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Milk processing-associated formation of Maillard reaction and lipid oxidation products during in vitro digestion

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Wien, 20.07.2026

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List of Abbreviations

AG	acylglycerol
AGEs	advanced glycation end products
ANOVA	analysis of variance
BGBL	Bundesgesetzblatt
CD-OH	hydroxy-conjugated dienes
CD-OOH	hydroperoxy-conjugated dienes
CEL	carboxyethyl-lysine
CML	carboxymethyl-lysine
DGs	diacylglycerols
e.g.	exempli gratia (for example)
EC	European Commission
EU	European Union
FA	fatty acid
FAMEs	fatty acid methyl esters
FID	flame ionization detector
GC	gas chromatography
GC-FID	gas chromatography with a flame ionization detector
GC-MS	gas chromatography coupled with mass spectrometry
G-H1	glyoxal-derived hydroimidazolone
GO	glyoxal
GOLD	glyoxal lysine dimer
HME	heptadecanoic acid methyl ester
5-HMF	5-hydroxymethylfurfural
9-HODE	9-hydroxyoctadecadienoic
13-HODE	13-hydroxyoctadecadienoic
HPLC	high performance liquid chromatography
9-HpODE	9-hydroperoxyoctadecadienoic
13-HpODE	13-hydroperoxyoctadecadienoic
HT	high-temperature pasteurization
LC	liquid chromatography
LC-MS	liquid chromatography coupled with mass spectrometry
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LOPs	lipid oxidation products
LOQ	limit of quantification
LOX	lipoxygenase
LTLT	long-term-low-temperature pasteurization
MeOX	methoxyamine hydrochloride
MFGM	milk fat globule membranes
MG-H1	methylglyoxal-derived hydroimidazolone
MGO	methylglyoxal
MGs	monoacylglycerols
MOLD	methylglyoxal lysine dimer
MR	Maillard reaction
MRM	multiple reaction monitoring
MRPs	Maillard reaction products
MS	mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MUFAs	monounsaturated fatty acids
NF- κ B	nuclear factor “kappa-light-chain-enhancer” of activated B-cells
NME	nonadecanoic acid methyl ester
NMR	nuclear magnetic resonance
oxDGs	oxidized diacylglycerols
oxFAs	oxidized free fatty acids
oxMGs	oxidized monoacylglycerols
oxTGs	oxidized triacylglycerols

PBDs	plant-based drinks/beverages
PBMAs	plant-based milk alternatives
PCA	principal component analysis
PTFE	polytetrafluoroethylene
PUFAs	polyunsaturated fatty acids
PVDF	polyvinylidene fluoride
PVF	polyvinylfluoride
QC	quality control
QqQ	triple-quadrupole
QTOF	quadrupole time-of-flight
RAGE	receptor for advanced glycation endproducts
RGE	rabbit gastric extract
ROS	reactive oxygen species
SD	standard deviation
sens	photosensitizer
SFAs	saturated fatty acids
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SPE	solid-phase extraction
SSF	simulated salivary fluid
TCA	trichloroacetic acid
TGs	triacylglycerols
TMS	tetramethylsilane
UFAs	unsaturated fatty acids
UHPLC-HRMS/MS	ultra-high performance liquid chromatography coupled with high -resolution tandem mass spectrometry
UHT	ultra-high-temperature pasteurization
α -DCs	α -dicarbonyls

Hilfsmittel

Bei der Erstellung dieser Masterarbeit wurden Werkzeuge der künstlichen Intelligenz (KI) unterstützend eingesetzt. Konkret wurden DeepL, ChatGPT und Gemini für die Übersetzung, die Formulierungshilfe sowie die sprachliche Korrektur genutzt. Die Generierung von Kerninhalten, die Datenanalyse und die wissenschaftliche Schlussfolgerung erfolgten ohne Einsatz von KI.

KI-Hilfsmittel	Firma	Einsatz
DeepL	DeepL SE	Übersetzung von wissenschaftlicher Literatur
ChatGPT (Version: 2026)	OpenAI	Lesbarkeit und Sprache der Masterarbeit
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1 Introduction

Milk is consumed worldwide as a beverage. It is a fluid produced by the mammary glands of female mammals. Depending on the mammal, different types of milk are distinguished, such as goat milk, sheep milk, mare milk, or cow milk. The latter is the most consumed type of milk.^{1,2} As a combination of water, carbohydrates, lipids, proteins, and minerals milk is an important component of the human diet.³

To protect the consumer from pathogenic microorganisms and to extend shelf life, milk must undergo several processing methods, such as heat treatments. The most common heat treatments used during milk processing are long term low temperature pasteurization, high temperature pasteurization and ultra-high temperature pasteurization.¹

In Austria, the processing of cow milk requires strict hygiene standards to prevent microbial spoilage and nutrition-related diseases.⁴

In recent years, consumer behavior has shifted towards plant-based milk alternatives (PBMA). In the European Union, they are legally referred to as plant-based beverages (PBDs).⁵ They are water-based extracts from various plant sources such as grains (oats), legumes (soy), and nuts (almonds). These beverages are becoming increasingly popular due to their environmental sustainability and animal welfare concerns. Moreover, these drinks provide options for people who are lactose intolerant or possess an allergy to milk proteins.⁶ Among plant-based milk alternatives, soy drinks are one of the most frequently consumed drinks.⁷

The professional production of soy drinks is governed by the EU-wide regulations of Regulation (EC) No. 1308/2013.⁸ To prevent spoilage and microbial growth on soybeans, strict hygiene measures and processing methods must be observed, as it occurs with milk.⁹

During milk and soy drink processing a chemical reaction, known as the Maillard Reaction (MR) takes place.^{7,10} This reaction is known as a non-enzymatic browning reaction between the amino group of proteins and the carbonyl group of reducing sugars under high temperatures. Maillard reaction products lead to a browning effect, and they are responsible for a variety of flavors and odors.^{2,10} By using these food processing techniques, not only Maillard reaction products (MRPs) but also lipid oxidation products (LOPs) can be generated.^{11,12}

Certain types of these products can have negative effects on our body. They can be harmful, causing inflammatory processes and cell damage.¹³

Likewise, it is known that human digestion also influences the chemical reactions behind these compounds. Human digestion takes place in three locations of the body: oral cavity, stomach and intestine. Moreover, enzymes, pH-value and temperature have a deep impact on digestion. Thus, simulated gastro-intestinal digestion methods are employed in many fields of nutritional sciences to evaluate the gastro-intestinal behavior of food.¹⁴ However, the extent to which different heat processing techniques used for milk affect the amount of these products during digestion is still unclear. To help closing this gap, this master's thesis investigates the occurrence of MRPs and LOPs in three different heat-treated milk types and one plant-based alternative during in vitro digestion.

2 Literature review

2.1 Milk

2.1.1 Composition

Whole milk is an opaque liquid and contains important nutrients. It is a complex mixture of various components, typically consisting of 87.1% water, 4.8% lactose, 3.5% fat, 3.4% proteins, 0.7% minerals and 0.5% other components (e.g. vitamins, enzymes).² In figure 1 the composition of milk is presented as a pie chart.

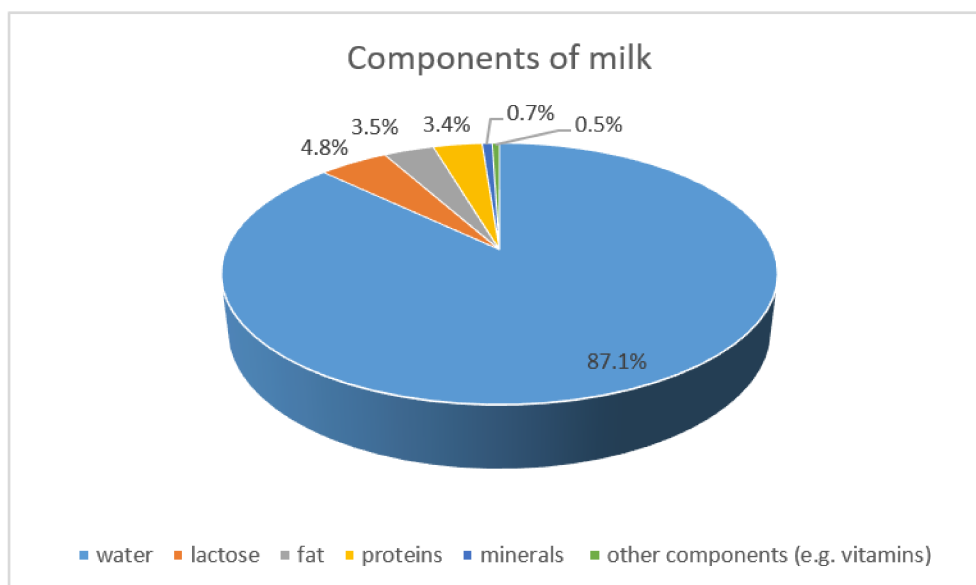


Figure 1: Components of milk (data according to Xiang et al., 2021)²

2.1.1.1 Proteins

Milk is known for its excellent amino acid profile and is considered as one of the highest quality protein sources for humans. Its most important proteins are casein and whey proteins (lactalbumin and lactoglobulin).¹⁵ Casein and whey protein make up approximately 80% (29.5 g/L) and 20% (6.3 g/L) of the total protein in cow milk, respectively. Casein is a phosphate-conjugated protein consisting primarily of calcium phosphate micelle complexes. It forms a heterogeneous family of four main components: α -casein, β -casein, γ -casein and κ -casein. One of its characteristic properties is its insolubility in the weakly acidic environment (pH 4.6 and 20°C). Under these conditions, it precipitates. Likewise, the main components of whey proteins are α -lactalbumin and β -lactoglobulins A and B. They are considered proteins with high biological value and are rich in essential amino acids.¹ In fact, milk contains all essential amino acids that cannot be produced by the body itself. The most important amino acids in milk protein (casein and whey) are lysine, leucine, isoleucine and threonine.

