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# The Effect of Cooking on Formation of Bioavailable Species of Iron from Chicken Breast Muscle

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**THE EFFECT OF COOKING ON FORMATION OF BIOAVAILABLE SPECIES OF  
IRON FROM CHICKEN BREAST MUSCLE**

A Thesis Presented

by

ADITYA S. GOKHALE

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

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Food Science

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## **ABSTRACT**

THE EFFECT OF COOKING ON FORMATION OF BIOAVAILABLE SPECIES OF IRON FROM  
CHICKEN BREAST MUSCLE

SEPTEMBER 2011

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Chicken breast muscle was cooked to an internal temperature of 165°F by four methods: boiling, baking, sautéing and deep-frying. All cooking methods led to a decrease in formation of dialyzable iron, formed by both extraction and digestion *in vitro*, compared to raw muscle. After cooking most of the dialyzable iron formed results from extraction and the formation of dialyzable iron by digestion is essentially eliminated. Cooking also decreased the levels of cysteine and histidine; these losses may contribute to the loss in dialyzable iron.

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## CHAPTER I

### INTRODUCTION

Iron is an important mineral required for functioning of cells. It plays a fundamental role in oxygen carrying proteins such as hemoglobin and myoglobin. From serving as a cofactor for the enzymes of catalase and peroxidase families to its role in DNA synthesis, iron is a critical factor for a healthy living.

Iron is present in heme and non-heme forms. The non-heme form is poorly bioavailable which is credited to its poor solubility. Most of the dietary iron is inorganic (non-heme) which is why it is important to study its bioavailability. Research has indicated that some food components like phytates, tannates, polyphenols inhibit iron absorption whereas meat promotes it.

Meat promotes iron bioavailability, which is an effect well known to researchers and is called the 'meat factor'. Several studies have suggested that the peptides formed by digestion of chicken muscle proteins could bind and/or reduce iron and keep it in a soluble state at the low intestinal pH, followed by its transfer to the mucosal receptors (Carpenter and Mahoney, 1992). Furthermore, digestion of chicken muscle enhances bioavailability of iron, some portion of iron is bioavailable even when there is no enzymatic digestion. It is believed that sulfhydryl groups and histidine contribute to the 'meat factor'. Levels of these amino acids drop on cooking at 195°F (Karava et al. 2008) which would have an impact on their contribution to formation of bioavailable forms of iron.

The impact of cooking before consumption of chicken on the non-heme iron bioavailability has not been paid attention to. The 'meat factor' would undergo a change upon various types of heat treatments to chicken such as boiling, baking, sautéing and deep frying etc. which are some of the ways in which chicken is cooked before consumption. Thus it is important to study the effects of various cooking methods on the iron bioavailability of chicken.

Objectives of this project are:

- To study the effect of various cooking methods on protein digestibility and production of bioavailable forms of iron.
- To study the effect of various cooking methods on critical amino acids such as histidine and sulfhydryl groups which may be involved in promoting iron bioavailability.

## CHAPTER II

### LITERATURE REVIEW

#### II.A. Iron

Iron is a group 8 and period 4 element with atomic weight 55.845. Iron catalyzes a great number of biochemical reactions, many of which are related to the chemical nature of the element characterized by two principle oxidation states. It exists in divalent iron (Fe II ( $d^6$ )) and trivalent (Fe III ( $d^5$ )) states.

#### II.B. History of iron

The relation of iron to blood formation did not become apparent until the seventeenth 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 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 the body tissue and fluids

The functions of iron in body such as oxygen transport and storage are regulated by hemoglobin and myoglobin which have iron as their principle component. Cytochromes are heme-containing compounds that are critical to cellular energy

production and therefore life, through their roles in mitochondrial electron transport. They serve as electron carriers during the synthesis of ATP, the primary energy-storage compound in cells. Nonheme iron-containing enzymes, such as NADH dehydrogenase and succinate dehydrogenase, are also critical to energy metabolism. Iron functions as a cofactor in various enzymes of the catalase and peroxidase families. These enzymes play an important role in protection of cells from damage due to accumulation of hydrogen peroxide. Iron is involved in DNA syntheses. Ribonucleotide reductase is an iron-dependent enzyme that is required for DNA synthesis. Thus, iron is required for a number of vital functions including growth, reproduction, healing, and immune function.

The levels of iron in the cell must be delicately balanced, as iron loading leads to free radical damage. Iron loading implies excess of iron in the cell which gives rise to the Fenton reaction i.e. excess iron reacts with oxygen to generate hydroxyl radicals. To achieve appropriate levels of cellular iron and to avoid iron loading, transport, storage and regulatory proteins are evolved (Dunn et al. 2006).

#### II.D. Dietary sources of iron

The RDA for iron is males- 8mg/day, females- 18mg/day [Food and Nutrition Board of the National Academy of Sciences (2008)]. According to the National Health and Nutrition Examination Survey (NHANES) III, intakes for men generally exceed the RDA, whereas most women consume lower than the RDA. A key point to remember is that the RDA for premenopausal women is 10 milligrams higher than the RDA for men,

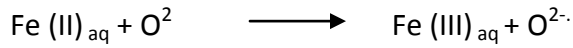
thus making it more challenging for women in this age group to consume an adequate amount of iron.

The overall iron intake from different diets varies with the proportion of iron-rich and iron-poor foods contained in the diet. It also depends on the degree to which the diet has been exposed to iron-rich contaminants. The average U.S. diet was reported to supply 14-20 mg iron per man. An Australian diet, which is typically high in meat, has been estimated to supply 14-20 mg iron. A typical poor India diet was shown to provide only 9 mg iron, whereas an 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%. 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. Iron from meats, poultry, and seafood is best absorbed by the body, only some of the iron content in plant-based foods is absorbed regardless of the amount of iron the food contains. 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 (Tseng et al, 1997). 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**

In water and in absence of oxygen, iron is present as the hexa-aqua Fe(II) ion. This ion is readily oxidized in the presence of oxygen to the hexa-aqua Fe(III) ion.



Except at very low pH values this ion undergoes hydrolytic polymerization reactions involving deprotonation to form hydroxo and oxo species leading progressively to more and more insoluble hydrated ferric oxides (Lippard, 1986).

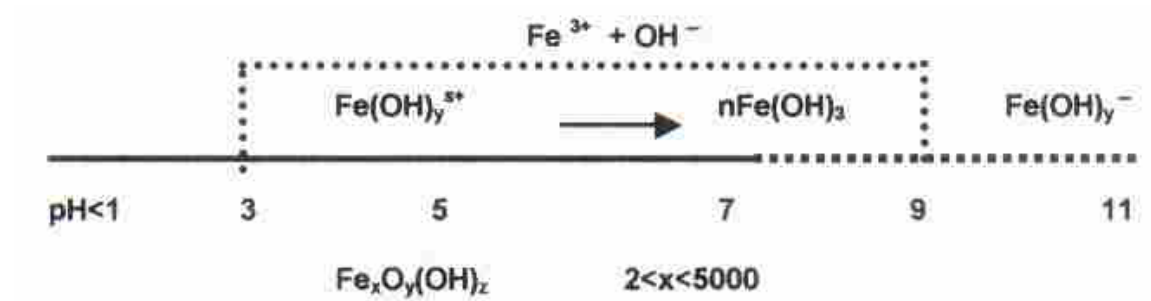


Figure I.1: Hydrolytic reaction of iron as function of pH. (Cremones et al., 2002)

As shown in Figure I.1, 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  $\text{O}^-$  and the  $(\text{OH})^-$  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 et al. 2002). In biological media at low oxygen tension,  $\text{Fe}(\text{OH}_2)_6^{2+}$  is the predominant species, while  $\text{Fe}(\text{OH}_2)_6^{3+}$  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 Fe(II) can be taken up more easily than Fe(III) by cell membrane as a consequences of more favorable solubility properties.



## II.F. Mechanism of iron absorption

In mammals, the majority of iron is present as hemoglobin in erythrocytes. Senescent erythrocytes are phagocytosed by macrophages and a significant portion of the iron is efficiently recycled. The daily loss of iron is then compensated for by the dietary iron.

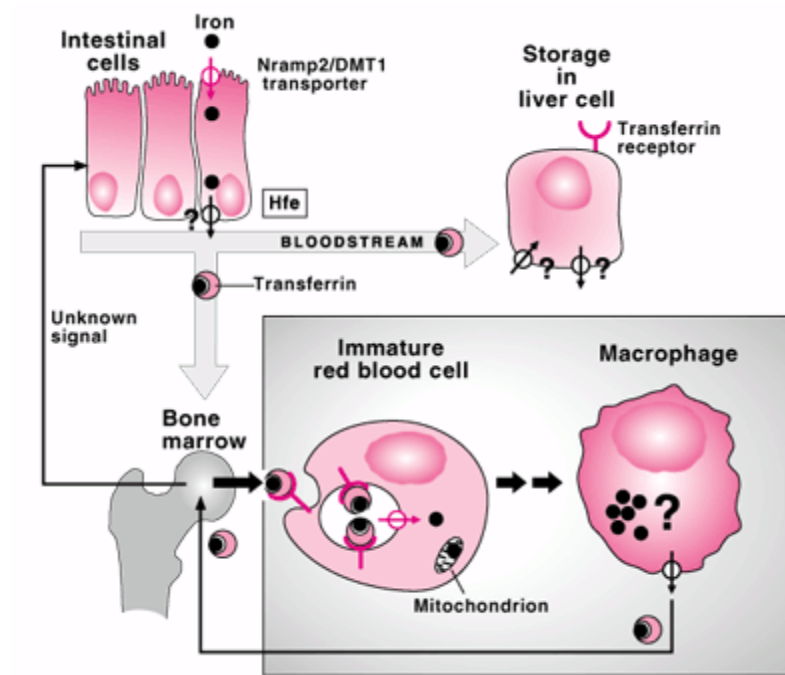


Figure I.2: Iron transport (Andrews, 1999).

Heme is a molecule that contains a protoporphyrin ring that binds iron. Heme results from the breakdown of hemoglobin and myoglobin found in meat products, and it is thought to be internalized through the recently identified receptor heme carrier protein-1 (HCP1).

Non-heme dietary iron is taken up in the enterocytes in the duodenum. Ferric iron needs to be reduced to the ferrous form before it is transported into the cell by divalent metal transporter-1 (DMT1) which also transports protons. A simple

diagrammatic transport mechanism is portrayed in Figure I.2. Animal models have shown that DMT1 is required for intestinal uptake of inorganic sources of dietary iron. Ferroportin facilitates the transport of iron across the basolateral membrane and passage through the intestinal space and capillary wall.

Figure I.3 shows the balance of iron in man.

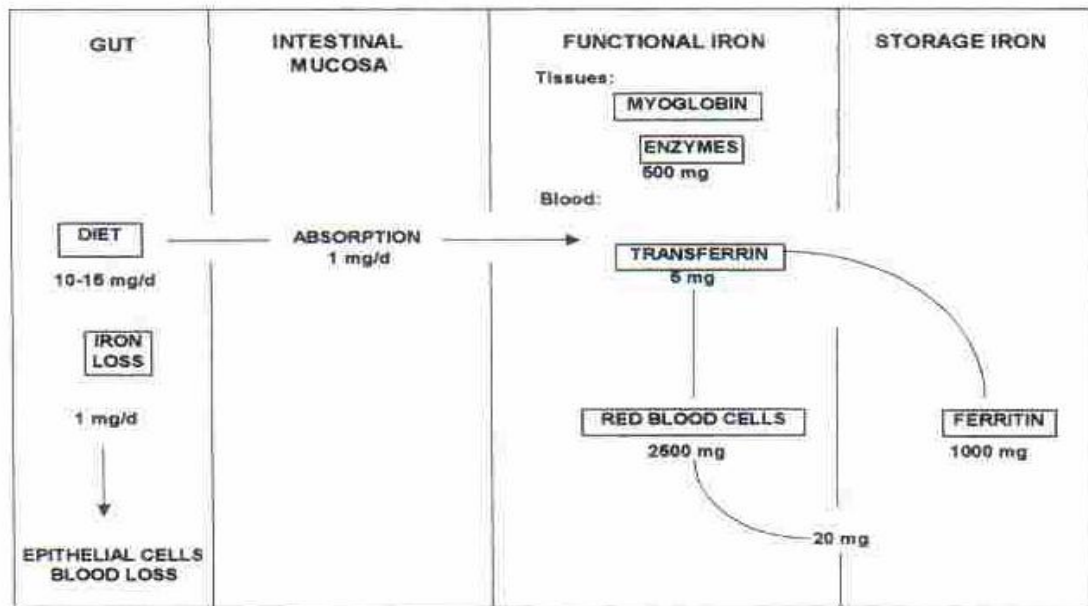


Figure I.3: Iron balance in man (Cremones et al. 2002)

Recently identified proteins for iron transport are as follows:

- Divalent metal transporter-1 (DMT-1): a ferrous iron transporter that absorbs dietary iron at the apical surface of the enterocyte and facilitates iron egress from endosomal vesicles.
- Ferroportin-1 (FPN-1): a transporter responsible for iron export from the basolateral membrane of enterocytes and the release of iron from hepatocytes and macrophages.
- Heme carrier protein-1 (HCP-1): a heme receptor that binds dietary heme on the apical surface of enterocytes and internalizes it.
- Duodenal cytochrome-b (Dcytb): a potential ferrireductase enzyme present on the apical surface of enterocytes that can reduce ferric iron to ferrous iron for absorption.
- Feline leukemic virus, sub-group C receptor (FLVCR): believed to export excess heme from developing erythrocytes and other cell types.
- ATP-binding cassette sub-family G member 2 (ABCG2): a breast cancer drug resistance protein that can protect cells from hypoxic conditions by preventing protoporphyrin IX accumulation.
- Mitoferrin: a mitochondrial iron transporter that could be responsible for the transport of iron into the mitochondrion.
- Sec15l1: a protein involved in the mammalian exocyst complex and suggested to be involved in the cycling of transferrin-containing endosomes and vesicle docking.

- Six-transmembrane epithelial antigen of the prostate-3 (Steap3): an endosomal ferrireductase responsible for transferrin-dependent iron uptake in erythroid cells.
- ABC-mitochondrial erythroid (ABC-me): an inner mitochondrial membrane transporter involved in heme biosynthesis in erythroid cells.
- ABCB7: a membrane transporter essential for [Fe-S] cluster transport in the mitochondria (Dunn et al. 2006).

