Inhibition of Maillard Reaction Pathways and Off-flavor Development in UHT milk: Structure Reactivity of Phenolic Compounds

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Dedication

To my family, for without them none of this would be possible.

Abstract

The Maillard reaction, a carbonyl-amine reaction, is an important food and biological reaction. In food, this reaction is known to impact color and flavor, alter the nutritional content, as well as generate therapeutic and toxic compounds. In biology, Maillard derived reactive carbonyl compounds, generated in vivo or from dietary exposure, have been associated with several pathological conditions. Consequently the ability to control the pathways/products of this reaction would be beneficial to the food and health related industries.

Recently phenolic compounds have been demonstrated to suppress the development of Maillard off-flavor compounds and browning in foods, as well as to reduce the levels of Maillard-derived reactive carbonyl species (RCS) and related advanced glycation end (AGEs) products in biological systems. The overall goal of this thesis was to build on this prior work by providing a more in-depth mechanistic study of phenolic structure-reactivity on the pathways of the Maillard reaction and product formation, specifically in UHT processed bovine milk during both thermal processing and storage.

In the first phase of this project, five phenolic compounds at an equivalent dose of 1.7mM were examined for structure-reactivity relationships (catechin, genistein, daidzein, 1,2,3-trihydroxybenene, and 1,3,5-trihydroxybenene) on Maillard pathways and product generation. Levels of transient Maillard reaction precursors (C_2, C_3, C_4, and C_5

α-dicarbonyls and α-hydroxycarbonyls) and select off-flavor markers (methional, 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline) were quantified by LC/MS/MS and GC/MS-TOF, respectively; stable isotope surrogates were utilized as internal standards. In general, the addition of phenolic compounds prior to UHT processing significantly reduced the concentration of MR precursors and off-flavor compounds compared to traditional UHT sample ($p<0.05$) and particularly after storage. However phenolic compounds with a more activated ring structure for aromatic electrophilic substitution reactions (1,3,5-trihydroxybenzene, catechin and genistein) were more reactive at suppressing Maillard pathways. Albeit unique structure reactivity was also noted among the different phenolic compounds analyzed. Sensory studies were in agreement with the analytical data; lower cooked flavor intensity was observed for the off-flavor recombination models of the catechin treated UHT milk (versus the control UHT milk). Furthermore consumer acceptability of the catechin treated UHT milk was rated significantly higher than the control sample (Fisher's LSD = 0.728) showing improved palatability.

In the final research phase, the application of Response Surface Methodology (RSM) for process optimization was evaluated. A Box-Benhken 3-factor (catechin, genistein and diadzein) 3-level (0.17, 0.645 and 1.12mM) design was employed and dose-response relationships of phenolic compounds/mixtures (added prior to thermal processing) on the levels of reactive carbonyl species (RCSs; glyoxal, methyglyoxal and 3-deoxyglucosone) in UHT milk were examined. In general, a range in RCSs reduction

was observed, with the most reactive phenolic mixtures reporting levels of RCS in UHT milk at or below those of pasteurized milk. Predictive models, with no significant lack of fit ($p>0.05$), high r^2-values (0.886-0.979) and good predictive power, were developed. ANOVA analysis for glyoxal levels reported only significant ($p<0.05$) linear effects of each factor indicating no significant interactions between the different phenolic compounds were observed. However for the levels of methylglyoxal, linear, cross product and quadratic effects were reported ($p<0.05$), indicating more complicated interactions existed. Similarly significant linear and quadratic effects ($p<0.05$) were also reported for 3-deoxyglucosone. Overall, based on canonical analysis, catechin was reported to be the most influential phenolic compound for the reduction of RCSs in UHT milk. The observed unique reactivity noted between select phenolic compounds and the suppression of the three RCS furthermore indicates the implementation of different phenolic structures can alter the appropriate dosage for tailored mixtures of phenolic compounds used for various food systems and processing conditions.

In summary, phenolic structures with a more activated ring structure for electrophilic aromatic substitution reactions were the most effective at reducing the levels of RCS and off-flavor compounds in UHT milk, particular during storage. The utilization of phenolic compounds as a pre-processing ingredient was demonstrated to improve the quality of UHT milk by increasing the palatability and simultaneously reducing the dietary load of these potentially toxic RCS compounds. Furthermore, statistical RSM provided an improved basis to understand phenolic structure reactivity to control

Maillard chemistry as well as to optimize the dose-response and therefore ingredient cost for effective utilization in UHT milk.

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Chapter 1

Introduction

Milk History

The use of milk as a beverage likely began with the domestication of animals. Goats and sheep were domesticated in the area of Iran and Afghanistan in about 9000 BC and by about 7000 BC cattle were being herded in what is now Turkey [1]. European dairy cows were brought to North America in the early 1600s but the origin of the dairy industry in the United States (U.S.) can be traced back to the 19th century [2–4], as fluid milk consumption became more common during that period.

Prior to the American Revolution most of the dairy products were consumed on the farm where they were produced. By about 1790, population centers such as Boston, New York, and Philadelphia had grown sufficiently to become an attractive market for larger-scale dairy operations. To meet the increased demand, farmers began importing breeds of cattle that were better suited for milk production. At that time, milk produced for human consumption was unpasteurized, and consequently contributed to childhood illnesses and even fatalities. It was not until the 1900s that the pasteurization of milk products became mandatory (written into the code of federal regulations; (21CFR131.3 and 131.110) [4] and milk became safer to drink [3]. In 1863, Louis Pasteur of France developed a method of heating wine to kill the microorganisms which was later adapted

to a number of food products and became known as pasteurization. The first milk processing plant in the United States installed pasteurizing equipment in 1891 and at the time many dairy operators opposed pasteurization as an unnecessary expense. Chicago became the first major city to require pasteurization of milk (1908) and New York and Philadelphia followed in 1914, and by 1917 most major cities had enacted laws requiring that all milk be pasteurized.

Nutritional properties

Milk is defined as, "… the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows. Milk that is in final package form for beverage use shall have been pasteurized or ultra-pasteurized, and shall contain not less than 8.25% milk solids not fat and not less than 3.25% milkfat." [4] For human and mammals milk is the first food, which is a testament of how important it is for growth and development as it contains all the necessary nutrients. Milk has been called the 'ideal food' [2] and is widely considered a key component of a healthy diet, as it is an important source of proteins, minerals and vitamins as well as energy (carbohydrates and fat).

Fat is present in milk in small globules suspended in water. Each globule is surrounded by a layer of phospholipids, which prevents the globules from clumping together by repelling other fat globules and attracting water. The majority of milk fat is in the form of triglycerols (95-96%) formed by the linking of glycerol and fatty acids. Milk contains a high content of low molecular weight fatty acids such as butyric, caproic, and capric acid, which account for approximately 11% of total fatty acid content. The most

important fatty acid from a quantitative viewpoint is palmitic acid, which accounts for approximately 25% of the total weight of fatty acids followed by stearic and myristic acids accounting for 13 and 9% respectively. Monounsaturated fatty acids make up approximately 28% of the total content with the predominant one being oleic (18-carbon chain), and polyunsaturated linoleic and linolenic acids, which make up approximately 3% of total fatty acid content, provide several health benefits and have been associated with prevention of atherosclerosis, hypertension, improved immune function and cancer [5], [6].

Milk contains approximately 4.9% carbohydrate that is predominately lactose with trace amounts of monosaccharides and oligosaccharides. Lactose is a disaccharide of glucose and galactose and is dissolved in the serum (whey) phase of fluid milk.

The concentration of protein in milk varies from 30-40 gr/l and there are two major groups of protein, casein and whey accounting roughly for 80% and 20% of total protein, respectively. The two major groups of milk protein are broadly defined by their chemical composition and physical properties where the casein family contains phosphorus and will coagulate or precipitate at pH 4.6. The whey proteins do not contain phosphorus, and they will remain in solution in milk at pH 4.6. The casein family of protein consists of several types of caseins (α-s1, α-s2, ß, and κ) and are suspended in milk in a complex called a micelle rich in phosphorus which enables the casein micelles to associate with calcium and form calcium phosphate salts. The whey protein family consists of approximately 60% β-lactoglobulin, 20% α-lactalbumin, blood serum albumin, immunoglobulins, lactoferrin, transferrin, and many minor proteins and enzymes.

Milk is also an excellent source of most minerals required for growth and maintenance of bone integrity, as well as enzyme function and oxygen transport. It also contains water- and fat-soluble vitamins such as A, D, E, K and B2, B3, B12 (Table 1.1) which are essential for proper metabolic function and balanced homeostasis. Other minerals of nutritional interest found in milk include iodine, zinc, sodium, potassium, chlorine and magnesium (Table 1.1).

Table 1.1. Mineral and vitamin concentrations in milk in ug/l and mg/l respectively adapted by source [7]

Minerals	**mg/l**	**Vitamins**	**mg/l**
Potassium	1,500	A	0.4
Calcium	1,200	D	0.001
Chloride	1,000	E	1
Phosphate	3,000	K	0.17
Sodium	500	B1	0.4
Sulfate	100	B2	1.7
Magnesiu	120	B6	0.6
Zinc	4	B12	0.005
Trace	<0.1	C	20

Consumption and market trends

Despite all nutritional and health benefits that milk delivers, the per capita consumption in the United States has decreased by approximately 15% since 1984 (Figure 1.1). In 2010 the USDA's Agricultural Marketing Service reported that total sales of conventional fluid milk products declined by 1.8 percent [8]. The decrease in milk consumption over the past few decades can be attributed to several factors most importantly, increased market competition from other beverage products. The market for carbonated soft drinks, energy drinks and still beverages such as flavored water and juice,

has grown tremendously. Numerous new products have been launched with a variety of flavors which appeal to a large pool of consumers and also offer convenience due to longer shelf life, ease of distribution and storage which is not limited by product perishability (i.e. pasteurized milk).

That decrease in milk consumption is negative for the dairy industry but also worrisome from a public health view point as the USDA's Economic Research Service (ERS) has shown that each 1- ounce decrease in milk consumption is accompanied by a 4.2-ounce increase in soft drink consumption, resulting in a gain of 31 calories and a loss of 34 mg of calcium, also raising nutritional concerns [9].

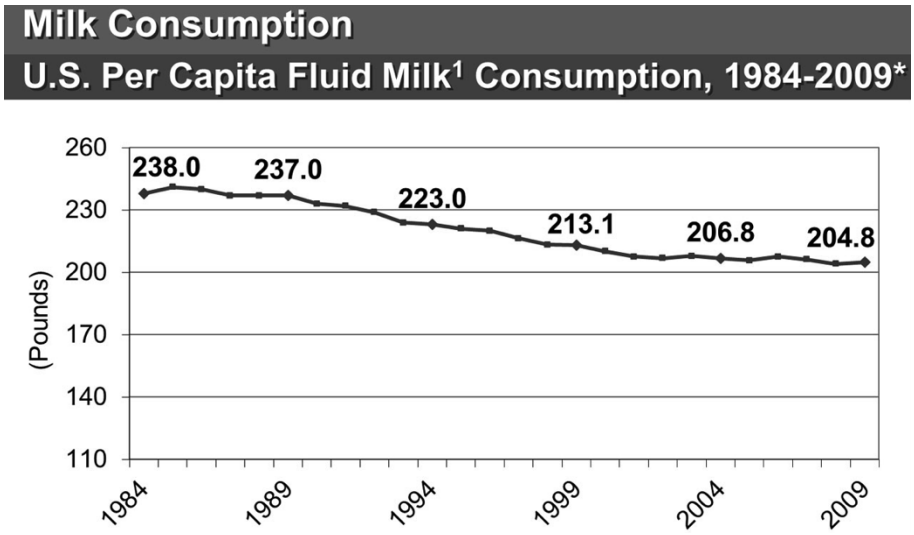


Figure 1.1. US per capita consumption of fluid milk from 1984 to 2009. Source: ERS/USDA Livestock, Dairy, Poultry outlook. [1]Includes fluid milk and cream.

Thermal processing

As mentioned earlier, milk in the US is mandated to undergo pasteurization for safety and stability and a number of different thermal processing techniques are available

that can also influence the shelf life of milk products [4]. Both pasteurization and sterilization heat treatments destroy pathogenic bacteria and increase the shelf life of milk by destroying spoilage bacteria and inactivating enzymes. The majority of milk consumed in the US is pasteurized or ESL (Extended shelf life)-treated as both of these processing methods ensure microbial safety and have minimal effects on the nutritional and flavor quality of milk. Extended shelf life (ESL) processing requires the milk to be heat-treated > 138°C for a minimum of 2sec is not packaged aseptically and requires refrigeration post processing. Extended shelf life milk produced in the U.S. and Canada averages a 45 to 60 day shelf life under refrigerated conditions [10] However, the drawback of these processing technologies is that they yield products with relatively short shelf life as compared to commercially sterilized products and additionally require refrigeration, as they are not shelf-stable.

To increase shelf-life and stability and produce milk that requires no refrigeration a heat processing method that achieves commercial sterility is required. Commercial sterility is achieved by application of heat, sufficient, alone or in combination with other ingredients and/or treatments to render the product free of microorganisms capable of growing in the product at non-refrigerated conditions (over 50^{o}F or over 10^{o}C) at which the product is intended to be held during distribution and storage (Definition from source, USDA, Food Safety and Inspection Service). Ultra High Temperature processing combined with aseptic filling (which will be referred to as UHT) is a commercial sterilization method that can yield a product with long shelf life and increased stability at ambient storage (6-12mo).

Aseptic UHT Processing

Aseptic UHT processing was introduced in 1948 and it involves heating milk in a continuous process to temperatures higher than 135^{o}C for a few sec (2-10sec) in order to inactivate spore-forming bacteria (more specifically B. subtilis, the most thermo-resistant mesophilic spores in raw milk), cooling rapidly, and aseptically packaging the milk into sterile containers [11]. This method has many advantages as it provides a product that has a shelf life of 6-12 months [12] and does not require refrigeration until after opening. In Based on the grade A pasteurized milk ordinance, aseptic UHT processing and packaging of milk shall be performed in accordance with the applicable requirements of 21 CFR Parts 108, 110 and 113 [13]. The increased stability of aseptically processed UHT milk also translates to energy efficiency as it saves 900 kJ/kg of energy due to efficient regeneration of thermal energy during processing as well as no required refrigeration during distribution and storage as compared to pasteurized or ESL processed refrigerated milk [14]. Additionally increased shelf-life and stability in ambient conditions translates to decreased inventory and distribution costs as well as financial losses related to expired products. Larger product inventories could be maintained at distribution centers and no refrigeration is required during transportation.

While UHT technology offers many advantages both from the dairy industry and a consumer perspective, it also causes several chemical changes that can be manifested in noticeable negative changes in flavor and color profile of milk thus consumer acceptability has been low.

Implications on sensory quality and consumer acceptability

Flavor is a key attribute in most food products, as it is one of the major factors influencing a consumer's choice of food. The negative changes in the flavor profile of milk caused by UHT processing are undesirable in some markets, such as the US where consumers are more familiar with the flavor profile of pasteurized milk. In the domestic market, consumers recognize milk as having a pleasing, slightly sweet flavor, with weak aroma characteristics, a pleasant mouth feel and aftertaste which are common characteristics of pasteurized milk [15] and any alteration to that profile is considered a defect. Consequently, although UHT milk products have been produced and distributed in North America for over 60 years, they have not seen growth as the undesired flavor quality of UHT milk is a strong barrier to consumer acceptance [16–18].

Research done by Cornell University and sponsored by New York State Milk Promotion Order, a division of the New York Department of Agriculture and Marketing, showed a direct correlation between the flavor quality of milk and level of consumption [19] . Blake et al. [20] also reported the negative effect of UHT processing on consumer liking compared to other (less severe) heat treatments. Based on a seven point hedonic scale, HTST (High Temperature Short Time) for pasteurized milk and UHT milk had an average score of 5.8 and 3.6 respectively (Figure 1.2). Additionally, UHT processing also affects the color of milk products [21–23], which is an important sensory attribute and greatly affects consumer acceptability. The extent of heat treatment and the storage conditions significantly affect browning which is more pronounced with increases in process thermal dose and storage temperature and time [12], [24].

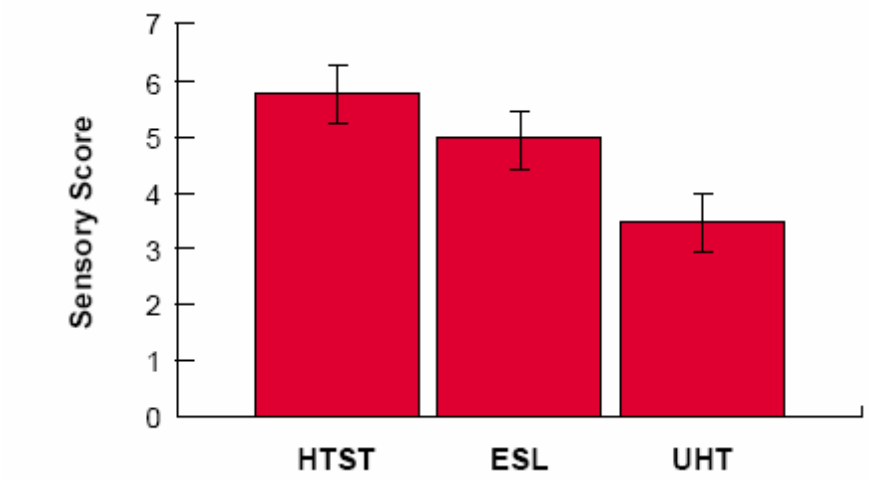


Figure 1.2. Least square mean (LSM) of consumer acceptance (hedonic rating with 1 associated with extreme dislike and 9 associated with extreme liking). HTST (74°C for 16 s), ESL (Extended Shelf Life) (l34°C for 4 s, direct steam injection), and commercial UHT (indirect plate-exchange heating) processed milk [20].

The increased consumption of UHT milk globally reflects the consumers demand for shelf-stable products. Since both flavor and convenience are important consumer drivers, the ability to suppress (control) off-flavor development during UHT processing of fluid milk will offer the advantage of product stability without the negative flavor aspects of traditional UHT milk and will result in a more competitive product; particularly in markets like the US.

A challenge to improving the flavor quality of UHT milk can be related to the lack of knowledge defining the key, or contributing off-flavor compounds and particularly the pathways involved. The negative flavor changes in UHT milk and particularly cooked flavor and staleness (both contribute to “lack of freshness”) have been previously reported to develop during heat treatment as well as during ambient storage and have been associated with the Maillard reaction and lipid oxidation [25–27].

More recent work by Colahan-Sederstrom and Peterson [18] identified important off-flavor compounds that contribute to the cooked flavor profile of UHT milk and unequivocally showed that the Maillard reaction was the main source of off-flavor development during thermal processing of UHT milk. Consequently, it can be derived that improving the flavor profile of UHT milk and milk-based beverages relies on developing technologies that allow inhibition or control of Maillard reaction pathways.

Implications on nutritional quality

Heat induced changes due to UHT processing aside from altering the flavor profile of milk can also have negative effects on the nutritional quality of the final product. Irreversible heat induced changes, which influence the nutritional quality of UHT processed milk include vitamin degradation and progression of the Maillard reaction, both during processing and storage.

The Maillard reaction can lead to increased generation of reactive carbonyl species, that contribute to “carbonyls stress” and ultimately lead to formation of Advance Glycation End products (AGEs). AGEs have been associated with the development of pathological conditions such as diabetes, Parkinson’s and Alzheimer’s and increased dietary intake of these compounds has been associated with increased risk of pathological conditions [28–31] thus, methods to reduce or inhibit their formation in heat-processed foodstuffs is of great importance.

Flavor profile and nutritional quality improvement strategies

Two different approaches have been previously utilized to reduce off-flavor development of UHT processed milk (1) improvement of thermal processing techniques and (2) development of pre-processing treatments- ingredient technology.

Regarding thermal processing techniques, new equipment was designed to reduce the overall thermal dose during processing required to produce a commercial sterilized product. There are two main commercial methods for the production of shelf-stable milk products: direct and indirect techniques. Both methods produce similar milk products in terms of stability, microbial safety and shelf life; however, there is a difference between those two methods in the overall thermal dose applied to the product, a variable that has been related to the final products flavor profile as well as cost and energy efficiency.

The 'newer' direct heating method has a much faster heating rate compared to indirect heating, thus the products undergo a less severe thermal dose which results in decreased off-flavor development [32]. The main methods of direct heating are steam injection and infusion.

During the injection process, high pressure steam is injected into pre-heated milk which causes to a very rapid rise in temperature. After holding at final temperature for a short time, the product is flash-cooled in a vacuum to remove water equivalent to the amount of condensed steam used. While this method allows fast heating and cooling, and volatile removal, it is only suitable for some products. It is energy intensive and because the product comes in contact with hot equipment, there is potential for flavor damage. When the infusion method is utilized, milk is pumped through a distributing nozzle into a

chamber of high-pressure steam. This method has several advantages including instantaneous heating and rapid cooling and no localized overheating.

In indirect heating systems the heating medium and product are not in direct contact, but separated by equipment contact surfaces. Heating occurs by a cross flow heat transfer resulting in a slower heating rate as compared to direct systems. There are several types of heat exchangers such as plate, tubular and scraped surface and each has advantages and disadvantages with tubular providing the most uniform heating for the product though not being the most efficient system for viscous products and are more susceptible to contamination, burn-on and fouling as compared to scrape surface systems.

Direct UHT processing techniques have many advantages but still produce milk that has lower flavor quality as compared to that of pasteurized and thus, less acceptable to consumers [32]. Additionaly the direct method, in comparison to the indirect, has been reported to be more expensive in terms of capital investment costs and energy efficiency. The indirect system can have regeneration of up to 90% of thermal energy as compared to approximately 50% for the direct processing system [12] thus, being overall more cost and energy efficient and a good canditate (from a financial standpoint) to be coupled with pre-UHT processing treatments that suppress the development of off-flavor.

Regarding pre-processing treatments, addition of ingredients prior to thermal processing and, specifically the addition of dietary phenolic compounds, have been previously examined and demonstrated to suppress the formation of off-flavor compounds formed via the Maillard reaction. Results obtained by a trained sensory panel also confirmed the reduced off-flavor generation [18]. This was a proof of concept study and demonstrated that natural products could potentially be utilized to develop an

ingredient technology for the production of higher flavor quality UHT milk. Additionally a study by G.P. Schamberger and T.P. Labuza [33], which was based on the work of Peterson, D.G., and Colahan-Sederstrom, P.M. [18], demonstrated the effectiveness of phenolic compounds (epicatechin and epigallocatechingallate) in reducing Maillard browning in UHT milk during processing and storage. This study provided further support to the idea that phenolic compounds can alter Maillard reaction pathways and thus improve flavor and color stability.

Moreover, addition of phenolic compounds has been shown to reduce the levels of reactive carbonyl species (sugar fragments) via trapping reactions and formation of adducts [34–36] in model reaction systems. A number of studies have focused on the effect of various phenolic compounds on scavenging reactive carbonyl species (RCSs) in different model systems, simulated physiological conditions and *in vivo* [37–40]. Results thus far suggest that phenolic compounds effectively scavenge RCSs, significantly inhibit the formation of AGEs and also inhibit Maillard reaction pathways in heat processed food systems these data suggest that addition of phenolic compounds prior to UHT processing could be potentially used to improve nutritional as well as flavor quality of UHT milk.

Considerations and Research Objectives

Development of shelf stable milk products with higher flavor quality would facilitate the dairy industry to be more competitive in the beverage market by providing milk beverages with increased shelf-life appealing to the need of consumers for high-convenience products. Stability in ambient temperature could also increase the ways by

which the products can be introduced to the market and reach the consumer (i.e. vending machines).

Additionally, increased shelf life could benefit the dairy industry financially as it translates into larger product inventories maintained at distribution centers which, decreased distribution and inventory costs for dairy processors, as well as financial losses related to expired products [41].

However, in order to develop strategies for flavor and potentially nutritional improvement of UHT processed milk a better understanding of how phenolic chemistry can be applied to develop a new UHT product technology is needed. The ultimate goal of this project is to improve our knowledge regarding the pathways related to off-flavor development in UHT milk and how dietary phenolic compounds can be utilized to provide cost effective and practical flavor quality (and ultimately nutritional quality) improvement for shelf-stable milk. This information would provide the dairy processing industry with a basis to develop energy-efficient processing technology that provides a convenient and more acceptable shelf-stable product.

In this study, we took a mechanistic approach to understand the thermally catalyzed pathways that affect the flavor quality of UHT milk and how addition of phenolic compounds alters the fate of off-flavor generation UHT during thermal processing and storage.

The main research objectives of this project were:

1. To characterize phenolic structure-reactivity relationships on Maillard pathways (intermediates and products) related to off-flavor development in UHT milk during thermal processing and subsequent storage.

   a. Monitor reactive carbonyl species (sugar fragments) and off-flavor compounds

2. Optimize the dose and composition of phenolic compounds to minimize levels of RCSs; glyoxal, methyglyoxal and 3-deoxyglucosone in UHT milk using response surface methodology (RSM) and thus, define dose-response relationships of phenolic compounds and RCSs.

Chapter 2

Literature Review

UHT Milk Flavor Characterization

The flavor of UHT milk has a distinctly different character from fresh pasteurized milk. UHT milk flavor is derived from a combination of increased thermal processing, increased storage time and different packaging materials. Unfortunately, the unique flavor properties of UHT milk have had a negative impact on its consumer acceptance in the United States. Research has been conducted to determine the identity and source of the compounds that cause the flavor of UHT milk and have examined the effects of thermal processing, packaging materials, storage and raw materials on UHT milk flavor.

UHT milk was first introduced in the United States in 1948, but the first published research study on UHT milk flavor in the 1960s, where Ashton [42] described the sensory properties of UHT milk throughout storage. It was reported that immediately after processing, the flavor and aroma was considered unpleasant and at its worst, boiled cabbage-like or sulfury. Two to three days post processing this character decreased, but a residual cooked flavor became more apparent. It was also noted that the flavor continued to change, and a flat or chalky flavor developed next. The final flavor discussed was the residual cooked flavors and oxidized and cardboard flavor character, which occurred after approximately 19 days. All of these stages were affected by storage temperature. At temperatures above 70°F the flavor changes progressed faster while at refrigerated

temperatures, it was much slower. Many of the above mentioned flavor descriptors identified in this study are still considered important and characteristic of UHT processed milk.

In 1962, Patel et al. [43] tentatively identified ethanal, dimethyl sulfide, acetone, pentyl acetate, 2-pentanone, as well as another peak that was possibly hexanal, 2-hexanaone, butyl acetate or dimethyl disulfide. This was one of the first publications which characterized volatile compounds in UHT processed milk.

Kirk et al. [44] also examined the flavor of UHT processed milk during storage and reported more compounds than previously identified. This group analyzed milk samples processed at 140.5°C ± 1°C for about 4 seconds that were stored for three months at three different temperatures (4.4, 22, 36°C). There were some compounds that had been tentatively identified by Patel et al. including ethanal, acetone, hexanal and 2-pentanone. Other compounds tentatively identified in this study included: butanal, propanal, heptanal, furfural, 2-butanone, 2-heptanone, ethanol and butanol. Furthermore, this study included sensory evaluation of the UHT milk samples using a trained panel. A caramel flavor was noted in the control and all samples at two months. This may be related to Maillard browning reactions, but furfural, which is related to this reaction, decreased in all samples throughout storage. Likely, there may have been other unidentified compounds (below detection limit) that contributed to the flavor and were not reported. The flavors described in the sensory evaluations are all common descriptors of UHT milk flavor.

One of the most comprehensive earlier studies done on UHT treated milk flavor was by Scanlan et al. [45]. Unlike the previous studies, this study examined the flavor

immediately after processing. Sensory evaluation, for levels of sulfury and heated/rich notes, was performed on samples at day 1 and 7. This study also compared volatile compounds found in both raw and heated milk. The results indicated increased levels of methyl ketones and lactones. There were four methyl ketones, 2-octanone, 2-decanone, 2-undecanone and 2-tridecanone, and one lactone, δ-octalactone, which were found in the heated milk but not in raw milk. Generation of Maillard reaction products such as maltol, furfural, acetophenone, diacetyl, and tentatively phenylacetaldehyde was also observed and their concentration increased with heating indicating that their formation was heat induced.

In the 1970's much of the milk flavor research was more focused on the influence of the dissolved oxygen content and subsequent changes over time. Zadow and Birtwistle [46] studied this relationship with regard to the headspace volume of packaging in correlation to the flavor properties of UHT milk which was conducted by a trained sensory panel. The samples were stored at three different temperatures, 2, 20 or 38°C, with varying headspace and analyzed after two time periods (4 – 21, and 26 – 84 days). There was also one set of samples that utilized de-aerated raw milk and were stored at 20°C. The final treatment involved samples processed using an indirect UHT method and subsequent storage at 20°C. Overall, Zadow and Birtwistle [46] concluded that samples with intermediate levels of dissolved O_2 were preferred, with the lowest cooked intensity shown in samples stored at refrigerated temperatures. Also, UHT milk with low O_2 content was reported to have poorer flavor performance if packaged with no-headspace volume instead of a moderate headspace volume. The results of this study have some importance because most UHT milk is packaged with low levels of headspace

although the levels of dissolve O_2 found in commercially processed UHT milk were not discussed.

In 1975, Thomas et al. [47] studied the effect of oxygen content on cooked flavor and free sulfhydryl groups (-SH) and chemical changes of whole UHT milk throughout storage. Milk was processed using both direct and indirect methods and stored at 20 ± 4°C, in the dark for 150 days. A trained 6-member panel was used to evaluate acceptability of the milk throughout storage and for the first time cooked flavor and cabbage-type flavor were distinguished (see Figure 2.1). This was not accounted for in the study by Zadow and Birtwistle [46] and previous studies thus, may have impacted their results. Results showed an increase in flavor acceptability throughout the first week but then the acceptability declined slowly whether the milk contained high (8.9 ppm), moderate (3.6 ppm) or low (1.0 ppm) initial levels of dissolved O_2. The milk scores ranged from approximately 6 (like moderately) to 3 (dislike moderately).

Although Zadow and Birtwistle [46] listed the indirectly heated milk as being very cooked, Thomas et al. [47] found it to be slightly preferred over the direct method initially although preference was independent of treatment throughout the remainder of storage. The cabbage-like flavor decreased in the first 2-3 d, which correlated with an increase in the flavor acceptability during the first week. The major defect associated with a decline in acceptability was the onset of staling. All differences perceived in the flavor based on the initial O_2 content were limited to the first 1-2 weeks of storage. Throughout this period (first two weeks), the samples with the lowest initial O_2 content were found to be the least acceptable. However, the cooked flavor was present throughout storage, and the flavor defects appeared to be similar to those reported by

Ashton [42]. Thomas et al. [47] found that the –SH levels correlated to the acceptability; as it was observed that as the –SH levels and cabbage descriptors decreased, the acceptability increased. Furthermore, acceptability also increased with higher O_2 content; for samples with the lowest levels of O_2, the –SH persisted the longest.

Jeon et al. [48] examined the formation of volatile flavor compounds in UHT milk during storage. Many important off-flavor compounds were identified and gas chromatography-olfactometry (GCO) was used in order to determine whether compounds were odor-active and whether the volatile concentration was above its odor threshold. This study showed dissolved O_2 decreasing slowly over time, with the slowest decline at 3°C. This would be expected because reaction rates are reduced as the temperature is decreased. Nonenzymatic browning (NEB) was limited at low storage temperatures (3 and 22^{o}C), but there was a slight increase in NEB in samples stored at 35°C. Figure 2.2 shows a typical chromatogram obtained by Jeon et al. and listed on it are identities of compounds and their aroma properties. Concentration of aliphatic aldehydes, methyl ketones and 1-butanone increased throughout storage.

