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CONSUMPTION OF IRON-FORTIFIED CHEESE AND LIPID PEROXIDATION IN FEMALES

by

Gene Joseph Giunti

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1994

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I would like to dedicate this to the memory of the late Dr. Arthur Mahoney. I would also like to thank Dr. Dejia Zhang, Mary Farley, Wendy Haws-Rice, Janet Wright, Boem Jun Lee, LeAnn Anderson, all those involved in the iron-fortified cheese project, and all those who helped me to complete this thesis. I would like to thank my major professor, Dr. Hendricks, for his inquisitive guidance, Noreen Schvaneveldt for her ubiquitous support and good cheer, Dr. Sisson for his statistical expertise and cookies, and Dr. Brown for approving the use of these data.

Gene J. Giunti

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ABSTRACT

Consumption of Iron-Fortified Cheese
and Lipid Peroxidation
in Females

by

Gene J. Giunti, Master of Science
Utah State University, 1994

Major Professor: Dr. Deloy G. Hendricks
Department: Nutrition and Food Sciences

Dairy products are important sources of calcium and other nutrients but are a poor source of dietary iron. Cheese comprises a substantial portion of dairy food consumption and has been determined an appropriate medium for iron-fortification. However, iron may promote the potentially harmful process in food and biological systems known as lipid peroxidation. Therefore, the safety of consuming iron-fortified cheese was examined.

Commercial-scale batches of Cheddar cheese were iron-fortified to a level of two milligrams of iron per ounce with either ferric chloride, ferric-casein complex, or ferric-whey protein complex. Fifty-four premenopausal females were divided into three treatment groups and supplemented one and one-half ounces of iron-fortified Cheddar cheese into their normal diet on a daily basis for six consecutive weeks. Lipid peroxidation was measured as thiobarbituric acid-reactive substances in serum, urine, and feces. A significant increase in serum thiobarbituric acid-reactive substances occurred in all treatment groups sixteen days after initiation of iron-fortified cheese consumption. Thiobarbituric acid-reactive substances in serum returned to baseline levels after thirty days of iron-

fortified cheese consumption. Thiobarbituric acid-reactive substances in serum, urine, and feces did not differ among iron-fortification methods.

Average daily intake of iron during the six weeks of iron-fortified cheese consumption significantly increased above baseline intake levels without cheese by the approximate amount of iron fortified into the cheese. Increased dietary iron intakes were not correlated with increased lipid peroxidation as measured by thiobarbituric acid-reactive substances in serum, urine, or feces.

These results indicated that the daily consumption of iron-fortified cheese increased dietary iron intake and produced a transient increase in lipid peroxidation as measured by thiobarbituric acid-reactive substances in human serum.

(137 pages)

INTRODUCTION

Inadequate iron intake can lead to iron deficiency anemia (IDA). IDA is generally recognized as the world's most prevalent nutritional deficiency and is estimated to affect more than one billion people in both developed and less-developed countries (Skikne, 1988). Children and women of childbearing age appear to be those most affected, and they continue to be the primary targets of efforts to decrease the prevalence of iron deficiency.

The primary preventable cause of IDA is insufficient dietary iron intake. Therefore, it has been the philosophy of the Joint FAO/WHO Experts to fortify foods with iron in order to increase dietary iron intake (Baynes and Bothwell, 1990). This intervention is applicable to large populations, relatively cost-effective, and has been successfully accomplished with infant formulas, cereal products, and breads.

Cheese has been determined to be an appropriate medium for iron-fortification and has been fortified with iron in pilot-scale studies without significant effect on quality. Cheese makes up a good portion of dairy food consumption; intake is increasing and is being used in adequate amounts by target populations with the potential for IDA. Since cheese and other dairy products are primary sources of dietary calcium but contribute practically no dietary iron, iron-fortified dairy products could improve both iron and calcium nutriture of individuals at risk for iron deficiency.

The research reported in this thesis was performed in conjunction with a feeding trial in a group at risk for IDA (premenopausal females) and examined the safety, absorption, and dietary contribution of iron from iron-fortified Cheddar cheese.

The purposes of this research were 1) to determine and compare the effects of consuming cheeses fortified with either ferric-casein complex, ferric whey-protein complex, or ferric chloride on *in vivo* lipid peroxidation, 2) to determine the effects of

iron absorption, iron status, and iron intake on *in vivo* lipid peroxidation, 3) to determine the effects of dietary antioxidant and lipid intakes on *in vivo* lipid peroxidation.

In this review of the literature, free radicals, iron and lipid peroxidation, dietary factors influencing lipid peroxidation, and methods used to measure lipid peroxidation are discussed, among others.

LITERATURE REVIEW

Iron, Free Radicals, and Lipid Peroxidation

Iron

Iron (Fe) is an essential micro nutrient involved in a broad spectrum of metabolic functions (Weinberg, 1989). A required component of the protein hemoglobin, it transports oxygen and carbon dioxide critical to cellular respiration. As an essential factor of tissue enzymes such as the cytochromes, it is needed for energy production. The importance of iron in biological systems derives from its reduction/oxidation (redox) reactivity because it exists in two stable but interchangeable forms: ferrous (Fe^{2+}) and ferric (Fe^{3+}) (Ramdath and Golden, 1989). These roles and others render iron an element most necessary for life. However, the redox reactivity of iron may also lead to the production of electron-deficient molecules known as free radicals. Free radicals may act to mediate tissue damage via the initiation and propagation of lipid peroxidation and are generally considered detrimental to health (Minotti and Aust, 1989). Antioxidant systems present *in vivo* to quench free radicals and minimize the havoc they can potentially wreak include decomposing enzymes (superoxide dismutases, catalase, and peroxidases), naturally occurring dietary components (vitamin E, vitamin C, beta-carotene, and phytic acid, and others), and small molecular weight substances synthesized by the body such as uric acid and glutathione (Vuillaume, 1987).

Free Radicals

Free radicals are defined as independent chemical species with one or more unpaired electrons. Because electrons are more stable when paired together, free radical species are more reactive than nonradical species and can interact with body tissues to chemically modify and damage essential biological constituents such as proteins, lipids, and nucleotides (Slater, 1984; Cochrane, 1991). Therefore, the most significant aspect of

free radicals is their ability to markedly alter function by changing the size and shape of molecules. An imbalance of free radicals beyond what tissue homeostatic responses can combat is known as oxidative stress and is believed to contribute to the pathogenesis of such varied disorders as atherosclerosis, cancer, alcoholic liver injury, intestinal diseases, Parkinson's disease, and myocardial reperfusion injury, among others (Shaw et al., 1988; Addis and Park, 1989; Adams and Odunze, 1991; Cochrane, 1991; Halliwell, 1991; Sies, 1991; Ceconi et al., 1992; Van Der Vliet and Bast, 1992). Although free radicals can be a pernicious by-product of normal cellular metabolism, they also serve an essential function by destroying pathogens via the oxidative burst of neutrophils and are, ironically, the body's first line of defense (Bendich et al., 1990).

Species of free radicals found in oxygen-based biological systems such as the human body are highly reactive oxygen-centered types including hydroxyl radical ($HO^{\cdot}$) and superoxide anion ($O_2^{\cdot -}$) (Kehrer, 1993). In addition to the true free radicals just mentioned, other free radical-generating species are produced from either normal cellular respiration or exposure to energy (ultraviolet light or radiation). These are hydrogen peroxide (H_2O_2) and singlet oxygen ($O_2^{\cdot}$) (Foegh et al., 1990). The most current and appropriate term to describe oxygen free radicals and other free radical-generating species is reactive oxygen species (ROS). *In vivo* reactions resulting in more reactive species than oxygen have been outlined by Bendich et al. (1990):

O_2 + energy ----------> $O_2^{\cdot}$ (Singlet Oxygen)

$O_2 + e^-$ ----------> $O_2^{\cdot -}$ (Superoxide Anion)

$2\ O_2^{\cdot -} + 2\ H^+$ ----------> $O_2 + H_2O_2$ (Hydrogen Peroxide)

$O_2 + 2\ e^- + 2\ H^+$ ----------> H_2O_2 (Hydrogen Peroxide)

$O_2^{\cdot -} + H_2O_2 + 4\ H^+$ --------> $O_2 + H_2O + HO^{\cdot}$ (Hydroxyl Radical)

Free Radicals and Iron

The transition metal iron is a primary culprit in the generation of ROS. Iron's variable oxidation numbers (specifically Fe^{2+} and Fe^{3+}) enable it to accept and donate single electrons and are the basis for the formation and propagation of many free radical reactions (Halliwell, 1991). One of the most important of these reactions is production of the extremely reactive hydroxyl radical. Although hydroxyl radical is theoretically formed from superoxide anion and hydrogen peroxide via the Haber-Weiss Reaction (Dunford, 1987),

$$O_2^{\cdot -} + H_2O_2 \text{ ----------} > \quad O_2 + OH^- + HO^{\cdot},$$

yield is virtually nonexistent. To drive the reaction and generate appreciable hydroxyl radical, iron is needed to shuttle one electron to hydrogen peroxide as occurs in Fenton's Reaction (Dunford, 1987):

$$H_2O_2 + Fe^{2+} \text{ ----------} > \quad Fe^{3+} + OH + HO^{\cdot}$$

These two reactions have been sequenced in a pathway where superoxide anion generates both hydrogen peroxide and ferrous iron (Fe^{2+}). This sequence of reactions exemplifies the role of iron in the production of hydroxyl radicals and is termed either the iron-catalyzed Haber-Weiss reaction or the superoxide-driven Fenton's reaction (Minotti and Aust, 1987; Minotti and Aust, 1989):

$$O_2^{\cdot -} + Fe^{3+} \text{ ----------} > \quad O_2 + Fe^{2+}$$

$$2O_2^{\cdot -} + 2\,H^+ \text{ ----------} > \quad O_2 + H_2O_2$$

$$H_2O_2 + Fe^{2+} \text{ ----------} > \quad Fe^{3+} + OH^- + HO^{\cdot} \text{ (Fenton's Reaction)}$$

$$\text{NET: } O_2^{\cdot -} + H_2O_2 \text{ ----------} > \quad O_2 + OH^- + HO^{\cdot} \text{ (Haber-Weiss Reaction)}$$

This sequence of reactions is thought to be mediated by the extent of the reduction of ferric iron and the oxidation of ferrous iron. A balance must be attained between oxidation and reduction to propagate the reaction. An excess in either direction will convert the iron to one form only (ferric or ferrous) and inhibit lipid peroxidation.

Evidence also indicates that oxygen may interact with Fe^{2+} and Fe^{3+} to form perferryl [$Fe^{2+}\ O_2^- <---> Fe^{3+}O_2^-$] intermediate, which may propagate these reactions (Minotti and Aust, 1987; Minotti and Aust, 1992).

Clearly, a potentially harmful situation exists if iron in the body is free to participate in the iron-catalyzed Haber-Weiss reaction. Presumably because free iron has the potential to be such a toxic oxidant, systems are present in the body to ensure that it is not found in its free ionic form. Upon entering the gastrointestinal tract, nonheme iron forms complexes with numerous food constituents such as fiber, phytates, and amino acids, or to various components of gastric secretions such as gastrin, gastric transferrin, and mucin (Carpenter and Mahoney, 1992). In the case of heme iron, it is complexed with its namesake, heme. These complexes influence the availability of iron to participate in ROS-generating reactions. In effect, the body is protecting itself from potential oxidative damage.

For absorption to occur, iron must be transported across the intestinal mucosa and into the plasma. Although knowledge of this mechanism is limited, it is known that iron-binding substances are involved. These substances, along with the better-known iron-binding proteins transferrin, ferritin, and hemosiderin, limit the availability of iron during transport and storage. Consequently, under normal physiological conditions, the concentration of iron capable of catalyzing free radical reactions should be low, if not negligible (Carpenter and Mahoney, 1992).

However, much is still unknown about the relationship of iron absorption, transport, storage, and how these interact to produce oxidative damage. *In vitro* evidence indicates that radical-mediated mechanisms do release iron from ferritin, transferrin, hemosiderin, and hemoglobin. This released iron has been shown to serve as a catalytic source for oxidative damage (Sadrzadeh et al., 1984; Brieland and Fantone, 1991; Minotti et al., 1991; Reif, 1992).

Other free radical species found in biological systems include carbon-centered radicals which may arise from the free radical attack on unsaturated bonds of the fatty acids found in all cell membranes. These radicals participate in a free radical-mediated process known as lipid peroxidation (Foegh et al., 1990).

Lipid Peroxidation

Lipid peroxidation is the oxidative deterioration of unsaturated lipids via a free radical chain reaction. Unsaturated fatty acids are especially liable to peroxidation because the presence of a double bond weakens the carbon-hydrogen bond of the carbon atom adjacent to a carbon with an unsaturated bond. The principal unsaturated fatty acids peroxidized in human cells are linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6) (Esterbauer et al., 1990). In food systems, lipid peroxidation causes rancidity of fats and oils. This involves the direct reaction of oxygen with long chain polyunsaturated fatty acids to form potentially damaging free radicals and unstable free radical precursors such as organic peroxides (Gutteridge and Halliwell, 1990).

Lipid peroxidation is initiated *in vivo* by environmental components inducing the formation of either oxygen or organic free radicals and begins with the abstraction of hydrogen from an unsaturated fatty acid. Chemical and physical changes to unsaturated fatty acids present in cellular membranes and increased levels of lipid peroxidation as indicated by formation of malondialdehyde have been found in human colorectal tumor tissues (Otamiri and Sjodahl, 1989). Besides cancer, lipid peroxidation is thought to play a causative role in the pathogenesis of several chronic and acute conditions such as atherosclerosis, inflammation, shock, and alcoholic liver injury, among others (Tappel, 1973). The free radical chain reaction process of lipid peroxidation has been outlined by Sevanian and Hochstein (1985):

Initiation: RH (unsaturated FA) + Initiator -----> R· (organic free radical)

Propagation: R· + O_2 ----------> ROO· (peroxyl radical)

	ROO· + RH ---------->	ROOH (lipid peroxide) + R·
Termination:	ROO· + ROO· ---------->	ROOR (tetroxide) + O_2
	ROO· + R· ---------->	ROOR
	R· + R· ---------->	RR (hydrocarbon)

Besides the direct production of membrane-damaging free radicals as outlined above, lipid peroxidation generates lipid peroxides (ROOH) during propagation and tetroxides (ROOR) upon termination. These products can either directly damage other cellular membrane components or decompose into peroxyl (ROO·) and/or alkoxyl (RO·) free radicals (Terao, 1990).

Iron Proteins and Free Radicals

Ferritin's primary function is to provide an intracellular store of iron which can be used for the synthesis of other iron-containing proteins. Iron is stored in the ferritin molecule in the ferric form but must be reduced to ferrous to be released. Small amounts of ferritin are normally present in the serum in equilibrium with intracellular ferritin and reflect body iron stores (Cook et al., 1974). Concentration of serum ferritin is usually between 10 to 250 µg/L for normal males and 10-150 µg/l for normal females (Leggett et al., 1990). In iron-deficient subjects, the serum ferritin concentration is usually below 10µg/L and, in iron overload, as high as 10,000 µg/L (Worwood, 1989).

Although ferritin acts as an antioxidant and protects against iron-catalyzed lipid peroxidation by binding iron, considerable evidence suggests that iron released from ferritin may in fact function *in vivo* as a source of iron for lipid peroxidation and free radical reactions (Biemond et al., 1984; Vile and Winterbourn, 1988). Superoxide-mediated iron release from ferritin has been found to stimulate lipid peroxidation as measured by malondialdehyde formation in liposomes under intralysomal conditions (O'Connell et al., 1985; Thomas et al., 1985). In a model of hypoxia and myocardial reperfusion injury, superoxide anion under aerobic conditions and xanthine oxidase

under anaerobic conditions released iron from ferritin, which further participated in radical-generating reactions (Bolann and Ulvik, 1987). Evidence also suggests that hydroxyl radical is produced as a byproduct of the oxidative deposition of ferric iron into the ferritin molecule (Grady et al., 1989).

Iron is transported within the body by binding to the extracellular protein carrier transferrin. Transferrin has the capacity to bind to more iron when needed, as well as precisely control the flow of transferrin iron into body cells. Because of these characteristics, unsaturated transferrin can have strong antioxidant activity by binding iron that might otherwise catalyze hydroxyl radical formation (Sullivan, 1989; Cook et al., 1993). Receptors on transferrin are normally 30% saturated with iron. Transferrin saturation percentage is the quotient of serum iron concentration and serum transferrin concentration and is a useful measure of transport iron status. A low transferrin saturation is usually associated with depleted body iron stores. Like ferritin, iron in transferrin is in the ferric form but must be reduced to the ferrous form in order to be released. Also like ferritin, transferrin may also act as a source of iron for generation of free radicals. Superoxide anion from stimulated neutrophils has been found to release ferrous iron from transferrin at physiologic levels of transferrin saturation (Brieland and Fantone, 1991).

Hemoglobin is the major iron-containing protein in the body and functions to transport oxygen and carbon dioxide. It contains 60-75% of the iron (approximately 2500 mg) found in the body (Weintraub, 1966). Its measurement is the most widely used screening test for iron-deficiency anemia (Lee and Nieman, 1993). Although hemoglobin represents a very large pool of iron, this iron is not usually free in the body unless it is released from red blood cells. However, *in vitro* evidence indicates that hydrogen peroxide can cause the removal of heme and subsequently free iron from hemoglobin.

This free iron can then participate in free radical-generating reactions (Sadrzadeh et al., 1984, Halliwell and Gutteridge, 1986; Harel et al., 1988; Prasad et al., 1989).

Serum iron is an index of iron status and represents the amount of iron bound to transferrin. Low levels indicate low iron status (Tilkian et al., 1987). Total iron-binding capacity (TIBC) measures the amount of iron capable of being bound to serum transferrin and is usually increased with depletion of body iron stores (Lee and Nieman, 1993).

Iron and Lipid Peroxidation

Iron is thought to play two important roles in lipid peroxidation (Minotti and Aust, 1987; Minotti and Aust, 1989; Miller et al., 1990; Minotti and Aust, 1992). As indicated previously, iron has the ability to catalyze the generation of damaging hydroxyl radicals. Hydroxyl radicals can then participate in the initiation event of lipid peroxidation. Perhaps more important is iron's ability to also propagate the lipid peroxidation chain reaction via metal-catalyzed decomposition of lipid peroxides into peroxyl and alkoxyl radicals (Aikens and Dix, 1991; Halliwell, 1991):

$$ROOH + Fe^{3+} \text{----------} > ROO\cdot \text{ (peroxyl)} + H^{+} + Fe^{2+}$$

$$ROOH + Fe^{2+} \text{----------} > RO\cdot \text{ (alkoxyl)} + OH^{-} + Fe^{3+}$$

Direct and indirect evidence indicate that cellular damage secondary to iron-induced lipid peroxidation is the underlying pathogenic mechanism of the diffuse tissue injury seen in conditions of iron overload (hemochromatosis) and has been described in detail *in vivo* and *in vitro* in animals (Dillard et al., 1984; Bacon et al., 1986; Britton et al., 1987; Gordeuk et al., 1987; Park et al., 1987; Sharma et al., 1990; Wu et al., 1990). High doses of oral iron have been documented to increase gastrointestinal formation of highly reactive hydroxyl radicals in animal models (Slivka et al., 1986; Kang et al., 1989). Physiologically normal iron concentrations have also been found to induce *in vitro* lipid peroxidation in microvillus membranes of the small intestine of laboratory animals (Fodor and Marx, 1988).

Studies in free-living human males indicated associations between high storage and dietary levels of iron and increased risk of cardiovascular disease. These associations may implicate high-normal iron levels in the pathogenesis of heart disease via a lipid peroxidation mechanism (Lauffer, 1990; Salonen et al., 1992; Beard, 1993). Based on data from the first National Health and Examination Survey, it has been hypothesized that increased body stores of iron may increase cancer risk to be secondary to free radical mechanisms (Stevens et al., 1980). However, whether currently accepted ranges of iron intake and status considered physiologically "normal" can induce lipid peroxidation of pathological significance has yet to be elucidated.

Measurement of Lipid Peroxidation

As described above, lipid peroxidation is the free radical chain reaction of unsaturated fatty acids yielding ROS and lipid peroxides. Lipid peroxides can then be further generated into ROS or be decomposed to form a variety of aldehydes. A major aldehyde product of lipid peroxides is malondialdehyde (MDA), and its formation is used as an index of lipid peroxidation in biological tissues and foods (Sinnhuber and Yu, 1958; Smith and Anderson, 1987). The main mechanism for formation of MDA and other aldehydes from lipid peroxides is the cleavage of two carbon-carbon bonds on either side of the hydroperoxy group (ß-cleavage). Besides ß-cleavage, MDA in biological samples can also be formed from hydroxy epidioxides arachidonic acid (20:4) and docosahexaenoic acid (22:6).

Although MDA is extensively metabolized by the liver to carbon dioxide, it can be relatively long-lived (12 hours) and mutagenic to biological tissues (Siu and Draper, 1982). A known mutagen found to be elevated in tissues from iron-overloaded experimental animals, it diffuses from its site of origin (cell membranes) to act as a toxic messenger of lipid peroxidation (Esterbauer et al., 1991; Esterbauer, 1993).

Malondialdehyde levels have been found to be responsive to MDA intake, conditions such as vitamin E deficiency and high tissue concentrations of polyunsaturated fatty acids, iron administration, and other conditions associated with increased lipid peroxidation (Draper et al., 1984; Berry et al., 1991; Haglund et al., 1991).

