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The Effect of Heating Chicken Muscle on Formation of Bioavailable Froms of Iron

Nilesh B. Karava
University of Massachusetts Amherst

THE EFFECT OF HEATING CHICKEN MUSCLE ON FORMATION OF BIOAVAILABLE FROMS OF IRON

A Thesis Presented

by

NILESH B. KARAVA

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Approved as to style and content by:

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Raymond R. Mahoney, Chair

_____

D. Julian McClements, Member

_____

Fergus M. Clydesdale, Member

_____

Fergus M. Clydesdale, Department Head
Department of Food Science

DEDICATION

To my Mom, Dad, Brother and Sister, who always taught me how to excel in life and have faith in myself to overcome difficult situations. Also, to my friend Kapil Shah and his family, who always supported me and inspired me to choose right path.

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CHAPTER I

INTRODUCTION:

An adequate supply of iron is essential for good health. Iron is required for many enzymes that are critical for cellular metabolism, and plays a fundamental role in oxygen carrying proteins such as hemoglobin and myoglobin. Iron also facilitates oxygen use and storage in muscle, interacts with cytochromes in cellular metabolism, and serves as cofactor for several tissue enzymes (Yip et al. 1998). Iron can also be toxic if present in excess as it is able to catalyze the formation of reactive oxygen species. Because of this dual nature, iron must be kept within defined limits and there are precise mechanism governing the regulation of the iron homeostasis.

Iron is present in the diet as one of the two forms: as inorganic (non-heme) iron or heme iron. Of these, inorganic iron is the predominant form of iron in dietary components of plant origin and accounts for 80-90% dietary iron (Hallberg, 1981; Carpenter and Mahoney 1992), with the remaining 10% as heme iron. The inorganic form of iron is poorly absorbed in the body because of its low solubility and hence it is desirable to make inorganic iron more potentially bioavailable (Berner and Miller, 1985). Extensive research has been done in last five decades to determine the components which can make the dietary iron more potentially bioavailable. A number of such identified carriers are ascorbic acid, amino acids and peptides.

Researchers have focused on several dietary components for their iron enhancing or inhibiting effect. Many plant components such as phytate, polyphenols (Gilloly et al. 1983) and soy protein (Cook et al. 1981) inhibit, whereas some animal tissues (Cook

et al. 1975, Hurrel et al. 1988) enhance non-heme iron absorption in humans. The enhancing effect of meat is attributed to unknown factors, usually referred as 'meat factor'. Meat tissue may also maintain the iron in soluble form, thereby increasing its bioavailability (Carpenter and Mahoney, 1992). Not all the animal proteins have enhancing effect on iron bioavailability. For example, beef, lamb, liver, pork, poultry and fish enhance non-heme iron absorption, but egg, cheese and milk do not (Layrisse et al. 1969; Cook et al. 1976; Rasmussen and Hallberg 1979).

However, not much attention has been given to what extent food preparation, such as cooking, affects the non-heme iron bioavailability. Cysteine containing peptides of meat, e.g. glutathione (Taylor et al. 1986) and sulfhydryl (-SH) and (disulphide groups) in meat (Hoffman and Hamm 1978), have been suggested to be responsible for 'meat factor', but the effect of cooking on these factors has not been given much consideration even though it is well known that sulfhydryls are heat labile (Taylor et al 1986). Baech et al. studied the effect of increasing cooking temperature on meat and concluded that increasing cooking temperature does not affect the non-heme iron absorption from a phytate rich meal (Baech et al. 2002). But the reference point in this study was meat cooked at 70^{o}C and not raw meat and also the meal was phytate rich.

Based on the literature review the objective of the present study is to investigate the effect of increasing cooking temperature of chicken muscle on *in-vitro* measure of non-heme iron bioavailability. The specific objectives are

- To study the effect of cooking temperature on production of potentially bioavailable iron species resulting from in-vitro pepsin and pancreatin digestion.
- To study the effect of cooking temperature on critical amino acids, such as, sulfhydryl and histidine content of chicken muscle.

CHAPTER II

LITERATURE REVIEW:

II.A Iron:

Iron is placed in 8^{th} group and 4^{th} period in periodic table and has atomic weight of 55.845. Iron is a relatively abundant element on earth and is the fourth most common metal on earth. Iron catalyzes great number of biochemical reactions, many of which are related to the chemical nature of the element characterized by two principle oxidation states: divalent iron (Fe II (d^{-6})) and trivalent (Fe III (d^{-5})) and their associated ability to form complexes.

II.B The history of Iron:

The relation of iron to blood formation did not become apparent until the seventh century when two English physicians, Sydenham and Willis, found simple salts of iron to be of value in treatment of chlorosis in women. This relationship was placed on more rational basis by the discovery that iron is a characteristic constituent of blood. After this Lecanu had shown that hemoglobin contains iron, and, in 1886, Zinoffsky had estimated the iron content of horse hemoglobin to be 0.335%. In 1937, McCance and Widdowson came up with new concept that the amount of iron in the body must be regulated by controlled absorption. (Underwood, 1971).

II.C Iron in Body Tissue and Fluids:

The total iron content of the animal body varies with species, age, sex, nutrition, and the state of health. Normal adult man is estimated to contain 4-5g of iron or 60-70ppm of whole body of a 70 kg individual (Lamb et al. 1958). Most of the body iron is present in complex form bound to proteins, either as porphyrin or heme compounds, particularly hemoglobin and myoglobin, or as non-heme protein-bound compound such as ferritin and transferrin. The hemoprotein and flavoprotein enzymes together constitute less than 1% of total body iron. Free, inorganic iron is present in negligible quantities (Lamb et al. 1958).

Among the organs and tissues of the body, the liver and spleen usually carry highest iron concentration, followed by kidney, heart, skeletal muscles, pancreas, and brain (Underwood, 1971).

II.D Dietary Sources of Iron:

The overall intake of iron from different diets varies greatly with the proportion of iron-rich and iron-poor foods that they contain, with the degree of contamination with iron to which they have been exposed, and, to some extent, with the locality from which they are obtained. Average U.S. diet was reported to supply 12-15 mg iron per day value, Australian diet, which is typically high in meat, have been estimated to supply 14-20 mg iron. A typical poor Indian diet was shown to provide only 9 mg iron, whereas improved diet containing less milled rice and more pulses and green vegetables could provide as much as 60 mg iron/day (Underwood, 1971).

The dietary source of iron influences the efficiency of iron absorption, which ranges from <1% to >20% (Underwood, 1971). Non-heme iron in food of vegetable origin is at lower end of the range, dairy products are in the middle, and meat is at the upper end. About 10% of the small amounts of iron in unfortified formulas or whole milk is absorbed. The iron content of the breast milk is same as that of cow's milk, but about 50% of the iron in the breast milk is absorbed, hence breast milk is better sources of iron than cow's milk and unfortified formulas (Ziegler and Filer, Jr. 1996). Its better absorption efficiency does not entirely make up for its low iron content, and after age of 6 months breast-fed infants require an additional sources of iron to meet their iron requirement (Ziegler and Filer, Jr. 1996)

Meat is good source of iron because much of it is in the form of heme-iron, which is absorbed 2-3 times more than non-heme iron completely than non-heme iron (Ziegler and Filer, Jr. 1996). In addition, it has been reported that factors in the meat promote non-heme iron absorption from the entire meal (Ziegler and Filer, Jr. 1996).

II.E Iron Chemistry and Biochemistry:

The study for mechanism of iron absorption has been controversial, probably due to the complex chemistry of the element and to the different capabilities of that biological membrane have for iron uptake. Iron catalyzes great number of chemical reaction and many of these reactions are related to the chemical nature of the element which is characterized by two principle oxidation states: divalent iron (Fe II (d^{-6})) and trivalent iron (Fe III (d^{-5})) and their associated ability to form complexes.

In water and in absence of oxygen, iron is present in the hexa-aqua complex divalent ion, which is readily oxidized upon increasing oxygen concentration to trivalent aqua

complex of similar structure according to the one electron reversible reaction shown below and oxygen become a source of free radicals:

$$Fe\ (II)_{aq} + O^{2} \longrightarrow Fe\ (III)_{aq} + O^{2-\cdot}$$

The water solubility of trivalent hexa aqua-complex is function of pH and rapidly decreased by increasing pH value from 1 to 9. Due to the complex hydroxylation reactions of deprotonation of the aqua-complex, oxo-hydroxy species of decreasing solubility are formed in water as shown in fig. below (Cremones P et al. 2002):

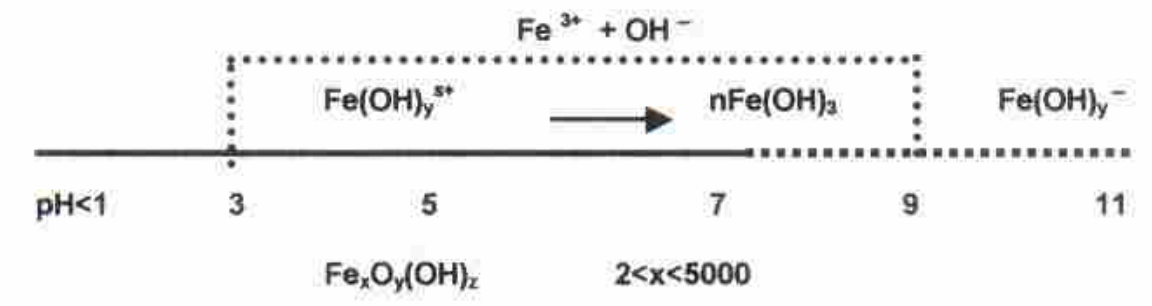

Fig. II.E.1. Hydrolytic reaction of iron as function of pH. At low pH values iron is present in solution as free ion. Aqua complex oligomers are generated at pH values higher than 2 and polymerization occurs by further increasing the pH; x is the estimation of number of iron atoms present in the aquated form, y and z are the O^{-} and the $(OH)^{-}$ in the bridging position of the polynuclear core bonded to x by the relationship 2y-z/p=n. Precipitation of these forms occurs at the x values higher than 20 (Cremones P et al. 2002).

In biological media at low oxygen tension, $Fe(OH_2)_6^{2+}$ is the predominant species, while $Fe(OH_2)_6^{3+}$ is minor species due to its low solubility (10^{-12}mol/l at pH 7) (Flynn, 1984; Cornell et al. 1989).

These chemical characteristics are suggestive, in principle that iron II derivative can be taken up more easily than iron III derivative by cell membrane as a consequences of more favorable solubility properties.

➢Iron balance and distribution:

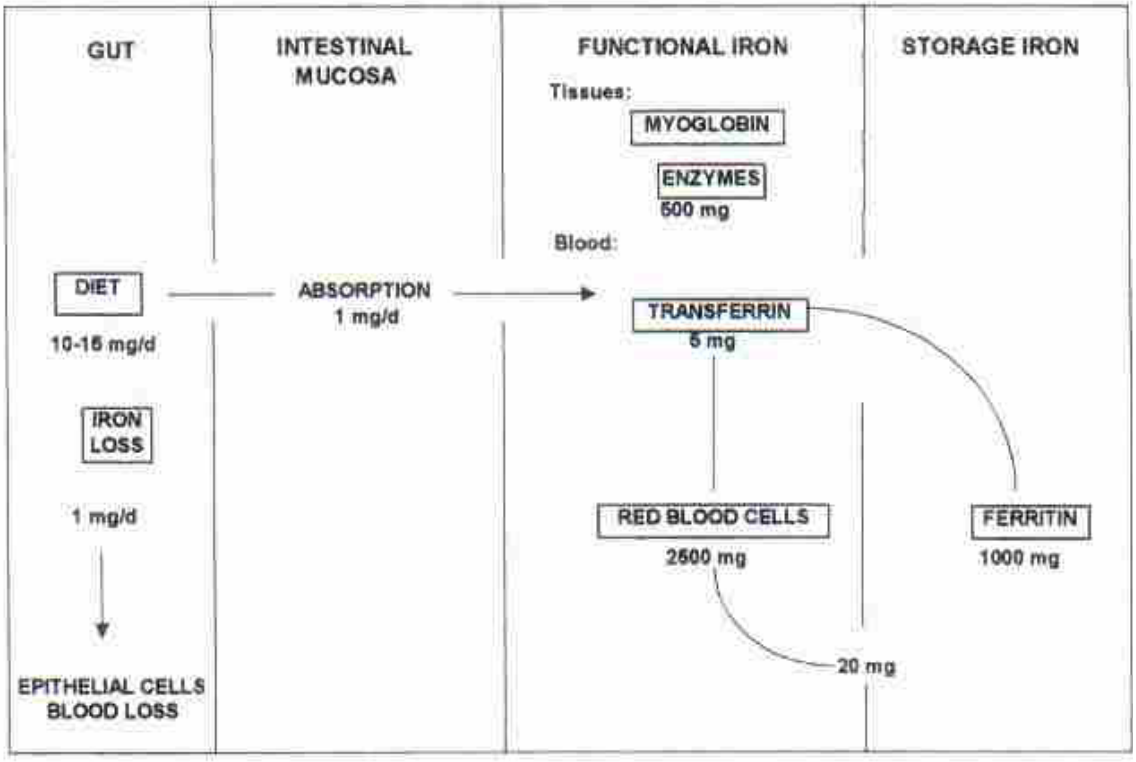

Fig. II.E.2. Iron balance in man.

In normal conditions the mammals are able to maintain iron homeostasis by controlling absorption from the diet and avoiding the overload (Fig 2). Appropriate amounts of iron (10-15mg) in the diet are taken up in the intestinal mucosa (normally 1 mg/day) and transported by plasma ferritin to the utilization compartment (mainly

red blood cells) and the storage protein (ferritin) from which it can be mobilized when required. Iron can be recycled within mammalian cells and any uncontrolled losses from epithetical cells or via blood loss is balanced by the amount of absorbed iron. The daily body turnover in healthy human is 1 mg and can reach 4-5 mg in menstruating woman. Figure II summarizes the iron balance in man (Cremones et al. 2002):

II.F Iron Absorption:

II.F.1 Introduction:

Iron absorption normally occurs in the duodenum and upper part of jejunum. The availability of ingested iron for absorption and the amount absorbed depends upon the chemical nature and quantity of iron the diet, the presence of other factors in ingested foods, the effect of gastrointestinal secretion and the absorptive capacity of the intestinal mucosa. The absorptive capacity is regulated by two internal factors, the size of the iron stores and the rate of erythropoiesis (Bothwell et al. 1979).

Iron absorption has been assumed to have an important role in regulation of iron balance. Iron balance in the normal adult male is limited with an exchange of ~1 mg/day. Absorption from the test meal is high if iron stores are depleted and is surpassed if iron stores are enlarged (Burke et al. 2001).

II.F.2 Mechanism of Iron Absorption:

Dietary iron compounds are divided into two types, heme and non-heme iron. In humans heme iron is absorbed more efficiently than non heme iron. Hence heme iron may contribute a large amount of absorbed iron even though it represents a lesser fraction of ingested iron. Heme and non-heme iron is absorbed in the body by different mechanisms, which are described below.

II.F.3 Non-heme Iron absorption:

Non-heme (inorganic) iron is present in the diet as either reduced ferrous (Fe II) or oxidized ferric (Fe III) form. Under normal physiological condition (i.e. neutral pH and in presence of oxygen) ferrous iron is rapidly oxidized to ferric form and precipitate as iron hydroxide. In the luminal contents of gut, iron is likely to be in the ferric form and therefore poorly potentially bioavailable. Non-heme iron is absorbed early in digestion mainly in the duodenum, where the low pH favors solubility. Further down the intestine it is likely that the formation of insoluble ferric complex reduces bioavailability. The transport of the non-heme iron across the duodenal mucosa has been studied intensively over the years and is highly adaptive to change in iron status (stores, erythropoiesis, and hypoxia). Much progress has been made in the last few years in identifying the proteins involved in this process (Miret et al. 2003).

Non-heme iron is transported into the cell in the ferrous form, mainly by the carrier DMT1 (Divalent Metal Transport 1), also known as natural resistance associated

macrophage protein (Nramp2). Several steps are involved in non-heme iron absorption, viz.

1. transport to erythrocytes across the brush border membrane
2. movement of the iron through the cell
3. transport across basolateral membrane (transfer step) and
4. passage through the interstitial space and capillary wall.

Two carriers are required, DMT1 for the uptake step and ferroportin 1/IREG1/MTP1 for the transfer step. Several other proteins have been shown to be involved in the absorptive process, either directly or indirectly. A ferric iron reductase on the brush border membrane, Dcytb, is believed to be responsible for the reduction of ferric to ferrous form before uptake by DMT1 (Morgan and Oates, 2002). For optimal transport of ferrous form, DMT1 also require a proton gradient provided by gastric acid to co-transport ferrous iron. The intracellular movement of iron is poorly understood but the copper containing ferroxidase, hephaestin (Hp) is involved (Valpe et al. 1999).

II.F.4 Heme iron absorption:

Heme iron derived from meat is an important source of iron, and is highly potentially bioavailable.

The absorption of hemoglobin and myoglobin derived iron differs from that of inorganic iron. Most heme-iron enters the small intestinal absorptive cells as an intact metalloporphyrin. Current evidence is that this is facilitated by heme receptor and heme enters the cell via vesicle (Grasbeck et al. 1979). Once heme is within the cell, iron is released from porphyrin by mucosal heme oxygenase so that it can enter the

circulation as inorganic iron. Unlike non-heme iron, heme iron is soluble in alkaline solutions and is precipitated as hematin in acidic milieu (Conrad et al. 1966). This makes the chelation less important to maintain solubility in duodenum. There is common intracellular pathway for heme and non-heme iron absorption, demonstrated by competitive inhibition between simultaneously and sequentially administered heme and non-heme iron (Hallberg and Solvell, 1967).

II.G Regulation of Iron Absorption:

Humans have limited capacity to excrete iron, so the amount or iron in the body is controlled at the point of absorption in the proximal small intestine. The iron uptake by mucosa cells and iron transfer into the carcass are significantly increased in iron deficient animal as compared to iron overloaded animals (Adams et al. 1991). Thus the uptake from the mucosa cell appears to be the main site of regulation of non-heme iron absorption. Storage iron status is also a well known factor for regulation of iron absorption. Enhanced erythropoiesis is another potent stimulus of iron absorption (Anderson et al. 2005).

Early autoradiographic studies by Conrad and Crosby showed that the mature epithelial cells of mid to upper villus are able to absorb iron from the diet; it is the cells of intestinal crypts that are able to take up iron from the body (Conrad and Crosby, 1963). The inference from this study is that the crypt cells are the ones that respond to the body signals to modulate the intestinal iron absorption.

The concentration of iron within the crypt cell is, thus, an important determinant of iron absorption. The crypt cell iron concentration must, thus, reflect body iron needs and must in turn modulate the expression of gene encoding proteins important for vectorial iron transport across the mature enterocyte. The mechanism by which the crypt cell's intracellular iron concentration may respond is poorly understood. Anderson studies the distribution and functions of the transferrin receptor in the small intestine and have shown that most receptors are located at the basolateral membranes of the intestinal crypt cells. These transferrin receptors are able to bind, internalize and recycle transferrin to the cell surface and, in short, the crypt cells appear to possess fully functional transferrin/transferrin receptor iron delivery system. These studies have indicated that the transferring receptor provides one pathway by which crypt cell could kept informed about body status, but one can not exclude the possibility that the crypt cell could be informed in other ways or by additional signals (Anderson, 1996).

The following figure shows the model for control if iron absorption.

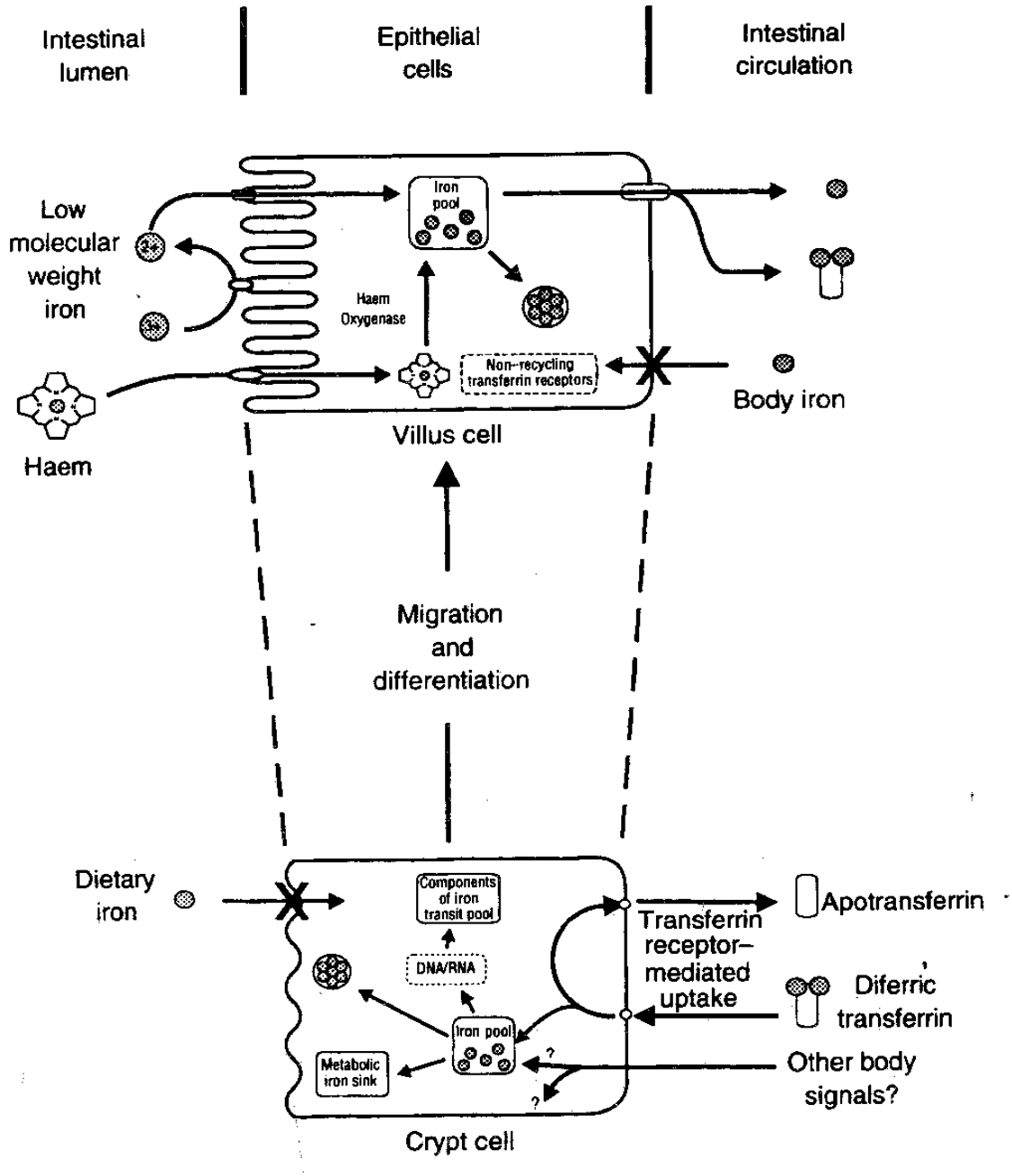

Figure II.G.1: Model for control of iron absorption. Signal from the body to modulated iron absorption are received by epithelial cells of the intestinal crypt. These cells are able to respond to the body signals but are unable to take up dietary iron themselves. The body signals, perhaps by altering the intracellular iron status of the crypt cells, determine the expression of the genes important for the absorption process. The proteins encodes by these genes are expressed as functional membrane transporters after the epithelial cells have migrated up the villus and matured into

absorptive enterocytes. These cells are now able to transport dietary iron to the circulation but appear to have little capacity to receive iron from the body (Anderson, 1996).