Aromagrams (Figure 2.3) also showed that diacetyl may be a key contributor to the flavor of UHT milk as it was present at the highest concentration to threshold ratio. Diacetyl compounds can be formed via the Maillard reaction and potentially contribute to the rich or heated note [45]. A drawback of the aromagram approach is that determination og the concentration of individual compounds and their odor threshold by GC/O may not be a valid way to describe the influence of that compound on the aroma and overall flavor profile of the finished product as interactions between volatiles and different food constituents can affect sensory outcomes.

Jeon et al. [48] also made synthetic solutions of fresh milk and compounds thought to be key to the flavor properties and they were found to be similar to the stored UHT milk indicating that the compounds evaluated may have played some role in the flavor of UHT milk. Overall, this study was very thorough in examining the volatiles present in UHT milk and considering the importance of defining aroma-active compounds.

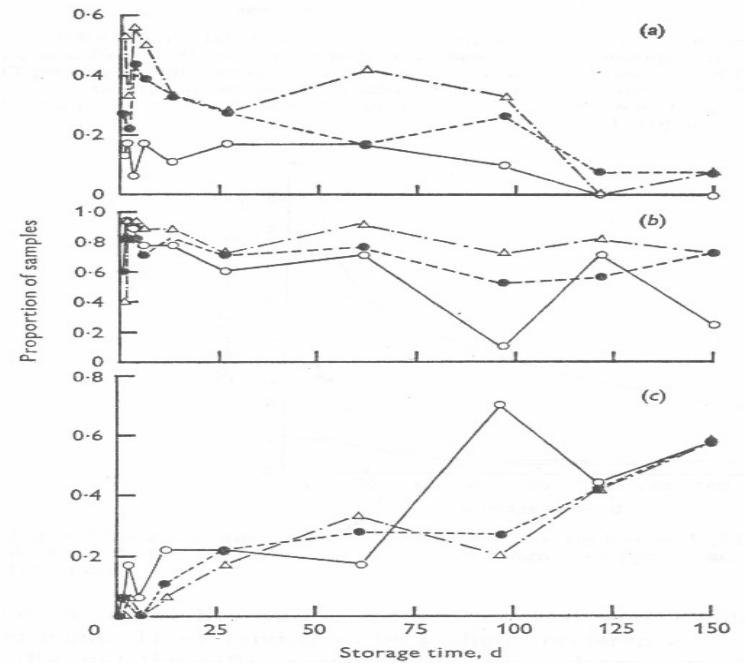


Figure 2.1. Changes in proportion of indirectly heated UHT processed milk samples judged to have (a) cabbage-like, (b) cooked and (c) stale + oxidized flavour during storage at 20 ± 4°C in the dark. [37] Initial O_2 levels: ○, high (8.9 ppm); ●, medium (3.6 ppm); Δ, low (1.0 ppm).

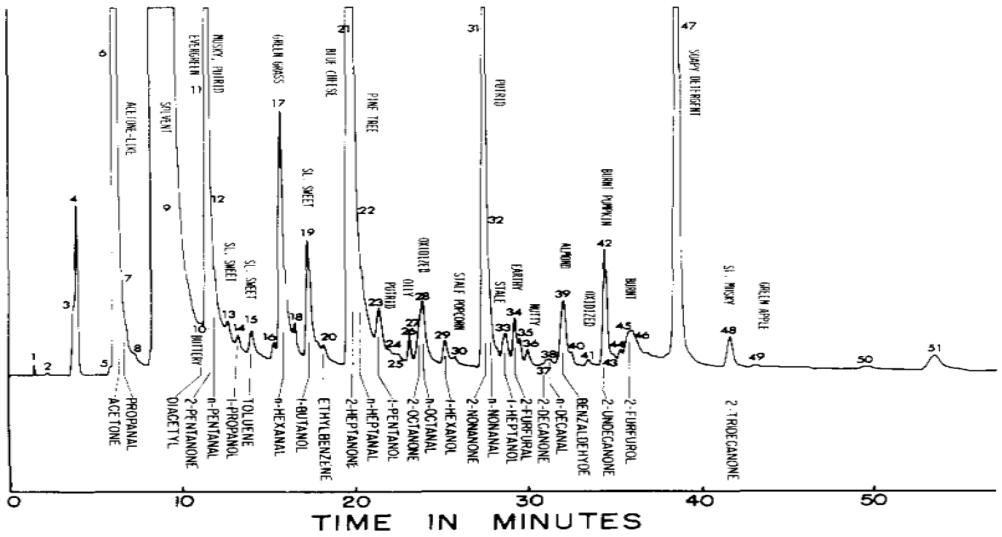


Figure 2.2. A typical gas chromatogram obtained from the flavor isolate of UHT milk stored 3 months at 35°C. [38]

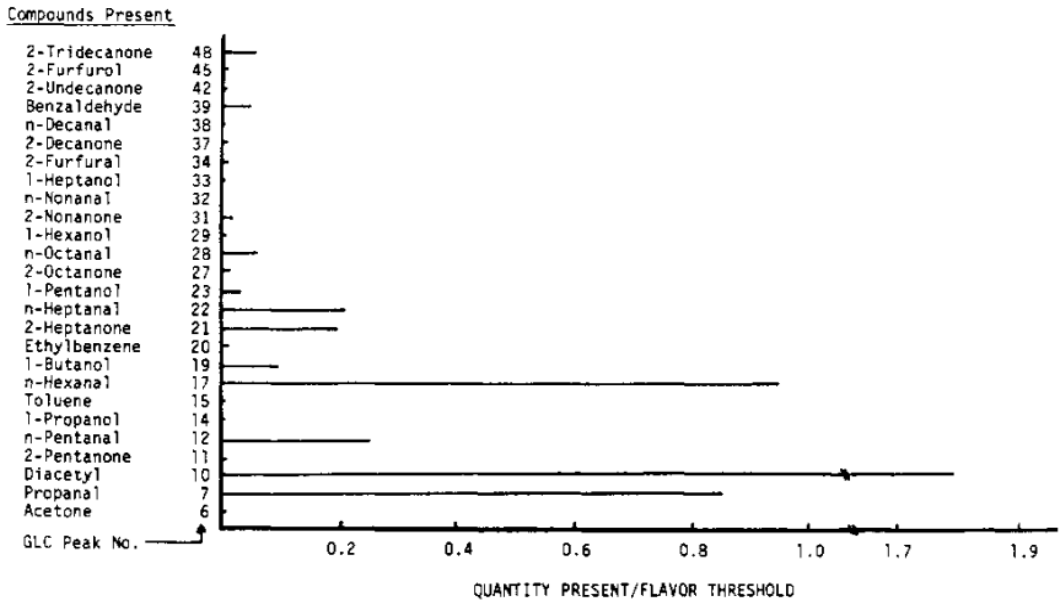


Figure 2.3. An aromagram obtained from UHT control milk stored at 35°C for 3 months [38]

In a similar study, Jaddou et al. [49] examined the volatile compounds present in heat-treated milk and raw milk. The data presented in Figure 2.4 illustrated that the concentration of dimethyl sulfide as well as other sulfur compounds increased right after processing in UHT processed and sterilized milk in comparison to raw milk. Hydrogen sulfide, carbonyl sulfide, methanethiol, dimethyl sulfide and carbon disulfide were found in UHT milk that was described by a sensory panel to have a cabbage-like aroma. After a month of storage, the milk became slightly stale and was described as having a cooked aroma. Throughout the storage period (112 days) all sulfur compounds decreased in concentration, with the exception of dimethyl sulfide, which increased slightly. Of all the off-flavors evaluated by panelists, astringent, stale, cooked and cabbage-like, decreased in UHT processed milk during storage. This study concluded the decrease in sulfur compounds was linearly related to the perceived cooked flavor intensity.

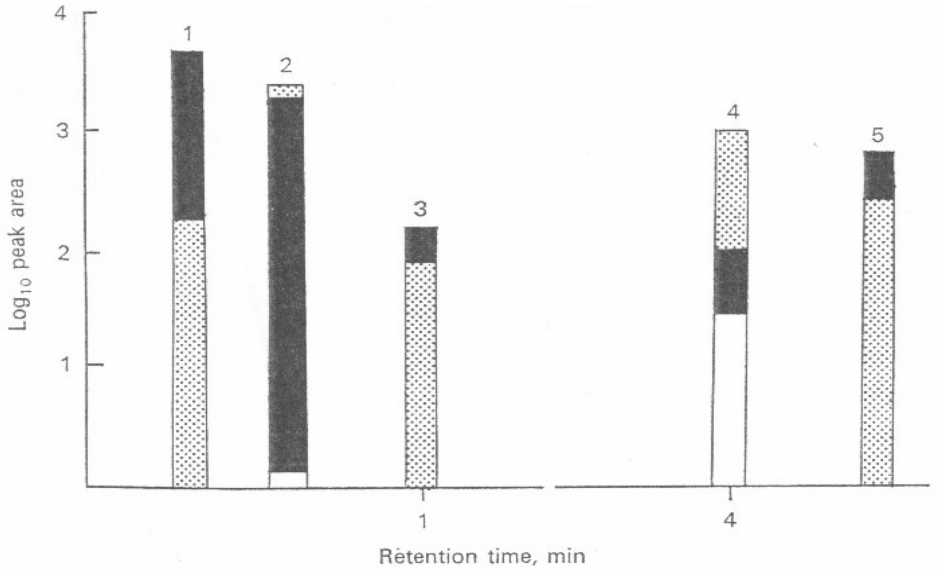


Figure 2.4. Effect of heat treatment on volatile S compounds in milk. 1, Hydrogen sulfide; 2, carbonyl sulfide; 3, methanethiol; 4, dimethyl sulfide; 5, carbon disulfide. Glass column 1.73 m x 4 mm packed with Carbopack B-HT-100. ■, 3 s ultra-high temperature; ▒, 90 s sterilized; □, raw milk. [29]

Research on UHT milk flavor expanded during the 1980s, including affects of packaging materials, microbial enzymes and raw milk quality in addition to the effects of storage. Badings et al. [50] compared the differences in key aroma compounds between pasteurized and UHT milk samples. Five levels of heat treatment were examined: low temperature-pasteurized (LP) milk heated to 75°C for 13 sec, high-pasteurized milk heated for 10 s at 82°C, two types of UHT processing, direct and indirect (UHT-d and UHT-i) heating, heated to 145 and 142°C respectively for 4.6 s, and in-bottle sterilization where UHT-indirect milk was heated for 30 min at 110°C. Both qualitative and quantitative differences between the low pasteurized, UHT-indirect and UHT-direct milk samples are shown in Table 2.1. A trained sensory panel was also used to monitor any differences in the flavor properties for these three milk samples. Overall, greater differences in the flavor properties were found between the UHT-indirect and LP milk than between the UHT-indirect and UHT-direct samples. Moderate differences in the volatile composition between UHT-indirect and low pasteurized milk samples were shown for: diacetyl, dimethyl disulfide, 2-hexanone, 2, 3, 4-trithiapentane, 2-undecanone, γ-dodecalactone and δ-dodecalactone, while larger differences were thought to be influenced by greater concentrations of 2-heptanone and 2-nonanone. Recombination models were created by addition of some of these compounds to low pasteurized milk and a flavor profile similar to that of UHT milk was observed. The flavor was furthermore examined over a period of 1 month for the direct-, indirect-UHT milks and the in-bottle sterilized milk. The only flavor to diminish was the cooked/cabbage-like flavor.

Table 2.1. List of components that contribute to differences in flavor between UHT-i. UHT-d and LP-milk [54]

Compound	LP [1]	UHT-i - LP [2]	UHT-i - d [3]
hydrogen sulfide		2	1
Acetaldehyde	1	-1	0
methane thiol		1	1
dimethyl sulfide	3	0	1
Diacetyl	2	2	1
3-methyl butanal	1	1	1
2-methyl butanal	1	0	1
Pentenone – 2		1	1
Pentanal		1	1
i-butyl mercaptan		1	1
methyl isothiocyanate		1	1
dimethyl disulfide		2	1
4-pentene nitril	1	-1	0
2-methyl butanol	2	-1	1
hexanone – 2	1	2	1
ethyl isothiocyanate		1	1
Hexanal	1	1	1
ethyl butyrate	1	-1	0
Furfural		1	1
2,4-dithiapentane	1	0	1
heptanone – 2		4	2
4-cis-heptanal	2	1	0
Benzaldehyde		1	0
2,3,4-trithiapentene		2	0
3-butenyl-1-isothiocyanate	2	-1	0
octanone – 2		1	0
Octanal		1	1
Unknown		1	0
Acetophenone		1	0
nonanone – 2		4	2
Unknown		1	1
Naphthalene		1	0
Unknown		1	1
2,4-trans, trans- nonadienial		-1	0
Benzthiazole		1	0
γ-octalactone		1	0
δ-octalactone		1	0
decanol – 1		1	1
undecanone – 2		2	1
γ-decalactone		1	0
δ-decalactone	1	1	0
tridecanone – 2		1	0
γ-dodecalactone		2	1
δ-dodecalactone	1	2	1

[1)] Only the most relevant aroma compounds of LP milk are given. Contribution: 1 = slight; 2 = moderate; 3 = strong; 4 = very strong. [2)] Difference in flavor contribution between UHT-i and LP milk (scale for difference): 1 = slight difference; 2 = moderate difference; 3 = strong difference; 4 = very strong difference. [3)] Difference in flavour contribution between UHT-d and UHT-i (for scale see 2). HT-i: UHT indirectly processed milk, UHT-d: UHT directly processed milk; LP: low temperature processed milk (75°C for 13 seconds)

The influence of the microbial load in raw milk prior to UHT processing on UHT milk flavor was also investigated. Gillis et al. [51] studied the effect of raw milk quality and off-flavor development in UHT processed milk, specifically looking at enzymes contributed by microbes in the milk prior to processing. McKellar [52] examined the relationship between off-flavor development in UHT milk and proteolysis from microbial enzymes and Andersson et al. studied the influences of microbial lipases and UHT milk flavor. Gillis et al. [51] observed that enzymatic activity was decreased by the UHT heat treatment, but it was not completely inhibited. As the raw milk quality decreased (in terms of initial microbial load), the number of samples that developed off-flavors within 70 days increased, indicating that the quality of raw milk influenced the shelf-life and flavor properties of UHT processed milk. Both Andersson et al. and McKellar [52] inoculated milk with *Pseudomonas fluorescens*, to examine the effect of active protein lipases. As a control, Andersson et al. [53] added a completely inactivated lipase enzyme (heated at 121°C for 4 h) to milk samples and performed sensory analysis, using triangle tests, which showed that after cold storage there was a significant difference between the treatment and control. Samples that contained active microbial lipases, post-processing, showed significant flavor changes at 8 -13 days, while the control did not show changes until 19 – 20 days. Bitterness developed in all treatment samples after 12 – 19 days, which suggested that residual heat stable lipases hydrolyzed the milk fat, resulting in a butyric/soapy off-flavor. Thus, it was concluded that the observed bitterness might have been caused by these lipases, although residual proteases activity could also contribute to the observed flavor changes.

McKellar [52] examined the effect of proteases in UHT and pasteurized milk as it related to off-flavor development. The microorganisms were added post UHT processing or pasteurization and results indicated that UHT milk was more susceptible to proteolysis than the pasteurized milk samples; potentially due to protein denaturation caused by the application of more severe heat treatment. Development of bitterness off-flavor in the UHT samples was observed, likely due to addition of proteases. Consequently it was suggested that the microbial quality of milk and presence of enzymes might have an impact on flavor development.

Consumer acceptability studies showed that UHT milk flavor is affected by different processing temperature and storage conditions. Hansen et al. [54] Bassette et al. [55], Jeon and Bassette [56] all studied these parameters with respect to UHT milk flavor. In all three of these studies, raw milk was processed at different time and temperature combinations, while Hansen et al. [54] and Bassette and Jeon [56] also investigated different storage temperatures on UHT milk flavor. While all three studies used sensory panels to evaluate flavor changes, Hansen et al. was the only one that used an untrained panel for acceptability of flavor attributes rather than measuring intensity. Hansen et al. [54] concluded that samples processed at 143°C for 6.9 sec or 149°C for 6.9 or 20.3 sec were considered acceptable by a consumer panel after 4 weeks of storage at room temperature and up to 20 weeks. Samples processed at 138 and 143°C for 20.3 sec were considered acceptable by the panel after 8 weeks and continued through 20 weeks. These results suggest that milk may be UHT processed using one of the above time-temperature combinations and remain acceptable to consumers.

In a further study on the characterization of UHT milk aroma Bassette and Jeon [56] showed there were very little differences in initial volatile content between the time-temperature profiles which were processed at 138-154^{o}C for 1.5-9.0 s. This could be due to the direct method, steam infusion, used for the heating milk samples, as it is considered a more 'gentle' processing method (in comparison to the conventional indirect procedure) for the production of UHT milk. As found in previous studies by Jeon et al. [48] the largest changes in concentrations (increase) throughout storage occurred in aliphatic aldehydes which showed a correlation with a decrease in consumer acceptability. The acceptability of UHT processed milk in this study dropped drastically during the 3 months of storage conflicting with the results reported by Hansen et al., [54] but the observed discrepancies may be due to differences in sensory panelists (trained versus untrained). Further studies done by Rerkrai, Jeon and Bassette [55] showed that as the cooked flavor dissipated, the stale flavor increased. There seemed to be no differences in off-flavor generation between processing at different temperatures (132.2, 143.3 and 137.2°C), but there were differences between storage temperatures (2 and 25°C), which had been shown previously [48]. Concentration of n-hexanal increased faster in samples stored at room temperature, as did acetaldehyde, 2-pentanone and 2-hexanone and 2-heptanone; once again there were not significant differences for the various processing temperatures within the same storage conditions. Aldehyde formation correlated well with stale flavor development and based on results, it was suggested that lipid oxidation might be an important off-flavor contributor.

Contarini et al., [57] using a different method for volatile isolation (dynamic headspace) than previously reported, characterized the volatile compounds in various

heat treated milk samples via a purge and trap technique. Overall, they found compounds similar to those previously identified by other researchers including: ketones, aldehydes, terpenes, and sulfur and aromatic compounds.

However, while previous research identified aldehydes as the most abundant class of compounds [55], this study found ketones to be the most abundant chemical class present. This may have been due to differences in processing or in the volatile extraction technique utilized. Methyl ketones typically form during β–oxidation of saturated fatty acids, followed by decarboxylation or through decarboxylation of β–ketoacids [58] whereas aldehydes can be produced from microbial growth, nonenzymatic browning or autoxidation of unsaturated fatty acids [45], [55], [59], [60].

Adhikari and Singhal [61] studied the changes in the flavor profile of UHT milk with respect to the Maillard reaction. In this study, the researchers examined 5-hydroxymethylfurfural (HMF), both free and total concentration during storage. They examined the relationship of dissolved oxygen and sulfhydryl compounds on the kinetics of the Maillard reaction. They reported a decrease in both total and free HMF initially during storage, but after 9 – 14 days the HMF levels increased to higher than the initial levels. The Maillard reaction continues throughout storage of a product and can be important for off-flavor generation of products stored at ambient or elevated temperatures (e.g. UHT milk). Thus, storage temperature seemed to have a positive effect on the rate of development of HMF, i.e. at 22°C the HMF developed slower than at 37°C. Adhikari and Singhal [61] also indicated that free sulfhydryl compounds seemed to have an inhibitory effect on HMF. Concentrations of both compounds (-SH and HMF) reduced sharply initially, but once levels of –SH groups reached the minimum levels the HMF

levels began to increase. Furthermore, the dissolved oxygen content was directly related to the reduction of the –SH groups. Adhikari and Singhal [61] also used a trained panel for sensory evaluation of the UHT milk and found that as HMF increased and –SH decreased the stale flavor profile increased. This indicated that stale flavor may be related to the Maillard reaction or possibly the flavors from the –SH compounds masked the stale flavors. Adhikari and Singhal [61] used HMF to determine the extent of the Maillard reaction in UHT processed milk, which is not always the most appropriate method, as the yield of HMF is only about 10% of the content of the Amadori compound [62], [63]. In addition, HMF may be formed during sample workup or analysis as an artifact skewing the results. The flavor compounds developed in the advanced stage of the Maillard reaction likely contribute to the characteristic flavor of UHT processed milk as well as off-flavors during storage [61].

More recently, Iwatsuki et al. [64] analyzed the aroma of raw, UHT and pasteurized milk using aroma extract dilution analysis (AEDA). Multiple pyrazine-type compounds were detected and found to be influenced by heat. Various Maillard reaction compounds were identified in both pasteurized and UHT milk samples and included 2, 6-dimethylpyrazine, 2-ethyl, 3-methylpyrazine, 2-ethylpyrazine, methional, furfural, skatole and other pyrazines. Concentrations of 2, 6-dimethylpyrazine, 2-ethyl, 3-methylpyrazine, 2-ethylpyrazine and methional increased with higher thermal treatment suggesting that they are heat induced. Other compounds identified in large quantities included hexanoic acid, octanoic acid and decanoic acid. AEDA was also used to evaluate differences between raw milk, LTLT milk, HTST milk and UHT milk. Changes were noted in 1-octen-3-one, nonanal and some methyl ketones and some lipid oxidation

products. This study effectively compared different heat treatments and the level of differences in odor active compounds.

As sulfhydryl compounds were thought to be responsible for the initial cooked flavor present in any thermally processed milk product, many studies focused on their effect on the flavor profile of heat treated milk. Heat treatment denatures serum proteins and liberates volatile sulfides and sulfhydryls [65].

Many researchers have identified numerous sulfur-based compounds in milk, both pasteurized and UHT. In 1956, Patton, Forss and Day [66], showed that as milk is treated more severely, the concentration of dimethyl sulfide increases (see Figure 2.5 [67]). Free sulfhydryls (-SH) were also studied by Adhikari and Singhal [68]and Andersson and Öste [69]. Both studies found –SH to be responsible for cooked flavor that arises during heat treatment.

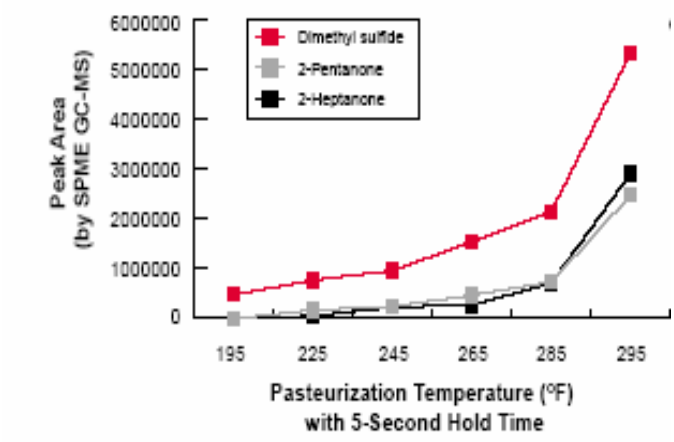


Figure 2.5. Production of methyl ketones and dimethyl sulfide as a function of heat treatment

Valero et al. [78] studied changes in flavor and volatile compounds throughout storage of both full fat and skim UHT milk investigating the effect of fat in off-flavor development. Using a sensory panel they found the full fat milk samples to be more acceptable initially and throughout storage. There were a greater number of volatile compounds identified and considered important in the skim UHT milk as compared to the full fat UHT milk. Valero et al. [78] determined the importance of compounds based on concentration found during GC analysis which can be misleading when it comes to flavor perception. Methyl ketones were the most abundant compounds in the full fat UHT milk, while hydrocarbons were considered most important in the skim milk samples. Although these compounds were present in the highest concentrations, as noted above, they are not necessarily the most important for ~~the~~ sensory perception. The concentration of methyl ketones was higher, while diacetyl was lower in the full fat milk samples. The volatiles identified in this study have been shown in previous research [29, 36, 47, 49, 50, 53, 54, 59-61, 65].

Simon, Hansen and Young [27] and Simon and Hansen [70] studied the impact of packaging material on shelf-life and flavor of ultra-pasteurized (UP) milk. The major difference between these two methods (UP vs. UHT) is how the product is packaged; UHT milk is filled aseptically thus maintaining commercial sterility while UP milk is not. UP milk possesses an extended shelf-life (up to 4 months) as compared to pasteurized milk. The flavor of UP milk has not been studied to the extent of UHT or pasteurized milk. However, information on the packaging materials and how they relate to the flavor may correlate between UHT and UP milk samples. Chemical interactions affecting flavor can arise through migration from outside into the package, migration from the package to

the food and from the food into the package. All of these may impact the flavor. Simon et al. [27] found that the higher the thermal processing temperatures, the greater number of sulfur compounds were present with the greatest differences in methanethiol, dimethyl disulfide and dimethyl trisulfide. Also, the levels of these compounds were found to decrease throughout storage. This correlates well with what has been shown by other researchers. With respect to packaging, they found that barrier and foil boards packaging retained the most sulfur compounds, because they minimize gas transfer through the packaging materials. The Maillard reaction and Strecker degradation products, 2-methyl propanal, methylpyrazines, and 2- and 3-methyl butanal, were also found in many samples and the concentration increased during storage. However, during storage certain aroma compounds, such as methylpyrazines may be absorbed into the packaging material, and this can impact flavor as well [27]. Simon and Hansen [70] examined how packaging materials (standard, barrier and foil boards) can affect shelf-life and flavor of UP milk. Ideally packaging will provide a barrier against transmission of light, organic flavor compounds and oxygen from the air into the package [70]. The taste panel showed the flavor of UP milk deteriorated fastest ($P>0.05$) in standard packaging, while UP milk packaged in barrier or foil board packaging maintained flavor longer. This information can be applied to UHT milk because it is packaged in similar types of material. One issue that may arise is the stability of the sulfur compounds, as they are most abundant in UHT processed milk samples.

In a more recent study Colahan-Sedestrom and Peterson [18] examined the effect of epicatechin addition prior to processing, on the inhibition of odor active compounds and the development of the characteristic cooked flavor associated with UHT milk. Odor

active compounds in this study were determined using GC/O as well as aroma extract dilution analysis. Aroma extracts of milk samples were separated into two fractions, acidic and neutral/basic and results from GC/O and AEDA are shown in Table 2.2 and 2.3.

Table 2.2. Aroma active compounds of UHT processed milk detected during Aroma Extracted Dilution Analysis- Neutral/basic Fraction [18]

		LRI			FD values	
no.	compound	DB-5	DB-Wax	odor[a]	control[b]	treatment[b] (0.1% EC)
1	methional[c]	909	1452	potato	128	4
2	2-acetyl-1-pyrroline[d]	922	1336	roasted/popcorn	32	8
3	1-octen-3-one[c]	978	1299	mushroom/earthy	1	0
4	Furaneol[d]	1056	2041	caramel	32	16
5	inknown 1	1083		foul	8	1
6	2-isopropyl-3-methoxypyrazine[d]	1098	1434	roasted/dairy	8	1
7	2-acetyl-2-thiazoline[c]	1100	1759	roasted/popcorn	32	8
8	(*E,Z*)-2,6-nonadienal[d]	1145	1577	fatty	4	2
9	(*E*)-2-nonenal[d]	1162	1535	oxidized	2	2
10	(*E,E*)-2,4-nonadienal[d]	1211	1688	cardboard	2	1
11	unknown 2	1220		acidic	1	1
12	unknown 3	1239		caramel-like	2	0
13	δ-octalactone[c]	1283	1973	peach	4	2
14	unknown 4	1298		floral	4	2
15	decanoic acid[c]	1382	2276	soapy	4	4
16	skatole[c]	1389	2504	foul	8	4
17	γ-decalactone[c]	1485	2139	sweet/perfumey	2	2
18	δ-decalactone[c]	1494	2204	coconut	64	32
19	γ-6-(*Z*)-dodecenolactone[c]	1651	2385	perfumey	32	16

[a] Odor descriptions at the GC-sniffing port during GCO. [b] Raw milk (with or without 0.1% EC) was preheated to 87.8 °C, homogenized (2500 psi) at a final heat of 141.1 °C, then held for 6 s (final temperature was 138.3 °C), and immediately cooled to 16.7 °C. [c] Compound positively identified (LRI, MS, authentic). [d] Compound tentatively identified (LRI, odor).

Table 2.3. Aroma active compounds of UHT processed milk detected during Aroma Extracted Dilution Analysis- Acidic Fraction [18]

		LRI			FD values	
no.	compound	DB-5	DB-FFAP	odor[a]	control[b]	treatment[b]
20	furfural[c]	833	1455	roasted	16	2
21	isobutyric acid[c]	792	1554	foul	4	4
22	butyric acid[c]	824	1628	sour	512	512
23	3-methylbutanoic acid[c]	875	1651	sour/acid type	128	128
24	hexanoic acid[c,d]	1020	1847	sour/cheesy	512	512
25	heptanoic acid[c]	1076	1946	sour	1	1
26	octanoic acid[c,d]	1186	2052	foul	1	1
27	nonanoic acid[c,d]	1277	2127	sour	1	1
28	homo-Furaneol[e]	1146	2079	burnt sugar	1	1
29	sotolon[e]	1099	2177	caramelly	32	32
30	4-chloro-3,5-dimethylphenol[c]	1384	2561	perfumey/floral	256	256
31	phenylacetic acid[c]	1255	2581	floral	32	32
32	3-phenylpropionic acid[c,d]	1344	2608	floral/perfumey	128	128

[a] Odor descriptions at the GC-sniffing port during GCO. [b] Raw milk (with or without 0.1% EC) was preheated to 87.8 °C, homogenized (2500 psi) at a final heat of 141.1 °C, then held for 6 s (final temperature was 138.3 °C), and immediately cooled to 16.7 °C. [c] Compound positively identified (LRI, MS, authentic). [d] Compound present in both N/B and acidic fractions. [e] Compound tentatively identified (LRI, odor).

Epicatechin was found to suppress the development of cooked flavor in UHT milk when added prior to processing. Looking at the suppression of specific odor active compounds epicatechin had little to no effect on the overall formation of lipid oxidation products (2,6-nonadienal, (E)-2-nonenal and (E,E)-2,4-nonadienal) but Maillard reaction derived compounds, such as methional, 2-acetyl-1-pyrroline and 2-acetyl-2-thiazoline were greatly inhibited, suggesting Maillard reaction as the main pathway of the formation of cooked off-flavor in UHT processed milk.

Maillard Reaction

History

The Maillard reaction was first observed in 1912 by the French physician and chemist Louis-Camille Maillard [71]. He noticed the formation of brown pigments as glucose reacted with glycine in aqueous systems and he suggested that the formation of pigments involved an initial interaction between amines and saccharides forming Schiff's base adducts. Maillard's discovery was recognized decades later, when researchers from University of California at Berkeley cited his work in their publication on non-enzymatic browning of fruit and fruit products in 1941. The complexity of Maillard reaction has had many food chemists dedicate their career to the elucidation of related reaction pathways and have focused on understanding the implications of this reaction on nutritional and sensorial quality of food products. More recently the implications of Maillard chemistry on regulatory biology and health have been an active area of research. For example

mutagenic and toxic Maillard Reaction Products (MRPs) formation, such as acrylamide [72] as well as protein modification via glycation (*in vivo*) and related pathological complications associated with diabetes and aging [22].