¹ Specifically, milk is well known as a very good source of lysine, which is only present in small amounts in many plant proteins. Lysine, along with arginine, is one of the most reactive amino acids due to its free amino groups. It reacts particularly readily with reducing sugars, such as glucose,

fructose, and lactose, thereby promoting the Maillard reaction. Heat treatments such as those involved in milk processing can reduce the amount of available lysine in milk, which leads to a loss of bioavailability.¹⁰

2.1.1.2 Carbohydrates

Carbohydrates in milk are present in the form of lactose. Lactose is a disaccharide generated from one molecule of glucose and one molecule of galactose linked by a 1,4- β -glycosidic bond. It can be further split up into glucose and galactose monosaccharides by the enzyme lactase.¹

In aqueous solution, the glucose molecule bound to galactose can be converted into a chain form and then re-establish itself as a ring. α -Glucose can then become β -glucose in a so-called mutarotation. In raw milk, α -lactose and β -lactose exist approximately in equilibrium with each other in this ratio. The ratio of the two anomers is temperature-dependent; the β -form decreases with increasing temperature.¹ The structure of lactose is shown in figure 2.

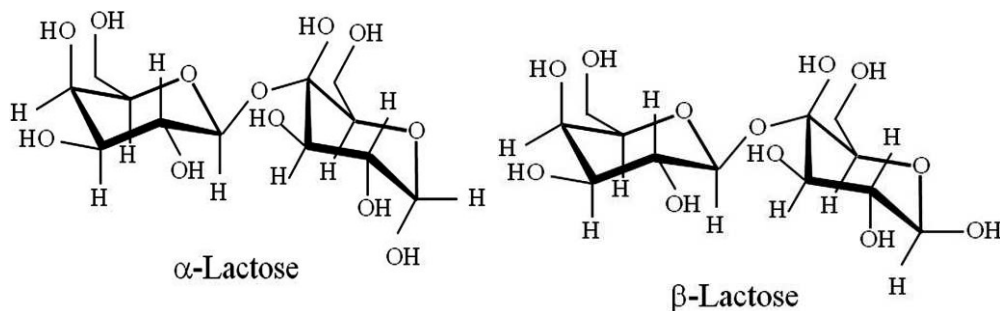


Figure 2: Molecular structure of α -Lactose and β -Lactose (according to Yamauchi et al., 2013)¹⁶

Lactose is part of the group of reducing sugars, which include all monosaccharides, as well as other disaccharides such as maltose. They are carbohydrates that, due to a free aldehyde or keto group, donate electrons in aqueous solution and thereby they are capable to reduce other compounds. In the hemiacetal structure, reducing sugars (e.g. lactose) possess a free anomeric hydroxyl group (OH-group), which allows a ring opening in solution to react with other components. Due to its reducing properties, lactose plays an important role in the Maillard reaction. This reaction leads to changes in odor and taste when milk is heated.^{2,10}

2.1.1.3 Fats

Milk fat mostly consists of 99.8% triacylglycerols and 0.2% phospholipids.^{12,17}

Milk fat has a relatively high content of esterified saturated fatty acids (SFAs) and a low content of unsaturated fatty acids (UFAs). According to Belitz et al. (2008) the most common fatty acids in milk are presented in table 1.

Table 1: Fatty acids in milk fat (according to Belitz et al. (2008)) ¹

Fatty acid	Number of carbon atoms: double bonds	Weight in percentage (%)
Butyric acid	4:0	2.79
Caproic acid	6:0	2.34
Caprylic acid	8:0	1.06
Capric acid	10:0	3.04
Lauric acid	12:0	2.87
Myristic acid	14:0	8.94
Palmitic acid	16:0	23.80
Stearic acid	18:0	13.20
Oleic acid	18:1	25.50
Linoleic acid	18:2	2.11
Linolenic acid	18:3	0.38

Saturated fatty acids (SFAs), especially palmitic acid and stearic acid dominate in milk fat. Among the monounsaturated fatty acids (MUFAs) oleic acid is predominant. Polyunsaturated fatty acids (PUFAs) such as linoleic acid and linolenic acid are present only in small amounts. ¹ The composition of fatty acids, however, can be variable and depends on the cow's diet. For instance, grazing increases the proportion of polyunsaturated fatty acids such as omega-3 fatty acids. ¹⁸ These lipids, however, are subject to oxidation processes in milk, largely driven by external and internal factors, such as light, oxygen, temperature, time and iron. This can lead to rancid or-, metallic off-flavors, thereby reducing the shelf life of dairy products. ¹⁹

Milk exists in the form of an oil-in-water emulsion. Milk fat is present in milk in the form of tiny droplets known as globules. These are surrounded by a coating called the milk fat globule membrane. Structurally, the milk fat globule membrane (MFGM) is a complex protein-lipid trilayer. It consists of an inner phospholipid monolayer flanking the triacylglycerol core, a narrow intermediate protein layer, and an outer lipid bilayer that interfaces with the continuous aqueous phase. Polar lipids, including sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, constitute the structural framework of the membrane.²⁰ During the heat treatment of milk (such as ultra-high temperature processing), structural, chemical, and physical changes occur in the milk fat globules (MFG) and their protective membrane (MFGM). Protein denaturation and membrane damage take place. The more intense the heat, the more unstable the membrane becomes.

Consequently, polar membrane lipids (such as sphingomyelin and phosphatidylcholine) and native membrane proteins detach from the MFGM and diffuse into the surrounding aqueous phase of the milk. The compromised and damaged membrane leads to the accumulation of fat globules, resulting in an increase in their average diameter. ²¹

2.1.1.4 Other components

Minerals abundantly found in milk are potassium, calcium, phosphate, and chloride.²² Particularly, calcium and phosphorus are responsible for the bone matrix and provide stability. In milk, both minerals are present in a bioavailable form, ensuring ideal absorption for bone formation in the body. Likewise, notable vitamins in milk are vitamin B₂ (Riboflavin), which acts as a sensitizer in the photo-oxidation of lipids and vitamin E (tocopherol), which is present in milk fat globule membranes (MFGM).^{23,24}

2.1.2 Milk processing

Due to hygienic and safety reasons, milk must be processed before it enters the food trade. First, raw milk is transported to the dairy in refrigerated trucks. In the dairy facilities the raw milk is put into a clarifier to remove sediment and impurities. After this step, milk is cooled and stored in refrigerated bulk tanks to prevent bacterial growth. The next step is the fat standardization, in which the desired fat content is set. Then, the crucial step of pasteurization takes place, which is a food preservation technique that involves applying heat to milk. After homogenization, milk is cooled back down to ~ 4° C and packaged into sterile cartons, pouches, or bottles.²⁵ In figure 3 these main steps of milk processing are presented.

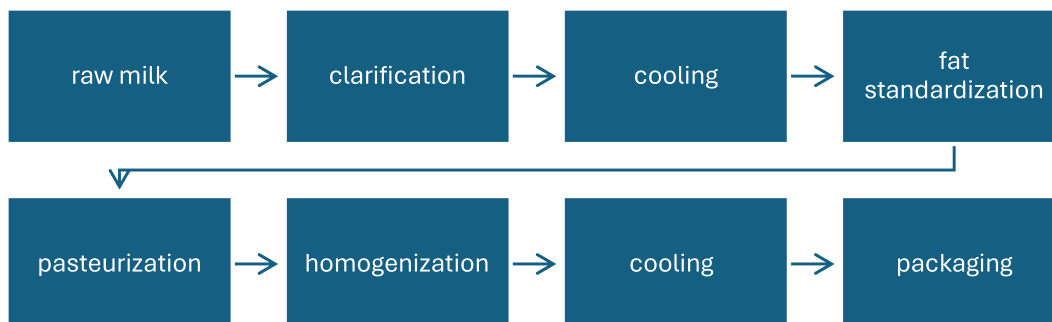


Figure 3: Milk processing (adapted from Brush et al., 2011)²⁵

As stated above, one of the key steps in milk processing is pasteurization, where raw milk is thermally treated, not only with the primary goal of eliminating pathogenic microorganism but also extending shelf life during storage.^{2,10} According to Belitz et al. (2008), the most common thermal treatments in milk processing currently used are:

- Long-Term-Low-Temperature pasteurization (LTLT): It takes place between temperatures from 63° to 66° C, for 30-32 minutes.
- High-temperature pasteurization (HT): It takes place by 85° C, for 2-3 seconds.
- Ultra-high-temperature pasteurization (UHT): This thermal treatment is carried out between temperatures from 136-138° C, for 5-8 seconds.¹

Table 2 gives an overview about these thermal processing methods.

