

**Evaluate the Validity of Two Popular Food Oxidation Markers  
(Hexanal & Limonene Oxide) in the Dry State**

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**Xiaohan Wu**

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**Dr. Gary A. Reineccius, Advisor**

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## **Dedication**

This thesis is dedicated to my parents, my grandparents, and every teacher that helped me become who I am today.

## Abstract

In research, it is common to use chemical indicators of oxidation to determine the oxidative state of a food. For example, hexanal formation/content is used to determine if lipids are oxidizing and limonene oxide (LO) serves a similar purpose in monitoring the oxidation of citrus oils. Recent research has shown that hexanal readily reacts with proteins and unpublished work suggests that limonene oxide also may react with proteins. Thus, these traditional indicators of oxidation may be inaccurate for foods that have higher protein levels or very reactive protein.

$\beta$ -Lactoglobulin (BLG) is a major whey protein in bovine milk and is also a prominent protein utilized in the food industry. Additionally, the structure and amino acid sequence of BLG is well characterized, and its molecular weight is appropriate for intact protein analysis. Therefore, BLG was selected as the model protein for this work.

This research studied the covalent reactions between BLG and the two popular oxidation markers (hexanal and LO) in the dry state by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and to determine the influence of temperature, pH, and water activity on the reactions. Results show that limonene oxide could form covalent bonds with the BLG. Moreover, the reaction rate increases with the increase of storage time, which could make limonene oxide inaccurate as an oxidation marker in food containing high levels of proteins. For hexanal, the increase of the protein adduct is not very significant during storage. Therefore, hexanal still can be considered as a usable oxidation marker for dry foods.

This research can help the food industry understand the validity of the two popular oxidation markers, choose better methods for food oxidation analysis, and improve their quality control system.

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## List of Abbreviations

APCI-TOF-MS - Atmospheric pressure chemical ionization time-of-flight mass spectroscopy

Auto-DHS-GC-MS - Automated dynamic headspace sampling gas chromatography mass spectrometry

p-AV – para anisidine value

a<sub>w</sub> - Water activity

BHA - Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

BLG -  $\beta$ -Lactoglobulin

CI-ELISA - Competitive indirect enzyme-linked immunosorbent assay

DHS - Dynamic headspace

DI - Direct injection

DSC - Differential scanning calorimetry

EDTA - Ethylenediaminetetraacetic

FID - Flame ionization detector

FTIR - Fourier-transform infrared spectroscopy

GC - Gas chromatography

GC-IMS - Gas chromatography ion mobility spectrometry

GC-MS - Gas chromatography mass spectrometry

GC-O - Gas chromatography-olfactometry

GC-O-MS - Gas chromatography-olfactometry-mass spectrometry

HAT - Hydrogen atom transfer

HPLC - High Performance Liquid Chromatography

HS-SPME - Headspace solid phase microextraction

HTST - High-temperature-short-time

Igs - Immunoglobulins

LC-ESI-MS - Liquid chromatography electrospray ionization mass spectrometry

LO - Limonene oxide

MALDI - Matrix-assisted laser desorption/ionization

NIR - Near-infrared spectroscopy

PV - Peroxide value

QTOF - Quadrupole time-of-flight

RP-HPLC - Reversed-phase high-performance liquid chromatographic

SA - Sinapic acid

SD - steam distillation

SDE - Simultaneous distillation and solvent extraction

SDG - Secoisolariciresinol diglucoside

SDS - Sodium dodecyl sulfate

SET - Single electron transfer

SHS - Static headspace

TAG - Triacylglycerols

TBARS - 2-thiobarbituric acid reactive substances

UHT - Ultra-high-temperature

WOF - Warmed over flavor

WPI - Whey protein isolate

## Chapter 1: INTRODUCTION

### 1.1 Background

Lipid oxidation is one of the main reasons causing food quality deterioration. The oxidation products can not only alter flavor, texture, and nutrients but also create health problems (Estévez et al., 2017). Therefore, it is important to develop reliable measuring methods to monitor lipid oxidation in food products. A commonly used method is testing the secondary oxidation products which are more stable than the primary oxidation products and are well related to sensory analysis (Pirnavatharsan et al., 2023). Many techniques have been developed including 2-thiobarbituric acid reactive substances (TBARS) test and gas chromatography-mass spectrometry analysis. Hexanal is one of the most common secondary lipid oxidation products, and due to its low odor threshold and the direct correlation of its content with oxidative off-flavors, has become a popular marker (Ha et al., 2011; Vičkačkaitė et al., 2020). Gas chromatography is one of the most common techniques to quantify hexanal in foods. There are different sample preparation techniques including dynamic headspace (DHS), static headspace (SHS), direct injection (DI), liquid extraction, and headspace solid phase microextraction (HS-SPME) for hexanal extraction (Azarbad & Jeleń, 2015). The headspace analysis is widely used because of simple sample treatment. However, hexanal has the potential to further react with other components in food. Recent research shows that covalent bonds form between hexanal and  $\beta$ -lactoglobulin (Anantharamkrishnan et al., 2020), which could make hexanal invalid as a marker of lipid oxidation when using volatile sample preparation methods.

Limonene oxide is the equivalent oxidation indicator for flavorings containing terpenes. It is the oxidative product of limonene which is a main component in orange oil. In flavor

encapsulation, published studies have noted that when a protein(s) is/are used as a component of encapsulation wall materials, the amount of limonene oxide decreases significantly compared to systems not using a protein component (Charve & Reineccius, 2009; Djordjevic et al., 2008; Marcuzzo et al., 2012). Therefore, protein materials can appear to provide effective protection against limonene oxidation. However, the possibility that limonene oxide and proteins can react has not been fully considered, which may make the oxidative marker unreliable.

Therefore, it is necessary to study the reactions between hexanal/limonene oxide and food proteins to determine if they are valid as oxidation markers.

## **1.2 Research Objectives**

This research is to evaluate the reliability of the two popular oxidation markers which are hexanal and limonene oxide.  $\beta$ -Lactoglobulin is chosen as the model protein because it is well characterized both in amino acid sequence and the structure, and it has suitable molecular weight for intact protein ESI MS analysis. However, for objective 2, BLG is used up and it is difficult to find a homozygous single cow and isolate a single variant. Therefore, whey protein isolate is used. However, WPI is not suitable for LC-ESI-MS analysis because of its large molecular weight, so the GC-FID method is adopted.

The objectives are:

1. Determine if limonene oxide reacts with beta lactoglobulins (BLG), and the reaction rates and extent of the interactions between hexanal/limonene oxide and BLG in the dry state.
2. Study the effect of pH, temperature, and water activity on the interaction between hexanal/LO and WPI in the dry state.

## **Chapter 2: LITERATURE REVIEW**

### **2.1 Food Oxidation**

Food oxidation can happen when food is exposed to oxygen, light, and/or heat during processing, production, and storage. It affects many interactions among food components and thus is a critical influencing factor of food quality. Among all food constituents, lipids are one of the most susceptible to oxidation (Jacobsen, 2019). Additionally, in food that has a citrus flavor, limonene oxidation can happen easily. Some of the oxidative products cause deterioration of food quality including flavor, texture, and nutrition. Moreover, consuming food with oxidation products can cause health problems (Estévez et al., 2017). Therefore, monitoring and preventing food oxidation have been two of the most important issues in the food industry.

#### **2.1.1 Lipid Oxidation**

Lipids are crucial components that influence the quality of food significantly. They not only provide energy to the human body but also contain essential fatty acids and fat-soluble vitamins which can only be obtained from diet. Moreover, one of the primary sources of odorants that give characteristic flavors to foods are lipids and lipid-derived compounds (Domínguez et al., 2019). The amount of fat in food is strongly related to consumer satisfaction and affects textural qualities including overall mouthfeel, juiciness, and tenderness (Frank, Joo, & Warner, 2016; Holman & Hopkins, 2021). In addition to the sensory qualities of foods, lipids play a significant role in the technological aspects of food processing. They influence the rheological and structural properties of food, assist fat-soluble ingredients such as antioxidants and vitamins dispersion, and can act as an emulsifier which improves emulsion stability (Domínguez et al., 2019; Domínguez, Bohrer, et al., 2021; Valenzuela, Delplanque, & Tavella, 2011). Lipids can be classified as simple lipids and complex lipids based on their structure.

Simple lipids are esters of fatty acids and alcohols that generate no more than two kinds of products (fatty acids and alcohols) by hydrolysis, while complex lipids produce three or more products including fatty acids, alcohols, phosphorus, amino acid, and carbohydrates (Domínguez et al., 2022). Triacylglycerols (TAG), which account for 99% of all lipids of both plant and animal origin, are the most common type of lipid found in foods. Additionally, phospholipids, which are important structural lipids, can constitute a big part of the lipids in foods such as eggs and meat. Because they are more unsaturated, phospholipids are more prone to oxidation than TAG (Jacobsen, 2019).

Lipid oxidation is the main reason for food quality deterioration. The oxidative products are responsible for off-flavors, texture loss, changes of color, and nutrient value (Ahmed et al., 2016). Rancid is the term usually used to describe the sensory alterations caused by lipid oxidation. Moreover, lipid hydrolysis can lead to rancidity, which is called hydrolytic rancidity. In unpasteurized milk, for instance, oxidative rancidity occurs because of lipid oxidation and hydrolytic rancidity happens due to lipolytic enzymes. Both reactions have a significant influence on unpasteurized milk. In addition, toxic compounds such as free radicals and reactive aldehydes are also produced during the reaction (Jacobsen, 2019). The products are harmful to human health due to their mutagenic, carcinogenic, and cytotoxic properties, which can cause health problems such as tumor cell growth and neuromyopathic disease (Ahmed et al., 2016).

Unsaturated fatty acids and oxygen are the basic components that interact during the oxidation of lipids. Other substances can also facilitate or inhibit the process (Domínguez et al., 2019). Unsaturated fatty acids cannot be directly oxidized by low energy, ground state oxygen (triplet oxygen  $^3\text{O}_2$ ) because of spin prohibition, however, this restriction can be removed by the presence of initiators that can generate radicals or by other methods (Jacobsen, 2019). The two

major lipid oxidation pathways are thermal oxidation, hydrolytic rancidity, and air oxidation. When lipid-containing food is processed under high temperatures, lipids will oxidize, decompose, polymerize, and produce compounds including fatty acids, aldehydes, and hydroperoxide which lead to a decrease in the quality of food (Wang et al., 2023). Hydrolytic rancidity is commonly caused by lipase enzymes, which leads to the release of short chain fatty acids from acylglycerols (Zeece, 2020). Air oxidation includes three mechanisms: autoxidation, which happens when free lipid radicals combine with oxygen; Photooxidation, which occurs when lipids are exposed to light in the presence of photosensitizers; And enzymatic oxidation (Jacobsen, 2019; Wang et al., 2023). Autoxidation is the most important process among the three mechanisms. Photooxidation and enzymatic oxidation are only different from autoxidation in the generation of hydroperoxides in the initiation phase (Domínguez et al., 2019). These reactions occur easily when food is subjected to air such as during storage and processing (Wang et al., 2023). The primary oxidation products are hydroperoxides which are not stable and break down into various small compounds including aldehydes, alcohols, and ketones (Ahmed et al., 2016). These volatile secondary oxidation products are the main reasons for flavor deterioration (Jacobsen, 2019). The significance of each compound in the final flavor is influenced by its concentration and olfactory threshold. Among these compounds, aldehydes are one of the most important products and play a major role in flavor alteration due to their low odor threshold and large quantities. Additionally, in food systems that contain proteins (e.g., meat), aldehydes can react with the proteins which result in modifications of nutritional and organoleptic characteristics (Domínguez et al., 2019).