Implications in Food and in vivo

The Maillard reaction is not one simple reaction but rather a very complex series of interconnected reactions pathways between reducing sugars and amino acids. A testament to its complexity is the fact that though it has been studied for almost a century still many pathways are unknown. It is a ubiquitous reaction in food, particularly during processing at elevated temperatures and during storage for prolonged periods of time. It influences the flavor development, color formation and nutritional value and its impact can be both desirable or not. For instance, the Maillard reaction is mainly responsible for the pleasant aromas developed when roasting cocoa and coffee, or when baking bread and meat but it can also lead to the formation of off-flavors such as stale or cooked notes [12]. Similarly, browning of cocoa and meat is usually a desirable quality, but color formation in spray-dried milk, UHT processed milk or dehydrated products, is undesirable. [21],[62],[72–74]. Apart from color and flavor generation, the Maillard reaction can also affect the nutritional quality of food. For example, the Maillard reaction can utilize lysine, which is an essential amino acid, in formation of ε-Amadori compounds and also render metals such as copper and magnesium biologically unavailable via formation of melanoidin\metal complexes [63]. Furthermore, Maillard reaction products (MRPs) have both toxicological and protective aspects. Numerous MRPs have been reported to be carcinogenic and mutagenic compounds. Very recently,

acrylamide formation emerged as a major focus of research regarding to the carcinogenicity of MRPs [72]. However, MRPs also have been found to have antioxidant activity, antimutagenic, antibiotic and antiallergenic effects [75]. Melanoidin can also complex with mineral ions, and in this way, prevent them from catalyzing lipid and other oxidation reactions. Consequently a better understanding of the mechanisms that influence MRP generation in processed food systems would provide the food industry with the ability to more effectively control food quality.

As mentioned earlier, owing to the abundant presence of proteins and carbohydrates in both living organisms and food, Maillard reaction is also known to play an important role in regulatory biology and age related pathology [74]. Maillard reaction or glycation occurs in our body when reducing sugars and amino compounds react and has been associated with the pathogenesis of cardiovascular diseases, diabetes, cataract as well as aging. The medical and technological implications have made it the subject of numerous studies. In the biomedical field, Maillard reaction products (MRPs) that occur *in vivo* are termed Advanced Glycation End products (AGEs). AGEs are thought to be related to the biochemistry of aging and diabetes [76].

In diabetics, AGE formation has been suggested to provide some explanation for the accelerated chronic complications in comparison to normal insulin functioning people. Elevated levels of sugars and reactive sugar fragments in diabetic patients results in increased formation and AGEs (Carbonyl stress), thus altering the functionality of biological molecules and resulting to uremic, renal and other pathological complications [77].

The theory of Maillard reaction induced aging has been hypothesized to have the similar process as in Maillard reaction in food. ARPs are generated by condensation reactions between sugars (mostly glucose) and amino acids in proteins. Then the degradation of ARPs leads to generation of reactive carbonyls such as 3-deoxyglucosone, 1-deoxyglucosone or 4-deoxyglucosone along with other small dicarbonyl compounds such as glyoxal and methylglyoxal via retroaldol mechanisms. These reactive carbonyl species attack amines, nucleic acids, peptides or proteins via Schiff's base formation leading eventually to altered functionality of long-lived biological molecules.

A pathway for the formation of N-ε-carboxymethyllysine (CML), a well-known AGE found in humans, is presented in Figure 2.6 as an example**.** Overall, reactive carbonyls can be biologically generated from oxidative reaction of numerous precursors. Upon formation, it can react with a lysine residue in a protein to generate CML. These reactive carbonyl compounds (i.e. glyoxal) are associated with structure-function alteration of protein *in vivo* [78].

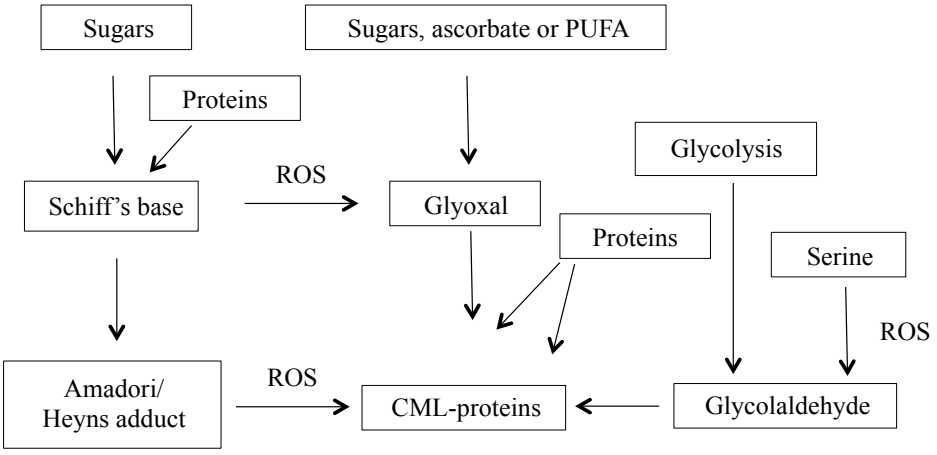


Figure 2.6. Formation of Carboxymethyllysine (CML) in vitro adapted from [79]. PUFA: polyunsaturated fatty acids.

Maillard reaction scheme and mechanism

The Hodge sequence (shown in Figure 2.7) is still widely accepted today as the general reaction mechanism of the Maillard reaction and is usually divided into 3 stages: (1) the initial stage, (2) Amadori degradation and (3) the advance stage (generation of color). The initial stage is begins by a condensation reaction between an amino acid and a reducing sugar followed by a series of transformations to yield an Amadori (N-substituted 1-amino-1-deoxy-2-ketose) or a Heyns product.

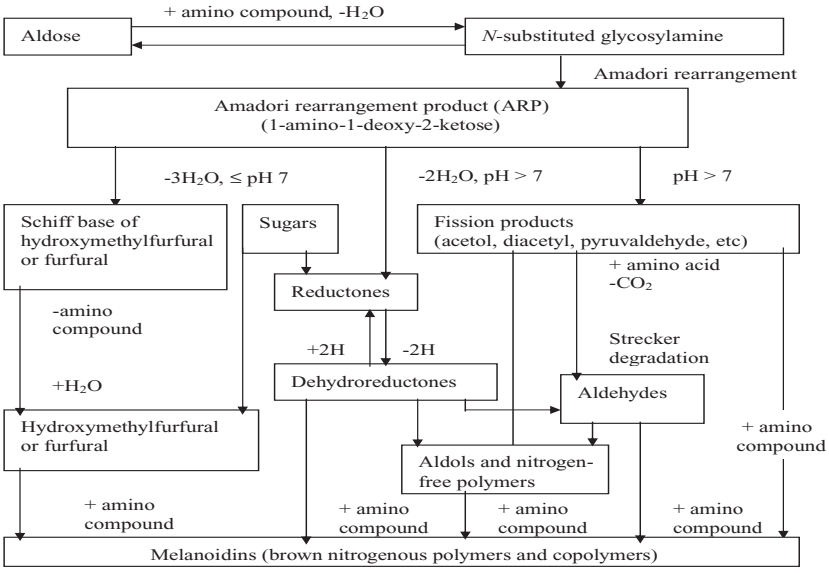


Figure 2.7. Hodge's scheme of Maillard reaction

In stage 2, Amadori degradation (Figure 2.8), is where flavor compounds are generated. Numerous reactions occur simultaneously thus, stage 2 is highly complex involving sugar dehydration, sugar fragmentation and amino acids degradation. This stage also yields products with absorbance or with yellow color. Briefly, Amadori compounds degrade to 1- or 3-deoxyosones by 1,2- or 2,3 enolization pathways

depending on the pH of the system. Acidic conditions favor 1,2 enolization and basic favor 2,3 enolization pathways. 1- or 3-deoxyosones then undergo further fragmentation by retroaldol condensation reactions (generally accepted as a major mechanism of sugar fragmentation) to yield reactive C_2, C_3, C_4, and C_5 α-dicarbonyl and α-hydroxycarbonyl sugar fragments. These sugar fragments are important precursors of Maillard reaction products (MRPs) that affect the three main pathways of flavor generation (A,B and C) in the Maillard reaction (Figure 2.8) [80–82] and thus, the sensorial and nutritional quality of thermally processed food can be associated with their fate. The reactivity of these carbonyl compounds has been largely related to their electrophilic properties.

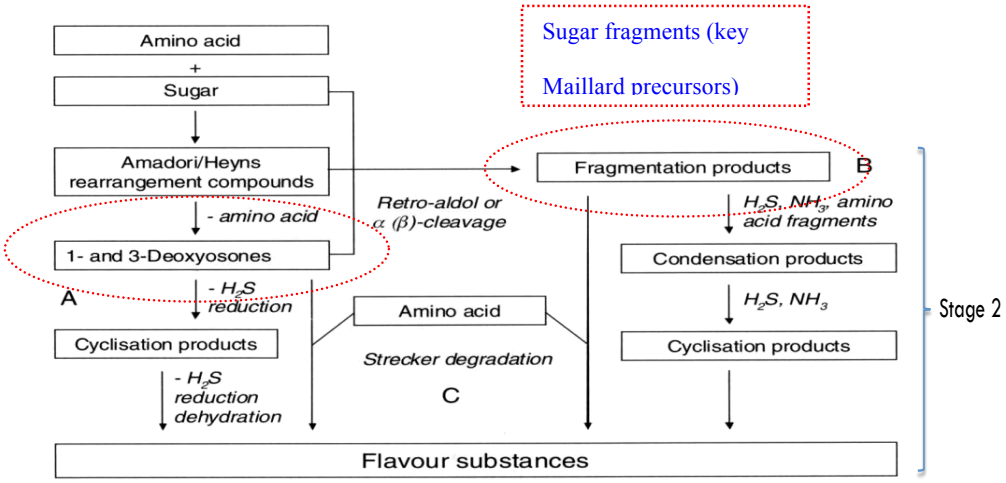


Figure 2.8. Hodge's scheme of Maillard reaction, Focus on Stage 2 and flavor generation. Pools generating reactive sugar fragments (circled) affect all three pathways of flavor generation.

A more recent reaction scheme (see Figure 2.9) of the Maillard chemistry has been proposed that describes the reaction through the formation and interaction of chemical pools [83]. This scheme was categorized based on the independent degradation of the amino acid and reducing sugar in addition to the conventional degradation in which

the Amadori (ARP) or Heyn's (HRP) rearrangement products are formed. Overall three primary fragmentation pools are formed from the specific precursors. Thus, the amino acids will generate an 'amino acid fragmentation pool'; and sugars and Amadori products will generate a 'sugar fragmentation pool' and an 'Amadori and Heyn's fragmentation pool' respectively. Progressive interactions between these pools lead to 'interaction pools' and three types of interactions pools have been identified: self-interactions, secondary interactions, and multiple interactions. It becomes apparent from this scheme that reactive C_2, C_3, C_4, and C_5 α-dicarbonyl and α-hydroxycarbonyl sugar fragments can be generated directly from sugar fragmentation as well as from degradation of Amadorri products and contribute to the reactive pools that will impact the pathways of Maillard reaction under specific conditions. Thus, providing further support to the notion that reactive carbonyl species (sugar fragments) as key intermediates will greatly affect the sensorial and nutritional quality of the final product.

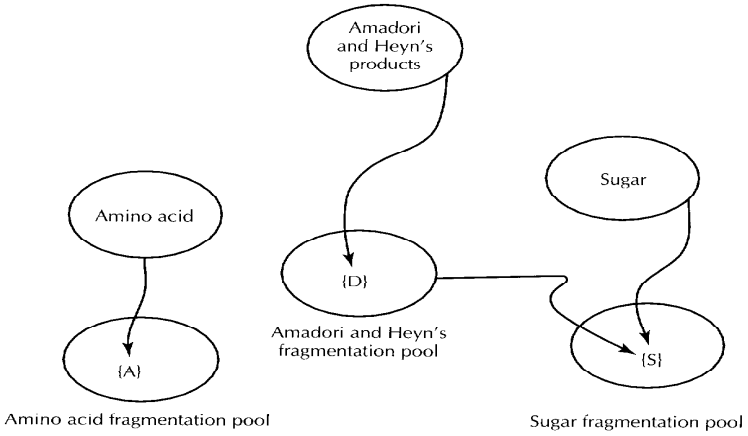


Figure 2.9. Generation of primary fragmentation pools- the building blocks of the Maillard reaction from the components of the parent pool. Source [83].

The advanced stage of the Maillard reaction involves the formation of brown nitrogenous polymer melanoidin by aldol condensation, aldehyde-amine condensation, etc. On a quantitative basis, melanodins species are the main MRPs generated and are additionally the least defined Maillard reaction products. The limited information regarding melanodins arises from two reasons, the lack of recognition of the significance of the products on food quality or biology as well as the lack of analytical technology to characterize such complex structures.

Early work by Tressl [84] assumed pyrroles and furans to be the major monomers that generate melanoidin compounds based on the high reactivity of these classes of compounds. On the other hand, Cämmerer [85] suggested that melanoidin species are composed of intact carbohydrates and non-aromatic carbohydrate fragments generated in the rather early stage of Maillard reaction (e.g. Amadori and 3-deoxyglucosone). These monomers were hypothesized to polymerize by aldol reactions. The most comprehensive information on melanodin structure was obtained recently by ^{13}C and ^{15}N nuclear magnetic resonance (NMR) studies [86], [87]. Based on this work, pyrrole or furan monomers might have been overemphasized in the past, and linear carbohydrate structures were supported as the major building blocks of melanodin subunits.

Polyphenols-Flavonoids

Phenolic compounds can be classified into simple phenols, hydroxybenzoic acid and hydroxyl cinnamic acid derivatives, flavonoids, stilbenes, lignans and tannins. Flavonoids include the following classes: flavones, flavonol, flavonones, flavononol, isoflavone, flavanol, anthocyanidin (Figure 2.10) [88]. Flavonoids are polyphenolics

compounds of plant origin that occur as secondary metabolites (compounds that are not essential nutrients in plant growth). However, they play an important role during the interaction of plants with their environment.

The bioactivity of phytochemicals includes functions such as protection against oxidants, microbial infection and visual attractors. The beneficial effects exerted by polyphenols on plants are known to extend to humans if consumed in adequate amounts. Numerous polyphenolic compounds have been associated with health-promoting properties such as anticarcinogenic, antibiotics, antidiarrheal, antiulcer, and anti-inflammatory agents and in treatment of hypertension and other diseases [89–91]. In foods, polyphenolic compounds have traditionally been researched with regard to the effect on nutrition or their sensory properties (astringent or bitter tastes). Recently attention has been focused on the abilities of flavonoids to reduce the formation of reactive carbonyl species generated by oxidative (lipid oxidation) and non-oxidative pathways (protein glycation). Wu and Yen [92] reported that protein glycation of human hemoglobin was reduced using a variety of flavonoids including catechin, epicatechin, epicatechin gallate, epigallocatechin gallate. The extent of glycation was followed in three different stages – early, middle and last stage. Glycation was dramatically reduced by the presence of flavonoids in all three stages with 66.1% reduction reported in first stage by rutin.

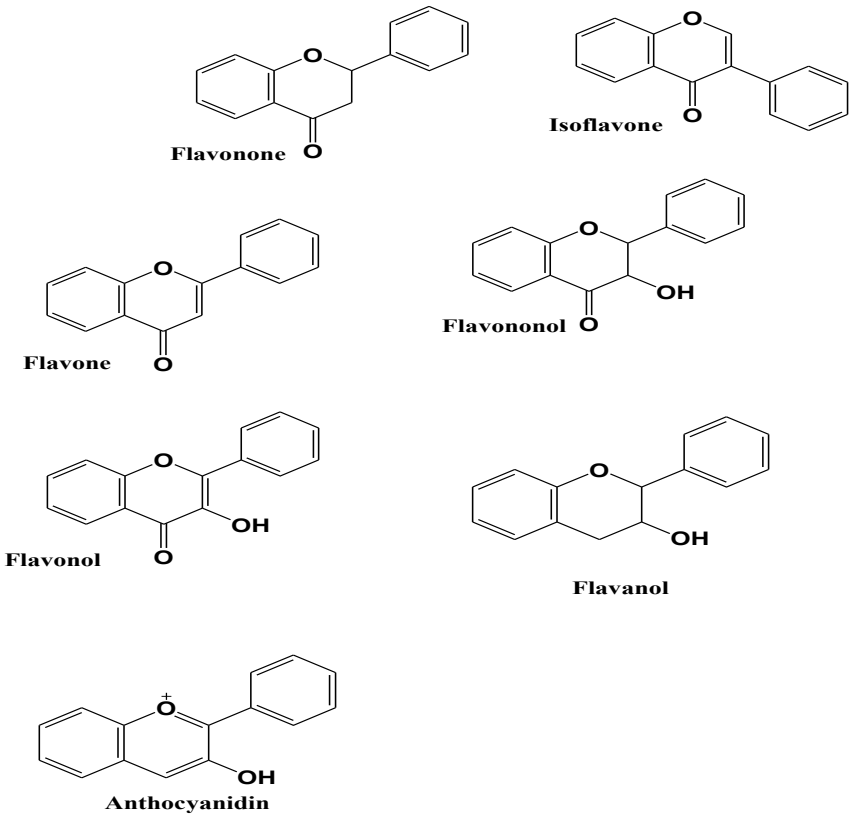


Figure 2.10. Structure of flavonoids adapted from source

Polyphenols and Maillard Chemistry

Polyphenols have also exhibited the ability to enter the Maillard reaction cascade and reduce the formation of MRPs. Flavonoids have been extensively reported to be trapping agents of reactive carbonyls species such as methylglyoxal and glyoxal which contribute to protein glycation in vitro and in vivo [37], [38], [40] thus, reducing the formation of AGEs. Structure requirements of flavonoids for inhibition of protein glycation were examined by [93] and it was proposed that the number of hydroxyl groups, especially those at 3'-, 4'-, 5- and 7-positions, are strongly related to their

inhibitory effects. Among different kinds of flavonoids, the activities of flavones were more potent than those of the corresponding flavonols, flavanones and isoflavones.

The effects of flavon-3-ols on MRPs generation has been extensively examined by Peterson et al. in both model systems [34–36], [94] and food [18], [95]. Epicatechin was shown to reduce flavor generation in a Maillard model reaction under both aqueous and low moisture conditions. Flavan-3-ols were suggested to function as carbonyl trapping agents via electrophilic aromatic substitution reactions – primarily on the A-ring (figure 2.11) going against the prevailing radical scavenging mechanism [80], [96], [97]. To further support the reaction mechanism of 'carbonyl trapping' by electrophilic aromatic substitution reactions, the influence of three phenolic structures (epigallocatechin, 1,2,3-trihydroxybenzene, and 1,3,5-trihydroxybenzene) on the time course generation of methylglyoxal, a reactive carbonyl, from a simple glucose/glycine model Maillard reaction over 30-min was monitored. The two simple phenolic compounds represent the A- and B-ring chemistry of the flavan-3-ol. 1,2,3-trihydroxybenzene would be more redox-active than 1,3,5-trihydroxybenzene [94] whereas, 1,3,5-trihydroxybenzene would be a more reactive trapping agent of sugar fragments based on the polyhydroxyl ring configuration. The meta-hydroxyl/alkoxyl configuration of 1,3,5-trihydroxybenzene activates the ortho- and para-positions resulting in increased reactivity of the electrophilic aromatic substitution reactions (e.g. with sugar fragment carbonyl compounds). 1,3,5-trihydroxybenzene was shown to be notably more reactive as compared to 1,2,3-trihroxybenzene for reducing the concentration of methylglyoxal strongly suggesting that phenolic-sugar trapping reactions were or are the main mechanism by which flavan-3-ols impacted Maillard chemistry. A suggested

mechanism, adduct formation between flavonoids and sugar fragments, is presented in Figure 2.11.

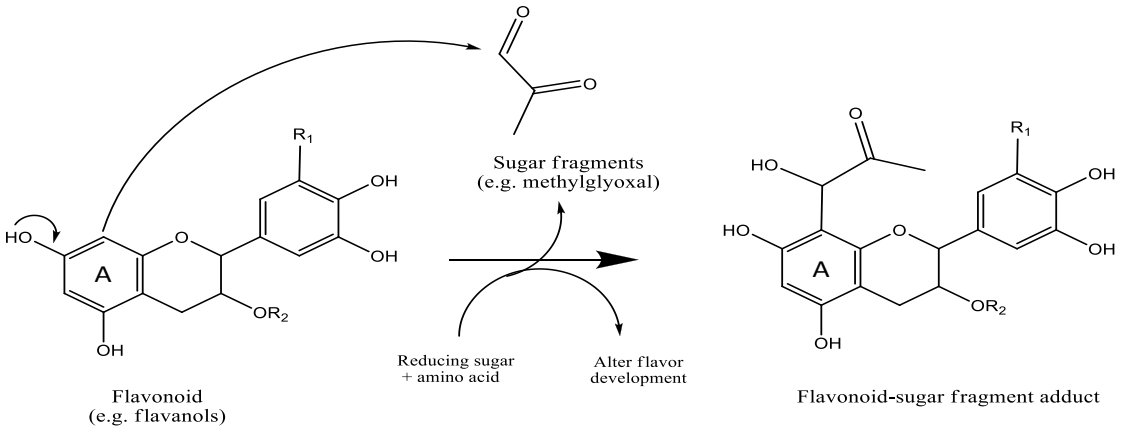


Figure 2.11. Flavonoid-sugar fragment reaction, R1 = H (epicatechin) or OH (epigallocatechin) , R2 = H (epicatechin) or gallate (epicatechin gallate).

These results were also applied to food systems; addition of epicatechin decreased the perception of off-flavors generated in processed milk, such as ultrahigh temperature (UHT) and spray-dried milk. Colahan-Sedestrom and Peterson 2005 [18] reported that the addition of 0.1% epicatechin to raw milk, prior to UHT processing, reduced the generation of Maillard-type aroma compounds that are responsible for imparting cooked aromas to the processed milk. After a comprehensive analysis of the flavor profile of treatment and control UHT milk samples (with and without added epicatechin respectively), methional, 2-acetyl-2-thiazoline and 2-acetyl-1-pyrroline were identified as the compounds of interest associated with the cooked flavor of UHT milk. Additionally it was shown that these important off-flavor contributors, generated mainly via Maillard reaction pathways, were reduced by 32-, 8-, and 4-fold, respectively, in treatment samples as compared to those of control. These results demonstrated the potential use of

phenolic compounds as pre-processing treatment, but further work is needed to understand the mechanisms involved and how phenolic compounds alter off-flavor generation in UHT milk during processing and storage. The addition of epicatechin at a 0.1% level prior to spray drying process was also shown to reduce the formation of ortho-aminoacetophenone in stored (17 months) milk powder. Ortho-aminoacetophenone is the primary compound responsible for stale off-flavor in SMP and it is Maillard-type product [95].

Besides flavonoids, other types of polyphenolic compounds such as caffeic, ferulic and chlorogenic acids were also shown to have dramatic inhibitory effects on aroma compound generation in a coffee roast model system [85].

Flavor Analysis

Food flavor can be defined as the combination of multiple sensations such as taste, smell (aroma), sight (food appearance), feeling (food texture and the trigeminal sensations). Aroma is the sense of smell perceived by the olfactory cells located in the nasal cavity and historically has been the focus of numerous flavor studies and without question is a critical component of flavor perception.

The analysis of volatile compounds in foods and beverages is an intricate task, as numerous chemical classes of compounds exist at minute concentrations and with different chemical properties and affinities, which greatly complicates the selection of the aroma isolation procedure.

The sample complexity can lead to poor chromatographic resolution hindering identification and quantification of important compounds and more complications arise

from the lack of detectors as sensitive to volatile compounds as the human nose (found to detect odors at concentrations of 10^{-19} moles [98]).

Methods of Aroma Extraction

Solvent extraction

Volatile compounds are extracted from the sample and partition into the solvent based on their solubility. The type of solvent chosen for extraction is dependent upon the solubility of the volatile compounds anticipated to be present in the sample. Isolation of volatiles from samples by solvent extraction has advantages and disadvantages.

One of the main advantages is that the extraction process can be carried out using simple devices such as, separatory funnels, or even using techniques such as agitation, centrifugation or an orbital shaking and no intricate equipment is required.

The limitation of solvent extraction arises from the fact that no solvent exists that will efficiently extract all classes of volatile chemical compounds of interest within a food product, thus it is extremely hard to obtain a comprehensive aroma profile. Another limitation of solvent extraction is that along with volatile compounds fat will also be extracted which further complicates the analysis steps as fat is decreases the life of the analytical columns and additionally results in poor chromatography. Emulsions can also be formed and must be broken in order for the volatile compounds to be successfully extracted, a task not always easy or feasible. Additionally, solvent extraction can be time consuming, and extracts may be contaminated with solvent contaminants.

Most foods contain fat, thus techniques such as Solvent Assisted Flavor Evaporation have been developed to overcome this obstacle. Emulsion formation may

also be resolved by adding salt to the sample (only if the solvent is less dense than water) or via centrifugation to aid in breaking the emulsion.

Solvent Assisted Flavor Evaporation

The SAFE method is a very effective isolation technique for trace analysis and addresses two important considerations during volatile extraction; minimal artifact formation and alteration of the native volatile compounds and non-volatile compounds will not be transferred. Figure 2.12 [99] shows an assembled SAFE system, the system is completely sealed and a vacuum is drawn through number 18. The left side (numbers 11, 15, 17) of the figure is placed in a warm water bath and sample is let in through the stopcock (number 5). Due to the high vacuum, all volatiles are immediately pulled to the right side of the system, where they are collected in a liquid nitrogen cold trap. The SAFE method was shown to be very efficient in extracting volatiles from solvent and systems containing 50% fat but it has major disadvantages as it is laborious, time consuming, requires complicated set-up and expensive glassware and it is difficult to reproduce as controlling the applied vacuum and flow rate of sample is nearly impossible with the current set up.

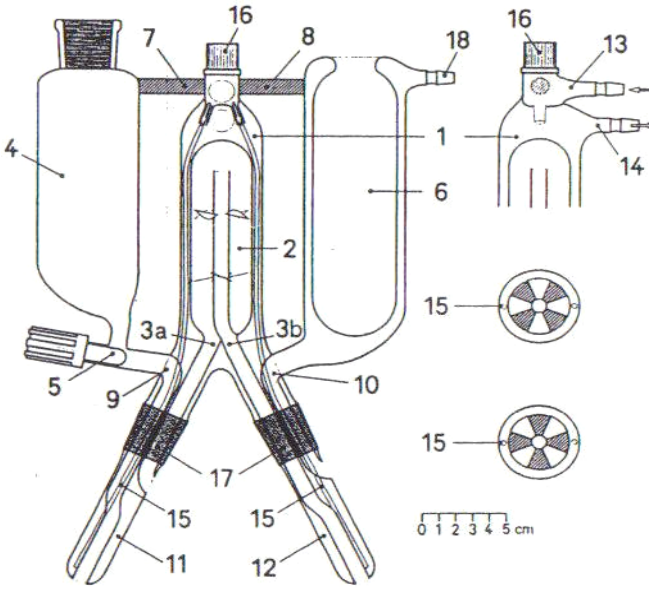


Figure 2.12. Schematic view of the distillation unit used for SAFE [1] Water jacketed area; [2] central head equipped with splash guard; [3a] vapor inlet; [3b] vapor outlet; [4] dropping funnel; [6] cooling trap; [7] connector; [8] connector; [9] inlet tube; [10] opening to vacuum; [11] inlet leg; [12] outlet leg; [13] water inlet; [14] water outlet; [15] polyethylene tubes to guide water flow; [16] cap; [17] ground joints; [18] vacuum pump hook-up to the apparatus. Source [100]

Methods for Aroma Characterization

Quantitation of any compound of interest can be done by performing recovery studies and utilizing techniques such as addition of internal or external standards, in order to test the precision of the quantitative analysis method. When the sample preparation methods applied prior to analysis are not robust and reproducible then it is impossible to determine the amount of the compound of interest the sample contained before extraction with the above-mentioned techniques. Unfortunately the latter is the case when high vacuum techniques such as SAFE are used for sample preparation, as it is nearly

impossible to control and exactly reproduce all the conditions. The best way to overcome this problem is the use of stable isotopes.

Isotope addition at known concentration prior to sample preparation is the best approach as the isotope is the most similar in terms of physical and chemical properties to the compound of interest. Although addition of stable isotopes for quantification has many advantages it also has one major drawback; buying these compounds can be extremely expensive to the point it becomes inhibitory and in-house synthesis can also be costly, laborious and time consuming.

Evaluation of aroma-active compounds can be done analytically or through sensory analysis. Regarding the analytical characterization, typically volatile aroma extracts from solvent extraction methods are analyzed by capillary gas chromatography. This technique is the most widely utilized method for identification and quantification of volatile compounds. For compound separation capillary columns that are coated with a thin layer of liquid stationary phase are used. Separation of volatile compounds is based on two mechanisms; the interaction of volatiles with the stationary phase of the column and the volatility of compounds. The type of detector used to distinguish the individual components of the mixture may vary based on the scope of the analysis and the required sensitivity.

Gas Chromatography-Flame Ionization (GC-FID) is the most commonly used GC detector for analysis of organic compounds as it responds to carbon-carbon and carbon-hydrogen bonds. It is a robust detector thus is a great tool for quantification but it provides no structural information making identification of compounds an intricate task [100], [101].

Gas Chromatography-Mass Spectrometry has assisted in the identification of volatile compounds in food products tremendously. Utilization of a mass selective detector allows molecular information about an unknown compound to be determined and later used for identification by using the characteristic ionization fragmentation pattern of a target compound [102]. It is important to note that the characteristic ionization fragmentation pattern does not result in a positive identification, and at least one other mode of analysis such as the compound's linear retention index and comparison with available standards is needed for conformation. The most commonly used ionization methods are Electron Ionization (EI) and Chemical Ionization (CI) [103] and the most common mass analyzers for GC/MS are quadropoles, ion traps and Time of Flight (ToF) instruments [100], [104].

Analysis of Precursors and Intermediates of The Maillard reaction

Characterization and quantification of flavor precursors and intermediate compounds usually involves a different approach as compared to aroma analysis since precursors and intermediate compounds are not always suitable for GC analysis or that approach might not be optimal depending on the scope of the study. Alternative sample preparation and analytical techniques that have been successfully used include Solid Phase Extraction (SPE) for sample clean up and concentration and Liquid Chromatography (LC) systems coupled with fraction collection systems for isolation and purification of analytes of interest and mass selective detectors for identification and quantification.

SPE uses the affinity of solutes dissolved or suspended in a liquid mixture for a solid (or commonly referred to as stationary phase) through which the sample is passed. The result is that, the desired analytes or undesired impurities in the sample are retained on the stationary phase and sample clean up and concentration is achieved prior to analysis [105].

LC chromatography techniques such as UPLC and HPLC are used to separate complex mixture of compounds with the purpose of identifying, quantifying and purifying the individual components and has been used extensively in analysis of food systems [106]. Both techniques rely on the pressure applied on a liquid solvent to load a sample mixture onto column packed with chemical absorbents, where the separation occurs. The difference between the two is the applied pressure as UPLC utilizes higher pressure to improve chromatographic separation and increase sensitivity. Both UPLC and HPLC separation is influenced by the liquid solvent (mobile phase) and the packing material on the column (stationary phase). Additionally factors such as pressure and temperature of the system can influence the separation efficiency thus; careful selection of those parameters and method development for optimal separation and subsequently isolation, identification and quantification.

Coupling of liquid chromatography with mass spectroscopy provides an excellent tool for quantification and identification of compounds and it can also provide information about chemical structures. For LC/MS analysis the ionization of the sample can be done in a variety of different ways but Electrospray Ionization (ESI) is the most commonly utilized method. ESI ionization is one of the ‘soft’ ionization processes, which means that predominantly the degradation of the analyte of interest is prevented. After

ionization the mass analyzer sorts the ions based on their mass to charge ratio. Single quadropole and triple quadropole mass analyzers are often used in this type of analysis with the latter allowing the study of fragmentation patterns of compounds of interest and provides invaluable structural information [104].

