Utilisation of bovine haemoglobin

UTILISATION OF BOVINE HAEMOGLOBIN

A doctoral thesis written by

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DEDICATED TO MY PARENTS

DIMITRA AND SPYROS

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Introduction

Various authors have asserted that there will be a serious worldwide shortage of proteins suitable for human consumption. For example as early as 1968 a United Nations Conference was warning about a food crisis emerging involving at least 300 million children, who lacked sufficient protein in their diet (U.N. Report 1968).

Vickery (1968) also reported that 300 to 400 million people in underdeveloped countries were suffering ill-health through a serious imbalance in their protein-calorie intake. He urged for new sources of food to be investigated and more specifically that a better utilisation of abattoir by-products be made.

In 1984-85 a large scale famine occurred in Central and East Africa involving millions of people. It could be said therefore that the production and distribution of protein-rich foods is not keeping pace with the steadily increasing world population.

Proteins are valued for two reasons:

- firstly because they contain the essential amino acids needed by man for his well-being and secondly not only their nutritional qualities are precious, but from a food manufacturers point of view, the functional properties they impart in a food system are of great importance. The latter implies that the production of a high quality protein, from a nutritional point of view, is only economical if the same protein has good functional properties. Hence there is a compromise to be achieved when

discussing the incorporation of a protein in a food formulation. It should be functional and sufficiently nutritious.

Meat proteins are both functional and of high nutritional value and this, coupled with their rising production costs, makes it vital that wastage of such material should be minimal.

Why process abattoir blood?

In Britain about 90000 tonnes of blood per year are potentially available from cattle, pigs and sheep slaughtered (MLC, 1982).

About 17% of this tonnage i.e. 15000 tonnes annually, is good quality protein which at present is not utilised to any significant extent. Akers (1973) reports that in 1975, 64,000 tonnes of dried plasma were imported, at a considerable cost in the U.K.

The bulk of blood produced in U.K. abattoirs is either processed for animal feed or used as a fertilizer or simply disposed of into the sewage system, at a cost.

This results in high quality protein being wasted which if processed could significantly contribute to the profitability of the industry. Also, these large amounts of biologically active materials which are released in the effluent streams, increase the levy the industry pays the Water Authorities to dispose of them. This way the operating costs of the slaughterhouse are increased.

The plasma fraction of blood is, still to a limited extent, commercially utilised (Bright, 1977), but the red cell fraction has not been investigated nearly as much. The main two problems associated with the direct incorporation of haemoglobin into foods are:

1. The dark colour this protein imparts into food, which is due to the iron-containing haem part of the haemoglobin molecule

2. The distinctive taste it imparts at haemoglobin concentrations higher than 3.5% (Slinde and Martens 1982).

The maximum level of haemoglobin addition depends on the product in which it will be incorporated. In meat loaves, produced by Oellingrath (1985), the acceptable level of haemoglobin was as high as 5%.

This project investigates the possibility of decolourising bovine haemoglobin, and the economics associated with the processes to accomplish this.

CHAPTER 2

Objectives of Research

The plasma fraction of animal blood has been the subject of much investigation. Research into its functional properties has been extensive, and process specifications, costings, and cost benefit studies have been undertaken. Both frozen plasma and spray dried plasma are commercially available in continental Europe, and to a lesser extent in the United Kingdom.

By way of contrast, the residual material when the plasma has been removed, namely the red cell fraction of the blood is still a relatively unknown quantity.

This study deals exclusively with the red cell fraction of bovine blood. The primary objective was to investigate the possibility of decolourisation of its main protein haemoglobin, and the development of processes to do so cost effectively.

The methods by which this objective was achieved consisted of

1. Laboratory scale investigations, to establish methods which would give a functional, decolourised protein product fit for human consumption.

2. To produce the necessary data for scale-up of these methods from laboratory to pilot plant scale. For each method examined, the following main variables were monitored:

   Protein content
   Solids content
   Protein yield
   Colour of final product

This information was combined with process mass balances.

3. Material produced from each of these methods at pilot scale was tested for its functionality and suitability for food systems. This enabled an approximate commercial value for the protein to be estimated, and in some cases this was confirmed by commercial food processing companies.

4. From data produced, a process specification, costing and profitability study could then be undertaken for the most promising process.

These steps in the study enabled process comparisons to be prepared which will enable large pilot plant/industrial scale work to proceed on the most favourable process.

CHAPTER 3

Literature Survey

3.1 Meat Industry: A short overview

Meat is defined as the flesh of animals used for food. This definition is expanded to include the organs such as liver and kidney, and other edible tissues.

The origins of the meat industry are lost in time. Slaughtering of meat animals in an organised way started just after their domestication by man. Sheep were the first mammals to be domesticated sometime before 7000 B.C. Cattle and pigs followed after there was a settled agriculture, circa 5000 B.C. Domestication caused an increase of their numbers for various reasons. Thus sheep, cattle and pigs were protected from predatory carnivores, except of course man, and they had access to regular supplies of food.

Over the years the necessity for more and better quality meat, and its more effective preservation has become apparent.

The small meat"shops" in the outskirts of villages grew as the village itself grew. The major meat processing factories originated in the large cities because of the availability of the labour force needed to operate the large plants.

The advent of refrigeration and new transportation systems contributed tremendously to the industry. The slaughterhouses of today utilise mechanised process lines open to automation and control and work under hygienic conditions which were unattainable in the earlier plants. This results in a high quality meat product. Automation and better designed processes have resulted in a considerable drop in the labour requirements of the plant.

The trend in the meat industry is that of more automation, resulting in more personnel being employed in the quality control and research and development areas.

Cattle and pigs still remain the main sources of meat in the world. The worlds stock for pigs and cattle is given by Filstrup (1980), to roughly be 500 million and 1000 million head respectively. To this total the stock of sheep, poultry and other minor meat sources such as goats, reindeer etc., must be added to give a total head figure of meat animals running into few thousand million heads. These figures have been more accurately estimated by FAO to be:

- 1,242,800,000 cattle and buffalo
- 1,477,700,000 sheep and goats
- 626,600,000 pigs
- 5,262,700,000 poultry (Filstrup 1980)

Filstrup has compiled a table which gives the percentage distribution of cattle and pigs.

From this table it is evident that Europe including USSR, and the Americas account for approximately 55% of the total stock.

Oceania has only 3% of the world's stock of cattle and pigs but it has the world's greatest concentration of sheep slaughtering.

In Asia, which accounts for 34% of total stock, special religious customs are assocated with slaughtering. Thus in India which has 20% of the world's total stock for cattle, only about a quarter of the population eats such meat.

Table 3.1

Area	%
Asia	34
Americas	30
Europe, including USSR	25
Africa	8
Oceania	3

Distribution of beef and pork (Filstrup 1980)

Meat products were staple foods for the western diet. In Britain according to the 1982 National Food Survey Committee, meat accounts for over a quarter of all domestic expenditure on food and contributes a similar proportion of protein and iron in the diet.

Meat is by far the biggest commodity of a slaughterhouse or meat packaging plant. The industry is very conservative in its approach to the use of their very expensive raw material. This is reflected in the technology they have developed over the years to use high and low value parts of the carcass for their best advantage. Hence the by-products play an important role in the profitability of the plant. Their commercial value nowadays has been calculated to be worth ten times the sum of the operating costs of a modern plant, thus providing the profit margin required by the slaughterhouse.

The overall market however still relies heavily on the main pieces of meat. The demand for particular types of meat on the retail market is normally related to the size of the individual piece of meat and its ease of preparation in the domestic kitchen.

The meat processing plants flourished as the per capita income of most countries increased during the period between 1945-1980. The consumption of meat products increased as did the proportion of animal protein to the total protein intake. This was reflected on the output of meat in the 34 main producing countries which increased from 51 million tonnes in 1965 to 58 million in 1969 and up again to 63 million in 1972. The trend is shown in figure 3.1.

However, at the end of the last decade and in the beginning of the 80's the consumption of animal protein has remained steady, even though the living standards have generally increased. This can be due to the substantial increase in the cost of production of meat for various reasons, such as the energy crisis. This cost rose more rapidly than the per capita income of most countries.

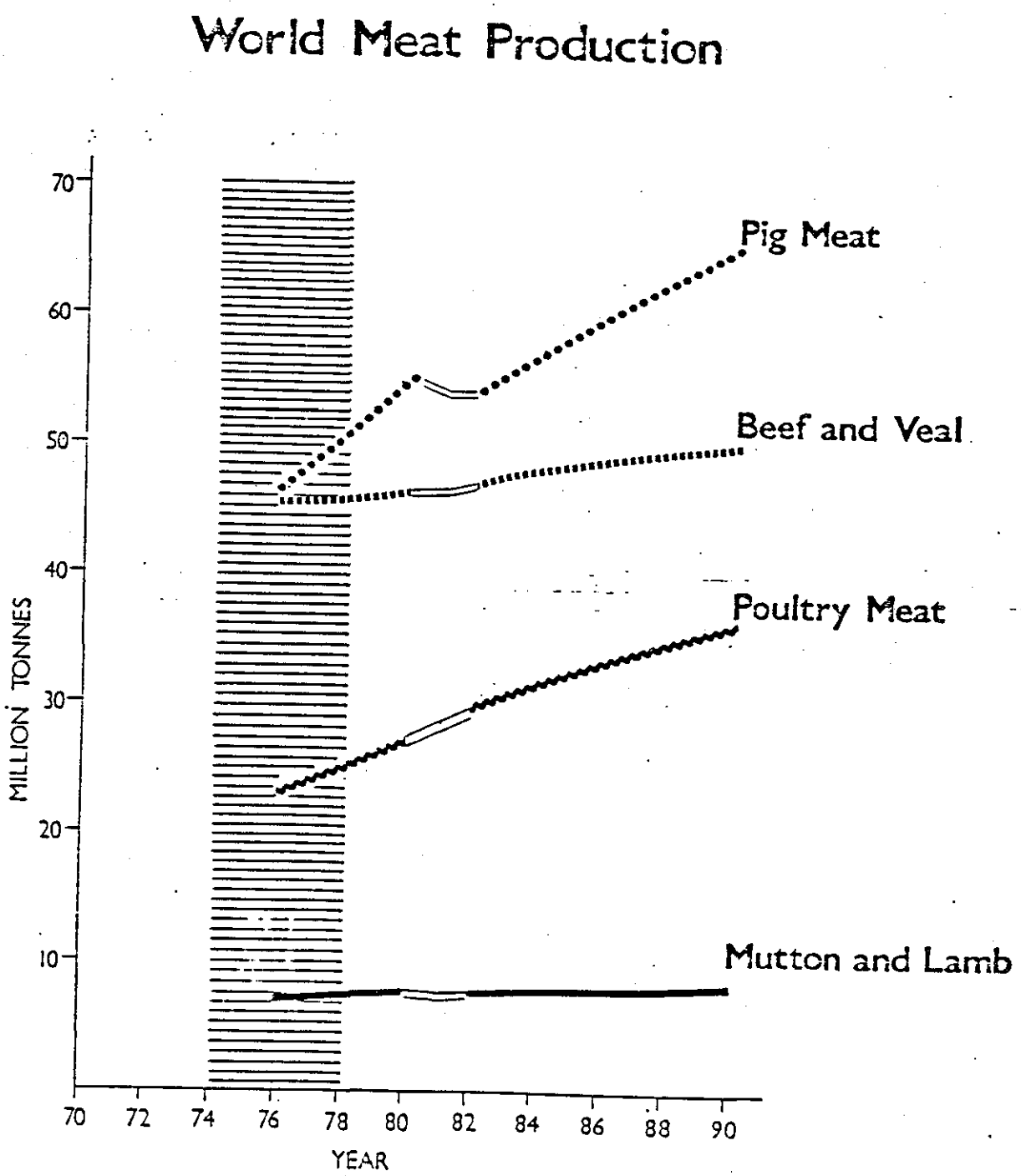
The outlook for the meat industry relies on reducing the cost of production, by perhaps more automated and efficient techniques, and at larger plants, centralised, with a higher level of slaughterings.

3.1.1 Meat Industry in the U.K.

The total consumption of meat in the UK was estimated to be 3,756 thousand tons (MLC 1981). The main type of meat consumed is beef and veal, about 1,141 thousand tons. The UK market share of each type meat is shown in figure 3.2. The order of importance, in descending order, is seen in this figure to be; beef and veal, poultry closely followed by pork bacon and ham, mutton and lamb and offal.

The production of meat between the years 1971 to 1984 followed the trend shown in figure 3.3. There is a slight upward trend in the curve correlating with the small increase of population of Great Britain during this time period.

Figure 3.1 Trends in world meat production and forecasts to 1990. The base period is 1974-78.



Meat consumption in the U.K.

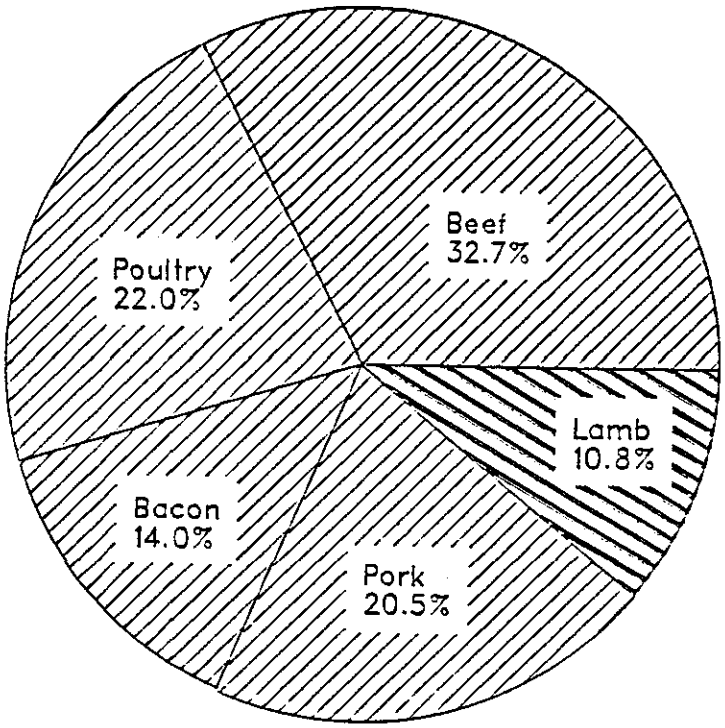


Figure 3.2

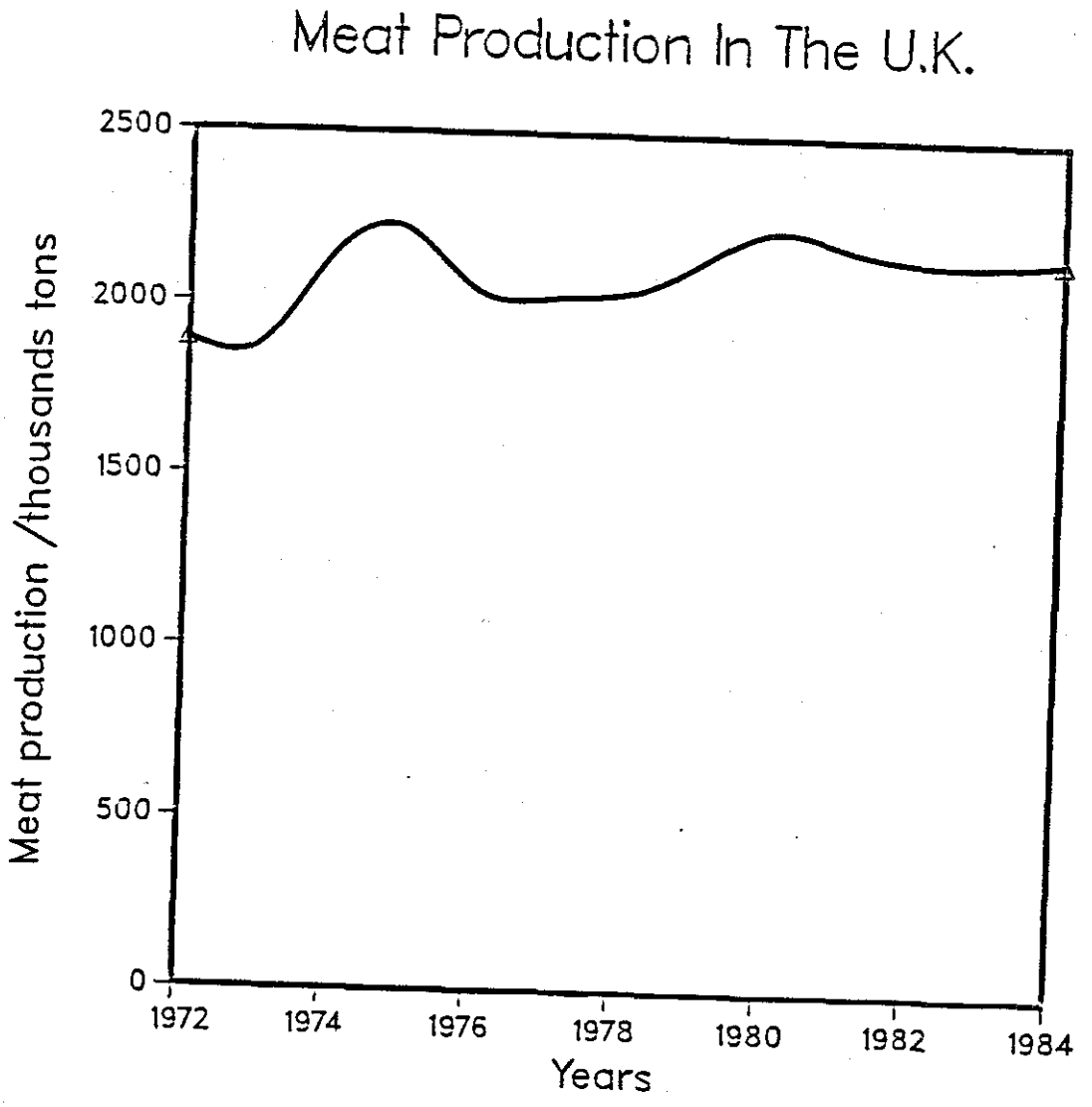

Meat Production In The U.K.
Meat production /thousands tons
2500
2000
1500
1000
500
0
1972
1974
1976
1978
1980
1982
1984
Years

Figure 3.3

From the meat consumption figure and the 1981 meat production one, there is a deficit of 1,371.4 thousand tons. This is of course the meat imported into the U.K. MLC and MAFF figures for 1981, show that meat imported constitutes 36.5% of all meat consumed. The percentage share of the imported meat changes very little annually (MLC economic reviews 1968-1981). This indicates that Great Britain is not yet self sufficient in meat and still depends on imports from various countries mainly from the EEC, and the Irish Republic in particular, New Zealand, Poland, Australia and some minor exporters.

The slaughtering industry in the UK has changed in the last fifteen years. While the number of slaughterhouses has dropped from 2,062 in 1969 to 1,135 in 1981, the average throughput in cattle units per year has increased twofold from 5,505 units to 11,594 units. The steady decline in the number of slaughterhouses, and the rise in throughputs is shown in figures 3.4, 3.5 This fall in the numbers of slaughterhouses could falsely lead to the assumption that no new plants have been built. This is not the case as the doubling of throughput indicates. The plants are now more fully automated and efficient than in 1969. Especially from 1977, new investment has resulted in more plants being modernised to meet the standards required for approval as export centres of meat to other EEC countries. This investment was both private and through the UK Government and the EEC.

The slaughterhouses whose numbers have decreased considerably over the years belong to the small to medium slaughterhouse throughput group.

Table 3.2 shows the distribution of slaughterhouses by size of throughput. The number of small abattoirs, i.e. up to 100 units annual throughput, has plummeted from 239 in 1969 to 79 in 1981.

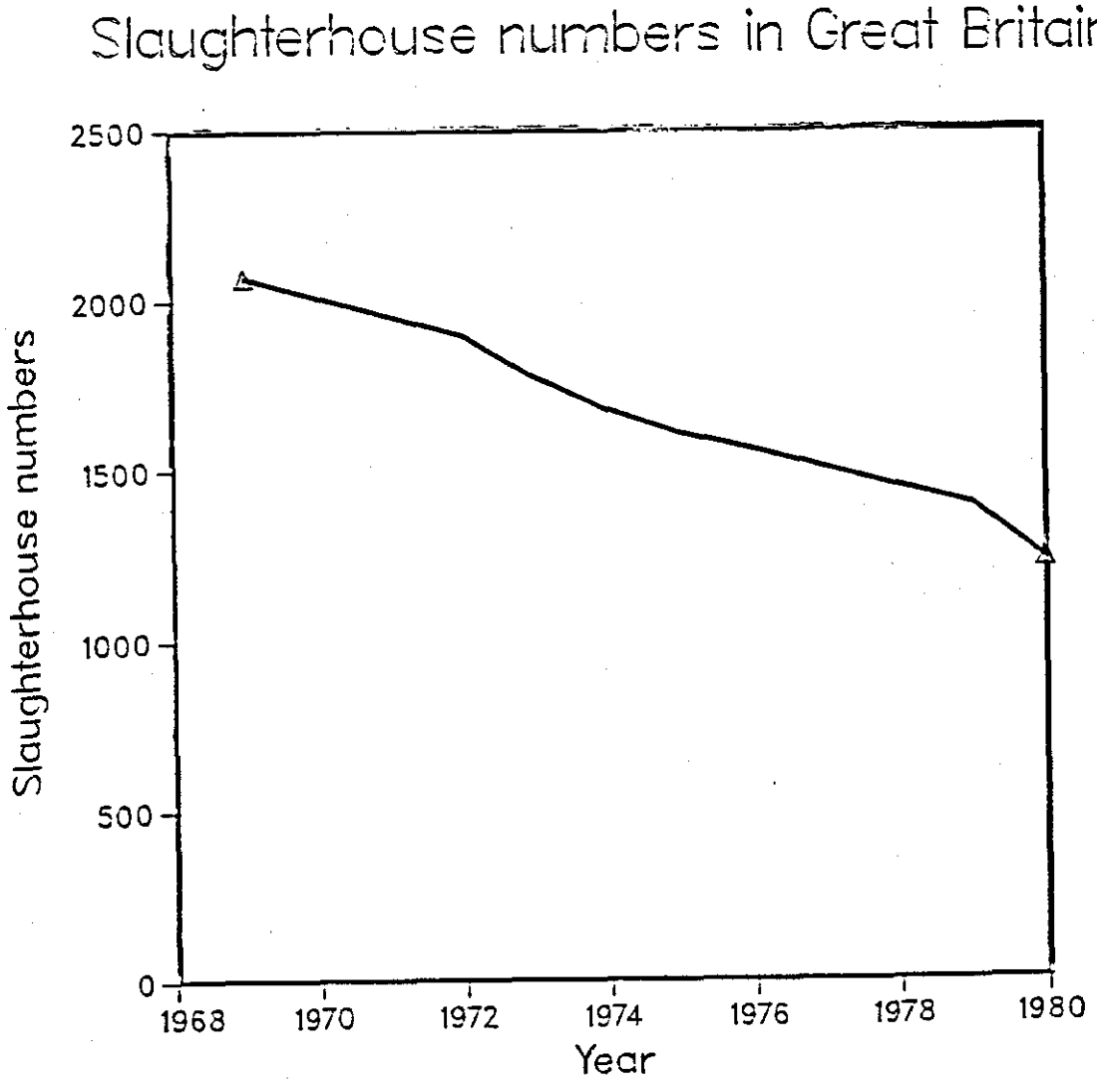

Slaughterhouse numbers in Great Britain
Slaughterhouse numbers
2500
2000
1500
1000
500
0
1968
1970
1972
1974
1976
1978
1980
Year

Figure 3.4

Changes in cattle units average throuputs in Great Britain

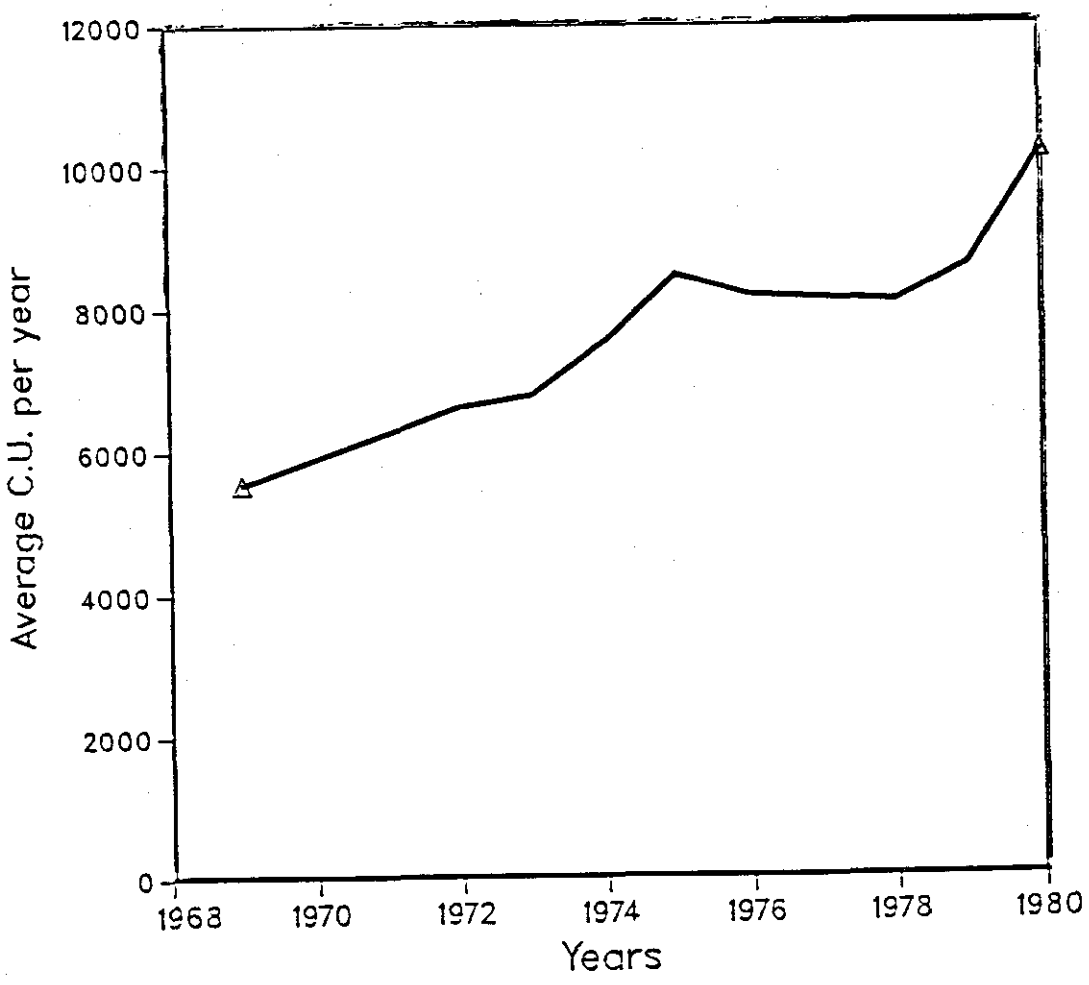


Figure 3.5

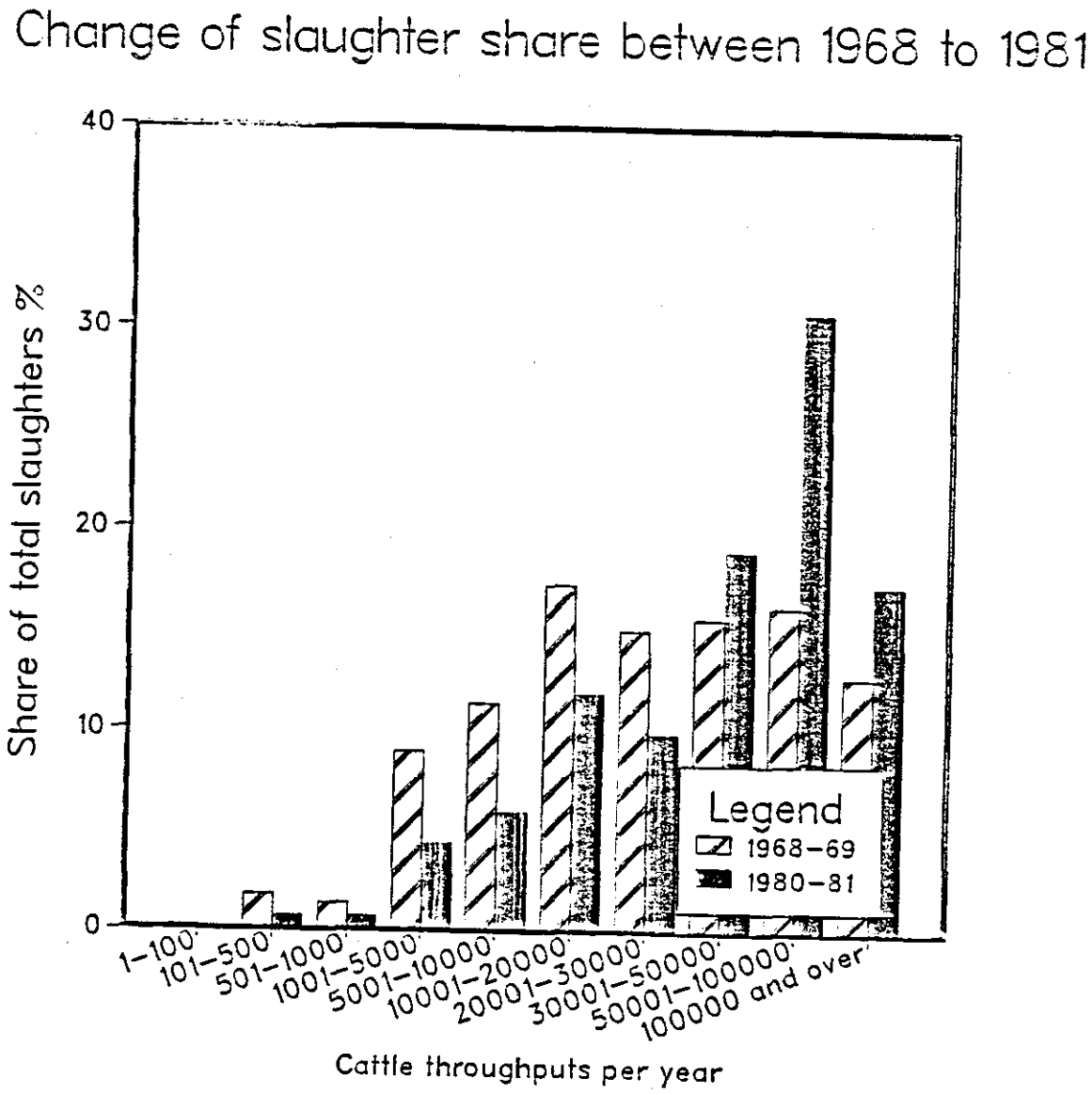

Change of slaughter share between 1968 to 1981
Share of total slaughters %
40
30
20
10
0
Legend
1968-69
1980-81
1-100
101-500
501-1000
1001-5000
5001-10000
10001-20000
20001-30000
30001-50000
50001-100000
100000 and over
Cattle throughputs per year

Figure 3.6

Slaughterhouse Numbers - by Size of Throughput: Great Britain

Slaughterhouse throughput (Cattle units per year)	*1968-69*	*1971-72*	*1972-73*	*1973-74*	*1974-75*	*1975-76*	*1977-78*	*1978-79*	*1979-80*	*1980-81*
	Number of slaughterhouses									
1 - 100	239	250	220	185	155	152	145	162	102	79
101 - 500	766	662	626	580	524	510	481	427	345	307
501 - 1,000	213	190	162	160	158	149	130	139	129	120
1,001 - 5,000	374	319	314	283	287	287	264	248	242	220
5,001 - 10,000	178	167	154	157	139	147	146	115	114	107
10,001 - 20,000	139	129	117	122	139	125	105	120	118	109
20,001 - 30,000	70	60	66	71	61	57	66	64	51	52
30,001 - 50,000	47	62	64	61	80	71	58	54	65	65
50,001 - 100,00	27	39	30	37	42	40	36	51	48	60
100,001 and over	9	12	13	15	16	16	13	12	17	16
TOTAL	2,062	1,890	1,766	1,671	1,601	1,554	1,444	1,392	1,231	1,135

Table 3.2

This change in slaughterhouse share is better shown in figure 3.6. In 1968 the groups with slaughter throughput up to 20,000 units a year had a 40.5% share of slaughterings. In 1981 the percentage share of this group has dropped by almost half, to 23%. In the same year the major share of slaughterings, 30.8% was achieved by the 50,000-100,000 group. This group has actually doubled its share of slaughtering in a period of 12 years. This is a reflection of the general trend, namely the concentration of slaughterings into fewer, bigger and fully modernised abattoirs.

3.1.2 Abattoir techniques

The slaughtering process of pigs and cattle will be briefly described. Under the heading cattle the following animals are included: steers, cows, heifers and calves. Table 3.3. gives the approximate live weight for the main meat animals.

Table 3.3

Animal	Live weight (kg)
Steers	400 - 600
Primegrade steers	450
Cows	350 - 400
Heifers	250 - 300
Calves	50 - 70
Pigs (bacon)	≈80
Pigs	110
Sheep	40 - 50
Lamb	25 - 30

Live weights of meat animals

Preslaughter handling of the animals is very important to the final quality of the meat product. During transportation death losses and bruising can occur. If the animal is exhausted prior to slaughtering rigor mortis sets very quickly (Lawrie, 1980); and the meat is difficult to keep fresh.

Exhaustion and the sense of danger causes the animal to consume its natural stock of glycogen in the muscles. The normal formation of lactic acid in the meat will not occur hence the meat will have a high ultimate pH and this promotes microbial contamination. A detailed description of the chemical and biochemical reactions that occur in the muscle after slaughtering is given by Lawrie (1980). Briefly glycogen in the muscle is converted to lactic acid due to anaerobic glycolysis and the pH of meat drops from the invivo level of 7.2 down to a final pH of 5.7. The pens should be sufficiently large and clean for the animals. Pigs are particularly sensitive to preslaughtering conditions.

The killing line in the abattoir is different for cattle, pigs and sheep. The slaughtering differs with each type of animal.

Bacon pigs are processed in the following way.

1. The pigs are stunned using either a bolt pistol or carbon dioxide and then the animal is hoisted onto an overhead conveyer rail. Electric stunning i.e. passing a current at 75 - 87 v, is also used. After stunning there is a period of one to two minutes in which the muscles of the pig are relaxed. During this period the second stage starts.

2. Sticking and bleeding. Sticking takes place by cutting the cervical vein and one of the arteries. After sticking, bleeding occurs. Traditionally the pig is left suspended to bleed for six minutes over a blood pit. New methods for aseptic collection of blood will be described later on.

3. Scalding and scraping. This ensures that the bacon sides will be free from any hair and also that all dirt and bacteria from the carcass will be removed. The temperature of the scalding water is about 60°C and this operation lasts for about six minutes. After scalding, the carcasses are transferred into the scraping machine. In there rotating cylinders equipped with specially formed flexible scrapers ensure efficient scraping.

4. Singeing. The carcasses are passed into a singeing oven; temperature is kept at 900-1000°C in which any residues of hair are removed.

5. Cutting up-evisceration-dressing. The carcass is opened by a cut along the centre of the abdomen. The intestines are then removed and inspected. Then the chest and neck are opened and the gall bladder and plucks are removed. The breast bone is cut, also the carcass is cut into two halves.

6. Cooling and storage. After weighing and grading the carcasses are conveyed into the cooling room, via a precooling stage, where they are rapidly cooled to about 0°C, in a forced air circulation at a relative air humidity of 85-90%.

Slaughtering of cattle follows a similar pattern:

1. Stunning. The animal is placed into a stunning pen i.e. a box with tilt table bottom and side which does not allow the animal to move and stunned using mechanically or pneumatically actuated bolt pistol.

2. Sticking and bleeding. The animal is then hoisted onto an overhead rail by a chain applied to one hind leg. Sticking takes place by piercing the jugular vein in the neck with a knife. Other blood collection methods will be discussed later.

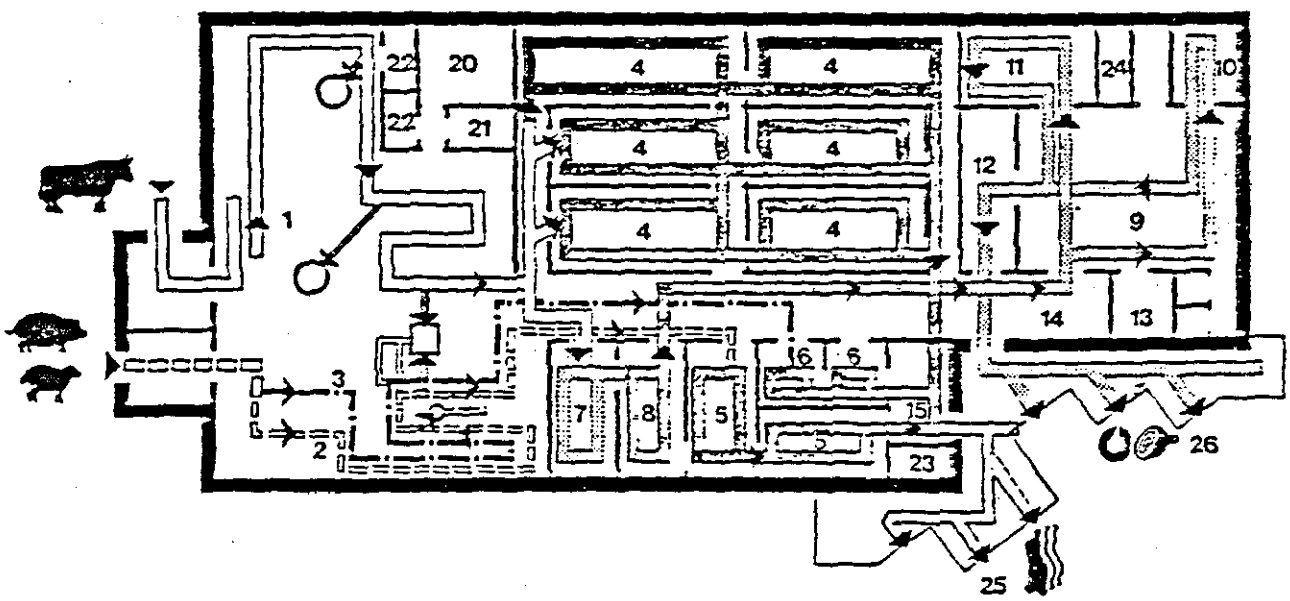
3. Dehiding. First the head is removed, washed and inspected. In the second stage the feet are skinned and removed. The carcasses are then hooked on to two roller hooks instead of the chain around the hind leg. The udder and pizzle are removed and sent for inspection. The hide is then removed from the carcass.

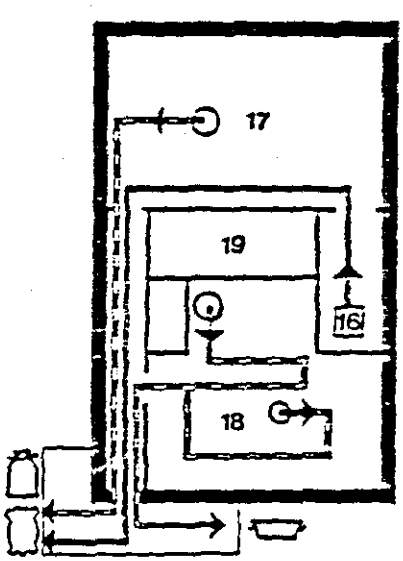
4. Cutting up-Evisceration-Dressing. After dehiding the brisket is opened and the viscera removed and inspected. The carcass is now cut into two halves. When all parts of the animal, including the carcass itself, have passed inspection the next stage consisting of weighing, classification and cooling starts.

5. Cooling and storage. After superficial cleaning the carcass is chilled for six hours, so bacterial growth is restricted. During the next ten to twelve hours the temperature of the carcass is reduced to $4^{o}C$. Ripening of beef reaches an optimum value after seventeen days. Storage takes place at a temperature of O - $1.5^{o}C$. If the temperature is not optimum ripening occurs much earlier.

The view plan of a modern slaughter house is shown in figure 3.7. Following slaughtering the animal is cut up an divided into different pieces. The following table gives the breakdown of live weights of sheep, lamb and cattle.

Typical Abattoir Layout.





1 Stunning, slaughtering and dressing of cattle
2 Stunning, slaughtering and dressing of hogs
3 Stunning, slaughtering and dressing of sheep
4 Chilling and freezing chambers for cattle
5 Chilling and freezing chambers for hogs
6 Chilling and freezing chambers for sheep
7 Processing of plucks (edible by-products)
8 Chilling chambers for plucks
9 Sausage-making plant
10 Cooking and smoking
11 Curing
12 Sausage store
13 Maturing chamber
14 Dispatching
15 Weighing
16 Processing of inedible by-products
17 Treatment of hides
18 Viscera treatment dept.
19 Storage of casings
20 Laboratory
21 Veterinary office
22 Sanitary facilities for operators
23 Office
24 Spice stores
25 Delivery of carcasses
26 Delivery of meat products

Plan view of killing line for cattle, pigs and sheep

Figure 3.7

Table 3.4

Parts	Cattle (%)	Sheep (%)	Lamb (%)
Carcasses and other edible products	62-64	61-63	62-64
Raw fat		4-5	5-6
Blood	3-4	4-4.5	3.5-4
Inedible raw material	8-10	7-8	6-7
Shrinkage	2-10	1-1.5	0.5-1
Stomach and Intestines	15	9.5	5.5
Pelt and Wool	-	11	15

Break down of live weights of animals.

3.1.3 Abattoir by products

As was mentioned before, the various by-products of a slaughterhouse contribute significantly to the profitability of the plant. In addition to this, the necessity of increasing the production of protein to meet man's growing needs, as discussed in the introduction, makes it essential to utilise more economically the products available and to use as much as possible the existing materials.

The following table gives most of the by products and the way they are utilised by the meat industry. A major by-product, namely blood, is not included as it will be discussed on its own more fully later.

Table 3.5

Raw by products	Processed by products	Uses
Edible raw fat	Edible fat Oleo Oil Oleo Stearin Cracklings	Frying purposes Margarine Shortening Sweets-Chewing gum
Inedible raw material	Inedible fat Meat and bone meal	Livestock and poultry feed
Inedible raw fat	Inedible fat	Lubricants, soap candle, glycerin livestock and poultry feed additive
Edible raw bone	Edible fat Bone pieces	Shortening Bone gelatine/urea
Inedible raw bone	Inedible fat Bone pieces	As above plus bone glue
Cattle feet	Neatsfoot oil	Fine lubricants
Hide and pelt	Prewashed hide Hair and wool Trimming for inedible rendering	Leather products Felt upholstery, fertilizers and textiles
Pig skin	Tanned skin Gelatine	Leather products Jellied food products
Horns and hoofs	Extracted protein	Foaming fire extinguishers
Toe-nails	Meat	Livestock feed or fertilizer
Glands, stomachs, Intestines	Pharmaceutical sausage casing	Medicine sausage skins
Trimmings from meat	Edible fat raw meat or crackling	As above pet food, meat meal.
Liver, hearts Kidney stomachs lungs	Edible products	Direct consumption or in comminuted meat products

By products of meat industry (Filstrup)

3.2 Blood recovery

Blood recovery is not a new concept. The collection and utilization of slaughterhouse blood is a process known for many years. Blood is already used in a range of industries including the production of glue, as a fertilizer, in animal feed and even for human consumption e.g. black puddings. As mentioned in the introduction blood is collected firstly for its protein value and secondly to reduce the load from the waste effluent of the meat processing plant.

In recent years a lot of research has gone in the collection, processing and utilization of blood in several countries.

The collection methods for the recovery of blood fall into three categories:

1. Traditional methods
2. Present improved collection methods
3. Aseptic collection of blood.

3.2.1 Traditional methods

These methods consist simply of a blood pit below ground level in which the blood is collected. The carcass is suspended from an overhead rail while it undergoes sticking. Two systems are used:

A. Open system, horizontal bleeding

In this, a shallow tray is paced beneath the carcass to catch the gush of blood after sticking. The collected blood is defibrinated by mechanical beating, anticoagulant rarely being used.

B. Open system, vertical bleeding

This is a slightly more sophisticated process whereby blood from the stick wound is directed into a small shoot which feeds a vertical trough. The collected blood then passes into a closed vessel like a milk churn containing an anticoagulant.

Both these methods are not hygienic. The blood is contaminated with urine, airborne bacteria, skin etc. Bacteriological counts of 10^5 to 10^6 ml/blood have been reported by Heinz (1969). Also the troughs are seldom cleaned down adequately between kills and so successive contamination from batch to batch of blood occurs.

However if the troughs are made of stainless steel or ceramic materials, the animal is cut open properly with the oesophagus tied and correct sticking procedure into the major blood vessels, the collected blood will contain less than 2% urine and stomach contents (Holt 1977).

3.2.2 Improved Collection methods

The Meat Plant Advisory Service is developing a system which pipes blood directly from the animal to the processing equipment This system consists of a stainless steel pipe shaped to cut the blood vessels and at the same time being able to direct the maximum blood in a hygienic way to a receiving tank or medium.

A closed system was developed consisting of a rubber or metal cup fitted around the base of a standard sticking knife. A flexible tube leads from this cup to a closed collecting vessel.

The cup is of suitable size to completely cover the wound and catch the gush of blood. Anti-coagulant is trickled into the cup via a feed tube and mixing occurs as a result of natural turbulence of the blood flow.

These are known as "closed systems" and have centered around blood collection direct from the sticking wound through a tube into a collecting vessel. As a consequence contamination can be drastically reduced (Akers 1973).

3.2.3 Aseptic collection of blood

These methods utilise a closed system in a form of a hollow knife. The systems consist of a set of blood taking knives which are connected with an intermediate tank with vacuum and blood is then further processed by passing through a blood pump in a heat exchanger and further on to be centrifuged or stored.

A. Rizzi knife

This was developed in Germany. It is a hollow knife of 2.5 cm internal diameter (figure 3.8). Sticking is achieved by plunging the sharpened knife through the skin of the animal in the sternum to sever the aorta or vena cava near the heart. The anticoagulant is present in the collecting vessel. Hygiene is good although contamination from the skin through sticking is possible.

B. Ekstam knife

This method was developed in Scandinavia. It consists of two blades of different lengths, figure 3.9 . The blades are secured to a large diameter stainless steel tube with a shield attachment to which is attached a flexible plastic tube. Blood flows through the tube into a collecting vessel. An anti-coagulant is added via a flexible feeding tube behind the shield.

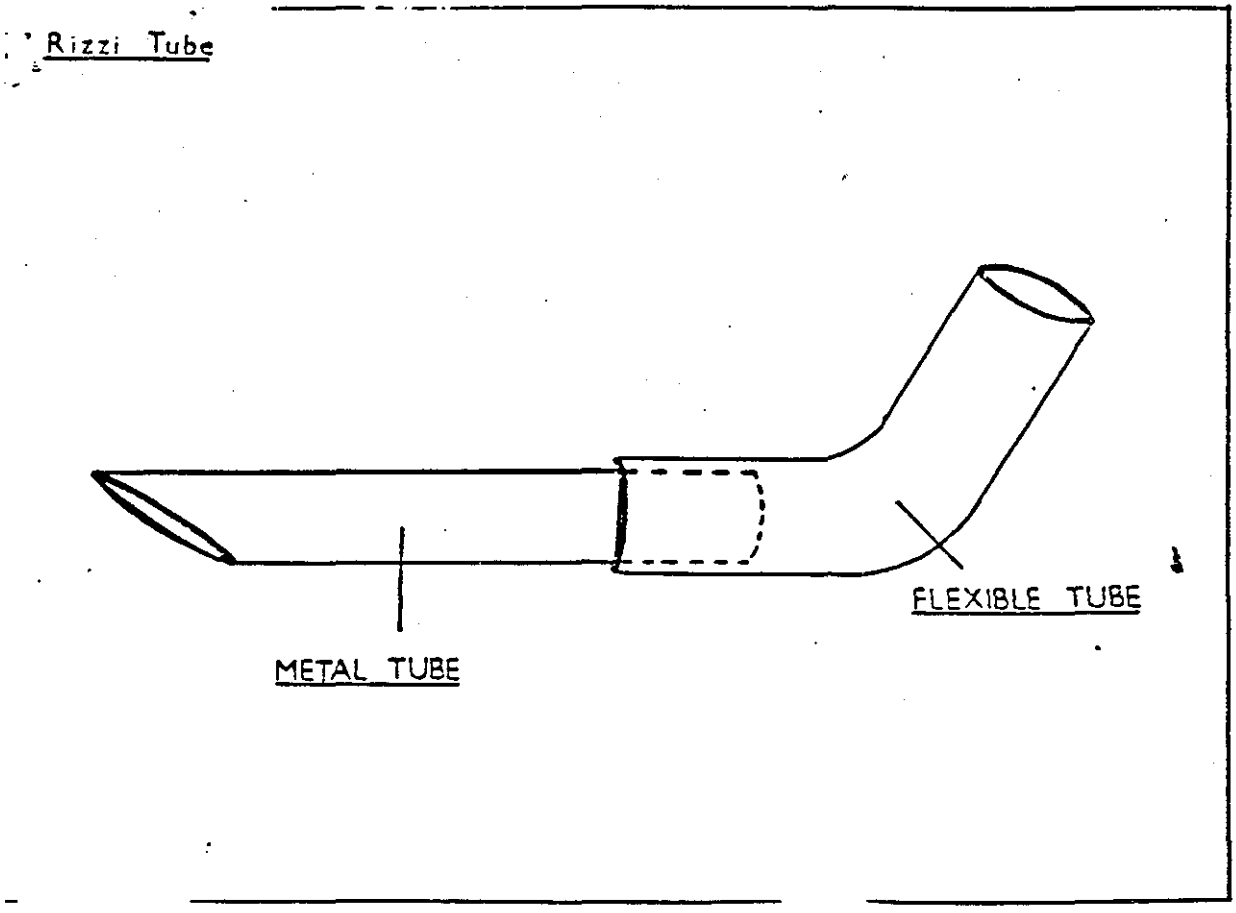


Figure 3.8

More recent models of this knife have replaced the short blade, by a thin metal bridge welded to the shield and either side of the long blade below the tip. This, effectively braces the blade and keeps the edges of the sticking wound open, allowing free flow of blood. The system utilises a minimum vacuum and contamination is minimal, particularly if a flap of skin is removed prior to sticking.

This knife can be used for both cattle and pigs.

C. Cannula knife

This is another simple method consisting of a 1.5 cm internal diameter tube, figure 3.10. One end is cut at an acute angle to give a very sharp point. Alternatively, the tube end is sealed and ground to form a sharp pointed fluted cone figure 3.11.

The system does not utilise vacuum; gravity flow is achieved by hanging the ox-head down and sticking the jugular vein using a downward stabbing motion. Thus this system is not suitable for other animals as the blood pressure in the hanging carcass is not high enough to sustain an economical collection of blood.

Obviously the closed systems utilising hollow knives are more hygienic over open systems. However the term 'aseptic collection' is somewhat misleading because asepsis is not achieved completely in the abattoir conditions. Other problems with these systems are:

a) It must be certain that the whole recoverable blood is drawn from the animal in minimum time, most systems needing about a minute. Care must be taken so no discolouration of the meat occurs due to excessive bleeding.

Ekstam Knife

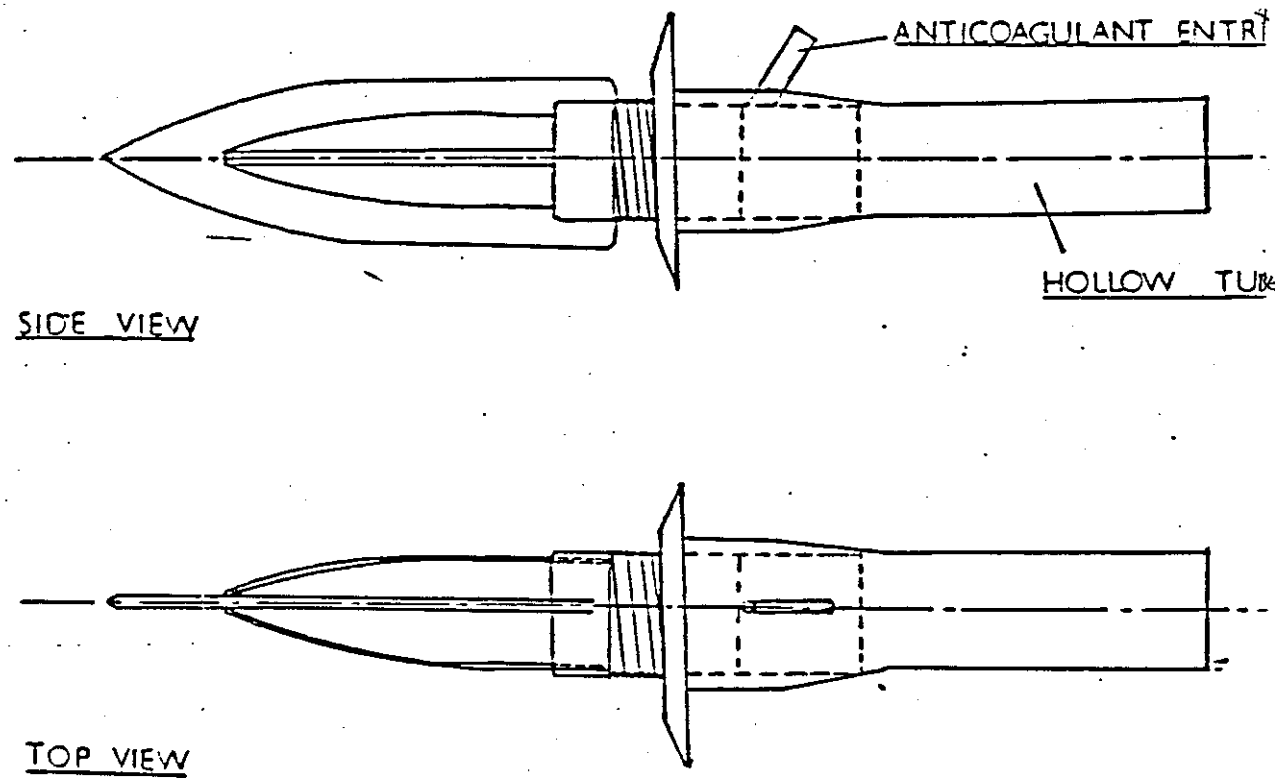


Figure 3.9

Cannula Device With Angled Blade

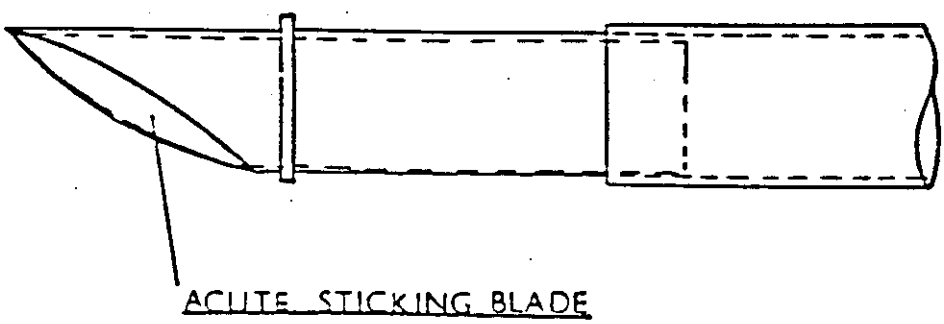


Figure 3.10

Cannula device with slits

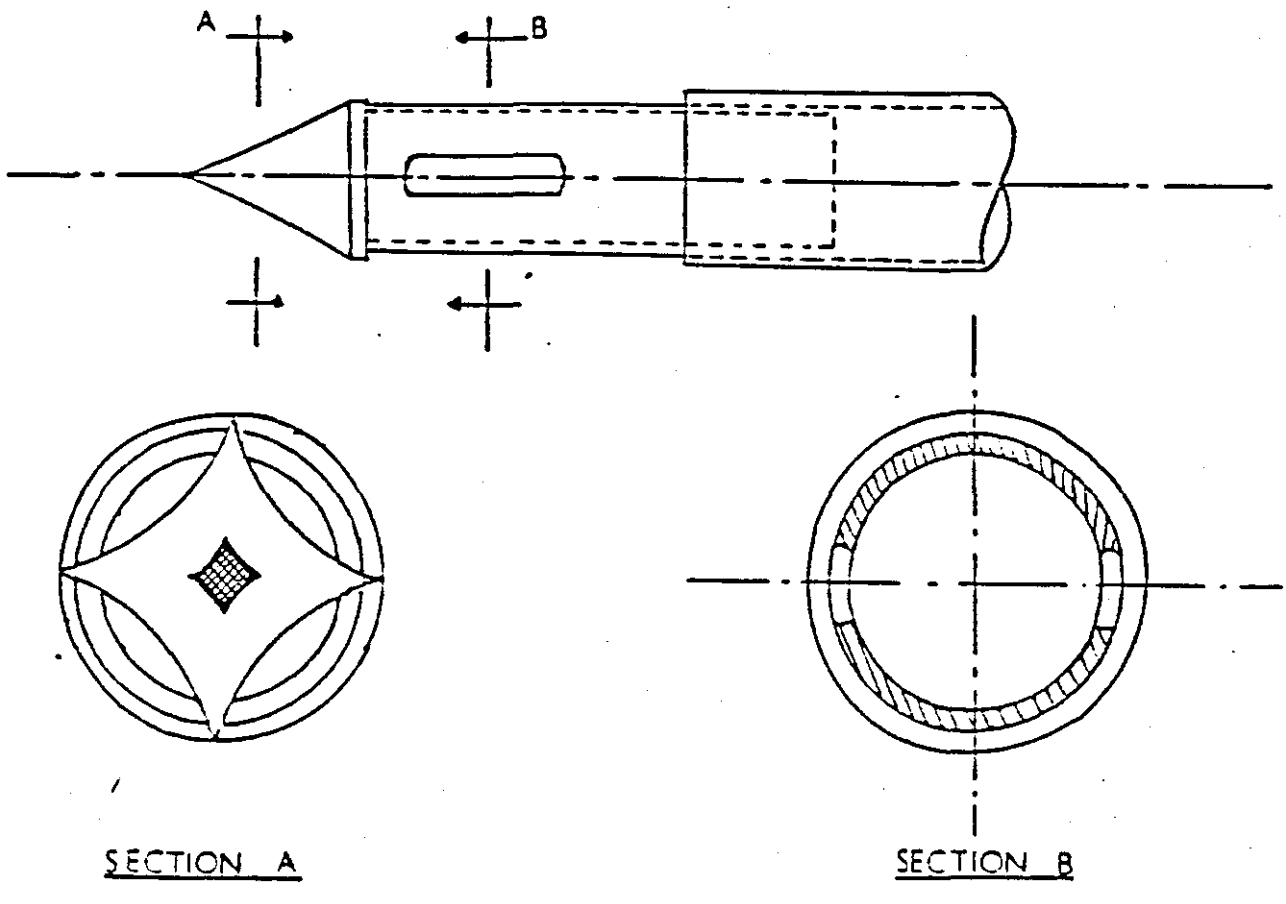


Figure 3.11

b) There can be a congestion at the sticking point in the slaughterhouse, due to interweaving of the pipe connecting the knives with the vacuum tank.

c) In small abattoirs with a kill rate of less than ten animals a day the expense cannot be justified.

