

METHODS TO DETECT AND QUANTITATE SPLEEN
ADDED TO GROUND BEEF

by

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1.0 INTRODUCTION

1.1 Background

The inclusion of "variety meats" or non-skeletal tissues in certain meat products has caused a great deal of consumer concern and public controversy. On December 23, 1972 the USDA published a proposed standard to ban meat by-products from frankfurters, bologna and similar cooked sausage products. An excellent editorial article in the February 3, 1973 issue of The National Provisioner (Anonymous, 1973) presents excerpts from numerous letters sent to the USDA hearing clerk by consumers supporting the proposed ban. It is apparent that many consumers object to "variety meats" and other animal tissue types for reasons of esthetics and fear that they are being fed these materials in disguised form, regardless of label declarations to the contrary. Concern is centered on the fact that the individual tissue types are not readily identified or recognized in meat items manufactured by grinding, chopping or emulsifying one or several animal tissues to yield a final product.

Discussing the comments (on the proposed ban of by-products) received by the USDA, Yeutter (1973) summarized a number of considerations that were submitted in support of provisions to permit the continued use of "by-products" or "variety meats" in sausages:

1. By-products are nutritious and wholesome. Analytical data show that sausages containing non-skeletal meat products are nutritionally equivalent and frequently superior to similar products that contain only skeletal meat.

2. A ban on by-products would increase the selling price of sausages, which would most affect low income families and further reduce their access to nutritionally beneficial protein foods.
3. The present opposition to the use of by-products is based on esthetics. Standards should not be used to control matters of esthetics. Such judgments should be made by individual consumers through informative labeling.
4. A ban on by-products would greatly stimulate competition for muscle meat and aggravate an already serious scarcity problem.

The current definition of many meat products specifically limits their composition to the skeletal muscle of one or several animal species. Examples of products so defined are: ground beef, hamburger, frankfurter, hot dog, wiener, vienna, and bologna. The presence of any non-skeletal or organ tissue in these products is prohibited.

Certain non-skeletal tissues are permitted in frankfurter, hot dog, wiener, vienna, bologna, and similar cooked sausages that are labeled with the phrase "with by-products" or "with variety meats" in the product name. The specific meat by-products used and their species of origin must be designated individually in the ingredients statement (USDA, 1973).

Splenic tissue is legally classified as a variety meat (USDA, 1973). It is a permissible ingredient of certain cooked sausages under the conditions stated above. However, spleen is prohibited from those products that are not labeled with the proper identifying

phrases. There is no provision for the legal addition of any non-skeletal tissue, including spleen, to ground beef or hamburger.

Although spleen is considered to be a great delicacy by many peoples of the world, it is rarely consumed in any form by people in the United States. In fact, spleen was among the most frequently and strongly objected to variety meats mentioned in the consumer letters referred to above. There is no public information available on the amount of spleen that is used for human food in the U.S.A. Splenic tissue is being included in some potted meat and scrapple products, and a very few "less expensive" hot dogs produced in certain areas of the country.

The major portion of available spleens are currently being processed into tankage and animal feed. As the world food and protein shortage worsens, an increased utilization of this high protein, low fat organ tissue for human consumption may become nutritionally and economically necessary. At that point, as well as for the present, it would be useful to have techniques available for detecting or quantitating the use or misuse of spleen in meat products. The ability to monitor the addition of any by-product should be instrumental in the determination of purchasing standards and the maintenance of confidence in the pricing and labeling of meat products.

1.2 Principles of Spleen Detection and Quantitation

This study was designed to explore the feasibility of modifying and adapting existing knowledge for the development of rapid tests able to identify or quantitate the presence of splenic tissue in meat products.

Ground beef was chosen as the specific product to be investigated because there are currently no provisions for the legal addition of spleen to it. However, ground beef was also viewed as a precursor to sausage and a logical starting point to be investigated before moving on to other more complex products.

The first step in developing a test to detect an organ's presence in a ground or emulsified meat block is to consider the physiological characteristics that contribute to that organ's differentiation, structure or function. The characteristic or indicator of choice should be organ specific, durable, and measurable.

Two of the iron containing compounds of the vertebrate body, hemosiderin and ferritin, satisfy these criterion reasonably well and appear to be useful as organ specific indicators. Under normal conditions of health, these compounds are predominantly found in the spleen, liver and bone marrow. Hemosiderin and ferritin are very durable and easily measured due to the presence of the transition metal iron.

The various iron forms of the vertebrate body can be physically separated or segregated into specific fractions based on their individual characteristics. The quantitation of iron in any fraction provides a measure of that iron form's presence in the original tissue.

This study was based on the assumption that the identification or determination of an organ-specific iron form in the proper fraction derived from a meat product gives an indication of that organ's presence in the product.

Hemosiderin is the only major iron form in the vertebrate body which is insoluble in water or dilute saline. In theory, a procedure based on saline extraction and centrifugation should remove the soluble iron forms from a tissue sample, leaving only hemosiderin iron in the insoluble tissue residue. Any iron measured in a tissue residue after extraction is assumed to be hemosiderin iron. The tissue residue derived from an unadulterated ground beef sample should be essentially iron-free. Therefore, the determination of hemosiderin iron should, under the conditions of this study, indicate the presence of spleen in the original product.

Of the soluble iron forms, ferritin is the only one which remains soluble after heating at 80⁰ C for 5 minutes. After separating the heat coaguable iron compounds from a tissue extract, any iron remaining in solution should be bound to ferritin protein. Under the conditions of this study, the separation of ferritin from a ground beef product should be indicative of spleen addition.

1.3 Objectives

In January, 1973 the USDA awarded this laboratory a grant to develop, or adapt from existing technology, rapid tests for the detection of objectionable inclusions in meat and meat products. The list of objectionable inclusions was far reaching; but the criterion for the test to be developed were very specific. Recommended tests were to discriminate among high, average and low levels of undesirable inclusions with adequate quantitative reliability to provide a definitive basis for acceptance or rejection of a product. Preference was to be given to objective procedures performable by an average

laboratory technician after receiving no more than 2 days instruction per test in a laboratory of a commercial food plant with the expenditure of no more than \$500 for specialized equipment for each test which will yield results within a normal working day.

This study was conducted to explore the feasibility of employing the storage iron compounds, hemosiderin and ferritin, as organ-specific indicators for the detection and quantitation of spleen added to ground beef.

The specific objectives of this research were:

1. To develop and test a method for the quantitative determination of spleen added to ground beef, based on the use of hemosiderin iron as an indicator.
 - a.) Determine the influence of product fat content on the ability to quantitate spleen addition by a method based on hemosiderin.
 - b.) Test a rapid, inexpensive, colorimetric iron measurement technique, using ferrozine as the chromogen.
 - c.) Measure iron by a more rapid and more costly technique, atomic absorption spectroscopy, with emphasis on direct aspiration.
2. To investigate a qualitative spot-test technique for the detection of spleen addition to ground beef, based on the use of ferritin iron as an indicator.
3. To perform a presumptive test for spleen addition on the raw product.

2.0 REVIEW OF LITERATURE

As the largest single collection of reticulo-endothelial cells in the body, the spleen functions as a blood filter, red cell graveyard, antibody production site and, in the bovine, a blood storage area (Frandsen, 1965). During adrenergic shock there is a massive capsular contraction which forces the reserve blood cells into circulation. This has a transfusion-like effect (Greep, 1966).

The reticulo-endothelial system plays a key role in the internal iron economy of the body. It is the primary organ concerned with the catabolism of hemoglobin, the supply of iron to the erythroid marrow, and the storage of iron which is not immediately required for the synthesis of metabolically active compounds. Under normal circumstances the major role is played by the reticulo-endothelial cells of the spleen, liver and bone marrow (Lynch et al., 1974).

2.1 Storage Iron

Biological iron in the mammalian body may be chemically classified into two groups: 1.) Heme compounds, such as hemoglobin, myoglobin, cytochrome, catalase and peroxidase; and, 2.) Non-heme compounds, such as the ferroflavin enzymes, transferrin, and the storage compounds ferritin and hemosiderin (Moore and Dubach, 1962). Most of the body iron is present in circulating hemoglobin (66%) (Lynch et al., 1974); about 3 to 5 percent is in myoglobin, while less than 1 percent is in heme-containing enzymes or is in transit through the plasma attached to transferrin (Bothwell and Finch, 1962). The iron storage compounds account for approximately 25 to 30 percent of iron in the body (Bothwell and Finch, 1962). They are predominantly present in the liver,

spleen and bone marrow, but are also found in smaller amounts in other parts of the body (Weinfeld, 1970; Underwood, 1971).

Storage iron forms a reserve which does not appear to participate in active metabolic processes, but can be drawn upon for the synthesis of functional compounds when needed (Lynch et al., 1974). Harrison et al. (1974) mention a number of physiological factors which emphasize the need for storage iron in higher animals:

1. The essential function of hemoglobin and other iron compounds in supporting life.
2. The advantage of having an internal reserve to protect against sudden losses by bleeding or a change to low dietary iron.
3. The need to provide a source of iron for the fetus during pregnancy.
4. The lack of an excretory mechanism for iron.
5. The relatively coarse control of iron absorption.
6. The tendency of free iron to cause precipitation of proteins and other toxic effects.

The amount of storage iron in the body, its distribution between hemosiderin and ferritin, and its relative concentration within a given tissue is dependent upon a number of factors including the species, sex, age, diet and health of the subject.

There is no complete study of iron metabolism in bovine animals available. Most of the important findings concerning the nutritional physiology of iron have been made with man and small laboratory animals. How far these physiological principles can be accepted as applying

to farm animals, especially ruminants with their markedly different digestive systems, is impossible to say (Underwood, 1966).

Species differences in body iron exist at birth, reflecting differences in liver and spleen iron stores and blood hemoglobin levels. The pig has relatively little iron in its body at birth because it is normally born with low liver iron stores, (Underwood, 1971) and lesser spleen stores (Furugouri, 1973a). By contrast, the newborn rabbit has an exceptionally high body iron concentration due to its large liver iron stores (Widdowson, 1950). Sow's milk is deficient in iron (Furugouri, 1973a), whereas rabbit's milk is higher and rat's milk is exceptionally rich (Underwood, 1971).

Female rats have a higher total body iron content than males. They accumulate iron in their livers as hemosiderin and ferritin about twice as rapidly as males on the same diet from 6 to 16 weeks of age (Vidnes and Helgeland, 1973). Female mice also carry greater concentrations of liver iron than males, but no such sex difference is apparent in rabbits or guinea pigs (Widdowson and McCance, 1948). Data on possible sex differences in iron of the larger farm animals have not appeared (Underwood, 1971).

The main factor affecting the relative distribution of iron between ferritin and hemosiderin in mammals is the total storage iron concentration (Underwood, 1971). It is generally stated that, under normal physiological conditions, there is a slight preponderance of ferritin iron over hemosiderin iron in storage. With increasing concentrations of iron this ratio is reversed and hemosiderin increases relative to ferritin (Bothwell and Finch, 1962; Moore and Dubach,

1962; Harrison et al., 1974).

Experimenting with rabbits, Shoden et al. (1953) found that, as iron loading was increased, hemosiderin stores predominated. All storage iron beyond a certain point, determined to be 200 mg of ferritin iron per 100 g of tissue in rabbit spleen and liver, was present as hemosiderin. The concentration of iron was reported to be greater in spleen than in liver. Similar results for human tissues were also reported in this study.

Studying the relative distribution between ferritin and hemosiderin in the splenic and hepatic storage iron of 130 human necropsies, Morgan and Walters (1963) found that in normal subjects somewhat more than half of the storage iron was present as ferritin. With total storage iron less than 500 μg per gram of tissue, more iron was stored as ferritin than hemosiderin, and with values above 1,000 μg per gram more was stored as hemosiderin.

The ratio of ferritin to hemosiderin iron is also affected by the rate of storage (Underwood, 1971). In iron loading or supplementation experiments, this rate is affected by the method and site of introduction as well as by the amount and form of iron administered.

Feeding pigs a basal diet supplemented with ferrous sulfate to provide levels up to 7,102 ppm of dietary iron, Furugouri (1972) found that total iron, non-heme iron, hemosiderin iron, and ferritin iron in the liver increased linearly as the amount of dietary iron increased. Ferritin iron content for the animals fed the elevated levels of iron was "markedly" greater than the hemosiderin iron content. The author noted that this relationship is peculiar to pigs.

In a later study of pigs from birth to 30 days of age, the same author (Furugouri, 1973a) found that iron supplementation by intramuscular injection of iron-dextran resulted in a rapid increase of non-heme iron in spleen and liver, with hemosiderin iron increasing to a "much greater" extent than ferritin iron. These results were consistent with those obtained by Shoden et al. (1953) with intravenous injections of saccharated iron oxide into rabbits. Furugouri (1973a) concluded that these discrepancies may be due to the difference in iron supplementation methods.

Under normal circumstances most of the visible (hemosiderin) iron is present in reticulo-endothelial cells; only small amounts are present in parenchymal cells (Wintrobe, 1967). The deposits in the reticulo-endothelial system are largely derived from the breakdown of red cells and hemoglobin, while parenchymal stores appear to derive their iron from transferrin (Bothwell and Finch, 1962).

Studying the liver, spleen and kidney of rats, Yoneyama and Konno (1953) found spleen to be the richest in non-heme iron and hemosiderin to be the predominant form in that organ. Drysdale and Ramsay (1965) reported that ferritin accounted for 79 percent of the storage iron in liver, but only 27 percent of that in spleen of mature male rats. The total storage iron of spleen was nearly five times greater than that of liver. Vidnes and Helgeland (1973) state that no previous attempts to purify hemosiderin from normal liver had been reported. They speculated that this was due to the very low content of hemosiderin in this tissue. They also noted the possibility that hemosiderin in normal liver contains less iron than hemosiderin from normal spleen.

Furugouri (1973a) found more total storage iron and more hemosiderin iron in spleens than livers of 30 day old pigs. In a later study of 60 kg pigs, Furugouri (1973b) reported slightly more storage iron in liver than spleen. Ferritin was dominant (60%) in liver; whereas hemosiderin was greatest (59%) in spleen.

Standish et al. (1969) microscopically observed iron stained sections of livers and spleens from beef calves (steers) fed graded levels of dietary iron for 84 days. A lesser amount of hemosiderin was observed in the liver than the spleen at each level of dietary iron. Small and moderate amounts of hemosiderin were observed in sections of spleens from animals fed 0 and 400 ppm iron, respectively. Liver sections of the animals fed 0 or 400 ppm iron rations did not contain hemosiderin. Much hemosiderin was seen as large accumulations in the spleens of cattle given 1,600 ppm iron diets, whereas liver sections from these same animals contained only a moderate amount.

Noting that the iron content of almost all tissues studied increased as dietary iron was increased, Standish et al. (1969) suggested that the mucosal block mechanism of the abomasum, if one exists in ruminants, was overcome by as little as 400 ppm supplemental iron.

Standish et al. (1971) found that increasing the phosphorous content from 0.23 to 0.46 percent in a 1,000 ppm iron diet caused a 50 percent decrease in microscopically observed hemosiderin of spleens and livers from beef calves fed for 77 days.

Iron absorption as well as the total iron status of a bovine animal depends upon the amount of iron in the diet (Standish et al.,

1969), the chemical form of the iron present (Ammerman et al., 1967), the quantity of other elements present (Standish et al., 1971), and the amounts and proportions of other components in the whole diet (Underwood, 1966). These facts can, in part, account for the wide range of iron content reported for bovine tissues under varied experimental conditions. Listed in Table 1 are the ranges of iron content reported by several authors. Although no author gives specific information as to the form of iron present in any tissue, it can be observed that the greatest amounts and ranges of iron were reported for those tissues known to hold the major portion of storage iron.

2.2 Ferritin

2.21 Characteristics

Ferritin serves to remove and store unneeded iron as well as to provide a source of iron which can be drawn on when required. It is an iron-protein complex with a well-defined iron-free protein moiety, apoferritin, forming a hollow, nearly spherical, shell which surrounds a core of six iron micelles (Underwood, 1971). The iron is present in the micelles as a hydrous ferric oxide-phosphate complex with an approximate composition of $(\text{FeOOH})_8\text{FePO}_3\text{H}_2$ (Granick, 1946). Ferritin is readily identified in electron micrographs as a characteristic cluster of four dots, representing the iron micelles, which can be seen without staining or shadowing (Bothwell and Finch, 1962). For this reason ferritin is frequently used as a marker in electron microscopy (Harrison et al., 1974).