Chapter 3

Control of Maillard-type Off-Flavor Development in Ultra High Temperature Processed Milk By Phenolic Chemistry

The application of phenolic compounds to suppress Maillard chemistry and related off-flavor development in UHT processed bovine milk during processing and storage was investigated. Five phenolic compounds at a dose of 1.7mM were examined for structure-reactivity relationships (catechin, genistein, daidzein, 1,2,3-trihydroxybenene, and 1,3,5-trihydroxybenene). The levels of key transient Maillard reaction (MR) precursors (C_2, C_3, C_4, and C_5 α-dicarbonyls and α-hydroxycarbonyls) and select off-flavor markers (methional, 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline) were quantified by LC/MS/MS and GC/MS-ToF, respectively and stable isotope surrogates were utilized as standards. The addition of phenolic compounds prior to UHT processing significantly reduced the concentration of MR precursors and off-flavor compounds compared to control sample ($p<0.05$). Notably, the food phenolic compounds with the more activated A-ring for aromatic electrophilic substitution reactions, catechin and genistein had the strongest effect on suppression the off-flavor markers as well as reactive carbonyl species in comparison to daidzein. Albeit unique structure reactivity was noted among the different phenolic compounds analyzed. Sensory studies were also in agreement with the analytical data; lower cooked flavor intensity was observed for off-flavor recombination model samples of the catechin treated UHT milk (versus the control UHT milk). Furthermore, based on consumer acceptability studies, the catechin treated

UHT milk was rated significantly higher than the control sample (Fisher's LSD = 0.728) showing improved palatability.

Introduction

Milk, like many foods and commodities, is mandated to undergo thermal processing during production as stated in the code of federal regulations (21CFR131.3 and 131.110) to improve microbial safety. Additional benefits of thermal processing are improved product stability and shelf life, which facilitates distribution and storage. For milk a number of different pasteurization methods are available such as, High Temperature-Short Time (HTST), Extended Shelf Life processing and Ultra High Temperature (UHT) processing, each technology utilizing different time-temperature profiles. Aseptic UHT processing of milk yields a commercially sterile product, with high stability in terms of microbial spoilage, and the longest shelf life of 6 months to 1 year. It also allows for non-refrigerated storage and transportation, widening distribution opportunities and reducing potential energy requirements. Despite the significant benefits of UHT products, high temperature treatment also yields a product with reduced flavor, and nutritional quality as well as undesirable color development creating a barrier to consumer acceptance [16–18]. Pasteurized milk, which is typically consumed in the US market, has as a slightly sweet flavor, with weak aroma attributes and a pleasant mouth feel and aftertaste [15]. Any alteration to this “clean” milk flavor profile is considered a product defect. Research done by researchers at Cornell University showed a direct correlation between flavor quality of milk and the level of consumption [19]. Blake, M. R. et al. [20] also reported UHT milk had a lower consumer liking compare to other less

severe thermally processed samples (i.e. pasteurized milk). A seven point hedonic scale was used and HTST and UHT milk had an average score of 5.8 and 3.6 respectively.

The negative flavor changes in UHT milk previously reported to develop during heat treatment and ambient storage have been associated with the Maillard reaction (MR) and lipid oxidation (LO), [26], [27], [107] Recent work by Colahan-Sederstrom, P.M. and Peterson, D.G. [18] identified three important off-flavor compounds that contributed to the "cooked" off-flavor of UHT milk, specifically 2-acetyl-1-pyrroline, 2-acetyly-2-thiazoline and methional. This indicated the MR was an important pathway of off-flavor development during thermal processing and a point of control in order to develop flavor improvement strategies for UHT milk and milk-based beverages. The MR is well-known to impact food flavor, color and moreover toxicity and nutritional value [12], [21–23], [73–75], [108]. Additionally it is known to play an important role in regulatory biology, diabetes and cardiovascular and age related pathology [74], [109]. Therefore, a better understanding of the mechanisms that influence Maillard reaction products (MRP) generation in processed food systems would provide the food industry with the ability to more effectively control quality and also potentially improve the nutritional attributes of food.

Phenolic compounds have been previously shown to inhibit Maillard-type flavor generation pathways and reduce off-flavors [18]. Totlani and Peterson [36] have shown that flavan-3-ols function as trapping agents (via electrophilic aromatic substitution reactions, primarily on the A-ring) of key transient Maillard precursors (reactive carbonyl species (RCSs)- C2, C3, C4, C6 sugar fragments) and consequently suppress the generation of MRPs. Flavanols such as catechin are found in several plant products, most

notably in tea, cocoa and wine. Their antioxidant capacity has been extensively studied and high consumption of those phenols has been associated with decreased risk of cardiovascular disease, cancer as well as metabolic regulation and weight loss, [110–115] The use of natural products to control food reactions that negatively impact flavor and nutrition would additionally benefit the food industry as consumers are increasingly aware of food labels and there is an increased demand for natural products.

The objective of the study was to investigate phenolic structure-reactivity relationships on the mechanisms of off-flavor development in 1% UHT milk.

Materials and Methods

Chemicals: (+)-catechin ≥ 97% (TCI America), genistein ≥ 98% (Shaanxi Schipar Biotechnology Co. Ltd., China), daidzein ≥ 98% (Shaanxi Schipar Biotechnology Co. Ltd., China), phloroglucinol 97% (1,3,5-trihydroxybenzene) (Aldrich, Saint Louis MO), pyrogallol (Fluka, Saint Louis MO), acetoin ≥ 96% FCC, food grade (FG) (Aldrich, Saint Louis MO), acetol 90% (Aldrich, Saint Louis MO), 2,3-butanedione 97% (Aldrich, Saint Louis MO), 3-deoxyglucosone (TRC, Toronto research chemicals, Ontario Canada), glyoxal 40% solution in water (Sigma, Saint Louis MO), methylglyoxal 40% solution in water (Sigma, Saint Louis MO), methional 97% FG (Aldrich, Saint Louis MO), 2-acetyl-2-thiazoline ≥ 96% FG (Aldrich, Saint Louis MO), o-phenylenediamine 99.5% (Aldrich, Saint Louis MO), o-ethylhydroxylamine hydrochloride (Aldrich, Saint Louis MO) re-purified (1×)diethyl ether (DEE) was prepared using 99.9+% anhydrous diethyl ether (Fisher Chemicals) and distilled via packed column distillation (30 cm, 4 mm glass beads, distilled in a 45 °C water bath). 2-acetyl-1-pyrroline was not commercially

available but was extracted from pandan leaves (*Pandanus amaryllifolius Roxb*.), which were obtained from the local market. The purification procedure is described below.

Milk processing conditions: Raw skim and whole milk were obtained from the University of Minnesota Joseph Warthesen Food Processing Center (Saint Paul, MN) and mixed to yield raw milk with 1% fat content. Samples were ultra high temperature aseptically processed using a MicroThermics HTST/UHT Direct Steam Injection & Indirect Tubular Processing System with water final heat (DIP-W), a homogenizer and aseptic filling station. Processing conditions were as follows: the unit was first sanitized using chlorine solution (100ppm) followed by a clean water wash at 121.1^{o}C (250^{o}F) for 1hr. The milk was homogenized, then processed at final temperature of 140^{o}C (284^{o}F), holding for 6sec to ensure commercial sterilization and immediately cooled to 15 °C, and filled in a sterilized laminar flow hood into previously sterilized 1L amber bottles with Teflon-lined lids.

Characterization of off-flavor generation in UHT processed milk: Identification and quantification of MR precursors: methylglyoxal, glyoxal, 3-deoxyglucosone and diacetyl (dicarbonyls) as well as glycolaldehyde, acetoin and acetol (hydroxycarbonyls) were quantified using the synthesized [116] stable isotope surrogate internal standards $^{13}C_4$-acetoin and $^{13}C_4$-diacetyl. 0.5mM of each internal standard was added to 5ml of milk, followed by 500uL of 10% trichloroacetic acid. The samples were then vortexed for 1 min, centrifuged at 3904g for 20min at 4^{o}C (Beckman Coulter, Allegra X-22R) and the

supernatant was collected. Solid Phase Extraction (SPE) method was used to isolate the compounds of interest according to Kokkinidou S., Peterson D.G., 2013 [117].

A derivatization method was used to produce the more stable quinoxilines and o-ethyloximes (derivatization products of dicarbonyl compounds) and samples were analyzed using an Acquity UPLC system (Binary solvent manager, Sample manager and Column heater) coupled with a Quattro Premier XE micromass mass spectrometer (Waters Co. Milford, MA). An Acquity UPLC 2.1x100mm BEH Phenyl 1.7um column with a VanGuard 2.1x5mm BEH Phenyl 1.7um pre-column were used for separation and all experiments were performed in triplicate. Mass spectrometric conditions were as follows: desolvation temperature, 300^{o}C; source temperature, 120^{o}C; capillary voltage, 3.8kV; desolvation gas, 600L/min; cone gas, 50L/min. Analytes were detected using electrospray positive ionization-multiple reaction monitoring (MRM) using methods previously developed and reported by Kokkinidou S., Peterson D.G., 2013 [117].

Generation of Phenolic-Maillard Reaction Standards: Model reactions were used to generate standards of Maillard-phenolic products as previously described by Totlani and Peterson (2006). A 50ml saline phosphate buffer (pH 7.4) reaction system containing 10mM of catechin and methylglyoxal, glyoxal, or 3-deoxyglucosone was incubated in a shaking water bath at 37^{o}C for 1hr. After incubation, 5ml of the reaction mixture was spiked with an internal standard (butyl paraben, 5uL, 2% solution in methanol) and loaded on a preconditioned 500mg DSC-18 cartridge (Supelco, Bellefonte, PA). The cartridge was then washed with 5ml of acidified water (0.1% acetic acid) and eluted with 1ml of methanol.

The isolate was subsequently filtered (iso-disk PTFE filter, 25mmx0.2um, Supelco, Bellefonte, PA) and further purified using a RP-HPLC/MS-ESI-fraction collection system that consisted of a Shimadzu, binary solvent management system (LC-10ADvp), degasser (DGU-14A), autosampler (SIL-10vp) UV detector (SPD-10Avp) (λ=254nm), a Waters ZMD 2000 mass spectrometer, a Waters Fraction Collector III (Waters Co. Milford, MA) and two semi-preperative LC columns, a Varian Dynamax Pursuit 5 Diphenyl (250x10.0mm) and a Varian Dynamax Prsuit 5 C18 (250x10.0mm).

Chromatography was performed with a flow rate of 3.7ml/min with the following linear gradient conditions (solvent A; 1mM ammonium acetate pH 5, Solvent B; methanol): solvent B at 20% (0-15min), 55% (15-61min), 80% (61-62min) 80% (62-66min), 100% (66-72min), 20% (72-73min) and maintain at 20% (73-81min). One hundred ul of isolate was injected and the target analytes were collected by MS guided fractionation (m/z reported in Table 3.1). The operational conditions of the mass spectrometer were as follows: desolvation temperature, 250^{o}C; source temperature, 100^{o}C; capillary voltage 3.2kV; and a scan range m/z 100-1200.

Fragmentation spectra of the purified analytes were determined by LC/MS/MS techniques and are shown in Table 3.1. Fragmentation spectra were obtained to monitor these analytes in milk by MRM methods as well as to support hypothesized structures of these adducts.

Isolation and Quantification of Off-Flavor Compounds In Milk: Three off-flavor compounds previously identified in UHT milk (3) were monitored and included methional, 2-acetyl-2-thiazoline (2A2T) and 2-acetyl-1-pyrroline (2AP). Quantification

was conducted by stable isotope surrogates standards of each compound. The labeled compounds were synthesized as previously described [$^2H_3$]-methional [118] , [$^2H_3$]-2-Acetyl-1-pyrroline [119] and [$^2H_4$]-2-Acetyl-2-thiazoline [120].

The identity and purity of each labeled standard was confirmed by MS analysis in both electron impact (EI, 70eV) and chemical ionization (CI, at 115eV with isobutane as reagent gas); analyzed with a Hewlett Packard GC (HP 5890 Series II) coupled with an MS detector (HP 5972). The concentration of each labeled standard was determined based on GC analysis in comparison to authentic compounds.

Volatile isolates of the samples were prepared by solvent extraction (adapted from [18]). In brevity, prior to extraction [$^2H_4$]-2A2T, [$^2H_3$]-2AP and [$^2H_3$]-methional were added as internal standards at levels of 5ug standard/kg milk. Each UHT milk sample (990 g) was extracted 5× with re-purified diethyl ether (5 × 175 mL) in a 250 mL Teflon centrifuge bottle (Nalgene, Rochester, NY). Initially sodium chloride (59.4 g/165 g of milk) was added to the milk samples, followed by solvent addition, and the mixture was subsequently agitated for 1 h with an orbit shaker table at 40% of maximum speed (model 3540, Labline Instruments, Inc., Melrose Park, IL). The samples were then centrifuged for 20 min at 4 °C and 5000g (IEC B-20A, Damon/IEC Division, Needham Heights, MA), the diethyl ether layer was carefully removed and extracts for each sample were pooled together, dried with anhydrous sodium sulfate, filtered, and concentrated to 100ml via packed column distillation (30 cm, 4 mm glass beads, distilled in a 40 °C water bath).

The concentrate was then subjected to high-vacuum distillation using a solvent-assisted flavor evaporation (SAFE) apparatus [121]. The isolate was further fractionated

into an Acidic (A) and Neutral/Basic (N/B) fraction. The N/B fraction was prepared by washing with 0.5 M NaHCO3 (3 × 120 mL). The organic layer was then collected, rewashed with a saturated NaCl solution (2 × 55 mL), dried with anhydrous sodium sulfate and it further concentrated to 350 µL using fractional distillation. The samples were stored at -80^{o}C prior to analysis by an Agilent 7890A GC (Agilent Technologies, Inc., Wilmington, DE) coupled with a TruTOF HT time of flight mass spectrometer (Leco Co. St. Joseph, MI).

Identification of Odorants: Positive identifications were made by comparing linear retention indices (LRI), mass spectra and were also compared to authentic compounds analyzed under identical conditions.

Purification of 2-acetyl-1-pyrroline: A 2AP standard was prepared (not commercially available) from an extract of pandan leaves (*Pandanus amaryllifolius Roxb*.) as previously described by Colahan-Sederstrom and Peterson (3). The reference compound was further purified using a two dimensional GC system coupled with a fraction collector.

The analytical system consisted of an Agilent 6890N GC equipped with two dean switch devices, a Gerstel Modular Accelerated Column Heater (MACH), an Agilent 7683 auto-sampler and injector and it was coupled with an Agilent 5973N MSD and a Gerstel preparative fraction collector (PFC). A RTX-5S ILMS (30mx0.25mmx0.25µm) was used as a primary column and a DB-wax (30mx0.25mmx0.25µm) was used as a secondary column. GC conditions for all runs were as follows: inlet temperature, 200 °C;

10 µL of sample injected in solvent vent mode (0kPa, 0.15 min); helium carrier gas ramp pressure mode (initial pressure 357kPa, hold 7.1min and then ramp to 10Pa at 999kPa/min) and a total flow of 3.9mL/min with a split 1:4 to MSD and 3:4 to PFC. MSD conditions were as follows: capillary direct interface temperature, 250 °C; mass range was 35-300 amu; The MACH heating profile for RTX-5S ILMS capillary column was 40 °C for 1 min, then ramped to 280 °C at 10 °C/min, and held for 2.35 min whereas for the DB-Wax the oven profile was 40 °C for 7.60 min and ramped to 230 °C at 10 °C/min, and held for 2 min. The valve-switching program for heart cutting and isolation of 2AP was as follows: valve 1 switched on at 6.29 min, directing the flow to the secondary column, and switched off at 7.10 min, then valve two switched on at 15.4min directing the flow to cryotrap of PFC for 2AP collection and switched off at 15.75min. PFC switching device was operated at 250 ^{o}C and the trap was kept at -70 ^{o}C. 2AP was extracted from the trap using food grade ethanol and further quantified using vinyl-pyrrolidine as a standard using an HP 5890 series II GC coupled with an FID.

Sensory analysis of UHT milk samples: Both consumer acceptability and descriptive analysis including off-flavor recombination models were conducted. For the consumer acceptability test, 49 panelists were recruited based on regular milk consumption and availability. One percent raw milk, with and without added 1.7mM catechin, was UHT processed and aseptically handled to produce a shelf-stable product. These samples were evaluated for acceptability after storing at 30C for 1 month. A commercial UHT 1% milk (Parmalat, Agropur Inc. Division Natrel, USA) was also used as an internal anchor for the evaluation. The two milk samples processed in our pilot plant were tested for

microbiological stability prior sensory testing. The samples were presented at room temperature in coded 3-ounce cups in randomized order. Room temperature water and unsalted crackers were used as palate cleansers. The panelists were asked to rank them on a nine-point hedonic scale (ranging from dislike extremely to like extremely). Differences among treatments were evaluated by analysis of variance (ANOVA) followed by Tukey's HSD pairwise comparison. All analyses were performed with Statistix Software (version 9). Significance was established at $P < 0.05$.

For the descriptive analysis trained panelists evaluated intensity of cooked off-flavor in milk samples. Seven graduate students from the Department of Food Science and Nutrition at the University of Minnesota were recruited for the analysis and were selected based on availability and willingness to participate. They received eight 1-hour training sessions focused on the cooked attribute of the milk samples. A cooked reference was prepared by heating 1% milk (Schroder Milk Co.) to 90°C and holding for 45 minutes. This cooked reference was mixed with varying amounts of pasteurized milk to create a range of cooked intensities. Samples were evaluated on a 15-point intensity scale, anchored on the left with "none" and on the right with "extreme". The panel agreed to use the following intensities as references: 100% cooked = 15; 75% cooked/25% pasteurized = 8; 50% Cooked/ 50% pasteurized= 4; 30% cooked/70% pasteurized = 2.

Panelists participated in three evaluation sessions, occurring over the period of one day. Five samples were presented to the panelists in randomized combinations of the following four samples: (1) pasteurized 1% milk (Schroder Milk Co.), (2) commercial 1% UHT milk (Parmalat), (3) off-flavor recombination model for simulation of control

UHT milk was prepared using pasteurized 1% milk (Schroder Milk Co.) spiked with 2AP (1.35ug/kg), 2A2T (0.95ug/kg) and methional (4.05ug/kg) and (4) off-flavor recombination model for simulation of UHT milk with added catechin was prepared using pasteurized 1% milk (Schroder Milk Co.) spiked with 2A2T (0.41ug/kg) and methional (0.88ug/kg) to simulate UHT milk (Figure 2) . The spiked-levels of off-flavor markers, in recombination models were determined after quantification in pasteurized 1% milk (Schroder Milk Co.) and addition of appropriate amounts for simulation of UHT processed milk samples.

The samples were presented at room temperature in coded 3-ounce cups with room temperature water and unsalted crackers as palate cleansers. Differences among treatments were evaluated by analysis of variance (ANOVA) followed by Tukey's HSD pairwise comparison. ANOVA was also used to monitor panelist performance. All analyses were performed with Statistix Software (version 9). Significance was established at $P < 0.05$.

Microbial Testing: Microbial analysis was conducted on both control and treatment samples using 3M Petrifilm, aerobic count (AC) plates, and coliform count (CC) plates, (3M Co., St. Paul, MN). For counts in 3M AC and CC plates, 1 mL of each dilution 1:10, 1:100, and 1:1000 of milk samples was plated using phosphate buffer, KH2PO4 (0.0425 g/L, adjusted to pH 7.2, Butterfield's buffer; Weber Scientific, Hamilton, NJ). Colonies were enumerated after incubating at 37^{o}C for 24h for coliforms and at 35^{o}C after 48h for aerobic bacteria.

Results and Discussion

Previously Colahan-Sederstrom and Peterson [18] used a trained sensory panel and demonstrated that addition of epicatechin to raw milk prior to UHT processing significantly reduced the cooked off-flavor intensity of the final product. The overall goal of the current study was to build on this prior work by investigating phenolic structure-function reactivity on related off-flavor pathways to further develop this ingredient technology as a strategy for product improvement. One percent milk was selected for evaluation, as it is the most consumed fluid milk product in the US. Although overall fluid milk consumption has steadily decreased over the past 3 decades, consumption of low fat milk has increased (in comparison to whole milk) as consumers became more aware of caloric intake and negative effects of saturated fat intake [122]. As an initial step, a consumer acceptability study was performed to assess the effectiveness of phenolic compound addition on increasing palatability and to test if the reduction in cooked flavor intensity previously observed by Colahan-Sederstrom and Peterson resulted in a positive change in the product acceptability, in comparison to a traditional aseptically UHT processed sample. Similar milk samples were prepared, as in the previous study [18], and further evaluated by a consumer panel (n= 49). Both the catechin containing UHT milk and control UHT milk were stored at 30^{o}C for 30 days, for accelerated aging (estimated 2-3 months at ambient storage conditions), prior to evaluation in the current study. A commercial UHT sample was also included as an internal anchor for the sensory evaluation. The liking and acceptability of the phenolic treated milk was reported to be significantly higher than both control and the commercial sample (see Table 3.2) indicating the negative flavor attributes of aseptic UHT milk are a

hurdle to utilizing UHT aseptic processing methods for fluid milk in the domestic market.

The observed increase in the liking score of UHT milk with catechin added prior to processing provided a further proof of concept for the application of phenolic compounds to improve UHT milk quality. To further develop this technology, it would be beneficial to understand more mechanistically the reactivity of phenolic chemistry related to off-flavor suppression for the optimization of the dose response. For example, select phenolic structures might be more reactive (effective in suppressing off-flavor) during thermal processing (ca. 140^{o}C) while others could be more reactive during ambient storage.

Five phenolic compounds (see Figure 3.1) were selected to examine phenolic structure function reactivity on the MR pathways and off-flavor development in UHT processed milk and the effectiveness of each structure on off-flavor suppression was evaluated immediately after processing as well as after storage. Three common food phenolic compounds catechin, (flavanol), genistein, daidzein (isoflavones), along with two simple phenols 1,2,3-trihydroxybenene, and 1,3,5-trihydroxybenene were selected.

The food phenolic compounds differ on the degree and position of hydroxyl substitution on ring A and B as well as the presence of a keto- group on the C-ring (isoflavones). The simple phenols were used to simulate different ring chemistry, more redox reactive (1,2,3-THB) or more 'trapping' reactive of carbonyl compounds by electrophilic aromatic substitution reactions (1,3,5-THB) to investigate the mechanisms by which phenolic compounds alter MR pathways. Among the food derived phenolic compounds, catechin would have the highest trapping reactivity, followed by genistein, then daidzein based on the position of the hydroxyl groups on the A-ring and electron

withdrawing groups on the neighboring C-ring (keto-group). Phenolic compounds were added at dose levels of 1.7mM, which was selected as this amount was reported [18] to be effective for epicatechin in order to suppress off-flavor development and not result in a detectable bitterness (a sensory attribute associated with polyphenols).

To quantitatively monitor off-flavor progression in UHT milk, products, the off-flavor compounds, as well as reaction intermediates of these compounds were targeted. For target off-flavor compounds, Colahan-Sederstrom and Peterson [18] identified off-flavor compounds utilizing gas chromatography-olfactometry dilution techniques and indicated three main Maillard-type compounds were related to the off-flavor of 1% UHT milk. These included methional, 2-acetyl-1-pyrroline and 2-acetyl-2-thiazoline. In the current study the contribution of these off-flavor compounds to the 'cooked' off-flavor attributes of UHT were further defined by sensory analysis using aroma recombination model systems.

The quantitative amount of each compound was determined immediately after processing (day 0) in UHT milk as well as in UHT milk manufactured with catechin (see Figure 3.2). Based on these off-flavor fingerprints, the corresponding aroma recombination models of each product were made in pasteurized (HTST) milk and evaluated for the cooked flavor intensity (see Table 3.3). The UHT aroma recombination model was rated very similar in cooked flavor intensity in comparison to commercial UHT milk and was significantly higher in ranking to both commercial pasteurized (HTST) milk and the model UHT milk that contained catechin. Overall these findings supported the utilization of these three Maillard-type off-flavor compounds as predictive chemical markers of cooked off-flavor associated with UHT processed milk.

The influence of different phenolic structures added prior to processing on the generation of cooked off-flavor both during thermal treatment (day 0) and after storage (30 day at 30^{o}C) is further illustrated in Figure 3.2; the concentration in the raw milk prior to UHT processing was also included for comparison.

Overall, the concentration of all three marker compounds (2AP, 2A2T, and methional) increased by both UHT processing and storage at 30^{o}C as compared to raw and pasteurized milk (Figure 3.2). Further comparison of the influence of phenolic addition on the suppression of off-flavor compounds, indicated the effects reported were variable and dependent on the type of off-flavor compounds, the phenolic structure added as well as on the temperature of the system (UHT processing temperature, 140^{o}C or storage, 30^{o}C).

For example regarding methional, catechin and daidzein slightly inhibited the amount generated during UHT processing, whereas the other three phenolics had no substantial effect. However, during storage, all phenolic compounds were reported to suppress the generation of methional, with genistein and catechin being the more effective, reducing methional by 80 and 75% respectively. This observation suggests that catechin is more reactive and thus effective for methional inhibition both during processing and storage. However, daidzein is more reactive during processing and genistein is more reactive during storage temperatures.

For 2A2T, the addition of phenolic compounds had little impact on off-flavor development during processing temperatures as compared to control UHT, but all effectively suppressed generation during storage. Catechin, genistein and daidzein reduced the concentration of 2A2T during storage by 42-53%, notably reaching

concentrations below the odor threshold of these compound (1ug/kg) with genistein being again the most reactive phenolic compound under storage conditions.

Finally for 2AP, most phenolic compounds had, again, negligible influence on off-flavor development during processing, albeit demonstrating a decreasing trend, except genistein, which, resulted in a slight increase. However, during storage, genistein and catechin were the most reactive and effective in suppressing off-flavor generation during storage; while daidzein, 1,2,3-THB, 1,3,5-THB had negligible effects.

It is also worth noting that overall 1,3,5 THB, which is more activated towards aromatic electrophilic substitution was more effective in reducing the concentration of off-flavor markers as compared to 1,2,3 THB thus, supporting the idea that one of the mechanisms by which phenolic compounds inhibit MR pathways and off-flavor generation is reactive carbonyl trapping. Based on these observations of unique structure reactivity of phenolic compounds the idea of utilizing phenolic mixtures as an off-flavor inhibition strategy seems promising but further work and optimization and validation experiments are needed.

To further explore the influence of phenolic structure reactivity on the generation of the Maillard-type off-flavor compounds (2AP, 2A2T, methional), RCSs, which are well-known intermediates of the Maillard reaction, were also quantified. The concentrations of dicarbonyl and hydroxylcarbonyl sugar fragments as influenced by UHT processing, phenolic addition, and storage are illustrated in Figure 3.3 and 3.4, respectively.

Overall UHT processing resulted in increased levels of RCSs when compared to both raw and pasteurized milk. This was expected as the ultra high temperature (UHT)

conditions would catalyze the MR and subsequently the generation of MR intermediates such as RCSs. Examination of the sugar fragments in the control UHT sample after storage indicated that two of the dicarbonyl compounds namely methylglyoxal and diacetyl increased whereas glyoxal and 3-deoxylglucosone decreased; all hydroxylcarbonyls increased (glycolaldehyde and acetol) or maintained similar levels (acetoin). The observed reduction in the concentration of glyoxal and 3-deoxyglucosone could be indicative of the high reactivity of glyoxal entering several reaction pathways leading to off-flavor formation and for 3-deoxyglucosone its further fragmentation to shorter chain sugar fragments further contributing to MRP generation.

In general, the addition of phenolic compounds prior to UHT processing resulted in reduced levels of MR precursors (RCSs) compared to the control UHT milk samples. The influence of phenolic addition on the reduction of RCSs, indicated that the different phenolic structures have unique reactivity and dependent on the structure of the dicarbonyl or hydroxycarbonyl compound, as well as on the temperature of the system (UHT processing temperature, 140^{o}C or storage, 30^{o}C).

All food phenolic compounds (catechin, genistein and daidzein) reported a significant effect ($p<0.05$) compared to control UHT milk on the levels of dicarbonyl compounds during processing and storage. Catechin, genistein and 1,3,5THB were the most effective phenolic treatments in reducing the concentration of glyoxal after 30 days of storage followed by diadzein. These observations were expected based on the activation of the phenolic structures towards aromatic electrophilic substitution (Figure 3.1). Genistein and 1,3,5THB were the most reactive phenolic compounds during UHT processing, demonstrating the highest reduction of glyoxal as compared to control UHT

milk.

Similarly for methylglyoxal, addition of catechin, genistein and 1,3,5THB had a significant impact after 30 days of storage and it is worth noting that both catechin and genistein were effective in reducing the concentration of methylglyoxal during storage to concentrations lower than observed right after processing (day 0) and markedly not significantly different than concentrations found in pasteurized milk demonstrating unique reactivity as compared to other phenolic treatments. 1,3,5THB was the most reactive phenolic compounds during UHT processing.

1,3,5THB was additionally the most reactive phenolic treatment in reducing the concentration of 3-deoxyglucosone both during UHT processing and storage, followed by catechin. Notably those two phenolic treatments resulted in 3-deoxyglucosone levels that were not significantly different or lower than these found in pasteurized and raw milk. The increased reactivity of 1,3,5THB as compared to catechin can also be explained by reduced steric hindrances. Daidzein and 1,2,3THB followed by genistein also resulted in a significant reduction of 3-deoxyglucosone during storage as compared to control UHT milk, but no effect during UHT processing. Overall genistein had reduced reactivity towards C6 sugar fragments. The reduced reactivity of both genistein and diadzein as compared to catechin and 1,3,5THB was expected due to the presence of a keto group on C-5 position on ring C that can decrease nucleophilicity of the activated ortho positions of ring A (C-8 and C-6) (Figure 3.1) and thus its sugar fragment trapping reactivity. The increased reactivity of daidzein as compared to genistein could potentially be attributed to reduced steric hindrance due to the absence of the hydroxyl group in C-5 position of the A-ring (Figure 3.1)

Diacetyl was effectively reduced during UHT processing by all phenolic treatments except diadzein, which was not significantly different as compared to control UHT milk demonstrating negligible reactivity for C4 sugar fragments. Catechin was the most reactive phenolic treatment in reducing diacetyl concentration during storage to levels below the observed ones at day 0 demonstrating again unique reactivity as for all other samples (control UHT and UHT with added phenolic treatments) diacetyl concentration continued to rises after day 0.

The effect of phenolic treatments on the levels of hydroxycarbonyl compounds also seemed to be guided by their unique structure reactivity but suppression effects of phenolic compounds on these RCSs was not as pronounced. All phenolic compounds significantly significantly ($p<0.05$) reduced the levels of glycolaldehyde with the more reactive phenols being catechin and genistein and 1,3,5-trihydroxybenzene. Acetoin was significantly reduced by only by catechin and genistein and 1,3,5-trihydroxybenzene. A reducing trend was observed when phenolic compounds were added prior to UHT processing but changes were not significant ($p>0.05$) as compared to control UHT milk. This reduced reactivity was anticipated since hydroxycarbonyl compounds are not as strong electrophiles thus are less likely to be trapped by phenolic compounds. From the three hydroxycarbonyls investigated, glycoldahyde is the most electrophilic and the one more likely to react with the activated nucleophilic sites and lead in adduct formation. That was supported by the results showing higher suppression effect of phenolic compounds on the levels of glycoaldehyde.