3.3 Legal status of blood and blood products

The Meat Inspection Regulations of 1963 require that blood intended for human consumption must remain identifiable until the carcass has been passed by the Meat Inspector. A direct consequence of this is the common practice to bulk up the blood of few animals together, i.e. 5 cattle, or 10 pigs into one container. If one carcass is deemed not fit for human consumption the whole tank of blood holding the blood of the condemned carcass is rejected.

The legal status of blood is uncertain at the present time. Blood is usually regarded as an offal by meat manufacturers. However, regulations such as the 'sausage and other meat products' (S.I. 1967 No. 862), the 'Canned Meat Products' and others do not define blood as an offal. In the list of prohibited offals contained in the 'Offals in Meat Products Order' (S.I. 1953, No. 246), blood is not included.

In the Food Standards Committee Report on Offals in Meat Products (1972) blood is often referred to as an offal. There is a general view therefore that blood can be considered as a 'permitted offal' and can be included in meat products.

The previous 'Labelling of Food Regulation' (S.I. No. 1970 No. 400) defined meat as including offal. However the true nature of blood must be indicated by a product name or "appropriate designation", to the intending purchaser. Thus labels such as 'cattle meat' or pork are not sufficient to describe a blood product.

The current 'Labelling and Composition Regulations' (S.I. 1984, No. 1566) keep the same meat definition and still do not refer to blood at all. It makes reference that the presence of any added substance, in whole meat products, other than additives, salt, herbs and spices must be indicated in the name of the product and the minimum meat content must be declared, which indicates that meat containing blood proteins must declare this clearly on the label. Similarly for products having the appearance of raw meat and containing added substances.

More problems of definition are created with fractions of blood such as haemoglobin and plasma. If blood is considered a permitted offal does not preclude that parts of it, such as haemoglobin, can be regarded similarly.

The Food Standards Committee Report on Offals in Meat Products (1971), makes a reference to blood plasma. The Committee does not object to the continuation of the use of plasma in sausage and luncheon meats.

Haemoglobin is not mentioned however. There is a generally accepted view that blood proteins can also be incorporated in non-meat food products, for their functional or nutritional properties, unless compositional requirements prescribe otherwise.

3.4 Blood

Blood is a specialised tissue which acts as a carrier for a range of body fluids. Nutrients are transported from their absorption site in the intestine, to be processed in the liver and then carried to other organs. Hormones are transported in blood, and waste products also are conveyed in the kidneys for excretion. Blood also transports oxygen to and from the tissues and lungs, and removes carbon dioxide which is generated during the respiratory metabolism.

In this thesis whenever the term 'blood' is cited, 'bovine blood' is actually described.

Blood constitutes about 3-4% in weight of a living animal. The structure of blood is shown below

Blood Structure

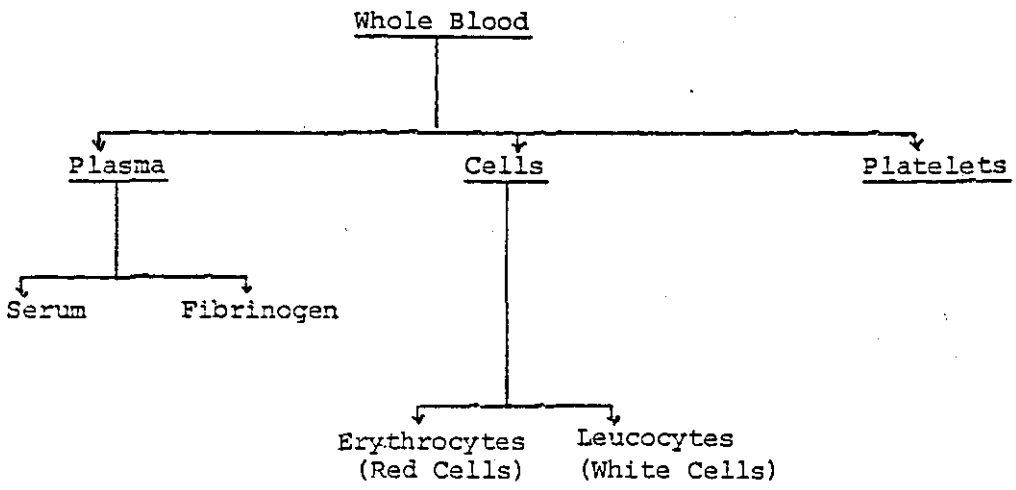


Figure 3.12

Nearly 45% of its volume consists of cells; erythrocytes which transport oxygen and some carbon dioxide, leucocytes which fight off invading microorganisms and viruses, and platelets. The rest consists of plasma which is mainly serum and 10% solutes, i.e. fibrinogen.

The composition of blood is shown below.

Composition of Blood (Ranken, 1977)

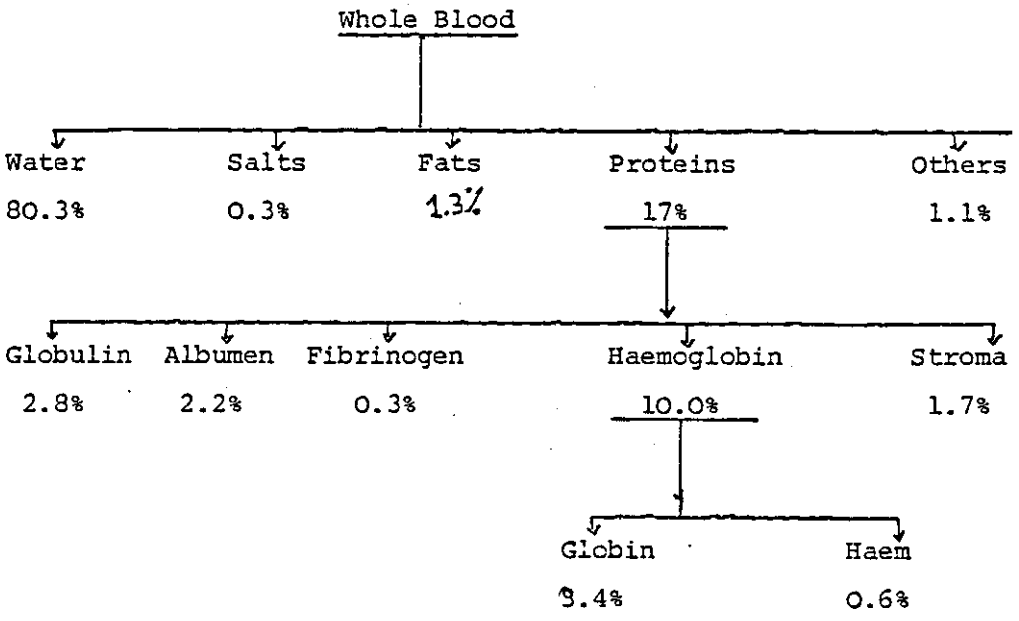


Figure 3.13

Evidence from electrophoresis and other chromatographic techniques has shown that there is intra- and inter- species variability in the composition of blood, but in general the overall percentages of the different components change only slightly.

If blood is left undisturbed it will coagulate forming a clot. This is a defence mechanism of the organism to prevent excessive blood loss. Essentially what occurs is the conversion of the soluble blood protein fibrinogen to insoluble fibrin thus forming a mesh on which blood cells adhere. The mechanism is shown below (Lehninger 1974).

Mechanism of Blood Coagulation

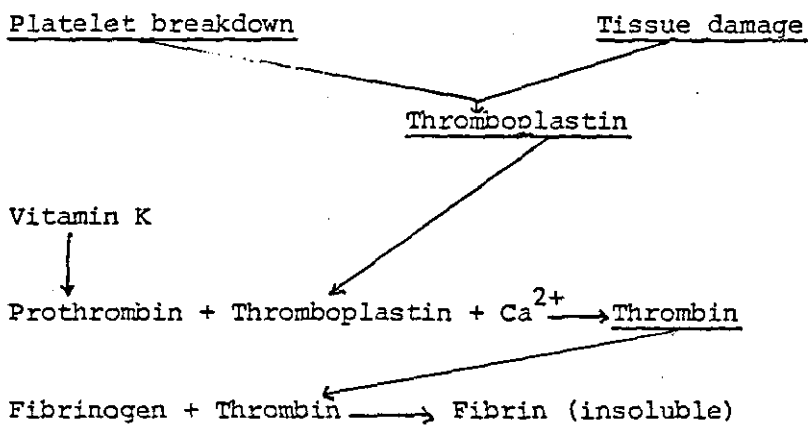


Figure 3.14

This clot can be prevented from forming by adding an anticoagulant. Bright (1977), reports on a wide range of anti-coagulants such as: phosphate preparations, EDTA, allyl isothiocynate heparin and sodium citrate. There are also other ways of preventing coagulation such as using mechanical means, bubbling ozone or by homogenising blood, also reported by Bright.

The most widely used anticoagulant is sodium citrate. This works by eliminating the calcium ions necessary for the clotting process. These ions are precipitated out by forming insoluble salts with sodium citrate.

Blood with anticoagulant added in, can be easily separated, see figure 3.15 (Ranken).

Separation of Whole Blood

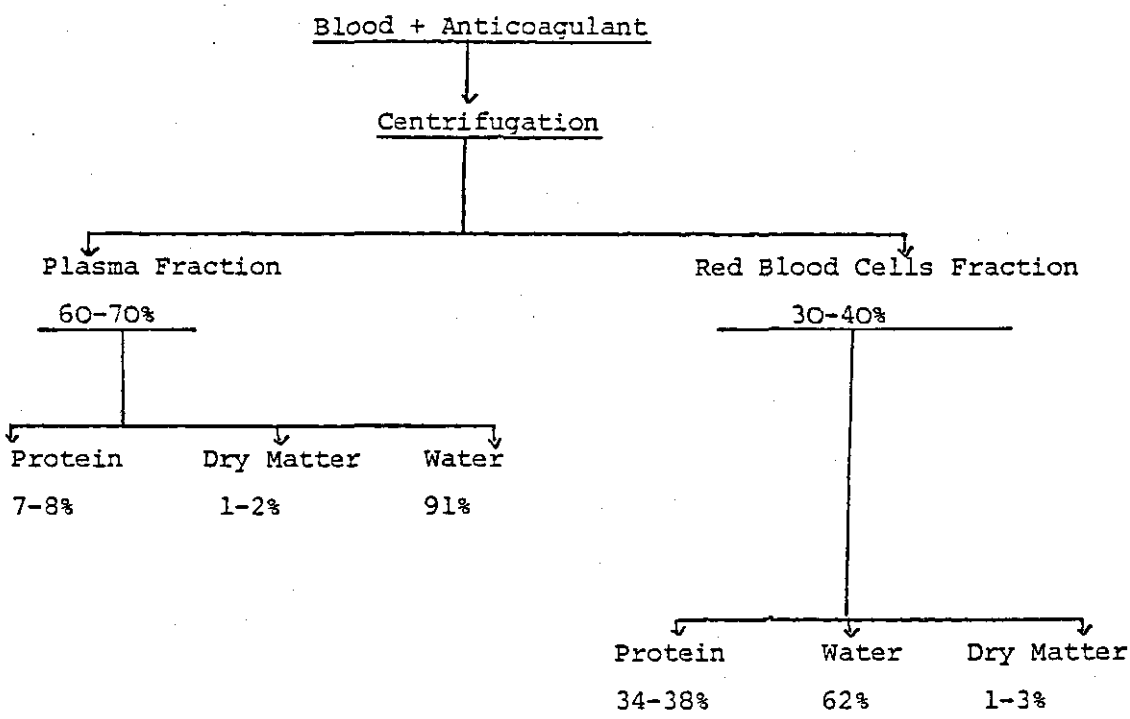


Figure 3.15

3.4.1 Plasma proteins

The composition of plasma is shown in the figure below (Hurst 1980).

Composition of plasma

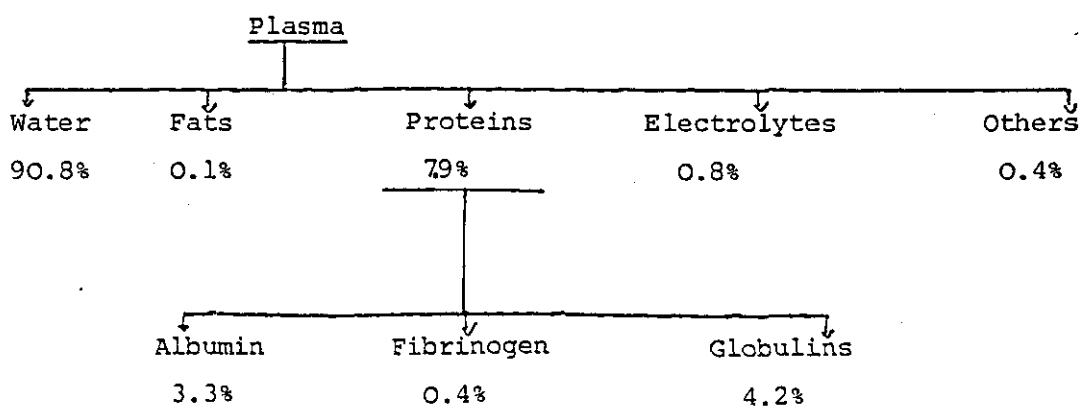


Figure 3.16

It is evident that the majority of solutes in plasma are proteins. Serum Albumin, with approximate molecular weight, (MW), of 68000 is responsible for osmotic regulation and transport of fatty acids.

Under the term globulins, a group of forty minor proteins are grouped. Most of them are conjugated with MW's ranging from 40,000 for α_1-globulin to 20 million for β_1-lipoproteins. Not all functions of globulins have yet been fully defined. The major function of globulins involves the transport of lipids, iron, copper. These are also involved in blood clotting and work as antibodies.

Fibrinogen, with MW of 340,000 is mainly involved in blood clotting.

Electrolytes in plasma include: Na^+, K^+, Cl^-, HCO_3^- and phosphates.

3.4.2 The Red Cell Fraction (RCF)

The deposit of centrifuged blood is almost entirely red cells. Above the red cells lies a thin layer containing the white cells and platelets. The red cells are by far the majority of the blood cells, for every white cell there are about 500 red cells and 30 platelets. The production of red cells, a process called erythropoiesis, occurs exclusively in the bone marrow.

The red blood cell count ranges from 3.9 - 6.5 x 10^{12}/l (Lehninger 1975). The mature red blood cell measures approximately 7 µm in diameter and appears as a biconcave disc with no nucleus.

The precise structure of the red blood cell and the forces which preserve its shape are not yet fully understood.

The cell is surrounded by a flexible elastic membrane which has a complex protein and lipid infrastructure.Only about 2% of the haemoglobin content of the red blood cell is near the membrane, while the remainder is uniformly distributed throughout the cell.

The red blood cell is the site of numerous metabolic activities, many of them are involved with the maintenance of the integrity of the cell membrane. However the presence of many enzymes and intermediary products without known function, suggests that the red cell has other functions as well as the transport of respiratory gases.

The circulating, nonnucleated, erythrocyte has a relatively simple respiratory mechanism. Glucose is transported across the cell membrane and about 90% of it is metabolised anaerobically to lactate via the Embden-Meyerhof pathway. The rest is aerobically metabolised via the hexose monophosphate pathway.

The composition of the red cell fraction of centrifuged blood, is shown in the figure below.

Composition of the Red Cell Fraction

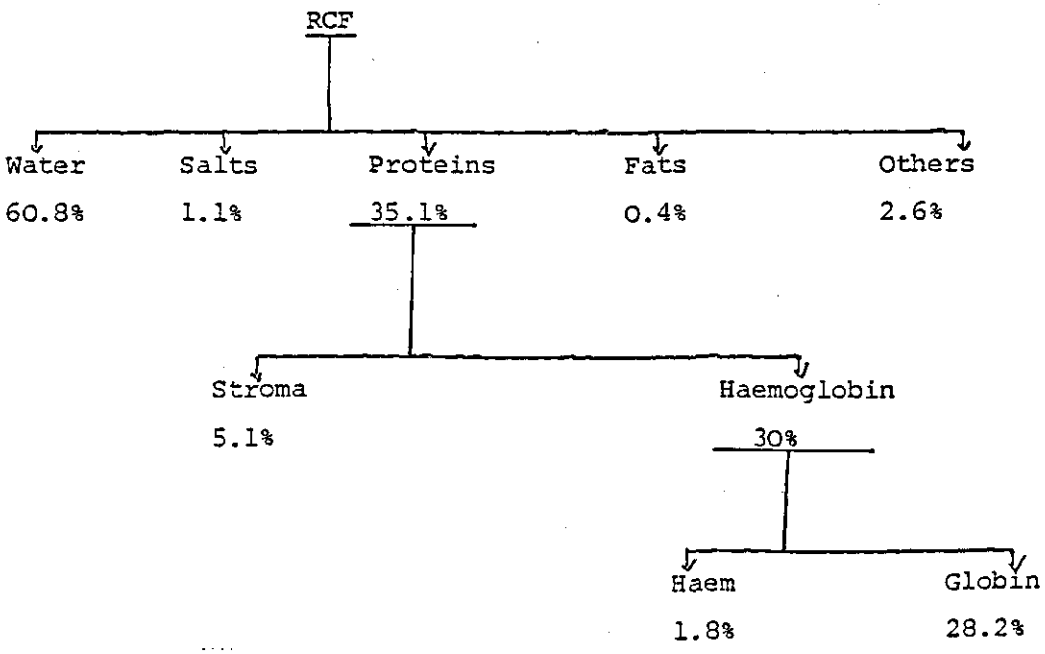


Figure 3.17

3.4.3 Haemoglobin

Haemoglobin is one of the most important proteins of vertebrates. Dickerson (1969) estimates that there are approximately 5 billion red cells in humans, each packed with 280 million molecules of haemoglobin. The main function of this protein is to act as the oxygen carrier for the organism.

Haemoglobin is a globular protein with molecular weight in the region of about 65,000. It contains something like 10,000 atoms, four of which are iron the rest being C, H, O, N,S.

Our knowledge of the three dimensional structure of haemoglobin comes from efforts started back in 1930's, and the use of x-ray analysis by Perutz (1961, 1968 a,b, 1969). The haemoglobin molecule was found to be roughly spherical with dimensions 64Å x 55Å x 50Å. It contains four polypeptide chains of roughly equal length, two α chains of 141 residues and two β with 146. They also differ in the sequence of amino acid residues. The haemoglobin molecule is shown in Fig. 3.18. It can be seen that the haemoglobin is a tetramer consisting of four subunits, designated α_1, α_2, β_1, β_2, the polypeptide chains mentioned above. There is a vertical line of symmetry in the molecule, this is the solid line in the figure. The four haem pockets are all exposed at the surface of the molecule. The haem groups of chains α_2 β_1 are particularly close as are those of α_1 and β_2. The packing of chains into the haemoglobin molecule is made in such a way that there is contact between unlike chains, i.e. α_1 β_2 or α_2 β_1 but not between α-α or β-β chains. There are hydrogen bond interactions but the majority of contact is due to hydrophobic interactions.

As seen, each chain engulfs a haem group of 12Å length and molecular weight of 600. The haem group is the functional part of the molecule and is described in detail here. It must be noted

The Haemoglobin Molecule

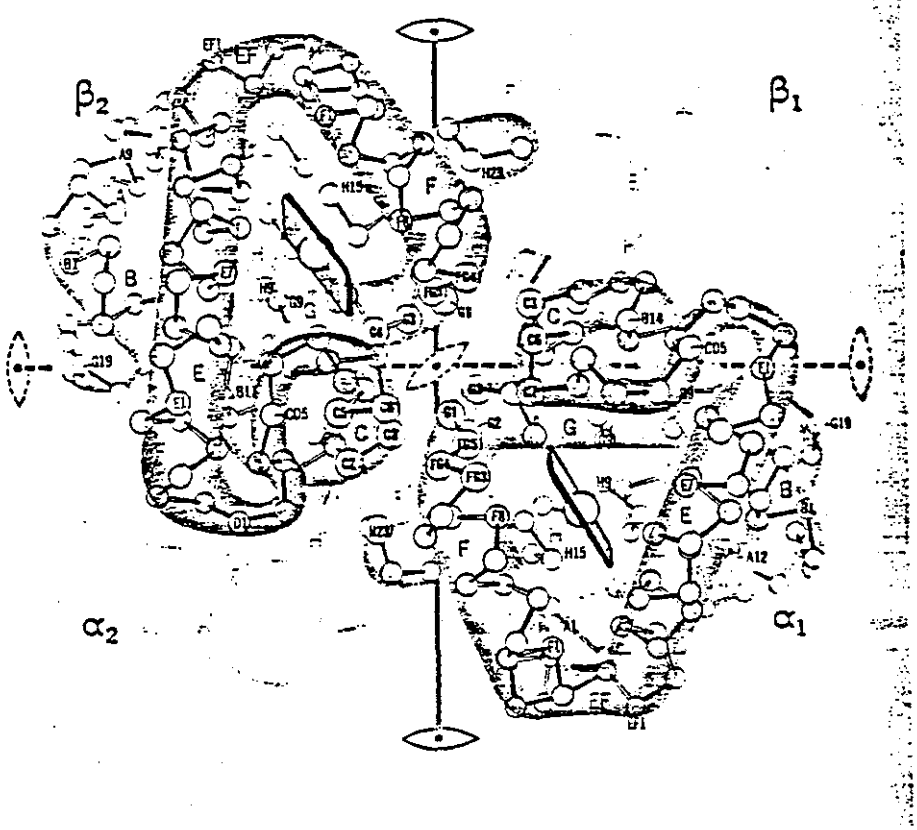


Figure 3.18 (Dickerson, 1969)

though that the haem functions in conjunction only with globin and no other protein.

A diagrammatic representation of the haem group is shown in Fig. 3.19. It consists of an iron atom attached to a porphyrin ring. In haemoglobin the porphyrin is protoporphyrin IX which is distinguished from other porphyrins but its side chains which are: four methyls, two vinyls and two propionic acids. Weissbluth (1974) describes the haem as follows. In the plane of the haem the iron atom is liganded to the four pyrrole nitrogens. In a direction perpendicular to the haem there is an attachment by means of a covalent bond between the iron and a nitrogen on the imidazole of histidine. This residue is known as the haem-linked histidine 87. Another attachment position is in the opposite direction in which various atoms or molecules are found. It is in this ligand in which O_2 is reversibly bound for transport. If O_2 is bound the molecule is called oxyhaemoglobin and has a bright red colour. If carbon monoxide is bound, carboxy-haemoglobin is produced. If the ligand is free deoxyhaemoglobin exists of dark red colour. When the haem is bound by a molecule the traditional description is that the iron is in the ferrous state (Fe^{2+}) hence the haem is reduced. The same applies when the sixth ligand is vacant i.e. in deoxyhaemoglobin. However Weissbluth suggests that the distinction between Fe^{2+} and Fe^{3+} in oxyhaemoglobin is rather blurred, and that there is considerable transfer of electronic charge from the iron to the oxygen.

The haem is mainly kept attached to the polypeptide chain by the histidine mentioned before. Perutz (1969) reports that except for the covalent bond in the haem-linked histidine, there are about sixty interactions between residues of the globin chains coming to within 4Å distance of the haems, all but one of those in the α chains and two in the β chain being non-polar.

As mentioned above the main function of the haemoglobin molecule is to transfer O_2 to the tissues for the purposes of

Attachment of Haem via Histidine to Globin

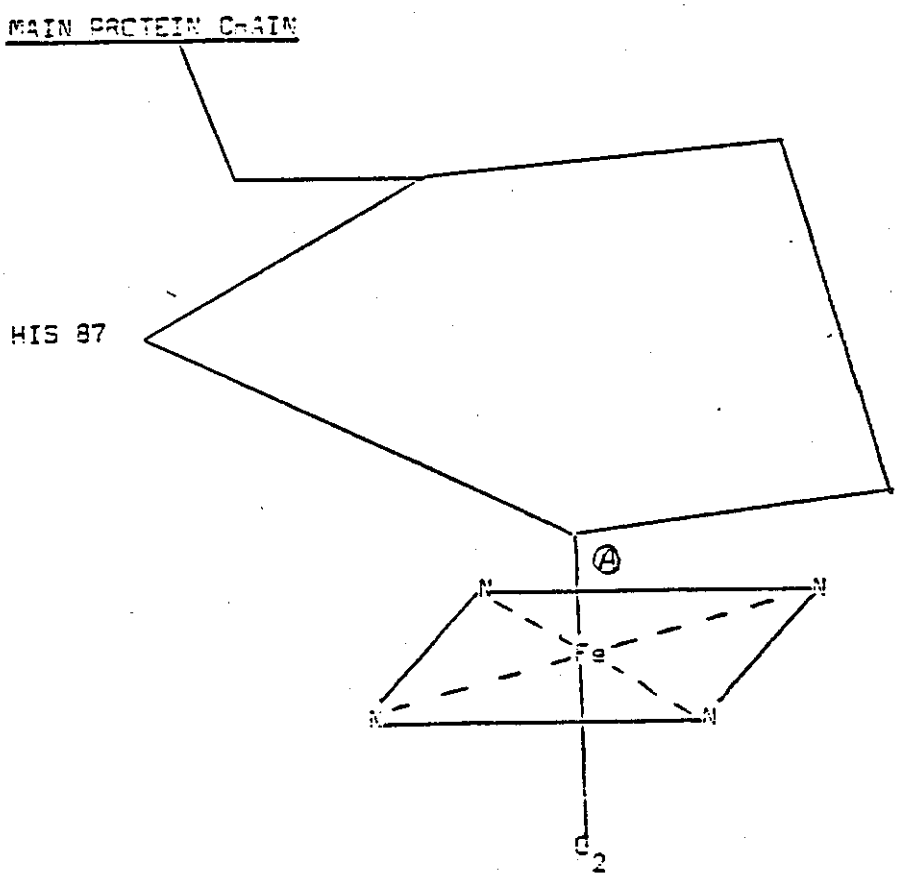


Figure 3.19

respiration. The four iron atoms which combine with oxygen, are not independent and the equilibrium constant of any of them is influenced by the state of oxygenation of the three others. Thus the oxygen dissociation curve of haemoglobin is sigmoid. This is advantageous since the partial pressure difference of oxygen between the tissues and the lungs is small, hence the sigmoidal equilibrium curve allows the haemoglobin to be charged with oxygen , in the lungs, and to be discharged completely, in the tissues, within this narrow difference.

3.4.4 Nutritional and functional properties of blood and its fractions

The content of essential amino acids in plasma and globin are given in Table 3.6 below. They are compared with the ideal values for a well balanced diet recommended by FAO. The concentrations of methionine and isoleucine are very low compared with the recommended value. The major contribution of nutritional value of blood and especially haemoglobin, is its high iron content, which is an important mineral in the diet.

Essential amino acids in blood

Amino acid	g/100 g protein			
	Whole blood	Plasma	Globin	FAO
Lysine	10.2	9.2	10.5	4.2
Threonine	4.3	6.3	3.8	2.8
Methionine	1.6	1.0	1.7	2.2
Valine	8.9	7.0	9.4	4.2
Phenylalanine	7.4	5.6	7.9	2.8
Leucine	13.0	10.1	13.8	4.8
Isoleucine	0.8	2.9	0.2	4.2
Tryptophan	2.0	1.9	2.0	1.4
Histidine	6.3	3.5	7.8	-

Table 3.6 (from Ranken, 1977)

However blood proteins are commercially more valuable for their functional properties. A summary of the functional properties of plasma and globin is given in Table 3.7 (Ranken 1977). The functional properties of globin have been further reported in a wide range of papers. Most workers agree that haemoglobin can provide bulk to a food system by binding fat and that it can form large, stable foams. (Hermansson 1976). It should be noted however, that the method by which globin is processed affects its functional properties.

Functional properties of blood fractions

Property	Globin	Plasma
Solubility	Good at pH < 6. Insoluble in 2% salt	Good Salt has no effect
Swelling in water	Good	Poor
Emulsifying capacity	Very good	Good
Emulsion stability	Good at pH < 6 improved with salt	Good, poorer with salt
Foaming	Very good	Good
Gel formation	None	Very good

Table 3.7 (Ranken, 1977).

3.5 Utilization of blood

The utilization of blood can be subdivided into three categories:

1. Whole blood utilization
2. Plasma utilization
3. Haemoglobin utilization

However when blood, or its fractions, is destined for human consumption certain provisions must be made. Ranken (1977) summarises what all blood collection systems should provide:

1. Hygienic collection
2. The rejection of blood from unfit carcasses
3. Addition of an anticoagulant
4. Minimal haemolysis
5. Facility to cool and store the blood at temperatures less than $4^{o}C$.
6. Cleaning of equipment

Hygienic collection is absolutely essential. Heinz (1969) reports that counts of up to 50 organisms/ft^3 of air of bacteria in the air have been recorded. Also contamination arises from the animal hide, regurgitated stomach contents and urine. Lawrie, (1978) also reports that the microbial load of blood from an excited or exhausted animal is increased.

Haemolysis must be minimal in order to stop development of 'off' flavours. This can be achieved by less mechanical agitation and by avoiding osmotic differences in the blood. Some haemolysis will however be inevitable in bulk collection systems, due to the mixing of blood from different animals.

Cleaning is important. Akers (1973) suggests a 1:20 dilution of hypochlorite solution followed by a hot water wash at $82^{o}C$ for the sticking knives. He also suggests stainless steel collecting equipment which can be sterilised with live steam. Cleaning in place systems are ideal.

Presently there are several blood collection systems available. Alfa-Laval, Nutridan Engineering and Bruendler International, A.G. all offer commercial blood recovery plants. All the systems

adhere to the principles outlined above, and are composed of a carousel blood collection system with sets of hollow sticking knives, dosing system for the anticoagulant, storage tanks, heat exchanger as a cooling system, and cleaning in place and sterilising facilities.

3.5.1 Whole blood utilization

Blood is a perishable product and must be processed soon after collection, and prior to any further usage.

Bright (1977) gives a detailed description of the methods used to preserve blood. These include coagulation of whole blood by means of chemicals, steam coagulation, dewatering using spray driers. The most widely used process is drying of blood in a spray drier.

Traditionally blood has been used for many years in the UK for the production of black puddings, blood sausage and blood and tongue sausage. The blood is generally only defibrinated, but lately there is a tendency to use the red cell fraction of blood only and not the plasma in these products (Bright, 1977).

Satterlee, Free and Levin (1973) have looked at the use of whole blood powder as a binder and extender in meat emulsions. They found that the powders have high emulsion capacity and stability.

Co-precipitates of blood and skimmed milk have been incorporated into a wide range of meat and other products, as well as in baby foods and dietetic foods (Bright, 1977).

Bright also reports on a series of other uses of whole blood in the Meat and Bakery Industry. These include the use of blood as a flavouring enhancer, colouring agent for meat and fish

sausages, as a meat substitute using blood meal. In Russia bread was prepared from rye flour and defibrinated ox blood, however that end product is of dark colour and has a crumb texture much coarser than the usual loaf.

Mitsyk et al (1975) and Osadchaya et al (1975) both used clarified blood as a replacement for bulk egg in the production of biscuits and flour types of dough. They report that in the range of 20-50% replacement level of blood to bulk egg, the final product was only slightly affected.

Blood can also be used in animal feeds. Blood meal is prepared by steam coagulation, dewatering and drying, and is used as an animal feed or as a component of animal feeds. Several workers however report problems such as unpalatability, poor digestibility and low biological value, with commercially available bloodmeal (Bright). Blood is also used in the pet food industry. Petfoods Inc. patented a coherent gel product which has the appearance of lung or liver tissue and is prepared from a mixture of aerated blood and egg white. Similarly Quaker Oats Co (1976) substituted some of the meat in the canned pet food by blood.

There is a wide range of non-food uses of blood reported by Bright (1977). The more well known ones are the use of blood as a fertiliser and filler in glues. However other uses such as additive to plaster, cement, in the production of fire-fighting foam and in oil recovery are reported.

In addition to veterinary therapy, animal blood can in certain cases be used in human medicine. Animal blood collected under strict hygienic conditions is treated to prepare various protein fractions such as thrombin.

3.5.2 Plasma utilization

Blood plasma is considered, at present, to be the most important fraction of blood, from a commercial point of view. It is devoid of the colour and flavour problems associated with whole blood or haemoglobin. It has good functional properties which makes it a good extender of meats in emulsion- based meat products and foaming agent in the bakery industry.

The present market outlets and future potential use of plasma are reviewed by Bright (1977), Howell (1981), Turner (1983) and Hill (1986).

3.5.3 Haemoglobin utilization

Outside its incorporation into traditional products, such as black pudding, liver sausage and 'rye bread', haemogoblin is hardly used in the food industry; in certain cases it was used as a colouring agent in mince meat products. Currently, haemoglobin is used by petfood manufacturers.

This is an underutilization of a protein with useful functional properties however. The problem, as mentioned before, lies with the intense dark colour and distinctive taste it imparts to foods. This is due to the haem part of the molecule. It was quite early that this was realised by food manufacturers. Hence work has started on the decolourisation of haemoglobin, that is, the removal of the haem molecule, while maintaining intact the functional properties imparted by globin.

3.5.4 Decolourisation of Haemoglobin

Presently the decolourisation methods can be divided into five groups:

1. Masking of colour
2. Enzymic decolourisation
3. Using organic solvents
4. Hydrogen peroxide decolourisation
5. Using ion-exchangers such as carboxymethyl cellulose (CMC)

3.5.4.1 Masking of colour

A method of 'masking' the colour of blood by producing emulsions consisting of blood, fat, water and sodium caseinate was described by Zayas, Klebamov and Zyrina (1976) and by Zayas, Zyrina and Sokolov (1975). These emulsions were prepared from: bovine blood 20%, pork lard - 45%, sodium caseinate -5%, water - 30%. These components were dispersed in a hydrodynamic sonic installation. Haemoglobin is an effective catalyst of lipid oxidation, however, Zayas found that in the presence of sodium caseinate this lipid oxidation was inhibited and by forming lipoprotein envelopes, oxidative processes on the surface of the fat globules were retarded. This, now discoloured, stable emulsion Zayas et al suggested could be used as a substitute for 10-15% of semi lean pork in meat products to improve the protein content and water binding capacity of the product.

Wismer-Pedersen (1979) also describes a similar process in which blood is decolourised by coating the blood corpuscles with a protein fat:water emulsion.

Bonneman (Nutridan Engineering A.S.) describes a plant for treating blood or/and haemoglobin and the production of blood emulsions. The plant consists of aseptic blood collecting

equipment, a mixing tank for fat, blood and water and an emulsifier and a high pressure pump. It has a capacity of 200 lt of blood/hour, producing an emulsion of about 600 lt/hour. Nonnemann claims that it is possible by adding the produced emulsions to meat products to incorporate 5-10% of blood without affecting the colour or taste of the final product.

The above methods rely on the production of small fat particles coating the whole haemoglobin molecule. This limits their uses because at high haemoglobin concentrations the taste and colour problems still exist.

3.5.4.2 Enzymatic decolourisation

Christensen (1978, 1979) has developed a process in which the decolourisation of haemoglobin is achieved by partial enzymatic hydrolysis followed by treatment with activated carbon. An outline of the method is shown in Figure 3.20.

The cell fraction is separated and diluted two to three times with water to achieve rupturing of the cells. The protein solution is adjusted with water to the desired substrate concentration defined as total protein N x 6.25, and the pH and temperature are regulated to give the optimum conditions for the addition of the enzyme. The enzyme used was Alcalase which was found most suitable for hydrolysing the haemoglobin into peptides. The enzyme is inactivated by lowering the pH to 4.2 and the reaction mixture is centrifuged. The supernatant contains the peptides of haemoglobin which are decolourised by the addition of activated carbon, in the range of 0.5 - 1% by weight addition. Better decolourisation results are obtained at temperatures around 55^{o}C. The activated carbon is removed by filtration and the final product is freeze dried. Final protein yield is claimed to be 60-70%.

This work has led to a patent taken out on the process by the Danish Meat Institute. In the patent the functional properties of the finished product are:

1 Solubility in a range of salt solutions
2 Emulsification properties
3 Reduction of fat loss in cooked meat formulation

However work carried out by Smith (1983) on the functionality of an enzyme-decolourised blood protein hydrolysate, reveals a different story. The protein was obtained from Novo Enzyme Products Ltd. It was powdery, white, hygroscopic and of low density. The only significant functional property reported by Smith was its high solubility unaffected by pH and presence of electrolytes. The emulsifying capacity of the protein was poor compared with egg albumin and haemoglobin powder. Also foam stability was very poor.

It appears that this method of decolourisation produces a protein powder which does not possess any significant functional properties. This may be due to the splitting of the haemoglobin molecule into smaller peptide chains.

Enzymatic decolourisation of slaughter blood

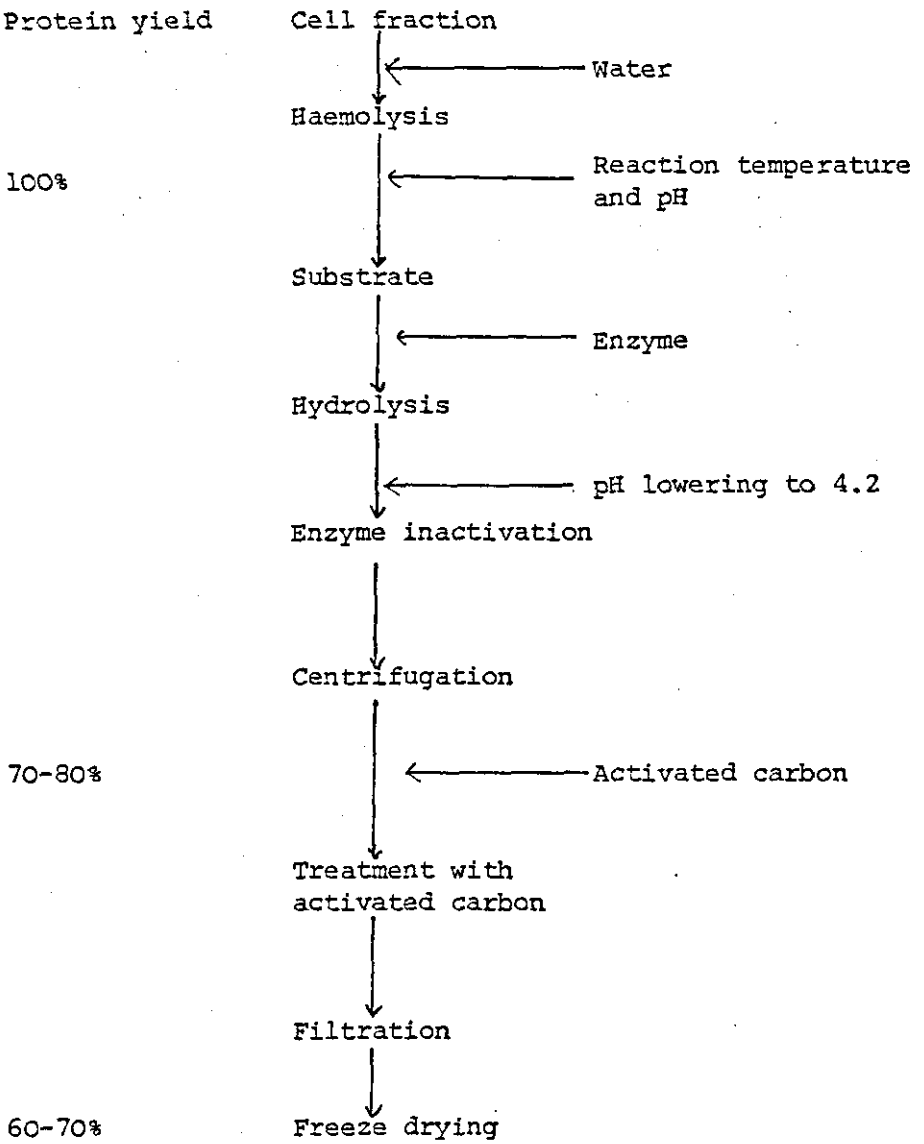


Figure 3.20 (after Christensen 1979)

3.5.4.3 Using Organic Solvents

Tybor et al (. , 1975) describe a process which separates globin from haem. A flow diagram of this process is shown in Figure 3.21. After centrifugation whole blood was separated into plasma and the red cell fraction. The red cells were then haemolysed by 100% addition of water. Chloroform was added to remove the stroma. Ascorbic acid was added to bring the pH of the haemoglobin solution down to 4.0. This suspension was pumped through a 1.5 in diameter pipe into a high speed turbomixer. There the haemoglobin was converted to choleglobin. The chromo protein solution was then passed into a second mixer, operating at 5000 rpm, and acidified acetone was introduced at a 4:1 (v:v) ratio. The prosthetic group of the chromo proteins was removed and the globin proteins were precipitated during this stage. The final product is a white free flowing protein isolate containing 91.22% protein.

Tybor (1975) examined the functional properties of proteins isolated from bovine blood using the above mentioned continuous pilot plant process. Globin showed maximum solubility (90-100%) at pH < 6.0. Emulsification capacity of globin was reported good, better than non-fat dry milk and cottonseed isolate. The foaming capacity of globin and its foam stability were better than plasma and egg albumin.

Shahidi et al (1984) describe two processes for decolourising bovine blood. In the first, red blood cells (100 ml) were slowly added to 1500-4500 ml boiling extraction solution prepared from one volume acetic acid containing 2% calcium or strontium chloride and 3-5 volumes acetone. The detached globin, which is insoluble in the extraction solution was filtered as a precipitate and air dried at room temperature.

Alternatively, a 20% red blood cell solution in water (500 ml) was added slowly to 600 ml glacial acetic acid saturated with NaCl at 90-100°C. The porphyrin materials were then filtered and the globin was precipitated using about 1000 ml of acetone. The globin was then filtered and air dried at room temperature.

Shahidi reports that the globin prepared with these methods was of light beige colour, containing 81% protein. The fat content of the product was below detection level and its iron content was 0.02 mg/g, indicating the presence of only traces of iron-porphyrin materials.

The author also reports good functional properties, much in agreement with the protein produced by Tybor. The globin product has high water solubility, excellent whippability and good foam stability. Mayonnaise type emulsions were stable over 8 weeks of storage at 4°C.

An Australian Patent Specification 502112 (CSIRO) describes a method in which red cell concentrate is injected under turbulent conditions into acidified acetone. The acetone which flows far more slowly, transports the haemoglobin away from the point of injection, decolourising it in the process. The temperature in which the reaction takes place must be kept constant and the optimum range is from -10°C to 35°C.

A process preparing globin from haemoglobin, using methanol and water and/or ethanol is described in European Patent Application (Publication number O 068537). In it red blood concentrate is contacted at increased temperature and under turbulent conditions with an organic solvent. This is achieved by mixing the cells vigorously with methanol and water, or ethanol, and keeping the mixture at 35°C. After mixing and heating with the organic solvent, a solution of methanol and hydrochloric acid is added under turbulent conditions. The solution of methanol and acid should be a pH below 4.5.

Decolourisation of Haemoglobin by the Tybor process

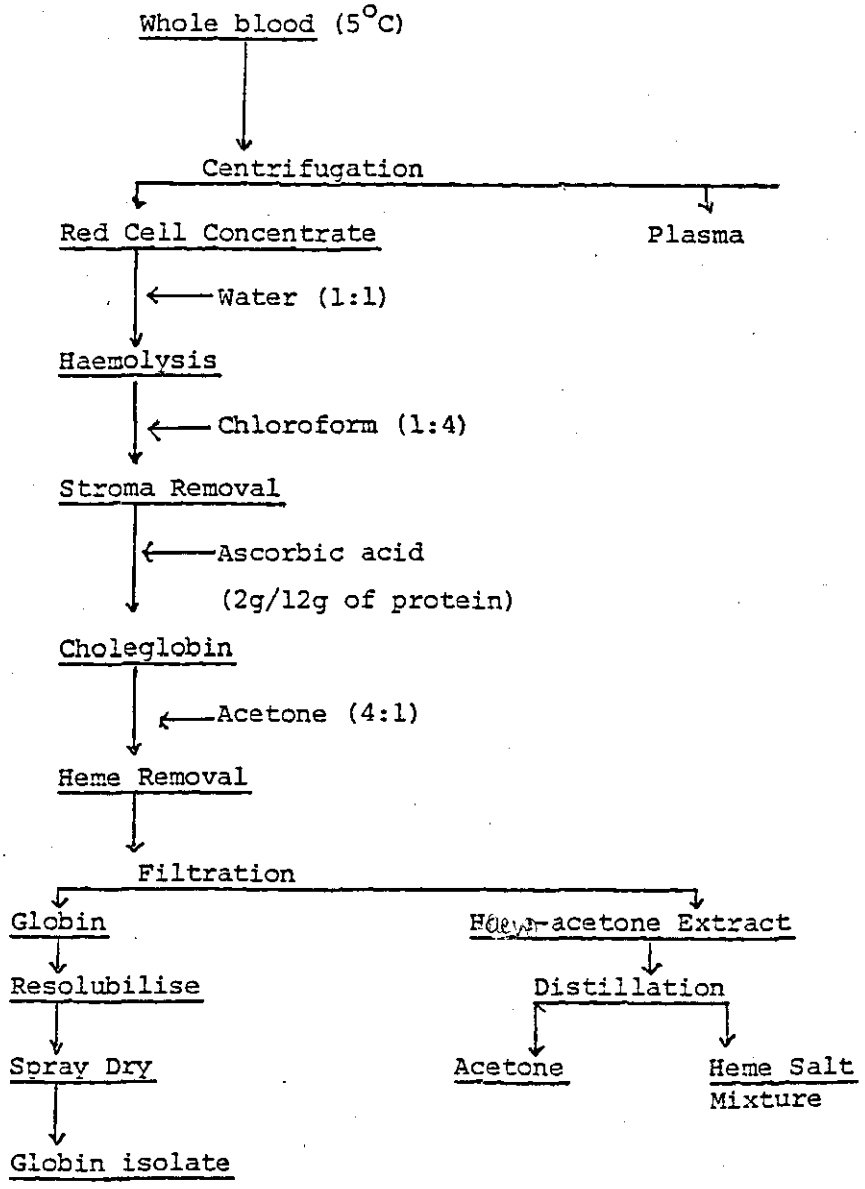


Figure 3.21 (Tybor 1973)

The dispersion of solvent, acid and split haemoglobin is separated by means of hydrocyclones into a haem rich phase and a globin rich phase. The globin can be then further processed by neutralization, separation and drying. This patent describes processes which can be either batch or continuous. It is claimed that the final protein product contains 350 ppm of iron, i.e. a 90% reduction of iron content.

Another patent, (European patent application; publication number 0 013 055) describes the following method for producing a tasteless - odourless globin isolate from bovine blood. The red cells are haemolysed and the converted into a solid form by dehydration, such as drying, e.g. air-drying, spray drying etc. The next stage is an acidified solvent extraction process. The organic solvent, methanol or a mixture of ethanol and methanol, is acidified with hydrochloric acid to a pH value below 3.5. The haem is carried over by the solvent and the globin is isolated and can be further processed. Another alternative, also described in the same patent, is to haemolyse and split the haemoglobin in one step i.e. the cell concentrate is added to strong acid, with vigorous stirring, and the resulting solution is dehydrated and subsequently subjected to solvent extraction as before. The patent hints that the globin isolate can be used as a foaming agent or emulsifier.

Overall, it is argued that solvent extraction of haem results in high quality globin isolates. However there are certain drawbacks with these methods. Firstly large quantities of expensive solvents must be used, secondly expensive solvent extraction methods are required to remove residual solvent from the protein product, and finally some solvents are not acceptable for use in processing food for human consumption.

3.5.4.4 Hydrogen peroxide decolourisation

The potential for using hydrogen peroxide to decolourise

bovine blood was known as early as 1942 in Germany. The German Patent (No. 744055) describes a process in which blood is heated to 70°C, so the catalase enzyme is destroyed, and hydrogen peroxide was added to oxidise the haemolysed blood.

Much later Van Den Oord and Wesdorp (1978) also described a decolourisation process using hydrogen peroxide. The red cell concentrate was diluted with seven volumes of water and heated up to 70°C. Then a 3% peroxide concentration solution was added under vigorous stirring. Complete decolourisation of haemoglobin was achieved in 10 minutes, at 70°C and by using 0.8 to 1 ml. peroxide per gram of red cell concentration. Wesdorp used different reaction temperatures and pH values. He concluded that the minimum temperature is 50°C, with the reaction needing 30 minutes to complete and the range of pH which gave a yellow cream product was above 6.0 and up to 7.1.

The peroxide was added in one portion to the hot haemoglobin solution, not as Brahm (1941) suggested. Brahm describes a similar method but using whole blood and without examining the final product. Wesdorp's product was 99% protein (on dry basis) and has no functional properties. The protein yield is 90%. However he speculates that treatment of haemoglobin with hydrogen peroxide might result in the formation of the bile pigment choleglobin and the formation of oxidized amino acids.

He speculated the formation of a growth depressant, methionine sulphone. Hence its toxicological safety must be assessed prior to its incorporation in human foods.

Two methods utilising hydrogen peroxide to decolourise bovine blood are described in a Mars patent (U.K. Number 1562 618) 1980.

The first method is called "pH reversal" technique. The following two procedures are described now using this pH reversal principle:

1. Whole blood, or red cell concentrate is made acid (pH 3) with hydrochloric acid. The resulting slurry is then brought up to pH 8 with sodium hydroxide. Hydrogen peroxide, 3-6% of 100 volume strength is vigorously stirred in, and after ten minutes a pale yellow curd is obtained which can be washed with water and further processed to remove any residual hydrogen peroxide.

2. Again whole blood is used but it is now made alkaline (pH 11) with sodium hydroxide. After five minutes it is neutralised to pH 6-8, with hydrochloric acid. Similar amounts and same strength of hydrogen peroxide is added, with vigorous stirring, and after fifteen minutes a similar product is obtained which can be further treated.

The generation of hydrogen peroxide from calcium or other alkali, or alkaline earth metal peroxide also has the effect to shift the pH of blood to become alkaline and decolourise the blood at the same time.

In the second method, instead of using a pH reversal process, a heat treatment is employed. In this whole blood is heated to at least 65°C. The temperature given in the patent as optimum is 75°C, quoting also temperatures up to 100°C. The blood is stirred slowly during this stage. The hydrogen peroxide is vigorously stirred in, at 100V strength, at levels quoted in the pH reversal method. The reaction takes about 15 minutes to complete. Then the mixture is cooled to below 50°C and any residual hydrogen peroxide is

decomposed by the addition of excess blood, about 2-6% (per weight) of whole blood. Instead of adding whole blood as excess, blood containing materials such as meat and offal can be added.

The colour of the product of both methods, is pale yellow granular and is similar to minced chicken. It can be used as meat replacement, and protein supplement. The protein however will be denatured with all this treatment and the functional properties of the product are expected to be minimal.

Some work was done at Loughborough University using the Mars patent (1983). It was found that the red cell fraction must be diluted with water prior to any hydrogen peroxide addition. He quotes a 40% (v/v) concentration of red cells to water as maximum for sufficient decolourisation. In the pH reversal methods he found inconsistent results.

3.5.4.5 Using Carboxy Methyl Cellulose (CMC)

The possibility of using an ion-exchanger such as CMC to isolate globin was first examined by Sato et al (1981). In this paper haemoglobin was prepared from bovine blood by a complicated process involving three stages of homogenization and sonication to remove all insoluble parts. Then 45 ml of 0.5% of the prepared haemoglobin was applied to a column containing 7g of CMC at acid conditions. The amounts of haemoglobin applied to 1g of CMC never exceeding 70 mg. The column was eluted with 1000 ml of 0.01N HCl having a linear gradient from 0 to 0.1 M NaCl. The elution was monitored by optical density at 280 nm and it showed a peak containing most of the globin and a second peak at 420 nm containing the haem. Sato found that the yields of globin using this method ranged from 67-80% with very small iron contents.

Hayakawa (1982) examined the functional properties of the globin prepared by the Sato method. He found the globin highly soluble in the pH regions below 6 and above 10. Also some gel forming properties were found in the globin, under well controlled heat conditions.

The problem with the Sato method is the low concentration of the haemoglobin solution, 0.5% (w/w), and the large amounts of CMC that it requires for separation of haem. This method does prepare a good globin product, but it would be difficult to scale it up to industrial scale.

A much more promising method, again using CMC is described by Autio (1983) in a patent (International patent No. 83/03198) In it the red cell fraction is haemolysed by diluting it with water. The haemolysed solution is acidified using 0.1N HCl to a pH less than 3, the optimum being around 1.5 in order to break the bonds between the globin and the haem. The acidified solution is then added to an aqueous solution of CMC at such rate that the weight ratio of CMC/globin lies in the range 1:10 up to 1:1000. The mechanism of separation probably is the formation of a covalent bond between the iron in the haem, with the carboxyl groups of CMC. The precipitated CMC-haem complex is separated from the globin by centrifugation. The centrifugation forces quoted by the patent are in the region of 8000 g for 15 minutes up to half an hour. The supernatant is a light coloured liquid containing 70-80% of the original globin and very small amounts of iron.

Autio (1984) examined the chemical and functional properties of porcine globin prepared by the above method. The globin again was found to be soluble at pH less than 6 and higher than 10. It also showed very good water binding capacity compared with soy protein, lactalbumin and glutein.

Autio also reports a great increase in viscosity of 5% globin solution occurring between pH 5.2 and 5.8 at 95°C and the formation of a firm gel.

CHAPTER 4

Materials and Methods

4.1 Whole blood collection and centrifugation

Bovine blood was collected from a slaughterhouse in Leicester (W & J. Parker Ltd). The blood was not aseptically collected. The cattle were hung and bled, the blood allowed to flow into 50 l and 25 l milk churns. In the churns, an anticoagulant, trisodium citrate of 5% w/v concentration was made up before collection. As soon as possible after collection the blood was brought to the Chemical Engineering Department in Loughborough.

The blood was first filtered through a 1 mm nylon mesh sieve to remove any residual hair and dirt as well as any small clots formed. The blood was then centrifuged at a temperature of 26° to 30°C in an Alfa Laval type BPB-204A-11 continuous disc centrifuge This centrifuge has a maximum speed of 7100 g and capacity of 150 l h^{-1}. Plasma constituted about 55% of the product and cell concentrate about 45%. This centrifuge gives a good cell-free plasma fraction and consequently a red cell concentrate 95% plasma free.

The red cell concentrate was then divided and poured into 1 l plastic containers and frozen at -25°C. The whole centrifugation process was completed within five hours after collection.

Bovine blood was also collected from the slaughterhouse of Barratts & Baird Ltd, West Bromwich. The blood was aseptically collected using a 'La Sangsue' vacuum assisted hollow knife, with metered anticoagulant solution (tri-sodium citrate). The blood was centrifuged at the point of slaughter, using either a Westfalia centrifuge, or the previously described Alfa Laval centrifuge.

Separation efficiency was increased to 60 : 40, plasma : red cell fraction. The red cell fraction was then stored in 25 l plastic containers, and transported to Loughborough either fresh or pre-frozen.

4.2 Specifications of equipment used

The decolourisation of bovine haemoglobin by the use of hydrogen peroxide, utilised a series of mixers which are specified below.

For small batches, up to 50 ml of blood, a Chiltern mixer was used, employing 3- and 4- bladed propellers. Power consumption was 100 W for this unit.

For batches up to 300 ml of blood solutions, a Morphy Richards, top mix food mixer Type 716, was used. The mixer utilised two z-blades. The motor consumed 100 W of power at 50 Hz.

Also for the hydrogen peroxide work, as well as for emulsifying, a Silverson multipurpose mixer, model L2R, with a 3/4 inch disintegrating head was used.

For batches of up to 2 litres of blood a Hobart mixer was used. This is a stationary pan mixer, in which the mixing elements move in a planetary path, visiting all parts of the mixing bowl. The capacity of the mixer was 5 litres, driven by a 100 W motor.

The decolourised product produced by the hydrogen peroxide process, was dried to a final moisture content of less than 8% in a freeze drier. The drier used was the Edwards EF4 Modulyo. Dimensions of the drier 332 x 535 x 622 (mm) Rated power consumption was 300 W. Minimum temperature under vacuum was $-55^{\circ}C \pm 5^{\circ}C$.

The vacuum ultimate in a clean dry system was 2 x 10^{-2} m bar. The samples to be dried were loaded in 500 ml conical flasks and frozen overnight. They were then attached to the drier unit. The total ice trapping capacity of the unit was 4 litres.

The decolourisation process based on the CMC and CMC-hydrogen peroxide methods, utilised a series of centrifuges.

For initial work on the Autio patent a MSE High Speed 25 super centrifuge was used. It employed an angle rotor, Catalogue Number 59584, head type with a load of 8 x 50 ml scaled tubes. Maximum revolutions per minute, using this head was 2,400g. The centrifugation process occurred at 4^{o}C. The centrifuge rotor was driven by a 110 volt, 50/60 Hz motor.

For small samples up to 100 ml a Band T. Mark IV, auto bench centrifuge with maximum speed of 5,000g at ambient temperature.

Most of the pilot plant work for the CMC process was done using an MSE "Magnum Refrigerator" centrifuge. The head type was a large, four place unit using 4 x 600 ml plastic bottles. Maximum rpm using this head was 2000 rpm (1180 g). The centrifugation was done at ambient temperature.

In the combined CMC-hydrogen peroxide process, the pilot plant work was carried out using a Sharples super centrifuge type AS16. Maximum speed 14100 g. The total bowl solids capacity was 3.5 litres. The unit was driven by a 1.5 kW motor. It is a high speed tubular bowl centrifuge with capacity in the range of 250-1000 litres per hour.