II.H The concept of Iron Bioavailability:

Iron bioavailability can be defined as the proportion of total mineral or total trace element in food, meal, or diet, which is utilized for normal physiological body functions. Various methods have been used to study the dietary bioavailability of iron in man. The chemical balance technique is the only method that directly measures the dietary iron absorption. That method, however, is insensitive, imprecise and time consuming, and it gives no information about the iron absorption from different meals.

The introduction of radioisotopes made it possible to label single food items biosynthetically with radioiron. Studies with labeled foods have shown that absorption from individual food differs markedly. These differences in the bioavailability are apparently related to differences in solubility, dissociation and uptake of chemically uncharacterized iron compounds in foods.

In recent years some unexpected observations have provided the important breakthrough and led to the development of extrinsic tag method. When single food biosynthetically labeled with radioiron (intrinsic tracer) was carefully mixed with a trace amount of iron salt labeled with another radioiron isotope (extrinsic tracer), the observations was made that the absorption of two tracers, from such doubly labeled

foods, was almost identical. The magnitude of absorption was different from different foods and in different subjects, but the absorption from the extrinsic and intrinsic tracers was the same in each subject. Based on these finding the concept of common non-heme iron was introduced. This concept assumes that the non-heme iron compound in different foods in a meal can be uniformly labeled by extrinsic inorganic radioiron tracer (Hallberg, 1981). Heme iron cannot be labeled by extrinsic inorganic tracer.

II.I Methods to Determine Iron Bioavailability:

Three methods are universally used for determination of iron bioavailability discussed below:

II.I.1. In-vitro Method:

In in-vitro method the sample is subjected to pepsin and pancreatin digestion with a dialysis bag of specific MWCO (Kane and Miller, 1984). The dialyzable iron is measured after pancreatin digestion and accounted for potentially bioavailable form of iron.

In this method the dialyzable iron is taken as potentially bioavailable form of iron, as it is necessary for the iron to get bound to a ligand and cross the dialysis tube membrane to get absorbed in human body.

II.I.2. Caco-2 Cell Method:

Caco-2 cell line has properties similar to human intestinal cells, which are utilized for determination of iron bioavailability. The caco-2 cultured plates are coupled with inserts carrying a dialysis membrane of specific MWCO and placed in the digestion sample (Swain et al. 2002). Once the digestion is over the uptake of iron by caco-2 cells is determined and accounted for potentially bioavailable forms of iron.

In this method the uptake of iron by Caco-2 cell is important as caco-2 cell line resembles to human intestinal cell.

II.I.3. In-vivo Method:

In-vivo method refers to the use of living subjects to study iron bioavailability. In case of animals, a specific diet, containing a stable isotope of iron called as radio labeled iron, is added to meal externally and given to the subjects during the study period. After the study period, the animal is sacrificed and hemoglobin concentration and plasma iron concentration is estimated.

In case of humans, healthy volunteers are selected randomly and their hemoglobin and plasma iron concentration is determined. Then the subjects are given the test meal containing radio labeled iron at regular interval. The blood and fecal samples are collected at regular intervals and analyzed for radio labeled iron. At the end of the study the data is analyzed for difference in blood iron before and after study and iron bioavailability is estimated by different available methods like WHO's method

(FAO/WHO 1988), Monsen's method (Monsen et al. 1978), Tseng's method (Tseng et al. 1997) etc.

II.J. Individual factors affecting non-heme iron bioavailability:

II.J.1. Iron status:

The absorption of non-heme iron is markedly influenced by the iron status of the subject – more iron is absorbed by the iron deficient and less by the iron-replete subjects. This leads to marked subject to subject variability, which makes it difficult to determine whether difference in test meal studied in different group subjects relate to properties of the meals or to the iron status of the subject.

The effect of difference in iron status among different subjects can be adjusted by obtaining independent measure of their absorptive capacity. This is accomplished by determining the absorption from the standard dose of inorganic radioiron given at physiological levels under standardized conditions (Hallberg, 1981).

There is good correlation between iron stores and serum ferritin, and it has been shown that there is good correlation between serum ferritin and non-heme iron absorption (Bezwoda et al. 1979). Therefore serum ferritin can also be used as an alternative to the reference dose absorption. However, serum ferritin is only an indirect measure of individual's ability to absorb iron, and extraneous factors such as minor infections may affect iron absorption and serum ferritin in opposite direction (Hallberg, 1981).

II.J.2. Pregnancy:

The bioavailability of dietary iron increases during pregnancy and is roughly parallel to the increased iron requirements.

II.J.3. Disease States:

In gastric achlorhydria, the absorption of dietary non-heme iron is reduced in relation to the absorption from ferrous iron salt (Rasmussen, 1981). After partial gastrectomy, a decrease in bioavailability of non-heme dietary iron is often observed. The magnitude of the decrease depends upon the type of the gastric operation performed (Magnusson, 1979). In idiopathic hemochromatosis, the absorption of non-heme iron marked increases in relation to the size of iron stores (Bezwoda, 1976).

II.K. Dietary factors influencing the bioavailability of non-heme iron:

II.K.1. Ascorbic acid:

It was shown early on that ascorbic acid or orange juice with a high content of ascorbic acid markedly increases the food iron absorption. This effect is due to promotion of non-heme iron absorption (Apte and Venkatachalam, 1965) and there is no effect on absorption of heme iron. The absorption increase is related to the amount of ascorbic acid. A significant effect can be observed with only 25 mg of ascorbic acid, which is amount present in a third of the glass of orange juice. Orange juice

containing 70 mg ascorbic acid increased iron absorption from breakfast meal 2.5 times (Rossander et al. 1979).

In summary, ascorbic acid is very potent promoter of non-heme iron absorption. Crystalline ascorbic acid and native ascorbic acid have about the same promoting effect. Cooking and baking can destroy the ascorbic acid and hence its effect on iron absorption. The effect of ascorbic acid seems to be independent of the effect of other promoters, such as meat. However when two promoters are present the effect of each promoter will be smaller as compared to when it is present alone. In the presence of inhibitor of non-heme iron, such as tea, the relative enhancing effect of ascorbic acid on non-heme iron absorption is same (Hallberg, 1979).

The effect of ascorbic acid may be related both to its reducing effect, preventing the formation of insoluble ferric hydroxide, and to its effect on forming soluble complexes with ferric iron, which preserve the iron solubility in more alkaline duodenal pH (Conrad and Schade, 1968).

In recent study (Engle-Stone et al. 2005) it has been shown that meat and ascorbic acid can promote iron availability from iron-phytate but not from iron-tannic acid complexes.

II.K.2. Meat and Fish:

An enhancing effect of meat and fish was first time reported by Layrisse et al. in 1968 and confirmed by many other studies (Taylor et al. 1986; Morrissey, 1998; Seth and

Mahoney, 2000; Swain et al. 2002). The absorption-promoting effect of meat is dose related (Underwood 1971). The enhancing effect of meat on iron bioavailability is termed as 'meat factor'. Several investors have tried to clarify the mechanism of this meat effect. Many components such as amino acids, histidine (Swain et al. 2002), histidine residue (Mulvihill and Morrissey, 1998; Seth and Mahoney, 2000), carbohydrate fractions (Rozo et al. 1986, Huh et al. 2004), sulfhydryl (Taylor et al. 1986) etc have been reported to be responsible for enhancing effect on iron absorption.

II.K.3. Tannates:

It has been reported that tea markedly reduced the iron absorption of non-heme iron absorption from foods. The absorption from bread was reduced to one third and from soup to one fourth when served with tea compared with water (Disler et al. I 1975). This effect has been attributed to the formation of iron-tannate complex. It has also been reported that the tannins may be partly responsible for low bioavailability of iron in many vegetable foods (Disler et al. II 1975). Tannates are also present in coffee and it is possible that inhibiting effect of coffee is due to tannates.

II.K.4. Phytates, phosphates and fibers:

Several studies have shown that the sodium phytate decreases iron absorption in man (Hallberg and Solvell, 1967; McCance et al. 1943). The lower fraction of iron absorbed from brown bread compared with white has been attributed to the high content of iron phytates in bran (Moore, 1968). Most of the phytate, however, is

broken down during leavening and baking of bread, with corresponding increase in inositol. The final content of phosphate in wheat is not such of magnitude that it can affect the iron absorption with increasing amounts of bran. It has been suggested that the inhibiting effect of bran is due to its content of fiber components (Rasmussen, 1974). Monoferric phytate, prepared from wheat, bran have been reported to have higher bioavailability for rats (Morris and Ellis 1976).

II.K.5. Egg and Milk:

Eggs have been reported to decrease the absorption of iron. Egg yolk has been reported to decrease the absorption of iron from inorganic salt (Elwood 1968).

Milk has been found to decrease the iron absorption from meals with low bioavailability.

II.K.6. Organic Acids:

Salovara et al. have observed that organic acids show a concentration-dependent influence on the uptake of ferrous and ferric iron in Caco-2 cells (Salovara et al. 2002). Results obtained by Salovara et al. showed a correlation between absorption pattern and chemical structure of the acids. Accordingly, four-carbon dicarboxylic acids, such as tartaric, malic, succinic, and fumaric acid, showed a positive effect on both ferric and ferrous iron absorption in the cells, but to varying degrees. The number of hydroxyl groups was shown to be important. Citric, lactic, and oxalic acid (2-, 3-, and 5-carbon carboxylic acids) had a similar and very negative effect on ferrous iron and a positive effect on ferric iron absorption. Acetic and propionic acid,

which are simple 2- or 3-carbon monocarboxylic acids, showed a positive effect on ferrous iron and no effect on ferric iron uptake (Salovara et al. 2002).

In a review, oxalate has been reported to decrease in iron bioavailability (Conrad, 1970).

Succinic acid, which increases the iron absorption from pharmaceutical doses of iron, has about the same 35% absorption-promoting effect on dietary non-heme iron in standard meal, when given in the amount of 150 mg (Hallberg, 1979).

II.L. The concept of 'Meat Factor':

The concept of 'meat factor' was established when Layrisse et al. showed that the absorption of iron from different vegetable foodstuff was markedly increased when they were served with meat and fish (Layrisse et al. 1969). This observation has been confirmed by number of studies both in-vitro and in-vivo (Amine and Hegsted, 1971; Monsen and Cook 1979; Kane and Miller, 1984; Slatkavitz and Clydesdale, 1988). It is evidence that meat and fish promote inorganic iron absorption. However, the mechanism by which meat acts to promote absorption of non-heme iron from diet is still unknown. Several factors have been proposed to be responsible for meat factor and are summarized below.

II.L.1. Sulphydryls:

Hamed et al. suggested that the sulphydryl (-SH) groups of cysteine and glutathione are capable of reducing Fe(III) to Fe(II) at low pH (Hamed et al. 1983). Kirwan et al.

suggests that –SH rich myofibrillar fractions of meat, in particular, the heavy meromyosin fractions may be a component responsible for meat factor (Kirwan et al. 1993). Mulvihill and Morrissey studied the effect of –SH content of animal proteins on production of dialyzable iron and showed that the –SH content of meat plays important role in this indicator of iron bioavailability (Mulvihill and Morrissey, 1998).

Mulvihill et al. showed that heavy meromyosin, which has 25 –SH residue per molecule, produces more amount of dialyzable iron than light meromyosin molecule, which has 4-5 –SH residue per molecule (Mulvihill et al. 1998).

Taylor et al. studied the effect of cysteine containing peptides released during the meat digestion on iron absorption in humans. In this study the sample was divided into two batches: in first batch the thiol groups of cysteine residues were preserved and in second batch the thiol groups were oxidized to cystine. The extracts were given to the subjects in form of a soup. The hemoglobin and serum ferritin concentration of the subjects was analyzed. The results obtained from this study suggested that the enhancing effect of meat on non-heme iron absorption is due to cysteine containing peptides, like glutathione, and not the free amino acids (Taylor et al. 1986).

II.L.2 Histidine:

Seth and Mahoney studied the role of histidine residue in chelation of iron by peptides from chicken muscle proteins in-vitro and concluded that histidine residue do contribute to iron chelation and could be involved in promotion of iron absorption by

muscle tissue (Seth and Mahoney, 2000). Swain et al. used Caco-2 cells method to study influence of beef protein on iron absorption and suggested that the enhancement of non-heme iron absorption of beef may be due to peptides produced during gastrointestinal digestion and that histidine content may be important factor contributing to this effect (Swain et al. 2002).

II.L.3. Protein Digestibility:

The importance of protein digestibility in promoting iron bioavailability has been addressed by many investigators (Bothwell et al. 1979; Kane and Miller, 1984; Slatkavitz and Clydesdale, 1988). Rasmussen and Hallberg studied the effect of animal proteins on the absorption of food iron in man and concluded that absorption-promoting effect of meat proteins may result from the peptides formed during digestion (Rasmussen and Hallberg, 1979). Kane and Miller (Kane and Miller, 1984) investigated the effect of selected proteins on iron-bioavailability in-vitro and came up with a conclusion that protein and iron interactions occur during the digestion and results in iron absorption.

Mulvihill and Morrissey showed that low molecular weight digestion products enhance iron bioavailability in-vitro, while large molecular weight products may bind iron and hence depress its bioavailability and concluded that the small molecular weight protein degradation products may contribute to ‘meat factor’ (Mulvihill and Morrissey, 1998). Mulvihill et al. suggested that the myofibrillar protein fraction, in part, may be responsible for the enhancing effect of meat on the in-vitro bioavailability of non-heme iron (Mulvihill et al. 1998). Kapsokefalou and Miller

concluded that the protein digestibility may be an important factor for iron bioavailability in-vitro as more completely digested the protein is, more thiol groups would be exposed and available for reducing iron (Kapsokefalou and Miller, 1991). Conflicting results were obtained by Huh et al., who stated that the protein digestion is not necessary for iron bioavailability and carbohydrate fractions, which are extracted at low pH, are responsible for iron bioavailability in cooked fish muscle (Huh et al. 2004)..

II.L.4. Carbohydrates:

It has been suggested that the absorption of radioiron is markedly influenced by the kind of dietary carbohydrate (Amine and Hegsted, 1971). Amine and Hegsted found that iron utilization is greatest with diets containing lactose, less in diets containing sucrose and least with diets in which carbohydrate supplied was starch. However the effect of carbohydrates was not uniform when different iron sources were used (Amine and Hegsted, 1975). Rozo et al. studied effect of some carbohydrates on iron absorption in rats and found observed that rats fed with high starch meal resulted in reduction in iron absorption, whereas glucose, fructose and lactose enhanced iron absorption (Rozo et al. 1986). Huh et al. studied the effect of cooked fish on iron uptake and found that the acid extract, which contained high amounts of carbohydrates and negligible amounts of proteins and amino acids, increased iron uptake up to 4.9 fold by Caco-2 cells (Huh et al. 2004). Huh et al. also proposed that the carbohydrates responsible for iron uptake may be oligosaccharides from glucosaminoglycans which is present in the extracellular matrix of muscle tissue (Huh et al. 2004).

II.M. Effect of Cooking on 'Meat Factor':

The literature review suggests that the effect of cooking on iron bioavailability has not been given much emphasis upon. The research by Baech et al. investigated the effect of cooking temperature of pork on iron bioavailability. Pork was prepared in the form of meat patties and packed into hermetically sealed aluminum cans. These cans were divided into three different batches and the cans were exposed to one of the three heat treatments as follows: by circulating the water at 70/95^{o}C for 60 mins till the final centre temperature reached 69.94^{o}C and maximal heat treatment at 120^{o}C in an autoclave. The patties were given along with a phytate rich meal to the test subjects and the serum ferritin and hemoglobin concentration was measured at the end of the study. The cysteine content of the heated samples dropped by 19%, but the iron bioavailability was not affected. The study concluded that the cooking temperature does not affect non-heme iron absorption (Baech et al. 2002). But in this study the meal was phytate rich, and it is well established that phytate is iron absorption inhibitor (Hurrel et al. 1992) and may interfere with the study.

Another study by Huh et al. suggested that carbohydrate fractions from cooked lyophilized fish promote iron bioavailability. The HPLC analysis of the extract showed that the content of protein and amino acid was negligible and the fraction was highly rich in carbohydrates (Huh et al. 2004). But the fish muscle was not heated under controlled temperature conditions, which may have destroyed thiol groups which are suggested to be responsible for meat factor. This study suggested that carbohydrates in chicken may also have some effect of iron bioavailability, and to

investigate the effect of carbohydrates on iron bioavailability, if there is any, is also one of the aims of this study.

Based on the literature findings, the aim of this work is to investigate the effect of heating temperatures on production of potentially bioavailable forms of iron and the specific components of chicken, if any, affecting the bioavailability of iron.

CHAPTER III

MATERIALS AND METHODS

III.A. Introduction:

Literature survey clearly mentioned that animal protein has a positive effect on iron absorption. But, there is very little evidence for effect of cooking chicken muscle on iron absorption. Using a standard model which involves only pepsin and pancreatin enzymes and water besides the different cooked samples was thought to be a better *in-vitro* model to determine the effect of cooking chicken muscle on iron bioavailability as compared to other *in-vitro* methods available. Production of dialyzable iron was determined for preliminary samples of cooked chicken to evaluate the consistency and reliability of the methodology.

III.B. Chemicals:

All chemicals were of analytical grade.

Water: Distilled-deionized water (DDW) was prepared using a Bantam Demineralizer Model BO-5 (Branstead Company, Boston, MA) with an ultrapure cartridge (Branstead International, Dubuque, IA, USA). DDW was used throughout the experiments.

Pepsin: Pepsin from porcine stomach mucosa (Sigma Chemical Co. St. Louis, MO, USA), P-7012, was prepared by dissolving 100mg in 5 ml of 0.01N HCl. Pepsin was added to the protein samples at a pepsin:protein ratio of 1:25 (w/w).

Pancreatin: Pancreatin from porcine pancreas (Sigma Chemical Co. St. Louis, MO, USA), P-1750, was prepared by suspending 200 mg in 50 ml of 0.1M PIPES/bile, at pH 6.5. Pancreatin was added to the protein samples at a pancreatin:protein ratio of 1:50 (w/w)

PIPES/Bile: PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) disodium salt (Sigma Chemical Co. St. Louis, MO, USA). PIPES, P-3768, was dissolved in DDW at a concentration of 0.1M and the final pH was adjusted to 6.5. BILE salts, at the concentration of 50mg/ml, were dissolved in 50 ml of this buffer.

Dialysis tubing (Spectrum Labs Inc. Gardena, CA, USA): Spectra/Pro®1 membrane tubing with a diameter of 20.4 mm and a MWCO of 6,000 - 8,000 Da was used for dialysis. Twenty centimeter length membrane tubes were cut and soaked in 5 mM EDTA solution in DDW for at least 2 hrs and rinsed several times with DDW to remove all the EDTA prior to use.

Reducing Protein Precipitant Solution: 100g of crystalline trichloroacetic acid (TCA), 50g of hydroxylamine • hydrochloride and 100 ml of 12N HCl were brought to 1 L with DDW.

Non-reducing Protein Precipitant Solution: 100g of TCA and 100 ml of 12N HCl were brought to 1 L with DDW.

Ferrozine Reagent: 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine monosodium salt. (Sigma Chemical Co. St. Louis, MO, USA P-9762). Ferrozine was dissolved in DDW to prepare a 9 mM solution.

Ammonium Acetate Buffer: Ammonium Acetate ACS reagent was dissolved in DDW and brought to a concentration of 10% (w/v).

Iron Solution (Fisher Chemical Fair Lawn, NJ, USA): Iron reference solution, suitable for atomic absorption spectroscopy, at a concentration of 1000 ppm (as ferric nitrate

in 2% of nitric acid), was used to prepare the standards as well as to add to the protein samples as the source of extrinsic iron.

Biuret Reagent: The reagent was prepared by separately dissolving 1.50g of $CuSO_4 \cdot 5H_2O$ in 250 ml of DDW and 6.00g of sodium potassium tartrate $(NaKC_4O_6 \cdot 4H_2O)_4$, in 250 ml of DDW. Both the solutions were mixed and 300 ml of 10% (w/v) NaOH was added and the final volume was made to 1 L with DDW.

Bovine Serum Albumin (BSA): Crystalline and lyophilized bovine serum albumin prepared from fraction V, essentially globulin-free. (Sigma Chemical Co. St. Louis, MO, USA A-7906). BSA was dissolved in DDW and made to concentrations ranging from 1-10 mg/ml when used as a reference protein in the protein standards.

III.C Apparatus

Glassware: All glassware was acid washed with 2N HCl, rinsed with DDW several times and oven dried.

Spectrophotometer: Perkin Elmer, Hitachi Model 200 UV-Vis, Coleman Instruments Division, Oak Broo, IL, USA.

Low Speed Centrifuge: Bench top centrifuge Damon IEC model HN-S II.

