International Journal of Nutrition and Food Sciences
2024, Vol. 13, No. 2, pp. 38-55
https://doi.org/10.11648/j.ijnfs.20241302.15

Research Article

L-proline Interaction with Methyl Linoleate Oxidation Products Formation in Dry System and at Temperatures: For Understanding in Low-Moisture Foods

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Abstract

Dry and low-moisture foods could experience a significant loss in nutritional value due to the process of methyl linoleate oxidation. L-proline could interact with lipid oxidation products, potentially modifying their formation and reaction path. However, there was a lack of research on the interaction between L-proline and methyl linoleate oxidation products in dry and low-moisture food matrices, which was a concern given the potential impact on food safety and nutrition. To address this knowledge gap, a study investigated the interaction between L-proline and the oxidation products of methyl linoleate in a dry system. The study examined the formation of methyl linoleate oxidation products such as conjugated dienes, hydroperoxide, and hexanal in the absence and presence of varying moles of L-proline at different temperatures. The formation of conjugated diene, hydroperoxide, and hexanal was analyzed using UV spectrometer analysis, xylenol orange, and DNPH derivatization HPLC-UV analysis. The results showed that adding proline to methyl linoleate samples stabilized conjugated diene and decreased hydroperoxide and hexanal levels as temperature increased, compared to the control sample. This suggests that L-proline effectively interacted with methyl linoleate oxidation products and altered their formation and oxidation path in the dry system. Overall, this study provided a basis for significantly enhancing understanding of the reactions between L-proline and methyl linoleate oxidation products in dry and low-moisture foods, offered practical implications for the food industry, and paved the way for future research.

Keywords

Oxidized Lipids, Amino Acid, Interaction, Co-oxidation, Arid System, High Oxidation, Autoxidation, Lipid Oxidation Markers

1. Introduction

Dry and low-moisture foods like edible nuts, seeds, meat, fish, and healthy food industrial products such as granola bars, cereals, and crackers are good sources of methyl linoleate (ML), providing numerous health benefits to consumers. However, their low water activity makes these foods more susceptible to ML oxidation than moisturized food [1, 2]. Therefore, it is crucial to understand how to provide consumers with the nutritional benefits of ML in dry and low-moisture foods [3]. The presence of L-proline amino acid can interact with ML oxidation product formation, reduce the

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Received: 20 March 2024; **Accepted:** 7 April 2024; **Published:** 29 April 2024

amount of ML oxidation product, and alter the ML oxidation path in dry systems at various temperatures. Therefore, understanding the interaction between L-proline and ML oxidation products in the dry system is crucial before using it in the dry and low-moisture food matrix. By initiating the use of L-proline in ML containing dry food products, it could be possible to prevent the loss of ML nutritional benefits, which can help food industries and food scientists to develop new dry food products with the use of L-proline and ML for the benefit of consumers.

1.1. Lipid Oxidation in Food

Lipid oxidation is a complex process that occurs in food via autoxidation, photooxidation, enzymatic oxidation, ionizing radiation, and many other processes [4, 5]. Several factors influence this process, such as temperature, oxygen concentration, and prooxidants including transition metals, singlet oxygen, light, and temperature [5]. Food properties such as low moisture, intermediate moisture, fluid water region, water activity, surface area, emulsion, pH, antioxidant location, and solid fat content also play a significant role [6, 7]. Food scientists need to understand lipid oxidation in dry and low-moisture food. Specific food components such as amino acids, proteins, natural polyphenols, and metal chelators can exhibit antioxidant activity and inherent oxidative stability. While antioxidant systems can effectively mitigate oxidation, they may be disrupted during food processing, so the strategic placement of free radical scavengers within the food system is crucial. It is important to note that lipid oxidation can differ based on the location of the lipid within a food product [8]. Lipid oxidation products can react with several food constituents during processing and storage, reducing the nutritional value of the food items. These reactions can negatively impact the sensory qualities of the food. Therefore, our collective responsibility is to maintain the nutritional value and sensory quality of processed foods. Given the demand for such foods, understanding the complexities of lipid oxidation pathways in low-moisture foods is essential. ML is the lipid of choice for this study due to its unique properties. Its oxidation is more stable than that of methyl linolenic acid and oxidizes more readily than that of methyl oleic acid, making it an ideal candidate for our research. Furthermore, it has been extensively studied, providing a substantial base of existing data to compare and validate our results.

1.2. Introduction of Proline: The Unique Amino Acid

Proline is known for its ability to protect against reactive oxygen species, hydroxyl radicals, and free radicals. It prevents oxidation and damage to lipids, proteins, and DNA under high temperatures, UV exposure, and other oxidative stress conditions. Proline exhibits a protective mechanism that involves quenching singlet oxygen rapidly by forming a charge transfer complex, where it provides an electron. Proline reacts with hydroxyl radicals under hydrogen abstraction, forming the most stable radical. It carries the spin on the C-5 atom, which is distanced from the carboxyl group and proximate to nitrogen. This reaction rate with proline surpasses that of most other amino acids. During stress, plants accumulate high proline levels, which shield them from reactive oxygen species due to their singlet-oxygen quenching properties. L-proline can react with lipid radicals, alkoxyl radicals, hydroxyl radicals, and lipid secondary products, which might affect the formation of lipid oxidation products [9-12, 14]. The free L-proline amino acid is the choice for this study because other amino acids like histidine, tryptophan, lysine, and cysteine were studied in muscle meat, plant, animal, and dairy protein. In contrast, other components like carbohydrates, cellulose, enzymes, vitamins, and flavor were present, which can interfere with the study results of lipid oxidation products and amino acid interaction [11]. Our study on the role of free L-proline in the ML oxidation process in a dry system is unique and remains unexplored. Although research articles on the role of free L-proline in plants under stress conditions are available, we chose the free L-proline amino acid for our study as it presents a novel and uncharted research opportunity.

1.3. Significance of the Study

This study investigates the development of ML oxidation products in a dry system, both with and without L-proline. The dry system is designed to mimic a dry and low-moisture food environment. The experiment takes place outside the food matrix to eliminate interference from other food components and ensure better clarity of the results. The study better understands the interaction between L-proline and ML oxidation products, particularly in the context of the temperature effect and the dry system. The findings of this study have significant practical implications for the dry and low-moisture food-producing industry. Gaining insights into ML oxidation in L-proline presence can help design more effective formulations for dry food products and processing. The research analyzes the major ML oxidation products, which are theoretically and practically significant for understanding. Overall, this study promises to enhance our understanding of ML oxidation and pave the way for using L-proline in dry products containing ML.

The hypothesis is that radicals form during the oxidation of ML in dry food systems. These radicals interact with other compounds within the food matrix, stabilizing and altering the reactions. This process can substantially impact the formation of ML oxidation products. Moreover, the temperatures and hydrogen donor compounds influence the degree of this impact in a dry system. L-proline can alter the oxidation product formation and reaction path by donating hydrogen to radicals during oxidation and interacting with the products formed in ML oxidization. To rigorously test this hypothesis, measuring these products in varying amounts of L-proline at different temperatures is crucial.

This study aims to provide evidence of changes in the amount of ML oxidation products in the presence and absence of different amounts of L-proline under various temperature conditions in a dry system. The study will analyze conjugated dienes, hydroperoxide, and hexanal during the oxidation of ML in dry samples. The focus will be on short reaction times to detect reactions early in the oxidation process. The study investigates how different reaction temperature conditions (freezer, refrigerator, 25 ℃, 37 ℃, and 65 ℃) affect the distribution of ML oxidation products in the presence and absence of L-proline. The samples will be tested in a closed headspace system. The results obtained from the freezer and refrigerator temperature samples will provide information about products stored at lower temperatures, which can be compared with those stored at 25 ℃, 37 ℃, and 65 ℃. This comparison will help evaluate and understand the effects of elevated temperatures on the product. The study also examines the effect of different amounts of L-proline by preparing samples for control ML, mole ratio ML:L-proline::1:5, and ML:L-proline::1:10. A control sample of ML is used to obtain a result in the absence of L-proline, which is compared to the results obtained in the presence of L-proline. To investigate L-proline amounts, a sample with a higher amount of L-proline in ML (ML: L-proline mole ratio of 1:10) is prepared and compared to the results obtained from a sample with a mole ratio of 1:5. L-proline control samples will be prepared and stored with other samples at all five temperature conditions to avoid interference with the L-proline products. L-proline control will help evaluate whether any products will be generated from L-proline only in the absence of ML. The results of a control L-proline sample will be compared with ML and L-proline (1:5 and 1:10) to evaluate the accurate product formation of ML oxidation and L-proline.