The iron content of ferritin is usually quoted as being about 20 percent by weight; however, ferritin as isolated from animal tissues

TABLE 1. TOTAL IRON CONTENT OF BOVINE TISSUES

	<u>SPLEEN</u>	<u>LIVER</u>	<u>KIDNEY</u>	<u>HEART</u>	<u>MUSCLE</u>
Ammerman et al. (1967) Fe, ppm dry wt.	1,067	233	479	223	85
Standish et al. (1969) Fe, ppm dry matter basis	1,219 2,671 8,941 5,479	185 269 605 496	315 360 410 326	291 291 329 287	91 81 98 87
Standish et al. (1971) Fe, ppm dry matter basis	1,521-6,725	256-552	320-400	257-327	73-94
Ammerman et al. (1974) Fe, ppm dry matter basis	750-2,763	227-445	331-358	251-261	127-152
Clement et al. (1972) steak Fe, ppm wet basis veal					30 28.6

consists of a mixture of molecules of continuously varying iron content (Weinfeld, 1970).

Iron can be removed from ferritin by chemical reduction of the ferric iron to the ferrous state. This has been accomplished with a number of reductants and several chelating agents (Harrison et al., 1974): The remaining iron-free apoferritin is not denatured or altered, but maintains the same electrophoretic mobility, isoelectric point, and immunochemical properties as iron-bearing ferritin (Mazur and Shore, 1950).

The protein portion of ferritin is currently thought to be a molecule consisting of 24 structurally identical subunits, each with a molecular weight of $18,500 \pm 500$. This leads to a best estimate of 445,000 for the molecular weight of the apoferritin monomer (Harrison et al., 1974).

Ferritin synthesis is induced by iron. This has been demonstrated in rat and guinea pig liver in vivo (Fineberg and Greenberg, 1955; Loftfield and Eigner, 1958), and in vitro in rat liver slices (Yu and Fineberg, 1965).

Apoferritin is thought to have catalytic ferroxidase activity which enables it to assimilate iron even when this element is present at very low concentrations. Thus, ferritin protein is not simply a passive covering for iron, but takes an active part in its accumulation (Harrison et al., 1974).

Ferritin is soluble in water and dilute salt solutions over a wide range of pH (Drysdale and Ramsay, 1965), and remains soluble after heating to 80° C (Granick, 1942). This red-brown protein is

precipitated by half saturation with ammonium sulfate (Gabrio et al., 1953), and crystallizes in the presence of zinc or cadmium sulfate (Granick, 1942). The stability of ferritin during frozen storage is mentioned by Gabrio et al. (1953) and implied by a number of investigators who have isolated and studied it from previously frozen samples (Morgan and Walters, 1963; Drysdale and Ramsay, 1965; Torrance et al., 1968).

2.22 Separation and Measurement

Ferritin is most commonly prepared for analysis by separation from tissues and other iron forms on the basis of its solubility and heat stability. The tissue is initially homogenized or otherwise treated with several volumes of water or dilute saline. If hemosiderin is to be measured separate from other iron forms, the homogenate is centrifuged at this point (Gabrio et al., 1953). The resulting supernatant fluid or original homogenate is heated to between 70 and 80⁰ C for 5 minutes. Hemoglobin and other proteins are precipitated during the heating; but the ferritin protein remains in solution (Bothwell and Finch, 1962). The coagulated proteins are removed from the ferritin-containing solution by filtration or centrifugation.

Gabrio et al. (1953) produced heat supernatant fluids that were free of hemoglobin by heating at 75⁰ C for 5 minutes. Granick (1942) noted that the required heating varies with the concentration of protein and the rate of heating.

Ferritin can be measured in the "heat supernatant fluid" by direct iron analysis or by immunochemical techniques (Gabrio et al., 1953).

It is more often measured after precipitation or concentration.

This has been accomplished by antiserum precipitation (Torrance *et al.*, 1968), heating to 100° C for 30 minutes (Yoneyama and Konno, 1953), or by half saturation with ammonium sulfate (Granick, 1942), followed by centrifugation.

The iron of the precipitated ferritin has been determined by a number of methods following its extraction by either the pyrophosphate method of Brückmann and Zondek (1940) or acid hydrolysis (Drysdaile and Ramsay, 1965; Torrance *et al.*, 1968).

Ferritin has also been estimated by submitting a concentrated aliquot to paper electrophoresis. The paper is stained for iron and the color is quantitated with a densitometer by comparison to standards (Bothwell and Finch, 1962).

2.23 Staining

Non-heme iron is often stained by the Prussian blue reaction. In this technique the iron is dissolved from its protein by hydrochloric acid and then reacted with potassium ferrocyanide to form a blue precipitate, ferric ferrocyanide (Humason, 1967). Iron that gives a positive Prussian blue test without the previous application of oxidizing agents must be in the ferric state (Richter, 1960). The iron present in hemosiderin granules and ferritin is in the trivalent state. Normal hemoglobin will not show a positive Prussian blue reaction because the iron is masked. The organic part of hemoglobin must be destroyed in order for the iron to react (Humason, 1967).

Ferritin takes the Prussian blue stain, but is too finely dispersed in most cells to be demonstrated by histochemical techniques evaluated with the light microscope (Underwood, 1971). When cells containing

excessive amounts of ferritin are stained, the ferritin can be visualized as a blue flush. Pure preparations of ferritin give a positive Prussian blue reaction (Bothwell and Finch, 1962).

2.3 Hemosiderin

2.31 Microscopic Observation

Hemosiderin, the second iron-containing storage complex, is easier to study because its golden-yellow granules, which range in size from a fraction to about 10 microns, can be visualized with the light microscope. Since these granules contain clusters of ferric hydroxide, they develop an intense Prussian blue when stained with potassium ferrocyanide.

A common method of assessing body iron stores employs the microscopic observation and grading of the hemosiderin present in fresh or stained bone marrow sections or smears (Weinfeld, 1970). Morgan and Walters (1963) reported an approximate agreement between stainable and chemically measured storage iron in human livers and spleens. Standish et al. (1969) microscopically observed increased hemosiderin in spleens and livers from cattle fed high levels of dietary iron. Richter (1960) monitored the fractionation of hemosiderin microscopically.

2.32 Separation and Measurement

The separation of hemosiderin from tissues and other biological iron forms is based on its insolubility. Most procedures are initiated by homogenizing the tissue of interest in several volumes of water or dilute saline. This is followed by centrifugation at forces ranging from 225 x g (Ludewig and Glover, 1966) to 72,000 x g (Drysdale and

Ramsay, 1965). Ferritin and hemoglobin are present in the supernatant fluid while the insoluble hemosiderin remains in the pellet of tissue residue (Gresham et al., 1971). The pellets are usually washed one or more additional times, followed by centrifugation, to insure complete removal of the soluble iron present.

Employing a centrifugal force of 1400 x g for 30 minutes, Gabrio et al. (1953) found that two water extractions of tissue, either high or low in iron content, removed 98 percent of the extractable iron from rat liver. Increasing the amount of water did not affect the extraction. They also determined that 94 to 97 percent of the hemosiderin was present in the tissue pellet. Clement et al. (1972) reported a 93 percent extraction of soluble iron from muscle tissue under similar circumstances.

The iron remaining in the pellet after extraction is often referred to as "insoluble iron" and is arbitrarily defined as, or assumed to be, hemosiderin. Many authors have reported values for the hemosiderin level of tissues based upon the chemical or radioactive iron content of the tissue residue remaining after extraction and centrifugation without attempting further purification.

Hemosiderin iron has been released from the tissue residue by 2 hours boiling in 1 percent sodium hydroxide (Gabrio et al., 1953), 15 minutes boiling in 0.1 N hydrochloric acid (Drysdale and Ramsay, 1965), and wet ashing in sulfuric and nitric acids (Torrance et al., 1968).

Fulton and Ramsay (1960) and Morgan and Walters (1963) reported hemosiderin values which they derived by subtraction of the ferritin

iron value from the total non-heme iron value determined for the sample.

Several authors have heated the initial tissue homogenate to 80⁰ C and then centrifuged. A separation of this type places ferritin in the supernatant fluid. Hemosiderin and the hemoproteins are in the heat coagulated pellet. The non-heme iron of the precipitate is then extracted by the pyrophosphate technique of Brückmann and Zondek (1940) and estimated by colorimetry or other methods of analysis. The hemosiderin iron values presented by Yoneyama and Konno (1953) and Furugouri (1973a) were determined by this method.

2.33 Characteristics

Hemosiderin is a poorly defined, amorphous compound of variable size and chemical composition (Weinfeld, 1970). It is primarily an insoluble agglomerate of hydrous iron oxide and assorted organic constituents.

The amount and form of iron and other compounds determined for a hemosiderin preparation is influenced by many factors. These include: the physiological iron balance and species of the donor animal (Underwood, 1971), the form of any administered iron, the rate of iron accumulation in storage (Harrison et al., 1974), the age of the granules, and the derivation of iron i.e., whether the hemosiderin resulted from the degradation of hemoglobin or the transformation of other iron compounds (Richter, 1960). It has also been demonstrated that the apparent properties of hemosiderin are dependent upon the method of purification (Ludewig, 1957), and that the hemosiderin from a single spleen may be fractionated into hemosiderins of different chemical composition (Ludewig and Franz, 1970).

While developing the first method for the quantitative separation of tissue ferritin, hemosiderin and hemoglobin iron, Gabrio et al. (1953) found that by varying the speed of centrifugation it was possible to change the proportion of hemosiderin and ferritin. They theorized that a graded series of molecular aggregates exist from ferritin to the visible particles of hemosiderin.

Ludewig (1957) qualitatively separated and purified hemosiderin from horse spleens for chemical analysis. Finding variations in the concentrations of iron, nitrogen, phosphorous and ash from different spleens, he concluded that hemosiderin does not have a constant composition. He also noted that hemosiderins prepared by different procedures yield different products. Measuring the rate of removal of iron from hemosiderin with EDTA, Ludewig (1959) indicated that iron may be present in more than one form in hemosiderin.

Although a number of researchers have demonstrated the presence of variable amounts of ferritin in hemosiderin, Shoden and Sturgeon (1960) have proven that hemosiderin is physio-chemically distinct from ferritin. They found the iron content of hemosiderin preparations to be substantially greater than that of ferritin. The iron-nitrogen and the phosphorous-nitrogen ratios were 4 times greater in hemosiderin while the sulfur-nitrogen ratio was twice as great as in ferritin. The magnetic moments of hemosiderin were of a wider and more variable range. When dissolved in mercaptoacetic acid the two compounds produced different light absorption spectra. Additionally, they were able to demonstrate that the presence of porphyrin in hemosiderin was dependent on the manner in which the hemosiderin deposits were estab-

lished; ie., whether by blood destruction or injection of iron preparations.

In a later study, Shoden and Sturgeon (1961) could not find the "tetrad" ferritin micelles in material which was extracted with water prior to examination. They recommended that the term "hemosiderin" be reserved to the water-insoluble tissue iron obtained after extraction of ferritin with water.

A wide range of iron content has been reported for purified hemosiderin. Ludewig (1957, 1959) reported iron values ranging from 19 to 36 percent for horse spleen hemosiderin. Ludewig and Franz (1970) stated that the iron content of hemosiderin fractions increases with the specific gravity of the sedimentation medium. Vidnes and Helgeland (1973), using a more elaborate purification scheme, found the iron content of rat liver hemosiderin to be relatively constant between 7.2 and 8.5 percent. They noted the possibility that hemosiderin in normal liver contains less iron than that from normal spleen.

2.4 Colorimetric Iron Determinations Using Ferrozine

Most colorimetric methods for determining iron have three steps in common: 1.) removal of the iron from the protein to which it is bound; 2.) reduction of the iron to an oxidation state of (II); and 3.) reaction of the Fe(II) with a chromogen to produce a colored complex which is measured photometrically (Plaut et al., 1972). On the basis of sensitivity, versatility, simplicity and cost, ferrozine [3-(2-pyridyl)-5, 6-bis(4-phenylsulfonic acid)-1,2,4-triazine, $C_{20}H_{14}N_4O_6S_2 \cdot 2Na$] became the chromogen of choice in this study.

2.41 Characteristics of Ferrozine

The synthesis of ferrozine (PDT-disulfonate or PDT) was first reported by Stookey (1970). Ferrozine is a light yellow powder which melts with decomposition above 350⁰ C and may be recrystallized from water. This compound reacts with divalent iron to form a stable magenta complex species which is very soluble in water and, in the visible absorption spectrum, exhibits a single sharp peak with maximum absorbance at 562 nm (Stookey, 1970; Carter, 1971; and Plaut et al., 1972). At this wavelength, the molar absorptivity is 27,900 (Stookey, 1970); and the Beer-Lambert law is obeyed to 4 µg Fe per ml, with slight deviation at 5 µg/ml (Yee and Zin, 1971). The magenta Fe (ligand)₃²⁺ species will form completely in aqueous solution between the pH values of 4 and 9 (Stookey, 1970; Plaut et al., 1972), thus making buffer selection less critical (White and Flashka, 1973). Plaut et al. (1972) stated that sodium acetate and ferrozine reagent could be mixed and stored at room temperature for extended periods with no loss in linearity or sensitivity of the subsequent reaction. White and Flashka (1973) reported that aqueous solutions of ferrozine appeared to be stable indefinitely.

The two major advantages of the use of ferrozine are its sensitivity and its low cost (Stookey, 1970). As shown by their molar absorptivities, ferrozine (27,900) holds an advantage over the conventional terpyridyl (22,600), bathophenanthroline (22,150) and 1,10-phenanthroline (11,100) (Carter, 1971). Stookey (1970) predicted the cost of ferrozine to be one half that of 1,10-phenanthroline, while Plaut et al. (1972) found it to be 1/20th the cost of bathophenan-

throline.

2.42 Applications

Since Stookey (1970) first used ferrozine to determine iron in potable water, manual techniques for the measurement of serum iron employing this chromogen have been reported by Carter (1971), Manasterski et al. (1971), and Plaut (1972). Automated procedures using ferrozine for the assay of serum iron and total iron-binding capacity have been developed by Yee and Zin (1971), Carter (1972), and White and Flashka (1973). Attari and Jaselskis (1972) spectrophotometrically determined micro amounts of sulfur dioxide in air and liquid based on the reduction of iron(III) to iron(II) in the presence of ferrozine, followed by measurement of the highly colored iron(II)-ferrozine complex at 562 nm.

Describing the difficulties in the precise quantitative determination of iron in plant materials by existing colorimetric procedures, Chen and Lewin (1972) tested the ferrozine procedure of Stookey (1970) against the commonly used 1,10-phenanthroline method. They concluded that the ferrozine method showed a higher sensitivity with greater simplicity than the 1,10-phenanthroline method and was thus a satisfactory reagent for colorimetric determination of iron in plant materials.

Mosandl (1973) states that all previously known methods for the photometric determination of iron in wine are applicable only after complicated decomposition procedures; but that the selective determination of iron(II) in wine with ferrozine is a simple and very sensitive method.

2.43 Interferences

Stookey (1970) investigated copper, cobalt, calcium, magnesium, lead, silver, molybdenum, aluminum, nickel, zinc, arsenic, manganese, chromium(VI) and chromium(III) and found cobalt(II) and copper(I) to be the only metals of this group forming colored species with ferrozine under the conditions of his test. The alkali metals and the alkaline earths had no effect on the determination of iron. The only anionic interferences were oxalate in concentration over 500 $\mu\text{g/ml}$, cyanide, and nitrite. The latter two were destroyed by heating and did not interfere with the final colorimetry (Stookey, 1970).

Each author reporting the determination of iron with ferrozine has presented a different procedure and combination of reagents. The major points of variation include the concentration of ferrozine, acid strength, reducing agent, and buffer system. All reports agree on the benefits of using ferrozine for iron measurement, however, there are a few points of disagreement which appear to be related to the varied chemical conditions in the measurement system. The major controversy centers around the significance of copper interference.

Employing hydroxylamine hydrochloride as the reducing agent and copper to iron ratios of 1:1, 2:1, and 3:1; Stookey (1970) found the respective error due to copper to be 3, 5 and 15 percent. Using 5 percent variance from expected absorbance as the criterion for interference, he concluded that, although monovalent copper forms a colored species with ferrozine, interferences were at a minimum.

Adding copper to an aqueous iron standard, Yee and Zin (1971) found a 4.2 percent deviation at a copper to iron ratio of 2:1.