High Speed Centrifuge: Sorvall Superspeed RC-58 Automatic refrigerated centrifuge, Ivan Sorvall Inc., Newton, CT, USA.

pH Meter: Corning, Model 125, Corning Medical, Medfield, MA, USA with an epoxi body combination electrode, Sensorex, Stanton, CA, USA.

Water Bath Shaker: Temperature controlled, Model 406015 Serial, American Optical, Buffalo, NY, USA.

Blender: Waring Commercial Blender, Model # 51BL31, Torrington, CT, USA.

Freeze Dryer: Virtis Company Inc, Model # 203314, Garinder, NY, USA.

Chest Freezer: So-Low, Environmental Equipment Company, Cincinnati, OH, USA.

III.D. Sample preparation:

III.D.1. Raw Chicken Muscle Sample:

Fresh, unfrozen chicken was purchased from local supermarket and the fat was removed. This sample was homogenized with twice the weight of water for 3 min with 1 min interval. After homogenization, 100g of sample was poured into container and frozen to -40^{o}C in a chest freezer.

III.D.2. Cooked Chicken Muscle Sample:

Fresh, unfrozen chicken was purchased from local supermarket and the fat was removed. This sample was homogenized with twice the amount of water for 3 min with 1 min interval. This slurry was heated in a boiling water bath till the temperature reached 165 or 195^{o}F with continuous stirring to maintain the uniform temperature of the sample. Then the sample was again homogenized for 30 sec and 100g of sample was poured into 1 pound plastic container, ~1 cm thick and frozen to -40^{o}C in a chest freezer.

The frozen samples were lyophilized using a Virtis company lyophilizer, at -40^{o}C with increase of 10^{o}C every hour till the temperature reached 20^{o}C and this temperature was maintained until the sample was dried thoroughly. The lyophilization

time for raw and cooked sample was 72 hrs and 48 hrs respectively. The end point was when the chicken layer started sticking (puffing) out from the surface of the container.

A detailed outline for the preparation of the sample is shown in following figure.

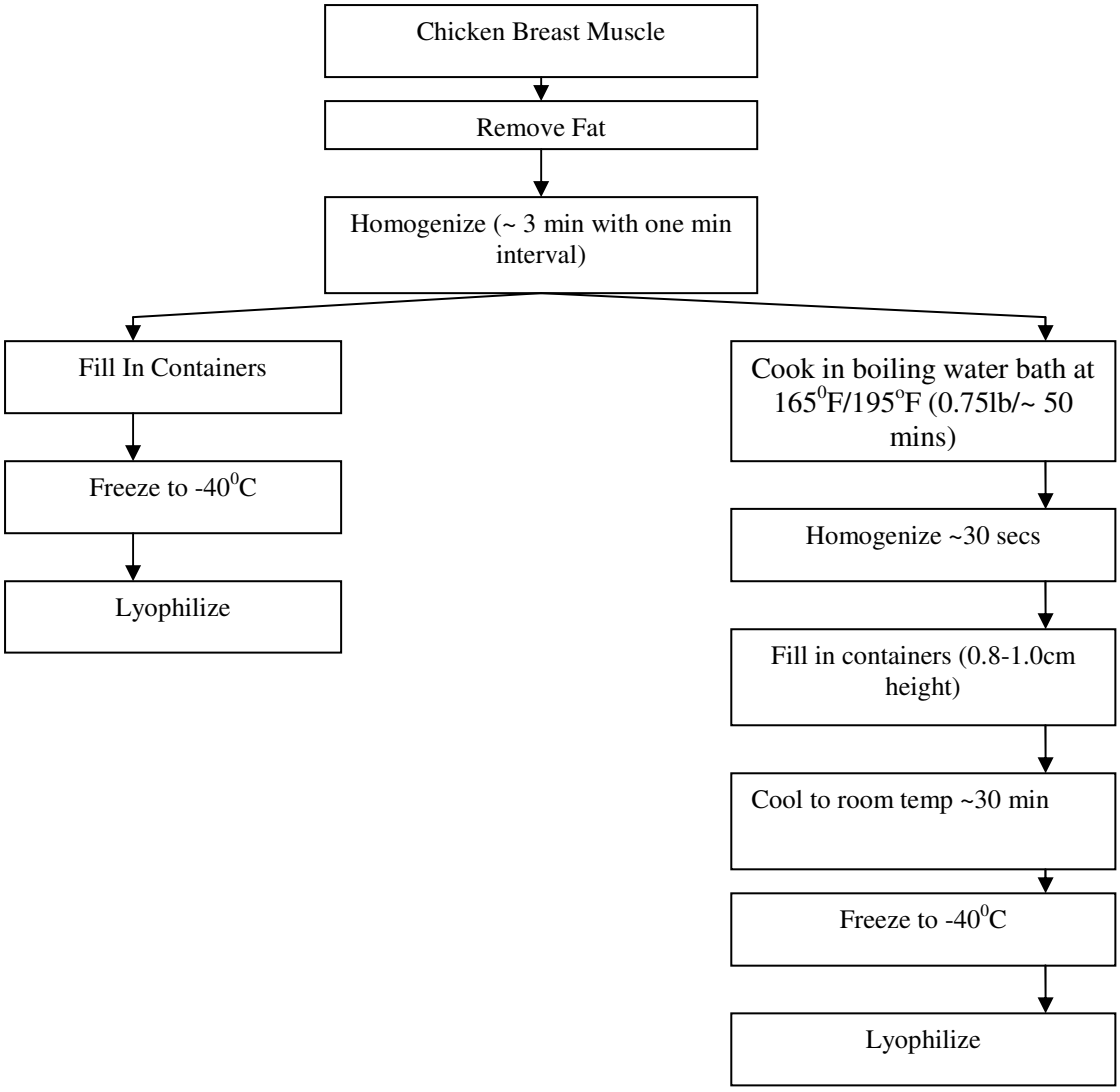

Figure III.D.2.1: Outline for lyophilization of raw and cooked chicken muscle sample

After lyophilization, the sample was removed from the container and blended for 1 min in a coffee blender and analyzed for protein content using Micro Dumas method (Ma and Rittner 1979).

III.E. Pepsin digestion:

The portion of the sample containing 2g of protein was mixed with DDW and the weight was adjusted to ~ 90g, following which the pH of the suspension was adjusted to 2.5 using 6.0 M HCl. 37.5 µM (1.4 ml) of Iron reference solution was added to this suspension. The pH of this suspension was adjusted to 2.0 and the weight was adjusted to 95g. This preparation was allowed to stand for 10mins. The final pH was rechecked and adjusted to 2.0, if required. The sample at pH 2.0 was then placed in a shaking water bath at 37^{o}C for 5 mins. After this, 5ml of pepsin was added and the suspension was incubated at 37^{o}C for 2 hrs. Three similar digests of 100g were incubated simultaneously in the same water bath. After pepsin digestion, the sample was removed from the water bath and placed in a ice bath to stop the pepsin digestion.

III.F. Titratable acidity:

Titratable acidity is the amount of 0.5 N NaOH required to bring the pH of pepsin-digested sample to 6.5. The equivalent moles of $NaHCO_3$ are then added to the actual digest to bring the pH to 6.5.

A 20g aliquot of the pepsin-digested sample from each of the 100g flask was taken and 5 ml of pancreatin and bile in PIPES at pH 6.5 was added. The pH of this suspension was then adjusted to 6.5 using 0.5N NaOH drop-wise. The suspension was

allowed to stand for 10 min and the pH was readjusted to 6.5. The total amount of NaOH required for this was used to calculate the equivalent moles of $NaHCO_3$ required in 20 ml of the solution to be added to the dialysis tubing to bring the pH to 6.5.

III.G. Pancreatin digestion:

For pancreatin digestion two 20g samples of the pepsin-digested sample from each flask were taken in 250 ml conical flasks, thus giving a total of six pancreatin digestions. Dialysis tubing, 20 cm in length and containing 20 ml $NaHCO_3$ solution at the concentration determined by titratable acidity were added to the flasks. After 30 min of incubation at 37^{o}C the pH was recorded and 5 ml of pancreatin/bile salt in PIPES was added to each of the flasks incubated at 37^{o}C for 2 hrs.

Following which the digests were removed and the contents inside and outside the dialysis bags were weighed. The final pHs of the dialyzate and non-dialyzate were recorded.

- Analysis:

After the completion of pancreatin digestion, both the dialyzate and the non-dialyzate were weighed and centrifuged at 1800 x g for 10 mins. Following which an aliquot from each of the samples was mixed with non-reducing solution at 1:1 ratio (v/v). These samples were used to quantify the amount of dialyzable ferrous and total ferrous iron produced during the digestion process. Similarly an aliquot of each of the samples was mixed with reducing solution at 1:1 ratio (v/v). These samples were used

to quantify the amount of total dialyzable and soluble iron produced during the digestion process. A reagent control was also prepared with the same ratio of reducing and non-reducing solutions with the dialyzate or non-dialyzate being replaced by DDW. These samples were covered and left on the bench at room temperature for analysis of iron and protein on the following day.

All the samples were centrifuged. The samples containing the dialyzable portion were centrifuged at 3500 RPM for 10 mins using the bench top laboratory centrifuge, while the samples containing the non-dialyzable portion were centrifuged at 9,000 x g for 20 mins.

III.H. General Analysis Methods:

III.H.1. Dialyzable Ferrous Iron:

This represents the amount of dialyzable iron present in the ferrous form. This is the most potentially bioavailable form of iron and so is the best indicator of potentially bioavailable iron in *in-vitro* studies.

For the determination of dialyzable ferrous iron, 1ml aliquot of the samples having a 1:1 ratio of dialyzable digest : non-reducing solution, were taken in acid washed test tubes. To this 2 ml of 10% ammonium acetate buffer was added, followed by the addition of 0.5 ml of Ferrozine reagent. The mixture was mixed with a vortex and the absorbance was measured immediately at 562 nm.

III.H.2. Dialyzable Total Iron:

This represents the amount of dialyzable iron present in both the ferrous and the ferric form. This is the indicator of the amount of absorbable iron as this is the iron bound to the low molecular weight ligands and so gets easily dialyzed through the 6,000 - 8,000 Da cut off dialysis tubing.

For the determination of dialyzable total iron, 1ml aliquot of the samples having a 1:1 ratio of dialyzable digest : reducing solution, were taken in acid washed test tubes. To this 2 ml of 10% ammonium acetate buffer was added, which was followed by the addition of 0.5 ml of Ferrozine reagent. The mixture was mixed using a vortex, allowed to stand at room temperature for one hour, and then the absorbance was measured at 562 nm.

III.H.3. Non-dialyzable Ferrous Iron:

This represents the amount of iron being converted to the ferrous form but was not dialyzable. This is an excellent indicator of the ability of the test protein or any other component of investigation, to convert ferric iron to the ferrous form, which is the most potentially bioavailable form of iron.

For the determination of non-dialyzable ferrous iron, 1ml aliquot of the samples having a 1:1 ratio of non-dialyzable digest : non-reducing solution, were taken in acid washed test tubes. To this 2 ml of 10% ammonium acetate buffer was added, followed

by the addition of 0.5 ml of Ferrozine reagent. The mixture was mixed with a vortex and the absorbance was measured immediately at 565 nm.

III.H.4. Non-dialyzable Total Iron:

This represents the amount of iron being converted to the soluble form but was not dialyzable. This is an excellent indicator of the ability of the test protein or any other component of investigation, to prevent polymerization of iron and convert it to the soluble form, which is one of the prerequisites for the iron to become potentially bioavailable.

For the determination of non-dialyzable total iron, 1ml aliquot of the samples having a 1:1 ratio of non-dialyzable digest : reducing solution, were taken in acid washed test tubes. To this 2 ml of 10% ammonium acetate buffer was added, followed by the addition of 0.5 ml of Ferrozine reagent. The mixture was mixed with a vortex, allowed to stand at room temperature for 1 hour and the absorbance was measured at 565 nm.

III.H.5. Dialyzable Protein:

This represents the amount of protein, which has been finely digested and converted into peptides within the 6,000 to 8,000 Da range or less, and hence could not be precipitated by trichloroacetic acid (TCA). These are referred to as Low Molecular

Weight Components (LMWCO), and it is a prerequisite to bind iron and carry it along with them through the 6,000 - 8,000 Da cut off dialysis tubing.

For the determination of dialyzable protein, 1 ml aliquot of the samples having a 1:1 ratio of dialyzable digests : non-reducing solution, were taken in clean test tubes. To this 4 ml of biuret reagent was added. The mixture is thoroughly mixed with a vortex, allowed to stand at room temperature for 30 mins and the final absorbance was measured at 540 nm. A blank value was obtained with 1 ml distilled water and 4 ml of biuret reagent.

III.H.6. Non-Dialyzable Protein:

This represents the amount of protein, which has been completely digested and is greater than the 6,000 - 8,000 Da range, but could not be precipitated by trichloro acetic acid (TCA) and hence remains soluble. These are referred to as High Molecular Weight Components (LMWCO), and have tendency to bind iron but cannot carry the iron along with them through the 6,000 - 8,000 Da cut off dialysis tubing.

For the determination of non-dialyzable protein, 1 ml aliquot of the samples having a 1:1 ratio of non-dialyzable digests : non-reducing solution, were taken in clean test tubes. To this 4 ml of biuret reagent was added. The mixture was thoroughly mixed with a vortex, allowed to stand at room temperature for 30 mins. and the final absorbance was measured at 540 nm. A blank value was obtained with 1 ml distilled water and 4 ml of biuret reagent.

III.H.7. Sulfhydryl Analysis:

The total sulfhydryl content of lyophilized raw and cooked (165^{o}F and 195^{o}F) sample was determined using Ellman's method (Ellman 1959). Protein at a concentration of 2mg/ml was dissolved in Na-phosphate buffer pH 8.0. 2ml of this sample was mixed with 18ml of 2.2% SDS in phosphate buffer pH 8.0. Ellman's reagent (DTNB) at the concentration 0.1% was dissolved in phosphate buffer pH 8.0 and 0.1ml was added to 3ml of blank/sample and the absorbance was read at 412 nm after 30 min against phosphate buffer. For the reagent blank, 3 ml of phosphate buffer was mixed with 0.1 ml of Ellman's reagent and the absorbance was read at 412nm against phosphate buffer and subtracted from each sample reading.

III.8. Histidine Analysis:

Histidine was analyzed using the method described by Seth and Mahoney (Seth and Mahoney, 2000). Protein at the concentration 10mg/ml was dissolved in phosphate buffer pH 6.5. 2ml of this sample was mixed with 18ml of 2.2% SDS in phosphate buffer pH 6.5. Diethyl-pyrocarbonate (DEPC) reagent at the concentration 20mM was dissolved in absolute (anhydrous) ethanol and 50 µl was added to the 1ml of blank/sample and the absorbance was read at 240nm after 30 min against phosphate buffer. For blank, 1 ml of phosphate buffer was mixed with 50 µl DEPC reagent and the absorbance was read at 240nm against phosphate buffer.

III.I. Controls:

III.I.1. Iron Only Control:

Digestion system without protein sample and enzymes was used carry out iron only control to compare the values with non-digested samples. The procedure was followed exactly as that mentioned above to obtain the values for dialyzable and non-dialyzable iron.

III.I.2. Iron-Pepsin control:

Digestion system with pepsin and iron but without protein sample and pancreatin enzyme was used to get the values for dialyzable ferrous, dialyzable total, total ferrous and total soluble iron. The procedure was followed exactly as that mentioned above to get the respective values.

III.I.3. Iron pepsin pancreatin control:

Digestion system with pepsin, pancreatin enzymes and iron but without protein sample was used as control to ascertain the authenticity of the digestion processes and to calculate the extrinsic amount of iron that contributes to the measured values of iron after digestion. The procedure was followed exactly as that mentioned above to obtain values for dialyzable and non-dialyzable iron.

III.I.4. Endogenous Control:

Endogenous control was used to estimate the contribution of intrinsic iron present in the samples. Digestion system with protein sample, pepsin and pancreatin enzymes and without standard iron solution was used to carry out endogenous control. The procedure was followed exactly as that mentioned above to obtain values for dialyzable and non-dialyzable iron and these values were subtracted from corresponding values for digested samples.

III.I.5. Non-digested control:

Non-digested control was used to estimate how much iron is produced without digestion of chicken muscle and to determine if digestion is important factor in iron bioavailability. For non-digested samples, the exact procedure as that of digestion was followed without adding any enzymes.

III.J. Calculations:

The amount of dialyzable and non-dialyzable matter obtained after each digestion were recorded and used to calculate dialyzable ferrous, total ferrous, total dialyzable and total soluble iron.

As the dialysis process is known to be an equilibrium process, the amount of dialyzable iron is distributed equally in the entire volume of liquid present inside the dialysis tubing and in the non-dialyzate during digestion. So the total volume for the

dialysis would be 45 ml (20 ml for dialysis bag content, 20 ml for non-dialyzate and 5 for PIPES/bile).

Therefore the total volume of dialyzate (V_D) = 45 ml.

The amount of non-dialyzate always varies depending upon the dynamics of the system. If the sample contains very high amount of carbohydrates, then the osmotic pressure is very high and amount of liquid coming out of dialysis bag is high and vice-versa. So this amount is determined by weighing non-dialyzate and dialysis bag content. Assuming the density to be one, the same amount is taken as volume of non-dialyzate and dialyzate produced. This volume generally varies from 26-28 ml.

Therefore the total volume of dialyzate (V_{ND}) = 26-28 ml.

The concentration of iron present in the dialyzate and non-dialyzate is calculated using ferrozine method as μg/ml, this can be represented as C_{Fe}^{F} for ferrous and C_{Fe}^{T} for total iron iron concentration.

Therefore,

Dialyzable ferrous iron = C_{Fe}^{F} dialyzate * V_D

Non-dialyzable ferrous iron = C_{Fe}^{F} non-dialyzate * V_{ND}

Total dialyzable iron = C_{Fe}^{T} dialyzate * V_D

Total non-dialyzable iron = C_{Fe}^{T} non-dialyzate * V_{ND}

And,

Total ferrous iron = Dialyzable ferrous iron + Non-dialyzable ferrous iron

Total soluble iron = Total dialyzable iron + Total non-dialyzable iron.

Similar calculation will be used to calculate the dialyzable protein and total protein content after TCA precipitation.

CHAPTER IV

EFFECT OF FREEZING AND LYOPHILIZATION ON PRODUCTION OF DIALYZABLE IRON BY RAW CHICKEN MUSCLE

IV.A. Introduction:

Literature survey showed that most of the researchers used lyophilized meat samples to maintain the uniformity of samples throughout the study (Baech et al. 2002). Also, there is no data showing the effect of individual processes like freezing and lyophilization on iron bioavailability of chicken muscle samples. Sulfhydryl groups (-SH) (Hoffman and Hamm 1978) and cysteine (Taylor et al. 1986) containing peptides of meat have been suggested to be responsible for 'meat factor'. The effect of freezing and lyophilization on these factors is not mentioned in the literature. Freezing and lyophilization processes are very complex, and may cause some structure-conformational changes and oxidation of –SH groups in the chicken muscle, because of which the ability of chicken muscle to produce potentially bioavailable forms of iron may be affected.

Taking above factors into account, this chapter will focus on the effect of freezing and lyophilization on production of dialyzable iron, an important marker of potentially bioavailable form of iron in *in-vitro* studies. Slurry of raw chicken muscle sample was formed as mentioned in methods and it was divided into three batches. One batch was kept refrigerated, one was frozen at -40^{o}C and one was lyophilized. The values for dialyzable iron were obtained and compared.

IV.B. Materials and Methods:

IV.B.1. Chemicals:

As discussed in Chapter III except for freshly prepared,

Pepsin: Pepsin from porcine stomach mucosa (Sigma Chemical Co. St. Louis, MO, USA), P-7012, was prepared by dissolving 100mg in 5 ml of 0.01N HCl. Pepsin was added to the protein samples at a pepsin:protein ratio of 1:25 (w/w).

Pancreatin: Pancreatin from porcine pancreas (Sigma Chemical Co. St. Louis, MO, USA), P-1750, was prepared by suspending 200 mg in 50 ml of 0.1M PIPES/bile, at pH 6.5. Pancreatin was added to the protein samples at a pancreatin:protein ratio of 1:50 (w/w)

PIPES/Bile: PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) disodium salt (Sigma Chemical Co. St. Louis, MO, USA). PIPES, P-3768, was dissolved in DDW at a concentration of 0.1M and the final pH was adjusted to 6.5. BILE salts, at the concentration of 50mg/ml, were dissolved in 50 ml of this buffer.

IV.B.2. Sample Preparation and Digestion Protocol:

As described in chapter III, except where specifically noted.

IV.C. General Analysis Methods:

As described in chapter III, except where specifically noted.

IV.D. Controls:

As described in chapter III, except where specifically noted.

IV.E. Results and Discussion

This section presents the iron and protein values of refrigerated, frozen and lyophilized raw and chicken samples in comparison with control. The endogenous control values were subtracted from each digested sample.

Table 1 shows the values for total sulfhydryl and histidine content of refrigerated, frozen and lyophilized raw chicken muscle sample.

SAMPLE	Total –SH Content in mM/g Protein	Total Histidine Content in mM/g Protein
Refrigerated	131.7 ± 7.96^{a}	161.6 ± 3.25^{c}
Frozen	124.8 ± 6.58^{a}	162.0 ± 1.99^{c}
Lyophilized	96.0 ± 4.23^{b}	150.0 ± 3.31^{d}

Table IV.E.1: Total sulfhydryl and histidine content for refrigerated, frozen and lyophilized raw chicken muscle samples. Mean ± SD of total sulfhydryl and histidine content of 6 samples (n = 6) in mM/g protein. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence.