A commonly utilized method to measure MDA in food and biological samples is the thiobarbituric acid (TBA) test. In this assay, a sample is heated with thiobarbituric acid at low pH to react one molecule of MDA with two molecules of TBA. The adduct forms a pink chromagen with an absorbance maxima at 532 nm (Dahle et al., 1962, Asakawa and Matsushita, 1979). However, depending on the length of the heating stage of the procedure, more MDA than originally present from the original peroxidation process may be formed from lipid peroxides during the TBA reaction procedure (Janero and Burghardt, 1989). Also, other components present in biological samples besides MDA may react with TBA. Therefore, this measure of lipid peroxidation is more accurately known as the thiobarbituric acid-reactive substances (TBARS) test (Gutteridge and Halliwell, 1990).

Lipid peroxidation measured by TBARS has been extensively utilized and well documented (Pryor and Godber, 1991). As an index in humans, urinary excretion of TBARS appears to most accurately reflect MDA ingested in the diet and MDA formed *in vivo* from lipid peroxidation in tissues; levels in plasma, serum, and feces are also utilized for site-specific measurements (Draper et al., 1984; Draper et al., 1986; Piche et al., 1988; Wade and van Rij, 1988).

Besides adding iron to the diet, iron-fortified Cheddar cheese also undergoes more extensive lipid peroxidation than unfortified Cheddar cheese as indicated by TBARS (Zhang and Mahoney, 1989a). Whether this added load of iron and TBARS in a normal mixed diet will influence *in vivo* lipid peroxidation is not known. TBARS measured in serum, urine, and feces will indicate if consumption of iron-fortified Cheddar cheese is

influencing *in vivo* lipid peroxidation or adding an increased load of TBARS to the diet. These factors are important in establishing the safety of iron fortification of cheese.

Antioxidants and Lipid Peroxidation

Antioxidants are molecules that function as blockers of radical processes, and, therefore, represent a defense system against free radical damage. As mentioned previously, antioxidants in biological systems are either enzymes, naturally occurring dietary factors, or small molecular weight substances synthesized by the body.

The three major dietary antioxidants acting to directly scavenge free radicals include alpha-tocopherol (vitamin E), vitamin C, and the precursor of vitamin A, beta-carotene. Shielding cells from peroxidation by becoming oxidized themselves, these three nutrients, taken in adequate amounts, are therefore thought to be protective against diseases such as cancer and atherosclerosis (Esterbauer et al., 1990; Canfield et al., 1992; Chakrabarty et al., 1992; Chen et al., 1992; Packer, 1992; Jackson et al., 1993).

In addition to vitamins, several nutritionally essential minerals are incorporated into antioxidant enzymes and include zinc, copper, manganese, selenium, and iron. Altering dietary intakes of these minerals may modify antioxidant enzyme systems and homeostatic responses to oxidative stress (Machlin and Bendich, 1987).

The antioxidants alpha-tocopherol and beta-carotene are lipid soluble and are important for protecting lipids in cellular membranes, whereas vitamin C is water soluble and traps free radicals in the aqueous portion of cells, the cytoplasm (Chow, 1979). Free radical trapping by major dietary antioxidants is outlined below (Krinsky, 1992; Bast et al., 1991):

alpha-tocopherol + ROO· ---------> alpha-tocopherol· (radical) + ROOH

alpha-tocopherol· + ROO· --------> ROO-alpha-tocopherol (stable)

beta-carotene + ROO· ----------> ROO-beta-carotene· (radical)

ROO-beta-carotene· + ROO· -----> ROO-beta-carotene-OOR (stable)

beta-carotene + O_2· ---------->"energized beta-carotene"

"energized beta-carotene" ----------> beta-carotene + heat

vit C + ROO· ----------> vit C· (radical) + ROOH

vit C· + ROO· ----------> ROO-vit C (stable product)

Besides acting as a scavenger of free radicals, the antioxidant vitamin C is also thought to perform the important function of recycling alpha-tocopherol (McCay, 1985):

alpha-tocopherol + ROO· ---------> alpha-tocopherol· (radical) + ROOH

alpha-tocopherol· + vit C-H^- -----> alpha-tocopherol + vit C·

vit C· + vit C· + H^+ ----------> vit C + vit C-H^- (anion)

The antioxidant abilities of alpha-tocopherol and beta-carotene have been widely studied in animal models. Supplementing these nutrients to rats has been shown to significantly decrease lipid peroxidation in tissues, erythrocytes, and plasma (Leibovitz et al., 1990; Zamora et al., 1991). Radical-initiated lipid peroxidation in rat microsomes is inhibited by alpha-tocopherol and beta-carotene (Palozza and Krinsky, 1991; Palozza et al., 1992). In addition, iron-induced lipid peroxidation in lab animals is inhibited by alpha-tocopherol (Hu et al., 1990; Sharma et al., 1990).

Iron Nutriture and Fortification

Iron Status

As previously mentioned, inadequate iron nutriture is recognized as the most common nutritional deficiency in developing and developed countries (Skikne, 1988). Consequently, iron research in the context of human nutrition has focused on the identification and resolution of problems related to deficiency. Incidence of iron deficiency varies widely with race, sex, age, and economic status (Food and Nutrition Board, 1989). In adult males (except for the elderly), iron deficiency is uncommon and is

usually precipitated by the growth spurts, chronic blood loss, or gastrointestinal malabsorption. Conversely, females (particularly those of childbearing age) are more frequently iron deficient. As menstruation essentially represents a form of chronic blood loss, and childbearing places an additional demand on iron requirements, the higher prevalence of iron deficiency in young women is understandable (Leibman, 1985). The Second National Health and Nutrition Examination Survey (NHANES II) estimated the prevalence of iron deficiency anemia in the U.S. to be approximately 5.7 % among young children and 5.9 % among teenage girls (Dallman et al., 1984). Prevalence in adult premenopausal females was 2.3% in 1986, which is a significant reduction from the prevalence rate of 8.4% in 1978 (Cook et al., 1986).

Iron-Fortification of Cheese

Public health efforts to improve the iron nutriture of at-risk groups have traditionally focused on the fortification of foods such as breads, breakfast cereals, and infant cereals. Farley et al. (1987) found that iron-fortified breads and cereals do in fact make a significant contribution to the daily iron intake of individuals consuming high-iron density diets. It was also determined that high-iron density diets contain the least amount of dairy products, whereas low-iron density diets contain more dairy products. These findings support the fact that although dairy products are an excellent source of protein, vitamins, and calcium, they are a poor source of iron (Blanc, 1981; Farley et al., 1987). It therefore appeared logical to increase dietary iron intake by increasing the amount of iron in dairy products.

Following this line of reasoning, Zhang and Mahoney accomplished small-scale iron-fortification of Cheddar cheese and produced a good-quality product (Zhang and Mahoney, 1989a; Zhang and Mahoney, 1989b). Fortification of cheese with 40 μg iron/g (approximately 1 mg iron/ounce) did not significantly affect oxidized flavor as compared with unfortified cheese. Also, bioavailability of iron from the cheese as determined in

laboratory animals was excellent. Further trials of small-scale production, accompanied by organoleptic evaluation and bioavailability studies in laboratory animals, identified Cheddar cheese fortified with either ferric chloride ($FeCl_3$), a ferric-casein (Fe-C) complex based on $FeCl_3$, or a ferric-whey protein (Fe-WP) complex based on $FeCl_3$ as the most suitable for proposed commercial-scale production trials, iron absorption, and safety studies in human subjects (Zhang and Mahoney, 1990).

MATERIALS AND METHODS

Experimental Design

This study was conducted in premenopausal females to examine the effect of iron-fortified cheese consumption on *in vivo* lipid peroxidation. Subjects were first blocked according to serum ferritin levels (low, average, high) and then assigned to one of three cheese treatments in order to balance the three experimental groups for iron status. After three weeks of baseline measurements, subjects in their respective treatment groups began consumption of Cheddar cheese fortified with either ferric-casein complex (Fe-C), ferric-whey protein complex (Fe-WP), or ferric chloride ($FeCl_3$). Consumption of cheese lasted from week 4 until 3 days before the end of the study period (week 9 or approximately 6 weeks of cheese consumption) as shown below:

Treatment :	Fe-C	Fe-WP	$FeCl_3$
Time: 3 weeks	Before Cheese	Before Cheese	Before Cheese
Time: 6 weeks	During Cheese	During Cheese	During Cheese
Subjects:	20	20	20

Volunteer Selection

Sixty female volunteers were identified through campus, newspaper, and television advertising. Prospective subjects were initially screened by telephone (Appendix A). If respondents were considered a potential volunteer, an additional health and medical history questionnaire was administered either by telephone or in person (Appendix B).

Participation Criteria

1. Subjects were determined to be at least 18 years of age.

2. Subjects were determined to be premenopausal and to have a regular menstruation cycle.

3. Subjects were determined not to be pregnant or lactating.

4. Subjects agreed not to pursue pregnancy until 6 months after completion of the study.

5. Subjects were not using an intrauterine device (IUD) form of birth control.

6. Subjects were determined not to have a medical problem affecting iron status.

7. Subjects were determined not to have an eating disorder.

8. Subjects were determined not to be regularly using any prescription or over-the-counter medication interfering with iron absorption or status.

9. Subjects were determined not to have donated blood within 3 months prior to the study and agreed not to donate blood during the study.

10. Subjects were determined not to be hypersensitive to dairy foods.

Pregnancy Testing

Because pregnancy was a contraindication for participation in the study, and because of the potential risks associated with fetal radioiron exposure, the gestational status of each subject was determined utilizing the Wampole® One-Step hCG Pregnancy Test distributed by Wampole Laboratories (Cranbury, NJ).

Informed Consent and Confidentiality

Permission for all experimental procedures, protocols, and privacy matters were obtained through the Institutional Review Board for the Protection of Human Subjects. After a detailed orientation by the principal investigator regarding the purposes of this investigation and the potential risks, all subjects signed a consent form prior to beginning the study (Appendix C). Subjects were allowed to voluntarily terminate their participation in the study at any time, and agreed to be involuntarily terminated from the

study at any time if the principal investigator either believed or was advised that the subject's continued participation in the study would be detrimental to the subject's well-being or would make the data obtained unusable. Data from subjects were stored in locked files and coded for analyses to maintain confidentiality.

Benefits and Risks

Benefits for participation were of four kinds. Subjects were given $100 as a "gift" for completing the study. Subjects received two Utah State University "A" academic credits of NFS 490 (Special Problems in Nutrition) free of charge. Subjects received information on their iron, biochemical, and nutritional status. Subjects received iron-fortified cheese as a free commodity.

The only perceivable risks to subjects were blood drawings by venipuncture and exposure to radioiron. These risks were minimized by employing skilled medical technologists to draw blood with disposable equipment and by employing minimal doses of radioiron in the absorption studies as outlined in the informed consent.

Subject Orientation

Prior to initiation of the study, each subject participated in a training and orientation session. A study schedule outlining dates for test cheese consumption, blood draws, and radioiron test meals was reviewed and provided to each subject. Procedures and equipment for specimen collections (Appendix D) and documentation instruments for dietary intake (Appendix E) were also reviewed and provided to each subject.

Iron-Fortified Cheese

Cheese Preparation

Iron-fortified and unfortified Cheddar cheeses were prepared in 5000-pound commercial batches by the Cache Valley Cheese Co. (Logan, UT). Cheeses were

manufactured, packaged, and stored according to the company's regular procedures except for the addition of the iron sources before the calf rennet in the cheese-making process.

Ferric chloride purchased from Sigma Chemical Co. (St. Louis, MO), and food grade milk products were used for the iron-fortification sources. Iron-fortified cheese was prepared using either ferric-casein complex, ferric-whey protein complex, or ferric chloride (Appendix F). The ferric-casein complex was made by adding ferric chloride to skim milk and then adjusting the pH to 4.6 (Zhang and Mahoney, 1989a). Ferric-whey protein complex was made by adding 0.5 M ferric chloride to cottage cheese whey and adjusting the pH to 3.5 with NaOH to precipitate the ferric-whey protein (Zhang and Mahoney, 1989b). Precipitates of both iron proteins were washed with lactic acid solution (pH 4.0) and then with deionized H_2O. $FeCl_3$ solution was made by adding ferric chloride to double-deionized water and then adjusting the pH to 2.0, resulting in a 0.2 molar solution of ferric chloride.

Iron content of cheeses was determined colorimetrically by the ferrozine method (Zhang and Mahoney, 1989a). Cheeses were fortified to a level of approximately 70-76 milligrams of iron per kilogram of cheese (Appendix G).

Cheese quality was determined chemically by thiobarbituric acid-reactive substances (TBARS) content (Appendix H) and organoleptically by taste panel judges (Appendix I).

Cheese Consumption

Iron-fortified cheese aged for 7 months was consumed daily for 6 weeks. Consumption of cheese began on day 20 (week 4) and continued through day 61 (week 9). Daily intake was 42.5 milligrams (1.5 ounces), and provided 3.15 milligrams of iron. Except for the supplementation of iron-fortified cheese and a fasting period before test meals and blood draws, normal daily dietary routines were continued by each subject.

Assessment of Lipid Peroxidation

Thiobarbituric acid-reactive substances (TBARS) are an indicator of lipid peroxidation. To determine if the consumption of iron-fortified Cheddar cheese had an effect on *in vivo* lipid peroxidation, levels of malondialdehyde (MDA) measured as TBARS were assayed spectrophotometrically with a UV 2100 U spectrophotometer from Shimadzu, Co. (Kyoto, Japan) in serum, urine, and feces using a modified procedure of Buege and Aust (1978) (Appendix J).

Urine TBARS

Throughout the 10-week study, one morning-void urine sample was collected weekly from each subject (weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) and assayed for TBARS. Samples were stored at 4°C and assayed within 48 hours. Specific gravity (dissolved substances in urine as compared to density of water) was also determined on each urine sample (Tilkian et al., 1987).

Fecal TBARS

Throughout the 10-week study, one fecal sample was collected weekly from each subject (weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) and assayed for TBARS. Samples were stored at 4°C and assayed within 48 hours. Moisture content was also determined on each fecal sample.

Serum TBARS

Serum was obtained from whole venous blood. Subjects were required to fast overnight (10 hours) before the day blood was drawn from each one by a licensed medical technologist/phlebotomist. Throughout the 10-week study, one serum sample was collected approximately biweekly from each subject (weeks 1, 3, 6, 8, 10) and assayed for TBARS. Samples were stored at 4°C and assayed within 24 hours.

Assessment of Iron Status

Iron status parameters were assessed from venous blood drawn by licensed medical technologists/phlebotomists. Approximately 45 milliliters (ml) of blood (two 15 ml clot tubes and three 5 ml EDTA tubes) were drawn from each subject on five separate occasions (weeks 1, 3, 6, 8, 10) after an overnight (10 hour) fast. Blood was drawn at the start of the study to evaluate initial iron status, three times during the study, and at the end of the study. Samples from the first and last blood draws were also analyzed by National Health Labs (Salt Lake City, UT) to verify results. National Health Labs also provided an analysis of serum lipids, uric acid, calcium, and bilirubin.

Hemoglobin was determined in duplicate samples of whole blood by the cyanmethemoglobin method (Crosby et al., 1954).

Packed cell volume (hematocrit) was determined in duplicate samples of whole blood by the microhematocrit method.

Serum ferritin was measured in duplicate samples of serum by radioimmunoassay with the Du Pont (Billerica, MA) RIANEN® Ferritin [^{125}I] RIA Kit (Catalog No. NEA-078).

Serum iron and unsaturated iron-binding capacity (UIBC) were determined colorimetrically at 560 nm in duplicate samples of serum with Sigma Diagnostics® (St. Louis, MO) Iron and Total Iron-Binding Capacity Kit (procedure No. 565).

Total iron-binding capacity (TIBC) was estimated from the sum of serum iron and UIBC (Tilkian et al., 1987).

Serum transferrin was estimated from the product of TIBC and 0.8 minus 43 (Tilkian et al., 1987).

Transferrin saturation percentage was estimated from the quotient of serum iron concentration and serum transferrin (Tilkian et al., 1987).

Assessment of Iron Absorption

Utilizing an extrinsic labeling technique, we determined the absorption of iron from the iron-fortified Cheddar cheeses at two separate radioiron absorption test meals. A third radioiron test meal was also given substituting the iron-fortified cheese with a solution of ferrous sulfate and ascorbic acid. Absorption values from the third test meal will be used as an adjustment factor for comparisons with other investigations of iron absorption.

Ferric chloride iron-59 radionuclide (^{59}Fe) purchased from the DuPont Co. (Boston, MA) was used as the extrinsic label. The usage of radioiron paralleled the procedures and methodologies utilized by James D. Cook, M.D., Leif Hallberg, M.D., and Torrence H. Bothwell, M.D. in previous iron absorption studies (Cook et al., 1972, 1973, 1981, 1983; Cook and Monsen, 1976; Forbes et al., 1989; Hallberg and Sölvell, 1967; Hallberg and Rossander, 1982; Hallberg et al., 1989; Jacobs et al., 1968; Lamparelli et al., 1987; Macfarlane et al., 1988).

Radioiron absorption test meals 1, 2, and 3 were administered on weeks 1, 6, and 8, respectively. Subjects consumed their respective iron-fortified cheeses for test meals 1 and 2. Test meals 1 and 2 each consisted of 42.5 grams of iron-fortified cheese (approximately 3.15 milligrams of iron) extrinsically labeled with 2.5 microcuries of ^{59}Fe. Test meal 3 occurred on week 8 and consisted of a reference dose of ferrous ascorbate (3 milligrams of iron) labeled with 2.5 microcuries of ^{59}Fe. All test meals were consumed on the morning after an overnight fast (10 hours). Nothing further by mouth was allowed for 3 hours after each test meal.

Blood samples were taken 14 days after each test meal and coincided with blood draws 2, 4, and 5. Samples were wet-ashed, and measurement of ^{59}Fe radioactivity in duplicate 5 ml samples of whole blood and measured portions of test doses were performed by liquid scintillation in a Beckman Instruments (Irvine, CA) LS 5801 Liquid

Scintillation Counter (Appendix K). To determine blood volume of the subjects, the height and weight of each subject was obtained. Iron absorption (the percentage absorption of the radioiron dose) for each test meal was then calculated as the proportion of radioactivity retained in the blood 14 days later, less the background radiation (Appendix K).

Because a major amount of dietary iron is unabsorbed and directly excreted via the feces, fecal material after the radioiron test meals contained radioactivity that was above background radiation levels. Feces were, therefore, considered to be hazardous radioactive waste. Consequently, for legal and environmental safety reasons, at least 3 consecutive days of fecal output were collected from each subject after each test meal and retained in storage until background radioactivity levels (10 half-lives or 450 days) were attained as determined by hand-held counter.

Intestinal Transit Time

Intestinal transit time was determined using ^{59}Fe as a fecal marker. Consecutive fecal samples were collected from each subject after radioiron test meal 2 and assayed in duplicate for ^{59}Fe radioactivity until background levels were attained. Detection of gamma radiation in counts per minute (cpm) was performed using a Packard Auto-Gamma® 5530 gamma counter from Packard Instrument Co. (Meriden, CT) with a pre-set region for measuring ^{59}Fe activity. Efficiency of the instrument for ^{59}Fe was determined as .13252 (Appendix L). Quantitation of gamma radiation was in cpm and microcuries. Transit time was determined to be the time period in hours from ingestion of the radioiron test meal (time=0) until at least 80% recovery of the originally ingested radioiron dose of 2.5 microcuries and peak excretion of radioiron in counts per minute per gram of feces (Appendix L).

Dietary Records

Dietary records were maintained by each subject in order to quantify nutrient intakes. These data were used to determine any relationships between *in vivo* lipid peroxidation and intakes of specific nutrients . Subjects documented their dietary intake for 3 days before consumption of iron-fortified cheese (two weekdays and one weekend day during week 3) and for 3 days during consumption of iron-fortified cheese (two weekdays and one weekend day during week 6). Dietary records included the use of vitamin/mineral supplements.

Quantitation of nutrient intakes was accomplished utilizing Food/Analyst Plus™ nutritional analysis software from Hopkins Technology (Hopkins, MN). Dietary records were reviewed for completeness prior to coding foods into the analysis program. After coding, the output was proofread before statistical analyses. Dietary records were quantified for energy, total fat, dietary fiber, iron, zinc, copper, calcium, vitamin A, vitamin C, å-tocopherol, and carotene.

Statistical Design and Analysis

The experiment was a repeated measures design. To balance each treatment group for iron status of subjects, experimental units were first stratified according to serum ferritin levels (iron status), and then assigned to an experimental condition (iron-fortified cheese) at the initiation of the study.

Although 60 subjects originally volunteered for the study, due to attrition through voluntary termination and/or incomplete participation, 54 subjects were used for statistical comparisons (19 subjects from treatments $FeCl_3$ and Fe-C, and 16 subjects from treatment Fe-WP).

Statistica/Mac™ statistical software package by Statsoft, Inc. (Tulsa, OK) was used for all statistical analyses. Repeated measures over time within the same subject

were taken for lipid peroxidation, iron status, iron absorption, and dietary intake across time (before cheese consumption versus during/after cheese consumption). Therefore, within subject variance was a component of the analysis of variance (ANOVA). ANOVA tables are reported for overall serum, urine, and fecal TBARS only.

Comparisons utilizing ANOVA were made between treatment groups, across time, and within treatment groups across time. When "F" was significant ($p<.05$), planned comparisons were performed using least significant difference. Means and standard deviations of most importance to interpretation of results are reported in table format in the "Results" section of this document. Otherwise, tables appear in the appendices, and are called out utilizing the letter corresponding to the appendix in which they appear, and a number corresponding to the order in which they appear in the appendix (i.e., Table M.1 is the first table in appendix M).