Autoxidation typically consists of three phases: Initiation, propagation, and termination (Domínguez et al., 2019). During the initiation, free radicals are generated. Unsaturated fatty

acids (LH) lose hydrogen and transform into alkyl radicals ( $L\cdot$ ) in the presence of initiators, such as metal ions, heat, or proteins. The alkyl radicals are the initial radicals that start lipid oxidation. Peroxyl radicals ( $LOO\cdot$ ) are created when alkyl radicals quickly react with oxygen. These peroxyl radicals are highly reactive. They interact with a new unsaturated fatty acid to produce hydroperoxides ( $LOOH$ ) and a new lipid radical, which subsequently propagates the chain reaction. Lipid hydroperoxides have no taste or odor due to their low volatility. The chain reaction terminates when the reactive compounds degrade or two free radicals combine and generate a non-radical product (Domínguez et al., 2019; Jacobsen, 2019).

Unsaturated fatty acids may undergo photooxidation in the presence of light and photosensitizers, such as myoglobin, hemoglobin, and riboflavin in milk and chlorophyll in unrefined oils. The photosensitizers absorb visible or near-UV light to transform from singlet to excited triplet sensitizers. Photosensitizers can be mainly classified into two types based on reaction mechanisms. Type I sensitizers react with lipids and produce lipid radicals which interact with oxygen in a similar way as autoxidation. Type II sensitizers can directly interact with triplet oxygen to produce singlet oxygen which is highly reactive through a triplet-triplet annihilation mechanism (Domínguez et al., 2019). The singlet oxygen then reacts with the double bond in the unsaturated fatty acids to generate hydroperoxides ( $ROOH$ ) (Frankel, 2012). This process is non-free-radical and forms non-conjugated hydroperoxides, while the free-radical oxidation process produces conjugated hydroperoxides (Jacobsen, 2019). Additionally, some excited sensitizers interact with triplet oxygen to form superoxide radical anion via electron transfer, which can abstract hydrogen from unsaturated fatty acids and thus start the oxidation reaction (Domínguez et al., 2019).

Lipoxygenase is the major enzyme that participates in the enzymatic oxidation. It has an active site that can remove a hydrogen atom from unsaturated fatty acids to create a conjugated diene system that interacts with oxygen. The peroxy radical subsequently abstracts hydrogen from another unsaturated fatty acid to produce a conjugated hydroperoxy diene and alkyl radical. The concentration of the enzyme has a significant influence on the rate of oxidation process (Domínguez et al., 2019).

The primary oxidation product, hydroperoxides, decompose into small volatile compounds which are responsible for the alteration of flavor in foods. Vinyl ketones and trans, cis-alkadienals have the lowest flavor thresholds in oils, while alkanes and alkenes have the highest flavor thresholds. Depending on its concentration and the food matrix in which it occurs, the same volatile oxidation product can result in a variety of off-flavors. In pure oil, the off flavors usually are described as nutty, grass, cucumber, rancid, and synthetic. In dairy products such as milk, cream, and butter, “oxidized flavor”, “sunlight flavor”, and “metallic” are normally used to describe the flavor flaws caused by lipid oxidation. In meat products, the off flavor is described as “warmed over flavor “(WOF) which is mainly caused by membrane phospholipids oxidation. Hexanal, (E)- and (Z)-2-octenal, 1-octen-3-one, (Z)-2-nonenal (E, E)-2,4-nonadienal, and trans-4,5-epoxy-(E)-2-decenal are the main compounds that lead to WOF (Jacobsen, 2019).

Numerous techniques, including peroxide value (PV), para-anisidine value (p-AV), thiobarbituric acid reactive substances (TBARS), HPLC, GC-MS, NIR, FTIR, and DSC, can be used to measure lipid oxidation. Moreover, sensory evaluation is a useful method to study the relationship between lipid oxidation and sensory properties of oxidized food. However, some methods that measure only peroxide value and hexanal or TBARS may not be accurate because of alternate pathways of lipid oxidation, which would result in missing a large number of

oxidation products and underestimating the extent of lipid oxidation. Furthermore, lipid oxidation radicals and secondary oxidation products including aldehydes can interact with other components in foods, especially proteins. As a result, these reactions deplete lipid oxidation intermediates and products and influence other components in food (Jacobsen, 2019)

Lipid oxidation not only causes flavor degradation but also leads to nutritional loss and toxic compound formation. Therefore, it is essential for academia and industry to control food lipid oxidation.

Antioxidants are chemical compounds that prevent, inhibit, or regulate the oxidation of a substrate (Zeb, 2020). Antioxidants such as chitosan, anthocyanins, secoisolariciresinol diglucoside (SDG), and sinapic acid (SA) have been discovered effective in lipid oxidation prevention (Wang et al., 2023). In food science, antioxidants are present in relatively small amounts in foods and can provide protection for food components from oxidation which helps maintain nutritional quality and extend shelf life (Zeb, 2020). They are generally classified as primary and secondary antioxidants based on their action mechanisms. The antioxidants that have more than one action mechanism are named multiple-function antioxidants (e.g., polyphenols). Primary antioxidants, also called chain-breaking antioxidants, react directly with free radicals and generate more stable products (Jacobsen, 2019). They neutralize free radicals by two mechanisms which are hydrogen atom transfer (HAT) and single electron transfer (SET). They are very effective and typically only need a low amount to neutralize many free radicals. This type of antioxidant is also easily reproduced. The mechanism of neutralizing prooxidant catalysts has been used to describe secondary antioxidants which normally includes chelation of transition metals, oxygen scavenging, and quenching of singlet oxygen, making them easily depleted (Jacobsen, 2019; Zeb, 2020). Metal chelators, which stop metal ions from breaking

down lipid hydroperoxides into reactive radicals, are crucial secondary antioxidants in food systems, such as synthetic ethylenediaminetetraacetic (EDTA), polyphosphates, and caseinate (Jacobsen, 2019).

In the industry, synthetic antioxidants, including butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT], and propyl gallate (Brewer, 2011), have long been applied to slow the reactions. These antioxidants contain one or more hydroxyl groups or phenol and can quench free radicals in foods, which effectively limits lipid oxidation. However, the potential health risks of synthetic antioxidants motivate the industry to find natural antioxidants. Natural phenolic compounds have attracted significant attention because of their widespread presence in plant foods and their effectiveness as antioxidants. Generally, phenolic compounds are biosynthesized from phenylalanine or tyrosine via the shikimic acid pathway. Their antioxidant capabilities are due to the hydroxyl group on the benzene ring (Zeb, 2020). The compounds limit lipid oxidation by inhibiting free radical formation and/or interrupting propagation of autoxidation (Brewer, 2011). They can donate their H-atoms to the free radicals and produce stable phenoxyl radicals (Jacobsen, 2019). In seafood, the effect of plant phenolic extract and compounds in retarding lipid oxidation has been proven. Antioxidants delay oxidation by preventing free radical formation or by breaking free radical propagation (Maqsood et al., 2014). The mechanisms include removing peroxidation initiative species, stopping the autoxidative chain reaction, and decreasing the concentration of localized O<sub>2</sub> (Brewer, 2011). Antioxidants that can interrupt the free radical chain reaction are the most effective, which usually contain phenolic or aromatic rings. They donate H<sup>+</sup> to the free radicals produced during oxidation and become radical themselves which are stable because of the electron resonance delocalization within the aromatic ring and synthesis of quinone structures. Therefore, phenolic compounds can

end the chain radical reaction to prevent lipid oxidation and preserve food quality, especially high lipid-containing foods. The potency of these compounds in retarding lipid oxidation is related to their metal-chelating activity or free-radical scavenging (Maqsood et al., 2014). Maqsood et al. (2010) discovered that ferulic acid, caffeic acid, and tannic acid are effective in preventing lipid oxidation in fish mince during iced storage (Maqsood & Benjakul, 2010). Sánchez-Alonso et al. (2007) studied the impact of adding grape antioxidant dietary fiber to minced fish muscle on the stability of lipids for 6 months of storage. The results showed that the added fiber significantly retarded lipid oxidation throughout the first 3 months of storage. The grape antioxidant dietary fiber could be applied as an ingredient to limit lipid oxidation in fish during frozen storage (Sánchez-Alonso et al., 2007; Zeb, 2020).

Lipid oxidation plays a critical role in the quality and reservation of food products, it is important to understand the mechanisms, develop reliable measuring techniques, and identify control methods.

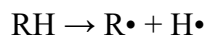
### **2.1.2 Limonene Oxidation**

Limonene is an unsaturated monocyclic monoterpene that has been found in more than 300 essential oils and mainly in citrus oil (Ibáñez et al., 2020). There are two optical isomers of limonene: L-limonene and D-limonene. L-limonene is the primary precursor of biosynthesis of L-menthol which has a mint flavor, and D-limonene can be found primarily in orange peels (Davoudi-Monfared et al., 2023). D-limonene has high volatility and low water solubility (Huang et al., 2022), and is frequently used as an important flavor and fragrance compound in food and cosmetics industries. It has antioxidant and anticancer properties. Although D-limonene is considered non-toxic, it can irritate and sensitize the skin after long-term exposure (Davoudi-Monfared et al., 2023). Nowadays, it is usually produced as a by-product of the processing of

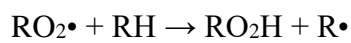
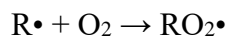
citric fruit juice by a cold process including centrifugal separation or steam distillation (Birk et al., 2022; Ciriminna et al., 2014; Davoudi-Monfared et al., 2023). Other important aroma compounds including limonene oxide, carvone, and menthol can be produced from limonene through natural oxidation reactions (Birk et al., 2022).

In the flavor industry, the deterioration of orange oils has been a problem for many years. Limonene can easily degrade when exposed to light, air, and high temperatures. Limonene autoxidation happens in the presence of air and light to produce various oxygenated monocyclic terpenes (NICNAS, 2002). Although complicated, similar to lipid oxidation, the autoxidation of limonene also includes free radical reaction chain initiation, chain propagation, and chain termination (Bernhard & Marr, 1960) (Figure 1). The primary oxidation products are hydroperoxides, which are unstable and can further react to produce secondary oxidation products including limonene oxide, carvone, and carveol (Huang et al., 2022; Kern et al., 2014). Limonene-2-hydroperoxide is the main hydroperoxide in the autoxidation of D-limonene while in photooxidation it only consists of a small fraction of the hydroperoxides (Karlberg et al., 1994). In the photooxidation of limonene, the singlet oxygen only reacts with the endocyclic double bond (Schieberle et al., 1987). Polymers are the end products of limonene oxidation. Carvone, carveol, limonene oxide, and other hydroperoxides are probably intermediates in the polymerization process (Karlberg et al., 1994).

Initiation:



Propagation:



Termination:

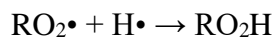
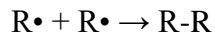


Figure 1: Free radical chain reaction (Source: Horne, 1950).