The decolourised protein solution was dried in a spray drier. Supplied by Spray Processes Limited, Bedford, type 3-8. The unit was specifically designed for drying blood and blood by-products. Optimum solution strength prior to drying is in the region of 20% (w/v). Heat requirements for the plant are in the region of 60,000 BTU/hr. and the residual moisture in the final product was quoted to be less than 5%. The heating medium was natural gas. The electrical load of the unit was 2 kW. The unit was interfaced with a Commodore Pet computer, and the whole process of drying was computer controlled.

The software used named "Emma" was developed by Dr. P. Phillipou of Spray Processes Ltd.

The ultrafiltration work was carried out using two units. Firstly an Amicon thin-channel ultrafiltration unit was used. This unit consists of a 500 ml feed reservoir pressurized by nitrogen or compressed air to a maximum of 3.5 kg cm^{-2}. The membrane, 90 mm in diameter is clamped between a porous backing plate, which carries away the permeate, and a ridged PTFE plate which forms the spiral channel over the membrane. Feed is recirculated through the 0.4 mm high channel, by a peristaltic pump of maximum capacity 1.5 l min^{-1}.

Amicon Ltd produce membranes with a range of molecular weight cut offs. In this thesis the following membranes were used: PM 10 with nominal cut off molecular weight of 10000 and PM30 with cut off of 30000. These membranes are operational at pH of 1 and a maximum pressure of 4.7 atm. The membranes were rinsed in distilled water for at least one hour prior to use.

The ultrafiltration unit employed with the CMC-hydrogen peroxide process was developed by Hurst (1981) in the Chemical Engineering Department at Loughborough University of Technology.

It was designed to utilize short lengths approximately 300 mm, of PCI (Paterson Candy International Ltd, Laverstoke Mill, Hampshire) tubular membrane. Effective membrane area of the unit is 0.00105 m^2. Maximum feed flow rate is 15.5 l min^{-1}.

The membrane used with this unit is the PCI Type T5/A. This cellulosic membrane has a 95% nominal cut off at 20000 MW. It can withstand low pH, down to 2 and the maximum operating pressure is 10 kg cm^{-2}.

All the membranes used, both from Amicon and in the PCI unit, were cleaned after each run as follows: Firstly tap water was pumped through the unit to rinse out the feed. The membranes were then washed in a 0.1% solution of the enzyme detergent 'Tergazyme', adjusted to pH 7.0-7.5 with concentrated HCl of a temperature of 30°C. The enzyme solution was recirculated over the membrane for two hours. The system was then thoroughly washed with water to drain until all visible traces of detergent were cleared. By running the ultrafiltration system under pressure with clean tap water, the membranes were flushed for a further half hour.

4.3 Analytical Methods

4.3.1 Protein Nitrogen

Protein nitrogen was determined by the semi-micro Kjeldahl method. This method is described in the manual supplied by Tecator Ltd. The Kjeltec System, incorporating a 1002 distilling unit was used for the nitrogen determination. The procedure was as follows. An aliquot of less than 1 ml of the sample to be examined, was placed in a digestion bottle. In the bottle 1g of selenium and cupric sulphate catalyst was added as well as 2-3 ml of concentrated sulphuric acid. The sample was then digested for two hours in a fume cupboard at 140°C. When the digestion was completed the samples were left to cool and 5 ml of distilled water were added.

The digestion bottles were then placed in the distilling unit and the protein nitrogen was collected in a 250 ml conical flask containing 20 ml of a 2% solution of boric acid. The final determination of nitrogen was done by titrating this solution against 0.1 N HCl and using screened methyl red as indicator.

When the amount of nitrogen was determined, in grams, the factor used to convert grams of nitrogen to protein was x 6.3.

In the titration of HCl against the boric-nitrogen solution, Pearson (1973) indicates that 1 ml of 0.1N is equivalent to 0.0014 g of protein.

Using the micro Kjeldahl method, all samples were duplicated and blank titrations were performed. Blank titrations did not exceed 0.05 ml of acid titre.

Protein was also determined by the Biuret method. The biuret reagent was prepared as follows: 1.5 g of cupric sulphate and 6 g of sodium potassium tartrate were dissolved in 500 ml of distilled water. In the solution, 300 ml of a 10% sodium hydroxide was added with swirling.

The solution was made up to 1 litre with distilled water.

One ml of the decolourised globin solution was mixed with 3 ml of the biuret reagent and left standing for 20 minutes. The absorbance of the sample was measured at 370 µm.

All the absorbance readings in this work were carried out in a Unicam SP600 Series 2 Spectrophotometer.

A total scan of the decolourised globin solution, by CMC method, was performed on a Unicam SP800 ultraviolet spectrophotometer.

4.3.2 Trace elements analysis

This analysis was carried out using an EEL 140 Atomic Absorption Spectrophotometer.

The standards for iron, sodium, magnesium and calcium were prepared from BDH Chemicals.

Sodium nitrate standard solution, for absorption spectroscopy 1000 ppm, BDH Chemicals Ltd. Poole, England.

Magnesium nitrate standard solution, for absorption spectroscopy, 1000 ppm, BDH Chemicals Ltd., Poole, England

Iron nitrate standard solution for absorption spectroscopy, 1000 ppm, BDH Chemicals, Ltd, Poole, England.

The standards were diluted in 100 ml volumetric flasks using distilled water.

4.3.3 Moisture analysis

The samples were weighed and placed on porcelain dishes. They were then placed in an oven at 100°C. The oven incorporated a fan, so that the temperature was uniform in all parts of the unit. The samples were left overnight and weighed again. After weighing they were returned to the oven for a further period of 4 hours. Then they were taken out and reweighed. If the weights were the same, constant weight had been attained. The difference in weight prior to drying and at constant weight gave the moisture of the sample.

Ash was determined as follows. The sample was weighed and placed on a porcelain dish. It was then dried to constant weight and it was then ignited using a bunsen burner. The sample was flamed for two minutes. After ignition it was reweighed and the difference between constant weight and ignition gave the ash content of the sample.

4.3.4 Salt and hydrogen peroxide determination

The estimation of salt was done by Mohr's method.

5 ml of a 10% decolourised globin powder solution were pipetted in 50 ml of deionised water. In the solution 1 ml of indicator was added. The indicator was prepared as follows: 4.2 g of potassium chromate and 0.7 g of potassium chromate were added in distilled water and diluted to 100 ml.

The powder solution with the indicator was titrated against standard 0.1 N silver nitrate until the first colour change from yellow to orange. Silver nitrate was added until the colour change was permanent. The titre is noted and 1 ml of 0.1 N silver nitrate is equivalent to 0.005845 of sodium chloride.

The hydrogen peroxide was determined by a method described by Pearson (1973). One gram of the sample was weighed into a clean dry boiling tube. 1g of powder KI and 20 ml of solvent mixture made by 2 volumes of glacial acetic acid and 1 volume of chloroform, were added. The tube was placed in boiling water for one minute. The contents of the tube were poured into a flask containing 20 ml of a 5% (w/v) potassium iodate solution, KI. The tube was washed twice with 25 ml of distilled water and titrated against 0.002 M sodium thiosulphate, 0.4964 g of thiosulphate made up to 1 litre solution with distilled water. Indicator used was made of 1g of starch diluted in 1000 ml of boiling water.

The titre was multiplied by 2 to give milli-equivalents of peroxide per kilogram of sample.

4.3.5 Amino acid analysis

The amino acid analysis of samples was carried out using a Chromakon 500 amino acid analyser connected with a Schimadzu recording data processor, type - Chromatopac C-RIB. This is a high pressure liquid chromatograph unit fully automated.

The samples were hydrolysed prior to loading by a method based on that of Moore and Stein (1963). This method however slightly underestimates the sulphur containing amino acids, and in particular cysteine.

4.3.6 Other analysis

Most of the bulk density work was performed by the particle sizing laboratory at Loughborough University of Technology. The particle size analysis was carried out by the same laboratory using a Malvern, 2200/3300 particle sizer V3.1.

The computer program was developed on a BBC microcomputer. The graphs were produced using a package called 'Tell-a-graph', property of Penn State University.

4.3.7 Lipids analysis

A colourimetric method for total lipids manufactured by Boehringer Mannheim GmbH, was employed. The principle of the test is the reaction between sulfuric and phosphoric acids and vanillin to form a pink coloured complex. This colour is measured at 530 nm. The calculation of total lipids is as follows:-

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of Standard}} \times 1000 = \text{lipids mg/100 ml.}$$

Further information Boehringer Catalogue number 124303 for 40-115 tests.

4.4 The procedure for the functionality tests employed

The following properties regarding the functionality of the protein powders were assessed.

- Gelation
- Emulsification (capacity and stabilisation)
- Foaming
- Fat absorbance
- Solubility

The methods are summarised below. For a fuller account of the tests see Hill (1986), Doctorate thesis, describes the development of these tests in Loughborough University of Technology.

For gelation 10% and 9% (w/v) solutions of dried globin were prepared. The solutions were kept in a water bath for half an hour at 80°C. They were then taken out of the water bath and left undisturbed for 18-26 hours before measurement. If the samples formed gels they were visually examined and also tested for solidity by inverting the bottle and observing movement of gel. The gel was tested using a Seta Penetrometer.

The bottle containing the gel was placed on the penetrometer so that the cone and plunger could enter the gel without fouling the bottle. The height of the cone was adjusted so it had just penetrated the gel. The cone and plunger were then released for 8 seconds. The depth, in mm, the cone had penetrated was recorded. If the cone reached the bottom of the gel the reading was recorded as 400. If no gel was produced an arbitrary value of 600 was assigned to the product.

The procedure for the estimation of an emulsion is as follows. A 2% (w/v) solution of globin powder was prepared. To a 25 ml aliquot of this solution 80 ml of corn oil was added. The oil and protein solution were mixed together for 2 minutes using a Silverson Mixer model L2R with 3/4 inch disintegrating head. The emulsion if produced, is seen clearly in the solution beaker. The stability of the emulsion was checked as follows. Two 10g samples of the emulsion were taken and put into two glass universals. The universals were then placed into a water bath at 80°C for one hour.

The universals were then left on the bench at ambient temperature, one for 24 hours, the other for 14 days. After the required time the samples were centrifuged at 1400g for 15 minutes. The weight of the free oil was noted and divided by the original weight of oil in sample, and the results expressed as a percentage oil released from the emulsion.

The capacity of the emulsion was measured using an LKB harloperpex peristaltic pump. A 25g sample of the emulsion was placed in a 100 ml plastic beaker. The pump was set up to deliver oil at the mixing head of the Silverson at a rate of 5 ml per minute. The emulsion was mixed using the Silverson as the oil was delivered and a visual examination of the emulsion was made. When the nature of the emulsion visually changed, by the decreased homogeniety and loss of viscosity, the pump was stopped. The oil feed container was weighed and the weight of oil required to break the 25g of emulsion was calculated and by extrapolation the amount of oil required to break 1g of emulsion then calculated.

Foaming was determined as follows. A 2% (w/v) solution of spray dried globin solution was prepared. A 50 ml aliquot of this solution was mixed using the Silverson for 2 minutes. The sample was then left undisturbed on the bench inside a plastic volumetric cylinder. The total volume of the sample was measured as well as

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the volume of the foam. Readings of foam volume were taken at 5, 25 and 60 minutes after the start of mixing. The percentage foam left after 55 minutes was used in evaluating data.

The fat absorbance procedure is summarised next. Silica glass tubes of internal diameter 14 mm and of known capacity were used. 2g of dried globin was added to 20 ml of corn oil of known specific gravity. The powder and oil were mixed using the Silverson for one minute. After homogenizing 10 ml aliquots of the mixture were transferred into the silica glass tubes and left in there for 10-15 minutes. The tubes were then centrifuged at 1400 g for five minutes. The height in mm of each layer was noted. By calculating the amount of bound oil in ml and the actual weight of powder in the tube, the amount of oil absorbed by 1g of powder was estimated. Also the percentage of the total volume each layer represented was noted.

Solubility was determined by calculating the amount of unsoluble material, as follows. A 10% (w/v) of dried globin was prepared. 20 ml aliquots were then centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 20ml distilled water. The sample was then centrifuged again at the same speed and time. The supernatant was again discarded and the residual pellet was placed on an aluminium dish and dried to constant temperature. The percentage unsoluble material was calculated as the weight of the final pellet multiplied by five.

4.5 Description of the pilot scale rig for decolourising red cell fraction

A rig was constructed for pilot plant work. The red cell fraction was decolourised using the CMC and CMC-hydrogen peroxide methods. Figure 4.1 is the flowsheet of the rig.

4.5.1 Equipment specification

T1 Red cell fraction preparation tank. Capacity 68 litres
Material of construction PVC

T2 Reaction vessel Capacity 400 litres. Skerman Pan
Material of construction Stainless Steel

T3 Decolourisation tanks. Three milk churns, capacity 12 litres each, made of stainless steel were used to collect the decolourised product.

P1 APV centrifugal pump. Power 210 W

P2 Alfa-Laval peristaltic pump Power consumption 370 W
Maximum speed 3000 rpm. Controlled by Rotary Regavolt

C1 Sharples super centrifuge Type M-4-P-240 T43
Maximum Bowl Speed 17000g.

4.5.2 Construction of the pilot plant

The equipment for the rig was installed in the main pilot plant area of the Department of Chemical Engineering. The following steps were undertaken, for the construction of the rig.

The reaction vessel was thoroughly cleaned. An 'on-off' valve was fitted at the bottom of the reaction vessel. The valve was checked for leaks by partially filling the vessel with water, with the valve shut. No leaks were observed.

The reaction vessel was calibrated every 10 litres with the zero set at 30 litres. The calibration points were marked by calibration tape and marker pen.

Flowsheet of the decolourisation rig

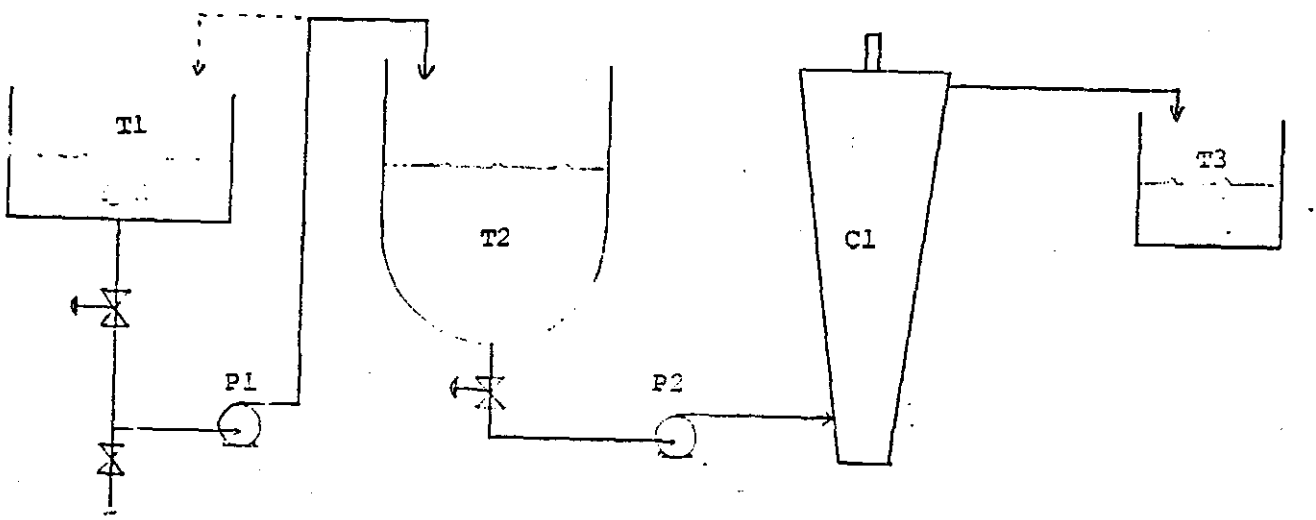


Figure 4.1

A PVC pipe was connected between the valve at the base of the reaction tank and the peristaltic pump.

A voltage regulator was fitted to the peristaltic pump so that the flowrate to the centrifuge could be controlled. The results are shown in Table 4.1. The fluid used was tap water at 15°C.

Calibration of voltage regulator

Input of voltage %	Time for 5 litres of water to flow (average of 6 runs) seconds	Volumetric flowrate $l\ s^{-1}$
55	88.25	0.0567
57.5	78.75	0.0635
60	66.00	0.0758
65	51.50	0.0971

Table 4.1

The red cell fraction preparation tank was cleaned and positioned next to the reaction vessel. Two 'on-off' valves were fitted. One valve leading to the drain, the other via a PVC pipe, leading to the centrifugal pump and then into the reaction vessel. This tank was calibrated for every 5 litres using a marker pen.

The flowrate generated by P1 centrifugal pump was measured. Results are shown in Table 4.2

Flowrate delivered by centrifugal pump

Volume of water delivered litres	Time taken secs	Volumetric flowrate $l\ s^{-1}$
10	10.7	0.93
10	10.3	0.97
10	10.0	1.00
10	10.3	0.97
10	9.9	1.01

Table 4.2

Average volumetric flowrate delivered by the pump is 0.98 ($l\ s^{-1}$).

The tank and vessel and all valves and pipes were checked for leaks, by pumping water through the system.

The peristaltic pump was connected to the inlet of the centrifuge, via a PVC pipe.

A photograph of the plant is shown in Figure 4.2.

Decolourisation Plant

Figure 4.2

4.6 Costing of a process

The economic appraisal of the three most promising decolourisation processes, namely hydrogen peroxide, CMC and the combined CMC-hydrogen peroxide method, was performed following a pattern first developed by Holland (1976) and adapted by Norwood and Sakellariou (1982).

A breakdown of the elements involved in the costing of a project is shown in figure 4.3.

The basic principle underlying any investment decision is the maximization of profit in relation to the investment employed. Hence the higher the profit on the capital spent on the process, the more attractive this process is.

Fixed capital costs include equipment costs, land purchase, site development, building costs, infrastructure costs, auxilliary buildings and utilities plus commissioning costs. A complete list of items for fixed capital cost estimates is given by Perry (1971).

The working capital, or "liquid investment", can be defined as the funds in addition to the fixed capital, which the company must contribute for the project. The main element of the working capital are the inventories.

Cost of working capital = (cost of unsold finished goods - cost of raw materials + goods in production).

The stocks of anticoagulants and cleaning agents are considered under the working capital.

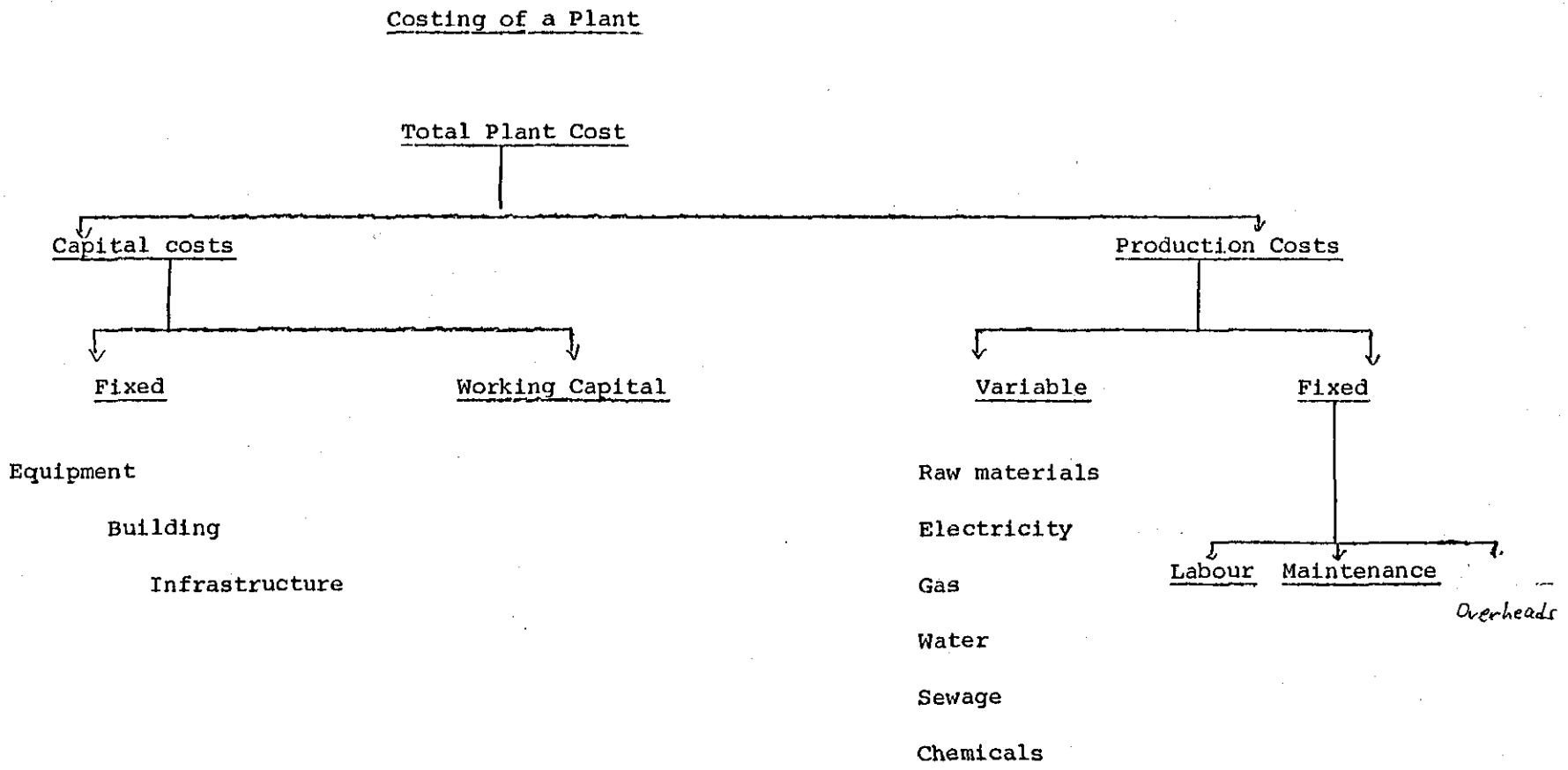


Figure 4.3 (Sakellariou 1982)

The variable production costs include raw materials, chemicals etc. All these costs are related to the amount, or volume, of product manufactured. Hence they increase with increasing production and decrease with less production.

The non variable costs are the ones not related to the amount of product manufactured. In today's automated plants, labour costs come under this heading, together with overheads i.e. administrative expenses, and maintenance. Maintenance and overheads are separately calculated as a percentage of the fixed capital costs.

The fixed capital costs depreciate with time. Depreciation is the yearly allocation of installed costs of fixed capital assets over the estimated useful life of plant. In this thesis a straight line depreciation and a ten year useful life of a plant is assumed, hence the annual depreciation is calculated as ten per cent of fixed capital costs.

At the end of its operating life, the plant and equipment can be sold, the money received being called the salvage value. In this thesis, the salvage value of the plant is assumed zero.

The criteria which are used to evaluate the profitability of a process fall under two groups.

A. Simple Interest Criteria

These are used when the annual profits are expected to be constant. The most common is the Return on Investment (ROI) calculated as:

$$\text{ROI} = \frac{\text{annual profit}}{\text{original capital cost}}$$

The annual profits are calculated from the difference of the income of sales minus the operating costs minus the depreciation of capital.

The second criterion used in this thesis is the Payback Time, T. In essence the payback time is the inverse of ROI and hence:

$$T = \frac{1}{ROI}$$

T is an indication of the time, in years, it will take for the project to pay back all the capital spent.

B. Compound Interest Criteria

These are used when the annual profits are expected to fluctuate. The main one is the Net Present Value (NPV). The NPV shows how much money the project is worth at the present time, after all capital and interest on it has been paid.

NPV is calculated from the following formula:

$$NPV = \sum_{o}^{n} P\,(1+i)^{-n} - C$$

where P = annual profit
C = initial capital investment
i = minimum rate of return for the project
n = project life (in years)

The annual profits, P, are the income from sales minus the operating costs. Depreciation is not taken into account. The minimum rate of return is decided by the manager and can vary from 10-15% in low risk ventures, to as much as 30% for high risk ventures.

Another useful criterion is the Discounted Cash Flow Rate of Return, (DCFRR) and is this value of i, i.e. the rate of return, which makes the NPV equal to zero i.e.

$$C = \sum_{o}^{n} P\ (1+i)^{-n}$$

The DCFRR indicates the rate of return the project produces so direct comparisons can be drawn with rates other institutes such as banks can give, if the capital was invested with them.

Other parameters used in this study are the terms capacity and capacity utilization.

The capacity of the blood decolourising plant is defined as the maximum amount of animals that can be processed per hour, and hence the maximum amount of blood which can be decolourised per hour.

The term capacity utilization, is defined as the sales achieved over the capacity of the plant or:

$$\mu = \frac{S}{K}$$

where μ = capacity utilization
S = sales volume
K = capacity

also $o < \mu < 1$

If $\mu = 1$ the plant is fully utilised.

Taxation and inflation indices are difficult to predict, so in this work they have been incorporated in the rate of return, i.

CHAPTER 5

Pepsin Decolourisation Method

Red cells, were firstly haemolysed with water. The resulting liquid was then hydrolysed with dilute, 1M hydrochloric acid, HCl. This would achieve the split between haem and globin, and because the acid is dilute it would not affect the protein by denaturing it. The second stage of the method is to use an enzyme to attack the globin in such a way as to help the release of the haem from the pockets existing at the surface of the molecule. Finally, the resulting haem-globin mixture can be separated by ultrafiltration thus utilising the molecular weight difference between haem, MW600, and globin MW18000. This ultrafiltration step will also affect the concentration of the globin solution.

Pepsin was chosen because it works optimally at low pH (Lehninger (1975). Pepsin breaks up only amino acids containing aromatic groups i.e. histidine, phenylalanine, tyrosine and tryptophan. Thus there is a clear advantage to use pepsin to help HCl to break up the histidine-haem bond. Other sites of pepsin attack are shown in Fig. 5.1.

The final product, i.e. globin must be as intact as possible to minimise loss of its functional properties.

Firstly the degree of haemoglobin digestion by pepsin with time was quantified.

Secondly different combinations of pepsin concentrations, reaction times, pH range and membrane types were tried to test their ability to isolate haem without denaturing the globin molecules.

Sites of Enzyme Attack

Sites marked in →

N-terminal ends

	α	β
	Val	Val
	→ His	
	Leu	Leu
	Ser	Thr
	Pro	Pro
	Ala	Glu
	Asp	Glu
	Lys	Lys
	Thr	Ser
	Asn	Ala
10	Val	Val
	Lys	Thr
	Ala	Ala
	Ala	Leu
	Trp	Trp
	Gly	Gly
	Lys	Lys
	Val	Val
	Gly	Asn
	Ala	
→ 20	His	
	Ala	Val
	Gly	Asp
	Glu	Glu
→	Tyr	Val
	Gly	Gly
	Ala	Gly
	Glu	Glu
	Ala	Ala
	Leu	Leu
30	Glu	Gly
	Arg	Arg
	Met	Leu
→	Phe	Leu
	Leu	Val
	Ser	Val
	Phe	→ Tyr
	Pro	Pro
	Thr	Trp
	Thr	Thr
40	Lys	Gln

	α	β
	Thr	Arg
	→ Tyr	→ Phe
	→ Phe	→ Phe
	Pro	Glu
	→ His	Ser
	→ Phe	→ Phe
		Gly
	Asp	Asp
	Leu	Leu
	Ser	Ser
60	→ His	Thr
	Gly	Pro
	Ser	Asp
	Ala	Ala
		Val
		Met
		Gly
		Asn
		Pro
	Gln	Lys
	Val	Val
	Lys	Lys
	Gly	Ala
	→ His	→ His
	Gly	Gly
80	Lys	Lys
	Lys	Lys
	Val	Val
	Ala	Leu
	Asp	Gly
	Ala	Ala
	Leu	→ Phe
	Thr	Ser
	Asn	Asp
	Ala	Gly
90	Val	Leu
	Ala	Ala
	→ His	→ His
	Val	Leu
	Asp	Asp
100	Asp	Asn

	α	β
	Met	Leu
	Pro	Lys
	Asn	Gly
	Ala	Thr
110	Leu	→ Phe
	Ser	Ala
	Ala	Thr
	Leu	Leu
	Ser	Ser
	Asp	Glu
	Leu	Leu
	→ His	→ His
	Ala	Cys
	→ His	Asp
120	Lys	Lys
	Leu	Leu
	Arg	→ His
	Val	Val
	Asp	Asp
	Pro	Pro
	Val	Glu
	Asn	Asn
	→ Phe	→ Phe
	Lys	Arg
130	Leu	Leu
	Leu	Leu
	Ser	Gly
	→ His	Asn
	Cys	Val
	Leu	Leu
	Leu	Val
	Val	Cys
	Thr	Val
	Leu	Leu
140	Ala	Ala
	Ala	→ His
	→ His	→ His
	Leu	→ Phe
	Pro	Gly
	Ala	Lys
146	Glu	Glu

	α	β
→	Phe	→ Phe
	Thr	Thr
	Pro	Pro
120	Ala	Pro
	Val	Val
→	His	Gln
	Ala	Ala
	Ser	Ala
	Leu	→ Tyr
	Asp	Gln
	Lys	Lys
→	Phe	Val
	Leu	Val
130	Ala	Ala
	Ser	Gly
	Val	Val
	Ser	Ala
	Thr	Asp
	Val	Ala
	Leu	Leu
	Thr	Ala
	Ser	→ His
	Lys	Lys
140 →	Tyr	→ Tyr
	Arg	→ His

C-terminal ends

Figure 5.1

5.1 Identification of reaction conditions between pepsin and Haemoglobin

Anson (1938) gave some data on the pepsin-haemoglobin reaction. Pepsin is most active at pH 1.82 and at 37°C. Anson, for a 20% red cell fraction solution, suggested a reaction time of 15 minutes, and a ratio of pepsin to haemoglobin of 1:100 by weight.

A 20% (w/v) solution of RCF in water was made up and acidified with 1N HCl to pH 2.1. A sample of this solution weighing 100g was reacted with 1.52g of a 0.05% (w/w) pepsin solution for 15 minutes at 37°C. (ratio 1:100). The reaction was stopped by adding 200g of a 20% (w/w) solution of trichloroacetic acid (TCA) This had the effect of precipitating out of solution all long chain proteins. The precipitate was removed by centrifuging at 3000 g for 4 minutes. The absorbance of the supernatant was recorded at 280 nm. This sample gave a reading of 1.29 units while a sample which had not been treated with pepsin gave a reading of 0.87 units. Distilled water was used to give zero absorbance units.

The greater absorbance value of the treated sample indicated that the long chains of haemoglobin have been broken up and some of these fragments were not precipitated by the TCA.

The next step was to find the effect of time on the extent of reaction. The RCF solution, pepsin and TCA were prepared as above. 40g of the TCA solution was weighed into each of fourteen beakers. The reaction was started by adding 4.75g of the pepsin solution to 360g protein solution. A 20g sample was taken every 2 minutes initially and added to a heater containing the TCA to stop the reaction. The sample was then centrifuged at 3000 g. for 4 minutes and the absorbance at 280 nm recorded in Table 5.1

Absorbance readings for various reaction times of haemoglobin pepsin

Reaction time (mins)	Absorbance units (at 280 nm)
0	0.640
2	0.880
4	1.085
6	1.045
8	1.120
10	1.090
12	1.160
14	1.295
15	1.420
30	1.480
45	1.580
60	1.720
90	1.690
108	1.640

Table 5.1

A graph of reaction time against absorbance units is shown in Fig. 5.2 This curve indicates that the reaction is 95% complete after 60 minutes.

The next series of experiments was carried out to determine if any colour could be separated out.

An initial breakdown of 50% was assumed. This takes place after about 10 minutes, see Fig. 5.2. The reaction was stopped by taking the pH of the RCF-pepsin solution to 7, thus inactivating the pepsin (Lehninger 197 5).

Absorbance for reaction times of haemoglobin–pepsin

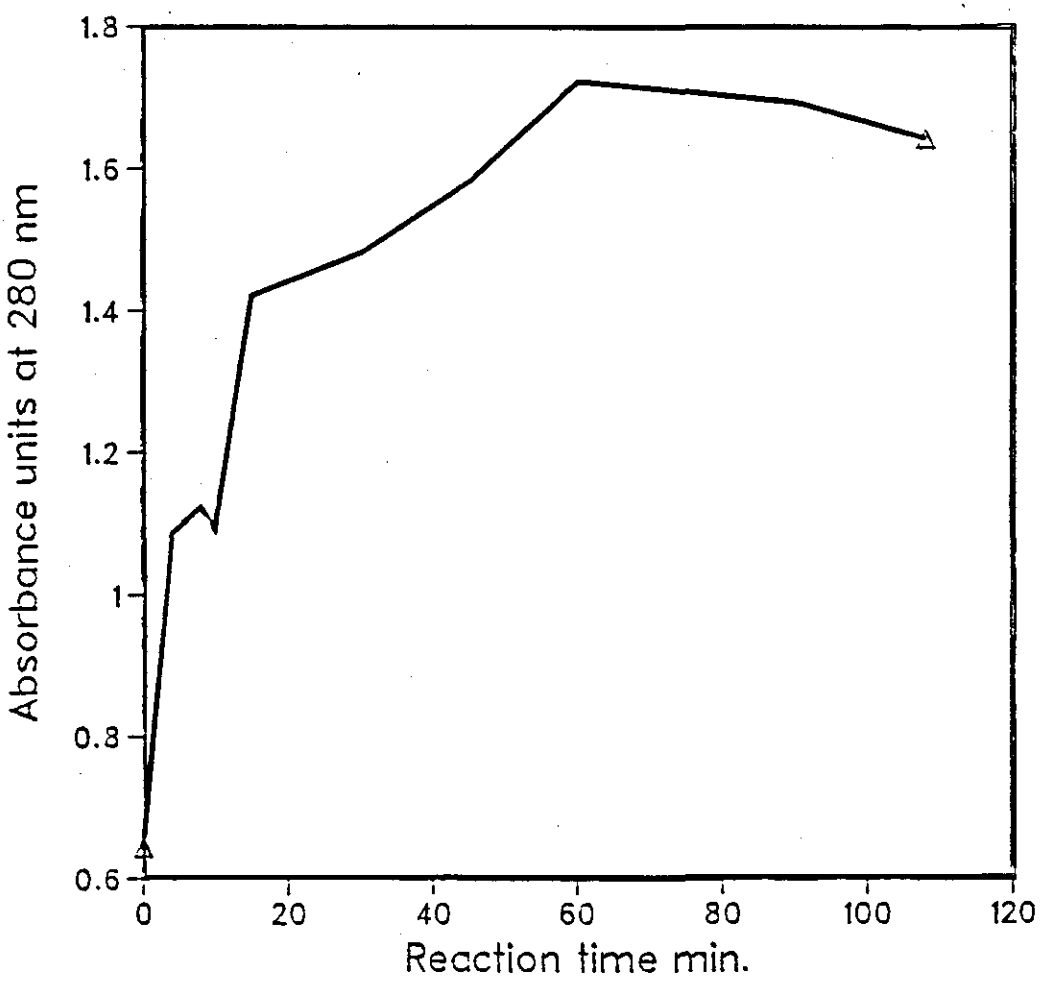


Figure 5.2

180g of RCF (20%) was prepared as before and reacted with 2.38g of pepsin solution (0.05%) for 10 minutes at pH 2 and temperature of 37°C. The reaction was stopped by altering the pH up to 7 using 1M NaOH. The resulting mixture included a thick dark precipitate. This dark red solid prevented the ultrafiltration of the solution. It was then filtered out using a grade 4 Whatmans filter paper and 130g of a light red filtrate recovered. The precipitate was analysed using the Kjeltec unit and found to contain 85% protein. The filtrate was centrifuged at 3000 g for 4 minutes and some particles were removed. However the supernatant was still red. It was evident that during the altering of pH from 2 to 7 most of the protein was precipitated out without the haem being separated from the globin.

It was then attempted to stop the haemoglobin-pepsin reaction by bringing the pH to 9.0. This would have the effect of stopping the enzyme hydrolysis and redissolving any protein which might precipitate at acidic pH.

Again a 20% RCF solution was treated with pepsin as before. The reaction was stopped by bringing the pH up to 8. with 1N NaOH.

The solution was then divided into five beakers and small amounts of 1N NaOH were added to take the pH up to 8.3, 8.5, 8.7, 9.0, 9.3 respectively. Aliquots were taken from the beakers and centrifuged at 3000g for 4 minutes. It was found that no precipitate was formed at pH of 8.7 and 8.9. By the addition of NaOH however the colour of the RCF-pepsin solution changed from light red to dark red with a green tinge.

Lehninger (1975), also reports that pepsin can be inactivated at temperatures higher than 50°C. The possibility of using heat to inactivate the pepsin instead of altering the pH was therefore investigated.

RCF solution and pepsin were prepared as above and allowed to react for 10 minutes at pH 2.0 and 37°C. The reaction was stopped by immersing the reaction flask in a water bath held at 55°C. When the RCF-pepsin solution reached 55°C it was held for a further 5 minutes. An aliquot of 20g of this solution was added to 40g of TCA.

The sample was centrifuged and the absorbance of the supernatant at 280 nm was noted. The absorbance reading was 1.42 representing a reaction of about 70%.

The rest of the solution was left overnight and a sample of 20g was again taken from it. It was added to 90g of TCA, centrifuged, and the absorbance was recorded. The value of the absorbance had risen to 1.67 units, corresponding to an extent of reaction of about 100%. This indicated that there is still residual enzymic activity in the solution. The heat treatment was not sufficient to inactivate the pepsin. However if higher temperatures were employed coupled with longer residence times, the haemoglobin will denature giving a non-functional product. Thus the temperature treatment was stopped since it was (a) unreliable in inactivating pepsin and (b) likely to affect protein functionality.

5.2 Estimation of Haem Concentration in the Permeate

The RCF solutions after acid hydrolysis and pepsin treatment were subjected to UF using an Amicon unit. In theory the permeate should contain the haem. In order to quickly estimate the concentration, in mg/l, of haem in the permeate the following method was developed.

A RCF solution was prepared 20% (w/w). RCF contains 33% (w/w) haemoglobin, the haemoglobin containing 6% (w/w) haem (Ranken, 1977). Therefore the haem concentration, in the 20% RCF solution is 3.96×10^{-3} per gramme of the RCF solution. Eleven solutions of RCF at different dilutions were made, as recorded in Table 5.2 . The dilution was calculated as parts of water added to one part of a 20% (w/w) solution of RCF. The haem concentration for each dilution was thus derived and a calibration graph was drawn, fig. 5.3. The absorbance was recorded at 540 nm. The permeate samples can be measured for absorbance directly or diluted if off scale, by adding 20 parts of water to 1 part of sample. For samples which were diluted, the haem concentration taken from the graph was multiplied by 20, to account for the dilution.

Determination of Haem Concentration

Dilution	Absorbance units (at 540 nm)	Haem Concentration (mg/litre)
1600	0.030	2.5
800	0.062	4.9
400	0.128	9.9
200	0.251	19.7
100	0.480	39.2
60	0.705	64.2
30	1.290	127.7
20	1.995	188.6
10	Off scale	-
3	Off scale	-
1	Off scale	-

Table 5.2

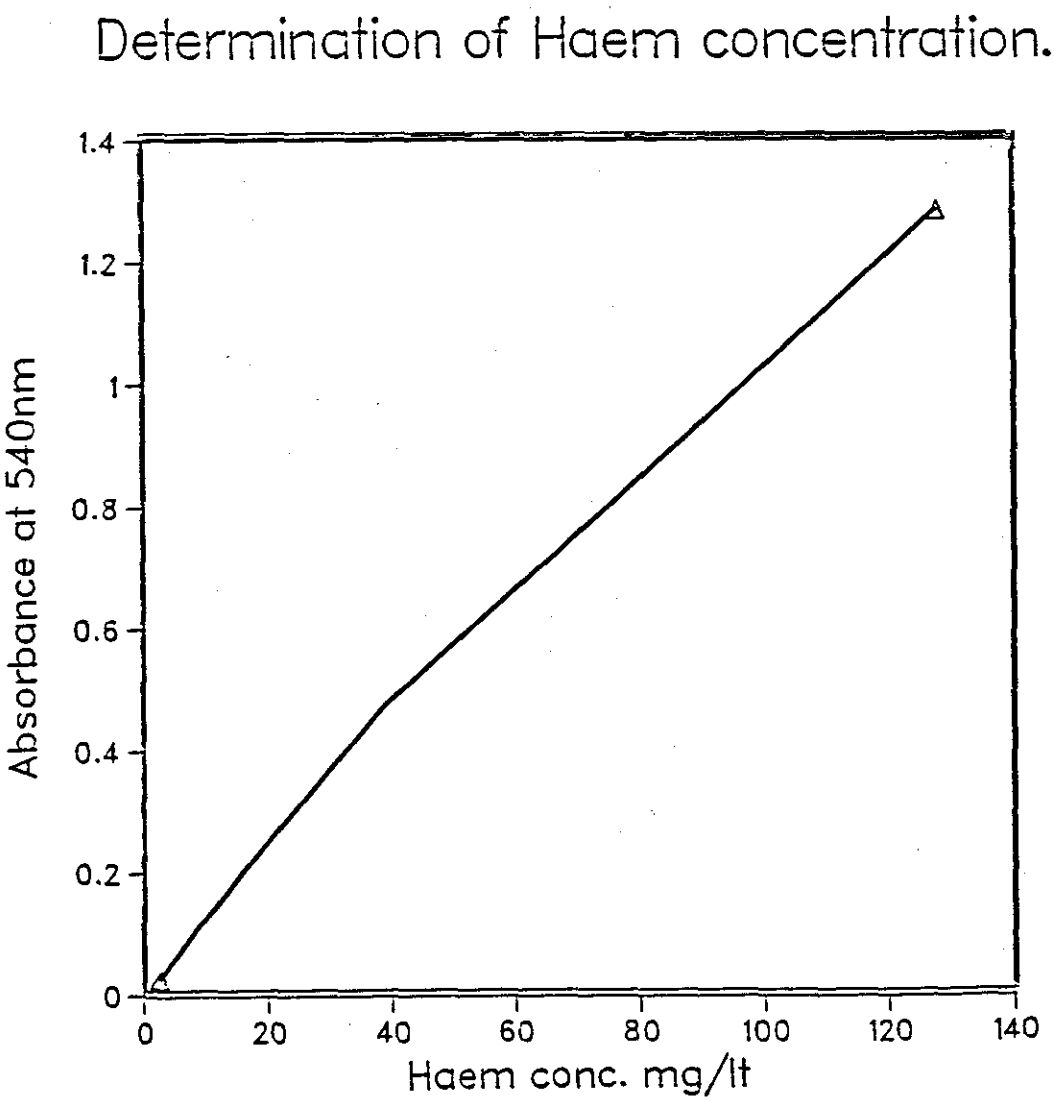

Determination of Haem concentration.
Absorbance at 540nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
Haem conc. mg/lt

Figure 5.3

5.3 Attempts to decolourise the red cell fraction of bovine blood

100g of RCF were added to 400g of distilled water in a one litre beaker to make up a 20% solution. 1N HCl was added to bring the pH to 1.9. The acidified solution was heated to 37°C. by immersion in a water bath. A 5% (w/w) pepsin solution was prepared and 5.4g of it was added to the acidified RCF solution. The mixture was left to react for 10 min. at 37°C. and the reaction was stopped by the addition of 1N NaOH until the pH of the mixture was 8.9. A 100 ml sample of this mixture was centrifuged at 3000 g for 4 minutes and no traces of solids were found. The rest of the mixture was loaded in the Amicon ultrafiltration unit.

The Amicon contained a UM 10 membrane. This has a nominal cutoff point of MW 10,000. It should allow haem to pass but not any long chain protein molecules.

The mixture was circulated in the Amicon unit for 2 hours. Conditions of the ultrafiltration were:

Pressure 25 psi, temperature 40°C, pump speed setting 6. Samples of permeate were taken at regular time intervals and a sample of the retentate was taken at the start of the process and after 2 hours. The results are summarised in Table 5.3.

Permeate flow rate was 0.2 ml/min. Total permeate volume collected was 72 ml.

Ultrafiltration data I

Sample number	Time period the sample was taken Start (min)	Finish	Protein conc. (%)	Haem concentration (mg/l)
0	0		5.65 R	2450 R
1	0	18	*	51 P
2	18	36	*	117 P
3	36	56	*	108 P
4	56	83	5.83 R	114 P
5	84	118	*	107 P
6	120		5.83 R	2583 R
			1.39 P	110 P

Table 5.3

* Protein determination was not carried out

P = sample taken from permeate

R = sample taken from retentate

As can be seen from the above table, some colouration was removed (haem concentration in permeate increased) but this was accompanied by some water. This resulted in an overall concentration of colour from a haem concentration of 2450 mg/l, to 2583 mg/l.

The retentate was concentrated from a protein content of 5.65% to 5.83% after 2 hours. The protein content of the permeate, after 2 hours operation, was 1.39% indicating that some small protein chains were getting through the membrane.

Total retentate volume 433 ml.

It seemed that the haem was not sufficiently separated from the globin to pass through the membrane without being obstructed. The retentate from the above experiment was diluted with an equal amount of distilled water and reloaded into the Amicon under the same operating conditions, in an attempt to wash out the haem. After 2 hours of ultrafiltration the haem concentration in the permeate was 23 mg/l. The retentate was further diluted with equal amount of water and reloaded in the Amicon. It was left running overnight in the Amicon. The permeate collected was of slight yellow colour and the retentate contained all the red colour. It appeared that the enzymic breakdown was not sufficient to release the haem. As a last attempt a further breakdown of the rententate RCF solution was tried.

A sample of 300 ml from the retentate was further treated with 3g of a 5% pepsin solution, at 37^{o}C and pH 1.9 for 10 minutes The pH was increased to 8.9 to stop the reaction using 1N NaOH and the mixture was reloaded in the Amicon. Operating conditions were the same as before. The ultrafiltration process lasted 3 hours. The haem concentration in the permeate rose to 1.8 mg/l in the first hour, and to 2.1 mg/l after the next two hours. However the retentate containing the globin solution remained red without any change.

A fresh sample of RCF was prepared. 180g of 20% RCF solution were acidified to pH 1.9 by the addition of 26 ml of 1N HCl. 10g of a 5% pepsin solution was added to the acidified RCF solution at 37^{o}C. and left to react for 60 minutes. The reaction was stopped by adding 31.2 ml of 1N NaOH to get the pH of the mixture to 8.9. This solution was now loaded in the Amicon, operating conditions 40^{o}C, 30 psi, pump speed 6. Samples of the permeate were taken every 5 minutes and their haem concentration monitored, as recorded in Table 5.4 below.

Ultrafiltration data II

Sample Number	Time period for sample taken		Haem concentration (mg/lt)
0	0		1979 (in retentate)
1	0	5 min	63 (in permeate)
2	5	10	294
3	10	15	273
4	15	20	231
5	20	30	168
6	30	40	147
7	40 min		1935 (in retentate)

Table 5.4

Again some colouration was removed but the retentate was not decolourised at all.

5.4 Conclusion

Acid hydrolysis followed by enzymic breakdown proved to be unsuccessful in decolourising bovine blood. Several reasons can be speculated for this.

The haem and globin were separated but attached to the haem are large portions of the protein molecule which hinder the ultrafiltration.

The above might be due to the denaturing of the protein, from the acid hydrolysis and the alkali treatment, and this would lead to a break up of the protein in a distribution of chain lengths.

Some of these fragments will pass through the membrane but most of them will not.

All the possibilities however were examined in the experimentation without success. This process of decolourisation was therefore abandoned.

CHAPTER 6

Hydrogen peroxide decolourisation process

6.1 Theory of reaction

The starting point for the development of this process was the Mars Patent, described in 3.5.4.4. This method does not depend on the separation of the haem group from the haemoglobin molecule. The mechanism of decolourisation using hydrogen peroxide has not yet been investigated. A probable mechanism will now be described.

As was already mentioned, haem contains the porphyrin ring system, see fig.3.18. The porphyrin ring contains four pyrrole nuclei connected by four C-H bridges and is highly aromatic in character. Haemoglobin shows absorption bands at about 430 nm and 560 nm (Rao 1961). When haemoglobin absorbs oxygen from the air, bright red oxyhaemoglobin occurs with absorption maximums at 415, 538 and 578 nm. Another spectrum shift occurs for carboxyhaemoglobin, absorption maxima 540 and 570 nm.

Hydrogen peroxide is a strong oxidising agent. The reaction is as follows:

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2$$

It is highly exothermic and proceeds rapidly at high temperatures.

According to Vogel (1959) rapid oxidation of ferrous to ferric compounds is effected with hydrogen peroxide. It is assumed therefore that hydrogen peroxide attacks the pyrrole ring of the haem oxidising iron (II) to iron (III). As a result of iron (III) being introduced haemoglobin precipitates out of solution as yellow solid with the simultaneous formation of water. There is the possibility of charge transfer occurring which results in a shift in the absorbance of the haemoglobin molecule showing a maximum in the yellow band of the spectrum.

This is a simple model of the reaction because to understand and analyse the mechanism of haem-protein catalyzed oxidation reactions, the knowledge of the nature of the reactive species, i.e. various forms of the haem-oxygen complexes is necessary.

Some recent work by Shiga and Imonizumi (1975) suggests that except iron being oxidised, a whole series of oxidation reactions occurs in the haemoglobin. They examined the reaction between horse radish peroxidase and methaemoglobin. They concluded that there is a one-electron oxidation reaction, in the H_2O_2 protein system. However they postulated the existence of a reactive intermediate complex which further degenerates to methaemoglobin. Also there is the oxidation of phenols, and napthols which takes place outside of the haem pocket of methaemoglobins, to consider.

Clearly the mechanism of hydrogen peroxide - haemoglobin needs to be extensively investigated before a definitive theory can emerge.

6.2 Process description

A flow diagram of this process is shown in Fig. 6.1. A solution of RCF with water is firstly prepared. This will achieve haemolysis and the proteins of the cells will go into solution. The next step is to heat the solution to a temperature above 65°C.

At the temperature selected, T_R, the solution is held for a minimum time of five minutes under slow stirring. This heat treatment is necessary so to deactivate the enzyme catalase present in the RCF. If the enzyme is not deactivated it will affect the hydrogen peroxide-haemoglobin reaction.

Flow diagram of the H_2O_2 decolourisation process

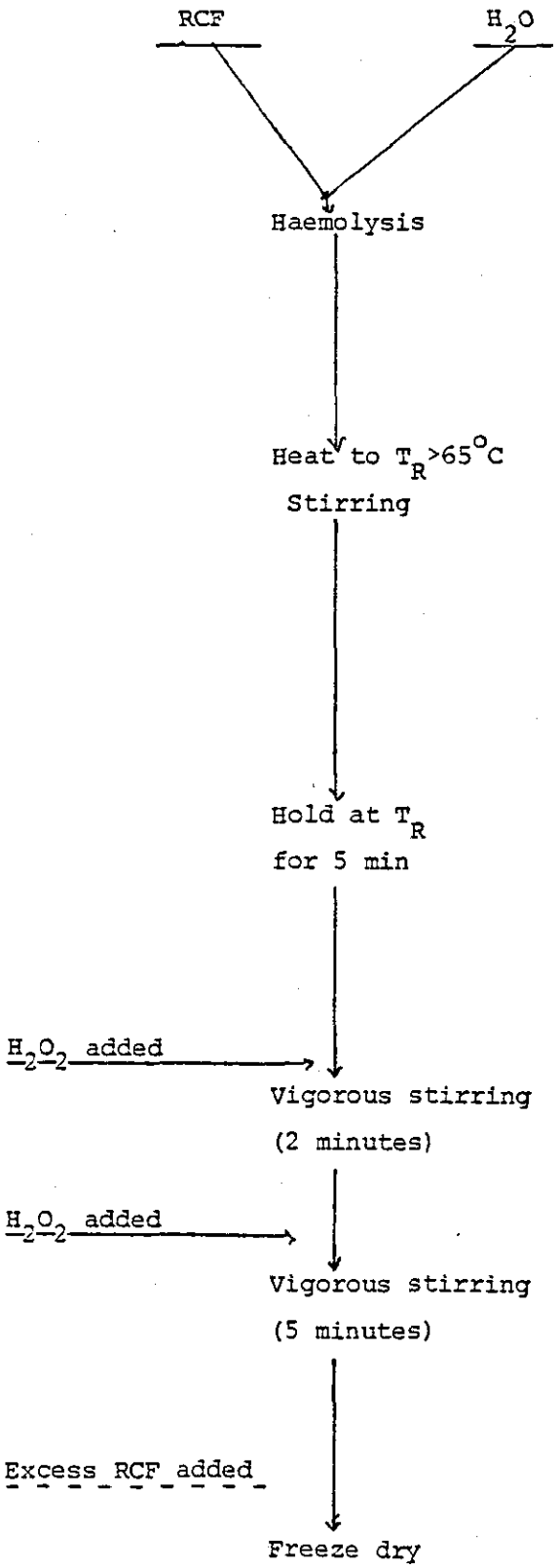


Figure 6.1

After the enzyme is deactivated the solution is stirred vigorously and half the quantity of hydrogen peroxide is added. The hydrogen peroxide is added at the top of the vortex created by the mixer. After about two minutes the rest of the hydrogen peroxide is added and the solution is left to react, under continuous stirring, for a further five minutes.

This completes the decolourisation process. There are two ways of further processing the product.

The first is to add excess RCF whilst stirring, for 'mopping up' any residual hydrogen peroxide still present in the product. The catalase in the RCF will ensure a product free of hydrogen peroxide. As already mentioned, hydrogen peroxide is toxic and Wesdorp (1978) has speculated that formation of growth depressants in products treated with this chemical may occur. After the addition of RCF the product is spray or freeze dried.

The second way of dealing with the decolourised product is to bypass the excess RCF addition step. The product is freeze dried and the volatile H_2O_2 is released during the drying procedure. Furthermore when the product is used as an additive in meatbased products, the catalase present in meat will also neutralise any residual H_2O_2. This way the product will obviously have a lower residual colour than if an excess of RCF is added.

The variables examined in this process are:

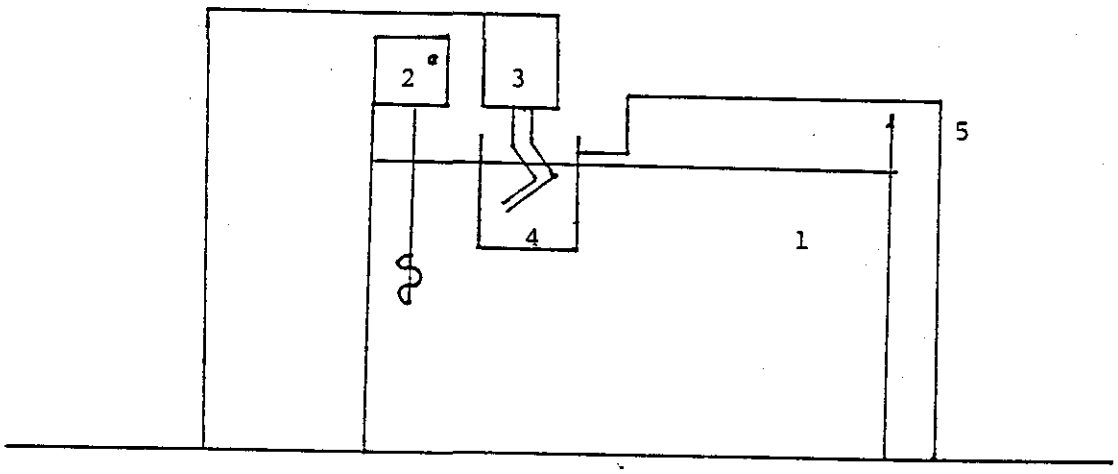
1. The optimum RCF : H_2O dilution
2. The effect and optimum addition of H_2O_2
3. The effect of mixing.

Temperature variations were not tried. The temperature was kept at the lowest possible value, i.e. 65-69^{o}C, in order to avoid heat coagulation of haemoglobin.

A schematic representation of the apparatus used is shown in Figure 6.2.

The main elements are a constant temperature water bath, a mixing device and a beaker in which the reaction takes place.

Schematic representation of H_2O_2 decolourisation plant



1. Water bath
2. Temperature controller and heating element
3. Food mixer
4. Solution to be decolourised
5. Stand

Figure 6.2

6.3 Experimentation

6.3.1 Hydrogen peroxide and excess RCF addition

The mixing device shown in Fig. 6.2 of this part of the work comprised an open three bladed propeller. The purpose of the first series of experiments was to establish the relationship between the RCF dilution and the amount of hydrogen peroxide needed to decolourise the RCF solutions.

Three solutions of RCF : water were prepared. These were

a) 20% (w/v) (RCF : water); 100 ml
b) 30% (w/v) (RCF : water); 100 ml
c) 40% (w/v) (RCF : water); 100 ml

The water bath temperature was set at 75°C, actual value 76°C. The solutions were placed in 250 cm^3 beakers. The beaker was placed in the water bath kept in place by clamps. The solution was stirred slowly. When the RCF solution reached the temperature of 66°C, it was held at that temperature for 5 minutes. The stirrer was speeded up and 2 cm^3 of H_2O_2 (strength 100 volumes) were introduced by means of a 5 ml plastic syringe. The flow of H_2O_2 was directed at the vortex formed by the propeller. At the point of contact between H_2O_2 and RCF, a creamy-yellow foam appeared. The foam was quite stable and needed further breaking up with the aid of a spatula. The rest of H_2O_2, another 2 cm^3 was added at this point. Using this type of mixing device some RCF solution was left unbleached at the bottom of the beaker.

After 5 minutes of reaction, 2 cm^3 of excess RCF, undiluted were added again by means of 5 ml plastic syringe. The excess RCF darkened the RCF solutions considerably. This step was complete in 5 minutes and all of the residual H_2O_2 was decomposed. The solutions of RCF prepared, the H_2O_2 and excess RCF added, and their appearance before and after bleaching are summarised in Table 6.1.

6.3 Experimentation

6.3.1 Hydrogen peroxide and excess RCF addition

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b) 30% (w/v) (RCF : water); 100 ml
c) 40% (w/v) (RCF : water); 100 ml

The water bath temperature was set at 75°C, actual value 76°C. The solutions were placed in 250 cm^3 beakers. The beaker was placed in the water bath kept in place by clamps. The solution was stirred slowly. When the RCF solution reached the temperature of 66°C, it was held at that temperature for 5 minutes. The stirrer was speeded up and 2 cm^3 of H_2O_2 (strength 100 volumes) were introduced by means of a 5 ml plastic syringe. The flow of H_2O_2 was directed at the vortex formed by the propeller. At the point of contact between H_2O_2 and RCF, a creamy-yellow foam appeared. The foam was quite stable and needed further breaking up with the aid of a spatula. The rest of H_2O_2, another 2 cm^3 was added at this point. Using this type of mixing device some RCF solution was left unbleached at the bottom of the beaker.