Correlation analysis was performed between lipid peroxidation measurements and parameters of iron status, iron absorption, dietary intake, fecal transit time, fecal moisture, and urine specific gravity. Significant correlations ($p<.05$ and $r>.30$) were chosen for analysis of covariance (ANCOVA).

Experimental Schedule

Prior to Day 1: Blood Test #1 for background radioactivity counting, initial iron status, pregnancy testing, and serum TBARS.

Day 1: Radioiron Test Meal #1. Urine and fecal samples for initial TBARS.

Day 6: Begin before cheese dietary record. Urine and fecal samples for TBARS.

Day 13: Urine and fecal samples for TBARS.

Day 15: Blood Test #2 for radioactivity counting, iron status, and serum TBARS.

Day 20: Begin consumption of test cheese. Urine and fecal samples for TBARS.

Day 27: Urine and fecal samples for TBARS.

Day 34: Begin during cheese dietary record.

Day 36: Radioiron Test Meal #2. Blood Test #3 for radioactivity counting, iron status, and serum TBARS. Urine and fecal samples for TBARS.

Day 41: Urine and fecal samples for TBARS.

Day 50: Radioiron Test Meal #3. Blood Test #4 for radioactivity counting, iron status, and serum TBARS. Urine and fecal samples for TBARS.

Day 56: Urine and fecal samples for TBARS.

Day 61: Discontinue consumption of test cheese.

Day 62: Urine and fecal samples for TBARS.

Day 64: Blood Test #5 for radioactivity counting, iron status, and serum TBARS.

RESULTS

Thiobarbituric Acid-Reactive Substances

Serum TBARS

Serum TBARS (S-TBARS) were assayed on five separate occasions approximately 2 weeks apart over the 10-week study period. S-TBARS on weeks 1 and 3 were determined in the time period before iron-fortified cheese consumption. S-TBARS on weeks 6 and 8 were determined in the time period during cheese consumption. S-TBARS on week 10 were determined 3 days after termination of cheese consumption.

Serum TBARS Before Cheese

Two-way ANOVA by treatment and S-TBARS on weeks 1 and 3 did not indicate significant main effects for treatment, S-TBARS, or a significant interaction of treatment and S-TBARS before cheese consumption (Table M.1).

Serum TBARS During Cheese

Two-way ANOVA by treatment and S-TBARS on weeks 6 and 8 indicated a significant ($p<.001$) main effect for S-TBARS but no main effect for treatment, or interaction of treatment and S-TBARS after initiation of cheese consumption (Table 1).

Column mean for S-TBARS on week 6 was significantly higher ($p<.001$) than column mean for S-TBARS on week 8. Within treatments, S-TBARS on week 6 were also higher than S-TBARS on week 8.

Serum TBARS Overall

Two-way ANOVA by treatment and S-TBARS on weeks 1, 3, 6, 8, and 10 indicated a significant ($p<.001$) main effect for S-TBARS but no main effect for treatment, or interaction of treatment and S-TBARS (Table 2 and Table N.1). Column mean for S-TBARS on week 6 was higher ($p<.001$) than the column mean for S-TBARS

Table 1. Serum TBARS During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	$6.6^{a,1}$ ±1.2	5.6^{b} ±1.1	6.1 ±1.3
Fe-C	6.2^{a} ±1.3	5.5^{b} ±1.0	5.8 ±1.1
Fe-WP	6.6^{a} ±1.2	5.2^{b} ±1.0	5.9 ±1.3
Pooled	6.4^{a} ±1.3	5.4^{b} ±1.1	

a,b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
1 Means and standard deviations in nanomoles/milliliter.

Table 2. Serum TBARS Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	5.7[1] ±1.1	5.4 ±1.1	6.6 ±1.2	5.6 ±1.1	5.1 ±1.1	5.7 ±1.2
Fe-C	5.7 ±0.9	5.5 ±1.1	6.2 ±1.3	5.5 ±1.0	4.8 ±0.6	5.5 ±1.1
Fe-WP	5.5 ±1.0	5.5 ±1.2	6.6 ±1.4	5.2 ±1.0	4.8 ±0.9	5.5 ±1.2
Pooled	5.6^{a} ±1.0	5.5^{a} ±1.1	6.4^{b} ±1.3	5.4^{a} ±1.1	4.9^{c} ±0.9	

a, b, c Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
1 Means and standard deviations in nanomoles/milliliter.

on weeks 1, 3, 8 or 10. Column mean for S-TBARS on week 8 was higher ($p<.05$) than column mean for S-TBARS on week 10 (after cheese consumption). Column mean for S-TBARS on week 10 was lower ($p<.05$) than column mean for S-TBARS on weeks 1, 3, or 8 and lower ($p<.001$) than column mean for S-TBARS on week 6. Planned

comparisons of column means before cheese (S-TBARS on weeks 1 and 3) and after cheese (S-TBARS on week 10) indicated S-TBARS on week 10 to be lower ($p<.025$) than S-TBARS on weeks 1 or 3.

Serum TBARS Before Versus During

Two-way ANOVA by treatment and S-TBARS on pooled S-TBARS before cheese (S-TBARS on weeks 1 and 3) and during cheese (S-TBARS on weeks 6 and 8) indicated a significant ($p<.025$) main effect for S-TBARS, but no main effect for treatment, or interaction of treatment and S-TBARS (Table 3). S-TBARS before cheese were higher than S-TBARS during cheese. Within treatment groups, S-TBARS increased ($p<.025$) only for $FeCl_3$.

Correlation with Serum TBARS

Serum TBARS determinations without statistically significant differences were pooled, and correlation analysis was performed with parameters of iron status, iron absorption, urine, and fecal TBARS, urine specific gravity, nutrient intake, fecal transit time, fecal moisture, serum lipids, serum calcium, serum bilirubin, and serum uric acid. The only significant correlation ($p<.05$ and $r>.30$) chosen for covariance analysis was with total iron-binding capacity (TIBC). Since column means for serum TBARS 3 ($r=.36$) and serum TBARS 5 ($r=.33$) were found to differ significantly from other serum TBARS and each other, an analysis of covariance was performed with TIBC. Results as reported above did not significantly differ with TIBC as a covariate.

Urine TBARS

Urine TBARS (U-TBARS) were assayed weekly and totaled ten determinations over the 10-week study period. U-TBARS on weeks 1, 2, 3, and 4 were before the cheese consumption period, and U-TBARS on weeks 5, 6, 7, 8, 9, and 10 were during the cheese consumption period.

Urine TBARS Before Cheese

Two-way ANOVA by treatment and U-TBARS on weeks 1, 2, 3, and 4 indicated a significant ($p<.025$) main effect for U-TBARS but no main effect for treatment, or interaction of treatment and U-TBARS before cheese consumption (Table M.2). Column mean for U-TBARS on week 1 was lower than the column mean for U-TBARS on weeks 3 ($p<.025$) or 4 ($p<.01$).

Urine TBARS During Cheese

Two-way ANOVA by treatment and U-TBARS on weeks 5, 6, 7, 8, 9, and 10 did not indicate significant main effects for treatment, U-TBARS, or interaction of treatment and U-TBARS during cheese consumption (Table M.3). However, planned comparisons of column means indicated that U-TBARS on week 10 were significantly lower ($p<.05$) than U-TBARS on week 5.

Table 3. Serum TBARS Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	$5.6^{a,1}$ ±1.1	6.1^{b} ±1.3
Fe-C	5.6^{a} ±1.0	5.8^{b} ±1.1
Fe-WP	5.5^{a} ±1.1	5.9^{b} ±1.3
Pooled	5.5^{a} ±1.1	5.9^{b} ±1.2

a, b Means in same row or column without common superscripts are different ($p<.05$); pooled means compared only to other pooled means in same row or column.

1 Means and standard deviations in nanomoles/milliliter.

Urine TBARS Overall

Two-way ANOVA by treatment and U-TBARS on weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 did not indicate significant main effects for treatment, U-TBARS, or significant interaction of treatment and time (Table M.4 and Table N.2).

However, planned comparisons of column means did indicate that U-TBARS on week 4 were significantly higher than U-TBARS on weeks 1 or 10 ($p<.01$) and U-TBARS on weeks 7 or 8 ($p<.025$). Also, U-TBARS on week 3 were significantly ($p<.025$) higher than U-TBARS on weeks 1 or 10.

Urine TBARS Before Versus During

Two-way ANOVA by treatment and pooled U-TBARS before cheese consumption (U-TBARS on weeks 1, 2, 3, and 4) and during cheese consumption (U-TBARS on weeks 5, 6, 7, 8, 9, and 10) did not indicate significant main effects for treatment, U-TBARS, or interaction of treatment and U-TBARS (Table M.5). One-way ANOVA found U-TBARS to become significantly lower ($p<.01$) within treatment group Fe-C across time.

Correlation with Urine TBARS

U-TBARS without significant differences were pooled and correlation analysis was performed with iron status, iron absorption, serum and fecal TBARS, urinary specific gravity, nutrient intake, intestinal transit time, fecal moisture, serum lipids, serum calcium, serum bilirubin, and serum uric acid. Urine specific gravity ($r=.46$) was the only significant ($p<.05$ and $r>.30$) correlation. ANCOVA with specific gravity altered results as reported above (Table 4). The main effect of U-TBARS overall became significant ($p<.025$). U-TBARS on weeks 3 ($p<.05$) and 4 ($p<.025$) were higher than all other U-TBARS except U-TBARS on week 5. Neither U-TBARS before versus during cheese consumption, nor the effect of treatment differed with specific gravity as a covariate.

Table 4. Urine TBARS with Specific Gravity as Covariate

Week	1	2	3	4	5	6	7	8	9	10
Treatment:										
$FeCl_3$	10.3[1] ±4.3	12.0 ±4.1	13.4 ±6.3	12.3 ±5.6	12.9 ±5.7	11.9 ±4.4	11.8 ±3.5	11.4 ±6.1	12.6 ±4.9	10.4 ±4.2
Fe-C	12.9 ±6.0	13.0 ±6.1	12.1 ±5.9	13.5 ±2.8	11.7 ±4.6	11.6 ±5.0	11.6 ±4.6	11.2 ±5.2	9.7 ±4.6	10.9 ±4.2
Fe-WP	8.9 ±4.2	9.2 ±3.8	11.8 ±4.4	12.6 ±4.3	11.4 ±5.3	10.7 ±4.2	9.8 ±4.6	10.6 ±6.4	11.8 ±6.1	10.1 ±4.1
Pooled	10.7^{a} ±5.1	11.4^{a} ±5.0	12.4^{b} ±5.6	12.8^{b} ±4.3	12.0^{ab} ±5.2	11.4^{a} ±4.9	11.0^{a} ±4.2	11.0^{a} ±5.8	11.4^{a} ±5.3	10.5^{a} ±4.1

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in nanomoles/milliliter.

Fecal TBARS

Fecal TBARS were assayed weekly and totaled ten determinations over the 10-week study period. Fecal TBARS on weeks 1, 2, 3, and 4 were before iron-fortified cheese consumption, and fecal TBARS on weeks 5, 6, 7, 8, 9, and 10 were during iron-fortified cheese consumption. Moistures were also determined for each fecal sample, and TBARS in feces were analyzed statistically on both a fecal wet TBARS (FW-TBARS) and fecal dry TBARS (FD-TBARS) basis.

Fecal Wet TBARS Before Cheese

Two-way ANOVA by treatment and FW-TBARS on weeks 1, 2, 3, and 4 indicated a significant ($p<.025$) main effect for FW-TBARS but no main effect for treatment, or interaction of treatment and FW-TBARS before cheese consumption (Table M.6). The column mean for FW-TBARS on week 2 was higher than the column mean for FW-TBARS on weeks 1 or 4.

Fecal Wet TBARS During Cheese

Two-way ANOVA by treatment and FW-TBARS on weeks 5, 6, 7, 8, 9, and 10 did not indicate significant main effects for treatment, FW-TBARS, or interaction of treatment and FW-TBARS during cheese consumption (Table M.7).

Fecal Wet TBARS Overall

Two-way ANOVA by treatment and FW-TBARS on weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 indicated significant main effects for treatment ($p<.05$) and FW-TBARS ($p<.001$) but no interaction of treatment and FW-TBARS (Table 5 and Table N.3). Row means indicated treatment $FeCl_3$ was higher than treatments Fe-C or Fe-WP. Column means for FW-TBARS on weeks 1, 2, 3, and 4 were higher than column means for FW-TBARS on weeks 5, 7, 8, 9, or 10.

Fecal Wet TBARS Before Versus During

Two-way ANOVA by treatment and pooled FW-TBARS before iron-fortified cheese consumption (FW-TBARS on weeks 1, 2, 3, and 4) and during cheese consumption (FW-TBARS on weeks 5, 6, 7, 8, 9, and 10) indicated significant main effects for treatment ($p<.05$) and FW-TBARS ($p<.001$) but no interaction of treatment and FW-TBARS (Table 6). Row means indicated treatment $FeCl_3$ was higher than Fe-C or Fe-WP. Column means indicated FW-TBARS were lower during cheese consumption. One-way ANOVA within treatment groups found lower ($p<.001$) FW-TBARS for all treatments across time.

Correlation with Fecal Wet TBARS

FW-TBARS without significant differences were pooled and correlation analysis was performed with iron status, iron absorption, serum and urine TBARS, urinary specific gravity, nutrient intake, fecal transit time, fecal moisture, serum lipids, serum calcium, serum bilirubin, and serum uric acid. Fecal moisture (r varied from -.35 to -.53)

was the only significant correlation ($p<.05$ and $r>.30$). ANCOVA with fecal moisture found the main effect for treatment to become nonsignificant (Table N.4). Also, the main effect of time before cheese consumption became nonsignificant. However, the overall main effect of time before cheese versus during cheese retained significance.

Table 5. Fecal Wet TBARS Overall

Week	1	2	3	4	5	6	7	8	9	10
Treatment:										
$FeCl_3$	12.1[1] ±4.7	13.5 ±5.7	12.8 ±4.1	11.1 ±2.8	11.2 ±3.3	11.5 ±3.4	10.4 ±2.3	10.9 ±2.8	10.2 ±2.0	10.6 ±2.5
Fe-C	11.0 ±5.1	11.8 ±2.9	11.4 ±3.1	11.1 ±3.0	8.9 ±2.2	11.2 ±3.8	9.8 ±2.9	8.3 ±2.3	8.3 ±3.0	8.3 ±2.0
Fe-WP	9.2 ±3.0	12.0 ±32.9	10.9 ±4.0	11.0 ±3.8	9.4 ±2.4	9.7 ±2.6	9.5 ±2.4	10.2 ±1.6	9.3 ±2.9	9.1 ±2.5
Pooled	10.8^{a} ±4.5	12.5^{a} ±4.1	11.7^{a} ±3.8	11.0^{a} ±3.1	9.8^{b} ±2.8	10.8^{ab} ±3.4	9.9^{b} ±2.5	9.8^{b} ±2.5	9.2^{b} ±2.7	9.3^{b} ±2.5

a, b Means in same row or column without common superscripts are different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in micrograms/gram.

Table 6. Fecal Wet TBARS Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	$12.4^{a,1}$ ±4.4	10.8^{b} ±2.8	11.4^{a} ±3.6
Fe-C	11.3^{a} ±3.6	9.1^{b} ±2.9	10.0^{b} ±3.4
Fe-WP	10.8^{a} ±3.5	9.5^{b} ±2.4	10.0^{b} ±3.0
Pooled	11.5^{a} ±3.8	9.8^{b} ±2.7	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in micrograms/gram.

Fecal Dry TBARS Before Cheese

Two-way ANOVA by treatment and FD-TBARS on weeks 1, 2, 3, and 4 did not indicate significant main effects for treatment, FD-TBARS, or interaction of treatment and FD-TBARS before cheese consumption (Table M.8).

Fecal Dry TBARS During Cheese

Two-way ANOVA by treatment and FD-TBARS on weeks 5, 6, 7, 8, 9, and 10 indicated a significant ($p<.05$) main effect for FD-TBARS but no main effect for treatment or interaction of treatment and FD-TBARS during cheese consumption (Table M.9). Column mean for FD-TBARS on week 6 was significantly higher than all other column means.

Fecal Dry TBARS Overall

Two-way ANOVA by treatment and FD-TBARS on weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 indicated a significant ($p<.001$) main effect for FD-TBARS but no main effect for treatment or interaction of treatment and FD-TBARS (Table 7 and Table N.5). Column means for FD-TBARS on weeks 1, 2, 3, and 4 were higher than column means for FD-TBARS on weeks 5, 7, 8, 9, and 10.

Fecal Dry TBARS Before Versus During

Two-way ANOVA by treatment and pooled FD-TBARS before cheese (FD-TBARS on weeks 1, 2, 3, 4) and during cheese (FD-TBARS on weeks 5, 6, 7, 8, 9, 10) consumption indicated a significant ($p<.001$) main effect for FD-TBARS, but no main effect for treatment or interaction of treatment and FD-TBARS (Table 8). By column mean and within treatment groups, FD-TBARS were higher before versus during cheese consumption.

Table 7. Fecal Dry TBARS Overall

Week	1	2	3	4	5	6	7	8	9	10
Treatment:										
$FeCl_3$	41.3[1] ±16.5	45.8 ±18.8	45.0 ±15.2	37.1 ±10.4	38.1 ±10.5	40.4 ±13.3	37.4 ±7.1	40.9 ±9.7	36.1 ±7.6	37.0 ±5.6
Fe-C	42.0 ±16.9	44.8 ±13.2	44.0 ±11.7	41.5 ±8.7	35.1 ±9.8	44.8 ±37.4	37.8 ±9.6	35.8 ±9.1	35.4 ±7.3	34.4 ±7.5
Fe-WP	36.6 ±10.4	44.4 ±13.0	41.8 ±14.7	44.4 ±11.3	34.1 ±6.2	39.8 ±12.2	35.1 ±8.6	36.7 ±9.9	37.5 ±6.1	35.7 ±4.5
Pooled	40.0[a] ±15.0	45.0[a] ±15.1	43.6[a] ±13.7	41.0[a] ±10.4	35.9[b] ±9.1	41.7[ab] ±11.2	36.7[b] ±8.4	37.5[b] ±9.6	36.3[b] ±7.0	35.7[b] ±6.1

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in micrograms/gram.

Table 8. Fecal Dry TBARS Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	42.3[a, 1] ±15.6	38.2[b] ±9.3	39.8 ±12.3
Fe-C	43.1[a] ±12.8	37.2[b] ±9.1	39.6 ±11.1
Fe-WP	41.8[a] ±12.6	36.6[b] ±8.3	38.6 ±10.5
Pooled	42.4[a] ±13.7	37.3[b] ±8.9	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in micrograms/gram.

Correlation with Fecal Dry TBARS

FD-TBARS without significant differences were pooled and correlation analysis performed with parameters of iron status, iron absorption, serum and urine TBARS, dietary intake, intestinal transit time, fecal moisture, serum lipids, serum calcium, serum

bilirubin, and serum uric acid. No significant ($p<.05$ with $r>.30$) relationships between FD-TBARS and selected parameters were found.

Iron Status

Iron status parameters included hemoglobin (HGB), hematocrit (HCT), serum iron (SFE), total iron-binding capacity (TIBC), transferrin saturation (TSAT), and serum ferritin (SFER). Parameters were assayed approximately every two weeks and totaled five determinations over the study period. Iron status parameters assayed on weeks 1 and 3 were before the period of iron-fortified cheese consumption. Iron status parameters assayed on weeks 6 and 8 were during the period of cheese consumption. Iron status parameters assayed on week 10 were 3 days after termination of cheese consumption.

Hemoglobin Before Cheese

Two-way ANOVA by treatment and HGB on weeks 1 and 3 indicated a significant main effect for HGB ($p<.01$) but no main effect for treatment or interaction of treatment and HGB before cheese consumption (Table O.1). Column mean for HGB on week 3 was significantly higher than the column mean for HGB on week 1. HGB on week 1 for treatment group Fe-C was significantly lower than all other determinations.

Hemoglobin During Cheese

Two-way ANOVA by treatment and HGB on weeks 6 and 8 did not indicate significant main effects for treatment, HGB, or an interaction of treatment and HGB during the cheese consumption period (Table O.2).

Hemoglobin Overall

Two-way ANOVA by treatment and HGB on weeks 1, 3, 6, 8, and 10 indicated a significant main effect ($p<.001$) for HGB but no main effect for treatment or interaction

of treatment and HGB (Table 9). Column mean for HGB on week 10 (after termination of cheese consumption) was lower than all other the column means. Planned comparisons indicated that the column mean for pooled HGB before cheese (HGB on weeks 1 and 3) was higher ($p<.001$) than the column for HGB on week 5. This also held true within treatment groups ($p<.01$)

Hemoglobin Before Versus During

Two-way ANOVA by treatment and pooled HGB before cheese (HGB on weeks 1 and 3) and during cheese consumption (HGB on weeks 6 and 8) indicated a significant ($p<.01$) main effect for HGB but no main effect for treatment or interaction of treatment and HGB (Table 10). The column mean for hemoglobin during cheese consumption was lower than the column mean before cheese consumption. Within treatments, significantly lower hemoglobin levels were found during cheese consumption for $FeCl_3$ ($p<.01$) and Fe-WP ($p<.025$) only.