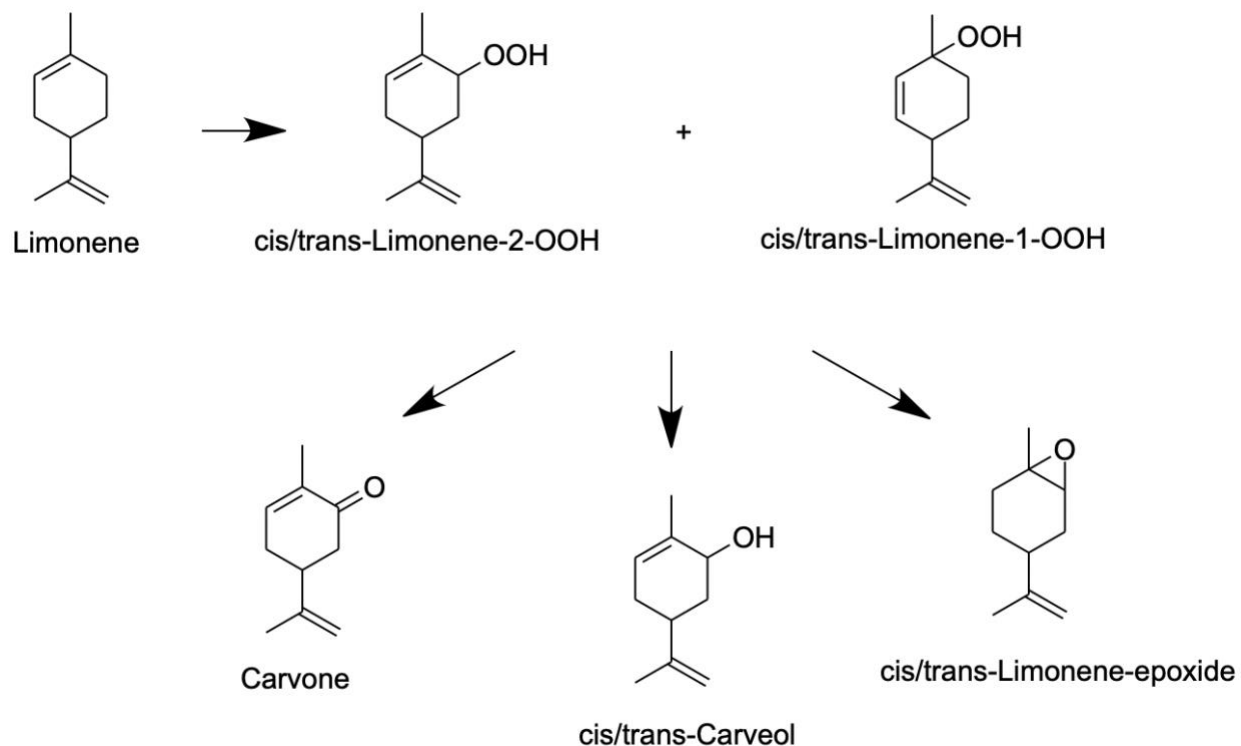


Figure 2: Main primary and secondary oxidation products of limonene. (Source: Kern et al., 2014 with slight modifications).

Since limonene is widely used in many industries (e.g., food and perfume), and is easily oxidized which causes rancid odor and skin irritants (Karlberg et al., 1992), methods are developed to protect it from the environment. One of the most effective and popular ways for improving flavor stability is encapsulation which active compounds (core material) are covered by wall materials, which protects flavorings against oxidative and thermal deterioration during processing. Common techniques include spray drying, freeze drying, coacervation, and fluidized

bed coating. A new technology named electrospinning/electrospraying appeared in the past few years. It creates continuous sub-micron or nanoscale fibers and capsules by applying high-voltage electrostatic fields to polymer solutions. The process doesn't involve heating which makes it ideal for encapsulating heat-unstable compounds (Premjit et al., 2022). Spray drying has been used to encapsulate limonene for a long time, and gum acacia and modified starches are the most commonly used wall materials. Other wall materials such as maltodextrin and whey/soy proteins are becoming popular, especially protein materials which show better protection for limonene from oxidation than the traditional materials (Charve & Reineccius, 2009).

Lipid oxidation and limonene oxidation can cause significant deterioration of food quality. Therefore, developing and utilizing proper methods to monitor the reaction is of great importance.

## **2.2 Hexanal and Limonene Oxide as Oxidation Markers**

Hexanal is one of the most common products of lipid oxidation, and because of its low odor threshold and the direct correlation of its content with oxidative off-flavors, it has become a popular marker (Ha et al., 2011; Vičkačkaitė et al., 2020). Limonene oxide is used as an oxidation marker for orange oils. Especially in flavor encapsulation studies, the amount of limonene oxide is an important index to evaluate the ability of wall materials to protect flavor compounds from oxidation.

### **2.2.1 Hexanal as an Oxidation Marker**

Lipid oxidation, also known as rancidity, is a main cause of fat-containing food product deterioration including off-flavors, decrease of nutrients, and generation of toxic compounds (Sanchez-Silva et al., 2004). Therefore, it is important to use an indicator to detect the reaction in food. Lipid oxidation happens when unsaturated fatty acids react with oxygen in a

photosensitized oxidation process or through a free radical mechanism (Vičkačkaitė et al., 2020). Hydroperoxides are the primary oxidation products which are highly reactive and quickly decompose to non-volatile and volatile compounds including aldehydes, ketones, acids, and alcohols. These volatile compounds are the reasons for flavor changes in food products (Panseri et al., 2011). Analyzing these volatile oxidation products can provide insights into the oxidation (Azarbad & Jeleń, 2015). Many methods have been applied to monitor lipid oxidation, and gas chromatography (GC) is one of the most popular ways. There are different sample preparation techniques including dynamic headspace (DHS), static headspace (SHS), direct injection (DI), liquid extraction, and headspace solid phase microextraction (HS-SPME) (Azarbad & Jeleń, 2015). The headspace analysis is widely used because of simple sample treatment, which limits the formation of artifactual volatile compounds. It is usually combined with solid phase microextraction (SPME) using the fiber coating material to adsorb analytes from samples (García-Llatas et al., 2007).

Sanches-Silva et al. (2004) applied two methods of analyzing hexanal which are headspace SPME (coated fused silica) -GC-MS and derivatization reversed-phase high-performance liquid chromatographic (RP-HPLC) to evaluate the suitability of hexanal as oxidation marker in potato crisps. The results show that hexanal is a good marker for the lipid oxidation state of potato crisps. The study conducted by Elisia and Kitts (2011) quantified hexanal in human milk by solid phase microextraction gas chromatography flame ionization detector (SPME-GC-FID). They found that hexanal was sensitive and reliable as an oxidation marker in human milk. In the study conducted by Bak et al. (2020), hexanal was also used as the oxidation marker to test the effectiveness of rosemary extract in preventing lipid oxidation in sliced and uncured deli turkey. The SPME headspace method was used to extract the hexanal.

The results showed that hexanal values were positively correlated with oxidation flavor scores during storage. Beltran et al. (2003) studied lipid oxidation of pressurized and cooked chicken by monitoring the amount of thiobarbituric acid reactive substances (TBARS) and hexanal. For processed meat, the most characteristic volatile compound that SPME can measure is hexanal. Both methods shows similar patterns throughout the experiment, which indicates that it is reliable to analyze hexanal levels by SPME as a measure of oxidation (Beltran et al., 2003). In some old studies, hexanal quantification by headspace gas chromatography was used successfully to monitor lipid oxidation in biological specimens as well as foods (Smith et al., 1999). Another study developed an HS-SPME-GC method to determine hexanal and pentane in liquid infant foods and infant formulas. Pentane is another volatile compound used as a lipid oxidation marker and is inert and stable under proper storage conditions. Interestingly, there were no variations in hexanal contents between the samples stored for four and seven months, while the amount of pentane increased with time. This result shows that hexanal can interact with other components in the samples (García-Llatas et al., 2007). Shahidi & Pegg (1994) studied the deterioration of meat flavor by using hexanal as the indicator via HS-GC analysis. During the early stages of storage, the concentration of hexanal increased rapidly. However, after six days, hexanal decreased significantly, which could be the result of reactions between hexanal and other meat components such as proteins. Hexanal therefore should be used with caution as a lipid oxidation indicator.

Goodridge et al. (2003) monitored lipid oxidation in freeze-dried chicken protein by three methods which are competitive indirect enzyme-linked immunosorbent assay (CI-ELISA), thiobarbituric acid reactive substances assay (TBARS), and SPME-GC/MS. TBARS is a common way of analyzing lipid oxidation. It is simple and highly correlated to sensory scores

but lacks specificity because other products in addition to malonaldehyde can also react with thiobarbituric acid (TBA). The CI-ELISA used the monoclonal antibody which could recognize hexanal conjugates to proteins, while free hexanal was not recognized. This method avoids the weakness of those conventional techniques, especially the headspace chromatographic methods which require volatilization of hexanal. Other components (e.g., proteins) in the food system may react with hexanal and form covalent bonds, which impedes hexanal volatilization and results in inaccurate quantification. Gremlı (1974) studied the interaction between soy protein and flavor compounds. Flavors added to soy protein products usually are lost or altered. The interactions can be reversible or irreversible and can be caused by physical sorption or chemical reactions. A reversible interaction, however, may be advantageous in some cases. For example, during food processing, this interaction may protect the flavors, and when the food product is masticated, the bound flavor compounds may be released and play roles in consumers' palates and contribute to flavor perception. This study developed an analytical technique to identify if the reaction between flavor compounds and soy protein is reversible or not, and the degree of binding. They measured the retention rates by the headspace method and the high vacuum transfer system. By comparing the results of these two methods, the reversibility of the reaction between a given flavor compound and soy protein can be determined. Aldehydes were found to strongly interact with soy proteins. 37-44% of hexanal was reversibly bound with soy protein while <5% was irreversibly bound. Gutheil and Bailey (1992) also developed an equilibrium headspace sampling method to measure the binding of hexanal to muscle myofibrillar proteins.

Hexanal has the potential to further react with other components in food, which can result in harmful effects, such as protein loss (García-Llatas et al., 2007). Saturated aldehydes are one of the main contributors of rancidity in foods, and hexanal and propanal are the two aldehydes

widely detected during lipid oxidation, which usually are used as oxidation indicators. However, some food components (e.g., proteins) can potentially interact with these indicators via processes such as Schiff base reactions (Zhou & Decker, 1999). Recent research shows that covalent bond forms between hexanal and  $\beta$ -lactoglobulin (Anantharamkrishnan et al., 2020), which makes hexanal invalid as a marker of lipid oxidation when using methods such as SPME and headspace GC, which measure only volatile hexanal. (Goodridge et al., 2003; Smith et al., 1999).

### **2.2.2 Limonene Oxide as an Oxidation Marker**

D-limonene is a main component of orange oil with low solubility in water (13.8 mg/L at 25 °C) and a boiling point of 176 °C. It has a pleasant orange aroma and thus has been widely used in food, perfume, and cleaning products. Additionally, D-limonene has numerous health benefits including anti-inflammatory, antioxidant, antibacterial, and antiviral properties. However, D-limonene is very unstable and can oxidize easily which generates an unpleasant flavor (Castel et al., 2023).

Encapsulation is a technology widely used to protect sensitive compounds from degradation (Castel et al., 2023). Spraying-drying is the most popular method in the industry due to its low cost, high throughput, and easy implementation (Castel et al., 2023; Potdar et al., 2020). Since D-limonene is easily oxidized which produces off-flavors, encapsulation has been a popular way to protect orange oil from oxygen. Limonene oxide is one of the most common oxidation products and is used as an oxidation marker, early detection of limonene oxide is critical for quality control (Marine & Clemons, 2003). Gas chromatography is considered an ideal method to test limonene oxide due to its volatile nature (Emberger et al., 2019). Emberger et al. (2019) developed an SPME-GC-MS/MS method to quantify limonene oxide in encapsulated orange oil. In the study conducted by Djordjevic et al. (2007), the stability of citral

and limonene in emulsions stabilized with a sodium dodecyl sulfate (SDS)-chitosan complex or gum acacia was compared. The deterioration of limonene was determined by measuring the reduction of limonene and the production of limonene oxide and carvone with gas chromatography. The results showed that SDS-chitosan multilayer emulsifier system could inhibit the oxidation of citral and limonene more effectively than GA-stabilized emulsions. Another study also used limonene oxide as the marker of limonene degradation to compare the protective ability of whey protein isolate (WPI) and gum acacia. The results showed that in the WPI emulsion, less limonene oxide was detected than in the gum acacia emulsion (Djordjevic et al., 2008). Soottitantawat et al. (2004) studied the oxidation stability of spray-dried encapsulated D-limonene with different wall materials (Gum arabic, soybean water-soluble polysaccharide, and modified starch) by using limonene oxide as the marker. The results showed that the oxidation rate rose as the water activity increased. However, at the glass transition temperature, the rates rapidly decreased before rising again as the water activity increased.