## **II.G. The concept of iron bioavailability**

“Bioavailability is key to nutrient effectiveness” (Blenford, 1995). Out of the total amount of the iron in a food only a portion is available to the body through absorption – only a certain amount is bioavailable. The term bioavailability was introduced to better distinguish between the chemical availability and the availability in the bioassays. The bioavailability of iron can be subdivided in three constituent phases viz. availability in the intestinal lumen for absorption, absorption and/or retention in the body and utilization by the body. Food processing can directly affect only the first phase.

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 (Hallberg, 1981). Balance studies represent the difference between intake and excretion. The primary advantages of chemical balance methods are that they do not expose subjects to ionizing radiation and are simple in concept. They are still useful in situations where radioisotope facilities are not available or

exposure to ionizing radiation is not advisable. Although simple in concept, in practice, balance studies require great care if valid results are to be obtained. Errors in determination of either intake or excretion can result in significant errors in absorption estimates. Hegsted (1973) suggested that intake is often overestimated (incomplete consumption) and excretion is often underestimated (incomplete fecal and urine collections) (Darrel *et al.* 1998). 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. The method works by labeling heme and nonheme iron with two radioiron tracers (as biosynthetically labeled hemoglobin, and as an inorganic iron salt). In all meals over several days the two kinds of iron are each labeled with regard to a uniform specific activity. New information can thus be obtained about, for instance, the average bioavailability of dietary iron in different types of diets, the overall effects of certain factors (e.g. Calcium) on iron nutrition, and regulation of iron absorption in relation to iron status. These differences in the bioavailability are apparently related to differences in solubility and dissociation of chemically uncharacterized iron compounds in foods (Garrow *et al.* 2000).

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 findings the concept of common non-heme iron pool 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.H. Methods to determine iron bioavailability**

### **II.H.1. In-vitro method**

In vitro dialyzability methods involve a two-step digestion process simulating the gastric and intestinal phase, and dialysis through a semi-permeable membrane with a selected molecular weight cut-off. Dialyzable iron is used to account for bioavailable form of iron. Final pH adjustment and use of a strict time schedule are critical factors for standardization of this method. In vitro bioavailability methods correlate in most cases with human absorption studies in ranking iron and zinc availability from different meals. Exceptions may be that effects of milk, certain proteins, tea, and organic acids cannot be predicted. The bioavailability methods exclude iron bound to large molecules, which in some cases is available, for instance the ferritin-bound iron which is readily absorbed but is too large to pass through the dialysis membrane; and include iron bound to small molecules, which is not always available: for instance egg protein which forms soluble complexes with iron and yet inhibits absorption (Sandberg, 2005). *In vitro*

solubility/dialyzability methods, nonetheless, correlate in most cases with human studies and can therefore be effective tools to understand factors that may affect subsequent iron absorption.

#### II.H.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 molecular weight cut-off (MWCO) and placed in the digestion sample. Once the digestion is over the uptake of iron by Caco-2 cells is determined and accounted for bioavailable forms of iron. The usefulness of Caco-2 cell method lies in its capability in assessing cellular iron absorption and the feasibility of this cell model in studying iron bioavailability from various food combinations, otherwise not easily performed in humans (Au and Reddy, 2000).

This method is cost effective as compared to the in vivo method discussed later and is also less time consuming.

#### II.H.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 ( $^{59}\text{Fe}$ ), 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  $^{59}\text{Fe}$  at regular intervals. The blood and fecal samples are collected at regular intervals and analyzed for radio labeled iron. At the end of the study the data are analyzed and iron bioavailability is determined 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. Cost and time factors are some of the disadvantages of the in vivo method.

## **II.I. Dietary factors influencing the bioavailability of non-heme iron**

Non-heme iron absorption is conditioned by a multitude of factors, such as the chemical form in which it is present in the food (in the ferrous state it is absorbed much better than in the ferric state), the iron content of the food, as well as by the presence of certain enhancers and inhibitors. Non-heme iron absorption will be conditioned by its solubility in intestinal lumen so all those substances that increase this solubility would be considered as enhancers whereas compounds that diminish solubility of this element would be considered as inhibitors.

### **II.I.1. Organic acids and Vitamins**

Ascorbic acid promotes iron absorption due to its ability to reduce ferric iron to the ferrous form (Van Dyck et al., 1996; Davidsson et al., 1998; Fidler et al., 2003). It can also counteract the inhibitory effect of phytic acid. Citric acid, on the other hand,



decreases the solubilization of iron. The combined effect of pH and organic acids on iron uptake by caco-2 cells was studied by Sandberg et al. (2003). The effect of five organic acids (tartaric, succinic, citric, oxalic, and propionic acid) on the absorption of Fe(II) and Fe(III) in Caco-2 cells and compared this with sample solutions without organic acids but set to equivalent pH by HCl. The results showed that the mechanisms behind the enhancing effect of organic acids differed for the two forms of iron. For ferric iron the organic acids promoted uptake both by chelation and by lowering the pH, whereas for ferrous iron the promoting effect was caused only by the lowered pH (Sandberg et al. 2003). Categorized as promoters, the action mechanism of vitamin A and beta carotene to enhance iron absorption is not clear, it is believed that these compounds could act as chelating agents, keeping iron soluble and preventing its capture by polyphenols and phytates (Lopez and Martos, 2004).

#### II.1.2. Meat and fish (covered in II J)

#### II.1.3. 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. 1975a). 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. 1975b). Tannates are also present in coffee and it is possible that inhibiting effect of coffee is due to tannates.

#### II.1.4. Phytates, phosphates, fibers, carbohydrates and EDTA

Phytic acid is a strong chelator and hence it hampers iron absorption. Phytate rich foods such as wheat bran do not promote iron absorption unless the phytate is broken down (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).

Disodium EDTA is a chelating agent which significantly improved the bioavailability in corn-masa tortillas (Walter et al. 2003). EDTA, on the other hand, does not increase iron uptake or ferritin synthesis as studied by caco-2 cells (Garcia-Casal et al. 2004).

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 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). Fructo-oligosaccharides are natural diet components that escape hydrolysis by mammalian digestive enzymes, but are largely fermented by colonic bacteria to produce a wide variety of compounds that may affect the gut as well as systemic physiology in both humans and rats. It has been observed in rats that the presence of fructo-oligosaccharides in diets with phytic acid neutralized all the inhibitory effects on iron status associated with this acid (Lopez and Martos, 2004).

## **II.J. The concept of 'meat factor'**

The concept of 'meat factor' was established when Layrisse et al. (1969) showed that iron from different vegetable foodstuff was markedly increased when they were served with meat and fish. 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 evident 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 the meat factor and are summarized below.

### II.J.1. Sulfhydryls

Hamed et al. (1983) suggested that the sulphhydryl (-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 in-vitro bioavailability of non-heme iron and showed that the -SH content of meat plays important role in iron bioavailability (Mulvihill and Morrissey, 1998).

Taylor et al. (1986) 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).

Mulvihill et al.(1998) 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).

Seth et al. (2001) investigated the role of sulfhydryl groups in chelation and reduction of iron by chicken muscle proteins. It was found out that cysteine and

glutathione bound iron, where cysteine bound three times more iron than glutathione. The reactive sulfhydryl groups in cysteine and glutathione were lost which indicated that they were involved in iron binding and reduction (Seth et al. 2001).

### II.J.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.J.3. Carboxyls

A study by Shears et al. (1987) showed that the number of titratable carboxyl groups was decreased after a pepsin and pancreatin digestion in presence of iron. This suggested that there was some iron complexation occurring with the carboxyl groups. An in vitro pepsin/pancreatin digestion study conducted by Hurrel et al. (2007) on meat, revealed that one group of iron binding peptides was enriched in glutamic and aspartic acids and contained potential peptide fragments from myosin. These low molecular

peptides are supposed to be released during pepsin digestion and are responsible for iron solubility.

#### II.J.4. Carbohydrates

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.K. Effect of heating on meat

##### II.K.1. General effect of heating on the iron composition in meat

The effects of cooking methods (electric oven, grill, microwave, and boiling) on total and heme iron contents of anchovy were found to be statistically significant by Turhan et al. (2004). The highest total and heme iron losses were found in grilled samples (52.6%, 70.4%) and the lowest were found in boiled samples (11.2%, 30.4%).

Beef semitendinosus muscle was fast, dry-heated in a Silex clam cooker (Set at 200°C) for 5.6 to 8.6 minutes for final internal temperatures of 60 and 85°C, respectively by Purchas et al. (2004). It was found that the changes in the proportions of the soluble

and insoluble non-heme iron were small, but increases in the percentages of insoluble non-heme iron with increasing final temperatures were significant.

#### II.K.2. Effect of heating on bioavailability of iron

An *in vitro* study conducted by Kapsokefalou and Miller (1991), showed that broiling and microwave cooking of beef did not decrease its ability to produce dialyzable ferrous iron, as compared to raw beef. It was concluded that the 'meat factor' was not affected by cooking procedures.

Pork meat was cooked at 95 and 120°C by Baech et al. (2003). It was found that cooking did not impair nonheme iron absorption from a phytate-rich meal that was cooked at 70°C. Non-heme iron absorption tended to increase at highest cooking temperature (120°C) compared with lower (70 and 95°C) cooking temperatures. The cysteine content of the meat decreased with increased cooking temperature but was not correlated with the degree of non-heme iron absorption.

Karava et al. (2008) investigated the effect of heating chicken muscle (at 130°F, 150°F, 165°F and 195°F) on formation of bioavailable forms of iron. Chicken muscle slurry was cooked in a boiling water bath, cooled to room temperature, and then frozen/lyophilized. After thawing overnight these samples were subjected to pepsin and pancreatin digestion and analyzed for dialyzable iron and critical amino acid values. They found:

The effect of freezing and lyophilization of chicken muscle sample (CMS) was as follows

- Compared to unprocessed chicken muscle protein the dialyzable iron values obtained after processing of the sample were lower. The values dropped after subjecting the chicken muscle sample to freezing and they fell drastically further down after lyophilization.
- The same trend was followed by total dialyzable and dialyzable ferrous iron values. There was, however, a significant four times change in the dialyzable ferrous iron value of lyophilized CMS as compared with a frozen CMS. Similarly, the total dialyzable iron values also dropped by a factor of two.
- Frozen CMS did not have significantly different total soluble iron and total dialyzable iron as compared to raw. On the other hand Lyophilized samples suffered from a significant reduction in both total soluble and total dialyzable iron values.
- There was 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.
- There was no significant difference between the –SH and histidine content in refrigerated and frozen chicken muscle protein but there was a significant loss in the total sulfhydryl content of the chicken upon lyophilization while a 6% drop in the histidine levels upon lyophilization.



The following conclusions were reached:

- Freezing and refrigeration do not affect the chicken muscle's ability of producing potentially bioavailable forms of iron.
- Lyophilization causes a significant loss in the total –SH and histidine content, which may be due to polymerization of chicken muscle proteins during lyophilization.
- The decrease in production of potentially bioavailable iron is well correlated with the loss in –SH and histidine on lyophilization of chicken muscle.
- The protein digestibility was found to be reduced which could be possibly due to polymerization of chicken muscle proteins causing an impediment to proteolytic digestion.

The effect of heating on iron bioavailability of chicken muscle was as follows:

- The solubility of iron (total soluble iron) dropped as the temperature at which the CMS was heated increased.
- Heating chicken muscle protein decreased the values of total dialyzable iron and dialyzable ferrous iron. Dialyzable ferrous levels dropped to ~25% and ~90% of its original value of a raw-digested chicken muscle sample when heated at 130°F and 195°F respectively. The total ferrous iron values also dropped as the heating temperature was increased.
- The total soluble protein level in raw and heated samples was not significantly different. Total dialyzable protein produced by raw chicken muscle is higher than

heated 130°F, 150°F and 165°F and it is not significantly different from that of heated 195°F chicken sample.

- There was a significant drop in the sulfhydryl and histidine content when the CMS was subjected to heating. An increase in the heating temperature furthermore dropped the –SH and histidine values in the chicken muscle.

The following conclusions were reached:

- Heating causes decrease in production of dialyzable iron in chicken muscle samples.
- The total dialyzable iron levels drop on heating chicken muscle sample, which can be well correlated with the drop in –SH and histidine content on cooking CMS.
- The “meat factor” in chicken was heat labile and could be well correlated in the drop in –SH and histidine levels on heating chicken muscle samples.

Conclusions on effect of heating on iron bioavailability: The conclusion by Garcia et al. (1996) does not match the findings of Karava et al. (2008) which showed that heating impairs the ability of chicken to promote the production of bioavailable forms of iron. However, Karava et al. found out that heating caused a progressive decrease in the total sulfhydryl and histidine content of chicken muscle, which was well correlated to their findings of a drop in total dialyzable iron levels. The study conducted by Baech et al. (2004) was based on a phytate rich meal which in itself is an inhibitor of iron bioavailability. Also, cysteine content was found to be decreased on increasing heating

temperature of pork meat but was not correlated with the increase in non-heme iron absorption.

## **II.L. Objectives**

The following objectives were set.

- To study the effect of various cooking methods on protein digestibility and production of bioavailable forms of iron from chicken breast muscle.

Chicken is consumed only after heat processing it such as boiling, baking, sautéing and deep frying. In order to have a true estimate of the potential of the chicken to form bioavailable iron, it is important to analyze it after the respective cooking treatments. An effect of various chicken cooking methods on the bioavailability of iron has not been paid much attention to and thus it is one of the objectives of this study. The enhanced iron bioavailability of enzymatically digested chicken has been linked to formation of peptides. Thus, it is important to understand the effect of various cooking methods on the digestibility of chicken muscle proteins.

- To study the effect of various cooking methods on histidine and sulfhydryl groups in chicken breast muscle.

A loss in the levels of total histidine and sulfhydryl content of chicken was followed by a decline in its ability to form bioavailable forms of iron. There has been no research work done on the effect of heating on this phenomenon. Thus, another aspect

of this study would be to study the effect of the previously mentioned cooking methods of chicken on histidine and sulfhydryl groups, on the bioavailability of iron.

## CHAPTER III

### METHODS

#### III.A. Introduction

A standard *in vitro* model was used to investigate the effect of boiling, deep frying, sautéing and baking chicken breast. The production of dialyzable iron due to extraction or digestion of fresh chicken containing 2 g protein was used as a baseline to determine the effect of cooking on chicken breast muscle. Amount of sample used was determined by the protein content in it.