Table 2: Types of milk processing ¹

Long-term-low-temperature pasteurization (LTLT)	High-temperature pasteurization (HT)	Ultra-high-temperature pasteurization (UHT)
63° to 66° C	85° C	136-138° C
for 30-32 minutes	for 2-3 seconds	for 5-8 seconds

However, the essential heat treatment during milk processing can cause detrimental changes in several of its components, like the degradation of lysine, as mentioned above. In addition, other chemical reactions, such as the Maillard reaction, occur. Furthermore, it is considered that lipid oxidation also takes place during the pasteurization process. ^{12,26} These two reactions, as a consequence, generate different flavors and odors in milk. ^{2,15}

In summary, heat treatment of milk is a necessary step in production process, but it leads to a loss partial of nutrients and a reduction in nutritional value. ^{2,10}

2.2 Soy drink

The plant-based beverage market is recently becoming more popular and the demand for plant-based milk alternatives is increasing. The consumer preferences have changed over the years for many different reasons. Searching for a sustainable diet, ethical reasons, animal welfare, lactose intolerance and a vegetarian or vegan diet are just some of the reasons why more people choose to replace cow milk with plant-based beverages. Commonly used plants and pulses for these drinks include almonds, hazelnuts, soybeans, rice, and coconut. Among plant-based beverages, soy drinks are the most popular and widely consumed. ^{9,27}

According to Olias et al., 2023 soy drink is an aqueous extract and consists of approximately 91.5% water. Further components are 3.4% protein, 3.1% carbohydrates (of which 0.6% fiber), 1.9% fat and 0.1% salt. The macro-nutrient composition of soy drink is presented in figure 4.

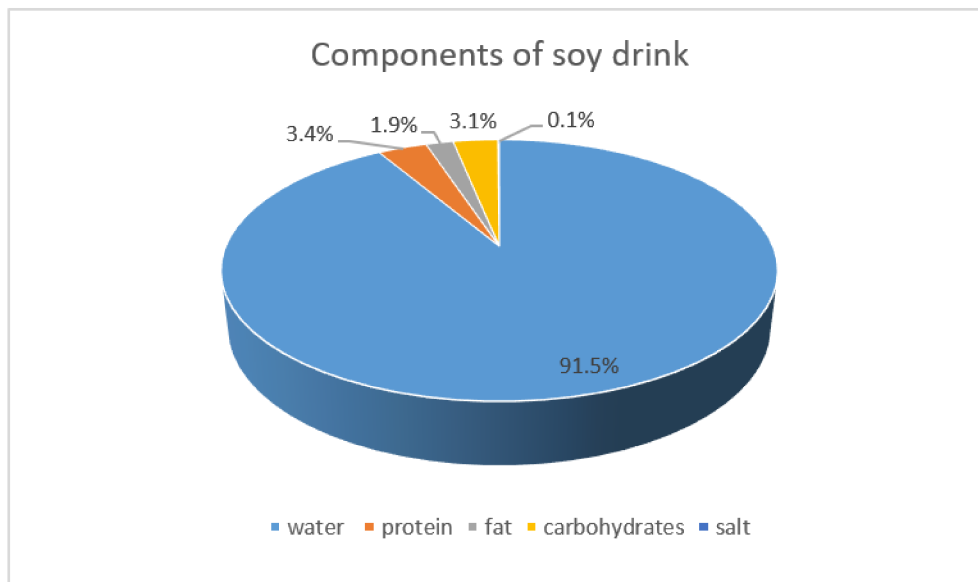


Figure 4: Components of soy drink (data according to Olias et al., 2023) ⁹

Regarding the fatty acid profile, predominantly polyunsaturated fatty acids (PUFAs) are present. Among them linoleic acid is most abundant in soy milk, followed by α -linolenic acid, oleic acid, palmitic acid and stearic acid. ^{9,28}

Contrary to the high content of carbohydrates in soybeans, the amount of carbohydrates in soy drinks is low. ⁹ Furthermore, soy beverages contain no lactose. Their sugar composition shows that the soluble sugars consist primarily of sucrose (46.8%), stachyose (31.7%), and raffinose (8.3%). All three are non-reducing sugars, while reducing sugars, such as glucose and fructose, are only present in smaller amounts (9.8%). ²⁹

Moreover, soy drinks consist of proteins that are obtained directly during the extraction of soybeans, from which glycinin represents approximately 80%. Regarding the amino acid profile, it is quantitatively similar to that of cow milk but differs in the quantity of certain amino acids. For instance, glycine and arginine are more abundant in soy drinks than in cow milk. ³⁰ In addition to storage proteins, soy drinks contain various albumins, lipoxygenase (LOX) and β -amylase. ⁹

To ensure product safety, prevent microbial growth and extend shelf life, the heat treatment of soy drinks is fundamentally necessary. ^{7,31}

The industrial production of soy beverages takes place in several steps. It starts with soaking in water, draining, blanching or cooking, wet milling and filtration. A colloidal suspension is created consisting of proteins, oils, and carbohydrates. In a further step many PBMA are fortified with calcium, vitamin B12, and riboflavin to reach levels comparable to cow milk. The final step involves heat treatment, homogenization, and sterile packaging. Figure 5 describes the production process of soy beverages. The graphic is taken from Olias et al., 2023 with minor modifications.

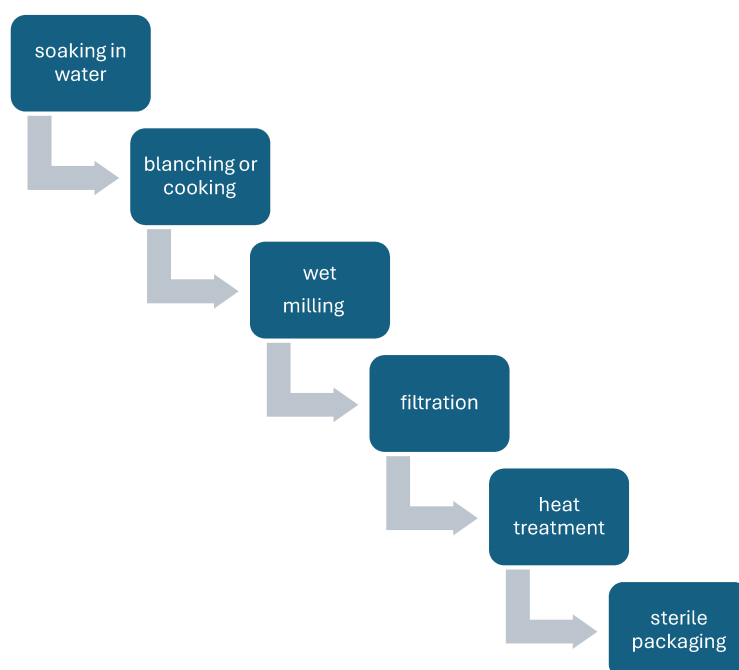


Figure 5: Main steps of soy beverage production (according to Olias et al., 2023) ⁹

During this manufacturing process, chemical reactions, such as Maillard reaction and lipid oxidation, occur. Because soy drinks consist mainly of unsaturated fatty acids, lipid oxidation is one of the most prominent chemical reactions during processing. ^{7,9}

Among the various types of lipid oxidation mechanisms, enzymatic oxidation is the most common type of oxidation in soy beverages, due to the high content of the enzyme lipoxygenase (LOX). The enzyme is responsible for off-flavors and odors and leads to a loss of quality. To avoid the enzymatic oxidation in soy drinks, soybeans are milled at high temperatures. This step leads to an inactivation of the enzyme. ^{9,32} In addition to enzymatic oxidation, the autooxidation of lipids, triggered by heat, can also occur during this process. Moreover, the Maillard reaction between proteins and reducing sugars is induced by thermal treatment. ⁷

2.3 Maillard Reaction

As mentioned above, during milk processing a reaction called Maillard reaction (MR) occurs, which is known as a non-enzymatic browning reaction that takes place between reducing sugars and amino acids. It is a common chemical process in foods that undergo thermal processing. ^{10,33}

It was discovered in 1912 by Louis Camille Maillard. The French chemist noticed browning effects on heated substances containing sugars and amino acids. It is well researched and widely used in food industry. However, the Maillard reaction and its products not only generate browning effects, but they are also responsible for an attractive variety of flavors and aroma compounds, such as aldehydes and furans. ¹⁰

Moreover, Maillard reaction products improve shelf life, appearance, and the taste of foods. Therefore, Maillard reactions and their products play an important role in food production, processing, and storage.^{10,33}

In contrast, Maillard reactions are also associated with numerous side effects such as quality down-grade and changes in taste, which can be sensed in the food. Furthermore, recent studies have claimed associations between dietary intake of Maillard reaction products and few diseases, like diabetes and arteriosclerosis.^{13,33}

2.3.1 Mechanism and products

The reaction cascade of the Maillard reaction can be structured into three stages: initial stage, intermediate stage, and advanced stage.^{33,34} Figure 6 shows a summarized version of this mechanism.