Limonene oxide has been widely used as an oxidation marker in flavor encapsulation to determine the oxidation stability of powders. Some studies discovered that when a protein(s) is/are used as a component of encapsulation wall materials, the amount of limonene oxide decreases significantly compared to systems not using a protein component (Charve & Reineccius, 2009; Djordjevic et al., 2008; Marcuzzo et al., 2012). Therefore, protein materials can appear to provide effective protection against limonene oxidation. However, the possibility that limonene oxide and proteins can react has not been fully considered, which may make the oxidative marker unreliable.

## 2.3 Food Proteins

Proteins are one of the four major biological macromolecules and have the most diverse functions. They play a significant role in many biological processes. They also serve a structural or mechanical purpose in muscles, connective tissue of animals and cell walls of plants, etc. Additionally, proteins are widely applied in areas such as food, medicine, cosmetics, and packaging (Phillips & Williams, 2011).

There are twenty amino acids that are the building blocks of proteins. Nine of them are essential amino acids that can only be obtained from dietary sources, five are non-essential since they can be synthesized by the human body, and the remaining six are conditional which are only necessary at specific life stages or in certain disease states (The Editors of Encyclopaedia Britannica, n.d.). The fundamental structure of amino acids is the same. In each amino acid, there is a hydrogen, an amine group, a carboxylic acid group, and a R group. The R group varies for different amino acids and confers polar, non-polar, anionic, or cationic characteristics on amino acids. These characteristics offer information on the temperature range for optimal protein function, solubility in water or lipids, and whether the protein performs better in acidic or basic environments (Sanvictores, 2022). Hydrophilic amino acids typically stay at the periphery of globular proteins while hydrophobic amino acids in the center. Amino acids form polypeptides via peptide bonds, and the two ends of every polypeptide are called the carboxyl terminus (C-terminus) and the amino terminus (N-terminus). Typically, proteins consist of 15–10,000 amino acids. The sequence of amino acids in the polypeptide chain determines the primary structure of proteins. The secondary structures, including  $\alpha$ -helix and  $\beta$ -sheet, are stabilized by hydrogen bonds. Some proteins are linear and some fold to form various globular structures. The whole shape of the protein is named the tertiary structure, which is maintained by interactions, such as

hydrogen bonding and disulfide bonding, of side chains from the polypeptide backbone. Some proteins also have a quaternary structure which is created by the side-chain interactions between two or more polypeptides (Philips & Williams, 2011; Sanvictores, 2022). Therefore, in the primary structure, the location of the amino acids that can form bindings such as ionic bonds and disulfide bridges can determine the secondary, tertiary, and quaternary structure. The shape of a protein is essential to its function. However, denaturation can be caused by alterations in temperature, pH, and chemical exposure, which results in permanent changes in protein shape, and thus leading to loss of function (Mattaini, 2020).

Proteins can be structural, contractile, regulatory, or protective, and function in transport, membranes, or storage (Mattaini, 2020). Linear proteins usually serve as structural components, for example in the connective tissue of animals. Collagen in tendons and bone, and keratin in skin, hair, and nails, are linear proteins that contain polypeptide chains organized in parallel to create long fibers. Globular proteins often participate in transport processes and dynamic functions in cells (Philips & Williams, 2011).

In addition to the functions in biological processes, proteins play a significant role as food ingredients and additives. Their ability in gelation, emulsion stabilization, and film formation leads to their wide application in the food industry. At high temperatures, globular proteins unfold and the denatured chains aggregate to produce gels that are thermally irreversible, while gelatin, which is a fibrous protein derived from collagen, can generate thermally reversible gels. Proteins are usually applied to stabilize oil-in-water emulsions and foams because of their ability to adsorb at the oil-water and air-water interfaces, especially those derived from milk and eggs. The molecular size and conformation decide the surface activity of proteins. The adsorption ability of proteins at interfaces and their overall amphiphilic properties

are determined by the composition of amino acids. Globular proteins unfold and expose hydrophobic amino acids to adsorb at interfaces, after which elastic networks can be formed by proteins via self-association. Moreover, proteins including whey, soy, and corn zein are used to make edible films and applied in biodegradable packaging. Various bioactive compounds, such as essential oils, bacteriocins, and enzymes, are added to protein films to give them more functional properties (Philips & Williams, 2011).

### **2.3.1 Whey Proteins**

Whey proteins are a byproduct from dairy products (e.g., cheese) processing, and have become an important ingredient in formulated foods. Typically, they are supplied as dry powders, and the two most common forms are whey protein isolates (90% protein) and whey protein concentrates (80% protein). Whey proteins are significant in muscle nutrition because they are a rich supply of branched-chain amino acids which are preferentially metabolized by muscle instead of liver. Branched-chain amino acids can facilitate protein synthesis by activating important enzymes related to muscle protein synthesis. In some specific nutritional applications, such as infant formulas, whey proteins are usually used as a balancer for other proteins. In addition, their functional properties, including the ability of gelling, foaming, water-binding, and stabilizing interfaces, are frequently utilized in food applications. They can function as valid emulsifiers at low concentrations such as 0.5% and generate thin interfacial layers. During heat treatment, whey proteins are vulnerable to denaturation since the majority of them are globular and have defined tertiary and quaternary structure (Philips & Williams, 2011).

### **2.3.2 $\beta$ -lactoglobulin**

$\beta$ -lactoglobulin is the most prominent protein in whey proteins. In natural cow milk, it accounts for about half the whey proteins or around 12% of the total protein. It is also considered

a major potential allergen in cow's milk.  $\beta$ -lactoglobulin belongs to the lipocalin family and has a globular conformation including seven main strands of  $\beta$ -sheet structure that generate a goblet shape. There are two genetically determined variants of BLG, which are known as variant A (18363 Da) and B (18276 Da) (Anantharamkrishnan & Reineccius, 2020b). The two variants are formed at different amounts in milk and have various characteristics because of a variation in the regulatory region of the gene (Philips & Williams, 2011). The protein consists of 162 amino acid residues and has a molecular weight of around 18,300. The composition of the amino acids is 22 Leu, 10 Ile, and 9 Val (or 10 in variant A), which imparts the molecule with some extremely hydrophobic regions. A variety of low molecular weight compounds, such as fatty acids, retinol, and flavor compounds can bind to the cavity in the protein. The sulfur chemistry is an important characteristic of beta-lactoglobulin. A single sulfhydryl group located at Cys 121 is buried in the protein and shielded by the  $\alpha$ -helix, in addition to the two internal disulfide bridges that stabilize the protein. This single sulfhydryl group can interact with other sulfhydryl groups if exposed due to high temperature or other disruption of the protein's secondary and tertiary structure, which can result in disulfide exchange and cross-links with other BLG molecules or other proteins (Creamer et al., 2004). BLG and its gelation properties are the main factors that influence whey protein gelling performance (Philips & Williams, 2011).

There are also some other proteins in whey proteins.  $\alpha$ -Lactalbumin is a component of the lactose synthase enzyme and has a molecular weight of 14,200. The disulfide bridges in the protein maintain the molecule structure effectively, allowing it to unfold and refold by itself. Additionally, it can cross-link with other reactive proteins (e.g.,  $\beta$ -lactoglobulin) resulting in instability. Immunoglobulins (Igs) in bovine milk are a combination of blood Igs and mammary gland-produced Igs. They are globular proteins and are heat-labile. The health of the cow and the

season both affect the levels of Igs in milk. The protein typically consists of two light chains and two heavy chains, and every light-heavy pair is cross-linked by disulfide bridges. Bovine serum albumin is another protein that usually appears in whey proteins but only in a small amount. It is well preserved in membrane processing due to its high molecular weight. There are numerous disulfide bridges in the molecule, which gives the protein the ability to interact with  $\beta$ -lactoglobulin and bind small molecule compounds (Philips & Williams, 2011).

## **2.4 Protein and Flavor Interactions**

The interactions between food proteins and flavor compounds have been an issue in the food industry for a long time. The binding can prevent some flavor molecules from partitioning into the gas phase which causes problems of perception by consumers. Moreover, it would also result in the loss of proteins. In general, there are two types of interactions: non-covalent interactions and covalent reactions. Reversible hydrophobic interactions, hydrophilic interactions, ionic bonds, and Van der Waal's forces are the main causes of flavor: protein interactions, while irreversible covalent binding can also happen (Snel et al., 2023). Equilibrium can be achieved over time for non-covalent interactions, but covalent bonding does not equilibrate and would continue until one of the reactants is consumed. In the case of food, the amount of protein usually is much higher than flavor, so it is more possible that flavor is consumed before protein (G. A. Reineccius, 2022). Therefore, it is important to understand the mechanisms of protein and flavor interactions to help with the flavor formulation.

### **2.4.1 Non-covalent Interactions Between Protein and Flavor**

Non-covalent interactions, also called reversible interactions, are considered predominant in nature since most aroma compounds are hydrophobic (Kühn et al., 2008; G. A. Reineccius,

2022). This type of interaction will achieve an equilibrium ultimately. Therefore, in the food industry, non-covalent interactions can be controlled by reformulation according to the flavor of the product after equilibrium (G. A. Reineccius, 2022).

Many studies have been conducted to understand interactions between protein and flavor for the past few decades, and most of them are focused on the total loss of flavor instead of the mechanisms. Evaluating equilibrium headspace concentration and expressing flavor retention as a percentage of decreased headspace concentration relative to the control is a popular method for studying the protein-flavor interaction. In addition, predicting flavor partitioning by modeling experimental data is also a method that has been commonly used (Snel et al., 2023). Whey proteins, especially  $\beta$ -lactoglobulin, are widely studied and used as the model protein for protein: flavor interactions research (Kühn et al., 2006). Kühn et al. (2008) studied the interactions of whey protein isolate (WPI) and flavor compounds (2-nonanone, 1-nonanal, and trans-2-nonenal) under heat and high pressure by headspace solid-phase microextraction (SPME) and gas chromatography (GC). Results showed that the binding decline in the order trans-2-nonenal > 1-nonanal > 2-nonanone, which can be interpreted as only hydrophobic interactions happened for 2-nonanone while covalent reaction can also occur in the case of the aldehydes (trans-2-nonenal and 1-nonanal). It was also observed that heat/pressure treatment has a different influence on the interactions depending on the structure of the flavor compound, which indicates that the three flavors react with whey proteins via different mechanisms and/or on different binding sites. Damodaran & Kinsella (1980) studied the binding between ketones (2-heptanone, 2-nonanone, and nonanal) and bovine serum albumin by a liquid-liquid partition equilibrium method. Viry et al. (2018) combined modeling and static headspace methods to predict flavor partitioning of a variety of flavor compounds above solutions of fat-free dairy protein mixture. Moreover, with

plant-based food becoming more and more popular, researchers have started paying more attention to plant proteins. Snel et al. (2023) conducted research on the interactions between esters and ketones and four plant proteins which are yellow pea, soy, fava bean, and chickpea, with whey as a reference. The flavor partitioning model was applied, and atmospheric pressure chemical ionization time-of-flight mass spectroscopy (APCI-TOF-MS) was used to measure the flavor concentration in the headspace. The results showed that hydrophobic interactions were highest for chickpeas, and decreased in the order of pea, fava bean, whey, and soy. However, this study interpreted the retention of flavor solely as hydrophobic interactions and did not take covalent reactions into account. Another study assessed the interactions between vanillin and barley proteins with pea protein and whey protein as two control proteins. Unbound vanillin at equilibrium was quantified, and the structure of protein-vanillin complexes was characterized by fluorescence spectrophotometry. Additionally, sensory evaluation was conducted to evaluate the interactions using a high-protein cookie. The sensory results correlated well with the Klotz plot predictions of unbound vanillin (Houde et al., 2018). Andriot et al. (2000) observed hydrophobic interactions between methyl ketones and  $\beta$ -lactoglobulin. They found that the addition of  $\beta$ -lactoglobulin into the aroma solutions can cause a huge decrease in odor intensity. The method of static headspace was used to study the release of these flavor compounds in water solutions with different  $\beta$ -lactoglobulin concentrations. The equilibrium partitioning characteristics and the release rate of the three methyl ketones were predicted using a mathematical model. Results showed that the increase of the hydrophobic chain can lead to a higher affinity constant, which resulted in lower release rates and a lower final headspace aroma concentration.

