products (Kinsella, 1976).

FPC showed capacity for forming a stable emulsion which was confirmed when FPC was successfully incorporated in a mayonnaise recipe without any adverse effects. This capacity can be utilized in processing of sausages, soups, fish and meat products (Kinsella, 1976).

It can be seen that the processing methods used in this project were successful in retaining the functional properties examined. Thus such functional protein will be valuable in many suggested food systems described above.

To extend FPC usage in the food systems which failed to function, it may be useful to modify protein to the specific requirements of the food system in which the protein is required to show functionality.

Finally, it is not within the scope of this investigation to determine a price which this functional protein would command on the open market, but comparing it, in broad terms with the functionality of competitor proteins described in Table 1.1., and

allowing for the fact that the present material is only about 60% protein, it could reasonably be expected to command perhaps around £1500/tonne.

Chapter Eight

Chapter Eight

Suggestions for Further Work

i) An electrophoretic investigation to examine the components of soluble fish protein extracted at temperatures ranging between $0^{o}C$ and $60^{o}C$ would be important in identifying the extracted protein species.

ii) Examination of Ca^{++}, Mg^{++}, Na^{+} and $C\bar{l}$ ionic transfer through an UF membrane during the concentration process at higher concentrations of fish protein extract, e.g. above 5% protein would be desirable.

iii) The effect of using a spray-drying or alternative technique for drying fish protein extracts, on the functional properties of fish protein concentrate is required.

iv) The effect of initiating SH-SS interchange mechanism on coagulating fish protein at different pHs, should be examined. An experiment was conducted to test this factor where $1cm^3$ of 0.1% L-Cysteine hydrochloride was added to 4 cm^3 of 1% fish protein extract. The pH of this mixture was 5.5 When this mixture was heated for 30 minutes in a boiling water bath, coagulation occurred. This test may explain that SS-SH amino acids in FPC were not available for reaction as predicted previously in section 7.9.3. It would be useful to examine the effect of different combinations of L-Cysteine hydrochloride and fish protein extract required to obtain coagulation at different pH and protein concentrations

This would explain the role of SH-SS amino acid of FPC in the coagulation process and also would explain the effect of added cysteine on the coagulation process generally.

v) Finally a full economic analysis of the proposed process is required not only to evaluate the present process, but also to compare it with the profitability of competing processes, taking into account the values of the corresponding products and their functionality,and the values of the residual materials.

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Appendices

Appendix I

The Statistical Analysis of the Experimental results described in Chapter 4.

1.1. 't'-test Calculation of S : L Ratios (Section 4.4)

The highest value 3.21 was compared with the other values (x).

The other values were 2.90, 3.05, 3.08, 2.9, 2.87.

$\bar{x}$ = 2.96 where $\bar{x}$ is the mean.

s = $\pm$ 0.097 where s, is the standard deviation.

$$t = \frac{\text{highest value} - \bar{x}}{S/\sqrt{n}} \quad \text{.....I.1}$$

$$= \frac{3.21 - 2.96}{0.097/\sqrt{5}}$$

t = 5.81

By comparing the calculated value of 't' with that in the table of the 't' distribution for (n-1) degrees of freedom (d.f.), t = 4.604

The calculated t is greater than the latter value

$\therefore$ with 99% confidence, a difference exists.

1.2 The Statistical Analysis of Protein Yield and $\frac{1}{d}$ of minced FW (section 4.6.1.)

	$\frac{1}{d}$ cm x	% Protein y	xy	x^2	Y
	1.03	0.48	0.49	1.06	0.57
	2.27	0.54	1.23	5.15	0.58
	3.03	0.54	1.64	9.18	0.59
	4.17	0.75	3.13	17.39	0.61
	11.11	0.75	8.33	123.43	0.69
	25.00	0.82	20.50	625.00	0.86
Σ	46.61	3.88	35.32	781.21	-

$n = 6$

$\bar{x} = 7.77$

$\bar{y} = 0.65$

$\frac{\Sigma x \Sigma y}{n} = 30.14$

$\frac{(\Sigma x)^2}{n} = 362.08$

$$b = \frac{\Sigma x y - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}} \quad \text{.....I.2}$$

$b = 0.012$

Applying the following linear equation, Y values were obtained

$$Y = \bar{y} + b (X - \bar{X}) \quad \text{.....I.3}$$

1.3 't'-test calculation for Type of Extraction Apparatus (section 4.7.2)

Rotary motion	Orbital motion
$\bar{X}_1 = 0.54$	$\bar{X}_2 = 0.47$
$S_1 = 0.02$	$S_2 = 0.04$

pool the standard deviation

$$S_p = \frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1 + n_2 - 2} \quad \text{.....I.4}$$

$S_p = 0.032$

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \quad \text{.....I.5}$$

$t = 4.375$

By comparing the calculated t with that in the table of the 't' distribution for $(n_1+n_2 - 2)$ d.f.,

$t_{0.995} = 2.977$

The calculated t is greater than the latter value

$\therefore$ with 99% confidence, a difference exists.

