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Palatable Foods for a Methionine Restricted Diet

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PALATABLE FOODS FOR A METHIONINE RESTRICTED DIET

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This dissertation is dedicated to my parents; their love, motivational words and support are the structural foundation of this work. To my mother, who set an example of continuous education and passion at every task we perform in our professional career; also for stimulating my creativity and research skills in my childhood. To my father, who everyday demonstrated me that hard work, planning and scheduling are key parameters for reaching our goals and success. To both my parent, who taught me the values of perseverance, patience, friendship and helping others.

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ABSTRACT

Protein oxidation results in loss of tryptophan, oxidation of cysteine to cysteic acid, oxidation of methionine to methionine sulfoxide and ultimately to methionine sulfone. Methionine sulfoxide has been shown to be completely unavailable to mammalian cells and promote improved insulin sensitivity, lifespan extension, weight loss induction and reduce tumor growth in rat studies. Casein was oxidized with ozone or hydrogen peroxide to reduce the cysteine and methionine levels for use in sulfur amino acid restricted diets. Casein was isoelectrically precipitated from skim milk and oxidized with hydrogen peroxide at various pH levels, from 2.5–11. Two strong oxidizers were used: hydrogen peroxide (H_2O_2) and ozone (O_3). Treatments with H_2O_2 were at 5, 10 and 20% concentration, heated to 90°C for 0.5, 1 and 2 hours to oxidize methionine. Hydrogen peroxide was removed by precipitation/washing or with catalase method. Ozone treatments were at 1, 3 and 12% concentration with a duration of 1-48 hours. Amino acid analysis was performed to determine extent of oxidation of methionine to methionine sulfone; cysteine to cysteic acid and tryptophan destruction. Hominex-2® a mixture of amino acids, has been used as a protein source to deliver sulfur amino acid restricted diets. Compliance has been difficult to maintain, because the mixture of amino acids is unpalatable. A sensory evaluation was conducted to assess the acceptability of the oxidized casein comparing it to Hominex-2®, methionine-free formula. An animal trial was performed with 45 week old male C57BL/6J mice to assess the metabolic and behavioral effects of oxidized casein to elemental methionine restricted supplementation. As a result of the oxidation treatments up to 91% of the methionine was oxidized by treatment at pH 2.5 for 2h at 10% H_2O_2. Treatments at alkaline pH were not successful in achieving desired levels of methionine oxidation in casein. Ozone treatments resulted in a partial oxidation (26%) of methionine to methionine sulfone.

Sensory evaluation demonstrated that the panelists showed a significantly higher acceptance ($p<0.05$) for a drink prepared with oxidized casein over Hominex-2®. The animal trial demonstrated increased food consumption concomitant with weight loss in the group of rats fed with oxidized casein. Oxidized casein offers a new alternative to other methionine-restricted nutritional mixes prepared with blends of free amino acids, which have unpleasant taste. The development of methionine restricted food ingredients offers a low-methionine protein source to formulate MR diets which help restrict tumor growth in cancer patients and for weight loss therapy in healthy patients.

CHAPTER 1.
LITERATURE REVIEW

1.1 Introduction

Methionine restricted (MR) diets have been shown to slow tumor growth in late stage cancer patients[1, 2]. More recently it has been shown that methionine restriction helps weight loss in obese animals and humans[3, 4, 5, 6]. The health benefits identified in diets with low-methionine content have suggested that there would be significant opportunities to develop and ultimately commercialize methionine restricted foods for nutritional studies and potentially as medical foods.

The formulation of MR diets represents a challenge for dietitians due to the presence of methionine in most foods. So far, all available *in vitro* and *in vivo* studies published on MR diets have been supplemented with blends of free amino acids as the "protein" source. The unpleasant flavor and texture of the free amino acids blends used to achieve the desired low levels of methionine, makes formulation of diets pleasant for human consumption nearly impossible when using amino acid mixtures. Free amino acids tend to have bitter or astringent flavors, making it difficult for subjects in the study to maintain compliance on the diet for extended periods of time. The result is that some well-planned studies have been prematurely aborted[3].

The lack of availability of methionine restricted foods added to the poor palatability of methionine restricted free amino acid blends have made the research on the health benefits of MR diets extremely complicated. In this dissertation we offer an alternative to free amino acid blends by developing methionine restricted food ingredients.

1.2 Methionine restricted diets for control of metastatic cancer

Diets with limited methionine content may be a key strategy in restricting metastatic cancer growth[7]. "Methionine dependence" has been identified as the growth requirement of several types of cancers such as recurrent gliomas and metastatic melanomas[8, 9, 10]. *In vitro* studies with malignant cell lines from kidney, colon, breast, bladder and prostate cancer have helped identified their methionine dependence[11, 12, 13]. The presence of methionine in the diet is required for these type of cancers to survive and propagate[14, 15, 16].

"Methionine dependence" of cancer cells was first identified by Sugimura and Birnbaum in 1959. When conducting feeding studies, each lacking in one essential amino acid, they observed growth of carcinosarcoma subcutaneous tumors was altered on Sprague-Dawley rats fed with diet lacking methionine[10]. In later research Halpern and Clark, 1974, showed the total dependence of carcinogenic cells on methionine. In the *in vitro* study they used cell cultures of Walker-256 mouse lymphatic leukemia and human monocytic leukemia cells growing in co-culture with non-tumorous human prostate and breast fibroblasts cells, in a zero methionine medium (Met^- Hcy^+). The absence of methionine in the medium selectively inhibited growth of carcinogenic cells while the non-carcinogenic cells remained unaffected[16].

Research on methionine dependent cancer has been widely studied *in vitro* and *in vivo*. The effects of methionine restriction on colonic tumors were investigated by Komninou 2006 in an animal trial by feeding six-week-old male F344 azoxymethane-treated rats with a blend of essential amino acids. The control diet had 86% methionine content and the MR diet contained 17% methionine. Formation of preneoplastic aberrant crypt foci (ACF) was monitored on the colon of the rats. After the 10 week study, formation of ACF was reduced by 80% in the group fed with MR diet compared to control. The proliferation of colonic cells was reduced by 12% in

the group fed with MR diet compared to control. The study showed that MR diet was effective in inhibiting proliferation of colonic cancer cells during the post-initiation phase of carcinogenesis[17].

MR diets can aid on the control and treatment of methionine dependent cancer. Studies in MR diets combined with chemotherapeutic drugs resulted in selective destruction of tumor cells in co-cultures of tumorous and normal cell lines. Stern *et al.* 1986 investigated the potentiated effect of chemotherapeutic drugs in combination with MR diets. They showed the synergistic effect of doxorubicin and vincristine when combined with MR diets to treat *in vitro* cell cultures. The study resulted in the selective killing of tumorous cells [18]. The same synergistic effect, between chemotherapeutic drugs and MR diets, was reported *in vivo* years later. Hoshiya 1995, conducted an animal trial with MX-1 human breast carcinoma cells in nude mice showing how the combination of MR diet and cisplatin resulted in death of carcinoma, but when the treatments were applied by separate no therapeutic effect was attained[19].

Even though the mechanisms responsible for methionine dependence in metastatic cancer cells have not yet been deciphered. The specificity of metastatic cancer for methionine offers a therapeutic approach to inhibit tumor growth and relapse[7]. Clinical trials indicated that enteral dietary methionine restriction in diets has limited harmful effects on regular healthy tissue but it is detrimental for oncogenic tissue[20, 21]. It has been observed that restriction of methionine in malignant cells causes the cell cycle to arrest in the late-S/G_2 phase (Figure 1). Cell cycle arrest before initiation of mitosis enhances cell sensitivity to chemotherapeutic drugs and may even lead to spontaneous death [22, 23]. The interaction of MR diets with chemotherapy treatment offers a promising strategic therapy for carcinogenesis and cancer growth control.

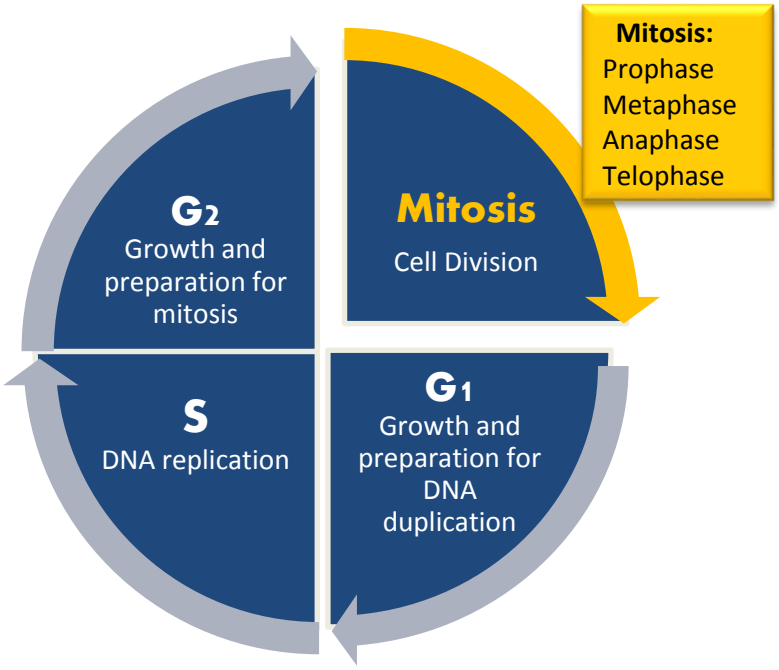


Figure 1. The cell life cycle: DNA replication and cell division

Clinical trials supplemented with MR diets have proven these diets to be safe and tolerable for adults with metastatic solid tumors[2]. Unfortunately, toxicological studies to determine safety limits of MR diets have not been performed yet due to the lack of availability of methionine restricted foods. The development of palatable methionine restricted food ingredients will potentiate the research in this area, by offering high protein foods with low methionine content. The new low methionine food ingredients will allow formulation of palatable MR diets to define the optimal level of methionine restriction for control of cancer growth, and for determination of its safety limits.

1.3 Methionine restricted diets for weight-loss and life-span extension

Methionine restricted diets have demonstrated enhanced weight-loss by reduction of abdominal fat deposition, and a prolonged healthy life-span in rats and mice studies[4, 24, 25]. Preclinical studies in flies, mice and rats have reported MR diets to enhance insulin sensitivity,

increase energy expenditure (EE), limit fat accumulation and reduce lipids in plasma[4, 25, 26, 27]. Figure 2 describes the health benefits reported in preclinical trials on MR diets on a chronological diagram.

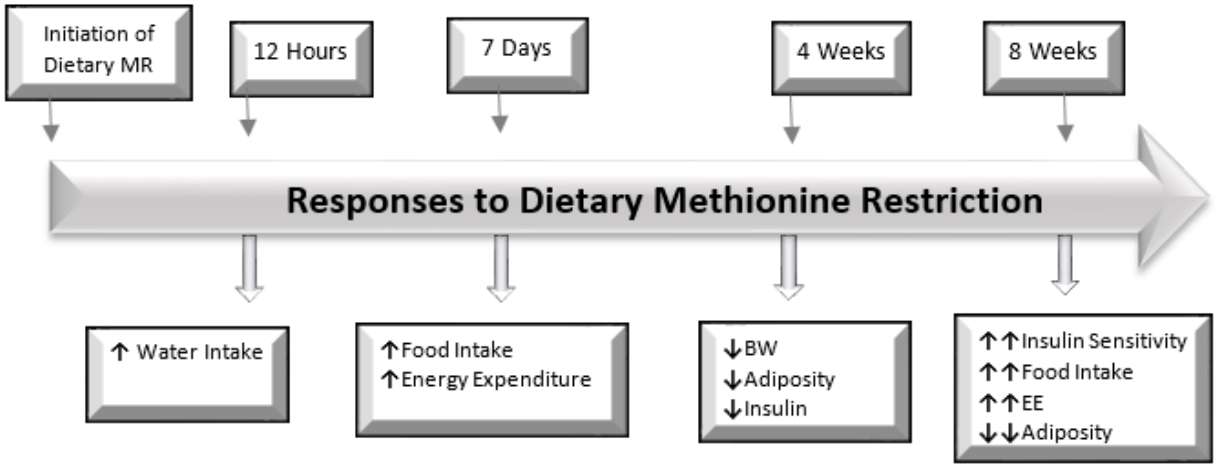


Figure 2. Chronological effects of methionine restricted diets[3]

Life-span extension has been observed repeatedly when methionine ingestion is restricted from 86% (control diet) to 0.17% of methionine in diet (1.7 g/kg). Orentreich 1993, reported a 30% life-span extension in group of 30 Fischer 344 male rats. In the 90 week study the group fed with a diet of 0.86% methionine content gained an average of 350g of weight in 50 weeks, while the group fed with 0.17% MR diet exhibited no weight gain during the same period of time. Another group of rats fed with 0.12% MR diet had no animals alive after 30 days. The data indicated that 0.17% MR diets were efficient in controlling weight gain and extending life-span in mammals, but lowering the content of methionine under the 0.17% level results in death. In order to differentiate if life-span extension was an isolated factor from restricted food intake, the 0.17% MR diet was compared with a food restricted diet. Results indicated that life-span extension was produced by the restriction of methionine only, highlighting it as an independent factor to overall food restriction [25].

Life-span extension has repeatedly been identified as a result of methionine restriction in diets. Sun and Miller 2005, reported life-span extension when methionine intake content was lowered on CB6F1 male mice study. One group of mice was fed a control diet with 0.43% methionine content and a second group was fed a MR diet with 0.15% methionine content. Administration of the diet started after 12 months of age. At the end of the study they documented life-span extension on the mice fed with the MR diet (0.15%). Longevity of the control group was 948 days of age and the MR diet group 1011 days of age. Also a higher rate of stress tolerance, slower development of cataracts and decreased rate of age-related change in T-cells and multiple tissues was observed in the MR diet group. The methionine restricted group of mice showed similarities with the caloric restricted group in the reduced serum levels of thyroid hormone, glucose, insulin and insulin-like growth factor-I (IGF-I), but they exhibited higher longevity [6, 28].

The biochemical mechanism by which methionine restriction causes weight-loss and life-span extension in mammals is still unconcise. It has been hypothesized that the loss of glutathione by reduction of methionine in diet can be the cause of a healthy life extension in animal trials. Methionine is a precursor of glutathione therefore a restriction of the intake levels of methionine should result in a change in glutathione levels. In 2004 Richie *et al*. monitored glutathione response in young and mature F344 rats subjected to methionine restriction. The control diet had a 0.86% methionine content and the MR diet was 0.17% methionine. The study resulted in a decrease in liver (66%) kidney (22%) and pancreas (80%) glutathione levels in the 11 week study[26]. In earlier research in methionine restricted intake Richie reported a decrease of 43% body weight and a 44% life-span increase in F344 rats fed with MR diet (0.17%)[4].