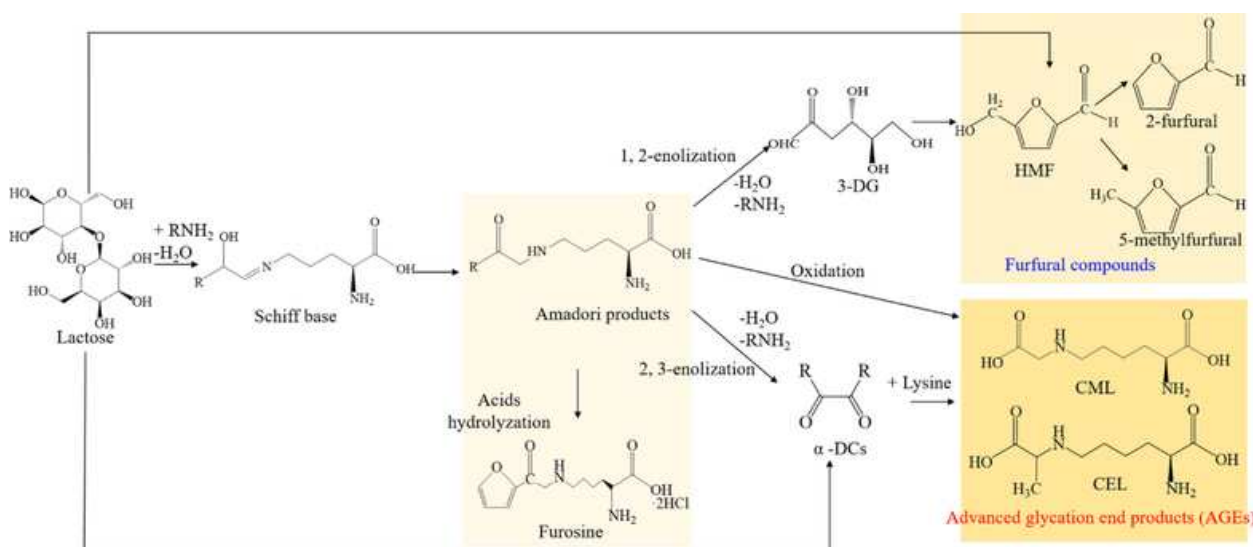


Figure 6: Simplified mechanism of the Maillard reaction (based on Li et al., 2022)³⁵

2.3.1.1 Initial stage

The initial stage takes place through a reaction between a carbonyl group (typically an aldehyde or ketone group) of a reducing sugar and an α -amino group in amino acids (mainly lysine and/or arginine), from which an unstable Schiff base is formed. The formation of this initial product is reversible and undergoes further transformations.^{7,10,33}

The Schiff base goes through double bond migrations and rearrangements, leading to the formation of an Amadori product.⁷ It is also known as an early glycation product. Amadori products have more stable ketoamine groups, but the reaction is still reversible.^{10,13}

2.3.1.2 Intermediate stage

The intermediate stage of the Maillard reaction is responsible for further conversion and degradation of Amadori products. A series of reactions such as dehydration, elimination, fragmentation, condensations and deamination, take place. These processes are mainly influenced by temperature and pH-value. At a pH-value greater than seven (pH > 7) the formation α -dicarbonyl compounds via 2,3-

enolization is favored. At a pH-value less than or equal to seven ($\text{pH} \leq 7$), the Amadori products are converted to furfural or hydroxymethylfurfural (HMF) via 1,2-enolization.^{7,33}

During this stage reactive intermediates are predominantly formed, such as the aforementioned α -dicarbonyl compounds and furanic compounds, via pathways such as the oxidative pathway. Some of the most common are glyoxal (GO) and methylglyoxal (MGO). Other ways in which these two Maillard reaction intermediates can be formed include the breakdown of glucose, the fragmentation of Schiff bases or lipid peroxidation.³⁶ Moreover, the reaction between α -dicarbonyl compounds and amino acids leads to the formation of Strecker aldehydes, which are associated with the characteristic aroma and flavor of MRPs.¹⁰ Figure 7 displays the structures of glyoxal and methylglyoxal.

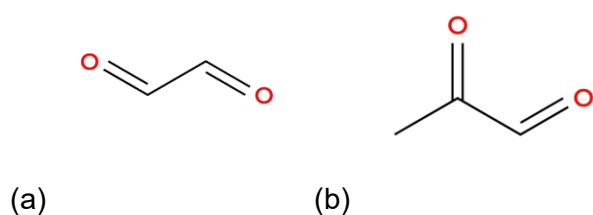


Figure 7: Chemical structure of a) glyoxal and b) methylglyoxal (self-drawn with Molview)

2.3.1.3 Advanced stage

In the advanced stage, Amadori products and subsequently formed products undergo various reactions including oxidation, condensation, cyclization, fragmentation, dehydration and rearrangement.^{33,34}

During this stage of the Maillard reaction advanced glycation end products (AGEs) and melanoidins are generated. The latter are high molecular weight polymers, responsible for the brown color in food products. The formation of both proceeds slowly and is irreversible.¹³

Advanced glycation end products (AGEs) are the other diverse group of compounds that are formed during the late stage of the Maillard reaction. They possess different molecular structures, molecular weights, and matrix-binding states. Moreover, they can accumulate and form crosslinked proteins. Therefore, AGEs can be classified into two categories based on their protein binding state: free AGEs and protein-bound AGEs. Both require different analytical strategies for their determination.³⁷ Among the most frequently identified AGEs are carboxymethyl-lysine (CML) and carboxyethyl-lysine (CEL).³⁸ The structures of CML and CEL are presented in figure 8.

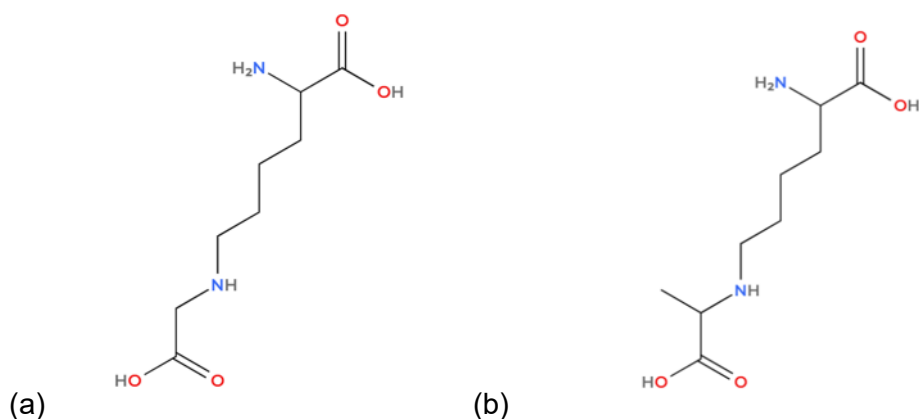


Figure 8: Chemical structure of (a) CML and (b) CEL (self-drawn with Molview)

Furthermore, at the advanced stage, α -dicarbonyl intermediates interact with amino acids of milk proteins (primarily lysine and arginine). The reaction between lysine and α -dicarbonyl intermediates leads to the formation of methylglyoxal lysine dimer (MOLD) and glyoxal lysine dimer (GOLD), which are typical representatives of protein-bound AGEs.

The interaction between arginine and α -dicarbonyl intermediates leads to the formation of imidazolones, such as glyoxal and methylglyoxal derived hydroimidazolones (G-H1, MG-H1) and pyrimidines. These substances are very stable and can further interfere with proteins to form crosslinked AGEs.^{10,13}

Advanced glycation end products (AGEs) are widely distributed in processed foods, but also in biological tissues. Some of them are regarded as harmful compounds formed during thermal processing or storage. Dietary AGEs contribute to the body's circulatory pool of AGEs. Increased AGE levels are associated with inflammation, oxidative stress, cardiovascular diseases and metabolic disorders, such as diabetes.^{13,37}

2.3.2 Influence of different parameters

Key parameters influencing the formation of Maillard reaction products in food are pH-value, temperature, reaction time, water activity and reactant concentration. In the case of milk processing, the pH-value and temperature are particularly important.¹⁰

The pH-value influences the reaction rate, the activity of the reactants and the formation of the intermediates for the different products. Under acidic conditions, the Maillard reaction runs slower due to protonation of the amino groups and the loss of nucleophilicity. Furthermore, fewer melanoidins are generated. Under alkaline conditions, the opposite takes place. Therefore, controlling the pH-value is an important parameter during food processing.¹⁰

It is generally known that temperature has a deep impact on milk processing. Harsher processing conditions generally lead to higher levels of AGEs modifications in proteins, reducing nutritional value, as essential amino acids, particularly lysine, are made unavailable for the body to absorb.¹⁰ Moreover, the molecular cross-linking of AGEs, that occur during the reaction, hinders nutrient breakdown by the digestive enzymes.³⁹

High-temperature treatments in milk increase AGE levels. Xie et al. (2024) demonstrated that the concentration of AGEs correlates with the temperature applied in milk treatments.⁴⁰ In another study by Li et al. (2022), it was shown that two key AGE markers, CML and CEL, exhibited a marked increase in UHT milk.³⁵

Increasing AGEs might be of concern due to their associated health-related issue, thereby making it relevant to consider the temperature in which milk processing techniques are carried out.^{10,13}

2.4 Lipid oxidation

In milk and soy drinks, lipids are present in the form of fat globules consisting of 99% triacylglycerols (TGs).^{41,42}

TGs are composed of a glycerol molecule bonded to three fatty acids (thereby containing three acyl chains). However, the glycerol molecule can form bonds with just one or two fatty acid moieties as well. Therefore, monoacylglycerols (MGs) and diacylglycerols (DGs) may be also present in milk and soy drink albeit in a minor proportion.^{41,43}

Fatty acids consist of a carboxylic acid group and a long aliphatic chain, which is structured straight or branched. When part of acylglycerol structures, they are joined via an ester bond between their carboxylic groups and one of the hydroxyl groups of the glycerol moiety. Depending on their chain length, they can be classified into short-chain fatty acids (2-6 carbon atoms), medium-chain fatty acids (7-12 carbon atoms), long-chain fatty acids (13-21 carbon atoms) and very long-chain fatty acids (more than 22 carbon atoms).⁴³

Moreover, depending on the presence of double bonds, fatty acids can be classified into saturated and unsaturated fatty acids. Saturated fatty acids do not possess double bonds and, regarding the presence of one or more double bonds, a distinction can be made between monounsaturated fatty acids or polyunsaturated fatty acids.¹

Namely, monounsaturated fatty acids possess only one double bond (e.g. oleic acid, C 18:1), while polyunsaturated fatty acids have more than one double bond, such as linoleic acid (C 18:2) and linolenic acid (C18:3).¹

Unsaturated fatty acids, especially polyunsaturated fatty acids, are susceptible to lipid oxidation as result of their C=C double bonds. This is due to the fact that the adjacent carbon-hydrogen bonds have significantly lower bond energy and are therefore more easily cleaved.⁴⁴