Overall based on predicted reactivity of the selected phenolic compounds towards aromatic electrophilic substitution reactions and RCSs trapping, catechin, as a flavanol,

was expected to be significantly more reactive as compared to isoflavones like genistein and daidzein. Based on the results in these study genistein demonstrated overall similar ability towards trapping RCSs as compared to catechin and this could potentially be explained by the increased auto-oxidation reactivity of catechin resulting in formation of quinones, which can lead to increased reactivity with milk proteins and other milk constituents and thus to reduced RCSs trapping reactivity.

Among the simple phenolic compounds, 1,3,5-THB was more effective in reducing the levels of RCSs, which is in agreement with previously reported findings [94]. 1,3,5-trihydroxybenzene was expected to be a more reactive trapping agent of sugar fragments than 1,2,3-THB based on the polyhydroxyl ring configuration. 1,3,5-THB has three electron-donating hydroxyl groups in the meta configuration on a benzene ring, which activate the ortho and para positions for electrophilic aromatic substitutions reactions at the three substituted carbon sites (e.g., RCSs). Consequently, the noted higher reactivity of 1,3,5-THB suggests that one of the mechanisms by which phenolic compounds alter the flavor generation pathways in UHT milk is the formation of adducts with these precursors as previously hypothesized by [35], [36]. The reduced observed levels of RCSs correlate with both analytical and sensorial data regarding the inhibition of off-flavor generation in UHT milk with added phenolic compounds as compared with traditional UHT milk.

The palatability of UHT milk treated with phenolic compounds (catechin) could be additionally improved due to reduction of RCSs concentrations, as these compounds are volatiles and have unique flavor characteristics. Diacetyl has been identified as an important contributor to the heated, cooked and fermented notes of UHT milk with a

flavor threshold of 12ppb (0.14uM) [123–125] and notably diacetyl concentration in control UHT milk after 30 days of storage was above its flavor threshold. Glyoxal and methyglyoxal have mild sour, pungent and slightly nutty aromas. Acetol is characterized as sweet and roasted or as having a yogurt-like flavor in emulsions and acetoin has a sweet, buttery, creamy aroma (34).

In order to further support the hypothesis of adduct formation as a mechanism for off-flavor inhibition, model reaction systems were prepared adducts between catechin and glyoxal, methylglyoxal and 3-deoxyglucosone were isolated and purified in order to develop analytical methods for their identification in UHT milk with added catechin. The most prominent products of these reactions were identified as catechin-glyoxal, catechin-glyoxal-catechin, catechin-methylglyoxal, methylglyoxal-catechin-methylglyoxal and catechin-3deoxyglucosone adducts. Their proposed structures based on reactivity and MS/MS fragmentation patterns (Figure 3.5) are shown in Figure 3.6. The parent ions of all purified products/adducts generated sibling ions of m/z 289, which corresponds to a catechin moiety along with fragments of m/z 245, 205 and 179 which are characteristic sibling ions of catechin fragmentation [126] confirming its presence in these products. The m/z 245 fragment may be generated after a neutral loss of CO_2, characteristic of negative ionization mode [127]. The m/z 205 fragment is most likely generated by loss of rings B and C [128] and m/z 179 is produced after an A-ring loss. This particular fragmentation pattern (loss of A-ring) supports the adduct formation between catechin and RCSs via electrophilic aromatic substitution at C-8 or C-6 of ring A as well as the proposed adduct structures since fragments that correspond to a loss of ring A with RCSs moieties were detected for all adducts. Namely, m/z 167 (A-ring-glyoxal), m/z 181 (A-

ring-methylglyoxal), m/z 253 (A-ring-methylglyoxal$_2$) and m/z 271 (A-ring-3-deoxyglucosone) were detected as sibling ions of catechin-glyoxal and catechin-glyoxal-catechin, catechin-methylglyoxal, methylglyoxal-catechin-methylglyoxal and catechin-3-deoxyglucosone adducts respectively. MRM methods were developed and the detection of all adducts in aseptically processed UHT milk with added catechin was achieved. An example chromatogram of detection of catechin-glyoxal and catechin-methylglyoxal adducts is shown in Figure 3.7. Multiple peaks in chromatograms are due to 4 diastereoisomers of catechin forming during thermal processing reacting with RCSs. Catechin-methylglyoxal adducts result in more than 4 peaks as addition of methylglyoxal added another chiral center to the molecule, thus increasing the number of possible products.

In summary, phenolic compounds were reported to significantly suppress MR off-flavor pathways in UHT milk during thermal processing and storage resulting in a product with increased palatability. Furthermore, the results of this study support phenolic trapping reactions of Maillard-generated sugar fragments as an important mechanism of off-flavor suppression. This work further defined the interplay between phenolic chemistry and the mechanisms of the MR in foodstuffs. Unique phenolic-structure reactivity was also reported in this study for the suppression of Maillard chemistry in UHT milk; different phenolic structures were more reactive during thermal processing versus storage in reducing both RCSs and off-flavor markers suggesting that we can effectively design the appropriate mixture of compounds, at the lowest levels, to optimize this technology for suppression off-flavor development.

Table 3.1. Mass fragmentation ions of main detected adducts between catechin (CAT) and glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3DG) in model systems[a].

Adduct/ Phenolic	**Parent ion (m/z)**	**Cone voltage (V)**	**Collision Energy (V)**	**Sibling Ion (m/z)**
CAT-GO	347	35	21	167
CAT_2-GO	619	35	20	329
CAT-MGO	361	35	20	181
CAT-MGO_2	433	35	27	235
CAT-3DG	451	32	22	289

a = Optimum Multi-Reaction-Monitoring (MRM) conditions used for their identification based on Mass Lynx (Waters, Milford, Ct)

Table 3.2. Consumer acceptability rating of 1% UHT milk manufactured with and without catechin plus a commercial UHT sample.

Sample	**Mean liking (9-pt hedonic scale)**
1% UHT-catechin containing (1.72mM)*	6.04[a]
1% UHT milk*	5.12[b]
Commercial UHT (Parmalot)	4.73[b]

* Samples prepared and produced at University of Minnesota Joseph Warthensen Processing Center facilities (processed to 140 ^{o}C for 6sec) and stored at 30^{o}C for 1month.. Different letters indicate statistically significant differences. Fisher's LSD = 0.728 (5% Significance Level)

Table 3.3. Average cooked flavor intensity of commercial milk and off-flavor recombination models

Sample	Mean Rating	Homogenous groups[a]
Commercial 1% UHT Milk (Parmalat)	5.5	A
Off-flavor Recombination Model of 1% UHT milk	5.3	A
Commercial 1% Pasteurized Milk	1.1	B
Off-flavor Recombination Model of 1% UHT-catechin containing (1.72mM)	0.8	B

a = Fisher's LSD = 0.319 (5% Significance Level), n = 8 in duplicate

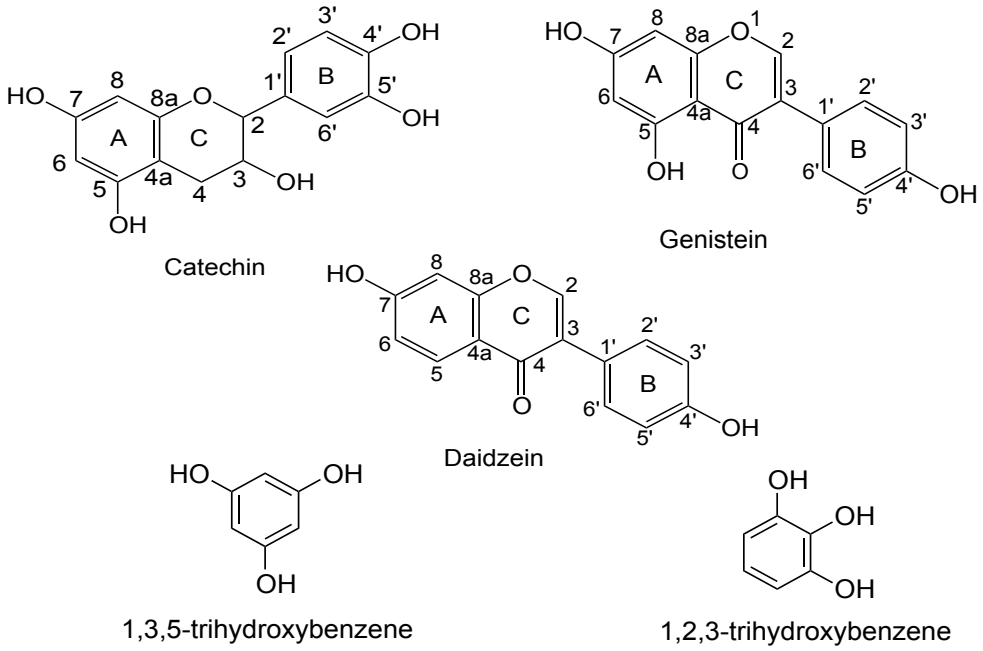


Figure 3.1. Select phenolic compounds evaluated in UHT milk

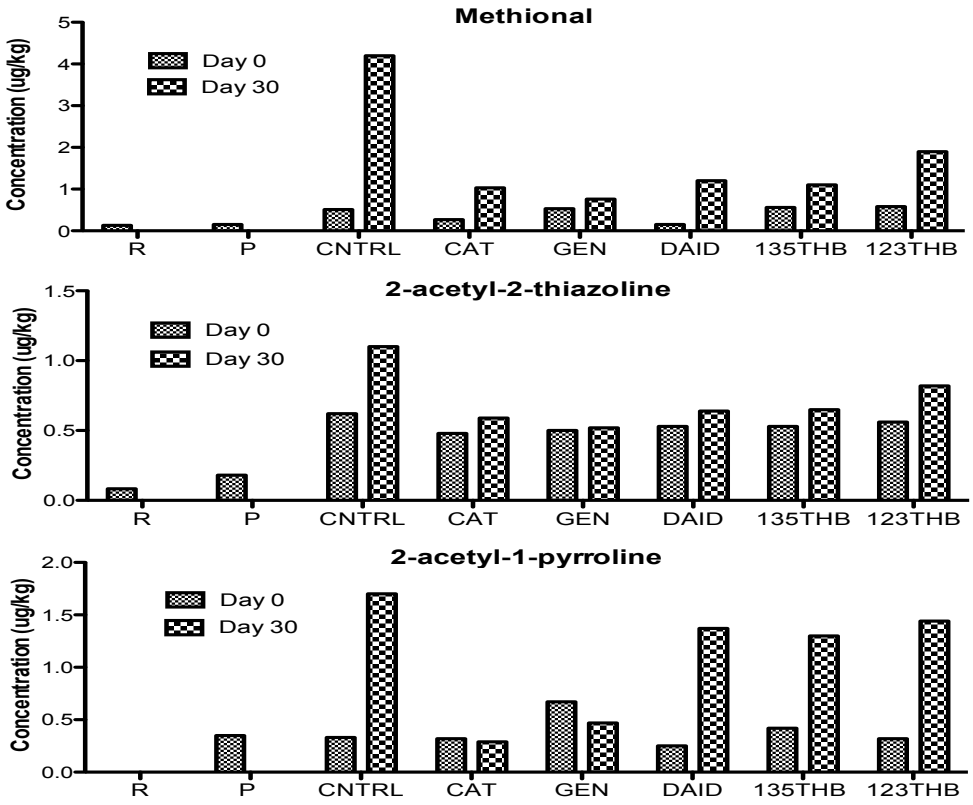


Figure 3.2. Detected concentration off-flavor markers 2-acetyl-1-pyrroline (2AP), methional and 2-acetyl-2-thiazoline (2A2T) in raw (R), pasteurized (P), control UHT milk (CNTRL), UHT milk with added catechin (CAT), genistein (GEN), daidzein (DAID), 1,3,5-trihydroxybenzene (135THB), 1,2,3-trihydroxybenzene (123THB) as determined right after UHT processing (day 0) and after 30days of storage in 30^{o}C (day 30).

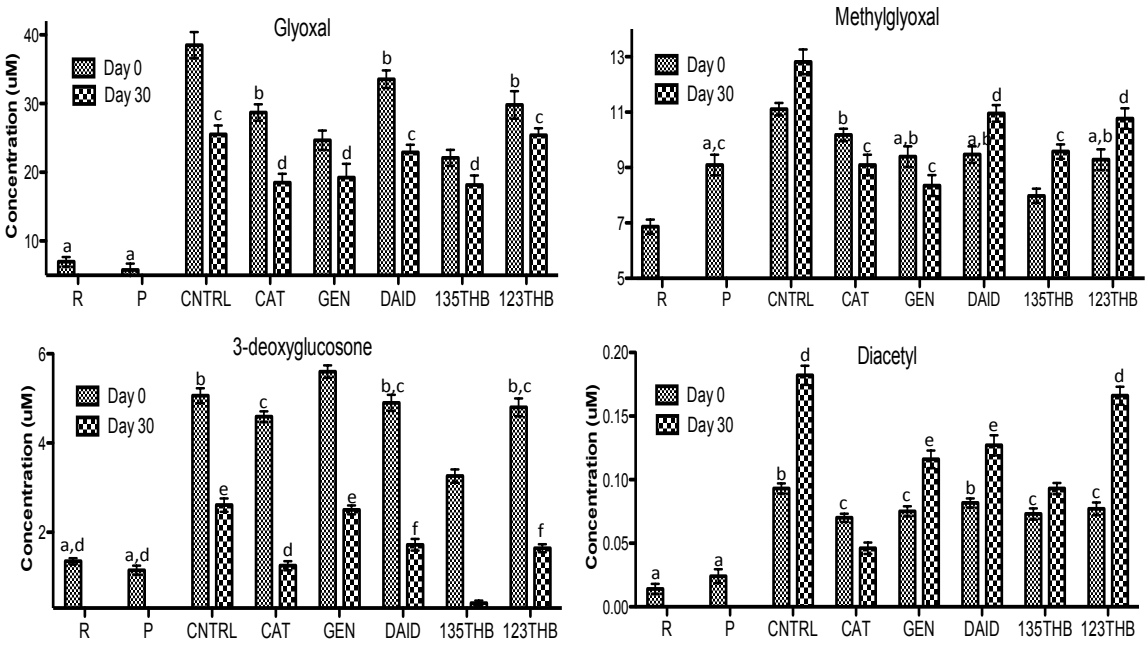


Figure 3.3. Detected concentrations of α-diacarbonyls (glyoxal, methylglyoxal, 3-deoxyglucosone and Diacetyl) in raw (R), pasteurized (P), control UHT milk (CNTRL), UHT milk with added catechin (CAT), genistein (GEN), daidzein (DAID), 1,3,5-trihydroxybenzene (135THB), and 1,2,3-trihydroxybenzene (123THB), as determined immediately after UHT processing, (day 0) and after 30days of storage in 30^{o}C (day 30). Each compound was monitored as quinoxaline-derivatives and run in triplicates. The same letters indicate no statistical significant differences ($p<0.05$)).

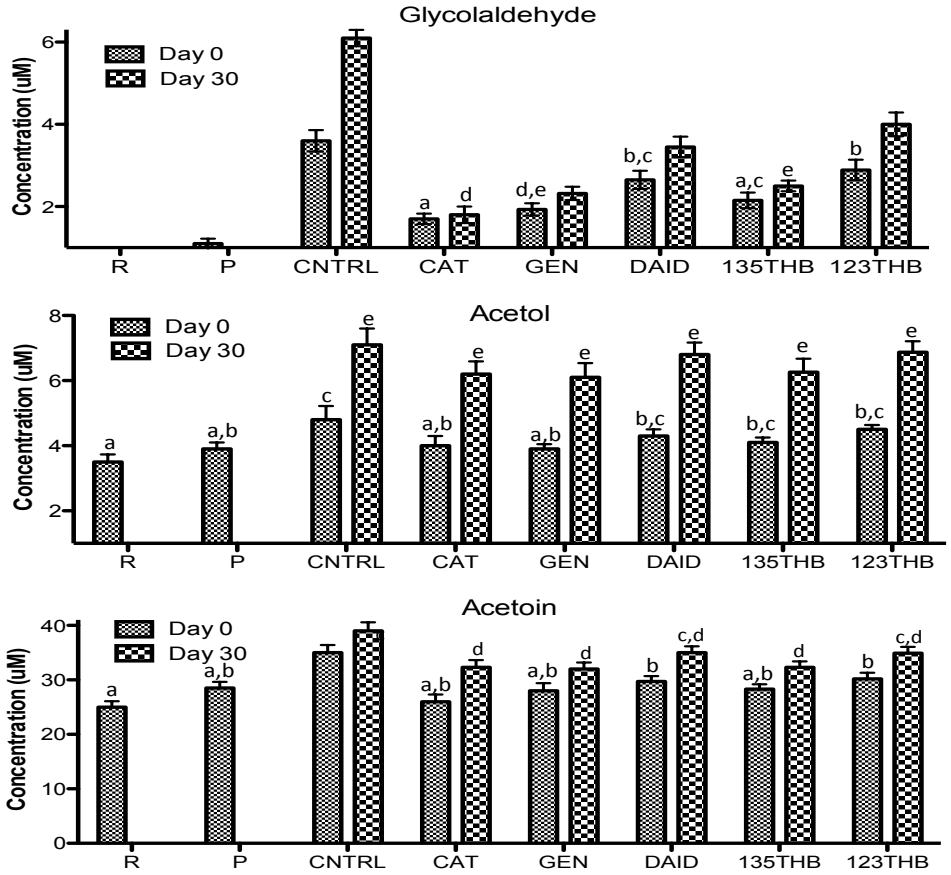


Figure 3.4. Detected concentrations of α-hydroxycarbonyls (glycolaldhyde, acetol and acetoin) in raw (R), pasteurized (P), control UHT milk (CNTRL), UHT milk with added catechin (CAT), genistein (GEN), daidzein (DAID), 1,3,5-trihydroxybenzene (135THB), 1,2,3-trihydroxybenzene (123THB), as determined immediately after UHT processing, (day 0) and after 30days of storage in 30^{o}C (day 30). Each compound was monitored as ethoxylamine-derivatives and run in triplicates. The same letters indicate no statistical significant differences ($p<0.05$).

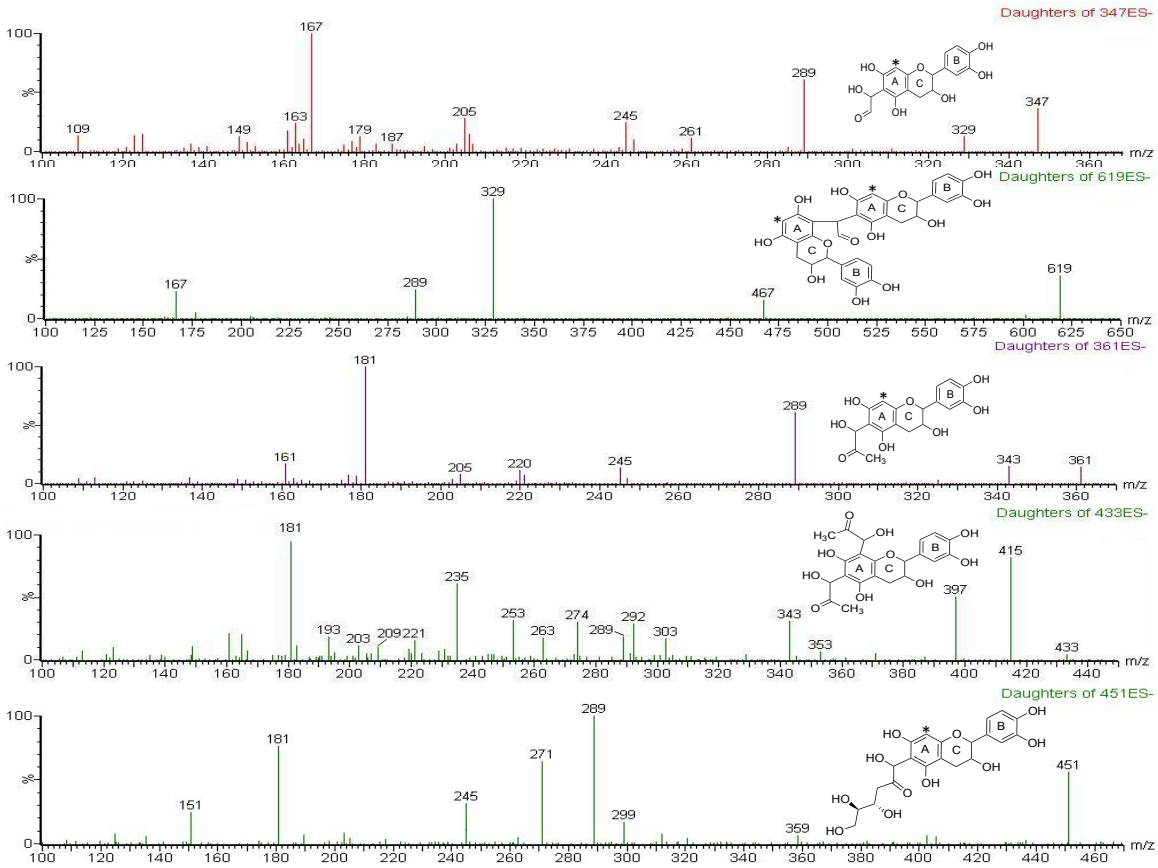


Figure 3.5. MS/MS fragmentation spectra for catechin-sugar fragment adduct reaction products Spectra correspond to: catechin-glyoxal, catechin-glyoxal-catechin, catechin-methylglyoxal, methylglyoxal-catechin-methylglyoxal and catechin-3-deoxyglucosone adducts from top to bottom. * denotes alternative position activated for aromatic electrophilic substitution. RCSs substitution can occur either at C-6 or C-8 position of A-ring. Only one structure is presented for simplicity.

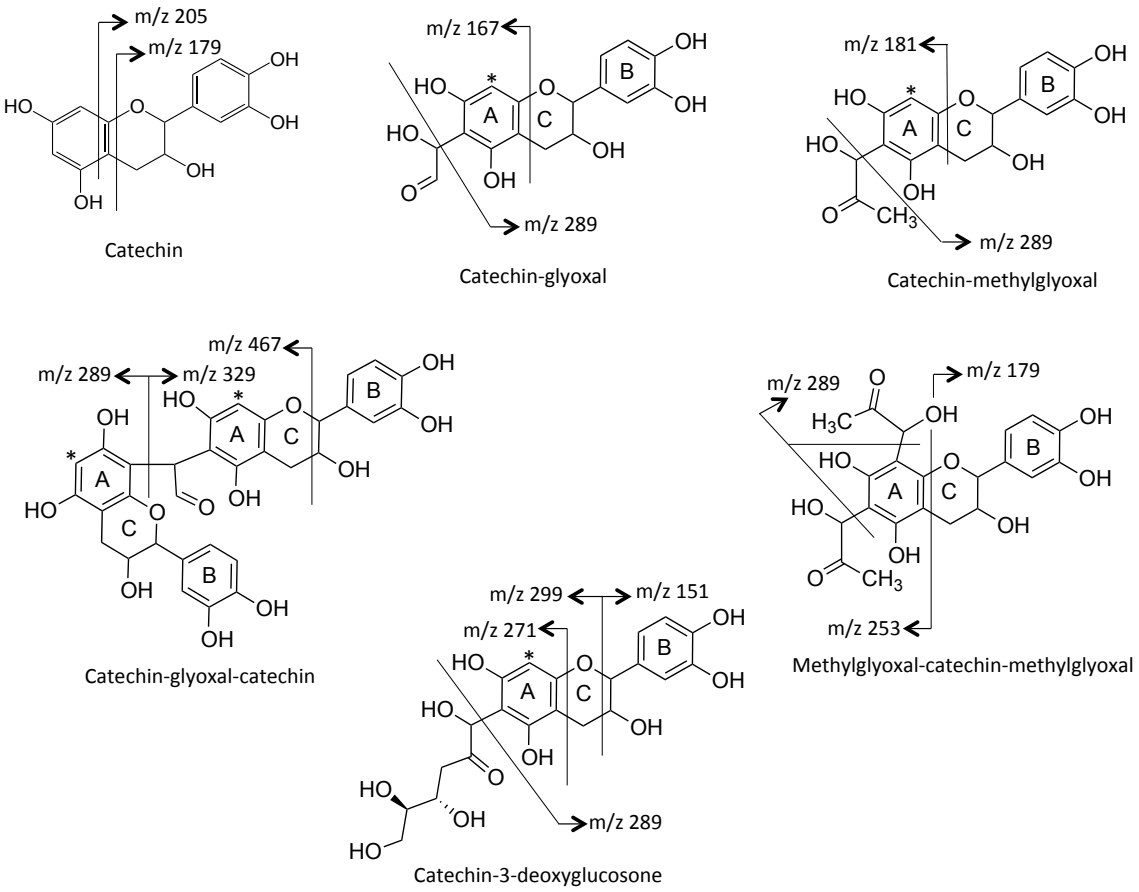


Figure 3.6. Hypothesized MS/MS fragmentation pattern of adducts between catechin and glyoxal, methylglyoxal and 3-deoxyglucosone. * denotes alternative position for aromatic electrophilic substitution. RCSs substitution can occur either at C-6 or C-8 position of A-ring. Only one structure is presented for simplicity.

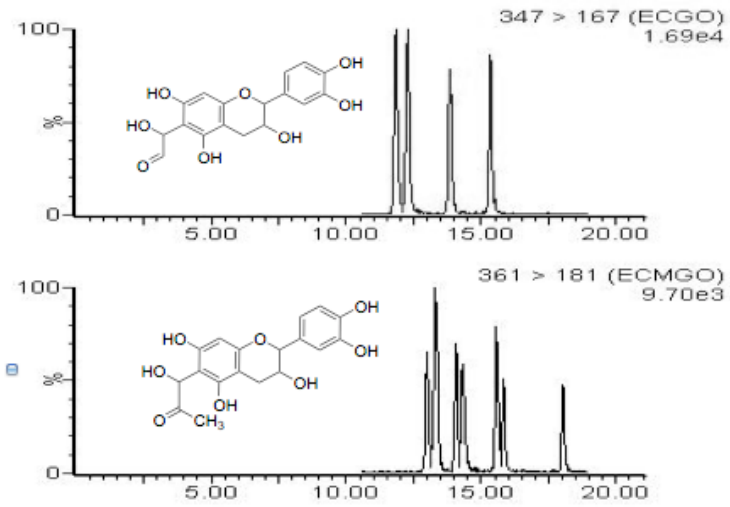


Figure 3.7. LC/MS-MS (ESI negative mode) chromatograms of detection of catechin adducts with glyoxal (top, 347m/z→167m/z) and methylglyoxal (bottom, 361m/z→181m/z) in UHT milk made with catechin.

Chapter 4

Response Surface Methodology as Optimization Strategy for Reduction of Reactive Carbonyl Species in Heat-processed Foods by Means of Phenolic Chemistry

Response surface methodology (RSM) was utilized to investigate the dose-response relationships of a phenolic mixture (catechin, genistein and diadzein) as a pre-thermal processing technique to reduce reactive carbonyl species (RCSs; glyoxal, methyglyoxal and 3-deoxyglucosone) in ultra-high temperature (UHT) bovine milk. A Box-Behnken 3-factor (catechin, genistein and diadzein) 3-level (0.17, 0.645 and 1.12mM) design was employed. In general, all phenolic mixtures were able to reduce RCSs in UHT milk; some compositions reported RCSs levels at or below levels reported in pasteurized milk. Predictive models with no significant lack of fit ($p>0.05$), high R^2-values (0.886-0.979) and good predictive power were developed. ANOVA analysis for glyoxal levels indicated that only linear effects of each phenolic compound had a significant effect ($p<0.05$) meaning that no significant interactions between the different phenolic compounds influenced glyoxal levels. Linear, cross product and quadratic effects of factors were reported ($p<0.05$) for methylglyoxal, indicating more complicated interactions between the phenolic compounds. Both linear and quadratic effects were also reported ($p<0.05$) for 3-deoxyglucosone. Overall, based on canonical analysis, catechin seemed to be the most influential factor for the reduction of RCSs in UHT milk. In summary RSM provided a basis to understand phenolic structure reactivity and to

optimize the composition of a tertiary mixture of phenolic compounds for reduction of RCSs in UHT milk.

Introduction

Reactions between amino and carbonyl groups are ubiquitous in nature and widely investigated in foodstuffs and biological systems. In biology, this reaction is mainly known as glycation and involves the non-enzymatic amino-carbonyl reaction between reducing sugars (or reactive carbonyl species) and long-lived molecules such as proteins, lipids and DNA, which leads to their chemical modification. The reaction products are called Advanced Glycation End products (AGEs) and are thought to have negative effects on regulatory biology due to altered functionality of the modified components [28], [77], [129]. Formation of AGEs *in vivo* has been observed to increase when the exposure of reducing sugars or reactive carbonyl species (RCSs) such as α-diacarbonyls increase from either metabolic dynfunction or from dietary exposure. This observation has lead to the development of the 'carbonyl stress' hypothesis stating that increased generation, intake and/or inadequate detoxification of carbonyl compounds may contribute to long-term complications such as cardiovascular diseases, diabetes, Alzheimer's and aging [109], [130–132].

In food, amino-carbonyl reactions are known as the Maillard reaction. Maillard reaction products (MRPs) have been broadly studied in foods for decades because of the importance on flavor/acceptability [133], [134], the nutritional value [135–137] and toxicity [72], [138–142]. MRPs in food have also been associated with increased biological exposure of carbonyl stress and AGEs [22], [73–75] that can increase the risk

of pathological conditions [28–31]. Examples of RCSs generated by the Maillard reaction (in food) or by glycation (in vivo) include compounds such as 3-deoxyglucosone, methylglyoxal, glyoxal and glycolaldehyde, among others.

The ability to control the generation of RCSs in foodstuffs would have obvious advantages for food processors to tailor product quality for acceptability (flavor development) as well as for beneficial health impact. Unfortunately, advancements in controlling Maillard chemistry in foods are limited. Recently, dietary phenolic compounds have been reported to suppress the levels of RCSs and subsequently AGEs formation. Totlani and Peterson [36] first reported the ability of phenolic compounds, and more specifically epicatechin, in model reaction systems to scavenge RCSs via adduct formation and alter Maillard reaction pathways/product generation. Several studies followed, investigating the effect of various phenolic compounds on scavenging RCSs in simulated physiological conditions and *in vivo* [37–40], [143], [144] as well as the effect of phenolic compounds on Maillard reaction products and how that translates to sensory properties of processed food systems [18], [33], [95].

Phenolic compounds have been previously reported to effectively lower the concentration of RCSs and suppress the formation of AGEs in biological systems or Maillard reaction products in heat processed food systems during thermal processing and subsequent storage. This property of phenolic compounds suggests they could be a viable candidate for the development of an ingredient technology for heat-processed foods aiming to reduce the dietary load of RCSs and AGEs as well as controlling the generation of unwanted Maillard reaction off-flavor compounds, thus resulting to more palatable and nutritious food products. The development of a pre-processing treatment

where natural dietary products are applied to control unwanted food reactions would benefit the food industry, as consumers have become increasingly aware of food labels.

The utilization of multiple phenolic compounds ranging in reactivity (versus a single compound) may be advantageous to control RCSs in a complex system (food) over different reaction conditions (i.e. temperature) during processing and subsequently storage. In the current study, statistical analysis was used to evaluate and optimize a tertiary mixture of phenolic compounds for suppression of RCSs levels in UHT milk. To monitor the reactivity of phenolic compound mixtures accurate quantification methods for RCSs are needed. Derivatization techniques are commonly utilized to monitor RCSs however the phenolic-RCS reaction products formed by adding the phenolic compounds were predicted to potentially interfere with the related quantitative analysis. Consequently, the main objectives of this study were to develop accurate RCSs quantification methods as well as to employ Response Surface Methodology (RSM), first described by Box and Wilson [145], to define dose-response relations and optimization of a tertiary phenolic mixture as a pre-processing treatment for the reduction of RCSs in heat-processed food, specifically UHT milk.