The first study on the metabolic effects MR diets have on human weight-loss was conducted by Plaisance and Greenway 2011. A clinical trial with 26 obese subjects (20 females and 6 males) was started in order to evaluate the effects of MR diets in humans with metabolic syndrome. Patients with metabolic syndrome suffer a cluster of pathologies involving an irregular metabolism of lipids and carbohydrates, obesity and insulin resistance. Twelve subjects were randomly assigned a capsule of placebo and the other 14 were provided with a capsule containing 33mg methionine/ kg body weight per day. All subjects in the study were fed with a MR diet containing 2 mg methionine/kg body weight per day and consumed their assigned daily capsule. Hominex-2®, a blend of free amino acids, was the food supplement used to replace the protein source in order to achieve the required low levels of methionine for the study. After 16 weeks of MR diet a high withdrawal rate of subjects from the study was reported due to the numerous complains on the poor palatability of Hominex-2. Methionine levels in plasma indicated that subjects in the study were not complying with the MR diet, since the projected levels of methionine reduction in plasma was 75% but only a 14% reduction was achieved. The 14% reduction of methionine in plasma produced a significant metabolic response on liver fat and fuel selection. No more conclusions could be reported due to the low compliance of humans to the MR diet. The health benefits reported on animal trials have become a challenge to reproduce in human clinical trials due to the low compliance of humans to diets of free amino acids blends, as Hominex-2[3]. The development of palatable low methionine food ingredients is necessary in order to allow researchers to formulate new palatable MR diets, for human consumption, capable of reproducing the certainty on compliance achieved in animal trials.

1.4 Metabolism of methionine

The *de novo* pathway is the biochemical cycle of methionine in mammalian cells (Figure 3).

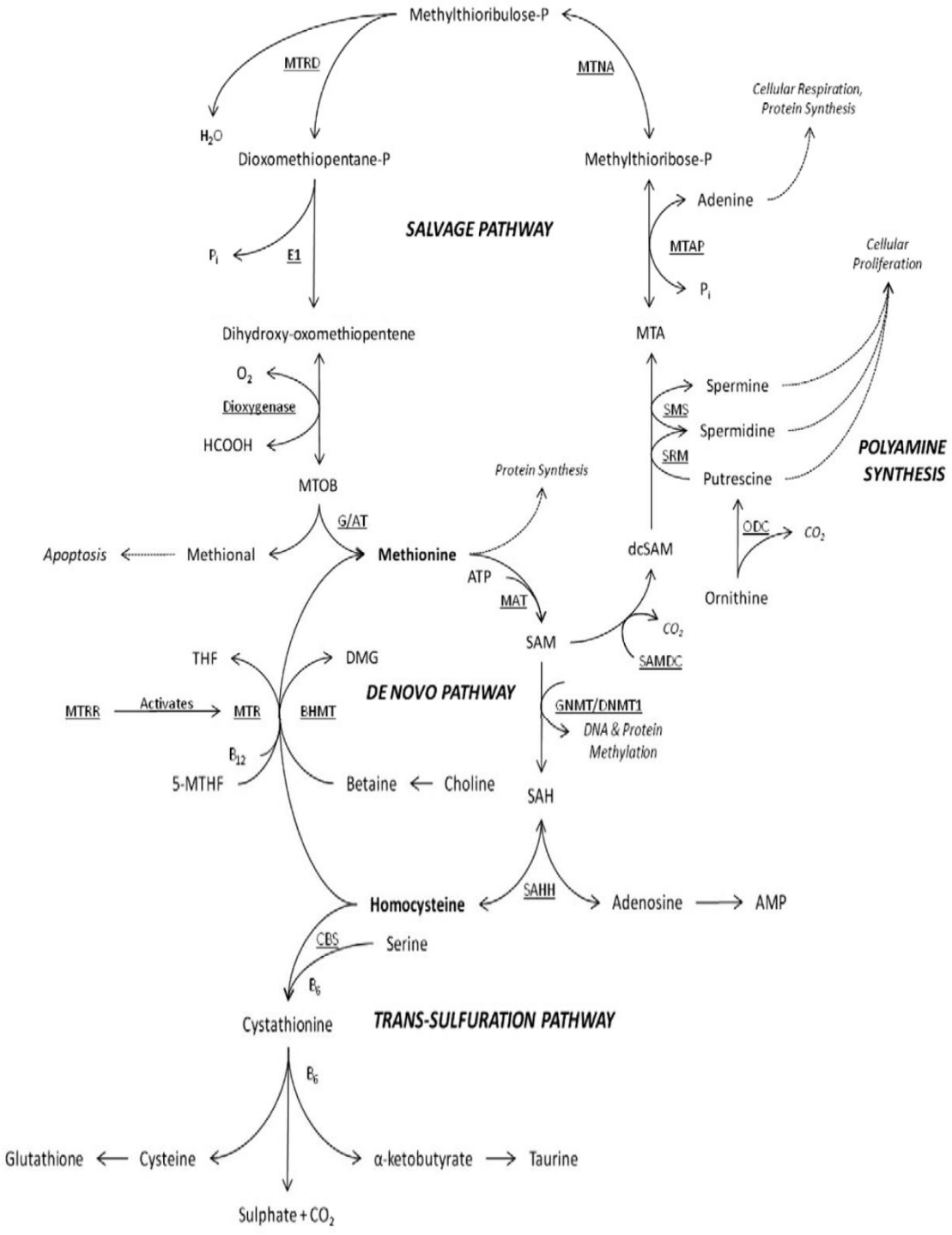


Figure 3. Methionine cycle and trans-sulfuration pathway[7]

The *de novo* pathway is also known as recycling or methylation pathway. Dietary methionine has two functions in the body. It's used for protein biosynthesis and as a metabolic source of methyl groups. The cycle of methionine in the *de novo* pathway starts with the conversion of methionine the methyl donor S-adenosylmethionine (SAM)[29]. SAM methylates DNA and the demethylated SAM is converted to S-adenosylhomocysteine (SAH) [30]. Homocysteine is formed from the hydrolysis of SAH, reversible reaction, and follows either the trans-sulfuration or the methylation pathway[29].

The methylation pathway leads to synthesis of methionine. During this pathway the homocysteine, previously synthesized in the *de novo* pathway, undergoes an enzymatic reaction and gets re-methylated to methionine by methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase MTR) or by betaine-homocysteine S-methyltransferase. An approximate of 50% homocysteine undergoes this pathway[31].

The trans-sulfuration pathway leads to the synthesis of glutathione. During this pathway homocysteine is first converted to cysteine ending in the synthesis of glutathione. It has been reported that during this pathway cysteine can be regenerated to form methionine, by re-methylation process, instead of synthesizing glutathione [32]. Elshorbagy 2011, reported a reversed effect on reduction of adipose deposits, after MR diet supplementation in rats, when cysteine was present in the diet. These results highlighted that in order to attain weight-loss therapy by MR diet supplementation the content of cysteine (cysteine) in the diet is also crucial. Cystine (cysteine) and methionine content need to be reduced in MR diets for weight-loss therapy purposes, since the sulfur content of both of these amino acids contributes to the regulation of Stearoyl-CoA desaturase-1 (SCD1), the enzyme controlling energy metabolism [33].

The sulfur content of MR diets needs to be monitored in order to successfully control the availability of methionine for metabolic cycles. The health benefits that MR diets provide get eliminated by the presence of cysteine in the diet. Regeneration of methionine from cysteine raised concern in the bioavailability of cysteine in the supplemented MR diets for animal and clinical trials.

1.5 Bioavailability of sulfonated amino acids

Methionine, cysteine and tryptophan are the three amino acids containing sulfur figuring in the list of essential amino acids for human consumption. These sulfonated amino acid residues are intrinsic to the protein in the food matrix, therefore their physical removal results impossible without eliminating or affecting the protein content of the diet. A reduction of the total sulfur content of diets has been achieved by controlled oxidation of the nutritionally essential sulfonated amino acid residues in the protein[34, 35]. Chemical modifications of these sulfonated amino acids have been widely researched to make them become unavailable for metabolism, without damaging the protein content of the food ingredient [34, 35, 36, 37].

The nutritional availability of methionine and cysteine has been observed to get adversely affected when these amino acids undergo oxidation[3, 38, 39]. The oxidation state of the sulfur group determines the amino acid characteristics, such as: solubility, pK values and steric configuration[40]. Therefore, the oxidation state influences the amino acid bioavailability during metabolic cycles. Methionine, cysteine and tryptophan are the three amino acids with highest susceptibility to oxidative processes. This high susceptibility to oxidation allows the reduction of the total sulfonated amino acid residues in the diet, by oxidizing them to their nutritional unavailable state without altering the total protein content.

Nutritionally available methionine oxidizes to methionine sulfoxide and to methionine sulfone, while nutritionally available cystine (cysteine) oxidizes to cysteic acid. Multiple studies on the availability of methionine and cysteine have reported methionine sulfone and cysteic acid to be completely nutritionally unavailable, while the availability of methionine sulfoxide is still debatable[35, 38, 39, 41]. Therefore, when developing methionine restricted food ingredients methionine must be oxidized to methionine sulphone to guarantee total unavailability of methionine in the diet. At the same time cysteine should be oxidized to cysteic acid to avoid its regeneration to methionine (Figure 4.) during the trans-sulfuration pathway.

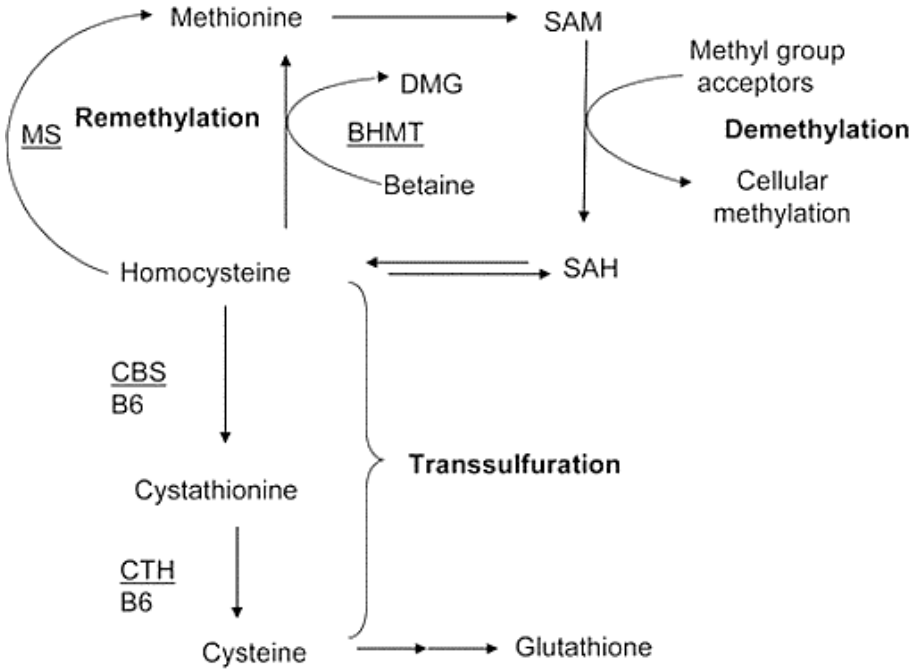


Figure 4. Cysteine remethylation to methionine during the trans-sulfuration pathway[42]

The reduction of nutritional availability of cysteine can be achieved by treatment of proteins with oxidizing agents. Finley and Wheeler 1981, developed a model system utilizing glutathione to estimate the state of oxidation of cysteine in proteins when subjected to oxidation with hydrogen peroxide (H_2O_2) for 60 min at a variable pH range (2.6-7.6). The results reported

a high susceptibility of the cysteine portion of the glutathione protein to undergo oxidation to cysteic acid (Figure 5) when treated with hydrogen peroxide[43]. The results were consistent with earlier studies were cysteine was treated with hydrogen peroxide and lipid hydroperoxides resulting in the formation of sulfinic acid and cysteic acid[44, 45].

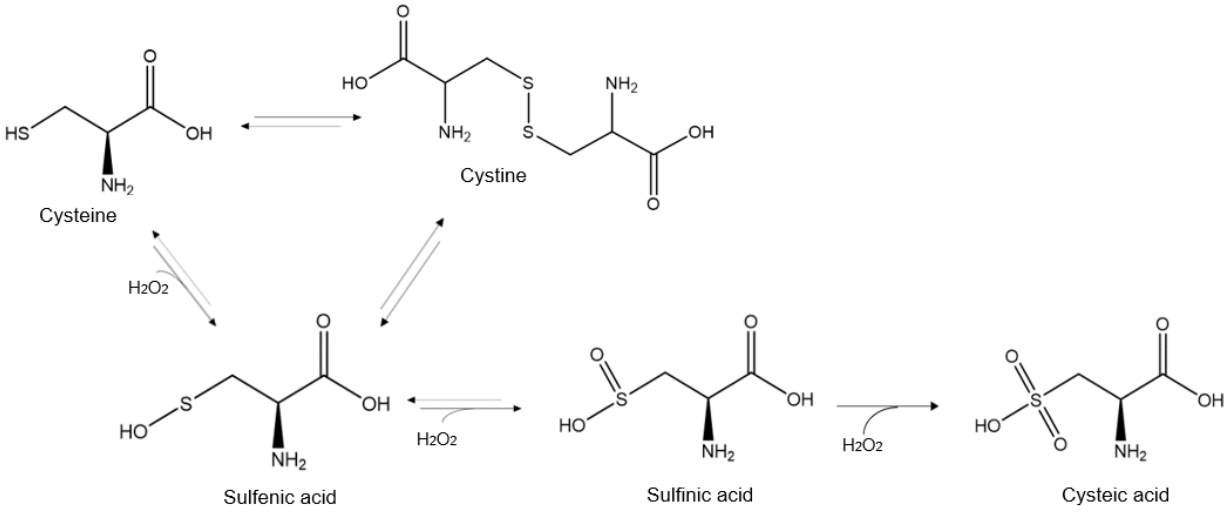


Figure 5. Molecular structure of the oxidation products of cystine (cysteine)

1.6 Oxidation of methionine

In the human body methionine undergoes oxidative reactions under natural conditions to control homeostasis. Methionine is a sulfur-containing amino acid with thioether linkage that allows oxidation to its sulfoxide and sulfone states. The sulfur atom has three valenced states: 2 for thiols, 4 for sulfoxides and 6 for sulfones (Figure 6). The sulfur content in amino acid residues of proteins creates a high sensitivity of the amino acid to oxidative reactions. This sensitivity allows the oxidative reactions to be highly specific to sulfonated amino acids residues (methionine, tryptophan and cysteine) of peptides and proteins[40].