#### 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<sup>®</sup> 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 0.5 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  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 250 ml of DDW and 6.00g of sodium potassium tartrate ( $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ), 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 Brook, 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 epoxy 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.

Spice Grinder: Krups Coffee/Spice Grinder, Model GX4100

Deep fat fryer: Waring Pro, Model DF250B, Torrington, CT, USA.

### **III.D. Cooking procedures**

The cooking methods whose effects were investigated were boiling, deep frying, sautéing and baking. Chicken breast was obtained from a local supermarket. Size of the chicken breast portion used for all the cooking methods was ~100g and 7-8 cm in diameter. It was made sure that all portions were similar in size and all the blood, skin and fat were removed. Details of cooking procedures are as follows:

#### **III.D.1. Raw chicken**

Two portions of chicken breast were chopped with a knife as finely as possible. On an average the size of one small piece was 4mm x 4mm x 4mm. Chopping the chicken into smaller pieces was avoided to prevent formation of a paste. The chicken was then packed in airtight Ziploc© bags and stored at -40°C in the chest freezer.

#### **III.D.2. Boiling**

Two portions of chicken breast were placed in a non-stick pot with boiling water. A meat thermometer was used to monitor the internal temperature of the chicken.



Chicken was cooked till the internal temperature reached 165°F. The chicken was then cooled down in a refrigerator till the other samples were ready.

#### III.D.3. Deep Frying

Two chicken breast portions were used for deep frying. A deep fat fryer was used for this method. Canola oil bought from a local supermarket was preheated to 400°F in the fryer. Samples were fried until the internal temperature reached 165°F, monitored using a frying thermometer. Samples were taken out of the fryer prior to reading internal temperature. Excess oil was removed by gently patting the sample with paper towels. The chicken was then temporarily stored in the refrigerator for 1 hr. After weighing the samples the rest of the surface oil was removed using hexane. Samples were dipped in hexane for one minute and then followed by another half minute dip after taking a break for one minute between the two dips. They were blotted dry with paper towels. The samples were stored in the fridge until other samples were ready. It was assumed that any residual hexane volatilized during the grinding process.

#### III.D.4. Baking

The oven was preheated till 365°F. Two standard sample size portions of chicken breast were placed in a baking sheet in an oven rack. The chicken was baked till the internal temperature reached 165°F, which was monitored with the help of a meat thermometer in a timely manner. In order to ensure even baking the samples were flipped over after every span of ten minutes. Samples were cooled in the refrigerator.

### III.D.5. Sautéing

Acid washed stainless steel pan was preheated with three tablespoonfuls of Canola oil bought from a supermarket. The temperature of the oil was not known but exceeded 400°F. Two standard sample size chicken portions were sautéed, while periodically flipping them over, until the internal temperature of the chicken reached 165°F. The chicken was then brought to room temperature for further processing. Excess oil was removed by gently patting the sample with paper towels. The chicken was then temporarily stored in the refrigerator for 1 hr. After weighing the samples the rest of the surface oil was removed by using hexane. Samples were dipped in hexane for one minute and then followed by another half minute dip after taking a break for one minute between the two dips. They were blotted dry with paper towels.

After cooking, all samples were let to cool down in the refrigerator. Samples were weighed and then ground in a spice blender to an approximate size of 2mm x 2mm x 2mm. The samples were well mixed to ensure representative sampling. All samples were packed in airtight Ziploc® bags and stored at -40°C in the chest freezer. A portion of the chopped/ground samples was analyzed for protein content using the Kjeldahl method (Helrich, 1990).

### III.E. Determination of dialyzable iron

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.

➤ Digestion:

### III.E.1. Pepsin digestion

The sample at pH 2.0 was then placed in a shaking water bath at 37°C for 5 mins. After this, 5 ml of pepsin at 20 mg/ml was added and the suspension was incubated at 37°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 an ice bath to stop the pepsin digestion.

### III.E.2. Titratable acidity

Titratable acidity is the amount of 0.5 N NaOH required to bring the pH of pepsin-digested sample, with pancreatin and bile in PIPES, to 6.5. The equivalent moles of NaHCO<sub>3</sub> 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  $\text{NaHCO}_3$  required in 20 ml of the solution to be added to the dialysis tubing to bring the pH to 6.5.

### III.E.3. 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  $\text{NaHCO}_3$  solution at the concentration determined by titratable acidity were added to the flasks. After 30 min of incubation at  $37^\circ\text{C}$  the pH was recorded and 5 ml of pancreatin/bile salt in PIPES was added to each of the flasks incubated at  $37^\circ\text{C}$  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.

#### ➤ Extraction:

The extraction involved the same procedure as the digestion except that the enzymes were omitted. 5 ml of pepsin suspension in the digestion was replaced by 0.01 N HCl in the extraction.

### **III.F. Controls**

#### **III.F.1. Iron only digestion**

This control was run the same way as the pepsin digestion (III.E.1.) except that the chicken muscle was excluded. This was used to determine amount of dialyzable iron that came from the added iron during the digestion process.

#### **III.F.2. Iron only extraction**

This control was run the same way as the extraction except that the chicken muscle was excluded. This was used to determine amount of dialyzable iron that came from the added iron during the extraction process.

### **III.I. Analyses**

After the completion of digestion or extraction, both the dialyzate and the non-dialyzate were weighed and centrifuged at 3000 x g for 10 mins. An aliquot (typically 5 ml) of the supernatant from each of the samples was mixed with non-reducing protein precipitant 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. Another aliquot (typically 5 ml) was mixed with reducing protein precipitant 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. All

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 3000 x g for 10 mins using the bench top laboratory centrifuge, while the samples containing the non-dialyzable portion were centrifuged at 8000 x g for 20 mins.

#### III.I.1. Dialyzable Ferrous Iron

This represents the amount of dialyzable iron present in the ferrous form. This 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.I.2. Total Dialyzable Iron

This represents the amount of dialyzable iron present in both the ferrous and the ferric form. For the determination of total dialyzable 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.I.3. Non-dialyzable Ferrous Iron

This represents the amount of iron being converted to the ferrous form but was not dialyzable. 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 562 nm.

#### III.I.4. Non-dialyzable Total Iron

This represents the amount of iron being converted to the soluble form but was not dialyzable. 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 562 nm.

### III.I.5. Dialyzable Protein

This represents the amount of protein, which has been 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).

For the determination of dialyzable protein, 1 ml aliquot of the samples having a 1:1 ratio of dialyzable digest : 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 542 nm. A blank value was obtained with 1 ml distilled water and 4 ml of biuret reagent.

### III.I.6. Non-Dialyzable Protein

This represents the amount of protein/peptide, 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 (HMWCO).

For the determination of non-dialyzable protein, 1 ml aliquot of the samples having a 1:1 ratio of non-dialyzable digest : 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 542 nm. A blank value was obtained with 1 ml distilled water and 4 ml of biuret reagent.

#### III.I.7. Total Sulfhydryl Analysis

The total sulfhydryl content of fresh and cooked samples was determined using the method described by Habeeb (Habeeb, 1973). Chicken sample at protein concentration of ~ 15 mg/ml was homogenized in Na-phosphate buffer pH 8.0 and 2% SDS. The extract was centrifuged at 7000 rpm for 10 min and protein in the supernatant was analyzed for protein using the biuret method. The supernatant was diluted with phosphate buffer to a final protein concentration of 0.2 mg/ml. 0.01 mM Ellman's reagent (DTNB) was prepared 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. The total sulfhydryl content was calculated using a molar extinction coefficient of  $13,500 \text{ M}^{-1}, \text{ cm}^{-1}$  (Habeeb, 1973).

#### III.I.8. Acid Extractable Non-Protein Sulfhydryl Analysis

Chicken sample containing 2 g protein was extracted in 0.01 N HCl with 0.1 mM EDTA for 5 minutes. Sample was kept on ice for 140 seconds after every minute of extraction. The extract was centrifuged at 3000 x g for 10 min and the supernatant was collected. A dialysis tubing (20 cm x 20.4 mm, 6-8 kD MWCO) with 20 ml of 0.01 N HCl

with 0.1 mM EDTA was prepared and was dialyzed with the supernatant. The dialysis was performed in a shaking Erlenmeyer flask at 4°C for 4 hr. The dialyzate was diluted (1:3) with 0.2 M sodium phosphate buffer (pH 8.0) resulting in a four dilution. Acid extractable non-protein sulfhydryl content was calculated using Ellman's reagent as described in III.I.7.

#### III.I.9. Histidine Analysis

Histidine was analyzed using the method described by Seth and Mahoney (Seth and Mahoney, 2000). Chicken sample at protein concentration of ~ 15 mg/ml was homogenized in Na-phosphate buffer pH 6.5 and 2% SDS. The extract was centrifuged at 7000 rpm for 10 min and protein in the supernatant was analyzed for protein using the biuret method. The supernatant was diluted with phosphate buffer to a final protein concentration of 1.0 mg/ml. Diethyl-pyrocabonate (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. The sulfhydryl content was calculated using an extinction coefficient of  $3,200 \text{ M}^{-1}, \text{ cm}^{-1}$ .

### III.J. Calculations

The amount of dialyzable and non-dialyzable matter obtained after each digestion/extraction 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 ( $V_{ND}$ ) 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 23-26 ml.

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

Therefore,

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

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

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.

One-way analysis of variance (ANOVA) was used to compare means with 95% confidence. Tukey 95% simultaneous confidence intervals were used for all pairwise comparisons among types of treatments.

## CHAPTER IV

### RESULTS

This chapter presents results from analyses of iron, protein and amino acids for all samples.

Table IV.1 shows total soluble iron and total dialyzable iron produced during digestion of control, raw and cooked samples.

Sample	Total Soluble Iron in $\mu\text{g}$	Total Dialyzable Iron in $\mu\text{g}$
Iron Only	$179 \pm 4^c$	$7.64 \pm 0.40^q$
Raw	$124 \pm 3^a$	$72.3 \pm 6.8^r$
Boiled	$52.0 \pm 21.4^b$	$9.19 \pm 1.04^{pq}$
Baked	$102 \pm 16.002^a$	$11.7 \pm 0.4^p$
Sautéed	$79.3 \pm 60.8^{ab}$	$7.60 \pm 2.08^q$
Deep Fried	$103 \pm 5^a$	$11.7 \pm 1.5^{pq}$

Table IV.1: Total soluble iron and total dialyzable iron produced during digestion of control, raw and cooked samples. Mean  $\pm$  SD of total soluble iron and total dialyzable iron in  $\mu\text{g}$  for total 10 pancreatin digestions ( $n = 10$ ). The total amount of iron used for each pancreatin digestion is 280  $\mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

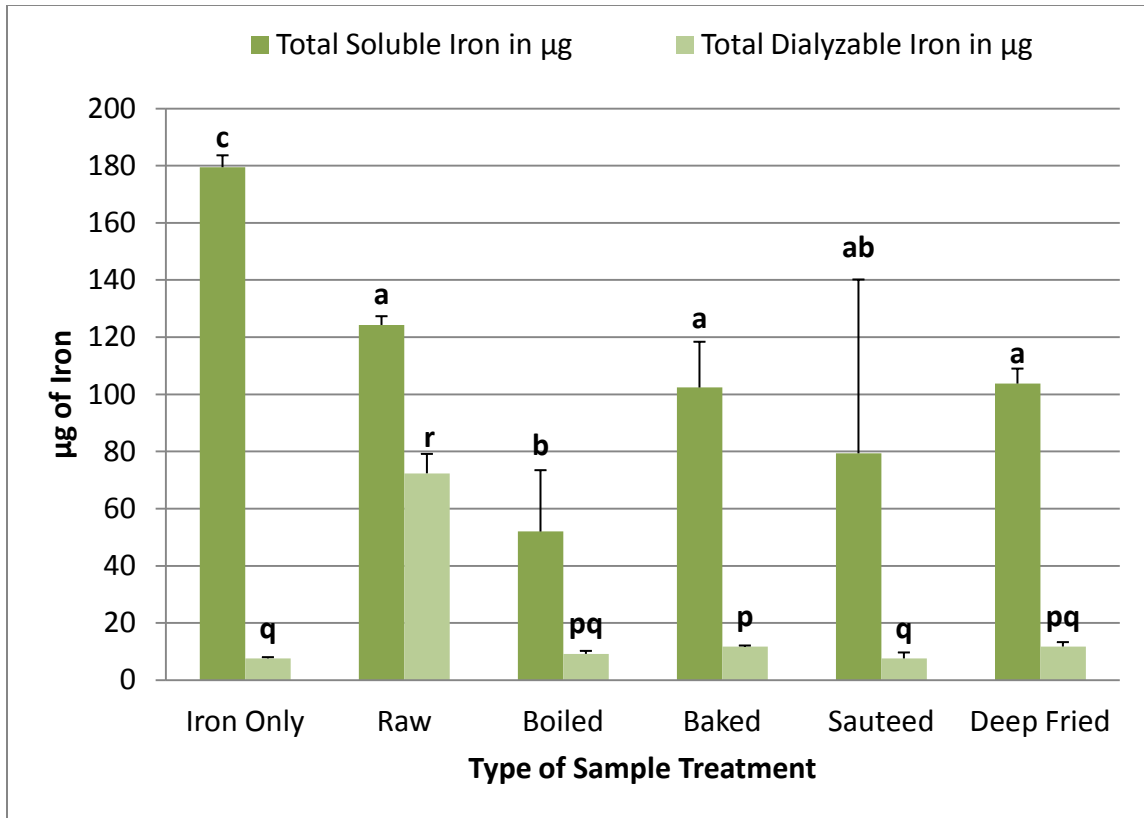


Figure IV.1: Total soluble iron and total dialyzable iron produced during digestion of control, raw and cooked samples. Graphical representation of data from table IV.1. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking chicken on production of soluble and dialyzable iron during digestion is shown in Figure IV.1. It is evident that not all soluble iron was dialyzable. In the iron only control only about 5% of the soluble iron was dialyzable. Digestion of raw muscle increased the amount of dialyzable iron by about 10 fold as compared to the control even though there was a decrease in total soluble iron. All cooking methods caused a decrease in both soluble and dialyzable iron. For boiled, baked and deep fried samples the level of dialyzable iron was decreased to that of the control, whereas in the baked sample it was only slightly higher. It is evident that cooking largely eliminated the effect of muscle on production of dialyzable iron.

Table IV.2 shows total dialyzable iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples.