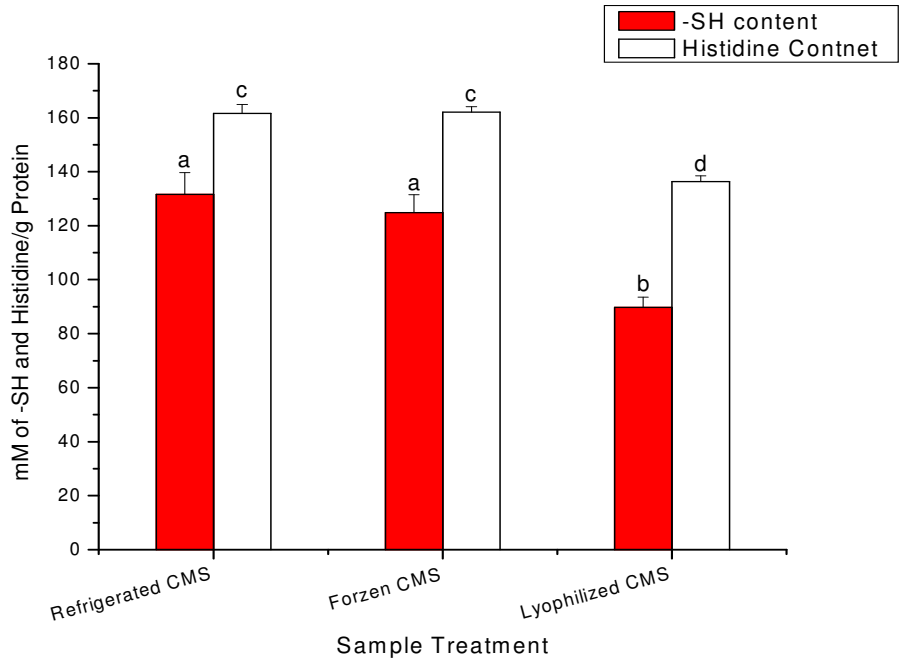

Figure IV.E.1: Total sulfhydryl and histidine content of refrigerated, frozen and lyophilized raw chicken muscle samples. Graphical representation of the data from table 1.

The total sulfhydryl and histidine content of refrigerated, frozen and lyophilized raw chicken muscle sample is shown in figure 1. There was no significant difference between total sulfhydryl and histidine content of refrigerated and frozen raw CMS. However, there was a significant loss of 32% for total sulfhydryl content after lyophilization of raw chicken muscle. Also, the histidine content of raw chicken dropped by ~6% after lyophilization. These results indicated that lyophilization causes a significant loss in sulfhydryl content, which may be either due to polymerization of proteins because of loss of water or due to oxidation of –SH groups during lyophilization. These results also showed that there was a drop in histidine content for raw chicken after lyophilization, which is the new finding as it is not mentioned anywhere in the literature.

Table 2 shows the values for total dialyzable iron and dialyzable ferrous iron produced by non-digested raw chicken sample for refrigerated, frozen and lyophilized treatments.

SAMPLE	Total Dialyzable iron in µg	Dialyzable Ferrous Iron in µg
Control	3.4 ± 0.52^{a}	2.0 ± 0.46^{d}
Refrigerated	38.1 ± 1.83^{b}	16.8 ± 1.21^{e}
Frozen	34.2 ± 0.89^{b}	15.1 ± 0.90^{e}
Lyophilized	19.4 ± 0.45^{c}	3.8 ± 0.96^{a}

Table IV.E.2: Total dialyzable iron and dialyzable ferrous iron produced by non-digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total dialyzable iron [Fe (II) + Fe (III)] and dialyzable ferrous iron in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

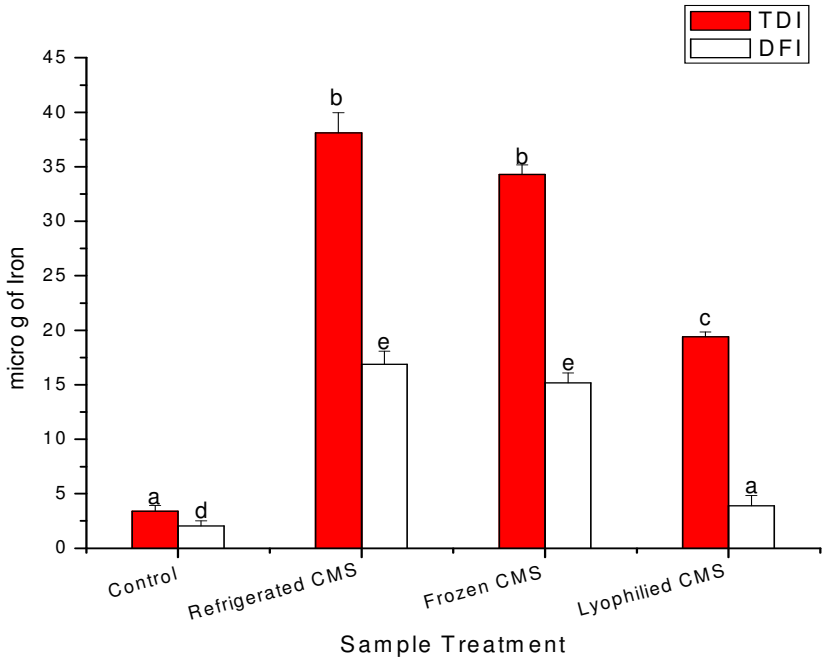

Figure IV.E.2: Production of total dialyzable iron and dialyzable ferrous iron by non-digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 2. TDI: Total Dialyzable Iron; DFI: Dialyzable ferrous iron.

The total dialyzable iron and dialyzable ferrous iron produced by non-digested samples of raw refrigerated, frozen and lyophilized CMS is shown in figure 2. Raw refrigerated and frozen chicken produced about 11 times higher amount of total dialyzable iron as that of control. Lyophilization of raw CMS caused a significant loss of ~50% for total dialyzable iron. Dialyzable ferrous iron produced by refrigerated and frozen raw chicken was 8 times higher than that of control. After lyophilization of raw chicken only 25% of dialyzable ferrous iron was produced as compared to refrigerated and frozen chicken sample. The values obtained for non-digested samples are much higher that control, which indicates that chicken muscle doesn't need digestion to produce potentially bioavailable forms of iron. These results also showed

that lyophilization of raw CMS caused a significant loss in total dialyzable iron and dialyzable ferrous. This implied that the factor(s) responsible for production of dialyzable iron, which does not need digestion, is sensitive to lyophilization. This result is in contradiction with one obtained by Huh et al. (Huh et al. 2005), who concluded that digestion of lyophilized fish muscle did not affect its ability to produce dialyzable iron. The loss in production of dialyzable iron for non-digested sample could be attributed to the 32% loss of sulfhydryls and 15% loss of histidine, if there are any low molecular weight peptides containing these amino acids are present in the raw chicken, as it is shown that low molecular weight peptides containing these amino acids have ability to chelate iron (Hamed et al. 1983; Mulvihill and Morrissey, 1998; Swain et al. 2002).

Table 3 shows the values for total soluble iron and total dialyzable iron produced by digested raw chicken sample for refrigerated, frozen and lyophilized treatments.

SAMPLE	Total Soluble iron in µg	Total Dialyzable iron in µg
Control	131.3 ± 9.67 [a]	6.5 ± 0.93 [d]
Refrigerated	121.3 ± 1.49 [a]	74.1 ± 3.21 [e]
Frozen	114.4 ± 2.64 [b]	70.4 ± 2.01 [e]
Lyophilized	88.7 ± 5.11 [c]	24.5 ± 1.73 [f]

Table IV.E.3: Total soluble iron and total dialyzable iron produced by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total soluble and total dialyzable iron [Fe (II) + Fe (III)] in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

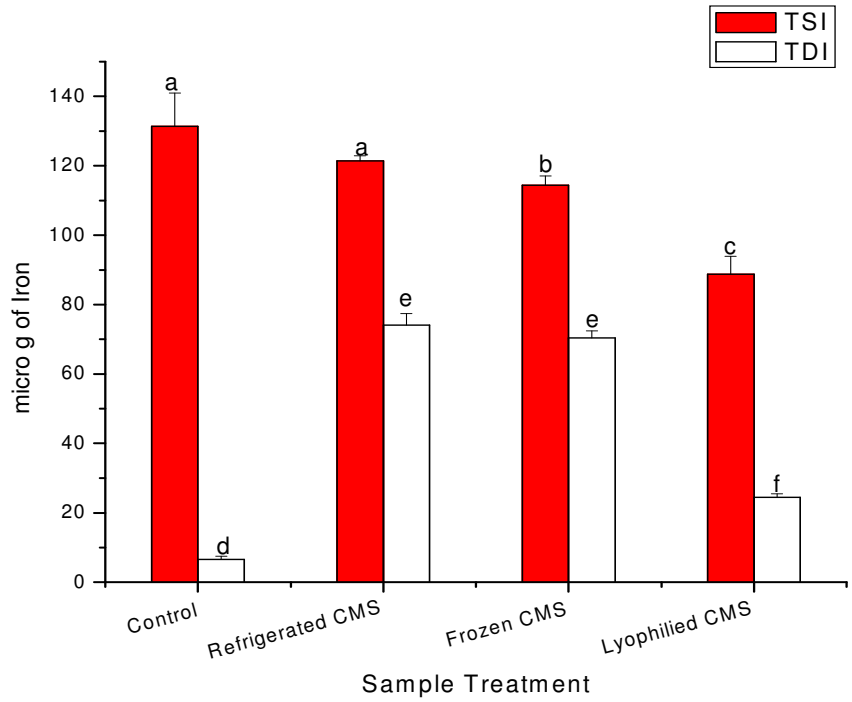

Figure IV.E.3: Production of total soluble iron and total dialyzable iron by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 3. TSI: Total Soluble Iron; TDI: Total Dialyzable Iron.

Figure 3, shows that the total soluble iron and total dialyzable iron produced by control and raw refrigerated, frozen and lyophilized chicken muscle sample (CMS). The total soluble iron produced by refrigerated raw CMS is not significantly different from frozen raw and CMS, which implies that freezing does not have any effect on chicken muscle's ability to keep iron soluble under gastric conditions. But, the total soluble iron for lyophilized raw CMS is significantly different from that of refrigerated and frozen CMS ($p<0.05$), which indicated that lyophilization affects production of total soluble iron by raw CMS. This might be due to the structure-conformational change in the protein structure caused by lyophilization. Total

dialyzable iron produced by refrigerated raw and frozen raw CMS is not significantly different from each other ($p>0.05$), which demonstrated that freezing does not affect digested chicken muscle's ability to chelate iron and carry it through a 6000 – 8000 Da dialysis membrane. But, there was ~65% drop in total dialyzable iron produced by lyophilized raw CMS when compared with refrigerated and frozen raw CMS, which implies that lyophilization has a major effect on chicken muscle's ability to chelate iron and carry it through 6000 – 8000 Da membrane. This might be because of polymerization of proteins, which might have resulted in loss of sulfhydryl (-SH) groups or oxidation of –SH groups during lyophilization. Also the total dialyzable iron produced after digestion of raw CMS is much higher that non-digested raw CMS. This implies that chicken muscle does not need digestion to produce potentially bioavailable iron but digestion enhances its production significantly.

Table 4 shows the values for total dialyzable iron and dialyzable ferrous iron produced by digested raw chicken sample for refrigerated, frozen and lyophilized treatments.

SAMPLE	Total Dialyzable iron in µg	Dialyzable Ferrous Iron in µg
Control	6.5 ± 0.93^{a}	2.8 ± 0.34^{d}
Refrigerated	74.1 ± 3.21^{b}	41.3 ± 2.04^{e}
Frozen	70.4 ± 2.01^{b}	40.9 ± 3.19^{e}
Lyophilized	24.5 ± 1.73^{c}	7.4 ± 1.38^{a}

Table IV.E.4: Total dialyzable iron and dialyzable ferrous iron produced by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total dialyzable iron [Fe (II) + Fe (III)] and dialyzable ferrous iron in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

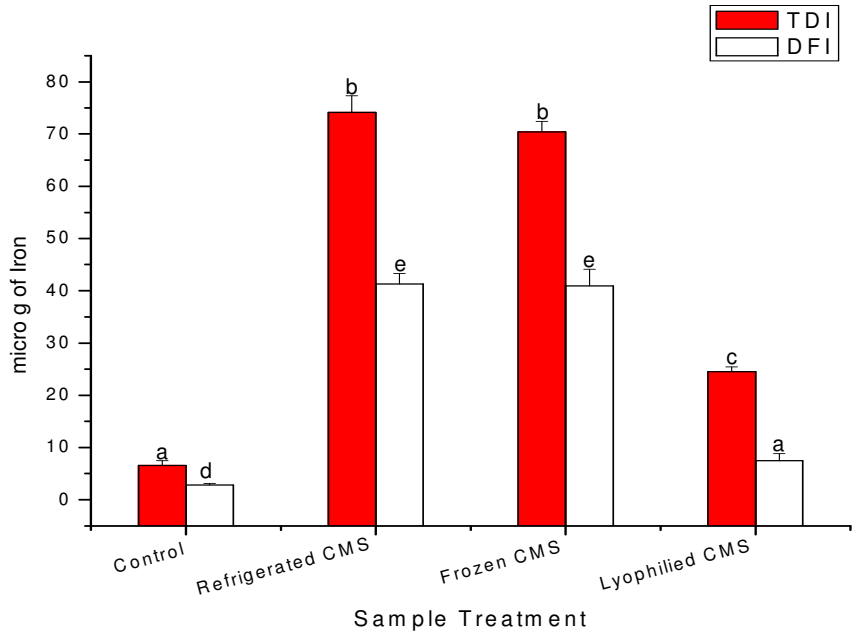

Figure IV.E.4: Production of total dialyzable iron and dialyzable ferrous iron by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 4. TDI: Total Dialyzable Iron; DFI: Dialyzable ferrous iron.

The total dialyzable iron and dialyzable ferrous iron produced by digested control and raw refrigerated, frozen and lyophilized CMS is shown in figure 4. Total dialyzable iron and dialyzable ferrous iron produced by refrigerated raw and frozen raw CMS is not significantly different from each other ($p>0.05$), which implies that freezing chicken muscle did not impair any change in raw chicken's ability to produce dialyzable ferrous iron and total dialyzable iron. But there was a drastic ~85% drop in dialyzable ferrous iron for lyophilized raw CMS as compared with refrigerated and frozen raw CMS, which indicated that lyophilization process almost destroyed the factor responsible for producing potentially bioavailable form of iron. It's shown in the literature that sulfhydryl have ability to convert ferric [Fe (III)] form of iron to

ferrous form [Fe (II)], and the drop in dialyzable ferrous iron can be correlated with loss of sulfhydryl (-SH) groups.

Table 5 shows the values for total ferrous iron and dialyzable ferrous iron produced by digested raw chicken sample for refrigerated, frozen and lyophilized treatments.

SAMPLE	Total Ferrous Iron in µg	Dialyzable Ferrous Iron in µg
Control	14.7 ± 0.85^{a}	2.8 ± 0.34^{d}
Refrigerated	76.1 ± 2.13^{b}	41.3 ± 2.04^{e}
Frozen	74.0 ± 1.51^{b}	40.9 ± 3.19^{e}
Lyophilized	23.2 ± 1.92^{c}	7.4 ± 1.38^{f}

Table IV.E.5: Total ferrous iron and dialyzable ferrous iron produced by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total ferrous iron and dialyzable ferrous iron in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

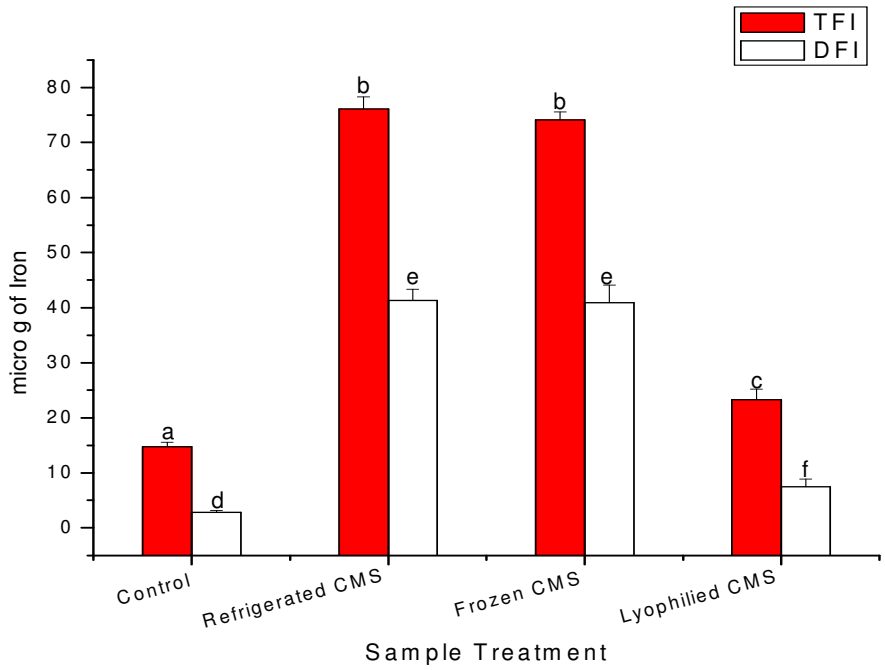

Figure IV.E.5: Production of total ferrous iron and dialyzable ferrous iron by digested control and raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 5. TFI: Total Ferrous Iron; DFI: Dialyzable ferrous iron.

Figure 5, shows that total ferrous iron and dialyzable ferrous iron produced by digested control and raw refrigerated, raw and frozen CMS. Dialyzable ferrous iron is already discussed in figure 5. The total ferrous iron produced by refrigerated and frozen raw CMS is not significantly different from each other, which again suggest that freezing does not impair any change in chicken muscle's ability convert ferric [Fe (III)] form to ferrous [Fe (II)] form of the iron. But, similar to dialyzable ferrous iron, there is ~73% drop in total ferrous iron for lyophilized raw CMS as compared with refrigerated and frozen raw CMS, which indicated that lyophilization affected conversion of ferric form to ferrous form of iron. This may be attributed to the loss of sulfhydryl group caused by lyophilization, as sulfhydryl groups have ability to convert

ferric form to ferrous form. This indicated that –SH groups may play important role in conversion of ferric form of iron to ferrous form, as there is very strong correlation between the drops of –SH content and drop in total and dialyzable ferrous iron.

Table 6 shows the values for total dialyzable iron and dialyzable ferrous iron produced due to digestion (Digested – Non-digested) by raw chicken sample for refrigerated, frozen and lyophilized treatments.

SAMPLE	Total Dialyzable iron in µg	Dialyzable Ferrous Iron in µg
Control	3.1 ± 0.93^{a}	0.7 ± 0.34^{d}
Refrigerated	36.0 ± 3.21^{b}	24.1 ± 2.04^{e}
Frozen	36.1 ± 2.01^{b}	25.7 ± 3.19^{e}
Lyophilized	5.1 ± 1.73^{c}	3.6 ± 1.38^{a}

Table IV.E.6: Total dialyzable iron and dialyzable ferrous iron produced due to digestion (Digested – Non-digested) by control and raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total dialyzable iron [Fe (II) + Fe (III)] and dialyzable ferrous iron produced due to digestion in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

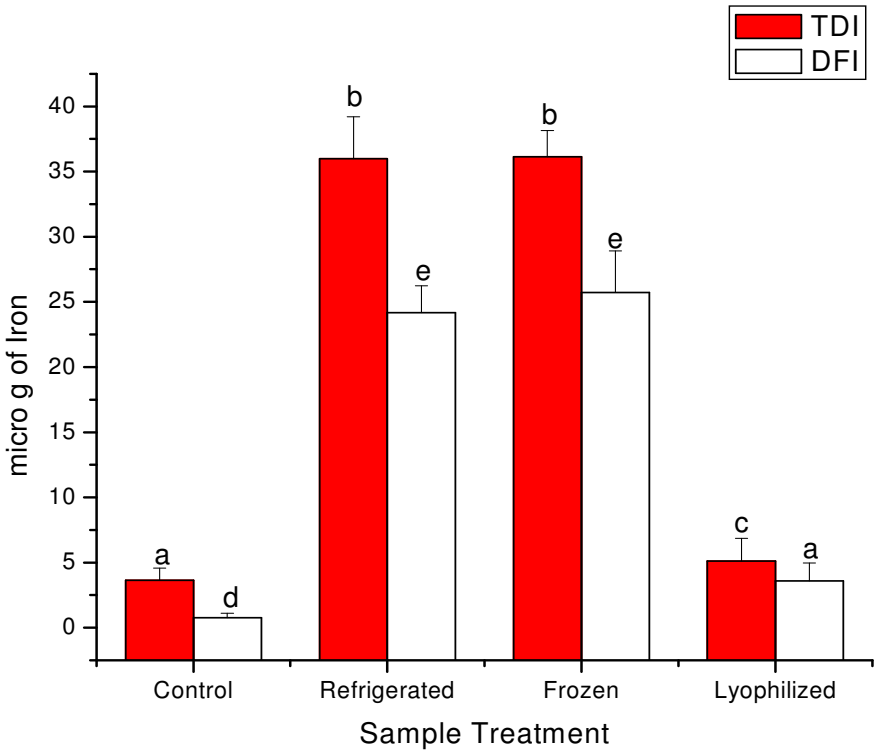

Figure IV.E.6: Production of total dialyzable iron and dialyzable ferrous iron due to digestion (Digested – Non-digested) by control and raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 6. TDI: Total Dialyzable Iron; DFI: Dialyzable ferrous iron.

Figure 6 shows the effect of digestion only (non-digested iron values are subtracted from digested iron values for corresponding chicken muscle sample) on production of dialyzable iron and non-dialyzable iron by control, refrigerated, frozen and lyophilized raw chicken muscle. Refrigerated and frozen chicken muscle produced about 12 times as much dialyzable iron as that of control, whereas lyophilized chicken produced 1.5 times dialyzable iron as that of control. Also, the dialyzable ferrous iron produced by refrigerated and frozen chicken was 32 times as much as that of control and lyophilized chicken produced about 5 times dialyzable ferrous iron as that of control.

These results showed that almost 70% of total dialyzable iron produced during digestion is ferrous refrigerated, frozen and lyophilized chicken muscle sample. There is huge drop in total dialyzable and dialyzable ferrous iron produced by lyophilized chicken as compared to refrigerated and frozen chicken. This implies that the factor responsible for producing dialyzable forms of iron was affected significantly during lyophilization, but it was not destroyed completely as the values are higher than control. This can be attributed to the loss of –SH and histidine during lyophilization process. These results clearly showed that lyophilization has significant effect on chicken muscle's ability to produce potentially bioavailable forms of iron. This might be due to the structure-conformational changes in chicken muscle protein because of loss of water during lyophilization process.