2. Materials

Methyl linoleate was purchased from NU-CHEK PREP INC. L-Proline was purchased from Alfa Aesar. Isooctane was purchased from Fisher Chemicals. PeroxiDetect™ kit (Catalog Number PD1), hexanal; 2,4-Dintrophenyl hydrazone of hexanal, 2,4-Dinitrophenyl hydrazine (DNPH), acetonitrile, dimethyl formamide (DMF), sulfuric acid and hydrochloric acid were purchased from Sigma Aldrich. All solutions were prepared using distilled water from the Milli-QTM water system.

Glass vials with cap, VWR; analytical balance, micropipette (200 µL, 100 µL, 2- 20 µL) and 1000 µL pipette, spectrophotometer (UV-5500 PC spectrophotometer Metash Program); freezer; refrigerator; oven; glass test tubes; Agilent HPLC 1260 Infinity II system (G711B Quat pump, G7129A 1260 vial sampler, G7116 1260 column compartment, UV detector), HPLC column Ultra C18 (150X 4.6 µm, 5 µm, cat# 9174565, Ser# 19052245), data acquire and analyze via Open LAB chromatography software, volumetric pipettes, volumetric glassware, centrifuge Allegra and HPLC vials.

3. Methods

3.1. Sample Preparation

For the control ML sample preparation, ML (8.9 mg) was weighed in a 5 ml glass vial and then capped. For the control L-proline sample preparation, L-proline (35 mg) was weighed in a 5 ml glass vial and then capped. For the ML:P:: 1:5 moles sample preparation, ML (8.9 mg) was weighed in a 5 ml glass vial, and L-proline (17.3 mg) was weighed in the same glass vial, which was then capped. ML and L-proline were mixed slowly with a vortex mixer. For the ML:P:: 1:10 moles sample preparation, ML (8.9 mg) was weighed in a 5 ml glass vial, and L-proline (35 mg) was weighed in the same glass vial, which was then capped. ML and L-proline were mixed slowly with a vortex mixer.

The samples were prepared in triplicate for each temperature condition and time point. The vials were placed in different temperature settings, including the freezer (-10 ℃), refrigerator (5 ℃), laboratory temperature (20 ℃), preset oven at 37 ℃, and preset oven at 65 ℃.

At each end of the day (days 1, 2, 3, 4, and 5), the sample vials were pulled out from each temperature and at time points. Then, triplicate vials were used for individual analysis for conjugate diene (using a UV spectrometer), hydroperoxide (using the PeroxiDetect™ kit), and hexanal (using the HPLC-DNPH assay).

3.2. Conjugate Dienes Analyses

Conjugated dienes were analyzed using a modified AOCS Ti 1a-64 [13]. 3 mL iso-octane was added into the sample vial and mixed well. ML in the mixed sample and control dissolved in iso-octane, while L-proline did not dissolve. So, all sample solutions were transferred into 5 mL centrifuge tubes and then centrifuged at 1430 RCF for 15 minutes to obtain a clear supernatant solution for analysis. A portion of the supernatant was retained for analysis, and the remainder was diluted serially by a factor of 50. The present sample solution of ML had a theoretical concentration of 200 nm/ml, while the serially diluted solution had a theoretical concentration of 4 nm/ml. The diluted sample solutions in iso-octane absorbance were collected from 200-300 nm and measured at 234 nm using a UV spectrophotometer [15]. The Iso-octane UV cut-off is 215 nm. The dilution factor was included in the calculation. The concentration was calculated using Beer's Law with an extinction coefficient of 29,500 L/mol cm in iso-octane [13, 20] and converted in mmol/ mol of ML the following equations the following formulas (1), (2).

$$A=\varepsilon lc \tag{1}$$

$$\frac{\text{mmol}}{\text{mol of ML}} = \frac{\text{Sample absorabce}}{29500\frac{\text{L}}{\text{mol}\times\text{cm}}\times\text{cm}} \times \frac{1000 \text{ X D}}{\text{mol of ML}} \tag{2}$$

A= absorbance
ε. = the extinction coefficient 29500 L / mol cm
l = path length in cm
C = is the concentration in mol/L
1000 = to convert mol to millimole
D = dilution factors

3.3. Methyl Linoleate Hydroperoxide Analyses

The xylenol Orange Assay method is used to detect peroxide values, specifically lipid hydroperoxides, in organic, aqueous, and biological samples. The xylenol orange assay is highly sensitive and can detect nanomoles of ML hydroperoxides concentration in a sample solution. In this method, a reaction occurs between ferrous (Fe^{2+}) ions and lipid hydroperoxide in the presence of acid, forming ferric (Fe^{3+}) ions. When ferric ions react with xylenol orange in acidic media, a blue-purple complex (XO, 3,3'-bis [N, N-bis (carboxymethyl)aminomethyl]-o-cresol sulfone phthalein, sodium salt) is formed, which can be measured for its absorbance maximum between 560 nm. The reaction maximum occurs near pH 1.8, so sulfuric acid (H_2SO_4) is used in this assay for better sensitivity and precision. The tert-butyl hydroperoxide (t-BuOOH) is used for a standard calibration curve to calculate peroxide value [16, 17]. However, the extinction coefficient varies with the R group of the hydroperoxide, and the bleaching effect is a limitation of this analytical method [18, 19].

Organic peroxide color reagent: The solution was prepared by mixing the contents of the vial (catalog number 08262 from PeroxiDetect™ kit) with 120 mL of a 90 % methanol solution in water and mixing it well. After reconstitution, the solution contained 4 mM BHT and 0.125 µmole xylenol orange. Working Color Reagent for lipid hydroperoxides: 100 volumes of the reconstituted organic peroxide color reagents were mixed with one volume of Ferrous Ammonium sulfate reagent to prepare the resulting reagent solution containing 0.25 mM ferrous ammonium sulfate in 0.025 M sulfuric acid. 200 µM (0.2 mM) tert-butyl hydroperoxide (t-BuOOH) Standard Solution: To prepare a 1 M solution of t-BuOOH, a 70% (7 M) solution of t-BuOOH (Catalog Number 458139 from PeroxiDetect™ kit) was diluted with methanol in a 7:1 ratio. For this, 0.5 mL of 7 M t-BuOOH was pipetted and transferred to a glass vial. Then, 3 mL of methanol was pipetted and transferred into the same glass vial. The total volume of the solution was 3.5 mL, and the vial was mixed well. Further, the 1 M t-BuOOH solution was diluted with three serial 10-fold dilutions in methanol to prepare a 1 mM solution. This 1 mM solution was then diluted 5-fold in methanol to prepare a 200 µM solution. To create the calibration curve, solutions for t-BuOOH, 0, 5, 10, 20, 40, 60, and 80 µL of the 200 µM t-BuOOH solution were pipetted and transferred to separate vials. The volume was adjusted to 100 µL using 90% methanol in water. Next, 1 mL of the working color reagent was added to each solution and mixed well. Then, the solutions were incubated at room temperature for 30 minutes to form the color. After that, the prepared standard solutions in the range of 1-16 nmoles of t-BuOOH per reaction volume were analyzed at 560 nm [17]. Table 1 presents the t-BuOOH linearity concentration range, correlation coefficient for the regression curve, slope, and intercept.