Sample	Total Dialyzable Iron in $\mu\text{g}$	Dialyzable Ferrous Iron in $\mu\text{g}$
Iron Only	$7.64 \pm 0.40^{\text{b}}$	$3.53 \pm 0.12^{\text{r}}$
Raw	$72.3 \pm 6.8^{\text{c}}$	$30.5 \pm 2.6^{\text{s}}$
Boiled	$9.19 \pm 1.04^{\text{ab}}$	$10.9 \pm 0.6^{\text{p}}$
Baked	$11.7 \pm 0.4^{\text{a}}$	$9.44 \pm 0.59^{\text{p}}$
Sautéed	$7.60 \pm 2.08^{\text{b}}$	$4.96 \pm 1.03^{\text{r}}$
Deep Fried	$11.7 \pm 1.5^{\text{ab}}$	$15.5 \pm 1.8^{\text{q}}$

Table IV.2: Total dialyzable iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples. Mean  $\pm$  SD of total dialyzable iron and dialyzable ferrous iron in  $\mu\text{g}$  for total 10 pancreatin digestions ( $n = 10$ ). The total amount of iron used for each pancreatin digestion is  $280 \mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

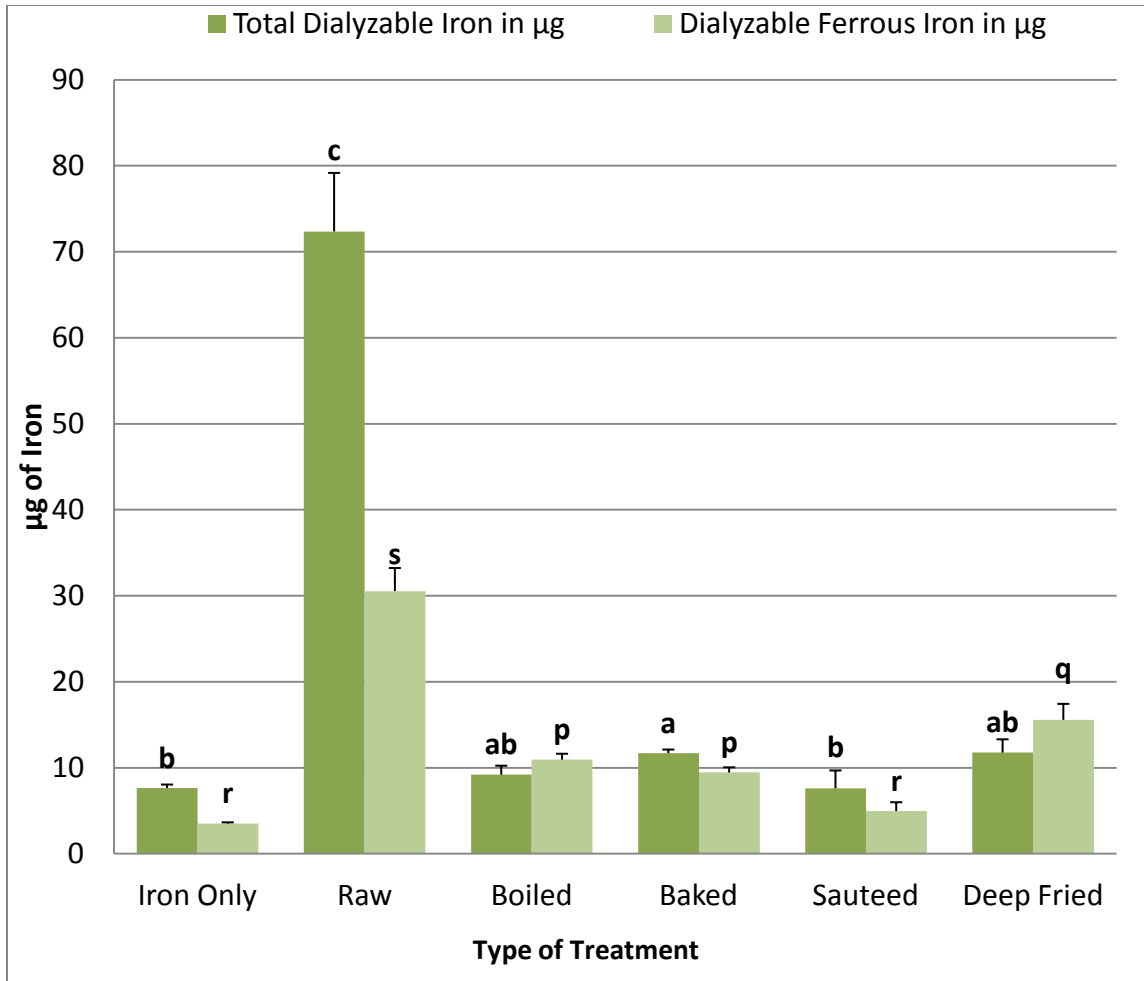


Figure IV.2: Total dialyzable iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples. Graphical representation of data from table IV.2. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking chicken on the production of dialyzable iron during digestion is shown in Figure IV.2. Raw chicken produced about a 10 fold increase in total dialyzable iron and about a 9 fold increase in dialyzable ferrous iron, compared to the iron only control. About 40% of the dialyzable iron in the raw sample was ferrous.

Cooking the chicken led to large decreases in total dialyzable iron; only the baked sample was significantly higher than the control. In the cooked samples most of



the iron was ferrous indicating that cooking destroyed the dialyzable ferric iron most of all.

Table IV.3 shows the total ferrous iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples.

Sample	Total Ferrous Iron in $\mu\text{g}$	Dialyzable Ferrous Iron in $\mu\text{g}$
Iron Only	$27.3 \pm 1.7^a$	$3.53 \pm 0.12^f$
Raw	$63.7 \pm 5.0^b$	$30.5 \pm 2.6^s$
Boiled	$41.2 \pm 3.2^a$	$10.9 \pm 0.6^p$
Baked	$33.9 \pm 11.0^a$	$9.44 \pm 0.59^p$
Sautéed	$25.9 \pm 11.2^a$	$4.96 \pm 1.03^f$
Deep Fried	$57.1 \pm 21.0^b$	$15.5 \pm 1.8^q$

Table IV.3: Total ferrous iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples. Mean  $\pm$  SD of total ferrous iron and dialyzable ferrous iron in  $\mu\text{g}$  for total 10 pancreatin digestions ( $n = 10$ ). The total amount of iron used for each pancreatin digestion is 280  $\mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

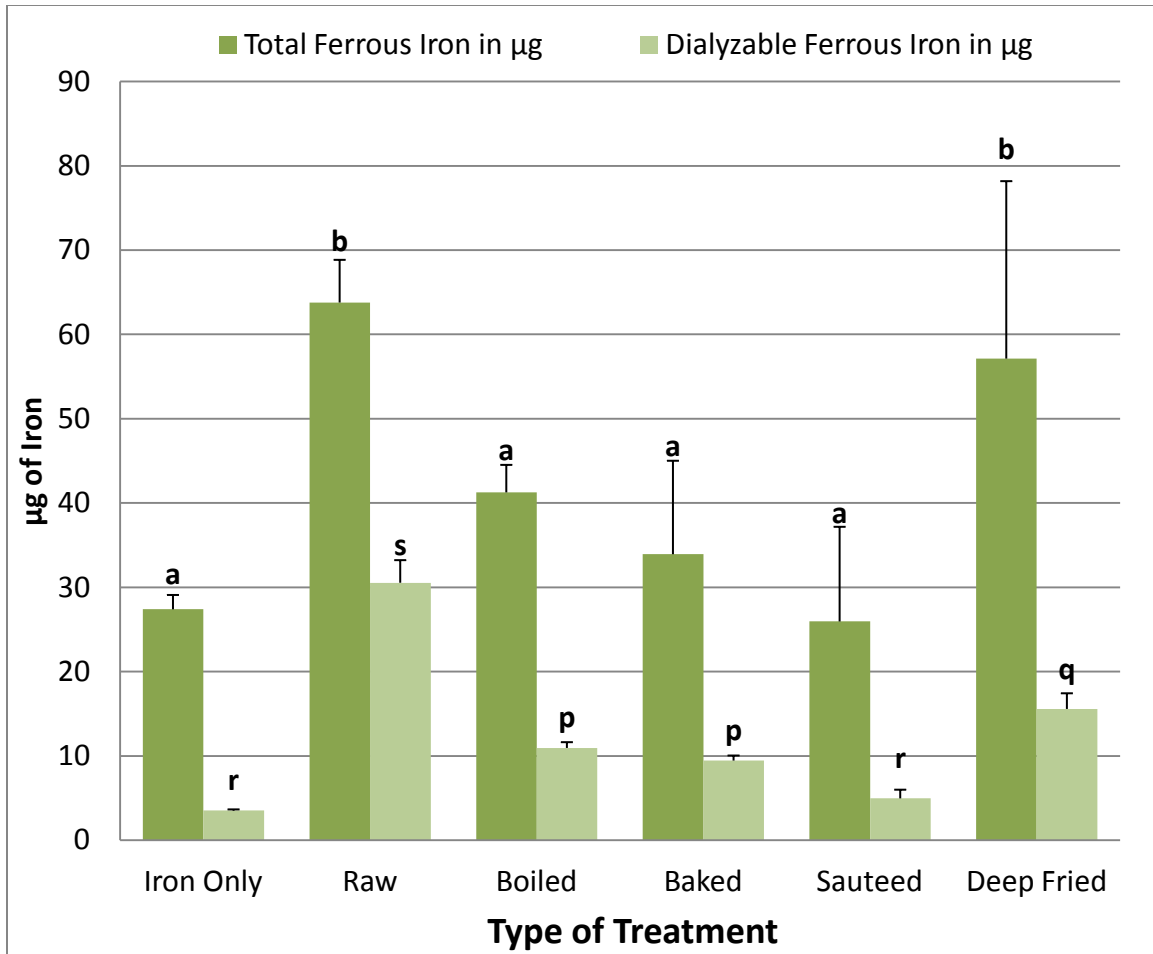


Figure IV.3: Total ferrous iron and dialyzable ferrous iron produced during digestion of control, raw and cooked samples. Graphical representation of data from table IV.3. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking muscle on production of ferrous iron is shown in Figure IV.3. Raw muscle produced about a 2 fold increase in the total ferrous iron and a 9 fold increase in dialyzable ferrous iron. 13% of the total ferrous iron produced by the control was dialyzable ferrous iron. About half of the ferrous iron produced by raw muscle was in the dialyzable form.

Cooking reduced levels of dialyzable ferrous iron for all treatments but the levels were still higher than the control except for the sautéed sample. These decreases ranged from about 49 to 84%. The results show that digestion of chicken muscle

increased the production of ferrous iron – both in the dialyzable as well as non dialyzable forms. Cooking procedures, on the other hand, reduced the ability of chicken to produce dialyzable ferrous iron during digestion.

Table IV.4 shows total soluble protein and total dialyzable protein produced during digestion of control, raw and cooked samples.

Sample	Total Soluble Protein in mg	Total Dialyzable Protein in mg
Raw	317 ± 24.2 <sup>a</sup>	247 ± 13.6 <sup>Dr</sup>
Boiled	419 ± 48.0 <sup>b</sup>	282 ± 11.7 <sup>q</sup>
Baked	337 ± 30.3 <sup>a</sup>	228 ± 12.5 <sup>p</sup>
Sautéed	269 ± 46.0 <sup>c</sup>	164 ± 43.0 <sup>s</sup>
Deep Fried	368 ± 28.3 <sup>a</sup>	269 ± 12.0 <sup>qr</sup>

Table IV.4: Total soluble protein and total dialyzable protein produced during digestion of control, raw and cooked samples. Mean ± SD of total soluble protein and total dialyzable protein in mg for total 10 pancreatin digestions (n = 10). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

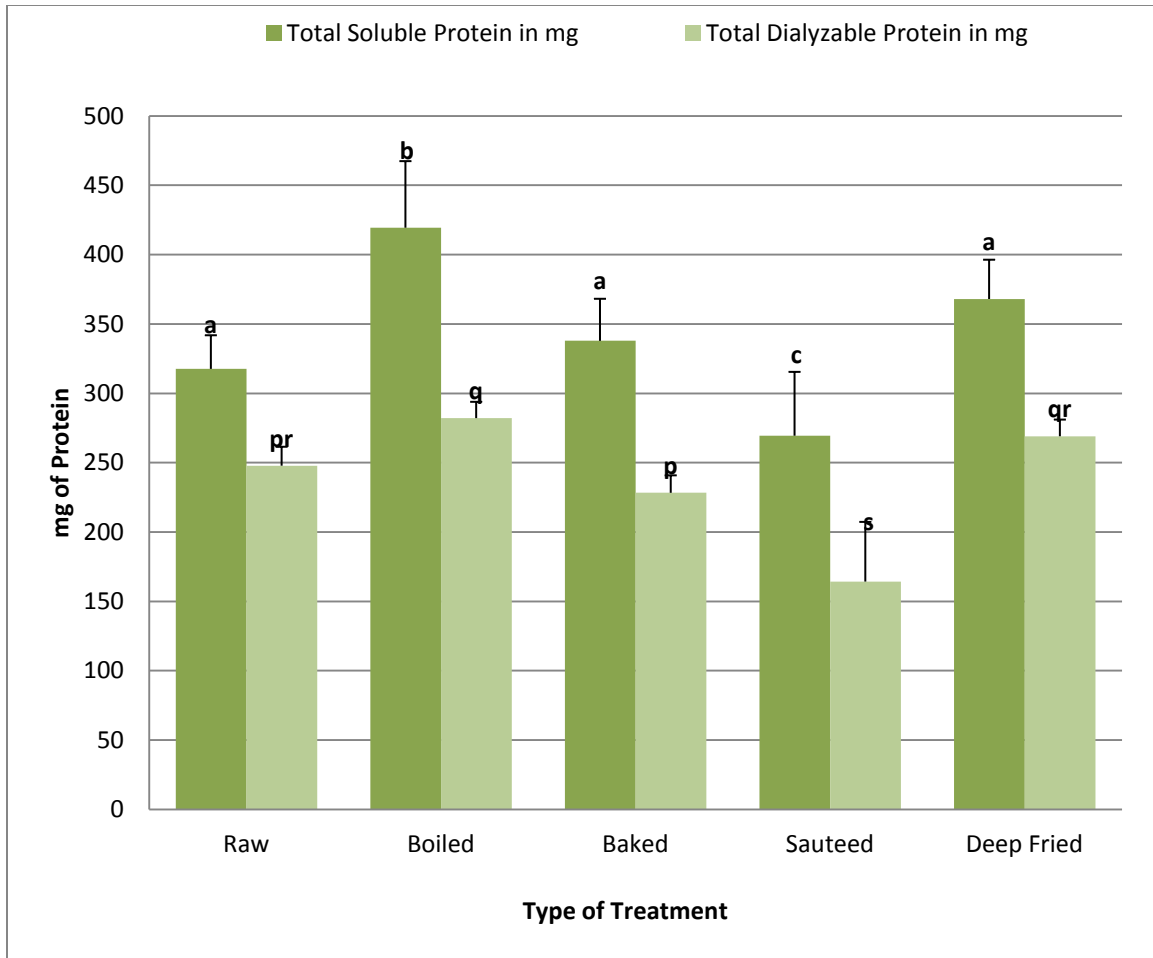


Figure IV.4: Total soluble protein and total dialyzable protein produced during digestion of control, raw and cooked samples. Graphical representation of data from table IV.4. Means without a common letter differ at  $p < 0.001$ .