After 5 minutes of reaction, 2 cm^3 of excess RCF, undiluted were added again by means of 5 ml plastic syringe. The excess RCF darkened the RCF solutions considerably. This step was complete in 5 minutes and all of the residual H_2O_2 was decomposed. The solutions of RCF prepared, the H_2O_2 and excess RCF added, and their appearance before and after bleaching are summarised in Table 6.1.

The H_2O_2 and excess RCF additions are calculated as percentages of the undiluted RCF.

H_2O_2 decolourisation and excess RCF data I

RCF solution % (v/v)	H_2O_2 added (%)	Appearance of powder	Excess RCF added (%)	Appearance of powder
20	20	Very pale, yellow fine powder	10	Yellow, fine powder
30	13.3	Granular, yellow brown, powder	6.6	Dark, coarse powder
40	10	Coarse, brown red powder	5	Dark red, coarse powder

Table 6.1

As expected the more dilute RCF solutions with high H_2O_2 addition, gave the lighter powders.

Also the 20% RCF solution was the easiest to mix and gave a fully decolourised product.

Another four RCF dilutions were decolourised under the same conditions as the above. The final product was freeze dried and tested for protein content. The results are summarised in Table 6.2

H_2O_2 decolourisation with excess added RCF data II

RCF solution % (v/v)	H_2O_2 added (%) (v/v)	Excess RCF % (v/v)	Protein content of product (%)
40	10	2	75.85
35	6	5	77.12
30	13.3	6.7	76.15
25	10	5	76.05
15	26.7	13.3	77.10

Table 6.2

The 15% RCF solution was kept as a reference. The protein in this solution decolourised easily, the product resembling "minced chicken". The H_2O_2 content was too high and the colour did not change significantly with the excess RCF.

The 25% RCF solution also decolourised well, producing small yellow particles.

The 30% RCF produced a brown-yellow protein powder. The mixture was fairly easy flowing and all of the solution was decolourised. The granules were not as light as the previous two solutions.

The 35% RCF conforms more closely with the Mars patent, described in 3.5.4.4. . The product was not decolourised completely. At the bottom of the beaker about 10% of the RCF solution was left untreated. This was attributed to insufficient mixing. The stirrer

used, coupled with the small amount of H_2O_2 employed resulted in a product very dark brown in colour.

The 40% RCF, after freeze drying, had a light brown-grey appearance, with a distribution of yellow granules mixed in. This product is considered optimum in terms of colour, hydrogen peroxide and excess RCF used. Using this dilution the whole RCF can be processed quickly.

The protein content, calculated as percentage of all five solutions was in the region of 75-77%. This was calculated before any excess RCF was added to the products, using the MicroKeltec method.

The 40% RCF product was analysed for its amino acid profile, and for its functionality properties. The results are summarised in sections 6.5 and 6.4, respectively.

The mixing devices used in this part of the work gave completely unsatisfactory results for solutions containing more than 40% RCF.

6.3.2 Other mixers employed

In an attempt to improve the mixing and decolourise samples containing more than 40% RCF, two more types of mixers were used.

The first type utilised a motor and an open four bladed propeller. This mixer gave similar results to the three bladed propeller used primarily. It did not decolourise satisfactorily any solutions containing more than 40% RCF.

The second mixer was a Silverson emulsifier, as described in Section 4. A 40% RCF solution was processed as described above. However the moment the hydrogen peroxide was introduced the granules

formed, blocked the head of the Silverson, thus stopping the decolourisation.

It was concluded that the most useful mixer will utilise either z-blades or a planetary action paddle, i.e. a Hobart mixer.

6.3.3 Decolourisation without addition of excess RCF

The possibility of not adding any excess RCF to the decolourised, solution was investigated. The mixing device used was a domestic food mixer described in 4,2. In this series of experiments it was attempted to determine the amount of hydrogen peroxide needed to decolourise a 40% RCF solution without leaving any excess H_2O_2 in the solution.

The RCF solution was prepared as follows. 60 ml of RCF were slowly added to 90 ml of distilled water. This gave a 40% (v/v) RCF solution.

This solution was decolourised in the apparatus shown in Fig. 6.2 and an amount of hydrogen peroxide (100 volumes) was added to effect the decolourisation.

The protein powders produced were examined visually for acceptability. The results are summarised in Table 6.3.

Decolourisation without addition of excess RCF

RCF solution	H_2O_2 (100v) added (ml)	Concentration of H_2O_2 in RCF (%) v/v	Comments on solution produced
150 ml of 40% RCF were used	12 Mixed for 5 minutes	8	Fast reaction. The solution decolourised quickly in pale yellow agglomerates
	10.5 (5minutes)	7	As above. Pale granules embedded in clear fluid
	9 Ten minutes mixing time required	6	Difficult decolourisation Requires twice mixing time as the solutions above. Brown-yellow granules.
	75 (10 minutes mixing)	5	Results as the solution above. Brown granules present.
	6 (20 minutes mixing)	4	Decolourisation difficult Final product of dark brown appearance

Table 6.3

The aim was to use as little hydrogen peroxide as possible to decolourise the most concentrated RCF solution, i.e. 40% RCF. It was found that the difference in colour between the sample decolourised with 6% H_2O_2 and the one with 5% H_2O_2 was minimal. The difference however between using 5% and 4% H_2O_2 was substantial.

Further investigation was carried out on 40% RCF solutions decolourised by adding 5% of H_2O_2 to the total RCF solution volume.

6.3.4 Investigation of 40% RCF decolourised by addition of 5% H_2O_2

A 40% RCF solution was prepared as follows: 80 ml of RCF were added to 120 ml of distilled water. The solution was placed in a beaker and attached to the apparatus shown in Fig 6.2. It was heated up to 65°C, the food mixer stirring the solution slowly and held for five minutes. Then the mixture was stirred vigorously and 10 ml of H_2O_2 (100 volumes) were added. This is equivalent to adding 5% H_2O_2 in the solution.

The sample decolourised completely after 10 minutes mixing time. The protein agglomerates were of brown-yellow colour embedded in clear fluid.

The mixture was vacuum filtered. The granules were not dried but tested for moisture and protein content, see Table 6.4.

The fluid was also checked for protein and for residual H_2O_2, as described in Table 6.4.

Analysis of the 40% RCF solution

Sample	Protein Content (%)	Moisture Content (%)	H_2O_2 present mEq /kg
Solids	13.44	80.6	8.0
Solids	13.05	80	8.0
Fluid	Negligible	-	10
Fluid	Negligible	-	10.5

Table 6.4

The protein content of the solids was 13.25% on wet basis or 68% on dry basis.

No protein was detected in the fluid.

The 40% RCF contained 14.04% protein. Hence the yield of protein is 94.37%. This method of decolourisation recovers most of the original protein. The levels of H_2O_2 detected in the solids were 8.0 mEq./kg, which is very low. The solids did not change colour over a period of a week, which indicates that no residual reaction between the haemoglobin and hydrogen peroxide exists.

A sample of this decolourised solution was freeze dried and analysed for its functional properties and amino acid profile.

6.3.5 Large batch decolourisation using the Hobart Mixer

A Hobart Mixer with a capacity of 1 litre, was used for preparing large amounts of decolourised haemoglobin solutions. No excess RCF was added, as part of the decolourisation process. Two batches of 1 litre solution were decolourised, both of 40% (v/v) concentration of RCF. The Hobart was placed in a water bath which was set at 78°C.

The first batch of solution was added to the bowl and the mixer was put in the slower gear. When the temperature of the solution reached 65°C and had been kept here for 5 minutes, the mixer was put into second gear. 50 ml of H_2O_2 (100v) were added to the solution i.e. 5% (v/v) of H_2O_2 RCF. After 10 minutes of vigorous mixing, the solution was decolourised. Pale-yellow protein agglomerates were produced. At the bottom of the bowl a film of dry undecolourised RCF remained. The weights of solutions prior to decolourisation, after the process, and the weight of undecolourised material were recorded as shown in Table 6.5. The protein content of the samples was also determined.

The residual film of RCF solution which was not properly decolourised, is defined in Table 6.5. The mixing pattern of the Hobart was such that in both batches, the ratio of residual to decolourised solution was 0.1, hence the mixing was quite efficient. The yield in Table 6.5, is calculated as the amount of protein recovered from both the decolourised and residual portions of the solution. In both batches it was high, around 80%. The rest of the protein was lost due to some material in the bowl of the mixer which was not collected.

The decolourised product in this larger scale process visually resembled the one prepared in small beakers. The analysis of the product was also similar to the smaller scale decolourisation reported in Table 6.4. However the yield achieved with the Hobart was 80%, lower than in the food mixer, which achieved 95% yield. This may be accounted for by the mixing pattern of the Hobart. It would appear that thick 'gel' type film which forms on the walls of the bowl, when using the Hobart, was lowering the protein recovery.

Decolourisation using the Hobart mixer

	Batch 1 1 ltr of 40% RCF solution			Batch 2 1 ltr of 40% RCF solution		
	Undecolourised	Decolourised	Residual	Undecolourised	Decolourised	Residual
Wt of sample (g)	1050	726.36	73.32	1010	720.15	71.65
Protein content(%)	14.0	11.75	42.5	14.0	11.68	41.28
Moisture content(%)	80.2	80.5	57.5	80.2	80.5	56.0
Protein content in wt (g)	147.0	85.35	31.16	141.4	84.12	29.58
Yield (%)		79.26%			80.41%	

Table 6.5

6.4 Analysis and functional properties of decolourised RCF by the H_2O_2 process

The two powders examined for their functional properties were:

1. Partially bleached powder. This is the 40% RCF solution decolourised by the addition of H_2O_2, 100 volumes. The total H_2O_2 addition was, in percentage terms, a volume equal to 4% of the whole RCF solution. The excess RCF added was of a volume equal to 2% of the whole RCF solution.

2. 'Bleached' powder This is the 40% RCF solution decolourised by the addition of H_2O_2, 100 volumes. The total addition of H_2O_2 was of a volume equal to 5% of the whole RCF solution. This sample had no excess RCF added.

The powder analysis is shown in Table 6.6

Analysis of powders

Parameter	Partially bleached powder	'Bleached' powder
Colour	Dark brown	Light brown
pH of 10% solution	6.00	6.00
Moisture %	6.00	6.00
Protein %	77.00	79.00
Salt %	1.40	1.30
Lipids %	0.77	0.65
Ash %	10.00	9.70
Other %	4.83	3.35

Table 6.6

The tests for functionality are described in section 4 The results for the 'bleached' and partially bleached powders are shown in Table 6.7

Functionality Tests

Property tested	'Bleached' powder	Partially bleached powder
Solubility	65.0%	64.5%
Fat binding	1.252 ml/g	1.250 ml/g
Gelation	No gel formed	No gel formed
Emulsification	No set emulsion formed	No set emulsion formed
Foaming (%) Stability	% foam remaining after time	
5 min	18.0	17.5
25 mins	8.5	8.5
60 mins	7.0	7.5

Table 6.7

The first conclusion from the tests was that the two powders have very similar properties. They do not exhibit any gelation or emulsification properties and their solubility and foaming properties are low. Fat binding at around 1.252 ml/g compares favourably with spray dried red cell fraction which binds 0.534 ml/g of powder. Blood cell hydrolysate, prepared by Novo Industries also has a fat binding capacity of 1.229 ml/g.

It appears on the basis of the tests carried out, that the powders are mainly of use as protein supplements in food products. There is some fat binding capacity which could be used in comminuted meat products.

6.5 Amino acid profile of decolourised powders

The amino acid profile of untreated RCF, 'bleached' and partially 'bleached' solutions was determined. The concentration of each amino acid is given in n moles/ml of solution. The purpose was not to examine the solutions in a nutritional context, but to see if the decolourisation process destroyed any amino acids.

The concentrations of the amino acids for each sample is given in Table 6.8.

The amino acid traces for the 'bleached' and partially bleached samples are shown in Figure 6.3.

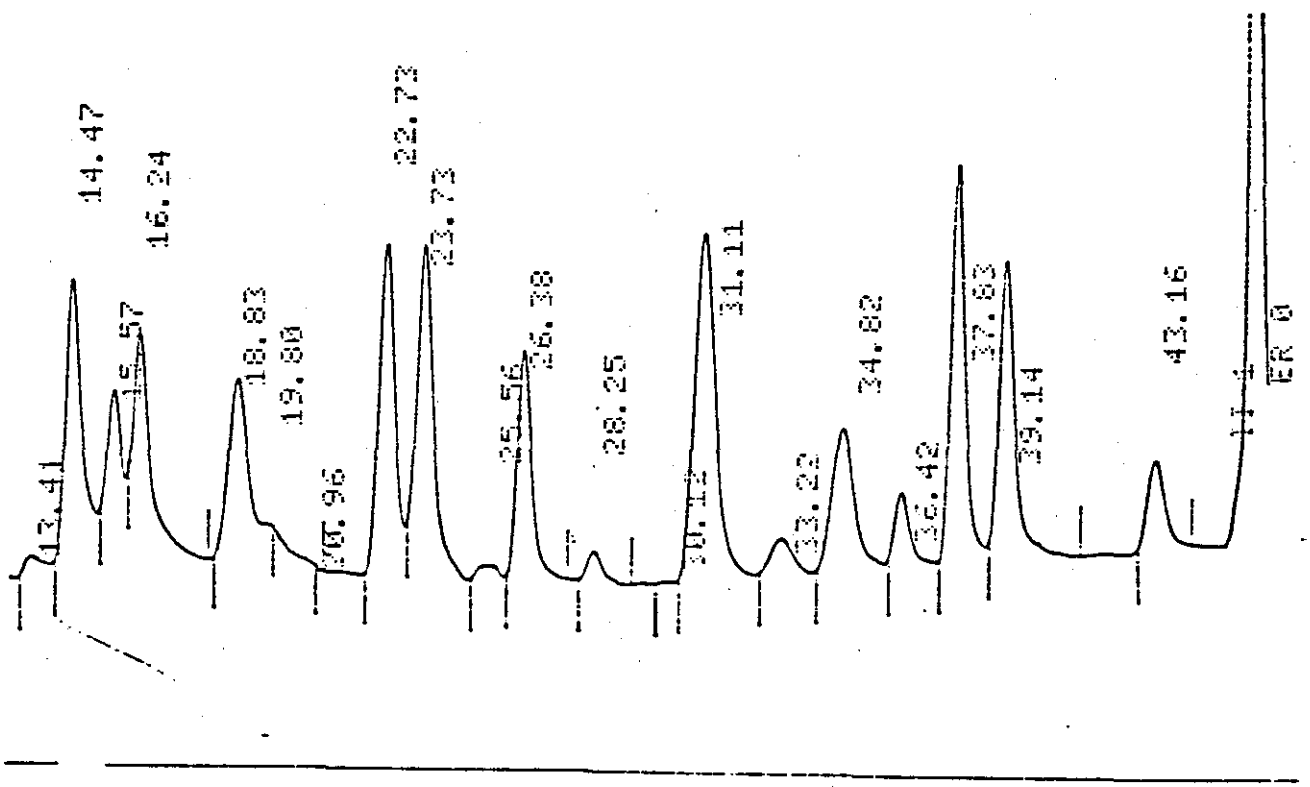
As a comparison, the amino acid trace of the 40% RCF is shown in Figure 6.4.

It can be seen from Table 6.8, that the amino acid concentrations of both the treated and untreated RCF is very similar.

This does not indicate that the haemoglobin has been unaffected by the process, but does suggest that the H_2O_2 decolourisation process does not destroy any amino acids in the haemoglobin molecule.

It was deduced therefore that the decolourised protein retains all the nutritional propertiesof the untreated red cell fraction of bovine blood, namely the high content of the essential amino acids lysine, valine, phenylalanine and leucine.

'Bleached Sample'



'Partially bleached' sample

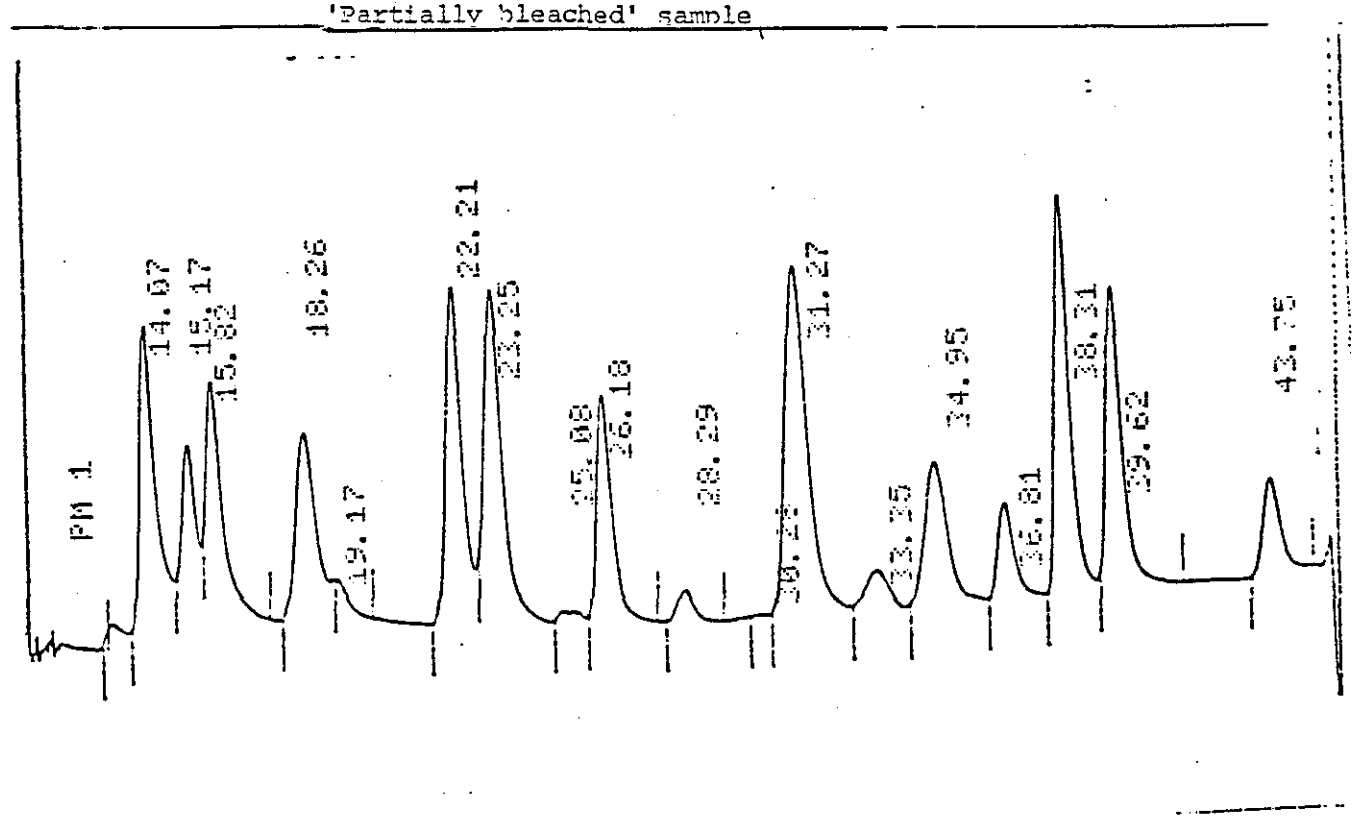


Figure 6.3

Amino acid profile of a 40% (v/v) RCF (untreated)

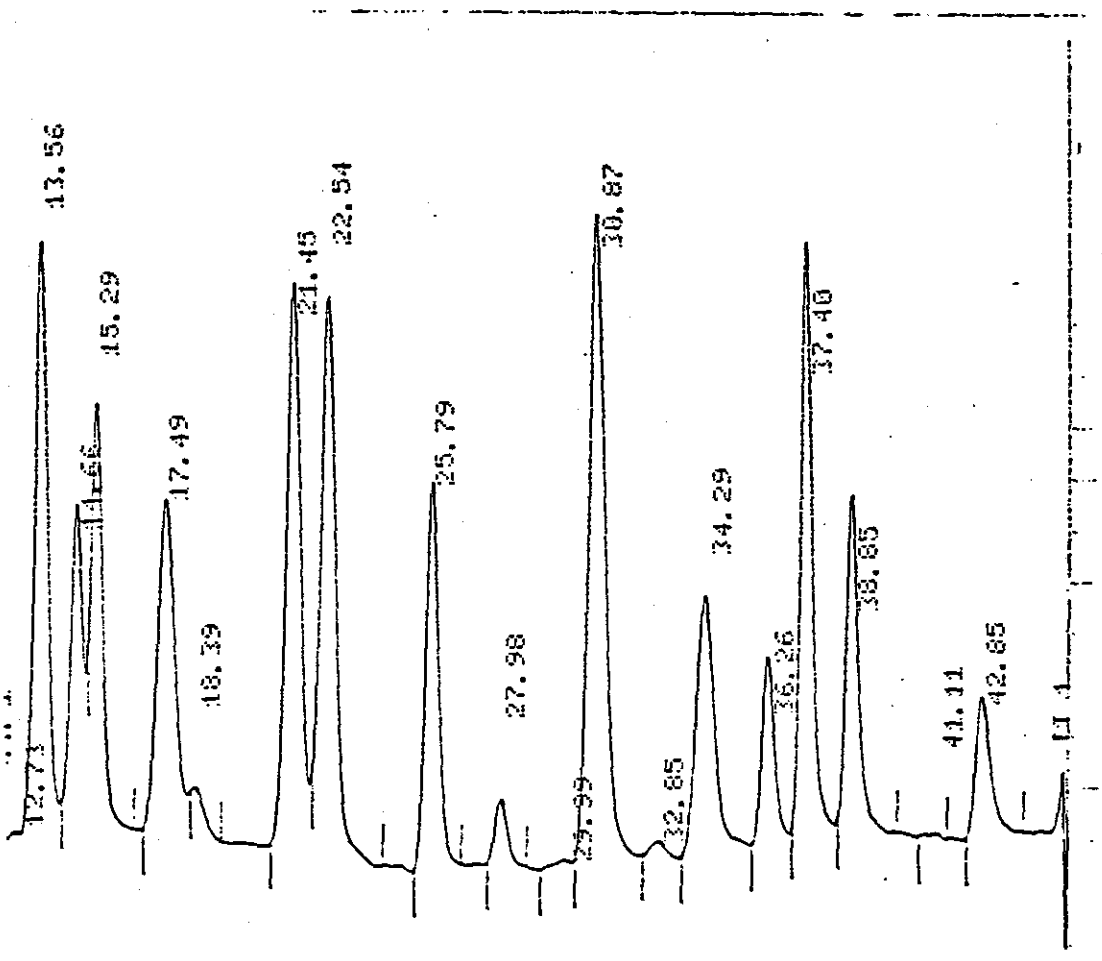


Figure 6.4

Amino acid profile of solutions

Amino acid (order appearing in trace)	Red cell fraction untreated (nmoles/ml.)	'Bleached' product (nmoles/ml)	Partially bleached product (nmoles/ml)
Aspartic acid (ASP)	115.83	119.82	118.75
Threonine (THR)	69.57	82.89	81.85
Serine (SER)	75.15	95.14	95.13
Glutamic acid (GLU)	93.75	49.08	48.95
Proline (PRO)	58.06	71.10	71.33
Glycine (GLY)	146.33	164.85	164.85
Alanine (ALA)	263.02	274.81	273.65
Cysteine (CYS)	0.43	0.67	0.59
Valine (VAL)	133.58	155.85	155.13
Methionine (MET)	25.20	27.99	27.65
Isoleucine (ILE)	44.36	53.36	52.15
Leucine (LEU)	207.86	232.80	232.82
Tyrosine (TYP)	12.62	16.31	16.58
Phenylalanine (PHE)	86.98	98.29	99.22
Ammonia trace	98.12	139.74	138.94
Lysine (LYS)	107.24	129.48	121.51
Histidine (HIS)	51.06	68.19	67.92
Arginine (ARG)	31.44	45.06	45.04

Table 6.8

6.6 Costing of the process

The hydrogen peroxide process does not require complicated equipment and is a relatively simple process. A simple costing of the method was attempted. However certain assumptions were made.

1. There are no building costs to house the decolourisation unit. The plant will be annexed to the main slaughterhouse.

2. The plant will receive the RCF immediately i.e. the blood will have already been centrifuged into its fractions. This eliminates the need for a low speed centrifuge.

3. The RCF will be coming from the same abattoir and thus will be free of charge and no big storage area will be required.

4. Labour costs will be minimal. Since it will take no more than two people to work the mixer which is the main unit of the plant, these people can be paid something extra and work part time.

The mass balance which follows will be the basis to estimate the cost of a kilogram of decolourised haemoglobin solution.

100 litres of RCF are to be processed by the hydrogen peroxide method. They are mixed with 150 litres of tap water to make up a 40% RCF (v/v) solution.

This solution is heated up to $65^{o}C$ in a jacketed mixer, by steam, or more simply hot water, and 10 litre of H_2O_2 (100v) are added. The total hydrogen peroxide added equals 4% by volume of the RCF solutions.

The product can be either dried in ovens or an alternative can be simple screening and sold wet containing 80.5% moisture.

Initially, 37.3464 kg of haemoglobin are contained in the RCF, the decolourisation process for a 4% H_2O_2 is 80% efficient, therefore in the final product 29.8771 kg of protein are retained.

The composition of the final product is

Water : 80.5% = 123.3388 kg
Protein : 19.5% = 29.8771 kg
Total weight of product = 153.2159 kg

As already shown by simply decanting the residual fluid of the decolourised mixture a further 10% of water can be removed. Therefore the final product weight = 140.88 kg

Capital costs for the process are

1. Mixer at £950
2. Centrifugal pump at £500

Capital costs £1450
Piping instalment at 2% of £1450 i.e. £29.
Total capital costs approximately £1479.

Operating costs are:

Hydrogen peroxide used : 10 litres BDH prices quoted for 100v hydrogen peroxide for large batches are as low as £1.75 per litre. Cost 10 x 1.75 = £17.5

Water : cost £0.005 per litre = 0.005 x 150 = £0.75
Electricity for 1.1 kwh pumps: Cost £0.0724 kWh
Total operating costs for producing 140.88 kg are £18.32 .
Therefore the cost per kilogram would be £0.13.

The product can be used as a protein supplement, with some fat absorbance properties. Pet food companies valued the product in the range £350-£450 per tonne i.e. £0.35-£0.45 per kilogram.

This will yield a profit, before depreciation of £0.23 - £0.33 per kilo or £230 to £330 per ton of decolourised product. This provides a considerable profit and could be a profitable venture, especially since the capital expenditure and operational costs are minimal.

CHAPTER 7

7. Decolourisation of RCF using Carboxymethyl Cellulose-CMC

The present work is loosely based on the Autio work described in section 3.5.4.5 . The RCF is haemolysed as in the hydrogen peroxide decolourisation process. Then a defined volume of HCl solution is added to the RCF solution to bring the pH to less than 3.

In the acidified RCF mixture a certain volume of weak aqueous solution of CMC is added. The mixture is allowed to react for fifteen minutes and then spun in a centrifuge.

The precipitate contains haem and CMC while the supernatant contains the decolourised globin, i.e. the final product.

The mechanism of reaction between the CMC and haemoglobin is not fully understood. Some insight into the possible reaction was provided by Sato (1981). He used CMC as a weak acidic ion exchanger to effect the separation of haem from globin. Even though the adsorbance of haem onto CMC cannot be completely explained using the general theory of ion exchange, this theory was the only one adequately describing the reaction. At a pH less than 3, haem is loosely bound to the globin molecule. The CMC at this pH might be its non-ionised H-form, Sato (1981). Thus a coordinate bond is formed between the non-ionised carboxyl group and Fe of the haem. Sato also showed that when simple cellulose was used in chromatography haem was not adsorbed. Thus the carboxymethyl group of CMC might be indispensable in the adsorption process of haem. Sato also showed, that when a haemoglobin solution contains Na ions, haem is more incompletely adsorbed with increase in concentration of Na ion. Thus absence of Na ion might also be indispensable for adsorption of haem. NaCl might interrupt the coordination between Fe and carboxyl group in a similar manner as carboxyl groups coordinated to chromium in chrome complexes. For example, Sato gives

the following reaction: $\{Cr.3\ H_2O.2COO\}^+\ Cl^-$, is exchanged with Cl^- ion in the presence of NaCl. However, without NaCl the complex remains intact. For this reason large volumes of water are used in the reaction in order to dilute the salt ions.

Temperature effects are not mentioned in the literature therefore the reactions have been carried out at room temperature.

The parameters examined in this section are:

1. The effects of using different concentrations of RCF and CMC solutions on decolourisation.

2. The effect of using different centrifugation speeds on the yield of protein.

3. The effect on hydrolysis at different pH

On the most promising samples, functionality tests and particle size analysis were carried out.

Pilot plant work was carried out on the best decolourised solution.

7.1 The Effect of Using Different Concentrations of RCF and CMC Solutions on Decolourisation

Initial attempts to effect decolourisation included the addition of low viscosity CMC powder added directly to acidified RCF solution with the aid of a mixer.

The RCF solutions were prepared as follows:
17 ml of RCF were added to 83 ml of water, i.e. 17% v/v solutions. The solutions were acidified with 1N HCl to a final pH of 2.8. The solution was placed in a 200 ml beaker and stirred by an open 3 bladed propeller attached to a mixer.

10g of CMC powder were added slowly in the acidified RCF solution. The mixer was geared at its highest speed and the CMC added in the vortex formed. The CMC powder however formed a stable agglomerate around the blade of the propeller. This white, paste-like agglomerate was wetted on the outside. When taken out of the beaker and cut with the aid of scissors, dry unreacted CMC was released.

The experiments were repeated with different amounts of CMC: 5, 7, 9 g., and with different propellers. The result was always the formation of these white-sticky filaments of CMC and no reaction between the CMC and haemoglobin occurred.

An alternative approach was to swell 5g of CMC in 20 ml of water and add to a 17% RCF solution. When the CMC was added to the acidified RCF it again formed a solid agglomerate.

More success was obtained when swelling 5g of CMC in 20 ml of concentrated HCl.

The CMC was thoroughly wetted but when added to a 17% RCF, acidified to pH 2.7, no reaction occurred.

The Autio patent was followed more closely in the next set of experiments. Two solutions of RCF were prepared, a 20% and a 25% (v/v). The solutions were acidified from an original pH of 7.3 to pH 1.5, using 1N HCl. The HCl added was metered as a percentage of the total volume of RCF solution. To the acidified solutions a low concentration of aqueous CMC solutions were added,

0.35% and 0.7% (w/v) respectively. The volume of CMC solution added in both experiments, was equal to three times the volume of the RCF solution.

The two mixtures were left at room temperature for fifteen minutes and then centrifuged for 30 minutes at 8000g. The supernatant was collected and its protein concentration determined using Kjeltec analysis. The results are summarised in Table 7.1

Decolourisation using Autio patent

RCF Conc (%) (v/v)	CMC Conc (%) (w/v)	1N HCl added (% of RCF volume)	pH of supernatant	g. of protein prior to centri-fugation	g. of protein in super-natant	Yield (%)	Colour of super-natant
20	0.35	20	2.9	9.214	5.368	58.25	Transparent brown
25	0.70	20	2.9	5.758	2.552	44.32	Transparent light yellow

Table 7.1

The supernatant, which contained the globin, was adequately decolourised when a 0.35% CMC solution was used. When 0.7% CMC was used the globin solution had a very light yellow colour. It would appear that the more concentrated the CMC solution the better the decolourisation. However the yields of globin obtained were low, 44% and 58%. The lower yield was obtained with the higher concentration CMC, (0.7%).

In order to examine the effect of CMC concentration and RCF concentration on the colour, the following twelve samples were prepared, Table 7.2.

RCF solution % (v/v)	CMC concentrations % (w/v)	1N HCl added (%) of total RCF	Sample Number
16	0.4		1
	0.6		2
	0.8	20	3
20	0.4		4
	0.6		5
	0.8	20	6
25	0.4		7
	0.6		8
	0.8	20	9
33	0.4		10
	0.6		11
	0.8	20	12

Sample preparation - Table 7.2

The RCF solutions were acidified to pH 1.5 using 1N HCl.

The CMC was then added to the samples. The total volume of CMC added equalled three times the volume of the RCF solution. The samples were left to react at room temperature for 15 minutes. They were then loaded into the bench centrifuge and spun at 4000 rpm for fifteen minutes. The samples therefore were centrifuged at less than the speed and time specified in the Autio patent.

After centrifugation all samples contained a definite precipitate occupying 0.1 of the total volume of the sample in the centrifuge tube. The precipitate was dark brown in colour and had a "rubbery" texture.

The supernatants and precipitates of all samples were examined for total solids and protein content, Tables 7.3, 7.4.

Total Solids Content of Samples

Sample	CMC-haem (%) solids	pH of Supernatant	Supernatant solids (%)
1	89.3	2.5	1.19
2	83.6	2.7	0.65
3	85.0	2.9	0.48
4	81.6	2.7	1.45
5	81.3	2.5	0.81
6	77.1	2.9	0.39
7	83.7	2.9	1.84
8	85.9	2.7	1.54
9	85.7	2.9	1.28
10	82.3	2.8	1.59
11	88.9	2.8	1.70
12	83.6	2.9	1.93

Table 7.3

Colour and protein analysis of decolourised protein solutions

Sample number	Colour solution (arbitrary * units)	Percentage protein prior to centrifugation	Protein in supernatant %	Yield (%)
1	2.5		0.593	33.79
2	2	1.755	0.335	19.09
3	1		Traces	-
4	5		0.713	33.86
5	4	2.194	0.713	32.50
6	3		Traces	-
7	7.5		1.669	60.80
8	7.0	2.742	1.389	50.60
9	6.5		0.971	35.37
10	9.0		1.456	40.22
11	8.5	3.620	1.415	39.09
12	8.0		1.164	32.15

Table 7.4

* Arbitrary units 1: water-clear
10 dark brown colour

It became apparent (Table 7.3) that the percentage total solids in the CMC-haem precipitate did vary in the different samples. All the precipitates were of dark brown colour and contained less than 20% moisture. The supernatant which contained the decolourised protein had a very low percentage solids content; maximum 1.93%, which may create problems when further processing the protein. The decolourised protein solution was at pH 3 approximately (Table 7.3). The decolourised solution will now be referred as the Decolourised Red Cell Fraction (DRCF)

The supernatants of the twelve samples were examined for protein content. The twelve samples were weighed prior to centrifugation and the supernatants were weighed following centrifugation. This enabled the protein yield of each sample to be determined. Nitrogen determinations were estimated using Kjeltec.

An arbitrary scale of 1 to 10 was developed for visual examination of the decolourised samples. At 1 a colour similar to water was produced, and at 10 a colour similar to a 50% solution of spray dried RCF in water was produced.

The results are summarised in Table 7.4. The percentage of solids supernatant in the (Table 7.3) indicate that when more concentrated CMC solution is used, the less solids appear in the decolourised solution. This is also shown in Table 7.4, where when the stronger CMC solution was used less protein appeared in the supernatant and hence the yield dropped.

It would seem from these results that the CMC is complexing not only the haem but also parts of the globin molecule.

This could have resulted from the acid hydrolysis step of the process, suggesting an incomplete split of the haemoglobin molecule. Sample number 1, 4, 7 and 10 which were solutions treated with 0.4% CMC showed a higher yield, 33.8%, 33.86%, 60.8% and 40.22% respectively.

The very dilute RCF solutions showed the lowest yield and no protein was detected when 0.8% CMC solution was used. The colour of the decolourised solutions was as expected, less light as less decolourisation occurs with increasing concentrations of RCF.

Absorbance of decolourised solutions at 470 nm (Biuret method)

Sample number	Absorbance at 470 nm (average)
1	0.480
2	0.355
3	0.o95
4	0.483
5	0.380
6	0.146
7	0.498
8	0.456
9	0.475
10	0.485
11	0.474
12	0.447

Table 7.5

The protein yields calculated in Table 7.4 were plotted against the CMC concentrations used for all samples. The graph obtained is shown in Figure 7.1. On the basis of the curves obtained it would appear that the 25% RCF solution produced better protein yields, with all three CMC solutions, than with the 33% RCF. It would seem therefore that any concentrations of RCF higher than 25% would have lower yields possibly because at the CMC concentrations used the complexing capacity of CMC may be exhausted.

In order to test this theory another series of twelve samples was prepared, under the same conditions but checked for protein content by the Biuret method instead of Kjeldahl. The results obtained are shown in Table 7.5.

The absorbance decreased with increasing CMC concentration in all RCF solutions. Also the highest absorbance obtained (indicated by the highest presence of solids) was given by sample 7, i.e. 25% RCF treated with 0.4% CMC solution. The absorbance was plotted against CMC concentration for all samples, Figure 7.2.

The curves obtained were similar to those in Figure 7.1. The highest absorbance (0.498) was shown by the 25% RCF, 0.4% CMC solution.

The 16% and 20% RCF solutions registered very little absorbance when treated with 0.8% CMC solution. Again the 33% RCF solution had lower absorbance than the 25% RCF, and did not alter with varying CMC concentration. The values obtained for the 33% solution ranged between 0.485 and 0.417, similar to those obtained from the 25% solution. This further supports the argument that at the CMC concentrations used, the 25% solution

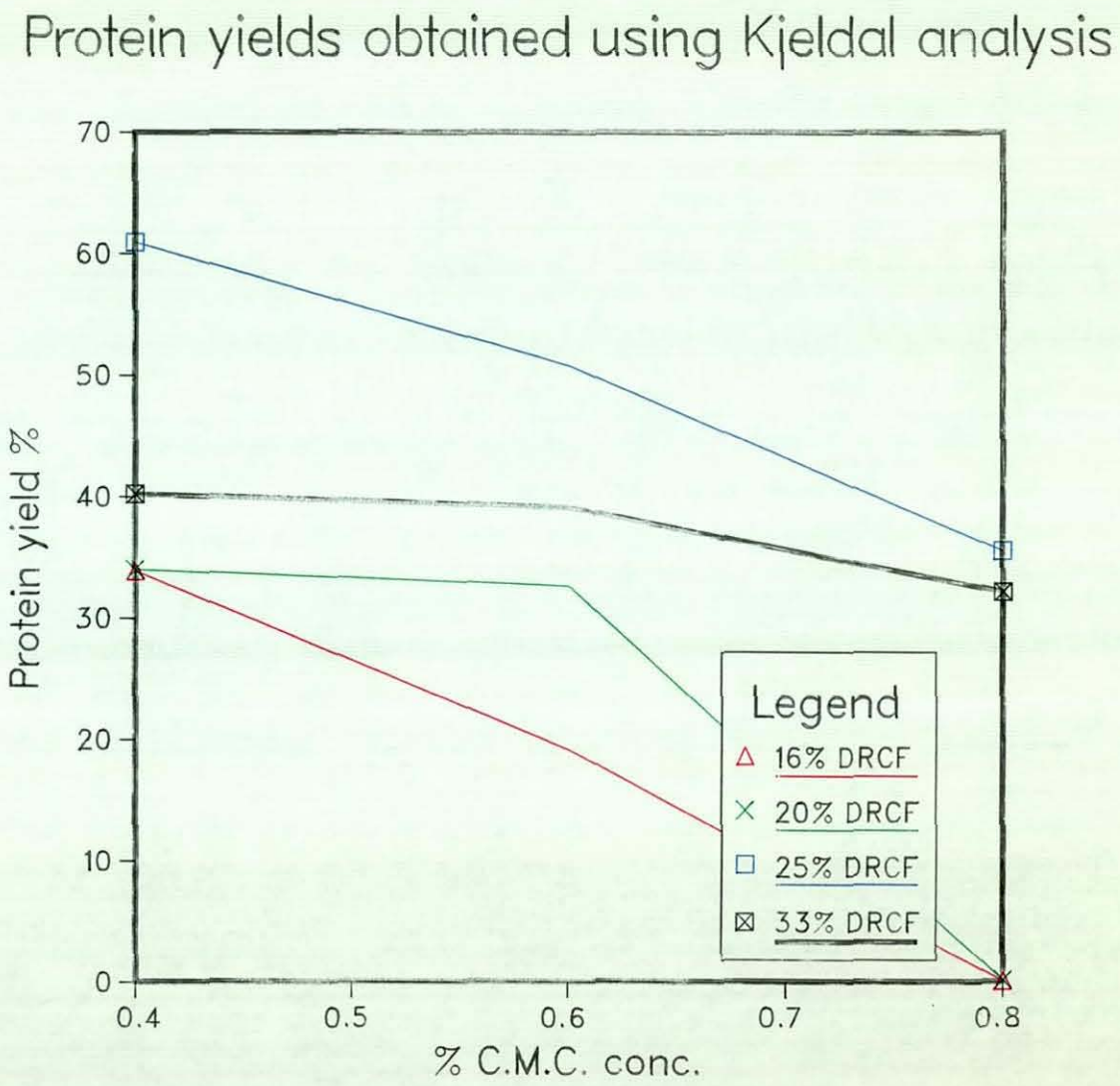

Protein yields obtained using Kjeldal analysis
Protein yield %
70
60
50
40
30
20
10
0
0.4
0.5
0.6
0.7
0.8
% C.M.C. conc.
Legend
16% DRCF
20% DRCF
25% DRCF
33% DRCF

Figure 7.1

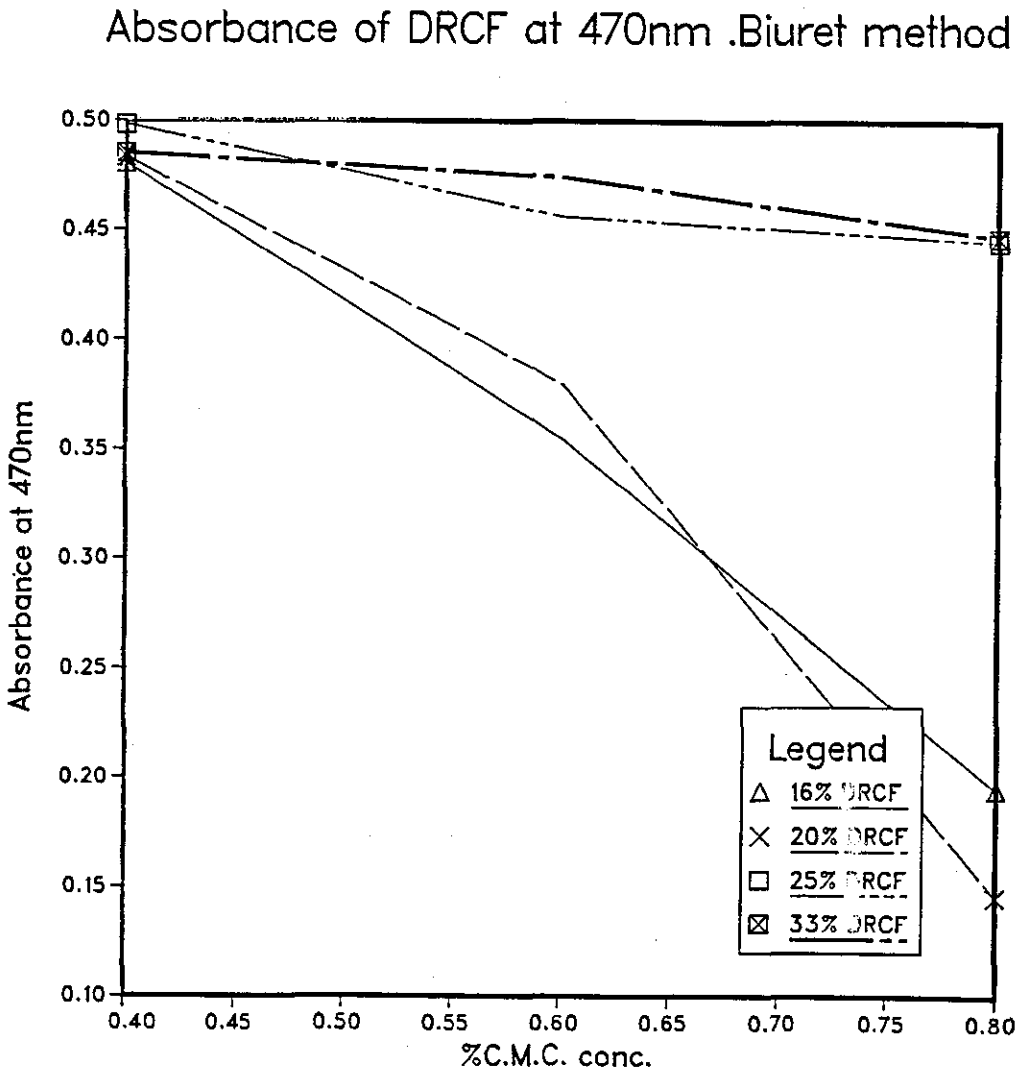

Absorbance of DRCF at 470nm .Biuret method
Absorbance at 470nm
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
%C.M.C. conc.
Legend
16% DRCF
20% DRCF
25% DRCF
33% DRCF

Figure 7.2

was optimum, with the more concentrated RCF solutions giving (a) incomplete decolourisation and (b) lower protein yields

The samples were examined for iron content using atomic absorption. The Fe atomic absorption standard calibration was first compiled, Table 7.6

Iron atomic absorption standard calibration

Absorbance	{Fe}/ppm
0.25	10
0.50	20
0.75	30
1.25	50
1.75	70
2.50	100

Table 7.6

Figure 7.3 is the standard iron curve used for the analysis of the samples. The slope of the curve was 0.025, hence the equation for the line was

$$A = 0.025\ \{Fe\}$$

The iron content of the 12 samples are shown in Table 7.7

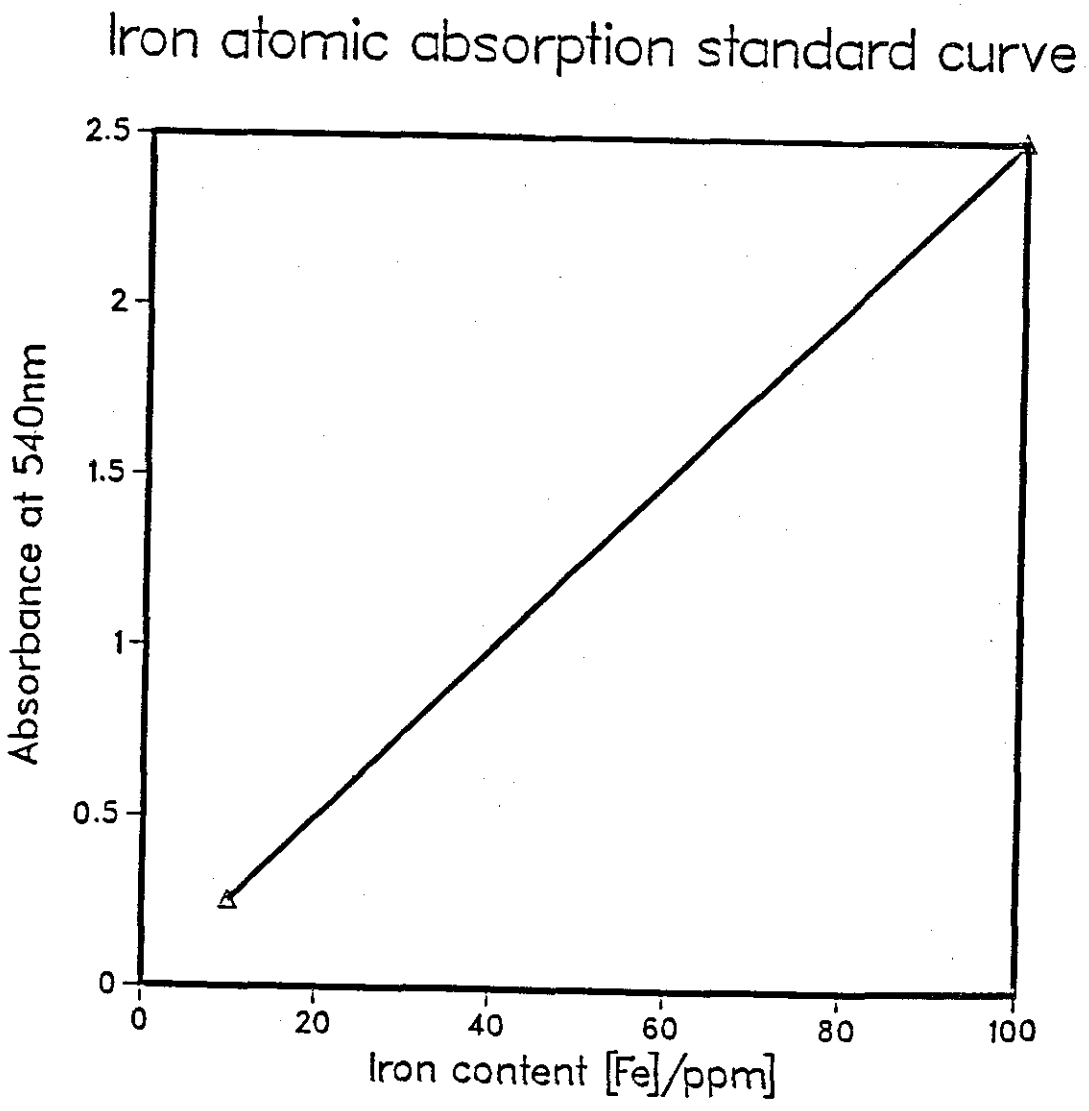

Iron atomic absorption standard curve
Absorbance at 540nm
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
Iron content [Fe]/ppm]

Figure 7.3

Iron content of 12 decolourised samples by CMC

Sample	Absorbance	{Fe}/ppm
1	0	0
2	0	0
3	0	0
4	0.25	10.0
5	0	0
6	0	0
7	0.35	14.0
8	0.15	6.0
9	0.05	2.0
10	0.38	15.2
11	0.32	12.8
12	0.30	12.0

Table 7.7

The undiluted RCF gave an absorbance of 37.5 i.e. 1500 ppm of iron. The iron content, in ppm, was plotted against the concentration of CMC used, for every RCF solution (Figure 7.4). The CMC had completely complexed all the Fe contained in the 16% RCF and therefore no readings were registered. Similarly most of the iron of the 20% RCF solution was complexed, with only the 0.4% CMC treated solution giving a reading of 10 ppm iron.

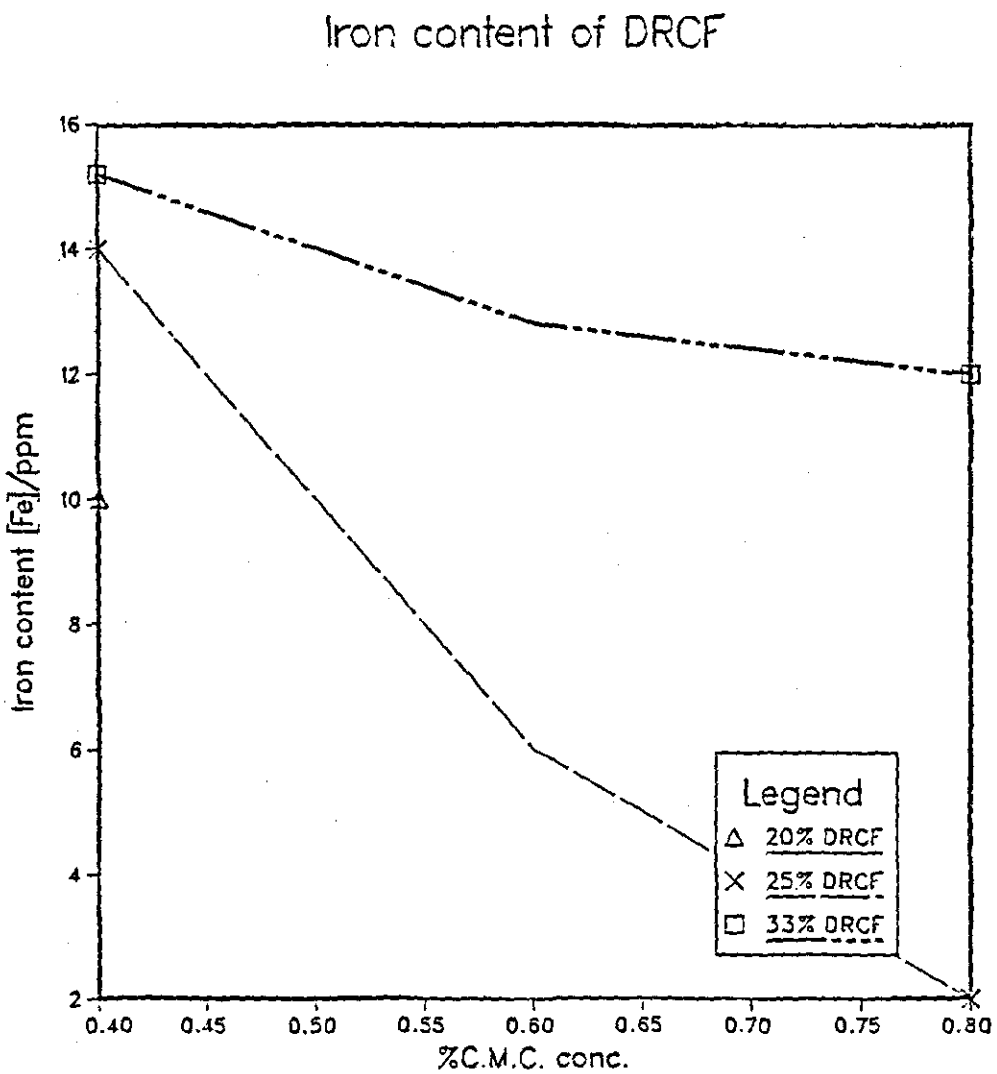

Iron content of DRCF
Iron content [Fe]/ppm
16
14
12
10
8
6
4
2
0.40
0.45
0.50
0.55
0.60
0.65
0.70
0.75
0.80
%C.M.C. conc.
Legend
20% DRCF
25% DRCF
33% DRCF

Figure 7.4

The 25% and 33% RCF gave the expected results i.e. the more concentrated the CMC solution used, the less iron existed in the final solution. It is worthwhile noting that the difference in iron content, with varying CMC concentrations at 33% RCF was small, the range of iron content being between 15 and 12.8 ppm. This compares with the much broader range obtained with the 25% RCF solution, in which the varying CMC concentration had a considerable effect on the iron content of the final decolourised solution. When treated with 0.4% CMC solution, the 25% RCF had an iron content of 14 ppm which was less than 1% of the initial iron content of the untreated RCF.

On the basis of the above results from solids, protein and iron content, it would seem that the optimum dilution for the red cell fraction is 25% and the optimum concentration of CMC is 0.4% (w/v).

The twelve samples were also examined for trace elements. The standard red cell fraction was found to contain 30 ppm of sodium and 16 ppm of calcium. The decolourised solutions contained less than 10 ppm of both sodium and calcium. There was no variation in the samples with the different CMC concentrations.

7.2 Spectrometric analysis of RCF

The Unicam SP800 was used to examine the spectrum from 200 to 450 of three samples. The first sample was untreated, undiluted RCF. The other two samples came from the decolourised globin solutions. In order to see the effect of decolourisation the two extreme solutions were used. These were the 10% RCF solution treated with 0.8% CMC, i.e. the most decolourised solution, and the 33% RCF treated with 0.4% CMC, i.e. the least decolourised one. The spectrum obtained is shown in Fig. 7.5.

Absorbance scan for RCFand two decolourised solutions.

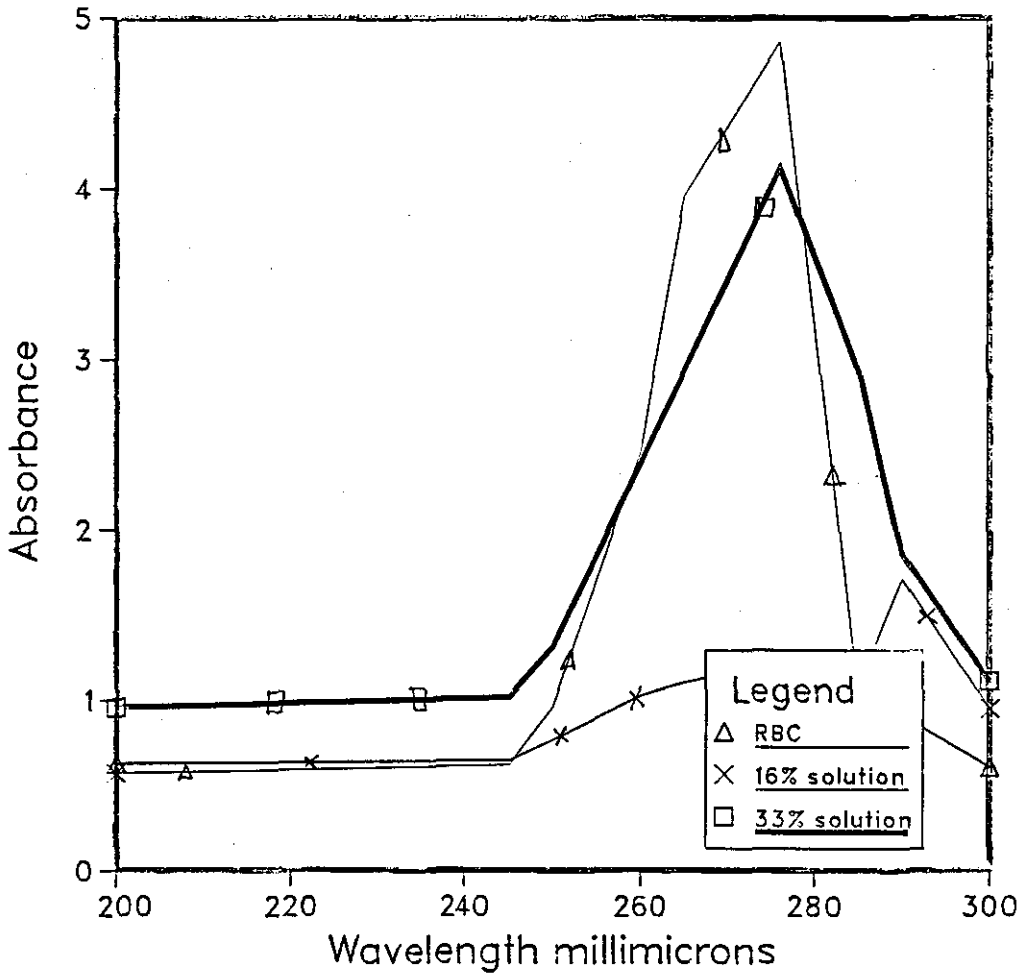


Figure 7.5

All three samples showed two peaks, one at 276 nm and the second at 372 nm. The first peak at 276 was attributed to the globin protein. The haem exhibited a peak at 372 nm i.e. close to the 'Soret' band, which is the band between 400-500 nm and is the range where porphyrins show highest absorbance (Rao (1961).