Appendix II

The Statistical Analysis of the Experimental results described in Chapter.5.

2.1 The Correlation Coefficient Test (r) for True Extracted Protein and NpN Content of F.W. for Samples Stored at $0^{o}C$. (Section 5.3.1.)

	% True Protein x	NPN y	xy	x^2	y^2
	0.45	0.14	0.06	0.20	0.02
	0.14	0.26	0.04	0.02	0.07
	0.14	0.67	0.09	0.02	0.45
	0.06	0.70	0.04	0.004	0.49
	0.08	1.14	0.09	0.006	1.30
	0.14	0.67	0.09	0.02	0.45
	0.15	0.62	0.09	0.02	0.38
	0.19	0.39	0.07	0.04	0.15
Σ	1.35	4.59	0.57	0.33	3.31

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.23$

$\frac{(\Sigma y)^2}{n} = 2.63$

$\frac{\Sigma x \Sigma y}{n} = 0.77$

$$r = \frac{\Sigma x y - \frac{\Sigma x \Sigma y}{n}}{\sqrt{(\Sigma x^2 - \frac{(\Sigma x)^2}{n})(\Sigma y^2 - \frac{(\Sigma y)^2}{n}}} \quad \text{.....I.6}$$

$r = 0.77$

By comparing r (the calculated value)with that in the table of the correlation coefficient for (n-2)df

$r_{0.5} = 0.707$

The calculated value of r is greater than the latter value

$\therefore$ with 95% confidence, a relationship exists.

2.2 't'-test for True Extracted Protein and NPN content of FW for Samples Stored at -15°C (section 5.3.1).

	% True Protein x	NPN y	xy	x^2	y^2
	0.45	0.14	0.06	0.20	0.02
	0.35	0.26	0.09	0.12	0.07
	0.11	0.55	0.06	0.01	0.30
	0.33	0.85	0.28	0.11	0.72
	0.35	1.15	0.40	0.12	1.32
	0.35	0.71	0.25	0.12	0.50
	0.38	0.74	0.28	0.14	0.55
	0.30	0.39	0.12	0.09	0.15
Σ	2.62	4.79	1.54	0.91	3.63

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.86$

$\frac{(\Sigma y)^2}{n} = 2.87$

$\frac{\Sigma x \Sigma y}{n} = 1.57$

Applying equation I.6

$r = 0.16$

The calculated r was found to be less than that in the table of correction coefficient for (n-2)df, $r_{0.10} = 0.621$

∴ No correlation could be established even at 90% confidence

2.3 'r'-test for True Extracted Protein and NPN content of FW for Samples Stored at $-30^{o}C$ (section 5.3.1.)

	% True Protein x	NPN y	xy	x^2	y^2
	0.45	0.14	0.063	0.203	0.0196
	0.35	0.26	0.091	0.123	0.0676
	0.33	0.48	0.1584	0.109	0.2304
	0.44	0.75	0.330	0.194	0.5625
	0.44	1.09	0.4796	0.194	1.1881
	0.46	0.64	0.2944	0.212	0.4096
	0.46	0.63	0.2898	0.212	0.3969
Σ	2.93	3.99	1.7062	1.247	2.8747

$n = 7$

$\frac{(\Sigma x)^2}{n} = 1.23$

$\frac{(\Sigma y)^2}{n} = 2.27$

$\frac{\Sigma x \Sigma y}{n} = 1.670$

Applying equation I.6

$r = 0.365$

The calcualted 'r' was found to be less than that in the table of correlation coefficient for (n-2) df, $r_{0.10} = 0621$

$\therefore$ No correlation could be established even at 90% confidence.

2.4 'r'-test for True Extracted Protein and NPN content in the Soluble Extract of FW Stored at 0°C (section 5.3.2)

	% True Protein x	NPN y	xy	x^2	y^2
	0.45	0.02	0.009	0.20	0.0004
	0.14	0.03	0.0042	0.02	0.0009
	0.14	0.04	0.0056	0.02	0.0016
	0.06	0.04	0.0024	0.004	0.0016
	0.08	0.05	0.004	0.006	0.0025
	0.14	0.03	0.0042	0.02	0.0009
	0.15	0.01	0.0015	0.02	0.0001
	0.19	0.02	0.0038	0.04	0.0004
Σ	1.35	0.24	0.0347	0.33	0.0084