Effect of cooking on the formation of soluble protein and dialyzable protein is shown in Figure IV.4. About 78% of soluble protein was dialyzable, during digestion of raw chicken muscle. Soluble protein was highest in the boiled sample and least in the sautéed sample. Dialyzable protein, which is a measure of digestion, was similar in baked and deep fried sample as compared to the raw muscle. It was slightly higher in the boiled sample whereas it fell by 34% in the sautéed sample.

Table IV.5 shows total soluble iron and total dialyzable iron produced during digestion of control, raw and cooked samples.

Sample	Total Soluble Iron in $\mu\text{g}$	Total Dialyzable Iron in $\mu\text{g}$
Iron Only	$19.8 \pm 0.3^c$	$2.82 \pm 0.0^f$
Raw	$24.5 \pm 1.3^d$	$19.3 \pm 2.0^s$
Boiled	$12.5 \pm 1.0^b$	$8.70 \pm 2.2^q$
Baked	$16.4 \pm 1.0^a$	$11.2 \pm 0.5^p$
Sautéed	$16.9 \pm 2.1^a$	$8.54 \pm 1.0^q$
Deep Fried	$14.7 \pm 2.0^a$	$8.24 \pm 0.4^q$

Table IV.5: Total soluble iron and total dialyzable iron produced during extraction of control, raw and cooked samples. Mean  $\pm$  SD of total soluble iron and total dialyzable iron in  $\mu\text{g}$  for total 10 no-pancreatin extractions (n = 10). The total amount of iron used for each no-pancreatin extraction is 280  $\mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

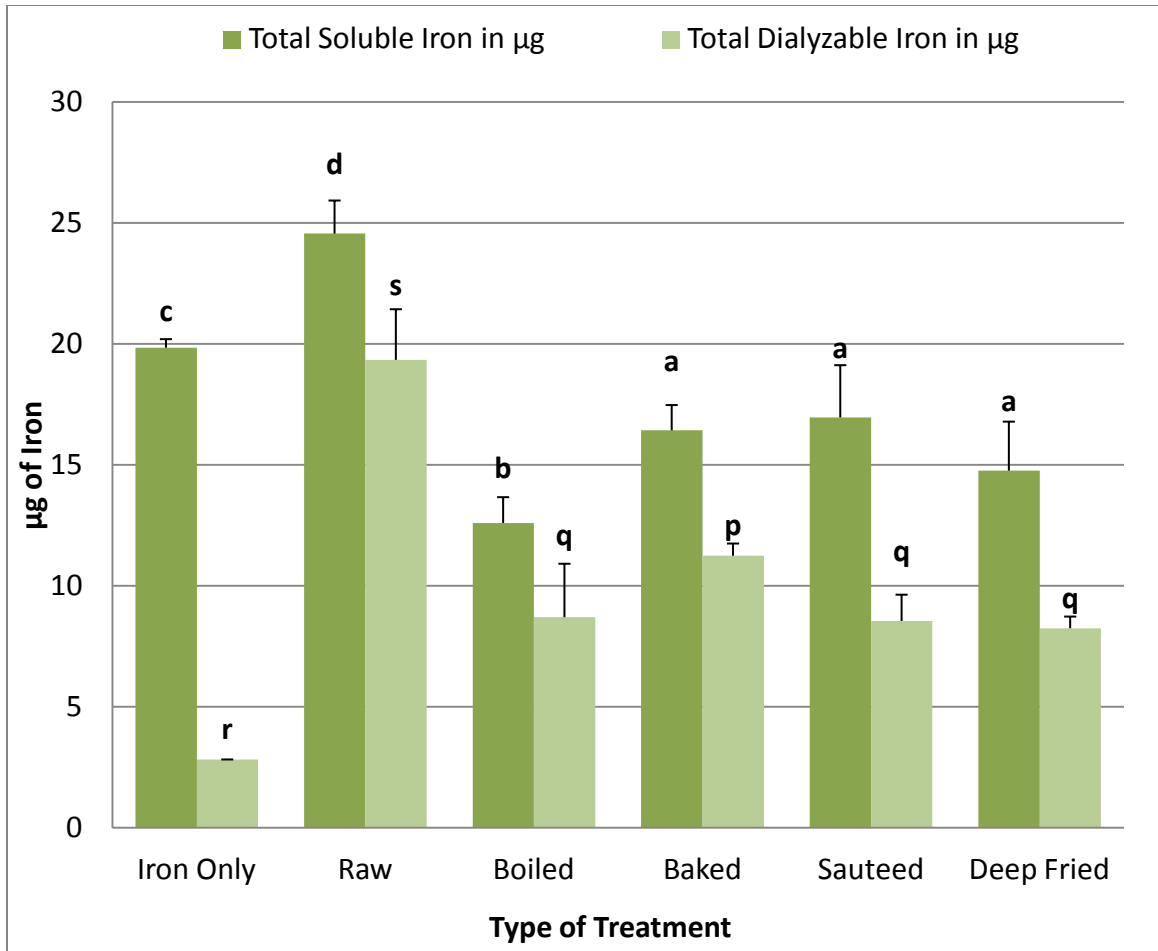


Figure IV.5: Total soluble iron and total dialyzable iron produced during extraction of control, raw and cooked samples. Graphical representation of data from table IV.5. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking chicken muscle on production of soluble and dialyzable iron by extraction is shown in Figure IV.5. It is evident that extraction alone is sufficient to increase the iron levels using raw muscle; furthermore most of the extracted iron was dialyzable, in contrast to the control where very little was dialyzable. Cooking the muscle decreased the amount of soluble iron for all treatments, compared to the raw sample. In addition to that, cooking also decreased the amount of dialyzable iron. These levels of dialyzable iron in boiled, sautéed and deep fried sample were similar to each other but significantly lower than baked sample.

Table IV.6 shows total dialyzable iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples.

Sample	Total Dialyzable Iron in $\mu\text{g}$	Dialyzable Ferrous Iron in $\mu\text{g}$
Iron Only	$2.82 \pm 0.0^c$	$2.19 \pm 0.0^r$
Raw	$19.3 \pm 2.0^d$	$13.5 \pm 2.4^s$
Boiled	$8.70 \pm 2.2^b$	$4.58 \pm 1.0^p$
Baked	$11.2 \pm 0.5^a$	$5.05 \pm 0.1^p$
Sautéed	$8.54 \pm 1.0^b$	$2.55 \pm 0.3^r$
Deep Fried	$8.24 \pm 0.4^b$	$7.94 \pm 1.2^q$

Table IV.6: Total dialyzable iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples. Mean  $\pm$  SD of total dialyzable iron and dialyzable ferrous iron in  $\mu\text{g}$  for total 10 no-pancreatin extractions (n = 10). The total amount of iron used for each no-pancreatin extraction is 280  $\mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

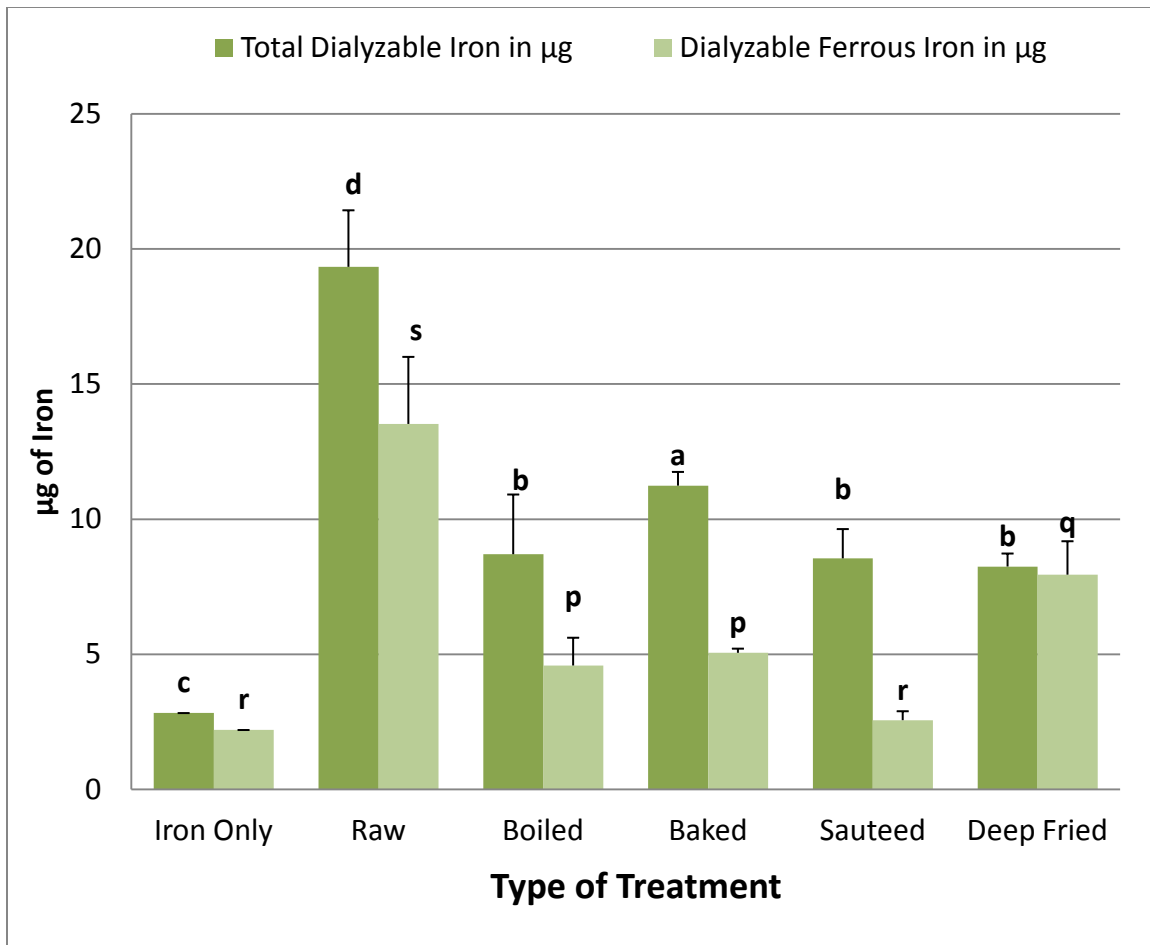


Figure IV.6: Total dialyzable iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples. Graphical representation of data from table IV.6. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking chicken muscle on the production of dialyzable iron after extraction is shown in Figure IV.6. All the dialyzable iron produced by the iron only control is in the ferrous form. Extraction of raw muscle produced about 85% more of both total dialyzable iron and dialyzable ferrous iron on extraction, compared to the iron only sample. About 70% of the total dialyzable iron produced by raw chicken was in the ferrous form.

Cooking chicken led to similar reduction in amounts of total dialyzable iron formed by extraction of boiled, sautéed and deep fried samples. The least reduction was



observed in the baked sample. Cooking also decreased dialyzable ferrous iron levels. The proportion of dialyzable iron that was ferrous was less in the cooked samples than in the raw sample, except for the deep fried sample. The amount of dialyzable ferrous iron produced by the sautéed sample was the same as the iron only control.

Table IV.7 show total ferrous iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples.