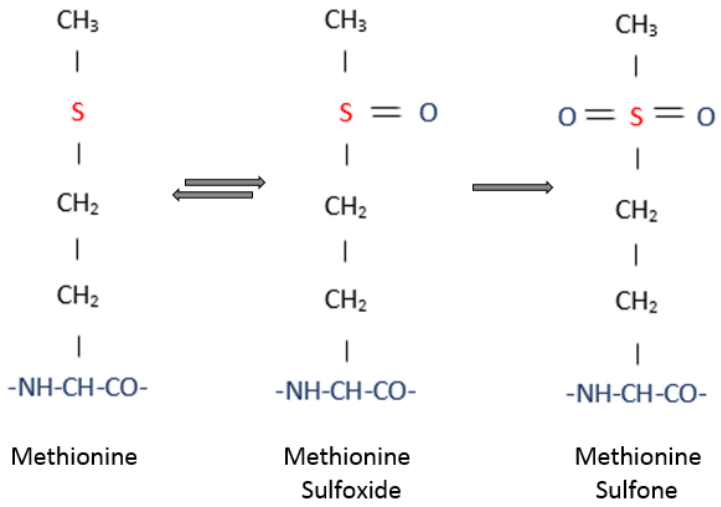


Figure 6. Molecular structure of sulfur oxidation states in methionine

Methionine loses its methyl group during oxidation to its sulfoxide state which can be reduced back to methionine. Methionine to methionine sulfoxide is a common oxidative process in biological cycles. It is the first step of oxidation of methionine since it needs low oxidative stress to react. Methionine sulfone is less commonly found in biological cycles, it is the final state of oxidation of methionine and cannot be reversed. Methionine sulfone is the stable oxidative state of methionine and has very rarely been identified in physiological processes[40, 46].

1.6.1 Methionine sulfoxide

Mild oxidizing conditions such as the exposure of diluted protein solutions to atmospheric oxygen results in the conversion of methionine to methionine sulfoxide[47]. As described by Li 1956, corticotropin isolation, a hormone composed of 39 amino acids and one residue of methionine, gets affected by this natural oxidative process. It causes the conversion of methionine to its inactive form, methionine sulfoxide, resulting in a great loss of hormonal activity due to atmospheric oxidation[48].

Methionine sulfoxide is very commonly found in biological cycles to control homeostatic balance of the system. For example, during the process of protecting the body by inflammatory

response or by tissue damage an oxidative burst starts the NADPH oxidase cycle. First O_2 gets reduced to its superoxide anion state by enzymatic oxidation[49, 50, 51, 52].

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\bullet -} + H^+ \quad 53$$

During the second step of the reaction, hydrogen peroxide is produced by a dismutation process enhanced by enzymatic reaction of superoxide dismutase [54, 55].

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad 53$$

The appearance of transition metal ions to catalyze the formation of $HO^{\bullet}$ from hydrogen peroxide is necessary for the development of the reaction [56, 57].

$$H_2O_2 + Me^{n+} \rightarrow Me^{n+1+} + OH^- + HO^{\bullet} \quad 53$$

The metal ions are key to these redox reactions. During a new cycle the reduced metal ions are oxidized through generation of $HO^{\bullet}$ [53].

$$Me^{n+1+} + O_2^{\bullet -} \rightarrow Me^{n+} + O_2$$

$$NADPH + H^+ + O_2^{\bullet -} \rightarrow NADP^+ + OH^- + HO^{\bullet}. \quad 53$$

In the presence of iron and copper the oxidation of methionine via $HO^{\bullet}$ is catalyzed even at very low concentrations of hydrogen peroxide. In the absence of hydrogen peroxide production the autoxidation reaction persists[58 59].

The defense activation reaction of phagocytic cells is another physiological cycle were spontaneous oxidation of methionine to methionine sulfoxide is used to control homeostasis balance. During this defense reaction PMN leukocytes release by-products in order to destroy any external organism attacking the host cell. The by-products of these reactions include: chloramines, hydroxyl radicals, superoxides and hypochlorous acids which are produced by

leukocytes to oxidize methionine to its sulfoxide state. The only by-product of a metabolic cycle that is strong enough to oxidize methionine all the way to its sulfone state is hydrogen peroxide and it is produced during the defense reactions of leukocyte cells [49].

1.6.2 Methionine sulfone

Experimental oxidation of methionine using chemical reagents has been widely studied. Production of methionine sulfone (Figure 7) has been reported only when methionine is reacted with very strong oxidizing agents as ozone (O3), hydrogen peroxide (H_2O_2) and in combination with catalyzer as selenite, copper, iron and iodide[60, 61].

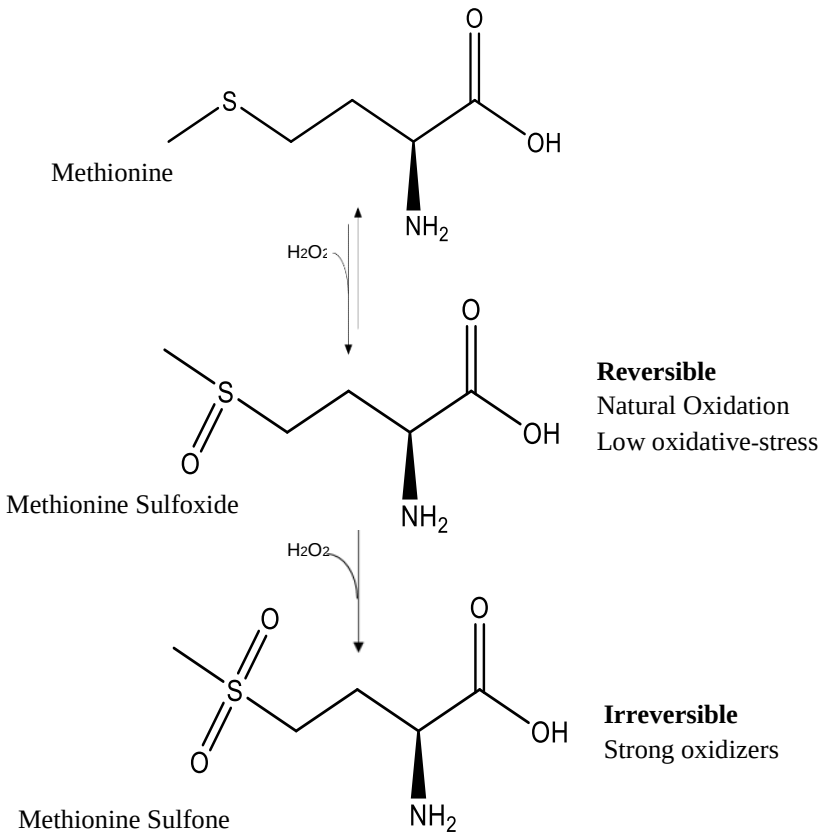


Figure 7. Molecular structure of the oxidation products of methionine

Hydrogen peroxide (H_2O_2) reacts with methionine residues of proteins under acidic conditions leaving most of its other constituents unaltered, indicating it as a relatively selective oxidizer[62]. The oxidation of methionine to methionine sulfone depends not only on the oxidative potential of the oxidizer utilized for the reaction. It has been reported that the physicochemical and biological properties of the protein also influence the oxidation of methionine to its sulfoxide and sulfone states[63].

The degree to which the methionine residues in proteins get oxidized can range from being a total oxidation of the protein, or it can become highly methionine selective. Highly specific methionine oxidants, which attack precisely methionine and cysteine, are N-chloro-succinimide (N-Cl-S) and chloramine T (CI-T)[64]. The oxidation reaction can be controlled to become even more specific when performed with dimethyl sulfoxide, it becomes highly selective for methionine leaving cysteine unaltered[61]. A wide variety of procedures available to regenerate methionine from methionine sulfoxide, but not from methionine sulfone, allows for verification of the stability of the molecule from specific steps in the oxidation process[65].

Oxidation of methionine to methionine sulfone has an extended array of nutritional applications. It has been reported to be unavailable for utilization in mammalian cells for growth, creating a deprivation of sulfur content in the diet [66 67, 68]. The presence of other sulfur amino acids as cysteine can serve to spare methionine. The methionine sparing effect of available cysteine in the diet can be avoided by oxidizing it to cysteic acid [69].

The following chapter will discuss methods to oxidize methionine to methionine sulfone, cysteine to cysteic acid and destruct tryptophan in food ingredients. The preliminary study will extensively discuss oxidation methodologies and results in order to obtain preliminary data needed for development of palatable methionine restricted food ingredients.

CHAPTER 2.
PRELIMINARY STUDY

2.1 Introduction

In the previous chapter the major health benefits of methionine restricted (MR) diets were highlighted. MR diet is a diet were the total content of methionine has been lowered considerably in order to achieve therapeutic purposes. A regular diet has a methionine content range of 0.7-0.8%. In a methionine restricted diet the amount of methionine is lowered to a range of 0.4-0.17%. The degree to which methionine is restricted in the supplemented diet depends on the therapeutic purpose of the diet. It has been reported repeatedly in cell culture studies and animal trials that for metastatic cancer control therapy the optimum level of methionine restriction is 0.4%[17, 18, 19, 20]. Studies on weight-loss and life-span extension indicated a higher level of methionine restriction (0.17%) needed to achieve the therapeutic purpose[3, 24, 25, 26].

Unfortunately, not enough research has been performed in the area of low-methionine food production for human consumption. In this preliminary study we will approach the mechanisms of methionine oxidation to produce palatable methionine restricted food ingredients. Early in history, the research on oxidation of amino acids was reported only as a merely activity-destructive process in physiological systems[53]. Later it started to be observed as a method for increasing functional activity of enzymes, by activating and deactivating their functional state with oxidation-reduction reactions[70]. Nowadays, the oxidation of methionine is widely researched as a therapeutic approach for diseases control.

Even though the health benefits of MR diets have been documented repeatedly in cell culture studies and animal trials. A vast and extensive research still needs to be performed in the production of palatable MR diets for human consumption, in order to reproduce the same effects of animal trials during clinical trials. All the available studies reporting oxidation of methionine

to its nutritionally unavailable state, methionine sulfone, have been performed to identify methionine in biological cycles or for protein stability studies[34, 35, 60, 71]. They reported the effect of the oxidative treatment on the molecular structure only, not on the food palatability (flavor, aroma, and texture).

The objective of this preliminary study is to extensively treat food samples to induce oxidation of methionine to methionine sulfone. We will report data on production of methionine sulfone (nutritionally unavailable) and the final palatability of each product after treatment. An MR diet proposal will be developed using the treated food ingredients to calculate the final total methionine content of the MR diet.

2.2 Materials and methods

Two strong oxidizers were selected for the study to be able to oxidize methionine to its methionine sulfone state, which were: ozone (O_3) and hydrogen peroxide (H_2O_2). When methionine sulfone is formed the molecule becomes stable, meaning it cannot be reduced back to methionine. This oxidation procedure will guarantee a food low in methionine by converting it to its biologically unavailable state, methionine sulfone. Via the described methodology we are looking forward to develop an array of food ingredients with low methionine content.

Ozone and hydrogen peroxide were selected for this experiment not only for their fast and high oxidative capacity, but also because they are both food grade processing agents. Ozone and hydrogen peroxide are classified as secondary direct food additive/ processing aids in the Code 21 of Federal Regulations of the United States Department of Agriculture (USDA).

2.2.1 Materials

An ozone reaction vessel was built using the following materials: two ozone generators ZO-103 portable purifier 120V/60hz with built-in air pump, and LG-7 Laboratory corona discharge were purchased from DEL Ozone (San Luis Obispo, CA). Oxygen and argon tanks were industrial grade 244 cu.ft. purchased from Air-liquide (Houston, TX). A Tygon chemical tubing 2001 was used to connect the ozone reaction vessel, purchased from Cole Parmer (Vernon Hills, IL). An ozone monitor and detector EZ-1X was installed for safety precaution, from Eco-Sensor (Santa Fe, NM).

Reagents as phenylisothiocyanate P1030-10ML, methanol, sodium acetate, thiodiethyleneglycol, hydrogen peroxide and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MS). Pierce vacuum acid hydrolysis glass tube #29572 from Thermo Scientific (Walthan, MA) as well as the Pierce amino acid standard H #20088 containing 2.5 umoles/ml in 0.1 N HCl for L-Alanine (Ala), L-Arginine (Arg), L-Asparatic Acid (Asx), L-Glutamic Acid (Glx), Glucine (Gly), L-Histidine (His), L-Isoleucine (Ile), L-Leucine (Leu), L-Lysine (Lys), L-Methionine (Met), L-Phenylalanine (Phe), L-Proline (Pro), L-Serine (Ser), L-Threonine (Thr), L-Tyrosine (Tyr), L-Valine (Val), and 1.25 umoles/ml of L-Cystine (Cys). All water utilized for the analysis was treated with Milli-Q filtering system (Millipore Corporation, MA).

2.2.2 Ozone (O_3) treatments

Two ozone generators were selected to perform the ozone treatments. Del Ozone ZO-103 which produces up to 3% ozone and Del Ozone Laboratory LG-7 connected to an oxygen tank to generate 12% ozone. Both generators were run at maximum capacity for all treatments. Tygon chemical 2001 tubing was installed to connect the ozone generator to the oxygen feed tank, and

to the stainless steel reaction vessel (Figure 8) were sample was pressurized. Tygon chemical 2001 tubing is ozone resistant and it has been approved by the FDA for food contact An oxygen tank was connected to the ozone generator which produced ozone to fill the stainless steel reaction vessel. All treatments were performed inside a gas hood and an ozone monitor was installed on the exterior of the hood as safety precaution to alert for leakage.

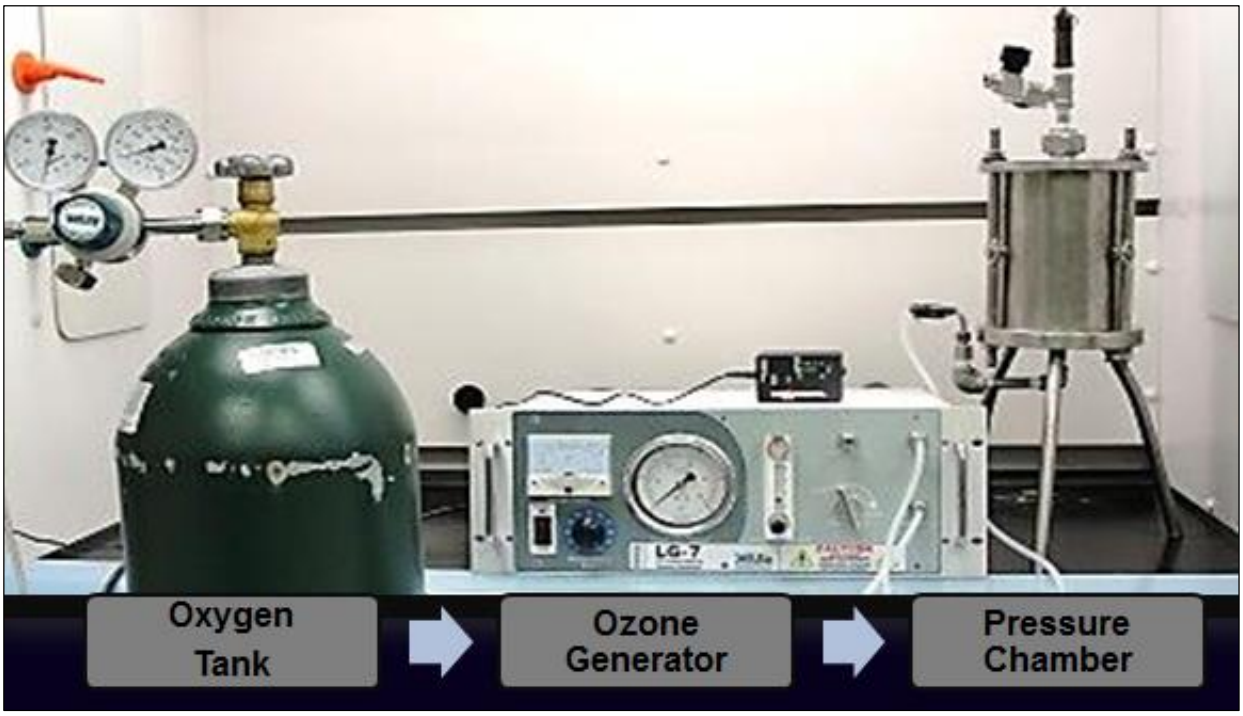


Figure 8. Ozone treatments reaction vessel

Samples were placed inside the stainless steel reaction vessel were ozone flowed in from the bottom and was exhausted on the top. The flow of ozone was calibrated by tightening the exhaust opening, at the top of the vessel, to keep a pressure of 50 psi. Pressure was monitored on the gauge of the ozone generator and it was kept constant throughout the system. Constant pressure was maintained during all ozone treatments. A compile of ozone treatments is shown in

(Table 1). The variables of the treatments were: time, dryness, and ozone [O_3] concentration. Ingredients with solid food matrix were treated for longer periods of time due to the low permeability the tightness of the matrix allows for penetration of ozone.