Table 9. Hemoglobin Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	14.9[1] ±1.0	15.1 ±0.8	14.6 ±0.8	14.9 ±0.8	14.3 ±1.0	14.8 ±0.9
Fe-C	14.3 ±0.9	14.7 ±0.8	14.5 ±0.8	14.6 ±1.0	14.2 ±1.0	14.4 ±0.9
Fe-WP	15.0 ±0.8	15.1 ±0.8	14.8 ±1.0	14.8 ±0.8	14.0 ±0.9	14.8 ±0.9
Pooled	14.7[a] ±0.9	15.0[a] ±0.8	14.6[a] ±0.8	14.6[a] ±0.9	14.2[b] ±0.9	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in grams/deciliter.

Table 10. Hemoglobin Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	15.0[a, 1] ±0.9	14.7[b] ±0.9
Fe-C	14.5[a] ±0.9	14.5[ab] ±1.0
Fe-WP	15.0[a] ±0.8	14.8[b] ±0.9
Pooled	14.9[a] ±0.8	14.5[b] ±0.9

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in grams/deciliter.

Hematocrit Before Cheese

Two-way ANOVA by treatment and HCT on weeks 1 and 3 did not indicate significant main effects for treatment, HCT, or interaction of treatment and HCT before cheese consumption (Table O.3).

Hematocrit During Cheese

Two-way ANOVA by treatment and HCT on weeks 6 and 8 did not indicate significant main effects for treatment, HCT, or an interaction of treatment and HCT during cheese consumption (Table O.4).

Hematocrit Overall

Two-way ANOVA by treatment and HCT on weeks 1, 3, 6, 8, and 10 indicated main effects for treatment ($p<.025$) and HCT ($p<.001$) but no interaction of treatment and HCT (Table 11). Row means indicated treatment group Fe-C had overall lower hematocrit levels. Column mean for HCT on week 5 was significantly higher than all other column means. Planned comparisons indicated that pooled HCT before the cheese

Table 11. Hematocrit Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	42.7[1] ±2.0	42.2 ±1.7	42.5 ±2.2	42.4 ±1.8	44.2 ±2.3	42.8[a] ±2.1
Fe-C	40.9 ±2.2	41.0 ±2.0	41.0 ±2.2	41.2 ±2.4	42.8 ±2.2	41.3[b] ±2.3
Fe-WP	42.9 ±2.4	42.2 ±2.2	43.3 ±1.9	42.6 ±2.1	43.6 ±2.4	42.9[a] ±2.2
Pooled	42.1[a] ±2.3	41.8[a] ±2.0	42.3[a] ±2.2	42.0[a] ±2.2	43.5[b] ±2.3	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in percent red blood cells.

consumption period (HCT on weeks 1 and 3) was lower ($p<.001$) than HCT on week 5 (after termination of cheese consumption) and also within treatment groups ($p<.025$).

Hematocrit Before Versus During

Two-way ANOVA by treatment and pooled HCT before cheese (weeks 1 and 3) and during cheese (weeks 6 and 8) consumption did not indicate significant effects for HCT, treatment, or an interaction of treatment and HCT (Table O.5).

Serum Iron Before Cheese

Two-way ANOVA by treatment and SFE on weeks 1 and 3 indicated a significant ($p<.025$) main effect of SFE but no main effect of treatment, or interaction of treatment and SFE prior to cheese consumption (Table O.6). Column mean for SFE on week 3 was higher than the column mean for SFE on week 1.

Serum Iron During Cheese

Two-way ANOVA by treatment and SFE on weeks 6 and 8 did not indicate main effects of treatment, SFE, or an interaction of treatment and SFE during the cheese consumption period (Table O.7).

Serum Iron Overall

Two-way ANOVA by treatment and SFE on weeks 1, 3, 6, 8, and 10 indicated a significant ($p<.01$) main effect of SFE but no main effect of treatment, or interaction of treatment and SFE (Table 12). Column mean for SFE on week 3 was lower than column mean for SFE on weeks 1, 8, or 10. Planned comparisons indicated that the column mean for pooled SFE before cheese consumption (SFE on weeks 1 and 3) was lower ($p<.01$) than SFE on week 10 (after the cheese consumption period). Within treatments, this comparison was only significant for Fe-C ($p<.05$).

Serum Iron Before Versus During

Two-way ANOVA by treatment and pooled SFE before cheese consumption (SFE on weeks 1 and 3) and during cheese consumption (SFE on weeks 6 and 8) did not indicate significant main effects of treatment, time, or an interaction of treatment and time (Table O.8). One-way ANOVA within treatment groups did not indicate significant differences between before and during cheese consumption.

Total Iron-Binding Capacity Before Cheese

Two-way ANOVA by treatment and TIBC on weeks 1 and 3 did not indicate significant main effects of treatment, TIBC, or an interaction of treatment and TIBC before the cheese consumption period (Table O.9).

Table 12. Serum Iron Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	112.5[1] ±49.8	82.9 ±41.8	100.2 ±32.0	97.6 ±29.6	109.2 ±44.4	100.5 ±40.7
Fe-C	97.9 ±47.4	87.4 ±27.9	91.0 ±40.9	108.1 ±39.2	109.5 ±38.2	98.8 ±39.4
Fe-WP	103.7 ±35.4	100.1 ±42.0	107.3 ±39.4	103.9 ±37.1	113.6 ±33.1	105.7 ±36.8
Pooled	104.7[a] ±44.7	90.2[b] ±37.5	99.5[ab] ±37.4	103.2[a] ±35.1	110.8[a] ±38.5	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in micrograms/deciliter.

Total Iron-Binding Capacity During Cheese

Two-way ANOVA by treatment and TIBC on weeks 6 and 8 indicated a significant ($p<.001$) main effect of TIBC but no main effect of treatment, or interaction of treatment and TIBC during cheese consumption (Table O.10). Column mean for TIBC on week 6 was significantly higher than the column mean for TIBC on week 8.

Total Iron-Binding Capacity Overall

Two-way ANOVA by treatment and TIBC on weeks 1, 3, 6, 8, and 10 indicated a significant ($p<.001$) main effect of TIBC but no main effect of treatment, or interaction of treatment and TIBC (Table 13). Column means for TIBC on weeks 1 and 3 were significantly higher ($p<.01$) than column means for TIBC on weeks 8 or 10. Column mean for TIBC on week 3 was higher ($p<.01$) than column mean for TIBC on week 8. Planned comparisons indicated that pooled TIBC before cheese consumption (TIBC on weeks 1 and 3) was higher ($p<.025$) than TIBC on week 10 (after cheese consumption period).

Table 13. Total Iron-Binding Capacity Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	351.2[1] ±42.4	338.3 ±55.7	359.6 ±61.2	325.0 ±42.4	331.6 ±52.9	341.2 ±51.9
Fe-C	332.1 ±55.3	319.8 ±45.7	331.0 ±62.4	312.4 ±50.9	317.3 ±59.5	322.5 ±54.4
Fe-WP	349.5 ±48.4	344.3 ±61.1	341.9 ±61.5	318.5 ±51.9	334.1 ±60.6	337.7 ±56.6
Pooled	344.3[a] ±48.9	334.1[ac] ±54.1	344.2[a] ±61.7	318.7[b] ±47.7	327.7[bc] ±57.0	

a, b, c Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in micrograms/deciliter.

Total Iron-Binding Capacity Before Versus During

Two-way ANOVA by treatment and pooled TIBC before cheese (TIBC on weeks 1 and 3) and during cheese (TIBC on weeks 6 and 8) consumption did not indicate main effects for treatment, TIBC, or interaction of treatment and TIBC (Table O.11). One way ANOVA within treatments indicated treatment Fe-WP had a lower ($p<.025$) TIBC level during the cheese consumption period.

Transferrin Saturation Before Cheese

Two-way ANOVA by treatment and TSAT on weeks 1 and 3 did not indicate significant main effects for treatment, TSAT, or an interaction of treatment and TSAT before the cheese consumption period (Table O.12).

Transferrin Saturation During Cheese

Two-way ANOVA by treatment and TSAT on weeks 6 and 8 did not indicate significant main effects for treatment, TSAT, or interaction of treatment and TSAT during the cheese consumption period (Table O.13).

Transferrin Saturation Overall

Two-way ANOVA by treatment and TSAT on weeks 1, 3, 6, 8, and 10 indicated a significant ($p<.001$) main effect of TSAT but no main effect of treatment or interaction of treatment and TSAT (Table 14). Column mean for TSAT on week 10 was significantly higher ($p<.01$) than the column means for TSAT on weeks 1, 3, or 6. The column mean for TSAT on week 8 was significantly higher ($p<.01$) than the column mean for TSAT on week 3. Planned comparisons indicated that pooled TSAT before cheese (TSAT on weeks 1 and 3) was higher ($p<.001$) than TSAT on week 5 (after cheese consumption period). However, within treatments, this comparison was significant for $FeCl_3$ ($p<.05$) and Fe-C ($p<.025$) only.

Transferrin Saturation Before Versus During

Two-way ANOVA by treatment and pooled TSAT before (TSAT on weeks 1 and 3) and during (TSAT on weeks 6 and 8) the cheese consumption period did not indicate main effects for treatment, TSAT, or interaction of treatment and TSAT (Table O.14).

Table 14. Transferrin Saturation Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	32.1[1] ±14.1	25.6 ±13.9	28.3 ±8.0	30.1 ±8.4	34.6 ±18.6	30.1 ±13.3
Fe-C	30.1 ±15.8	28.0 ±10.2	28.5 ±13.0	35.6 ±13.7	35.9 ±14.1	31.6 ±13.6
Fe-WP	29.4 ±8.8	29.0 ±10.4	32.8 ±14.5	33.7 ±13.7	34.4 ±9.8	31.9 ±11.6
Pooled	30.5^{ab} ±13.2	27.5^{a} ±11.6	29.9^{ab} ±11.9	33.1^{bc} ±12.1	35.0^{c} ±14.6	

a, b, c Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in percent saturation.

Serum Ferritin Before Cheese

Two-way ANOVA by treatment and SFER on weeks 1 and 3 indicated a significant ($p<.001$) main effect for TSAT but no main effect for treatment or interaction of treatment and TSAT before the cheese consumption period (Table O.15). Column mean for SFER on week 1 was higher than column mean for SFER on week 3.

Serum Ferritin During Cheese

Two-way ANOVA by treatment and SFER on weeks 6 and 8 indicated a significant main ($p<.025$) effect for SFER but no main effect for treatment, or interaction of treatment and SFER during the cheese consumption period (Table O.16). Column mean for SFER on week 6 was higher ($p<.01$) than the column mean for SFER on week 8. SFER on week 6 for treatment group $FeCl_3$ was significantly higher ($p<.001$) than all other SFER determinations during the cheese consumption period.

Serum Ferritin Overall

Two-way ANOVA by treatment and SFER on weeks 1, 3, 6, 8, and 10 indicated a significant ($p<.001$) main effect of SFER but no main effect of treatment, or interaction of treatment and SFER (Table 15). Column mean for SFER on week 1 was significantly higher ($p<.01$) than all other column means. Column mean for SFER on week 6 was significantly higher ($p<.01$) than column mean for SFER on week 8. Planned comparisons indicated that pooled SFER before the cheese consumption period (SFER on weeks 1 and 3) was higher ($p<.001$) than SFER on week 10 (after the cheese consumption period). However, within treatments, this comparison was significant for treatment Fe-C ($p<.05$) only.

Table 15. Serum Ferritin Overall

Week	1	3	6	8	10	Pooled
Treatment:						
$FeCl_3$	37.4[1] ±34.3	28.4 ±29.2	39.5 ±56.4	26.8 ±30.1	29.9 ±29.2	32.4 ±36.9
Fe-C	37.5 ±32.0	28.7 ±24.8	25.8 ±22.7	23.5 ±22.6	27.0 ±24.1	28.5 ±25.4
Fe-WP	39.6 ±37.7	27.0 ±31.0	27.2 ±26.3	20.8 ±25.0	25.1 ±28.6	28.0 ±30.0
Pooled	38.2[a] ±33.9	28.0[bc] ±27.8	30.8[bc] ±38.6	23.7[b] ±25.8	27.4[bc] ±26.9	

a, b, c Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in micrograms/liter.

Serum Ferritin Before Versus During

Two-way ANOVA by treatment and pooled SFER before (SFER on weeks 1 and 3) and during (SFER on weeks 6 and 8) cheese consumption indicated a significant ($p<.01$) main effect for SFER but no main effect for treatment or interaction of treatment and SFER (Table O.17). Although column means indicated a significant decrease in SFER, within treatment groups, only Fe-C and Fe-WP had significantly ($p<.01$) lower SFER levels during cheese consumption.

Iron Absorption

Iron absorption from iron-fortified cheese was determined twice during the 10-week experimental period. Absorption was measured on week 1 before cheese consumption (Test Meal 1) and on week 6 during cheese consumption (Test Meal 2).

Two-way ANOVA by treatment and iron absorption did not indicate significant main effects for treatment, iron absorption, or interaction of treatment and iron absorption

(Table 16). One-way ANOVA within treatment groups did not indicate any significant difference in iron absorption before versus during cheese consumption.

Intestinal Transit Time

Intestinal transit time was determined on week 6 using ^{59}Fe as a fecal marker, and coincided with test meal 2. One-way ANOVA by treatment did not indicate a significant effect of treatment (Table 17). However, the difference between treatments $FeCl_3$ and Fe-C did approach significance ($p<.08$) .

Correlation analysis was performed with intestinal transit time and parameters of iron status, iron absorption, serum, urine, and fecal TBARS, dietary intake, and fecal moisture. Correlations were performed by pooling together all fecal transit time determinations across treatment groups. The only significant correlation ($p<.05$ and $r >.30$) chosen for covariance analysis was fecal moisture ($r = -.60$) which indicated an inverse relationship. Analysis of covariance with fecal moisture as the covariate showed the effect of treatment to remain nonsignificant ($p<.66$).

Table 16. Iron Absorption from Iron-Fortified Cheeses

Test Meal:	1	2	Pooled
Treatment:			
$FeCl_3$	8.5[1] ±6.9	7.1 ±5.7	7.8 ±6.3
Fe-C	8.2 ±6.6	8.2 ±4.7	8.2 ±5.6
Fe-WP	7.5 ±9.8	6.3 ±6.3	6.9 ±8.1
Pooled	8.1 ±7.6	7.2 ±5.5	

[1] Means and standard deviations in percent retained radioiron.

Fecal Moisture

Fecal moisture (FMOIST) was determined on all fecal samples and also on week 6 to coincide with transit time measurements. One-way ANOVA by treatment did not indicate a significant difference in FMOIST between treatments at week 6 (Table 17). However, two-way ANOVA performed by treatment and all fecal moisture determinations (FMOIST ALL) indicated a significant ($p<.05$) main effect of treatment. Treatment $FeCl_3$ was lower ($p<.05$) than Fe-C. Also, the difference between $FeCl_3$ and Fe-C approached significance ($p<.09$).

Dietary Intakes

Two separate 3-day intake periods were analyzed for each treatment group (Table 18, Table 19, Table 20). Intake periods were before and during cheese consumption.

Iron Intake

Two-way ANOVA by treatment and iron intake before versus during the cheese consumption period indicated a significant ($p<.01$) main effect for iron intake but no main effect for treatment or interaction of treatment and iron intake (Table 21). Although column means indicated that iron intake increased during cheese consumption, within treatment groups, group $FeCl_3$ had a statistically significant increase in iron intake across time.

Table 17. Intestinal Transit Time and Fecal Moisture

	Transit Time	Fecal Moisture Week 6	Fecal Moisture Overall
Treatment:			
$FeCl_3$	79.2[1] ±39.7	71.0[2] ±6.3	70.9 ±5.9
Fe-C	60.5 ±20.0	75.1 ±6.0	74.5 ±5.7
Fe-WP	64.7 ±26.7	74.6 ±7.0	73.4 ±6.7

[1] Means and standard deviations in hours.
[2] Means and standard deviations in percent moisture.

Table 18. Dietary Intakes for Subjects in Treatment Ferric Chloride

	Before Cheese			During Cheese		
	Mean	SD	Range	Mean	SD	Range
Iron (mg)	13.1[1]	8.8	1.6-62.4	16.3	8.7	4.1-57.4
Energy (Kcal)	1923	826	309-4802	1973	801	616-5505
Fat (g)	74.0	36.8	5.5-159.7	77.8	32.7	18.9-166.3
Dietary Fiber (g)	13.0	6.2	1.1-30.7	13.2	11.4	1.4-84.7
Vitamin A (IU)	6678	6733	187-31744	6589	7473	722-44175
Carotene (RE)	420	567	7-3010	348	516	9-2292
å-tocopherol (mg)	11.0	19.7	0.8-146.6	17.6	55.9	0.9-428.1
Vitamin C (mg)	107.4	96.7	0.2-403.3	113.7	170.6	2.8-1224
Calcium (mg)	854	469	70-2360	1109	490	398-2742
Copper (mg)	1.1	0.6	0.1-3.7	1.2	0.8	0.2-4.0
Zinc (mg)	11.8	12.8	0.7-61.1	12.8	13.2	2.9-65.2

[1] Values are for three days from 19 subjects (57 subject-days)

Table 19. Dietary Intakes for Subjects in Treatment Ferric-Casein

	Before Cheese			During Cheese		
Treatment:	Mean	SD	Range	Mean	SD	Range
Iron (mg)	15.1[1]	7.9	4.1-38.0	17.4	12.3	4.6-95.1
Energy (Kcal)	2212	780	875-4441	2112	851	405-4596
Fat (g)	82.9	38.6	10.0-165.0	83.8	35.6	22.0-208.7
Dietary Fiber (g)	15.3	8.5	4.3-43.2	14.4	7.5	1.9-35.3
Vitamin A (IU)	6094	9275	112-68587	7932	9193	450-38093
Carotene (RE)	442	867	10-5854	628	958	9-3674
å-tocopherol (mg)	22.2	74.6	1.5-409.5	22.3	74.6	0.6-409.3
Vitamin C (mg)	273.5	838.9	2.3-6252.3	243.6	447.8	2.6-2183.8
Calcium (mg)	1023	641	192-2752	1138	478	240-2364
Copper (mg)	1.3	0.7	0.4-4.1	1.2	0.7	0.2-3.8
Zinc (mg)	11.0	6.2	1.7-33.4	11.4	5.3	1.8-22.5

[1] Values are for three days from 19 subjects (57 subject-days).

Table 20. Dietary Intakes for Subjects in Treatment Ferric-Whey Protein

	Before Cheese			During Cheese		
Treatment:	Mean	SD	Range	Mean	SD	Range
Iron (mg)	13.9[1]	6.6	5.4-36.2	16.2	7.8	4.5-49.9
Energy (Kcal)	2098	782	654-4899	2068	738	371-3953
Fat (g)	89.0	42.8	4.0-206.5	87.9	35.5	16.6-178.0
Dietary Fiber (g)	13.6	6.9	1.5-34.4	12.7	7.3	0.4-38.0
Vitamin A (IU)	7668	8060	913-49512	4809	3570	477-18483
Carotene (RE)	478	760	25-4139	225	281	12-1755
å-tocopherol (mg)	7.8	4.3	1.2-19.9	7.9	5.1	0.7-31.3
Vitamin C (mg)	79.1	62.8	0.4-257.6	105.8	92.3	0.2-356.7
Calcium (mg)	1035	588	257-3772	1099	468	215-2007
Copper (mg)	1.2	0.5	0.5-2.9	1.1	0.4	0.3-1.9
Zinc (mg)	10.0	4.1	2.7-20.9	11.8	5.9	2.1-34.1

[1] Values are for three days from 16 subjects (48 subject-days).

Table 21. Iron Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	13.1[a, 1] ±8.8	16.3[b] ±8.7	14.7 ±8.9
Fe-C	15.1[a] ±7.9	17.4[ab] ±12.3	16.2 ±10.4
Fe-WP	13.9[a] ±6.6	16.2[ab] ±7.8	15.1 ±7.3
Pooled	14.0[a] ±7.9	16.7[b] ±9.9	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in milligrams/day.

å-Tocopherol Intake

Two-way ANOVA by treatment and å-tocopherol intake before versus during the cheese consumption period did not indicate significant main effects for treatment, å-tocopherol intake, or interaction of treatment and å-tocopherol intake (Table 22). However, planned comparisons indicated that treatment group Fe-C had a higher ($p<.05$) å-tocopherol intake than treatment Fe-WP both before and during cheese consumption.

Vitamin C Intake

Two-way ANOVA by treatment and vitamin C intake before versus during the cheese consumption period indicated a significant ($p<.01$) main effect for treatment but no main effect for vitamin C intake or interaction of treatment and vitamin C intake (Table 23). Treatment Fe-C had a higher vitamin C intake both before and during cheese consumption.

Table 22. å-Tocopherol Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	$11.0^{ab, 1}$ ±19.7	17.6^{ab} ±55.9	14.3 ±41.9
Fe-C	22.2^{b} ±74.6	22.3^{b} ±74.6	22.3 ±74.2
Fe-WP	7.8^{a} ±4.3	7.9^{a} ±5.1	7.8 ±4.7
Pooled	14.0 ±46.0	16.4 ±55.4	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
1 Means and standard deviations in milligrams/day.

Table 23. Vitamin C Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	$107.4^{a, 1}$ ±96.7	113.7^{a} ±170.6	110.5^{a} ±138.1
Fe-C	273.5^{b} ±838.9	243.6^{b} ±447.8	258.5^{b} ±669.6
Fe-WP	79.1^{a} ±62.8	105.8^{a} ±92.3	92.4^{a} ±79.6
Pooled	157.4 ±506.6	157.0 ±294.1	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
1 Means and standard deviations in milligrams/day.

Calcium Intake

Two-way ANOVA by treatment and calcium intake before cheese versus during cheese consumption indicated a significant ($p<.01$) main effect for calcium intake but no main effect for treatment or interaction of treatment and calcium intake (Table 24).