The RCF, untreated, exhibited two small peaks at 276 nm and 372 nm, (absorbance 1.22 (a.u.) and 0.70 (a.u.) respectively). However, both the 16% and the 33% RCF solutions showed considerable peaks at 276 nm, 4.85 and 4.11 absorbance units respectively . At 372 nm the 16% RCF showed a very small absorbance peak, 0.48 units, compared to the peak obtained by the 33% RCF which was 1.42 absorbance units (a.u.) . This indicated that the best decolourisation gave the highest difference between the two peaks. Thus the 16% RCF, which was the best decolourised solution, gave a difference of 4.29 a.u. while the 33% RCF gave 3.17 a.u. Therefore the greater the difference in height between the peaks at 276 and 372 nm, the better the decolourisation i.e. the clearer the solution.

7.3 The effect of using different centrifugation speeds on protein yield

A series of RCF solutions were prepared and acidified to pH 1.5 using 1N HCl. The samples were centrifuged at 8000g for 30 minutes. The supernatant was weighed and its protein content was determined using the Kjeltec. The protein yield was thus calculated and compared with samples centrifuged at 4000 g. The results are summarised in Table 7.8

Effect of centrifugation

RCF solution (%)	CMC conc (%)	Centrifuged at	Protein content (%)	Protein Yield (%)
	0.4		0.821	46.48
20	0.7		-	-
	0.4	8000g	1.796	70.01
25	0.7		1.496	45.60
	0.4		0.743	33.86
20	0.7	4000 g	-	-
	0.4		1.669	60.80
25	0.7		1.023	37.31

Table 7.8

At higher speeds of centrifugation there is a general increase in protein yield of the decolourised samples. As can be seen in Table 7.8, the 25% RCF decolourised with 0.4% CMC and centrifuged at 8,000 g had a yield of 70% compared with the similar sample centrifuged at 4,000 g which had a yield of 61%. On average about 8-10% better protein yields were achieved using higher centrifugation rates. The difference in yields were not however considered to be sufficiently great and subsequent experiments were carried out in an MSE centrifuge at a speed of 1150 g.

7.4 Changing other parameters

The first parameter examined was the addition of CMC. A 25% RCF solution was prepared, 400 ml total volume, and acidified to pH 1.5 using 1N HCl and was added to a solution of 2% (w/v) CMC. This was equivalent to adding one volume of CMC at five times its usual concentration.

The mixture was left to react undisturbed for fifteen minutes at ambient temperature. It was centrifuged at 1150 g, with some small aliquots being spun at 4000 g, but in both cases no clear CMC-haem precipitate was formed. Centrifugation was performed for 15 minutes.

A similar procedure was followed for a further three solutions of RCF (25%). CMC concentrations of 1%, 1.5% and 2.5% (w/v) were added. The CMC solutions were added at a ratio of one volume of CMC to one volume of acidified RCF solution. No precipitation of CMC-haem was observed at either 2000g or 4000g.

It appeared that decolourisation was not effected by using less volumes of higher concentration CMC solutions.

The other parameter examined was the pH at which hydrolysis occurred. Three 200 ml of 25% RCF solutions were prepared, and acidified using 1N HCl to a pH value of 2.0, 2.5 and 2.8 respectively. To each acidified solution 600 ml of 0.4% CMC solution was added. The mixtures were left undisturbed for 15 minutes and then centrifuged at 2000 and 4000 g for 15 minutes.

The mixture which contained the RCF solutions acidified to pH 2.8 and 2.5, did not decolourise. No clear CMC-haem precipitate was formed. The samples were centrifuged for a further fifteen minutes at 4000 g, but still no precipitation occurred.

The mixture containing the RCF solution acidified to pH 2.0 did react. After centrifugation the supernatant was examined visually and its protein yield was recorded. The colour of the supernatant was dark brown, scoring 8 on the arbitrary colour scale. The protein yield was approximately 40%, compared with 61% of the same solution treated with 0.4% CMC but acidified to pH of 1.5

Thus a much lower protein yield and a product with a very dark colour was achieved. It was decided that the optimum pH of hydrolysis was 1.5

An attempt to dispense with the centrifugation step was tried. A 20% RCF solution was acidified to pH 1.5 using 1N HCl The solution was then reacted with a 0.6% CMC solution addition of 3 volumes of CMC to one volume of acidified RCF. The mixture was left to react for 15 minutes. A flocculant of CMC with the solution was formed. The whole mixture was squeezed through a cheese mesh. The red transparent liquid obtained was vacuum filtered through a Number 1 Whatman paper. The filtrate collected was of light red colour. To this solution 1N NaOH was added to bring the pH to 7. This had the effect of precipitating out the protein as light yellow brown particles.

A solids determination of this mixture at pH 7.0, gave a solids content of approximately 1% and a protein content of 0.35% i.e. protein recoveries were very low. The colour was also unsatisfactory although the experiment did indicate a"bleaching" effect taking place when the pH of the mixture was altered to pH 7.0. Therefore it was deemed necessary to centrifuge the reaction mixture to achieve the best results.

7.5 Solubility of the decolourised red cell fraction

Titration curves were determined for untreated red cell fraction and for decolourised red cell fraction. The RCF used was 0.25% (v/v) solution, total volume 50 ml. The decolourised RCF was prepared as follows. A 25% RCF solution was acidified to pH 1.5 with 1N HCl and added to a 0.6% CMC solution. The volume of CMC solution added was three times the volume of the acidified RCF giving a total volume of 100 ml

Both solutions were titrated against 0.1N HCl and 0.1N NaOH. The titration curve for the untreated RCF is shown in Figure 7.6. The titration curve for the decolourised sample is shown in Figure 7.7.

For the DRCF sample, it can be seen from Figure 7.7 that an adjustment of about 5% with 0.1N NaOH is necessary to bring the pH of the product to 3.0, (the necessary minimum pH for most ultrafiltration membranes). An addition of between 15-20% of 0.1N NaOH is required to bring the product to a neutral pH. Also precipitation of proteins takes place when the solution reaches a pH of 5.0.

The solubility of DRCF, prepared as described above, was determined as follows:

The percentage protein of the DRCF was determined by Kjeldahl. This gave an average value of 1.39% protein.

Twelve samples, volume 20 ml each were placed in sample test tubes. The pH of the samples was adjusted to range from 2.8 to 12.7 with 6N NaOH. Concentrated sodium hydroxide was used to keep the volume added to a minimum. The samples were left for one day to stabilise. Readings of the pH's were taken again, the samples

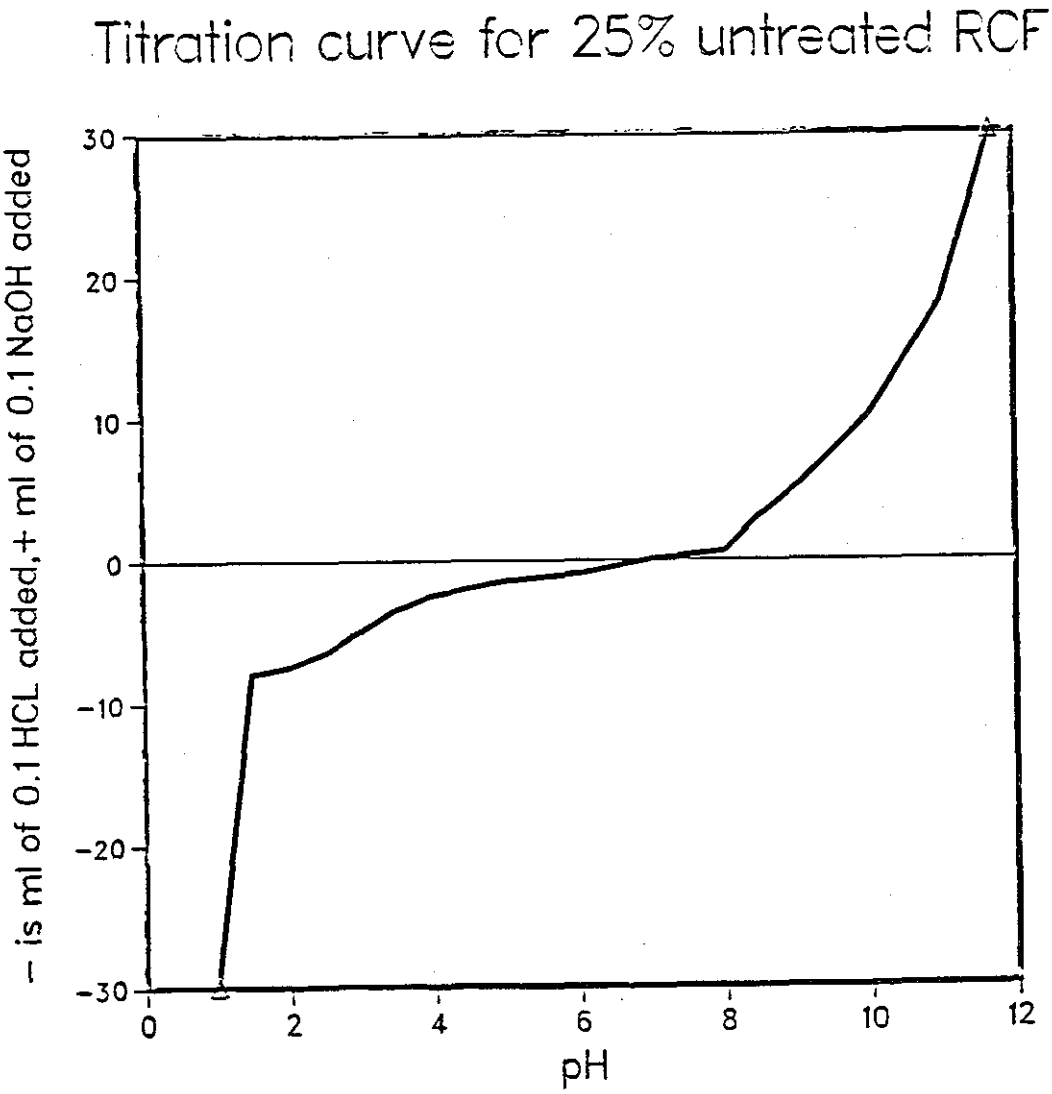

Titration curve for 25% untreated RCF
- is ml of 0.1 HCL added,+ ml of 0.1 NaOH added
30
20
10
0
-10
-20
-30
0
2
4
6
8
10
12
pH

Figure 7.6

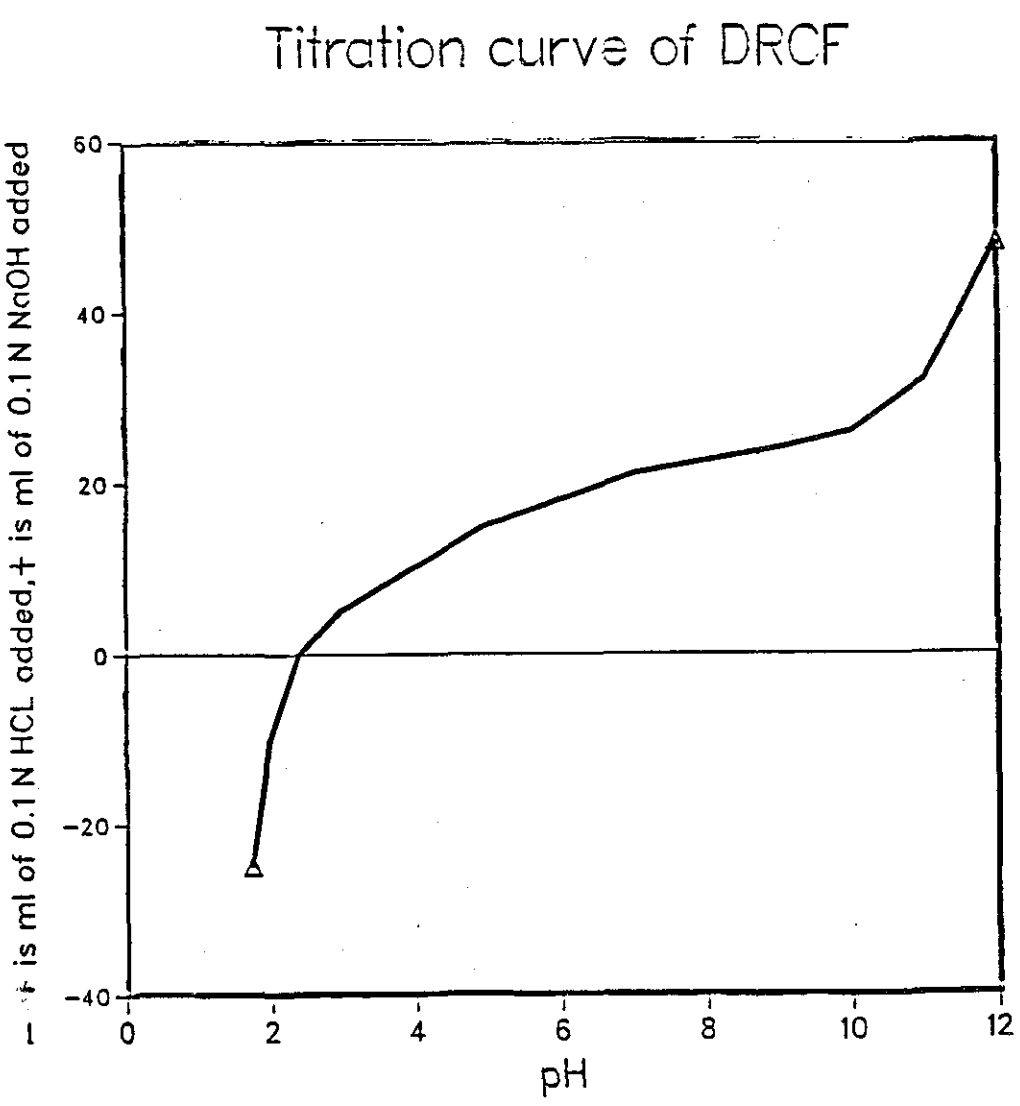

Titration curve of DRCF
60
40
20
0
-20
-40
0
2
4
6
8
10
12
pH
- is ml of 0.1 N HCL added,+ is ml of 0.1 N NaOH added

Figure 7.7

were thoroughly mixed and 10 ml aliquots were taken and placed in centrifuge tubes, which had been cleaned, dried and weighed prior to loading.

All twelve tubes were centrifuged at 3000 g. for 25 minutes on the bench centrifuge. The supernatants were decanted and the tubes and precipitates were placed in the oven, at 100°C for 24 hours. The tubes and precipitates were then weighed and the percentage protein calculated.

The average moisture of the precipitates was 2.5% after drying. The initial percentage protein content was 1.39% as mentioned above. The results are summarised in Table 7.9.

The above procedure was repeated for a further twelve samples but left to stabilise for two days. The results are summarised in Table 7.10.

From the tables it can be seen that the pH of the samples does not alter to any significant extent over the 2 day period. Also between pH 7 and 8 most precipitation occurred. Maximum protein precipitation (Table 7.9) was 82.0% at pH 8.3. When the samples were left to stabilise for 48 hours maximum precipitation (68.3%) occurred at pH 7.2. Precipitation started at a pH value of 5.5 for both sets of samples.

The results are represented graphically in Figure 7.8.

Solubility of DRCF I

pH value Day 1	Day 2	Weight of precipitate (gr)	Protein content of precipitate (%)
2.8	2.9	0.000	0
3.9	3.9	0.000	0
4.9	4.8	0.003	2.2
5.8	5.9	0.021	15.1
6.3	6.4	0.091	65.5
7.2	7.2	0.103	74.0
7.6	7.7	0.108	78.0
8.3	8.1	0.114	82.0
10.0	10.1	0.029	20.0
11.2	11.3	0.007	5.0
12.2	12.3	0.005	4.3
12.7	12.7	0.004	2.9

Table 7.9

Solubility of DRCF II

pH value Day 1	Day 3	Weight of precipitate (g)	Protein content of precipitate (%)
3.2	3.3	0.000	0
3.6	3.6	0.001	0
4.3	4.2	0.009	1.80
5.3	5.1	0.003	2.20
5.5	5.7	0.0032	23.0
5.9	5.9	0.084	60.4
7.2	7.1	0.095	68.3
9.4	9.3	0.081	58.3
10.4	10.4	0.029	20.1
10.7	10.8	0.002	1.90
11.8	11.8	0.002	0
12	12.3	0.000	0

Table 7.10

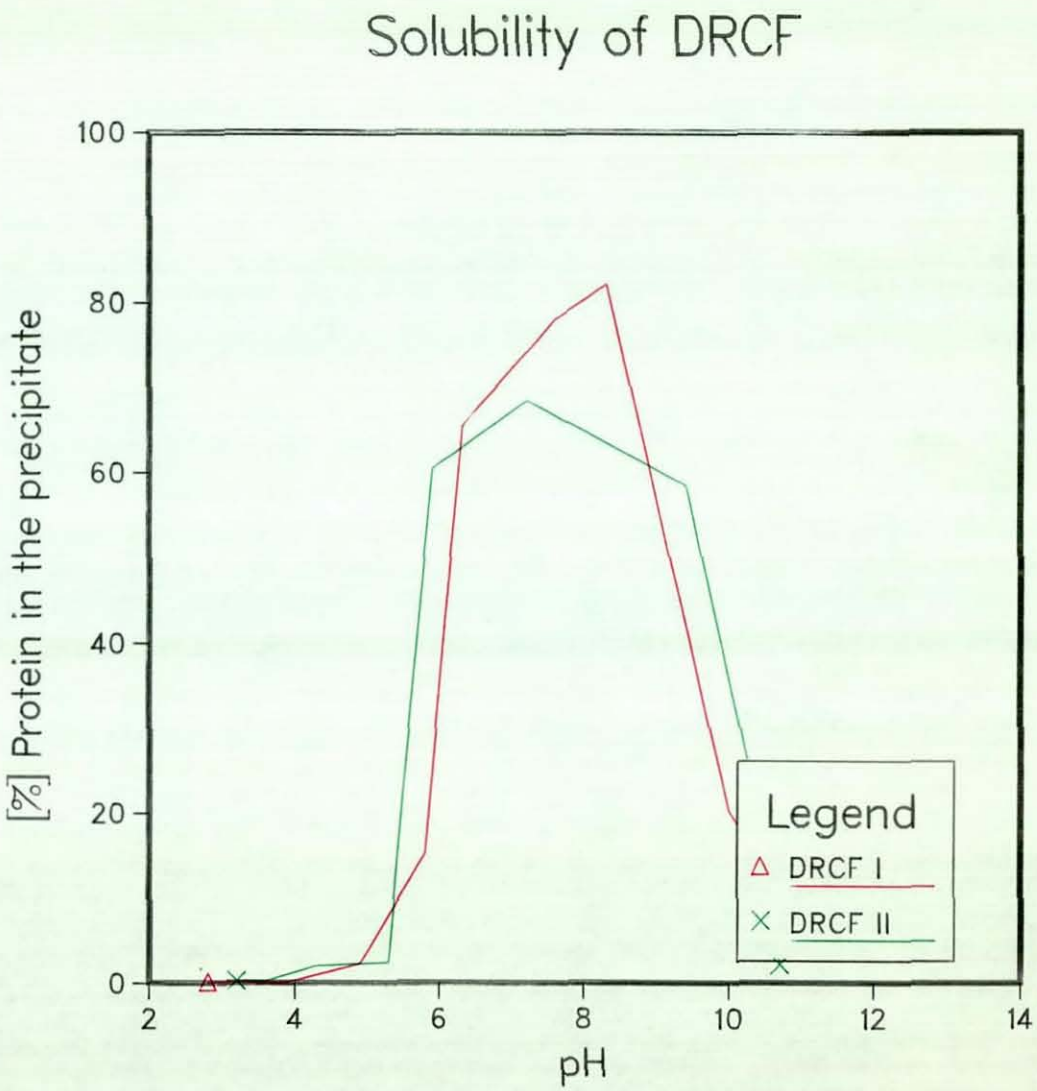


Fig. 7.8

Figure 7.8

7.6 Ultrafiltration of decolourised red cell fraction using the Amicon ultrafiltration unit

The solution used was prepared as follows. A 25% RCF was acidified to pH 1.5 and three times its total volume of CMC (0.4% (w/v) aqueous solution) was added. The mixture was allowed to react for fifteen minutes and then centrifuged for fifteen minutes at 4,000 g. The supernatant was collected. The pH of supernatant was adjusted with 1N NaOH to 3.0.

The Amicon unit was used with a PM30 and a PM10 membrane.

In the first run the PM30 membrane was used. The operating pressure was 0.17×10^{6} NM^{-2} and the area of the membrane was 63.62 cm^2. Three solutions were ultrafiltered: water at 39°C, the decolourised solution at 39°C, and the same solution at a slightly higher temperature of 45°C. The results are shown in Figure 7.9. The run for each solution lasted 140 minutes.

Nitrogen analysis on the decolourised samples showed that after 140 minutes the protein concentration of the solution, was 5.3%, a substantial increase from the original 1.7%.

The membrane was then changed and a PM10 membrane was fitted. This has a nominal molecular weight cut off point of 10000. Three solutions were run with this membrane. The decolourised solution at temperatures of 39°C and 45°C and a plasma solution containing 1.7% protein at 39°C. The results are shown in Figure 7.10.

The flux achieved by the decolourised solution at 39°C and 45°C is not significantly different. If a higher temperature is used a very slight increase in flux occurs. Also from Fig. 7.10 it can be seen that the 1.7% plasma solution behaved very similarly to the decolourised product.

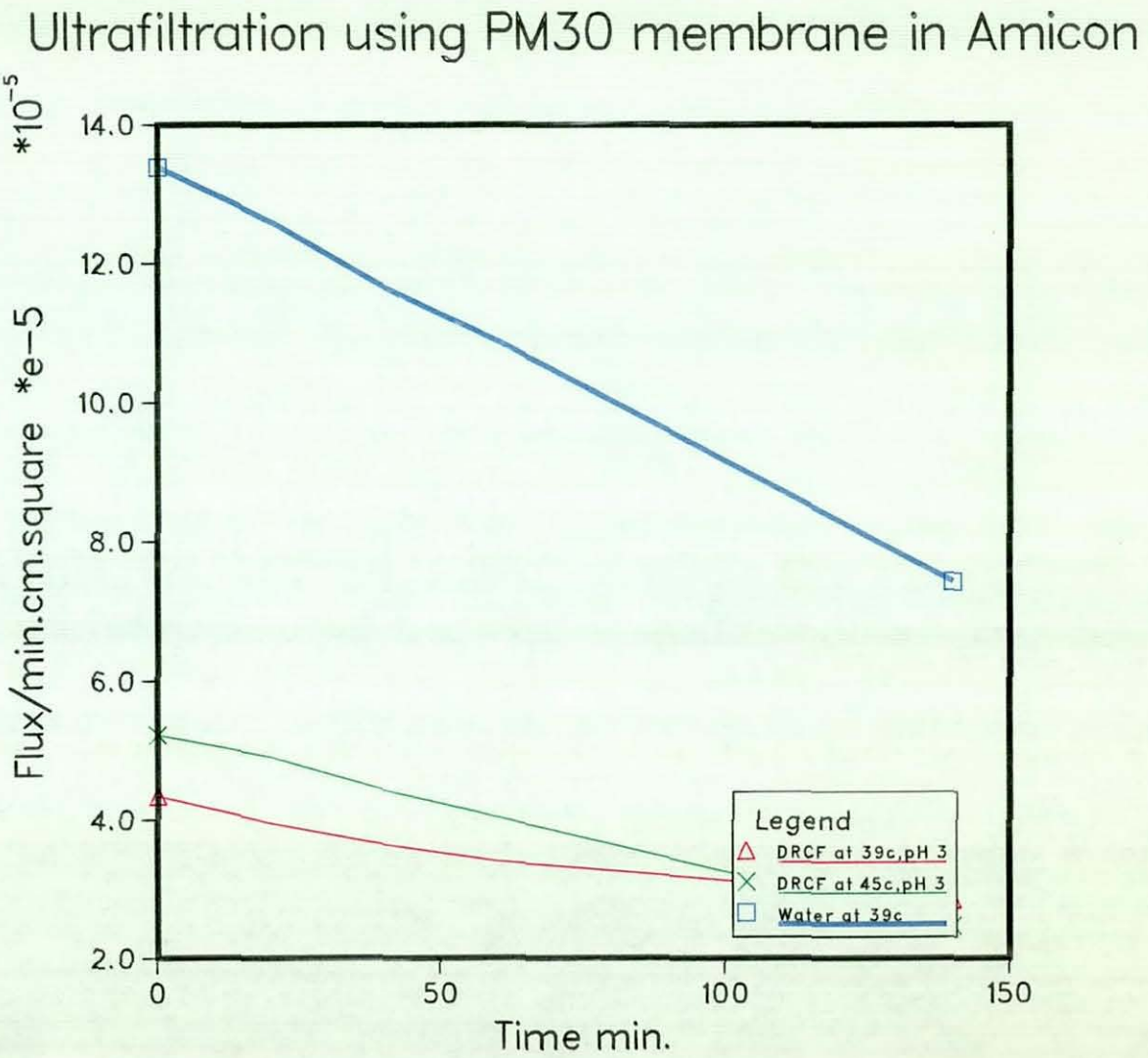

Ultrafiltration using PM30 membrane in Amicon
Flux/min.cm.square *e-5 *10^-5
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
50
100
150
Time min.
Legend
DRCF at 39c,pH 3
DRCF at 45c,pH 3
Water at 39c

Figure 7.9

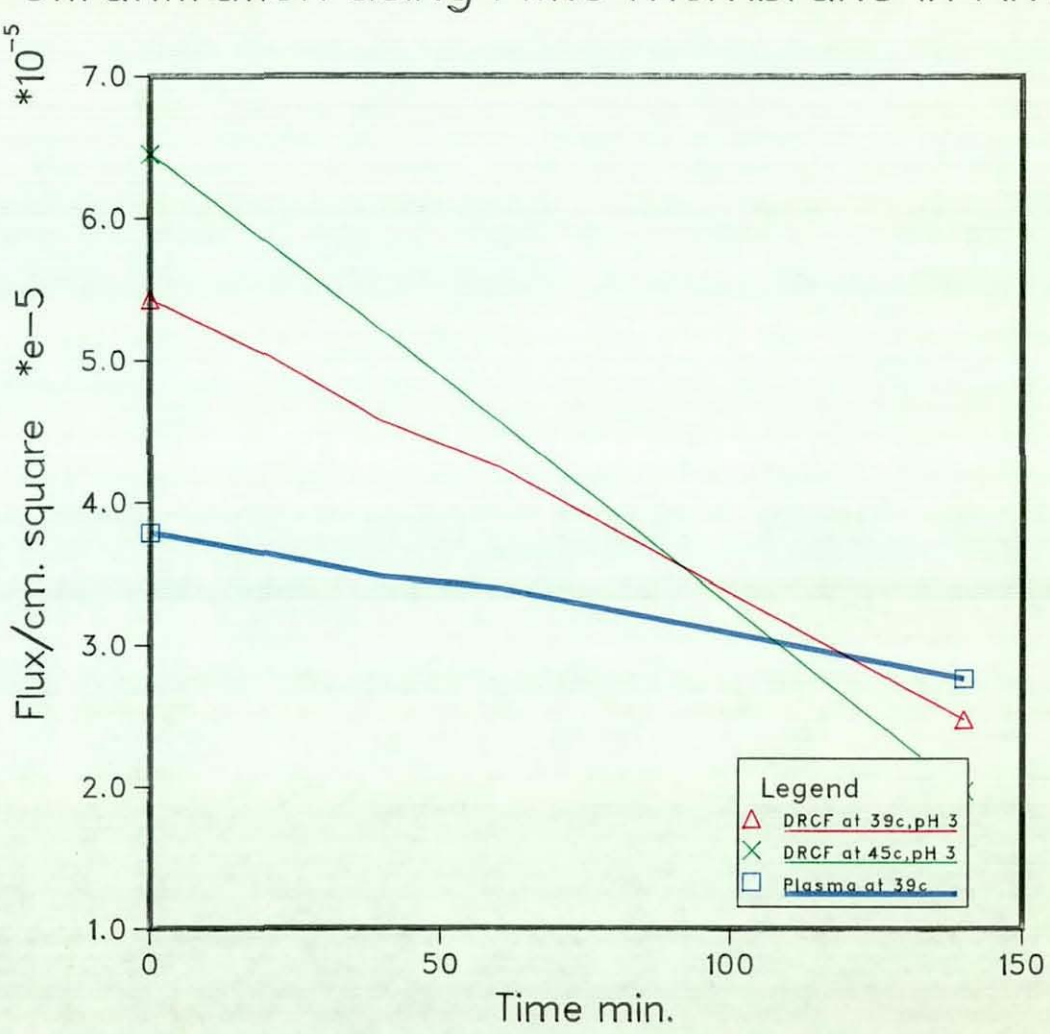


Fig. 7.10

Figure 7.10

7.8 Spray drying of solutions

A series of powders were produced by using a combination of CMC and the pH swing method. The procedure was as follows. An aqueous solution of red cell fraction was prepared and acidified to pH 1.5 using 1N HCl. It was then mixed with CMC solution, concentration 0.4 or 0.6 % (w/v), and then centrifuged for twenty five minutes at 1150 g using the MSE equipment. The precipitate was then filtered off using cheese cloth mesh. The filtrate was collected and its pH adjusted with 1N NaOH to pH 7.5 where maximum precipitation of protein occurred.

At this point some solutions were homogenised using a Silverson emulsifier.

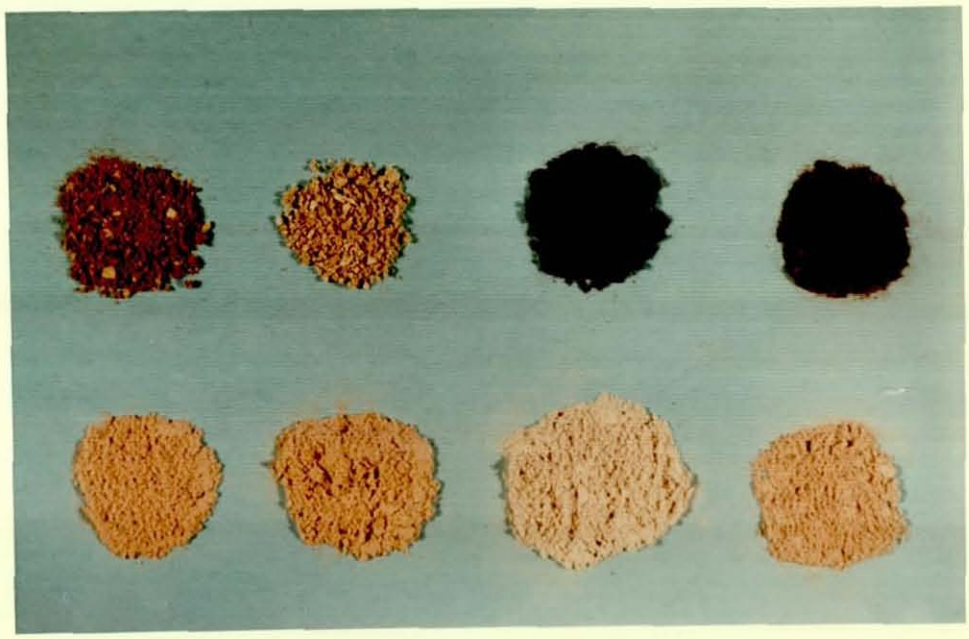
The neutral solutions were then spray dried. The powders were examined for protein content, particle size analysis, and on the most promising, functional tests were performed.

The solutions had a total volume of six litres prior to centrifugation. The results are summarised in Table 7.11. The powders produced were photographed also with a sample of spray dried, untreated red cell fraction, and two hydrogen peroxide treated samples. The photograph is shown in Figure 7.11.

The protein content of the powders was between 60-70%, with the lowest value given by the 33% RCF solution. The samples which were mixed using the Silverson produced very fine powders. This was also reflected by the particle size analysis results. Figure 7.12 shows the results of the analysis of two powders 20% and 25% RCF decolourised by 0.4% CMC. The 25% RCF was mixed using the Silverson.

Spray dried protein samples

1. 20% RCF + 0.4% CMC
2. 20% RCF + 0.6% CMC
3. 25% RCF + 0.4% CMC
4. 25% RCF + 0.6% CMC
5. 33% RCF + 0.6% CMC
6. Spray dried RCF untreated
7. 'Bleached' RCF by H_2O_2 method
8. 'Partially bleached' RCF by H_2O_2 method



1 2 3 4

5 6 7 8

Figure 7.11

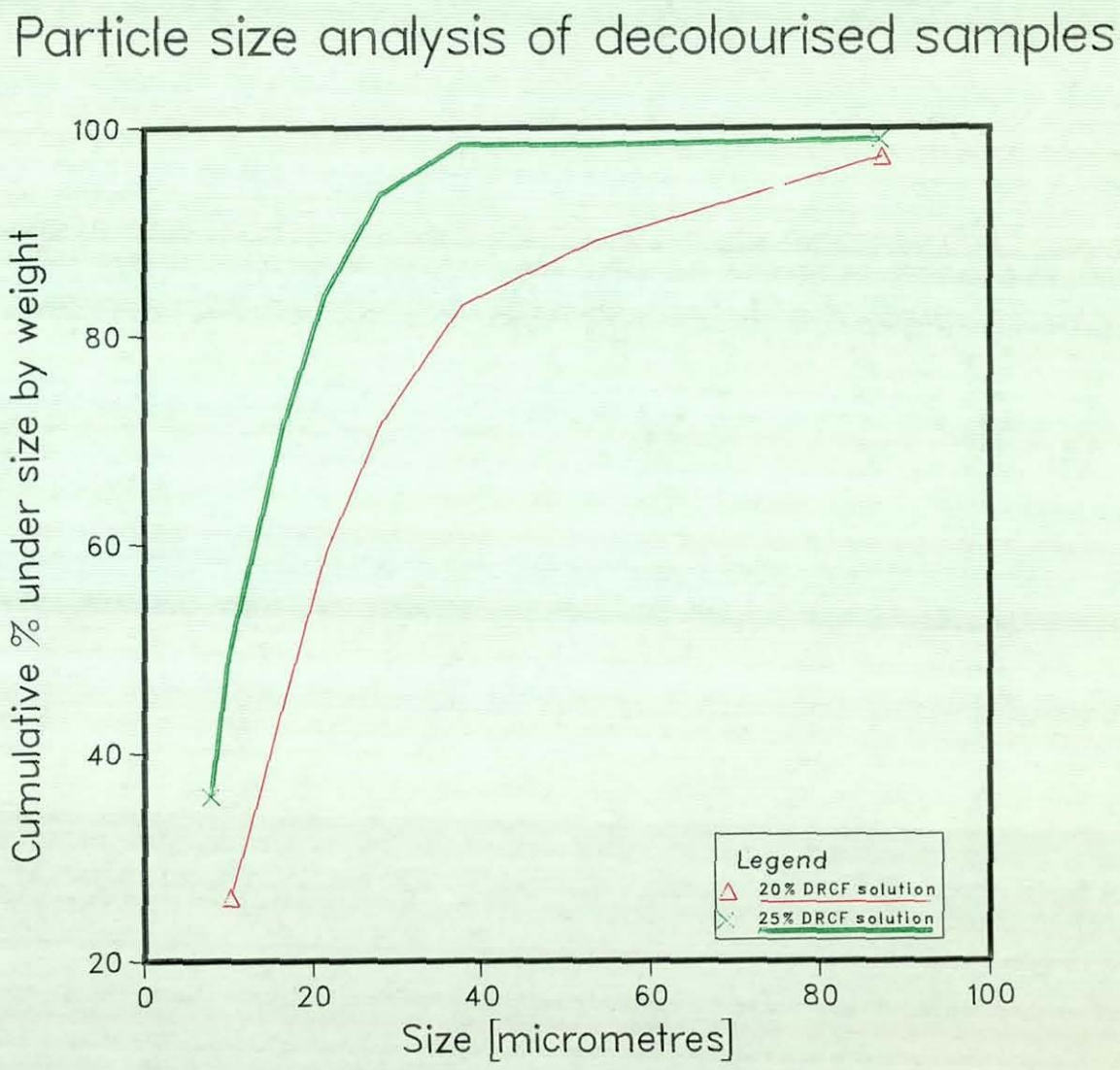

Particle size analysis of decolourised samples
Cumulative % under size by weight
100
80
60
40
20
0
20
40
60
80
100
Size [micrometres]
Legend
20% DRCF solution
25% DRCF solution

Figure 7.12

The results shown in Figure 7.12, indicate 50% of the particles of this powder have a size less than 8 μm, while in the 20% powder which was not mixed only 15% have a size less than 8 μm.

The most decolourised powder was produced, as expected, from a 20% RCF solution decolourised by 0.6% CMC, Figure 7.11.

A set of functional tests were performed on the 20% and 25% powders decolourised by 0.4% CMC. As a control a 25% RCF powder decolourised using 0.1% CMC was produced in the pilot plant (see section 4.5.2) and tested for functionality. This powder of rather dark brown colour was produced from a solution which had its pH unaltered. The results of the functionality tests are summarised in Table 7.12. It was clear that the powders produced from the solutions of pH to 7.5 have less functionality. It would appear that the spatial configuration of the protein had been altered by the sodium hydroxide, resulting in poor functionality. Hence the pH swing method will not produce a powder of significant commercial value. A slight adjustment of the decolourised globin solution to pH 3.0 will then only be necessary for the ultrafiltration step.

Spray drying of decolourised red cell fraction

Red cell fraction solution % v/v	CMC conc. % w/v	Silverson used (5 minutes)	Protein content % (average)
20	0.4	No	67.82
20	0.6	Yes	63.20
25	0.4	Yes	65.82
25	0.6	No	69.60
33	0.6	No	60.93

Table 7.11

Functionality tests on powders produced

Powder nature	pH (10% soln)	Solubility (% Unsoluble material)	Gelation	Foaming (after 60 min)	Fat binding (ml/g)	Emulsion (g)
20% RCF + 0.4% CMC	7.5	55.05	None	48%	1.480	None
25% RCF + 0.4% CMC Silverson used	7.2	64.03	None	51%	1.285	None
25% RCF + 0.1% CMC	2.4	62.10	442	55%	1.550	1.21

Table 7.12

The RCF which was decolourised with 0.1% CMC, and had a pH of 2.4, produced an emulsion, (Table 7.12) with 1.21g of oil being needed to break 25 g of emulsion.

Also, this decolourised protein produced a weak gel, value 442. (Table 7.12.) refer to section 4.

7.9 Mass balances on the spray dried product

A 25% RCF solution was acidified to pH 1.5 using HCl acid and centrifuged in the MSE centrifuge for 10 minutes at 1150 g . The quantity was prepared as follows:

2.7 litres of RCF were added to 8.3 litres of water. The solution was acidified with 37.5g of 1NHCl acid.The CMC solution was prepared as follows: 33.33 litres of H_2O was heated to 60°C and 200 g of CMC powder was dissolved into it (i.e. a 0.6% (w/v) solution).

The two solutions were mixed and left to stand for 15 minutes. The mixture was then divided into 600 ml plastic centrifuge containers and centrifuged. The supernatant was collected after it was filtered through a cheese mesh cloth. The pH of the supernatant was adjusted to 7.0 by 2N NaOH, the addition being 0.64g of NaOH per litre of supernatant.

The whole mixture was placed in the Silverson and mixed for two minutes. A sample was taken to be analysed for solids and protein content. The rest of the mixture was spray dried.

Samples were also taken from the initial acidified solution with CMC added, the precipitate of CMC-haem from the centrifugation, and of the solution prior to spray drying. The final spray dried powder was also analysed for protein and moisture.

1. Initial solution

2.7 litres RCF	Protein	Protein	Solids
4.24 litres Water	(%)	(g)	(%)
200 g CMC	2.54	947.7	2.79
37.5 g HCl			

2. Centrifugation

2.1 Precipitate (CMC-haem)	Moisture	Protein	Protein
6333.8 g water	(%)	(%)	(g)
200.0 g CMC			
466.2 g Protein	90.48	6.66	466.2

2.2 Supernatant	Moisture	Protein	Protein
	(%)	(%)	(g)
35120 g water	98.5	1.37	481.5
481.5 g protein			

3. Neutralisation	Solids	Protein	Moisture
	(%)	(%)	(%)
35120 water			
481.5 g protein	1.69	1.37	98.31
22.5 g NaOH			

This solution was spray dried.

4. Final product analysis

Total powder collected = 300 g

Protein		Moisture		Ash	
(%)	(g)	(%)	(g)	(%)	(g)
75	225	4	12	21	63

Initial protein content = 947.7 g
Final protein content = 225.0 g

Percentage protein recovery = 23.7%

The CMC-haem precipitate contains 466.2 g of the protein or 49.2% of the total. This is a highly proteinaceous product. However this protein content will decrease when high speed centrifuge is employed.

The low percentage protein recovery can be also attributed to another reason. The minimum solids content for efficient use of the spray drier is around 15-20%. The solution which was spray dried contained only 1.65%.

7.10 Amino acid analysis of the decolourised RCF by the CMC method

A 25% RCF was acidified to pH 1.5 with 1N HCl. Three times its total volume, of a 0.4% CMC solution was added. The mixture was centrifuged at 3030 g for 15 minutes. The supernatant was collected and analysed using the Amino acid analyser described in Chapter 4.

The printout obtained is shown in Figure 7.13. The concentration of each amino acid in the solution is given in table 7.13. The print out obtained is very similar to untreated RCF and RCF decolourised by hydrogen peroxide. The decolourisation process developed does not seem to breakdown amino acids in the haemoglobin molecule.

Amino acid profile of 25% RCF decolourised by 0.4% CMC

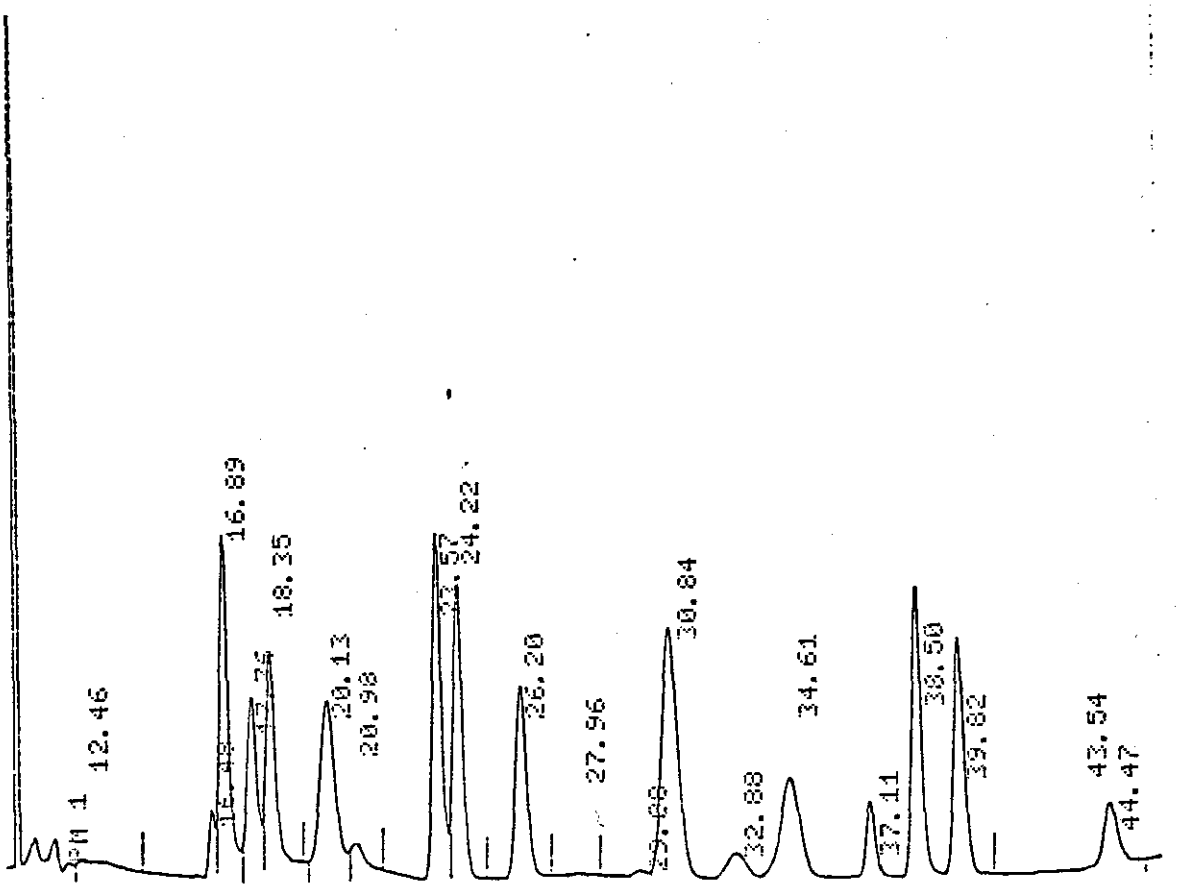


Figure 7.13

Amino acid profile of a 25% RCF solution decolourised by 0.4% CMC solution

Amino acid	Retention time (RT)	Concentration of amino acid in solution (µg/ml)
Aspartic	16.89	6.855
Threonine	17.76	3.201
Serine	18.35	2.666
Glutamic	20.13	5.337
Proline	20.98	1.855
Glycine	23.57	3.112
Alanine	24.22	6.094
Valine	26.20	5.795
Methionine	27.96	0.071
Isoleucine	29.88	0.239
Leucine	30.84	8.845
Tyrosine	32.88	1.243
Phenylalanine	34.61	5.624
Ammonia trace	37.11	2.451
Lycine	38.5	6.649
Histidine	39.82	4.715
Arginine	44.42	2.732

Table 7 .13

CHAPTER 8

Process and Economic Analysis of a Plant Capable of Producing Globin from Whole Blood Using Carboxymethyl Cellulose

8.1 Process description

The process description and technical assessment of the method using CMC for decolourisation will now be discussed.

1. Blood collection unit. Suitable production units for collecting 750 litre/yr to 150 tonnes/yr of blood are available from a series of companies such as Alfa-Laval, Nutridan Engineering, Bruendler International A.G. and Simon Foods Ltd.

Throughput is limited by two factors (i) The one minute bleeding time per carcass. (ii) A minimum of thirty minutes 'holding time' while the carcasses are inspected.

Thus the blood collection unit capacity needs to be large enough to handle large throughputs of cattle in cases of temporary shut-down or extended holding times. Installation of a secondary holding tank, into which the temporary holding tanks drain, of a sufficient size to absorb large volumes of blood resulting from high slaughter rates will allow the blood processing plant to operate at a constant rate of throughput.

2. Separation of the plasma and RCF

This can be easily achieved by a low rate centrifuge. The proportion of plasma to RCF collected depends on the processing conditions. In this model an average value of 35% for the red cell fraction has been chosen giving the following composition:

Water	60.8%
Protein	35.1%
Salt	1.1%
Other	3.0%

3. Decolourisation using CMC

This is achieved using the process described in Section 7, and in the Autio patent.

4. Separation of the CMC-haem fraction from Globin

A high rate centrifuge operating at 8000g for thirty minutes is employed to separate the two fractions. The globin fraction i.e. the supernatant, contained 1.6% solids and its specific gravity was assumed to be 1.

The CMC-haem fraction contained 25-35% solids.

The supernatant was adjusted with NaOH to pH 3.0.

5. Ultrafiltration stage

A simple plate heat exchanger was necessary to heat the globin to 45°C to facilitate optimum flux rates for the U.F. stage.

The globin solution was concentrated to 25% solids, the optimum for spray drying. The optimum conditions for ultrafiltration of globin, and operating constants are derived from Hurst (1981).

6. Spray drying of protein

This method of drying minimises protein damage. The final product had a light beige/cream colour and contained 95% solids and 5% water.

7. Packaging and storage

The dried product was slightly hygroscopic and must be packaged in an impermeable material. A plastic, or plastic lined sack, is ideal. Low volumes of product are likely, therefore manual filling is suggested.

The CMC-haem fraction contains 80-90% solids, and can be sold for animal feed. It can be packaged in large sacks without further processing.

8. Labour requirements

In this economic model a supervisor, two labourers and a technician, working part time, are required to run the plant.

Figure 8.1 shows the process flow diagram of the plant.

8.2 Mass balance calculation

The mass balance calculations required to size up the equipment needs of the decolourisation plant is computed now.

The overall calculations are based on a kill rate of twenty cattle per hour and a blood yield of eleven litres per cattle head.

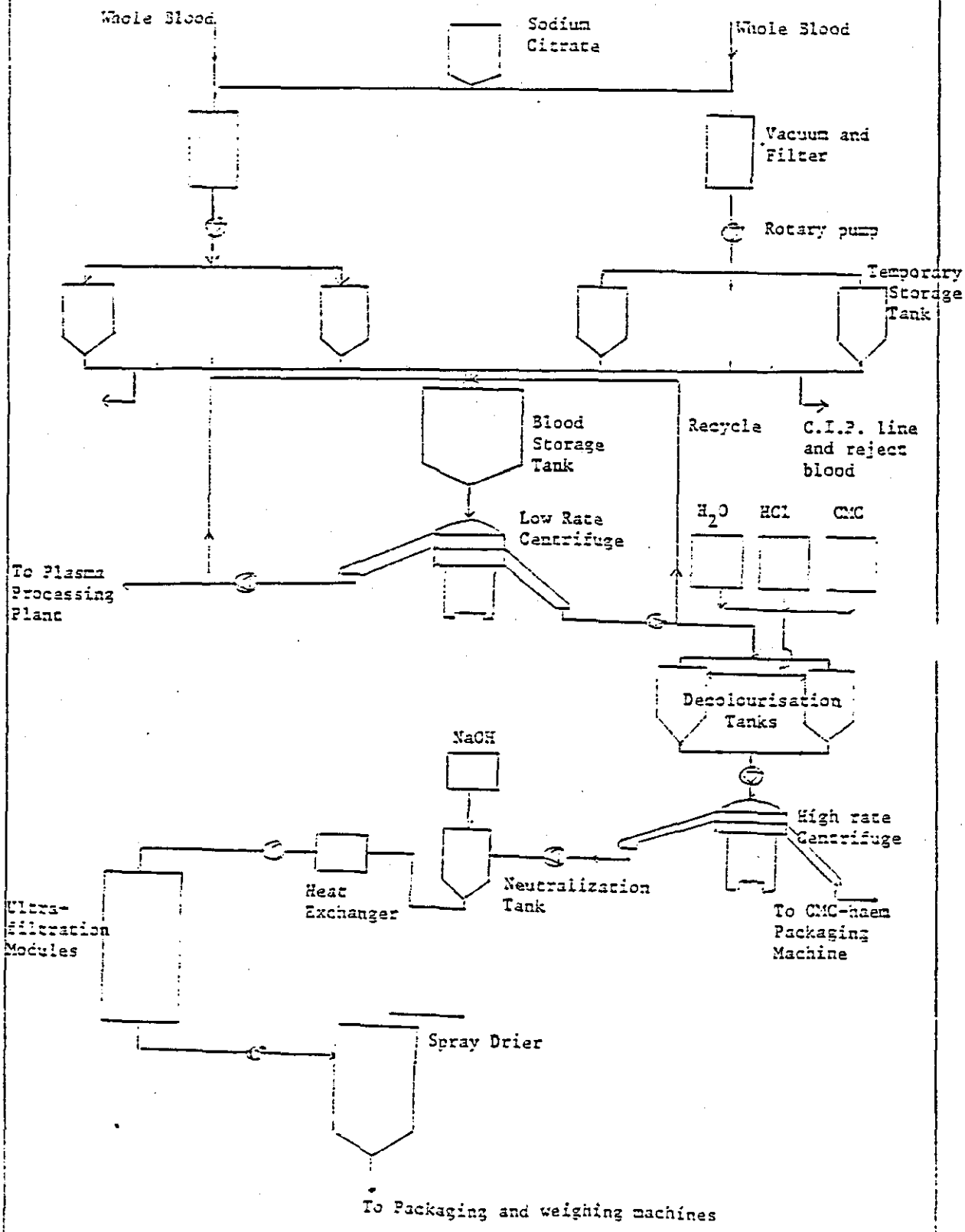
8.2.1 Aseptic collection

Assumptions:

(i) Working week = 35 hours

(ii) Required concentration of sodium citrate 1% in blood

Figure 8.1 Process Flow Diagram



(iii) Maximum 800 carcasses per working week.

(iv) Concentration of sodium citrate solution 40% (w/v)

(v) Addition of sodium citrate in blood is 40% (v/v)

Blood yield per hour = $\frac{800}{35}$ x 250 litre/hr.

Volume of sodium citrate added x; required concentration 1% is

$$x = 0.1\ (250 + x)\ \frac{100}{40} = 6.3 \text{ litre/hr}$$

Volume of blood leaving the collecting unit = 250 + 6.3 = 256.3 litres/hr

Mass flowrate of blood = 256 x 1.06 = 271.6 kg/hr

8.2.2 Centrifugal Separation

Assumptions:

(i) The fractions of the centrifuge are:
35% the red cell concentrate and
65% plasma

(ii) Protein concentration in the RCF is 35%

Quantity of plasma fraction = 256.3 x $\frac{65}{100}$ = 166.6 litres/hr

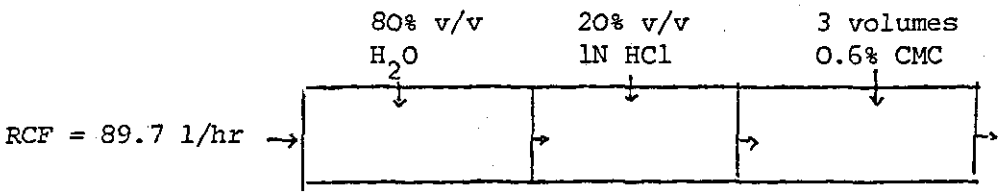
Mass flowrate of plasma = 166.6 x 1.034 = 172.3 kg/hr.

Quantity of red cell fraction = 256.3 x $\frac{35}{100}$ = 89.7 litres/hr

Mass flowrate of RCF = 89.7 x 1.064 = 95.4 kg/hr

Protein quantity of RCF = 95.4 x 0.35 = 33.4 kg/hr

8.2.3 Decolourisation



volume of added water = $89.7 \times \frac{80}{20} = 358.8$ l/hr

Volume of added 1N HCl = $89.7 \times \frac{20}{100} = 17.94$ l/hr

Volume of added CMC = $3(89.7 + 358.8 + 17.94) = 1399.3$ l/hr

Volume of decolourised blood leaving mixer
= 89.7 + 358.8 + 17.94 +1399.3 = 1865.8 l/hr

Mass flowrate = 95.4 + (358.8 + 17.94 + 1399.3) x 1 = 1871.7 kg/hr

Protein concentration = $\frac{33.4}{1871.7} = 1.78\%$

8.2.4 Centrifugation

Assumptions:

(i) Separation efficiency of centrifuge 95%

(ii) The fractions are split as follows, volume basis:

CMC-haem	10%
Globin	90%

The solution to be centrifuged is = 1865.8 l/hr

Volumetric flowrate of CMC-haem = $1865.8 \times \frac{10}{100} = 186.58$ l/hr

Volumetric flowrate of globin = $1865.8 \times \frac{90}{100} = 1679.2$ l/hr

Mass flowrate of globin = 1679.2 x 1 = 1679.2 kg/hr.

Weight of protein in globin fraction = 33.4 x 0.8 x 0.95 = 25.38 kg/hr

Protein concentration in globin $= \frac{25.38}{1679.2} \times 100 = 1.5\%$

8.2.5 Adjustment to pH 3.0

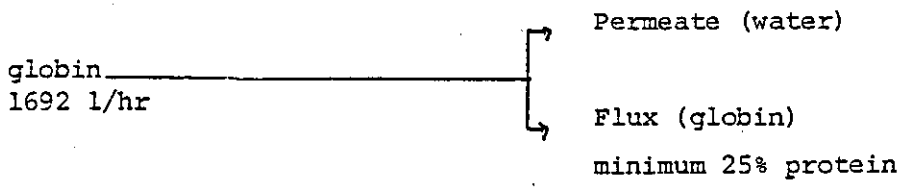
Amount of NaOH required to adjust solution is 0.77% on weight basis of total solution.

Quantity of NaOH required = 1679.2 x 0.77 = 12.92 kg/hr

Solution to be passed through the ultrafiltration plant is = 1679.2 + 12.92 = 1692 l/hr.

8.2.6 Ultrafiltration

Target protein concentration is 25% of the flux.



Protein in feed = 25.38 kg/hr

Mass flowrate of flux = $25.38 \times \frac{100}{25} = 101.5$ kg/hr

Amount of permeate = 1692 - 1015 = 1590.6 kg/hr

8.2.7 Spray drier

The globin powder contains 95% protein and 5% moisture.

Amount of protein in feed = 25.38 kg/hr

Mass flowrate of product = $25.38 \times \frac{100}{95} = 26.72$ kg/hr

Mass flowrate of water evaporated = 101.5 - 26.72 = 74.8 kg/hr

8.3 Equipment specification

The equipment specification is shown in Appendix II based on information compiled by Sakellariou (1981).

It will include (i) an aseptic collection unit, (ii) a low rate centrifugal separation unit of plasma and RCF, (iii) A high rate centrifugal separation unit for the globin and CMC-haem fractions, (iv) A heat exchanger, (v) Ultrafiltration plant (vi) Spray drier, (vii) Packaging machine, (viii) C.I.P Cleaning unit, (ix) Mixing tank and pumps and fitting, (x) storage facility, laboratory building.

8.4 Plant capacity and variability

The plant is designed with a capacity of

1. 800 cattle a week
2. 11 litres blood per carcass

A degree of flexibility has been written into the computer program to assess the profitability of different plant capacities of

1. 0 to 1000 cattle a week.
2. 5 to 15 litres of blood per head

The aseptic collection unit limits the capacity of the plant, allowing a maximum throughput of 40 cattle per hour. The capacity of the blood processing plant is limited by the spray drier.

Maximum plant capacity is 450 litre of whole blood per hour.

The number of ultrafiltration modules required is particularly sensitive to the capacity. The computer program adjusts the plant hardware to handle all capacities up to 450 l/hr of whole blood.

Figure 8.2 shows the proposed plant layout for the blood processing unit.