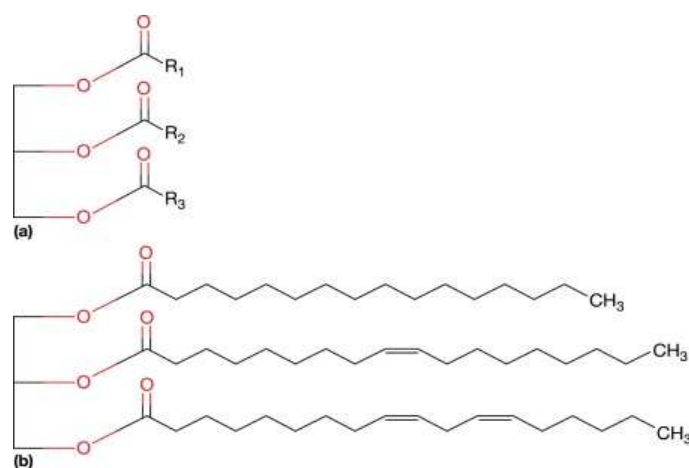


Figure 9: (a) General chemical structure of a triacylglycerol. (b) Chemical structure of a specific triacylglycerol – representing R₁ as palmitic acid (C16:0); R₂ as oleic acid (C18:1); and R₃ as linoleic acid (C18:2). (based on Marini, 2016) ⁴⁵

Differences in susceptibility to lipid oxidation arise from the distinct fatty acid profiles of milk and soy drink. The fat profile of cow milk is dominated by saturated fatty acids (SFA), but its unsaturated fatty acids (UFA) can still undergo oxidation primarily when exposed to light (photo-oxidation). In contrast, soy drink contains predominantly polyunsaturated fatty acids (PUFA). Hence, why soy drink is more sensitive to oxidation. ⁴⁶

Thermal processing methods for milk and soy drinks extend shelf-life and prevent spoilage. However, they simultaneously affect the lipid content of milk with lipid oxidation playing a crucial role. ³ Therefore, it is important to face the fundamentals of this type of oxidation. Lipid oxidation is a complex process influenced by oxygen, light, heat, and enzymes. The chemical process is a radical chain reaction and proceeds in three stages: initiation, propagation, and termination. Depending on which trigger mechanism initiates the process, it is possible to differ between autoxidation, photooxidation and enzymatic oxidation. ⁴⁷

2.4.1 Autoxidation

During the initiation stage, a radical, usually a reactive oxygen species (ROS), interacts with an unsaturated fatty acid and through the abstraction of a hydrogen atom linked to the carbon atom adjacent to the double bond, a fatty acid radical is formed. This lipid radical reacts with oxygen, producing a lipid peroxy radical. In the propagation stage, the lipid peroxy radical reacts with another unsaturated fatty acid, resulting in a lipid hydroperoxide and a lipid radical. ⁴⁸ During this stage the number of reactive compounds increases. Finally, during the termination stage, reactive compounds degrade or react with each other, and non-reactive compounds are formed. ⁴⁷ The reaction mechanism of autoxidation is shown in figure 10.

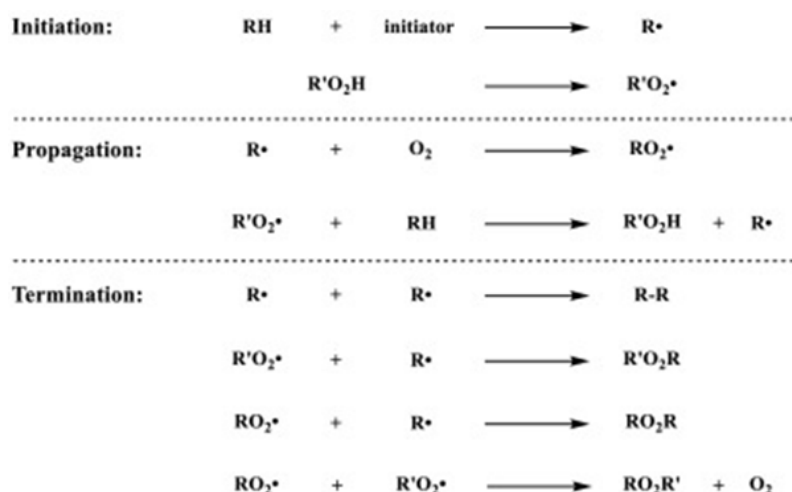


Figure 10: Lipid oxidation – mechanism of autooxidation (based on Wang et al. 2023) ⁴⁷

2.4.2 Photooxidation

In photooxidation, the initial mechanism is triggered by light and photosensitizers. Riboflavin is the most important photosensitizer in milk. It is naturally present in relatively high concentrations and is highly photosensitive. ^{49,50}

During photooxidation type I photosensitizers (sens) can be excited into a singlet state under the presence of light and directly initiate lipid oxidation. In contrast, during photooxidation type II the sensitizer (sens) absorbs light and is thereby brought into a higher energy state, and then oxygen in the ground state (triplet oxygen) is converted by the sensitizer into a reactive state (singlet oxygen), which represents an important reactive oxygen species (ROS) and initiates the lipid oxidation process. In this type of photooxidation, the singlet oxygen reacts directly with the C-C double bonds of the unsaturated fatty acid and can thus generate fatty acid hydroperoxides. ^{1,47} The reaction mechanism of photooxidation is shown in figure 11.

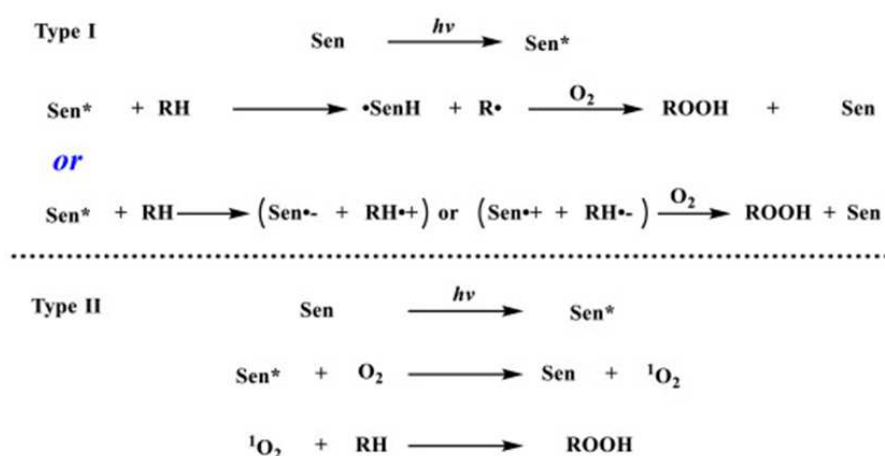


Figure 11: Lipid oxidation - mechanism of photooxidation (based on Wang et al. 2023) ⁴⁷

2.4.3 Enzymatic oxidation

Enzymatic lipid oxidation is largely controlled by the enzyme lipoxygenase (LOX). This enzyme is present in high concentrations in plants, such as soybeans, and plays a significant role in quality reduction, particularly through the development of off-flavors.^{51–53}

Lipoxygenase oxidizes unsaturated fatty acids (such as linoleic acid and linolenic acid) in the presence of oxygen. More precisely, the enzymatic oxidation pathway of fatty acids is initiated by the iron (III)-hydroxide (Fe(III)-OH) coenzyme factor and takes place in four reaction steps: hydrogen abstraction, radical rearrangement, oxygen insertion, and radical reduction-protonation.^{47,51} This process produces lipid hydroperoxides, which are considered primary products of oxidation and are further broken down into non-volatile and volatile compounds, leading to a rancid odor.^{1,54,55} In food industry, heat treatment is an effective method to prevent enzymatic oxidation by lipoxygenase.⁵⁶ However, heat can promote autoxidation in turn. The reaction mechanism of enzymatic oxidation is represented in figure 12.

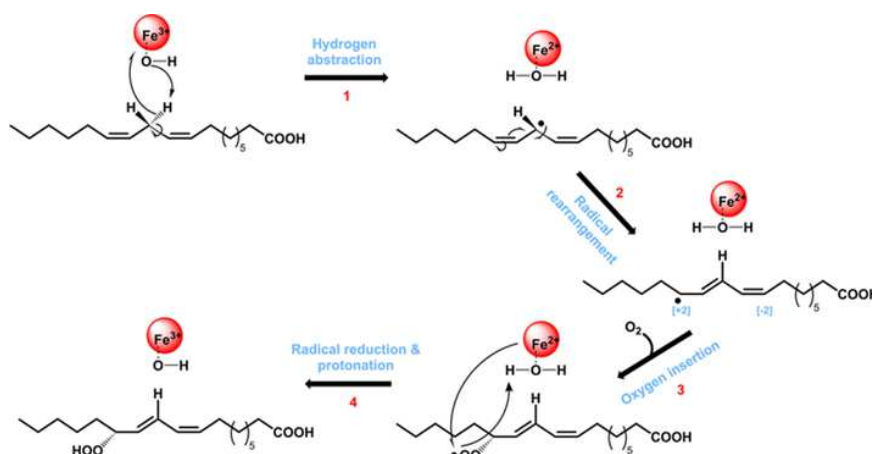


Figure 12: Lipid oxidation - mechanism of enzymatic oxidation (based on Chrisnasari et al., 2024)⁵¹

2.4.4 Products of lipid oxidation

The primary products of lipid oxidation in milk are hydroperoxides and conjugated dienes. They are mostly odorless and tasteless, but act as indicators of incipient spoilage. They are unstable and decompose into secondary products such as aldehydes, ketones, and alcohols. These products are responsible for changes in taste and odor, such as rancid taste and a pungent odor.⁵⁵ Figure 13 shows typical secondary lipid oxidation products of linoleic acid.