Sample	Total Ferrous Iron in $\mu\text{g}$	Dialyzable Ferrous Iron in $\mu\text{g}$
Iron Only	$5.70 \pm 0.01^{\text{ac}}$	$2.19 \pm 0.0^{\text{r}}$
Raw	$14.9 \pm 2.6^{\text{d}}$	$13.5 \pm 2.4^{\text{s}}$
Boiled	$6.53 \pm 0.82^{\text{a}}$	$4.58 \pm 1.0^{\text{p}}$
Baked	$6.80 \pm 0.77^{\text{a}}$	$5.05 \pm 0.1^{\text{p}}$
Sautéed	$4.14 \pm 0.35^{\text{a}}$	$2.55 \pm 0.3^{\text{r}}$
Deep Fried	$12.2 \pm 3.5^{\text{b}}$	$7.94 \pm 1.2^{\text{q}}$

Table IV.7: Total ferrous iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples. Mean  $\pm$  SD of total ferrous iron and dialyzable ferrous iron in  $\mu\text{g}$  for total 10 no-pancreatin extractions ( $n = 10$ ). The total amount of iron used for each no-pancreatin extraction is 280  $\mu\text{g}$ . Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

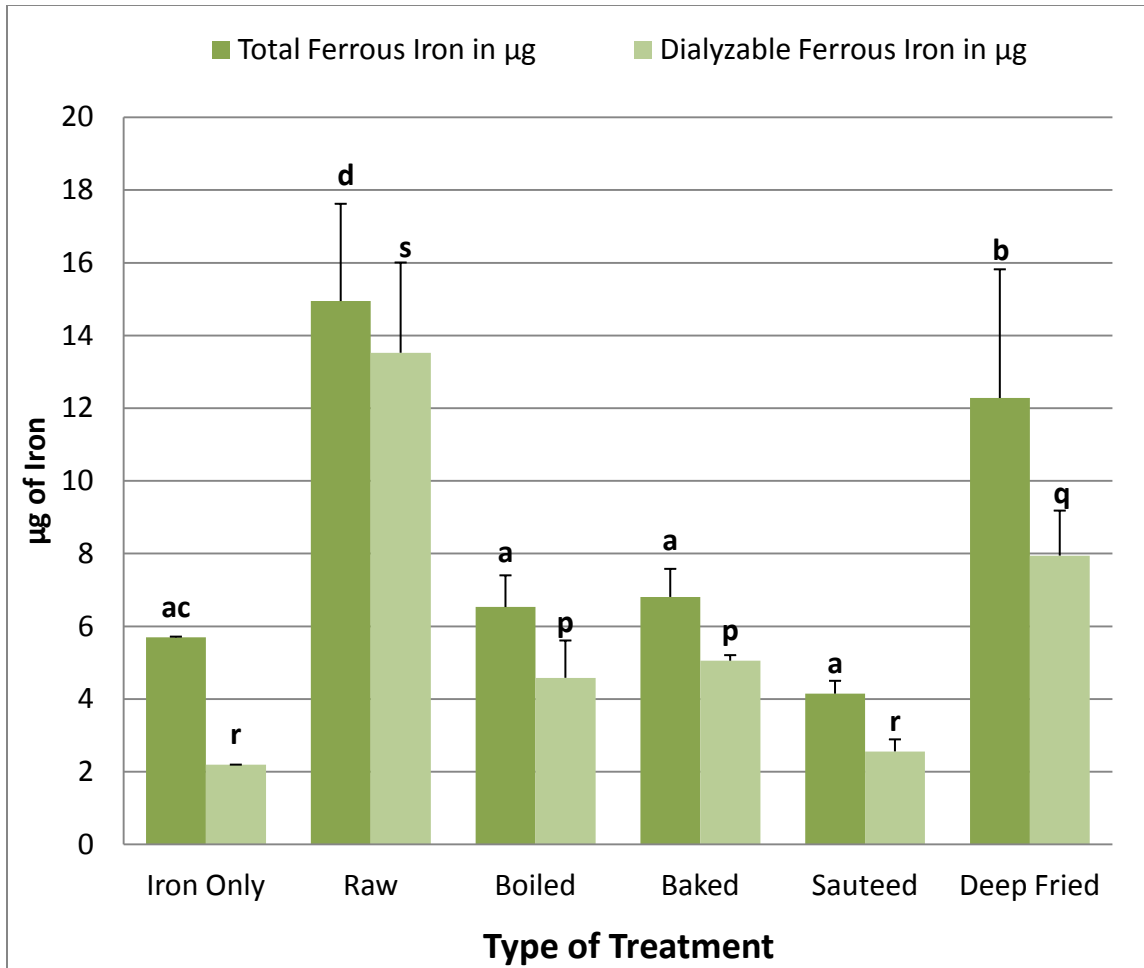


Figure IV.7: Total ferrous iron and dialyzable ferrous iron produced during extraction of control, raw and cooked samples. Graphical representation of data from table IV.7. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking on production of ferrous iron during extraction is shown in Figure IV.7. The raw chicken produced more than twice as much ferrous iron as the control and almost all of it was dialyzable. Cooking the muscle reduced the amount of ferrous iron for all treatments but less for deep fried than the others. In all cooked samples most of the ferrous iron produced by extraction was dialyzable.

Table IV.8 shows total soluble protein and total dialyzable protein produced during extraction of control, raw and cooked samples.

Sample	Total Soluble Protein in mg	Total Dialyzable Protein in mg
Raw	51.0 ± 8 <sup>a</sup>	44.8 ± 8.8 <sup>p</sup>
Boiled	80.2 ± 12 <sup>b</sup>	72.7 ± 16 <sup>q</sup>
Baked	48.3 ± 3.1 <sup>a</sup>	48.5 ± 12 <sup>p</sup>
Sautéed	63.5 ± 2.6 <sup>c</sup>	52.6 ± 5.2 <sup>pr</sup>
Deep Fried	74.7 ± 16 <sup>c</sup>	67.4 ± 16 <sup>qr</sup>

Table IV.8: Total soluble protein and total dialyzable protein produced during extraction of control, raw and cooked samples. Mean ± SD of total soluble protein and total dialyzable protein in mg for total 10 no-pancreatin extractions (n = 10). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

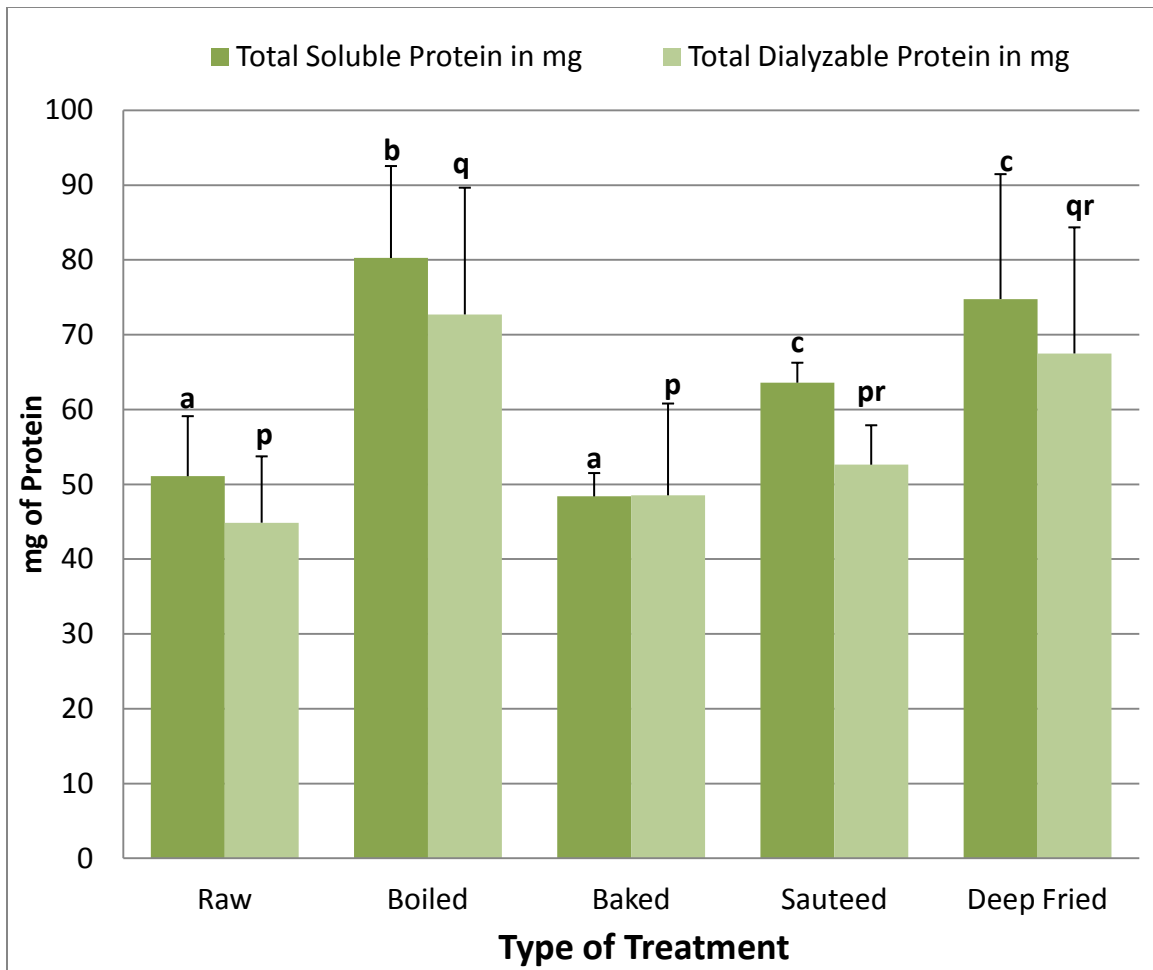


Figure IV.8: Total soluble protein and total dialyzable protein produced during extraction of control, raw and cooked samples. Graphical representation of data from table IV.8. Means without a common letter differ at  $p < 0.001$ .

The effect of production of soluble and dialyzable protein during extraction is shown in Figure IV.8. In the raw sample all of the protein was dialyzable. Cooking led to increases in extractable protein for all treatments except for baked. Most of the soluble protein was dialyzable in the boiled and sautéed samples. As with the raw sample all the extracted protein, which are peptides, in the baked and deep fried samples was dialyzable.

Table IV.9 shows total dialyzable iron and dialyzable ferrous iron produced only due to enzymatic digestion (Digestion – extraction) of control, raw and cooked samples.

Sample	Total Dialyzable Iron in $\mu\text{g}$	Dialyzable Ferrous Iron in $\mu\text{g}$
Iron Only	$4.82 \pm 0.40^b$	$1.33 \pm 0.12^r$
Raw	$53.0 \pm 6.8^c$	$17.0 \pm 1.5^s$
Boiled	$0.493 \pm 2.7^a$	$6.36 \pm 1.4^{pq}$
Baked	$0.459 \pm 0.9^a$	$4.39 \pm 0.66^p$
Sautéed	$-0.944 \pm 1^a$	$2.40 \pm 1.3^{pr}$
Deep Fried	$3.52 \pm 1.6^{ab}$	$7.62 \pm 2.9^q$

Table IV.9: Total dialyzable iron and dialyzable ferrous iron produced due to enzymatic digestion only (Digestion – extraction) by control, raw and cooked samples. Mean  $\pm$  SD of total dialyzable iron and dialyzable ferrous iron produced due to enzymatic digestion only (n = 10). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

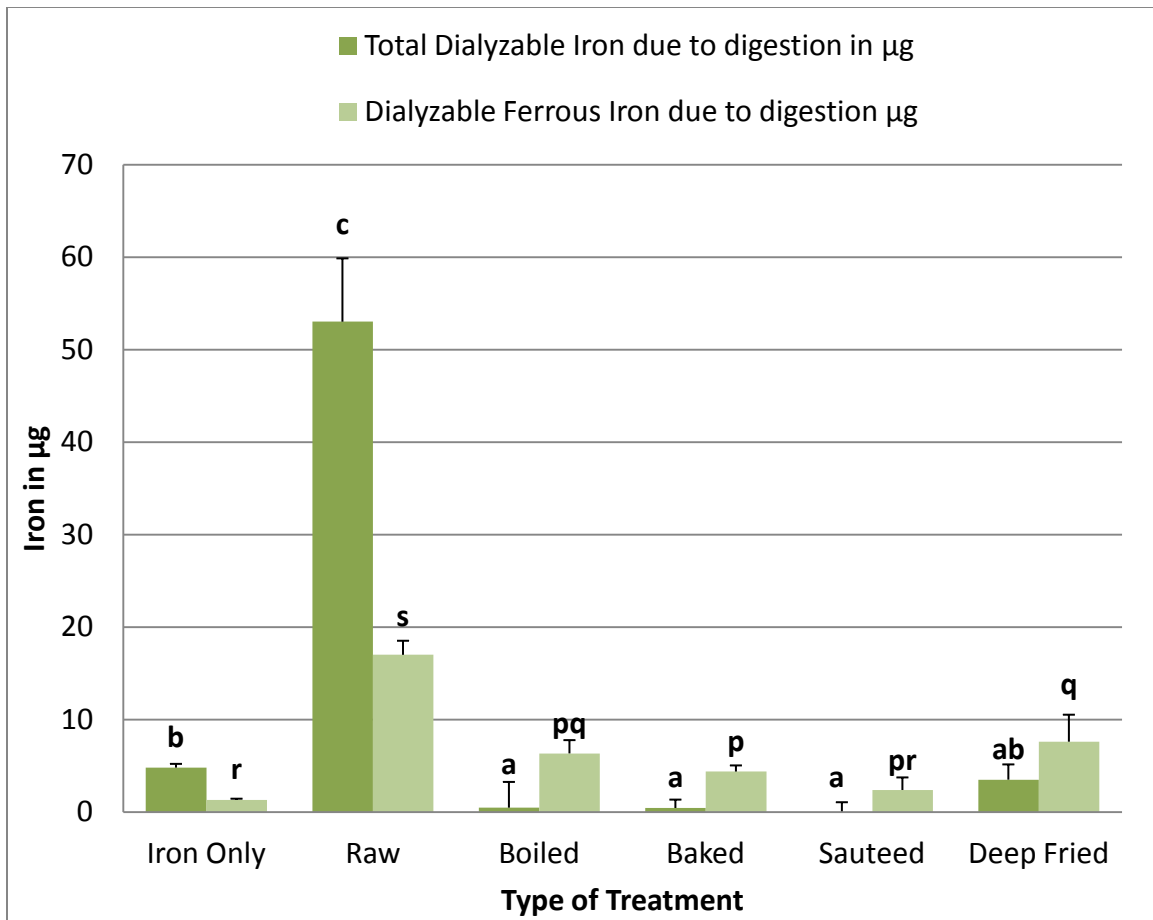


Figure IV.9: Total dialyzable iron and dialyzable ferrous iron as a result of enzymatic digestion (Digestion – extraction) of control, raw and cooked samples. Graphical representation of data from table IV.9. Means without a common letter differ at  $p < 0.001$ .

The amount of iron produced by enzymatic digestion only can be estimated by subtracting the values for extraction from those from digestion, since the latter process includes extraction. The results are shown in Figure IV.9. For the raw muscle there is an 11 fold increase in dialyzable iron and a 13 fold increase in dialyzable ferrous iron, compared to the control. Cooking treatments reduced the levels of dialyzable iron to that of the control or even lower. This indicates that cooking eliminated the effect of digestion. Cooking also reduced the levels of dialyzable ferrous iron but the levels remained above those of the control except for sautéed muscle.

Table IV.10 shows total sulfhydryl content of raw and cooked samples.