Materials and Methods

Chemicals: (+)-catechin ≥ 97% (TCI America), genistein ≥ 98% (Shaanxi Schipar Biotechnology Co. Ltd., China), daidzein ≥ 98% (Shaanxi Schipar Biotechnology Co. Ltd., China), 3-deoxyglucosone (TRC, Toronto research chemicals, Ontario Canada), glyoxal 40% solution in water (Sigma, Saint Louis MO), methylglyoxal 40% solution in water (Sigma, Saint Louis MO), o-phenylenediamine (o-PD) 99.5% (Sigma-Aldrich,

Saint Louis MO), formic acid (MS grade 98%) (Fluka, Switzerland), methanol (MS grade) (Fisher Chemicals, Fair Lawn, NJ), sodium phosphate, potassium phosphate, sodium chloride (Sigma-Aldrich, Saint Louis MO), acetic acid (Sigma-Aldrich, Saint Louis MO), sodium bicarbonate, sodium carbonate (Fluka, Switzerland), bismuth (III) oxide (Aldrich, Germany), diethyl ether (Fisher Chemicals, Fair Lawn, NJ), [13C2]-acetaldehyde (Aldrich, Germany), triethylamine (Sigma-Aldrich, Saint Louis MO), 3-benzyl-5- (2-hydroxyethyl)-4-methylthiazolium chloride (Aldrich, Germany).

Bench-top UHT milk processing: Raw skim and whole bovine milk was obtained from the University of Minnesota Joseph Warthensen Processing Center (Saint Paul, MN) and mixed to yield raw milk with 1% fat content. A Combi-Pal auto-sampler (CTC Analytics, Switzerland) equipped with a cooling tray and agitated heating station was used as a bench-top UHT processing system. The method utilized small sample volumes thus being more cost and time efficient as compared to using an industrial or pilot plant scale UHT processing unit for optimization purposes. Briefly, 5ml of 1% raw milk in headspace vials was placed in a cooling vial holder held at 8^{o}C. Vials were transferred to an agitator (150 ^{o}C) and held for 265sec to allow the sample to reach a final temperature of 140-141^{o}C for 6sec and subsequently returned to the cooler. The time-temperature profile of the sample was monitored by a thermocouple located in the liquid sample in the vial.

The time-temperature profile of the simulated method was very similar to that of a commercial indirect tubular UHT processing method (see Figure 4.1) thus supporting the validity and the use of the simulated UHT method for process optimization

experiments. The UHT processed milk samples with and without added phenolic mixtures were stored at 30^{o}C immediately after processing for 3 weeks (accelerated shelf-life – equivalent to 6-8 weeks at ambient) before analysis.

Response Surface Methodology: RSM was used and its potential as optimization strategy for the RCSs reduction in heat-processed foods was evaluated. RSM, is a collection of mathematical and statistical techniques, which evaluates the effects of factors on response variables, searching for the optimum conditions and building an empirical model that approximates the true relationship between factors and response variables. In the present study the three select phenolic compounds are the factors and the dependent variables are the three select RCSs. Assuming that a function (f) exists that links the factors (Xi) with the response y, a relationship between factors and response can be identified. Nonetheless the true function is either unknown or too complex so a non-linear quadratic model was considered by RSM to approximate the true function. The nonlinear quadratic model is given in Eq. (1)

Eq (1)

1

$$y = bo + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_{11}^{\ 2} + b_{22}x_{22}^{\ 2} + b_{33}x_{33}^{\ 2}$$

Where Y is the measured response associated with each factor level combination, b_0 is the intercept, b_1 to b_{33} are the regression coefficients and x_1, x_2, x_3 are the independent variables.

A 3-factor, 3-level Box-Behnken statistical design was used to investigate and evaluate main effects, interaction effects, and quadratic effects of the addition of different concentrations of catechin, genistein and daidzein on the concentration of RCSs in UHT processed milk. This statistical design was selected since it was suitable for exploring quadratic response surfaces and constructing second-order polynomial models. The design generated 15 runs and low, central and high factor (phenolic compounds) levels are indicated in Table 4.1 for each of them by the coded values -1, 0 and +1, respectively. Statistical analysis was performed using SAS v 9.2 (SAS Institute Inc. Carry, NC) and JMP 10 (SAS Institute Inc. Carry, NC).

RSM Validation Experiment: The predictive model developed for RCSs by RSM was further validated by direct comparison to experimental values. A phenolic mixture of catechin, genistein and daidzein at levels of 0.17, 1.12 and 0.4mM was added to raw 1% milk, subsequently UHT processed and the concentrations of glyoxal, methylglyoxal and 3-deoxyglucosone were determined and compared to the predicted values (t-test p-value<0.05).

Quantification of RCSs: The influence of phenolic-carbonyl compounds on the quantification of RCSs by derivatization techniques was determined. A 10ml phenolic-carbonyl reaction model in phosphate buffer (pH 7.4) consisting of equimollar amounts of catechin and methyglyoxal (10mM) was incubated in a shaking water bath at 37^{o}C for 1hr. Two 5ml aliquots of the reaction mixture were placed into separate vials and analyzed by two procedures. For procedure A the sample was immediately derivatized

by adding a methanolic solution of o-PD (0.5mol/L) following incubation at 37^{o}C in the dark for 1hr. For procedure B, the sample was further fractionated by Solid Phase Extraction (SPE) preparation step in order to isolate free RCSs from free phenolic compounds and phenol-RCSs adducts prior to derivatization. Briefly, a 500mg DSC-18 cartridge (Supelco, Bellefonte, PA) was preconditioned with methanol (3ml) and nanopure water (3ml), an aliquot of 5ml of reaction mixture, spiked with internal standard (butyl paraben, 5uL, 2% solution in methanol) was loaded on the cartridge. The aqueous eluent from the sample was collected and further washed with a 1ml of 95:5 0.1%formic acid: methanol solution. Both eluents were combined (the free dicarbonyls), then derivatized as reported above and subsequently filtered through Iso-Disk PTFE filters (25mmx0.2um, Supelco, Bellefonte, PA) using 1ml syringe (Millipore, Bedford, MA). The phenolic compounds and phenolic-adducts were eluted from cartridge using methanol (acidified with 0.1% formic acid).

The levels of methylglyoxal and catechin for both aliquot A and B were quantified using LC/MS/MS. An Acquity UPLC system (Binary solvent manager, Sample manager and Column heater) coupled with a Quattro Premier XE Waters mass spectrometer (Waters Co. Milford, MA) was employed. An Acquity UPLC 2.1x100mm BEH Phenyl 1.7um column with a VanGuard 2.1x5mm BEH Phenyl 1.7um pre-column were used and all experiments were performed in triplicates. Mass spectroscopic conditions were as follows: desolvation temperature, 300^{o}C; source temperature, 120^{o}C; capillary voltage, 3.8kV; desolvation gas, 600L/min; cone gas, 50L/min. Analytes were detected using electrospray positive ionization-multiple reaction monitoring (MRM) and optimum conditions are reported in Table 4.2.

Isolation and purification of methylglyoxal-catechin adducts: Standards were prepared as previously described in Chapter 3.The purified analytes were used to determine the response factor by LC/MS/MS techniques and determine the fragmentation pattern of each product as well as the best MRM conditions for optimum sensitivity. Fragmentation spectra were also used to support hypothesized structures of these adducts.

Quantification of Reactive Carbonyl Species in UHT Milk: The select RCSs (glyoxal, methylglyoxal and 3-deoxyglucosone) were quantified in the fifteen UHT milk samples listed in Table 4.3. $^{13}C_4$-Diacetyl was synthesized according to Schieberle, P., Hofmann, T. [116] and used as an internal standard for accurate quantification. 0.5 mM of the internal standard was added to five ml of milk, followed by 500uL of 10% trichloroacetic acid for protein precipitation and vortexed for 1min. The sample was centrifuged at 3904g for 20min at 4^{o}C (Beckman Coulter, Allegra X-22R) and the supernatant was collected. Hexane was used to remove fat from the samples and then prepared according to procedure B (list above) and analyzed by LC/MS/MS methods. In brevity, an aliquot of 0.5ml of the free dicarbonyls SPE eluent was taken, a methanolic solution of o-PD (0.5ml, 0.5M) was added for derivatization and the mixture was maintained in the dark at 37^{o}C for two hours prior to analysis.

In order to calculate the recovery efficiency of free dicarbonyls for procedure B, a control UHT milk and a UHT milk sample spiked with known amounts of dicarbonyls (0.3mM) were analyzed. Previously mentioned SPE sample preparation method (see Quantification of RCSs) was used for both control and spiked samples. Total free

dicarbonyl levels in each sample were calculated by addition of the amounts quantified in both aqueous and methanolic SPE eluents to account for dicarbonyls not eluting for SPE cartridge with the aqueous wash step. Experiments were replicated (6x), the response of the dicarbonyl amounts that were spiked in UHT milk was also determined and the recovery efficiency of free dicarbonyls was estimated. Recovery percentages for glyoxal, methylglyoxal and 3-deoxyglucosone were 89 (±9) 72.3 (±12) and 91.4 (±11.5), which indicated that the isolation method has a high recovery efficiency and good reproducibility.

Results and Discussion

The dose-response reactivity of tertiary mixtures of dietary phenolic compounds as trapping agents of Maillard derived RCSs in UHT processed milk (1%) was investigated by RSM statistical design. The Maillard reaction is known to contribute to off-flavor generation, protein modification and browning for this product both during processing and storage [18], [25], [57], [63], [109], [130–132], [146–148]. The fat content was selected as 1% milk it is the most consumed fluid milk product in the US. Overall fluid milk consumption has steadily decreased over the past 3 decades but consumption of low fat milk has increased as consumers became more aware of caloric intake and negative effects of saturated fat intake [122].

Three food phenolic compounds catechin, (flavanol), genistein, daidzein (isoflavones) (Figure 4.2) were selected in order to examine phenolic structure/mixture reactivity, determine any additive or synergistic effects on the RCSs generation in UHT milk and examine their potential use for process optimization. These phenolic compounds

differ on the degree and position of hydroxyl substitution on ring A and B as well as the presence of a keto- group on ring C (isoflavones) and thus, vary on both antioxidant capacity and sugar fragment trapping reactivity [94]. Phenolic compounds were added at dose levels below 1.7mM, as this amount was reported [18] to be effective for epicatechin in order to suppress of off-flavor development and not result in a detectable bitterness (a sensory attribute associated with polyphenols).

Three α-dicarbonyl compounds, glyoxal, methyglyoxal and 3-deoxyglucosone (Figure 4.2) were selected as important RCSs markers and as response variables for optimization purposes because they have been extensively studied and their contribution to AGE formation as well as (off)-flavor development is well documented.

The effect of phenolic compounds on the derivatization of RCSs using o-phenylendiane (o-PD) to produce more stable quinoxaline derivatives was tested, as accurate quantification of free RCSs is dependent on that step. For comparison reasons, RCSs levels were also quantified in 1% control UHT milk (no added phenolic mixture) and 1% pasteurized milk (Schroder Milk Co. Maplewood, MN).

The trapping reactivity of the phenolic mixtures was characterized by monitoring the residual levels of carbonyl compounds in the milk samples. For comparison, RCSs levels were also quantified in a 1% control UHT milk (no added phenolic mixture) as well as a local 1% pasteurized milk sample. Quantification of RCSs in food, biological systems [149], [150] and models simulating physiological conditions [39] has been typically performed by derivatization techniques utilizing o-phenylenediamine (o-PD) to generate more stable quinoxaline moieties and facilitate detection. In the current study, the phenolic-RCSs adducts in the UHT milk samples (as a result of phenolic addition)

were predicted to be susceptible to the derivatizing agent (o-PD) resulting in the liberation the RCSs moieties from adducts to generate quinoxaline derivative compounds and a proposed mechanism is presented in Figure 3.

To test the reactivity of phenolic-RCSs adducts on quinoxaline generation during o-PD derivatization, a purified catechin-methylglyoxal adduct was analyzed by LC/MS before and after derivatization (see Figure 4.4). Before derivatization, only the catechin-methylglyoxal adduct could be detected however after derivatization, the adduct could no longer be detected while both the 2-methylquinoxaline (derivative of methylglyoxal) and the liberated phenolic compound (catechin) were detected. The detection of the 2-methylquinoxaline after o-PD derivatization, indicated the phenolic-RCSs adducts would contribute to the concentration of RCSs determined. To further quantitatively determine the effect of phenolic-RCSs adducts on the derivatization accuracy of RCSs analysis, a model reaction with 10mM of catechin and methylglyoxal was analyzed; yield of catechin-methylglyoxal products was predicted to be approximately 70% [34]. The reaction mixture was separated into two aliquots (A and B in Aliquot A the derivatizing agent (o-PD) was added directly to the reaction mixture, while aliquot B underwent solid phase extraction (SPE) preparation step to separate free methylglyoxal from the catechin-methylglyoxal adducts; the methylglyoxal fraction was derivatized.

The observed levels of methylglyoxal as well as catechin were higher for aliquot A as compared to aliquot B (see Figure 4.5); for aliquot B the levels of both compounds were also adjusted for the % recovery for the extra SPE step. Therefore the presence of phenolic-RCSs adducts in the reaction mixture during derivatization resulted in concentration of catechin and methylglyoxal to be overestimated by 76.6±10.8% and

65.3±12.2% respectively. Overall, these findings indicated the previously used protocols for RCSs quantification by o-PD derivatization [27-31] could lead to overestimated levels of 'free' RCSs. An additional SPE isolation step, to separate RCSs and phenolic-RCSs adducts or other potentially reversible reaction products, is consequently recommended for accurate quantification of s by o-PD techniques in food or in biological systems. This modified procedure was subsequently used for quantification of RCSs in the current study.

RSM was used to investigate the relationship between phenolic compounds and RCSs. RMS designs have many desirable properties such as adequate distribution of information across the experimental region (rotatability), good lack of fit detection, the fitted values are as close as possible to the observed ones and they require the minimum number of treatment combinations, which is cost and time effective. There has been a growing and successful application of RSM in the areas of foods, tobaccos, military research, pharmaceuticals, petroleum, electronics and many other fields [151] and many researchers have employed RSM to optimize process variable conditions in food systems [152–155].

For the statistical optimization a Box-Benhken response surface design was employed to determine the effect of catechin, genistein and daidzein on the levels of RCSs in UHT milk; the design consisted of 15 different phenolic mixture combinations (shown in Table 4.1). Treatment samples as well as control were UHT processed and subsequently stored for 3 weeks at 30^{o}C prior to analysis. The concentration of RCSs in the milk samples was subsequently determined and the effect of each phenolic compound on the levels of glyoxal, methylglyoxal and 3-deoxyglucosone was examined.

Overall phenolic mixtures were effective in reducing the concentration of RCSs as compared to control UHT milk sample and in certain cases were below the quantified RCSs levels founds in pasteurized milk (see Table 4.2). These findings support the concept that phenolic compounds could be applied as ingredients to effectively reduce the dietary load of RCSs or suppress the formation of undesirable Maillard reaction products of heat-processed foods.

The predictive models that describe the relationship between each dicarbonyl compound and the selected food phenolic compounds were developed (Table 4.4). These models can be used to estimate the level of RCSs levels in UHT milk when adding phenolic mixtures at known concentrations prior to processing. The lack of fit test and R^2 value (Table 4.5), were used to evaluate the adequacy of the developed models and their predictive power. The lack of fit test helps to assess the correctness of the model and how well the function fits the experimental data. The coefficient of determination R^2, describes the percentages of variability explained by the predicted model and thus the higher the values the better the model. The lack of fit test for the predicted models for methylglyoxal, glyoxal and 3-deoxyglucosone was not significant ($p>0.05$) and R^2 were relatively high ($r^2 > 0.88$) thus, supporting the correctness of the model and the good fit on the experimental data. The residual versus predicted values plots (data not shown) were also supported the models had a good fit as they exhibited a random pattern.

Further evaluation of the response surface was conducted to identify influential factors for reducing the levels of each RCSs, to provide information regarding phenolic structure reactivity. ANOVA analysis was used to determine significant factors and Pareto charts that were used in combination with the eigenvalues and eigenvectors

generated by the canonical analysis to determine the sensitivity of RCSs reduction to variations in concentration of the different phenolic compounds. Eigenvalues and eigenvectors characterize the shape of the response surface. The eigenvectors point in the directions of principle orientation of the surface, and the signs and magnitudes of the associated eigenvalues give the shape of the surface in these directions. The larger the absolute value of an eigenvalue, the more distinct is the curvature of the response surface in the associated direction and thus the higher the sensitivity of the response to the factor associated with that eigenvector.

ANOVA analysis revealed that only linear effects significantly ($p<0.05$) affected the concentration of glyoxal in UHT processed milk, showing that no significant interactions occur between the different phenolic compounds in the employed mixtures. Based on the eigenvalues and eigenvectors (Table 4.6) catechin and daidzein were the most influential factors followed by genistein and Pareto charts (Figure 4.6a) also demonstrated these results. The eigenvalues suggested that the stationary point of the response surface was a saddle point (both negative and positive eigenvalues) meaning that as we move away from the stationary point the height of the response can either increase or decrease. The presence of a saddle point can denote two distinct regions containing maxima, which can imply the existence of two distinct mechanisms of action or unique reactivity of factors and interactions between them. Nonetheless in the case of glyoxal the stationary point was located outside the experimental range, which made extrapolation unsafe and thus inconclusive. A safe conclusion regarding the stationary point could be drawn with further experimentation and expansion of the experimental range towards that point but this was outside of the scope of this study as an optimum

(minimum) within the experimental region could still be approached using ridge analysis. Ridge analysis locates the overall or local optimal regions and indicated a minimum response region for glyoxal was determined at factors levels of 0.97, 0.86 and 0.91uM for catechin, genistein and daidzein respectively (Table 4.7). The response surface plots (Figure 4.7a) illustrate these results, as it was apparent that as concentration of phenolic compounds increased glyoxal levels decreased linearly thus concluding that within the chosen experimental range, an optimum region is achieved towards high factor levels. It is noteworthy that the predicted levels of glyoxal at the optimum region based on ridge analysis is 10.38uM, a concentration well bellow the control UHT milk and more comparable to concentrations found in pasteurized milk demonstrating the potential for dose-process optimization.

Linear, cross product and quadratic effects of factors were reported to have a significant effect on methylglyoxal concentration, indicating more complicated interactions between phenolic compounds themselves and methylglyoxal. Linear effects of catechin, genistein and daidzein as well as interaction effects between catechin and genistein and quadratic effects of catechin were significant (p-value<0.05) based on ANOVA analysis and included in the predictive model. Canonical analysis of eigenvalues and eigenvectors showed that the most influential factor was catechin as was expected since linear, quadratic and interaction effects of that factor were significant. Genistein was the second most influential factor followed by daidzein. Pareto chart (Figure 4.6b) demonstrated the influence and significance of the individual linear, interaction and quadratic effects of each factor. The linear effect of genistein was the most significant followed by the quadratic effect of catechin and linear effect of daidzein,

catechin and the interaction effect of catechin and genistein. The stationary point of the response surface was a saddle point and again it lied outside the experimental space thus ridge analysis was employed in order to determine a minimum region for the response. A minimum response for methylglyoxal based on ridge analysis was determined at factor concentration of 1.10, 0.75 and 0.68uM for catechin, genistein and daidzein respectively (Table 4.7) and over all a minimum region lied when the phenolic dose ranged between 0.645-1.10, 0.645-0.78 and 0.645-0.71uM for catechin, genistein and daidzein respectively. The response surface plots (Figure 4.7b) constructed based on these trends further depicted that a number different combination of factors could minimize the levels of methylglyoxal, as the response surface resembles a saddle point. Minimum concentrations of methylglyoxal in UHT milk (comparable to levels found in pasteurized milk) were also predicted when catechin and daidzein were used in mixtures at concentrations of 0.17-0.41uM and genistein 0.8-1.12uM showing a potential for dose and cost optimization for phenolic mixtures to be used as treatments for RCSs reduction.

Similar to the previous two RCSs, linear effects of all phenolic compounds had a significant effect on the concentration of 3-deoxyglucosone. Quadratic effects of catechin and daidzein were also found to be significant ($p<0.05$) thus, were included in the predictive model. Canonical analysis of eigenvalues and eigenvectors showed that overall catechin was the most influential factor, closely followed by daidzein (based on absolute magnitude of eigenvalue associated with that factor) and genistein. Pareto charts (Figure 4.6c) displayed that the linear term of catechin had the most significant effect on reducing the levels of 3-deoxyglucosone followed by the quadratic terms of daidzein and catechin and the linear terms of daidzein and genistein. Additionally, the eigenvalues showed that

the stationary point of the response surface was a maximum meaning that the response was shaped as a hill and when moving away from the stationary point the height of the response decreased. Ridge analysis was similarly performed as the stationary point was not within the experimental space and a minimum response region for 3-deoxyglucosone was determined at factors levels of 1.05, 0.69 and 0.87uM for catechin, genistein and daidzein respectively (Table 4.7). Response surface plots (Figure 4.7c) demonstrated the effect of the different factors on the concentration of 3-deoxyglucosone in UHT milk processed samples. The significance on both quadratic terms was apparent as both catechin and daidzein exhibited diminishing return effects and at low and mid concentrations the levels of 3-deoxyglucosone approached maximum. At higher concentration of the phenolic compounds (>0.8uM) the response approached the minimum predicted values within the experimental range. The levels of genistein had a significant linear effect and for increasing concentrations of genistein the achieved minimum concentration of 3-deoxyglocosone was decreased. Based on numerical optimization, using the predictive model, at all levels of phenolic compounds within the experimental range the predicted concentration of 3-deoxyglucosone was significantly lower as compared to the concentration found in control UHT as well as pasteurized milk demonstrating the efficiency of these phenolic mixtures in reducing 3-deoxyglucosone in UHT processed milk and the potential of dose and cost optimization as an ingredient technology.

The observed quadratic effects mean that an interaction of that factor with itself exists. More specifically, as the level of a factor (in this case concentration) changes the nature of the influence of that factor on the response also changes and this interaction can

be visualized as a curvature in the response surface. Quadratic effects of catechin for methylglyoxal and catechin and daidzein for 3-deoxygluocsone were found to be significant and that could potentially be explained by the lower levels of these RCSs in aseptic UHT milk as compared to glyoxal (only linear effects were significant – Figure 7a-c). Lower concentrations of RCSs could lead to dilution effects and when at low levels of added phenolic compounds would be expected to be less effective. As the concentration of phenolic compounds increases the effectiveness would likely increase thus, the observed influence on the response will change leading to the observed quadratic effects.

To test the predictive power of the developed models and thus, RMS as optimization tool for the development of a pre-processing treatment for the reduction of RCSs levels in food, such as milk, a validation experiment was run using catechin, genistein and daidzein at 0.17, 1.12 and 0.40uM respectively. Based on the developed RSM models the predicted values along with 95% confidence intervals for glyoxal, methylglyoxal and 3-deoxyglucosone were 16.64 (14.65-18.14), 7.20 (6.35-8.06) and 0.56 (0.42-0.68) uM respectively. The actual quantification of the RCSs in these samples showed that the actual and predicted values for glyoxal (15.31uM), methylglyoxal (6.46uM) and 3-deoxyglucosone (0.59uM) concentration were not significantly different (p-value<0.05) and thus, that the predictive models were illustrated to adequately predict the levels of RCSs in UHT milk when these three phenolic mixture treatments are used. Though these results are promising there is still a need to further validate the developed models using industrial and/or pilot plant scale UHT processors in order to translate the findings from these model systems.

In summary based on an improved derivatization method to quantitatively monitor RCSs, RSM was reported to be an effective optimization strategy for phenolic mixtures as a pre-processing treatment for the reduction of RCSs in UHT milk. The developed predictive models had a good fit/power and provided insight into phenolic structure reactivity for the reduction of levels of RCSs in this food sample. Certain phenolic mixtures were able to reduce RCSs levels close to or below levels found in pasteurized milk, demonstrating the potential of this ingredient technology to suppress off-flavor during processing in storage (by trapping key precursors) as well as limit the dietary intake of these potential deleterious compounds.

Table 4.1. Sample set for 3-factor 3-level Box-Behnken statistical design including the coded levels of factors; 1, 0 and -1 represent high (1.12mM), mid (0.645mM) and low (0.17mM) dose level added to milk prior UHT processing respectively.

Run	Catechin	Genistein	Daidzein
1	-1	-1	0
2	-1	1	0
3	1	-1	0
4	1	1	0
5	0	-1	-1
6	0	-1	1
7	0	1	-1
8	0	1	1
9	-1	0	-1
10	1	0	-1
11	-1	0	1
12	1	0	1
13	0	0	0
14	0	0	0
15	0	0	0

Table 4.2. Concentration of glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3-DG) in milk samples when corresponding phenolic mixture treatments were applied prior to UHT processing.

Run	Catechin (uM)	Genistein (uM)	Daidzein (uM)	GO (uM)	MGO (uM)	3-DG (uM)
1	0.17	0.17	0.645	24.723	11.094	0.873
2	0.17	1.12	0.645	12.472	6.553	0.540
3	1.12	0.17	0.645	14.774	8.811	0.381
4	1.12	1.12	0.645	9.650	7.075	0.319
5	0.645	0.17	0.17	19.734	11.768	0.782
6	0.645	0.17	1.12	12.727	9.507	0.486
7	0.645	1.12	0.17	17.626	8.378	0.609
8	0.645	1.12	1.12	12.728	7.688	0.294
9	0.17	0.645	0.17	24.319	9.728	0.660
10	1.12	0.645	0.17	14.441	7.282	0.325
11	0.17	0.645	1.12	15.683	7.793	0.566
12	1.12	0.645	1.12	10.346	6.901	0.212
13	0.645	0.645	0.645	16.352	9.316	0.707
14	0.645	0.645	0.645	16.769	9.038	0.747
15	0.645	0.645	0.645	15.236	8.775	0.672
Control UHT	N.A.	N.A.	N.A.	27.516	13.240	2.611
Pasteurized	N.A.	N.A.	N.A.	5.841	7.296	1.150

Table 4.3. Parent and main sibling ions detected for optimum Multi-Reaction-Monitoring (MRM) conditions for glyoxal, methylglyoxal, 3-deoxyglucosone, catechin as well as prominent adducts between catechin (CAT) and methylglyoxal (MGO) and internal standards [$^{13}C_4$]- diacetyl and butyl-paraben.

Analyte	**Parent Ion(m/z)**	**Cone voltage (V)**	**Collision Energy (V)**	**Sibling Ion (m/z)**
Catechin[a]	289	35	23	205
CAT-MGO[a]	361	35	20	181
CAT-MGO_2[a]	433	35	27	235
Glyoxal[b]	131	35	27	77
Methylglyoxal[b]	145	35	26	77
3-deoxyglucosone[b]	235	35	28	199
[$^{13}C_4$]- Diacetyl[a]	163	35	28	77
Butyl-paraben[a]	193	35	21	92

a = ESI (+ve)
b = ESI (-ve)

Table 4.4. Response surface models predicting the concentration of glyoxal, methylglyoxal and 3-deoxyglucosone in UHT processed milk when catechin, genistein and daidzein are added prior to processing at levels between 0.17uM and 1.12uM.

RCSs	**Predictive model**
Glyoxal (GO)	GO = 28.077 – 7.365xCAT - 5.127xGEN - 6.483xDAID
Methylglyoxal (MGO)	MGO = 12.266 + 2.688xCAT - 5.0273xGEN - 1.3860xDAI - $4.677xCAT^2$ + 3.108xCATxGEN
3-deoxyglucosone (3-DG)	DG = 0.707 + 0.421xCAT - 0.200xGEN + 0.492xDAID -$0.612xCAT^2$ - $0.548xDAID^2$

Table 4.5. Coefficients of determination and lack of fit test results for the predictive models generated by RSM for glyoxal, methylglyoxal and 3-deoxyglucosone.

Predictive Model	R-square	Lack of fit (P-value)
Glyoxal	0.806	0.1047
Methylglyoxal	0.924	0.1950
3-deoxyglucosone	0.916	0.1922

Table 4.6. Eigenvalues and eigenvectors of response surfaces of glyoxal, methylglyoxal and 3-deoxyglucosone. Same superscripts indicate association of the coefficient of a specific eigenvector with an eigenvalue. The largest the eigenvalue the higher the sensitivity of the response to the factor associated with that eigenvector.

	Glyoxal			**Methylglyoxal**			**3-deoxyglucosone**		
Eigenvalue	1.521	-0.249	-0.795	0.547	-0.152	-1.137	-0.029	-0.127	-0.151
Catechin[a]	0.659[a]	0.411	-0.629	0.244	0.053	0.968[a]	0.289	-0.053	0.956[a]
Genistein[b]	0.458	0.443	0.771[b]	0.902[b]	-0.379	-0.206	0.957[b]	0.0474	-0.287
Daidzein[c]	0.595	-0.797[c]	0.103	0.357	0.924[c]	-0.139	-0.030	0.9975[c]	0.064

Table 4.7. Ridge analysis results for predicted responses glyoxal, methylglyoxal and 3-deoxyglucosone, which locates a region (radius around the center of experimental design) were the optimum response of predicted variables, in this case minimum, lies.

Radius	Predicted GO (uM)	S.E.	Catechin (uM)	Genistein (uM)	Daidzein (uM)
0	15.119	1.442	0.645	0.645	0.645
0.1	14.608	1.438	0.676	0.667	0.672
0.2	14.123	1.425	0.708	0.689	0.700
0.3	13.678	1.405	0.739	0.711	0.728
0.4	13.258	1.380	0.771	0.733	0.755
0.5	12.869	1.353	0.803	0.755	0.783
0.6	12.511	1.330	0.835	0.777	0.810
0.7	12.187	1.315	0.867	0.799	0.838
0.8	11.885	1.318	0.899	0.821	0.865
0.9	11.617	1.345	0.931	0.843	0.892
1	11.380	1.304	0.965	0.865	0.918
Radius	**Predicted MGO (uM)**	**S.E.**	**Catechin (uM)**	**Genistein (uM)**	**Daidzein (uM)**
0	9.043	0.208	0.645	0.645	0.645
0.1	8.877	0.207	0.664	0.684	0.663
0.2	8.722	0.205	0.687	0.721	0.680
0.3	8.574	0.202	0.720	0.754	0.696
0.4	8.431	0.199	0.767	0.776	0.707
0.5	8.283	0.195	0.826	0.783	0.709
0.6	8.122	0.193	0.887	0.781	0.707
0.7	7.943	0.193	0.945	0.775	0.702
0.8	7.744	0.197	1.001	0.767	0.696
0.9	7.524	0.207	1.054	0.758	0.690
1	7.281	0.223	1.106	0.749	0.684
Radius	**Predicted 3DG (uM)**	**S.E.**	**Catechin (uM)**	**Genistein (uM)**	**Diadzein (uM)**
0	0.708	0.039	0.645	0.645	0.645
0.1	0.685	0.039	0.683	0.663	0.667
0.2	0.659	0.039	0.722	0.677	0.690
0.3	0.631	0.038	0.762	0.687	0.714
0.4	0.601	0.038	0.804	0.694	0.737
0.5	0.568	0.037	0.845	0.698	0.761
0.6	0.533	0.036	0.888	0.699	0.784
0.7	0.495	0.036	0.930	0.700	0.806
0.8	0.454	0.037	0.973	0.697	0.829
0.9	0.410	0.038	1.017	0.695	0.850
1	0.364	0.040	1.060	0.690	0.871

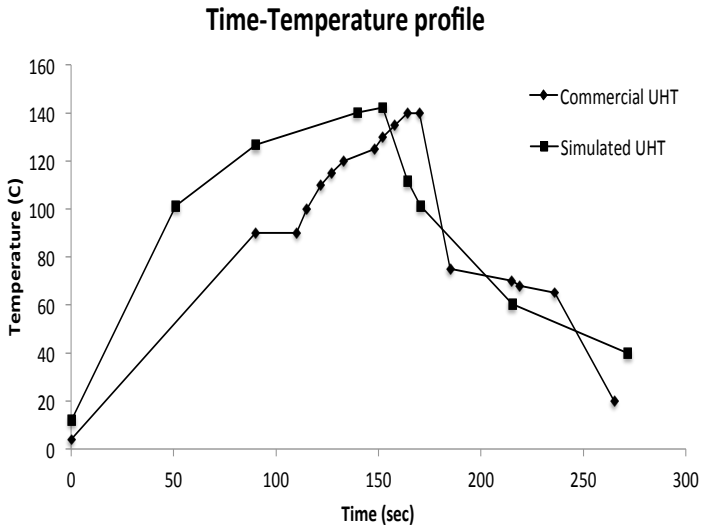


Figure 4.1. Time-temperature profile of the bench-top UHT processing method developed with a CTC Analytics auto-sampler system equipped with a cooling vial holder and a heated vial agitator; a commercial indirect tubular profile is display for comparison.