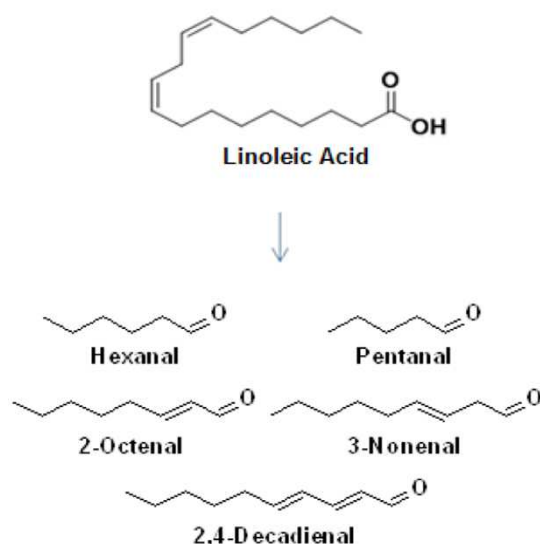


Figure 13: Secondary lipid oxidation products of linoleic acid (based on Clarke et al., 2021) ⁵⁷

2.4.4.1 Lipid oxidation products in cow milk

Milk fat is susceptible to lipid oxidation, following formation of kinetically unstable hydroperoxides and subsequent breakdown into a variety of secondary lipid oxidation products. These include aldehydes, such as hexanal (from linoleic acid) and other n-alkanals (such as pentanal, heptanal, and octanal), which cause metallic and grassy off flavors. Among other products, ketones in milk are, for instance, 2-nonanone and 2-heptanone, their formation often intensified by thermal degradation or concomitant lipolysis. ⁵⁷

2.4.4.2 Lipid oxidation products in soy drinks

Soybeans have a very high content of polyunsaturated fatty acids (particularly linoleic acid). In addition, raw soybeans contain the enzyme lipoxygenase (LOX), which operates with a high degree of regio- and stereospecificity. Taking linoleic acid as an example, four specific stereoisomers of the conjugated diene hydroperoxides are therefore formed. The main enzymatic isomer corresponds to a 13-hydroperoxyoctadecadienoic acyl chains (13-HpODE). The kinetic primary product is the *cis*-, *trans*-13-HpODE, which is formed in 95% yield. It is unstable and, over the course of the reaction, converts to the thermodynamically more stable *trans*-, *trans*-13-HpODE. The minor enzymatic isomer is the 9-hydroperoxyoctadecadienoic acyl chain (9-HpODE), which also initially appears in the *cis-trans* configuration (*cis*-, *trans*-9-HpODE) and subsequently turns into the *trans-trans* configuration. Upon further progression of the reaction, stable hydroxy fatty acyl chains (13-HODE and 9-HODE) are formed, as well as aldehydes and alcohols as secondary degradation products. Among the aldehydes, hexanal is formed from linoleic acid in large quantities and is responsible for the beany off note. In the case of alcohols, 1-hexanol and 1-octen-3-ol can be highlighted. The latter has a strong mushroom-like note. ⁵⁸

2.5 Digestion

The digestion of cow milk and soy drink takes place primarily in the stomach and small intestine. It is influenced by the simultaneous presence of lipid oxidation products and Maillard reaction products, as these interact chemically in biochemical systems, such as the digestive tract.⁵⁹

Contrary to the earlier assumption that these processes occur only during heat-based food processing, the human gastrointestinal tract is highly conducive to these chemical reactions.

Recent research indicates that lipid oxidation and the Maillard reaction progress and interact with one another during gastrointestinal digestion.^{60,61}

Human digestion can be simulated using an in vivo or in vitro model. In vitro methods are frequently used in many areas of food and nutritional science to study the gastrointestinal behavior of food. Since studies on humans are ethically problematic and require many resources (costly), in vitro digestions are a good tool to investigate food components and their digestion products. These methods simulate, under controlled conditions, the human digestive tract, especially the biochemical breakdown of food by using specific enzymes, pH-values and temperatures.^{62,63}

According to Brodkorb et al. (2019), the process of in vitro digestion takes place in 3 phases:

Oral phase: The sample is crushed and saliva (containing the enzyme amylase) is added for carbohydrate digestion.

Gastric phase: During this phase, the acidic environment (hydrochloric acid) is simulated, and pepsin is added to break down proteins.

Intestinal Phase: In this phase, the pH value is typically neutralized and digestive juices from the pancreas (e.g. pancreatin) as well as bile salts are added to finally break down fats, proteins and carbohydrates into absorbable building blocks (e.g. amino acids).⁶²

On the one hand, in vitro methods offer many advantages: they are fast and cost-effective. Furthermore, they enable measurements on a larger number of samples under controlled conditions, higher reproducibility, and easy on-site sampling.⁶⁴

On the other hand, a static model is commonly used, which implies certain limitations. For instance, gastric peristalsis, gastric acid dosage, and continuous intestinal emptying are missing. Moreover, there are now various digestion models that use numerous enzymes from different sources (e.g., pig, rabbit, human), varying pH values, digestion times, and temperatures. This has made it more difficult to compare results.⁶⁴

Therefore, recognized, standardized, static in vitro digestive models were developed by the INFOGEST COST network (an international research network) to ensure reproducible results. The INFOGEST 2.0. model is the most frequently used standardized, static in vitro digestive model, widely present in food research-related scientific work. The term "static" in this case refers to the fact that the ratio of food to digestive juices, as well as the pH value, is kept constant during the respective digestive phase.⁶³ Bieck et al. (2025) used the INFOGEST 2.0. model in a recent study examining

how Maillard reaction products are released during simulated digestion of plant-based dairy alternatives.⁶⁵ Furthermore, Zhao et al. (2023) also used INFOGEST 2.0. protocol to assess the bioaccessibility of different nutrients in soybean and cow milk.⁶¹

Despite this, prior research has not simultaneously addressed the digestive dynamics of both the Maillard reaction and lipid oxidation, nor have applied these considerations to cow milk. This thesis, therefore, aims to tackle these research questions for the first time.

3 Goals of the thesis and hypotheses

It is commonly known that many food products undergo different heat treatments, which can lead to the formation of certain types of Maillard reaction products (MRPs) and lipid oxidation products (LOPs). The heating procedures during milk processing can stimulate the production of these products. Additionally, it is known that human digestion has an impact on the chemical reaction of these compounds. However, the extent to which different heat processing techniques used for milk influence the amount of MRPs and LOPs during digestion is unclear. Thus, the following goals were proposed for the present thesis:

One aim of this thesis was to demonstrate the influence of processing and origin (animal/plant based) of cow milk and soy beverage on MRPs and LOPs.

Furthermore, this study aimed to evaluate the impact of digestion on MRPs and LOPs to ascertain if processing-induced effects remain detectable post-digestion.

The objective was to comprehensively characterize these chemical processes and to describe substances previously undetected in these beverages and their digestates, with a particular focus on lipid oxidation products.

It is expected that higher thermally treated milk will generate more MRPs and LOPs during digestion. In addition, since cow milk and soy drink differ in lipid composition and protein content, it can be hypothesized that these reactions will progress differently during the digestion of this food. Moreover, another hypothesis is that an increase in free MRPs and free oxidized fatty acids is awaited after gastrointestinal digestion, the latter supported by a measurable decrease in unsaturated acyl groups.

4 Methods and materials

4.1 Samples

The cow milk and soy drink samples were bought at a local supermarket chain. The different heat-treated cow milk samples were from the same brand, while the soy drink was from a different one. For this study three different types of cow milk were investigated: one long-term low-temperature pasteurized (LT-treated) milk, one high-temperature pasteurized (HT-treated) milk and one ultra-high-temperature pasteurized (UHT-treated) milk; as well as one UHT-treated plant-based milk alternative (soy drink). Table 3 provides an overview of information for all milk types and the soy drink, related to their composition and nutritional value.

Table 3: Nutritional value table of the milk types and soy drink used according to the manufacturer

	FrISChe Vollmilch 3,5% Fett	LÄnger frISChe Vollmilch 3,5% Fett	Haltbare Vollmilch 3,5% Fett	voll mit Protein Soja
Processing	LT	HT	UHT	UHT
Energy	266kJ	266kJ	267kJ	163kJ
Fat	3.5g	3.5g	3.5g	1.8g
Saturated fatty acids	2.1g	2.1g	2.1g	0.3g
Carbohydrates	4.7g	4.7g	4.7g	2.5g
Sugars	4.7g	4.7g	4.7g	2.5g
Proteins	3.3g	3.3g	3.4g	3.0g
Salt	0.10g	0.10g	0.10g	0.09g
Vitamins				D ₂ : 0.75µg B ₂ : 0.21µg B ₁₂ : 0.38µg
Fibers				0.5g

To point out the main differences in appearance, taste and smell between the three cow milk types and the soy drink, all three milk types were white-colored, had a creamy taste and a fresh smell. In contrast, the soy drink appeared yellowish and had a nutty, beany flavor with a slight vanilla touch.

4.2 Study design

The four beverages described above were the samples of the study, which were then subjected to *in vitro* digestion, yielding the corresponding digestates. The study contained different extraction methods and analysis, which were carried out to monitor the extent of lipid oxidation and Maillard reaction both in the beverages and their digestates. Figure 14 provides a general overview of the study design. It summarizes the determinations and which analysis techniques were used.