Table 1. Ozone (O_3) treatments

Food	dry/wet	Time	[O_3]
Egg whites	wet	0.5 hour	1%
Egg whites	wet	1.5 hour	1%
Egg whites	wet	2 hour	1%
Casein	dry	3 hour	1%
Casein	wet	3 hour	1%
Egg whites	wet	3 hour	3%
Beans	dry	2 days	3%
Beans	dry	4 days	3%
Beans	dry	7 days	3%
Beans	wet	4 days	3%
Pasta	dry	4 days	3%
Pasta	dry	7 days	3%
Spaghetti	dry	4 days	3%
Spaghetti	dry	7 days	3%
Macaroni	dry	7 days	3%
Noodles	wet	3 days	3%
Noodles	dry	3 days	3%
Noodles	dry	6 days	3%
Chili	dry	3 days	3%
Chili	dry	6 days	3%
Beef	wet	3 days	3%
Beef	dry	3 days	3%
Beef	dry	6 days	3%
Textured soy	dry	3 days	3%
Ice cream	dry	3 days	3%
Ice cream	dry	6 days	3%
Casein	dry	3 days	3%
Beans	dry	2 days	12%
Beans	dry	4 days	12%
Beans	wet	4 days	12%

(Table 1 Continued)

Food	dry/wet	Time	$[O_3]$
Rice	wet	2 days	12%
Egg whites	dry	12 hours	12%
Egg whites	dry	2 days	12%
Omelet	dry	2 days	12%
Chili	dry	6 days	12%
Beef	dry	1 day	12%
Beef	dry	2 days	12%
Beef	wet	2 days	12%
Ham	dry	2 days	12%
Ham	wet	2 days	12%
Casein	wet	2 days	12%
Skim milk	wet	3 days	12%
Noodle	wet	2 days	12%
Noodle	dry	2 days	12%
Mini wheat	dry	3 days	12%
Potatoes	wet	2 days	12%
Wheat flour	dry	2 days	12%
Couscous	dry	2 days	12%
Spaghetti	dry	2 days	12%
Macaroni	dry	2 days	12%
Pasta	dry	3 days	12%
Pasta	dry	6 days	12%
Macaroni	dry	2 days	12%
Rice noodle	dry	2 days	12%
Bean noodle	dry	2 days	12%

2.2.3 Hydrogen peroxide (H_2O_2) treatments

Egg whites were treated in a pyrex glass beaker, 30ml of egg whites were mixed with 30 ml of 20% hydrogen peroxide to reach a 10% hydrogen peroxide dilution (v/v). The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer inside a fume hood for 2 h. The sample was let cool down for 2 hours at room temperature (25°C). Hydrogen

peroxide removal consisted on an enzymatic catalysis: 24000 units of catalase were used for hydrogen peroxide removal. Catalase was added to the egg whites-hydrogen peroxide solution at 25°C with constant medium-low stirring (300 rpm). Every 30 minutes 4000 more units of catalase were added for a total of 4 hours of treatment with catalase.

Tilapia was treated in a pyrex glass beaker, 300 g of tilapia fillet were mixed with 400ml of 10% hydrogen peroxide. The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer under a fume hood for 1 h. The sample was let cool down for 2 hours at room temperature. Hydrogen peroxide was removed by washing the sample with 400ml of tap water. On each wash the tilapia was thoroughly mixed with water and let precipitate for 10 min, cheese cloth was used to prevent filtration during removal of the liquid phase. Washing procedure was repeated 4 times. The sample was freeze dried 24hrs for amino acid analysis.

Casein was treated after acid precipitation. Non-fat dry milk (NFDM) powder was hydrated in a gallon of water and the casein was precipitated using 1N hydrochloric acid to reach pH 4.5, then pH was adjusted to 2.5 with 1M hydrochloric acid. In a beaker, 30 ml of precipitated casein was mixed with 30ml of 20% hydrogen peroxide to reach a 10% hydrogen peroxide dilution (v/v). The mixture was heated to 90°C and stirred at medium speed on a hot plate magnetic stirrer under a fume hood for 2 h. The sample was let cool down for 2 hours at room temperature. Hydrogen peroxide was removed by washing the sample with 60ml of tap water. On each wash the casein was thoroughly mixed with water and let precipitate for 1 hour, cheese cloth was used to prevent casein filtration during removal of the liquid phase. Washing/precipitating procedure was repeated 4 times. The sample was freeze dried 24 hrs for amino acid analysis.

Chicken was treated in a pyrex glass beaker, 300 g of diced (1 in^2) chicken breast was mixed with 400ml of 10% hydrogen peroxide. The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer under a fume hood for 1 h. The sample was let cool down for 2 hours at room temperature. Hydrogen peroxide was removed by washing the sample with 400ml of tap water. On each wash the chicken was thoroughly mixed with water and let precipitate for 10 min, cheese cloth was used to prevent filtration during removal of the liquid phase. Washing procedure was repeated 4 times. The sample was freeze dried 24hrs for amino acid analysis.

Mushrooms were treated in a pyrex glass beaker, 300 g of canned mushrooms were mixed with 400ml of 10% hydrogen peroxide. The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer under a fume hood for 1 h. The sample was let cool down for 2 hours at room temperature. Hydrogen peroxide was removed by washing the sample with 400ml of tap water. On each wash the mushrooms were thoroughly mixed, a strainer was used to collect the mushrooms during removal of the liquid phase. Washing procedure was repeated 4 times. The sample was freeze dried 24hrs for amino acid analysis.

Ham was treated in a pyrex glass beaker, 300 g of grinded virginia ham were mixed with 400ml of 10% hydrogen peroxide. The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer under a fume hood for 1 h. The sample was let cool down for 2 hours at room temperature. Hydrogen peroxide was removed by washing the sample with 400ml of tap water. On each wash the ham was thoroughly mixed with water and let precipitate for 10 min, cheese cloth was used to prevent filtration during removal of the liquid phase. Washing procedure was repeated 4 times. The sample was freeze dried 24hrs for amino acid analysis.

2.2.4 Amino acid analysis

Samples were freeze dried for 24 hours and amino acid analysis was perform by acid digestion and quantification in UHPLC system. One gram of sample was weighted into a glass hydrolysis tube and then added 7ml 6N HCl containing 0.25% phenol. Each sample was frozen with liquid nitrogen and the hydrolysis tube was connected to vacuum for 1 minute. The tube was sealed before removing vacuum. Then the sample was let thaw. This step was repeated three times to assure vacuum is formed inside the tube before hydrolysis. The sealed tubes were placed in a heating block at 110 ℃ for 24 h. The hydrolysis tubes were let cool down to room temperature and then slowly opened to release vacuum. The hydrolysate was filtered with paper filter and washed with distilled water into a 50 ml volumetric flask and brought to 20 ml volume. Then 20 ul of hydrolysates were transferred to microcentrifuge tubes and dried with speed vac at 35ºC for 5 hr.

Tryptophan analysis was performed by alkaline digestion. One gram of sample was hydrolyzed with 2ml 5M NaOH with 3% thiodiethylene glycol in screw cap teflon tubes. Each sample was covered with argon to form vacuum, then capped tightly and heated to 160 °C. Then 2 ml of 25% acetic acid were added to neutralize the sample.

Samples were transferred to microcentrifuge tubes and 10ul of 2.5 mM norleucine (Nle) solution were added to the microcentrifuge tube and freeze dried for 24hr. Samples were derivatized in a microcentrifuge tube with 50 ul derivatization solution (EtOH:water: triethylamine:phenyl isothiocyanate = 7:1:1:1 by volume). Vortexed and reacted for 30 min at room temperature, then dried in the freeze drier for 24hrs. The amino acid standard and Nle internal standard and/or the sample was dissolved into 1000 ul diluent (5 mM Na_2HPO_4 buffer, pH 7.4 containing 5% acetonitrile) and filtered with 0.2 um syringe filter. Injection volume for

HPLC analysis was 20 ul. The reference standard was 10 ul AA standard + 10 ul (2.5 mM Nle). Dried with speed vacuum at 35ºC for 5 hr, and derivatized with PITC. Then the sample was dried in freeze drier for 24 hr, and dissolved into 1000 ul diluent for injection.

UHPLC analysis was performed with Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC (RSLC) system controlled by Chromeleon software. The separation was performed on a Waters Pico-Tag C18 column (4 um, 3.9 × 150 mm) with Nova-Pak guard column (4 um, 3.9 × 20 mm) maintained at 38°C by a gradient resulting from mixing eluents A and B. Eluent A consisted of 140 mM sodium acetate, 0.05% triethylamine, titrated to pH 6.40 with glacial acetic acid, then addition of 60 ml/L acetonitrile. Eluent B consisted of 60% acetonitrile in water.

The PITC labeled amino acids eluting from the column were detected at 254 nm and recorded. The column was regenerated and equilibrated with eluent A for 5 min. A new and freshly reconstituted sample was injected and analyzed every 30 min. Injection amount was 20uL. Eluent gradient was controlled with the following time flow:

Table 2. Amino acid analysis: UHPLC eluent gradient

Time	Flow	% B	% A	Curve
0.01	1.00	0.0	100.0	
5.00	1.00	13.0	87.0	5
6.20	1.00	13.0	87.0	6
9.00	1.00	28.0	72.0	6
13.00	1.00	28.0	72.0	6
16.00	1.00	80.0	20.0	6
16.20	1.00	100.0	0.0	6
20.00	1.00	0.0	100.0	11

2.3 Results and discussion

Ozone is known to be a strong oxidizer, as observed in the following results table ozone treatments achieved oxidation of methionine to methionine sulfone in freeze dried beans, ice

cream, omelet, beef, ham, noodle and potatoes. Wet ozone treatments were more effective for rice and casein.

Table 3. Methionine sulfone content after ozone (O_3) treatments

#	Food	dry/wet	Time	O_3	Methionine % in total AA** (*w/w**)	Methionine Sulfone % in total AA (*w/w*)	Oxidation
1	Egg whites	wet	0.5 hours	1 %	4.98	n/a	0 %
2	Egg whites	wet	1.5 hours	1 %	4.76	n/a	0 %
3	Egg whites	wet	2 hours	1 %	4.10	n/a	0 %
4	NFDM***	dry	1 hour	1 %	3.22	n/a	0 %
5	NFDM	wet	1 hour	1 %	2.59	n/a	0 %
6	Egg whites	wet	3 hours	3 %	3.56	n/a	0 %
7	Beans	dry	2 days	3 %	0.89	n/a	0 %
8	Beans	dry	4 days	3 %	1.41	n/a	0 %
9	Beans	dry	7 days	3 %	0.30	2.47	90 %
10	Beans	wet	4 days	3 %	0.79	n/a	0 %
11	Pasta	dry	4 days	3 %	1.92	n/a	0 %
12	Pasta	dry	7 days	3 %	1.36	n/a	0 %
13	Spaghetti	dry	4 days	3 %	0.38	n/a	0 %
14	Spaghetti	dry	7 days	3 %	0.54	n/a	0 %
15	Macaroni	dry	7 days	3 %	1.38	n/a	0 %
16	Noodles	wet	3 days	3 %	3.15	n/a	0 %
17	Noodles	dry	3 days	3 %	3.48	n/a	0 %
18	Noodles	dry	6 days	3 %	3.08	n/a	0 %
19	Chili	dry	3 days	3 %	2.41	n/a	0 %
20	Chili	dry	6 days	3 %	0.62	n/a	0 %
21	Beef	wet	3 days	3 %	3.79	n/a	0 %
22	Beef	dry	3 days	3 %	3.46	n/a	0 %
23	Beef	dry	6 days	3 %	2.88	n/a	0 %
24	Texturized soy	dry	3 days	3 %	1.90	n/a	0 %
25	Ice cream	dry	3 days	3 %	1.06	5.61	84 %
26	Ice cream	dry	6 days	3 %	1.03	7.72	88 %
27	Casein	dry	3 hours	3 %	3.17	n/a	0 %

**w/w*: weight by weight
**AA: amino acid
***NFDM: non-fat dry milk

(Table 3 continued)

#	Food	dry/wet	Time	O_3	Methionine % in total AA** (*w/w**)	Methionine Sulfone % in total AA (*w/w*)	Oxidation
28	Rice	dry	2 days	12 %	2.30	2.28	50 %
29	Egg whites	dry	12 hours	12 %	4.42	n/a	0 %
30	Egg whites	dry	2 days	12 %	3.42	n/a	0 %
31	Beans	dry	2 days	12 %	1.17	n/a	0 %
32	Beans	dry	4 days	12 %	1.27	n/a	0 %
33	Beans	wet	4 days	12 %	1.47	n/a	0 %
34	Omelet	dry	2 days	12 %	3.55	0.96	21 %
35	Chili	dry	6 days	12 %	1.85	n/a	0 %
36	Beef	dry	1 days	12 %	1.78	0.45	20%
37	Beef	dry	2 days	12 %	0.93	1.34	60 %
38	Beef	wet	2 days	12 %	1.79	n/a	0 %
39	Ham	dry	2 days	12 %	2.46	0.98	28 %
40	Ham	wet	2 days	12 %	2.78	0.91	25 %
41	NFDM	wet	2 days	12 %	3.62 ±	1.28	26 %
42	Skim milk	wet	3 days	12 %	4.46	n/a	0 %
43	Noodle	wet	2 days	12 %	0.94	n/a	0 %
44	Noodle	dry	2 days	12 %	1.16	1.59	58 %
45	Mini wheat	dry	3 days	12 %	1.89	n/a	0 %
46	Potatoes	dry	2 days	12 %	1.20	2.50	68 %
47	Wheat flour	dry	2 days	12 %	1.22	n/a	0 %
48	Couscous	dry	2 days	12 %	1.41	n/a	0 %
49	Spaghetti	dry	2 days	12 %	0.99	n/a	0 %
50	Macaroni	dry	2 days	12 %	0.92	n/a	0 %
51	Pasta	dry	3 days	12 %	1.38	n/a	0 %
52	Pasta	dry	6 days	12 %	2.68	n/a	0 %
53	Macaroni	dry	2 days	12 %	0.92	n/a	0 %
54	Corn meal	dry	1.5 hours	12 %	2.34	0.69	22 %
55	Rice noodle	dry	2 days	12 %	n/a	n/a	0 %
56	Bean noodle	dry	2 days	12 %	n/a	n/a	0 %

**w/w*: weight by weight
**AA: amino acid
***NFDM: non-fat dry milk

Hydrogen Peroxide oxidation offered a fast and effective treatment for developing methionine restricted food ingredients. The downtime of this procedure is due to the peroxide removal step needed in order to make these food ingredients palatable after treatment. The

objectionable (peroxide flavor) was removed by either the enzymatic or the washing method, or the combination of both procedures, depending on the food product requirement. Table 4 shows the percentage of methionine oxidation achieved for each food ingredient after hydrogen peroxide treatment.