Although column means indicate that calcium intake increased during the cheese

Table 24. Calcium Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	$854^{a,\ 1}$ ±469	1109^{b} ±490	981 ±494
Fe-C	1023^{ab} ±641	1138^{ab} ±478	1080 ±566
Fe-WP	1035^{ab} ±588	1099^{ab} ±468	1066 ±530
Pooled	967^{a} ±572	1116^{b} ±476	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in milligrams/day.

consumption period, within treatment groups, only $FeCl_3$ had a significant ($p<.01$) increase.

Total Fat Intake

Two-way ANOVA by treatment and total fat intake before cheese versus during cheese consumption indicated a significant ($p<.05$) main effect for treatment but no main effect for total fat intake or interaction of treatment and total fat intake (Table 25). Row means indicated treatment $FeCl_3$ was lower than treatment Fe-WP before cheese and pooled.

Copper Intake

Two-way ANOVA by treatment and copper intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, copper intake, or interaction of treatment and copper intake (Table P.1).

Table 25. Total Fat Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	74.0[a, 1] ±36.8	77.8[ab] ±32.7	75.9[a] ±34.7
Fe-C	82.9[ab] ±38.6	83.8[ab] ±35.6	83.3[ab] ±37.0
Fe-WP	89.0[b] ±42.8	87.9[ab] ±35.5	88.5[b] ±39.1
Pooled	81.6 ±39.5	82.9 ±34.6	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.
[1] Means and standard deviations in grams/day.

Zinc Intake

Two-way ANOVA by treatment and zinc intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, zinc intake, or interaction of treatment and zinc intake (Table P.2).

Dietary Fiber Intake

Two-way ANOVA by treatment and dietary fiber intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, dietary fiber intake, or interaction of treatment and dietary fiber intake (Table P.3).

Energy Intake

Two-way ANOVA by treatment and energy intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, energy intake, or interaction of treatment and energy intake (Table P.4).

Vitamin A Intake

Two-way ANOVA by treatment and vitamin A intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, vitamin A intake, or interaction of treatment and vitamin A intake (Table P.5).

Carotene Intake

Two-way ANOVA performed by treatment and carotene intake before cheese versus during cheese consumption did not indicate significant main effects for treatment, carotene intake, or a significant interaction of treatment and carotene intake (Table P.6).

DISCUSSION

Thiobarbituric Acid-Reactive Substances

Serum TBARS

Serum TBARS measured before cheese consumption (S-TBARS on weeks 1 and 3) did not differ between treatment groups or by time, indicating no differences in lipid peroxidation before cheese consumption. However, S-TBARS on week 6 of the study period (16 days after initiation of cheese) indicated a significant increase in lipid peroxidation within all treatment groups, but without any differences among treatment groups (Fig. 1).

Increased lipid peroxidation in serum on week 6 may have been due to an increased dietary intake of TBARS from the supplementation of iron-fortified cheese. However, because dietary TBARS consumed from all foods besides the iron-fortified cheese were not determined; whether this indicates increased *in vivo* lipid peroxidation or merely increased dietary levels of lipid peroxidation products/precursors cannot be determined. Malondialdehyde in the diet is known to be absorbed by the gastrointestinal tract and transported to the liver for subsequent metabolization by hepatic mitochondria into carbon dioxide and acetate (Siu and Draper, 1982). Therefore, the increase in serum TBARS 16 days after initiation of iron-fortified cheese consumption may represent increased dietary levels being transported in the blood.

Despite continued consumption of iron-fortified cheese, S-TBARS on week 8 (30 days after initiation of cheese) significantly decreased from levels on week 6 and were comparable to S-TBARS levels before cheese consumption (S-TBARS on weeks 1 and 3). S-TBARS measured at the end of the study on (week 10 or 3 days after termination of cheese consumption) were significantly lower than S-TBARS on weeks 1, 3, 6, or 8.

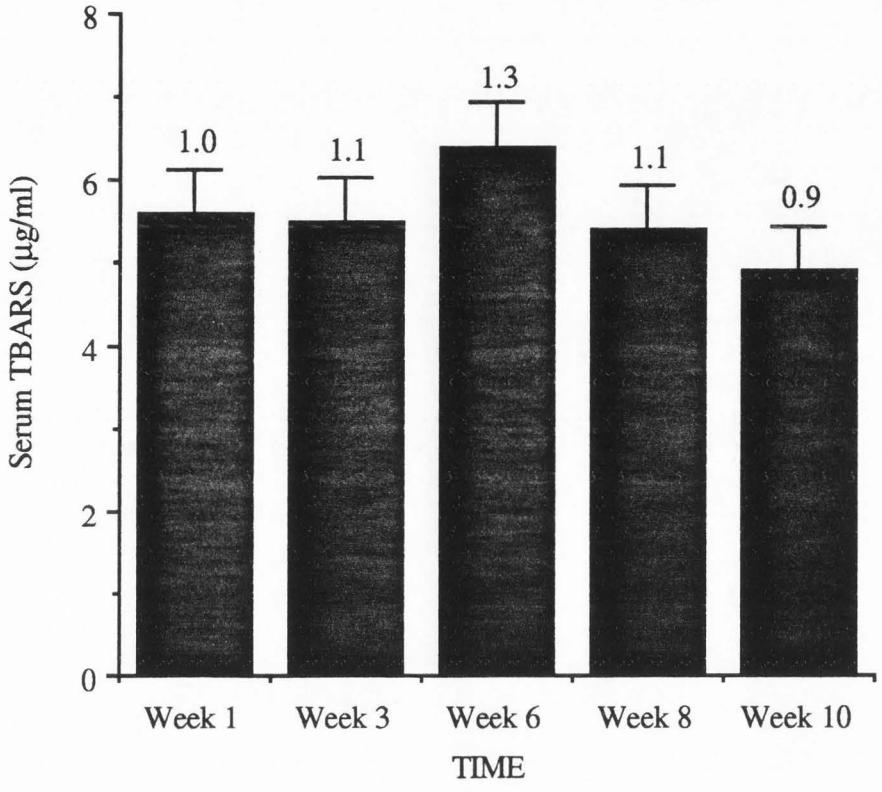

Fig.1. Serum TBARS Across Time. Column values are standard deviations.

The pattern of increased and then decreased lipid peroxidation in serum is hypothesized to possibly be secondary to some type of antioxidant enzymatic induction mechanism in response to an increased oxidative load or stress from the supplementation of iron-fortified cheese.

Numerous antioxidant enzymes are present in the body whose primary functions appear to be to decrease the amount of oxidants or potential oxidants and, therefore, serve a protective function against reactive oxygen species and lipid peroxidation. Among the most important of these are superoxide dismutase (occurring mostly in the cytosol and mitochondria of hepatic cells), which reacts with superoxide radical to produce hydrogen peroxide; catalase, which subsequently decomposes hydrogen peroxide into harmless products; and the peroxidases (present in peroxisomes), such as glutathione peroxidase,

which also decompose hydrogen peroxide (Bast et al., 1991; Krinsky, 1992).

Experimental evidence in animals and humans indicates that antioxidant enzyme production (superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase) is induced upon exposure to increased levels of oxidative stress (hypoxia , exercise, or ethanol administration), which may increase the formation of the superoxide anion radical and hydrogen peroxide (Allen, 1991; Sies, 1991; Adams and Odunze, 1991; Iarema et al., 1992; Ji and Fu, 1992; Burmistrov et al., 1992; Reddy et al., 1992). MDA is apparently oxidized in hepatic mitochondria by mitochondrial aldehyde dehydrogenase (Siu and Draper, 1982). Whether this enzyme is induced by an increased dietary load of TBARS or MDA has not yet been determined.

Ionizing radiation from gamma particles also produces free radicals *in vivo* which can alter DNA, RNA, and proteins via lipid peroxidation. Radiation-induced lipid peroxidation is also augmented by the presence of iron (Stevens and Kalkwarf, 1990). Reif et al. (1988) investigated the release of iron from ferritin by radiolytically generated reducing radicals using ^{137}Cesium gamma radiation. It was found that radiolytically generated superoxide anion increased lipid peroxidation in liposomes as measured by malondialdehyde formation only when ferritin was included as an iron source in the experimental conditions.

Theoretically, cells exposed to ionizing radiation as present in the radioiron test meals may have generated oxygen radicals such as superoxide and hydroxyl, which lead to an increased level of oxidative stress and lipid peroxidation (Vuillaume, 1987; Stark, 1991). Therefore, iron combined with radiation as the ferric chloride iron-59 radionuclide may have contributed to an increased level of oxidative stress in the study subjects.

This suggests that the stimulus of iron-fortified cheese consumption and/or radioiron test meals may have augmented lipid peroxidation, resulting in the increased

levels of S-TBARS observed on week 6 of the study. These stimuli may have also induced an enzymatic adaptation responsible for the decrease to before-cheese levels of S-TBARS on week 8. When the stimulus of cheese was removed 3 days before the final S-TBARS determination on week 10, TBARS may have been reduced below before-cheese levels because of a time lag in the down regulation of endogenous antioxidant enzymatic systems.

Serum TBARS measurements did not correlate with other parameters measured in blood, urine, or feces except for a weak ($r = .33$ to $.36$) but significant positive correlation with total iron-binding capacity. However, analysis of covariance with TIBC and serum TBARS did not alter results. TIBC is the sum of serum iron (the amount of iron actually bound to transferrin) plus the unsaturated iron-binding capacity (the number of iron-binding sites which are not occupied) and, therefore, estimates the amount of iron that would be present if all the binding sites on the iron-transport protein transferrin were occupied. Since the binding sites on transferrin are normally 30% occupied, an increased TIBC (>400) is indicative of a disruption in iron transport secondary to iron deficiency (i.e. transferrin saturation or serum iron is decreased). Both of these situations indicate more binding sites are available on transferrin to bind iron, which could potentially participate in reactions producing reactive oxygen species or lipid peroxides. Therefore, increased lipid peroxidation as TIBC increases is not an expected result. Since the coefficient of determination for this relationship was very low (0.11 to 0.13), it will not be further addressed.

Urine TBARS

Urine TBARS measured before cheese consumption (U-TBARS on weeks 1, 2, 3, and 4) did not differ among treatment groups. Differences across time indicated U-TBARS on weeks 3 and 4 were higher than U-TBARS on week 1. This difference over time occurred without the stimulus of iron-fortified cheese consumption. However, the

potential stimulus for increased oxidative stress of radioiron test meal 1 may have contributed to this increase in TBARS over time.

Urine TBARS measured during cheese consumption (U-TBARS on weeks 5, 6, 7, 8, 9, and 10) did not differ among treatment groups. Although there was no effect of time, planned comparisons indicated U-TBARS on week 10 (after 6 weeks of cheese consumption) were lower than U-TBARS on week 5 (after 1 week of cheese consumption).

Comparison of all urine TBARS (U-TBARS on weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) did not indicate differences among treatment groups, or a main effect of time. Planned comparisons indicated that U-TBARS on weeks 3 and 4 were higher than U-TBARS on weeks 1 or 10, and that U-TBARS on week 4 were also higher than U-TBARS on weeks 7 or 8. Similar to serum TBARS, urine TBARS appeared to increase and then decrease over time. However, the increase in urine TBARS did not coincide with cheese consumption. To suggest that increased urine TBARS was due to the radioiron test meal on week 1 and, therefore, had a more rapid response to this stimulus than serum, is conjecture at best.

Urine TBARS had a significant positive correlation ($r = .46$) with urine specific gravity. Specific gravity represents dissolved substances in the urine, and this result may be expected. Analysis of covariance with specific gravity and urine TBARS altered results for overall urine TBARS. With specific gravity as a covariate, U-TBARS on weeks 3 and 4 were higher than all other U-TBARS except for U-TBARS on week 5 (measured one week after the initiation of cheese consumption). Therefore, once cheese consumption began (U-TBARS on week 5), lipid peroxidation did not increase above levels before cheese consumption and eventually decreased below the maximum levels noted at U-TBARS on weeks 3 and 4.

According to the results above for U-TBARS, it appears that the consumption of iron-fortified cheese did not increase dietary TBARS above before-cheese levels, which would have resulted in increased TBARS in the urine.

Fecal TBARS

Fecal wet TBARS measured before cheese consumption (FW-TBARS on weeks 1, 2, 3, and 4) did not differ among treatment groups. Across time, FW-TBARS on week 2 were higher than FW-TBARS on weeks 1 or 4. This change over time occurred without the stimulus of iron-fortified cheese consumption. However, a potential stimulus for increased oxidative stress at FW-TBARS on week 2 may have been the the radioiron from test meal 1.

Fecal wet TBARS measured during cheese consumption (FW-TBARS on weeks 5, 6, 7, 8, 9, and 10) did not differ among treatment groups or across time. However, comparison of all fecal wet TBARS (FW-TBARS on weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) did indicate differences among treatment groups and across time. Column means for FW-TBARS on weeks 1, 2, 3, and 4 (before cheese) were found to be higher than all TBARS determinations during cheese consumption except for FW-TBARS on week 6. Comparisons of pooled determinations from before and during cheese consumption indicated higher TBARS before cheese consumption.

Treatment $FeCl_3$ had higher overall fecal wet TBARS levels. However, fecal wet TBARS had a significant negative correlation ($r = -.35$ to $-.53$) with fecal moisture. Analysis of covariance with fecal moisture and FW-TBARS altered results for the main effect of time before cheese and treatment overall, which indicated an influence of fecal moisture on fecal TBARS levels as expressed on a wet weight basis. This is strengthened by the fact that the ANOVA for fecal dry TBARS produced similar results. In addition, ANOVA results for fecal moisture indicated treatment $FeCl_3$ had statistically significant ($p<.05$) lower fecal moisture (71% moisture) content than did treatment Fe-C

(74% moisture). Treatment $FeCl_3$ also resulted in a lower fecal moisture level than treatment Fe-WP (73% moisture), which approached statistical significance ($p<.09$).

Although the difference among treatments with respect to FW-TBARS does not appear due to differences in lipid peroxidation, this finding may still be of physiological significance. MDA has been reported to be mutagenic in bacterial systems, cytotoxic to mammalian cells, and an initiator of carcinogenic events, with increased levels having been detected in colorectal tumors (Jacobson et al., 1983; Otamiri and Sjodahl, 1989). Subsequently, exposure to increased concentrations of MDA secondary to decreased fecal moisture may be of some pathological significance in the etiology of diseases of the lower intestinal tract. By increasing stool moisture levels, intestinal mucosa may be exposed to lower concentrations of a potent mutagen. Also, the effect of increased fecal moisture on fecal wet TBARS may indicate some relationship between dietary fiber intake and concentrations of MDA since increased intakes of dietary fiber are generally associated with increased stool weight, increased stool moisture, decreased intestinal transit time, and decreased risk for colon cancer (Eastwood et al., 1973; Cummings et al., 1976; Cummings et al., 1992). In this investigation, however, dietary fiber intakes were not found to be significantly different among treatment groups, or to correlate with fecal moisture content or fecal wet TBARS.

Fecal TBARS on a dry weight basis had similar results as fecal wet TBARS with fecal moisture as a covariate.

TBARS in Iron-Fortified Cheese

Thiobarbituric acid-reactive substances in iron-fortified Cheddar cheeses at 8 months of age were higher than unfortified Cheddar cheese of the same age (Appendix H). Unfortified cheese contained .24 µg/g TBARS, whereas Fe-C, Fe-WP, and $FeCl_3$ contained .66 µg/g, .51 µg/g, and .63 µg/g TBARS, respectively. The statistical significance of these differences was not determined. Compared to other cheeses, levels

of TBARS found in the iron-fortified cheeses were much lower than levels reported for American cheese (3.6-5.7 μg/g TBARS), but they were higher than levels reported for Swiss (.3 μg/g TBARS), mozzarella (.5 μg/g TBARS), and ricotta (.4 μg/g TBARS) (Shamberger et al., 1977). Reported TBARS content for meats are .95 to 13.7 μg/g for beef, .24 to 4.1 μg/g for pork, 1.3 to 7.7 μg/g for chicken, and 5.6 to 13.5 μg/g for turkey (Wilson et al., 1976; Shamberger et al., 1977; Igene and Pearson, 1979). Wide ranges in meats are secondary to cooking and storage conditions. Values for cheeses and meats suggest that dietary TBARS may vary greatly depending on what foods are eaten. Values also suggest that the contribution from iron-fortified cheese to total TBARS in a mixed diet is similar to the contribution from other cheeses.

Intestinal Transit Time

Slow intestinal transit time is thought to be associated with an increased risk for colon cancer (Cummings et al., 1992). Because of the high concentration of iron in feces (90-95% of dietary iron is unabsorbed) and iron's ability to initiate free radical production, it has been suggested that intracolonic production of oxygen radicals may actually contribute to the etiology of colon cancer (Babbs, 1990). Also, oxidative stress in the large intestine has been found to disturb intestinal smooth muscle motility (Van Der Vliet et al., 1989).

Intestinal transit time was measured after radioiron test meal 2 to determine any relationships with parameters of lipid peroxidation. Although no significant differences were found among treatments with regards to transit time, the difference between treatment $FeCl_3$ and Fe-C approached significance ($p<.08$). Despite lack of statistical significance possibly due to small sample sizes (n=18, 17, and 15), mean values for intestinal transit time in hours appeared to indicate a trend towards a slower transit time

in treatment $FeCl_3$ (79.2 hours) as compared to treatments Fe-C (60.5 hours) and Fe-WP (64.7 hours).

Intestinal transit time did not correlate with any measurements of lipid peroxidation but did have a significant negative correlation ($r = -.60$) with fecal moisture. Because intestinal transit time has previously been found to be related to fecal moisture, it is speculated that high fecal moisture may have a beneficial effect by lowering fecal TBARS concentrations and, consequently, decreasing exposure time of MDA with the intestinal mucosa.

Iron Status

Hemoglobin

After 6 weeks of cheese consumption, hemoglobin levels were found to be significantly below levels before cheese consumption. The final hemoglobin level (HGB on week 10 taken 3 days after consumption of cheese ended) was significantly lower than all other column means either before or during cheese consumption, but it did not approach levels consistent with iron-deficiency (<12.0 g/dl). Hemoglobin levels decreased despite an increased iron intake of approximately 2.6 mg/d over the 6 weeks of iron-fortified cheese consumption. Since day-to-day variation of hemoglobin is low, only a single determination is needed to accurately determine serum levels. Therefore, the decreases observed appear to be due to decreased synthesis or increased losses of hemoglobin (Borel et al., 1991).

Since blood was taken throughout the study (approximately 160 milliliters taken prior to HGB on week 10), decreases in hemoglobin may have been secondary to iron losses with insufficient replacement. Based on a mean hemoglobin level pooled for all subjects throughout the study of 14.6 g/dl and an iron content of hemoglobin of 0.34%, 160 ml of blood loss represents an average iron loss of 78 milligrams by each subject

over the course of the study up to the last blood draw (Baynes and Bothwell, 1990). Iron lost from blood was compensated for by an increased iron intake during cheese consumption, representing an effective total contribution of 10.1 milligrams of iron from cheese during the entire 6 weeks of consumption (based on total intake of 132.3 mg iron from iron-fortified cheese and 7.65% mean iron absorption for all subjects at radioiron test meal 2). In addition, serum ferritin levels decreased by an average of 10.8 µg/l over the study period, which represents mobilization of approximately 86.4 milligrams of storage iron (8 mg of iron per µg/l of serum ferritin). This totals 96.5 mg of additional iron mobilized and ingested to replace losses of 78 mg from hemoglobin.

Serum ferritin is known to have significant day-to-day variation with 3 to 10 measurements needed to obtain an accurate determination. The high levels seen on the first serum ferritin determination may have been false, suggesting that less storage iron was utilized than calculated above. Due to the question regarding the accuracy of serum ferritin, replacement iron may have been less than losses and may give some credence to the speculation that iron from blood loss may have contributed to decreased hemoglobin levels.

Hemoglobin levels did not correlate with TBARS. It appeared that at physiologically normal levels, hemoglobin was not related to lipid peroxidation.

Hematocrit

Hematocrit levels were within normal reference ranges (38%-47%) for females. (Tilkian et al., 1987). As opposed to hemoglobin, hematocrits were higher after cheese consumption. This may have been due to reticulocytosis in response to blood loss. Hematocrit did not correlate with TBARS in serum, urine, or feces, and does not appear to be related to lipid peroxidation at physiologically normal levels.

Serum Iron

Serum iron levels were within normal reference ranges (50-150 μg/dl) for females (Tilkian et al., 1987). Levels were found to be higher after the cheese consumption period, which could be interpreted as an increase in iron transport secondary to blood loss. Serum iron did not correlate with TBARS in serum, urine, or feces, and does not appear to be related to lipid peroxidation at physiologically normal levels.

Total Iron-Binding Capacity

TIBC was within normal reference ranges (300-400 μg/dl) on all determinations (Tilkian et al., 1987). Levels were found to be lower after cheese consumption. TIBC levels correlated with serum TBARS but did not appear to be related to lipid peroxidation at physiologically normal levels.

Transferrin Saturation

Transferrin saturation was within normal reference ranges (30-50%) on weeks 1, 8, and 10 with levels significantly increased after cheese consumption (Tilkian et al., 1987). Saturation was slightly decreased below reference ranges on weeks 6 and 8. Decreases in saturation below 30% may indicate iron-limited erythropoiesis. Transferrin saturation did not correlate with TBARS in serum, urine, or feces, and did not appear to be related to lipid peroxidation at physiologically normal levels.

Serum Ferritin

Serum ferritin was within normal reference ranges for females (10-150 μg/l) on all determinations, and approximated the median value (25 μg/l) for females aged 18-45 (Sullivan, 1981; Leggett et al., 1990). Levels on week 1 were significantly higher than all other determinations. Serum ferritin did not correlate with TBARS and did not appear to be related to lipid peroxidation at physiologically normal levels.