Thioglycolic acid was the reductant. They observed the same amount of interference with copper added to sera and concluded that their results essentially agreed with those of Stookey (1970). They were satisfied that, at normal physiologic concentrations of 70-150 μg of Cu per 100 ml, copper interference with the determination of serum iron by ferrozine is less than 5 percent.

Carter (1971), using ascorbic acid as the reductant, reported the error due to copper to be 12, 16, 25 and 32 percent at copper to iron ratios of 1:1, 2:1, 3:1 and 4:1, respectively. He noted that this error could be serious in serum iron determinations if hypercupremia exists as associated with infectious diseases or iron-deficiency anemia. In this study neocuproin was effectively used to complex copper, thereby reducing the severity of error.

Manasterski et al. (1971) also noted that the serum concentration relationship of copper to iron is often greater than 1:1 in certain pathological circumstances and investigated possible interferences at higher levels of copper. Using ascorbic acid as the reducing agent, they found that when the copper to iron ratio exceeded 1:1, the error due to copper in the ferrozine procedure became "formidable". This interference was first overcome by destroying the copper complex with cyanide, and also by complexing copper with ethylenediaminetetraacetic acid (EDTA). Both methods were successful in removal of the copper interference without effect on the very strong Fe(II)-ferrozine complex. Since the addition of a chemical was proven necessary to avoid copper interference, the authors substituted a complexing ligand for copper which is sensitive, specific, and compatible for reaction

in the same medium, bathocuproine sulfonate (BCS). This reagent successfully maintained the sensitivity of the ferrozine procedure without interference from copper, while allowing the quantitative photometric determination of a second important and biologically related trace metal, copper, in the same test solution.

In an automated procedure employing thioglycolic acid as the reductant, Carter (1972) found it unnecessary to utilize either a masking or sequestering agent. He demonstrated that even at copper concentrations four times normal ($\text{Cu:Fe} = 4:1$) the error is only about 2 percent when thioglycolic acid rather than ascorbic acid is used.

Plaut et al. (1972) proposed a method for determining serum iron with ferrozine in which thiourea was employed as the reducing agent instead of the more commonly used reductants. They experienced less interference from copper than previously reported and theorized that the effect might be due to an inability of the thiourea to reduce Cu(II) to Cu(I) .

By comparing results obtained by atomic absorption and by ferrozine for identical serum samples, White and Flashka (1973) determined that normal copper concentrations caused no detectable interference in their procedure. Investigating the effects of abnormally high copper concentrations, they added copper to aqueous solutions and serum pools to yield samples containing $500 \mu\text{g}$ of Cu per deciliter. At this level of copper and with ascorbic acid as the reductant, the authors observed an increase in apparent iron concentration of about $50 \mu\text{g/dl}$. They were able to eliminate the copper interference by adding thiourea immediately after adding the ferrozine reagent. The resulting

Cu(I)-thiourea complex effectively masked the copper against ferrozine. Samples treated in this way showed no absorbance due to a ferrozine-Cu(I) complex. Since it is impractical to determine what copper concentrations might be encountered, the authors recommended thiourea addition as part of the routine procedure. In difference to Manasterski *et al.* (1971), White and Flashka (1973) found EDTA to cause a decrease of about 25 percent in the apparent iron concentration.

2.44 Ferrozine Purity

As previously mentioned, Attari and Jaselskis (1972) used ferrozine in the spectrophotometric determination of micro amounts of sulfur dioxide in air and liquid samples. These authors reported that, as purchased from Hach Chemical Co., the reagent ferrozine was grossly impure and had to be recrystallized from distilled water at least four times. With pure ferrozine samples, their blanks remained low and changed small amounts on standing, while with impure ferrozine their blanks were in the order of 0.1 absorbance unit and changed appreciably on standing. They also found the absorbance of the iron(II)-ferrozine chelate to increase after 5 minutes with the magnitude of change primarily depending on the purity of the ferrozine reagent. Under the conditions of the sulfur dioxide test, it was possible to minimize the absorbance change by the addition of sodium fluoride to the blank and unknown after the development of color. It was concluded that, with sodium fluoride treatment, even impure ferrozine can be used with a reasonable amount of success.

Carter (1971) used the ferrozine reagent of Hach Chemical Co. without further purification and reported the colored complex to remain

stable at room temperature for at least 30 minutes. Yee and Zin (1971) stated that ferrozine reagent purity was excellent (Hach Chemical Co.) and made no mention of absorbance increase with time. Working with an automated system, their samples were read within a constant short time after ferrozine addition and consequently presented no opportunity for absorbance vs. time evaluation. Developing a similar but more rapid automated procedure, White and Flashka (1973) specifically mention that absorbance readings were taken 5 minutes after ferrozine-buffer solution introduction. Again, with the one point automated reading, no influence of time is considered, nor is it necessary under constant conditions.

2.5 Iron Analysis by Atomic Absorption Spectroscopy

2.51 Background

The basic principle of atomic absorption spectroscopy (AAS) is often described as being the inverse of emission methods for determining metallic elements (Kahn, 1968). In all emission techniques, energy is applied to an atomic cloud, causing some of the atoms to be excited to higher energy levels. When these atoms return to their lower energy or ground state, the energy must be released (Slavin, 1968). That portion of this released energy which appears as light is manifest in the form of an emission spectrum which consists of a number of discrete wavelengths.

When energy is transferred to a population of atoms by means of thermal or electrical excitation, as in the various forms of emission spectroscopy, the amount of energy transferred may vary considerably from atom to atom. This results in a number of different excitation

states throughout the population. Emission by these higher and lower energy level atoms results in the radiation of a number of different frequencies (Price, 1972). Thus, the emission spectrum of any given element may be highly complex and variable (Walsh, 1955). An appropriate filtering system is necessary to select the useful radiation. The intensity of this emitted radiation is measured and compared with standards to determine the concentration of the desired element in the sample (Kahn, 1968).

In atomic absorption, the opposite process is employed. The element of interest is not excited, but is merely dissociated from its chemical bonds, and placed into an unexcited, un-ionized ground state (Kahn, 1968). This dissociation is most commonly achieved by spraying a sample solution into a flame (Allan, 1959). The element is then capable of absorbing light radiation at discrete wavelengths equivalent to the energy required to raise the atom from its minimum energy state to some higher level. The required discrete lines of narrow band width are the same lines as would be emitted if the element were excited. Therefore, the atomic cloud is illuminated by a lamp which has a cathode made of the element being sought. Such a lamp emits only the spectrum of the desired element, together with that of the filler gas, neon or argon (Kahn, 1968). A monochromator is employed to isolate and allow passage of the resonance wavelength of interest and reject all others. A photodetector, seeing only the resonance radiation, is used to measure the absorption of light by the atoms of interest in the atomic cloud. Monitoring an excitation wavelength, diminished by sample absorption, is the basis for atomic

absorption spectroscopy (Slavin, 1968).

The theoretical factors governing the relationship between atomic absorption spectra and atomic concentration were first published by Walsh (1955). He showed that atomic absorption spectra provided a promising method of chemical analysis with vital advantages over emission methods. Allan (1959) was the first to use the atomic absorption principle to determine iron. He demonstrated that the most sensitive absorption line for iron is 2483.3 Å. Commercial equipment capable of routine operation was first introduced in 1963 (Kahn, 1968). The development of AAS has made it possible to perform rapid, convenient, and highly accurate analysis for many metallic elements in biological materials with a minimum of chemical pretreatment (Harrison *et al.*, 1968). Detection limits, precision, and accuracy are at useful levels for about 65 elements in a large number of matrices (Kahn, 1968).

2.52 Sample Preparation

The major requirement of samples submitted to flame atomic absorption analysis is that they be a homogeneous, low viscosity liquid (Rowe, 1973). Once a sample is in solution, AAS procedures often require only the dilution of the sample to the optimum analytical range for the element of interest and the preparation of standards for that range (Slavin, 1968). Several authors have reported the direct determination of various elements in samples which characteristically occur as liquids, such as waters, juices, beverages and body fluids (Price, 1972).

The direct determination of iron in a biologic liquid was well demonstrated by Zettner and Mansbach (1965). They analyzed

urine for iron by aspiration with no prior sample preparation. The method was reported to be accurate, rapid and relatively free of interference. They also reported that the absorption of iron in aqueous solutions was the same whether the metal was present as simple ferric or ferrous salt, or as an organic chelate.

Investigating the possibility of measuring hemoglobin iron by the direct aspiration of aqueous solutions into the flame, Zettner and Mensch (1967) found that the iron analysis on identical directly-diluted and wet-ashed samples yielded identical values. They concluded that toluene hemolysates or whole blood required no treatment before analysis except dilution with water. The technique was recommended on the basis of accuracy, high precision, speed and convenience.

Van Assendelft et al. (1968) have since suggested that both the presence of inorganic substances and the handling of blood samples influence the results obtained by direct analysis of hemoglobin iron to a degree which makes it unsatisfactory for the establishment of the cyanmethemoglobin extinction coefficient (E_{540}^{HICN}).

Sprague and Slavin (1965) determined serum iron directly by atomic absorption after diluting the serum 1:1 with water. Noting that direct aspiration of serum (or a dilution thereof) into the flame gave erroneous results due to the high protein content and the impossibility to distinguish between serum ferric (transferrin) iron and hemoglobin ferrous iron; Zettner et al. (1966), Olson and Hamlin (1969), and Tavenier and Hellendoorn (1969) investigated procedures to eliminate these analytical errors and improve sensitivity. Zettner et al. (1966) freed ferric iron from serum with 1.0 N HCl and thiogly-

colate. After deproteinization with trichloroacetic acid (TCA), the freed iron was chelated with bathophenanthroline and extracted into a concentrating volume of methylisobutyl ketone prior to aspiration. The method was reported to be precise (better than 1 $\mu\text{g/ml}$), sensitive and free of interferences. Olson and Hamlin (1969) investigated a technique wherein equal volumes of serum and 20 percent TCA were mixed and heated at 90⁰ C for 15 minutes, followed by centrifugation. They reported the method to be highly specific, accurate, and more rapid than colorimetric or atomic absorption methods employing chelation and extraction.

A large number of pretreatments have been reported for the measurement of trace elements in plant and animal tissues. Although the literature on sample preparation is very extensive and often conflicting, it has been implied that any method by which the element of interest is quantitatively removed to a low viscosity liquid phase constitutes an acceptable pretreatment (Rowe, 1973). The choice of pretreatment is based on the type of sample, the element to be analyzed, its concentration, and the precision and accuracy needed (Christian and Feldman, 1970).

Simpson and Blay (1966) developed a method for the elemental analysis of canned foodstuffs based on hydrochloric acid hydrolysis. They boiled a 5 g sample for 5 minutes with 6 N or 8 N HCl. This was followed by dilution and filtration. The resulting solution was analyzed for calcium, iron, copper, tin and zinc directly in the flame atomizer. The method was reported to give excellent results for these elements.

Studying trace elements in plant materials and organic residues, Premi and Cornfield (1968) theorized that organic materials would be destroyed by the flame in atomic absorption analysis. Thus, prior destruction of organic matter by dry-ashing or digestion with strong oxidizing acid mixtures would not be necessary, provided that the elements of interest could be quantitatively extracted from the sample. The authors tested a procedure wherein plant materials and organic residues were boiled in 6 N HCl for 15 minutes followed by filtration, washing and dilution. The elements were determined directly in the filtrate by aspiration into the flame. By comparing the results obtained with their HCl method to those determined by a nitric-perchloric wet-ashing technique, they concluded that complete recovery of copper, zinc, iron, manganese and chromium could be achieved by boiling the tested materials in 6 N HCl. This procedure allows considerable saving of time when compared with dry or wet-ashing methods.

Stating that dry or wet-ashing techniques are complicated and extremely tedious for red meat samples, Rowe (1973) recommends the HCl hydrolysis method as the simplest and most useful. He also noted that platinum crucibles are destroyed by high-iron samples such as red meats.

2.53 Comparison to Colorimetry

Relatively few authors have directly compared the determination of iron by colorimetric and atomic absorption procedures. None of those reporting has employed ferrozine as the chromogen in their colorimetry.

In the first study of iron determination by AAS, Allan (1959) analyzed three different plant samples after wet-ashing. He concluded that, although the method is not as sensitive as some colorimetric methods, its freedom from interferences, rapidity and sensitivity make it particularly convenient and attractive.

In developing a serum iron technique, Zettner et al. (1966) compared a conventional colorimetric method based on bathophenanthroline with their atomic absorption method. They reported "reasonable" agreement between the methods. Atomic absorption presented no apparent advantage over the colorimetric determination.

Bowen (1967) reported a collaborative study by 29 laboratories which compared different techniques for the determination of 40 elements in a standard plant material. The author reported that consistent results for iron were obtained by the laboratories with several different techniques. No significant differences were found between activation analysis, AAS, colorimetry or spectrometry; however, the precision of the atomic absorption method was superior to the colorimetric method. Details of the methods used were not given.

Tavenier and Hellendoorn (1969) measured iron in deproteinized serum by a colorimetric (bathophenanthroline) method and atomic absorption. Comparison of the results showed a "striking agreement".

Evaluating various methods for the determination of iron in hematinic preparations, Tarlin and Batchelder (1970) found that iron-carbohydrate complexes required ashing prior to assay by AAS, whereas, iron chelates or simple salts could be determined directly. The standard deviation of the AAS method was greater than that of the

colorimetric or gravimetric methods used, but the analysis of variance indicated there was no significant difference between the methods.

Studying the determination of iron in low-iron basal diets, Davies et al. (1972) investigated several wet-ashing techniques and measured iron by atomic absorption and two colorimetric techniques: 1,10-phenanthroline and bathophenanthroline. In the comparison study, analysis by atomic absorption and bathophenanthroline were performed on identical digests prepared with nitric and perchloric acids. The authors reported that, even though the two methods yielded essentially the same amount of iron, the atomic absorption method was considerably more variable than the colorimetric method.

Speaking in general, Price (1972) notes that spectrophotometry usually covers a similar, if not smaller, concentration range than atomic absorption with the advantages that its calibration curves shift less, the contents of the measurement cell are static, and its precision and accuracy depend critically upon the quality of the instrument, the chemical separations, and the preparation of the sample.

2.54 Interferences

The literature is in general agreement that iron determinations by AAS in an air-acetylene flame are essentially interference-free. In the initial publication of iron determination by AAS, Allan (1959) found complete lack of interference from Mn, K, Ca, Na, Mg and phosphate. Zettner and Mansbach (1965) investigated the single and combined action of Na, K, Ca, Mg, Cu, phosphate, sulfate, chloride, TCA, thioglycolic acid, sulfosalicylic acid, sodium citrate, glucose and

sucrose, and observed no effect in aqueous solutions. These authors also studied the effects of certain chelators in aqueous solutions and found no effect on absorption by EDTA, desferrioxamine, ammonium pyrrolidine dithiocarbamate, and bathophenanthroline. Zettner et al. (1966) investigated the action of several additional substances when added to aqueous iron solutions and saw no effect from concentrations of up to 1 percent of hydrochloric, phosphoric, nitric, ascorbic and citric acids; and the chelator diethylenetriaminepentaacetic acid. The authors noted that the prolonged aspiration of acids of greater normality than 1.0 resulted in erratic and rising absorption readings, apparently caused by the acid attack on steel parts of the burner.

Although the determination of iron by atomic absorption is free of spectral and chemical interferences, bulk or matrix interferences have been noted. These are changes in the analytical result influenced by the number of atoms actually entering the resonance beam rather than their effectiveness once there. Matrix effects usually arise from differences in the physical properties of the sample which influence their flow or nebulization rate such as viscosity, surface tension, vapor pressure and temperature (Price, 1972).

Organic solvents have been used to increase the sensitivity of various elements including iron. This enhancement of sensitivity is attributed to decreased viscosity, increased flow rate and lower heat of vaporization as compared to water (Christian and Feldman, 1970).

Dissolved solids tend to concentrate solutions, causing them to flow more slowly through the burner. A depressed flow rate is accompanied by a decreased absorption (Kahn, 1968). Zettner and

Mansbach (1965) observed a mild depression only when salt concentrations exceeded 1.5 percent. The effect was attributed to the effect of solids on the aerosol phase. Zettner and Mensch (1967) reported that mild matrix effects were seen only when the total concentration of inorganic solids in aqueous solution exceeded 1 percent. In the serum iron study of Zettner et al. (1966) protein concentrations of less than 500 mg per 100 ml showed a mildly enhancing effect on absorption, whereas, concentrations greater than 1 percent led to moderate absorption depression resulting from the viscosity effect on aspiration and nebulization.