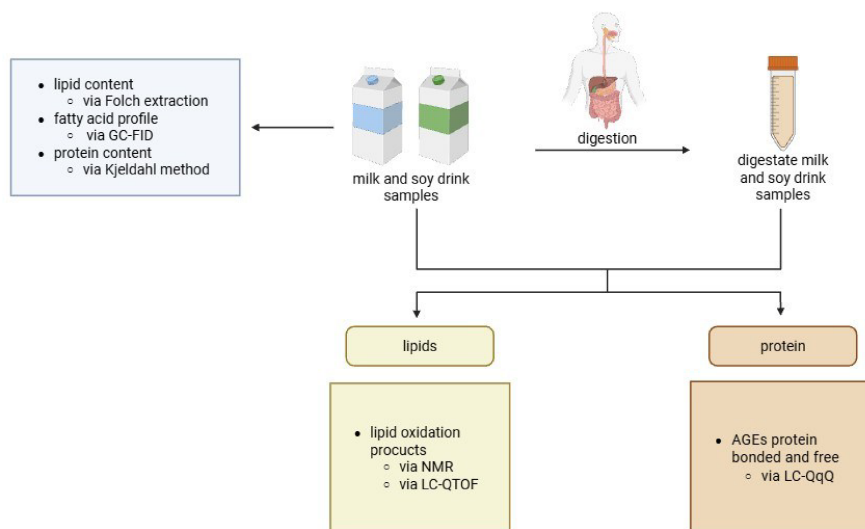


Figure 14: Overview of the study design (created in BioRender.com)

4.3 List of chemicals, devices and consumables

Meanings of pictograms:  = health hazard,  = serious health hazard,  = acute toxicity,  = flammable,  = hazardous to the environment,  = corrosive,  = compressed gas

Table 4: Chemicals for *in vitro* digestion

Chemical	Purity	Supplier	CAS-Number	GHS
Pancreatin from porcine pancreas		Sigma -Aldrich	8049-47-6	 
Bile bovine		Sigma-Aldrich	8008-63-7	 
Pepsin from porcine gastric mucosa		Sigma-Aldrich	9001-75-6	 
Rabbit Gastric Extract (RGE 15 – 1G)		Lipolytech		
Calcium chloride dihydrate	≥99%	Carl Roth	10035-04-8	
Sodium taurodeoxycholate hydrate	≥95%	Sigma-Aldrich	207737-97-1	

Albumin Fraction V, NZ-Origin	≥98%	Carl Roth	90604-29-8	
Sodium chloride CELLPURE®	≥99,5%	Carl Roth	7647-14-5	
Glyceryl tributyrat	≥99%	Sigma-Aldrich	60-01-5	
Hemoglobin from bovine blood		Sigma-Aldrich	9008-02-0	
Potassium chloride	≥99,5%	Carl Roth	7447-40-7	
Potassium dihydrogen phosphate	≥99%	Carl Roth	7778-77-0	
Sodium hydrogen carbonate		Sigma-Aldrich	144-55-8	
Magnesium chloride hexahydrate	≥99%	Carl Roth	7791-18-6	
Ammonium carbonate		Sigma-Aldrich	506-87-6	
Hydrochloric acid		Carl Roth	7647-01-0	

Table 5: Chemicals for extraction and determination of oxidized lipids

Chemical	Purity	Supplier	CAS-Number	GHS-Hazard Pictogram
Chloroform D + 0,03% TMS V/V	99,80%	Eurisotop	865-49-6	
n-Hexan Rotipuran	≥99%	Carl Roth	110-54-3	
LiChrosolv®methanol		Sigma-Aldrich	67-56-1	
LiChrosolv®acetonitrile hypergrade for LC-MS		Sigma-Aldrich	75-05-8	
LiChropur®acetic acid 100% for LC-MS	100%	Sigma-Aldrich	64-19-7	
Sodium methoxide solution 0,5M in methanol		Sigma-Aldrich	124-41-4	
Diethyl ether	≥99%	Sigma-Aldrich	60-29-7	
Petroleum ether 40-60°C		Carl Roth	64742-49-0	
Supelco 37 Component FAME Mix		Sigma-Aldrich	2566-90-7	
Methyl heptadecanoate	95%	Sigma-Aldrich	1731-92-6	
Glyceryl trionadecanoate	≥99%	Sigma-Aldrich	26536-13-0	
Pyrogallol	≥99%	Sigma-Aldrich	87-66-1	
Toluene	≥99,9%	Sigma-Aldrich	108-88-3	
Potassium chloride	≥99,5%	Carl Roth	7447-40-7	

Sodium sulfate	≥99%	Sigma-Aldrich	7757-82-6	
Ammonium acetate LiChropur™, eluent additive for LC-MS		Sigma-Aldrich	631-61-8	
Argon		Linde		
Nitrogen (N ₂)		Linde		
Synthetic air		Linde		
Hydrogen (H ₂)		Linde		 

Table 6: Chemicals for the determination of reducing sugars

Chemical	Purity	Supplier	CAS-Number	GHS-Hazard Pictogram
N-methyl-N-(trimethylsilyl)-tri-fluoroacetamide	≥99%	Carl Roth	24589-78-4	 
Adonitol	≥99,0%	Sigma-Aldrich	488-81-3	
Pyridine	≥99%	Sigma-Aldrich	110-86-1	 
D(+)-glucose monohydrate	≥99,5%	Carl Roth	77938-63-7	
D(+)-maltose monohydrate	≥92%	Carl Roth	6363-53-7	
D-lactose monohydrate	≥99%	Sigma-Aldrich	64044-51-5	
D-(+)-galactose	≥99%	Sigma-Aldrich	59-23-4	
2-propanol		VWR Chemicals	67-63-0	 
Acetone	≥99,7%	Carl Roth	67-64-1	 
Methoxyamine hydrochloride		Sigma-Aldrich	593-56-6	   

Table 7: Chemicals for determination of the protein content

Chemical	Purity	Supplier	CAS-Number	GHS-Hazard Pictogram
Sulfuric acid Rotipuran ® 98%, for nitrogen determination	98%	Carl Roth	7664-93-9	
Boric acid	≥99,8%	Carl Roth	10043-35-3	
Sodium hydroxide	≥99%	Carl Roth	1310-73-2	
Tashiro-indicator solution		Carl Roth	64-17-5	
Kjeldahl catalyst tablets Missouri 5g		Büchi	44647	

Table 8: Chemicals for determination of the AGEs

Chemical	Purity	Supplier	CAS-Number	GHS-Hazard Pictogram
Trichloroacetic acid	≥99%	Carl Roth	76-03-9	
Perfluoropentanocid acid	97%	Sigma-Aldrich	2706-90-3	
Hydrochloric acid Rotipuran ®37% fuming		Carl Roth	7664-93-9	
Ammonium formate LiChro-pur™, eluent additive for LC-MS	≥99%	Sigma-Aldrich	540-69-2	
Sodium borohydride	≥97%	Carl Roth	16940-66-2	
Sodium borate buffer ultra pure		VWR Chemicals	1303-96-4	

Table 9: List of devices

Device	Supplier
Digest Automat K-438	BÜCHI
Distillation Unit K-355	BÜCHI
Interface I-100	BÜCHI
Vacuum Pump V-100	BÜCHI
Rotavapor R-100	BÜCHI
Heating Bath B-100	BÜCHI
pH-meter pHenomenal™	VWR
pH-electrode PHENOMENAL 221	VWR
Ultrasonic Cleaner	VWR
Hotplate/Stirrer	VWR
Balance Entris	Satorius
Water distillation system arium® pro	Satorius
Balance Pioneer™	OHAUS
Centrifuge 5415 R	Eppendorf
Centrifuge 5810 R	Eppendorf
Thermomixer C	Eppendorf
Concentrator plus	Eppendorf
Pipette Research plus (2.5µL, 10µL, 20µL, 100µL, 200µL, 1000 µL, 5000µL)	Eppendorf
CHROMABOND® SPE vacuum manifold for 12 positions	Macherey-Nagel
Vacuum pump M2K3	SCHEGO
Incubator	DRY-Line
HPLC System: UFLC XR SIL-20AC HT prominence AUTO SAMPLER LC-20AD prominence LIQUID CHROMATOGRAPH DGU-20A5 prominence DEGASSER CTO-20AC prominence COLUMN OVEN	SHIMADZU
Mass spectrometer LCMS-8040	SHIMADZU
Mass spectrometer 6546 LC/Q-TOF	Agilent
Vanquish Column Compartment	Thermo scientific
Vanquish Autosampler	Thermo scientific
Vanquish Pump	Thermo scientific

Incubator	Heraeus Instruments
GC for MS: Gas chromatograph system GC2010 Plus Mass Spectrometer GCMS-QP2010 Ultra Auto sampler AOC-5000 plus	SHIMADZU
GC for FID: Gas chromatograph system GC-2010 Plus Injector system AOC-20i Auto Injector Auto sampler AOC-20s Auto Sampler	SHIMADZU
Minichiller (cooling system/heating system/pump)	Huber
Vortex	Star Lab
TipOne®, 200 µL Yellow Tip	Star Lab
TipOne®, 1250 µL XL Graduated Tip	Star Lab
TipOne®, 10/20 µL XL Graduated Tip	Star Lab
Vortex RS-VA 10	PHÖNIX Instrument
Overhead rotator RS-RR 5	PHÖNIX Instrument
Tube 15ml, 120x17 mm, PP	SARSTEDT AG
Tube 50ml, 114x28 mm, PP	SARSTEDT AG
Safe-Lock Tubes 2.0 mL	Eppendorf
Rotilabo®-Reaktionsgefäße PP; 1.5 mL	Carl Roth
Microtube 5 mL	ARGOS Technologies
Parafilm M IS PM-999	Carl Roth
-80°C freezer	VWR
Sublimator VaCo 5-II	ZIRBUS Technology
Needle Sterican® 21G 0,80x25mm	BRAUN
Syringe Inject®-F Luer Solo 1ml	BRAUN
Fridge -20°C, Comfort NoFrost	LIEBHERR

4.4 Methods

In this section of the thesis, the preparation step before each measurement and the important parameters for each analysis are described.