Cheese Iron Absorption

All three iron-fortified Cheddar cheeses were similarly absorbed in radioiron test meals 1 and 2. Iron absorption from cheese did not correlate with TBARS and did not appear related to lipid peroxidation.

Dietary Intake

Iron Intake

No differences were found among treatment groups before or during iron-fortified cheese consumption. Intakes of iron approximated the recommended daily allowance for females (15 mg/d). However, iron intake did significantly increase during cheese consumption by approximately 2.6 mg/d (all three treatment groups pooled). Iron intakes were not correlated with TBARS. Iron intake at levels approximating the recommended daily allowance do not appear to be related to lipid peroxidation.

å-Tocopherol Intake

Intakes of the antioxidant nutrient å-tocopherol were significantly higher in treatment Fe-C both before and during cheese consumption. This difference was due to supplement use by one subject in treatment Fe-C, which increased mean intake of å-tocopherol by approximately 14 mg and 14.5 mg daily before and during cheese consumption, respectively (Table P.7 and Table P.8). Intakes of å-tocopherol were not correlated with TBARS.

Vitamin C Intake

Intakes of the antioxidant nutrient vitamin C were significantly higher in treatment Fe-C both before and during cheese consumption. This difference was due to supplement use by two subjects in treatment Fe-C, which increased mean intake of vitamin C by approximately 150 mg and 126 mg daily before and during cheese

consumption, respectively (Table P.7 and Table P.8). Intakes of vitamin C were not correlated with TBARS.

Calcium Intake

Intakes of calcium were significantly higher during iron-fortified cheese consumption. This result may be expected from daily supplementation of a calcium-rich product such as cheese. Intakes of calcium were not correlated with TBARS.

Total Fat Intake

Total fat intake was lower in treatment $FeCl_3$ than in treatment Fe-WP before cheese consumption and when both diet periods were pooled. Total fat intakes were not correlated with TBARS. Due to database problems, polyunsaturated, monounsaturated, and saturated fatty acid intakes could not be accurately quantified.

Methodology Limitations

Although the TBARS method for detection of lipid peroxidation in biological samples is simple, inexpensive, and sensitive, it is thought to lack specificity for MDA. When applied to biological samples such as urine, serum, and feces, the TBA reaction may measure more MDA than originally present in the sample. Several non-lipidic compounds have been found to form "MDA-TBA-like" chromagens under the conditions of the TBARS assay or interfere with absorbance spectra at 532 nm. These compounds include nonvolatile lipid peroxide precursors, glycoproteins, sialic acid, derivatives of glucose, albumin, biliverdin, and bilirubin (Gutteridge and Tickner, 1978; Bird et al., 1983; Knight et al., 1988).

Quantification of *in vivo* lipid peroxidation requires measurement of preformed lipid hydroperoxides *in vivo*. Current evidence indicates that up to 90% of TBARS in the TBARS test may be formed during the decomposition of lipid peroxides produced *in*

vitro during the incubation stage of the assay (Gotz et al., 1993). Therefore, TBARS, as reported in this investigation, may not have accurately measured lipid peroxidation. An assay to accurately measure *in vivo* lipid peroxidation should avoid the presence of interfering compounds and reaction conditions in order to truly reflect MDA as opposed to a broad range of oxidation products (Ohkawa et al., 1979; Kosugi et al., 1989; Valenzuela, 1991).

There is no standardized methodology for measuring MDA. Since TBARS methods appear to be greatly influenced by sample type and reaction procedures, comparisons between studies are difficult. Measurement of MDA by high performance liquid chromatography (HPLC) has been found to remove the effect of non-MDA TBARS (Wade et al., 1985; Tatum et al., 1990). Comparisons between MDA measured by direct HPLC quantitation and the TBARS-test indicates that thiobarbituric acid-reactive substances assay is not a reliable index of MDA formation in vivo (Ceconi et al., 1992). Use of HPLC to identify MDA under various conditions of oxidative stress should yield a better understanding of the mechanisms and pathophysiology of lipid peroxidation *in vivo*.

Since diet appears to be the main source of MDA under most conditions, it is necessary to preclude the effects of dietary MDA when attempting to evaluate MDA excretion as an indicator of *in vivo* lipid peroxidation (Draper et al., 1984; Dhanakoti and Draper, 1987). Although an objective of this examination was to determine changes in *in vivo* lipid peroxidation, dietary MDA effects were not precluded, and *in vivo* lipid peroxidation could not be assessed from TBARS.

Overall Impressions

Iron-fortified cheese consumption was associated with a transient increase in serum TBARS, which eventually decreased to below baseline levels after 6 weeks of

cheese consumption. Urine and fecal TBARS (on a dry weight basis) did not increase during the period of iron-fortified cheese consumption, but they were associated with decreased levels in comparison to before-cheese consumption. Theoretically, supplementing an equivalent amount of unfortified cheese or meat daily into an otherwise mixed diet may have produced the same results.

Although this investigation was performed to examine *in vivo* lipid peroxidation in females in response to iron-fortified cheese consumption, it fell short of this goal. Due to the methodology employed, measurement of a change in *in vivo* lipid peroxidation was not quantified. Evidence in human subjects indicates that urine TBARS respond very efficiently and rapidly (4 hours) to intake of dietary MDA or MDA precursors and that levels can also decrease to baseline fasting levels just as quickly (Jacobson et al., 1983). Since dietary MDA or MDA precursors actually ingested prior to each TBARS determination were not assayed, the generation of MDA *in vivo* as determined by the difference between MDA ingested and MDA excreted could not be quantitated. Accurate quantification of changes in *in vivo* lipid peroxidation produced by the addition of iron-fortified cheese to a mixed diet would require controlling the dietary intake of subjects for TBARS besides those contained in the iron-fortified cheese.

Despite these shortcomings, lipid peroxidation with and without iron-fortified cheese as part of a mixed diet could be measured. Thiobarbituric acid-reactive substances did have qualitative value by comparing lipid peroxidation in the presence and absence of iron-fortified cheese supplemented into normal mixed diets.

CONCLUSIONS

Daily supplementation of iron-fortified cheese into a normal mixed diet was associated with a significant but transient increase in lipid peroxidation as measured in serum by the thiobarbituric acid-reactive substances assay. This transient increase in serum TBARS suggests either a short-term increase in total dietary TBARS or an increase in *in vivo* lipid peroxidation. However, neither of these was verified.

TBARS levels in iron-fortified cheese were equivalent to levels found in other cheeses and lower than TBARS levels reported in meats. Therefore, contributions from iron-fortified cheese to total TBARS in a normal mixed diet are not significantly higher than other foods.

TBARS in urine and feces did not increase in response to iron-fortified cheese consumption, but decreased during the cheese consumption period. From a toxicological perspective, these findings suggest that the addition of iron-fortified cheese for 6 weeks to a normal mixed diet is safe.

Supplementation of iron-fortified cheese increased dietary iron intakes by approximately 2.6 milligrams daily. This increase did not correlate with measurements of lipid peroxidation in serum, urine, or feces. Iron status parameters were within fairly narrow and normal physiologic ranges throughout the investigation, and differences in lipid peroxidation as related to iron status were not found.

Because a transient increase was found in serum TBARS, future examinations measuring the effect of iron-fortified cheese on *in vivo* lipid peroxidation are warranted. Also, comparisons of iron-fortified cheese to regular cheese, and other foods with similar TBARS levels, may lead to more concrete conclusions regarding the safety of iron-fortified cheese.

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APPENDICES

APPENDIX A

Telephone Screening Instrument

Eligibility for the iron-fortified cheese study conducted by the Utah State University Department of Nutrition and Food Sciences depends on six conditions as follows:

1) Participants must be menstruating regularly.
2) Participants must be at least 18 years old .
3) Participants must not be pregnant.
4) Participants must not be breast feeding.
5) Participant must not be an insulin-dependent diabetic.
6) Participants must not have gastrointestinal disease.

If these conditions are not satisfied then the prospective volunteer is not a candidate. We want to send all respondents a letter of gratitude so please obtain their name and address. Check off "No" next to "Candidate for Study" and ask how they were informed about the study and write it down next to "Source of Information"

Brief Description of Study
This is a nutrition research study examining the absorption of iron from iron fortified cheese. The study is ten weeks long and the approximate starting date is the beginning of October, although participants will be accepted at later dates.

Participant Activities include:
1) Consumption of cheese for six weeks including three test meals with tracer iron.
2) Six blood drawings throughout the study period.
3) Weekly urine and stool samples.
4) Daily diet records for four separate weeks.
5) A pregnancy test at the beginning of the study

Participant Benefits include:
1) $100 cash gift for completing the study.
2) 2 free university credits in Nutrition and Food Science.
3) Training in diet monitoring techniques.
4) Information on your iron and nutritional status.

If you are interested in participating in this study please leave your name, address, and telephone number and we will contact you to arrange an interview. Thank you.

Prospective Volunteer Personal Data:

NAME: _____ Candidate for Study: Yes___

ADDRESS:_____ No___

Source of Info: _____

TEL#: Home_____Work_____ Date:_____ Time:_____

APPENDIX B

Health Screening Questionnaire

1. Do you menstruate on a regular basis? Yes__ No__
   If No, please specify _____

2. Are you currently breast feeding? Yes__ No__
   If Yes, when do you plan to wean? _____

3. Are you currently trying to become pregnant? Yes__ No__

4. Do you plan on becoming pregnant within the next six months?
   Yes__ No__

5. If you are sexually active, do you use any method of birth control?
   Yes__ No__ Not Applicable__
   If Yes, please specify:
   __ Oral Contraceptives(Birth Control Pills)
   __ Condom
   __ Intra-Uterine Device(IUD)
   __ Diaphragm
   __ Rhythm Method
   __ Surgical Sterilization
   __ Partner Vasectomy
   __ Other(please specify)_____

6. If you are not sexually active, do you use oral contraceptives(birth control pills)?
Yes__ No__

7. Excluding oral contraceptives, do you use any other female hormones?
   Yes__ No__

8. Have you been diagnosed with any chronic illnesses(such as cancer, diabetes, heart disease, kidney disease, hypertension, etc.)? Yes__ No__
   If Yes, please specify _____

9. Have you been diagnosed with gastrointestinal disease(such as ulcers, diverticulosis, gastritis, etc.)? Yes__ No__
   If Yes, please specify _____

10. Do you have a history of gastrointestinal surgery? Yes__ No__
    If Yes, please specify _____

11. Do you currently have any chronic bowel infections(such as Giardia, Amebiasis, Clostridium Difficile, etc.)? Yes__ No__
    If Yes, please specify _____

12. Do you have a past history of any chronic bowel infections? Yes__ No__
    If Yes, please specify _____

13. Do you have a history of Lactose Intolerance? Yes__ No__

14. Do you have any food allergies? Yes__ No__
If Yes, please specify _____

15. Have you ever been diagnosed with any type of anemia? Yes__ No__
If Yes, please specify _____

16. Have you ever been diagnosed with hemosiderosis or hemochromatosis?
Yes__ No__

17. Are you currently taking any prescription medications? Yes__ No__
If Yes, please specify:

Medication	Amount/Day	Date Started	Date Expected to Finish
1.			
2.			
3.			
4.			

18. Are you currently using any non-prescription medications? Yes__ No__
If Yes, Please Specify:

Medication	Amount/Day	Date Started	Date Expected to Finish
1.			
2.			
3.			
4.			

19. Do you use any non-prescription medications on a chronic basis?
Yes__ No__
If Yes, please specify:

Medication	Amount/Day	Date Started
1.		
2.		
3.		

20. Do you use antacids(such as Tums, Maalox, Mylanta, etc.)? Yes__ No__
If Yes, please specify:

Antacid	Amount/Week
1.	
2.	
3.	

21. Are you currently taking any vitamin, mineral or other nutritional supplements?
Yes__ No__
If Yes, please specify:

Supplement Name	Amount/Day	Length of Use

1._____
2._____
3._____

22. Do you smoke cigarettes? Yes__ No__
    If Yes, please specify:
    Brand Name:_____
    Amount smoked per day: _____

23. Have you significantly changed your dietary habits within the past six months?
    Yes__ No__
    If Yes, Please Specify_____

24. Do you exercise three or more times per week? Yes__ No__
    If Yes, please specify:

Activity	Duration	Times/Week
1._____		
2._____		
3._____		

25. What is your current body weight? _____pounds

26. What is your usual body weight? _____pounds

27. How tall are you? ____feet ____inches

28. What is your marital status? Single____ Married____

29. Do you have any children? Yes__ No__
    If Yes, please specify:
    How Many:_____
    Ages:_____
    Birth Date of most recent child:_____

Your Name:_____
Your Soc. Sec. Number:_____
Your Date of Birth:_____

Additional Information: _____
_____
_____

APPENDIX C

Informed Consent Form

VOLUNTEER AGREEMENT TO PARTICIPATE IN THE BIOAVAILABILITY STUDY OF IRON FORTIFIED CHEESE

Arthur W. Mahoney, Professor, Principle Investigator

Department of Nutrition and Food Sciences
Utah State University
Logan, UT 84322-8700

BACKGROUND INFORMATION:

This is a nutrition study of dietary iron and prevention of iron deficiency. The three objectives are 1) to determine normal dietary iron and other nutrient intakes, 2) to determine human absorption of iron from fortified cheese, and 3) to determine if iron-fortified cheese improves the body's iron status. For the study information to be accurate and beneficial to the general public, it is necessary for you as a participant to be candid and adhere to the details of the research methods. These details are simple but necessary.

Iron and calcium are two critical nutrients in the human diet. Iron deficiency and anemia are common ailments of women of child-bearing age, young children, and the elderly. Osteoporosis (calcium loss from bone) and high blood pressure are two common ailments believed to be caused by low dietary calcium intakes. Iron and calcium are a nutritional dilemma; foods rich in iron are poor in calcium such as whole grain products, brans, meats, and certain vegetables. On the other hand, foods rich in calcium are poor in iron; these foods are primarily dairy products. To solve this nutritional dilemma, we are developing iron-fortified dairy products. In this study, iron-fortified cheddar cheese is being evaluated.

We have extensively studied the iron-fortified cheeses to be used in this study. We found that people like them as well as unfortified cheeses. We evaluated the iron bioavailabilities of these fortified cheeses in rats and found that absorption of the fortified cheese iron is similar to meat iron (the best source of dietary iron). To move towards marketing these products, we need human bioavailability data of iron fortified cheese. Your participation in this study will serve the public by assisting us in the development of iron-fortified cheese.

Participant activities

In order to enter the study, you will be given a pregnancy test before the study starts. Only menstruating women aged 18 or older are eligible to participate in the study.

You will be asked to keep accurate and complete records of your normal diet for four weeks during the study. We will provide a detailed daily activity sheet to facilitate

your following the activities of the study. We will also provide you with charts and instruct you on recording dietary data. We will be in regular contact by telephone and visitations to accommodate your needs.

We ask that you do not change your normal eating habits in any way except to consume the test cheese. If you are taking minerals (iron, calcium, zinc, copper, etc.) and/or vitamin supplements before the study, keep taking the amount as you have been taking, and record them in your dietary data sheet. If you have not been taking minerals and/or vitamin supplements before the study, then do not take them during the study. We need to know how fortified cheese affects iron metabolism in normal dietary situations.

To determine the absorption of the ingested fortified cheese iron in normal dietary situations, you will take three very small doses of radioactive iron (2.5 microcuries each time making a total of 7.5 microcuries); the first dose early in the study, the second dose about two-thirds way through the study, and the third dose at the beginning of the final two weeks of the study.

You will allow us to take blood six times during the study (30 milliliters - about one fluid ounce - of blood each time) from the arm by a licensed medical technologist or nurse. You are required to fast overnight, no food after 11 PM, before each blood draw. You will provide morning urine and stool samples weekly during the study.

You will be given an exit interview at the end of the study for us to determine your feelings about participating in the study. This information will be kept confidential and may be used to improve future studies.

<u>What does it mean to consume 7.5 microcuries of iron-59?</u>

Iron-59 is a radioactive form of iron. A microcurie is a measure of radiation activity. The absorbed dose equivalent for the human body consuming 7.5 microcuries of iron-59 is about 50 millirems. A rem is a measure of the absorbed dose equivalent. One millirem is one thousandth of one rem. The International Commission on Radiological Protection (ICRP) recommended 500 millirems per year as the radiation dose limit for members of the general public. The ICRP also recommended that 25 microcuries is the maximum activity per subject for iron-59. Compared to those recommendations by the ICRP, the radioactive dose from consuming 7.5 microcuries of iron-59 in this study is very low. The radiation you will receive from all three doses of iron-59 is about equal to one chest X-ray, or one-eighth of a head or neck X-ray, or one-twentieth of an abdominal X-ray, or one-half of an X-ray of an extremity. Your total radiation exposure in this study is also about equal to the radiation received in five round-trip flights from New York to Paris.

We believe that health risks to the participants will be minimal. In iron absorption studies reported over last thirty years, thousands of human volunteers all over the world have consumed radioactive iron and no known adverse effects have been reported. This methodology for assessing human iron absorption from food and supplements continues to be the method of choice in the United States and around the world. James D. Cook, M.D., Clinical Hematologist and authority on iron nutrition at the University of Kansas Medical School in Kansas City, has published results from approximately 30 studies during the past 20 years in which humans were given iron-59; six of these studies were published as recently as 1988 through 1991.

Based on the scientific information we know, the effect of consuming 7.5 microcuries of iron-59 (a total dose equivalent about 50 millirems to the mother) is negligible to the human fetus. A fetus receives approximately 75 millirems from its mother's normal environment during pregnancy. Compared with the radiation a fetus receives from its mother's normal environment, the exposure of a fetus to the radiation when its mother consumes 7.5 microcuries iron-59 is very low. However, we recommend that you do not become pregnant during the study.

Benefits from Participating in the Study

You will be given $100 as a benefit for completing the study. The benefit schedule will be $25.00 at the end of the first absorption period (about 14 days after the first test meal), $25.00 at the end of the second absorption period (about 14 days after the second test meal), and $50.00 on completing the study.

By participating in the study, you will be learning about nutrition evaluation. Therefore, you will have 2 credits for NFS 490 (special problems) in nutrition and food science when you complete the study. We will reimburse you the credits for this special course.

You will receive information on your iron and nutritional status. Our dietitians will provide you with a complete nutritional analysis and make recommendations for any diet improvements.

Privacy

All records and personal information about an individual participant will be kept confidential. After seven years, all records of personal information will be destroyed. Diet and metabolism information without names may be kept indefinitely for research use. No data will be published or shared with other researchers in a manner that any individual can be identified.

Termination

Your participation in the study may be terminated at any time by the principle investigator, (e.g., if you do not keep daily records of your diet or do not provide blood and other necessary samples). If you become ill and the principle investigator believes that your continued participation would be either detrimental to your well-being or that your data would be unusable, your participation in the study will be terminated.

You may terminate your participation from the study for any reason. However, you will only receive whatever benefit would be due you at the end of the period in which the participation is terminated based on the benefit schedule described above.

AGREEMENT TO PARTICIPATE:

I, with complete understanding of the purpose of this study, my responsibilities as a participant, and the risks involved, agree to participate in the bioavailability study of iron fortified cheese . I will follow all instructions, keep all records, provide urine and fecal samples, consume iron-59 labeled samples, allow blood to be drawn as needed according to the study plan, and keep the study personnel informed of my daily health situation to the best of my abilities. I acknowledge I should not become pregnant during

the study. I understand that I may stop participating in the study at any time for any reason. I understand that Dr. Arthur W. Mahoney, Professor of Nutrition and Food Sciences, Utah State University, Logan, UT 84322-8700 (office telephone 750-2125, home telephone 752-9538), is the principle investigator responsible for the study.

Participant: _____ Witness: _____
(Printed) (Printed)

_____ _____
(Participant's signature) (Witness' signature)

_____ _____
(Date) (Date)

Principle Investigator: _____ _____
(Signed) (Date)

Arthur W. Mahoney, Ph.D, Fellow American College of Nutrition
Department of Nutrition and Food Sciences, Rooms 302 and 303
Utah State University
Logan, Utah 84322-8700

APPENDIX D

Urine and Stool Sample Collection

GUIDELINES FOR COLLECTION OF URINE AND STOOL SAMPLES

URINE:

COLLECTION TIME: MORNING VOID upon rising.

Test Meal #1: You do not need to collect urine on the first day of this test meal. However, we would like your MORNING VOID for the following two days after the test meal which would be Friday (4/3/92) and Saturday (4/4/92).

Test Meal #2: When you come in for the second test meal please bring your morning void for that day (Wednesday, 5/6/92), and then collect your morning void for the following two days according to the study calendar (Thursday, 5/7/92 and Friday, 5/8/92).

Test Meal #3: When you come in for the third test meal please bring your morning void for that day (Wednesday, 5/20/92) and then collect your morning void for the following two days according to the study calendar (Thursday, 5/21/92 and Friday, 5/22/92).

OTHER TIMES: You will also collect your morning void six (6) times on a Monday and one (1) time on a Tuesday according to the study calendar.

MONDAY COLLECTION DATES: April 6, April 13, April 20, April 27, May 11, and June 1.