Table 4. Methionine sulfone content after hydrogen peroxide (H_2O_2) treatment

#	Food	Time	H_2O_2	Methionine % in total AA** (*w/w**)	Methionine Sulfone % in total AA (*w/w*)	Oxidation
57	Egg whites	1 hour	10 %	0.367	4.251	92 %
58	Tilapia	1 hour	10 %	3.033	n/a	0 %
59	Chicken	1 hour	10 %	2.474	1.049	29 %
60	Mushroom	1 hour	10 %	0.096	0.659	87 %
61	Casein	1 hour	10 %	2.914	n/a	0 %
62	Casein	1 hour	10 %	2.908	n/a	0 %
63	Casein	1 hour	10 %	2.801	n/a	0 %
64	NFDM***	0.5 hour	05 %	2.340	0.696	23 %
65	NFDM	1 hour	05 %	1.821	1.052	37 %
66	NFDM	2 hour	05 %	0.352	2.743	89 %
67	NFDM	0.5 hour	10 %	2.512	0.875	25 %
68	NFDM	1 hour	10 %	0.348	2.195	86 %
69	NFDM	1.5 hours	10 %	1.435	2.152	60 %
70	NFDM	2 hours	10 %	0.266	2.705	91 %
71	NFDM	2 hours	20 %	0.283	4.125	93 %

**w/w*: weight by weight
**AA: amino acid
***NFDM: non-fat dry milk

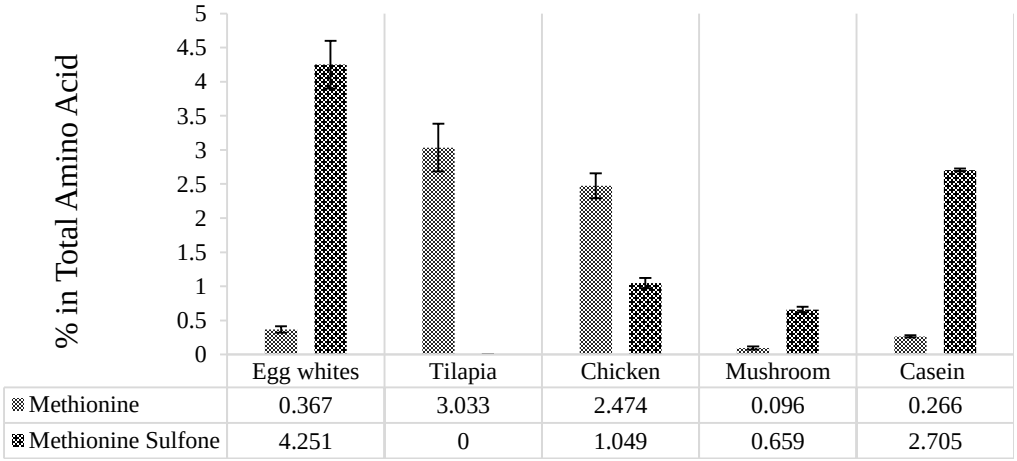


Figure 9**.** Methionine to methionine sulfone ratio after hydrogen peroxide treatment

The bar graph displays the amount (% in total AA (*w/w*)) of methionine left in samples after treatment, comparing it with amount of methionine sulfone produced by the hydrogen peroxide oxidation reaction. Hydrogen peroxide had a time of treatment that represents a huge advantage on the overall process when compared to ozone treatments. However, for treatment selection it is important to keep in mind that not all foods react to oxidation with hydrogen peroxide, as shown in the following table. A summary of all treatments where methionine was successfully oxidized to methionine sulfone is shown in Table 5.

Table 5. Hydrogen peroxide (H_2O_2) and ozone (O_3) treatments yielding methionine sulfone

Food	Treatment	Methionine % in total AA** (*w/w**)	Methionine Sulfone % in total AA (*w/w*)	Oxidation	Palatable
Casein NFDM***	10% peroxide 2h, pH 2.5	0.26	2.70	91 %	Yes
Casein NFDM	12% ozone 2 days, Wet	3.62	1.28	26 %	Yes
Egg whites Liquid	10% peroxide 1hour	0.36	4.25	92 %	No
Omelet Freeze dried	12% ozone 2 days, Dry	3.55	0.96	21 %	Yes
Beans Freeze dried	3% ozone 7 days, Dry	0.30	2.47	90 %	Yes
Ice Cream Freeze dried	3% ozone 3 days, Dry	1.06	5.61	84 %	Yes
Beef stew Freeze dried	12% ozone 3 days, Dry	0.93	1.34	60 %	Yes
Beef stew Freeze dried	12% ozone 3 days, Wet	1.78	0.45	20 %	Yes
Rice Freeze dried	12% ozone 2 days, Dry	2.30	2.28	50 %	Yes
Ham Freeze dried	12% ozone 2 days, Dry	2.46	0.98	28 %	Yes
Ham Freeze dried	12% ozone 2 days, Wet	2.78	0.91	25 %	Yes

(Table 5 continued)

Food	Treatment	Methionine % in total AA** (*w/w**)	Methionine Sulfone % in total AA (*w/w*)	Oxidation	Palatable
Noodle Freeze dried	12% ozone 2 days, Dry	1.16	1.59	58 %	No
Potatoes Freeze dried	12% ozone 2 days, Dry	1.20	2.50	68 %	Yes
Chicken Fresh	10% peroxide 1 hour	2.47	1.04	29 %	Yes
Mushroom Canned	10% peroxide 1 hour	0.09	0.65	87 %	Yes
Corn meal	3% ozone 1.5 hours	2.34	0.69	23 %	Yes

**w/w*: weight by weight
**AA: amino acid

Even when hydrogen peroxide treatments have been proven to be of high efficiency, research with other food grade oxidizers is encouraged due to the lack of targeted oxidation in other food products. For example, no methionine sulfone conversion was achieved in Tilapia, a fish with high methionine content, after 1 hour of hydrogen peroxide treatment.

It can be observed in Table 5 that the only two food ingredient products that lost their palatability during treatment were egg whites when oxidized with hydrogen peroxide, and noodles when oxidized with ozone. Egg whites were not able to be separated from hydrogen peroxide by the precipitation/washing method nor the enzymatic method. The precipitation/washing method did not work with egg whites because there is no natural precipitation occurring when egg whites are mixed with hydrogen peroxide. Further research needs to be performed on the removal of hydrogen peroxide from treated egg whites by precipitation. Effects of variables as temperature, isoelectric point, salt concentration and pH need to be analyzed.

The enzymatic hydrogen peroxide removal did not achieve the required catalysis of hydrogen peroxide for a clean flavor. During the enzymatic treatment egg whites were denaturized to form a foam, folding their volume 10x, since catalase treatment needs high shearing to react. The shearing caused egg whites to increase their volume extremely causing them to overflow out of the container. The technical difficulties associated with the shearing of egg whites makes the precipitation method analysis more attractive for further research.

Other than oxidizing methionine, the production of a palatable final product is of extreme importance in this study; this not only includes removing the residual peroxide after treatment but also maintaining the texture matrix of the treated food ingredient appealing for human consumption. This resulted in one of the biggest challenges during the oxidation reaction. Hydrogen peroxide treatments were more damaging than ozone treatments to the texture of the food samples.

Mushrooms conserved their turgid texture after treatment and allowed an easy hydrogen peroxide removal procedure with the washing/precipitating method. The diced chicken protein, on the other hand, was dissolved by the hydrogen peroxide treatment, but it can be combined with extensors and used to form restructured meat products as hams, sausage, nuggets, etc. Maintaining the texture in the treated food ingredients is of extreme importance in order to achieve palatability of the final product.

It is also preferred to maintain consistency with their original appearance, for example: casein micelles physicochemical properties were not affected with H_2O_2 treatment. Treated casein micelles can be reconstituted in water after treatment and with addition of cream and lactose they can be standardized to milk's original composition; or desired composition for production of cheese and other dairy products.

Cysteine/cystine content was monitored. The process of transulfuration during the *de novo* cycle can convert cysteine to methionine[29], eliminating by this way all benefits of a MR diet. In order to ensure the low content of methionine in plasma during clinical trials the amount of cysteine (Table 6) of the treated food ingredients was quantitated.

Table 6. Cysteine content before and after treatment

	g/100g Protein		g/100g Food		
Treatment	Cysteine Untreated	Cysteine Treated	Cysteine Untreated	Cysteine Treated	Palatable
Casein 10% H_2O_2, 2h, pH 2.5	0.27	n/a	0.25	n/a	Yes
Egg whites 10% H_2O_2, 1h	3.14	1.59	2.01	1.02	No
Omelet 12% ozone, 2 days, Dry	0.32	n/a	0.23	n/a	Yes
Beans 3% ozone, 7 days, Dry	2.18	n/a	0.24	n/a	Yes
Ice Cream 3% ozone, 3 days, Dry	1.28	n/a	0.09	n/a	Yes
Beef stew 12% ozone, 3 days, Dry	1.06	n/a	0.47	n/a	Yes
Beef stew 12% ozone, 3 days, Wet	1.06	n/a	0.47	n/a	Yes
Rice 12% ozone, 2 days	3.20	n/a	0.32	n/a	Yes
Ham 12% ozone, 2 days, Dry	0.51	n/a	0.48	n/a	Yes
Noodle 12% ozone, 2 days, Dry	1.60	n/a	0.32	n/a	No
Potato 12% ozone, 2 days, Dry	1.09	n/a	0.12	n/a	Yes
Chicken 10% H_2O_2, 1h	1.61	n/a	1.52	n/a	Yes
Mushroom 10% H_2O_2, 1h	0.18	n/a	0.07	n/a	Yes
Corn meal 3% ozone, 1.5h	1.24	6.47	0.21	1.10	Yes

Table 7. Tryptophan content before and after treatment

Food	Treatment	g/100g Food Trypt. Untreated	Trypt. Treated	Palatable
Casein	10% H_2O_2 2h, pH 2.5	0.652	0.075	Yes
Egg whites	10% H_2O_2 1h, Wet	0.104	n/a	No
Omelet	12% ozone 2 days, Dry	0.210	0.026	Yes
Beans	3% ozone 7 days, Dry	0.067	n/a	Yes
Ice Cream	3% ozone 3 days, Dry	0.032	n/a	Yes
Beef stew	12% ozone 3 days, Dry	0.421	0.356	Yes
Beef stew	12% ozone 3 days, Wet	0.421	0.075	Yes
Rice	12% ozone 2 days, Wet	0.129	n/a	Yes
Ham	12% ozone 2 days, Dry	0.152	0.020	Yes
Noodle	12% ozone 2 days, Dry	0.117	n/a	No
Potato	12% ozone 2 days, Dry	0.038	n/a	Yes
Chicken	10% H_2O_2 1h, Dry	0.135	n/a	Yes
Mushroom	10% H_2O_2 1h, Wet	0.109	n/a	Yes
Corn meal	3% ozone 1.5h, Dry	0.048	n/a	Yes

*Trypt: tryptophan

The effectiveness of ozone and hydrogen peroxide to oxidize sulfur amino acids has been quantitated in Table 5, 6 and 7 for methionine, cysteine and tryptophan respectively. The food ingredients with more than 50% oxidized methionine will be used to develop a proposal of entrees that can be manufactured with a final methionine content of ≤0.17%.

The proposal of low-methionine entrees is developed under the nutritional guidelines of the United States Department of Agriculture (USDA) for the average American population. The Center for Disease Control and Prevention (CDC) describes the average weight and height of an American man over 20 years old to be 5 foot 9 and 195.5 pounds. The average American man consumes a daily diet of 3000 calories approximately. The daily recommended intake is 282g of grains, 198g of protein, 3 cups of dairy, 4 cups of vegetables, and 2.5 cups of fruits.

The total of 6.5 recommended cups of fruits and vegetables daily intake will be delivering an approximate of 600 Cal to the diet, making fruits and vegetables accountable for 20% of the total caloric intake on the diet. For the following table we have selected the fruits and vegetables with lowest methionine content to be used in this proposal.

Table 8. List of fruits and vegetables with low-methionine content

Fruits	g/100g	Vegetables	g/100g
Tangerine	0.002	Iceberg	0.006
Strawberry	0.003	Carrot	0.002
Orange	0.002	Celery	0.002
Peach	0.001	Cucumber	0.006
Pear euro	0.002	Bell pepper	0.001
Plum	0.003	Tomato	0.006
Apple	0.001		
Raisin	0.001		
Average	0.002	Average	0.002

A daily intake of three cups of dairy products has an average of 900 Cal, accounting for another 30% of the total daily caloric intake. The methionine content of dairy products in this diet can very easily be reduced by utilizing our oxidized casein to produce cheese,yogurt and reconstitute it back to milk. The treated casein micelles preserved their ability be precipitated at pH 4.5, which permits cheese production; and are soluble in water at pH higher than 4.5, which allows for yogurt and sour cream production as well as regular milk. Any dairy product

developed with the oxidized casein will have a final methionine reduction of 91%. For example, the daily recommended dairy intake of three cups of milk have a methionine content of 0.61g, by using oxidized casein to reconstitute milk the methionine content gets reduced to 0.05g in three cups of milk (732g). The percentile methionine content drops from 0.08% to 0.006%.

The methionine content of protein foods can be lowered a 27% by producing a sausage using the treated chicken and ham. Reconstituted meat products are very common nowdays and have gained a high acceptability for daily consumption. Examples of reconstituted or reshaped meat products are hamburger patties, hot dogs, chicken nuggets, surimi (imitation crab sticks), ham with water added, and sausages.

The production of an Andouille sausage using our low-methionine ham and chicken proteins will make possible the incorporation of meats to methionine restricted diets. Examples of proposed low-methionine entrees to be developed with our treated food ingredients are: jambalaya, red beans and rice, boudin balls, cream of mushrooms, mashed potatoes with beef gravy, hash browns, omelet, etc. Noodles made of rice or beans starches can also be combined with any of the following dishes due to their zero methionine content.