3.0 MATERIALS AND METHODS

3.1 Sample Preparation

3.11 Ground Beef Samples

Once a workable method for extracting the soluble iron from a muscle-spleen mix had been developed, its completeness had to be evaluated. In theory, the insoluble tissue residue remaining after saline extraction of a spleen-added product should contain only hemosiderin iron. The residue from an unadulterated product should be iron-free. Since less than 100 percent removal of iron from muscle sample residues had been experienced in preliminary tests, it was necessary to determine a quantitative value for iron remaining in the residue from representative ground beef samples after treatment by the chosen extraction procedure. These values should produce a base line, above which spleen addition would be indicated.

A total of seven packages of ground beef, weighing one pound each, were purchased from three retail stores in the Blacksburg, Virginia area. The packages were selected to provide a wide range of fat content. This sampling was assumed to provide a cross-section of typical, non-spleen-added ground beef offered for sale.

The contents of each package were mixed until it appeared homogeneous. Small amounts were alternately placed in three lots until the entire block had been equally divided. All samples were frozen in double Saran and plastic bags. One of the three samples was randomly chosen for proximate analysis and the remaining two were retained for analysis by the procedures listed below. Duplicate analysis for each store package were obtained by testing each of the two subsamples.

3.12 Spleen-added Ground Beef Samples

Three 10 lb. lots of coarse broken ($\frac{1}{2}$ in. plate) ground beef were purchased from a local packing house. These were to provide three fat levels, assumed to be 10, 20 and 30 percent.

Packing house personnel randomly selected three bovine spleens from typical market weight animals. A composite spleen preparation was produced by removing the capsule from each organ and homogenizing the combined pulp in a silent cutter.

Weighed amounts of ground beef and spleen, totaling 1,000 g, were combined to yield products with 0, 2.5, 5 and 10 percent spleen in the final mix. A graded series of spleen addition was prepared at each of the three fat levels, yielding a total of 12 products. All samples, including those with no added spleen, were thoroughly mixed in the silent cutter.

Each 1 kg mix was randomly broken into seven subsamples and frozen. One subsample was randomly chosen for proximate analysis and the remaining six were retained for analysis by the procedures listed below. Triplicate analysis for each fat and spleen level were obtained by testing three of the subsamples.

3.2 Apparatus

3.21 Atomic Absorption Spectrophotometer

A Perkin-Elmer Model 403 atomic absorption spectrophotometer was used to quantitate iron. Instrument settings were as follows: iron hollow cathode lamp, operating current 30 mA; analytical resonance line $\lambda = 248.3$ nm, monochromater slit in position 3; flame, air-acetylene.

3.22 Centrifuge

All centrifugations were performed by an International Model UV centrifuge equipped with a No. 279-12 place head and 50 ml cups ($r = 22.5$ cm).

3.23 Spectrophotometer

Colorimetric determinations were made with a Perkin-Elmer, Coleman Model 124 double beam, grating spectrophotometer equipped with a cell programmer and a Perkin-Elmer Model 56 recorder.

3.24 Silent Cutter

The ground beef-spleen mixes, as well as their unadulterated counterparts were homogenized in a Hobart Model 8142 Food Cutter. This machine operates on the same principle as an industrial-size silent cutter or bowl chopper.

3.3 Prepared Reagents

3.31 Saline

The soluble iron forms were extracted with a 0.85 percent (w/v) saline solution which was prepared by placing 17 g of certified A.C.S. grade (Fisher) sodium chloride in a 2 liter erlenmeyer flask and filling to the mark with distilled, deionized water.

3.32 Digestion-Reduction Reagent

The digestion-reduction reagent was prepared in a 1 liter volumetric by dissolving 20 g of thiourea (Baker Analyzed), without aeration, in about 300 ml of distilled, deionized water; adding 81 ml of concentrated (37.8%) A.C.S. certified grade hydrochloric acid (Baker); mixing; and filling to volume with distilled, deionized water. The finished reagent, 2 percent (w/v) thiourea in 1.0 N HCl, was stored in a

refrigerator.

This reagent was used to remove iron from the insoluble tissue residue (Drysdale and Ramsay, 1965; Simpson and Blay, 1966) and reduce it to the ferrous state (Plaut et al., 1972).

3.33 Protein Precipitating Reagent

The protein precipitating reagent was prepared in a 100 ml volumetric by dissolving 35 g of certified A.C.S. grade (Baker) trichloroacetic acid (TCA) in distilled, deionized water and filling to the mark. This 35 percent (w/v) solution of TCA was stored in a refrigerator.

3.34 Buffered Chromogen

The buffered chromogen was prepared in a 100 ml volumetric by dissolving 200 mg of ferrozine (PDT-disulfonate; Nutritional Biochemicals Corp., Cleveland, Ohio) and 35 g of certified A.C.S. grade crystalline sodium acetate (Fisher) in distilled, deionized water and filling to the mark.

This reagent, prepared according to Plaut et al. (1972), was stored in the dark at room temperature. The ferrozine was used as purchased.

3.35 Prussian Blue Iron Stain

Concentrated HCl was added dropwise, with constant mixing, to 5 ml of a 20 percent potassium ferrocyanide stock solution. Acid addition was stopped just before formation of a permanent white precipitate. About 23 drops of HCl are required. This clear yellow stain was prepared daily.

The stock solution, prepared from 4 g of technical crystal

potassium ferrocyanide (Fisher) and 20 ml of distilled, deionized water, was prepared weekly.

3.4 Procedures

3.41 Proximate Analysis

Proximate analysis were performed in duplicate on a randomly chosen subunit from each of the 12 - 1 kg samples in the spleen-addition series and each of the seven store packages. Moisture was determined by lyophilization. Crude protein, ash and ether extract determinations were made on the dry samples by A.O.A.C. (1970) methods.

3.42 Quantitative Test for Spleen Based on Hemosiderin

3.421 Extraction of Soluble Iron

A sample of ground beef was removed from frozen storage, partially unwrapped, and trimmed of ice crystals and any freezer burn with a stainless steel scalpel. Once sufficient area had been cleared, very thin slices were shaved from the frozen sample into a tared, plastic petri dish. A total of 10 g, as determined by a triple beam balance (Ohaus) with a sensitivity of 0.1 g, was collected.

The shaved meat was then quantitatively transferred, with 90 ml of 0.85 percent saline, to a 150 ml beaker which held a teflon-coated stirring bar. The beaker was then placed on a water-powered magnetic stirrer that was submerged in a 45⁰ C water bath. The meat slurry was stirred, with a 3/4 inch vortex, for 20 minutes to allow leaching of the soluble iron forms and melting of the tallow from the insoluble tissue.

Upon completion of stirring, the contents of the beaker were quantitatively, with the aid of additional saline, transferred to 3 polypropylene, conical, 50 ml centrifuge tubes (Kimble No. 58300).

The tubes were then warmed in a 45⁰ C water bath for 10 minutes to promote migration and localization of the fat in the upper zone.

After removal from the water bath, the tubes were centrifuged at 1448 x g (2400 rpm) for 35 minutes. Each centrifuged tube had three zones: a white, hardened fat cap; a red supernatant fluid; and a pellet of insoluble tissue residue. The fat cap was transferred to a 50 ml round-bottom, screw cap centrifuge tube (polypropylene) and retained. The supernatant fluid was carefully decanted and discarded. The pellet contained in each tube was dispersed and thoroughly mixed in fresh saline.

The tubes were then placed in the 45⁰ C water bath for 10 minutes to allow additional iron leaching and fat migration before re-centrifugation 1448 x g for 35 minutes. After re-centrifugation, any fat cap present was removed to the screw cap centrifuge tube, the supernatant was discarded, and the pellet of insoluble tissue residue was retained.

The fat caps had been collected and retained because a small amount of protein-like material was consistently carried up by and trapped in the fat during the saline extraction and centrifugation steps. This protein material had previously been shown, by microscopic observation, to contain hemosiderin when spleen-added samples were being tested. The combined fat cap, contained in the screw cap tube, was extracted of most lipids by vigorously shaking with certified A.C.S. grade petroleum ether (Fisher) and decanting from the insoluble protein. A total of two extractions were used. This extraction was deemed necessary because excessive fat had been observed to interfere in the

later filtration step.

3.422 Acid Hydrolysis: Iron Removal and Reduction

Once the ether had been evaporated from the screw cap tube, the insoluble tissue residues were quantitatively transferred to it from the three conical tubes with a glass rod and a total of 30 ml of the digestion-reduction (D-R) reagent. A reagent blank was prepared by adding 30 ml of the D-R reagent to a clean screw cap tube. An iron standard was prepared by adding 0.15 ml of a 1 mg/ml FeCl_3 in dilute HCl stock iron solution (Harleco Chem. Co.) and 30 ml of the D-R reagent to a second clean tube.

All tubes, caps on, were placed in an autoclave. Steam was injected until an autoclave temperature of 121°C was attained (about 1 minute). At this point the steam was shut off (exhaust closed) and the pressure was allowed to come down over a period of 10 minutes. The tubes were removed from the autoclave and vigorously shaken to disperse the acid-coagulated protein of the tissue pellets. After shaking, the tubes were placed back in the autoclave where they were held at 121°C for 20 minutes. The steam was shut off and the pressure was allowed to drop with the exhaust closed (10-15 minutes). Upon removal from the autoclave, the tubes were shaken and allowed to cool to room temperature. Successful hydrolysis has also been achieved by a one time autoclaving at 121°C for 30 minutes.

The contents of each autoclaved tube were gravity filtered through ashless filter paper (Whatman No. 41) into a 50 ml volumetric flask. Distilled, deionized water was used to wash the tubes and residue on the filter until a total of 50 ml of filtrate had been collected.

3.423 Iron Analysis by AAS

Each filtrate was mixed in the 50 ml volumetric and analyzed for iron by AAS. The filtrates derived from samples containing 10 percent spleen were diluted 1:10 with distilled, deionized water. All other filtrates, including blanks and standards, were aspirated directly into the flame for analysis.

3.424 Iron Analysis by Colorimetry

A 10 ml disposable glass pipet (Corning No. 7079) was used to transfer 10 ml of each filtrate, including blank and standard, to a 50 ml conical centrifuge tube. Five milliliters of 35 percent TCA were pipetted into each tube before centrifugation at 1448 x g for 20 minutes. The deproteinized supernatant was then decanted into another 50 ml conical tube.

A 2 in 1/100 ml disposable glass pipet (Corning No. 7079) was used to transfer 1.50 ml of the deproteinized filtrate to a spectrophotometer absorption cell (cuvette) containing 1.50 ml of the buffered ferrozine reagent. The contents of each cuvette were mixed by inversion five times.

The absorbance (562 nm) of each sample was determined within a period of 5 to 7 minutes after introduction of the iron-containing filtrate into the ferrozine-buffer reagent. The linear optical response of ferrozine to iron had previously been demonstrated by direct atomic absorption analysis of cuvette contents. Therefore, the iron content of each unknown was calculated by comparing its absorbance to the absorbance of the iron standard. The iron content of the standard had been determined by atomic absorption (Sec. 3.423).

3.425 Calculations: Atomic Absorption

The following formula was used to calculate the concentration of "insoluble iron" in the original ground beef sample:

$$\mu\text{g Fe/g sample} = \frac{(C) \times (V) \times (\text{d.f.})}{(W)}$$

where "C" is the concentration of iron in $\mu\text{g/ml}$ in the D-R reagent filtrate, as determined by AAS; "V" is the volume, in ml, of the filtrate (50 ml); "W" is the original sample weight in grams (10 g); and "d.f." is the dilution factor.

The dilution factor, if necessary, is computed as described below:

$$\text{d.f.} = \frac{(\text{Volume of dilute sample in ml})}{(\text{Volume of aliquot taken for dilution in ml})}$$

The concentration of iron expected to be in the spectrophotometer cell can be calculated as follows:

$$\mu\text{g Fe/ml solution in light path} = (\mu\text{g Fe/ml of filtrate, as measured by AAS}) \times (K_1)$$

where K_1 is a constant equal to $1/3$. This value for K_1 is derived from the various dilutions of the original filtrate before it reaches the cuvette:

$$\mu\text{g Fe/ml filtrate} \times 10 \text{ ml filtrate removed to be deproteinized} = \mu\text{g Fe in centrifuge tube}$$

$$\mu\text{g Fe in tube} \div 15 \text{ ml (total volume after TCA addition)} = \mu\text{g Fe/ml deproteinized filtrate}$$

$$\mu\text{g Fe/ml deprot. filtrate} \times 1.5 \text{ ml removed to cuvette} = \mu\text{g Fe in cuvette}$$

$$\mu\text{g Fe in cuvette} \div 3 \text{ ml (total volume in cuvette)} = \mu\text{g Fe/ml in cuvette}$$

$$\text{Thus: } \mu\text{g Fe/ml in cuvette} = \mu\text{g Fe/ml filtrate} \times \frac{10 \times 1.5}{15 \times 3}$$

$$\mu\text{g Fe/ml in cuvette} = \mu\text{g Fe/ml filtrate} \times 1/3$$

$$K_1 = 1/3$$

3.426 Calculations: Colorimetry

The concentration of iron ($\mu\text{g/ml}$) in the cuvette was calculated by this formula (Plaut *et al.*, 1972):

$$\mu\text{g Fe/ml unknown in the cuvette} = \frac{\text{O.D. Unknown}}{\text{O.D. Known}} \times \text{concentration } (\mu\text{g Fe/ml}) \text{ known.}$$

The following formula was used to calculate the concentration of "insoluble iron" in the original ground beef sample:

$$\mu\text{g Fe/g sample} = \frac{(\text{Cc}) \times (\text{K}_2) \times (\text{V})}{\text{W}}$$

where "Cc" is the concentration of iron in $\mu\text{g/ml}$ in the cuvette; "V" is the volume in ml of the original filtrate (50 ml); "W" is the original sample weight in grams (10 g); and "K₂" is a constant equal to 3.

The value for K₂ is derived from the inverse of the dilutions of the original filtrate before it reaches the cuvette; i.e., $K_2 = 1/K_1$. This value is derived as follows:

$$\frac{\mu\text{g Fe/ml in cuvette} \times 3 \text{ ml volume in cuvette}}{\mu\text{g Fe in cuvette}} =$$

$$\frac{\mu\text{g Fe in cuvette} \div 1.5 \text{ ml of deprot. filtrate in cuvette}}{\mu\text{g Fe/ml deprot. filtrate}} =$$

$$\frac{\mu\text{g Fe/ml deprot. filtrate} \times 15 \text{ ml volume of deprot. filtrate}}{\mu\text{g Fe in deprot. filtrate}} =$$

$$\frac{\mu\text{g Fe in deprot. filtrate} \div 10 \text{ ml of original filtrate used}}{\mu\text{g Fe/ml of original filtrate}} =$$

$$\text{Thus: } \mu\text{g Fe/ml of original filtrate} = \mu\text{g Fe/ml in cuvette} \times \frac{3 \times 15}{1.5 \times 10}$$

$\mu\text{g Fe/ml of original filtrate} = \mu\text{g/ml in cuvette} \times 3$

$$K_2 = 3$$

3.43 Qualitative Spot Test for Spleen Based on Ferritin

A sample of ground beef was removed from frozen storage and shaved, with a stainless steel scalpel, into a plastic petri dish as described above (3.421). The 10 g sample of shaved meat was then quantitatively transferred, with 55 ml of 0.85 percent saline, to a 150 ml beaker. The beaker was placed on a magnetic stirrer in a 45⁰ C water bath. The meat slurry was stirred, with a 3/4 inch vortex, for 20 minutes to allow leaching of the soluble iron, including ferritin, from the insoluble tissue.

Upon completion of stirring, the contents of the beaker were gravity filtered through Whatman No. 4 filter paper into a clean 150 ml beaker. Successful results are also achieved if this filtration step is omitted and the entire meat slurry is treated as the filtrate.

The beaker containing the filtrate of soluble iron was placed on a magnetic stirrer in a water bath which was held at 89⁰ C. The filtrate was stirred until it reached 80⁰ C. Stirring was continued for 5 minutes at 80⁰ C. The beaker was then removed from the water bath and placed in a refrigerator for 20 minutes.