Table 7 shows the values for total soluble protein and total dialyzable protein produced by digested raw refrigerated, frozen and cooked chicken muscle sample.

SAMPLE	Total Soluble Protein in mg	Total Dialyzable Protein in mg
Refrigerated	380.5 ± 5.32^{a}	295.3 ± 10.12^{c}
Frozen	384.8 ± 7.88^{a}	306.4 ± 11.81^{bc}
Lyophilized	320.5 ± 4.11^{b}	223.4 ± 12.48^{d}

Table IV.E.7: Total soluble protein and total dialyzable protein values for digested raw refrigerated, frozen and lyophilized chicken muscle samples. Mean ± SD of total soluble protein and total dialyzable protein in mg for total 6 pancreatin digestions (n = 6). Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence.

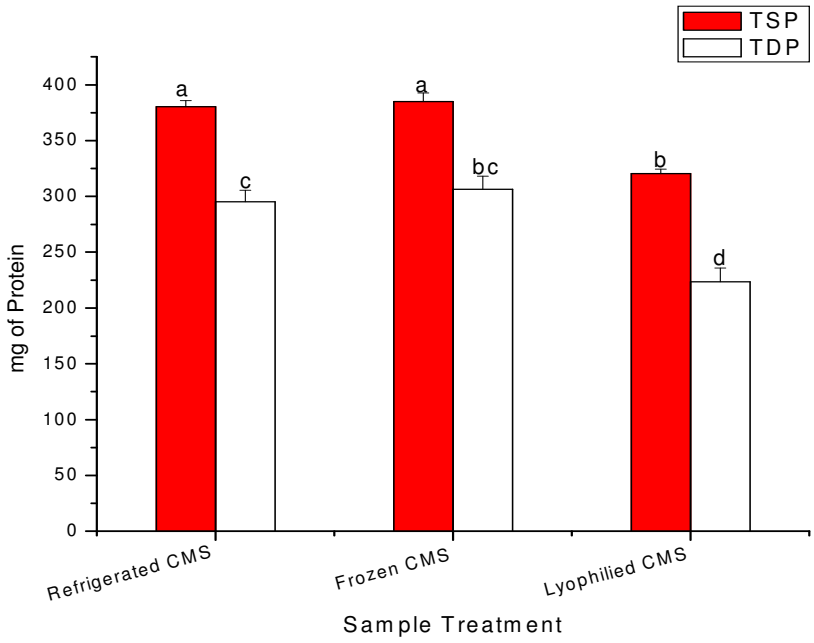

Figure IV.E.7: Production of total soluble protein and total dialyzable protein by raw refrigerated, frozen and lyophilized chicken muscle samples. Graphical representation of the data from table 6. TSP: Total Soluble Protein; TDP: Total Dialyzable Protein.

The production of total soluble protein and total dialyzable protein by digested raw refrigerated, frozen and lyophilized CMS is shown in figure 7. There is no significant difference between refrigerated and frozen raw CMS on production of total soluble protein and total dialyzable protein. However, lyophilization caused small but significant change in production of dialyzable protein. However, lyophilization caused ~15% and ~25% drop in total soluble protein and total dialyzable protein, respectively as compared to refrigerated and frozen CMS. This indicates that lyophilization affects digestibility of chicken muscle. This may be due to the polymerization of chicken muscle protein during lyophilization, which made it difficult for the enzymes to break it down into small peptides and this may be one of the factor responsible for producing different amounts of potentially bioavailable iron.

IV.F. Conclusions:

After this study, following conclusions can be made:

- Chicken muscle does not need digestion to produce potentially bioavailable forms of iron. However digestion enhances its production significantly.

- Freezing does not affect chicken muscle's ability to produce potentially bioavailable forms of iron.

- Lyophilization causes significant loss in total sulfhydryl and histidine content, which may be due to the polymerization of muscle proteins during lyophilization.

- Lyophilization decreases chicken muscle's ability to produce potentially bioavailable forms of iron and this is well correlated with decrease in –SH and histidine content.

- The digestibility of chicken muscle affected significantly after lyophilization. This may be due to the polymerization of chicken muscle protein, which made it difficult for the enzymes to break it down in smaller peptides.

CHAPTER V

EFFECT OF COOKING ON IRON BIOAVAILABILITY OF CHICKEN MUSCLE

V.A. Introduction:

From preliminary work, it is clear that lyophilization has a significant effect on sulfhydryl content and ability of chicken muscle to produce potentially bioavailable forms of iron. However, freezing chicken muscle slurry did not cause any significant effect on sulfhydryl content and production on potentially bioavailable species of iron. As we want to maintain the uniformity of the sample throughout the study, chicken muscle slurry can be frozen as it does not affect its ability to produce potentially bioavailable forms of iron. It will be interesting to cook the chicken muscle sample at different temperatures and obtain the values for amino acid content and dialyzable iron and to find the correlation between them.

This chapter will focus on effect of cooking chicken muscle at four different temperatures. Chicken muscle slurry was cooked at 130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F in a boiling water bath, cooled to room temperature, poured into 1 pound plastic container and frozen at -40^{o}C in a chest freezer. These samples were thawed overnight in the refrigerated and subjected to pepsin and pancreatin digestion and analyzed for dialyzable iron values. The dialyzable iron values will be compared with amino acid content to see if there is any correlation between them. Detailed procedure for sample preparation and analysis is described in Chapter III.

V.B. Materials and Methods:

V.B.1. Chemicals:

As discussed in Chapter III except for freshly prepared solutions described below:

Pepsin: Pepsin from porcine stomach mucosa (Sigma Chemical Co. St. Louis, MO, USA), P-7012, was prepared by dissolving 100mg in 5 ml of 0.01N HCl. Pepsin was added to the protein samples at a pepsin:protein ratio of 1:25 (w/w).

Pancreatin: Pancreatin from porcine pancreas (Sigma Chemical Co. St. Louis, MO, USA), P-1750, was prepared by suspending 200 mg in 50 ml of 0.1M PIPES/bile, at pH 6.5. Pancreatin was added to the protein samples at a pancreatin:protein ratio of 1:50 (w/w)

PIPES/Bile: PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) disodium salt (Sigma Chemical Co. St. Louis, MO, USA). PIPES, P-3768, was dissolved in DDW at a concentration of 0.1M and the final pH was adjusted to 6.5. BILE salts, at the concentration of 50mg/ml, were dissolved in 50 ml of this buffer.

V.B.2. Sample Preparation and Digestion Protocol:

As described in chapter III, except where specifically noted.

V.C. General Analysis Methods:

As described in chapter III, except where specifically noted.

V.D. Controls:

As described in chapter III, except where specifically noted.

V.E. Results and Discussion

Table 1 shows the values for total sulfhydryl and histidine content of raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle sample (CMS).

Sample Treatment	Sulfhydryl content µM/g protein sample	Histidine content µM/g protein sample
Raw Chicken Muscle Sample	124.8 ± 6.58^{a}	162.0 ± 1.99^{f}
Cooked (130^{o}F) Chicken Muscle Sample	73.0 ± 1.89^{b}	129.0 ± 1.57^{a}
Cooked (150^{o}F) Chicken Muscle Sample	55.5 ± 0.97^{c}	124.0 ± 0.32^{a}
Cooked (165^{o}F) Chicken Muscle Sample	31.5 ± 2.65^{d}	101.6 ± 11.27^{g}
Cooked (195^{o}F) Chicken Muscle Sample	27.3 ± 0.93^{e}	83.2 ± 0.65^{h}

Table V.E.1: Total sulfhydryl and histidine content for raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples in µM/g protein. Mean ± SD of total sulfhydryl and histidine content of 6 samples (n = 6). Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence.

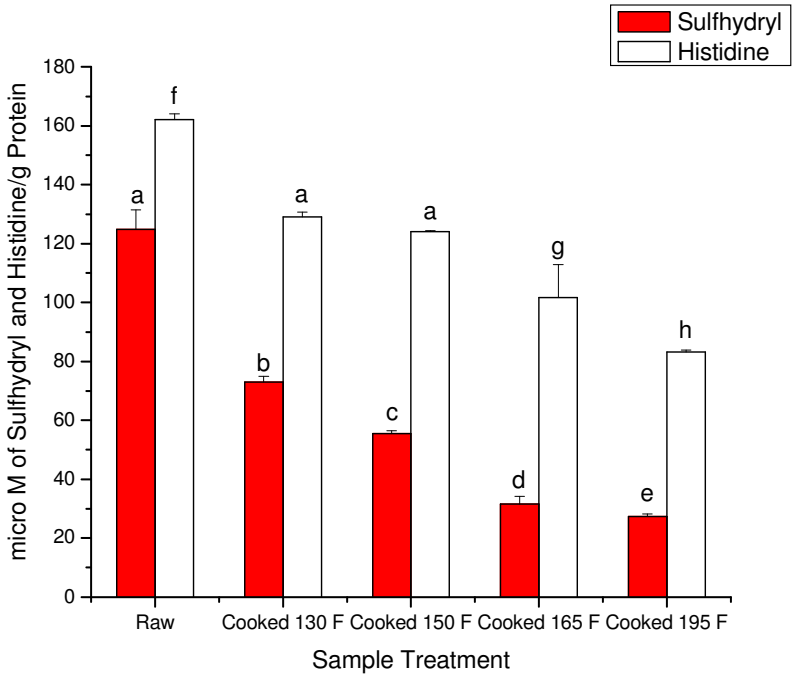

Figure V.E.1: Total sulfhydryl and histidine content of raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples. Graphical representation of the data from table 1.

The effect of heating on the total sulfhydryl (-SH) and histidine content of raw and cooked CMS is shown in figure 1. There was a significant ~40% drop in total –SH and ~20% drop in histidine content was observed when the chicken muscle was heated to 130^{o}F. When the chicken muscle was heated to 150^{o}F, the total –SH and histidine content dropped further, by ~55% and ~23% respectively, as compared to raw. Heating chicken muscle to 165^{o}F resulted in further reduction of 75% in total –SH content and ~37% in histidine content as compared to raw CMS. Only 20% of original –SH content was left i.e. ~80% was destroyed, when the cooking temperature of 195^{o}F was used. Also, the histidine contend was dropped to half of the original histidine content after heating the chicken muscle to 195^{o}F.

These result indicated that sulfhydryls are very sensitive to heat. Heating chicken muscle at 130^{o}F and 150^{o}F caused a significant reduction in total –SH content. These results are in contradiction with those mentioned by Hoffman and Hamm (Hoffman and Hamm, 1978), who measured the accessible –SH content and stated that heating myofibrils from 86^{o}F to 158^{o}F causes increase in accessible –SH group because of unfolding of protein molecules. The increase in cooking temperature of chicken muscle from 165^{o}F to 195^{o}F caused further destruction of –SH content. These results are consistent with those obtained by Hoffman and Hamm (Hoffman and Hamm, 1978), which mentioned that heating chicken muscle protein above 160^{o}F results in the loss of –SH content. The results obtained for histidine content showed that histidine is also heat sensitive, but not as much as that of –SH. There is no data to compare the histidine results in literature as no one mentioned effect of heat on histidine content.

Table 2, gives the values for total soluble iron and total dialyzable iron produced by digested control, raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total Soluble Iron in μg	Total Dialyzable iron in μg
	Mean ± SD [Fe (II) + Fe (III)]	
Control	131.3 ± 9.67 [a]	6.5 ± 0.93 [g]
Raw Chicken	114.4 ± 2.64 [b]	70.4 ± 2.01 [f]
Cooked (130°F) CMS	98.8 ± 2.49 [c]	55.0 ± 2.16 [h]
Cooked (150°F) CMS	89.8 ± 1.92 [d]	43.4 ± 1.14 [i]
Cooked (165°F) CMS	85.1 ± 1.56 [e]	37.0 ± 0.99 [j]
Cooked (195°F) CMS	70.6 ± 5.04 [f]	9.5 ± 0.76 [k]

Table V.E.2: Total soluble iron and total dialyzable iron values for digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Mean ± SD of total soluble and total dialyzable iron [Fe (II) + Fe (III)] in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

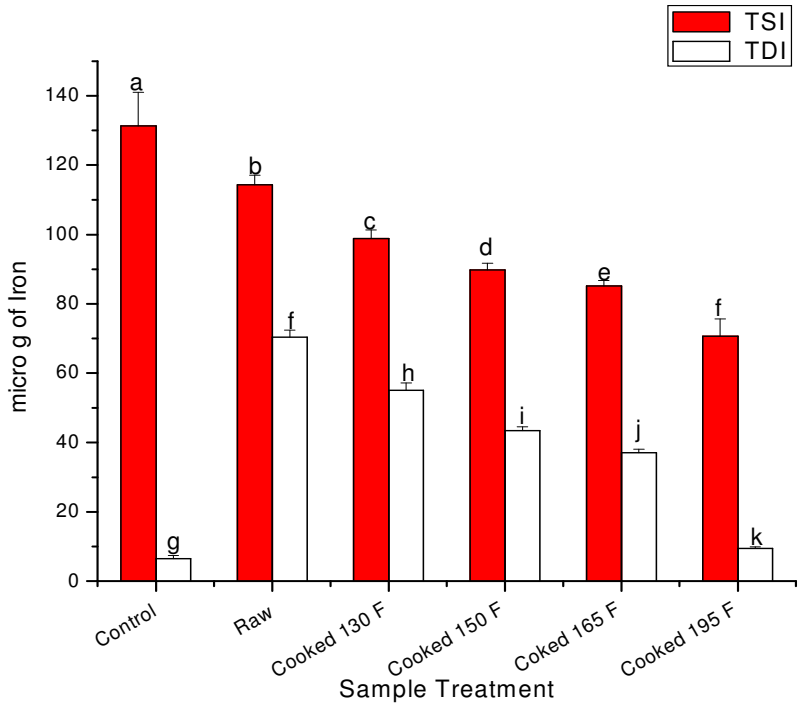

Figure V.E.2: Production of total soluble iron and total dialyzable iron by control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 2. TSI: Total Soluble Iron; TDI: Total Dialyzable Iron.

The total soluble iron and total dialyzable iron produced by raw and cooked chicken muscle is shown in figure 2. Results showed that the total soluble iron produced by raw chicken was only slightly less than the control. In case of cooked 131°F chicken muscle, there was a significant ~25% drop in total soluble iron as compared to control. The total soluble iron decreased further with the increase in cooking temperature. There was ~41%, ~35% and ~46% drop in total soluble iron for cooked 150°F, 165°F and 195°F chicken muscle respectively, as compared to control. The total dialyzable iron for raw chicken was 12 times higher than that of control, which

showed chicken muscle's ability to promote iron bioavailability. Cooking caused drop in production of total dialyzable. Total dialyzable iron is further discussed in figure 3. These results indicated that cooking chicken muscle results in the loss of its ability to keep iron in the soluble form, which is a primary requirement to make iron potentially bioavailable. This may be due to the ability of soluble high molecular weight fraction (HMWF), which chelates iron and is then precipitated by TCA. This may be because the HMWF might contain histidine and cysteine containing peptides, as it is previously shown that they have ability to bind iron and keep it in the soluble form (Mulvihill and Morrissey, 1997; Seth and Mahoney, 2000; Swain et al. 2001) and figure 1 shows that histidine and –SH content drops with increase in cooking temperature. In conclusion, heating chicken muscle affects its ability to keep iron in soluble form.

Table 3 gives the values for total dialyzable iron and dialyzable ferrous iron produced by digested control, raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total Dialyzable Iron in μg	Dialyzable Ferrous Iron in μg
	Mean ± SD	
Control	6.5 ± 0.93 [a]	2.8 ± 0.34 [g]
Raw Chicken	70.4 ± 2.01 [b]	49.9 ± 3.19 [c]
Cooked (130°F) CMS	55.0 ± 2.16 [c]	36.4 ± 1.43 [e]
Cooked (150°F) CMS	43.4 ± 1.14 [d]	16.6 ± 0.92 [h]
Cooked (165°F) CMS	37.0 ± 0.99 [e]	13.4 ± 0.45 [i]
Cooked (195°F) CMS	9.5 ± 0.76 [f]	5.5 ± 0.32 [a]

Table V.E.3: Total dialyzable iron and dialyzable ferrous iron values for digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Mean ± SD of total dialyzable iron and dialyzable ferrous iron in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

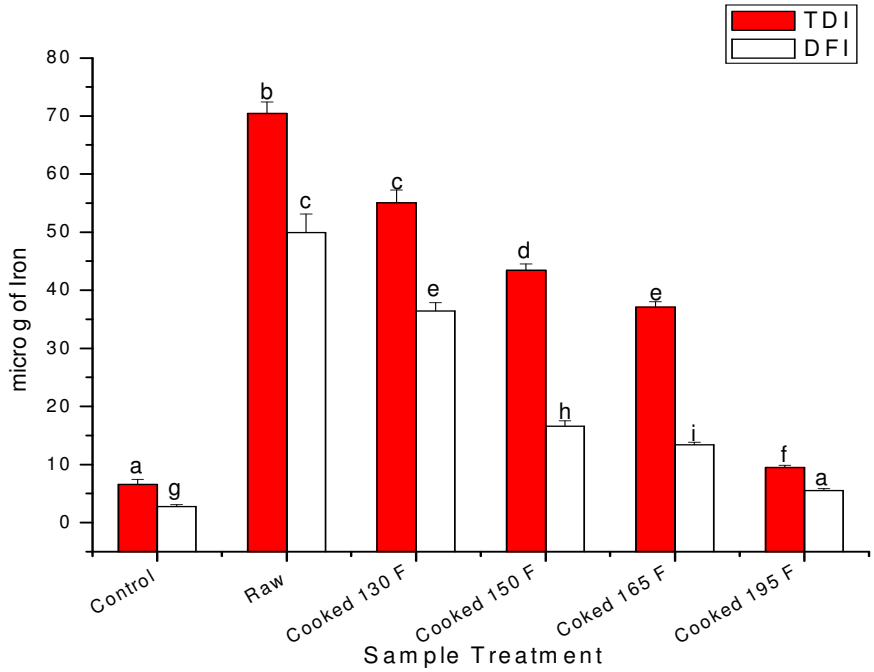

Figure V.E.3: Production of total dialyzable iron and dialyzable ferrous iron by control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 3. TDI: Total Dialyzable Iron; DFI: Dialyzable Ferrous Iron.

Total dialyzable iron and dialyzable ferrous iron produced by raw and cooked chicken muscle samples is shown in figure 3. Raw chicken produced about 11 times as much total dialyzable iron and 17 times dialyzable ferrous iron as that of control, which clearly shows that chicken muscle is very good enhancer of iron bioavailability. Heating chicken muscle to 130°F caused ~40% drop in total dialyzable iron and ~26% drop in dialyzable ferrous iron, as compared to raw chicken sample. When the cooking temperature was increased to 150°F, this resulted in 50% drop in total dialyzable iron and 60% drop in dialyzable iron as compared to raw chicken sample. Further heating the sample to 165°F and 195°F caused a significant ~70% and ~77% drop in total dialyzable iron respectively. The dialyzable ferrous iron decreased by 75% and 90%, when the samples were heated to 165°F and 195°F.

These results clearly show that heating chicken muscle has very strong effect on its ability to produce potentially bioavailable forms of iron. The drop in total dialyzable and dialyzable ferrous iron at 130^{o}F shows that the factor(s) responsible for production of potentially bioavailable forms of iron is very heat labile and gets affected even if the sample is heated to 130^{o}F. Also, the drop in total dialyzable and dialyzable ferrous iron continues when further addition of heat. Figure 1 shows that total sulfhydryl (-SH) and histidine content is also affected with increasing cooking temperature. So there is a very strong correlation between the drops of –SH, histidine and potentially bioavailable forms of iron. As mentioned in the literature –SH groups have ability to reduce ferric form of iron to ferrous form, which is the most potentially bioavailable form of iron (Taylor et al. 1986; Kirwan et al. 1993; Mulvihill and Morrissey, 1998; Mulvihill et al. 1998). Also histidine containing peptides have ability to chelate iron (Seth and Mahoney, 2000). In conclusion, these results indicate that sulfhydryl and histidine containing peptides might be involved production of potentially bioavailable forms of iron, as there is a very strong correlation between the drops of –SH, histidine and iron values.

Table 4 gives the values for total ferrous iron and dialyzable ferrous iron produced by digested control, raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total Ferrous Iron in μg	Dialyzable Ferrous Iron in μg
	Mean ± SD	
Control	14.7 ± 0.85 [a]	2.8 ± 0.34 [g]
Raw Chicken	90.0 ± 1.51 [b]	49.9 ± 3.19 [d]
Cooked (130°F) CMS	55.4 ± 1.63 [c]	36.4 ± 1.43 [h]
Cooked (150°F) CMS	46.1 ± 1.39 [d]	16.6 ± 0.92 [a]
Cooked (165°F) CMS	25.7 ± 1.52 [e]	13.4 ± 0.45 [a]
Cooked (195°F) CMS	20.3 ± 1.39 [f]	5.5 ± 0.317 [i]

Table V.E.4: Total ferrous iron and dialyzable ferrous iron values for digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Mean ± SD of total ferrous iron and dialyzable ferrous iron in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

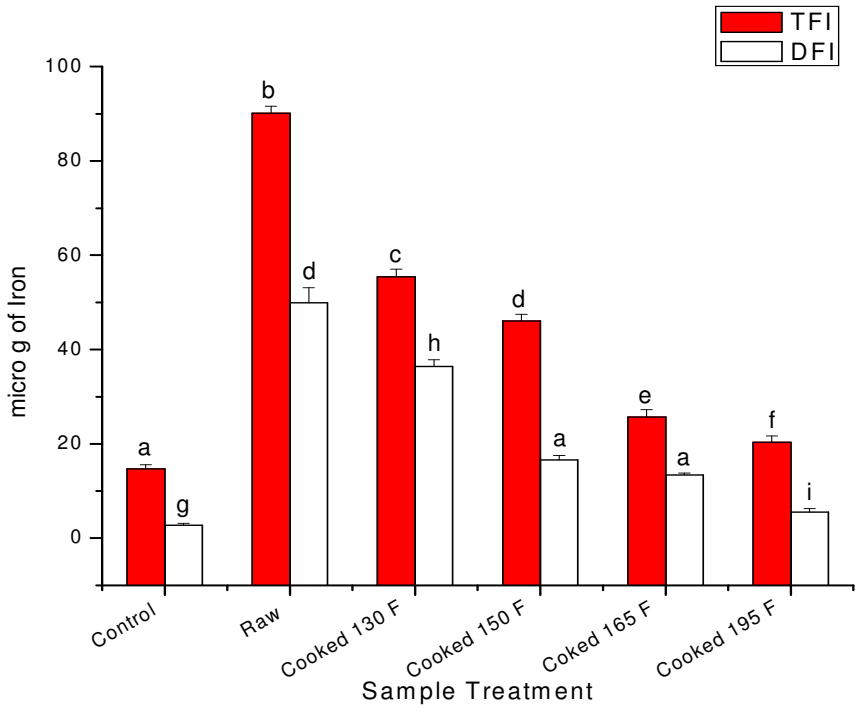

Figure V.E.4: Production of total ferrous iron and dialyzable ferrous iron by control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 4. TFI: Total Ferrous Iron; DFI: Dialyzable Ferrous Iron.