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.23$

$\frac{(\Sigma y)^2}{n} = 0.0072$

$\frac{\Sigma x \Sigma y}{n} = 0.041$

Applying equation I.6

$r = 0$

2.5 'r'-test for True Extracted Protein and NPN content in Soluble Extract of FW stored at $-15^{o}C$ (section 5.3.2)

	% True Protein x	NPN y	xy	x^2	y^2
	0.45	0.02	0.009	0.20	0.0004
	0.35	0.02	0.007	0.12	0.0004
	0.11	0.07	0.0077	0.01	0.0049
	0.33	0.03	0.0099	0.11	0.0009
	0.35	0.02	0.007	0.12	0.0004
	0.35	0.02	0.007	0.12	0.0004
	0.38	0.02	0.0076	0.14	0.0004
	0.30	0.01	0.003	0.09	0.0001
Σ	2.62	0.21	0.0582	0.91	0.0079

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.86$

$\frac{(\Sigma y)^2}{n} = 0.0055$

$\frac{\Sigma x \Sigma y}{n} = 0.0687$

Applying equation I.6

$r = 0$

2.6 'r'-test for True Extracted Protein and NPN content in Soluble Extract of FW Stored at -30^{o} (section 5.3.2)

	% True Protein N	NPN y	xy	x^2	y^2
	0.45	0.02	0.009	0.203	0.0004
	0.35	0.02	0.007	0.123	0.0004
	0.33	0.05	0.0165	0.109	0.0025
	0.44	0.04	0.0176	0.194	0.0016
	0.44	0.03	0.0132	0.194	0.0009
	0.46	0.02	0.0092	0.212	0.0004
	0.46	0.01	0.0046	0.212	0.0001
Σ	2.93	0.19	0.0771	1.247	0.0063

n =7

$\frac{(\Sigma x)^2}{n} = 1.23$

$\frac{(\Sigma y)^2}{n} = 0.005$

$\frac{(\Sigma \Sigma x\, y)^2}{n} = 0.079$

Applying equation I.6

r = 0

2.7 'r'-test for True Extracted Protein and FFA Content of FW. for Samples Stored at $0^{o}C$ (section 5.4)

	% True Protein x	FFA y	xy	x^2	y^2
	0.45	2.75	1.24	0.20	7.56
	0.14	3.11	0.44	0.02	9.67
	0.14	3.69	0.52	0.02	13.62
	0.06	3.12	0.19	0.004	9.74
	0.08	2.41	0.19	0.006	5.81
	0.14	3.06	0.43	0.02	9.36
	0.15	3.40	0.51	0.02	11.56
	0.19	2.90	0.55	0.04	8.41
Σ	1.35	24.44	4.07	0.33	75.73

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.23$

$\frac{(\Sigma y)^2}{n} = 74.66$

$\frac{\Sigma x \Sigma y}{n} = 4.13$

Applying equation I.6

$r = -0.18$

The calculated 'r' was found to be less than that in the table of correlation coefficient for (n-2)df, $r_{0.10} = 0.621$

∴ No correlation could be established even at 90% confidence.

2.8 'r'-test for True Extracted Protein and FFA content of F.W. for Samples Stored at $-15^{o}C$ (section 5.4)

	% True Protein x	FFA y	xy	x^2	y^2
	0.45	2.75	1.24	0.20	7.56
	0.35	2.70	0.95	0.12	7.29
	0.11	3.96	0.44	0.01	15.68
	0.33	2.49	0.82	0.11	6.20
	0.35	2.40	0.84	0.12	5.76
	0.35	3.30	1.16	0.12	10.89
	0.38	2.40	0.91	0.14	5.76
	0.30	2.13	0.64	0·09	4.54
Σ	2.62	22.13	7.00	0.91	63.68

$n = 8$

$\frac{(\Sigma x)^2}{n} = 0.858$

$\frac{(\Sigma y)^2}{n} = 61.21$

$\frac{\Sigma x \Sigma y}{n} = 7.25$

Applying equation I.6

$r = -0.6055$

The calculated r was found to be less than that in the table of correlation coefficient for (n-2) d.f, $r_{0.10} = 0.621$

∴ No correlation could be established even at 90% confidence.

2.9 'r'-test for True Extracted Protein and FFA Content of F.W. for samples Stored at $-30^{o}C$ (section 5.4)

	% True Protein x	FFA y	xy	x^2	y^2
	0.45	2.75	1.24	0.203	7.56
	0.35	2.67	0.93	0.123	7.13
	0.33	2.53	0.83	0.109	6.40
	0.44	2.19	0.96	0.194	4.80
	0.44	2.38	1.05	0.194	5.66
	0.46	2.61	1.20	0.212	6.81
	0.46	2.96	1.36	0.212	8.76
Σ	2.93	18.09	7.57	1.247	47.12

$n = 7$

$\frac{(\Sigma x)^2}{n} = 1.23$

$\frac{(\Sigma y)^2}{n} = 46.75$

$\frac{\Sigma x \Sigma y}{n} = 7.57$

Applying equation I.6

$r = 0$

Appendix III

The Statistical Analysis of the Experimental Results described in Chapter 6.