Sample	Total -SH Content ( $\mu\text{M/g}$ )
Raw	$56.6 \pm 0.8^c$
Boiled	$47.7 \pm 0.0^b$
Baked	$34.5 \pm 0.3^a$
Sautéed	$35.4 \pm 2.1^a$
Deep Fried	$32.1 \pm 2.9^a$

Table IV.10: Total sulfhydryl content of raw and cooked samples. Mean  $\pm$  SD of total sulfhydryl content (n = 6). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

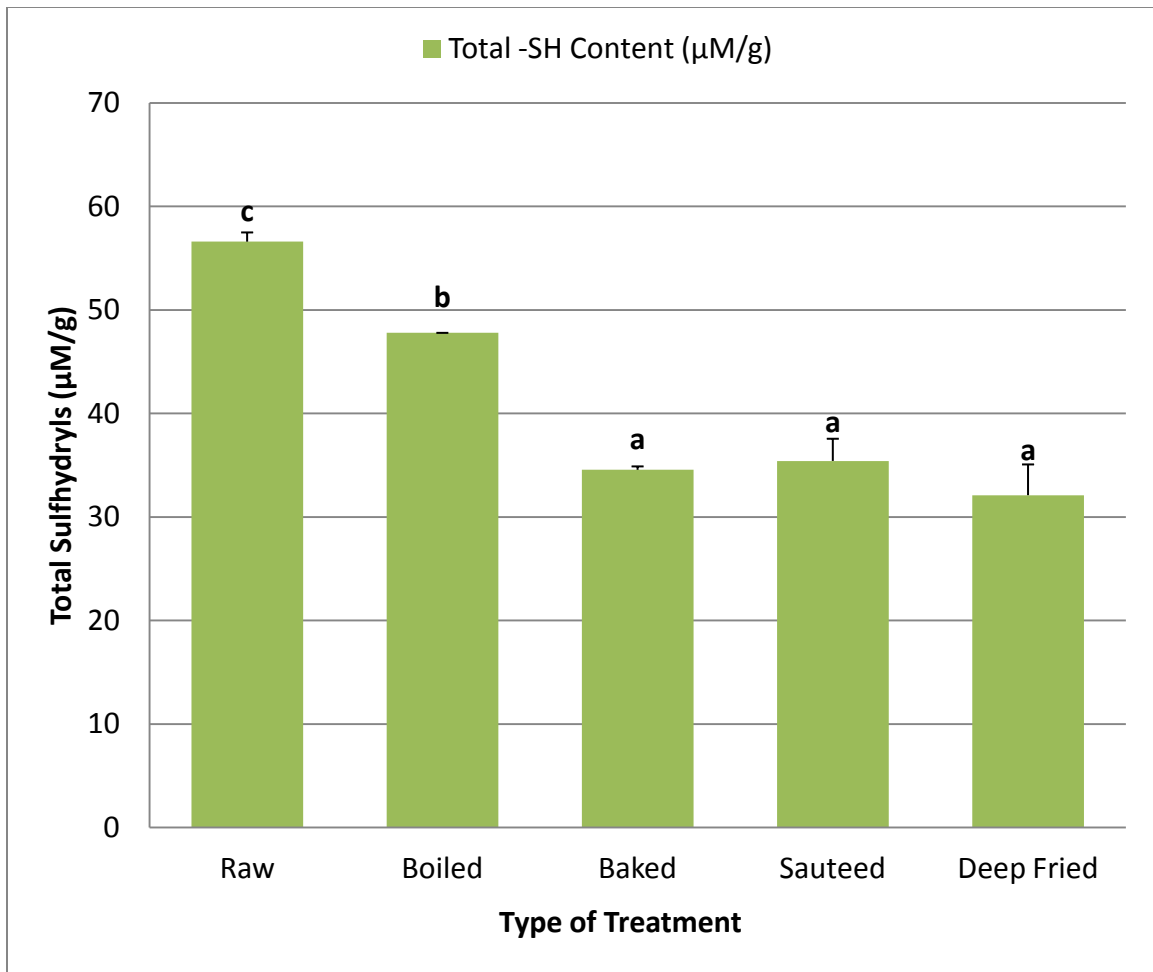


Figure IV.10: Total sulfhydryl content of raw and cooked samples. Graphical representation of the data from table IV.10. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking on the total sulfhydryl content is shown in Figure IV.10. Compared to raw muscle, boiled chicken observed a 16% drop in total sulfhydryls. Baked, sautéed and deep fried samples underwent a more significant drop of about 40% in total sulfhydryls.



Table IV.11 shows acid extractable non-protein sulfhydryl content of raw and cooked samples.

Sample	$\mu\text{moles SH}/100\text{g tissue}$
Raw	$46.2 \pm 0.9^{\text{d}}$
Boiled	$13.6 \pm 0.7^{\text{b}}$
Baked	$24.8 \pm 0.7^{\text{a}}$
Sautéed	$26.9 \pm 1.3^{\text{a}}$
Deep Fried	$1.3 \pm 0.2^{\text{c}}$

Table IV.11: Acid extractable non-protein sulfhydryl content of raw and cooked samples. Mean  $\pm$  SD of acid extractable non-protein sulfhydryl content (n = 6). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

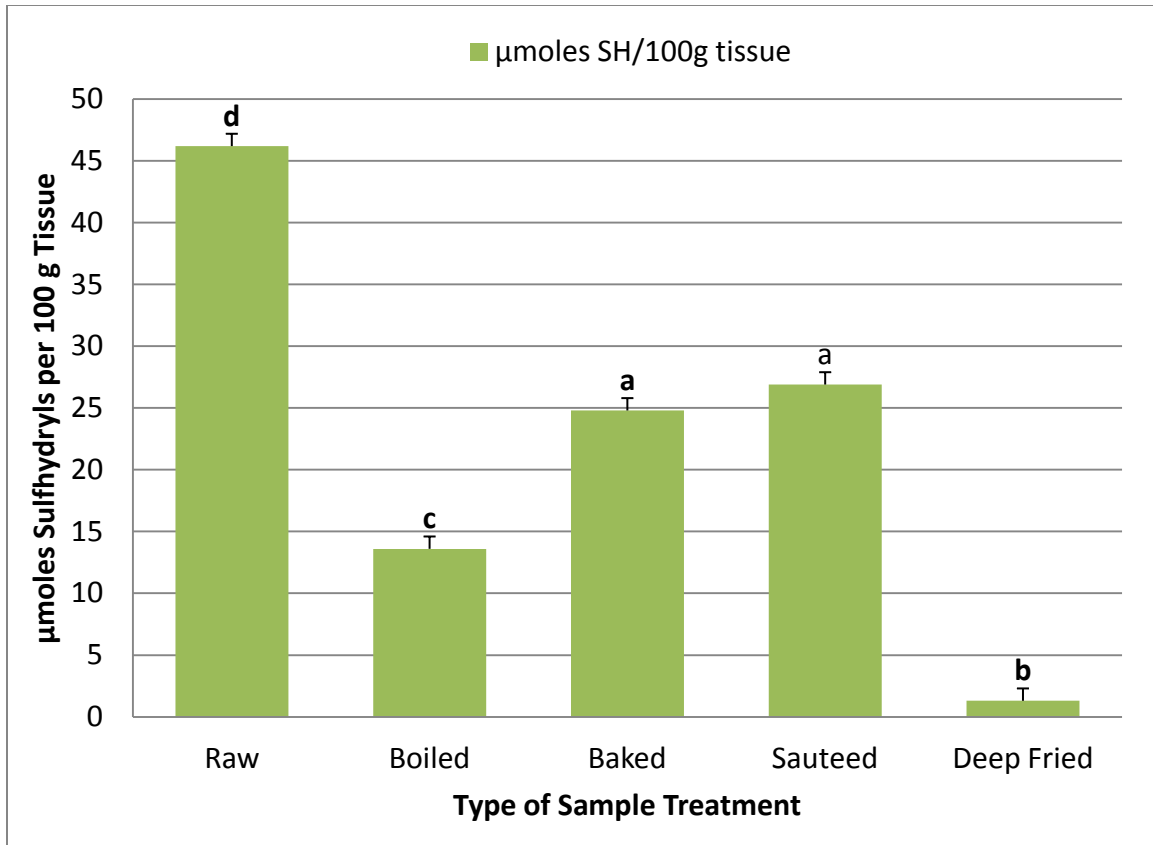


Figure IV.11: Acid extractable non-protein sulfhydryl content of raw and cooked samples. Graphical representation of the data from table IV.11. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking on production of acid extractable non-protein sulfhydryls is shown in Figure IV.11. All cooking treatments caused a loss of acid extractable non-protein sulfhydryls but the effect was most marked in the deep fried samples where more than 95% of the sulfhydryls were lost.

Table IV.12 shows total histidine content of raw and cooked samples.

Sample	Total Histidine Content ( $\mu\text{M/g}$ )
Raw	$200 \pm 1.6^c$
Boiled	$156 \pm 0.3^b$
Baked	$140 \pm 2.0^a$
Sautéed	$163 \pm 9.4^b$
Deep Fried	$161 \pm 0.1^b$

Table IV.12: Total histidine content of raw and cooked samples. Mean  $\pm$  SD of total histidine content (n = 6). Means without a common letter differ at  $p < 0.001$  using one-way analysis of variance.

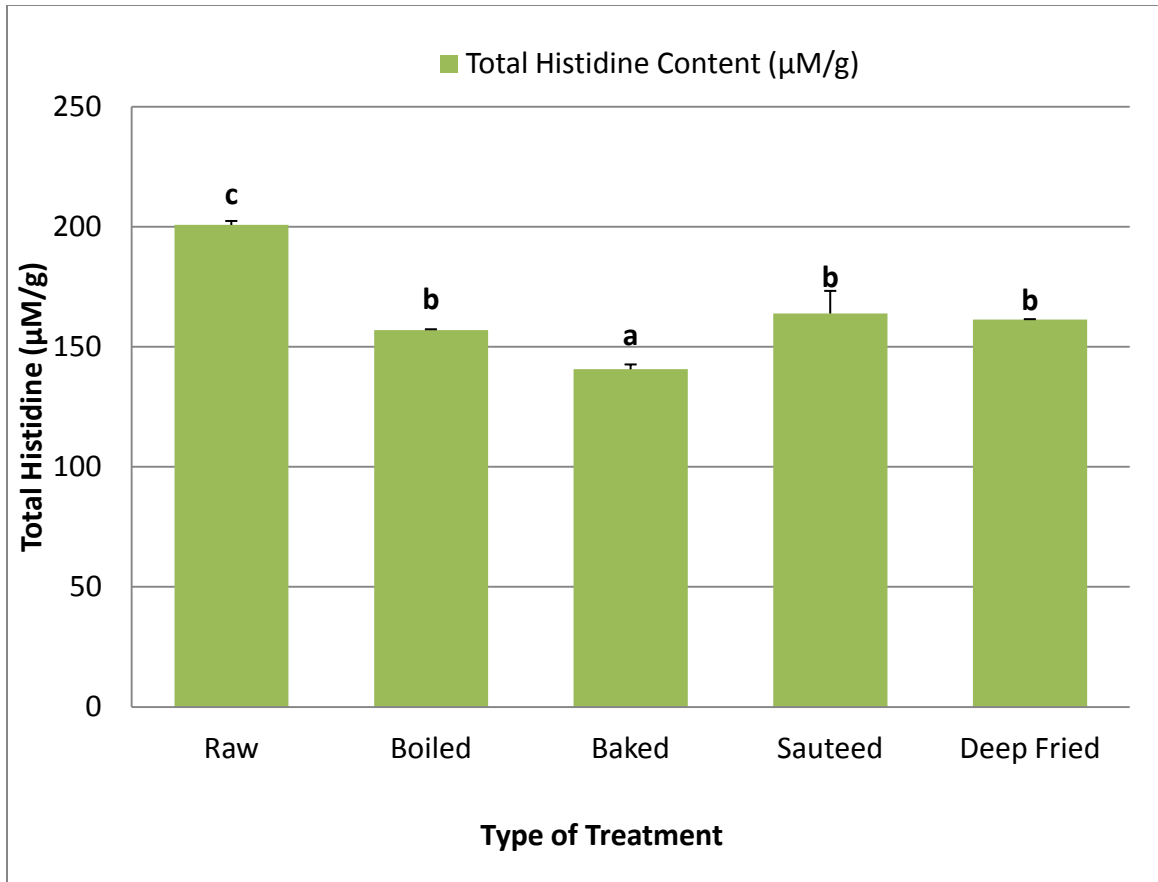


Figure IV.12: Total histidine content of raw and cooked samples. Graphical representation of the data from table IV.12. Means without a common letter differ at  $p < 0.001$ .

The effect of cooking on the histidine content of muscle is shown in Figure IV.12.

All treatments caused a loss in histidines varying from 20 to 40%. The greatest loss (40%) was in the baked sample.

## CHAPTER V

### DISCUSSION

Studies have shown that *in vitro* dialyzability of iron can be a reliable predictor of iron bioavailability (Miller, 1989; Kapsokefalou, 1991). Meat proteins enhance dialyzability of non-heme iron when it undergoes digestion. Vatterm and Mahoney (2005) have indicated that digestion of chicken muscle produces peptides up to molecular weight 10kDa which may play an important role in iron chelation and its solubility at intestinal conditions *in vitro*. This study confirmed the enhancing effect of chicken muscle in production of dialyzable iron during digestion. Digestion of raw muscle produced a lot more dialyzable iron compared to the iron only control. In this study, about 42% of the dialyzable iron was found to be in the ferrous form – which may be a better predictor of iron bioavailability than total dialyzable iron (Kapsokefalou, 1991). Digestion was not necessary for production of dialyzable iron; it was also produced during extraction. About 70% of the dialyzable iron produced during extraction was found to be in the ferrous form. This confirmed earlier findings of Karava *et al* (2008) indicating two distinct sources of dialyzable iron – dialyzable iron formed due to enzymatic digestion and dialyzable iron formed due to non-enzymatic extraction. Of the total dialyzable produced during digestion, about 27% was due to extraction.

Dialyzable iron levels formed during digestion of cooked chicken were not significantly different from the iron only control (Figure IV.2). This suggested that the enhancing effect of raw chicken was destroyed upon application of all cooking methods. Since all the dialyzable iron in cooked samples was ferrous (Figure IV.2), it means that

cooking destroyed production of dialyzable ferric iron, most of all. This change is very significant compared to raw chicken, which means that cooking impaired the 'meat factor'. Cooking caused reduction in the formation of ferrous iron. Significant losses were found in total ferrous iron and dialyzable ferrous iron (Figure IV.3) which may be related to the drop in total sulfhydryls (Figure IV.10) and dialyzable sulfhydryls (Figure IV.11).

Karava *et al.* (2008) found that increase in heating temperature of chicken muscle slurry progressively decreases the amount of dialyzable iron, total sulfhydryls and histidines produced. Therefore, in my study, I expected more losses in dialyzable iron as the external set temperature increased. However, all cooking methods had a set cooking temperature of at least 212°F, and yet there was only little difference in the dialyzable iron levels between cooking methods (Figure IV.2). It seems that the only parameter that had a profound effect on the ability of all samples to produce dialyzable iron was the internal temperature to which they were cooked (165°F).