The total ferrous iron and dialyzable ferrous iron produced by digested control, raw and cooked chicken muscle samples is shown in figure 4. Raw chicken produced about six times as much total ferrous iron as that of control and 17 times dialyzable ferrous iron as that of control, which clearly showed chicken muscle's ability to reduce ferric form of iron to ferrous form and make it potentially bioavailable. Cooking the sample at 130°F resulted in ~38% drop in total ferrous iron and ~25% loss in total dialyzable iron. Further cooking the sample at 150°F resulted in drop of total ferrous iron to half and dialyzable ferrous iron to one third as compared to raw chicken sample. Cooking temperatures of 165°F and 195°F resulted in collapse of total ferrous iron by approximately 75%, as compared with raw chicken. The

dialyzable ferrous iron dropped by 73% and 90% for cooked 165°F and 195°F chicken muscle respectively, as compared to raw chicken muscle.

These results shows that cooking chicken even at 130°F results in the loss of potentially bioavailable forms of iron, which implies that the meat factor(s) is heat labile. The increase in cooking temperature from 130°F to 195°F results in significant drop in production of potentially bioavailable forms of iron. Dialyzable ferrous iron produced by cooked 195°F sample is higher than that of control, which implies that heating chicken muscle at 195°F did not destroy the meat factor(s) completely. From figure 1, it is clear that –SH and histidine content drops significantly and show that similar trend as that of total ferrous iron and total dialyzable iron. It is well know that –SH group has ability to reduce ferric form of iron to ferrous form (Taylor et al. 1986; Kirwan et al. 1993; Mulvihill and Morrissey, 1998; Mulvihill et al. 1998) and histidine containing peptides can chelate iron (Seth and Mahoney, 2000). So the loss of chicken muscle's ability to produce potentially bioavailable forms of iron is well correlated with that of –SH and histidine content.

In conclusion heating chicken muscle results in loss of its ability to produced potentially bioavailable forms of iron and –SH and histidine containing peptides might be responsible for 'meat factor'.

Table 5 gives the values for total dialyzable iron and dialyzable ferrous iron produced by non-digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples.

Sample	Total Dialyzable Iron in μg	Dialyzable Ferrous Iron in μg
	Mean ± SD	
Control	2.9 ± 0.44 [a]	2.0 ± 0.46 [a]
Raw Chicken	34.2 ± 0.89 [b]	15.1 ± 0.90 [g]
Cooked (130°F) CMS	30.0 ± 1.70 [c]	20.9 ± 1.11 [d]
Cooked (150°F) CMS	20.5 ± 0.73 [d]	10.9 ± 0.65 [h]
Cooked (165°F) CMS	6.0 ± 1.18 [e]	2.0 ± 0.85 [a]
Cooked (195°F) CMS	4.0 ± 0.39 [f]	2.5 ± 0.39 [a]

Table V.E.5: Total dialyzable iron and dialyzable ferrous iron values for non-digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Mean ± SD of total dialyzable iron and dialyzable ferrous iron in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

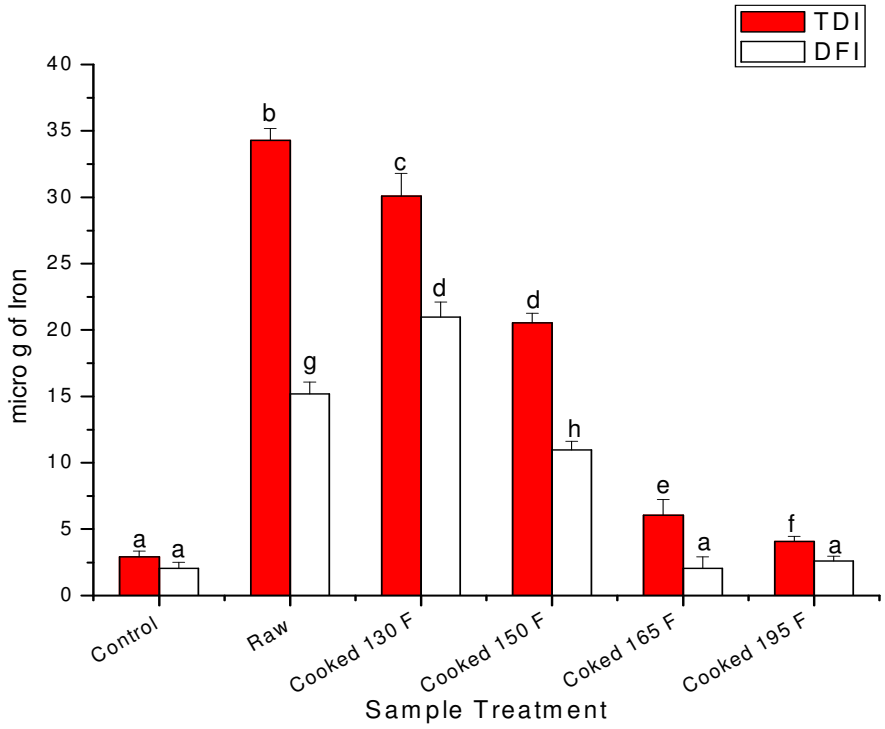

Figure V.E.5: Production of total dialyzable iron and dialyzable ferrous iron by non-digested control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 5. TDI: Total Dialyzable Iron; DFI: Dialyzable Ferrous Iron.

The total dialyzable iron and dialyzable ferrous iron produced by non-digested raw and cooked sample is shown in figure 5. Non-digested raw chicken produced about 12 times total dialyzable iron and 7 times dialyzable ferrous iron as that of control. The total dialyzable iron produced by cooked 130°F chicken sample dropped slightly by insignificant 10% as compared to control. The dialyzable ferrous iron increased by ~30% as compared with raw chicken, which may be attributed to the unfolding of protein molecules and exposure of accessible –SH groups to the iron. There was drop in both total dialyzable iron and dialyzable ferrous iron by approximately 40% and 33%, respectively for cooked 150°F chicken sample as compared with raw chicken.

When the sample was cooked at 165^{o}F and 195^{o}F, the total dialyzable iron collapsed by 82% and 88% respectively, as compared with raw chicken. The total ferrous iron was dropped by a very significant amount and was similar to that produced by control.

These results show that chicken muscle does not need digestion to produce potentially bioavailable forms of iron. This implies that some of the 'meat factor(s)' is present in raw meat, which does not need digestion to produce potentially bioavailable forms of iron. However, from figure 3 it's also clear that digestion helps to produce more potentially bioavailable iron, which implies that some of the 'meat factor(s)' is produced during digestion. The drop in total dialyzable iron and dialyzable ferrous iron for cooked samples indicates that the 'meat factor(s)' present in raw meat is very heat sensitive. The dialyzable ferrous iron for cooked 165^{o}F and 195^{o}F sample dropped to the level which is not significantly different from control.

This implies that the 'meat factor(s)' present in raw meat, which does not need digestion to produce potentially bioavailable forms of iron, was destroyed completely when chicken was cooked at 165^{o}F. However, from figure IV.E.3 it is clear that the total dialyzable iron and dialyzable ferrous iron produced by cooked 165^{o}F and 195^{o}F chicken sample is much higher than control. This implies that most of the 'meat factor(s)' for cooked 165^{o}F and 195^{o}F chicken sample was produced during digestion.

In conclusion, the 'meat factor' present in raw meat, which does not need digestion to produce potentially bioavailable forms of iron, was destroyed completely when chicken was cooked at 165^{o}F.

Table 6 gives the values for total dialyzable iron and dialyzable ferrous iron produced because of digestion (non-digested iron values are subtracted from the digested iron values for the corresponding sample) by control, raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples.

Sample	**Total Dialyzable Iron in μg**	**Dialyzable Ferrous Iron in μg**
	Mean ± SD	
Control	3.6 ± 0.93 ^{a}	0.7 ± 0.34 ^{f}
Raw Chicken	36.1 ± 2.01 ^{b}	34.7 ± 3.19 ^{b}
Cooked (130^{o}F) CMS	25.0 ± 2.16 ^{c}	15.4 ± 1.43 ^{g}
Cooked (150^{o}F) CMS	22.8 ± 1.14 ^{c}	5.6 ± 0.92 ^{h}
Cooked (165^{o}F) CMS	31.0 ± 0.99 ^{d}	11.3 ± 0.45 ^{i}
Cooked (195^{o}F) CMS	5.4 ± 0.76 ^{e}	2.9 ± 0.32 ^{a}

Table V.E.6: Total dialyzable iron and dialyzable ferrous iron produced due to digestion (digested iron – non-digested iron values) of control, raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples. Mean ± SD of total dialyzable iron and dialyzable ferrous iron in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

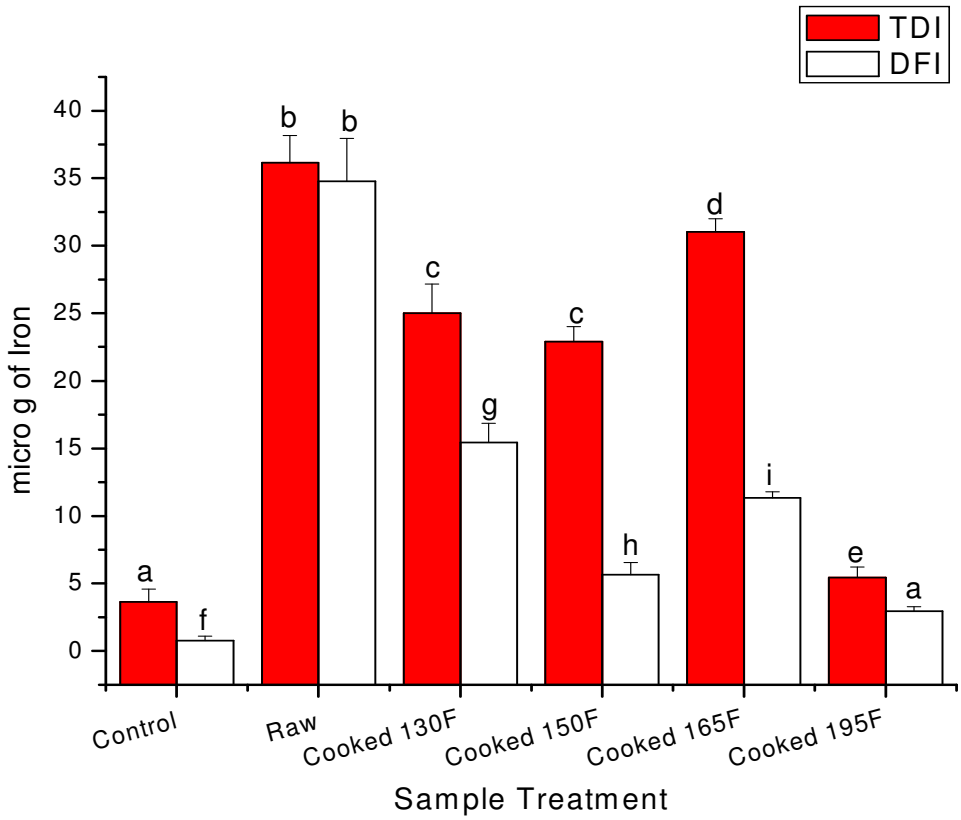

Figure V.E.6: Production of total dialyzable iron and dialyzable ferrous iron due to digestion (digested iron – non-digested iron values) of control, raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 6. TDI: Total Dialyzable Iron; DFI: Dialyzable Ferrous Iron.

The total dialyzable iron and dialyzable ferrous iron produced because of digestion (non-digested iron values are subtracted from the digested iron values for the corresponding sample) is shown in fig 6. Raw and cooked (130°F) chicken samples produced about 10 times and 7 times as much dialyzable iron as that of control, respectively, whereas cooked 150°F and 165°F chicken samples produced about 6 times and 8 times total dialyzable iron as that of control, respectively. The total dialyzable iron produced by cooked 195°F chicken sample was slightly higher than control. In case of dialyzable ferrous iron, almost 100% dialyzable iron produced is

ferrous for raw chicken sample. But, the percentage of dialyzable ferrous iron produced by cooked samples decreases with temperature, except for cooked 165^{o}F. These results clearly showed that digestion of chicken muscle does produce potentially bioavailable forms of iron and it's in contradiction with the results obtained by Huh et al. (Huh et al. 2005) who suggested that digestion did not produce potentially bioavailable forms of iron. Also, the drop in production of total dialyzable and dialyzable ferrous iron with temperature is well correlated with the drop in –SH and histidine content for corresponding samples, which are suggested to be responsible for 'meat factor'.

Table 7 gives the values for total soluble protein and total dialyzable protein produced by lyophilized raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total Soluble Protein in mg	Total Dialyzable Protein in mg
	Mean ± SD	
Control	-	-
Raw Chicken	384.8 ± 7.88 [a]	306.4 ± 11.81 [d]
Cooked (130°F) CMS	364.0 ± 8.67 [b]	235.8± 8.24 [e]
Cooked (150°F) CMS	340.7 ± 3.56 [c]	237.5 ± 8.46 [e]
Cooked (165°F) CMS	378.5 ± 10.29 [ab]	265.0± 18.49 [e]
Cooked (195°F) CMS	386.3 ± 3.59 [a]	322.5 ± 21.27 [d]

Table V.E.7: Total soluble protein and total dialyzable protein values for digested raw and cooked (130°F, 150°F, 165°F and 195°F) chicken muscle samples. Mean ± SD of total soluble protein and total dialyzable protein in mg for total 6 pancreatin digestions (n = 6). The total amount of protein used for each pancreatin digestion is 400 mg. Different subscripts indicates difference using One Way ANOVA test for α = 0.05 or 95% confidence.

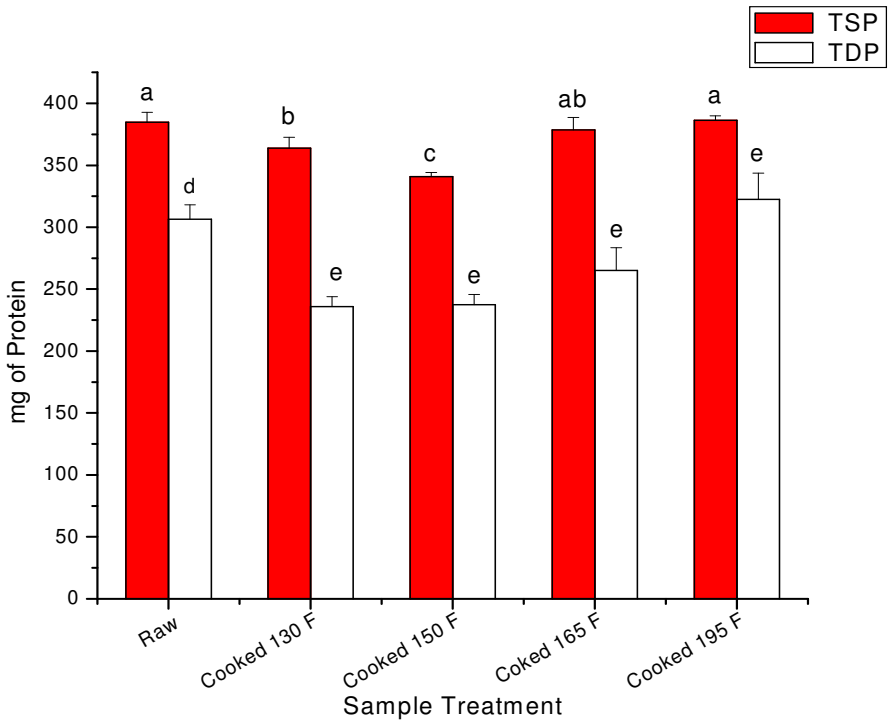

Figure V.E.7: Production of total soluble protein and total dialyzable protein by digested raw and cooked (130^{o}F, 150^{o}F, 165^{o}F and 195^{o}F) chicken muscle samples. Graphical representation of the data from table 7. TSP: Total Soluble Protein; TDP: Total Dialyzable Protein.

The production of total soluble protein and total dialyzable protein by digested raw and cooked chicken muscle samples is shown in figure 7. Even if there is slight variation in total soluble protein produced by raw and cooked samples, there is no significant difference between them. Total soluble protein produced by raw and cooked chicken muscle samples are not significantly different from each other, which suggests that digestion of chicken muscle was uniform and was not a factor responsible for difference in the dialyzable iron produced. Total dialyzable protein produced by raw chicken muscle is higher than cooked 130^{o}F, 150^{o}F and 165^{o}F and it is not significantly different from that of cooked 195^{o}F chicken sample. These results suggest that cooking temperature plays important role in digestibility of chicken

muscle samples. At 195^{o}F, the proteins may be denatured well enough to get digested; whereas at lower cooking temperature, the digestibility might have affected because of polymerization of proteins.

V.F. Conclusions:

After this study, following conclusion can be made:

- Chicken muscle does not need digestion to produce potentially bioavailable forms of iron. But digestion increases the amount of potentially bioavailable forms of iron significantly.

- Heating causes progressive decrease in production of dialyzable iron by both digested and non-digested chicken muscle samples.

- The total dialyzable iron, which is an important marker of potentially bioavailable forms of iron in *in-vitro* study, dropped significantly with increase in cooking temperature and this drop is well correlated with the drop in sulfhydryl and histidine content. This suggests that sulfhydryl and histidine play an important role in 'meat factor'.

- The factor(s) responsible to convert ferric from of iron to ferrous is very heat labile. This can be correlated with the drop in total -SH content as –SH groups have ability to reduce ferric iron to ferrous form.

CHAPTER VI

PRODUCTION OF DIALYZABLE IRON BY LYOPHILIZED RAW AND COOKED SAMPLES

VI.A. Introduction:

Literature survey suggests that effect of cooking chicken on production of potentially bioavailable forms of iron has not been given much attention. Cysteine containing peptides of meat, e.g. glutathione (Taylor et al. 1986) and sulfhydryl (-SH) and disulphide groups in meat (Hoffman and Hamm 1978), have been suggested to be responsible for 'meat factor', but the effect of cooking on these factors has not been given much emphasis. It is well known that sulfhydryls are heat labile and heating temperature plays important role in sulfhydryl degradation. Literature review indicates no such study where the effect on sulfhydryl and histidine content under cooking conditions is measured. We selected heating temperature as 165^{o}F and 195^{o}F. 165^{o}F is minimum temperature to kill microbes and 195^{o}F is used for further heating reference temperature.

Chicken muscle slurry was heated at 165 and 195^{o}F in a boiling water bath, cooled to room temperature and 100g of raw and heated slurry was poured in 1 pound containers to the thickness of 1 cm. These samples were frozen to -40^{o}C and lyophilized in a commercial lyophilizer. After lyophilization, the sample were stored at -15^{o}C and used as required for the experiments. The amount of dialyzable iron formed, -SH and histidine content of each sample was analyzed using methods described in Chapter III and the results were compared with raw chicken values.

VI.B. Materials and Methods:

VI.B.1. Chemicals:

As discussed in Chapter III except for freshly prepared solutions below:

Pepsin: Pepsin from porcine stomach mucosa (Sigma Chemical Co. St. Louis, MO, USA), P-7012, was prepared by dissolving 100mg in 5 ml of 0.01N HCl. Pepsin was added to the protein samples at a pepsin:protein ratio of 1:25 (w/w).

Pancreatin: Pancreatin from porcine pancreas (Sigma Chemical Co. St. Louis, MO, USA), P-1750, was prepared by suspending 200 mg in 50 ml of 0.1M PIPES/bile, at pH 6.5. Pancreatin was added to the protein samples at a pancreatin:protein ratio of 1:50 (w/w)

PIPES/Bile: PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) disodium salt (Sigma Chemical Co. St. Louis, MO, USA). PIPES, P-3768, was dissolved in DDW at a concentration of 0.1M and the final pH was adjusted to 6.5. BILE salts, at the concentration of 50mg/ml, were dissolved in 50 ml of this buffer.

VI.B.2. Sample preparation:

As described in chapter III, except where specifically noted.

VI.C. Analysis:

As discussed in chapter III, except where specifically noted.

VI.D. Controls:

As described in chapter III, except where specifically noted.

VI.E. Results and Discussion

This section presents the amino acid, iron and protein values of lyophilized raw and cooked (165^{o}F and 195^{o}F) samples in comparison with control. The endogenous control values were subtracted from each digested sample to obtain the values shown in this section.

Table 1 gives the values for total sulfhydryl and histidine content of lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples (CMS).

Sample	Sulfhydryl content µM/g protein sample	Histidine content µM/g protein sample
Lyophilized Raw CMS	89.7±3.78 [a]	136.3±2.03 [d]
Lyophilized Cooked (165°F) CMS	64.0±3.94 [b]	99.7±3.72 [e]
Lyophilized Cooked (195°F) CMS	40.2±2.17 [c]	82.6±2.66 [f]

Table VI.E.1: Total sulfhydryl and histidine content for lyophilized raw and cooked (165°F and 195°F) chicken muscle samples in µM/g protein. Mean ± SD of total sulfhydryl and histidine content of 6 samples (n = 6). Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence.