Table 1. *The concentration range, the correlation coefficient for regression curve, slope, and intercept for the t-BuOOH calibration curve.*

Name	t-BuOOH
Concentration range (nmol)	1 to 16
Intercept	- 0.095
Slope	0.0571
Coefficient of determination (R^2)	0.9961
Regression equation	Y= 0.0571 X - 0.095

Lipid hydroperoxides, the primary oxidation products, were measured in triplicate using a xylenol orange commercial kit (PeroxiDetect™ kit). Each sample was equilibrated to room temperature, followed by the addition of 200 µL of 90% methanol in water, vortexed briefly, and then centrifuged at 1430 RCF for 15 minutes to extract the hydroperoxide fraction. A volume of extract (80 µL) was removed and diluted to 100 µL using 90% methanol in water. Next, 1 mL of the working color reagent was added to each solution. The final mixture was mixed well and covered to prevent evaporation, and the solutions were allowed to incubate at room temperature for 30 minutes to form the color. Absorbance was measured at 560 nm on a spectrophotometer. Hydroperoxide concentration was calculated from a t-BuOOH calibration curve, and the results were converted in mmol/ mol of ML the following formulas (3).

$$\frac{\text{peroxide}\ \frac{\text{mmol}}{\text{mol of ML}}}{} = \frac{\left(\frac{\text{Sample absobance} - \text{Blank absorbance}}{}\right) - \text{Intercept}}{\text{Slope}} \text{X} \frac{D}{10^6 \times \text{mol of ML}} \quad (3)$$

Where,
10^6 = conversation of nmole to mmol

3.4. Hexanal DNPH (2,4-Dintrophhenyl hydrazine) Assay Analyses

The aim here was to optimize reverse-phase high-pressure liquid chromatography (RP-HPLC) conditions and hydrazone solubility. The solubility of commercial C6-C10 DNPH carbonyls was evaluated in acetonitrile, acetonitrile: water:: 70:30, and dimethylformamide (DMF). Hydrazones were found to be soluble only in DMF. The DNPH hydrazone mixture was made up of saturated aldehyde C6-C10 and formaldehyde. Lipid oxidation produces various carbon

chain-length carbonyl products. These could react with DNPH in the mixture and provide chromatographic interference at the hexanal-DNPH peak retention time. Therefore, it was necessary to separate other hydrazone peaks chromatographically from the hexanal-DNPH peak, which was crucial for accurate and precise analysis. DNPH-derivatized saturated aldehyde C6-C10 and formaldehyde were prepared under acidic conditions with DMF diluent, separated using acetonitrile-water gradients via RP-HPLC, and detected via UV. Literature-reported chromatographic conditions were used for sample analysis [20]. The RP-HPLC acquisition chromatographic conditions are presented in Table 2. The hydrazones eluted in sequence by carbon chain length. The shorter chain length eluted first, and the longer chain length eluted last.

DNPH acidic solution preparation: DNPH weighed 0.70 g and was transferred into a glass vial. DMF 9.9 mL was pipetted and transferred into the same vial. Mix well to dissolve. 0.100 ml Conc. HCl was pipetted and added to the DNPH solution, and the solution was mixed well. The DNPH concentration was 70 grams/little (357 mmole) in DMF. They were mixed well.

Table 2. *Instrument condition for data acquisition.*

Quaternary Pump (G7111B module), Autosampler (G7129A module), Column Compartment (G7116A module) and UV wavelength detector (G7114A module)			
Flow (ml/min)	**1.200 ml/min**		
Mobile phase A Channel	Water		
Mobile phase B Channel	Acetonitrile		
Gradient Timetable	Time (min)	A (%)	B (%)
	0.0	30	70
	15.00	0	100
	17.00	0	100
	18.50	30	70
	20.00	30	70
Stop Time (min)	20.00		
Injection volume (µL)	10.00		
Column	Ultra C18 5 µm 150 X 4.6 mm, Cat # 9174565		
Signal wavelength	360 nm		

The blank chromatogram shows no chromatography interference at the retention time for the peak of interest. The hexanal DNPH peak retention time was 7.06 min in the chromatogram. Table 3 presents the hexanal linearity concentration range, correlation coefficient for regression curve, slope, and intercept.

Table 3. *The concentration range, the correlation coefficient for the regression curve, the slope, and the intercept for the hexanal calibration curve.*

Name	**Hexanal**
Concentration range (µmol)	14 to 91
Intercept	31.77
Name	**Hexanal**
Slope	22979.60
Coefficient of determination (R^2)	0.9929
Regression equation	Y= 22979.60 X + 31.77

200 µL DNPH acidic was added to each sample vial and mixed well. They were kept in the dark for 30 minutes. Then, each sample solution was transferred to a separate microcentrifuge tube and centrifuged at 17530 RCF for 15 minutes. The supernatant solution was transferred to HPLC vials and analyzed via HPLC with detection at 360 nm wavelength [20]. The present ML sample solution had a theoretical concentration of 150 µmol/ml. A standard curve of µmol of hexanal was

generated and used to calculate the hexanal results and converted the following formulas in mmol/ mol of ML (4).

$$\frac{mmol}{\text{mol of ML}} = \frac{\left(\begin{matrix}peak\ area\\ in\ sample\end{matrix}\right) - Intercept}{Slope} X \frac{\text{D}}{\text{1000 X mol of ML}} \quad (4)$$

Where,
1000 = to convert µmol to mmol.
D = dilution factor.

3.5. Statistical Analysis

All sample analysis experiments were conducted in triplicate, and the mean was compared using variance analysis. A significance level of $p < 0.05$ was defined as being statistically different. All calculations were performed using XLSTAT Excel data analysis software (version 2020.1.3, Addinsoft, New York, NY).

4. Results

4.1. Conjugate Diene Analyses

4.1.1. Conjugated Diene of Samples at Freezer and Refrigerator Temperature

The ML control and L-proline added ML samples (1:5 and 1:10) exhibited identical UV spectra at 234 nm when stored at freezer and refrigerator temperature conditions. There was no change in the absorbance at 234 nm wavelength for five days in ML control, L-proline control, and samples containing L-proline with ML from initial to five days. The concentration of conjugated dienes (mmol/mol ML) was calculated for the samples of ML control and L-proline added in ML (1:5 and 1:10) under freezer and refrigerator conditions with variable time points and results presented in Table 4 and Table 5. Results indicated the samples stored at freezer and refrigerator temperatures did not show a significant change in the formation of conjugated dienes in the presence or absence of L-proline in ML and support use of these temperature conditions samples served as temperature controls for the study of the room and elevated temperature conditions samples.

Table 4. *Average conjugated dienes concentration (mmol/ mol ML) determined in samples stored at freezer temperature conditions.*[*]

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	5.15	6.97	6.98
1	6.05	6.15	6.16
2	6.32	6.00	7.44
3	5.76	5.89	7.41
4	6.43	6.55	7.36
5	6.87	6.03	6.50

[*]Table Footnote. The standard deviation of triplicate sample results was < 0.001.

Table 5. *Average conjugated dienes concentration (mmol/ mol ML) determined in samples stored at refrigerator temperature conditions.*[*]

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	5.15	6.97	6.98
1	6.15	7.04	6.38
2	6.20	6.74	7.34
3	6.01	6.70	7.36
4	6.84	7.00	7.29
5	6.87	6.72	7.76

[*]Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.1.2. Conjugated Diene of Samples at Room Temperature

The accumulation of conjugated dienes had an induction period of nearly two days. The formation of conjugated dienes in the samples gradually increased at room temperature. The highest amounts of conjugated dienes were found on the fifth day in control samples of ML, as well as in samples of ML and L-proline at ratios of 1:5. From the initial to the fifth day, the accumulation of conjugated dienes in samples of ML and ML: L-proline ratio 1:5 followed similar patterns, with a linear increase over the five days.

The sample with the methyl linoleate: proline ratio of 1:10 had a parallel pattern up to the second day, but the accumulation of conjugated dienes increased from the third day to the fifth day. However, on the fifth day, the UV spectrum of the methyl linoleate: proline:1:10 sample significantly changed, indicating almost double the formation of conjugated dienes compared to the fourth day. The average conjugated dienes concentrations (mmol/mol of ML) are presented in Table 6. No absorbance was observed at 234 nm for the proline control sample stored at room temperature.