8.5 The computer program

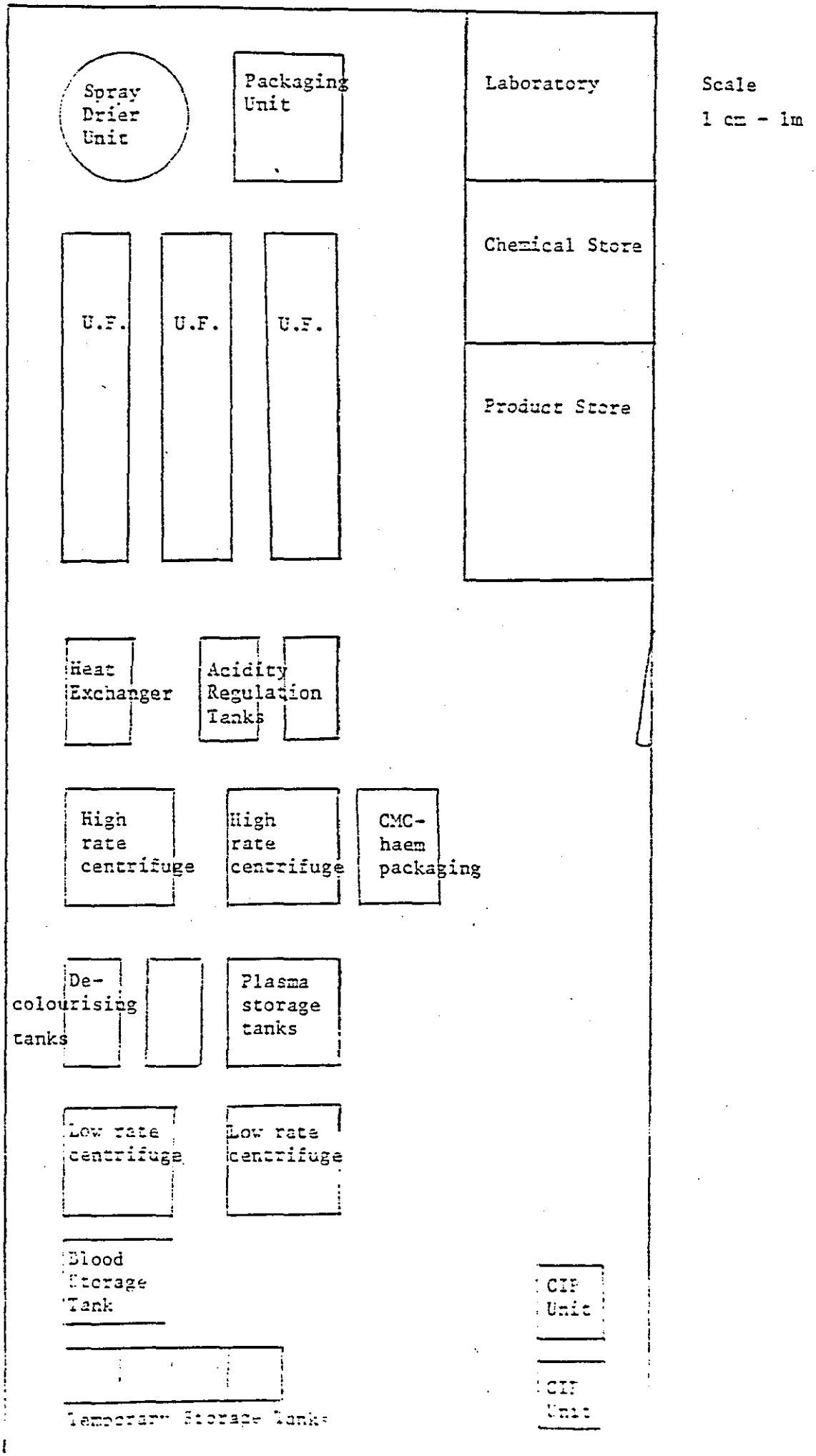
The computer program gives a complete process costing and sensitivity analysis for the CMC-decolourisation process.

The project life is assumed to be 10 years.

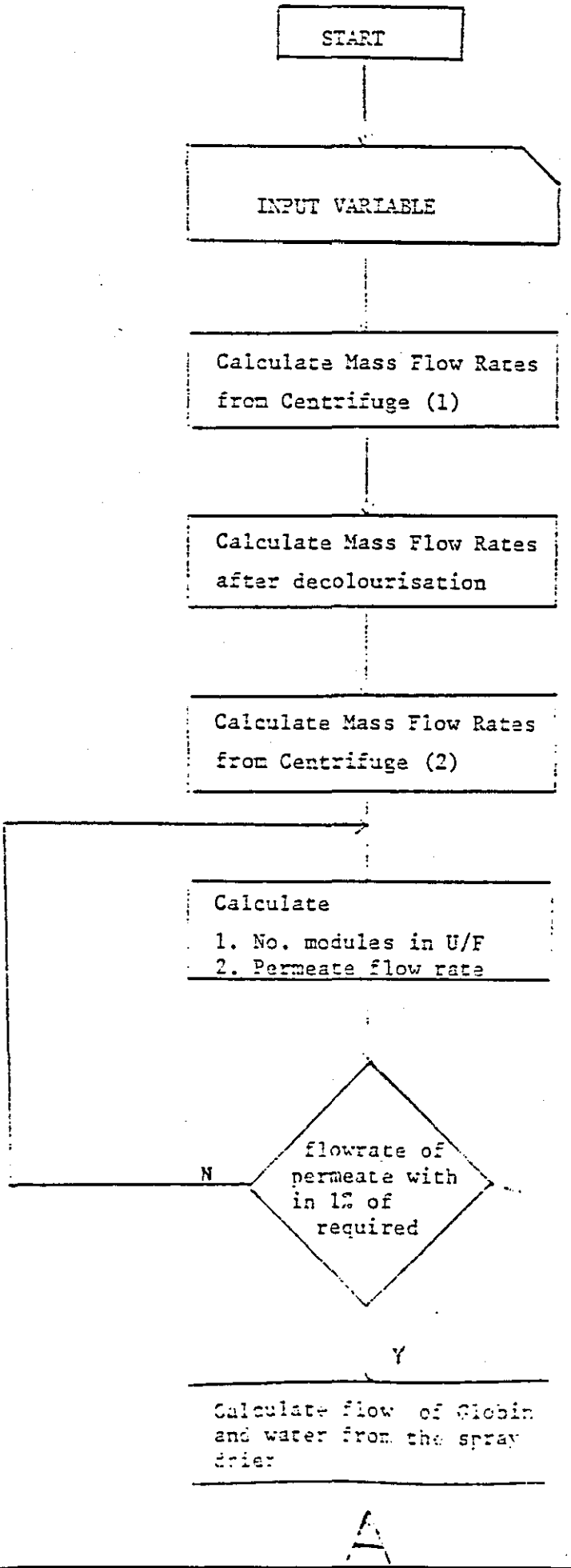
8.5.1 Users guide

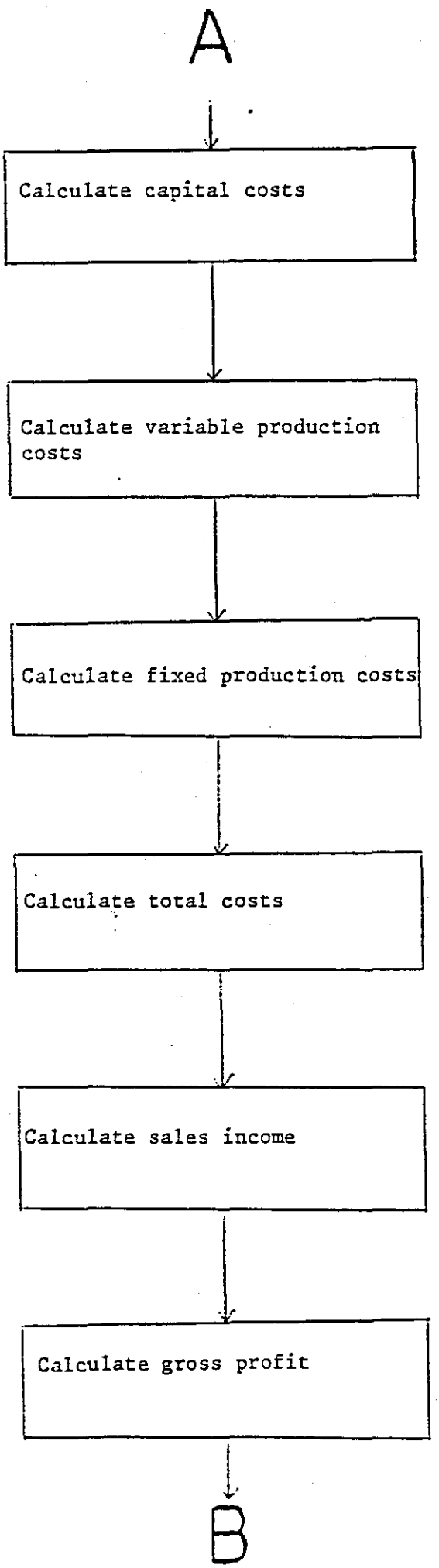
The computer flow diagram is shown in Figure 8.3. The program was developed in BBC basic and is stored on a 40k disc. Program title 'Jenny'.

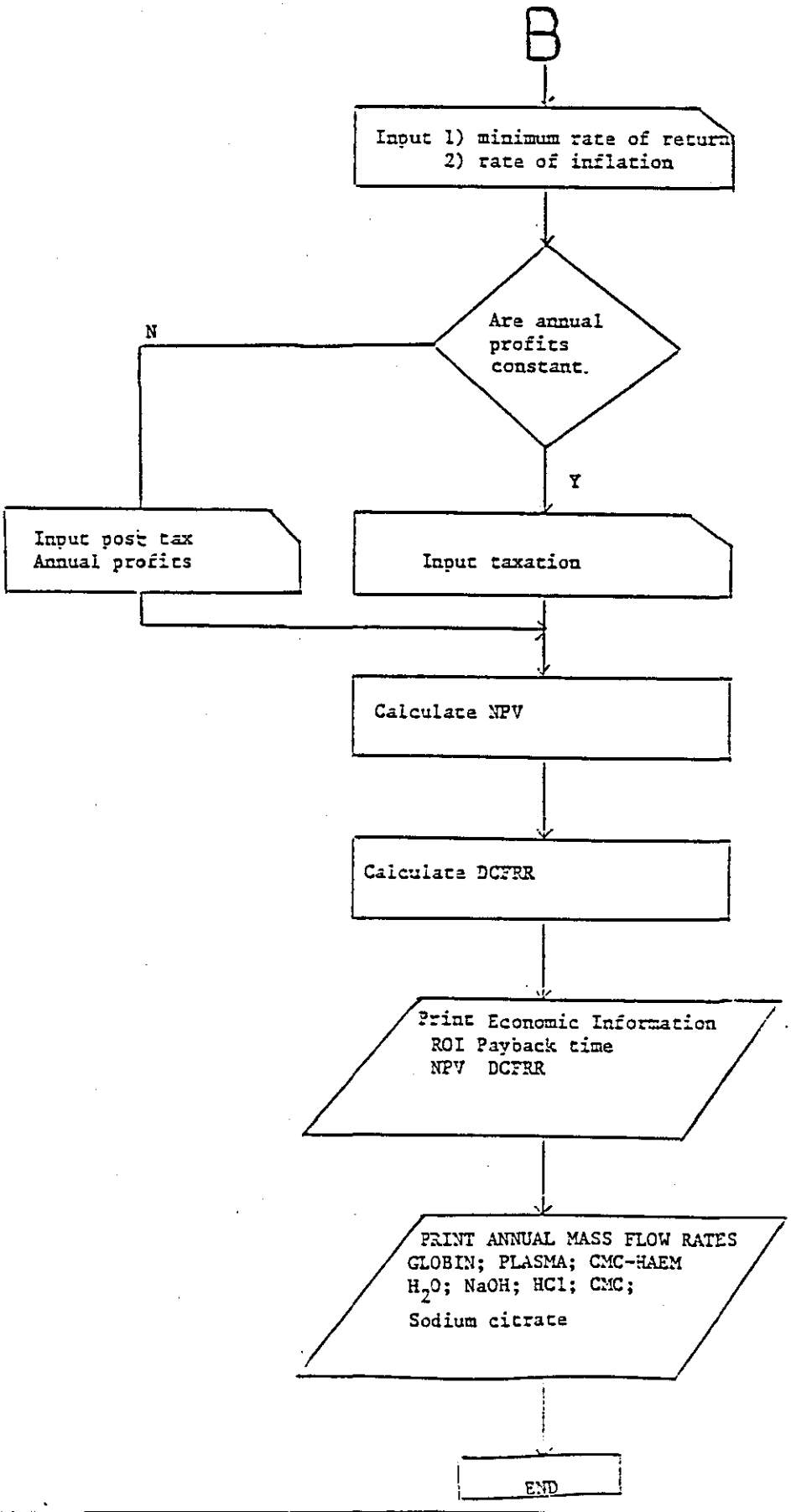
Figure 8.2 Plant Layout



COMPUTER FLOW DIAGRAM




A
Calculate capital costs
Calculate variable production costs
Calculate fixed production costs
Calculate total costs
Calculate sales income
Calculate gross profit
B


B
Input 1) minimum rate of return
2) rate of inflation
Are annual profits constant.
N
Y
Input post tax Annual profits
Input taxation
Calculate NPV
Calculate DCFRR
Print Economic Information
ROI Payback time
NPV DCFRR
PRINT ANNUAL MASS FLOW RATES
GLOBIN; PLASMA; CMC-HAEM
H_2O; NaOH; HCl; CMC;
Sodium citrate
END

Input Section

The programmer is asked to input the blood collecting process conditions and selling price variables. The recommended and limiting values of these variables appear in the Tables 8.1 to 8.5.

Table 8.1 Blood Collection Variables

	Minimum	Maximum	Recommended
Heads of cattle killed/hr	10	30	20
Blood yield (l/animal)	5	15	11
Number of operating hours per day	0	24	5

Table 8.2 shows a series of decolourisation process conditions both recommended in the Autio patent and the reactions conditions utilised at Loughborough University, see Section 7.0.

The ultrafiltration step in the program is based on work carried out on the PCI equipment at Loughborough University by Hurst (1981).

The flux rate is related to the protein concentration as follows:

$$Y = (-0.067x + 9.5)\ T$$

where Y = flux rate ($lm^{-2}\ hr^{-1}$)

x = protein concentration (mg/ml)

T = correction factor for temperature, 1.5

A loop within the computer program calculates the number of modules required to concentrate the globin to 25% solids. According to the UF specification maximum capacity of each module is 15.5 l/min. The UF modules will operate in series. A 30 kW pump recommended for the UF unit should minimise any pressure drop.

Table 8.2 Recommended Reaction Conditions

Reaction	Water Volume addition RCF:Water v/v	1N Hydrochloric Acid (Ultimate pH of RCF)	Volume addition (RCF:Acid v/v)	Carboxymethyl Cellulose Concn of solution (Wt %)	Volume addition (RCF inclusive of water & acid) v/v
1	1:2	< pH 3	-	RCF : CMC =	1:10
2	1:2	pH 1.5	-	0.3	1:4
3	1:3	pH 1.5	-	0.3	1:4
4	1:4	pH 1.5	-	0.35	1:3
5	1:5	pH 1.4	-	0.06	1:3
Research conditions at Loughborough					
6	1:4	pH 1.5	(5:1)	0.6	1:3
7	1:4	pH 1.5	(5:1)	0.1	1:3

Table 8.3 Other Processing Conditions

	Recommended
Addition of 1N NaOH (litres/litres of globin)	0.077
UF membrane life (yrs)	1

Table 8.4 Selling Prices

	Recommended Value (£/tonne)
Selling price globin	2500
selling price plasma	2500
Selling price CMC-haem	350

Table 8.5 Profitability Criteria

	Minimum	Maximum	Recommended
Minimum Rate of Return	0	0.99	0.15
Rate of Inflation	0	0.99	0.05
Rate of Taxation (constant annual profits only)	0	0.99	0.4
Variable Annual profits	input annual profits (post-tax) for the next 10 years		

8.5.2 COMPUTER PRINTOUT

The computer prints out the below information

1. Total Capital Costs
2. Total Annual Production Costs
   a) Variable production costs
   b) fixed production costs
3. Total Annual Income
   a) Income from globin sales
   b) Income from plasma sales
   c) Income from CMC-haem sales
4. Profitability
   a) Gross annual profit
   b) return on investments
   c) payback time
   d) net present value
   e) discounted cash flow rate of return
5. Annual Material Usage & Production
   a) mass of globin produced
   b) mass of plasma produced
   c) mass of CMC-haem produced
   d) required mass of pure water
   e) required mass of 1N HCl
   f) required mass of CMC
   g) required mass of 1N NaOH
   h) required mass of sodium citrate
6. Listing of the plant hardware required for each production capacity.

DATA FILE

All the economic and processing equipment data are stored in the data file. These can easily be updated to suit alternative processing conditions.

8.6 COMPUTER LISTING

```
 VDU3
>LIST
    1 DIM A(10)
    2 MODE 7
    3 PRINT"PROCESS AND ECONOMIC EVALUATION OF THE PRODUCTION OF GLOBIN FROM WHO
LE BLOOD"
    4 PRINT   "**********************************************************************
**********"
    5 PRINT "USING CMC"
    6 PRINT "*********"
    7 PRINT"================'
    8 PRINT"INPUT DATA"
    9 PRINT"================'
   10 PRINT"Input Blood Collection Data:"
   11PRINT"============================"
   12 PRINT
   13 GOSUB 2215
   14 PRINT
   17 PRINT "NO OF COWS PROCESSED PER HOUR ?"
   18 PRINT
   20 INPUT A1
   29 PRINT
   30 PRINT"BLOOD YIELD (LITRES/ANIMAL)"
   31 PRINT
   40 INPUT A2
   41 PRINT
   45 A4=A1*A2
   46 IF A4>450 THEN 4920
   50 PRINT'"NO OF OPERATING HOURS A DAY ?"
   52 INPUT A3
   53 PRINT
   80 PRINT"Select Processing Conditions from the Users Guide"
   81 PRINT"================================================"
   90 PRINT''"Addition of water (litres/litre of blood)?"
   92 PRINT: INPUT D1
  100 PRINT'"Addition of 1N HCL (Litres/Litres of blood)?"
  102 PRINT':INPUT D2
  110 PRINT''"Concentration of CMC solution (WT.%)?"
  112 INPUT D4
  120 PRINT''"ADDITION OF CMC (LITRES/LITRE OF BLOOD)"
  122 PRINT'' :INPUT D3
  126 PRINT''
  140 PRINT'"ADDITION OF 1N NaOH TO ADJUST GLOBIN pH TO REQUIRED VALUE (1/1 OF G
LOBIN FRACTION)?"
  150 PRINT':INPUT C1
  152 PRINT "EXPECTED MEMBRANE LIFE (YEARS)?"
  156 PRINT':INPUT U9
  160 PRINT''"COST OF PROCESSING PLASMA ('/TONNE)?"
  161 PRINT''"VALUE IS ASSUMED TO BE '1100 PLEASE INPUT THIS OR AN ALTERNATIVE V
ALUE."
  163 PRINT'':INPUT A4
  166 PRINT "Input Selling Data:"
  167 PRINT "===================="
  180 PRINT''"SELLING PRICE OF DRIED PLASMA ('/TONNE)?"
  190 PRINT':INPUT V5
  200 PRINT''"SELLING PRICE OF DRIED GLOBIN  ('/TONNE)?"
  210 PRINT':INPUT V4
  220 PRINT''"SELLING PRICE OF CMC-HAEM ('/TONNE)?"
  230 PRINT':INPUT V6
  231 PRINT''
  300 REM ========================
  301 REM MASS BALANCE
  302 REM ========================
```

```
330 P0=(B1*(1-B2)-B1*C3/C2)*P5
340 P1=P0*P3/950
350 REM RED CELL FRACTION
360 R0=B0*B2
365 R1=B0*R2
370 R4=R1*0.606
380 R5=R1*0.351
390 R6=R1*0.011
400 R7=R1-R4-R5-R6
410 D1=D1*R0
420 D2=D2*R0
430 D3=D3*(D1+D2+R0)
440 R8=R0+D1+D2+D3
450 R9=D3*D4/100
500 G0=R8*I0
510 G5=G0+C1*G0
530 G6=B0*I1/G5
540 G6=R5*I1*I2/G5
560 H0=R8*(1-I0)
570 H1=R5-G6*G5
580 H2=R8-G8*G0
590 H3=H1-H2-R9
600 H4=R9
610 H5=H0*A3*252/1000
625 REM ======================
626 REM ULTRA - FILTRATION
627 REM ======================
630 G4=R5*I1*I2
640 G11=G8*1000
650 G12=G5
660 G13=(-0.067*G11+9.5)*1.5VDU2
670 G14=G13*G0*3
680 G12=G12-G14
690 G11=G4*1000/G12
700 U8=1-U2
708 IF G11<0 THEN 750
710 IF G11<250 THEN 660
715 IF G11>250 THEN G11=0.25
750 PRINT
760 G15=G4*4
900 T0=T8-G15
910 T2=T0*A3*252/1000
1295 REM =================
1296 REM SPRAY DRIER
1297 REM =================
1300 S2=G4*100/95
1310 S3=S2*A3*252/1000
1315 REM =================
1316 REM WASTE WATER
1317 REM =================
1320 S4=G15-S2
1330 S5=S4*A3*252/1000
1395 REM =================
1396 REM CAPITAL COSTS
1397 REM =================
1400 K0=J0
1410 K1=J2*N0
1420 N1=INT(R8/1200)+1
1430 K2=J3*N1
1440 K3=J4*N2*C2-J14*3
1450 K4=J4*N3*C+J15
1460 K5=J5
1470 K6=J6
```

```
1510 K9=J10*N4
1520 K10=J10*N5+J13*N6
1530 K11=J15*N7+J16
1540 K12=K0+K1+K2+K3+K4+K5+K6+K7+K8+K9+10
1550 K13=K12+K11
1560 K14=K12*1.2
1570 K15=K13+K14
1590 REM ==========================
1591 REM PRODUCTION COSTS
1592 REM ==========================
1593 REM
1594 REM "FIXED COSTS"
1600 Q0=M0+M1*S3+M2
1610 Q1=K14*0.025
1620 Q2=K14*0.001
1630 Q3=Q0+Q1-Q2
1635 REM
1636 REM PRODUCTION COSTS
1637 REM
1640 REM raw materials
1641 REM
1650 E0=D1*L0*A3*252
1660 E1=D2*L1*A3*252
1670 E2=D3*D4*L2*A3*252/100
1680 E4=C3*B0*L4*A3*252
1690 E3=C1*G0*L3*A3*252/100
1700 E5=L5*50
1710 E6=S2*L10*252*A3+H5*L10*10
1715 REM
1716 REM operating equipment
1717 REM
1718 F10=P1*A4
1720 F0=(L7*1.1+L8*1.02+L3*1.2+L9*1.2)*A3*252
1730 F1=(L7*2.2*A3*252*N0)
1740 F2=(L7*6.5*A3*252*N1)
1750 F3=(L3*0.002*G3*A3*252)
1760 F4=(L7*30*A3*252)+L9*T2-U2*L11/U9
1770 F5=L8*0.071*S5
1780 F6=(L7*1.1*N4*A3*252)
1790 F7=(L7*3.5+L8+L9)*A3*252
1800 F8=F0+F1+F2+F3+F4+F5+F6+F7+F10+E0+E1+E2+E3+E4+E5+E6
1805 F9=F8+Q3
1810 REM
1811 REM=============================
1812 REM PROFIT
1813 REM=============================
1814 REM
1820 V0=V4*S3
1830 V1=V5*P1
1840 V2=V6*H5
1850 V3=V0+V1+V2
1860 V7=V3-F9
1870 V8=V7-(0.1*Q3)
1880 V9=V8*100/K14
1890 V10=K14/V8
1895 GOTO 4000
1900 END
2200 PRINT
2201 REM ================================================
2202 REM DATA FILE:MASS BALANCE AND COSTING FIGURES
2203 REM ================================================
2210 PRINT
2211 REM MASS BALANCE
2212 PRINT
```

```
,U1.S1
 2220 REM SPECIFIC GRAVITIES;WHOLE BLOOD.PLASMA,RED CELL FRACTION.GLOBIN(1%SOL.)
 2230 DATA 1.06,1.064,1.064,1
 2240 REM CONC. AND VOL. ADDITION OF REAGENTS:WATER, 1N HCL. CONC.CMC.VOL.CMC, 1
N NaOH
 2250 DATA 4,0.2,0.6,3,0.077
 2260 REM SODIUM CITRATE: CONC. OF SALT, FINAL CONC.IN BLOOD
 2270 DATA 0.4,0.01
 2280 REM FLOW RATES OF BLOOD: BLOOD/COW(1),COWS/hr hrs/DAY
 2290 DATA 11,20,5
 2300 REM FRACTION RCF FROM CENTRIFUGE 1
 2310 DATA 0.4
 2320 REM CENTRIFUGE 2; VOL.SEPARATION. EFFICIENCY,%PROTEIN IN GLOBIN
 2330 DATA 0.9,0.95,0.9
 2340 REM U/F: TEMP.(C),MEMBRANE AREA(M),EXPECTED LIFE (YRS)
 2350 DATA 45,2.6,1
 2360 REM FRACTION OF PROTEIN IN GLOBIN LEAVING THE U/F.
 2370 DATA 0.25
 2380 REM PROTEIN CONTENT OF SPRAY DRIED GLOBIN
 2390 DATA 0.95
 2400 REM U/F CORRECTION FACTOR. SPRAY DRIER CORRECTION FACTOR
 2410 DATA 1,1
 2420 REM COST OF UTILITIES, SERVICES AND CHEMICALS
 2425 READ L0,L1,L3,L3,L2,L4,L6,L7,L8,L9,L11,L10
 2440 REM COSTS: WATER(/L),1N HCL(/L),1N NaOH(/L),CLEANING FLUID(/WEEK)
 2450 DATA 0.005,0.3,0.25,100
 2460 REM COST('/KG):CMC,SODIUM CITRATE
 2470 DATA 2.275,1.055
 2480 REM COST('):PROCESS WATER(CU.M),ELECTRICITY(KWH),GAS(THERM),EFFLUENT(CU.M)
 MEMBRANE REPLACEMENT
 2490 DATA 0.298, 0.0724,0.37,0.303,153
 2500 REM PACKING MATERIAL ('/KG PRODUCT)
 2510 DATA 0.05
 2520 REM COST LABOURER, SUPERVISOR, PART TIME TECHNICIAN
 2525 READ M1,M0,M2
 2540 DATA 8500,8000,4000
 2550 REM CAPITAL COSTS
 2555 READ J0,J2,J3,J5,J4,J10,J9,J8,J17,J7,K11,J12,J13,J15,J16,J14
 2557 REM COST OF ASCEPTIC COLLECTION FOR 30 CATTLE/HR.
 2570 DATA 20000
 2580 REM CENTRIFUGES: BPB207,BTPPX206
 2585 DATA 9500,35000
 2600 REM HEAT EXCHANGER, TANKS (STAINLESS STEEL '/L), 1.1KW PUMP, PACKING MACHI
NE
 2610 DATA 1000,1.4,393,500
 2620 REM U/F FRAME, COST OF ADDITIONAL MODULES
 2630 DATA 45000,731
 2640 REM COST OF SPRAY DRIER (CAP.200KG/HR)
 2650 DATA 70000
 2660 REM CIP UNIT, ZEPHYR VALVES B3,B4
 2670 DATA 5000,440,464
 2680 REM BUILDING COST,CU.M), AUXILLARIES(INC.LAB.),FEED TANK ACCESSORIES.
 2690 DATA 230,3000,20
 2704 REM NO. CENTRIFUGES,VOL. DECOLOURISING TANKS, VOL. NEUTRALISING TANKS, NO.
 ZEPHYR VALVES B3,B4, NO. PUMPS, AREA BUILDING (SQ.M)
 2705 READ N0,N2,N3,N5,N6,N4,N7
 2706 DATA 1.2000,2000,6,3,6,500
 2710 REM SELLING
 2715 READ V4,V5,V6,P6,P3,S3,U5
 2720 REM SELLING PRICE ('/TONNE),GLOBIN,PLASMA,CMC-HAEM
 2730 DATA 2500,2500,250
 2740 REM COST TO PROCESS PLASMA PER TONNE
 2750 DATA 1100
```

```
 4000 PRINT "        ECONOMIC EVALUATION"
 4001 PRINT "        ====================="
 4005 PRINT''"MINIMUM RATE OF RETURN ?(INPUT VALUE BETWEEN 0 AND 0.99)."
 4010 PRINT'':INPUT K3
 4020 PRINT''"RATE OF INFLATION ?(INPUT VALUE BETWEEN 0 AND 0.99 . SUGGESTED VA
LUE IS 0.05 ."
 4040 PRINT'':INPUT K4
 4050 K0=K3+K4
 4060 PRINT''"ARE ANUAL PROFITS CONSTANT ?(Y/N)?"
 4070 PRINT'':INPUT L$
 4080 IF L$="N" THEN 4200
 4100 PRINT''"RATE OF TAXATION ON PROFITS ?(INPUT A VALUE BETWEEN 0 AND 0.99)."
 4110 PRINT'':INPUT K5
 4120 V7=V7*(1-K5)
 4128 PRINT''
 4130 Z0=(V7*(1-(1-K0)^(-10))/K0)-K14
 4140  X1=0
 4145 X11=1
 4150 REPEAT
 4155 ZZ=(X1+X11)/2
 4160 Z1= ((1-(1-ZZ)^(-10))/ZZ*V7)-K14
 4165 IF Z1<0 THEN X11=ZZ
 4170 IF Z1>0 THEN X1=ZZ
 4175 UNTIL ABS(Z1)<10
 4180 GOTO 4400
 4200 PRINT''
 4210 PRINT"INPUT ANNUAL PROFITS (POST TAX) FOR NEXT 10 YEARS IN CHRONOLOGICAL O
RDER SEPARATED BY COMMAS."
 4220 PRINT'':INPUT A(1),A(2),A(3),A(4),A(5),A(6),A(7),A(8),A(9),A(10)
 4270 IF L$<>"N" THEN 1900
 4280 REM NPV PROCEDURE
 4290 Z0=A(1)*((1+K0)^-1)+A(2)*((1+K0)^-2)+A(3)*((1+K0)^-3)+A(4)*((1+K0)^-4)+A(5
)*((1+K0)^-5)+A(6)*((1+K0)^-6)+A(7)*((1+K0)^-7)+A(8)*((1+K0)^-8)+A(9)*((1+K0)^-9
)+A(10)*((1+K0)^-10)-K14
 4375 X1=0
 4377 X11=0.5
 4378 REPEAT
 4379 ZZ=(X1+X11)/2
 4380 Z1=A(1)*((1+ZZ)^-1)+A(2)*((1+ZZ)^-2)+A(3)*((1+ZZ)^-3)+A(4)*((1+ZZ)^-4)+A(5
)*((1+ZZ)^-5)+A(6)*((1+ZZ)^-6)+A(7)*((1+ZZ)^-7)+A(8)*((1+ZZ)^-8)+A(9)*((1+ZZ)^-9
)+A(10)*((1+ZZ)^-10)-K14
 4385 IF Z1<0 THEN X11=ZZ
 4390 IF Z1>0 THEN X1=ZZ
 4395 UNTIL ABS(Z1)<10
 4400 REM PRINTOUT SECTION
 4405 PRINT''" PROJECT ECONOMIC ASSESSMENT"
 4406 PRINT   "============================="
 4480 PRINT'''"CAPITAL COSTS"
 4440 PRINT   "=============="
 4470 PRINT'''"Total capital costs inclusive of installation and comissioning co
sts:"
 4490 PRINT''"    ";K14
 4496 PRINT''''"PRODUCTION COSTS"
 4499 PRINT    "================"
 4502 PRINT '''"ANNUAL PRODUCTION COSTS"
 4503 PRINT    "======================"
 4506 PRINT''"1. Variable costs ($/yr)       = ";INT(F8)
 4507 PRINT''"2. Fixed costs ($/yr)          = ";INT(Q8)
 4509 PRINT''"3. Total prod. costs ($/yr)    = ";INT(F8+Q8)
 4530 PRINT''''"ANNUAL INCOME"
 4531 PRINT    "==============="
 4534 PRINT'''"Annual income from sales are:"
 4540 PRINT'''"GLOBIN              ";INT(V0)
 4550 PRINT''"SLAZMA              ";INT(V1)
```

```
 4581 PRINT'' " DO YOU WISH TO CONTINUE ?"
 4590 INPUT C
 4610 PRINT "PROFITABILITY"
 4611 PRINT "=============="
 4630 PRINT''' "GROSS ANNUAL PROFIT =";INT(V7)
 4637 PRINT''' "ROI and payback time assuming simple interest criteria and const
ant annual profits:"
 4641 PRINT''"ROI          =";INT(V9)"    %"
 4650 PRINT''"PAYBACK TIME (YRS)=";V10"     YRS"
 4670 PRINT'''"NPV and DCFRR assuming compound interest criteria for either con
stant or variable annual profits:"
 4680 PRINT''' "1. NPV     =";Z0
 4690 PRINT''   "2. DCFRR  = ";Z2*100" %"
 4700 PRINT "DO YOU WISH TO CONTINUE ?"
 4701 INPUT C
 4730 PRINT'' "ANNUAL PRODUCTION AND UTILISATION OF PRODUCTS AND REAGENTS"
 4731 PRINT"================================="
 4740 PRINT''"MASS OF PRODUCT PER YEAR."
 4780 PRINT''"1.  GLOBIN          =";INT(S3);"          (TONNES/YEAR)"
 4790 PRINT''"2.  PLASMA          =";INT(P1);"          (TONNES/YEAR)"
 4800 PRINT''"3.  CMC-HAEM        =";INT(H5);"          (TONNES/YEAR)"
 4820 PRINT'''"QUANTITY OF REAGENTS USED PER YEAR"
 4830 PRINT'''"MASS OF PURE WATER  =";INT(D1*A3*252/1000)"    (TONNES/YEAR)"
 4840 PRINT "MASS OF 1N HCL     =";INT(D2*A3*252/1000)"    (TONNES/YEAR)"
 4850 PRINT'   "MASS OF CMC          =";INT(D3*D4*A3*252/100000)"    (TONNES/YEAR)"

 4860 PRINT'   "MASS OF 1N NaOH      =";INT(C1*A3*252)"    (KG/YR)"
 4870 PRINT' "MASS OF Na CITRATE  =";INT(C3/100*A1*A2*A3*252)"    (KG/YR)"
 4889 PRINT"LIST OF MAJOR PLANT HARDWARE"
 4900 PRINT "============================"
 4903 PRINT"NO. OF ASCEPTIC COLLECTION UNITS                =1"
 4904 PRINT"NO OF C.I.P. UNITS                              =1"
 4905 PRINT"NO OF LOW RATE CENTRUFUGES                      =2"
 4906 PRINT"NO OF HIGH RATE CENTRUFUGES                      =";N1
 4907 PRINT"NO OF UF MODULES                                =";U2
 4908 PRINT"SPRAY DRIER CAPACITY                            =200 KG/HR"
 4912 PRINT ''
 4913 GOTO 1900
 4920 PRINT "Rate of blood collection exceeds the max. plant capacity of 450 l/h
r. Please input a lower throughput of cattle."
 4921 GOTO 10
 4930 PRINT "An excessive number of modules are required to provide the required
 globin concentration. Please input new blood collection data."
 4931 GOTO 3
 4978 PRINT
>VDU2
>
```

8.7 Economic criteria

8.7.1 Capital Costs

In this computer program the following assumptions about capital costs were made.

1. Cost of piping and fitments is calculated as the 10% of capital costs
2. Cost of commissioning is 20% of capital costs.
3. A working capital of £2000 is assumed
4. The capital outlay takes place at the beginning of the project.

8.7.2 Production costs

Fixed production costs are calculated as follows:

1. Maintenance costs = 2.5% of capital cost
2. Overhead costs = 0.01% of capital cost
3. Wages Supervisor = £8000 per annum
   Labourer = £6000 per annum
   Technician (part time) £3000 per annum

The variable costs are calculated as follows:

1. Chemicals used. These are worked out based on the annual flowrate of blood
2. Utilities. The program calculates all utilities as follows:
   Electricity = based on an hourly rate
   Gas, process water, steam based on the requirements of processing per litre of blood.

8.7.3 Other Economic Variables

Taxation on a new venture was assumed to 0%. However in the computer program this variable can be altered.

A 5% general rate of inflation is also assumed. This too can be altered if necessary.

As minimum acceptable rate of return anything above 15% will be sufficient.

8.8 Example of computer runs

The computer program gave the following 4 runs, which are included in the following pages.

The conditions for Run 1 were:

30 cows/hour, slaughter rate
11 l./animal, blood yield
5 operating hours a day
The economic evaluation parameters were:
15% minimum. rate of return
No inflation or taxation rates included
Constant yearly profits

The conditions for Run 2 were:
20 cows/hour, slaughter rate
11 l/animal, blood yield
5 operating hours a day
The economic evaluation parameters were:
20% minimum rate of return
5% inflation rate
20% taxation rate
Constant annual profit

```
>RUN 1
PROCESS AND ECONOMIC EVALUATION OF THE PRODUCTION OF GLOBIN FROM WHOLE BLOOD
**********************************************************************
USING CMC
*********
===============
INPUT DATA
===============
Input Blood Collection Data:
============================


NO OF COWS PROCESSED PER HOUR ?

?30

BLOOD YIELD (LITRES/ANIMAL)

?11


NO OF OPERATING HOURS A DAY ?
?5

Select Processing Conditions from the Users Guide
=================================================


Addition of water (litres/litre of blood)?

?4

Addition of 1N HCL (Litres/Litres of blood)?


?0.2


Concentration of CMC solution (WT.%)?
?0.6


ADDITION OF CMC (LITRES/LITRE OF BLOOD)



?3




ADDITION OF 1N NaOH TO ADJUST GLOBIN pH TO REQUIRED VALUE (1/1 OF GLOBIN FRACTIO
N)?


?0.077
EXPECTED MEMBRANE LIFE (YEARS)?


?1
```

COST OF PROCESSING PLASMA (₤/TONNE)?

VALUE IS ASSUMED TO BE ₤1100 PLEASE INPUT THIS OR AN ALTERNATIVE VALUE.

?1100
Input Selling Data:
==========

SELLING PRICE OF DRIED PLASMA (₤/TONNE)?

?2500

SELLING PRICE OF DRIED GLOBIN (₤/TONNE)?

?2500

SELLING PRICE OF CMC-HAEM (₤/TONNE)?

?350

ECONOMIC EVALUATION
==========

MINIMUM RATE OF RETURN ?(INPUT VALUE BETWEEN 0 AND 0.99).

?0.15

RATE OF INFLATION ?(INPUT VALUE BETWEEN 0 AND 0.99 . SUGGESTED VALUE IS 0.05).

?0

ARE ANNUAL PROFITS CONSTANT ?(Y/N)?

?Y

RATE OF TAXATION ON PROFITS ?(INPUT A VALUE BETWEEN 0 AND 0.99).

?0

PROJECT ECONOMIC ASSESSMENT
==========

CAPITAL COSTS
==========

Total capital costs inclusive of installation and comissioning costs:

₦221060

PRODUCTION COSTS
==========

ANNUAL PRODUCTION COSTS
==========

1. Variable costs (₦/yr) = ₦100486

2. Fixed costs (₦/yr) = ₦38355

3. Total prod. costs (₦/yr) = ₦138842

ANNUAL INCOME
==========

Annual income from sales are:

GLOBIN ₦134229

PLASMA ₦38570

CNT HyEM ₦172,799

TOTAL SALES ₦301330

DO YOU WISH TO CONTINUE ?
?
PROFITABILITY
=============

GROSS ANNUAL PROFIT =₦136086

ROI and payback time assuming simple interest criteria and constant annual profi
ts:

ROI =51 %

PAYBACK TIME (YRS)=1.96114873 YRS

NPV and DCFRR assuming compound interest criteria for either constant or variabl
e annual profits:

1. NPV =₦321966.382

2. DCFRR = 51.4678886 %
DO YOU WISH TO CONTINUE ?
?

ANNUAL PRODUCTION AND UTILISATION OF PRODUCTS AND REAGENTS
==========

MASS OF PRODUCT PER YEAR.

1. GLOBIN =49 (TONNES/YEAR)

2. PLASMA =22 (TONNES/YEAR)

3. CMC-HAEM =045 (TONNES/YEAR)

QUANTITY OF REAGENTS USED PER YEAR

MASS OF PURE WATER =865 (TONNES/YEAR)
MASS OF 1N HCL =33 (TONNES/YEAR)

[illegible]

MASS OF Na CITRATE =41 (KG/YR)

LIST OF MAJOR PLANT HARDWARE
==========

NO. OF ASEPTIC COLLECTION UNITS	=1
NO OF C.I.P. UNITS	=1
NO OF LOW RATE CENTRIFUGES	=2
NO OF HIGH RATE CENTRIFUGES	=3
NO OF UF MODULES	=19
SPRAY DRIER CAPACITY	=200 KG/HR

>

```
RUN 2
PROCESS AND ECONOMIC EVALUATION OF THE PRODUCTION OF GLOBIN FROM WHOLE BLOOD
********************************************************************************
USING CMC
*********
==============
INPUT DATA
==============
Input Blood Collection Data:
============================

NO OF COWS PROCESSED PER HOUR ?

?220

BLOOD YIELD (LITRES/ANIMAL)

?11

NO OF OPERATING HOURS A DAY ?
?5

Select Processing Conditions from the Users Guide
=================================================

Addition of water (litres/litre of blood)?

?4

Addition of 1N HCL (Litres/Litres of blood)?

?0.2

Concentration of CMC solution (WT.%)?
?0.6

ADDITION OF CMC (LITRES/LITRE OF BLOOD)

?3

ADDITION OF 1N NaOH TO ADJUST GLOBIN pH TO REQUIRED VALUE (1/1 OF GLOBIN FRACTI
N)?

?0.077
EXPECTED MEMBRANE LIFE (YEARS)?
```

?1

COST OF PROCESSING PLASMA (£/TONNE)?

VALUE IS ASSUMED TO BE £1100 PLEASE INPUT THIS OR AN ALTERNATIVE VALUE.

?1100
Input Selling Data:
==========

SELLING PRICE OF DRIED PLASMA (£/TONNE)?

?2500

SELLING PRICE OF DRIED GLOBIN (£/TONNE)?

?2500

SELLING PRICE OF CMC-HAEM (£/TONNE)?

?350

ECONOMIC EVALUATION
==========

MINIMUM RATE OF RETURN ?(INPUT VALUE BETWEEN 0 AND 0.99).

?0.2

RATE OF INFLATION ?(INPUT VALUE BETWEEN 0 AND 0.99 . SUGGESTED VALUE IS 0.05):

?0.05

ARE ANUAL PROFITS CONSTANT ?(Y/N)?

?Y

RATE OF TAXATION ON PROFITS ?(INPUT A VALUE BETWEEN 0 AND 0.99).

?0.2

PROJECT ECONOMIC ASSESSMENT
==========

CAPITAL COSTS
==========

Total capital costs inclusive of installation and comissioning costs:

£79360

PRODUCTION COSTS
==========

ANNUAL PRODUCTION COSTS
==========

1. Variable costs (£/yr) = £70742

2. Fixed costs (£/yr) = £32263

3. Total prod. costs (£/yr) = £103005

ANNUAL INCOME
==========

Annual income from sales are:

GLOBIN ₦82819

PLASMA ₦37713

CMC-HAEM ₦80720

TOTAL SALES ₦201253

DO YOU WISH TO CONTINUE ?
?
PROFITABILITY
==========

GROSS ANNUAL PROFIT =₦78598

ROI and payback time assuming simple interest criteria and constant annual pro-
ts:

ROI =34 %

PAYBACK TIME (YRS)=2.93996079 YRS

NPV and DCFRR assuming compound interest criteria for either constant or varia
e annual profits:

1. NPV =₦1275.87732

2. DCFRR = 25.14953a1 %
DO YOU WISH TO CONTINUE ?
?

ANNUAL PRODUCTION AND UTILISATION OF PRODUCTS AND REAGENTS
==========

```
MASS OF PRODUCT PER YEAR.

1.  GLOBIN          =33          (TONNES/YEAR)

2.  PLASMA          =15          (TONNES/YEAR)

3.  CMC-HAEM        =230          (TONNES/YEAR)


QUANTITY OF REAGENTS USED PER YEAR


MASS OF PURE WATER  =443    (TONNES/YEAR)
MASS OF 1N HCL      =22    (TONNES/YEAR)

MASS OF CMC           =10    (TONNES/YEAR)

MASS OF 1N NaOH       =97    (KG/YR)

MASS OF Na CITRATE  =27    (KG/YR)

LIST OF MAJOR PLANT HARDWARE
============================
NO. OF ASCEPTIC COLLECTION UNITS              =1
NO OF C.I.P. UNITS                            =1
NO OF LOW RATE CENTRUFUGES                    =2
NO OF HIGH RATE CENTRUFUGES                    =2
NO OF UF MODULES                              =13
SPRAY DRIER CAPACITY                          =200 KG/HR


>RUN 3
PROCESS AND ECONOMIC EVALUATION OF THE PRODUCTION OF GLOBIN FROM WHOLE BLOOD
*****************************************************************************
USING CMC
*********
===============
INPUT DATA
===============
Input Blood Collection Data:
============================


NO OF COWS PROCESSED PER HOUR ?

?30

BLOOD YIELD (LITRES/ANIMAL)

?11


NO OF OPERATING HOURS A DAY ?
```

?6

Select Processing Conditions from the Users Guide
==========

Addition of water (litres/litre of blood)?

?4

Addition of 1N HCL (Litres/Litres of blood)?

?0.2

Concentration of CMC solution (WT.%)?
?0.1

ADDITION OF CMC (LITRES/LITRE OF BLOOD

?3

ADDITION OF 1N NaOH TO ADJUST GLOBIN pH TO REQUIRED VALUE (1/1 OF GLOBIN FRACT
N)?

?0.077
EXPECTED MEMBRANE LIFE (YEARS)?

?1

COST OF PROCESSING PLASMA (£/TONNE)?

VALUE IS ASSUMED TO BE £1100 PLEASE INPUT THIS OR AN ALTERNATIVE VALUE.

?1100
Input Selling Data:
==========

SELLING PRICE OF DRIED PLASMA (£/TONNE)?

?2500

SELLING PRICE OF DRIED GLOBIN (£/TONNE)?

?2500

SELLING PRICE OF CMC-HAEM (£/TONNE)?

?350

ECONOMIC EVALUATION
==========

MINIMUM RATE OF RETURN ?(INPUT VALUE BETWEEN 0 AND 0.99).

?0.13

RATE OF INFLATION ?(INPUT VALUE BETWEEN 0 AND 0.99 . SUGGESTED VALUE IS 0.05).

?0.404

ARE ANUAL PROFITS CONSTANT ?(Y/N)?

?Y

RATE OF TAXATION ON PROFITS ?(INPUT A VALUE BETWEEN 0 AND 0.99).

?0

PROJECT ECONOMIC ASSESSMENT

==========

CAPITAL COSTS
==========

Total capital costs inclusive of installation and comissioning costs:

£221360

PRODUCTION COSTS
==========

ANNUAL PRODUCTION COSTS
==========

1. Variable costs (£/yr) = '83586

2. Fixed costs (£/yr) = '33355

3. Total prod. costs (£/yr) = '116941

ANNUAL INCOME
==========

Annual income from sales are:

GLOBIN £149074

PLASMA £57884

CMC-HAEM £145297

TOTAL SALES £362256

DO YOU WISH TO CONTINUE ?
?

PROFITABILITY
==========

GROSS ANNUAL PROFIT =£245514

ROI and payback time assuming simple interest criteria and constant annual pro
ts:

ROI =75 %

PAYBACK TIME (YRS)=1.3280473E YRS

NPV and DCFRR assuming compound interest criteria for either constant or varia
e annual profits:

1. NPV =£821464.697

2. DCFRR = 76.0681152 %
DO YOU WISH TO CONTINUE ?
?

ANNUAL PRODUCTION AND UTILISATION OF PRODUCTS AND REAGENTS
==========

MASS OF PRODUCT PER YEAR.

1. GLOBIN =59 (TONNES/YEAR)

2. PLASMA =27 (TONNES/YEAR)

3. CMC-HAEM =415 (TONNES/YEAR)

QUANTITY OF REAGENTS USED PER YEAR

MASS OF PURE WATER =799 (TONNES/YEAR)
MASS OF 1N HCL =39 (TONNES/YEAR)

MASS OF CMC =3 (TONNES/YEAR)

```
MASS OF 1N NaOH      =116    (KG/YR)

MASS OF Na CITRATE  =49    (KG/YR)

LIST OF MAJOR PLANT HARDWARE
============================
NO. OF ASCEPTIC COLLECTION UNITS              =1
NO OF C.I.P. UNITS                            =1
NO OF LOW RATE CENTRUFUGES                    =2
NO OF HIGH RATE CENTRUFUGES                    =3
NO OF UF MODULES                              =19
SPRAY DRIER CAPACITY                          =200 KG/HR


>RUN 4
PROCESS AND ECONOMIC EVALUATION OF THE PRODUCTION OF GLOBIN FROM WHOLE BLOOD
*****************************************************************************
USING CMC
*********
===============
INPUT DATA
===============
Input Blood Collection Data:
============================


NO OF COWS PROCESSED PER HOUR ?

?25

BLOOD YIELD (LITRES/ANIMAL)

?11


NO OF OPERATING HOURS A DAY ?
?5

Select Processing Conditions from the Users Guide
=================================================


Addition of water (litres/litre of blood)?

?4

Addition of 1N HCL (Litres/Litres of blood)?


?0.2


Concentration of CMC solution (WT.%)?
?0.2

ADDITION OF CMC (LITRES/LITRE OF BLOOD)
```

?43

ADDITION OF 1N NaOH TO ADJUST GLOBIN pH TO REQUIRED VALUE (1/1 OF GLOBIN FRACT
N)?

?0.077
EXPECTED MEMBRANE LIFE (YEARS)?

?1

COST OF PROCESSING PLASMA (£/TONNE)?

VALUE IS ASSUMED TO BE £1100 PLEASE INPUT THIS OR AN ALTERNATIVE VALUE.

?1100
Input Selling Data:
==========

SELLING PRICE OF DRIED PLASMA (£/TONNE)?

?2500

SELLING PRICE OF DRIED GLOBIN (£/TONNE)?

?2500

SELLING PRICE OF CMC-HAEM (£/TONNE)?

?350

ECONOMIC EVALUATION
==========

MINIMUM RATE OF RETURN ?(INPUT VALUE BETWEEN 0 AND 0.99).

?.0.15

RATE OF INFLATION ?(INPUT VALUE BETWEEN 0 AND 0.99 . SUGGESTED VALUE IS 0.05 .

?0.04

ARE ANUAL PROFITS CONSTANT ?(Y/N)?

?N

INPUT ANNUAL PROFITS (POST TAX) FOR NEXT 10 YEARS IN CHRONOLOGICAL ORDER SEPARATED BY COMMAS.

?20000
?30000
?30000
?30000
?400000
?45000
?50000
?50000
?45000
?39000

PROJECT ECONOMIC ASSESSMENT
==========

CAPITAL COSTS
==========

Total capital costs inclusive of installation and comissioning costs:

₹279360

PRODUCTION COSTS
==========

ANNUAL PRODUCTION COSTS
==========

1. Variable costs (₹/yr) = ₹65642

2. Fixed costs (₹/yr) = ₹32263

3. Total prod. costs (₹/yr) = ₹97905

ANNUAL INCOME
==========

Annual income from sales are:

GLOBIN ₹103524

PLASMA ₹47142

CMC-HAEM ₹100900

TOTAL SALES ₹251567

DO YOU WISH TO CONTINUE ?
?
PROFITABILITY
==========

GROSS ANNUAL PROFIT = ₹153661

ROI and payback time assuming simple interest criteria and constant annual profits:

ROI =53 %

PAYBACK TIME (YRS)=1.85701396 YRS

NPV and DCFRR assuming compound interest criteria for either constant or varia
e annual profits:

1. NPV =-122515.994

2. DCFRR = 5.31005859 %
DO YOU WISH TO CONTINUE ?
?

ANNUAL PRODUCTION AND UTILISATION OF PRODUCTS AND REAGENTS
==========

MASS OF PRODUCT PER YEAR.

1. GLOBIN =41 (TONNES/YEAR)

2. PLASMA =18 (TONNES/YEAR)

3. CMC-HAEM =288 (TONNES/YEAR)

QUANTITY OF REAGENTS USED PER YEAR

MASS OF PURE WATER =834 (TONNES/YEAR)
MASS OF 1N HCL =27 (TONNES/YEAR)

MASS OF CMC =4 (TONNES/YEAR)

MASS OF 1N NaOH =97 (KG/YR)

MASS OF Na CITRATE =34 (KG/YR)

LIST OF MAJOR PLANT HARDWARE
==========
NO. OF ASCEPTIC COLLECTION UNITS =1
NO OF C.I.P. UNITS =1
NO OF LOW RATE CENTRUFUGES =2
NO OF HIGH RATE CENTRUFUGES =2
NO OF UF MODULES =16
SPRAY DRIER CAPACITY =200 KG/HR

The conditions for Run 3 were:
30 cows/hour, slaughter rate
11 l:/animal, blood yield
6 operating hours a day
0.1% concentration of CMC used
The economic evaluation parameters were:
13% minimum rate of return
4% inflation rate
0% taxation
Constant annual profits

The conditions for Run 4 were:
25 cows/day, slaughter rate
11 l:/animal, blood yield
5 operating hours a day
0.2% concentration of CMC used

The economic evaluation parameters were:
15% minimum rate of return
4% inflation rate
No taxation

The annual profits were assumed variable. The values inputed in the model are hypothetical.

8.9 Sensitivity analysis

The feasibility of this decolourisation process is affected by both processing and economic factors. The sensitivity of the project to different variables is analysed below.

In each case, the values assumed for the basis of comparison is detailed in Table 8.6

Values of variables taken as basis for sensitivity analysis

Variable	Units	Value
Cattle throughput	Nos/hr	20
Blood yield	l/animal	11
Hours of operation	hr/day	5
Addition of water	(l/l blood)	4
1N HCl	(l/l blood)	0.2
CMC conc.	% (w/v)	0.6
CMC addition	l/l of haemolysed acidified blood	3
1N NaOH	l/l globin	0.077
Protein yield	%	80
Min. rate of return	%	15
Inflation rate	%	0
Taxation rate	%	0
Selling price : globin	£	2500
plasma	£	2500
CMC-haem	£	350
Processing price plasma (cost)	£	1100

Table 8.6

The sensitivity analysis of the decolourisation process is conducted with respect to the following factors.

1. Capacity utilisation
2. Blood yield per animal
3. Operating hours a day
4. Protein yield
5. Selling price of the globin
6. Minimum rate of return
7. Decolourisation conditions
8. Selling price of CMC-haem

The results are tabulated in Appendix IV, Figures 8.3, 8.4, 8.5 8.6, 8.7, 8.8, 8.9, and 8.10 are a graphical representation of the NPV of the project against each factor described above. Minimum rate of interest for the NPV calculation was 15%.

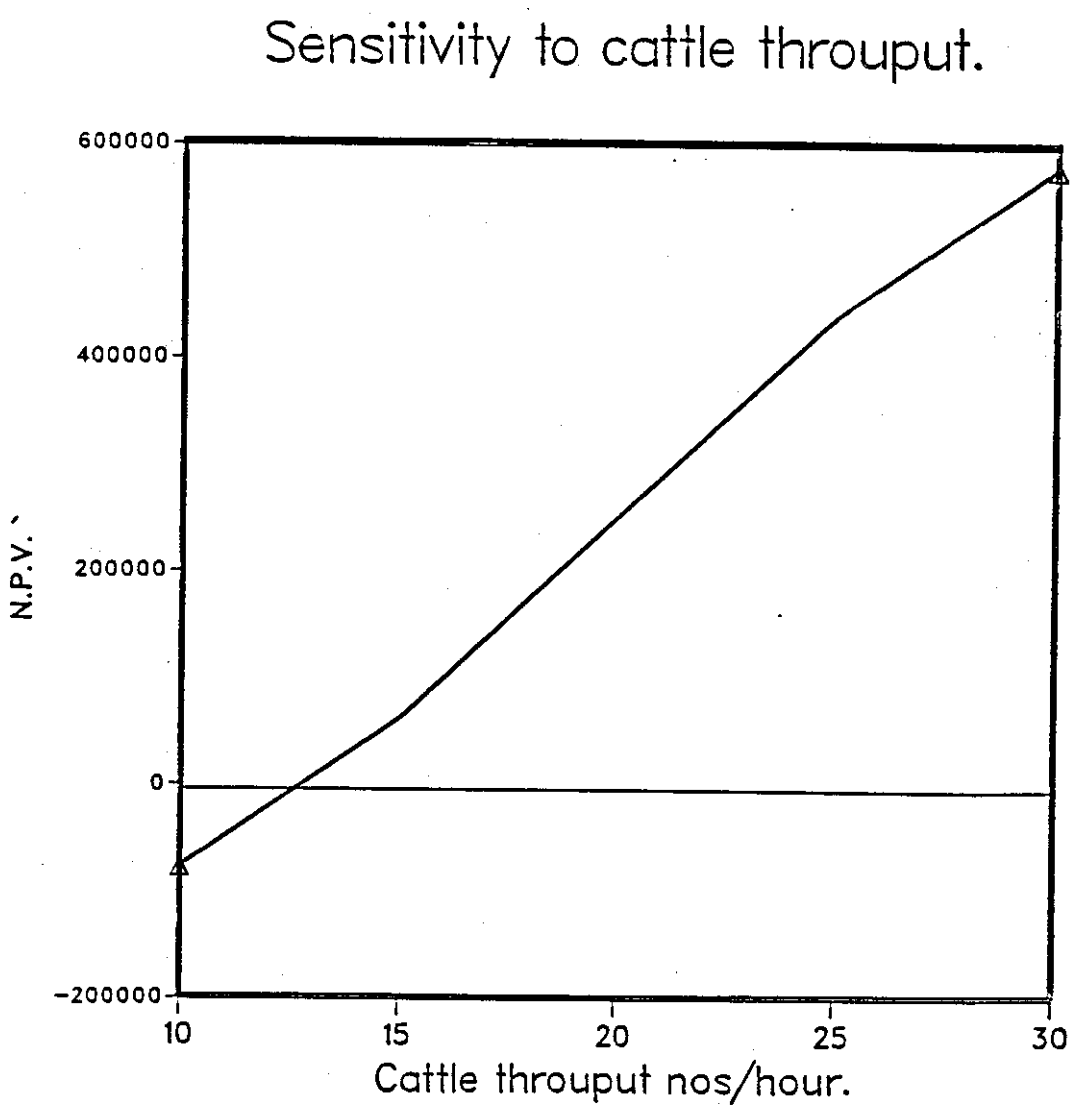

Sensitivity to cattle throuput.
600000
400000
200000
0
-200000
N.P.V.
10
15
20
25
30
Cattle throuput nos/hour.