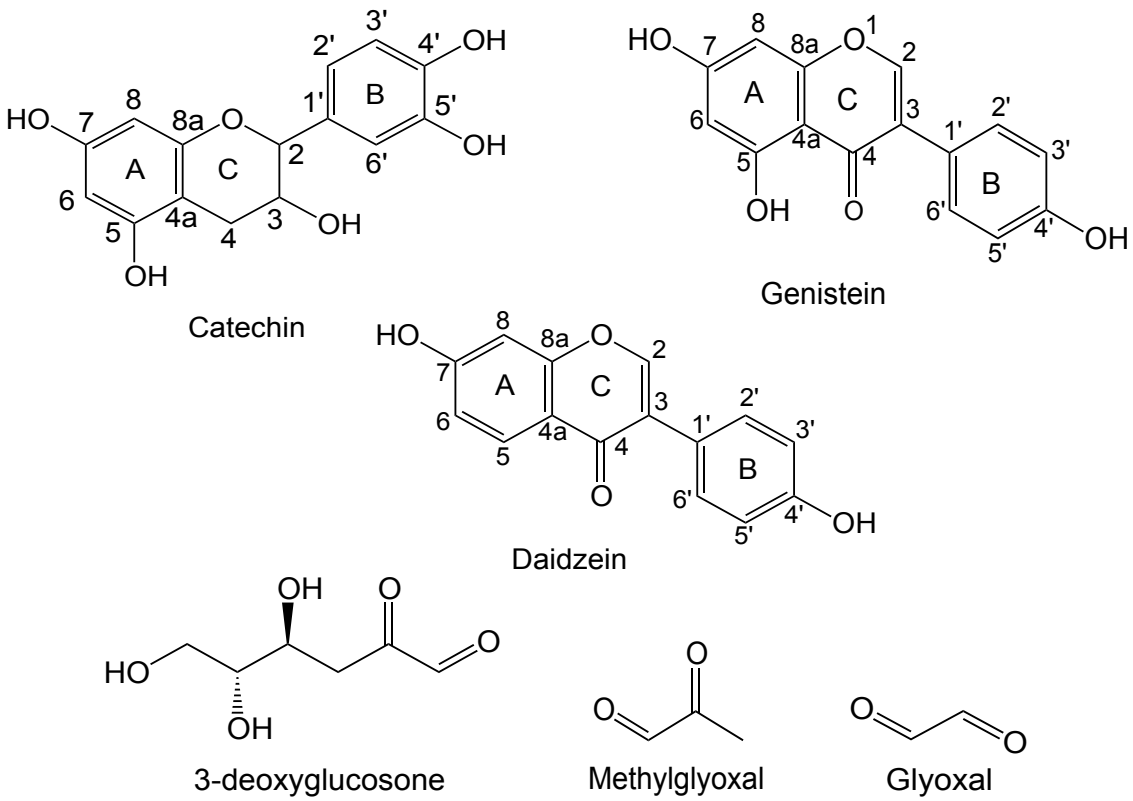


Figure 4.2. Structure of select food phenolic compounds (catechin, daidzein, and genistein) and reactive carbonyl compounds (glyoxal, methylglyoxal and 3-deoxyglucosone).

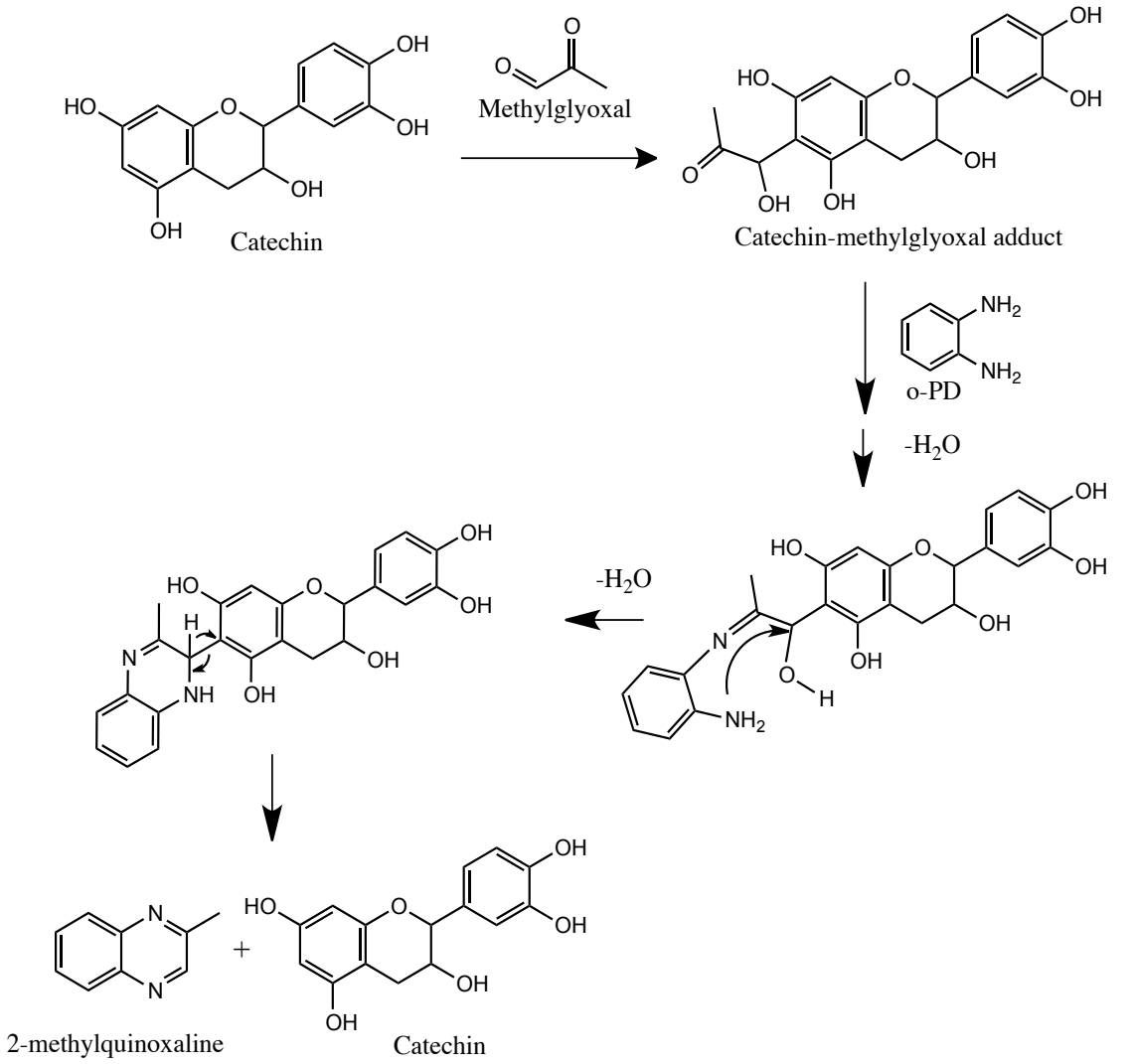


Figure 4.3. Proposed mechanism for adduct formation between phenolic compounds catechin) and RCSs (methylglyoxal) and subsequent liberation of RCSs moieties from adducts by addition of derivatizing agent o-phenylendiamine (o-PD) and formation of quinoxilines derivatives (2-methylquinoxaline).

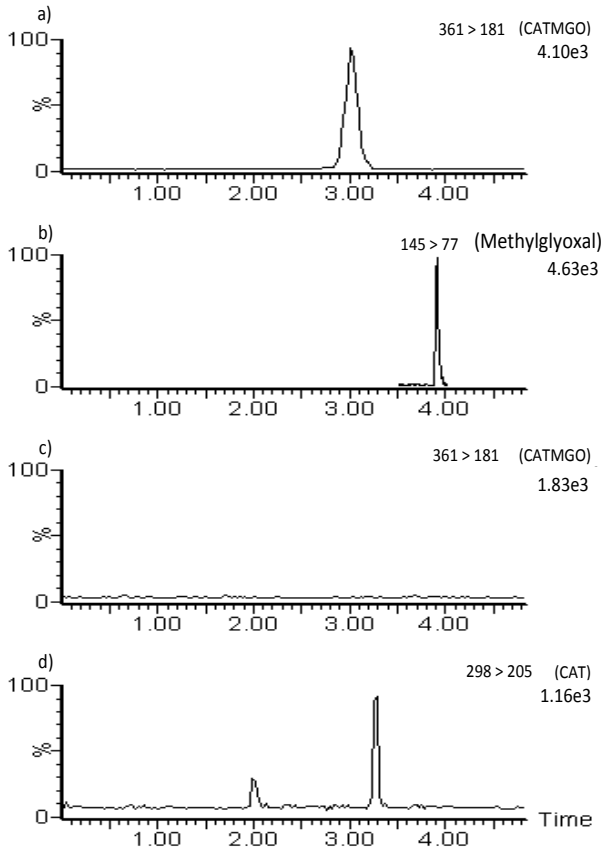


Figure 4.4. UPLC/MS/MS analysis of purified catechin-methyglyoxal adduct (CATMGO) solutions before and after derivatization with o-PD. Prior to derivatization only the adduct was detected (chromatogram a); whereas after derivatization methylglyoxal as 3-methylquinoxaline (chromatogram b) and catechin (chromatogram d, 3.15min) are detected while the CATMGO adduct is not detectable (chromatogram c)

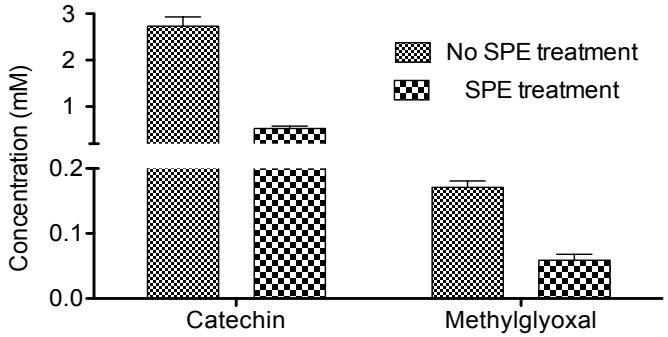


Figure 4.5. Concentration of catechin and methylglyoxal determined in a model reaction of 10mM catechin and methylglyoxal by two different analytical methods; (1) derivatization of the model reaction by o-phenylenediamino and (2) fractionation of solid phase extraction of model reaction followed by derivatization; average with 95% confidence intervals in triplicate.

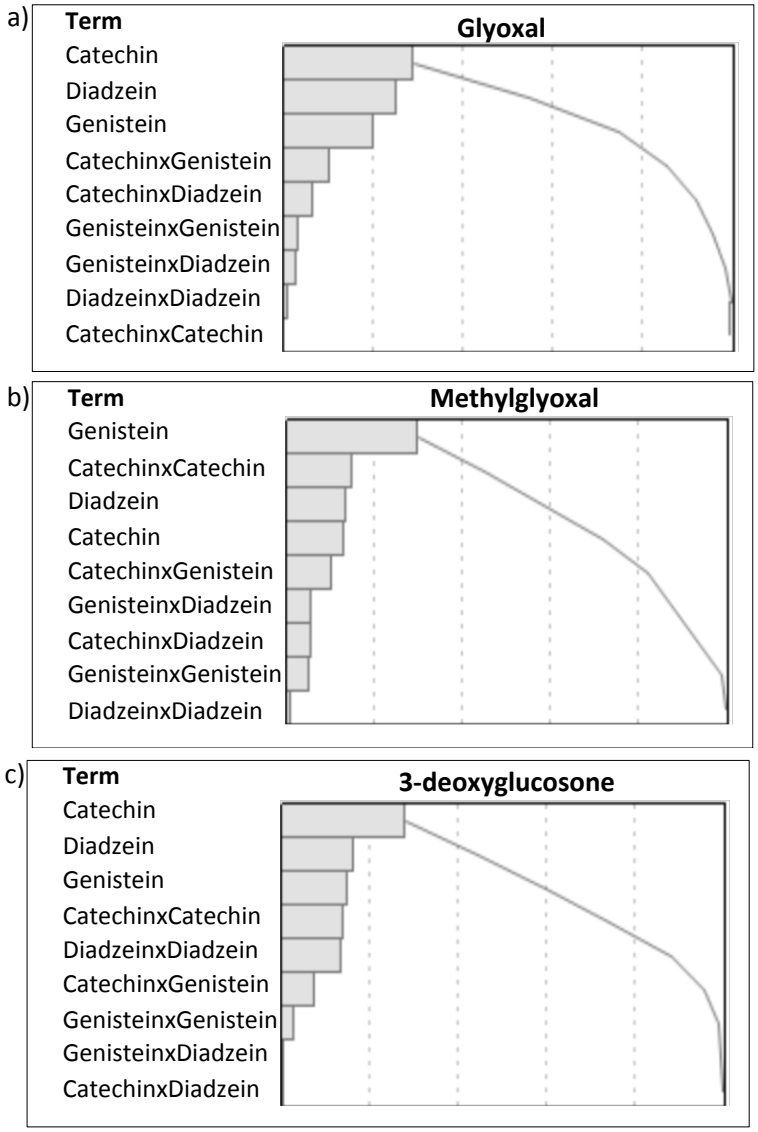
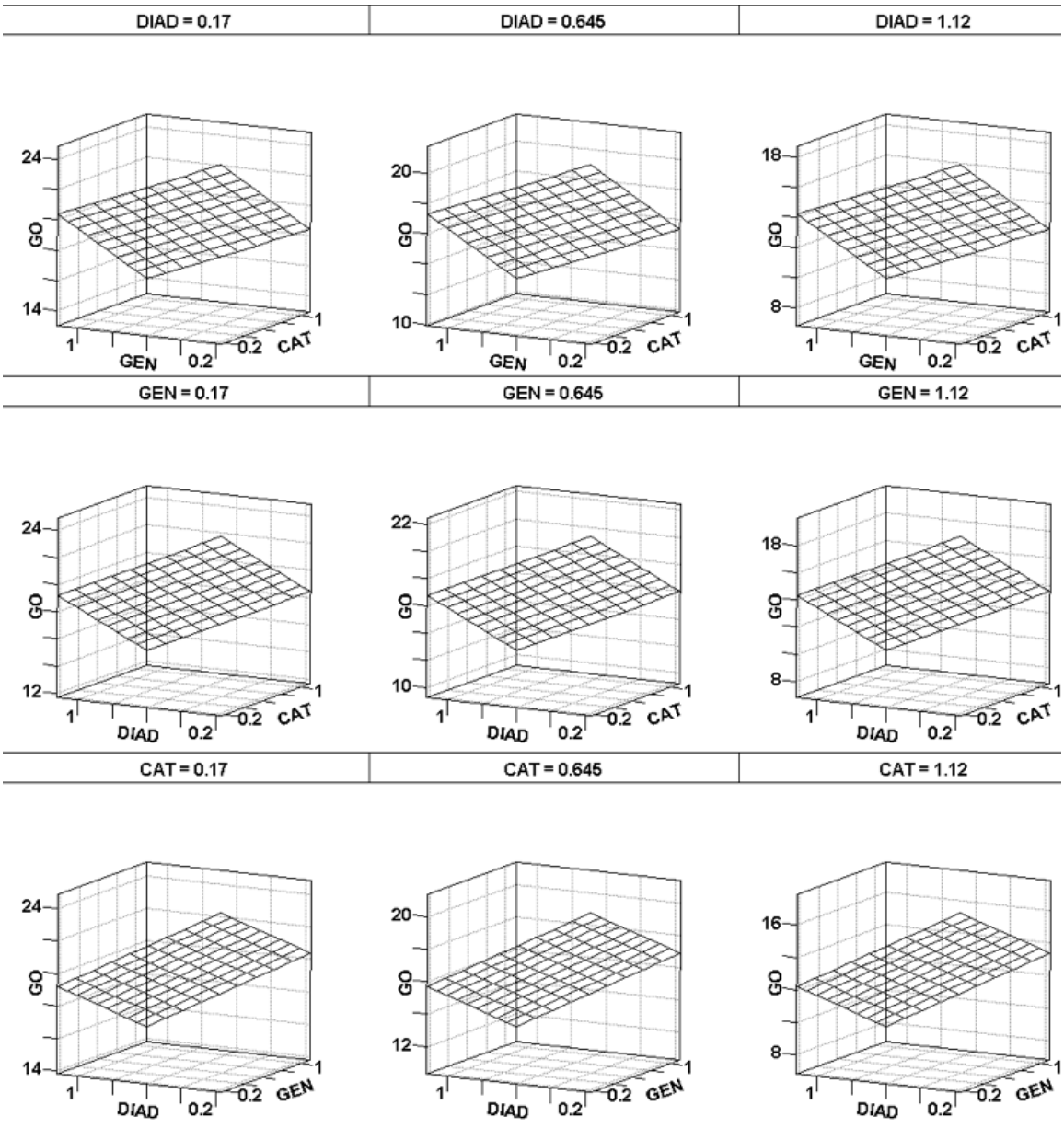
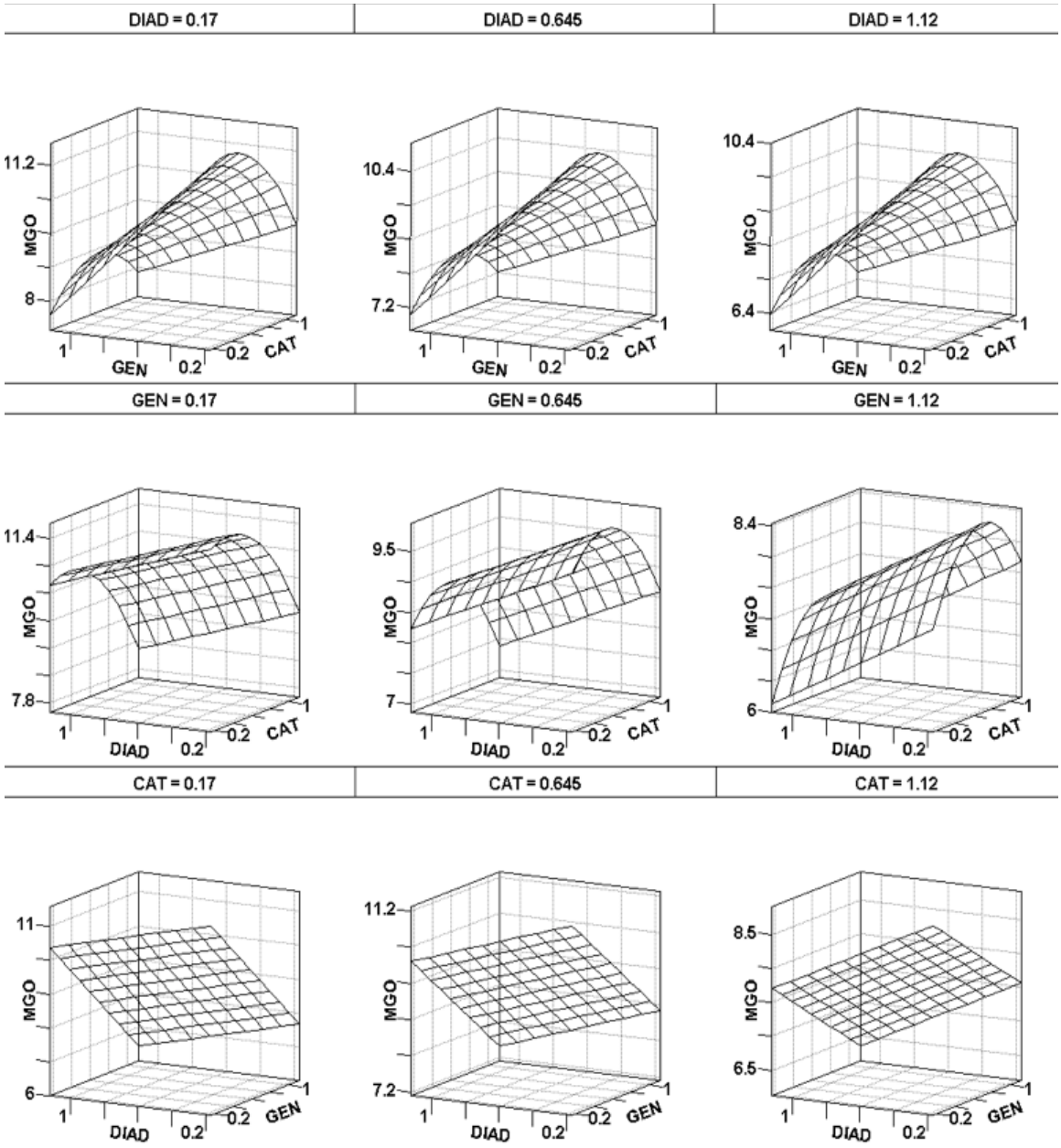


Figure 4.6. Pareto Charts for glyoxal, methylglyoxal and 3-deoxyglucosone reflecting the impact and significance of factors linear, quadratic and interaction effects.

a)


DIAD = 0.17
DIAD = 0.645
DIAD = 1.12
GO
GEN
CAT
GEN = 0.17
GEN = 0.645
GEN = 1.12
DIAD
CAT = 0.17
CAT = 0.645
CAT = 1.12

b)


DIAD = 0.17
DIAD = 0.645
DIAD = 1.12
MGO
11.2
8
10.4
7.2
10.4
6.4
GEN
CAT
1
0.2
GEN = 0.17
GEN = 0.645
GEN = 1.12
11.4
7.8
9.5
7
8.4
6
DIAD
CAT = 0.17
CAT = 0.645
CAT = 1.12
11
6
11.2
7.2
8.5
6.5

c)

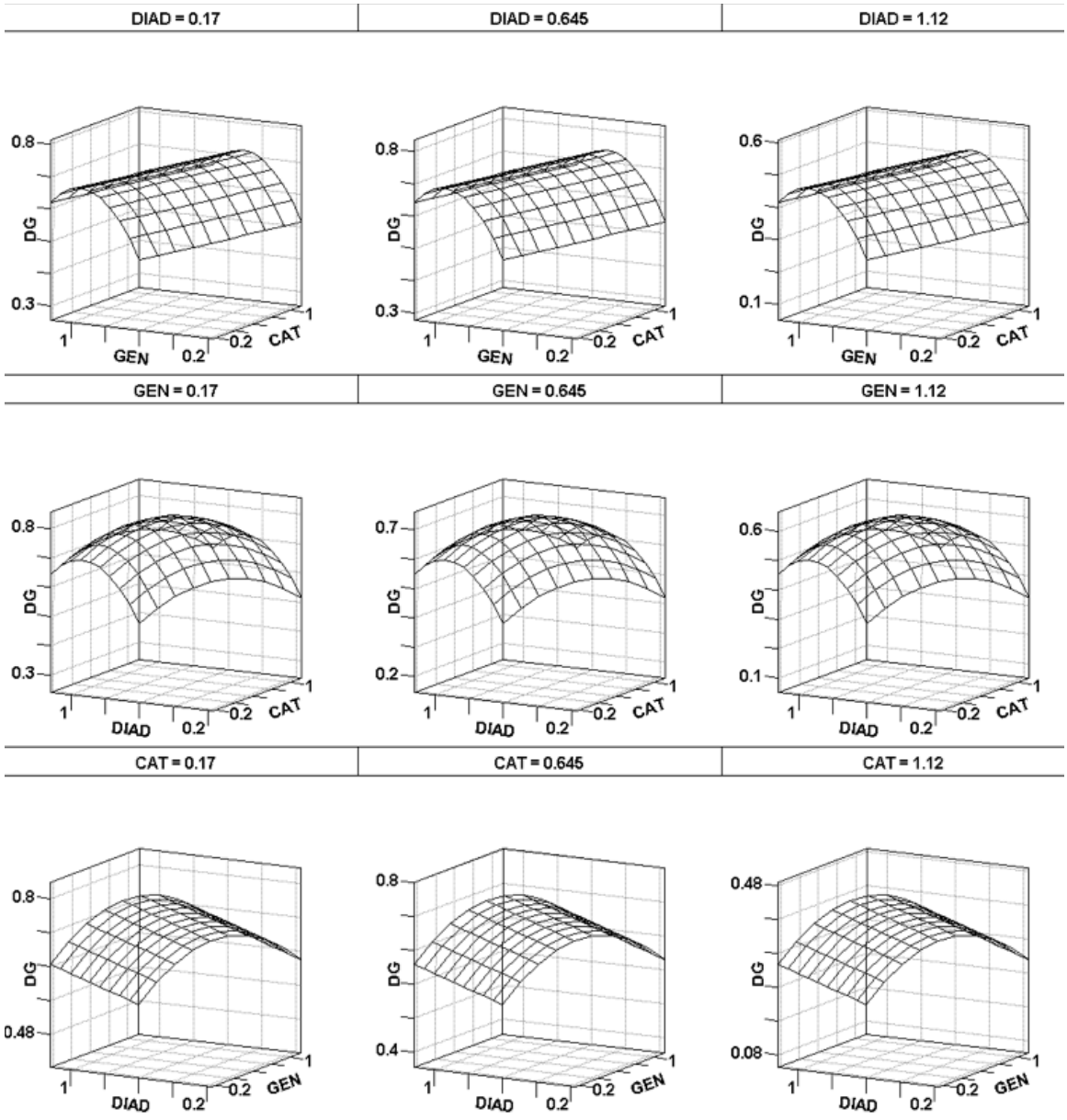


Figure 4.7. Response surface plots generated from RSM analysis for a) glyoxal (GO), b) methylglyoxal (MGO) and c) 3-deoxyglucosone (3DG) when daidzein, genistein and catechin are fixed factors at 0.17, 0.645 and 1.12uM respectively.

Chapter 5

Suggested Future Work

Overall, three main suggestions are discussed as proposed future work. First, based on the observed results regarding structural reactivity of phenolic compounds and how it relates to inhibition of RCSs and important off-flavor markers in UHT processed milk work, a logical next step would be to further investigate, characterize in-depth and confirm the mechanisms of off-flavor generation. Thus far our results suggest that one the mechanisms of action of phenolic compounds in MR inhibition is aromatic electrophilic substitution reactions and RCSs trapping that ultimately lead to adduct formation between phenolic compounds and RCSs. In order to achieve a more comprehensive analytical characterization of off-flavor generation and further elucidate the mechanistic pathways in UHT milk it would be beneficial to perform isotope-labeling studies to define precursors/intermediates and end products and the mechanisms involved.

Select C-13 labeled precursors can be infused into the milk before UHT processing (140C for 6 sec) and based on methods previously used by our laboratory [36] isotopomeric analysis of the important off-flavor compounds identified in these study can be used to elucidate precursors and the pathways of generation. The use of the small scale simulated UHT processing system developed in this study would be recommended as an optimal UHT processing approach as isotope-labeling experiments can be cost

prohibiting when large-scale experiments (i.e. industrial scale UHT processing) need to be performed.

For Maillard reaction pathways, quantification of reducing sugars and monosaccharide levels in UHT milk using LC/MS/MS methods would be the first logical step. Next, appropriate aqueous model system should be used to simulate the milk matrix and similar quantities of sugars detected should be added back one at a time as labeled isotopes. A mass balance study regarding reactions between phenolic compounds and RCSs and would help understand how the concentration of phenolics and phenolic-RCSs adducts is changing during UHT processing and storage and provide valuable information regarding the fate of these chemical species and mechanistic pathways they enter.

Furthermore, understanding the effect of the end products of the reaction between different phenolic structures with RCSs or other food constituents on human health is of vital importance, thus microbial and mammalian cell studies and eventually animal studies exploring potential adverse or beneficial health effects are recommended.

Lastly, validation of the predictive models for process optimization developed using Response Surface Methodology using an industrial scale UHT processing method is also needed to test both the efficiency of the model but also the efficiency of Response Surface Methodology as a food process optimization strategy for reducing RCSs concentration.

References

[1] P. Bellwood, “The Beginnings of Agriculture in Southwest Asia,” in *First Farmers: the origins of agricultural societies*, Malden, MA: Blackwell Publushing, 2005, pp. 44–68.

[2] E. M. Dupuis, *Nature’s Perfect Food How Milk Became America's Drink*. New York: New York University Press, 2002.

[3] Internatiol Dairy Foods Association, “Milestones of Milk History in the United States.” 2004.

[4] FDA, “Milk.” p. 21CFR131.110, 2005.

[5] L. Clement, J. Scimeca, and H. Thompson, “Conjugated linoleic acid. A powerful anticarcinogen from animal fat sources,” *Cancer*, vol. 74, no. 3, pp. 1050–4, 1994.

[6] D. Kritchevsky, “Antimutagenic and some other effects of conjugated linoleic acid,” *The British Journal of Nutrition*, vol. 83, no. 5, pp. 459–65, 2000.

[7] H. D. Belitz, W. Grosch, and P. Schieberle, “Milk and Dairy Products,” in *Food Chemistry*, 3rd editio., Springer-Verlag, Ed. Berlin: , 2004, pp. 505–550.

[8] U. Agricultural Marketing Resource Center, “AgMRC Report,” 2011.

[9] S. Yen and B. Lin, "Beverage Consumption Among U.S. Children and Adolescents: Full-Information and Quasi Maximum-Likelihood Estimation of a Censored Syste," *European Review of Agricultural Economics*, vol. 29, no. 1, pp. 85–103, 2002.

[10] D. K. Henyon, "Extended Shelf-Life Milks in North America: A Perspective.," *International Journal of Dairy Technology*, vol. 52, no. 3, pp. 95–101, 1999.

[11] T. E. Galesloot, *The sterilization of milk, Milk Hygiene*, WHO Monogr. Geneva: WHO, 1962, pp. 269–302.

[12] H. Burton, *Ultra-high-temperature processing of milk and milk products*. London: Elsevier Applied Science, 1988.

[13] U.S. Department of Health and Human Services, "Grade ' A ' P asteurized Milk Ordinance."2011.

[14] D. W. Sun, *Thermal Food Processing, New Technologies and Quality Issues*. Boca Raton: CRC Press Taylor and Francis Group, 2006.