It has been established previously that this experimentation found two distinct sources of dialyzable iron – dialyzable iron formed due to enzymatic digestion and dialyzable iron formed due to non-enzymatic extraction. Cooking decreased dialyzable iron formed during digestion (Figure IV.2) but it did not decrease dialyzable iron formed during extraction in the same degree (Figure IV.6). Compared to raw muscle, ability of cooked samples to produce dialyzable iron during digestion was almost completely destroyed. On the other hand, compared to raw muscle, ability of cooked samples to produce dialyzable iron during extraction was decreased by only about 50%. This may

mean that cooking decreased the 'meat factor' more during digestion than extraction (Figure IV.6). However, in both cases, sautéed samples showed the lowest dialyzable ferrous iron production – equal to the iron only control; which means that sautéing completely destroyed the ability of chicken to produce dialyzable ferrous iron. This may be explained by the external cooking temperature during sautéing which was the highest amongst all cooking procedures.

Results of this study show that digestion of raw muscle not only increased the dialyzability of non-heme iron but also reduced ferric iron to the ferrous form (Figure IV.2), because all the original iron in the system was non-heme ferric iron. While that is so, extraction of raw muscle also produced dialyzable ferrous iron (Figure IV.6) to a lesser degree, compared to digestion. The contribution of enzymatic digestion towards production of total dialyzable iron and dialyzable ferrous iron can only be estimated by subtracting extraction numbers from digestion numbers. This arithmetic subtraction is valid because the extraction process is exactly the same as the digestion process without enzymes. Results from this calculation (Figure IV.9) suggested that raw muscle produced 11 fold more dialyzable iron and 13 fold more dialyzable ferrous iron due to enzymatic digestion, compared than the iron only control. Also, cooking eliminated the ability of chicken to form dialyzable iron due to enzymatic digestion. While it may appear (Figure IV.9) as if dialyzable ferrous iron produced due to enzymatic digestion is greater than the total dialyzable iron in the cooked samples, this is just an artifact of the calculation and is physically impossible.

Cooking chicken muscle also had an impact on its digestibility. Sautéing resulted in decreased digestibility of muscle while boiling increased it. Baking and deep frying did not alter the digestibility of muscle. Similar trend was observed in formation of peptides, in the range of 6000 to 8000 Da MWCO, during digestion of cooked samples. Boiling, sautéing and deep frying muscle increased extractable proteins and peptides in the range of 6000 to 8000 Da. It can be concluded that the cooking method and time of cooking affected the digestibility and extractability of muscle proteins/peptides.

Cooking decreased total sulfhydryls (Figure IV.10), dialyzable sulfhydryls (Figure IV.11) and total histidines (Figure IV.12) because they are heat labile – which was expected from the findings of Karava *et al.* (2008). However, because some of the cooking treatments in my study were more severe in terms of external temperature than those used by Karava, a greater drop was expected. Total sulfhydryl content (Figure 10) in baked (external temperature = 375°F), sautéed (external temperature > 400°F) and deep fried (external temperature = 400°F) samples were not found to be significantly different from each other ( $p < 0.05$ ) but were significantly different ( $p < 0.05$ ) from the boiled sample (external temperature = 212°F). Dialyzable sulfhydryls were acid extractable non-protein sulfhydryls. Cooking decreased total histidine content of all samples (Figure IV.12). There was no significant difference ( $p < 0.05$ ) between the total histidine content of boiled, sautéed and deep fried samples. The greatest loss of about 40% total histidines, compared to the raw sample, was observed in the baked sample. This again suggests that external set cooking temperature and method of cooking had little effect on loss of total sulfhydryls and histidines. Also, a major factor that governed



the drop in their levels may have been the internal temperature attained (165°F). Method of cooking may have dictated the trend in loss of dialyzable sulfhydryls (Figure IV.11). Similar to the total sulfhydryl content results, histidines were not completely destroyed either and their residual levels could not be correlated with the destruction of the ability of cooked muscle in formation of dialyzable ferrous iron. This may suggest that sulfhydryls and histidines did not play a major role in formation of dialyzable ferrous iron.

Available evidence on cooking/heating meat and its effect on iron bioavailability are conflicting. Karava *et al.* (2008) correlated decreased iron dialyzability during digestion to the drop in levels of total sulfhydryls and total histidines in the muscle upon cooking. They observed a progressive drop in iron dialyzability (total and ferrous) upon increase in heating temperature of chicken muscle slurry. The highest losses were found at 195°F – where the residual dialyzable iron levels (total and ferrous) were just above the iron only control. This differed from my results where all cooking treatments led to destruction of all or most of dialyzable ferric iron (Figure IV.2), and the very little residual dialyzable iron left was in the ferrous form which may be available for uptake. Also, there was a decrease in levels of sulfhydryls but they were not completely destroyed (except for dialyzable sulfhydryls in the deep fried sample). Therefore, no clear-cut correlation was found between dialyzable sulfhydryls and dialyzable iron formed by cooked samples during extraction (Figure IV.6). This suggests that dialyzable sulfhydryls may not play a role in production of dialyzable iron during extraction and that other elements (explained later by the Huh study) may be responsible for the same.

In contrast with the above findings of Karava *et al.*, Baech *et al.* (2003) found that increase in cooking temperature of meat did not impair non-heme iron absorption from a phytate-rich meal *in vivo*; but cysteine content of meat decreased with increasing cooking temperatures. These findings do not support a role of sulfhydryl groups in increasing absorption of non-heme iron. My results do not agree with conclusions of the Baech *et al.* (2003) study because all cooking methods decreased the ability of muscle to produce dialyzable ferrous iron which is accepted to be a good predictor of iron bioavailability. On the contrary, cysteine was found to be heat labile, but was not completely destroyed, in all cooked samples which were consistent with the findings of the Baech study.

Conclusions drawn by Baech *et al.* (2003) do not agree with the findings of current study. This may be because of two reasons: (a) The 'meat factor' that is responsible for iron uptake is due to extraction; (b) The 'meat factor' in red meats reacts to cooking through a different mechanism which may involve a role of sulfhydryl groups that were not destroyed due to cooking. Huh *et al.* (2004) strongly suggested that low molecular weight carbohydrate fractions from fish muscle tissue are responsible for increased non-heme iron uptake by Caco-2 cells. This may explain the formation of dialyzable ferrous iron from extraction of raw as well as cooked chicken muscle samples.

Kapsokefalou and Miller (1991) showed that raw, broiled and microwave cooked beef did not produce significantly different amount of dialyzable ferrous iron. While cooking did not affect dialyzable ferrous iron formation, it increased non dialyzable ferrous iron formation. The amount of non-dialyzable ferrous iron formed by cooked

samples was in the order: Broiled > Microwave cooked > Raw. This suggests that dialyzability of non-heme iron was decreased as beef cooking temperature increased. Conclusively, the fact that this study found no change in dialyzable ferrous iron formation after cooking meat means that my results are strikingly different. Because the methodology used in my study and the Kapsoketalou study is very similar, this may mean that – unlike chicken breast muscle - beef plays a role in preserving the ‘meat factor’ even after undergoing broiling and microwave cooking.

## CHAPTER VI

### CONCLUSIONS

The following conclusions were reached:

1. Dialyzable iron was produced by both digestion (73%) and extraction (27%) of raw muscle.
2. Cooking muscle by all methods decreased production of dialyzable iron to levels similar to the iron only control. There was little difference between cooking methods despite large differences in external temperature. Most of the remaining dialyzable iron was ferrous.
3. Cooking by all methods decreased the amount of dialyzable iron obtained by extraction by about one half but essentially eliminated the production of dialyzable iron due only to enzymatic digestion.
4. Cooking reduced levels of total sulfhydryls, acid extractable non-protein sulfhydryls and histidine residues. This reduction may account for some of the loss of dialyzable iron.
5. Our results indicate that cooking severely reduces the ability of chicken muscle to produce dialyzable iron and that what little remains is due largely to extractable non-protein components rather than to products of proteolytic digestion. Accordingly, the "meat factor" in cooked chicken may be due to extractable muscle components that produce dialyzable iron.

## APPENDIX A

### STANDARD CURVE FOR PROTEIN

Table A.1 shows results of total protein content of bovine serum albumin.

Bovine Serum Albumin (mg/ml)	Final Absorbance (542 nm)
0.0	0.000
0.5	0.029
1.0	0.057
2.0	0.103
3.0	0.155
4.0	0.206
5.0	0.247

Table A.1: Final absorbance of dilutions of bovine serum albumin (n = 3).

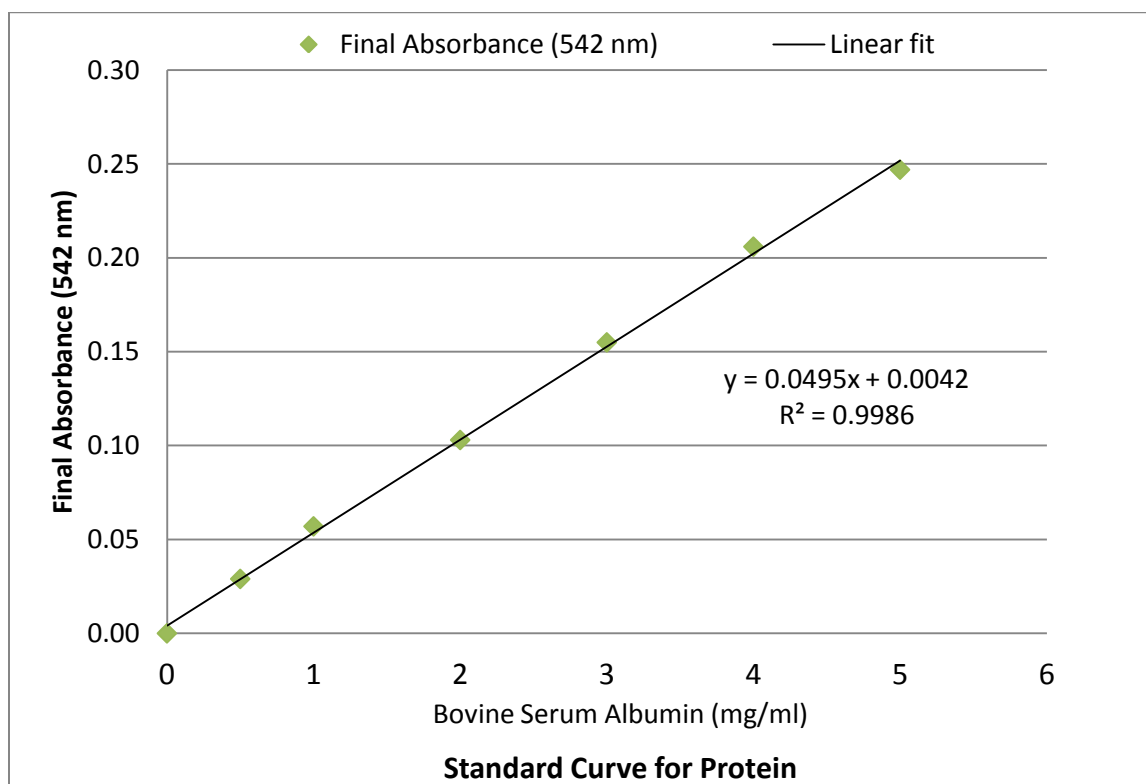


Figure A.1: Standard curve for protein from bovine serum albumin. Graphical representation of results from table A.1.

## APPENDIX B

### STANDARD CURVE FOR IRON

Table B.1 shows results of total iron content from standard iron solution (n = 3).

Iron ( $\mu\text{g/ml}$ )	Final Absorbance (562 nm)
0	0.000
1	0.072
2	0.147
3	0.219
4	0.294
5	0.367

Table B.1: Final absorbance of dilutions of reference iron solution (n = 3)

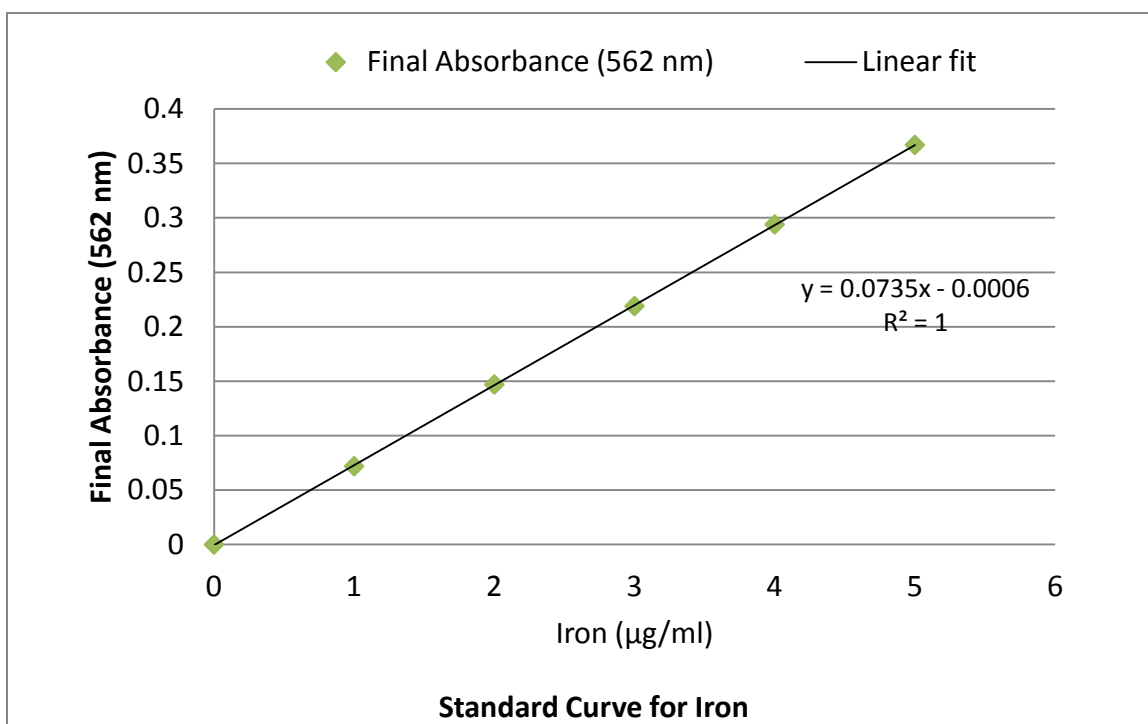


Figure B.1: Standard curve for iron from reference iron solution. Graphical representation of results from table B.1.

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