Figure 8.3

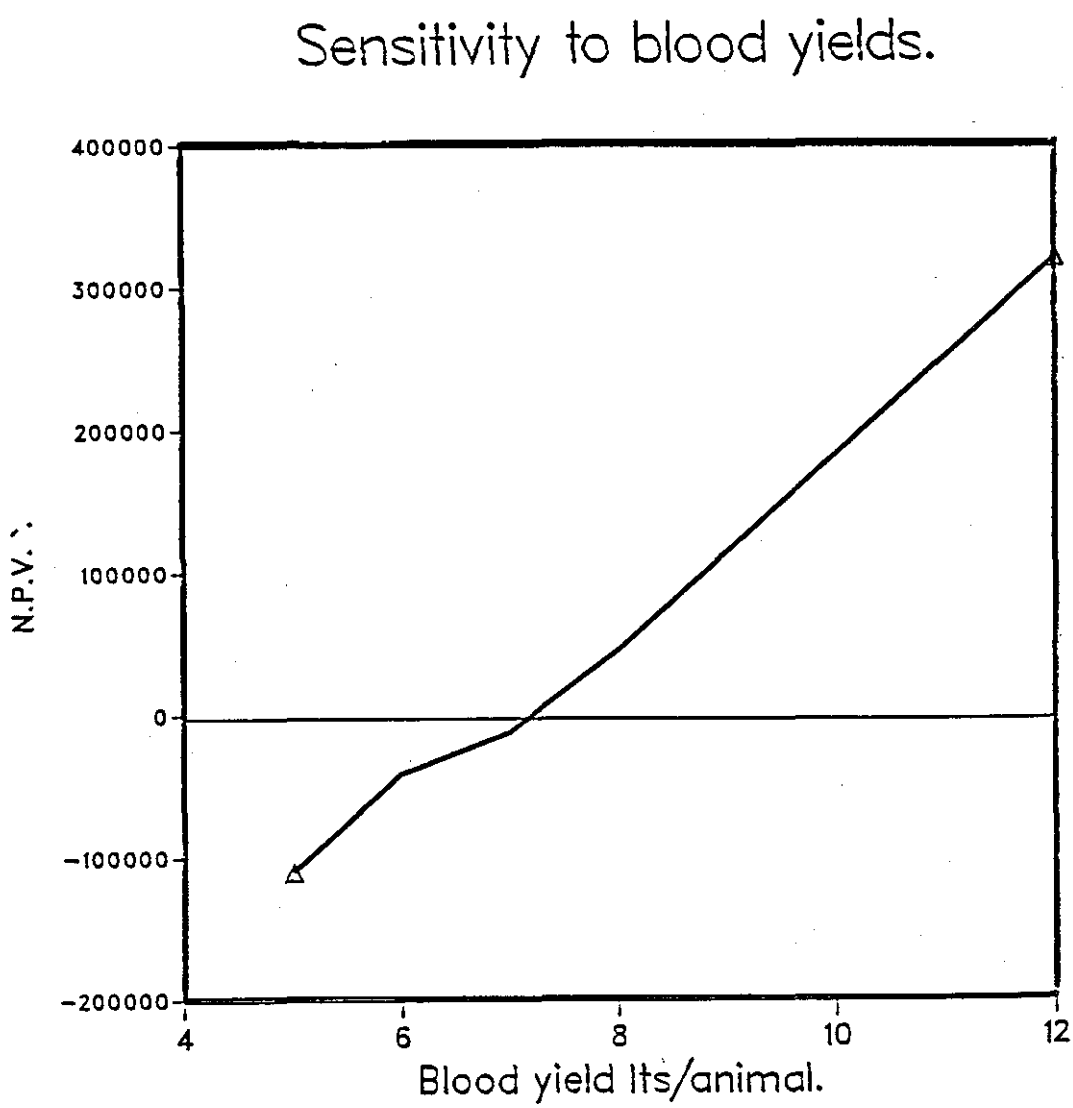

Sensitivity to blood yields.
400000
300000
200000
100000
0
-100000
-200000
N.P.V.'.
4
6
8
10
12
Blood yield lts/animal.

Figure 8.4

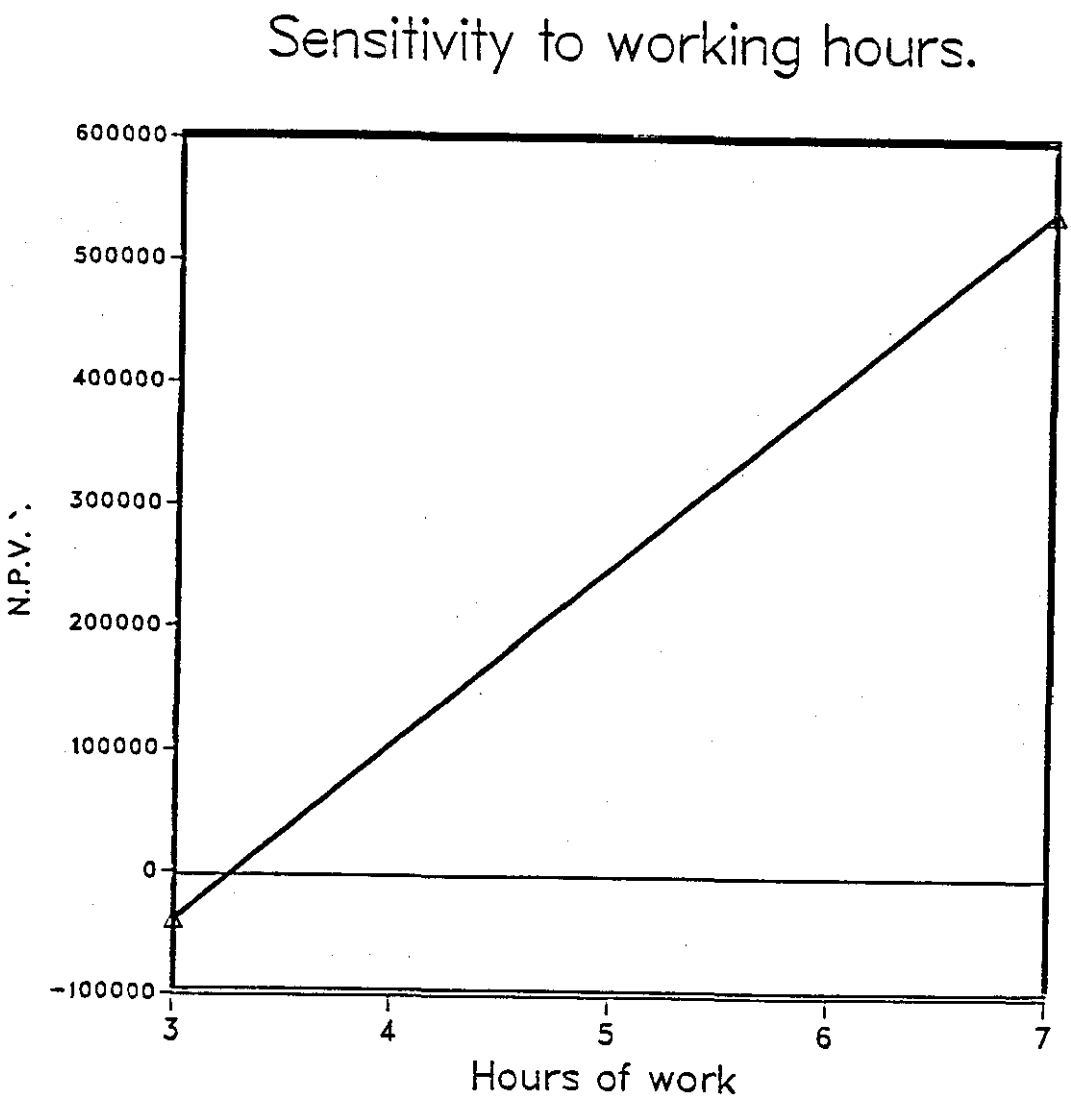

Sensitivity to working hours.
600000
500000
400000
300000
200000
100000
0
-100000
N.P.V.
3
4
5
6
7
Hours of work

Figure 8.5

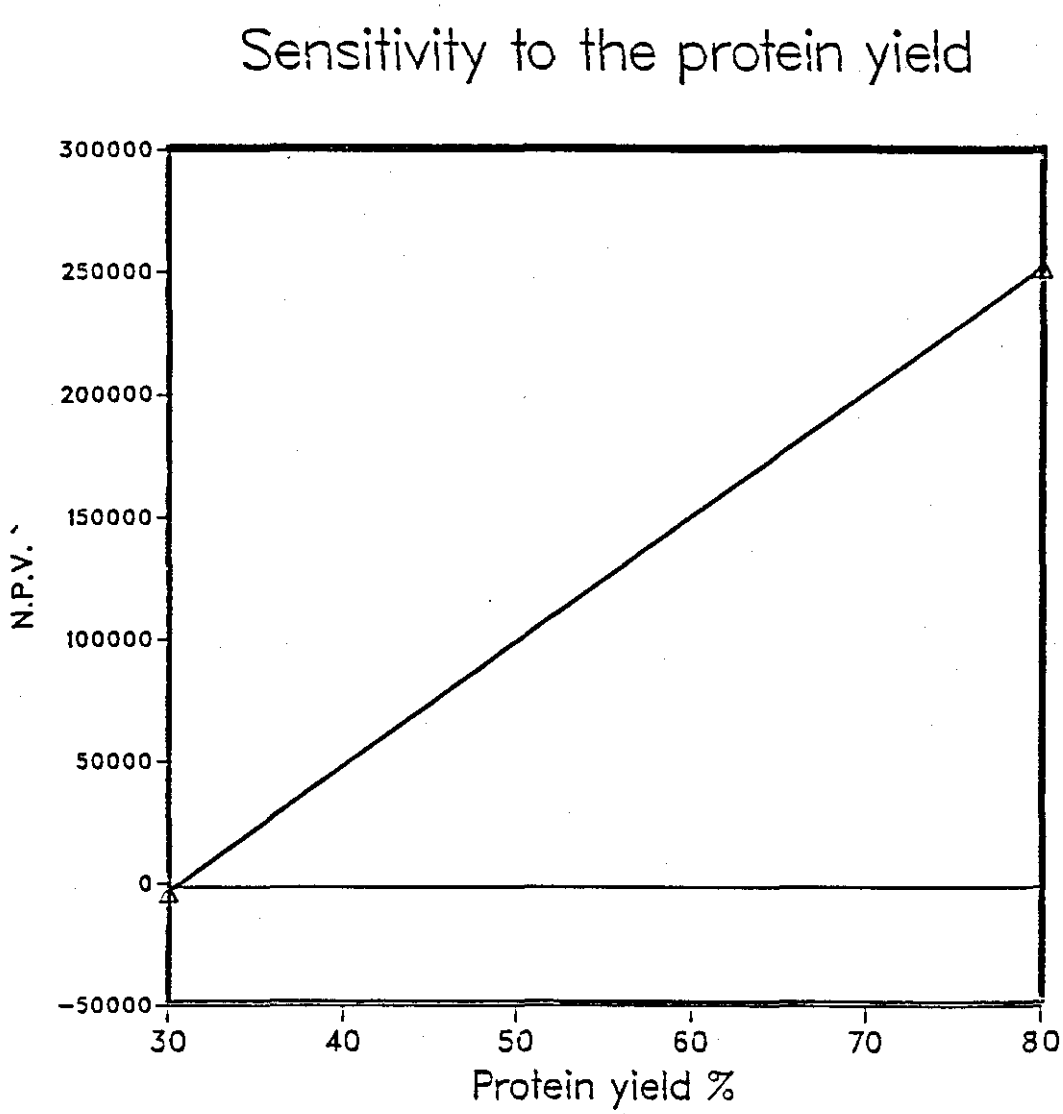

Sensitivity to the protein yield
300000
250000
200000
150000
100000
50000
0
-50000
N.P.V.
30
40
50
60
70
80
Protein yield %

Figure 3.6

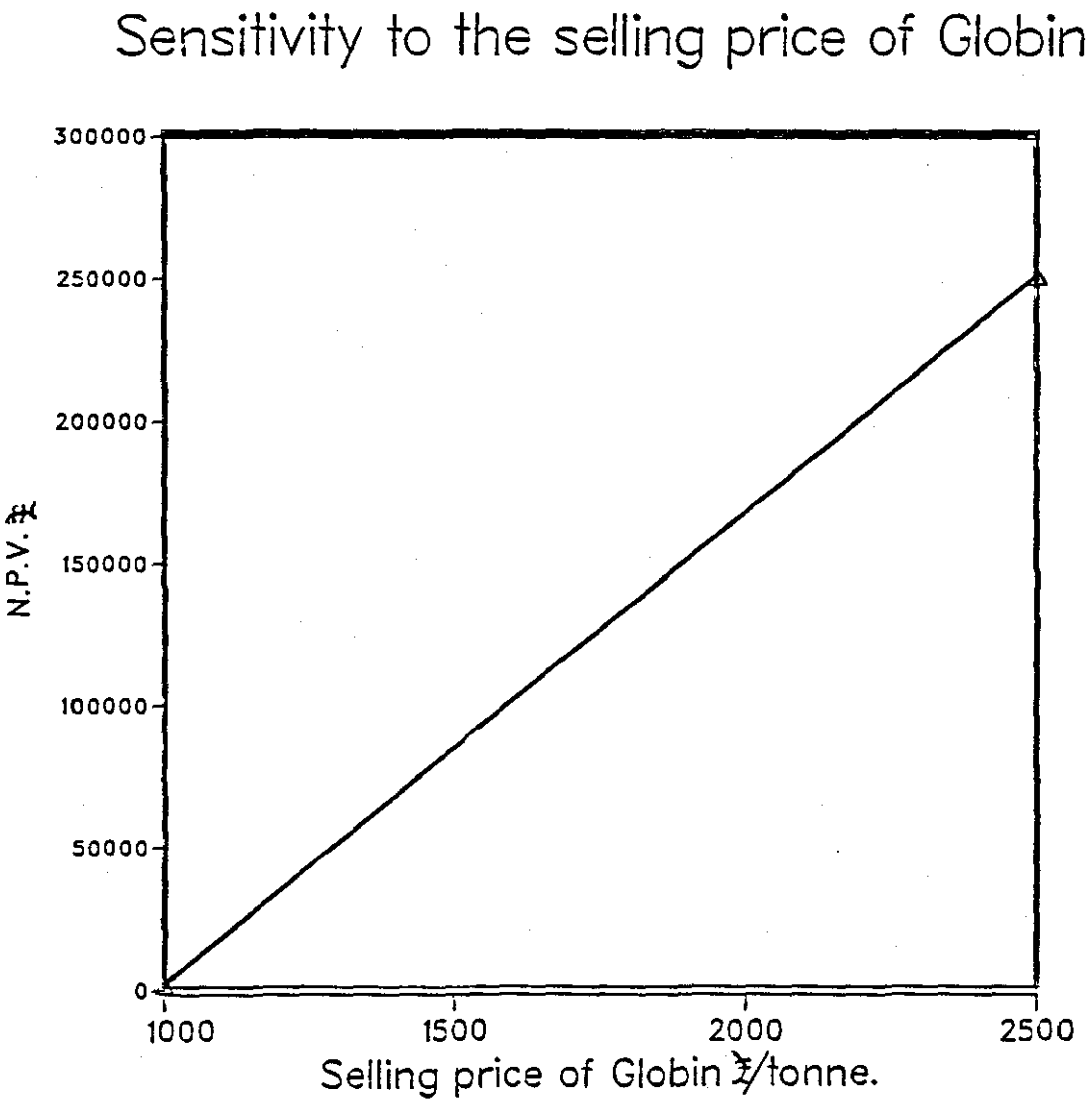

Sensitivity to the selling price of Globin
300000
250000
200000
150000
100000
50000
0
N.P.V. £
1000
1500
2000
2500
Selling price of Globin £/tonne.

Figure 8.7

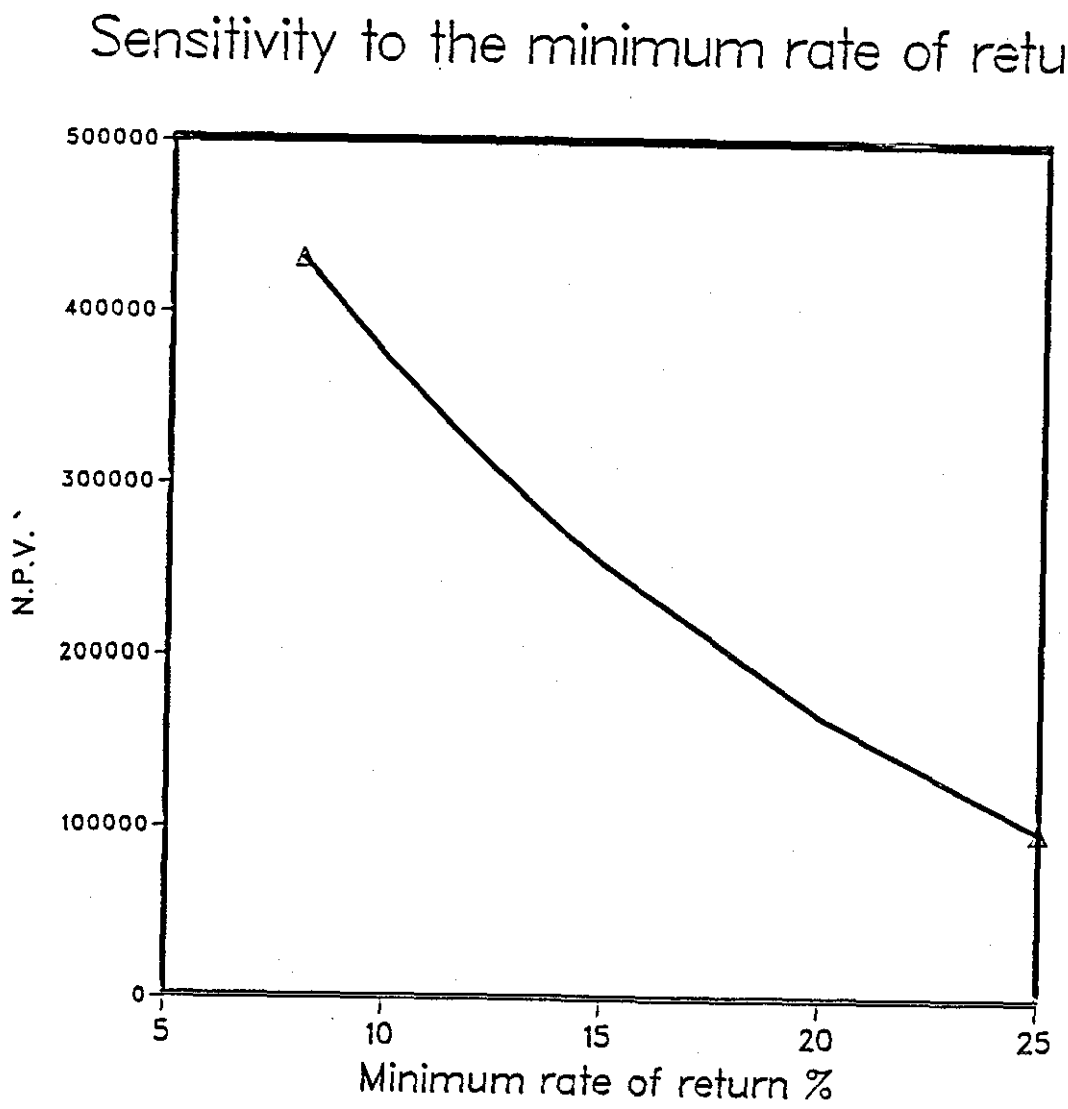

Sensitivity to the minimum rate of return
500000
400000
300000
200000
100000
0
N.P.V.
5
10
15
20
25
Minimum rate of return %

Figure 8.8

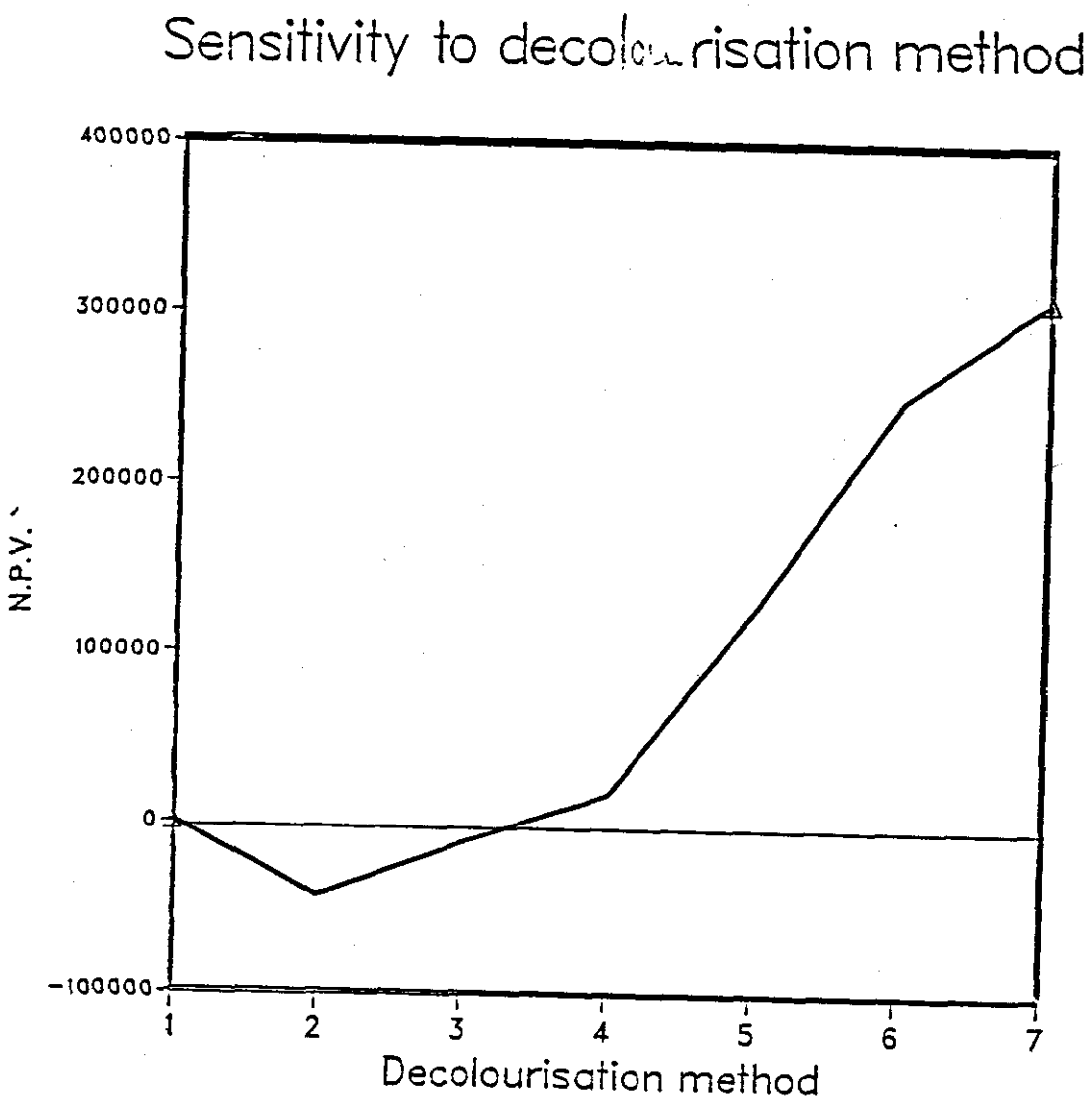

Sensitivity to decolourisation method
400000
300000
200000
100000
0
-100000
N.P.V.
1
2
3
4
5
6
7
Decolourisation method

Figure 8.9

Sensitivity to the selling price of CMC–Haem byproduct.

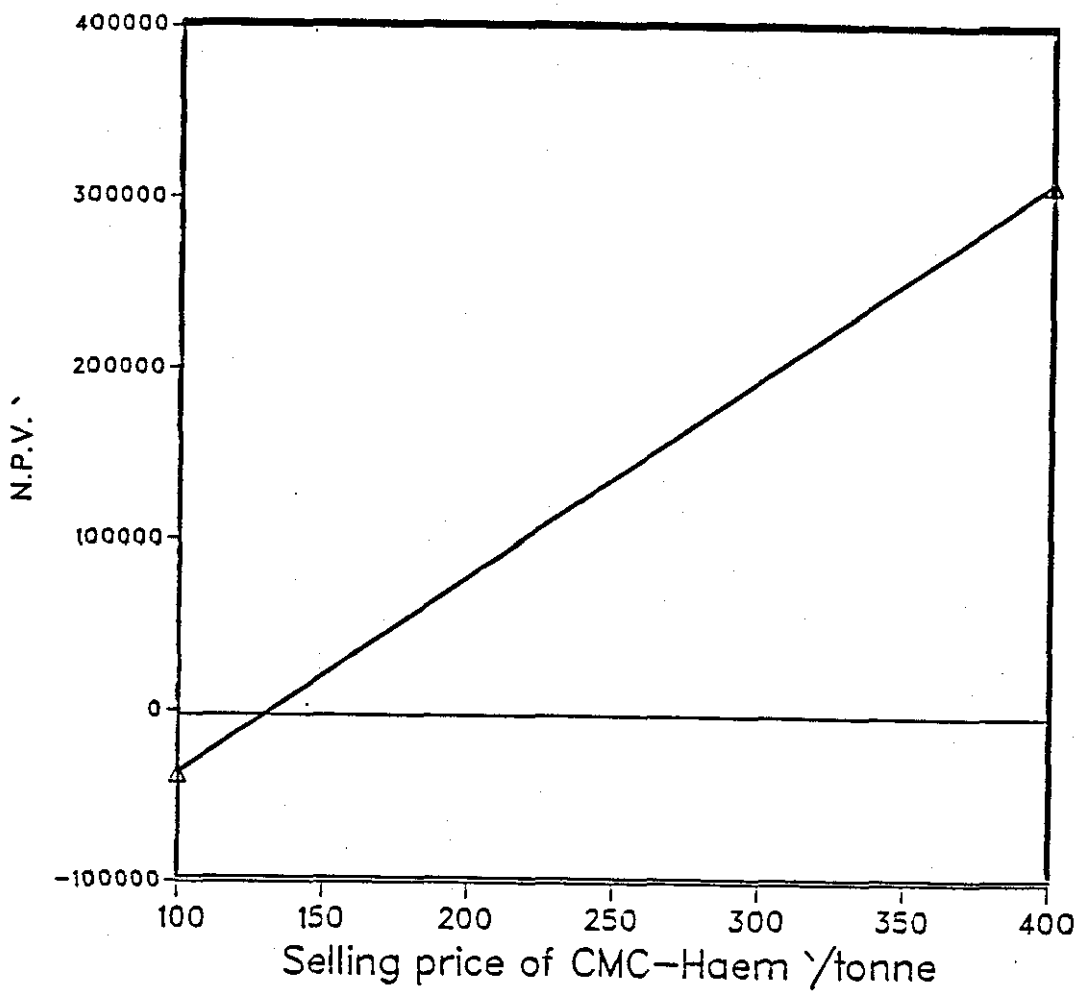


Figure 8.10

Figures 8.3 to 8.10 show a linear relationship between the parameter examined and N.P.V. This shows that for example the more hours of slaughter per day at constant slaughter rates the higher the value of the project, as would be expected. (Figure 8.5). Similarly the higher the selling price for the globin product, the more NPV of the project increase (Figure 8.7).

The minimum conditions for a N.P.V. worth of zero pounds are the following:

Parameter	Minimum Value
Cattle throughput (nos/hr)	13
Blood yield (lts/animal)	7
Hours of work per day	3.25
Protein yield (%)	30
Selling price of Globin (£/tonne)	1000
Selling price of CMC-haem (£/tonne)	135

These parameters relate to the standard conditions set in Table 8.6. Therefore when for example the cattle throughput is 13 animals/hour, the rest of the variables are taken from Table 8.6. The parameters here are independent of one another.

Even with as high as 25% minimum rate of return the NPV of the project stays at £100,000, Fig. 8.8. This indicates that the project will stay profitable at rate of returns higher than 30%.

The parameters which affect the profitability the most are: the killing rate of cattle, Fig. 8.3 and the amount of working hours, Fig. 8.5 These parameters at their maximum give the best NPV figures of £600,000 and £550,000 respectively.

The following parameters affect the NPV of the project almost identically: blood yield per animal, protein yield and the CMC-haem byproduct. All these give at their maximum a value of £300,000.

It is noticeable that the CMC-haem by-product is very important in the profitability of the plant and indeed it affects NPV more than the selling price of the globin product. Fig. 8.7 shows that at its maximum selling price, £25·00 globin gives a project worth of £250,000. The CMC-haem byproduct at its maximum selling value, £400, gives a project worth of £300,000.

This can be explained by the large amounts of by-product produced by the process. However, the maximum price assumed for the globin, £2500 per tonne, could possibly be an under-estimate. The equation of the line is $y = mx$ where y = NPV in pounds and x = selling price of globin (£/tonne) $m = \tan 45^{\circ}$ as derived from Fig. 8.7. Therefore if a price of £3000 per tonne of globin is feasible the NPV figure of the project will increase to £300,000.

Figure 8.9 compares the decolourisation methods tabulated in Table 8.2. Numbers 1,2,3,4 and 5 refer to decolourisation methods, and are described in the Autio patent . These methods give NPV figures of less than £100,000. By contrast the methods developed in this work, 6 and 7 give NPV of £250,000 and £300,000 respectively. The downstream processing conditions of the five methods described by Autio were assumed the same as the ones developed for methods 6 and 7. Method 7 utilises a CMC solution of only 0.1% concentration. This produces the most functional and proteinaceous globin powder of all, but the least decolourised one. Therefore method number 6 utilising a 0.6% CMC emerges as the optimum decolourisation process.

CHAPTER 9

CMC and Hydrogen peroxide combined decolourisation method

This process of decolourisation was developed as a natural progression of the hydrogen proxide and CMC methods. As seen (Ch. 6) the hydrogen peroxide 'bleaching' process produces a protein powder with very few functional properties but with excellent colour.

The CMC method on the other hand produces a decolourised dilute globin solution with functional properties. The colour and protein yield of the final product are determined by the concentration of the CMC solution. The more concentrated CMC solution used, the better decolourised is the product but less protein is recovered, Chapter 7.

The combined CMC-hydrogen peroxide method has the following advantages.

1. Utilises much less concentrated solution of CMC thus improving the protein recovery.

2. The controlling step of the decolourisation process now becomes the addition of hydrogen peroxide solution. Thus, depending on the final destination of the protein powder, its colour can be controlled more accurately.

3. Concentration of RCF of higher than 25% (v/v) can now be decolourised, due to the strong action of the hydrogen peroxide.

This combined method of decolourisation is as follows:

An aqueous solution of RCF is prepared. It is then acidified to pH 1.5 using HCl acid. The acidified solution is then added to a dilute aqueous solution of CMC, the total volume of CMC solution is three times the acidified RCF solution. The mixture is centrifuged at 3000g for 15 minutes. The precipitate which contains most of the haem and all the CMC is discarded.

The supernatant is collected.

Hydrogen peroxide is added to the supernatant, about 1% of the total volume of the supernatant. The decolourised solution is left to stabilise overnight or is heated up to 37°C. The decolourised globin can then be passed through an ultrafiltration unit and then spray dried.

9.1 Preliminary work using the combined method

A 25% RCF solution was prepared. It was acidified to pH 1.5 using 1N HCl. The RCF was added to a beaker containing 0.1% CMC solution, 3 volumes of CMC to 1 volume of acidified RCF. The solution was centrifuged at 2000 g for 15 minutes. The supernatant was collected and a protein determination was performed. This gave an average of 1.41% protein content. 1150 cm^3 of this decolourised solution were loaded into the Amicon unit, fitted with 10K membrane, at 37°C and 25 psi pressure. The solution was concentrated to 150 ml and a protein content, average, of 10-15%.

Four 20 ml samples of this retentate were taken. Three were treated with hydrogen peroxide, strength 100 volumes. The amounts of hydrogen peroxide added were 0.2 ml, 0.6 ml and 0.8 ml respectively.

i.e. addition of 1%, 3% and 4% of total volume.

The fourth sample was kept as control.

The colour of all three bleached samples was bright yellow in comparison with the dark brown colour of the untreated sample. However the decolourised samples contained bubbles due to the decomposition of the hydrogen peroxide which did not settle out. It appeared that a better product would result if the hydrogen peroxide addition step was taken before the ultrafiltration process.

9.2 Rate of reaction between hydrogen peroxide and RCF

An investigation was conducted on the rate of reaction between hydrogen peroxide and RCF treated with 0.1% CMC.

A 25% RCF solution was acidified to pH 1.5 using 1N HCl and 0.1% CMC added, three volumes. It was then centrifuged at 3030g for 15 minutes. The supernatant was collected and filtered through a cheese mesh cloth. Hydrogen peroxide strength 100 v was added to the solution at a concentration of 10% of the total supernatant volume.

Aliquots of this solution were taken every twenty minutes and their absorbance at 372 nm was measured.

An investigation, using Minitab, a computer package, showed that plotting the inverse of absorbance, A, against time, t, was a straight line, see figure 9.1. This is consistent with with a rate of reaction being proportional to the square of the absorbance, as shown below:

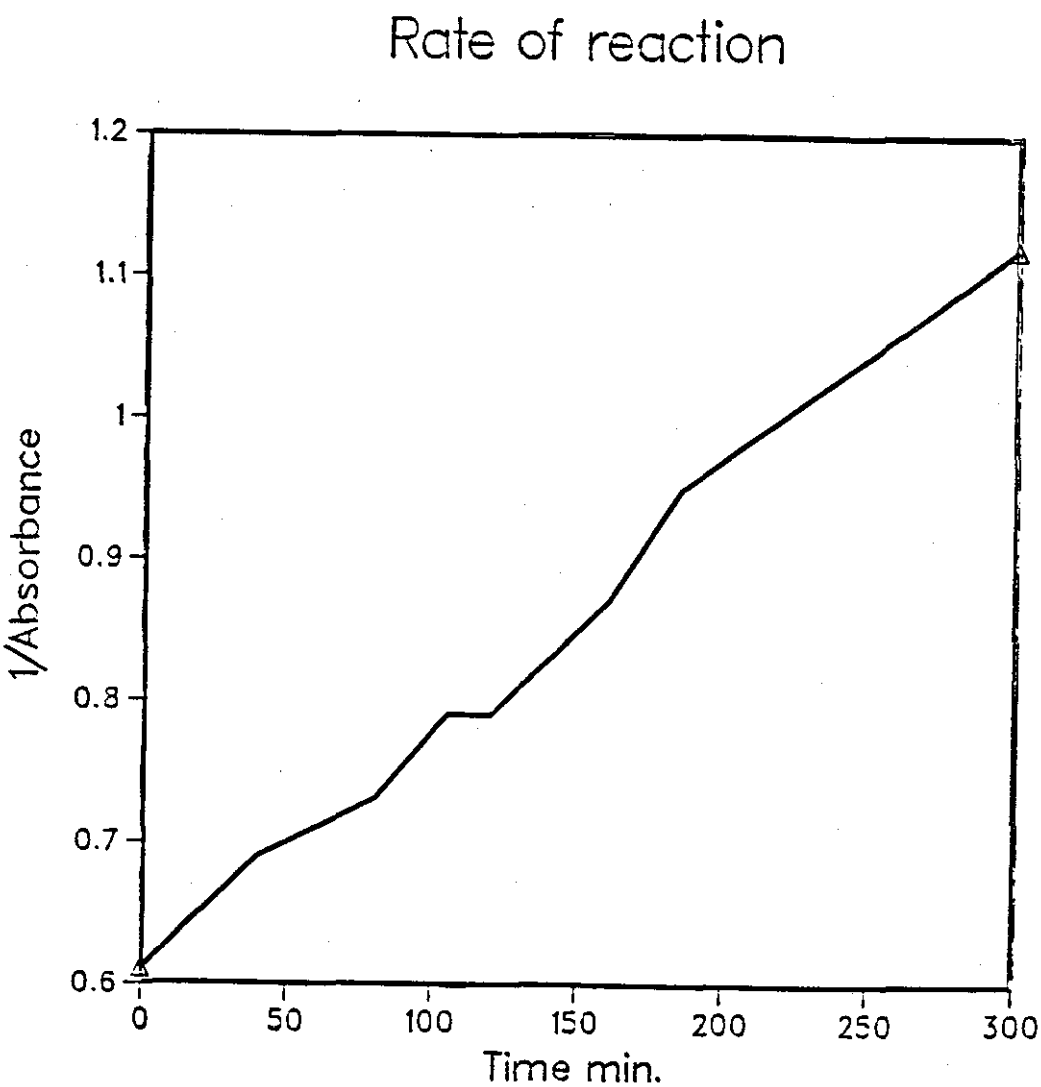

Rate of reaction
1.2
1.1
1
0.9
0.8
0.7
0.6
1/Absorbance
0
50
100
150
200
250
300
Time min.

Figure 9.1

$$\int \frac{d\{A\}}{dt} = \int k\{A\}^2 \rightarrow$$

$$kt = \frac{1}{\{A\}_t} - \frac{1}{\{A\}_o} \rightarrow$$

$$\frac{1}{A} = kt + \frac{1}{Ao}$$

The regression equation is

$$\frac{1}{A} = 0.0017\ t + 0.613$$

where $k = 0.0017\ A^{-1}\ min^{-1}$

The results correlate to 99.3%

9.3 Optimisation of hydrogen peroxide amount added

The amount of hydrogen peroxide added was optimised as follows. A 25% RCF solution was prepared and acidified to pH 1.5. To this 0.1% CMC solution was added, 3 parts of CMC to 1 art of RCF and the mixture centrifuged at 3030g for 15 minutes. The supernatant was collected and seven samples of 20 ml each were taken. To each sample a certain amount of hydrogen peroxide was added and the absorbance of the sample was measured at 372 nm.

The samples were left undisturbed at room temperature for 24 hours and again readings of their absorbance were taken. The results are summarised in Table 9.1.

Absorbance of decolourised solutions

RCF used	H_2O_2 added (ml)	H_2O_2 added (% of total volume)	Absorbance (372 nm) at 0 hours	at 24 hours
Water	0	0	0	0
	0.00054	0.0027	1.475	1.460
25% RCF +	0.03	0.15	0.90	0.89
0.1% CMC	0.05	0.25	0.85	0.83
20 ml each	0.1	0.5	0.80	0.79
sample	0.2	1.0	0.75	0.73
	0.3	1.5	0.73	0.72
	0.4	2.0	0.71	0.70
20% RCF + 0.6% CMC	None	None	0.559	0.540

Table 9.1

The results are represented in figure 9.2. It became apparent that addition of hydrogen peroxide at more than 0.5% (v/v) of the total volume of supernatant, did not produce any significant reduction in the absorbance i.e. a less coloured product. The optimum amount of hydrogen peroxide added was in the region of 0.5 - 1% (v/v). Also there was very little residual activity of hydrogen peroxide after 24 hours, see absorbance values tables 9.1.

The samples stabilised in 24 hours.

9.4 Pilot Plant Work

Using the pilot plant constructed (see section 4) three batches of blood were decolourised.

In the first run a 20% RCF solution was used. 30 litres of water, at 45°C, were poured from the mains hot water tap into the reaction tank and 36g of CMC powder were slowly added. The tank was stirred continuously to ensure that all CMC was dissolved. A further 6 litres of water were added to give a total solution of 0.1% (w/v) concentration of CMC.

24 litres of mains water and 6 litres of RCF were mixed in the preparation tank, (i.e. a 20% v/v solution was prepared. Concentrated HCl acid was poured into the RCF solution until the pH reached 1.5. About 400 ml of conc HCL (6N) were used.

The acidified RCF solution was stirred for 10 minutes. After this period the pH was measured to ensure that it was 1.5.

The centrifugal pump was started up, and initially fed to drain to remove any residual water in the system. The RCF was then pumped into the reaction tank. Flowrate of the centrifugal pump was 1 litre s^{-1}, hence it took 12 seconds for all the RCF solution to transfer to the reaction vessel.

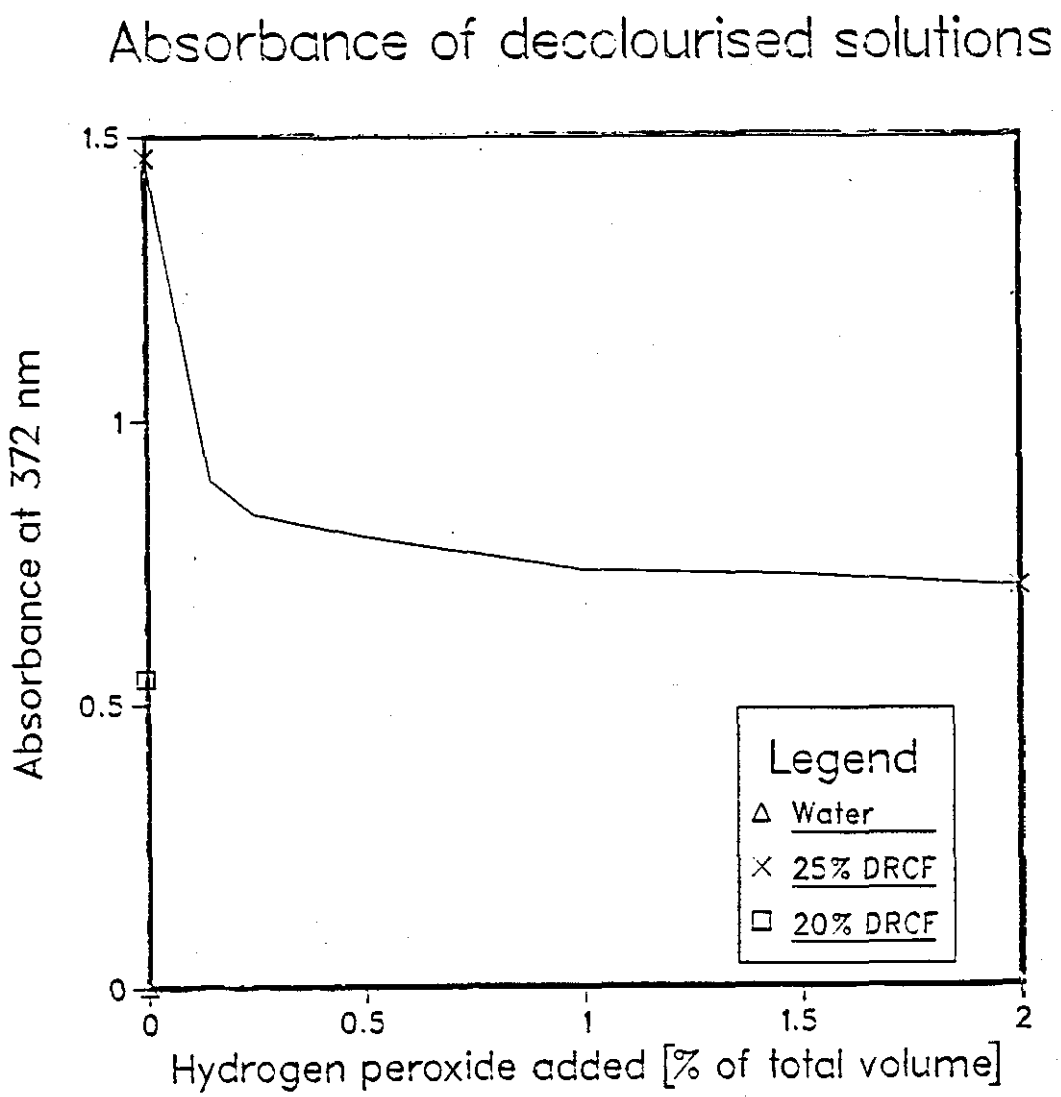

Absorbance of decclourised solutions
Absorbance at 372 nm
1.5
1
0.5
0
0
0.5
1
1.5
2
Hydrogen peroxide added [% of total volume]
Legend
Water
25% DRCF
20% DRCF

Figure 9.2

The CMC-RCF mixture was stirred for 5 minutes and left undisturbed for 1½ hours in the reaction vessel. After this period of time a stable foam had formed on the mixture. The valve on the reaction vessel was opened and the peristaltic pump was started up, set at 55% input of volts.

All the water in the system was first flushed out before the pump was connected to the centrifuge.

The Sharples centrifuge took 5 minutes to fill up before any supernatant could be collected. By timing how long it took to collect 1 litre of supernatant it was calculated that the flowrate out of the centrifuge was 4 litres per minute.

The supernatant was collected in 3 milk churns, and each batch of 10 litres was treated with different amounts of hydrogen peroxide and left overnight. The batches were spray dried.

Before spray drying a small amount of supernatant from each batch was tested in the spectrophotometer at 372 nm.

The second run incorporated a 25% RCF solution, i.e. 6 litres of RCF and 18 litres of tap water. The pH was dropped to 1.5 by using 450 ml of concentrated HCl acid.

Since the 25% RCF solution had a greater viscosity than the 20% RCF solution to obtain a steady pH the centrifugal pump was started with the outlet pipe leading back to the preparation tank. This recycle ensured adequate mixing of the solution.

The acidified RCF was then pumped into the reaction vessel containing 72 litres of 0.1% CMC solution, as in the first run. The peristaltic pump was set at 55% voltage input giving a flowrate out of the centrifuge of 5 litre/min. The supernatant produced was not clear. It contained CMC-haem particles.

This indicated that the peristaltic pump had been set at a too high flowrate, not allowing enough residence time in the centrifuge for adequate separation.

No spray drying or further analysis of the supernatant was carried out.

The third run was a repeat of run 2. However the peristaltic pump was set at 45% input voltage giving a volumetric flowrate out of the centrifuge of 2.4 l/min. The supernatant was free of any particles. It was collected in 3 batches and different amounts of hydrogen peroxide were added. The solutions were spray dried.

The results of the two runs are summarised in Table 9.2.

The colour of the solutions was dark brown for samples not treated with hydrogen peroxide to bright yellow transparent solutions for samples treated with 1% hydrogen peroxide.

The absorbance of the solutions against the amount, in percentage terms of hydrogen peroxide added is shown in Fig. 9.3. From this figure it can be seen that the operating conditions of Run 3 gave slightly lower absorbance reading and hence produced a better decolourised product.

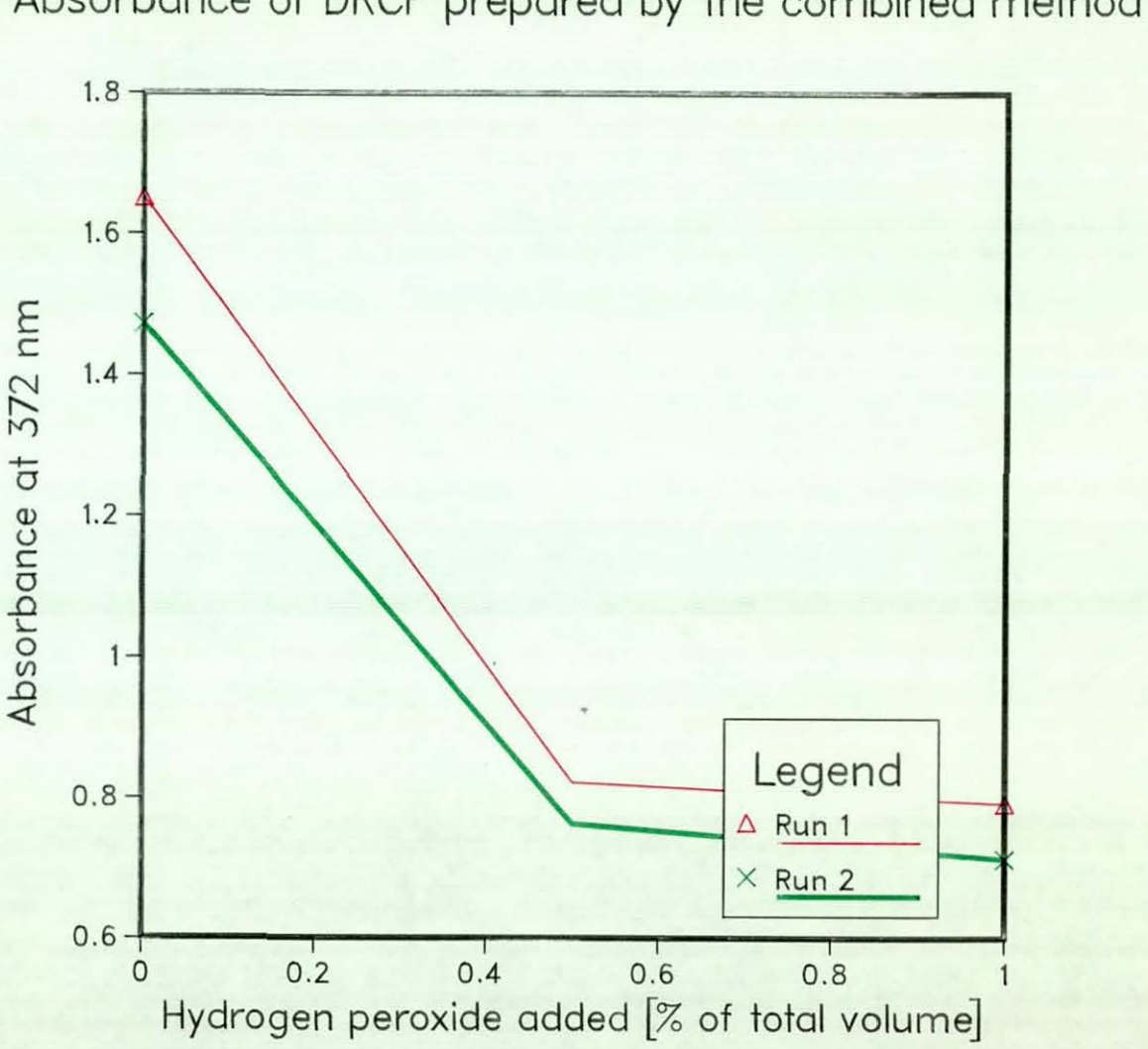

Absorbance of DRCF prepared by the combined method
Absorbance at 372 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0
0.2
0.4
0.6
0.8
1
Hydrogen peroxide added [% of total volume]
Legend
Run 1
Run 2

Figure 9.3

Solutions prepared by the combined CMC-H_2O_2 method

Run No.	Batch No.	H_2O_2 added (% of total volume)	Absorbance at 372 nm	pH of supernatant
1	1	0	1.65	
	2	0.5	0.82	2.8
	3	1.0	0.79	
3	1	0	1.47	
	2	0.5	0.76	2.9
	3	1.0	0.71	

Powder collected from Run 1 = 67.3 g x 3 = 202.9 g

Powder collected from Run 3 = 59.2 g x 3 = 177.6 g

Table 9.2

The protein recovery from both Run 1 and 2 was very low, less than 10%. This can be explained by the low solids solutions fed to the spray drier. Approximate solids content were 1.8 - 1.9% while the minimum solids content for spray drying should be 15-20%.

The optimum RCF solution still remained 25% v/v because it did not create any handling problems. Attempts to load higher concentrations of RCF ended up with difficulties in pumping, mixing and decolourising.

The optimum amount of hydrogen peroxide was 0.5% added to the supernatant. Any larger amounts did not give better decolourisation products.

9.5 Functional properties of protein products

The following powders were tested for functional properties, as prepared from the pilot plant.

1. A 20% RCF solution decolourised by 0.1% CMC and 0.5% H_2O_2, Powder A.

2. A 20% RCF solution decolourised by 0.1% CMC and 1% H_2O_2, Powder B.

3. A 25% RCF solution decolourised by 0.1% CMC and 0.5% H_2O_2 Powder C.

4. A 25% RCF solution decolourised by 0.1% CMC and 1% H_2O_2 Powder D.

The pH of the powders, 10% v/v in water solution, as well as their protein contents are summarised in Table 9.3.

Table 9.3 - Protein analysis of decolourised powders

Powder	pH of solution	Protein content (%)
A	2.65	83.13
B	2.60	82.50
C	3.4	83.60
D	3.4	82.4

The powders were also tested for foaming, emulsion, gelation and fat absorbance properties. The results are summarised in Table 9.4. Emulsions were tested as grams of oil required to break 1g of emulsion. Foaming was tested as the total foam volume remaining after 60 minutes.

The results of the functional tests suggested the following:

The powders produced did not have as good foaming properties as the ones produced by the hydrogen peroxide method and CMC only method. An average value of 55% foam was achieved by CMC-decolourised powders, but less than 20% was obtained by these powders.

The fat absorbance was quite high at 1.5 ml/g average, considerably better than all the previous powders. However, when only 0.1% CMC is used for decolourising, a fat absorbance of 1.55 was also achieved.

Functional properties of powders produced by the combined decolourisation method

Powder	Foaming %	Emulsion (g)	Fat Absorbance (ml/g)	Gelation
A	25	1.53	1.20	420
B	18.5	1.51	1.50	480
C	26.7	1.22	1.50	370
D	18.9	1.24	1.50	393

Table 9.4

From the results there is an indication that some gels may be produced but they are very weak.

The emulsion capacities of the powders were also much better than other powders tested.

In conclusion, this method of decolourisation produced the most functional powders. However, very good functional powders were also produced from the pilot plant when using 0.1% CMC with no further treatment.

9.6 Amino acid analysis of a RCF solution decolourised by the CMC-H_2O_2 combined method

The solution analysed was prepared as follows. A 25% RCF was acidified to pH 1.5 using 1N HCl acid. A 0.1% CMC solution was added at a concentration of three times its total volume. The solution was centrifuged at 3030 g for 15 minutes.

The supernatant was collected. An aliquot of 20 ml of the supernatant was treated with 0.2 ml hydrogen peroxide strength 100 volumes, i.e. 1% of volume to be decolourised. The sample was left undisturbed for 48 hours and then analysed in the Amino Acid Analyser, as in Chapter 4.

The print out obtained is shown in Figure 9.4. The concentration, of each amino acid in the solution is shown in Table 9.5. The results obtained were very similar to those obtained by hydrogen peroxide and CMC decolourised products, Chapters 6 and 7. This verifies that the CMC-hydrogen peroxide method does not affect the haemoglobin molecule to the point of breaking down amino acids.

Amino acid profile of 25% RCF decolourised by 0.1% CMC and 1% H_2O_2

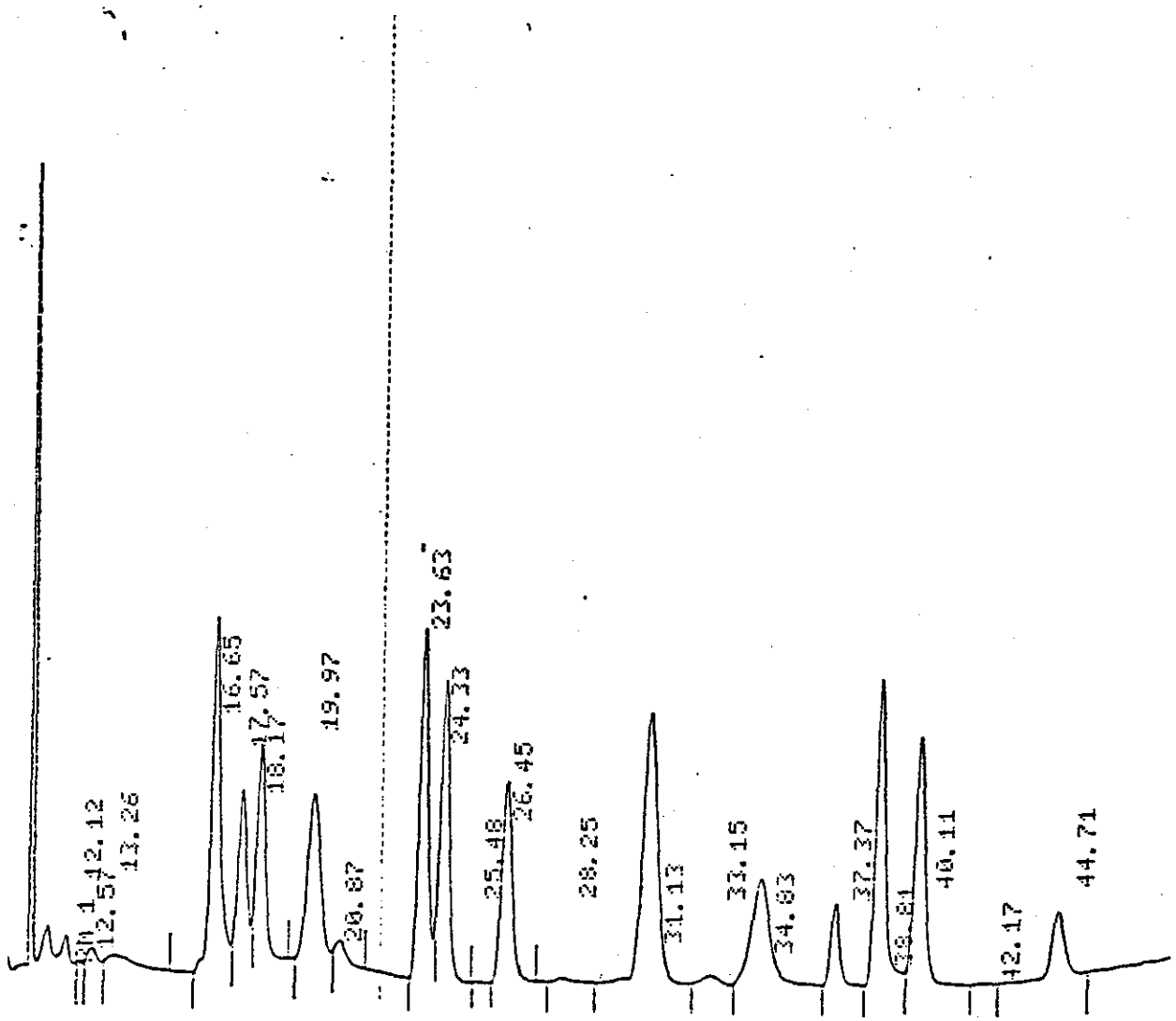


Figure 9.4

Amino acid profile of a 25% RCF solution decolourised by 0.1% CMC and 1% H_2O_2

Amino Acid	Retention time (R.T.)	Concentration of amino acids in the solution µg/ml
Aspartic	16.65	8.299
Threonine	17.57	3.472
Serine	18.17	2.86
Glutamic	19.97	5.93
Proline	20.87	2.103
Glycine	23.63	3.527
Alanine	24.33	6.927
Cysteine	25.48	0.03
Valine	26.45	6.652
Methionine	28.25	0.144
Leucine	31.13	10.471
Tyrosine	33.15	0.454
Phenylalanine	39.83	6.539
Ammonia Trace	37.37	2.838
Lysine	38.31	7.954
Histidine	40.11	5.544
Arginine	44.71	2.782

Table 9.5

10. DISCUSSION AND CONCLUSIONS

The possibility of decolourising bovine haemoglobin in a way such as to produce a functional and valuable product was investigated experimentally and financially, and compared in this project. Four methods were applied to achieve this.