TUESDAY COLLECTION DATE: May 26

COLLECTION METHOD:

1. Place urine collection hat on commode with the seat up and then lower the seat onto the hat.
2. Collect your full void.
3. Read the TOTAL VOLUME of the void from the numbers on the inside of the urine hat and write this on one of the small vials used for urine collection along with the DATE, TIME, and your NAME.
4. Fill up one of the small vials with urine up to the top of the tape. Remember to pour most of the urine into your commode prior to filling up the small urine vial so that only 50 to 100 cc's of urine remains in the hat. This will prevent spilling the urine while filling the small vial.
5. Securely place the cap on the top of the small urine vial (turn it tightly).
6. Place the small urine vial into a small Ziploc bag and seal the bag. This will prevent any mess from contaminating the inside of your cooler or the ice packs if the urine vial leaks (it should not leak). The Ziploc bags can be reused and we will provide you with more if needed.
7. Place the urine sample into the cooler along with the reusable ice packs that you should keep in your freezer.

8. Call us at 750-2450 and we will pick-up your sample; or you may bring the sample to the Nutrition and Food Science building, room #332.

STOOL:

TEST MEAL #1: ALL stool samples should be collected AFTER your first test meal for the next three (3) days. These days include the entire day following the test meal (Thursday, 4/2/92) and then the next two days (Friday, 4/3/92 and Saturday, 4/4/92) up until midnight on Saturday. Remember to write the DATE, TIME, and your NAME on your stool collection container.

TEST MEAL #2: ALL stool samples should be collected AFTER your second test meal for the next three (3) days. These days include the entire day following the test meal (Wednesday, 5/6/92) and then the next two days (Thursday, 5/7/92 and Friday, 5/8/92) up until midnight on Friday. You DO NOT need to bring a stool sample with you when you come to the test meal. Remember to write the DATE, TIME, and your NAME on your stool collection container.

TEST MEAL #3: ALL stool samples should be collected AFTER your third test meal for the next three (3) days. These days include the entire day following the test meal (Wednesday, 5/20/92) and then the next two days (Thursday, 5/21/92) and Friday, 5/22/92) up until midnight on Friday. You DO NOT need to bring a stool sample with you when you come to the test meal. Remember to write the DATE, TIME, and your NAME on your stool collection container.

OTHER TIMES: You will also collect a stool sample six (6) other times on a Monday and one (1) other time on a Tuesday. You will only need to collect ONE SAMPLE on these days. If you do not have a bowel movement on these days then just collect your sample for the following day. Remember to write the DATE, TIME, and your NAME on your stool collection container.

MONDAY COLLECTION DATES: April 6, April 13, April 20, April 27, May 11, and June 1.

TUESDAY COLLECTION DATE: May 26

COLLECTION METHOD:
1. Place stool collection apparatus onto the commode with the seat up, and then lower the seat.
2. Void into the container.
3. Place the lid securely onto the container.
4. Label the container with your NAME, the DATE, and the TIME.
5. Place the container into a large Ziploc bag and seal it. This will prevent the sample from contaminating the inside of your cooler if the container happens to leak (highly unlikely). The bags may be reused and we will provide you with more if needed.
6. Place the sample into the cooler along with the reusable ice packs.
7. Call us at 750-2450 and we will pick-up your sample; or you may bring it to NFS 332.

APPENDIX E

Dietary Intake Documentation

GUIDELINES FOR RECORDING DIETARY DATA

1. This book is to record your food intake. Three pages have been provided for each 24 hour period. We suggest that you either carry this book or a small pad for recording foods eaten away from home and then transfer the information into the diet record. Record everything you have eaten according to the number of days indicated on the study calendar. The first recording period is for 14 consecutive days. Begin each day's food record on a new page. Remember to record the day and date on each page of the diet record.

2. Everything that you put in your mouth and swallow should be included except for tap water. Tea, coffee, diet soda, bottled water, other similar beverages, breath mints, and chewing gum should also be recorded in the diet record. Do not eat any differently than usual, and remember to write it in your diet record.

3. A diet scale has been provided for accurate measurement of certain foods. BE SURE TO TARE THE SCALE TO ZERO BEFORE YOU WEIGH FOOD. We prefer that you record foods in GRAM amounts that can be easily read off the diet scale. Preferably, the scale should be used for weighing: 1) Meats either before or after cooking (just make sure you specify cooked or uncooked in the diet record along with the weight), 2) Cheeses that cannot be accurately measured by any other method, 3) Raw, cooked or canned fruits and vegetables that are difficult to measure using any other method, 4) Odd size foods without a serving size on the package such as unsliced bread, cakes, pies, muffins, bagels, etc., and 5) Homemade foods.

4. Be very specific when recording the foods you eat and always include BRAND NAMES when available. For example:

Soup:	Campbell's prepared, canned chicken noodle soup
Cereal:	Post brand iron-fortified bran flakes
Sweets:	Mounds candy bar
Cheese:	Kraft, American Processed Cheese
Meat/Fish:	Mrs. Pauls Fish Sticks
Fruit:	Dole Pineapple slices in natural juice
Vegetables:	Green Giant canned green beans, low sodium pack
Bread:	Pepperidge Farms, white sandwich slices
Other:	Rice-a-Roni, beef flavor
Fast Foods:	Kentucky Fried Chicken, extra crispy
Beverages:	Diet Coke
Alcohol:	Coors Light Beer

5. Describe the PREPARATION of foods. For example:

egg:	scrambled
hamburger:	fried in non-stick pan
broccoli:	steamed

condensed soup: one cup water added

6. Record the AMOUNT of each food consumed. Use either your diet scale, measured amounts in cups, teaspoons, tablespoons and ounces, or estimated size. Again, remember to weigh foods in GRAM amounts which can be easily read off the scale. Please use your diet scale for foods that cannot be easily measured using any other method. For example:

Red Delicious Apple:	one medium size
Cheerios Breakfast Cereal:	one cup
Broiled Steak:	180 grams, cooked
2% Milk:	12 ounces
Canned Corn:	1/2 cup, drained
Bread:	one slice, 3/4 ounce per slice
Canned Pears:	two halves, drained
Butter:	one tablespoon
Corn Oil:	one teaspoon
Cherry Pie:	1/6 of a 12 inch round pie
Macaroni and Cheese:	two cups, prepared
Oreos:	three cookies
Carrot:	medium size, peeled and trimmed
Homemade Cookie:	30 grams
Oatmeal:	1/2 cup, dry
Potato:	one medium, baked
Kentucky Fried Chicken:	one thigh, one breast, one leg

7. When SIMPLE combination foods are consumed, list each separate ingredient and circle the ingredients to indicate they were eaten as a unit. For Example:

Salad:
60 grams Iceberg lettuce, 1/3 cup diced tomato
1/3 cup sliced fresh mushrooms
2 Tbsp. Kraft fat-free French dressing

Toasted Ham and Cheese sandwich:
2 slices toasted white Wonder bread (one ounce per slice)
30 grams Swiss cheese, 30 grams sliced Ham
1 romaine lettuce leaf, 1 tsp. French's Dijon mustard

Enchilada:
One 10 inch flour tortilla
60 grams filling prepared from one pound lean ground beef, 6 ounces tomato paste, 1/2 cup chopped onions and spices
1 Tbsp shredded Monterey Jack Cheese
1/8 cup shredded lettuce
1/8 cup chopped tomato

8. When recording foods prepared from a package such as Macaroni and Cheese, StoveTop Stuffing, Rice-a-Roni, Hamburger Helper, etc., you should record how much you ate and also include any ingredients you added that did not come in the package. For Example:

Hamburger Helper: one cup eaten, total package prepared with one pound extra lean beef and 1/4 cup margerine.
Macaroni and Cheese: one cup eaten, total package prepared with 1/4 cup 2% milk and 1/4 cup margerine.

9. When recording homemade foods such as casseroles, lasagna, soups, potato salad, pies, cakes, etc, please record your serving size by weight and provide us with your recipe on the back of your diet record sheet for that day or staple a copy of the recipe onto your diet record sheet for that day. For Example:

Lasagna: 240 gram piece prepared from recipe on back of page
Recipe: one pound lasagna noodles, dry
one pound extra lean ground beef, browned in 1 tsp oil
8 oz. container Ricotta cheese
16 oz. tomato sauce
2 large white eggs
1 tsp. salt
1 tsp black pepper

Potato Salad: 180 gram serving from recipe attached to page
Recipe: 2 pounds red potatoes, boiled
one cup Best Foods regular mayonaise
1/2 cup chopped onions
1/2 cup chopped celery
2 Tbsp. mustard

10. When eating out please be as detailed as possible about the foods you eat. Estimate the portion sizes based on your experiences at home using your diet scale and measuring instruments. If you are unsure about a serving size at a restaurant you may ask your waiter or waitress.

11. Remember to record ingredients or condiments added to foods at the table or during preparation. For Example:

Butter, margerine, oil used on foods or while cooking
Syrups, sugars, jam, honey, jelly, artificial sweeteners added to foods or beverages
Salt, pepper, Accent, etc., used while cooking
Milk or cream added to foods or beverages

12. List any vitamin and/or mineral supplements taken along with the brand name and dosage. REMEMBER, DO NOT TAKE SUPPLEMENTS DURING PERIODS WHEN YOU SHOULD BE FASTING.

PLEASE call us at 750-2450 at any time if you have any questions about recording your dietary intake. If we are not available, our answering machine will give you a number where we can be reached.

APPENDIX F

Methods for Preparing Iron-Fortified Cheddar Cheese

Ferric Chloride cheese. Cheese was made by adding a 0.2 molar solution of the ferric chloride salt directly to the pasteurized cheese milk before renneting. Iron is added to the milk assuming a 65 percent recovery in the cheese, the rest of the iron is lost in the whey.

Fe-Casein complex fortified cheese. Fe-Casein complex was made by mixing 0.5 M $FeCl_3$ with skim milk at a ratio of 1:10 and adjusting pH to 4.6, isoelectric point for casein, using 12N NaOH. The iron-casein slurry can be stored at refrigeration temperature until use, normally within 24 to 48 hours. About 99% of iron is recovered in the precipitate. The Fe-Casein fortified cheese was made by adding Fe-Casein complex into the pasteurized cheese milk before renneting.

Amount of skim milk for Fe-Casein needed to fortify 55,000 pounds milk:

Amount milk to fortify:	55,000 lb = 25,000 kg
Assuming 10% yield of cheese, then	
Total amount of cheese:	2,500 kg
Designed iron content:	60 mg/kg cheese
Total amount of Fe in cheese:	60 x 2,500 / (1000 mg/g) = 150 g
Iron needed based on 65% recovery:	150 g / 0.65 = 230 g
Iron recovered in Fe-Casein:	99%
Iron to skim milk ratio:	0.5 M $FeCl_3$:Skim milk = 1:10
Total iron needed:	230g / 0.99 = 232.3 g
Total $FeCl_3 \cdot 6H_2O$ needed:	232 g x 270.206 / 55.874 = 1122 g
Mole of this amount iron:	232.3 / 55.847 = 4.16 mole
How much water needed to make 0.5 M of $FeCl_3$:	4.16 / 0.5 = 8.3 liter
How much skim milk needed to make Fe-Casein:	8.3 x 10 = 83 liter which is 83 liter/3.785 liter/gallon = 22 gallons

If skim milk powder: powder : water = 1:9, then need 8.3 kg = 18.26 lb SMP

Fe-Whey protein (Fe-WP) complex fortified cheese. Fe-WP will be made by adding 0.5 M $FeCl_3$ into a Swiss cheese whey at a ratio of 1:10 and adjusting the pH to 4.5 with 12 N NaOH to precipitate Fe-WP. The iron-whey protein slurry can be stored at refrigeration temperature before use, usually 24 to 48 hours. The recovery of iron in the precipitate is expected to be about 99%. Fe-WP fortified cheese will be made by adding Fe-WP complex into the pasteurized cheese milk before renneting.

APPENDIX G

Iron Content of Iron-Fortified Cheddar Cheese

Cheese:	Control	Fe-C	Fe-WP	$FeCl_3$
Fe Content[1]:	2.3	75.1	70.4	76.7

[1] Iron content values in g/kg are means of 8 samples from different 640-pound blocks and during different aging periods.

APPENDIX H

TBARS of Iron-Fortified and Control Cheddar Cheeses

Cheese:[1]	Control	Fe-C	Fe-WP	$FeCl_3$
Age:				
1 day	0.27	0.35	0.36	0.41
20 days	0.50	0.73	0.68	0.85
1 month	0.35	0.71	0.93	0.82
2 months	0.30	0.68	0.80	0.65
3 months	0.28	0.58	0.57	0.59
5 months	0.41	0.60	0.59	0.56
8 months	0.24	0.66	0.51	0.63
10 months	0.29	0.55	0.62	0.60

[1] TBARS in ug/g

APPENDIX I

Organoleptic Evaluation of Cheeses

Cheese aged one month[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD[2]
Flavor (192)[3]	6.88	6.73	6.19	5.68	5.91	5.56	0.33
Texture (192)	7.14	6.63	6.21	5.64	5.31	5.72	0.34
Overall (192)	6.90	6.63	6.09	5.54	5.51	5.51	0.32

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

Cheese aged two months[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD
Flavor (104)	6.27	6.88	5.91	6.34	5.40	5.37	0.46
Texture (104)	6.20	7.18	6.27	6.71	5.07	5.39	0.45
Overall (104)	6.18	7.00	5.98	6.43	5.22	5.41	0.43

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

Cheese aged three months[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD
Flavor (107)	6.53	6.40	6.09	6.22	5.07	5.75	0.47
Texture (107)	6.31	6.42	6.51	6.54	5.68	6.06	0.44
Overall (107)	6.42	6.33	6.04	6.29	5.28	5.75	0.44

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

Cheese aged five months[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD
Flavor (107)	6.51	6.41	6.36	5.63	6.40	5.66	0.44
Texture (104)	6.21	5.78	6.87	6.14	6.75	6.23	0.45
Overall (104)	6.32	6.05	6.41	5.71	6.42	5.67	0.43

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

Cheese aged eight months[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD
Flavor (56)	6.00	5.95	5.77	6.14	6.28	6.04	NS
Texture (56)	6.30	6.13	6.54	6.64	6.68	6.46	NS
Overall (56)	6.13	5.96	5.93	6.42	6.49	6.14	NS

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

Cheese aged ten months[1]

Treat	Control1	Control2	$FeCl_3$1	$FeCl_3$2	Fe-WP	Fe-C	LSD
Flavor (84)	5.61	6.20	5.87	6.08	5.92	5.59	NS
Texture (84)	5.88	6.48	6.24	6.49	6.30	6.42	NS
Overall (84)	5.53	6.21	5.82	6.19	5.92	5.73	NS

[1] Control1 and control2 or $FeCl_3$1 and $FeCl_3$2 were randomly picked samples from control or $FeCl_3$ fortified cheese. Only one sample was randomly picked from Fe-WP or Fe-C fortified cheese.

[2] LSD stands for least significant difference at the level of å=0.05. NS: not significant.

[3] Values in the parenthesis are number of cheese samples evaluated.

APPENDIX J

TBARS Assay for Cheese, Serum, Urine, and Feces

Malondialdehyde (MDA) is formed from the breakdown of polyunsaturated fatty acids and has been identified as a product of lipid peroxidation. MDA reacts with 2-thiobarbituric acid to give a red chromogen absorbing at 532 nanometers (nm) and serves as a convenient index for determining the extent of the peroxidation reaction.

Trichloroacetic Acid-Thiobarbituric Acid-Hydrochloric Acid (TCA-TBA-HCL) reagent:

1. Dissolve 15 grams trichloroacetic acid (TCA) in 30 ml of deionized water.
2. Dissolve 0.375 grams 2-thiobarbituric acid (4, 6-dihydroxypyrimidine-2-thiol) to 20ml of deionized water. Heat gently to assist dissolution and cool to room temperature.
3. Add 2.083 ml of concentrated hydrochloric acid (12N) to a 100 ml volumetric flask.
4. Combine 1 and 2 into 3 and take it to a volume of 100 ml with deionized water for a final concentration of 0.25N hydrochloric acid, 15% w/v trichloroacetic acid, and 0.375% w/v 2-thiobarbituric acid.
5. Store in a sealed brown glass container at room temperatureuntil ready for use.

Procedures:

Cheese:

1. Hand chop cheese sample into very fine pieces using a stainless steel spatula or razor.
2. Weigh 0.5 g of sample into a 10 ml glass screw-cap test tube.
3. Add 4.5 ml of TCA-TBA-HCL stock reagent to test tube, cap, and vortex thoroughly.
4. Incubate in a hot water bath (95^0C) for 60 minutes.
5. Cool under running water.
6. After cooling, centrifuge the red colored turbid solution at 4000 rpm for 10 minutes to obtain a clear supernatant.
7. Absorbance of the supernatant is determined in a spectrophotometer at 532 nm against a blank that contains 4.5 of stock reagent plus 0.5 ml of deionized water.

Standard curve:

The procedure for standards are the same as for samples except samples are replaced with equivalent weights of known standards. The standard curve is made by determining the absorbance of a known amount of 1,1,3,3-tetraethoxypropane (TEP), the precursor of malonaldehyde. The absorbance (X) of 1.5, 3.0, and 6.0 nanomoles (nmoles) TEP (MDA) is plotted against their respect amounts (Y).

Calculation:

nmoles MDA per gram = sample concentration (nmoles MDA)/sample weight
micrograms MDA per gram = (nmoles MDA per gram x 72.07)/1000
(Note: molecular weight of MDA equals 72.07)

Serum:

1. Pipet 0.3 ml of serum into a 10 ml glass screw-cap test tube.
2. Add 2.0 ml of TCA-TBA-HCL stock reagent to test tube. Cap and vortex thoroughly.
3. Incubate in a hot water bath (95^0C) for 45 minutes.
4. Cool under running water.
5. After cooling, centrifuge the red colored turbid solution at 4000 rpm for 10 minutes to obtain a clear supernatant.
6. Absorbance of the supernatant is determined in a spectrophotometer at 532 nm against a blank that contains 2.0 of stock reagent plus 0.3 ml of deionized water.

Standard curve:

The procedure for standards are the same as for samples except samples are replaced with equivalent weights of known standards. The standard curve is made by determining the absorbance of a known amount of 1,1,3,3-tetraethoxypropane (TEP), the precursor of malonaldehyde. The absorbance (X) of 0.6, 1.2, 2.4, and 4.8 nmoles TEP is plotted against their respect amounts (Y).

Calculation:

nmoles MDA per ml = sample concentration (nmoles MDA)/sample volume(ml)
micrograms MDA per ml = (nmoles MDA per ml x 72.07)/1000
(Note: molecular weight of MDA is 72.07)

Urine:

1. Pipet 0.5 g of sample into a 10 ml glass screw-cap test tube.
2. Add 4.5 ml of TCA-TBA-HCL stock reagent to test tube. Cap and vortex thoroughly.
3. Incubate in hot water bath (95^0C) for 60 minutes.
4. Cool under running water.
5. After cooling, centrifuge the red colored turbid solution at 4000 rpm for 10 minutes to obtain a clear supernatant.
6. Absorbance of the supernatant is determined in a spectrophotometer at 532 nm against a blank that contains 4.5 of stock reagent plus 0.5 ml of deionized water.

Standard curve:

The procedure for standards are the same as for samples except samples are replaced with equivalent weights of known standards. The standard curve is made by determining the absorbance of a known amount of 1,1,3,3-tetraethoxypropane (TEP), the precursor of malonaldehyde. The absorbance (X) of 1.25, 2.5, 5.0, 7.5, and 10.0 nmoles TEP (MDA) is plotted against their respect amounts (Y).

Calculation:

nmoles MDA per ml = sample concentration (nmoles MDA)/sample volume (ml)
micrograms MDA per ml = (nmoles per ml x 72.07)/1000
(Note: molecular weight of MDA is 72.07)

Feces:

1. Weigh 0.2 to 0.5 g of feces into a 10 ml glass screw-cap test tube.
2. Bring weight of sample to one gram with an appropriate amount of deionized water.
3. Add 4.0 ml of TCA-TBA-HCL reagent to test tube. Cap and vortex thoroughly.
4. Incubate in a hot water bath (95^0C) for 60 minutes.
5. Cool under running water.
6. After cooling, centrifuge the red colored turbid solution at 4000 rpm for 15 minutes to obtain a clear supernatant.
7. Remove a 1.0 ml aliquot of supernatant and pipet into a glass test tube. Dilute with a 5.0 ml aliquot of deionized water and vortex thoroughly.
8. Absorbance of the diluted supernatant is determined in a spectrophotometer at 532 nm against a blank that contains 4.0 of stock reagent plus 1.0 ml of deionized water.

Standard curve:

The procedure for standards are the same as for samples except samples are replaced with equivalent weights of known standards. The standard curve is made by determining the absorbance of a known amount of 1,1,3,3-tetraethoxypropane (TEP), the precursor of malonaldehyde. The absorbance (X) of 2.0, 4.0, 8.0, and 15.0 nmoles TEP (MDA) is plotted against their respect amounts (Y).

Calculation:

nmoles MDA per gram = sample concentration (nmoles MDA)/sample weight (g)
micrograms MDA per gram = (nmoles per gram x 6 x 72.07)/1000
(Note: molecular weight of MDA is 72.07)

APPENDIX K

Liquid Scintillation Method for Blood Radioiron

1. Measure 5 ml human whole blood into glass scintillation vial and add 2 to 5 ml concentrated nitric acid (HNO_3). Heat vials on a hot plate at low temperature until dry. If the ash is not white, add additional HNO_3 and 30% hydrogen peroxide (H_2O_2) until the ash is white. (NOTE: The ashing process may take 2 days to complete.)

2. Add 1 ml of 1N HCl to each vial to dissolve the ash; then, add 10 to 15 ml of liquid scintillation counting-cocktail (Hionic-Fluor™ LSC-cocktail, Packard Instrument B.V. Chemical Operations, Groningen, The Netherlands)

Count Determination:

Place the vials with samples in a scintillation spectrometer and count for 1 to 5 minutes (according to activity) with the window range from 0 to 466 nm.