Table 6. Average conjugated dienes concentration (mmol/ mol ML) determined in samples stored at room temperature. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	5.15	6.97	6.98
1	6.25	6.84	6.28
2	6.70	7.65	7.70
3	6.40	8.03	11.37
4	7.46	8.61	13.41
5	9.06	9.79	27.07

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.1.3. Conjugated Diene of Samples at 37 ℃

While the ML control sample was stored at 37 ℃, the UV absorbance at 234 nm increased from the first day. This indicates a double-bond conjugated pi-bond system formation in the ML control sample. On the third day, ML samples with L-proline ratios of 1:5 and 1:10 showed the highest formation of conjugated double bonds. However, the formation of conjugated double bonds decreased after the third day in samples of ML with L-proline ratios of 1:5 and 1:10. Interestingly, the decrease in the formation of conjugated double bonds was more significant in the ML: L-proline::1:10 sample than in the ML: L-proline::1:5 sample. There was no absorbance at 234 nm for the L-proline control sample stored at 37 ℃.

Table 7. Average conjugated dienes concentration (mmol/ mol ML) determined in samples stored at 37 ℃. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	5.15	6.97	6.98
1	7.41	8.76	14.96
2	12.08	60.82	103.38
3	33.43	307.38	319.17
4	125.31	304.01	202.11
5	291.38	200.43	157.48

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.1.4. Conjugated Diene of Samples at 65 ℃

The study found that ML and L-proline in ML samples (1:5 and 1:10) produced conjugated diene after one day at 65 ℃. This reaction occurred quickly without an induction period. However, the ML control samples showed a significant decrease in conjugated diene absorbance value after the first days at 65 ℃, which continued to decrease for up to five days. This indicated that the conjugated dienes system was unstable at 65 ℃ temperature after day one. On the other hand, ML: L-proline:1:5 and 1:10 samples reached a plateau after the first day and showed a minor decrease for days 4 and 5. This indicated that its concentration reached a stable point at lower levels after the incubation. From the second day, it declined in ML samples while accumulation plateaued in the L-proline present samples. The study also found no absorbance at 234 nm for the L-proline control sample stored at 65 ℃.

Table 8. Average conjugated dienes concentration (mmol/ mol ML) determined in samples stored at 65 ℃. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	5.15	6.97	6.98
1	87.62	118.12	117.88
2	24.66	104.04	116.99
3	21.26	111.82	113.54
4	17.01	101.07	99.10
5	18.86	109.43	111.55

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.2. Hydroperoxide Analyses

4.2.1. Hydroperoxide of Samples at Freezer and Refrigerator Temperature

Table 9. Average Hydroperoxide concentration (mmol/ mol ML) determined in samples stored at freezer temperature conditions. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.75	0.60	0.70
1	0.75	0.56	0.68
2	0.70	0.55	0.67
3	0.81	0.58	0.65
4	0.71	0.55	0.65
5	0.72	0.61	0.62

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

Table 10. *Average Hydroperoxide concentration (mmol/ mol ML) determined in samples stored at refrigerator temperature conditions.* [*]

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.75	0.60	0.70
1	0.80	0.57	0.73
2	0.75	0.59	0.67
3	0.78	0.54	0.68
4	0.75	0.57	0.68
5	0.76	0.62	0.71

[*]Table Footnote. The standard deviation of triplicate sample results was < 0.001.

The ML control and L-proline that were added in ML samples (1:5 and 1:10) did not result in a significant change in hydroperoxide amount from the initial to the end period, close container at freezer and refrigerator temperatures. The concentration of Hydroperoxide (mmol/mol ML) was calculated for the samples of ML control and L-proline added in ML (1:5 and 1:10) under freezer and refrigerator conditions with variable time points, and the results presented in Table 9 and Table 10. The results indicated that the samples stored at freezer and refrigerator temperatures did not show a significant change in hydroperoxide formation in the presence or absence of L-proline in ML. This finding underscores the crucial role of these temperature samples as temperature controls for the study of the room and elevated temperature samples, enhancing the reliability and validity of our research.

4.2.2. Hydroperoxide of Samples at Room Temperature

Table 11. *Average Hydroperoxide concentration (mmol/ mol ML) determined in samples stored at room temperature.* [*]

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.75	0.60	0.70
1	0.75	0.59	0.68
2	0.88	0.59	0.66
3	0.95	0.76	0.72
4	1.18	0.81	0.80
5	1.34	0.84	0.83

[*]Table Footnote. The standard deviation of triplicate sample results was < 0.001.

Initial to the fifth day, hydroperoxide (mmol/mol of ML) amounts in samples of ML control and proline added in ML samples are presented in Table 11. The accumulation of hydroperoxide had an induction period of nearly one day in ML control samples and two days in samples of ML and proline at ratios (1:5 and 1:10) stored at room temperature compared to samples stored at freezer and refrigerator temperatures. Hydroperoxide formation in the ML control samples gradually increased from the first day to the end of the study period at room temperature. Hydroperoxide formation in the samples with proline added gradually increased from the second day to the end of the study period at room temperature. The hydroperoxide amounts were higher in ML control samples compared to proline added in ML samples during the study period. The highest amounts of hydroperoxide were found on the fifth day in control samples of ML. The accumulation of hydroperoxide in samples of ML followed patterns, with a linear increase over the five days.

4.2.3. Hydroperoxide of Samples at 37 ℃

During the five-day experiment, the accumulation of hydroperoxide (mmol/mol of ML) was measured in ML samples and ML samples with added proline. The results, presented in Table 12, showed that the hydroperoxide amount increased in the ML control sample stored at 37 ℃ from the first day until the end of the experiment, similar to the pattern observed in the ML control samples stored at room temperature. However, the highest hydroperoxide amount in the ML control sample stored at 37 ℃ was lower than the ML control sample stored at room temperature. L-proline added in ML samples with mole ratios of 1:5 and 1:10 showed the highest formation of hydroperoxide bonds on the fourth and third days of the study, respectively. However, the hydroperoxide amounts decreased after the fourth and third days in ML samples with L-proline ratios of 1:5 and 1:10. Moreover, the highest hydroperoxide amount in the proline added in the ML sample stored at 37 ℃ was lower than the proline added in the ML sample stored at room temperature.

Table 12. *Average hydroperoxide concentration (mmol/ mol ML) determined in samples stored at 37 ℃.* [*]

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.75	0.60	0.70
1	0.74	0.62	0.66
2	0.82	0.58	0.77
3	1.03	0.65	0.79
4	1.05	0.73	0.68
5	1.19	0.53	0.69

[*]Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.2.4. Hydroperoxide of Samples at 65 ℃

Table 13. Average hydroperoxide concentration (mmol/ mol ML) determined in samples stored at 65 ℃. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.75	0.60	0.70
1	1.25	0.72	0.79
2	1.02	0.61	0.71
3	0.97	0.58	0.68
4	0.96	0.61	0.70
5	0.88	0.64	0.77

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

Hydroperoxide amounts (mmol/mol of ML) in samples of ML and proline added in ML samples were presented in Table 13. The study found that ML control and L-proline in ML samples (1:5 and 1:10) produced hydroperoxide on one day at 65 ℃. This reaction occurred quickly without an induction period. However, the ML control samples showed a decrease in hydroperoxide value after the first days at 65 ℃, which continued to decrease for up to five days. This indicated that the hydroperoxide system was unstable at 65 ℃ temperature after day one. On the other hand, ML: L-proline:1:5 and 1:10 samples reached a plateau after the first day and showed a minor decrease on the third day. This indicated that hydroperoxide concentration reached a stable point at lower levels after incubating in proline present samples.

4.3. Hexanal Analyses

4.3.1. Hexanal of Samples at Freezer and Refrigerator Temperature

Table 14. Average hexanal concentration (mmol/ mol ML) determined in samples stored at freezer temperature conditions. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.00	0.00	0.00
1	0.00	0.01	0.01
2	0.02	0.03	0.04
3	0.02	0.03	0.02
4	0.03	0.05	0.04
5	0.03	0.09	0.03

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

Table 15. Average hexanal concentration (mmol/ mol ML) determined in samples stored at refrigerator temperature conditions. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.00	0.00	0.00
1	0.01	0.04	0.03
2	0.05	0.05	0.04
3	0.02	0.06	0.06
4	0.02	0.06	0.06
5	0.06	0.05	0.07

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

The study found that when ML control and L-proline added ML samples (at ratios of 1:5 and 1:10) were stored at freezer and refrigerator temperatures, hexanal had no significant formation for five days. The hexanal concentration (mmol/mol ML) calculated for the samples of ML control and L-proline added in ML under freezer and refrigerator conditions with variable time points are presented in Table 14 and Table 15. This indicates that the samples stored at freezer and refrigerator temperatures did not show a significant formation of hexanal in the presence or absence of L-proline in ML. It indicated that these temperature samples served as temperature controls for the study of the room and elevated temperature samples.