After chilling, the filtrate was gravity filtered through two (Whatman No. 4) filter papers (stacked funnels) to free it of the heat coagulated proteins, including hemoglobin and myoglobin. A total of 35 ml of this filtrate, containing any ferritin that was present in the original sample, was collected in a 50 ml conical

centrifuge tube.

Twelve milliliters of a 35 percent TCA solution were added to each tube before centrifugation at 1448 x g for 25 minutes. The deproteinized supernatant was decanted and discarded. The pellet of precipitated protein, which should include any ferritin from the original sample, was retained.

Each pellet of precipitated protein was quantitatively removed to a glass microscope slide. After adding three drops of the Prussian blue iron stain, the pellet was flattened with a glass cover slip. The rate of development and intensity of any blue color that formed in the pellet was observed and photographed.

3.44 Presumptive Test for Spleen on the Raw Product

Small sections were cut from each of the 12 ground beef samples of the spleen-addition experiment and placed on microscope slides. Prussian blue stain was applied directly to the meat. The samples were then observed and photographed.

As stated previously, the Prussian reaction produces a blue color in the presence of trivalent iron as complexed in hemosiderin and ferritin. This stain does not react with the iron of hemoglobin or myoglobin. Thus, any "blueing" of the product would be a result of greater than normal amounts of trivalent iron being present. Under the conditions of this experiment, the source of trivalent iron is known and the blue color is indicative of spleen addition. In uncontrolled situations, this reaction must be evaluated cautiously.

4.0 RESULTS AND DISCUSSION

4.1 Preliminary Observations

Hematoxylin-eosin stained sections and Prussian blue-safranin stained sections were prepared from the liver and spleen of a typical market weight steer killed at the VPI & SU meat laboratory. These sections provided experience in the microscopic observation of stained and unstained hemosiderin, verified its presence in the two organs, and allowed an appraisal of the relative amount of hemosiderin contained in each. An extremely small number of isolated hemosiderin granules were observed in the iron-stained liver sections, whereas a great number of granules and clumps of granules were observed in the spleen sections. These observations are in agreement with those of Standish et al. (1969).

The spleen offers a great abundance of intense red color to anyone wishing to improve upon the natural color of a product such as ground beef. Developing a method for the determination of blood deceptively added to ground beef to conceal excess fat, Hankin (1965) noted that any level of added blood over 4 percent made the meat too moist. The spleen-added samples prepared for this study (Sect. 3.12) demonstrated spleen to be an effective fat disguising agent with the advantage that, at substitution levels as high as 10 percent, it had no significant effect on the visually or tactually apparent moisture level of the products.

4.2 Quantitative Test for Spleen Based on Hemosiderin

4.21 Tests on Unadulterated Ground Beef

The quantity of lean or muscle contained in a ground beef sample is inversely related to the fat content. Since the iron compounds are

predominantly held in the lean portion, it was necessary to test the ability of the soluble iron removal procedure (Sect. 3.421) to bring all unadulterated ground beef samples, regardless of fat level, to a narrow base-line iron value.

4.211 Proximate Composition

The seven store packages, representing typical unadulterated ground beef, were specifically selected to provide a wide range of fat contents. The ether extracts of these samples, as shown in Table 2, ranged from 3.77 to 28.69 percent. (For the remainder of Section 4.21, these seven samples will be collectively referred to as group A.) The three blocks of ground beef purchased for use in the spleen-addition portion of this study, collectively referred to as group B in this section, were to have contained 10, 20, and 30 percent fat. As shown in Table 2, these lots actually contained 8.38, 32.48, and 39.02 percent fat and were thereafter referred to only as fat levels 1, 2, and 3 respectively. Together, these ten unadulterated ground beef samples presented as wide a range in fat content (3.77 to 39.02 percent) as could be expected to be encountered in the market under normal conditions.

4.212 Iron Analysis

Duplicate samples from each of the seven store packages were exposed to the hemosiderin procedure. The iron remaining in the insoluble tissue residue was determined by AAS and PDT. The results of the individual analyses and the means determined for each package by both measurement techniques are listed in Table 3. The mean iron value for the population of seven samples in group A, as determined by AAS, was 2.91 $\mu\text{g Fe/g sample}$ with a range of 2.43 to 3.38 $\mu\text{g Fe/g sample}$.

TABLE 2. PROXIMATE COMPOSITION OF UNADULTERATED GROUND BEEF ^a

<u>Store Sample</u>	<u>Moisture</u>	<u>Ether Extract</u>	<u>Protein</u>	<u>Ash</u>
1	73.80	3.77	19.63	1.25
2	61.76	18.37	17.74	0.93
3	65.46	14.10	18.61	0.92
4	66.05	18.65	14.83	0.73
5	54.49	28.69	15.70	1.54
6	65.21	14.39	18.09	0.80
7	60.40	20.31	17.38	0.94

<u>Fat Level</u>	<u>Moisture</u>	<u>Ether Extract</u>	<u>Protein</u>	<u>Ash</u>
1	69.57	8.38	21.22	1.01
2	51.75	32.48	15.31	0.65
3	46.62	39.02	14.39	0.65

^a All values are expressed as percentage on a wet basis.

TABLE 3. IRON VALUES DETERMINED FOR UNADULTERATED GROUND BEEF
(STORE SAMPLES).^a

Store Sample	Measurement Technique	
	AAS	PDT
1	3.35	3.79
	<u>3.40</u>	<u>3.85</u>
mean	<u>3.38</u>	<u>3.82</u>
2	2.70	2.97
	<u>2.30</u>	<u>2.50</u>
mean	<u>2.50</u>	<u>2.74</u>
3	2.90	3.29
	<u>2.70</u>	<u>3.00</u>
mean	<u>2.80</u>	<u>3.15</u>
4	2.30	2.50
	<u>2.55</u>	<u>2.89</u>
mean	<u>2.43</u>	<u>2.70</u>
5	3.10	3.58
	<u>3.55</u>	<u>3.89</u>
mean	<u>3.33</u>	<u>3.74</u>
6	3.40	3.79
	<u>3.30</u>	<u>3.67</u>
mean	<u>3.35</u>	<u>3.73</u>
7	2.55	2.89
	<u>2.60</u>	<u>2.94</u>
mean	<u>2.58</u>	<u>2.91</u>
MEAN	2.91	3.26
RANGE	2.43-3.38	2.70-3.82
S.E. ^b	0.40	0.46

^a All values expressed as $\mu\text{g Fe/g}$ original sample.

^b Standard error.

The comparable values determined by PDT were: mean = 3.26 $\mu\text{g Fe/g}$ sample; range = 2.70 to 3.82 $\mu\text{g Fe/g}$ sample. The correlation coefficient comparing the AAS and PDT values for these seven samples was .9538.

It was necessary to determine how well the samples of group B fit the population of unadulterated ground beef as defined by the samples of group A. Triplicate samples from each fat level in group B were exposed to the hemosiderin procedure. The iron content of the tissue residue was determined by AAS and PDT. The mean iron value for the 3 fat levels in group B, as determined by AAS, was 2.78 $\mu\text{g Fe/g}$ sample with a range of 2.43 to 3.32 $\mu\text{g Fe/g}$ sample. The comparable values determined by PDT were: mean, 3 levels = 2.97 $\mu\text{g Fe/g}$ sample; range, 3 levels = 2.62 to 3.29 $\mu\text{g Fe/g}$ sample.

Statistical analyses performed on the ten iron values obtained by each measurement technique are summarized in Table 4. The F tests indicated that the iron values determined for the unadulterated ground beef samples were significantly different at the 1 percent level within each measurement technique. A Duncan's multiple range test was performed to locate the differences and determine how the samples of group B fit the population of ground beef defined by the samples of group A.

The Duncan's test showed a high degree of overlap with the values from fat levels 1 and 3 (sample no. 8 and 10, respectively, in Table 4) centrally placed in the distribution. The value determined for fat level 2 (sample no. 9 in Table 4) was at the low end of the range, but it was not different from the preceding four values.

TABLE 4. STATISTICAL SUMMARY OF IRON VALUES FOR UNADULTERATED GROUND BEEF

		<u>Iron Values by AAS</u>									
		F = 11.34** ^a					CV = 7.6384				
Fat Content ^b		3.77	14.39	28.69	8.38	14.10	39.02	20.31	18.37	18.65	32.48
Sample No. ^c	1	6	5	8	3	10	7	2	4	9	
Iron Value ^d		3.38	3.35	3.33	3.32	2.80	2.58	2.50	2.43	2.43	

		<u>Iron Values by PDT</u>									
		F = 7.78**					CV = 6.7216				
Fat Content		3.77	28.69	14.39	8.38	14.10	39.02	20.31	18.31	18.65	32.48
Sample No.	1	5	6	8	3	10	7	2	4	9	
Iron Value		3.82	3.74	3.73	3.29	3.15	3.00	2.91	2.74	2.70	

^a Significant at the 1 percent level.
^b Expressed as percent on a wet basis.
^c No. 1-7 indicate the store samples.
^d No. 8-10 indicate the unadulterated ground beef samples used in the spleen addition study.
 Expressed as $\mu\text{g Fe/g}$ original sample.

Since the objective of this portion of the study was to determine a base line iron value for unadulterated ground beef after soluble iron extraction, a low value is desirable.

By matching the fat content of the individual samples to the iron values as presented in the Duncan's test, it was determined that the amount of iron measured in a sample was not directly related to its initial fat content.

The total range in iron value for the unadulterated samples, considering all individual determinations by both measurement techniques, was 1.6 $\mu\text{g Fe/g}$ sample. This value corresponds to a total range of 0.32 ppm Fe in the original filtrate and 0.11 ppm Fe in the cuvette. A range of this magnitude is probably within the precision limits allowed by the various steps in the procedure. Thus, no real difference in iron value for unadulterated ground beef can be reported.

Coefficient of variation was used to compare the precision of the iron measurement techniques. Iron measurements made on the deproteinized filtrates (Sect. 3.424) with PDT (C.V. = 6.7216) were more precise than those made on the non-deproteinized filtrates (Sect. 3.423) by AAS (C.V. = 7.6384).

4.22 Tests on Spleen-Added Ground Beef

4.221 Proximate Composition

Weighed amounts of the composite spleen had been combined with the ground beef of fat levels 1, 2 and 3 to yield products with 0, 2.5, 5 and 10 percent spleen in the final mix (Sect. 3.12). The proximate composition of the 12 products used in this portion of the

study are reported in Table 5.

The addition of spleen to the ground beef of fat level 1 had no effect on its proximate composition. This can be explained by the similarity between the proximate composition of this ground beef and spleen. The samples in fat level 2 showed a small but progressive increase in moisture and protein, and a decrease in ether extract with increased spleen addition. The samples of fat level 3 demonstrated a greater increase in moisture and protein, combined with a substantial decrease in ether extract.

The changes in composition of the ground beef of fat levels 2 and 3 with spleen addition were caused by the dissimilarities in the composition of the two materials. Considering the compositional data presented by Watt and Merrill (1963), bovine spleen is higher in moisture (76.9%) and protein (18.1%), but lower in ether extract (3%) than the unadulterated ground beef samples of fat levels 2 and 3. Based on this data, the addition of spleen to a high-fat ground beef produces an effect similar to that expected from the addition of lean muscle.

4.222 Iron Analysis

Three samples from each of the 12 products were exposed to the hemosiderin procedure. An iron value was determined for each tissue residue by AAS and PDT. Results of the individual analysis and the means determined for each of the 12 products by both techniques are reported in Table 6. Comparison of the iron values from AAS and PDT for each of the samples and products gives an indication of the agreement between the two techniques. The overall coefficients of

TABLE 5. PROXIMATE COMPOSITION OF GROUND BEEF: SPLEEN ADDITION EXPERIMENT ^a

	<u>Spleen (%)</u>	<u>Moisture</u>	<u>Ether Extract</u>	<u>Protein</u>	<u>Ash</u>
Fat Level 1	0	69.57	8.38	21.22	1.01
	2.5	69.19	9.11	21.33	1.05
	5	69.43	8.55	21.55	1.04
	10	69.51	8.66	20.71	1.03
Fat Level 2	0	51.75	32.48	15.31	0.65
	2.5	52.56	31.38	15.27	0.71
	5	53.16	29.96	15.96	0.74
	10	55.48	27.17	16.52	0.77
Fat Level 3	0	46.62	39.02	14.39	0.65
	2.5	50.69	33.75	15.06	0.74
	5	51.13	32.60	15.30	0.74
	10	54.25	29.37	16.09	0.80

^a All values are expressed as percentage on a wet basis.

TABLE 6. IRON VALUES: SPLEEN ADDITION EXPERIMENT ^a

	PERCENT SPLEEN											
	0%		2.5%		5%		10%					
	AAS	PDT	AAS	PDT	AAS	PDT	AAS	PDT	AAS	PDT	AAS	PDT
Fat Level 1	3.25	3.29	13.25	14.36	22.80	24.97	53.15	52.00				
	3.35	3.23	13.50	14.45	24.40	27.26	55.45	52.14				
	3.35	3.35	13.15	14.10	23.50	26.47	52.00	51.26				
mean	<u>3.32</u>	<u>3.29</u>	<u>13.30</u>	<u>14.30</u>	<u>23.57</u>	<u>26.23</u>	<u>53.53</u>	<u>51.80</u>				
Fat Level 2	2.70	2.64	12.80	13.89	23.80	26.50	52.25	49.50				
	2.35	2.61	12.80	13.87	24.05	26.88	52.25	49.21				
	2.25	2.61	13.25	14.45	24.15	27.05	53.35	49.94				
mean	<u>2.43</u>	<u>2.62</u>	<u>12.95</u>	<u>14.07</u>	<u>24.00</u>	<u>26.81</u>	<u>52.62</u>	<u>49.55</u>				
Fat Level 3	2.70	2.93	12.90	14.27	21.95	24.38	49.95	47.90				
	2.80	3.47	11.80	13.32	21.60	24.00	50.50	49.31				
	2.25	2.61	15.40	16.54	21.70	24.38	49.95	48.88				
mean	<u>2.58</u>	<u>3.00</u>	<u>13.37</u>	<u>14.68</u>	<u>21.75</u>	<u>24.25</u>	<u>50.13</u>	<u>48.70</u>				

^a All values expressed as $\mu\text{g Fe/g}$ original sample.

variation for the 36 iron determinations made by AAS and PDT on the 12 products indicate that the PDT measurements (C.V. = 2.0449) were somewhat more precise than those made by AAS (C.V. = 2.1618).

The mean iron value and standard deviation determined from the individual samples of each of the three fat levels within a spleen addition level are presented in Table 7. The standard deviation of the mean iron value reported for a given fat and spleen level, as measured by either technique, gives an indication of how homogeneous the products were and how repeatable the entire procedure was in separating and measuring the iron from the three separate samples tested for each product.

Table 7 also shows a composite mean iron value and standard error for each of the spleen addition levels. The standard errors determined for each spleen addition level by both AAS and PDT are small in relation to the total iron value at each level. There is no overlap of iron values between the spleen addition levels and the amount of iron measured increased as spleen addition increased.

The data listed in Table 7 are graphically presented in Figures 1-4. The set of iron values determined for fat levels 1, 2 and 3 are presented in Figures 1, 2 and 3, respectively. The composite mean values from all fat levels are presented in Figure 4.

These graphs illustrate that the iron values were linearly related to the spleen addition level within and across all fat levels. In all cases the iron values determined by PDT more closely fit a straight line than those determined by AAS. The AAS values for the 2.5 and 5 percent addition levels appear somewhat depressed, whereas those for

TABLE 7. MEAN IRON VALUES: SPLEEN ADDITION EXPERIMENT ^a

<u>Spleen Added</u>	<u>Fat Level</u>	<u>AAS</u>	<u>PDT</u>
0%	1	3.32 ± 0.06 ^b	3.29 ± 0.06
	2	2.43 ± 0.24	2.62 ± 0.02
	3	2.58 ± 0.29	3.00 ± 0.43
	mean	2.78 ± 0.39 ^c	2.97 ± 0.27
2.5%	1	13.30 ± 0.18	14.30 ± 0.18
	2	12.95 ± 0.26	14.07 ± 0.33
	3	13.37 ± 1.84	14.68 ± 1.70
	mean	13.21 ± 0.18	14.35 ± 0.25
5%	1	23.57 ± 0.80	26.23 ± 1.16
	2	24.00 ± 0.18	26.81 ± 0.23
	3	21.75 ± 0.18	24.25 ± 0.22
	mean	23.11 ± 0.98	25.76 ± 1.10
10%	1	53.53 ± 1.76	51.80 ± 0.47
	2	52.62 ± 0.64	49.55 ± 0.37
	3	50.13 ± 0.32	48.70 ± 0.72
	mean	52.09 ± 1.44	50.02 ± 1.31

^a All values expressed as µg Fe/g original sample.