Calculation for Iron Absorption from Test Meals

$$\text{Percent Absorption} = \frac{(\text{radioactivity per ml of blood} - \text{background}) \times \text{Blood Volume (ml)}}{\text{radioactivity in test dose}}$$

APPENDIX L

Calculations for Intestinal Transit Time

1. Efficiency of Detection System:

$$\text{Efficiency} = \frac{\text{counts per minute of known amount of isotope (cpm) - background}}{\text{disintegrations per minute of known amount of isotope (dpm)}}$$

Example for 0.1 microcurie sample of ^{59}Fe: (one microcurie equals 2.22×10^6 dpm)

Efficiency = $29419/(0.1 \times 2.22 \times 10^6) = .13252$ or 13.252%

2. cpm per gram of counted sample =

   cpm in counted sample/ counted sample weight in grams

3. cpm per total fecal sample =

   (cpm/gram counted sample x total weight of fecal sample in grams) - background

4. dpm per total fecal sample =

   cpm per total fecal sample/efficiency

5. microcuries per total fecal sample =

   dpm per total fecal sample/2.22×10^6

APPENDIX M

Serum, Urine, and Fecal TBARS

Table M.1. Serum TBARS Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	5.7[1] ±1.1	5.4 ±1.1	5.6 ±1.0
Fe-C	5.7 ±0.9	5.5 ±1.1	5.6 ±1.0
Fe-WP	5.5 ±1.0	5.5 ±1.2	5.5 ±1.1
Pooled	5.6 ±1.2	5.5 ±1.1	

[1] Means and standard deviations in nanomoles/milliliter.

Table M.2. Urine TBARS Before Cheese Consumption

Week	1	2	3	4	Pooled
Treatment:					
$FeCl_3$	10.3[1] ±4.3	12.0 ±4.1	13.4 ±6.3	12.3 ±5.6	12.0 ±5.2
Fe-C	12.9 ±6.0	13.0 ±6.1	12.1 ±5.9	13.5 ±2.8	12.9 ±5.3
Fe-WP	8.9 ±4.2	9.2 ±3.8	11.8 ±4.4	12.6 ±4.3	10.6 ±4.4
Pooled	10.7[a] ±5.1	11.4[ab] ±5.0	12.4[b] ±5.6	12.8[b] ±4.3	

[a, b] Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in nanomoles/milliliter.

Table M.3. Urine TBARS During Cheese Consumption

Week	5	6	7	8	9	10	Pooled
Treatment:							
$FeCl_3$	12.9[1] ±5.7	11.9 ±4.4	11.8 ±3.5	11.4 ±6.1	12.6 ±4.9	10.4 ±4.2	11.8 ±4.8
Fe-C	11.7 ±4.6	11.6 ±5.0	11.6 ±4.6	11.2 ±5.2	9.7 ±4.6	10.9 ±4.2	11.1 ±4.7
Fe-WP	11.4 ±5.3	10.7 ±4.2	9.8 ±4.6	10.6 ±6.4	11.8 ±6.1	10.1 ±4.1	10.7 ±5.1
Pooled	12.0^{a} ±5.2	11.4^{ab} ±4.9	11.0^{ab} ±4.2	11.0^{ab} ±5.8	11.4^{ab} ±5.3	10.5^{b} ±4.1	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in nanomoles/milliliter.

Table M.4. Urine TBARS Overall

Week	1	2	3	4	5	6	7	8	9	10
Treatment:										
$FeCl_3$	10.3[1] ±4.3	12.0 ±4.1	13.4 ±6.3	12.3 ±5.6	12.9 ±5.7	11.9 ±4.4	11.8 ±3.5	11.4 ±6.1	12.6 ±4.9	10.4 ±4.2
Fe-C	12.9 ±6.0	13.0 ±6.1	12.1 ±5.9	13.5 ±2.8	11.7 ±4.6	11.6 ±5.0	11.6 ±4.6	11.2 ±5.2	9.7 ±4.6	10.9 ±4.2
Fe-WP	8.9 ±4.2	9.2 ±3.8	11.8 ±4.4	12.6 ±4.3	11.4 ±5.3	10.7 ±4.2	9.8 ±4.6	10.6 ±6.4	11.8 ±6.1	10.1 ±4.1
Pooled	10.7^{a} ±5.1	11.4^{ab} ±5.0	12.4^{b} ±5.6	12.8^{b} ±4.3	12.0^{ab} ±5.2	11.4^{ab} ±4.9	11.0^{a} ±4.2	11.0^{a} ±5.8	11.4^{ab} ±5.3	10.5^{a} ±4.1

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in nanomoles/milliliter.

Table M.5. Urine TBARS Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	12.0[1] ±5.2	11.8 ±4.8	11.9 ±5.0
Fe-C	12.9[a] ±5.3	11.1[b] ±4.7	11.8 ±5.0
Fe-WP	10.6 ±4.4	10.7 ±5.1	10.7 ±4.8
Pooled	11.8 ±5.0	11.2 ±4.8	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] Means and standard deviations in nanomoles/milliliter.

Table M.6. Fecal Wet TBARS Before Cheese Consumption

Week	1	2	3	4	Pooled
Treatment:					
$FeCl_3$	12.1[1] ±4.7	13.5 ±5.7	12.8 ±4.1	11.1 ±2.8	12.4 ±4.4
Fe-C	11.0 ±5.1	11.8 ±2.9	11.4 ±3.1	11.1 ±3.0	11.3 ±3.6
Fe-WP	9.2 ±3.0	12.0 ±2.9	10.9 ±4.0	11.0 ±3.8	10.8 ±3.5
Pooled	10.8[a] ±4.5	12.5[b] ±4.1	11.7[ab] ±3.8	11.0[a] ±3.1	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/gram.

Table M.7. Fecal Wet TBARS During Cheese Consumption

Week	5	6	7	8	9	10	Pooled
Treatment:							
$FeCl_3$	11.2[1] ±3.3	11.5 ±3.4	10.4 ±2.3	10.9 ±2.8	10.2 ±2.0	10.6 ±2.5	10.8 ±2.8
Fe-C	8.9 ±2.2	11.2 ±3.8	9.8 ±2.9	8.3 ±2.3	8.3 ±3.0	8.3 ±2.0	9.1 ±2.9
Fe-WP	9.4 ±2.4	9.7 ±2.6	9.5 ±2.4	10.2 ±1.6	9.3 ±2.9	9.1 ±2.5	9.5 ±2.4
Pooled	9.8 ±2.8	10.8 ±3.4	9.9 ±2.5	9.8 ±2.5	9.2 ±2.7	9.3 ±2.5	

[1] Means and standard deviations in micrograms/gram.

Table M.8. Fecal Dry TBARS Before Cheese Consumption

Week	1	2	3	4	Pooled
Treatment:					
$FeCl_3$	41.1[1] ±16.5	45.8 ±18.8	45.0 ±15.2	37.1 ±10.4	42.3 ±15.6
Fe-C	42.0 ±16.9	44.8 ±13.2	44.0 ±11.7	41.5 ±8.7	43.1 ±12.8
Fe-WP	36.6 ±10.4	44.4 ±13.0	41.8 ±14.7	44.4 ±11.3	41.8 ±12.6
Pooled	40.0 ±15.0	45.0 ±15.1	43.6 ±13.7	41.0 ±10.4	

[1] Means and standard deviations in micrograms/gram.

Table M.9. Fecal Dry TBARS During Cheese Consumption

Week	5	6	7	8	9	10	Pooled
Treatment:							
$FeCl_3$	38.1[1] ±10.5	40.4 ±13.3	37.4 ±7.1	40.2 ±9.7	36.1 ±7.6	37.0 ±5.6	38.2 ±9.3
Fe-C	35.1 ±9.8	44.8 ±7.4	37.8 ±9.6	35.8 ±9.1	35.4 ±7.3	34.4 ±7.5	37.2 ±9.1
Fe-WP	34.6 ±6.2	39.8 ±12.2	35.1 ±8.6	36.7 ±9.9	37.5 ±6.1	35.7 ±4.5	36.6 ±8.3
Pooled	35.9[a] ±9.1	41.7[b] ±11.2	36.7[a] ±8.4	37.5[a] ±9.6	36.3[a] ±7.0	35.7[a] ±6.1	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/gram.

APPENDIX N

Analysis of Variance Tables

Table N.1. ANOVA for Serum TBARS

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Treatment	2	1.024	51	2.274	0.451	0.639
Time (Week)	4	16.538	204	0.897	18.444	0.000
Treat x Time	8	0.481	204	0.897	0.536	0.828

Table N.2. ANOVA for Urine TBARS

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Treatment	2	76.151	51	104.069	0.732	0.486
Time (Week)	9	29.685	459	15.672	1.894	0.051
Treat x Time	18	18.365	459	15.672	1.172	0.281

Table N.3. ANOVA for Fecal Wet TBARS

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Treatment	2	119.103	51	31.921	3.731	0.031
Time (Week)	9	61.183	459	7.974	7.672	0.000
Treat x Time	18	7.853	459	7.974	0.985	0.476

Table N.4. ANCOVA for Fecal Wet TBARS and Fecal Moisture

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Treatment	2	21.441	51	19.793	1.083	0.346
Time (Week)	9	22.871	459	5.616	4.073	0.000
Treat x Time	18	7.912	459	5.616	1.409	0.122

Table N.5. ANOVA for Fecal Dry TBARS

Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
Treatment	2	65.581	51	297.831	0.221	0.803
Time (Week)	9	611.735	459	101.839	6.007	0.000
Treat x Time	18	84.141	459	101.839	0.826	0.669

APPENDIX O

Iron Status Parameters

Table O.1. Hemoglobin Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	14.9[a, 1] ±1.0	15.1 ±0.8	15.0[3] ±0.9
Fe-C	14.3[b] ±0.9	14.7[a] ±0.8	14.5[3] ±0.9
Fe-WP	15.0[a] ±0.8	15.1 ±0.8	15.0[5] ±0.8
Pooled	14.7[a] ±0.9	15.0[b] ±0.8	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in grams/deciliter.

Table O.2. Hemoglobin During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	14.6[1] ±0.8	14.9 ±0.8	14.7 ±0.9
Fe-C	14.5 ±0.8	14.6 ±1.0	14.5 ±1.0
Fe-WP	14.8 ±1.0	14.8 ±0.8	14.8 ±0.9
Pooled	14.6 ±0.8	14.6 ±0.9	

[1] Means and standard deviations in grams/deciliter.

Table O.3. Hematocrit Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	42.7[1] ±2.0	42.2 ±1.7	42.4 ±1.9
Fe-C	40.9 ±2.0	41.0 ±1.7	40.9 ±2.1
Fe-WP	42.9 ±2.4	42.2 ±2.2	42.5 ±2.2
Pooled	42.1 ±2.3	41.8 ±2.0	

[1] Means and standard deviations in percent red blood cells.

Table O.4. Hematocrit During Cheese Consumption

Week	5	7	Pooled
Treatment:			
$FeCl_3$	42.5[1] ±2.2	42.4 ±1.8	42.4 ±2.2
Fe-C	41.0 ±2.2	41.2 ±2.4	41.2 ±2.4
Fe-WP	43.3 ±1.9	42.6 ±2.1	42.9 ±2.1
Pooled	42.3 ±2.2	42.0 ±2.2	

[1] Means and standard deviations in percent red blood cells.

Table O.5. Hematocrit Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	42.4[1] ±1.9	42.4 ±2.2
Fe-C	40.9 ±2.1	41.2 ±2.4
Fe-WP	42.5 ±2.2	42.9 ±2.1
Pooled	42.0 ±2.1	42.2 ±2.2

[1] Means and standard deviations in percent red blood cells.

Table O.6. Serum Iron Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	112.5[1] ±49.8	82.9 ±41.8	97.7 ±47.8
Fe-C	97.9 ±47.4	87.4 ±27.9	92.6 ±38.7
Fe-WP	103.7 ±35.4	100.1 ±42.0	101.9 ±38.2
Pooled	104.7[a] ±44.7	90.2[b] ±37.5	

[a, b] Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/deciliter.

Table O.7. Serum Iron During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	100.2[1] ±32.0	97.6 ±29.6	98.9 ±30.8
Fe-C	91.0 ±40.9	108.1 ±39.2	99.5 ±40.0
Fe-WP	107.3 ±39.4	103.9 ±37.1	105.6 ±38.0
Pooled	99.5 ±37.4	103.2 ±35.1	

[1] Means and standard deviations in micrograms/deciliter.

Table O.8. Serum Iron Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	97.7[1] ±47.8	98.9 ±30.8
Fe-C	92.6 ±38.7	99.5 ±40.0
Fe-WP	101.9 ±38.2	105.6 ±38.0
Pooled	97.4 ±41.7	101.1 ±36.2

[1] Means and standard deviations in micrograms/deciliter.

Table O.9. Total Iron-Binding Capacity Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	351.2[1] ±42.4	338.3 ±55.7	344.7 ±49.2
Fe-C	332.1 ±55.3	319.8 ±45.7	325.9 ±50.4
Fe-WP	349.5 ±48.4	344.3 ±61.1	346.9 ±54.3
Pooled	344.3 ±48.9	334.1 ±54.1	

[1] Means and standard deviations in micrograms/deciliter.

Table O.10. Total Iron-Binding Capacity During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	359.6[1] ±61.2	325.0 ±42.4	342.3 ±51.8
Fe-C	331.0 ±62.4	312.4 ±50.9	321.7 ±56.7
Fe-WP	341.9 ±61.5	318.5 ±51.9	330.2 ±56.7
Pooled	344.2[a] ±61.7	318.7[b] ±47.7	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/deciliter.

Table O.11. Total Iron-Binding Capacity Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	344.7[a, 1] ±49.2	342.3[ab] ±51.8
Fe-C	325.9[a] ±50.4	321.7[ab] ±56.7
Fe-WP	346.9[a] ±54.3	330.2[b] ±56.7
Pooled	339.2 ±51.1	331.5 ±54.9

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

1 All means and standard deviations in micrograms/deciliter.

Table O.12. Transferrin Saturation Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	32.1[1] ±14.1	25.6 ±13.9	28.9 ±14.2
Fe-C	30.1 ±15.8	28.0 ±10.2	29.0 ±13.2
Fe-WP	29.4 ±8.8	29.0 ±10.4	29.2 ±9.5
Pooled	30.5 ±8.8	27.5 ±10.4	

1 Means and standard deviations in percent saturation.

Table O.13. Transferrin Saturation During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	28.3[1] ±8.0	30.1 ±8.4	29.2 ±8.2
Fe-C	28.5 ±13.0	35.6 ±13.7	32.0 ±13.3
Fe-WP	32.8 ±14.5	33.7 ±13.7	33.3 ±14.1
Pooled	29.9 ±11.9	33.1 ±12.1	

[1] Means and standard deviations in percent saturation.

Table O.14. Transferrin Saturation Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	28.9[1] ±14.2	29.2 ±8.2
Fe-C	29.0 ±13.2	32.0 ±13.3
Fe-WP	29.2 ±9.5	33.3 ±14.1
Pooled	29.0 ±12.4	31.4 ±11.7

[1] Means and standard deviations in percent saturation.

Table O.15. Serum Ferritin Before Cheese Consumption

Week	1	3	Pooled
Treatment:			
$FeCl_3$	37.4[1] ±34.3	28.4 ±29.2	32.9 ±31.8
Fe-C	37.5 ±32.0	28.7 ±24.8	33.1 ±28.6
Fe-WP	39.6 ±37.7	27.0 ±31.0	33.3 ±34.5
Pooled	38.2[a] ±33.9	28.0[b] ±27.8	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/liter.

Table O.16. Serum Ferritin During Cheese Consumption

Week	6	8	Pooled
Treatment:			
$FeCl_3$	39.5[a, 1] ±56.4	26.8[b] ±30.1	33.1 ±43.3
Fe-C	25.8[b] ±22.7	23.5[b] ±22.6	24.6 ±22.7
Fe-WP	27.2[b] ±26.3	20.8[b] ±25.0	24.0 ±25.7
Pooled	30.8[a] ±38.6	23.7[b] ±25.8	

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

[1] All means and standard deviations in micrograms/liter.

Table O.17. Serum Ferritin Before Versus During Cheese Consumption

Time Period	Before	During
Treatment:		
$FeCl_3$	32.9[a, 1] ±31.8	33.1[ab] ±43.3
Fe-C	33.1[a] ±28.6	24.6[b] ±22.7
Fe-WP	33.3[a] ±34.5	24.0[b] ±25.7
Pooled	33.1[a] ±31.5	27.4[b] ±30.8

a, b Means in same row or column with different superscripts are significantly different ($p<.05$); pooled means compared only to other pooled means in same row or column.

1 All means and standard deviations in micrograms/liter.

APPENDIX P

Dietary Intake Parameters

Table P.1. Copper Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	1.2[1] ±0.6	1.2 ±0.8	1.2 ±0.7
Fe-C	1.3 ±0.7	1.2 ±0.7	1.3 ±0.7
Fe-WP	1.2 ±0.5	1.1 ±0.4	1.2 ±0.4
Pooled	1.2 ±0.6	1.2 ±0.6	

[1] Means and standard deviations in milligrams/day.

Table P.2. Zinc Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	11.8[1] ±12.8	12.8 ±13.2	12.3 ±13.0
Fe-C	11.0 ±6.2	11.4 ±5.3	11.2 ±5.8
Fe-WP	10.0 ±4.1	10.8 ±5.9	10.4 ±5.1
Pooled	11.0 ±8.7	11.7 ±9.0	

[1] Means and standard deviations in milligrams/day.

Table P.3. Dietary Fiber Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	13.0[1] ±6.2	13.2 ±11.4	13.1 ±9.1
Fe-C	15.3 ±8.5	14.4 ±7.5	14.8 ±8.0
Fe-WP	13.6 ±6.9	12.7 ±7.3	13.2 ±7.1
Pooled	14.0 ±7.3	13.5 ±9.0	

[1] Means and standard deviations in grams/day.

Table P.4. Energy Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	1924[1] ±826	1974 ±801	1949 ±811
Fe-C	2212 ±780	2122 ±851	2162 ±814
Fe-WP	2098 ±782	2067 ±738	2082 ±756
Pooled	2077 ±802	2050 ±799	

[1] Means and standard deviations in kilocalories/day.

Table P.5. Vitamin A Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	6678[1] ±6733	6589 ±6589	6634 ±7134
Fe-C	6095 ±9275	7932 ±9193	7013 ±9240
Fe-WP	7668 ±8060	4809 ±3570	6238 ±6365
Pooled	6766 ±8066	6534 ±7392	

[1] Means and standard deviations in international units/day.

Table P.6. Carotene Intake Before Versus During Cheese Consumption

Time Period	Before	During	Pooled
Treatment:			
$FeCl_3$	421[1] ±567	348 ±516	384 ±541
Fe-C	442 ±867	628 ±958	535 ±914
Fe-WP	478 ±760	225 ±281	351 ±584
Pooled	445 ±737	410 ±681	

[1] Means and standard deviations in retinol equivalents/day.

Table P.7. Dietary Supplement Usage Before Cheese Consumption

Nutrient	Vitamin C	Vitamin A	å-Toc	Calcium	Iron	Zinc
Treatment:						
$FeCl_3$	4[1, 2]	456[3]	0.9[2]	26[2]	0.6[2]	2.9[2]
Fe-C	150	526	14.0	38	0.9	0.8
Fe-WP	3	208	0.3	0	0.8	0.0
Pooled	55	407	5.4	23	0.7	1.3

[1] All values are mean daily intakes.
[2] Values in milligrams/day.
[3] Values in international units/day.

Table P.8. Dietary Supplement Usage During Cheese Consumption

Nutrient	Vitamin C	Vitamin A	å-Toc	Calcium	Iron	Zinc
Treatment:						
$FeCl_3$	23[1, 2]	1158[3]	8.9[2]	52[2]	1.2[2]	3.7[2]
Fe-C	126	526	14.5	41	1.2	1.1
Fe-WP	11	351	0.7	0	1.1	0.5
Pooled	56	716	8.4	33	1.2	1.8

[1] All values are mean daily intakes.
[2] Values in milligrams/day.
[3] Values in international units/day.

VITA

Gene Joseph Giunti
Hamden, Connecticut
(January, 1995)

CAREER OBJECTIVES:

1) to enhance and strengthen my knowledge in all aspects of nutrition science, associated disciplines such as physiology, biochemistry, and medicine; and their interrelationships; 2) to develop proficiencies in the areas of experimental design and methodology, laboratory procedures, epidemiology, and statistical analysis; 3) to refine my teaching skills in order to foster an instructional environment leading to a progressive exchange of ideas; and, 4) to maintain and improve my skills as a clinician for effective assessment and delivery of health care. These objectives continue to be my guideline as I aspire to be a Physician, the endpoint being competent autonomy as a clinical and research professional.

EDUCATION:

BS in Medical Dietetics CUP, Utah State University, 1986.
MS in Nutrition and Food Sciences, Utah State University, 1994.
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EXPERIENCE:

Research Assistant, Iron Cheese Project, Utah State University, 1991-93.
Research Associate Hip Fracture Pilot Studies, Utah State University, 1993-94.
Research Assistant, Alzheimer's Disease Pilot Study, Utah State University, 1993.
Clinical Instructor, Medical Dietetics CUP, Utah State University, 1991-94.
Instructor, Nutrition in the Life Cycle, Utah State University, 1993.
Registered Dietitian, Youville Hospital, Cambridge, MA, 1986-91.
Nutrition/Food Consultant , Voyager World Flight, 1985-86.