We have already mentioned how to reduce methionine on the dairy, fruit and vegetables food groups. It can be observed in Table 9 and 10 the amount of methionine reduction achieved in the remaining carbohydrate and protein food groups. This gives an example of the wide array of entrees that can be prepared by using the low methionine food ingredients we produced *via* ozone or hydrogen peroxide methionine oxidation treatments. The following proposal of methionine restricted entrees was developed using only the food ingredients that maintained their palatability after treatment.

Table 9. Methionine content of proposed protein entrees before and after treatment

	grams	Cream of mushrooms		Red beans & rice		Boudin balls	
		UT*	T**	UT	T	UT	T
Rice	195	0	0	0.31	0.15	0.31	0.15
Sausage	200	0	0	1.66	1.21	0	0
Milk	200	0.2	0.01	0	0	0	0
Chicken	100	0	0	0	0	0.83	0.05
Corn meal	10	0	0	0	0	0.23	0.17
Potatoes	100	0	0	0	0	0	0
Omelet	100	0	0	0	0	0	0
Beef stew	100	0	0	0	0	0	0
Beans	100	0	0	0.07	0.00	0	0
Mushroom	153	0.03	0.003	0	0	0	0
Total		0.23	0.01	2.04	1.36	1.37	0.37

*T= treated (g)
**UT= untreated (g)

Table 10. Methionine content of proposed carbohydrate entrees before and after treatment

	grams	Jambalaya		Mashed potatoes & gravy		Omelet		Hash browns	
		UT*	T**	UT	T	UT	T	UT	T
Rice	195	0.31	0.15	0	0	0	0	0	0
Sausage	200	1.66	1.21	0	0	0	0	0	0
Milk	200	0	0	0	0	0	0	0	0
Chicken	100	0.83	0.58	0	0	0	0	0	0
Corn meal	10	0	0	0	0	0	0	0	0
Potatoes	100	0	0	0.03	0.01	0	0	0.03	0.01
Omelet	100	0	0	0	0	0.31	0.25	0	0
Beef stew	100	0	0	0.09	0.03	0	0	0	0
Beans	100	0	0	0	0	0	0	0	0
Mushroom	153	0	0	0	0	0	0	0	0
Total		2.8	1.41	0.12	0.04	0.31	0.25	0.03	0.01

*T= treated (g)
**UT= untreated (g)

Table 11. Final methionine content in diet developed with low-methionine ingredients

Food Group	DVs*	Energy	Methionine (g)
Grains (rice)	282 g	600 Cal	0.423
Protein (chicken)	198 g	400 Cal	1.14
Dairy	738 g	900 Cal	0.044
Vegetables	800 g	150 Cal	0.16
Fruits	500 g	450 Cal	0.01
Added fats	42 g	360 Cal	0
Added sugars	35 g	140 Cal	0
Total	2595 g	3000 Cal	1.777
		g/100g	0.068

*DVs: daily values

As shown on Table 11, the methionine content of a diet can be reduced to the desired ≤0.17% level for weight loss therapy. By utilizing the treated casein, rice, chicken and selecting low methionine fruits and vegetables a 3000 Cal diet meeting the USDA recommended daily values can be produced, containing a final methionine content of 0.068%.

2.4 Conclusion

A high reduction of methionine content in food ingredients was achieved by oxidizing the food matrix with hydrogen peroxide. The desired palatability was reached by removal of hydrogen peroxide with water or by enzymatic treatment with catalase or the combination of both. Hydrogen peroxide works as a food processing aid in the production of methionine restricted food ingredients (casein, mushrooms, chicken, egg whites). Hydrogen peroxide treatments yield a high percentage of methionine sulfone which is unavailable to the human body metabolism, meaning it has no dietary value.

The high oxidative rate of hydrogen peroxide reactions makes it a great oxidizer for weight loss studies, which need an extreme reduction of available methionine in diet to be able to induce weight loss. Dietary methionine reduction therapy requirements vary depending on the purpose, for metastatic cancer treatment methionine reduction targets a 30-40% range and goes up to 80% reduction for weight loss induction therapy[3]. These range of oxidation can be

achieved with both, hydrogen peroxide or ozone. Specifically hydrogen peroxide produced an extreme reduction of methionine content of the food ingredients, which is projected to translate to a high reduction of methionine content in plasma during clinical trials.

The low methionine food ingredients we developed represent an innovative opportunity to develop palatable methionine restricted entrees for animal and clinical studies. They will allow to approach the therapeutic effects that dietary methionine reduction has on weight-loss, metastatic cancer and other metabolic disorders as homocystinuria and methaioninemia.

Further research needs to be performed on how ozone and hydrogen peroxide oxidize high methionine foods, and on the effects it causes on the food flavor and matrix in order to improve production of palatable MR diets for human consumption. Published research, available in methionine oxidation, focuses only on the quantification of methionine sulfoxide and sulfone in biological processes or analytical methods, but the effect that this same oxidation reaction has on food ingredients has not yet been explored sufficiently. Further research needs to be performed in the area of low-methionine food production including:

- Exploring alternative oxidizers, other than ozone and hydrogen peroxide, which comply with the Food and Drug Administration (FDA) regulation as food ingredients and will accelerate methionine oxidation in amino acid residues of proteins.
- Optimize the production process of methionine restricted food ingredients by monitoring the response of food ingredients to time of reaction and concentration of oxidizer.
- Conduct sensory evaluations on the proposed methionine restricted food entrees described in this chapter.
- Perform an economic analysis of the production of methionine restricted food ingredients.

CHAPTER 3.
DEVELOPMENT OF LOW-METHIONINE MILK

3.1 Introduction

As proof of concept on our ability to successfully produce a palatable methionine restricted food *via* the proposed oxidizing methodology, the production, sensory evaluation and clinical trial of our low-methionine food prototype was required. In this chapter we show the overall development of a low methionine food prototype. It will discuss the oxidation of casein, its sensory evaluation and clinical trial.

Oxidized casein was selected from the 71 treatments in the preliminary study for its high methionine oxidative rate. As described in Chapter 2, hydrogen peroxide treatment achieved 91% conversion rate of methionine to methionine sulfone after 2 hours of treatment. In this chapter we will show the results of an acceptance sensory evaluation test and a brief summary of the clinical trial performed at Pennington Biomedical Research Center (PBRC) to evaluate the ability of methionine restricted casein to reproduce the same health effect benefits of methionine restricted supplement Hominex-2®.

Hominex-2 ® is one of the few methionine-free formulas available in the market for the nutrition support of children and adults with proven vitamin B6-nonresponsive homocystinuria or hypermethioninemia. Due to these amino acid disorders patients are required to keep a strictly limited diet avoiding high methionine foods as: meat, fish, cow's milk, eggs, cheese, flour, beans, and nuts. Other foods as fruits and vegetables can be consumed in measured amounts. Hominex-2® supplements all amino acids, except methionine, to these strictly controlled diets. Patients have complained in previous clinical trials of its low palatability.

3.2 Casein oxidation

We have already mentioned the oxidation of casein in the previews chapter, but here we will give an extended recompilation of all the methodology, ozone and hydrogen peroxide, used for oxidation of casein only. Casein was selected from the preliminary study because the oxidized casein micelles maintained their physico-chemical properties after treatment, and were able to be reconstituted to pH 6.7, milk's pH, to produce a low methionine milk product. By adding lactose and cream to reconstitute milk's original composition a low-methionine milk product can be developed using the oxidized casein.

3.2.1 Materials and methods

An ozone reaction vessel was built using the following materials: ozone generators ZO-103 portable purifier 120V/60hz with built-in air pump and LG-7 Laboratory corona discharge were purchased from DEL Ozone (San Luis Obispo, CA). Oxygen and argon tanks were industrial grade 244 cuft purchased from Air-liquide (Houston, TX). A Tygon chemical tubing 2001 was used to connect the ozone reaction vessel, purchased from Cole Parmer (Vernon Hills, IL). An ozone monitor and detector EZ-1X was installed for safety precaution, from Eco-Sensor (Santa Fe, NM). Casein powder was purchased from the National Casein Company (Chicago, IL) and skim milk from Saco Foods (Middleton, WI). Lactose from LD Carlson (Kent, OH) and cream from Brown's Dairy (New Orleans, LA) was used to reconstitute milk to its original composition using the oxidized casein.

Reagents as phenylisothiocyanate P1030-10ML, methanol, sodium acetate, thiodiethyleneglycol, (30%) hydrogen peroxide and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MS). Pierce vacuum acid hydrolysis glass tube #29572 from Thermo Scientific (Walthan, MA) as well as the Pierce amino acid standard H #20088 containing 2.5

umoles/ml in 0.1 N HCl for L-Alanine (Ala), L-Arginine (Arg), L-Asparatic Acid (Asx), L-Glutamic Acid (Glx), Glucine (Gly), L-Histidine (His), L-Isoleucine (Ile), L-Leucine (Leu), L-Lysine (Lys), L-Methionine (Met), L-Phenylalanine (Phe), L-Proline (Pro), L-Serine (Ser), L-Threonine (Thr), L-Tyrosine (Tyr), L-Valine (Val), and 1.25 umoles/ml of L-Cystine (Cys). All water utilized for the analysis was Milli-Q grade (Millipore Corporation, MA).

Ozone treatments were performed using two ozone generators: Del Ozone ZO-103 which produces up to 3% ozone and Del Ozone Laboratory LG-7 connected to an oxygen tank to generate 12% ozone. Both generators were run at maximum capacity for all treatments. Tygon chemical 2001 tubing was used to connect the ozone generator to the pressurized stainless steel reaction vessel. An oxygen tank was connected to the ozone generator which produced the ozone that filled the stainless steel reaction vessel. All treatments were performed inside a gas hood with the ozone monitor on the exterior to alert for any leakage.

Table 12. Ozone (O_3) casein treatments

Food	dry/wet	pH	Time	O3
NFDM*	dry	6.6	1h	1 %
NFDM	wet	6.6	1h	1 %
NFDM	dry	6.6	3h	3 %
Skim M.	wet	6.6	48h	12 %
NFDM	wet	2.5	48h	12 %

*****NFDM: non-fat dry milk

Hydrogen peroxide treatments started with hydration of non-fat dry milk (NFDM) powder in a gallon of water and the casein was precipitated using 1N hydrochloric acid, then pH was adjusted as shown in Table 12. In a beaker, 30 ml of precipitated casein was mixed with 30ml of hydrogen peroxide to reach the specified hydrogen peroxide dilution. The mixture was heated to 90°C and stirred at medium speed (720 rpm) on a hot plate magnetic stirrer under a fume hood. The sample was let cool down for 2 hours at room temperature.

Hydrogen peroxide was removed by washing the sample with 60ml tap water. On each wash the casein was thoroughly mixed with water and let precipitate for 1 hour, cheese cloth was used to prevent casein filtration when removing the liquid phase. Washing procedure was repeated 4 times. Sample was freeze dried 24 hr for amino acid analysis. The following, Table 8, shows the different treatments performed using hydrogen peroxide as oxidizing agent.

Table 13. Hydrogen peroxide (H_2O_2) casein treatments

Food	pH	Time	[H_2O_2]
Casein	11.0	1.0 h	10 %
Casein	9.0	1.0 h	10 %
Casein	2.5	1.0h	10 %
NFDM*	2.5	0.5 h	05 %
NFDM	2.5	1.0 h	05 %
NFDM	2.5	2.0 h	05 %
NFDM	2.5	0.5 h	10 %
NFDM	2.5	1.0 h	10 %
NFDM	2.5	1.5 h	10 %
NFDM	2.5	2.0 h	10 %
NFDM	2.5	2.0 h	20 %

*NFDM: non-fat dry milk

3.2.2 Results and discussion

Casein micelles oxidized in the presence of hydrogen peroxide only under an acidic pH environment. Under alkaline conditions the oxidation reaction did not occur, no oxidation was achieved at pH 9 and 11. Hydrogen peroxide was completely removed by the washing/precipitation method which eliminated completely the peroxide flavor. As mentioned in the previous chapter, it was at pH 2.5 were all the treatments produced methionine sulfone. The denaturation of casein under acidic environment allowed the oxidation reaction of methionine to take place.

Table 14. Methionine sulfone production with hydrogen peroxide (H_2O_2)

Food	pH	Time (*h*)	% H_2O_2	Methionine % in total AA*** (*w/w***)	Methionine Sulfone % in total AA (*w/w*)	% Oxidation
Casein	11	1.0	10	2.914 ± 0.20	n/a	0
Casein	9	1.0	10	2.908 ± 0.28	n/a	0
Casein	2.5	1.0	10	2.801 ± 0.01	n/a	0
NFDM*	2.5	0.5	05	2.340 ± 0.05	0.696 ± 0.09	23
NFDM	2.5	1.0	05	1.821 ± 0.02	1.052 ± 0.43	37
NFDM	2.5	2.0	05	0.352 ± 0.10	2.743 ± 0.11	89
NFDM	2.5	0.5	10	2.512 ± 0.03	0.875 ± 0.13	25
NFDM	2.5	1.0	10	0.348 ± 0.14	2.195 ± 0.81	86
NFDM	2.5	1.5	10	1.435 ± 0.03	2.152 ± 0.04	60
NFDM	2.5	2.0	10	0.266 ± 0.06	2.705 ± 0.02	91
NFDM	2.5	2.0	20	0.283± 0.01	4.125 ± 0.36	93

*NFDM: non-fat dry milk
***w/w*: weight by weight
***AA: amino acid

Methionine to methionine sulfone conversion rate was directly proportional to the total time of treatment. Especially the 2h treatments were always yielding the highest methionine sulfone concentration. This observation is of high importance on the economic aspect of methionine restricted food ingredients production. The above table demonstrates how the concentration of hydrogen peroxide has a direct effect on the reaction rate, but it is NOT a determining factor. The treatment at *5% H_2O_2 for 2h* yields 89% methionine sulfone and treatment at *10% H_2O_2 for 2h* yields 91% methionine sulfone. The total effect of adding 100% more (duplicating) hydrogen peroxide concentration gave an overall output of only 2% difference on the conversion rate of the reaction. The exact same effect on conversion rate was observed when hydrogen peroxide concentration was increased from 10 to 20 %.

Ozone treatments displayed the same behavior that hydrogen peroxide treatments had to variation of pH. Even though ozone is known to be a relatively stronger oxidizing agent, compared to hydrogen peroxide, a much lower conversion rate of methionine to methionine

sulfone was achieved with the ozone treatments. As shown in Table 15, only 26% methionine sulfone was produced after two days of sample treatment with a constant ozone stream flow of 12% ozone.

Table 15. Methionine sulfone production with ozone (O_3)

Food	dry/wet	pH	Time	O_3	Methionine % in total AA*** (*w/w***)	Methionine Sulfone % in total AA (*w/w*)	Oxidation
NFDM*	dry	6.6	1 hour	1 %	3.22 ± 0.31	n/a	0 %
NFDM	wet	6.6	1 hour	1 %	2.59 ± 0.22	n/a	0 %
NFDM	dry	6.6	3 hour	3 %	3.17 ± 0.62	n/a	0 %
Skim M.	wet	6.6	2 days	12 %	4.46 ± 0.47	n/a	0 %
NFDM	wet	2.5	2 days	12 %	3.62 ± 0.10	1.28 ± 0.33	26 %

*NFDM: non-fat dry milk
**_w/w_: weight by weight
***AA: amino acid

The pH used for both, ozone and hydrogen peroxide, treatments ranged from very acidic to highly basic (pH 2.5 - 9). Casein was first adjusted to each different pH, these variations were performed in order to monitor to what degree basic and acidic environments can affect the oxidation reaction of methionine to methionine sulfone. The following scanning electron microscope (SEM) images helped identify what changes the pH variations cause in casein micelles which generate a different outcome on the oxidative rate for each treatment.