[15] E. L. Thomas, "Trends in Milk Flavor," *J. Dairy Res.*, no. 42, pp. 285–295, 1981.

[16] J. G. Zadow, "The development of UHT processing in Australia," *Aust. J. Dairy Technol.*, vol. 53, no. 3, pp. 195–198, 1998.

[17] H. C. Perkins, M. L. and Deeth, “A survey of Australian consumers’ attitudes towards UHT milk,” *Aust. J. Dairy Technol.*, vol. 56, no. 1, pp. 28–34, 2001.

[18] P. M. Colahan-Sederstrom and D. G. Peterson, “Inhibition of key aroma compound generated during ultrahigh-temperature processing of bovine milk via epicatechin addition.,” *Journal of agricultural and food chemistry*, vol. 53, no. 2, pp. 398–402, Jan. 2005.

[19] D. K. Bandler, “Rancidity: an icreasingly common milk flavor problem,” *Dairy Food Sanit.*, vol. 2, pp. 312–315, 1982.

[20] M. R. Blake, B. C. Weimer, D. J. Mcmahon, and P. A. Savello, “Sensory and Microbial Quality of Milk Processed for Extended Shelf Life by Direct Steam Injection t,” vol. 58, no. 9, pp. 1007–1013, 1995.

[21] S. Patton, “Browning and associated changes in milk and its products: A review,” *Journal of Dairy Science*, vol. 38, pp. 457–478, 1955.

[22] M. Namiki, “Chemistry of Maillard Reactions: Recent studies on the Browning Reaction Mechanism and the Development of Antioxidant and Mutagens.,” *Advances in Food Research*, vol. 32, pp. 116–170, 1988.

[23] Ames, *The Maillard reaction, in Biochemistry of food proteins*. London: , 1992, pp. 95–153.

[24] K. E. Dunkley, W.L. and Stevenson, "Ultra-High Temperature Processing and Aseptic Packaging of Dairy Products," *Journal of Dairy Science*, vol. 70, no. 10, pp. 2190–2202, 1987.

[25] Z. Al-Attabi, B. R. D'Arcy, and H. C. Deeth, "Volatile sulphur compounds in UHT milk.," *Critical reviews in food science and nutrition*, vol. 49, no. 1, pp. 28–47, Jan. 2009.

[26] E. L. Thomas, H. J. Burton, E. Ford, and A. G. Perkin, "The effect of oxygen content on flavour and chemical changes during aseptic storage of whole milk after ultra-high-temperature processing.," *Journal of Dairy Research*, no. 42, pp. 285–295, 1975.

[27] M. Simon, a P. Hansen, and C. T. Young, "Effect of various dairy packaging materials on the headspace analysis of ultrapasteurized milk.," *Journal of dairy science*, vol. 84, no. 4, pp. 774–83, Apr. 2001.

[28] F. G. Tessier and I. Birlouez-Aragon, "Health effects of dietary Maillard reaction products: the results of ICARE and other studies," *Amino Acids*, vol. 42, no. 4, pp. 1119–1131, 2012.

[29] J. Uribarri, W. Cai, O. Sandu, M. Peppa, T. Goldberg, and H. Vlassara, "Diet-derived advanced glycation end products are major contributors to the body's AGE

pool and induce inflammation in healthy subjects.," *Annals of the New York Academy of Sciences*, vol. 1043, pp. 461–6, Jun. 2005.

[30] J. Uribarri, "Restriction of Dietary Glycotoxins Reduces Excessive Advanced Glycation End Products in Renal Failure Patients," *Journal of the American Society of Nephrology*, vol. 14, no. 3, pp. 728–731, Mar. 2003.

[31] T. Koschinsky, C. J. He, T. Mitsuhashi, R. Bucala, C. Liu, C. Buenting, K. Heitmann, and H. Vlassara, "Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 12, pp. 6474–9, Jun. 1997.

[32] N. Datta, A. J. Elliott, M. L. Perkins, and H. C. Deeth, "Ultra-high-temperature (UHT) treatment of milk: comparison of direct and indirect modes of heating," *Australian Journal of Dairy Technology*, vol. 57, pp. 211–227, 2002.

[33] G. P. Schamberger and T. P. Labuza, "Effect of green tea flavonoids on Maillard browning in UHT milk," *LWT - Food Science and Technology*, vol. 40, no. 8, pp. 1410–1417, Oct. 2007.

[34] V. M. Totlani and D. G. Peterson, "Epicatechin carbonyl-trapping reactions in aqueous maillard systems: Identification and structural elucidation.," *Journal of agricultural and food chemistry*, vol. 54, no. 19, pp. 7311–8, Sep. 2006.

[35] V. M. Totlani and D. G. Peterson, “Influence of epicatechin reactions on the mechanisms of Maillard product formation in low moisture model systems.,” *Journal of agricultural and food chemistry*, vol. 55, no. 2, pp. 414–20, Jan. 2007.

[36] V. M. Totlani and D. G. Peterson, “Reactivity of epicatechin in aqueous glycine and glucose maillard reaction models: quenching of C2, C3, and C4 sugar fragments.,” *Journal of agricultural and food chemistry*, vol. 53, no. 10, pp. 4130–5, May 2005.

[37] C. Y. Lo, W. T. Hsiao, and X. Y. Chen, “Efficiency of trapping methylglyoxal by phenols and phenolic acids,” *Journal of Food Science*, vol. 76, no. 3, pp. 90–96, 2011.

[38] X. Peng, K. Cheng, J. Ma, B. Chen, C. T. Ho, and C. Lo, “Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advance glycation end products,” *Journal of agricultural and food chemistry*, vol. 56, no. 6, pp. 1907–1911, 2008.

[39] C. Y. Lo, S. Li, D. Tan, M. H. Pan, S. Sang, and C. T. Ho, “Trapping reactions of reactive carbonyl species with tea polyphenols in simulated phisiological conditions,” *Molecular Nutrition and Food Research*, vol. 50, no. 12, pp. 1118–1128, 2006.

[40] W. Wang, Y. Yagiz, T. J. Buran, C. D. N. Nunes, and L. Gu, "Phytochemicals from berries and grapes inhibited the formation of advanced glycation end-products by scavenging reactive carbonyls," *Food Research International*, vol. 44, no. 9, pp. 2666–2673, Nov. 2011.

[41] DMI, "Extending Shelf Life in Dairy Foods. Dairy Industry Technology Review," 1998.

[42] T. Asthon, "Practical Experience: The processing and Aseptic packaging of sterile milk in the United Kindom," *Journal of Society of Dairy Technology*, vol. 18, pp. 65–85, 1965.

[43] T. Patel, D. Morgan, H. Calbert, and F. Strong, "Changes in Volatile Flavor Components of Sterilized Concentrated Milk During Storage," *Jornal of Dairy Science*, vol. 45, no. 601–606, 1962.

[44] J. Kirk, T. Hedrick, and C. Stine, "Gas Chromatographi Study of Flavor deterioration in High-Temperature Short-Time fluid sterile milk," *Journal of Dairy Science*, vol. 51, pp. 169–177, 1968.

[45] R. Scanlan, R. Lindsay, L. Libbey, and E. Day, "Heat-Induced Volatile Compounds in Milk," *Journal of Dairy Science*, vol. 51, pp. 1001–1007, 1968.

[46] J. Zadow and R. Britwistle, “The effect of dissolved O2 on the changes occuring in the flavour of ultra-high-temperature milk during storage,” *Journal of Dairy Research*, vol. 40, pp. 169–177, 1973.

[47] E. Thomas, H. Burton, J. Ford, and A. Perkins, “The effect of oxygen content on flavour and chemical changes during aseptic storage of whole milk after ultra-high-temperature processing,” *Journal of Dairy Research*, vol. 42, pp. 285–295, 1975.

[48] I. Jeon, E. Thomas, and G. A. Reineccius, “Production of Volatile Flavor Compounds in Ultra High-Temperature Processed Milk During Aseptic Storage,” *Journal of Agricultural and Food Chemistry*, vol. 26, pp. 1183–1188, 1978.

[49] H. A. Jaddou, J. A. Pavey, and D. J. Manning, “Chemical Analysis of Flavour Volatiles in Heat-Treated Milks,” *Journal of Dairy Research*, vol. 45, pp. 391–403, 1978.

[50] H. T. Badings, J. J. P. van der Poll, and R. Neeter, “Aroma Compounds which Contribute to the Difference in Flavour Between Pasteurized Milk and UHT Milk,” in *Weurman Flavour Symposia*, 1981.

[51] W. T. Gillis, M. F. Cartledge, I. R. Rodriguez, and E. J. Suarez, “Effect of Raw Milk Quality on Ultra-High Temperature Processed Milk,” *Journal of Dairy Science*, vol. 68, pp. 2875–2879, 1985.

[52] R. C. McKellar, “Developemnt of Off-Flavors in Ultra-High Temperature and Pasteurized Milk as Function of Proteolysis,” *Journal of Dairy Science*, vol. 64, pp. 2138–2145, 1981.

[53] R. E. Anderson, G. Danielsson, C. B. Hedlund, and S. G. Svensson, “Effect of a Heat-Ressistant Microbial Lipase on Flavor of Ultra-High-Temperature Sterilized Milk,” *Journal of Dairy Science*, vol. 64, pp. 375–379, 1981.

[54] A. P. Hansen, K. R. Swartzel, and F. G. Giesbrecht, “Effect of Temperature and Time of Processing and Storage on Consumer Acceptability of Ultra-High Temperature Steam Injected Whole Milk,” *Journal of Dairy Science*, vol. 63, pp. 187–192, 1980.

[55] S. Rerkrkai, I. J. Jeon, and R. Bassette, “Effect of Various Direct Ultra-High Temeprature Heat Treatments on Flavor of Commercially Prepared Milks,” *Journal of Dairy Science*, vol. 70, pp. 2046–2054, 1987.

[56] R. Bassette and I. J. Jeon, “Effect of Process- and Storage-Times on Concentration of Volatile Materials in Ultra-High Temperature Steam-Infusion Processed Milk,” *Journal of Food Protection*, vol. 46, pp. 950–953, 1983.

[57] G. Contarini, M. Povolo, R. Leardi, P. M. Toppino, I. S. Lattiero-caseario, V. Lombardo, and V. B. Salerno, “Influence of Heat Treatment on the Volatile Compounds of Milk,” pp. 3171–3177, 1997.

[58] W. Grosch, “Lipid Degradation Products and Flavour,” in *Food Flavours*, I. D. Norton and A. J. MacLeod, Eds. Amsterdam: Elsevier Science Publishers, 1982.

[59] L. Moio, P. Etievant, D. Langlois, J. Dekimpe, and F. Addeo, “Detection of Powerful Odorants in Heated Milk by Use of Extract Dilution Sniffing Analysis,” *Journal of Dairy Research*, vol. 61, pp. 385–394, 1994.

[60] E. H. Ramsaw and E. A. Dunstone, “No Title,” *Volatile Compounds Associates with the Off-Flavour in Stored Casein*, vol. 36, pp. 215–223, 1969.

[61] A. K. Adhikari and O. P. Singhal, “Changes in Flavor Profile of Indirectly Heated UHT Milk During Storage: Effect of Maillard Browning and Some Factors Affecting it.,” *Indian Journal of Dairy Science*, vol. 44, pp. 422–448, 1991.

[62] A. J. Furth, “Methods of Assaying Nonenzymatic Glycosylation,” *Analytical Biochemistry*, vol. 175, pp. 347–360, 1988.

[63] M. A. J. S. Van Boekel, “Effect of heating on Maillard reactions in milk,” vol. 62, no. 4, 1998.

[64] K. Iwatsuki, Y. Mizota, T. Kubota, O. Nishimura, H. Masuda, K. Sotoyoma, and M. Tomita, “Evaluation of Aroma of Pasteurized and UHT Processed Milk by Aroma Extract Dilution Analysis,” *Journal of the Japanese Society fir Food Science and Technology*, vol. 46, pp. 587–597, 1999.

[65] J. T. Huton and S. Patton, “The Origin of Sulfhydryl Groups in Milk Proteins and their Contribution to Cooked Flavor,” *Journal of Dairy Science*, vol. 35, pp. 699–705, 1952.

[66] S. Patton, D. A. Forss, and E. A. Day, “Methyl sulfide and the Flavor of Milk,” *Journal of Dairy Science*, vol. 39, pp. 1469–1470, 1956.

[67] R. T. Marsilli, “Flavours and Off-Flavours in Dairy Foods,” in *Encyclopedia of DAiry Sciences*, H. Roginski, J. Fuquay, and P. Fox, Eds. Boston: Academic Press, 2002.

[68] A. K. Adhikari and O. P. Singhal, “Effects of Dissolved-Oxygen Content on the Flavor Profile of UHT Milk During Storage,” *Australian Journal of Dairy Technology*, vol. 47, pp. 1–6, 1992.

[69] I. Andersson and R. Oste, “Sensory Changes and Free Sh-Groups in UHT Milk,” *Milchwissenschaft-Milk Science International*, vol. 47, pp. 438–441, 1992.

[70] M. Simon and A. P. Hansen, “Effect of Various Packaging Materials in the Shelf Life and Flavor of Ultrapasteurized mIlk,” *Journal of Dairy Science*, vol. 84, pp. 784–791, 2001.

[71] C. Billaud and J. Adrian, “Louis-Camille Maillard, 1878-1936,” *Food Reviews International*, vol. 19, no. 4, pp. 345–74, 2003.

[72] D. S. Mottram, B. L. Wedzicha, and A. T. Dodson, “Acrylamide is formed in the Maillard reaction.,” *Nature*, vol. 419, no. 6906, pp. 448–9, Oct. 2002.

[73] G. Schneeman, B. O. & Dunaif, “Nutritional and gastrointestinal response to heated non fat dry milk,” *J. Agri. Food Chem*, vol. 32, pp. 477–480, 1984.

[74] et al. Baynes, J. W., “The Maillard reaction in food and biological sciences,” *Annals of the New York Academy of Sciences*, p. 1043, 2005.

[75] H. Steinhart, “The Maillard Reaction. Chemistry, Biochemistry and Implications. By Harry Nursten.,” *Angewandte Chemie International Edition*, vol. 44, no. 46, pp. 7503–7504, 2005.

[76] J. A. Gerrad, “New Aspects of an AGEing chemistry- Recent developments concerning the Maillard reaction,” *Aust. J.Chem*, vol. 55, pp. 299–310, 2002.

[77] R. Lehmann and E. D. Schleicher, “Molecular mechanism of diabetic nephropathy.,” *Clinica chimica acta; international journal of clinical chemistry*, vol. 297, no. 1–2, pp. 135–44, Jul. 2000.

[78] T. P. Degenhardt, *Role of carbonyl stress in aging and age-related diseases, in The Maillard Reaction in Food and Medicine*. Science Park, Cambridge: The Royal Society of Chemistry, 1998, pp. 1–10.

[79] V. Šoškić, K. Groebe, and A. Schrattenholz, "Nonenzymatic posttranslational protein modifications in ageing," *Experimental Gerentology*, vol. 43, no. 4, pp. 247–257, 2008.

[80] A. Arnoldi and E. Corain, "Effects of free radicals on pyrazine formation in the Maillard reaction," in *Recent Developments in Flavour Science*, A. J. Taylor and D. S. Mottram, Eds. Science Park, Cambridge: The Royal Society of Chemistry, 1996, pp. 217–220.

[81] H. Weenen and W. Apeldoorn, "Carbohydrate cleavage in the Mailland reaction," in *Flavour science: recent developments*, Science Park, Cambridge: The Royal Society of Chemistry, 1996, pp. 211–216.

[82] M. Villamiel, D. M. Del Castillo, and N. Corzo, "Browning Reactions," in *Food Biochemistry and Food Processing*, W.-K. Nip, Ed. Somerset, NJ: Blackwell Publishing, 2006, pp. 71–101.

[83] V. A. Yaylayan, "Classification of the Maillard reaction: A conceptual approach," *Trends Food Sci. Tech.*, vol. 8, pp. 13–18, 1997.

[84] R. Tressl, "Pentoses and Hexoses as Sources of New Melanoidin-like Maillard Polymers," *J. Agric. Food. Chem.*, vol. 46, pp. 1765–1776., 1998.

[85] B. Cämmerer, W. Jalyschko, and L. W. Kroh, “Intact carbohydrate structures as part of the melanoidin skeleton.,” *Journal of Agricultural and Food Chemistry*, vol. 50, no. 7, pp. 2083–2087, 2002.

[86] X. Fang and K. Schmidt-Rohr, “Alkyl and Other Major Structures in 13C-Labeled Glucose-Glycine Melanoidins Identified by Solid-State Nuclear Magnetic Resonance,” *Journal of Agricultural and Food Chemistry*.

[87] X. Fang and K. Schmidt-Rohr, “Fate of the Amino Acid in Glucose- Glycine Melanoidins Investigated by Solid-State Nuclear Magnetic Resonance (NMR),” *Journal of Agricultural and Food Chemistry*, vol. 57, no. 22, pp. 10701–10711, 2009.

[88] M. Shahidi, F.; Naczk, “Biosynthesis, classification, and nomenclature of phenolics in foods and nutraceuticals,” in *Phenolics in Foods and Nutraceuticals*, New York: CRC press, 2004, pp. 1–16.

[89] V. L. Singleton, “Naturally Occuring Food Toxicants: Phenolic Substances of Plant Origin Common in Foods,” *Advances in Food Research*, vol. 27, pp. 149–242, 1981.

[90] J. Kuhnau, “The Flavonoids: A Class of Semi-Essential Food Components: Their Role in Human Nutrition,” *World Review Nutrition and Dietetics*, vol. 24, pp. 117–191, 1976.

[91] M. Saito, H. Hosoyama, T. Ariga, S. Kataoka, and N. Yamaji, "Antiulcer Activity of Grape Seed Extract and procyanidins," *J. Agric. Food Chem.*, vol. 46, pp. 1460–1464, 1998.

[92] C.-H. Wu and G.-C. Yen, "Inhibitory effect of naturally occurring flavonoids on the formation of Advanced Glycation Endproducts," *J.Agric. Food Chem.*, vol. 53, pp. 3167–3173, 2005.

[93] H. Matsuda, "Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities," *Bioorganic & medicinal chemistry*, vol. 11, no. 24, pp. 5317–5323, 2003.

[94] Y. Noda and D. G. Peterson, "Structure-reactivity relationships of flavan-3-ols on product generation in aqueous glucose/glycine model systems.," *Journal of agricultural and food chemistry*, vol. 55, no. 9, pp. 3686–91, May 2007.

[95] S. L. Schwambach and D. G. Peterson, "Reduction of stale flavor development in low-heat skim milk powder via epicatechin addition.," *Journal of agricultural and food chemistry*, vol. 54, no. 2, pp. 502–8, Jan. 2006.

[96] A. D'Agostina, M. Negroni, and A. Arnoldi, "Autoxidation in the formation of volatiles from glucose-lysine," *J. Agric. Food Chem.*, vol. 46, pp. 2554–2559, 1998.

[97] L. J. W. Haffenden and V. A. Yaylayan, “Mechanism of formation of redox-active hydroxylated benzenes and pyrazine in 13C-Labeled glycince/D-gLucose model systems,” *J. Agric. Food Chem.*, vol. 53, pp. 9742–9746, 2005.

[98] G. Reineccius, *Flavor Chemistry and Technology*, 2nd ed. New York: Taylor & Francis Group, 2005, p. 465.

[99] W. Engel, W. Bahr, and P. Schieberle, “Solvent assisted flavour evaporation, a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices,” *Eur. Food Res. Technol.*, vol. 209, pp. 237–241, 1999.

[100] D. Skoog, J. Holler, and S. Crouch, “Gas Chromatography,” in *Principles of Instrumental Analysis*, 6th ed., Thomson Brooks/Cole, 2007, pp. 788–815.

[101] F. Rouessac and A. Rouessac, *Chemical Analysis Modern Instrumentation Methods and Techniques*. New York: John Wiley & Sons, LTD:, 2000, p. 433.

[102] M. S. Feather, “Amine assisted sugar dehydration reactions,” *Prog. Food Nutr. Sci*, vol. 5, pp. 37–45, 1981.

[103] R. Davis and M. Frearson, “Mass Spectrometry,” in *Analytical Chemistry*, London: Open Learning, John Wiley & Sons, 1987.

[104] D. A. Skoog, J. F. Holler, and S. R. Crouch, “Molecular Mass Spectroscopy,” in *Principles of Instrumental Analysis*, 6th ed., 2007, pp. 550–585.

[105] Nigel J.K. Simpson, *Solid-Phase Extraction: Principles, Techniques, and Applications*. 2000.

[106] L. M. L. Nollet, *Food Analysis by HPLc*, 3rd ed. CRC Press, 2012.

[107] A. K. Adhikari, D. Sahai, and O. N. Mathur, “A rapid spectrophotometric method for quantitative determination of lactulose in heated milk and milk products,” *Le Lait*, vol. 71, no. 5, pp. 555–564, 1991.

[108] R. Ikan, “Maillard Reaction - Consequences for the Chemical and Life Sciences.” John Wiley & Sons.

[109] K. J. Wells-knecht, E. Brinkmann, M. C. Wells-knecht, J. E. Litchfield, M. U. Ahmed, S. Reddy, D. V Zyzak, S. R. Thorpe, and J. W. Baynes, “Nephrology Dialysis Transplantation New biomarkers of Maillard reaction damage to proteins,” vol. 1, pp. 41–47, 1996.

[110] T. Murase, a Nagasawa, J. Suzuki, T. Hase, and I. Tokimitsu, “Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver.,” *International journal of obesity and related metabolic disorders : journal*

of the International Association for the Study of Obesity, vol. 26, no. 11, pp. 1459–64, Nov. 2002.

[111] M. Hasan and A. Nihal, "Tea polyphenols: prevention of cancer and optimizing health," *American Society of Clinical Nutrinion*, vol. 71, no. 6, pp. 1698–1702, 2000.

[112] T. Z. Nurulain, "Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications," *Life Sciences*, vol. 78, pp. 1073–2080, 2006.

[113] B. Anandh, P. V. Liu, and Dongmin, "Green tea catechins and cardiovascular health: an update," *Current Medicinal Chemistry*, vol. 15, no. 18, pp. 1840–1850, 2008.

[114] S. C. Yang, X. Wang, G. Lu, and C. S. Pinich, "Cancer prevention by tea: animal studies, molecular mechanisms and human relevance," *Nature Reviews Cancer*, vol. 9, pp. 429–439, 2009.

[115] K. et al Maki, "Green tea catechin consumption enhances exercise-induced abdominal fat loss in overweight and obese adults," *Journal of Nutrition*, vol. 139, no. 2, pp. 264–270, 2009.

[116] P. Schieberle and T. Hofmann, "Evaluation of the Character Impact Odorants in Fresh Strawberry Juice by Quantitative Measurements and Sensory Studies on

Model Mixtures," *Journal of Agricultural and Food Chemistry*, vol. 45, no. 1, pp. 227–232, Jan. 1997.

[117] S. Kokkinidou and D. G. Peterson, "Response Surface Methodology as Optimization Strategy for Reduction of Reactive Carbonyl Species in Heat-processed Foods by Means of Phenolic Chemistry," *Food and Function*, 2013.

[118] A. Sen and W. Grosch, "Original paper Synthesis of six deuterated sulfur containing odorants to be used as internal standards in quantification assays ~ O- ~ cS -- SC ~ - ~," pp. 541–547, 1991.

[119] N. G. . De Kimpe, C. V. . Stevens, and M. A. Keppens, "Synthesis of 2-acetyl-1-pyrroline, the principal rice flavor component," *J. Agric. Food Chem.*, vol. 41, pp. 1458–1461, 1993.

[120] C. Cerny and W. Grosch, "Quantification of character-impact odour compounds of roasted beef," *Zeitschrift f*[illegible]*r Lebensmittel-Untersuchung und -Forschung*, vol. 196, no. 5, pp. 417–422, May 1993.

[121] W. Engel, W. Bahr, and P. Schieberle, "Solvent assisted flavour evaporation – a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices," *European Food Research and Technology*, vol. 209, no. 3, pp. 237–241, Aug. 1999.

[122] USDA/ERS, “Livestock, Dairy and Poultry outlook,” 2010.

[123] R. A. Scanlan, R. C. Lindsay, L. M. Libbey, and E. A. Day, “Heat-induced volatile compounds in milk,” *Journal of Dairy Science*, vol. 51, pp. 1001–1007, 1968.

[124] T. Shibamoto, “Flavor volatiles formed by heated milk,” in *The Analysis and Control of Less Desirable Flavors in Foods and Beverages*, New York: , 1980, pp. 241–265.

[125] I. J. Jeon, *Food Taints and Off-Flavours*. New York: Blackie Academic & Professional, 1996, pp. 139–167.

[126] J. Sun, F. Liang, Y. Bin, P. Li, and C. Duan, “Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries.,” *Molecules (Basel, Switzerland)*, vol. 12, no. 3, pp. 679–93, Jan. 2007.

[127] N. Fabre, I. Rustan, E. de Hoffmann, and J. Quetin-Leclercq, “Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry.,” *Journal of the American Society for Mass Spectrometry*, vol. 12, no. 6, pp. 707–15, Jun. 2001.

[128] W. M. Stöggl, C. W. Huck, and G. K. Bonn, “Structural elucidation of catechin and epicatechin in sorrel leaf extracts using liquid-chromatography coupled to

diode array-, fluorescence-, and mass spectrometric detection," *Journal of Separation Science*, vol. 27, no. 7–8, pp. 524–528, May 2004.

[129] M. Brownlee, "biology of diabetic complications," vol. 414, no. December, pp. 813–820, 2001.

[130] D. Suzuki and T. Miyata, "Carbonyl stress in the pathogenesis of diabetic nephropathy.," *Internal medicine (Tokyo, Japan)*, vol. 38, no. 4, pp. 309–14, Apr. 1999.

[131] G. Basta, A. M. Schmidt, and R. De Caterina, "Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes.," *Cardiovascular research*, vol. 63, no. 4, pp. 582–92, Sep. 2004.

[132] B. Kuhla, H.-J. Lüth, D. Haferburg, K. Boeck, T. Arendt, and G. Münch, "Methylglyoxal, glyoxal, and their detoxification in Alzheimer's disease.," *Annals of the New York Academy of Sciences*, vol. 1043, no. 0, pp. 211–6, Jun. 2005.

[133] D. S. Mottram, "Flavor Compounds Formed during the Maillard Reaction," 1994.

[134] G. A. Reineccius, "The influence of Maillard reactions on the sensory properties of foods," in *The Maillard Reaction in Food Processing, Human Nutrition and Physiology*, Basel, Boston,Berlin: Birkhauser Verlag, 1990, pp. 157–170.

[135] I. Seiquer, J. Díaz-Alguacil, C. Delgado-Andrade, M. López-Frías, A. Muñoz Hoyos, G. Galdó, and M. P. Navarro, "Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11-14 y.," *The American journal of clinical nutrition*, vol. 83, no. 5, pp. 1082–8, May 2006.

[136] F.-L. Gu, J. M. Kim, S. Abbas, X.-M. Zhang, S.-Q. Xia, and Z.-X. Chen, "Structure and antioxidant activity of high molecular weight Maillard reaction products from casein–glucose," *Food Chemistry*, vol. 120, no. 2, pp. 505–511, May 2010.

[137] R. Oste and P. Sjödin, "Effect of Maillard reaction products on protein digestion. In vivo studies on rats.," *The Journal of nutrition*, vol. 114, no. 12, pp. 2228–34, Dec. 1984.

[138] M. Jägerstada and K. Skogb, "Genotoxicity of heat-processed foods," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 574, no. 1–2, pp. 156–172, 2005.

[139] E. Capuano and V. Fogliano, "Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies," *LWT - Food Science and Technology*, vol. 44, no. 4, pp. 793–810, 2011.

[140] C. Crews and L. Castle, “A review of the occurrence, formation and analysis of furan in heat-processed foods,” *Trends in Food Science & Technology*, vol. 18, no. 7, pp. 365–372, Jul. 2007.

[141] J.-K. Moon and T. Shibamoto, “Formation of carcinogenic 4(5)-methylimidazole in Maillard reaction systems.,” *Journal of agricultural and food chemistry*, vol. 59, no. 2, pp. 615–8, Jan. 2011.

[142] E. Tareke, P. Rydberg, P. Karlsson, S. Eriksson, and M. Törnqvist, “Analysis of acrylamide, a carcinogen formed in heated foodstuffs.,” *Journal of agricultural and food chemistry*, vol. 50, no. 17, pp. 4998–5006, Aug. 2002.

[143] L. Lv, X. Shao, L. Wang, D. Huang, C. T. Ho, and S. Sang, “Stilbene glucoside from polygonum multiflorum thund: A novel natural inhibitor of advanced glycation end product formation by trapping of methyglyoxal,” *Journal of agricultural and food chemistry*, vol. 58, no. 4, pp. 2239–2245, 2010.

[144] H. Y. Lui, H. W. Liu, W. Wang, C. Khoo, J. Taylor, and L. W. Gu, “Cranberry phytochemicals inhibit glycation of human hemoglobin and serum albumin by scavenging reactive carbonyls,” *Food and Function*, vol. 2, no. 8, pp. 475–482, 2011.

[145] G. E. . Box and K. B. Wilson, “On the experimental attainment of optimum conditions,” *Journal of Royal Statistical Society*, no. 13, pp. 1–45, 1951.

[146] E. Enright, A. P. Bland, E. C. Needs, and A. L. Kelly, “Proteolysis and physicochemical changes in milk on storage as affected by UHT treatment , plasmin activity and KIO addition,” vol. 9, 1999.

[147] A. J. Elliott, N. Datta, B. Amenu, and H. C. Deeth, “Heat-induced and other chemical changes in commercial UHT milks,” pp. 442–446, 2005.

[148] C. M. Oliver, “Insight into the glycation of milk proteins: an ESI- and MALDI-MS perspective (review).,” *Critical reviews in food science and nutrition*, vol. 51, no. 5, pp. 410–31, May 2011.

[149] P. J. Beisswenger, S. K. Howell, a D. Touchette, S. Lal, and B. S. Szwergold, “Metformin reduces systemic methylglyoxal levels in type 2 diabetes.,” *Diabetes*, vol. 48, no. 1, pp. 198–202, Jan. 1999.

[150] D. Dobler, N. Ahmed, L. Song, K. E. Eboigbodin, and P. J. Thornalley, “Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification.,” *Diabetes*, vol. 55, no. 7, pp. 1961–9, Jul. 2006.

[151] R. H. Myers, A. I. Khuri, and H. C. J. Carter, “Response Surface Methodology: 1966–88.,” *Technometrics*, vol. 31, pp. 137–157, 1989.

[152] Y. Ge, Y. Ni, H. Yan, Y. Chen, and T. Cai, “Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using responsesurfacemethodology,” *Journal of Food Science*, vol. 67, pp. 239–243, 2002.

[153] C. Liyana-Pathiranaa and F. Shahidi, “Optimization of extraction of phenolic compounds from wheat using responsesurfacemethodology,” *Food Chemistry*, vol. 93, no. 1, pp. 47–56, 2005.

[154] E. Valduga, A. Valerio, H. Treichel, A. Furigo, and M. Di Luccio, “Optimization of the production of total carotenoids by sporidiobolus salmonicolor (CBS 2636) using response surface technique.,” *Food and Bioprocess Technology*, vol. 2, pp. 415–421, 2009.

[155] Y. S. Kumar, R. S. Prakasam, and O. V. S. Reddy, “Optimisation of fermentation conditions for mango (Mangifera indica L.) wine production by employing response surface methodology,” *International Journal of Food Science & Technology*, vol. 44, pp. 2320–2327, 2009.