4.3.2. Hexanal of Samples at Room Temperature

During the experiment, it was observed that the samples of ML control and L-proline added in ML samples stored at room temperature showed hexanal peaks comparable to samples stored at freezer and refrigerator temperatures. The formation of hexanal in the samples gradually increased marginally at room temperature. The highest amounts of hexanal were observed on the fourth day in the sample of ML and L-proline at a ratio of 1:10 and in the samples of ML and L-proline at a ratio of 1:5 than ML during a five-day study. From the initial to the fifth day, the accumulation of hexanal in samples of ML and ML: L-proline ratio 1:5 followed similar patterns, with a linear increase over the five days, results presented in Table 16. No peak interference

was observed at hexanal retention time in the L-proline control sample stored at room temperature.

Table 16. Average hexanal concentration (mmol/ mol ML) determined in samples stored at room temperature. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.00	0.00	0.00
1	0.02	0.07	0.07
2	0.02	0.10	0.18
3	0.04	0.09	0.27
4	0.05	0.16	0.36
5	0.05	0.14	0.22

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.3.3. Hexanal of Samples at 37 ℃

The study found that the level of hexanal (mmol/mol mL) increased more rapidly and to a greater extent in the sample stored at 37 ℃ than at room temperature. This increase was observed in both the ML control and the ML mixed with L-proline in ratios of 1:5 and 1:10. The ML control sample showed a significant increase in hexanal formation starting from the second day, which continued until the end of the experiment. The samples of ML with L-proline ratios of 1:5 showed the highest formation of hexanal on the fourth day, whereas samples of ML with L-proline ratios of 1:10 showed the highest formation of hexanal on the third day. However, the formation of hexanal marginally decreased after the fourth day in the sample of ML with L-proline 1:5 and after the third day in samples of ML with L-proline ratios of 1:10. The results are presented in Table 17. Still, there was no interference at hexanal peak retention time from the L-proline control sample stored at 37 ℃.

Table 17. Average hexanal concentration (mmol/ mol ML) determined in samples stored at 37 ℃ temperature. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.00	0.00	0.00
1	0.05	0.24	0.29
2	0.27	0.23	0.70
3	6.04	3.99	7.58
4	14.84	8.08	6.17
5	17.41	6.87	3.53

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

4.3.4. Hexanal of Samples at 65 ℃

Table 18. Average hexanal concentration (mmol/ mol ML) determined in samples stored at 65 ℃ temperature. *

Days	ML sample	ML:P::1:5 sample	ML:P::1:10 sample
0	0.00	0.00	0.00
1	14.62	1.38	0.71
2	10.87	0.27	0.15
3	12.61	0.37	0.39
4	7.52	0.41	0.40
5	4.77	0.44	0.41

*Table Footnote. The standard deviation of triplicate sample results was < 0.001.

The study found that ML and L-proline in ML samples (1:5 and 1:10) had hexanal formation on the first day at 65 ℃. This suggested that hexanal formation started quickly without an induction period at 65 ℃ temperature. However, in the ML control samples, the hexanal value decreased gradually after the first day. It continued to decrease through the end of the experiment. The results are presented in Table 18. On the other hand, samples of ML: L-proline:1:5 and 1:10 had a plateau from the second day sample and showed a minor decrease from the third day until the end of the experiment time. This indicated that the hexanal concentration reached a stable point at lower levels after the initial day of incubation. After the first day, it declined in ML control samples while the amount plateaued in the L-proline present samples. Furthermore, no chromatographic interference was observed at hexanal retention time from the L-proline control sample stored at 65 ℃.

5. Discussion

5.1. Conjugated Diene, Hydroperoxide, and Hexanal in Freezer and Refrigerator Samples

ML tends to exist as solids at freezer temperature

sufficiently below its melting point. The freezer and refrigerator temperatures impact the ML physical properties and attractive interaction between the molecules significantly changing by thermal behavior, density, and rheological properties [21, 22]. The physical state of ML at a particular temperature depends on its free energy, which is contributed by both enthalpy and entropy. At low temperatures, the enthalpy dominates the entropy term ($\Delta G = \Delta H - T\Delta S$, where $\Delta H > T\Delta S$), resulting in ML having more oxidative stability, less diffusion, and less interaction with L-proline in freezer and refrigerator conditions compared to elevated temperatures.

The ML primary and secondary oxidation products have a higher lag phase at the freezer and refrigerator temperature. Under the freezer and refrigerator conditions, it was observed that there was no formation of conjugated dienes, hydroperoxide, and hexanal in the control sample of ML, as well as in the samples of ML mixed with L-proline in ratios of 1:5 and 1:10. The interaction between L-proline and conjugated diene, hydroperoxide and hexanal interaction could not be assessed during in these conditions. This suggested that temperature played a crucial role in the interaction of L-proline and ML oxidation products and the formation of the ML oxidation products. It suggested that freezing and refrigerator temperature are proven to be the most effective methods for preserving and protecting from destructive changes in dry food that contains ML. It is important to consider these methods when storing dry food items to maintain their quality and nutritional value [22, 23].

5.2. Conjugated Diene, Hydroperoxide, and Hexanal in Room Temperature Samples

ML transitions from freezer to refrigerator to room temperature as the temperature increases from solid to liquid. At a specific temperature, the entropy ($T\Delta S$) dominates the enthalpy (ΔH), resulting in the liquid state having the lowest free energy ($\Delta G = \Delta H - T\Delta S$, where $T\Delta S > \Delta H$). This is significant because it indicates the system is endothermic, requiring energy to pull the molecules apart [22, 23]. The results of the samples at this temperature indicated that thermodynamic behavior initiated the formation of the ML oxidation products and the interaction between them and L-proline.

At room temperature, conjugated diene formation was observed in ML control and ML: L-proline::1:5 and 1:10, indicating the initiation of ML oxidation [24]. In lipid oxidation, conjugated diene is usually converted to the following oxidation product to continue the lipid oxidation reaction. When more L-proline was added to the sample of ML in a 1:10 ratio, it resulted in a higher formation of conjugated dienes at room temperature. This observation indicates that L-proline had the potential to interact with conjugated dienes radical and that a higher concentration of L-proline stabilized the structure of conjugated dienes (Figure 1 to Figure 3). Another possibility was that the free radicals of ML may have directly abstracted hydrogen from the L-proline before conjugated diene forms, leading to free radical scavenging (Figure 4) [24]. The L-proline directly interacts with the free radical or donates a proton to stabilize the conjugated diene structure, which can be evaluated using mass spectrometry [25]. Research suggested that L-proline has strong free radical scavenging properties, which could have been one way in which it interacted with the free radicals generated during the reaction.

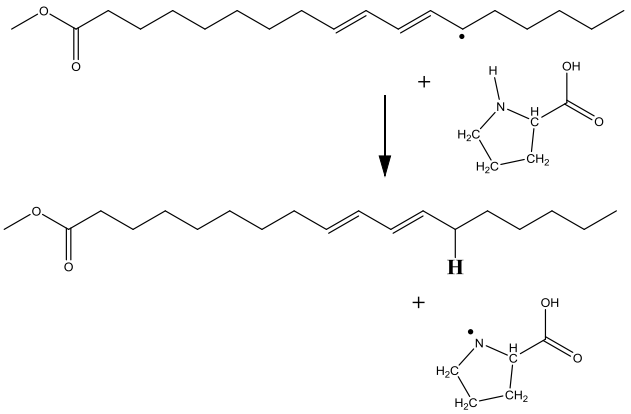

Figure 1. *Alternatively, the direct hydrogen abstraction by ML-conjugated diene radical might lead to a radical scavenging reaction.*