^b Standard Deviation.

^c Standard Error.

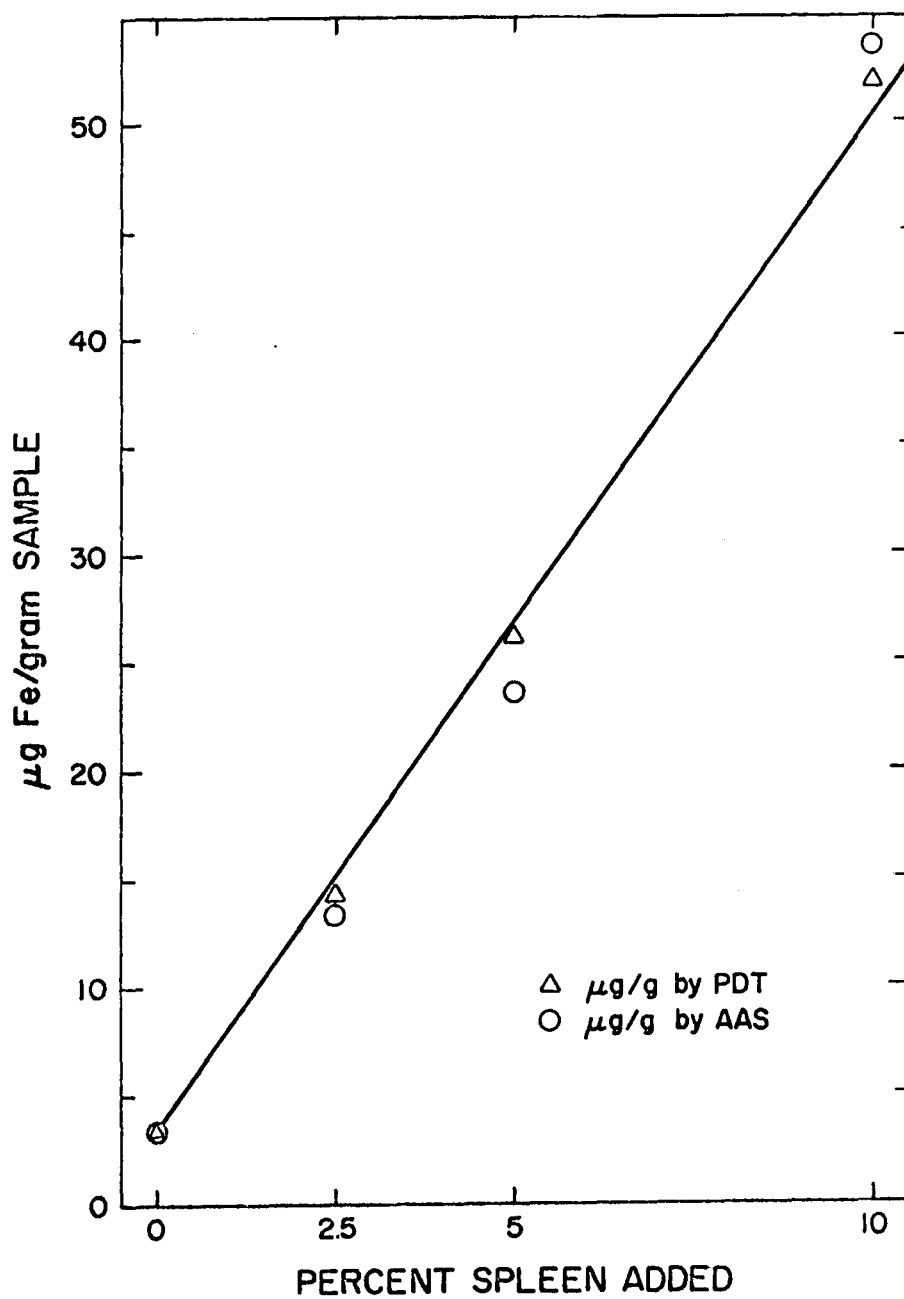


Figure 1. MEAN IRON VALUES FOR EACH SPLEEN ADDITION LEVEL WITHIN FAT LEVEL 1.

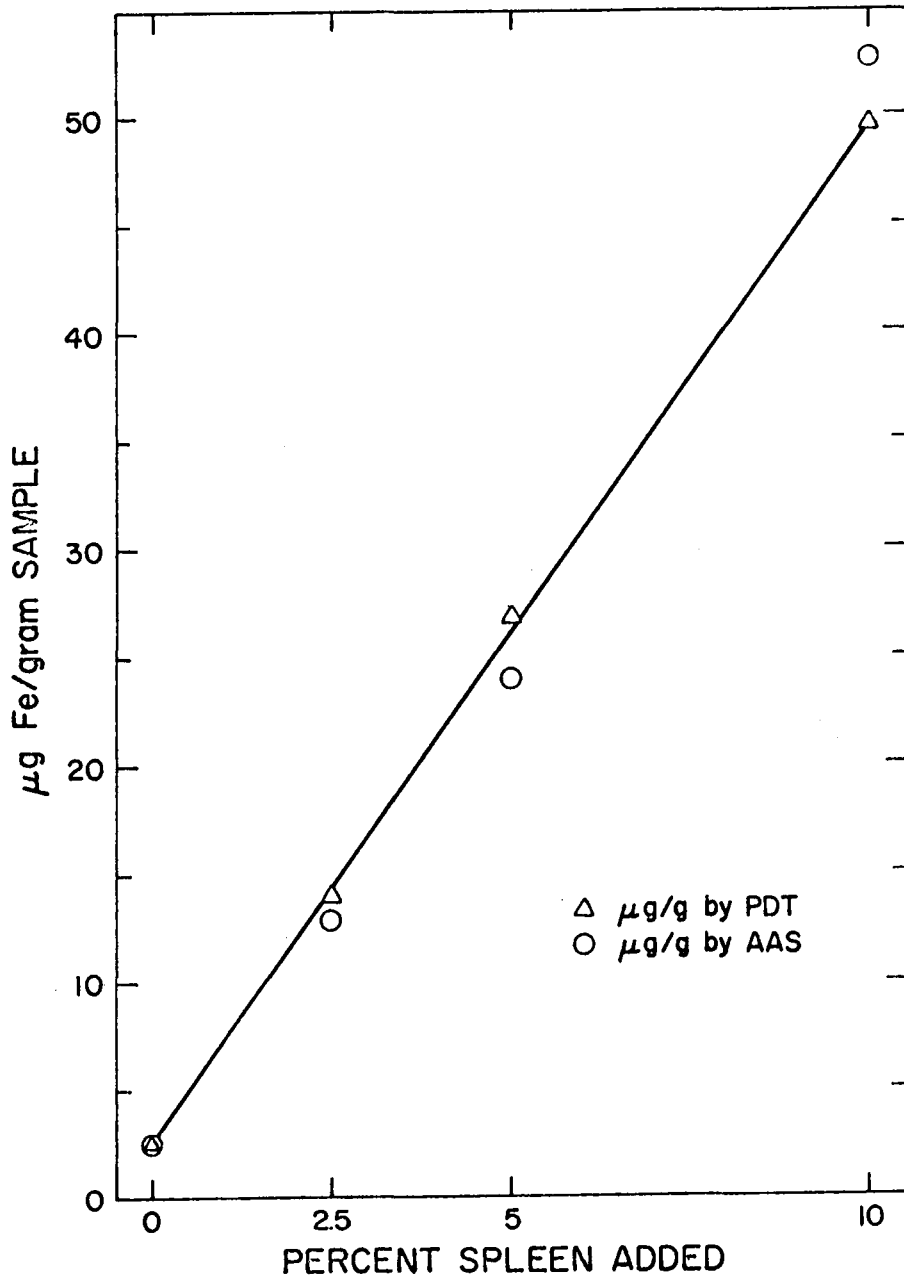


Figure 2. MEAN IRON VALUES FOR EACH SPLEEN ADDITION LEVEL WITHIN FAT LEVEL 2.

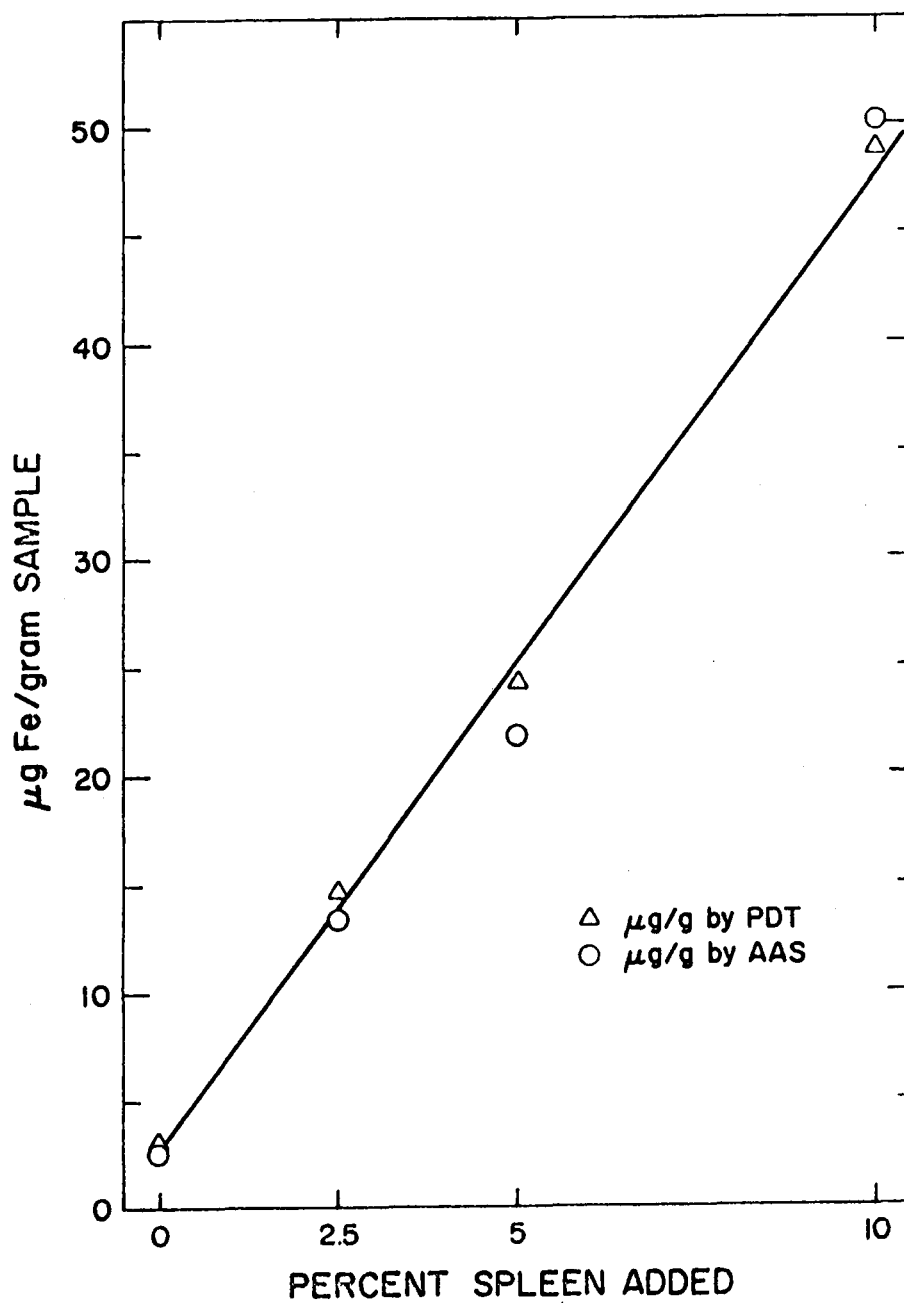


Figure 3. MEAN IRON VALUES FOR EACH SPLEEN ADDITION LEVEL WITHIN FAT LEVEL 3.

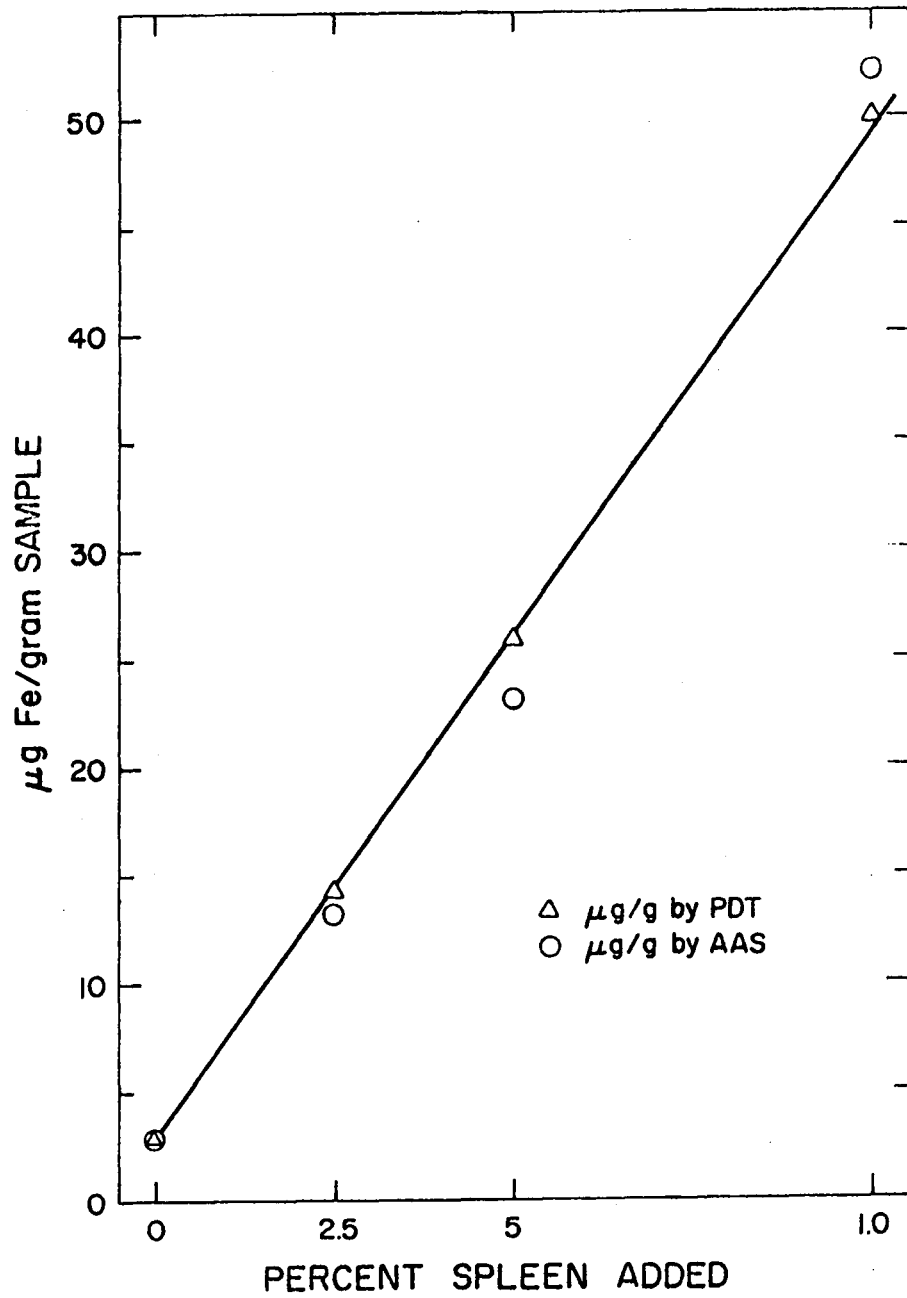


Figure 4. COMPOSITE MEAN IRON VALUE FOR EACH SPLEEN ADDITION LEVEL.

the 10 percent spleen samples appear elevated.

It should be recalled that the AAS values for the 0, 2.5 and 5 percent spleen samples were determined by direct aspiration of the non-deproteinized filtrates and that the filtrates of the 10 percent spleen-added samples were diluted 1:10 before aspiration (Sect. 3.423).