The pH dependent dissociation of casein micelles induced oxidation of methionine at different pH than those reported in literature. The presence of catalyzers as iodide, selenite[60], cupper or zinc[72] in other studies, reported successful oxidation of methionine to methionine sulfone under alkaline conditions. The results of this study showed a pH dependent reaction. Figure 10 and 11 the show behavior of casein micelles under alkaline and acid environments, respectively.

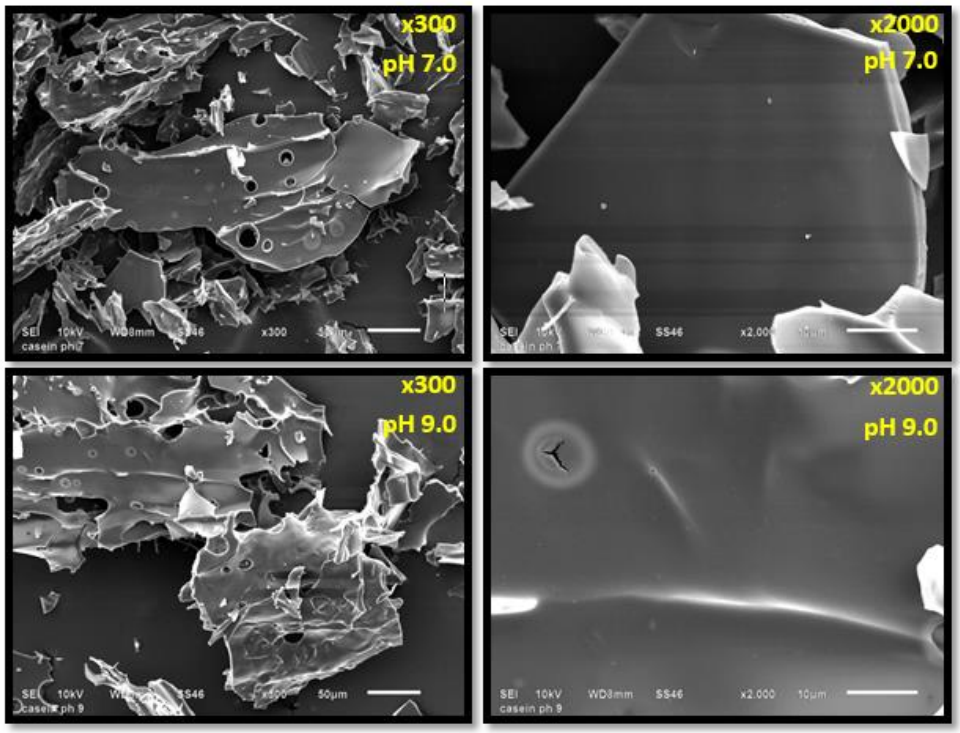


Figure 10. SEM imaging of casein micelles at basic pH

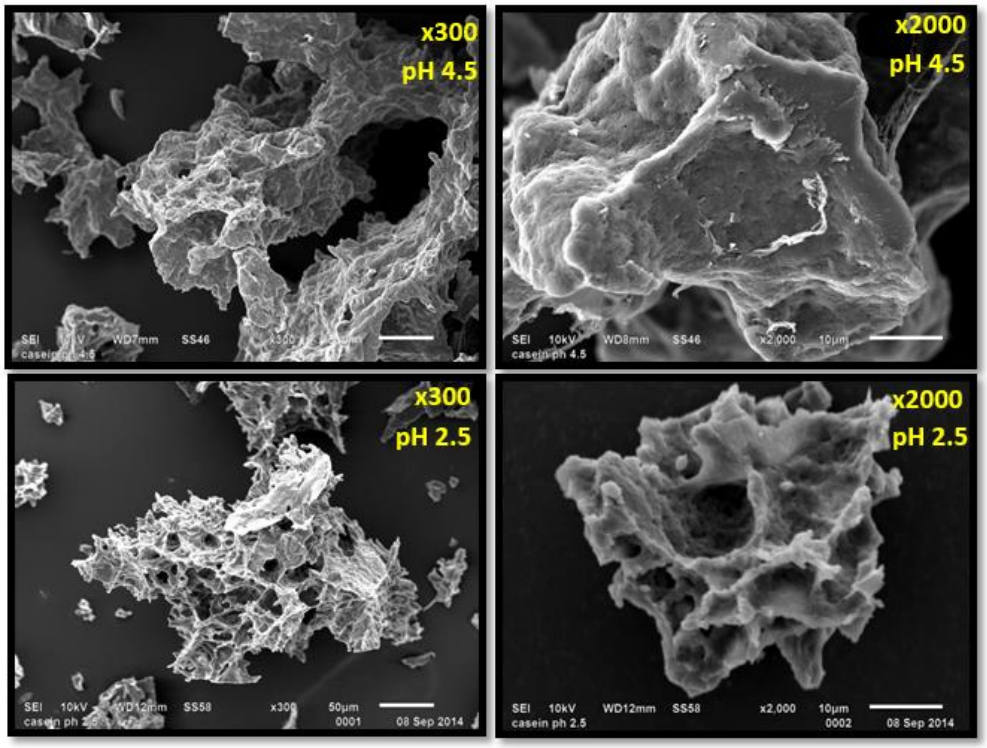


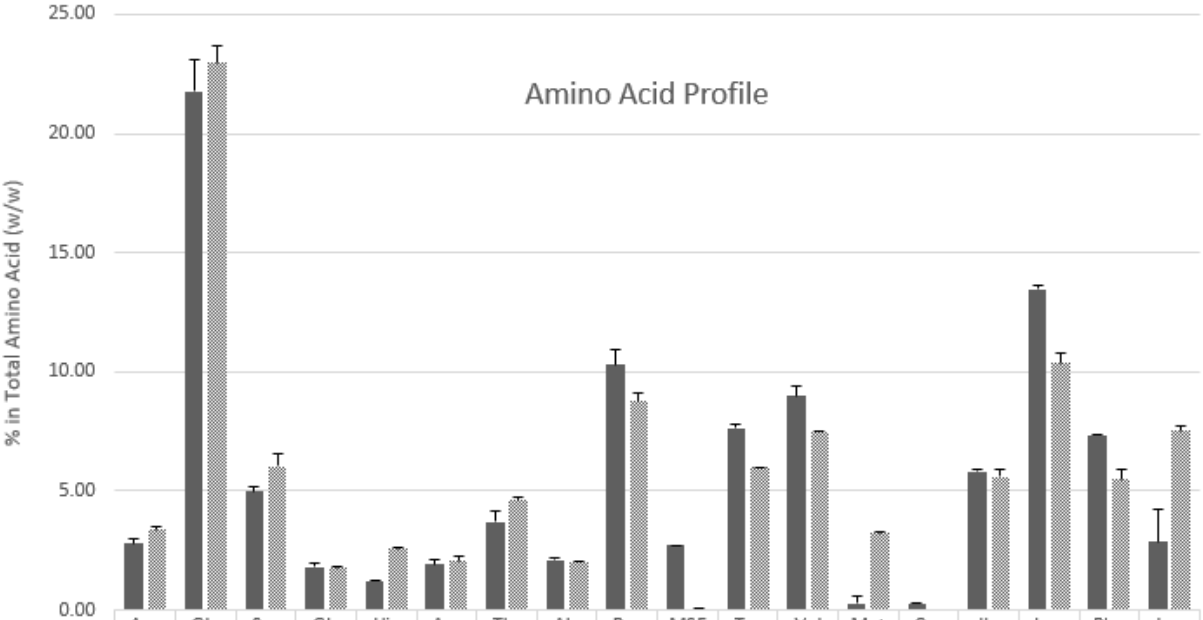
Figure 11. SEM imaging of casein micelles at acidic pH

At pH 7, close to the natural pH at which casein is found in fresh milk, laminar structure was observed. When increasing the pH to 9, casein starts to form a clear solution in water and its laminar structure was observed to be thinner under SEM imaging. Casein reaches its isoelectric point at pH 4.5, and precipitates out of solution. Structural changes were observed going from laminar to micellar structure formation. The most acidic, pH 2.5, shows casein micelle unfolding into a highly porous matrix.

The high degree of transformation observed on casein micelles, after SEM imaging at basic and acidic pH range, indicates this could the cause of the strong influence of pH on the rate at which the oxidation reaction occurred. The percentage of oxidation achieved after variations in pH, time and amount of oxidizer ranged from 0 to 93%, a considerable outcome. The highest oxidation rate was achieved at pH 2.5, yielding a conversion of methionine to 93% methionine sulfone after 2 hours of H_2O_2 treatment. The amino acid profile (Table 16) of this treatment was compared to control casein.

Table 16. Amino acid profile of casein and oxidized casein

Amino Acid		Control Casein	Oxidized* Casein
Asparagine	Asp	3.363 ± 0.23	2.787 ±0.14
Glutamine	Glu	22.990 ± 1.32	21.790 ±0.67
Serine	Ser	6.050 ± 0.20	4.984 ±0.52
Glycine	Gly	1.791 ± 0.20	1.803 ±0.03
Histidine	His	2.582 ± 0.02	1.215 ±0.07
Arginine	Arg	2.080 ± 0.18	1.924 ±0.16
Threonine	Thr	4.625 ± 0.41	3.718 ±0.14
Alanine	Ala	2.045 ± 0.11	2.105 ±0.23
Proline	Pro	8.774 ± 0.62	10.300 ±0.34
Methionine sulfone	Msf	n/a	2.705 ±0.07
Tyrosine	Tyr	6.000 ± 0.18	7.642 ±0.97
Valine	Val	7.506 ± 0.43	9.001 ±0.36
Methionine	Met	3.220 ± 0.31	0.266 ±0.05
Cysteine	Cys	n/a	0.272 ±0.03
Isoleucine	Ile	5.587 ± 0.09	5.805 ±0.92
Leucine	Leu	10.370 ± 0.12	13.470 ±1.40
Phenylalanine	Phe	5.480 ± 0.03	7.340 ±0.94
Lysine	Lys	7.538 ± 1.34	2.879 ±0.20



	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	MSF	Tyr	Val	Met	Cys	Ile	Leu	Phe	Lys
OxCasein	2.79	21.79	4.98	1.80	1.22	1.92	3.72	2.11	10.30	2.71	7.64	9.00	0.27	0.27	5.81	13.47	7.34	2.88
Casein	3.36	22.99	6.05	1.79	2.58	2.08	4.63	2.05	8.77	0.00	6.00	7.51	3.22	0.00	5.59	10.37	5.48	7.54

Figure 12. Amino acid profile: control casein *vrs.* oxidized casein

The difference produced on the amino acid profile by the hydrogen peroxide oxidation treatment can be observed on the above graph. As it can be observed in the previous graph, oxidized casein amino acid profile does not vary much from casein amino acid profile. Indicating that the hydrogen peroxide did not affect the majority of amino acids other than lysine, methionine and cysteine. Lysine was the amino was the amino acid with highest variation, but its original value can be reconstituted by supplementing it back to the diet if considered necessary.

3.3 Sensory evaluation

A preliminary sensory evaluation study was conducted to estimate the acceptance of a panel group for Hominex-2 ® and low methionine milk. Oxidized casein was used to prepare a reconstituted milk drink by bringing it to its original composition, 3.8% fat and 5% lactose.

3.3.1 Materials and methods

Hominex-2® was prepared according to the manufacturer instructions on the back of the can. Samples for sensory evaluation were prepared to a final concentration of 10% protein in water. Formulations of the methionine free protein drinks used in the sensory evaluation are shown in the Table 17.

Table 17. Methionine-free protein drinks formulations

Ingredient	Hominex-2®	Low Methionine Milk
Oxidized Casein	0	153g
Lysine	0	11.1g
Methionine	0	0
Tryptophan	0	1.6g
Hominex-2 ®*	25.90g	0
Water	751.10g	1424.50g
Total	777.00g	1591.60g

*Hominex-2 ® is 30% amino acids

All samples were labeled randomly and served cold (8 ºC) to the panelists. The panel was formed with 10 healthy adult males, ages 34 ± 14.0 years old, samples were presented and they were asked to taste the samples and rate their overall acceptance on a hedonic scale (Figure 13) ranging from 1 to 5. Anchor 1 indicated least acceptance starting at "dislike very much" and anchor 5 indicated the greatest acceptance ending with "like very much", as shown on the following scale:

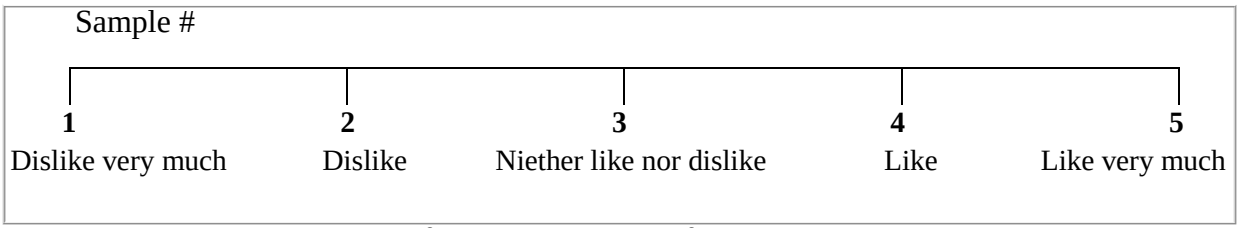


Figure 13. Hedonic scale for acceptance test of methionine restricted protein drinks

3.3.2 Results and discussion

The acceptance test rating results (Table 18) showed how the oxidation treatment produced no off-flavors on casein. It also demonstrates the efficiency of the washing/precipitating hydrogen peroxide removal method we used. The panelists showed a significant greater acceptance for oxidized casein with added AA than for Hominex-2®. The following group of tables show data for ratings panelists assigned to each of the samples with their respective Tukey's mean separation.

Table 18: Sensory evaluation samples ratings

	Panelist	1	2	3	4	5	6	7	8	9	10	Mean	SD*
Hominex	Appearance	5	4	5	5	2	2	8	3	7	3	4.4	2.01
	Flavor	4	1	2	1	2	1	2	2	7	1	2.3	1.89
	Liking	5	1	2	1	2	1	2	2	7	2	2.5	1.96
L**. Milk	Appearance	5	5	5	8	5	6	8	5	3	4	5.4	1.58
	Flavor	4	4	4	3	5	3	7	5	4	3	4.2	1.23
	Liking	4	5	4	3	5	3	7	5	5	3	4.4	1.26
Preferred		LM	LM	LM	LM	LM	LM	LM	LM	HX	LM		
Age		58	27	32	27	27	24	62	28	28	25	33.8	14

*SD: standard deviation
**L: low methionine

Table 19. Tukey's mean separation

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
389 vs 495	1.500	4.666	2.101	0.000	≥0.0500
Tukey's d critical value:			2.971		

Category	Mean	Groups	
LM milk	3.100	A	
Hominex-2®	1.600		B

Hominex-2® obtained a mean rating of 1.6 and oxidized casein with added AA obtained a mean rating of 3.1, $p<0.000$. Panelists "neither like nor dislike" oxidized casein with added AA, but "dislike" Hominex-2®. The sensory evaluation demonstrated the panelists' higher significant acceptance ($p<0.05$) for oxidized casein with added AA drink over Hominex-2®.