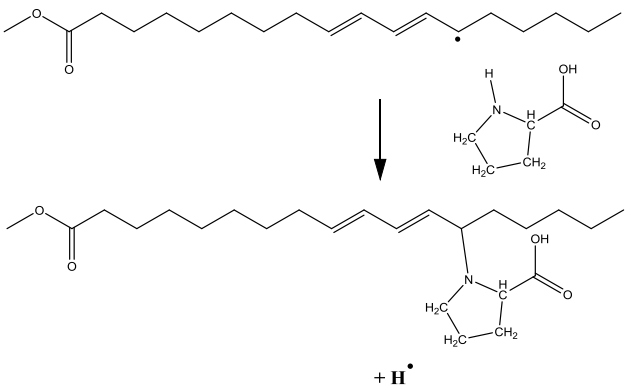

Figure 2. *Alternatively, the direct hydrogen abstraction by ML-conjugated diene radical might lead to a radical scavenging reaction and form an L-proline ML-conjugated diene complex.*

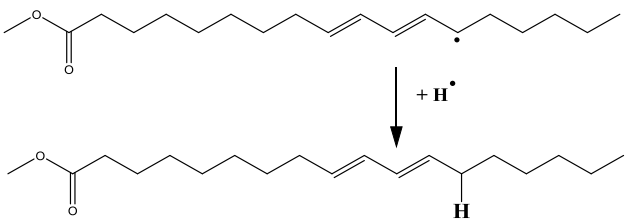

Figure 3. *Alternatively, the direct reaction with hydrogen radical and ML-conjugated diene radical leads to a radical scavenging reaction.*

ML oxidation depends upon how ML is distributed in the dry system and interacts with L-proline at room temperature. The ML hydroperoxide lag phase exhibited differences in dry model system samples of ML compared to the ML emulsion system and ML present in the meat [26, 27]. The formation of ML hydroperoxide could be reduced by scavenging the ML radical when L-proline was present in the samples. As a result, the L-proline in the ML:P:1:5 mole sample showed a lower amount of ML hydroperoxide than the ML control sample for five days at room temperature. However, increasing the amount of L-proline in the ML:P:1:10 mole sample did not reduce further ML hydroperoxide formation in the dry model system. This was because L-proline and ML were not easily diffusible in the dry model system, so their interaction was limited in very dry systems.

At room temperature, hexanal formation was detected in ML control and ML: L-proline::1:5 and 1:10, indicating the scission of ML oxide radical. When more L-proline was added in the sample of ML in a 1:10 ratio, it resulted in a marginally higher formation of hexanal at room temperature compared to ML control and ML: L-proline ratio:: 1:5. The hexanal incubation was slow, and the lag phase was longer. Further studies are needed to understand how more L-proline in the sample detects hexanal formation than the control ML and L-proline added in the ML 1:5 sample.

5.3. Conjugated Diene, Hydroperoxide, and Hexanal at 37 ℃

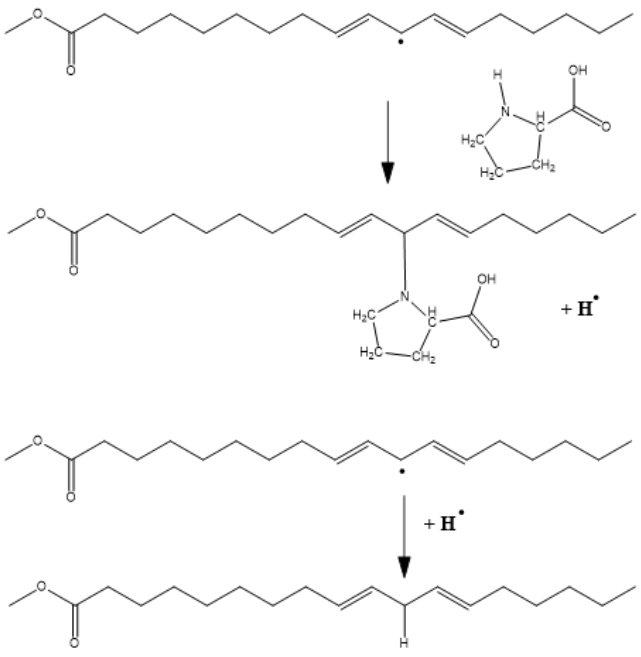

Figure 4. *A possible way in which L-proline could interact with the ML free radical formed before conjugated diene form and for the radical scavenging.*

The amount of conjugated diene (mmol/mol mL) increased at a higher rate and to a greater extent in the sample stored at 37 ℃ compared to the sample stored at room temperature. This increase was observed in the ML control and the ML mixed with L-proline in a ratio of 1:5 and 1:10. The observed increase in conjugated diene in the ML control was in line with the prediction made by the Arrhenius equation. The higher formation of conjugated diene (mmol/mol ML) in the presence of L-proline indicates that L-proline is responsible for the stabilized conjugated diene structure in the ML. The conjugated diene formation in the ML control sample can be converted to further oxidation products is typically converted into the next oxidation product, such as hydroperoxide, to propagate the oxidation chain reaction. A conjugated diene with a lower amount of found after the third day indicates distortion of a conjugated diene. It indicated that if L-proline stabilizes conjugated diene, it can stabilize conjugated up to a certain day limit. After the third day of stored samples at 37 ℃, it is also possible that L-proline scavenging activity may be lost, or ML and L-proline amount reduced in the samples or started to convert to other lipid oxidation products in the presence of L-proline or the reaction pathway may be changed (Figure 4). This could be why Arrhenius's relation was not established in ML and L-proline mix samples in a ratio of 1:5 and 1:10 at 37 ℃ after the third day [28].

For ML hydroperoxide: Temperature plays a crucial role in the decomposition of ML hydroperoxide. The reaction rate and hydroperoxide formation should also increase as the temperature increases. However, an interesting observation was made in the 37 ℃ sample, where hydroperoxide formation was lower than at room temperatures. This suggests hydroperoxide decomposition was also increased, occurring at a higher rate than hydroperoxide formation. Consequently, a lower remaining amount of hydroperoxide was found after decomposition. This underscores the importance of temperature in the decomposition process. If lipid hydroperoxide does not decompose, then no secondary oxidation product can form. A trace amount of transition metals, oxygen, light, and temperature was assumed to contribute to lipid hydroperoxide decomposition into secondary products or lipid oxide radicals in a dry model system. Lipid peroxide decomposes rapidly to form secondary products such as carbonyls, epoxides, alkanes, polymers, and other unidentified reactions that can transform ML hydroperoxide into an unknown product or ML peroxide radical reacts with L-proline and forms >N-OOL adducts [5, 12, 35]. Therefore, the detected ML peroxide level represents only the amount of ML peroxide remaining after the decomposition [5]. Prooxidants, such as transition metals like iron and copper, play a significant role in the decomposition of lipid peroxide [32]. These prooxidants were not readily diffused in dry model systems, limiting their interaction with lipid hydroperoxide [38]. However, the transition metals in a dry system were not hydrated, making them more reactive. This reactivity could lead to the quick decomposition of lipid hydroperoxide. The matrix in a dry system was also open and porous, enabling the prooxidant to flow freely and react with lipid hydroperoxide

[5]. On the other hand, in dry systems, the molecular sites of oxidation were exposed, making it more straightforward to reach ML. Lipid hydroperoxide was about ten times more reactive than hydrogen peroxide and is thus more likely to decompose [5, 32]. Moreover, superoxide anion (O_2^-) was formed by adding an additional electron to triplet oxygen. This anion could be transferred to a transition metal, causing its reduction. This redox cycling of transition metal could promote decomposing lipid hydroperoxide [32]. However, light promoted lipid hydroperoxide decomposition in a dry model system. Light exposure can also catalyze lipid hydroperoxide decomposition, generating free radicals [32, 33]. They could significantly increase the rate of generating free radicals such as alkoxyl and hydroxyl from lipid hydroperoxide [5, 32-34].