The first was the 'pepsin hydrolysis method' described in Chapter 5. The aim was to split the haem from the globin molecule by using pepsin under acidic conditions. However, all attempts to achieve a process based on this were unsuccessful. The reasons put forward for this are either the non-specifity of the enzyme ending up in splitting the globin molecule and attaching these fragments to the haem group or the denaturation of the protein which occurs at the acidification step, resulting in the break up of the protein in a distribution of chain lengths, thus stopping the decolourisation.

When all the possibilities were exhausted this line of experimentation was terminated.

The second method of decolourisation was using H_2O_2, Chapter 6. This method does not depend on the separation of the haem group from the haemoglobin molecule. H_2O_2 oxidises the haemoglobin molecule giving a decolourised product. There were two types of powders produced. In the first type any residual H_2O_2 is reacted with excess RCF added after the decolourisation process. This powder, called the 'partially bleached' product, is of a rather dark brown appearance, see Table 6.1. The optimum RCF solution is of a 25% concentration and is decolourised by adding 5% H_2O_2 (v/v).

The protein content achieved in all these powders was consistently in the region 75-77%.

The other type of powder was produced without adding any excess RCF after the decolourisation step. This resulted in lighter powders than the previous ones, see Table 6.3. The aim was to use as little hydrogen peroxide as possible to decolourise the most concentrated RCF solution, i.e. 40% RCF and to rely on the drying step to dissociate any residual H_2O_2. It was found (Table 6.4) that very little residual H_2O_2 is present in the decolourised product. The optimum RCF solution had a 40% concentration and was decolourised by adding 5%, H_2O_2 (v/v) solution (100 volumes strength). This gave a brown-yellow product containing 13-14% protein in solution. These resulted in the 'bleached' powders examined in Table 6.6.

The most important feature of the H_2O_2 decolourisation plant will be the mixer used. It was found that vigorous and adequate mixing is of paramount importance for well decolourised products. Using the Hobart mixer a protein yield of 80% was achieved, see Table 6.5. This is lower than the 95% yield achieved with smaller batches of solution and with a fast food domestic mixer.

Both 'bleached' and 'partially bleached' powders showed poor functionality, Table 6.7. There is some fat binding capacity, about 1.252 ml/g, but very little else. These powders are cheap to produce, cost less than 13 pence per kilogram, and can be used in comminuted meat products for their protein value and fat binding properties.

The third method developed was the CMC decolourisation process described in Chapter 7. This relies on the splitting of the haem group from the globin molecule and the subsequent separation of the CMC-haem complex by centrifugation from the decolourised globin solution. The different parameters affecting the decolourisation were examined in Chapter 7. It was found that the more concentrated the CMC solution used, the less solids and hence protein, appeared in the decolourised solution. Therefore the protein yield was dropping when high CMC concentrations were utilised, Figs 7.1, 7.2. This indicated that the CMC is complexing not only the haem but also parts of the globin molecule, which on centrifugation were included in the CMC haem precipitate. The more concentrated the RCF solution was, the less decolourisation was achieved, the optimum RCF solution being 25%, decolourised using 0.4% CMC. Table 7.7 shows that most of the Fe content of the haemoglobin went to the CMC-haem precipitate leaving less than 1% of the initial iron content in the final product. The centrifugation step in this process is quite important. At higher speeds of centrifugation there is an increase in protein yield of the decolourised samples. As seen in Table 7.8, about 8-10% better protein yield was achieved using high centrifugation rates, i.e. in the region of 8000g.

Attempts were made to by stop the addition of so much water to the RCF solution, by using a high concentration of CMC solution, section 7.4. However, this did not produce any decolourisation. Also, the RCF solution must be acidified to pH 1.5 prior to any CMC addition for optimum results.(see Section 7.4). The ultrafiltration work carried out using the Amicon, revealed that the DRCF behaves similarly to a 1.7% plasma solution, Figures 7.9, 7.10.

A series of powders were produced for spray drying, Section 7.10. The protein solutions were adjusted to pH 7.5 with 1N NaOH prior to loading to the spray drier.

The protein content of the powders was in the region of 60-70%. The most promising powders were produced from a 20% RCF decolourised by 0.4% CMC and a 25% RCF decolourised also by 0.4% CMC, see Fig. 7.11.

The powders showed very good foaming properties and adequate fat binding capacities, see Table 7.12. Also some powders produced by using a minimum CMC concentration, (only 0.1%) and no pH adjustment prior to spray drying showed the best functionality. Even some gel forming ability was detected in contrast with everything reported in the literature, Table 7.2. When large scale decolourisation was attempted using the facilities already existing in the department a protein recovery of only 23.7% was achieved, Section 7.11.

This was however using a low rate centrifuge and also a substantial loss of product occurred in the spray drier, due to feeding the drier with a very dilute solution well below its recommended concentration. The dilute solution would result in an exceptionally fine powder, with resulting reduction in cyclone efficiency.

For this method of decolourisation a computer model was developed to provide the mass balance, costing and complete evaluation of a major plant decolourisation unit. This is described in Chapter 8, where the plant layout and design are also detailed. Using the computer program, a profitability analysis of the different decolourising methods was undertaken. The most profitable

method was shown to be the one utilising 0.1% CMC followed by the 0.6% CMC without any pH adjustment of the solution as it leaves the ultrafiltration stage, Fig. 8.9. This model also showed that the process is very profitable within a wide range of process conditions, Fig. 8.3 - 8.10.

The final method of decolourisation was using CMC and H_2O_2 combined, see Chapter 9. This showed that RCF solution can be firstly treated with 0.1% CMC and after centrifugation an addition of H_2O_2 at no more than 0.5 1% (v/v) of the total volume of supernatant did produce a very decolourised, functional globin solution, Table 9.2. This process was also tested in the pilot plant and a series of products were developed. The optimum RCF solution, prior to decolourisation, still remained at 25% because it did not create any handling problems. Attempts to load higher concentrations of RCF ended up with difficulties in pumping, mixing, and decolourising. Protein recovery was again low, less than 10% because the decolourised solution was not concentrated to optimum level prior to spray drying. The dilute solution resulted in a lot of powder being lost in the cyclone exhaust of the spray drier. No significantly better decolourising results were given when more than 0.5% H_2O_2 was added to the supernatant. Hence the optimum H_2O_2 addition is 0.5% of the total supernatant volume.

The H_2O_2 added did decrease the foaming properties of the powders as compared with the ones produced when CMC alone was used. See Table 9.4. However, some gelation was again recorded, Table 9.4.

In all three successful methods of decolourisation, an amino acid profile analysis was performed, and the results compared with untreated RCF. All the profiles obtained were similar, indicating

that no breaking down of amino acids was occurring by any of the processes used.

In conclusion, the most functional product was given by treating a 25% RCF with a 0.1% CMC solution. This was the most profitable process as well. The next most functional products were given by the CMC-H_2O_2 combined method, then by the CMC method using higher concentrations of CMC, 0.4-0.6%, and finally the least functional products were given by the H_2O_2 method.

However it should be noted that the H_2O_2 method is a very simple, easy process. It does not only decolourise the haemoglobin efficiently, but by changing the amount of H_2O_2 used the colour can be adjusted to the needs of the final food system in which the treated haemoglobin is to be included. Furthermore, the method is a means of quickly dewatering a RCF or whole blood solution. It also leaves a substantial room for profit due to low capital and operating costs for slaughterhouses of any size.

The other two methods are more sophisticated and are to be applied in abattoirs with large throughputs of animals to justify their expenditure.

11. SUGGESTIONS FOR FUTURE WORK

The CMC-H_2O_2 decolourisation method needs further development at large pilot plant scale. The ultrafiltration step of both CMC and the combined method must be closely examined using appropriate membranes and large scale UF modules. At the rig constructed for the purposes of this work only about 70 litres of RCF could be processed in one batch. Larger volumes are needed to check the ultrafiltration stage.

More market-orientated work must be carried out for potential outlets. Ultimately the degree of decolourisation needed as well as the functionality required by the globin, or haemoglobin is the case of the H_2O_2 method, will depend on the food system in which these products will be included.

Some formulations may require little decolourisation, for example in comminuted meat products or tinned meats. Others may require an acidic protein solution and not a powder.

These products are highly dependent on the food formulation that will follow after they have left the decolourisation plant.

Reference List

1. Akers, J.M. (1973). "Utilisation of Blood". Food Manufacture, 47, 4, p 31-2.

2. Altschul, A.M. Editor, (1978). 'New protein foods'. Volume 3, Academic Press.

3. Anson, M.L. (1932) 'Estimation of pepsin, trypsin, papain and cathepsin with haemoglobin'. J. Gen. Physiology 22, 79-89.

4. Antonini, E.; Brunori, M. (1970). 'Haemoglobin'.

5. Autio, K.; Kiesvaaro, M.; Malkki, Y.; Kanko, S. (1984). 'Chemical and Functional properties of blood globin prepared by a new method'. J. of Food Science, 49, 859-862.

6. Birch, G. G.; Parker, K.J.; Worgan, J.T. Editors (1976). 'Food from Waste'. Applied Science.

7. Borchers, C.W.A. (1944). Patenschrift. German patent office number 744055.

8. Brahn, B. (1941). 'Decolourisation of blood'. Voeding, 2, 141.

9. Bright,A.M. (1977). 'Recent Developments in the collection processing and utilization of blood'. Meat and Livestock Commission project number 603.

10 Bulchar, R.; Nutten, A.J. (1960). Quantitative Inorganic Analysis 206, Butterworths.

11. Buckley, K.; Vernon, A.; Lowe, P.J. (1976). Patent specification. The patent office London 1562618.

12. Christensen, V.H.; (1979). 'Decolourisation of slaughter blood by partial enzymatic hydrolysis'. The Meat Hygienist, August-September 22-26.

13. Christensen-Hald, V. (1978). Paper H5. 24th European Meeting of Meat Research Workers.

14. Cremwelge, D.D.; Dill, C.W.; Tybor, P.T.; Landmann, W.A. (1974). 'A comparison of the emulsification capacities of some protein concentrates'. J. Food Science, 39, 175-177.

15. Delaney, R.A.M. (1977). 'Protein concentrates from slaughter animal blood. 1. Preparation and purification of red blood cell concentrates'. J. Food Technology, 12, 339-354.

16. Dickerson, R.E.; Geis, I. (1969). 'The Structure and action of Proteins'. Harper and Row.

17 Doty, D.M. (1972). 'Developments in processing meat and blood by-products'. Conf. Symposium on alternative sources of protein for animal production. Virginia Polytechnic Institute and State University, July 1972, 61-72.

18. European patent application number 82200672.2. Unilever N.V. 'Process for the manufacture of globin or blood cell protein and haem from haemoglobin'.

19. European patent application number 792007833. Unilever N.V. 'Process for preparing blood cell protein and haem from haemoglobin'.

20. Filstrup, P. (Alfa Laval Sept A/S, Copenhagen Den.) (1980). 'Processes and equipment for protein byproducts in the meat industry'. Applied Protein Chemistry, 181-222. Edited by Grant R.A., Applied Science.

21. Forrest, J.C.; Aberle, E.D.; Hendrick, H.B.; Judge, M.D.; Merkel, R.A. (1975) 'Principles of meat science'. Freeman and Company.

22. Grant, R.A. (1980). 'Applied protein chemistry'. Applied Science.

23. Hansen, P.M.T.; Chang, J.C. (1968). 'Quantitative recovery of carboxymethyl cellulose from milk'½ J. Agricultural Food Chemistry, 16, 77-79.

24. Hayakawa, S.; Ogawa, T.; Sato, Y. (1982). 'Some functional properties under heating of the globin prepared by carboxy methyl cellulose procedure. J. of Food Science, 47, 1415-1418.

25. Heinz, G. (1969). "Bleeding pigs via a closed circuit and the use of blood obtained in this way in the preparation of plasma". Fleischwirtschaft, 49 (5), 613-9.

26. Hermansson, A.M. and Tornberg, E. (1976). "Functional properties of some protein precipitations from blood". 22nd European Meeting of Meat Research Workers. Sweden 11:1 - 11:6.

27. Hill, S. (1986). Functional properties of blood plasma. Ph.D. Thesis submitted. Personal communication.

28. Holland, F.A.; Watson, F.A.; Wilkinson, J.K.; (1983). 'Introduction to process economics'. 2nd edition. John Wiley & Sons.

29. Holt, B.W.; M.L.C. Marketing Services (1977). "Collection Techniques at the Abattoir". Proceeding of an MLC Seminar Sept. 1977.

30. Howell, N.K. (1981) "Separation and Functional properties of blood plasma proteins". Ph.D. Thesis, Nottingham University,

31. Hurst, N.W. (1980). 'Ultrafiltration of bovine blood plasma' Ph.D. Thesis Loughborough University.

32. International Patent number WO 83/03198. "A method for dividing blood haemoglobin into haem and globin'.

33. International Patent number WO 74/1562618. Australian Specification Patent 502112 (CSIRO).

34. Lawrie, R.A. (1978) 'Meat Science'. 3rd Edition Pergamon Press.

35. Ledward, D.A.; Taylor, A.J.; Lawrie, R.A. (1983) 'Upgrading waste for feeds and food'. Butterworths.

36. Lehringer, L.A.; (1975). 'Biochemistry'. 2nd Edition Worth. Publishers Ltd.

37. Mitsyk, V.E.; Osadchaya, I.F.; Kostyuk, E.A.; Pugachevskaya, R.I.; Moroz, A.M. and Reznichenko, L.Z. (1975). "Quality of clarified animal blood and of food products with additions of this blood". Fd Sci. and Tech. Abstracts, 1976, 8, No. 2S. 228.

38. Moore, S.; Stein, W.H. (1963) 'Methods in Enzymology', Volume 6, Editors Colowick S.P.; Kaplan, N.O. Academic Press New York.

39. Nonnemann, K. (1979). 'Plant for bleaching of blood and production of blood emulsions'. Nutridan Engineering, Denmark.

40. Oellingrath, I.M.; Slinde, E. 'Iron content and colour of meat loaves'. Norwegian Food Research Institute. Private communication.

41. Osadchaya, I.R.; Moroz, A.M. and Kostyk, E.A. (1975). 'Use of animal protein in the manufacture of dough products'. Tovarovedenie, 8, 24-27.

42. Passmore, R. Editor,(1976). 'A companion to medical studies'. Volume 1. Davis, J.R. 'Blood' Chapter 4-8 Second edition, Blackwell.

43. Pearson, D. (1976). 'The chemical analysis of Foods'. Seventh edition, Churchill Livingstone.

44. Perry, R.H.; Chilton, C.H.; (1973). 'Chemical Engineers Handbook'. 5th Edition. McGraw Hill.

45. Perutz, M.F. (1968). 'The haemoglobin molecule'. The Groonian Lecture 1968. Proc. Roy. Soc. 173, 113-140.

46. Perutz, M.F.; Muirhead, H.; Mazzarelloi. L.; Crowther, R.A.; Greer, J. ; Kilmartin, J.V. (1969). "Haemoglobin" Nature, 222, 1240.

47. Perutz, M.F.; Muirhead, H.; Cox, J.M.; Coaman, L.C.G.; Mathews, F.S.; McGandy, E.L.; Webb, L.E.(1968b). "The haemoglobin molecule". Nature, 219, 39

48. Perutz, M.F. (1961). 'Proteins and nucleic acid' Elsevier.

49. Quaker Oats Co. (1976). "Access for producing a can pet food". British patent, 1414, 107.

50. Ranken, M.D. (1977) "Food Ingredients from Animal Blood" Chemistry and Industry, 18, 498.

51. Rao, C.N.R. (1961). 'Ultra violet and visible spectroscopy chemical applications'. Butterworths.

52. Sakellariou, E.S.; Norwood, D. (1982). 'Design of an animal blood processing plant'. Final year project, Loughborough University.

53. Sato, Y.; Hayakawa, S.; Hayakawa, M. (1981). 'Preparation of blood globin through carboxymethul cellulose chromatography'. J. Food Technology, 16, 81-91.

54. Satterlee, L.D., Free, B. and Levin, E. (1973). "Utilisation of high protein tissue powders as bidner/extender in meat emulsions". J. Fd Sci., 38 (2), 306-309.

55. Shahidi, F.; Naczk, M.; Rulin, L.J. and Diosady, L.L. (1984). 'Functional properties of blood globin'. J. of Food Science, 49, 370-372.

56. Shiga, T.- Imaizumi, K. (1975). 'Electron spin resonance study on peroxidase and oxidase - Reactions of Horse Radish peroxidase and methaemoglobin'. Archives of Biochemistry and Biophysics, 167, 469-479.

57. Smith, D.L. (1983). 'Functionality of an enzyme decolourised blood protein hydrolysate'. Report, LUT.

58. Turner, D.P. (1983). 'Collection; processing and uses of slaughterhouse blood'. Final Year Dissertation, Loughborough University.

59. Tybor, P.T., Gill, C.W.; Landmann, W.A. (1975). 'Functional properties of proteins isolated from bovine blood by a continuous pilot process'. J. of Food Science, 40, 155-159.

60. Van Den Oord, A.H.A.; Wesdorp, J.J. (1978). 'Decolourisation of slaughterhouse blood by treatment with hydrogen peroxide'. Report. Unilever Research, The Netherlands.

61. Vickery, J.R. (1968). 'Better utilisation of abattoir by-products'. Institute Meat Bulletin, 62, 21-26.

62. Vickery, J.R. (1968). 'The recovery and utilization of edible proteins from blood and fresh fish'. Food Technology in Australia. July 1968, 315-319.

63. Vogel, A.I. (1959). 'A text book of macro and semimicro qualitative inorganic analysis'. Fourth edition, Longman).

64. Weissbluth, M. (1974). 'Haemoglobin Cooperativity and Electronic Properties'. Chapman and Hall Limited, London.

65. Wismer-Pedersen, J. (1979). 'Ultrafiltration of animal blood in meat products' J. Fd Tech. 33, 76.

66. Zayas, Yu, F.; Klebanov, G.I. and Zyrina, L.K. (1976). "A chemiluminescent study into the effect of lipids peroxidation of a blood-fat emulsion". 22nd European Meeting of Meat Research Workers, Sweden, E3: 1-7.

67. Zayas, Y.; Zyrina, L. and Sokolov, A. (1975). "Physical method of purifying blood". Myas Ind. SSSR, 53 (1) 20-22.

APPENDIX I

Physicochemical properties of blood and its fractions

Specific gravity

Whole blood (bovine)	1.060
Plasma	1.034
Red Cell Fraction	1.064

Freezing point of plasma is -0.5 to -0.6^{o}C

Whole blood in vivo has a pH of 7.2

APPENDIX II

Equipment specification for the CMC decolourisation plant

1. Aseptic Collection

The following information is provided by Simon-Iwel U.K.

Equipment listing

	Item	Units required
1	Carousel blood collection system (includes 4 X100 litre tanks, pneumatically operated controls, valves and electrical control panels)	1
2.	Set of hollow sticking knives (includes citrate flow control, lockable sterilising cabinets and blood delivery hoses)	2
3.	Citrate anticoagulant unit (includes closing system complete with tank circulating pump, temperature controls, pipework and valves).	1
4.	Carcass marking set (Digital branding equipment)	1
5.	Condemned blood removal (includes pump, interlock arrangement delivery piping and controls)	1

4. Heat exchanger

Recommended equipment = APV paraflow plate heat exchanger.

Material of construction = Stainless Steel
Heating medium = Water preheated by steam.
Price £1000

5. Ultrafiltration plant

Recommended Membrane type = PCI model T5/A - Price £45,000
Membrane specification
nominal 95% cut off for 20,000 MW dextran
and 50% cut off for 10,000 MW dextran
Operation temperature 20-50°C
U.F. module specification = B1 UF module with twin entry and caps containing 18 x 8 membranes
Modules connected in series or parallel
Additional equipment = Pump = 30 kW
Pressure control valves
Feed tank and recycle

6. Spray Drier

Recommended equipment. Spray Processes Ltd type 616

Heat requirements = 60800 BTU/hr
Electrical requirements 14 kW
Material of construction = stainless steel
Solid collecting: through a rotary valve at the base of the drying chamber.
Inlet temperature 200°C
Outlet temperature 90°C
Dimensions 6'x 16'
Maximum capacity 200 kg/hr
Price £70,000

6. Edible blood delivery system 1
(includes piping and control valves)

7. Edible blood storage tanks 2
(Tank capacity 2000 litres
complete with valve & fittings)

Materials of construction = Stainless Steel
Utilities required

Process water = 30 l/min
Steam (at 4 bar) = 75 kg/hr
Compressed air (4 bar) = 0.2
Electricity = 1.5 kW
Price £20,000

2. Low rate centrifuge

Recommended Equipment = Alfa Lavel BPB 207 Centrifuge

Capacity = 300-500 l/hr
Material of construction = Stainless Steel
Power requirements = 2.2 kW
Price £9500

3. High rate centrifuge

Recommended equipment = Alfa Laval BTP x 205

Max Capacity = 1.2 m^3/hr (water)
Power requirements = 6.5 kW
Maximum g-force = 12,800 g
Material of construction = Stainless Steel
Price £35,000

7. Packaging Machine

Gravity feed from a cyclone shaped storage tank through a manually operated valve into 25 kg sacks is recommended. Material of construction should be stainless steel.

Price £500

8. C.I.P cleaning unit

Recommended Equipment : APV CIP Unit
Electrical requirements 1.1 kW
Utilities steam, gas (as heating medium)
process water, compressed air.

Additional equipment
Control valves 6 x B3 Zephyr valves
3 x B4 Zephyr valves
2 storage tanks for rinse water and detergent solution

Price: £5,000

9. Mixing Tank

Size : 1. Decolourisation 2 x 2000l
2. pH adjustment 2 x 2000 l

Material of construction - Stainless steel
Auxiliary equipment: mixing paddle, control valves and fitting.
Price £2800 each

10. Pumps and piping

Recommended pump : APV rotary pump

Power 1.1 kW Price £895

Recommended pipe diameter 1½"

Material of construction : Stainless Steel

11. Storage facility, laboratory building

These have been included in the computer program. Cost was calculated per cubic meter for the storage facility and building, £280. The area of the building including stores is calculated to 500 m^2

The laboratory cost is of the order of £8,000

APPENDIX III

Computer Variables Index

Computer Variable Index

A_1 No. cattle killed/per hr
A_2 Blood yield (ℓ/animal)
A_3 No. of operating hours per day
A_5 Blood flowrate (ℓ/hr)

B_o Average blood flowrate (ℓ/hr)
B_1 Blood flowrate (ℓ/yr)
B_2 Fraction of RCF from centrifuge (1)
B_3 Specific gravity of blood

C_1 Volume of 1N NaOH required (ℓ)
C_2 Concn of sodium citrate solution (wt-fraction)
C_3 Final concn of sodium citrate in the blood (ℓ/ℓ blood)

D_1 Ratio of water added in decolourisation operation
D_2 Ratio of 1N HCl added in decolourisation operation
D_3 Ratio of CMC added in decolourisation operation
D_4 Concn of CMC added in decolourisation operation

E_o Cost of H_2O (£)
E_1 Cost of 1N HCl (£)
E_2 Cost of CMC (£)
E_3 Cost of 1N NaOH (£)
E_4 Cost of Sodium citrate (£)
E_5 Cost of cleaning fluid (£)
E_6 Cost of packaging material (£)

F_o Variable cost of operating asceptic collection unit (£)
F_1 Variable cost of operating low rate centrifuge (£)
F_2 Variable cost of operating high rate centrifuge (£)
F_3 Variable cost of operating heat exchanger (£)
F_4 Variable cost of operating ultrafiltration unit (£)
F_5 Variable cost of operating spray drier (£)
F_6 Variable cost of operating pumps (£)
F_7 Variable cost of operating building services (£)
F_8 Total variable costs (£)
F_9 Total production costs (£)
F_{10} Variable cost of processing plasma fraction (£)

G_o Mass/Volumetric flowrate of globin prior to neutralisation (ℓ/hr)
G_1 Fraction of protein in globin prior to neutralisation (ℓ/hr)
G_5 Mass/Volumetric flowrate of globin after neutralisation (ℓ/hr)
G_{11} Protein concn of globin (mg/ml)
G_{12} Flux rate of permeate(ℓ/hr)
G_{13} Flux rate of water/series of modules/hr
G_{14} Flux rate of permeate/series of modules/hr
G_{15} Fluxrate of globin through module/sec.
G_{19} Fraction of water in the ultrafiltrated globin
G_{11} Fraction of protein in the ultrafiltrated globin
G_{21} Fraction of salt in the ultrafiltrated globin
G_{23} Specific gravity of globin

H_o Volumetric flowrate of CMC-haem (ℓ/hr)
H_1 Fraction of CMC-haem in CMC-haem
H_2 Fraction of protein in CMC-haem
H_3 Fraction of water in CMC-haem
H_4 Mass flowrate of CMC-haem (kg/hr)
H_5 Mass flowrate of CMC-haem (kg/hr)

I_o Volume fraction of globin from centrifuge (2)
I_1 Efficienty of centrifuge (2)
I_2 Fractional protein loss to haem-CMC fraction

J_o Capital cost of asceptic collection unit for 30 cattle (£/hr)
J_1 Capital cost of asceptic collection auxilliary equipment (£)
J_2 Capital cost of low rate centrifuge (£)
J_3 Capital cost of high rate centrifuge (£)
J_4 Capital cost of stainless steel tank (£/ℓ)
J_5 Capital cost of heat exchanger (£)
J_6 Capital cost of ultra filtration unit inclusive of auxiliary equipment (£)
J_7 Capital cost of spray drier (£)
J_9 Capital cost of packing machine (£)
J_{10} Capital cost of 1.1 kw pump (£)
J_{12} Capital cost of Zephyr control valve B3 (£)
J_{13} Capital cost of Zephyr control valve B4 (£)
J_{14} Capital cost of support, piping and control valves (£)
for H_2Om HCl, NaOH & CMC lines (£)
J_{15} Capital cost of building (£)
J_{16} Capital cost of auxiliaries (£)

K_o Capital cost of asceptic collection unit for 30 cattle/hr (£)
K_1 Capital cost of low rate centrifuge process (£)
K_2 Capital cost of high rate centrifuge process (£)
K_3 Capital cost of decolourisation unit (£)
K_4 Capital cost of sodium hydroxide addition unit (£)
K_5 Capital cost of heat exchanger (£)
K_7 Capital cost of UF unit (£)
K_8 Capital cost of packing machine (£)
K_9 Total capital cost of 1.1 kw pumping network (£)
K_{10} Capital cost of control system (£)
K_{11} Capital cost of CIP unit (£)
K_{12} Capital cost of building and auxiliaries (£)
K_{13} Capital cost of piping and installation (£)
K_{14} Total fixed capital costs including commissioning a laboratory (£)

L_o Cost of water (£/ℓ)
L_1 Cost of 1N HCl (£/ℓ)
L_2 Cost of CMC (£/kg)
L_3 Cost of 1N NaOH (£/ℓ)
L_4 Cost of sodium citrate (£/kg)
L_5 Cost of cleaning fluid (£/weeks supply)
L_6 Cost of process water (£/m^3)
L_7 Cost of electricity - day rate (£/kwh)
L_8 Cost of gas (£/therm)
L_9 Cost of effluent (£/m^3)
L_{10} Cost of packaging material (£/kg dried globin)
L_{11} Cost of membrane replacement (£/18 membranes)

M_o Supervisor salary (£)
M_1 Labourer salary (£)
M_2 Part-time technician salary (£)

N_o No of low rate centrifuges
N_1 No. of high rate centrifuges
N_2 Volume of decolourising tank (ℓ)
N_3 Volume of NaOH treatment tank (ℓ)
N_5 No. of APV B3 Zephyr valves
N_6 No. of APV B4 Zephyr valves
N_4 No. of 1.1 kw pumps
N_7 Building area (m^2)

P_o Mass flowrate of plasma solution (ℓ/yr)
P_1 Mass flowrate of dried plasma (kg/yr)
P_2 Concentration of H_2O in plasma fraction (inclusion of sodium citrate)
P_3 Concentration of protein in plasma fraction (inclusion of sodium citrate)
P_4 Concentration of salt in plasma fraction (inclusion of sodium citrate)
P_5 Specific gravity of plasma
P_6 Cost of processing plasma (£/tonne)

Q_o Cost of labour (£/yr)
Q_1 Cost of maintenance (£/yr)
Q_2 Cost of overheads (£/yr)
Q_3 Total fixed production costs (£/yr)

R_o Volumetric flowrate of RCF from low rate centrifuge (ℓ/yr)
R_1 Mass flowrate of RCF from low rate centrifuge (ℓ/yr)
R_2 Specific gravity of RCF from low rate centrifuge (ℓ/yr)
R_3 Mass flowrate of RCF from low rate centrifuge (kg/hr)
R_4 Mass flowrate of H_2O in RCF
R_5 Mass flowrate of protein in RCF
R_6 Mass flowrate of salt in RCF
R_7 Mass flowrate of other in RCF
R_8 Mass flowrate of decolourised RCF (kg/hr)
R_9 Fraction of protein in decolourised RCF
R_{10} Fraction of haem-CMC in decolourised RCF
R_{11} Fraction of H_2O in decolourised RCF
R_{12} Fraction of salt in decolourised RCF
R_{13} Fraction of other in decolourised RCF

S_o Concentration of protein in spray dried globin
S_1 Spray drier correction factor
S_2 Mass flowrate of spray dried globin (kg/hr)
S_3 Fraction of water in spray dried globin
S_4 Fraction of protein in spray dried globin
S_5 Fraction of salt in spray dried globin
S_6 Mass flowrate at spray dried globin (kg/hr)

T Temperature of feed entering the UF unit (^oC)
T_1 Mass flowrate of water evaporated from spray dried (kg/hr)
T_2 Mass flowrate of water UF from spray drier (tonnes/yr)

U_o Area of U/F membrane (m^2)
U_1 U/F flux rate correction factor
U_2 No. U.F. modules in the plant
U_3 No. of U.F. modules in series in the plant
U_4 Fraction of water in globin leaving U.F.
U_5 Fraction of protein in globin leaving the U.F.
U_6 Fraction of salt in globin leaving U.F.
U_7 Control on the maximum flowrate of globin through U.F.
U_8 Control on calculations to produce similar flux rates
U_9 Expected membrane life (yrs).

V_o Annual sales income from dried globin (£/yr)
V_1 Annual sales income from dried plasma (£/yr)
V_2 Annual sales income from CMC-haem (£/yr)
V_3 Total sales income (£/yr)
V_4 Selling price of dried globin (£/tonne)
V_5 Selling price of dried plasma (£/tonne)
V_6 Selling price of CMC-haem (£/tonne)
V_7 Gross annual profit (£)
V_8 Net annual profit (£)
V_9 Return on investment (%)
V_{10} Payback time (yrs)

X_o Minimum rate of return (inclusive of inflation)
X_1 Iterative rate of return
X_2 Annual discounted cash flow
X_3 Min rate of return (exclusive of inflation)
X_4 Inflation rate
X_5 Rate of taxation

Y_o loop counter
Y_1 discounted factor

Z_o Net present value
Z_1 DCFRR
Z_2 NPV at variable annual profits
Z_5 DCFRR for variable annual profits

APPENDIX IV

Sensitivity Analysis Data

Sensitivity Analysis Data

Table 1 Sensitivity to cattle throughputs of 10 to 30 cattle/hr

Cattle throughput Nos/Hour	ROI %	Payback Time hrs	NPV (h)	DCFRR (%)
10	12	8.1	-74788	6.09
15	23	4.2	63988	20.78
20	36	2.7	253223	36.26
25	50	1.9	442457	50.62
30	54	1.8	581239	55.26

Table 2 Sensitivity to blood yields of 5-12 litres/animal

Blood Yield ltr/animal	ROI %	Payback Time hrs	NPV (h)	DCFRR (%)
5	9	10.55	- 108845	1.90
6	15	6.50	- 39963	10.42
7	21	4.75	- 11023	13.76
8	22	4.52	46576	19.25
9	27	3.70	115458	25.18
10	31	3.13	184340	30.82
11	36	2.71	253223	36.26
12	41	2.39	322105	41.57

Table 3 Sensitivity to Operating time

Hours of Operation (hrs/day)	ROI (%)	Payback Time (yrs)	NPV (h)	DCFRR (%)
3	16	6.24	- 38605	11.26
4	26	3.78	107306	24.49
5	36	2.71	253223	36.26
6	47	2.11	399139	47.39
7	57	1.73	545055	58.20

Table 4 Sensitivity to the Protein Yield

Protein yield (%P	ROI (%)	Payback Time (yrs)	NPV (h)	DCFRR (%)
30	18	5.4	- 3011	14.71
40	22	4.49	48496	19.43
50	25	3.86	99636	23.84
60	29	3.38	151343	28.14
70	33	3.01	202283	32.25
80	36	2.71	253223	36.26

Table 7 Sensitivity to the Selling Price of Globin

Selling Price of Globin (£/tonne)	ROI (%)	Payback Time	NPV (£)	DCFRR (%)
1000	19	5.25	3822	15.34
1250	22	4.54	45397	19.14
1500	29	4.0	86962	22.76
1750	27	3.58	128527	26.27
2000	30	3.23	170099	29.67
2250	33	2.95	211657	32.99
2500	36	2.71	253223	36.99
2750	39	2.51	294788	39.48
3000	42	2.34	336353	42.65

Table 8 Sensitivity to the Selling price of CMC-haem

Selling price of CMC-haem £/tonne	ROI (%)	Payback Time (yrs)	NPV (£)	DCFRR (%)
100	16	6.17	- 36147	11.51
150	20	4.92	21726	17.01
200	24	4.10	79600	22.14
250	28	3.50	137475	27.00
300	32	3.05	195349	31.70
350	36	2.71	253223	36.26
400	40	2.44	311097	40.73

APPENDIX V

Particle size analysis of decolourised RCF by the CMC method

The following figures are of the samples described below.

Figure 1	Untreated red cell fraction
Figure 2	A 20% RCF solution decolourised by 0.6% (w/v) CMC, mixture homogenized
Figure 3	A 25% RCF solution decolourised by 0.6% (w/v) CMC
Figure 4	A 33% RCF solution decolourised by 0.6% (w/v) CMC

MALVERN [illegible] PARTICLE SIZER MK1.1

MALVERN INSTRUMENTS LTD.SPRING LANE,MALVERN,WORCS.

PRINTING RESULTS FROM DATA BLOCK 1

TIME 09:12:30 RUN NO. 26 LENS ERROR = 0.03

SAMPLE CONCENTRATION = 0.012% BY VOLUME
OBSCURATION = 0.20

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY CONFIRMED	LIGHT ENERGY MEASURED
188.0	87.2	[illegible]	5.8	0.0	714	711
87.2	53.8	75.4	18.0	5.8	957	981
53.8	37.6	61.8	13.4	24.6	1153	1139
37.6	28.1	47.7	14.2	38.1	1318	1329
28.1	21.5	35.4	12.3	52.3	1549	1512
21.5	16.7	25.9	9.5	64.6	1777	1803
16.7	13.0	18.2	7.7	74.1	1960	1961
13.0	10.1	11.1	5.1	81.9	2047	2047
10.1	7.9	7.9	3.1	87.2	2041	2031
7.9	6.2	5.2	1.8	93.0	1939	1909
6.2	4.8	3.6	1.6	94.8	1737	1717
4.8	3.8	2.7	0.9	95.4	1538	1499
3.8	3.0	2.7	0.0	97.3	1419	1348
3.0	2.4	1.9	0.8	97.3	1409	1241
2.4	1.9	1.1	0.7	98.2	1417	1266

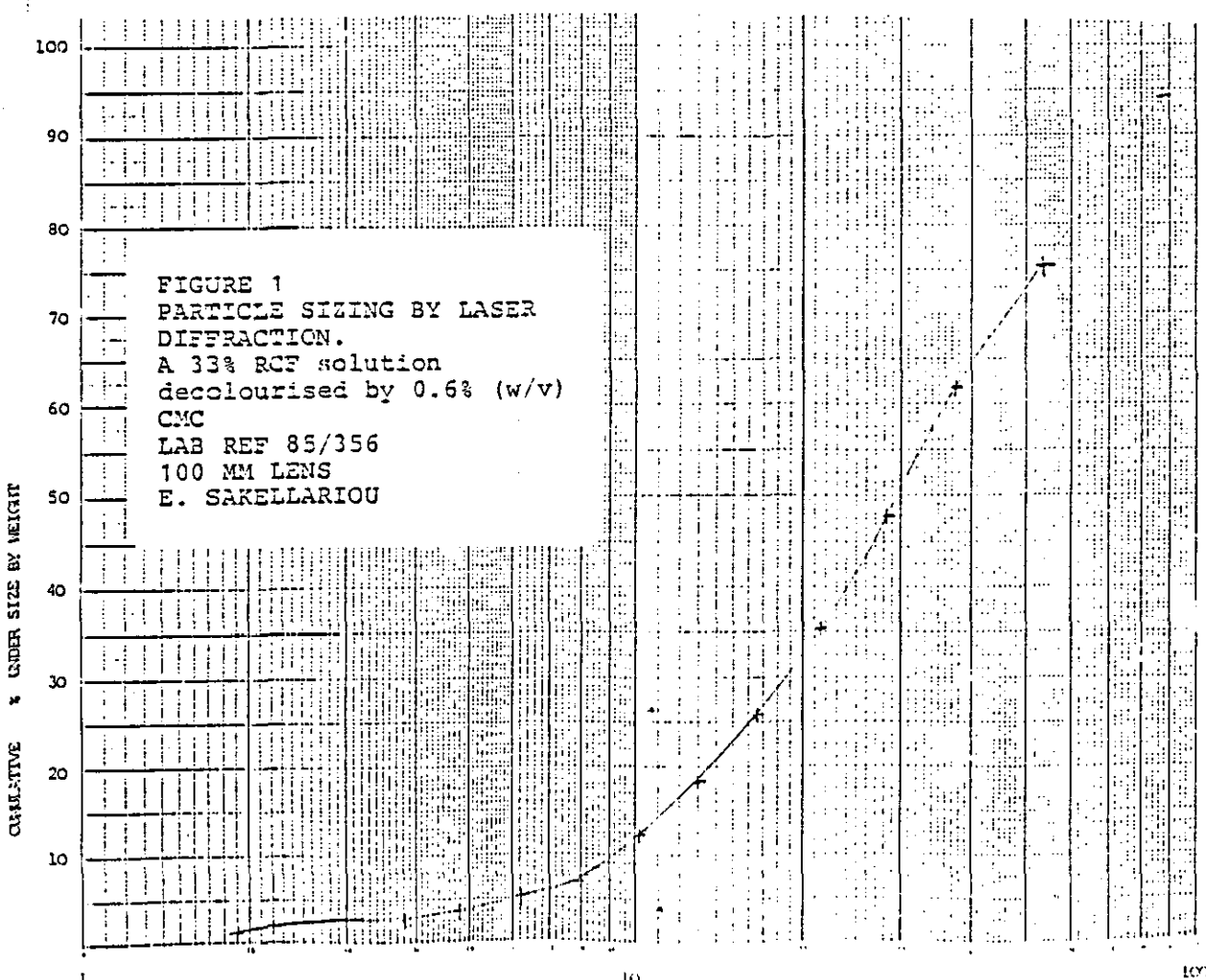


FIGURE 1
PARTICLE SIZING BY LASER DIFFRACTION.
A 33% RCF solution decolourised by 0.6% (w/v) CMC
LAB REF 85/356
100 MM LENS
E. SAKELLARIOU

MALVERN 2600 PARTICLE SIZER V3.1

MALVERN INSTRUMENTS LTD.,SPRING LANE,MALVERN,WORCS.

PRINTING RESULTS FROM DATA BLOCK: 1

TIME 09-27-30 RUN NO. 25 LOG ERROR = 3.70

SAMPLE CONCENTRATION = [illegible] % BY VOLUME
OBSCURATION = 0.29

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE % BELOW	HEIGHT IN BAND	CUMULATIVE % ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	87.2	96.6	3.4	0.0	311	213
87.2	53.8	86.3	10.3	3.4	299	302
53.8	37.6	81.4	4.9	13.7	294	291
37.6	28.1	74.1	7.4	18.6	289	289
28.1	21.5	66.9	7.1	25.9	691	692
21.5	16.7	57.2	9.8	34.5	939	950
16.7	13.0	46.6	10.6	42.8	1225	1227
13.0	10.1	39.3	7.3	53.4	1525	1533
10.1	7.9	26.4	12.9	60.7	1804	1807
7.9	6.2	16.6	9.8	73.6	2047	2044
6.2	4.8	12.7	3.9	83.4	2047	2047
4.8	3.8	11.3	1.4	87.3	1944	1962
3.8	3.0	7.3	4.0	88.7	1751	1748
3.0	2.4	6.9	0.3	92.7	1702	1614
2.4	1.9	4.7	1.8	93.5	1768	1765

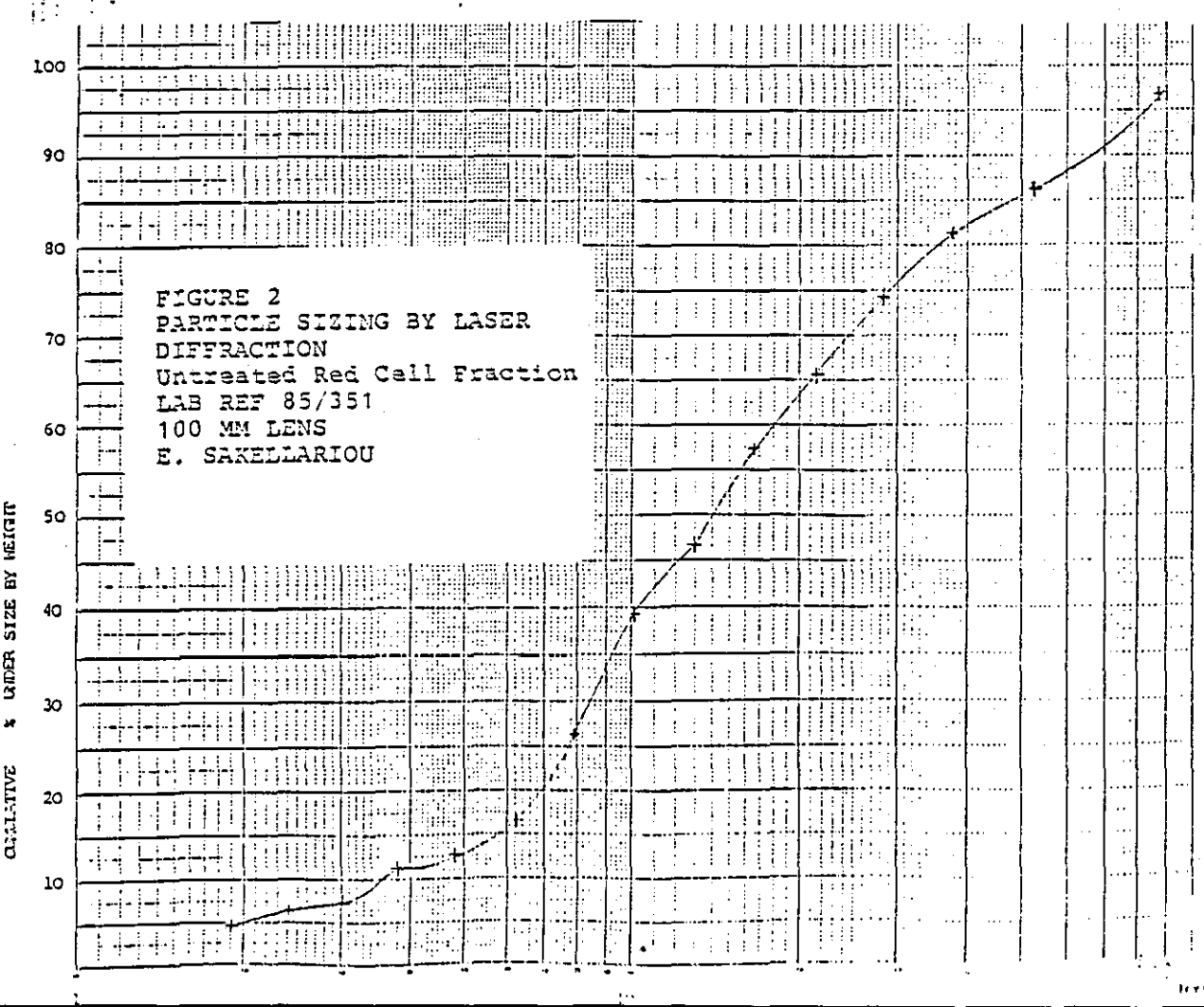


FIGURE 2
PARTICLE SIZING BY LASER DIFFRACTION
Untreated Red Cell Fraction
LAB REF 85/351
100 MM LENS
E. SAKELLARIOU

MALVERN 2600 ANALYSIS PARTICLE SIZER MK. 1

MALVERN INSTRUMENTS LTD.ARTHUR ROAD.MALVERN.WORCS.

PRINTING RESULTS FROM DATA BLOCK 1

TIME 02-14-30 RUN NO. 13 LOG ERROR = 3.69

SAMPLE CONCENTRATION = 0.0001 % BY VOLUME
OBSCURATION = 0.16

SIZE BAND UPPER	LOWER	CUMULATIVE % BY BELOW	WEIGHT % IN BAND	CUMULATIVE % BY ABOVE	LIGHT ENERGY CONCENTRATED	MEASURED
188.8	87.2	100.0	0.0	0.0	151	144
87.2	53.7	96.5	3.5	3.5	352	350
53.5	37.6	95.1	1.3	4.9	377	378
37.6	28.1	87.4	7.8	12.6	530	546
28.1	21.5	75.7	11.6	24.3	774	759
21.5	16.7	63.3	12.4	36.7	1061	1072
16.7	13.0	50.8	12.5	49.2	1399	1384
13.0	10.1	39.1	11.7	60.9	1651	1662
10.1	7.9	27.1	12.0	72.9	1911	1913
7.9	6.2	14.6	12.4	85.4	2047	2047
6.2	4.8	13.0	1.6	87.0	2011	2010
4.8	3.8	11.1	2.2	88.7	1837	1854
3.8	3.0	9.7	1.4	90.3	1679	1674
3.0	2.4	6.8	2.9	93.0	1663	1686
2.4	1.9	4.7	2.1	95.2	1811	1794

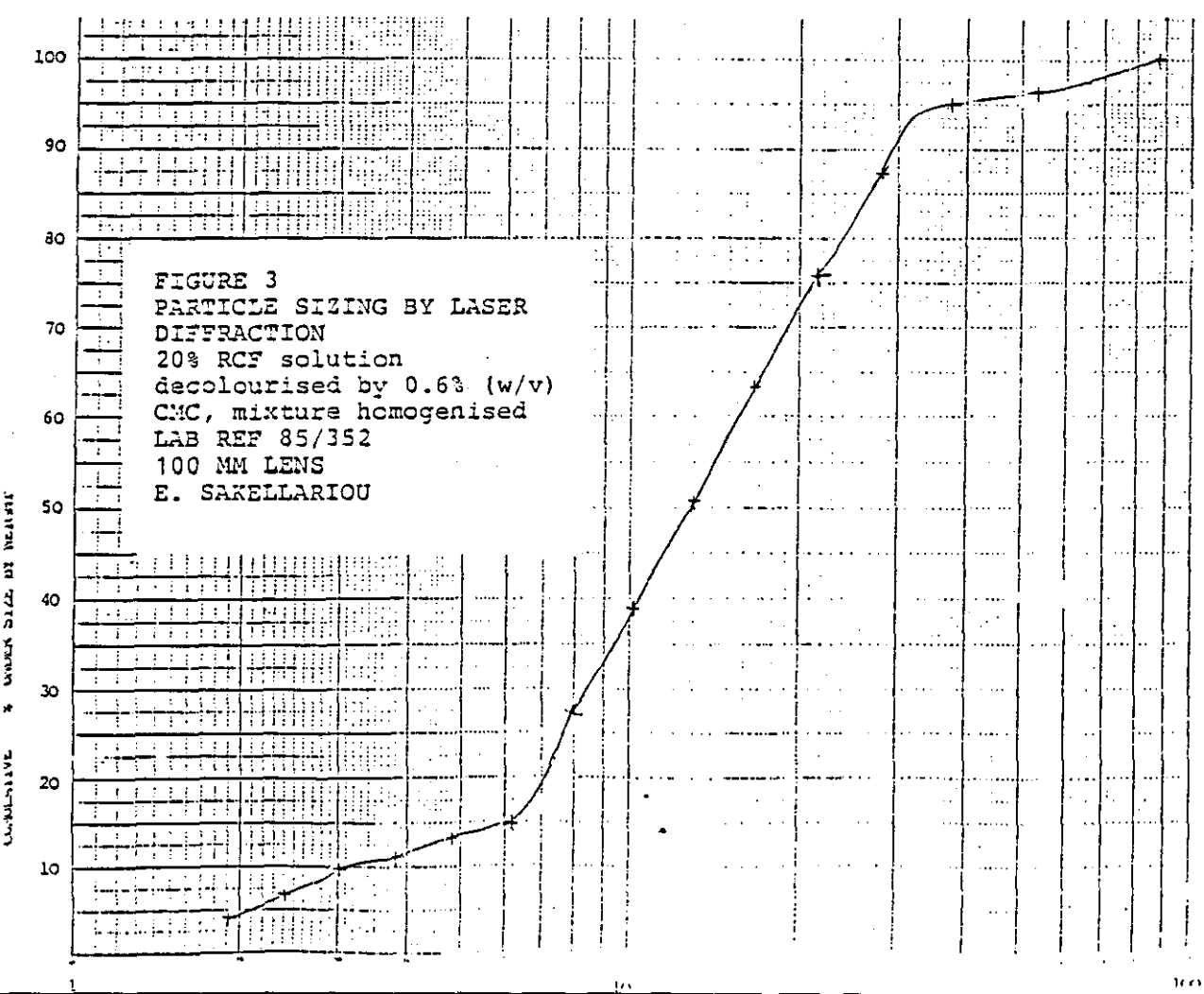


FIGURE 3
PARTICLE SIZING BY LASER DIFFRACTION
20% RCF solution
decolourised by 0.6% (w/v)
CMC, mixture homogenised
LAB REF 85/352
100 MM LENS
E. SAKELLARIOU

MALVERN 2600/3600 PARTICLE SIZER V3.1

MALVERN INSTRUMENTS LTD.SPRING LANE,MALVERN,ENGLAND.

PRINTING RESULTS FROM DATA BLOCK 1

TIME 03-12-90 RUN NO. 22 LOG ERROR = 3.61

SAMPLE CONCENTRATION = 0.0057 % BY VOLUME
OBSCURATION = 0.07

SIZE BAND UPPER	SIZE BAND LOWER	CUMULATIVE WT BELOW	WEIGHT IN BAND	CUMULATIVE WT ABOVE	LIGHT ENERGY COMPUTED	LIGHT ENERGY MEASURED
188.0	87.2	99.9	0.6	0.0	163	163
87.2	53.3	98.7	0.7	0.6	267	267
53.3	37.6	94.2	1.6	4.3	326	329
37.6	28.1	86.3	7.8	5.8	564	573
28.1	21.3	74.6	11.8	13.7	895	903
21.3	16.7	62.8	10.6	25.4	1101	1104
16.7	13.0	51.4	11.4	36.0	1402	1401
13.0	10.1	38.8	9.0	52.4	1715	1720
10.1	7.9	25.8	12.8	61.7	1931	1931
7.9	6.2	14.2	9.0	74.4	2047	2047
6.2	4.8	10.0	3.8	83.8	2027	2019
4.8	3.8	10.1	2.7	87.0	1917	1899
3.8	3.0	8.4	2.0	89.6	1749	1749
3.0	2.4	5.8	1.9	91.6	1718	1697
2.4	1.9	4.2	2.4	93.5	1789	1762

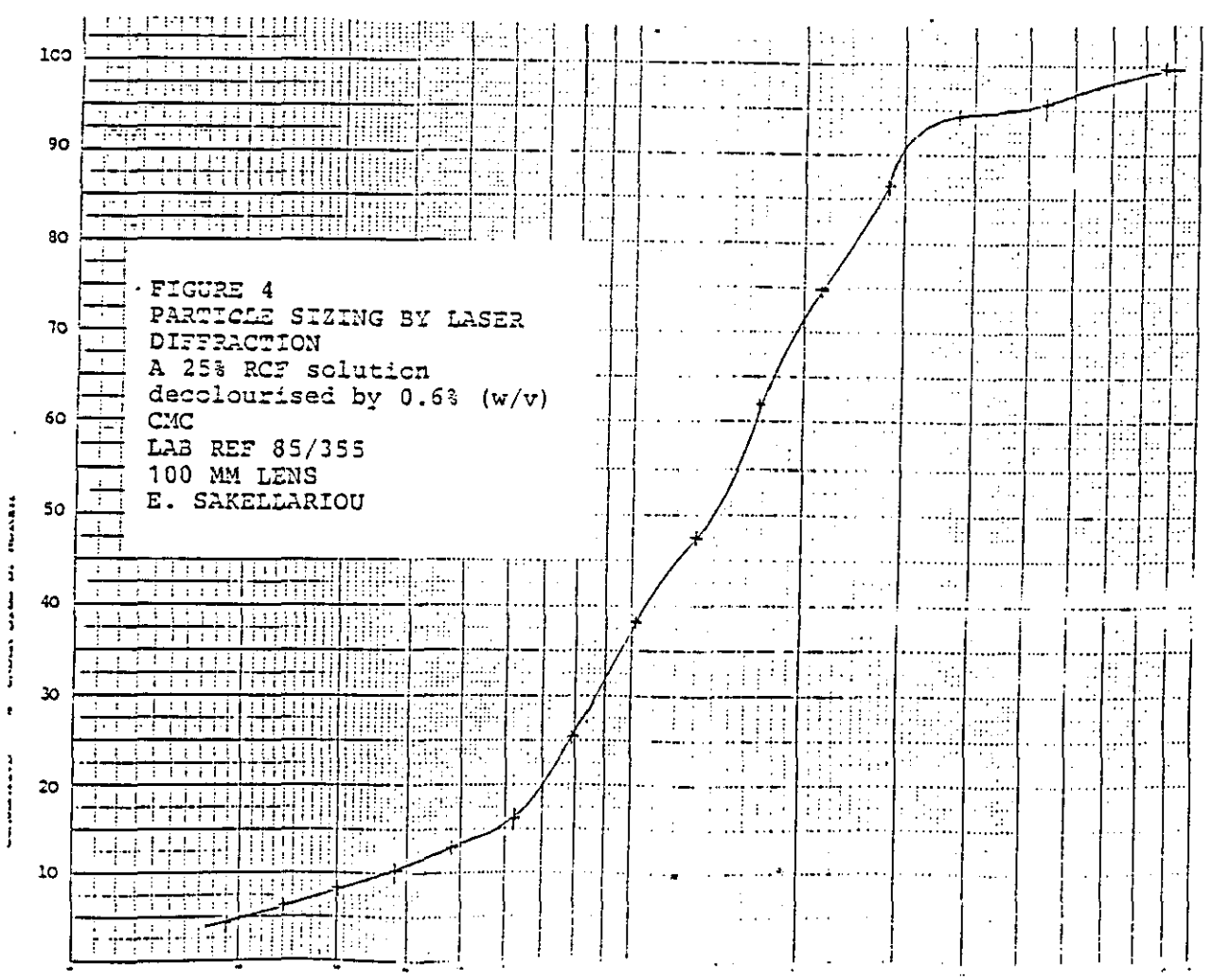


FIGURE 4
PARTICLE SIZING BY LASER DIFFRACTION
A 25% RCF solution decolourised by 0.6% (w/v) CMC
LAB REF 85/355
100 MM LENS
E. SAKELLARIOU

APPENDIX VI

Amicon membranes used

The membranes used for UF in the Amicon were of the PM series. They were made from an inert, non-ionic polymer and provide high flow.

Membrane type	Nominal MW cutoff	Typical Clean Water flow* ($ml/cm^2/min$)
PM10	10,000	2.5 - 4.0
PM30	30,000	6.0 - 10.00

* Flow rates after 5 minutes pressure at 55 psi (3.8 kg/cm^2)

The PM membranes can withstand 1.0 N hydrochloric acid.

Information supplied by Amicon Co.

APPENDIX VIII

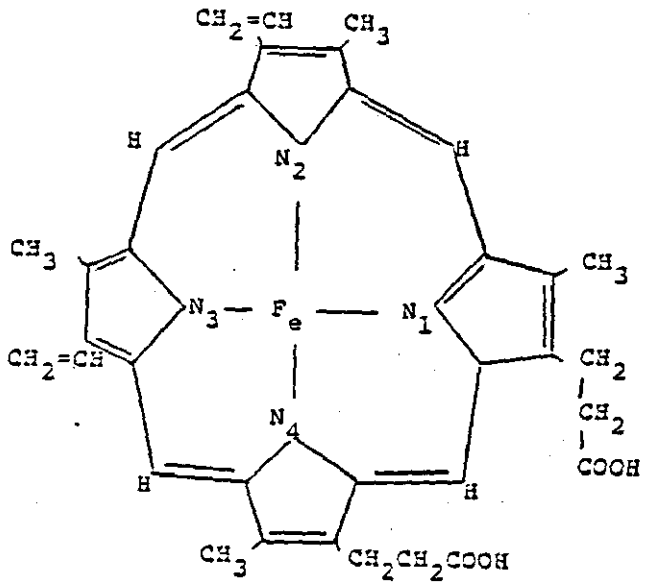
The haem molecule

APPENDIX VIII

The haem molecule

The haem molecule and its bonding to the polypeptide chain are shown below

The haem molecule



Attachment to polypeptide chain.