### 2.4.2 Covalent Reactions Between Protein and Flavor

Covalent bonds are chemical bonds in which atoms share electrons to form electron pairs. In protein: flavor interactions, certain flavor compounds (e.g., aldehydes) can form covalent bonds with proteins. Groups such as  $-NH_2$ ,  $-SS-$ , and  $-SH$  in proteins are most likely to form covalent bonds with flavor compounds (G. Reineccius & Heath, 2006). Common reactions include Schiff base formation, Michael addition, and the formation of amide and ester (Kühn et al., 2008). These bonds are strong and irreversible and can result in permanent loss of flavor, significantly shortening the shelf life of the food. Early studies had difficulties in distinguishing covalent reactions and non-covalent reactions between flavor and protein because of a lack of methodologies. Recently, with the development of instrumentation, researchers have been able to discriminate the mechanisms. The main methods are MALDI, LC-ESI-MS, proteomics, and C14 isotope binding (G. A. Reineccius, 2022).

Anantharamkrishnan & Reineccius (2020b) developed a method to characterize and monitor covalent reactions between BLG and flavor compounds by electrospray ionization mass spectrometry (ESI/MS) and proteomics. This method allows measurement of increases in  $\beta$ -lactoglobulin molecular weight when the reaction occurs (BLG + flavor) and allows identification of the reaction sites. After developing this method, Anantharamkrishnan et al. (2020) conducted research on the covalent reactions between 47 flavor compounds and  $\beta$ -lactoglobulin. The reaction mechanisms observed were Schiff base, Michael addition, and disulfide linkages. Results showed that the most reactive flavor compounds are aldehydes, functional group-containing furans, and sulfur-containing molecules (especially thiols). Furthermore, the influence of pH, temperature, and water activity on the formation of covalent bonds between  $\beta$ -lactoglobulin and flavor was studied. Higher pHs displayed higher reaction

rates, and high temperatures also led to more covalent adduct formation. For certain flavor compounds (allyl isothiocyanate), obvious increases in covalent bonds were observed at high water activities, while no big difference in the reaction rate was noticed for benzaldehyde, citral, and dimethyl disulfide (Anantharamkrishnan & Reineccius, 2020a). Additionally, another study was conducted to investigate the reaction under thermal treatments (pasteurization and sterilization). Covalent reactions between 46 flavor compounds and  $\beta$ -lactoglobulin were examined by UPLC–ESI–QTOF–MS under pasteurization and sterilization conditions, and three flavor compounds (eugenol, 4-vinyl phenol, and 3nonen-2one) were discovered covalently react with  $\beta$ -lactoglobulin which previously did not show any reactivity at room temperature. The data showed that the high-temperature-short-time (HTST) process had a minimal effect on the degree of the reaction compared to in-container pasteurization and ultra-high-temperature (UHT) process (Yuan et al., 2023).

The radioisotope (C-14) label method is ideal for studying covalent reactions because of its simplicity and the high-quality data it gives. This method involves using a labeled flavor compound, allowing reaction with protein forming covalent bonds. The unreacted labelled flavor compounds will then be solvent extracted to leave a protein: flavor compound solution which goes into a scintillate counter to measure the amount of isotope reacted with the protein,  $^{14}\text{C}$ -isotope marker tracking flavor-protein reactions. The  $^{14}\text{C}$ -isotope flavor compound is mixed and incubated in a protein solution, then the free  $^{14}\text{C}$ -isotope is extracted with hexane and NaCl and quantified by the scintillation counter. However, it hasn't been widely applied probably because of high cost and complicated work environment requirements (G. A. Reineccius, 2022).

In conclusion, the interactions between flavor and protein can influence flavor perception significantly and thus affect the shelf life of food products. Non-covalent interactions will

achieve equilibrium so they can be controlled by reformulation. However, covalent reactions are irreversible and will continue until either of the reactants (flavor compounds and proteins) is consumed. Therefore, it is important to distinguish the type of interactions and monitor the covalent reactions.

## **2.5 Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS/MS) for Measuring Protein Adducts**

LC-ESI-MS/MS, MALDI-MS, C14 isotope, and proteomics are the main methodologies for studying the covalent bonding of proteins and small molecules. The C-14 isotope labeling method uses C-14 labeled molecules to monitor reactions with proteins and gives high-quality data. However, the cost and availability of the isotopes, and the training and workplace requirements make this method less popular. Proteomics is a method for determining the location of the bonding in proteins. The creation of MALDI and ESI makes intact protein analysis possible. These two techniques allow the ionization of large protein molecules without enzymatic treatment. The matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has properties including simple sample introduction and data interpretation, but the resolving power of MALDI-TOF is not adequate for detecting the reaction in most animal and plant proteins. LC-ESI-MS has been used widely in studying the reactions between proteins and small molecules (e.g., phenolic compounds and flavor compounds). Although the data interpretation requires a complex deconvolution step because of multiple charges, with the development of computer technology, the powerful MaxEnt-1 mass deconvolution software makes the data interpretation much more convenient. Moreover, the resolution of this method is

sufficient to detect the addition of small molecules to many animal and plant proteins (Alu'datt et al., 2019; Anantharamkrishnan & Reineccius, 2020b; Reineccius, 2022; Rouse et al., 2005)

Electrospray ionization mass spectrometry is an important technique used widely in protein studies. It allows the study of covalent bonds between proteins and small molecules. Mass spectrometry can give both qualitative and quantitative information about molecules. The target molecules are initially introduced to the mass spectrometer's ionization source, where they are ionized to pick up positive or negative charges. After passing through the mass analyzer, the ions reach various locations within the detector based on their mass/charge ( $m/z$ ) ratio. A computer system generates and records usable signals once the ions meet the detector. The signals are graphically shown by the computer as a mass spectrum, which indicates the signals' relative abundance based on their  $m/z$  ratio. Liquid chromatography facilitates quick and quantitative compound separation by the physico-chemical properties of compounds, when combined with ESI-MS, a powerful technology that can rapidly analyze both small and big molecules for a wide range of applications is created (Ho et al., 2003; Sargent et al., 2013).

During the ESI process, electrical energy helps ions transfer from the solution into the gas phase in three steps: dispersing a tiny spray of charge droplets, evaporating the solvent, and ejecting the ions from the highly charged droplets (Figure 3). A sampling skimmer cone collects the released ions, which are then accelerated into the mass analyzer for molecular mass analysis and ion intensity measurement. The quadrupole mass analyzer is one of the most used. Usually, three quadrupoles are arranged in a linear pattern making up a tandem quadrupole system, known as a "triplequad". The result is shown as a mass spectrum which is a graph of the relative abundance of ion signals against the  $m/z$  ratios. For ESI, multiple protonations of proteins can

happen, so a deconvolution step is often applied to convert the result to a single charged format for easy interpretation (Ho et al., 2003).

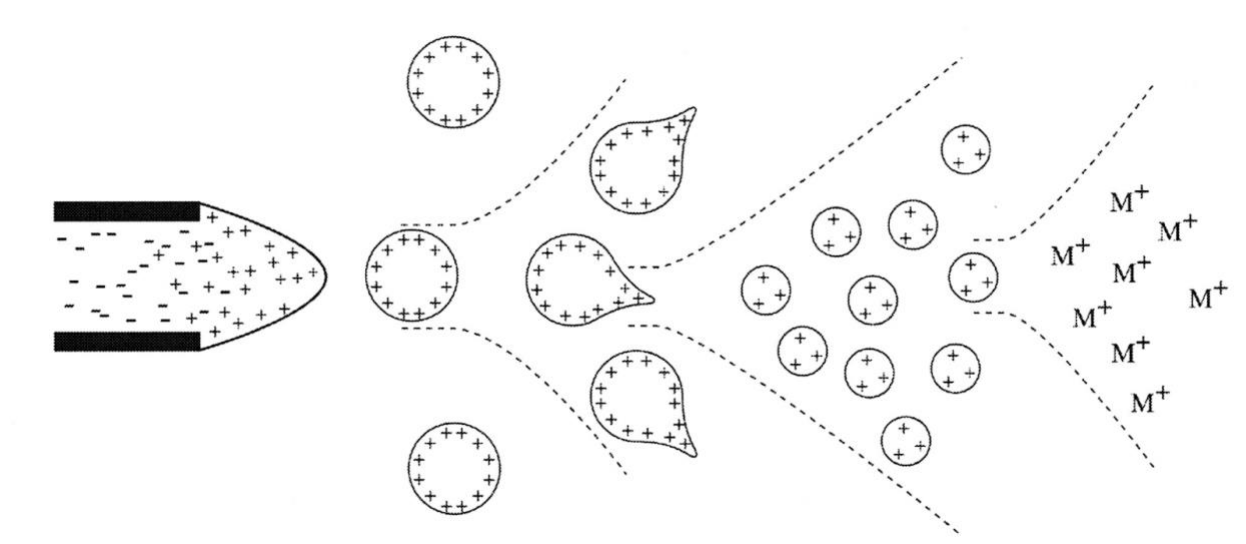


Figure 3: Mechanism of electrospray ionization. (Source: Ho et al., 2003).

Anantharamkrishnan & Reineccius (2020b) developed a method using LC-ESI-MS and proteomics to study covalent reactions between BLG and flavor compounds of 13 functional groups (Anantharamkrishnan et al., 2020). The effects of pH, water activity, and temperature on the reactions were also investigated by the LC-ESI-MS method (Anantharamkrishnan & Reineccius, 2020a). Moreover, the covalent adduct formation between the protein and flavor compounds under thermal treatments (pasteurization and sterilization) was explored (Yuan et al., 2023). Poojary et al. (2023) developed a stable isotope dilution LC-ESI-MS method to identify and quantify adducts of polyphenols and proteins in dairy beverages. ESI-MS was also used to monitor the reactions between a drug named Auranofin and a group of representative proteins. The method provided details of the reactions and allowed the determination of the properties of the adducts at the molecular level (Zoppi et al., 2020). Furthermore, the adducts of proteins ( $\beta$ -lactoglobulin B, human hemoglobin, cytochrome c, and insulin) and lipid oxidation products (4-

hydroxy-2-nonenal (HNE), hexanal, and 2(E),4(E)-decadienal (DDE)) were analyzed by ESI-MS (Bruenner et al., 1995; Fenaille et al., 2003; Tang et al., 2011).

LC-ESI-MS has been widely used in clinical studies and very recently was applied to determine covalent reactions between proteins and flavor compounds. For decades, an enormous number of studies on interactions between flavor and proteins were conducted, however, most of them only measured the total loss of flavor without considering the mechanisms of interaction. Covalent bonding was observed in some studies (Alaiz & Giron, 1994; Kühn et al., 2008; Meynier et al., 2004) but was not proven until LC-ESI-MS was applied (Anantharamkrishnan & Reineccius, 2020b). LC-ESI-MS is an effective method for measuring protein adducts with small molecular weight. Therefore, this research used this technology to study the covalent reaction between BLG and the two oxidation markers which are hexanal and limonene oxide.

One weakness of this technology is the resolution of ESI-MS decrease with the increase in the molecular weight of analytes. Therefore, it is not suitable for large protein (e.g. plant proteins) analysis.