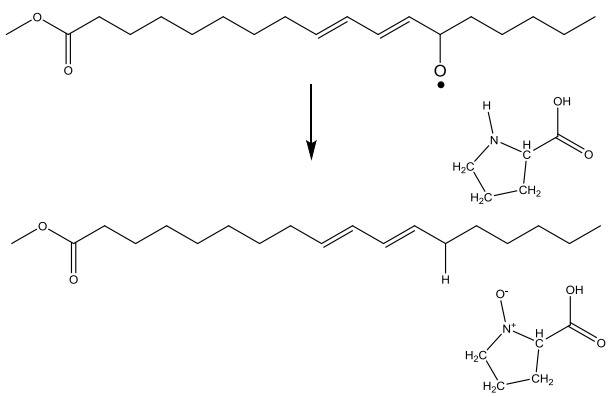

Figure 5. *A possible way in which L-proline could interact with the ML alkoxyl radical formed proline N-Oxide to prevent the scission of alkoxyl radical.*

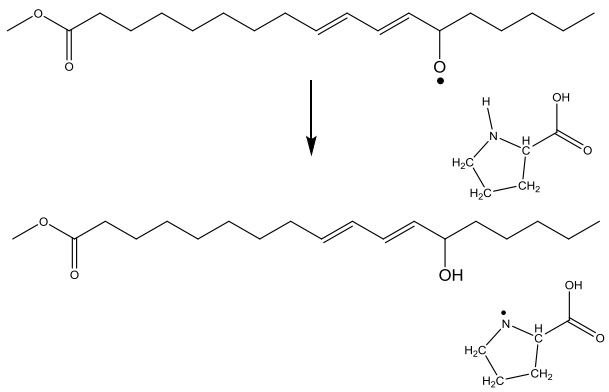

Figure 6. *A possible way in which L-proline could interact with the ML alkoxyl radical formed alcohol before scission reaction and for the hexanal.*

The hexanal compound is considered the secondary product in lipid oxidation. It is expected to be seen at the end of lipid oxidation as it forms after alkoxyl radical formation and undergoes a scission reaction. This scission reaction is energy intensive and increases at 37 ℃ compared to room temperature. The study found that hexanal formation began accumulating as early as two days during the lag phase at 37 ℃ in the ML samples with and without L-proline. This indicates a higher lipid oxidation rate in a dry model system [37]. The maximum hexanal formation was observed in the ML control sample stored at 37 ℃ on the fifth day. The observed increase in hexanal in the ML control was in line with the prediction made by the Arrhenius equation. The sample of ML with L-proline added at mole ratios of 1:5 and 1:10 showed a maximum amount of hexanal (mmol/mol ML) on the third and fourth day, which was about half the amount compared to the control sample of ML maximum amount of hexanal formation in the experimental. However, after that, the amount of hexanal started to decrease. This may indicate that L-proline is involved in co-oxidation with oxidized ML (Figure 5). L-proline can donate hydrogen to the alkoxyl radical, which reduces the scission of the alkoxyl radical without significant redistribution of molecular charge corresponding to small reorganization energies [36, 39]. The alkoxyl radical initiates the oxidation of L-proline via hydrogen abstraction from L-proline, and this process stabilizes the alkoxyl radical and converts it into alcohol (Figure 6). However, it does not stop the overall hexanal formation, as observed hexanal formation on the fourth and fifth days in L-proline-added samples. This indicates that the oxidation pathways were rerouted to other products (Figure 7). Further studies are needed to determine how scission of the alkoxyl radical decreases in the presence of L-proline and what structure forms to reduce scission of lipid oxide radical. The structure information can be achieved using the high-resolution mass spectrometry technique.

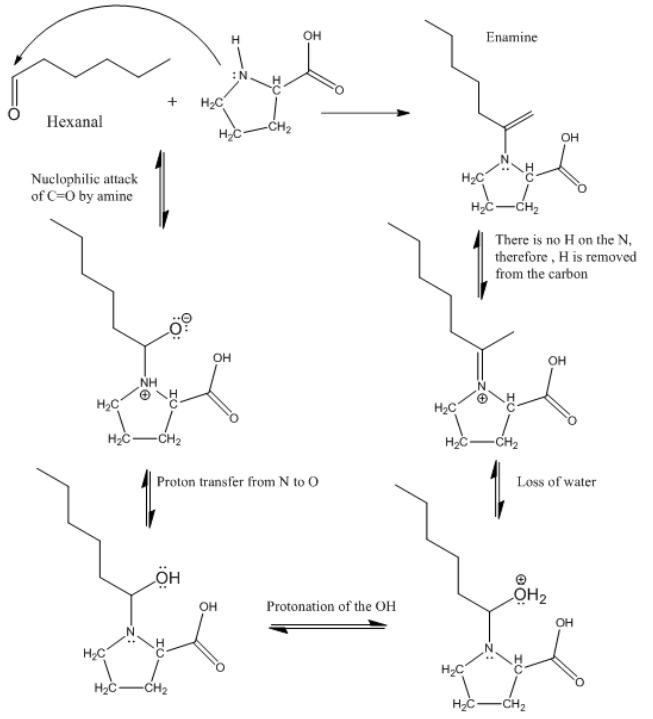

Figure 7. *A possible way in which L-proline could interact with the hexanal and formed enamine which impact on hexanal analytical results.*

5.4. Conjugated Diene, Hydroperoxide, and Hexanal at 65 ℃

The second day at 65 ℃, the ML control sample did not show the conjugated diene system at 234 nm. This suggested that the conjugated diene system was converted, and other lipid oxidation products were formed in the sample at 65 ℃ after the second day. In the experiments conducted on ML with L-proline in ratios of 1:5 and 1:10, it was observed that the samples at 65 ℃ showed stabilization of the conjugated diene system. However, the concentration of conjugated diene (measured in mmol/mol of ML) was lower than that at 37 ℃, indicating that L-proline ability reduced to stabilize conjugated diene formation by directly interacting with the free radical or donating a proton. As a result, conjugated diene was converted to an oxidation product that initiated an oxidation chain reaction. It is worth noting that the absorbance level recorded around 270 nm was approximately 0.22 AU in the sample containing ML and L-proline with mole ratios of 1:5 and 1:10. This indicated that additional conjugated or UV absorbance systems were formed at 65 ℃ in the presence of L-proline. The samples stored at 65 ℃ exhibited a rapid initial rate of oxidation, which led to a decrease in the induction period and eventually reached a lower accumulation and plateau. This suggested the unidentified reaction that transformed conjugated dienes, leading to increased transformation rates to ML oxidation products or unknown products, resulting in the decomposition of conjugated dienes. It was easier for initiation to occur at elevated temperatures in the presence of thermal energy, which increased oxidation. However, higher temperatures resulted in secondary transformations. Conjugated dienes could be found in peroxides, alkoxyl, and scission products of alkoxyl radicals, and they could be used to determine the oxidation level comparatively (Figure 8) [5].

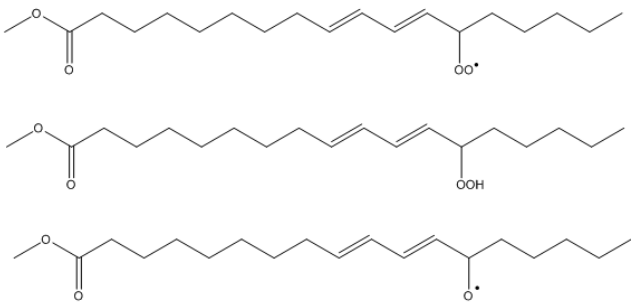

Figure 8. *A possible ML conjugated diene structure is maintained in the ML peroxide radical, ML hydroperoxide and ML alkoxyl radical.*