The depression of the AAS values below the PDT values increased as spleen increased from 0 to 2.5 to 5 percent. Very little depression was noted for the unadulterated, ie., 0 percent spleen-added, samples. This progressive depression of the iron values determined for the undiluted filtrates by AAS was probably caused by increased levels of protein in the filtrates of the spleen-added samples. It is possible that the non-saline soluble proteins of spleen are simpler and more easily hydrolyzed to a point allowing passage through a filter than are the non-saline soluble proteins of muscle. Thus, as spleen increased, the protein content of the filtrate increased. Zettner et al. (1966) noted that protein concentrations greater than 1 percent led to a moderate absorption depression resulting from the viscosity effect on aspiration and nebulization.

Since the filtrates of the 10 percent spleen-added samples were diluted 1:10, the protein concentration was reduced, the flow rate increased and the absorption increased. Zettner et al. (1966) also noted that protein concentrations less than 500 mg/100 ml had a mild enhancing effect on iron absorption.

The AAS values were determined by direct aspiration of the non-deproteinized filtrates (Sect. 3.423), whereas the PDT measurements were made after deproteinization of the filtrates (Sect. 3.424).

Considering the matrix effects of protein on AAS, it is probable that the values determined by PDT are more nearly correct. More accurate AAS values may have been achieved if the measurements had been made after deproteinization. However, it was desirable to make the AAS determinations at the first possible point in the procedure before any further dilutions or reagents were introduced.

Although the colorimetric iron determinations by PDT satisfied the inexpensive criterion of this study, it was also desirable to determine if direct AAS readings could be useful to a laboratory with AAS equipment available. The ability to aspirate a non-deproteinized filtrate represents a considerable saving of time and reagents. The results of this study indicate that, even though the iron values determined on the non-deproteinized filtrates were not exact, they were reproducible and within the accuracy required, considering the natural biological variability of the samples.

Statistical analyses were performed to demonstrate that the mean iron values determined for each spleen addition level were different from one another within each of the fat levels. These analyses are summarized in Table 8.

Within each fat level the F value was significant at the 1 percent level for each iron measurement technique, indicating that the iron value was different for each spleen addition level. The Duncan's multiple range tests showed no overlap between spleen levels in any cell. These tests statistically prove that the relation between iron value and spleen addition is real within the samples used in this study.

The coefficient of variation was smaller for PDT than AAS at each

TABLE 8. STATISTICAL SUMMARY OF IRON VALUES FOR THE SPLEEN-ADDITION LEVELS WITHIN EACH FAT LEVEL

Fat Level	Iron Measurement Technique					
	AAS			PDT		
1						
2						
3						

a Significant at the 1 percent level.
 b Expressed as µg Fe/g original sample.

fat level, indicating that the iron measurements on the deproteinized filtrates with PDT were more precise than those made on the non-deproteinized filtrates by AAS.

Statistical analyses were performed to determine if the mean iron values for each spleen addition level were the same at all fat levels. These analyses are summarized in Table 9.

Significant differences in iron value, as determined by both AAS and PDT, were indicated between the fat levels for the 0, 5 and 10 percent spleen-added samples. No difference was detected by either technique for the 2.5 percent spleen samples.

The Duncan's multiple range test on the 0, 5 and 10 percent spleen samples indicated that pairs of fat levels were the same. These pairs were different from a third fat level. However, the pairs that were indicated to be the same changed between the spleen addition levels and the iron measurement techniques within a level.

Although these statistics would appear to indicate that fat level had a variable effect on the ability to measure spleen added to ground beef, careful appraisal of the entire study leads to the conclusion that fat wasn't really a significant factor. Since the differences in iron value between the fat levels within a spleen addition level were small and variable (no difference with 2.5% spleen) in relation to the differences in iron value between spleen levels within all fat levels, it was concluded that the effect of fat on spleen determination was outweighed by the variability of iron in the spleen and the inability to prepare a constant sample each time.

To evaluate the efficiency of the procedure in recovering the

TABLE 9. STATISTICAL SUMMARY OF IRON VALUES FOR EACH FAT LEVEL WITHIN A SPLEEN-ADDITION LEVEL

Spleen (%)	Iron Measurement Technique						
	AAS			PDT			
0	Fat Level	F = 18.53** ^a			F = 5.59* ^b		
	Iron Value ^c	1 3.32	2 2.58	3 2.43	1 3.29	2 3.00	3 2.62
		CV = 8.8739			CV = 7.7110		
2.5	Fat Level	F = —			F = —		
	Iron Value	2 12.95	3 13.37	1 13.30	2 14.07	1 14.30	3 14.68
		CV = 2.8322			CV = 2.5362		
5	Fat Level	F = 33.67**			F = 10.00**		
	Iron Value	2 24.00	1 23.57	3 21.75	2 26.81	1 26.23	3 24.25
		CV = .5361			CV = .8628		
10	Fat Level	F = 8.25**			F = 30.00**		
	Iron Value	1 53.53	2 52.62	3 50.13	1 51.80	2 49.55	3 48.70
		CV = .5250			CV = .2800		

^a Significant at the 1 percent level.

^b Significant at the 5 percent level.

^c Expressed as µg Fe/g original sample.

hemosiderin added to a ground beef sample as spleen, it was necessary to determine an insoluble or hemosiderin iron value for the composite spleen. A 10 g and a 1 g sample of the composite spleen were exposed to the hemosiderin procedure. Due to the high concentration of iron, measurements were made on diluted filtrates by AAS only. The average hemosiderin value calculated for the composite spleen was 530.6 $\mu\text{g Fe/g spleen}$.

By subtracting the AAS composite mean iron value of the unadulterated ground beef (2.78 $\mu\text{g Fe/g sample}$) from the AAS composite mean for the 10 percent spleen-added samples (52.09 $\mu\text{g Fe/g sample}$), the approximate amount of added iron is determined (49.31 $\mu\text{g Fe/g sample}$). This iron was contributed by the 0.1 g of spleen in each gram of the 10 percent spleen-added samples. Thus, 1 g of spleen would be predicted to contain 493.1 $\mu\text{g Fe/g}$. The ratio of 493.1 $\mu\text{g Fe}$ to 530.6 $\mu\text{g Fe}$ indicates that approximately 93 percent of the hemosiderin recoverable from spleen was recovered from the spleen-added ground beef.

4.3 Qualitative Spot Test for Spleen Based on Ferritin

The results of the ferritin spot test performed on the samples of fat levels 1, 2 and 3 are pictorially presented in Figures 5, 6 and 7, respectively. The protein pellets were prepared and stained as described in Section 3.43. In each photograph the first digit denotes the fat level, the second and third digits denote the spleen addition level. For example: 100 is fat level 1 with no spleen, 102 is fat level 1 with 2.5 percent spleen, etc.

After staining with Prussian blue, the protein pellets prepared from the unadulterated ground beef samples maintained a white to

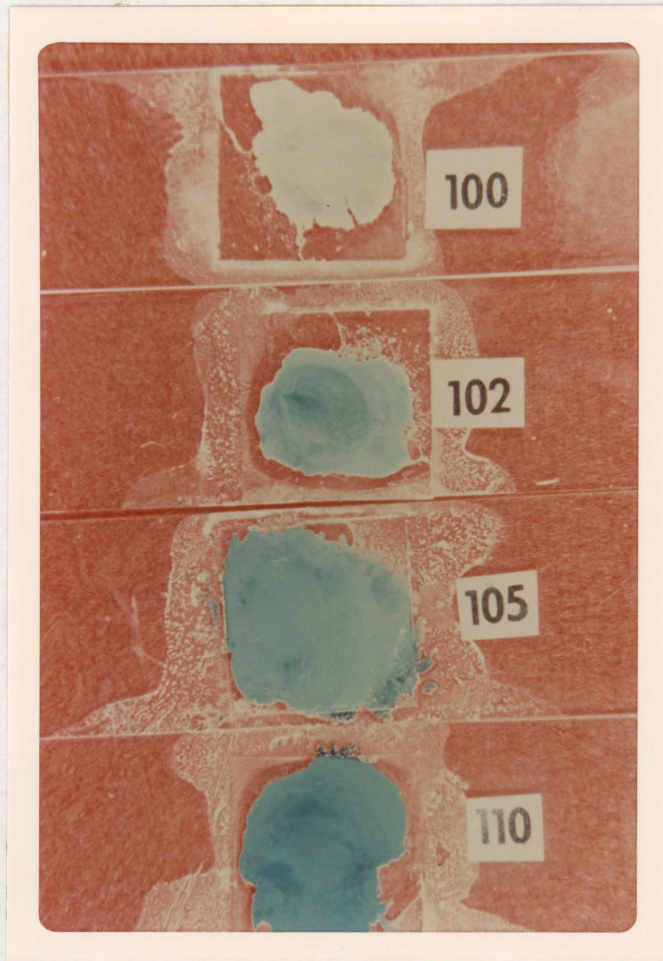


Figure 5. FERRITIN SPOT TEST ON THE SAMPLES OF FAT LEVEL 1.

Note: Due to the technical difficulties encountered in color printing from slides, this print contains a gray overcast as observed on the background for the numbers. Yet, a progressive change in blue color can be seen with increased spleen addition.

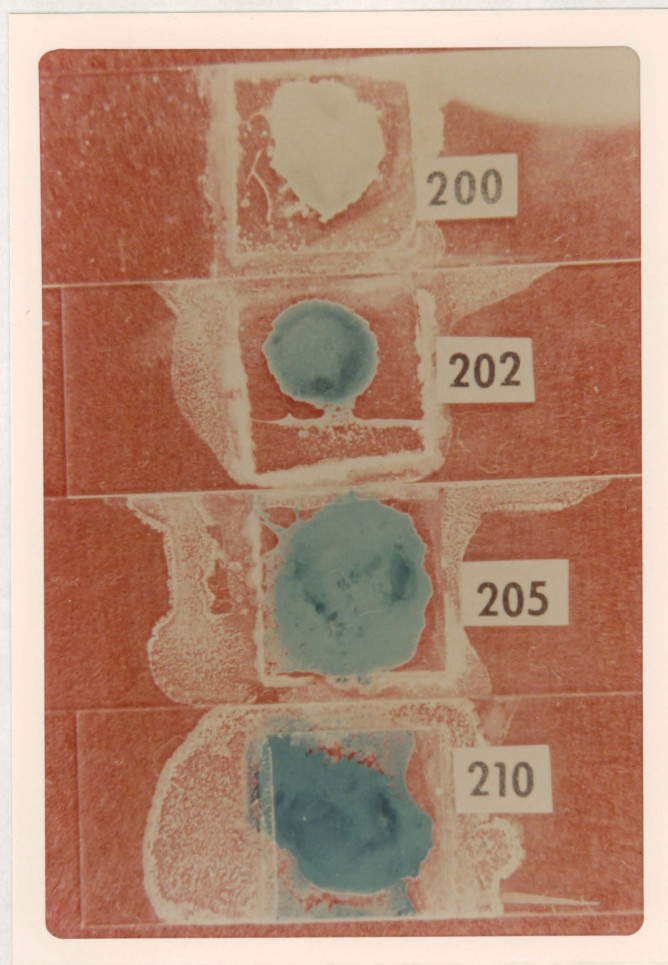


Figure 6. FERRITIN SPOT TEST ON THE SAMPLES OF FAT LEVEL 2.

Note: Due to the technical difficulties encountered in color printing from slides, this print contains a gray overcast as observed on the background for the numbers. Yet, a progressive change in blue color can be seen with increased spleen addition.

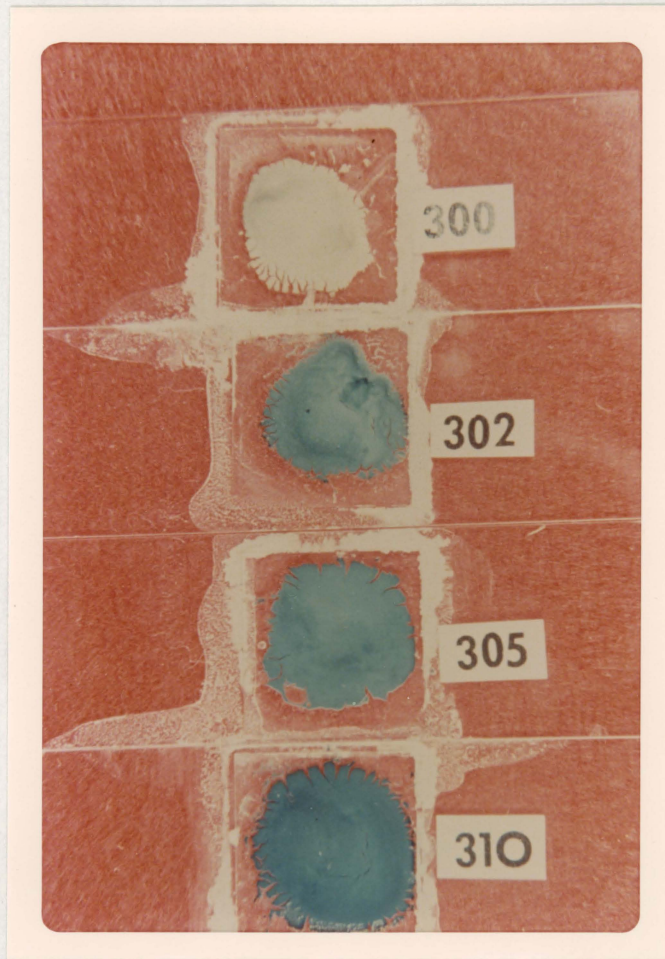


Figure 7. FERRITIN SPOT TEST ON THE SAMPLES OF FAT LEVEL 3.

Note: Due to the technical difficulties encountered in color printing from slides, this print contains a gray overcast as observed on the background for the numbers. Yet, a progressive change in blue color can be seen with increased spleen addition.

grey-white color, whereas those from the spleen-added samples developed a blue color. The amount and intensity of blue developed in the pellets, indicating ferritin iron, increased as the amount of spleen in the original sample increased. The results were similar across all fat levels.

4.4 Presumptive Test for Spleen on the Raw Product

Small sections from each of the 12 ground beef products used in the hemosiderin test are shown in Figure 8. The spleen content is listed across the top and the fat level is denoted along the side. These same samples, after staining with Prussian blue, are shown in Figure 9.

Some bleaching or greying was observed on some unadulterated products. A faint, but visible, blue color developed on the 2.5 percent spleen samples. Significant "blueing" occurred on the 5 and 10 percent spleen-added samples.

Under the conditions of this experiment, the source of the trivalent iron causing the positive Prussian reaction is known, and the blue color is indicative of spleen addition. In uncontrolled situations other possible sources of trivalent iron must be considered, and this reaction must be evaluated cautiously.

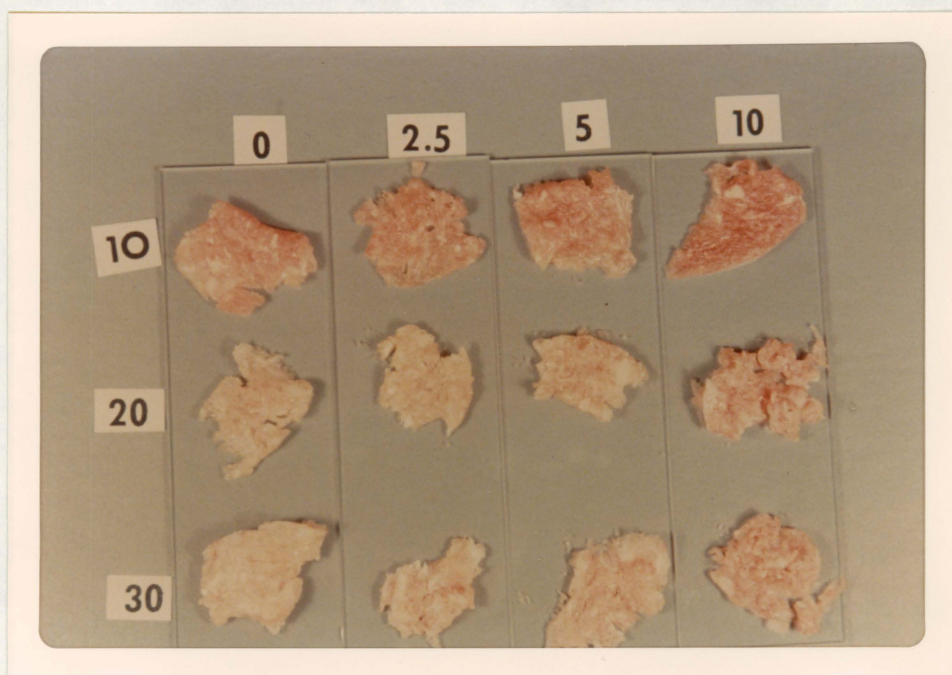


Figure 8. PRESUMPTIVE TEST ON RAW PRODUCT - BEFORE STAINING.
Rows of fat levels and columns of spleen addition.