## **2.6 Gas Chromatography (GC) for Measuring Flavor Compounds**

Gas chromatography is a technique for separation and analysis of volatile compounds. It is used in qualitative and/or quantitative analysis of food composition, food additives, pesticides, natural toxins, flavor and aroma, packaging materials, etc. (Lehotay & Hajšlová, 2002). For flavor compound identification, gas chromatography–mass spectrometry (GC–MS), gas chromatography–olfactometry–mass spectrometry (GC–O–MS), and electronic nose are the most commonly used techniques (Wang et al., 2020).

When a sample is introduced into the GC, it will be vaporized and carried by the mobile phase, which is also called the carrier gas, passing through the stationary phase. The carrier gas is often an inert gas such as helium, nitrogen, and hydrogen. The stationary phase is set inside a separation column. When a sample passes through the stationary phase, due to different chemical and physical properties, the compounds will pass the column at different rates and exit at different times, and then be tested by a detector. Two common detectors are the thermal conductivity detector (TCD) and the flame ionization detector (FID). For TCD, analyte molecules elute from the column and mix with carrier gas, causing a drop in thermal conductivity as well as an increase in filament temperature and resistivity. These changes in filament temperature and resistivity eventually lead to oscillations in voltage, which trigger a detector response. The sensitivity of the detector is directly proportional to the filament current and inversely proportional to the temperature of the detector's immediate surroundings and the carrier gas flow rate. For FID, near the exit of the column, electrodes are positioned next to a hydrogen/air-fueled flame, which pyrolyzes compounds containing carbon as they escape the column. Due to the carbons' capacity to produce cations and electrons during pyrolysis, a current between the electrodes can be created. The increase in current is translated and shows up as a peak in a chromatogram. This detector is limited to organic and hydrocarbon-containing substances (Harris & Lucy, 2016). TCD is not as popular as FID mainly because of its poor sensitivity for most analytes (Harvey, 2000).

In a study conducted by Hawthorne et al. (1988), GC-FID was applied to analyze flavor and fragrance compounds in various natural products including orange peel, cedar wood, and spices. Additionally, flavor features of vegetable oils can also be assessed effectively by GC (Dupuy et al., 1977). In flavor encapsulation, GC was used to determine the amounts of aroma

compounds maintained by the encapsulation operations. The ability of wall materials to prevent flavors from oxidation was also evaluated by GC by measuring the amount of the oxidation product (Charve & Reineccius, 2009). Vazquez-Landaverde et al. (2005) developed a headspace solid-phase microextraction (HS-SPME) GC-FID method to quantitatively analyze the thermally derived off-flavors in milk under different thermal treatments. For complex food samples that have various flavor compounds, two-dimensional GC (GC×GC) is applied for its fortified separation abilities (Wang et al., 2020; Zhu et al., 2007).

GC usually is combined with mass spectrometry (MS) for compound identification. Especially in flavor analysis, the GC-MS method has been widely used in identifying flavor compounds in foods. GC-MS identified 183 volatile compounds in 11 types of sauce spareribs, and results showed that there was a good separation among groups. The inherent flavors in oat groats were also characterized by GC-MS (Heydanek & McGorin, 1981). Qian & Reineccius (2003) quantified neutral aroma compounds in Parmigiano Reggiano cheese by the dynamic headspace GC-MS technique and free fatty acids by the ion-exchange chromatography and GC-FID. A relatively new technology named gas chromatography ion mobility spectrometry (GC-IMS) shows a good ability to measure flavor compounds accurately. It integrates the fast response of IMS and the high separation capacity of GC. GC-IMS separates and analyzes ionized molecules based on their mobilities in an electric field under ambient temperatures and pressures. IMS works well for trace gas analysis and has low detection limits, excellent sensitivity, low cost, and easy operation (Wang et al., 2020; Yao et al., 2022). Yao et al. (2022) used headspace gas chromatography ion mobility spectrometry (HS-GC-IMS) to study the flavor formation during Dezhou braised chicken processing, and 37 compounds were identified successfully by this method. A better understanding of the influence of processing on the flavor formation in the

chicken was gained, which can benefit the improvement of processing and products for the poultry industry. There is also a study comparing sampling techniques including steam distillation (SD), simultaneous distillation and solvent extraction (SDE), and headspace solid-phase microextraction (HS-SPME) for Korean garlic flavor determination via GC-MS. HS-SPME shows several advantages compared to other methods. It features fast solvent-free extraction, no significant thermal degradation, labor-saving operation and low sample requirements. (Lee et al., 2003).

GC-MS is effective in flavor compound identification, but it cannot determine the odor properties of the compounds. Additionally, certain volatile chemicals with strong odors have concentrations too low for GC-MS to identify (H. Song & Liu, 2018). Therefore, an olfactometry technique is integrated with GC. Gas chromatography-olfactometry (GC-O) incorporates an olfactometer (human assessor) that detects odor, describes the odor perceived, and evaluates the odor intensity and duration (Brattoli et al., 2013). An odor port is added to a standard GC, and the eluate splits to the odor port and the traditional detector. At the odor port, assessors sniff the eluting volatiles. This method allows the connection of a volatile compound to its sensory properties (Delahunty et al., 2006). GC-O offers both instrumental and sensorial analysis and has been widely used in flavor studies (d'Acampora Zellner et al., 2008). Jonsdottir et al. (2004) studied the flavor profiles of processed ripened roe via several methods including GC-O, electronic nose, and sensory analysis. GC-O was able to detect compounds with very low thresholds and showed good correlations with both the electronic nose measures and sensory evaluation. In the study conducted by Moon et al. (2006), the flavor of simulated beef was analyzed via GC-O and GC-MS and compared with roasted and boiled beef. GC-O has also been applied in cheese aroma characterization (Frank et al., 2004). Furthermore, the combination of

GC-O and GC-MS creates a powerful technique to study food flavors. GC-O-MS can quickly map aroma-active compounds, identify key aroma-active compounds, study the connection between odorants and sensory attributes, and clarify the formation mechanism of significant odorants (H. Song & Liu, 2018). GC-O-MS has many applications, and it has been used to investigate the effect of free fatty acids on the odor of pork (Aaslyng & Schäfer, 2008), identify the aroma-active compounds of Beijing roast duck, olive oils, and muskmelon juice (Chen et al., 2009; Kesen et al., 2013; Pang et al., 2012), determine the migration of odor compounds from adhesives used in food packaging materials (Vera et al., 2014), etc.

Gas chromatography is a significant technique in food flavor study, when combined with MS and/or olfactometry, many flavor problems and challenges in the food industry can be solved. This study used GC-FID to quantify the unbound oxidation markers (hexanal and limonene oxide) in the samples during storage to study the reactions between the proteins and the two markers.

## Chapter 3: MATERIALS AND METHODS

### 3.1 Materials

Whey protein isolate (BIPRO 9500) was provided by Agropur (Eden Prairie, MN, USA). The single variant  $\beta$ -lactoglobulin (BLG) was obtained from a previous work in the lab by isolating the protein from a homozygous, single cow's milk (Anantharamkrishnan & Reineccius, 2020b). (+)-limonene oxide (97%, mixture of *cis* and *trans*) and ethyl butyrate (99%) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Ethyl heptanoate (98%) was bought from BeanTown Chemical (Hudson, NH, USA). Hexanal (97%) was purchased from Chem Impex International (Wood Dale, IL, USA). Hydrochloric acid (37%) was bought from Mallinckrodt Baker (Paris, KY, USA), sodium hydroxide was from Greener Life Essentials (Sebring, FL, USA), and magnesium chloride hexahydrate was from Sigma-Aldrich (St. Louis, MO, USA). The water used for the gas chromatography analysis was deionized, and for the electrospray ionization mass spectrometry analysis was double distilled.

### 3.2 Methods

#### 3.2.1 Determination of Covalent Reactions with Proteins in Dry State

##### 3.2.1.1 UPLC ESI MS Conditions

For UPLC/MS analysis of proteins, a Waters Acquity UPLC coupled to a Waters Synapt G2 HDMS quadrupole orthogonal acceleration time of flight mass spectrometer was used (Waters Corp., Milford, MA, USA). A Waters Acquity UPLC Protein BEH C<sub>4</sub> 2.1 mm  $\times$  100 mm column (1.7  $\mu$ m diameter particles) maintained at 50°C was used for a 30 min linear gradient separation at a flow rate of 0.400 mL/min using A: water containing 0.1% formic acid and B: methanol

containing 0.1% formic acid: 3% B, 0 min to 5 min; 3% B to 97% B, 5 min to 15 min; 97% B, 15 min to 16 min; 97% B to 55% B, 16 min to 17 min; 55% B 17 min to 24 min.; 55% B to 3% B, 24 min to 25 min. Mass spectra were collected in profile mode over the range  $m/z$  300-2500 every 0.1s during the chromatographic separation. MS parameters in positive electrospray ionization mode were as follows: capillary, 0.6 kV; sampling cone, 30.0 V; extraction cone, 5.0 V; desolvation gas flow, 800 L/h; source temperature, 100°C; desolvation temperature, 350°C; cone gas flow, 20 L/h; trap CE, off. Lockspray (on-the-fly mass calibration) configuration consisted of infusion of a 5  $\mu\text{g}/\text{mL}$  solution of leucine-enkephalin and acquisition of one mass spectrum (0.2s scan,  $m/z$  50-1200) every 10s. Three lockspray  $m/z$  measurements of protonated (positive ionization mode) leucine-enkephalin were averaged and used to apply a mass correction to measured  $m/z$  values during the course of the analysis.

### **3.2.1.2 Sample Preparation for UPLC ESI MS Storage Analysis**

A 20% w/w beta lactoglobulin solution was prepared with double distilled water. An aliquot of the protein solution was spiked with either limonene oxide or hexanal (0.06% on a protein basis). The solution was mixed well and then frozen with liquid Nitrogen and freeze dried by a VirTis benchtop 3L freeze dryer (SP Industries, Inc., Warminster, PA, USA) for 5 days. The freeze-dried powder was divided into aliquots (1g) and stored at 45°C in a desiccator containing saturated  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  salt. This saturated salt provided an air environment of  $a_w$  0.3. Sample aliquots were taken at different storage time points (Day 0, 1, 3, 7, 14, 28) for LC MS analysis. The samples for LC-MS analysis were prepared at 50  $\mu\text{M}$  protein. The solution was filtered using a 0.22  $\mu\text{m}$  syringe filter. The solution was then sent to the Mass Spectrometry Lab in the Department of Chemistry at the University of Minnesota, Twin Cities for analysis.

## **3.2.2 Gas Chromatography FID Analysis for Studying the Influence of Reaction Conditions on Protein: Marker Reaction**

### **3.2.2.1 Gas Chromatography FID Conditions**

A HP-5890A Series II GC (Hewlett-Packard, Alto Palo, CA, USA) with a DB-WAX capillary column (30 m × 0.25 mm × 0.25 μm) (J&W Scientific, Folsom, CA) and a flame ionization detector (FID) was used in analysis. The oven program was 40°C initial temperature, increasing at 7°C/min to 96°C, and then increasing at 20°C/min to final temperature of 220°C, holding for 5min. The injection port temperature was 220°C, and the detector temperature was 250°C. Two μL of sample were injected using an autosampler with the GC operated in split mode (60:1). Hydrogen was the carrier gas, and the column head pressure was 12.0 psi. The flow through the column was ca. 2 ml/min.

### **3.2.2.2 Reacting Proteins and Oxidation Markers**

A 25% w/w whey protein isolate solution was prepared with deionized water. The pH was adjusted to 4 or 7 with 1M HCl and/or 1M NaOH. Nine g limonene oxide and 9 g hexanal were added to 800 g of the solution and homogenized by PowerGen 700 Homogenizer (Fisher Scientific, Hampton, NH) for 10 min. The “flavored” solution was then freeze dried. The resultant powders were separated into aliquots (1g) for storage and stored at different temperatures (25°, 40°, and 60°C) and water activities (0.23, 0.53, 0.75) in desiccators. The stored powders were analyzed at different reaction time points (Day 0, 1, 3, 7, 14, 28) for Gas Chromatography analysis.