For ML hydroperoxide, the sample showed a plateau in lipid hydroperoxide amount after the first day for proline presence or absence in the samples at 65 ℃. It indicated the decomposition of the ML hydroperoxide rather than formation. The elevated temperatures promoted lipid hydroperoxide decomposition in a dry model system. It was worth noting that lipid hydroperoxide accumulation was typically not observed in frying oils because hydroperoxides quickly degraded upon formation [32, 38]. Furthermore, lipid peroxide formation depended on the availability of oxygen, which was present in the headspace of closed vials. Another source of oxygen was the trace amount already dissolved in the ML [20]. However, both sources provided limited oxygen amounts. Moreover, at elevated temperatures such as 65 ℃, the dissolved oxygen and the pressure of oxygen were reduced, which could explain the plateau observed in ML peroxide formation due to the equivalent amount of oxygen available in the samples in the closed container and dry system from the second day to the remaining study at 65 ℃. It also indicated that the L-proline could not provide a physical barrier to the ML hydroperoxide that might have influenced lipid oxidation at 65 ℃ [27, 38]. L-proline oxide and alcohol could be generated via a reaction between ML hydroperoxide and L-proline at elevated temperatures [12, 39]. That was why the ML: P::1:10 mole sample, where the L-proline amount was higher than ML: P::1:5, did not further reduce the decomposition of the ML hydroperoxide samples for a five-day lag phase at elevated temperatures. While the findings suggested that L-proline may not significantly prevent the breakdown of lipid hydroperoxide in a dry system, especially in the presence of prooxidants like transition metal, light, oxygen, and elevated temperature, they also highlighted the need for further research. Understanding the impact of L-proline in the dry model system on the formation or decomposition of lipid hydroperoxide was a crucial and complex process, as lipid oxidation mechanisms differed significantly in the dry model system due to the absence of an aqueous phase. The study findings and discussions were based on an in-depth analysis of samples using Fe(III)-xylenol redox reaction and ultraviolet detection and observed the sample results during a five-day lag phase. However, there was still much to be identified regarding the impact of L-proline on ML hydroperoxide in dry model systems. To further enhance the understanding of specific hydroperoxides and oxidation pathways, additional studies for about twenty days using advanced chromatographic techniques with UV and mass spectrometry are planned [26]. These efforts were expected to lead to innovative findings in lipid oxidation in the presence of L-proline in the dry and low-moisture food system.

The hexanal formation exhibited a rapid initial rate in the samples stored at a high temperature of 65 ℃. This led to a decrease in the induction period, the time taken for the hexanal formation to reach a certain level. On the first day, the maximum hexanal formation was observed in the ML control sample, which gradually reduced over time. This suggested that the hexanal amount was either reduced, converted to other small compounds of the lipid oxidation process, or formed enamine (Figure 8). In the experiments with L-proline added to ML in mole ratios of 1:5 and 1:10, the formation of

hexanal was significantly reduced by 9% and 5%, respectively, compared to the ML control on the first day. This indicates the potential of L-proline in mitigating hexanal formation, which could have significant implications for lipid oxidation in dry and low-moisture foods. L-proline interacts directly with the alkoxyl radical when introduced to oxidized ML at elevated temperatures. It donates hydrogen to stabilize the alkoxyl radical or forms an N-Oxide complex, thereby reducing scission (Figure 6) and hexanal formation [5, 35, 39]. This interaction with the alkoxyl radical is a key mechanism through which L-proline mitigates hexanal formation. Ultimately, L-proline leads to a lower accumulation and plateau of hexanal after the first day at 65 ℃. The chromatograms of the ML control sample revealed the presence of unknown peaks at 65 ℃. These peaks indicate the existence of additional, yet unidentified, products. This discovery is particularly exciting as it deepens our understanding of lipid oxidation and opens new avenues for research. Further investigation using techniques such as high-resolution mass spectrometry with Liquid chromatography and UV acquisition can provide more insight into the nature and significance of these unidentified peaks [26].

6. Conclusions

The research in this study focused on understanding low-moisture foods by systematically investigating L-proline and temperatures influencing ML oxidation in a dry system. The results showed that L-proline impacts ML oxidation product formations, such as conjugated diene, hydroperoxide, and hexanal, in the dry system and at various temperatures. L-proline was found to interact with oxidized methyl linoleate products, reducing the amount of oxidation product formation and altering the oxidation reaction path in the dry system. It acted as a free radical scavenger in a dry system, where ML undergoes high oxidation and limited diffusion occurs. L-proline stabilized ML-conjugated diene radicals, resulting in a higher amount of ML-conjugated diene at elevated temperatures. This reduced ML hydroperoxide formation and further ML oxidation reaction. L-proline was also found to stabilize the ML alkoxyl radical and reduce its decomposition. This resulted in a lower amount of hexanal in the ML oxidation in a dry system at elevated temperatures. Thus, we conclude that L-proline reduces ML oxidation in the dry system. The study opens up new avenues for exploration and understanding, inviting further engagement from the scientific community. We plan to conduct further studies on the co-oxidation of L-proline by oxidizing ML, unoxidized ML and L-proline, identify the unknown compounds and perform studies in low-moisture food matrices. Overall, this study provides an experimental basis for significantly enhancing our understanding of the reactions between L-proline and methyl linoleate oxidation products in dry and low-moisture foods, providing practical implications for the food industry and paving the way for future research.

Abbreviations

ML: Methyl Linoleate
P: L-Proline
DNPH: 2,4-Dinitrophenyl Hydrazine
DMF: Dimethyl Formamide
RP-HPLC: Reverse-Phase High-Pressure Liquid Chromatography
UV: Ultraviolet
M: Mole
mmol: Millimole
μmol: Micromole
nmole: Nanomole
μL: Microliter
t-BuOOH: Tert-Butyl Hydroperoxide
BHT: Butylated Hydroxytoluene
mg: Milligram
g: Gram
DNA: Deoxyribonucleic Acid

Acknowledgments

I appreciate Dr. Karen M. Schaich from the Department of Food Science and Nutritional Sciences at Rutgers, The State University of New Jersey, for providing technical guidance and sharing her research teamwork publication on the methyl linoleate oxidation study. Her contribution has been valuable to our research.

Author Contributions

Viral Shah: Conceptualization, Data curation, Software, Formal Analysis, Validation, Methodology, Writing – original draft

Gerald Buonopane: Conceptualization, Data curation, Resources, Supervision, Investigation, Project administration, Writing – review and editing, Methodology

Louis Fleck: Resources, Investigation, Writing – review & editing

Funding

This work is not supported by any external funding.

Data Availability Statement

The data is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Biography

Vial Shah is a part-time student pursuing further study in Dr. Buonopane's research group at Seton Hall University. He is a scientist in Intertek, Whitehouse, NJ, USA. He acquired his Master of Science in Organic Chemistry from Sardar Patel University, Gujarat, India. He has diversified work experience in the research area and experience in analytical method development, validation, and analysis of finished products, stability samples, and raw materials in the cGMP area. He has analytical instrumental skills: High-Performance Liquid Chromatography (HPLC), Gas Chromatography Flame Ionization (GC-FID), and Ultra-Violet (UV) spectrophotometer. Synthesis of small organic molecules identified them by NMR, IR, MS, HPLC, and another analytical method.

Reverend Gerald J. Buonopane, Ph.D. is a Senior Lecturer in the Department of Chemistry and Biochemistry at Seton Hall University. He acquired his Ph.D. at Pennsylvania State University and Master of Science at the University of Connecticut. Rev. Buonopane's area of specialization is food chemistry. Prior to seminary and the priesthood, Fr. Buonopane held several positions in academia, the federal government (USFDA), and the food and pharmaceutical industries. Among the courses Fr. Buonopane teaches and has taught are graduate-level courses in food chemistry, as well as Core curriculum courses, including a Core 3 course, "Science and Theology of Food." His research areas of interest are Chemical Deterioration of Food Lipids: Oxidative Reactions, Essential Oils as Natural Antioxidants, and Cold Plasma Treatment of Botanicals and Essential Oils. In addition to this work, Fr. Buonopane was appointed Minister to the Priest Community on June 1, 2020.

Louis Fleck is the Trace Organic Analytical Manager at Intertek Whitehouse, NJ, USA. He directs and coordinates lab operations activities and supports the development of proposals, protocols, and reports for extractable and leachable (E&L) projects. Louis has 20+ years of experience in contract laboratories performing and managing activities to directly support the pharmaceutical industry, which includes chromatographic method development and validation and E&L testing for container closure systems and finished pharmaceutical products.

Research Field

Viral Shah: Food lipid, Lipid oxidation, Food analyses, Food chemistry, Analytical chemistry, Liquid Chromatography, Gas chromatography Flame Ionization detectors, Liquid Chromatography-Mass Spectrometry, Gas Chromatography-Mass Spectrometry

Gerald J. Buonopane: Food chemistry, Food Analysis, Low-moisture food, Lipid Oxidation, Co-Oxidation of amino acids and proteins, Water activity in food, Cold plasma in Food

Louis Fleck: Trace Organic Analysis, Analytical method development, Extractable and Leachable, Liquid Chromatography-Mass Spectrometry, Gas Chromatography-Mass Spectrometry, Liquid Chromatography, Gas chromatography Flame Ionization detectors