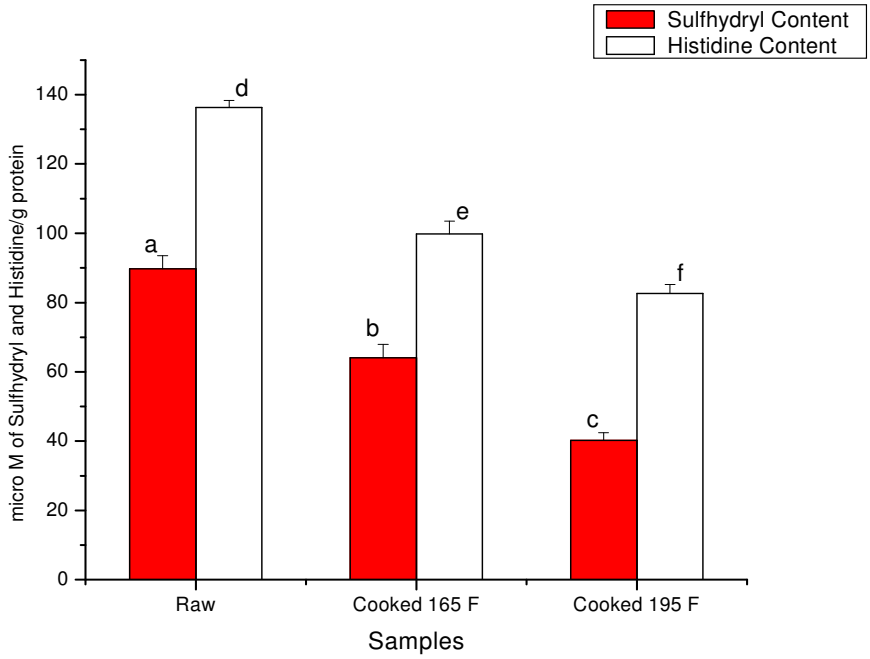

Figure VI.E.1: Total sulfhydryl and histidine content of lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples. Graphical representation of the data from table 1.

Figure 1, shows the sulfhydryl content for lyophilized raw and cooked samples (165^{o}F and 195^{o}F) is significantly different from each other ($p<0.05$). Sulfhydryl content of lyophilized cooked 165^{o}F and 195^{o}F CMS dropped by ~30% and ~55%, as compared with lyophilized raw samples respectively. This implies that sulfhydryls are heat labile and were degraded at 165^{o}F and the degradation continued till 195^{o}F. The effect of further heating is unknown to us as 195^{o}F was highest temperature of heating for our study. The histidine values were also dropped by ~30 % and ~40% for lyophilized cooked 165^{o}F and 195^{o}F CMS respectively, when compared with raw CMS. The effect of heating on histidine is not mentioned in anywhere in literature. This implies that histidine is also heat labile and may be a factor responsible for drop in dialyzable total iron.

Table 2, gives the values for total soluble iron and total dialyzable iron produced by digested control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples.

Sample	**Total soluble iron in μg**	**Total dialyzable iron in μg**
	Mean ± SD	
Control	131.3 ± 9.67 ^{a}	6.5 ± 0.93 ^{d}
Raw Chicken	88.7 ± 5.11 ^{b}	24.5 ± 1.73 ^{e}
Cooked (165^{o}F) chicken	79.6 ± 6.87 ^{b}	21.7 ± 0.90 ^{f}
Cooked (195^{o}F) chicken	64.1 ± 3.36 ^{c}	21.1 ± 1.27 ^{f}

Table VI.E.2: Total soluble iron and total dialyzable iron values for no protein control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples. Mean ± SD of total soluble and total dialyzable iron [Fe (II) + Fe (III)] in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of samples has been subtracted from all the values.

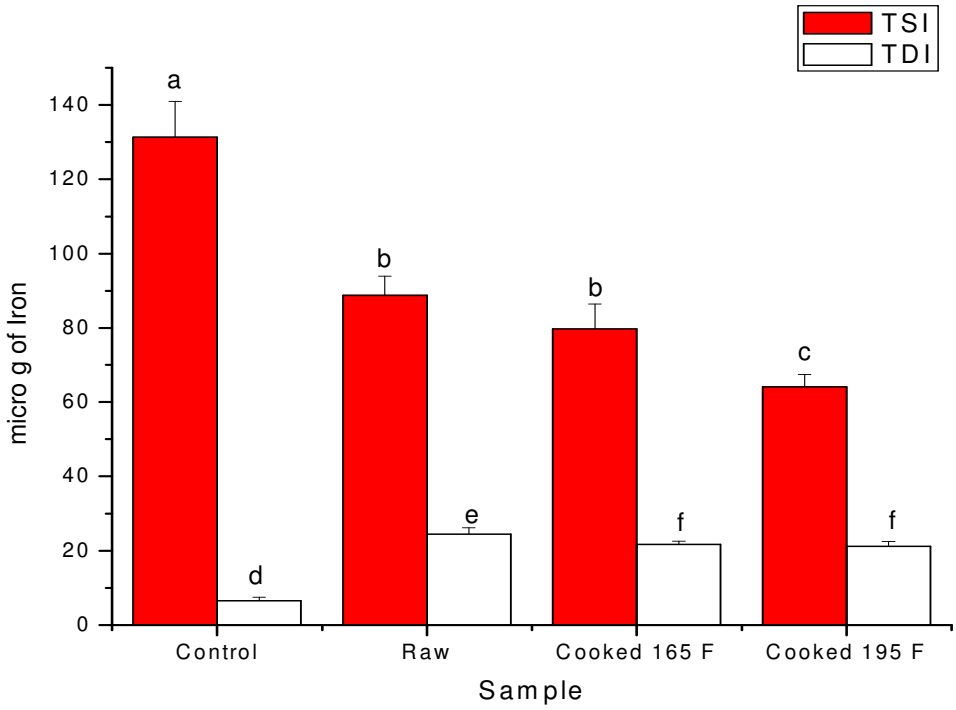

Figure VI.E.2: Production of total soluble iron and total dialyzable iron by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 2. TSI: Total Soluble Iron; TDI: Total Dialyzable Iron.

Figure 2, indicates the total soluble iron produced by digested control and lyophilized raw and cooked (165°F and 195°F) are significantly different ($p<0.05$). The total soluble iron is dropped by ~32% in case of lyophilized raw, ~40% in case of cooked (165°F) and ~50% in case of cooked (195°F) chicken muscle sample as compared to control. This can be attributed to the high molecular weight fractions from chicken muscle samples, which bind iron and then precipitated when TCA was added. This suggests that the fraction which was not digested completely (high molecular weight fraction) bound large amount of inorganic iron and it was precipitated by TCA. This

may be due to heat induced polymerization of proteins, which reduced the digestibility of chicken. The total dialyzable iron values for lyophilized raw and cooked (165^{o}F and 195^{o}F) are very distinct from control ($p<0.05$). The total dialyzable iron produced by uncooked chicken is slightly higher than cooked CMS. However, the total dialyzable iron produced by cooked 165^{o}F and cooked 195^{o}F CMS is not significantly distinct from each other. This suggested that heating chicken from 165 to 195^{o}F did not cause any further drop in total dialyzable iron, even though the total sulfhydryl and histidines dropped. Total dialyzable iron is further discussed in figure 3.

Table 3 gives the values for total dialyzable iron and dialyzable ferrous iron produced by no protein control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total dialyzable iron in μg	Dialyzable ferrous iron in μg
	Mean ± SD	
Control	6.5 ± 0.93 [a]	2.8 ± 0.34 [c]
Raw Chicken	24.5 ± 1.73 [b]	7.4 ± 1.38 [d]
Cooked (165°F) chicken	21.7 ± 0.90 [b]	7.2 ± 0.55 [d]
Cooked (195°F) chicken	21.1 ± 1.27 [b]	4.2 ± 1.50 [c]

Table VI.E.3: Total dialyzable iron and dialyzable ferrous iron values for digested control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Mean ± SD of total dialyzable [Fe (II) + Fe (III)] and dialyzable ferrous iron [Fe (II)] in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of sample has been subtracted from all the values.

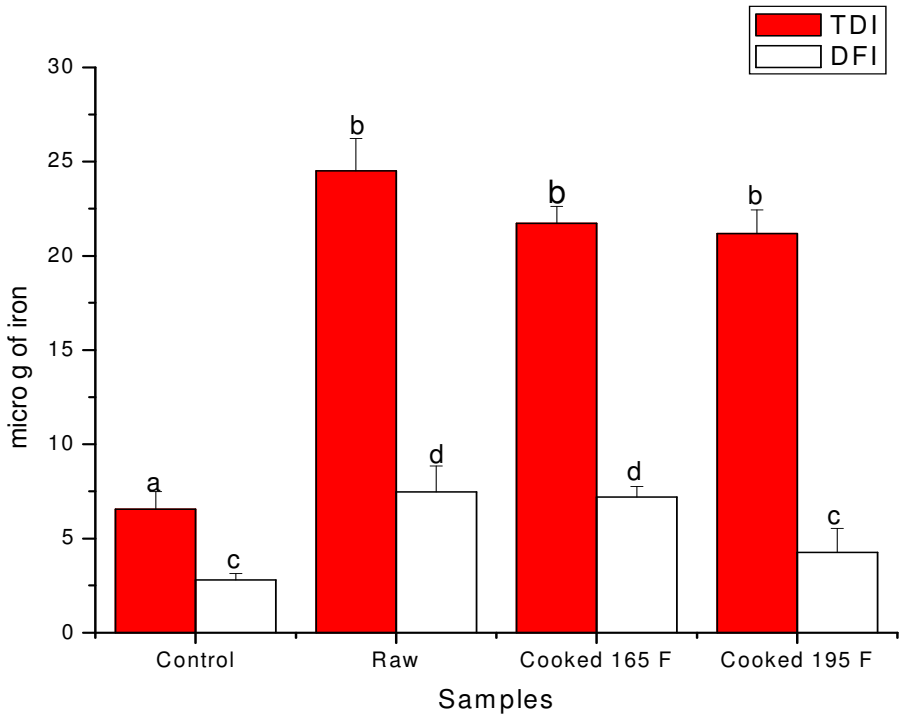

Figure VI.E.3: Production of total dialyzable iron and dialyzable ferrous iron by digested control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples. Graphical representation of the data from table 3. TDI: Total Dialyzable Iron; DFI: Dialyzable ferrous Iron.

Figure 3, indicates the comparison between total dialyzable iron and dialyzable ferrous iron produced by digested chicken muscle samples. Total dialyzable iron is ~4 times higher for lyophilized raw chicken muscle samples and ~3 times higher for lyophilized cooked chicken muscle samples (165^{o}F and 195^{o}F). This proves the previous findings that chicken muscle plays important role in production of potentially bioavailable forms of iron (Hallberg, 1981; Berner and Miller, 1985; Carpenter and Mahoney 1992; Seth and Mahoney, 2000). The total dialyzable iron values for lyophilized cooked (165^{o}F and 195^{o}F) chicken muscle samples (CMS) are not distinct from lyophilized raw CMS ($p<0.05$), which indicates that heating does not

affects chicken muscle's ability to produce potentially bioavailable forms of iron and the factor(s) responsible for producing potentially bioavailable forms of iron is not heat labile. Total dialyzable iron values for lyophilized cooked 165^{o}F sample and lyophilized cooked 195^{o}F sample are not distinct ($p>0.05$) from each other. This suggests that the factor(s) responsible for producing potentially bioavailable forms of iron was degraded at 165^{o}F, but heating it further to 195^{o}F has no effect on these factor(s). The findings for cooked samples are in consistent with those obtained by Baech et al. (Baech et al. 2002), which suggested that increasing cooking temperature of ham does not have any effect on iron bioavailability *in-vivo*. But the values for total dialyzable iron for cooked samples are ~3 times higher than that of no protein control, which implies that the factor(s) responsible for producing potentially bioavailable forms of iron was affected slightly. Nearly 30% of the total dialyzable iron is ferrous in case of lyophilized raw and cooked (165^{o}F) CMS and nearly 20% of total dialyzable iron is in ferrous form for cooked 195^{o}F CMS. This indicates that only one third of the total dialyzable iron was reduced to ferrous form, which is the most potentially bioavailable form of iron and two third remained ferric. The marked decrease in dialyzable ferrous iron for 195^{o}F cooked sample may be correlated with the ~55% drop in sulfhydryl content.

Overall, these results show that cooking does not have any prominent effect on production of dialyzable iron species, except for dialyzable ferrous iron at 195^{o}F, despite the drop in –SH and histidine content.

Table 4 gives the values for total ferrous iron and dialyzable ferrous iron produced by no protein control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total ferrous iron in µg	Dialyzable ferrous iron in µg
	Mean ± SD	
Control	14.7 ± 0.85 [a]	2.8 ± 0.34 [c]
Raw Chicken	23.2 ± 1.92 [b]	7.4 ± 1.38 [d]
Cooked (165°F) chicken	22.5 ± 1.54 [b]	7.2 ± 0.55 [d]
Cooked (195°F) chicken	20.7 ± 3.76 [b]	4.2 ± 1.50 [c]

Table VI.E.4: Total ferrous iron and dialyzable ferrous iron values for digested control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Mean ± SD of total ferrous [Fe (II)] and dialyzable ferrous iron [Fe (II)] in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of sample has been subtracted from all the values.

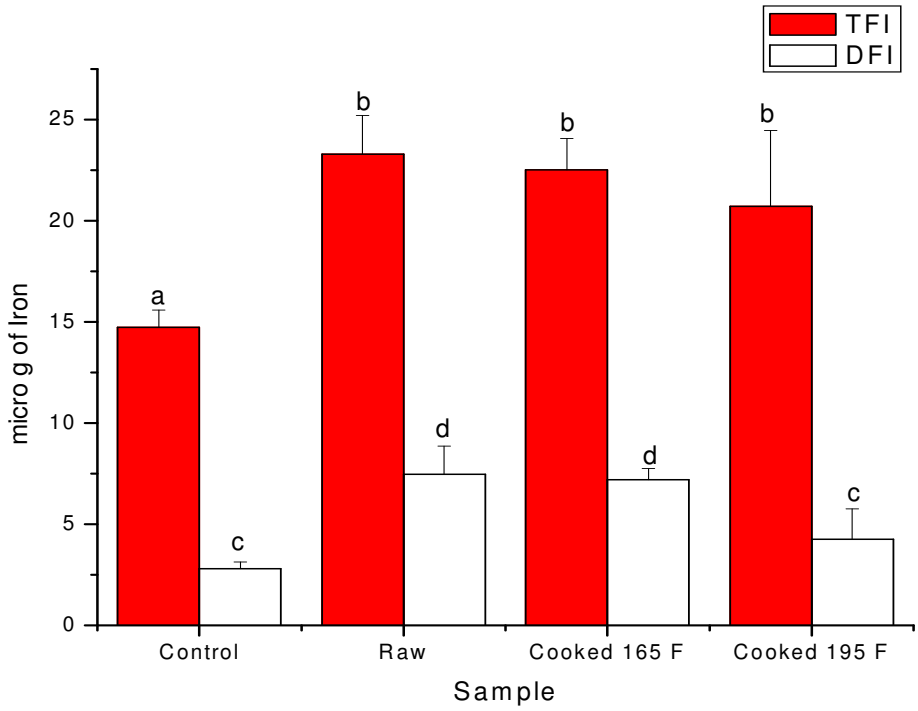

Figure VI.E.4: Production of total ferrous iron and dialyzable ferrous iron by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Graphical representation of the data from table 4. TFI: Total Ferrous Iron; DFI: Dialyzable ferrous Iron.

Figure 4, shows that the total ferrous iron values obtained for digested lyophilized raw and cooked samples (165°F and 195°F) are distinct from control ($p<0.05$). The total ferrous iron values for chicken muscle samples are ~1.5 times higher than no protein control, which suggests that chicken muscle produced higher amounts of total ferrous iron as compared to no protein control. The total ferrous iron values for lyophilized raw and cooked samples (165°F and 195°F) are not distinctly different from each other when analyzed using one way ANOVA ($p>0.05$). This implies that the factor(s) responsible for producing total ferrous iron is not destroyed by cooking. The values for dialyzable ferrous iron for lyophilized raw and cooked (165°F) are distinct from

no protein control ($p<0.05$), but for cooked (195^{o}F) it is not distinct from control ($p>0.05$). Lyophilized raw and cooked (165^{o}F) sample produced ~2.5 times higher values for most potentially bioavailable form of iron i.e. dialyzable ferrous iron as compared to no-protein control. This suggests that the factor(s) responsible for producing the most potentially bioavailable form of iron and carry it through 6000-8000 Da dialysis membrane was not destroyed after heating the chicken muscle sample at 165^{o}F. The dialyzable ferrous iron value for cooked (195^{o}F) was much lower than lyophilized raw and cooked (165^{o}F) CMS, and slightly higher than no protein control but not significantly different. This implies that the factor(s) responsible for producing most potentially bioavailable form of iron is also heat labile and destroyed completely at 195^{o}F. The major component suggested to produce ferrous iron in meat is sulfhydryl (Taylor et al. 1986; Kirwan et al. 1993; Mulvihill and Morrissey, 1998; Mulvihill et al. 1998) and it is well know that sulfhydryl is heat labile. So the drop in dialyzable ferrous iron could be attributed to the loss of sulfhydryls during heating the chicken muscle sample.

Table 5 gives the values for total dialyzable iron for control, lyophilized raw and cooked (165^{o}F and 195^{o}F) samples for iron only, iron pepsin and iron pepsin pancreatin bile treatments.

Sample	Iron Only (No Enzymes)	Iron/Pep (No PB)	Iron/Pep/PB
	Mean ± SD Total Dialyzable Iron [Fe(II) + Fe (III)] µg		
Control (No Sample))	2.9 ± 0.44 [a]	3.4 ± 0.52 [a]	6.0 ± 0.70 [c]
Raw Sample	8.4 ± 0.49 [b]	19.4 ± 0.45 [d]	24.5 ± 1.73 [f]
Cooked 165°F Sample	6.0 ± 0.70 [c]	14.6 ± 0.75 [e]	21.7 ± 0.90 [f]
Cooked 195°F Sample	4.0± 0.95 [a]	15.5 ± 0.79 [e]	21.1 ± 1.27 [f]

Table VI.E.5: Total dialyzable iron produced by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples for Iron only (no enzymes), iron pepsin (no pancreatin) and iron/pep/PB treatments. Mean ± SD of total dialyzable [Fe (II) + Fe (III)] in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of sample has been subtracted from all the values. Pep: Pepsin; PB: Pancreatin Bile.

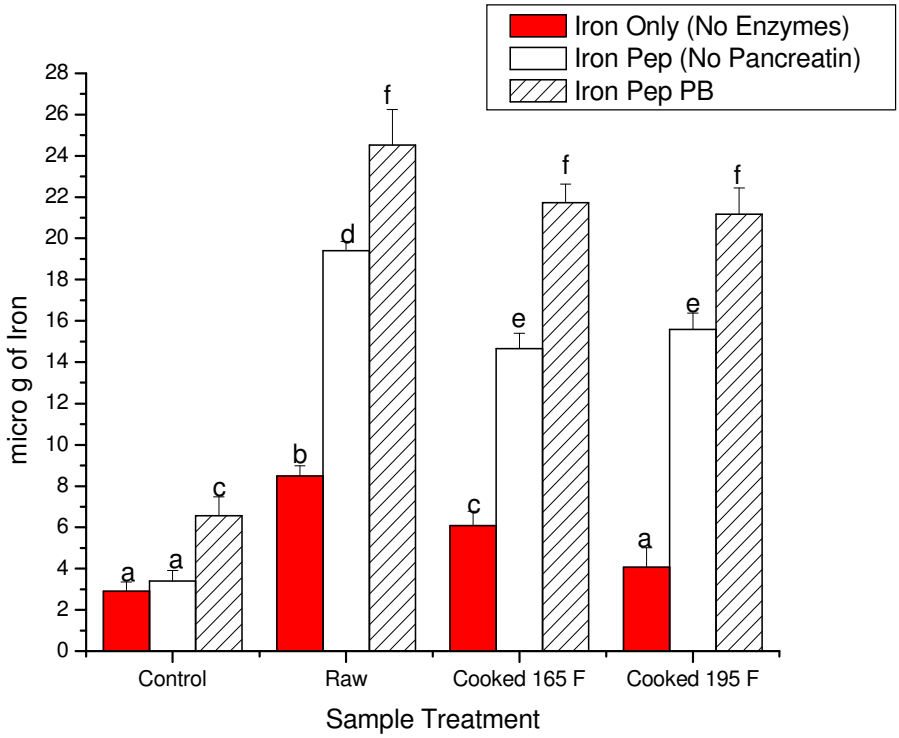

Figure VI.E.5: Production of total dialyzable iron by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples for iron only (no enzymes), iron-pep (no pancreatin) and iron pepsin pancreatin bile. Graphical representation of the data from table 5.

Figure 5, indicates the effect of iron only (no enzymes), pepsin (no pancreatin) and pepsin-pancreatin/bile digestion by lyophilized raw and cooked (165°F and 195°F) CMS on production of total dialyzable iron and the values are significantly distinct from each other ($p<0.05$). The iron only (no enzymes) treatment values for total dialyzable iron produced by lyophilized raw and cooked (165°F and 195°F) chicken muscle samples are slightly higher than control, which suggests that chicken muscle does not need digestion to produce potentially bioavailable forms of iron. The values obtained for total dialyzable iron for lyophilized raw and cooked (165°F and 195°F) CMS show ~2.5 and ~3 times increase when pepsin (no pancreatin) and pepsin-

pancreatin/bile were used for the digestion respectively, as compared with iron only (no enzymes). This shows that chicken muscle do not need digestion to enhance iron bioavailability but this also suggests that digestion further enhances production of potentially bioavailable forms of iron by ~3 times. This result is in contradiction with that obtained by Huh et al., which suggested that digestion is not necessary to produce potentially bioavailable forms of iron in heated lyophilized fish muscle (Huh et al. 2005).

Table 6 gives the values for dialyzable ferrous iron for lyophilized raw and cooked (165^{o}F and 195^{o}F) samples for iron only, iron pepsin and iron pepsin pancreatin bile treatments.