### **3.2.2.3 Sample Preparation for Gas Chromatography FID Analysis to Measure the Unbound Hexanal/LO**

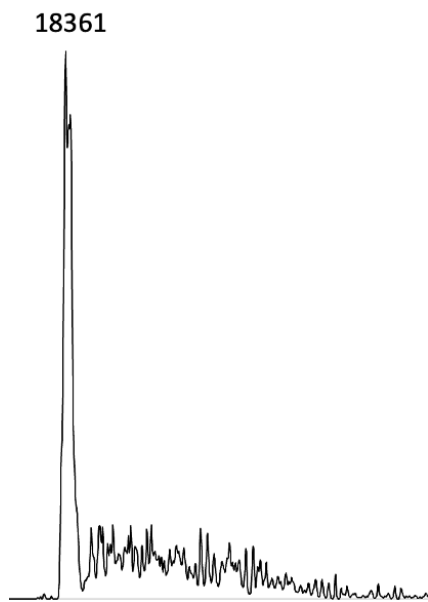
A 15% w/w solution (6.67g) of each aliquot of the stored sample powder was prepared in deionized water. Three ml of the solution was transferred to a vial, then 12 ml of an acetone solution containing ethyl butyrate (500 ppm) and ethyl heptanoate (500 ppm) as internal standards was added to the vial with stirring. The mixture was allowed to settle for 30min and then centrifuged if necessary to produce a clear solution. The clear fraction (1.5 mL) was transferred to 2mL autosampler vials and loaded into a HP7673A automatic sampler (Hewlett-Packard, Wilmington, DE), and 2  $\mu$ L of sample was injected into the GC. A standard sample was prepared by adding limonene oxide (500 ppm), hexanal (500 ppm), ethyl butyrate (500 ppm) and ethyl heptanoate (500 ppm) into acetone. The standard sample was used to calculate the response factor. Data collection and peak area integrations were done. The experiment was run in duplicate.

## Chapter 4: RESULTS AND DISCUSSION

### 4.1 Reaction of BLG with Limonene Oxide

After incubating for 28 days, a significant increase in peak intensity was observed at a molecular mass of 18516 Da compared to the peak area on Day 0. The adduct peak has a mass shift of 152 Da which is consistent with the addition of limonene oxide. The change of the adduct peak size is shown in Table 1. On Day 0, the adduct peak exists probably because the reaction largely happens during sample preparation (mixing and freeze drying). During the second 14 days of incubation, the peak approximately doubles in size. Epoxides can react with nucleophiles in an  $S_N2$  reaction. Thiols are the most likely nucleophilic functional group in a protein. In BLG, free cysteines are usually buried deeply in the protein, making it difficult for limonene oxide to access, which could lead to the slow reaction at the beginning. With an increase in reaction time, limonene oxide can access the cysteines and formed covalent adducts, so the reaction is much faster in the Day 14 ~ 28 interval than in the Day 0 ~ 14 period.

a) Day 0



b) Day 28

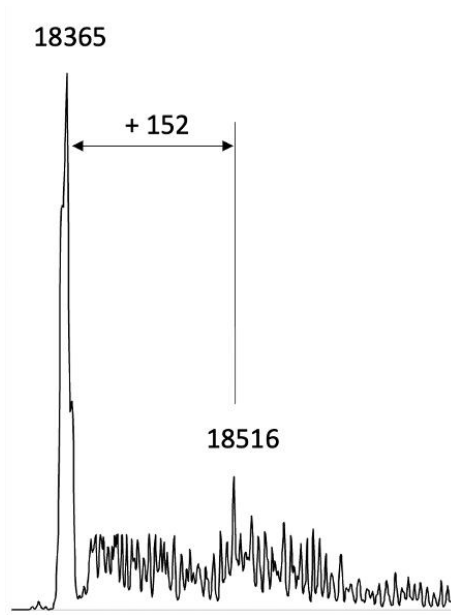


Figure 4: Deconvoluted ESI mass spectra of BLG and limonene oxide incubated at 45°C,  $a_w$  0.33 for 0 and 28 days.

Table 1: The intensity of the adduct peak on different incubation days.

Incubation Time (Days)	0	14	28
Adduct Peak Intensity	60,450	82,020	175,700



Figure 5: Proposed mechanism of the reaction between limonene oxide and BLG.

#### 4.2 Reaction of BLG with Hexanal

Previous work (Anantharamkrishnan et al., 2020) has shown that hexanal readily reacts with BLG through a Schiff base reaction (Figure 6). This reaction happens quickly in liquid state: large adduct peaks are observed after only 24 hours of incubation. The single Schiff base adduct (BLG + 82 Da) has the biggest peak which means it is the main product. In the case of dry state, only the peak of the single Schiff base adduct (BLG + 82 Da) appears obviously on the spectrum.

Table 2 shows the change in intensity of the adduct peak with storage time. The dry state makes it difficult for hexanal to access the reaction sites (lysine) in the protein, so the reaction rate is slow. In industry, if hexanal is used as an oxidation marker in a dry food that contains protein and the storage time is months, the influence of the reaction between hexanal and protein on the reliability of this marker could be insignificant.

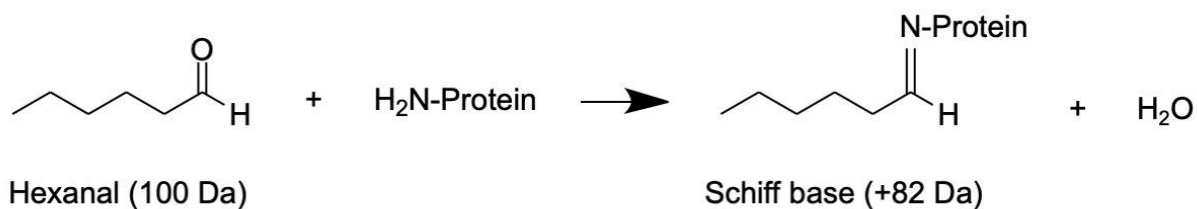


Figure 6: Mechanism for the formation of Schiff base by hexanal and a free amine-containing group in a protein.

a) Day 0

b) Day 28

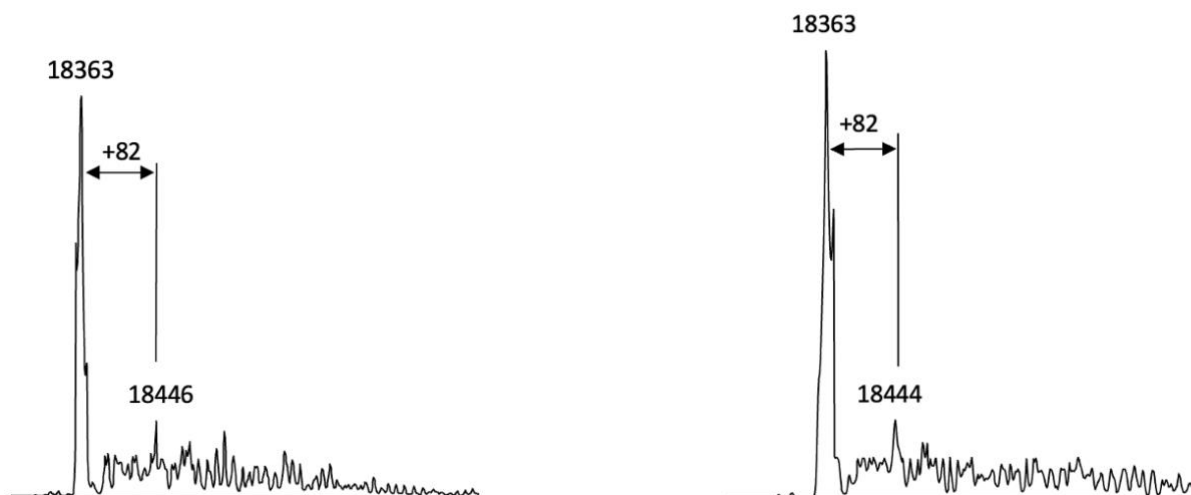


Figure 7: Deconvoluted ESI mass spectra of BLG and hexanal incubated at 45°C,  $a_w$  0.33 for 0 and 28 days.

Table 2: The intensity of the adduct peak on different incubation days.

Incubation Time (Days)	0	14	28
Adduct Peak Intensity	173,000	193,000	188,100

### **4.3 Effect of Temperature, Water Activity, and pH on the Reaction Between Whey Protein Isolate (WPI) and Hexanal/LO**

#### **4.3.1 WPI and Limonene Oxide**

The amount of extractable limonene oxide in the samples was determined after selected storage times. Theoretically, if the extractable amount decreases, this may suggest that more limonene oxide is bonded with the protein. The data do show a reduction in the amount of LO present in samples with storage. The rate of loss increasing with increased temperature and increased water activity is consistent with expectations for chemical reactions. *Unfortunately, there is another potential factor that could lead to the decrease of extractable LO – i.e., evaporation.* The samples had to be stored in open vials when in the desiccators to properly adjust the water activity. This could result in the marker undergoing some loss due to evaporation.

Interestingly, on Day 0 when freeze drying was finished, the amount of extractable limonene oxide remaining in the sample was substantially different between samples held at two different pHs (pH 4 and 7) (Fig. 8). It is well known that pH changes the structure and properties of a protein which can alter both evaporative losses and reaction rates. The unfolding of the protein at pH 4 may have favored the evaporative loss of small molecules (i.e., LO). The alternative hypothesis is that the reaction rate between volatiles and the protein increased at low pH. One finds that losses increased at elevated water activities and storage temperatures. There are no data to differentiate between these loss mechanisms. These observations may be explained/are consistent with either factor (water activity or temperature). Again, there is no basis to choose mode of loss based on the data.