3.1 'r'-test for the protein concentration and the vescosity (section 6.6)

	% Protein x	Vescosity ηcp y	xy	x^2	y^2	Y *
	0.89	1.015	0.9034	0.7921	1.0302	1.00
	1.0	1.030	1.03	1.00	1.0609	1.03
	1.17	1.124	1.3151	1.3689	1.2634	1.07
	2.05	1.176	2.4108	4.2025	1.383	1.28
	3.25	1.629	5.2942	10.5625	2.6536	1.57
Σ	8.36	5.974	10.9536	17.926	7.3911	

$n = 5$

$\frac{(\Sigma x)^2}{n} = 13.9778$ $\qquad$ $\frac{\Sigma y}{n} = 1.19$

$\frac{(\Sigma y)^2}{n} = 7.138$ $\qquad$ $\frac{\Sigma x}{n} = 1.67$

$\frac{\Sigma x \Sigma y}{n} = 9.9885$

Applying equation I.5

$r = 0.9656$

By comparing the calculated 'r' with that in the table of correction coefficient for (n-2)df, $r_{0.01} = 0.959$

The calculated value of 'r' is greater than the latter value.

∴ with 99% confidence, a relationship exists.

Y * values were obtained from equation I.3

Appendix IV

The Statistical Analysis of Experimental Results described in Chapter 7.

4.1 'r'-test for water holding capacity (Section 7.4)

	gram F.P.C. x	cm^3 water y	xy	x^2	y^2
	0.05	0.06	0.003	0.0025	0.0036
	0.10	0.09	0.009	0.01	0.0081
	0.15	0.16	0.024	0.0225	0.0256
	0.20	0.20	0.040	0.04	0.040
	0.25	0.22	0.055	0.0625	0.0484
Σ	0.75	0.73	0.131	0.1375	0.1257

$n = 5$

$\frac{(\Sigma x)^2}{n} = 0.1125$

$\frac{(\Sigma y)^2}{n} = 0.1066$

$\frac{\Sigma x \Sigma y}{n} = 0.1095$

Applying equation I.6

$r = 0.9834$

By comparing the calculated 'r' with that in the table of correlation coefficient for (n-2)df $r_{0.01} = 0.959$

The calculated value of 'r' is greater than the latter value

$\therefore$ with 99% confidence, a relationship exists.

4.2 'r'-test for F.P.C. Coagulation (section 7.5)

	% Protein x	% Coagulation y	xy	x^2	y^2
	0.09	33	2.97	0.0081	1089
	0.21	39	8.19	0.04	1521
	0.31	43	13.33	0.0961	1849
	0.42	48	20.16	0.1764	2304
	0.92	62	57.04	0.8464	3844
	1.40	66	92.40	1.96	4356
Σ	3.35	291	194.09	3.127	14963

$n = 6$

$\frac{(\Sigma x)^2}{n} = 1.8704$

$\frac{(\Sigma y)^2}{n} = 14113.5$

$\frac{\Sigma x \Sigma y}{n} = 162.475$

Apply equation I.6

$r = 0.9676$

By comparing the calculated r with that in the table of correlation coefficient for (n-2)df, $r_{0.01} = 0.917$

The calculated value of r is greater than the latter value.

∴ With 99% confidence, a relationship exists.

4.3 'r'-test for Blood Albumin Coagulation (section 7.5)

	% blood albumin x	% coagulation y	xy	x^2	y^2
	0.10	69	6.9	0.01	4761
	0.16	78	12.48	0.0256	6084
	0.26	82	21.32	0.0676	6724
	0.28	87	24.36	0.0784	7569
	0.44	92	40.48	0.1936	8464
Σ	1.24	408	105.54	0.3752	33602

$n = 5$

$\frac{(\Sigma x)^2}{n} = 0.31$

$\frac{(\Sigma y)^2}{n} = 33293$

$\frac{\Sigma x \Sigma y}{n} = 101.18$

Applying equation I.6

$r = 0.938$

By comparing the calculated 'r' with that in the table of correlation coefficient for (n-2)df, $r_{0.01} = 0.917$

The calculated value r is greater than the latter value .

. . with 99% confidence, a relationship exists.

Abbreviations

n =	Number of samples
$\bar{x}$ =	The average values of x
$\bar{y}$ =	The average values of y
b =	The slope of the line
r =	Correlation coeeficient
df=	degree of freedom
s =	standard deviation
Sp=	pool standard deviation

The statistical analysis were conducted according to the methods listed in Hinchen (1969).