The highest oxidation rate of methionine to methionine sulfone occurred at pH 2.5, yielding a conversion of methionine to 93% methionine sulfone after 2 hours of H_2O_2 treatment at 90°C. By reconstituting this oxidize casein a palatable low methionine milk can be produced *via* methionine oxidation process reaching a higher acceptability from the panelists than methionine-free formulas (Hominex-2®) available in the market.

3.4 Clinical trials

Oxidized casein was sent to Pennington Biomedical Research Center (PBRC) for animal trial, conducted by Thomas Gettys Ph.D. and Frank Greenway Ph.D[73]. The objective of this study was to test the efficacy of the oxidized casein (methionine depleted casein) we produced with the above discussed methodology, to reproduce the metabolic response of Hominex-2®. The methionine depleted casein used for this animal study was treated with 10% hydrogen peroxide at 90°C for 2h under continuous stirring.

3.4.1 Materials and methods

Five rodent diets were formulated with Dyets, Inc. (Bethlehem, PA). A mixture of individual amino acids was used to formulate the following two diets: Elemental Methionine-restricted diet and the Elemental Control diet. A third diet consisted of regular casein (un-oxidized) for the Control Diet.

The methionine depleted diet was used to formulate diets number four and five which were: the Oxidized Control Casein and the Oxidized Casein Methionine-restricted diet. The

Oxidized Control Casein diet was prepared by adding back the original levels of individual amino acids (e.g., tryptophan, methionine, lysine). For the Oxidized Casein Methionine-restricted diet only tryptophan and methionine were added back to their original level, final concentration of methionine in this diet was brought up to 1.7g/kg of diet.

Table 20. Amino acid composition of formulated diets

Amino Acid	Control Casein	Ox*-Casein Control	Ox-Casein MR**	Elemental Control	Elemental MR
Alanine	3.8	4.0	4.0	0	0
Arginine	3.9	8.4	8.4	11.2	11.2
Asparagine	0	0	0	0	0
Aspartate	8.5	9.2	9.2	0	0
Cysteine	0	0	0	0	0
Glutamate	27.5	26.6	26.6	0	0
Glutamic acid	0	0	0	27.1	33.9
Glycine	2.3	2.4	2.4	23.3	23.3
Histidine	3.4	2.3	2.3	3.3	3.3
Isoleucine	6.9	5.8	5.8	8.2	8.2
Leucine	11.8	12.5	12.5	11.1	11.1
Lysine	18.0	18.0	18.0	18.0	18.0
Methionine	8.6	8.6	1.7	8.6	1.7
Phenylalanine	6.3	6.7	6.7	11.6	11.6
Proline	14.7	13.8	13.8	0	0
Serine	7.1	6.3	6.3	0	0
Threonine	5.6	4.7	4.7	8.2	8.2
Tryptophan	1.8	1.8	1.8	1.8	1.8
Tyrosine	7.2	7.8	7.8	0	0
Valine	7.8	8.0	8.0	8.2	8.2
Casein	141	0	0	0	0
Oxidized casein	0	153	153	0	0
Dextrose	200	200	200	200	200
Dyetrose	50	50	50	50	50
Cornstarch	419.9	399	405.9	432.5	432.5
Corn oil	80	80	80	80	80
Cellulose	50	50	50	50	50
Mineral mix #200000	35	35	35	35	35
Vitamin mix #300050	10	10	10	10	10
Choline bitartrate	2	2	2	2	2
Total	1000	1000	1000	1000	1000

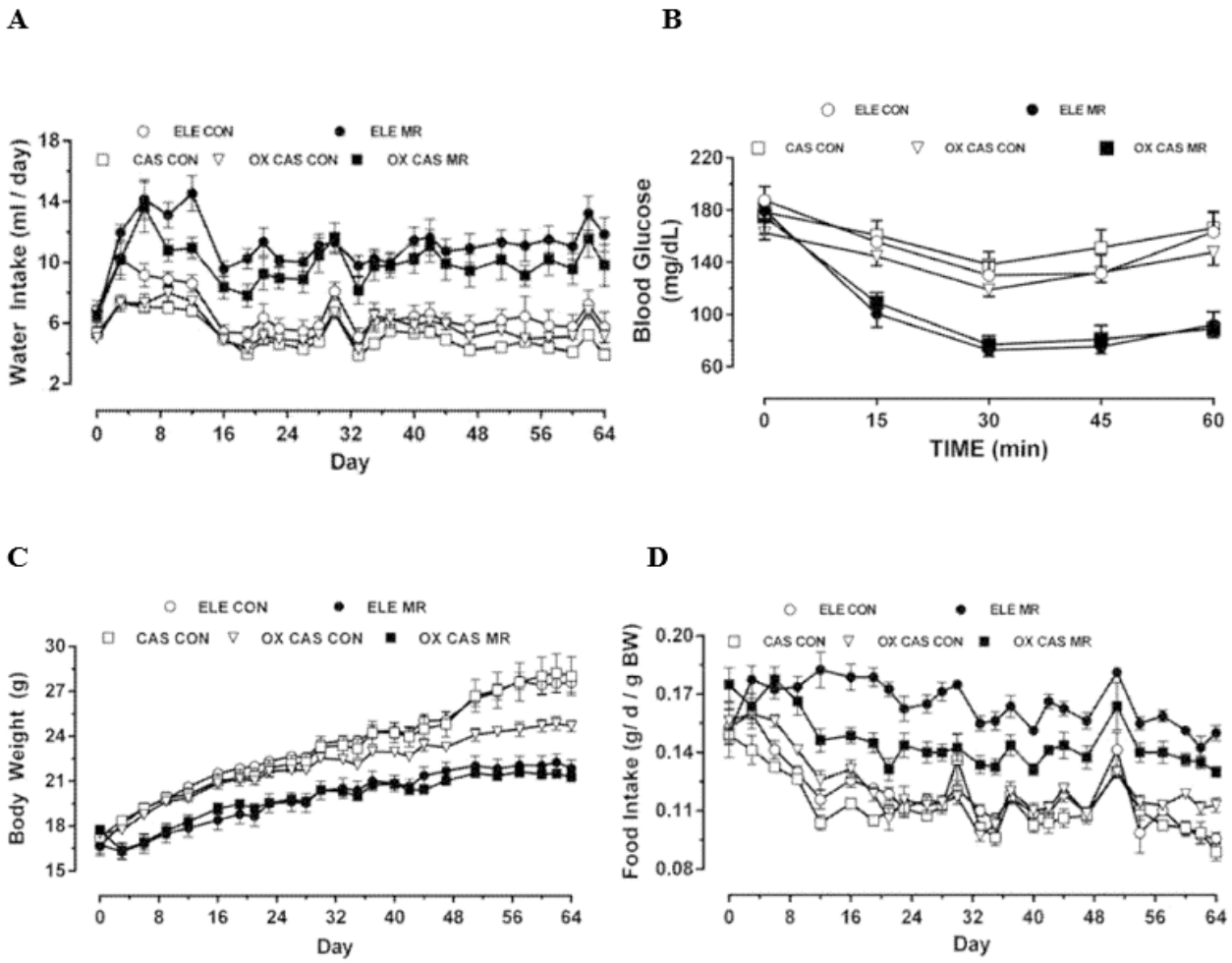
*Ox: oxidized
**MR: methionine restricted

Rats were obtained from Jackson Labs when they were forty five weeks old. The rats were all male C57BL/6J. Upon arrival they were divided into one group of 16 and another group of 24 and set for a 10 day quarantine, adaptation period. During quarantine the group of 16 was fed with Elemental Control diet and the group of 24 with the Casein Control diet. By the end of the quarantine period the group of 16 mice was divide into two groups of 8, one continued to receive Elemental Control diet and the other half were switched to Elemental MR diet. The group of 24 mice was divided to form 3 groups of 8 as well. One was continued to be fed with Casein Control diet, the other two groups of 8 were switched to Oxidized Casein Control diet or Oxidized Casein Methionine-restricted diet.

The groups of rats, eight rats per group, were destined for each of the five diets, and fed *ad libitum* with their respective diet for 9 weeks after weaning. After one week of evaluation of energy expenditure by indirect calorimetry, all rats were killed and their tissue and blood were examined for gene expression and measurement of lipids. Insulin, FGF-21 and triglyceride were measured from the collected serum.

3.4.2 Results and discussion

The five diets were denoted Elemental Control (ELE CON), Elemental methionine restricted (ELE MR), Casein Control (CAS CON), Oxidized Casein Control (OX CAS CON), and Oxidized Casein Methionine-restricted (OX CAS MR). The first effect observed on mice subjected to dietary methionine restriction of 0.17% was an increase in water intake right at the first week as shown in chart A. Groups of ELE MR and ELE CAS MR showed the same increase in water intake. The behavioral and metabolic responses of the mice grouped on the different diets are shown in the following charts.



A. Change in water intake over time in mice consuming control and methionine-restricted diets (n=8 mice/grp).
B. Change in insulin sensitivity in mice after consuming control or methionine-restricted diets for 9 wks (n=8/grp).
C. Change in body weight in mice after consuming control or methionine-restricted diets for 9 wks (n=8/grp).
D. Change in food intake over time in mice consuming control and methionine-restricted diets (n=8 mice/grp).

Figure 14. Clinical trial results of methionine depleted casein

The change in food intake was comparable for the three control groups, as expected. For the OX CAS MR diet the change in food consumption started with a decrease in day 1, but it rapidly increased and even surpassed the control groups' food consumption by day 7. ELE MR and OX CAS MR group showed the same behavior during the 64 days study.

It is important to highlight that the rate of food consumption of the OX CAS MR and ELE MR groups was considerably higher than the control groups. On the other hand, the

adiposity and body weight of OX CAS MR and ELE MR were reduced to a similar degree by the end of the study. Both MR groups showed hyperphagia relative to their control groups.

It can be observed that energy expenditure increased by 41% on the ELE MR group compared to the ELE CON group. The increase in energy expenditure of OX CAS MR group compared to CAS CON group was of 35%. The above data supports the conclusion that OX CAS MR diet is a replica of ELE MR diet metabolic and behavioral responses.

3.5 Conclusion

Casein micelles oxidized in the presence of hydrogen peroxide only at acidic pH. The high degree of transformation observed on casein micelles, after SEM imaging at basic and acidic pH range, indicates this can be the cause of the strong influence of pH on the rate at which the oxidation reaction occurs. The highest oxidation rate was achieved at pH 2.5, yielding a conversion of methionine to 93% methionine sulfone after 2 hours of H_2O_2 treatment. Methionine to methionine sulfone conversion rate was directly proportional to the total time of treatment.

Both hydrogen peroxide and ozone, successfully oxidized methionine to methionine sulfone. The concentration of hydrogen peroxide has a direct effect on the oxidation rate of the reaction, but it is not a determining factor. The total effect of adding 100% more (duplicating) hydrogen peroxide concentration gave an output of only 2% increase on the conversion rate of the reaction.

The preliminary sensory evaluation study showed how the oxidation treatment produced no off-flavors on casein. Hominex-2® obtained a mean rating of 1.6 and oxidized casein with added AA obtained a mean rating of 3.1, $p<0.000$. Panelists “neither like nor dislike” oxidized casein with added AA, and “dislike” Hominex-2®. The sensory evaluation indicated that

panelists demonstrated a higher significant acceptance ($p<0.05$) for oxidized casein with added AA drink over Hominex-2®.

The clinical trial conducted at Pennington Biomedical Research Center with a final concentration of methionine in the MR diet of 1.7g/kg, proved the concept that the effects of an elemental methionine restricted diet can be fully replicated by using the oxidized casein we produced. The study culminated with increase in food consumption concomitant to weight loss for both, oxidized casein and elemental methionine restricted diet. The results of this study revalidate the notion that methionine restriction in the diet has a direct effect on weight loss.

Published research available in methionine oxidation focuses only on the quantification of methionine sulfoxide and sulfone in biological processes or analytical methods, but the effect that this same oxidation reaction has on food ingredients has not yet been explored sufficiently. Further research needs to be performed on how ozone and hydrogen peroxide oxidize high methionine foods, and on the effects it causes on the food flavor and matrix in order to be able to produce palatable MR diets for human consumption. The production of palatable methionine restricted food ingredients will allow to expand research on the following areas:

- The health benefits of low methionine diets on: control of cancer metastasis and carcinogenesis, weight-loss, insulin resistance and metabolic syndrome.
- Toxicological studies of MR diets to stablish optimum levels of methionine restriction for the different therapeutic purposes.
- Replicate animal studies in clinical trials to monitor the health benefits of MR diets on humans and determine the sustainability of MR diets for long term consumption.
- Synergism of MR diets in combination with chemotherapy for cancer control.

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VITA

Gabriela Melissa Crespo Gutierrez was born, and raised, in the city of San Pedro Sula, Honduras to Jose Luis Crespo Hernandez and Maria del Pilar Gutierrez Elvir. The younger of two siblings, she attended Saint Peter´s Academy bilingual school (Spanish – English) where she graduated with honors by receiving the bronze medal (December 2004). She learned her third language, Italian, by living in the Sardinia island, Italy for six months in 2005 through participating in the American Flight Service (AFS) intercultural program. Prior to graduating as a B.S. in Food Science and second best student of her class of 300 students at Zamorano University in 2009, Gabriela spent a semester as an intern in Purdue University where she worked in carotenoids HPLC analysis with Dr. Mario Ferruzzi. Gabriela´s undergraduate project at Zamorano University entitled "Development of a prototype of Tilapia *(Oreochromis spp.)* nugget with sensory evaluation of flavor and appearance", was supervised by Professor Flor Nuñez Ms. and is the conclusion of the research she performed for Aquafinca Saint Peter Fish, a company dedicated to the cultivation of tilapia in the Yojoa Lake, Honduras. After graduation Gabriela went to Louisiana State University (LSU) where she was granted an assistantship at the Food Science graduate program. She enrolled in August 2010 as a graduate student in the Food for Health Program. She wrote her dissertation on "Palatable Foods for a Methionine Restricted Diet" doctoral student under the mentorship of Dr. John Finley. Gabriela shared her research in the 2012 annual meeting of the American Chemical Society (ACS) and the 2013 annual meeting of the Institute of Food Technologists (IFT). Gabriela is a candidate to receive the degree of Doctor of Philosophy in Food Science during the May 2015 commencement ceremony at LSU. Besides her passion for scientific research, she enjoys the dance arts, oil painting and has tutored underprivileged children in Honduras.