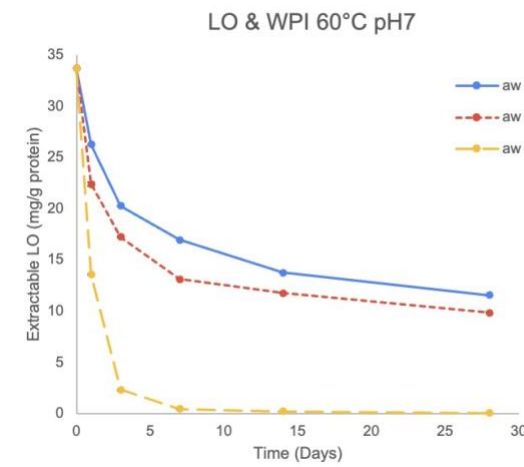
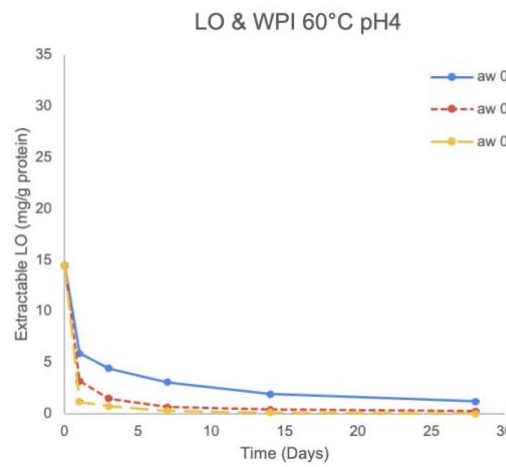
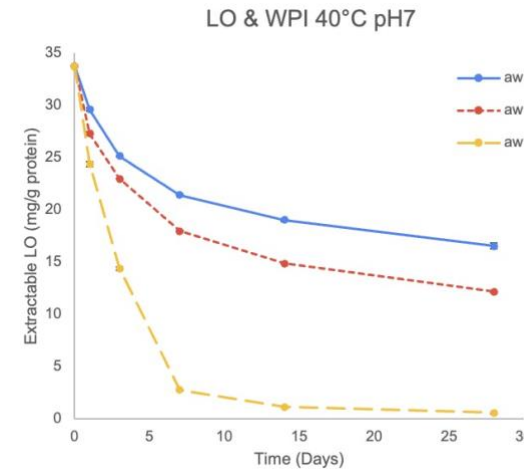
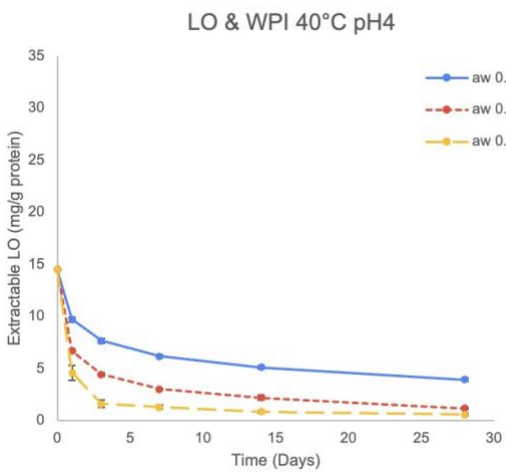
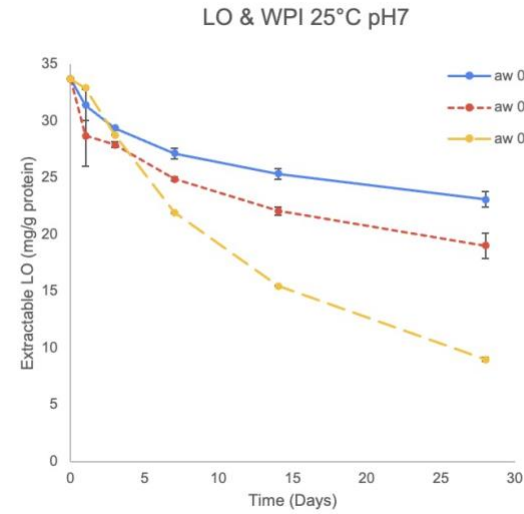
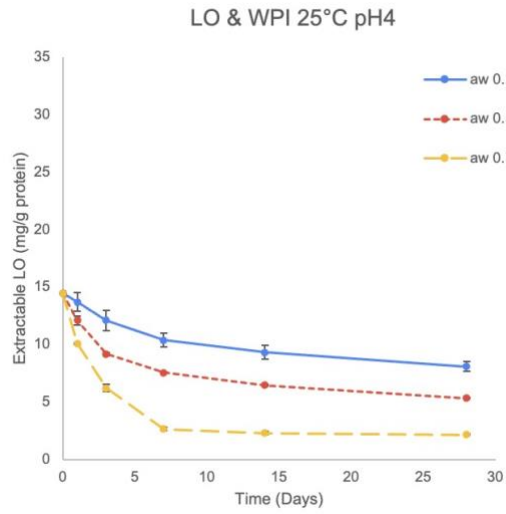
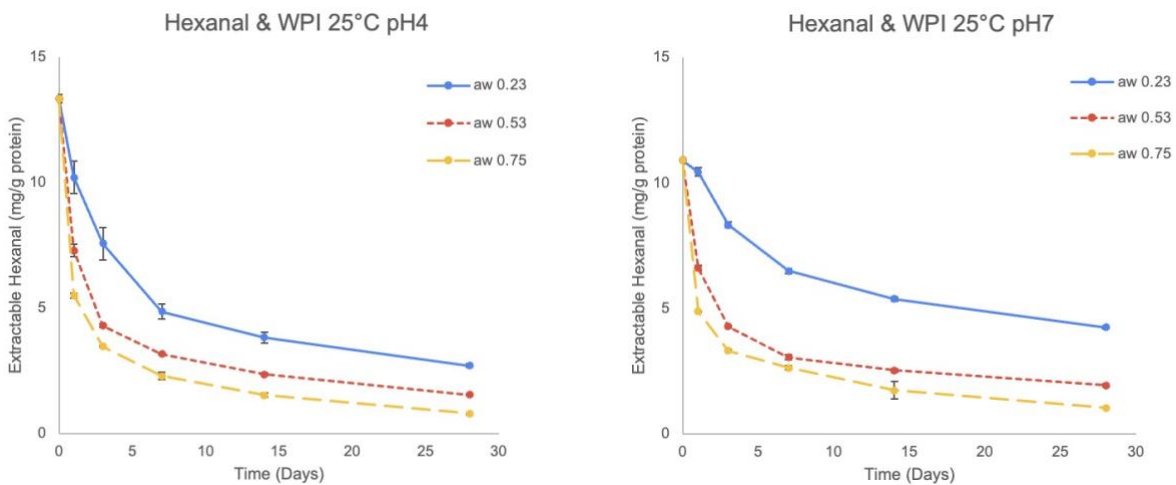


Figure 8: The extractable limonene oxide in the samples during incubation.

### 4.3.2 WPI and Hexanal

The amount of extractable hexanal decreases with time, and the rate increases with an increase in temperature or water activity. Similar to limonene oxide data, during the first few days of incubation, a sharp decrease in the amount of extractable hexanal probably is due to the evaporation of hexanal instead of the reaction with the protein. The trends that the experimental variables show for the loss of hexanal are very similar to those observed for limonene oxide. However, there are some minor differences. The protein shows different ability to maintain limonene oxide vs hexanal at pH 7. On Day 0 (the time that freeze drying was finished), at pH 7, the amount of extractable limonene oxide is 34 mg/g protein while extractable hexanal is 11 mg/g protein. This could be related to the solubility and vapor pressure of the two compounds.

In conclusion, because of the influence of evaporation, solubility, and vapor pressure, this data is hard to interpret, and not valid to show the effect of reaction conditions (temperature,  $a_w$ , and pH) on the reaction between LO/hexanal and WPI.



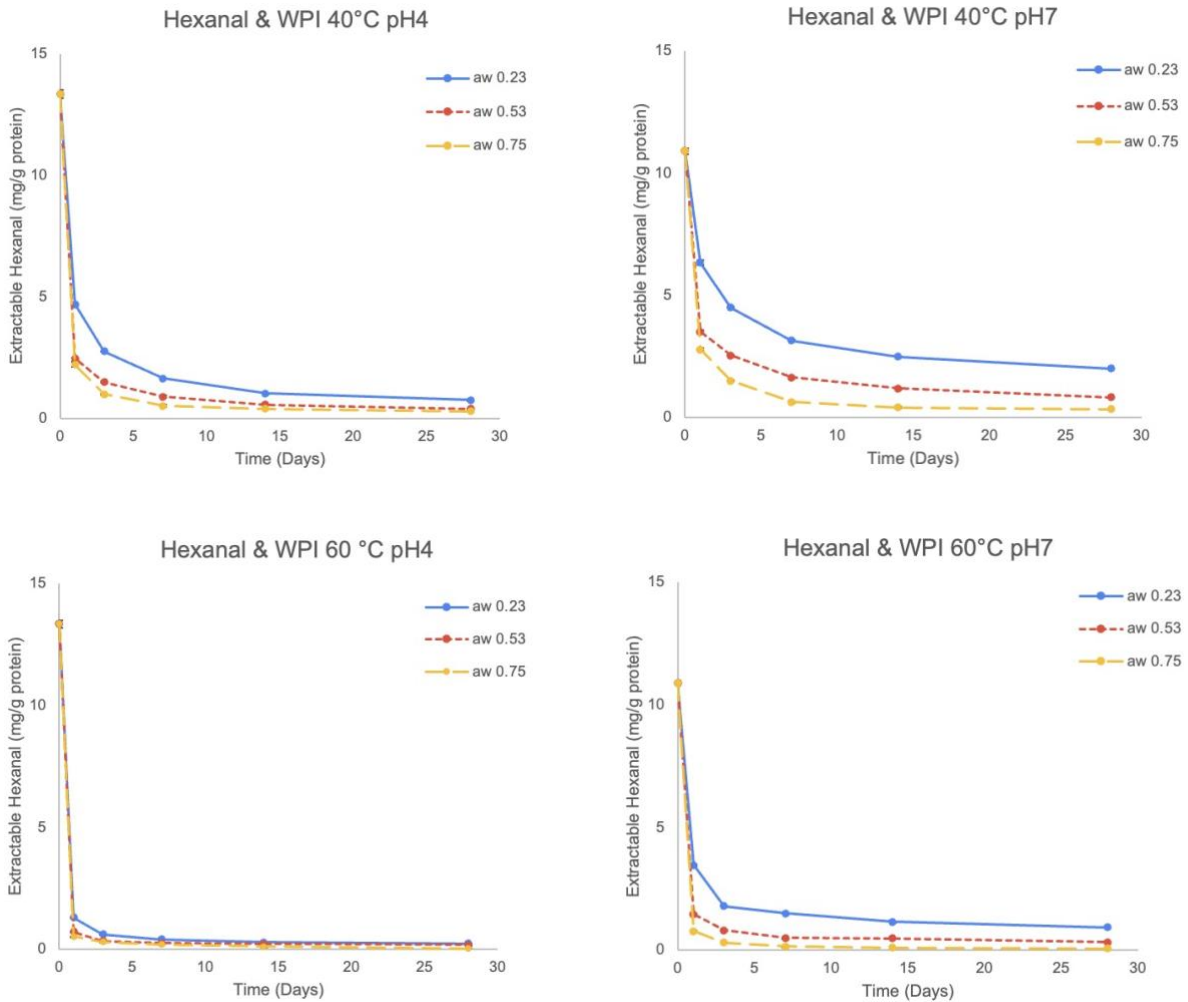


Figure 9: The extractable hexanal in the samples during incubation.

#### 4.4 Discussion

The MS results indicate limonene oxide could react with  $\beta$ -lactoglobulin and form covalent adducts. The possible reaction mechanism could be the  $S_N2$  nucleophilic substitution reaction between the compound and free cysteines in the protein. The reaction rate increases significantly after storing for 14 days which could be due to the accessibility to the cysteines buried in the protein. These results suggest that limonene oxide may not be a very valid

oxidation marker for foods containing high levels of proteins, but more data are needed to determine the importance of this reaction in undermining the use of LO as an indicator of orange oil oxidation. The reaction between hexanal and  $\beta$ -lactoglobulin is relatively slow in the dry state during storage, which could make hexanal a usable marker of lipid oxidation in dry food when the storage time is within months.

For objective 2, pure  $\beta$ -lactoglobulin variant A is exhausted and it is difficult to find a homozygous single cow and isolate a single variant. Therefore, whey protein isolate is used. However, WPI is not suitable for LC-ESI-MS analysis because of its large molecular weight, so the GC-FID method is adopted. It is an indirect measurement that measures the free markers in the samples. Theoretically, if the amount of the free markers decreases, it would indicate that more are binded with the protein. However, the evaporation makes it hard to determine the mechanism of the loss of limonene oxide/hexanal. During storage, the sample vials are stored in desiccators without caps to adjust the water activity, which leads to evaporation. Moreover, different temperatures, water activities, and pH influence the vapor pressure and solubility of the two compounds, as well as the ability of the protein to maintain the compounds during freeze-drying. Unfortunately, all these factors cause the GC results uninterpretable and invalid to support the second objective of this research.

In conclusion, this research shows that limonene oxide could form covalent bonds with the model food protein ( $\beta$ -lactoglobulin). Moreover, the reaction rate would increase with the increase of storage time, which could make limonene oxide inaccurate as an oxidation marker in food containing high levels of proteins. For hexanal, the increase of the protein adduct is not very significant during storage, so it still can be considered as a usable oxidation marker for dry foods. Temperature, pH, and water activity have an influence on the reaction rate, but the experiment

design for objective 2 is problematic leading to uninterpretable GC results. For future work, using pure  $\beta$ -lactoglobulin for sample preparation and LC-ESI-MS for analysis to study the effect of reaction conditions is a better choice.

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