Note: The background in this photo is a neutral gray test card - 18% reflectance.

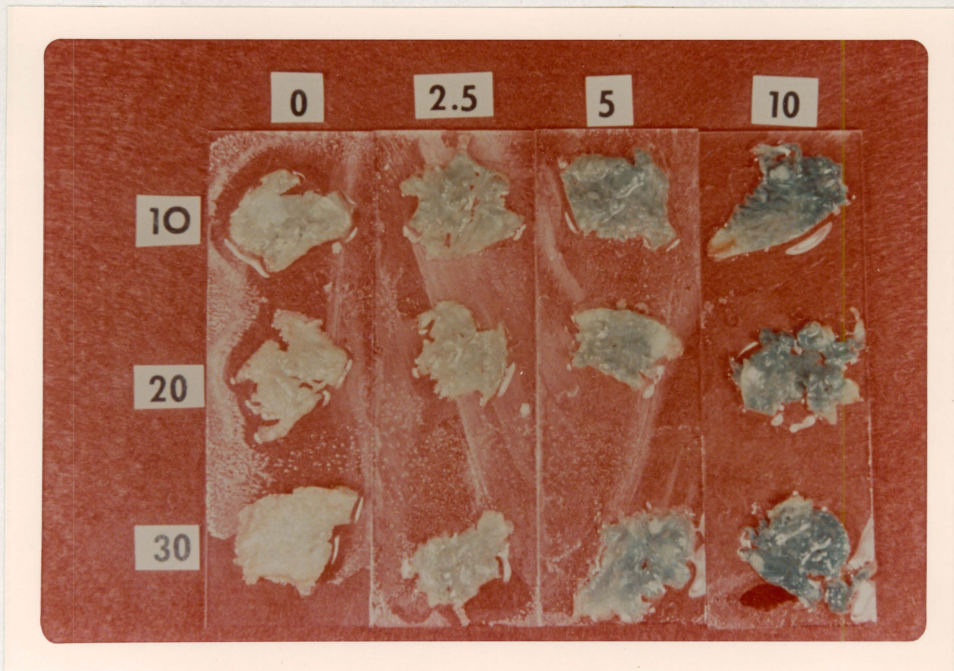


Figure 9. PRESUMPTIVE TEST ON RAW PRODUCT - AFTER STAINING WITH PRUSSIAN BLUE. Rows of fat levels and columns of spleen addition.

Note: Due to the technical difficulties encountered in color printing from slides, this print contains a grey-blue overcast as observed on the background for the numbers. Yet, a progressive change in blue color can be seen with increased spleen addition.

5.0 SUMMARY AND CONCLUSIONS

A series of experiments were conducted to explore the feasibility of employing the storage iron compounds, hemosiderin and ferritin, as indicators for the detection and quantitation of spleen added to ground beef.

Seven samples of ground beef were purchased at local stores and assumed to be representative of typical ground beef as offered for sale. These samples were used to determine the characteristics of unadulterated ground beef as effected by the procedures being developed. Weighed amounts of ground beef and spleen were combined to yield products with 0, 2.5, 5 and 10 percent spleen in the final mix. A graded series of spleen addition was prepared at each of three fat levels, yielding a total of 12 products.

A procedure, based on saline solubility and centrifugation, had been developed for the extraction of soluble iron compounds from a ground beef sample. In the case of a spleen-added product, the resulting residue of insoluble tissue was shown, by microscopic observation, to contain hemosiderin granules. Excepting that iron which could not be washed from an unadulterated ground beef sample, any iron remaining in the tissue residue was assumed to be hemosiderin iron and indicative of spleen addition.

The iron retained in the tissue residue was extracted into solution by acid hydrolysis. After filtration, the iron concentration of this solution was determined by atomic absorption spectroscopy. Deproteinization preceded the colorimetric iron determinations made with PDT. Iron values were calculated and reported as $\mu\text{g Fe/g}$ original

sample.

The extraction procedure was shown to be capable of removing a major portion of the soluble iron from a ground beef sample. A narrow range of iron values was determined for the unadulterated ground beef samples, regardless of initial fat content. All of the iron values for unadulterated samples were less than 4 $\mu\text{g Fe/g}$ original sample.

Spleen-added samples, treated by the same procedure, produced higher iron values than their unadulterated counterparts. The iron retained in the insoluble tissue residue of the spleen-added samples, defined as hemosiderin iron, increased linearly as the amount of spleen in the original sample increased. No overlapping of iron values between the spleen-addition levels was observed. Statistical analysis indicated that the differences in iron value between the addition levels were significant at the 1 percent level.

The fat content of the ground beef had very little effect on the ability to quantitate the added spleen. The differences in iron value between the addition levels were great in relation to the differences between the three fat levels within a spleen addition level. It is probable that an inability to prepare a constant sample for each fat level caused the relatively small differences in iron value noted between the fat levels.

The coefficients of variation for the two iron measurement techniques indicated that the determinations made on the deproteinized filtrates with PDT were somewhat more precise than those made on the non-deproteinized filtrates by AAS.

The results of this experiment indicate that, under controlled conditions, the proposed procedure was able to detect and quantitate spleen added to ground beef on the basis of hemosiderin iron. Evidence is given that approximately 93 percent of the hemosiderin recoverable from spleen was recovered in the tissue residue of a ground beef sample after soluble iron extraction.

Although positive results are reported, the reader is cautioned that this was merely a feasibility study. It is widely recognized that the amount of hemosiderin iron in spleens of bovine animals is variable. A composite spleen homogenate, prepared from randomly selected spleens, was used in an attempt to approximate the characteristics of a typical spleen. Since no attempt was made to quantitate the hemosiderin iron level of a large number of spleens, the author cannot guarantee or imply that another spleen homogenate would contain an equivalent amount of hemosiderin and produce iron values comparable or comparable with those reported in this study. Therefore, these results should be viewed cautiously. Further work must be done to determine if the principles set forth are useful or practical for products of unknown history.

The procedure for hemosiderin localization used in this study was specifically developed to fit the equipment most probably available to an average laboratory in the meat industry. As presented, it is somewhat cumbersome and time consuming; however, given the proper equipment, it could be streamlined and shortened considerably. It appears reasonable that the saline extraction of soluble iron could be carried out in one 250 ml centrifuge tube instead of three

50 ml tubes. In addition to reducing the number of transfers, this system would result in an overall increase in the volume of saline used in each wash. An increased volume of saline may allow the successful extraction of soluble iron with just one wash. With a centrifuge capable of developing forces of 10,000 to 14,000 x g, the time of each centrifugation could be reduced to approximately 10 minutes.

Since hemosiderin is visible under the light microscope, the presence of spleen in ground beef can be visually confirmed, even at levels where the total amount of hemosiderin iron is below the detection limits allowed by the quantitative procedure. Hemosiderin granules have easily been identified in ground beef samples containing as little as 0.1 percent spleen. All tests for spleen should be accompanied by microscopic confirmation of hemosiderin in sample homogenates or slurries after Prussian blue staining.

A qualitative method of spleen detection based on ferritin was also tested. Ferritin was extracted from the ground beef samples with saline and then separated from the other soluble iron forms on the basis of its heat stability. The ferritin was then precipitated, along with other heat stable proteins, by TCA and stained with Prussian blue.

The protein pellets prepared from the unadulterated ground beef samples maintained a white to grey-white color, whereas those from the spleen-added samples developed a blue color. The amount and intensity of blue color developed in the pellets increased as the amount of spleen in the original sample increased.

A preliminary presumptive test was performed by applying Prussian blue iron stain directly to the raw products. After staining, the spleen-added products developed a blue color. Under the controlled conditions of this experiment, the source of trivalent iron causing the positive Prussian reaction was known to be hemosiderin and ferritin. Thus, the blue color was indicative of the presence of spleen in a product.

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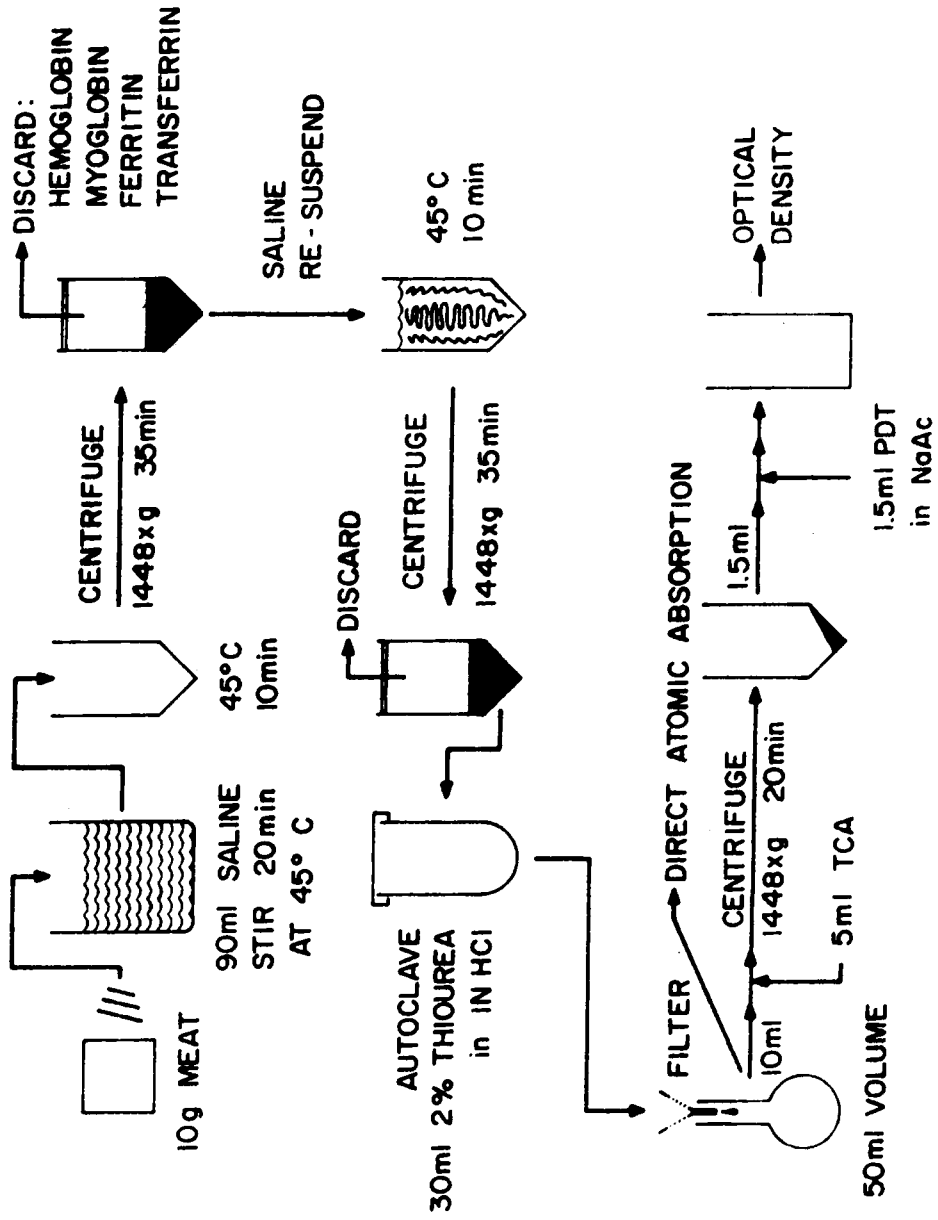
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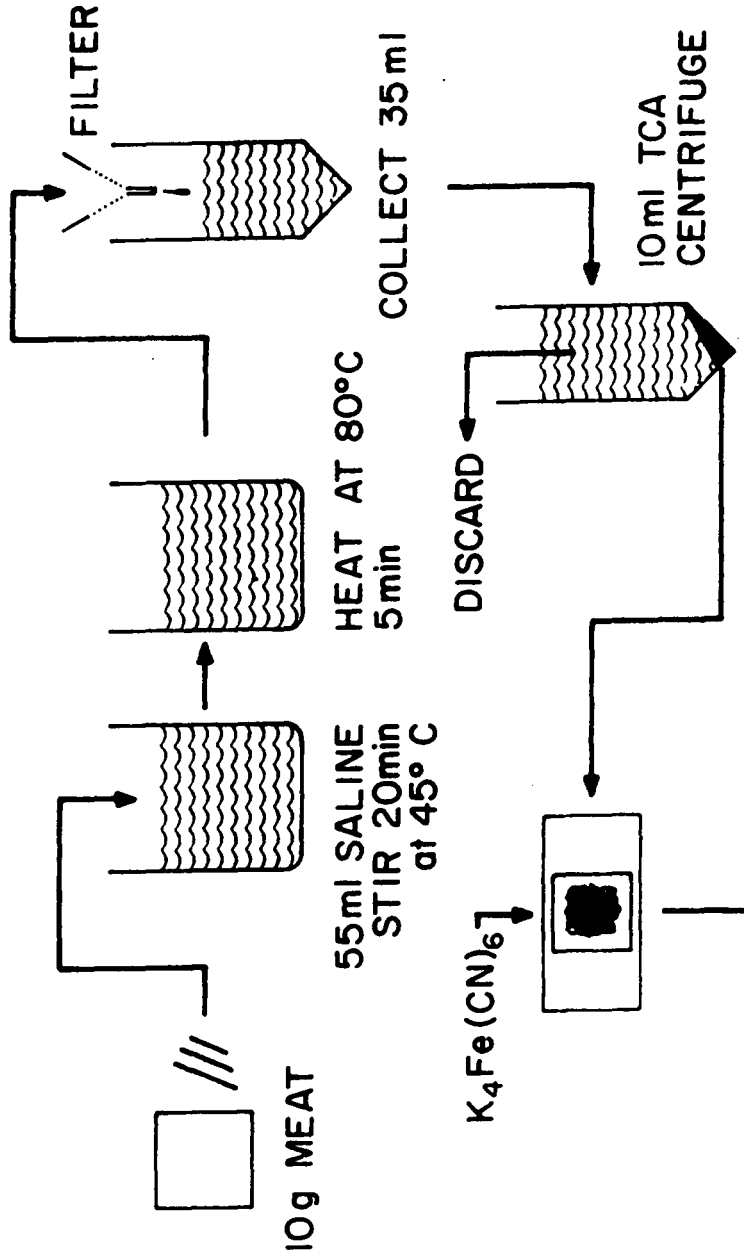
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No. 1. FLOW DIAGRAM FOR THE QUANTITATIVE TEST FOR SPLEEN BASED ON HEMOSIDERIN.



No. 2. FLOW DIAGRAM FOR THE QUALITATIVE SPOT TEST FOR SPLEEN BASED ON FERRITIN.

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METHODS TO DETECT AND QUANTITATE SPLEEN
ADDED TO GROUND BEEF

by

Ralph J. Bittel

(ABSTRACT)

Experiments were conducted to explore the feasibility of employing the storage iron compounds, hemosiderin and ferritin, as indicators for the detection and quantitation of spleen added to ground beef.

Weighed amounts of ground beef and a composite spleen homogenate were combined to yield products with 0, 2.5, 5 and 10 percent spleen. A graded series was prepared at each of three fat levels.

A quantitative procedure based on the organ specificity and insolubility of hemosiderin was tested. The soluble iron forms were removed by saline extraction and centrifugation. Any iron remaining in the residue was assumed to be hemosiderin iron.

The retained iron was extracted by acid hydrolysis and quantitated colorimetrically with ferrozine and by atomic absorption. The residues from unadulterated samples held less than 4 μg Fe/g original sample. Insoluble iron increased linearly (Slope = 4.83) as the level of spleen in the product increased. The fat content of the products had little effect on the quantitation of added spleen.

A qualitative spot test based on the solubility and heat stability of ferritin was investigated. Protein pellets prepared from unadulterated samples maintained a white to grey-white color after Prussian blue iron staining. Pellets from spleen-added samples developed a blue

color. The amount and intensity of blue increased as the amount of spleen in the product increased.

A presumptive test was performed by applying Prussian blue directly to the raw products. The spleen-added products developed a blue color.