Sample	Iron Only (No Enzymes)	Iron/Pep (No PB)	Iron/Pep/PB
	Mean ± SD Dialyzable Ferrous Iron [Fe(II)] µg		
Control (No Sample))	2.0 ± 0.46 [a]	1.8 ± 0.50 [a]	2.8 ± 0.34 [a]
Raw Sample	3.8 ± 0.96 [b]	8.4 ± 0.32 [c]	7.4 ± 1.38 [c]
Cooked 165°F Sample	3.2 ± 0.42 [b]	6.4 ± 1.08 [c]	7.2 ± 0.55 [c]
Cooked 195°F Sample	2.6 ± 0.42 [a]	3.2 ± 0.60 [ab]	4.2 ± 1.50 [ab]

Table VI.E.6: Dialyzable ferrous iron produced by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples for Iron only (no enzymes), iron pepsin (no pancreatin) and iron/pep/PB. Mean ± SD of total dialyzable [Fe (II) + Fe (III)] in µg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 µg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of sample has been subtracted from all the values. Pep: Pepsin; PB: Pancreatin Bile.

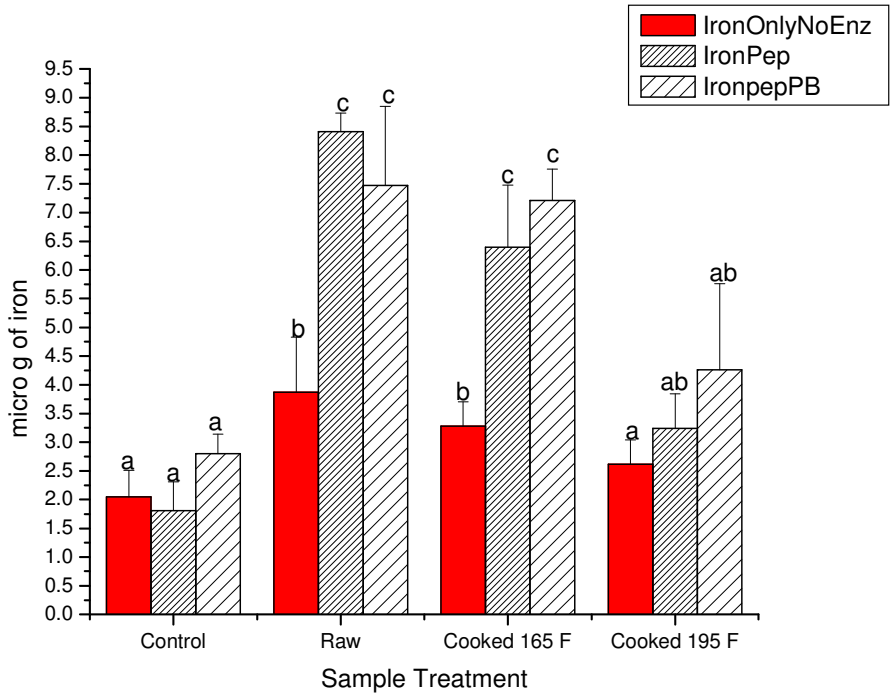

Figure VI.E.6: Production of dialyzable ferrous iron by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples for iron only (no enzymes), iron-pep (no pancreatin) and iron pepsin pancreatin bile. Graphical representation of the data from table 6.

The effect of iron only (no enzymes), pepsin (no pancreatin) and pepsin-pancreatin/bile digestion treatments on production of dialyzable ferrous iron by lyophilized raw and cooked (165°F and 195°F) CMS is shown in figure 6. There was no difference on dialyzable ferrous iron produced by lyophilized raw and cooked 165°F CMS for iron only treatment, but these values were ~1.5 times higher than control. There was no significant difference between control and lyophilized cooked 195°F CMS, suggesting that the factor responsible for production of dialyzable ferrous iron for iron only treatment was destroyed completely at 195°F. The

dialyzable ferrous iron produced by raw chicken was five times higher than that of control for iron pepsin treatment but heating chicken to 165^{o}F did not cause any drop. However when the chicken was heated to 195^{o}F, a significant change was observed, suggesting that the factor(s) responsible for production of dialyzable ferrous iron was destroyed completely at 195^{o}F, as the value is equal control. Also, these results indicated that digestion is not necessary for production of dialyzable ferrous iron but it helps to increase the amount of dialyzable ferrous iron significantly.

Table 7 gives the values for total dialyzable iron and dialyzable ferrous iron produced because of digestion (non-digested iron values are subtracted from the digested iron values for the corresponding sample) by control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total dialyzable iron in μg	Dialyzable ferrous iron in μg
	Mean ± SD	
Control	3.6 ± 0.93 [a]	0.7 ± 0.34 [c]
Raw Chicken	16.0 ± 1.73 [b]	3.6 ± 1.38 [d]
Cooked (165°F) chicken	15.6 ± 0.90 [b]	3.9 ± 0.55 [d]
Cooked (195°F) chicken	17.1 ± 1.27 [b]	1.6 ± 1.50 [d]

Table VI.E.7: Total dialyzable iron and dialyzable ferrous iron produced because of digestion (digested iron – non-digested iron values) by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Mean ± SD of total dialyzable [Fe (II) + Fe (III)] and dialyzable ferrous iron [Fe (II)] in μg for total 6 pancreatin digestions (n = 6). The total amount of iron used for each pancreatin digestion is 279 μg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence. Endogenous iron produced by the same amount of sample has been subtracted from all the values.

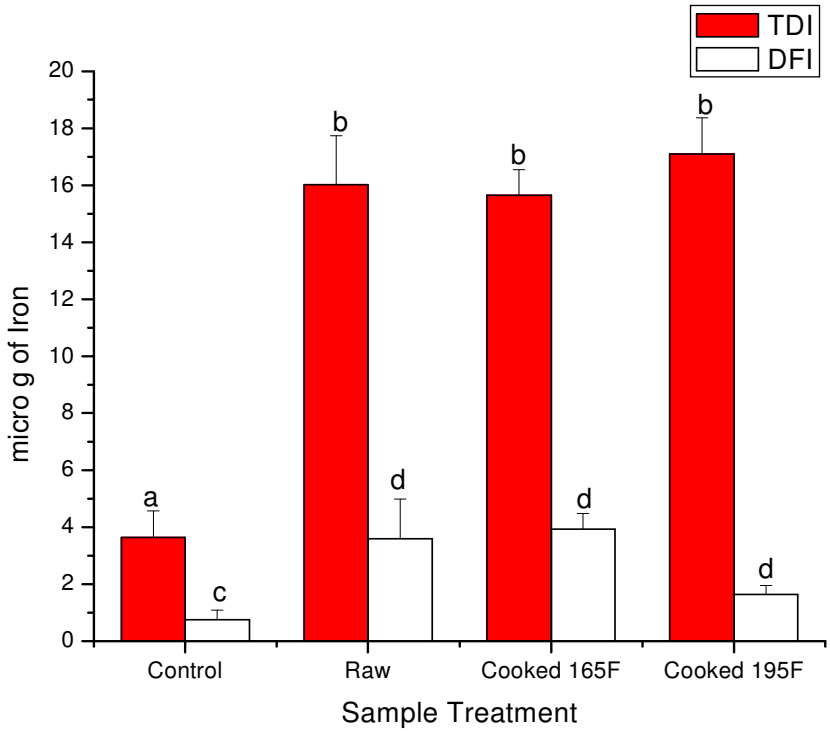

Figure VI.E.7: Production of dialyzable ferrous iron by control, lyophilized raw and cooked (165°F and 195°F) chicken muscle samples for iron only (no enzymes), iron-pep (no pancreatin) and iron pepsin pancreatin bile. Graphical representation of the data from table 7.

The total dialyzable iron and dialyzable ferrous iron produced because of digestion (non-digested iron values are subtracted from digested iron values for the corresponding sample) is shown in fig 7. The total dialyzable iron produced because of digestion for raw and cooked chicken sample is ~5 times greater than that of control. Also, the dialyzable ferrous iron produced because of digestion for raw and cooked 165°F chicken sample is ~4 times higher than control, whereas for 195°F sample, it is two times as much as that of control.

These results clearly showed that majority of total dialyzable iron are produced during digestion. So digestion is very critical for production of potentially bioavailable forms of iron. Also, it can be seen that, there is no effect of cooking on production of total

dialyzable iron. As far as the dialyzable ferrous iron is concerned, there is not significant difference for raw and cooked sample and heating does not have any effect on it.

It can be concluded from these results that digestion is very necessary for production of potentially bioavailable forms of iron and heating does not have any effect on ability of chicken to produce potentially bioavailable forms of iron.

Table 8 gives the values for total soluble protein and total dialyzable protein produced by lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples.

Sample	Total soluble protein in mg	Total dialyzable protein in mg
	Mean ± SD	
Raw Chicken	320.5 ± 4.11 [a]	223.4 ± 12.48 [d]
Cooked (165°F) chicken	362.6 ± 10.92 [b]	260.7 ± 16.3 [e]
Cooked (195°F) chicken	385.4 ± 9.63 [c]	293.4 ± 14.75 [f]

Table VI.E.8: Total soluble protein and total dialyzable protein values for lyophilized raw and cooked (165°F and 195°F) chicken muscle samples. Mean ± SD of total soluble protein and total dialyzable protein in mg for total 6 pancreatin digestions (n = 6). The total amount of protein used for each pancreatin digestion is 400 mg. Different subscripts indicates difference using One Way ANOVA test for $\alpha = 0.05$ or 95% confidence.

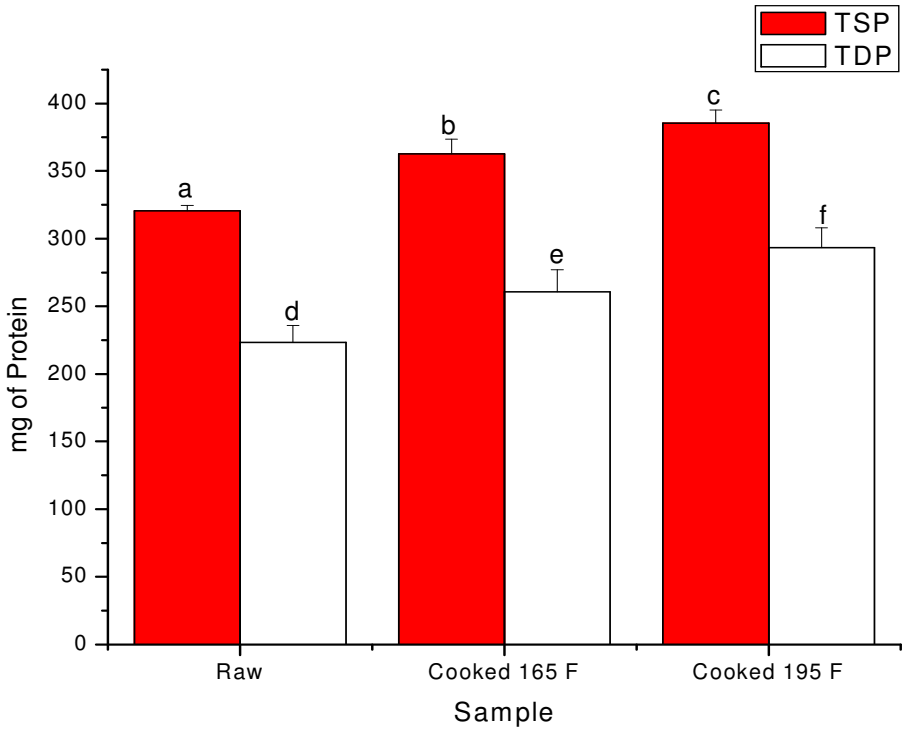

Figure VI.E.8: Production of total soluble protein and total dialyzable protein because of digestion (digested iron – non-digested iron values) by control, lyophilized raw and cooked (165^{o}F and 195^{o}F) chicken muscle samples. Graphical representation of the data from table 8. TSP: Total Soluble Protein; TDP: Total Dialyzable Protein.

Figure 8, indicates that the total soluble protein and total dialyzable protein values for lyophilized raw and cooked (165^{o}F and 195^{o}F) is significantly different from each other ($p<0.05$). This suggests that as the temperature of heating increases, the digestibility of protein improves, may be because of denaturation of proteins, which helps for better digestion. This also implies that the digestion process was adequate and is not the component of variation responsible for production of different amount of dialyzable and soluble iron.

VI.F. Conclusions:

After this study, we came to following conclusions:

- Sulfhydryl and histidines are very heat labile and drops significantly after cooking at 195^{o}F

- The total dialyzable iron was not affected after cooking the sample at 165^{o}F, even though there was significant drop in sulfhydryl and histidine content.

- Dialyzable ferrous iron dropped to the level which is not significantly different from control after heating the sample to 195^{o}F, which is well correlated with the drop in total sulfhydryl content.

- Chicken muscle does not need digestion to produce potentially bioavailable forms of iron, but digestion further enhances its production.

APPENDIX A:

STANDARD CURVE FOR PROTEIN

Obs. No.	BSA (mg)	Abs at 542nm		Final Abs
		I	II	
1	0.0	0.053	0.052	0.00
2	1.0	0.107	0.104	0.052
3	2.0	0.162	0.164	0.110
4	4.0	0.268	0.268	0.216
5	6.0	0.370	0.370	0.318
6	8.0	0.475	0.474	0.422
7	10.0	0.560	0.560	0.508

Table A.1: Observations for standard protein (BSA) sample.

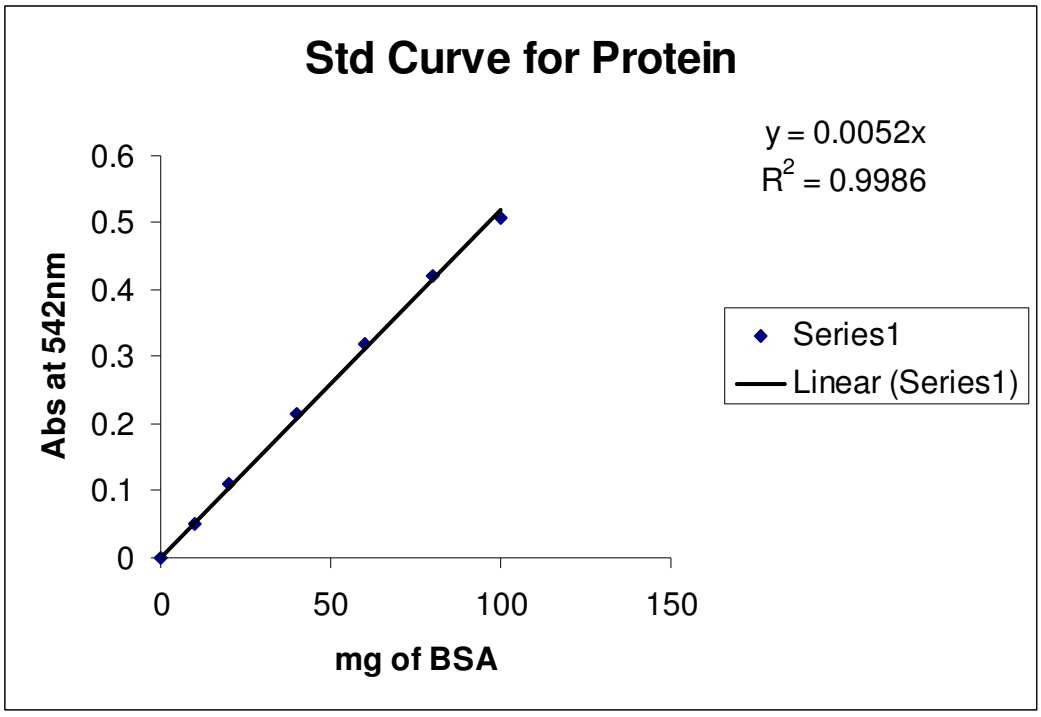

Fig A.1. Protein standard graph. Standard curve using bovine serum albumin as a standard protein.

APPENDIX B:

STANDARD CURVE FOR Fe

Obs No.	Fe Conc. (ug)	Abs at 562nm			Final Abs.
		I	II	III	
1	0.0	0.037	0.032	0.037	0.000
2	1.0	0.205	0.206	0.203	0.168
3	2.0	0.365	0.368	0.395	0.328
4	3.0	0.544	0.547	0.546	0.504
5	4.0	0.700	0.707	0.692	0.670
6	5.0	0.853	0.830	0.860	0.823

Table B.1: Observations for standard iron sample.

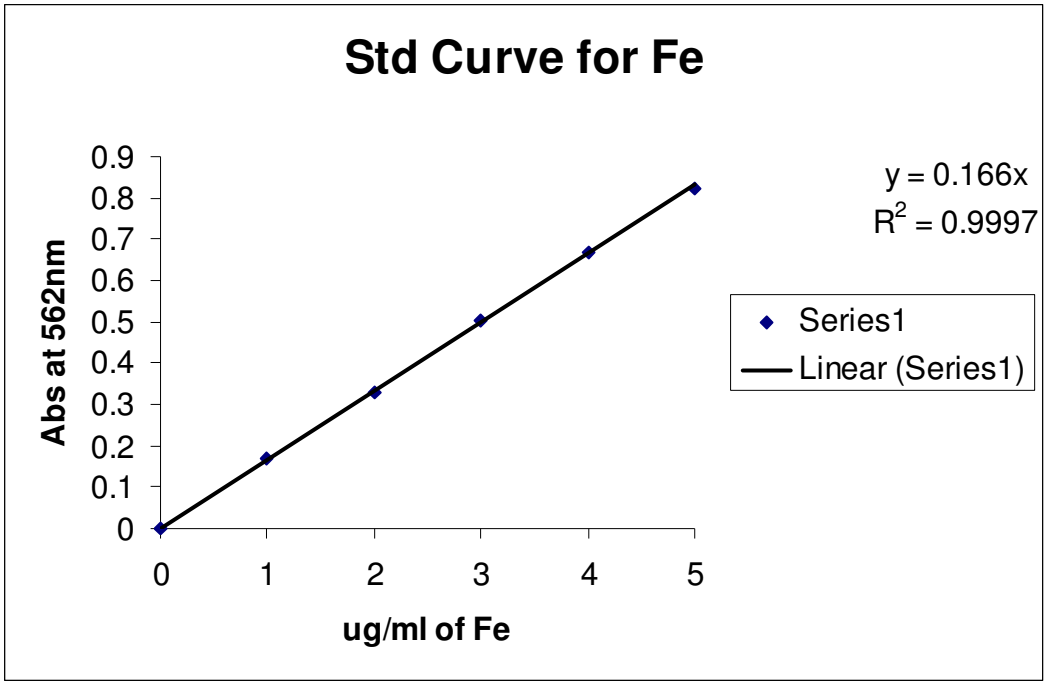

Fig B.1. Iron standard graph. Standard graph for iron using a standard iron solution.

APPENDIX C.

FLOW DIAGRAM FOR THE DIGESTION PROTOCOL

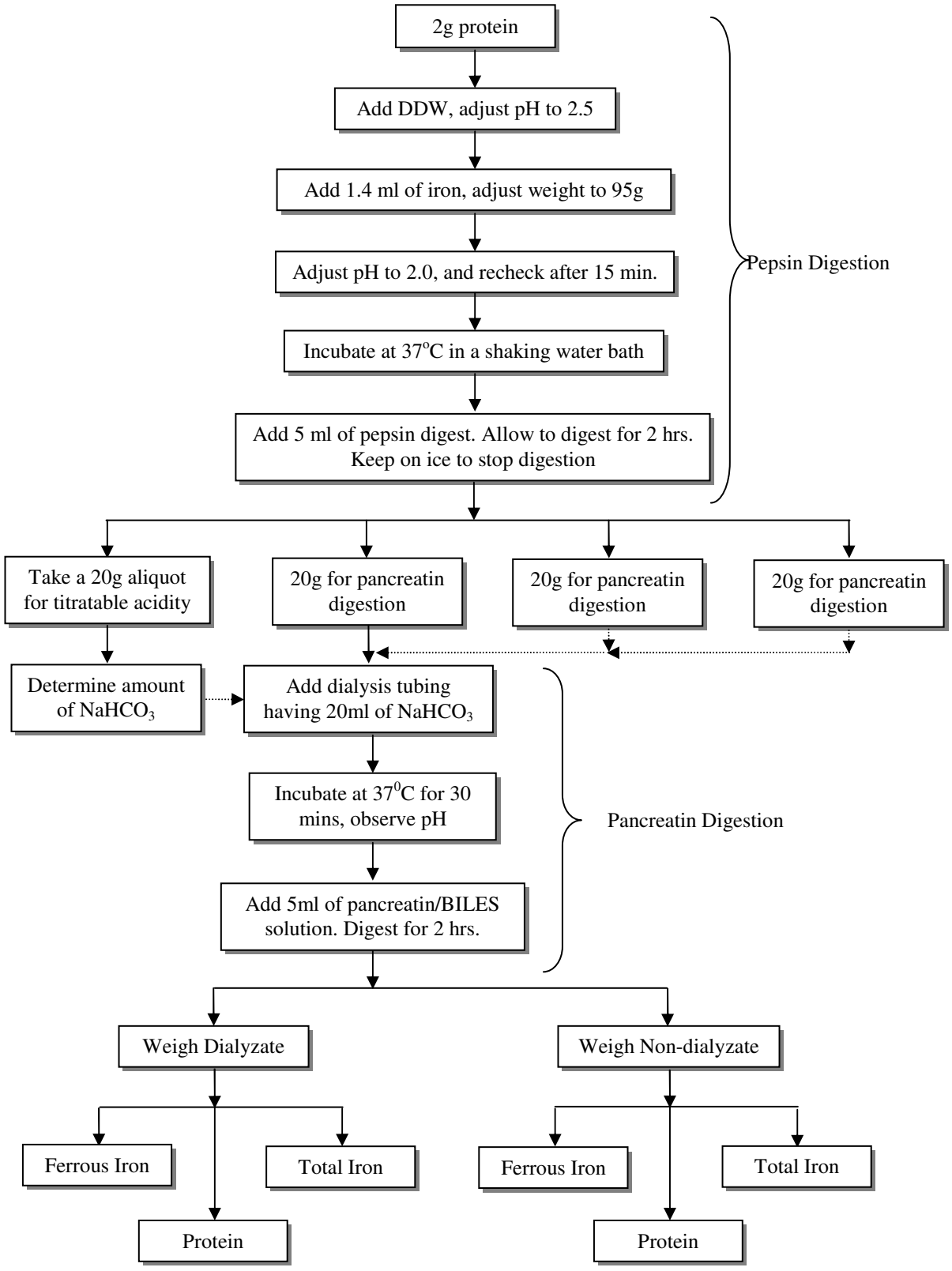

Fig C.1: Flow diagram for digestion protocol.

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