4.4.1 Procedure of the in vitro digestion

The procedure was followed according to the protocol of INFOGEST 2.0⁶² implementing some modifications. Before starting the simulated digestion, the digestion fluids and enzyme solutions with their specific activity for the different phases of in vitro digestion were prepared. On the one hand the fluids were prepared before starting the experiment and then they were stored in a -40°C freezer for no more than 1 year, as stated by the INFOGEST 2.0. On the other hand, the enzyme and bile salt solutions were always prepared freshly on the day of the experiment, because that ensured no loss of enzyme activity. In table 10 the stock concentration of each salt used was summarized according to Brodkorb et al. (2019).⁶²

Table 10: Overview of the salt solutions and their stock concentration for the digestion fluids (according to Brodkorb et al. 2019) ⁶²

salt solution	stock concentration	
	g/L	Molar (M)
KCl	37.3	0.5
KH ₂ PO ₄	68	0.5
NaHCO ₃	84	1
NaCl	117	2
MgCl ₂ (H ₂ O) ₆	30.5	0.15
(NH ₄) ₂ CO ₃	48	0.5
HCl	-	6
CaCl ₂ (H ₂ O) ₂	44.1	0.3

After the stock solution, the digestion fluids were prepared. There were three different digestion fluids, one for each phase. Firstly, the oral phase was represented by the simulated salivary fluid (SSF). Secondly, the gastric phase had the simulated gastric fluid (SGF) as its medium. Lastly, the intestinal phase corresponded with the simulated intestinal fluid (SIF). In the following table 11 the volume of each electrolyte stock solution and their final concentration in each fluid juice were calculated for a total volume of 400 mL. This resulted in 1.25x concentrated storage solutions. This higher concentration accounted for the subsequent dilution by enzymes, CaCl₂, and pH-adjusting reagents, ensuring that the final digestion mixture reached the intended 1x physiological concentration. The corresponding amount of water was added to reach the desired total volume of 400 mL.

Table 11: Volumes and final concentration of the electrolyte stock 1.25x solutions of the digestive fluids (modified according to Brodkorb et al. 2019) ⁶²

salt solution	SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
	volume of stock [mL]	final salt conc. [mM]	volume of stock [mL]	final salt conc. [mM]	volume of stock [mL]	final salt conc. [mM]
KCl	15.1	15.1	6.9	6.9	6.8	6.8
KH ₂ PO ₄	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO ₃	6.8	13.6	12.5	25	42.5	85
NaCl	-	-	11.8	47.2	9.6	38.4
MgCl ₂ (H ₂ O) ₆	0.5	0.15	0.4	0.12	1.1	0.33
(NH ₄) ₂ CO ₃	0.06	0.06	0.5	0.5	-	-
HCl	0.09	1.1	1.3	15.6	0.7	8.4
CaCl ₂ (H ₂ O) ₂	0.025	1.5	0.005	0.15	0.04	0.6

The necessary assays to warrant the required enzymatic activity were performed in advance. They were executed according to the supplementary information of the INFOGEST 2.0 protocol Brodkorb et al. (2019).⁶⁶

Likewise, the in vitro digestion was carried out according to protocol of Brodkorb et al. (2019).⁶² An overview of the experiment is given in table 12.

Table 12: Example of an in vitro digestion experiment with 5 g of sample (according to Brodkorb et al. 2019, with slight modifications)⁶²

Input	5 g of liquid milk sample				
Digestion	Oral (SSF)	Gastric (SGF)		Intestinal (SIF)	
Food or digestate	5 g of sample	10 mL from oral phase		20 mL from gastric phase	
1.25x electrolyte stock solution [mL]	4	8		16	
CaCl₂ (H₂O)₂ (0.3 M) [mL]	0.025	0.005		0.04	
Enzymes	-	pepsin	RGE	pancreatin	bile salt
Enzyme activity [U/mL] or bile conc. [mM]	-	2,000	60	100	10
Volumen of enzyme or bile to be added [mL]	-	0.5	0.5	5.0	2.5
Final volume [mL]	10	20		40	

Table 12 does not show the amount of water being added for the final volume, because it varied from sample to sample and it depended on the volume used for the pH adjustment with HCl and NaOH in each phase.

The in vitro digestion procedure was performed by weighing 5 g of liquid milk or soy drink sample in a 50 mL falcon tube. All fluids were pre-heated in a 37°C water bath.

Firstly, the oral phase started by adding 4 mL of the SSF juice to the sample. Immediately, 0.025 mL of the 0.3 M CaCl₂ solution was added to the tube, to achieve the final concentration of 1.5 mM in the SSF, as shown in table 11. Next would be the addition of the salivary amylase in an activity of 75 U/mL, but since there is no starch in milk or soy drink, its addition was not necessary. The amount of the amylase solution was therefore replaced by water. The final volume of 10 mL was reached by adding 0.975 mL of distilled water, in this way a 1x concentration of the SSF could then be achieved. The 50 mL tubes were put on an overhead rotator to mix the digestive mixture for two minutes at 37°C in an incubator.⁶²

Afterwards, the gastric phase was carried out. The pre-heated SGF juice was added (8 mL) to each tube, to achieve a final ratio of 1:1 (vol/vol) with the SGF electrolyte stock solution and the oral bolus. The adjustment of the pH to 3.0 in the gastric phase was performed with a 2 M HCl solution, which

was verified with a pH-electrode. The volume of HCl had to be considered to calculate the proper addition of water. A volume of 0.005 mL of a 0.3 M CaCl_2 solution was added to the tubes after the pH adjustment. Then the first enzymes were added to the tubes. Namely, 0.5 mL of the pepsin solution with an enzyme activity of 2,000 U/mL, which was dissolved in water, was added to the tubes. Afterwards, 0.5 mL of the gastric lipase solution was added to the tube to achieve an enzyme activity of 60 U/mL in the final volume in this phase. In this case, rabbit gastric extract (RGE) was used as lipase, which contains pepsin as well. This already had to be taken into consideration during the calculation for the weighing of both enzymes, to reach the target pepsin enzymatic activity. The pH in the tubes had to be re-checked and re-adjusted to 3.0 with the 2 M HCl solution when needed. This additional volume was taken as well into consideration for the final addition of water to reach the indicated final volume in this phase (20mL), achieving a 1x concentration of SGF. The tubes were placed in the incubator with the overhead rotator. The samples were incubated at 37°C for two hours.⁶²

The last phase the sample went through was the intestinal phase. The pancreatin and bile salt solution were dissolved in SIF juice. The SIF electrolyte stock solution was pre-warmed in a 37°C water bath and then added (8.5 mL) to the gastric chyme. Then the pH was adjusted to 7.0 by adding a certain amount of 2 M NaOH solution, which differed from sample to sample. After the pH was adjusted in the tubes, 2.5 mL of the bile solution dissolved in stock SIF was added to reach a final concentration of 10 mM once the rest of the components were added. Additionally, 0.04 mL of the 0.3 M CaCl_2 was added to reach a final concentration of 0.6 mM in the SIF (see table 11). A total of 5mL from the pancreatin solution in SIF was then added, to reach a final trypsin activity of 100 U/mL in the mixture. After the addition of the pancreatin and bile salt solution the pH of 7.0 had to be verified again. Finally, water was added to the tube until reaching a total volume of 40 mL, so that the SIF juice achieved a 1x concentration. The tubes were once again placed on the overhead rotator in the incubator at 37°C and there they were incubated for two hours. After those two hours the tubes with the final digestate were stored immediately in the -80°C freezer.

4.4.2 Determination of the lipid content by Folch method

To determine the fat content for the milk, soy drink and digestate samples, extractions were performed according to Folch et al. (1957) with some slight modifications.⁶⁷ Based on the expected fat content of milk and soy drink and considering the amount of fat required for subsequent analyses, the total amount of sample needed was 20 g. Thus, 10 g of sample were weighed in two 50mL tubes and dissolved in 30 mL of methanol (ratio of 1:3 (w/v)). This solution was vortexed for one minute. Then the tubes were stored overnight in the -20°C freezer for protein precipitation. On the next day the tubes were centrifugated at a speed of 3.220 x g for 10 minutes. Both supernatants and precipitates from each of the 2 tubes were combined in the same (250 mL) glass beaker. Then 120 mL of chloroform was added to the beaker (chloroform/methanol 2:1(v/v) ratio). This mixture was flushed with argon and the beaker was sealed with parafilm. This solution was magnetic stirred for one hour

under inert atmosphere. In the meantime, 60 mL of an 8.8 g/L KCl solution was added into a separation funnel (500 mL), this volume was equivalent to a third of the chloroform/methanol extract. After one hour the extract was added to the separation funnel. For the phase separation the funnel was shaken vigorously and left to rest for 15 minutes afterwards. The lower organic phase was collected in an empty round flask with tip bottom (250 mL). Rotatory evaporation was used to remove the organic solvent until only an oily residue remained. The remaining residue was pipetted into a pre-weighted 2 mL Eppendorf tube. The Eppendorf tube was dried under nitrogen to dry out any remaining chloroform, so that only the fat was left in the Eppendorf tube. The Eppendorf tube was weighed again for the determination of the fat and subsequently flushed with argon and stored in the -20°C freezer.

For the lipid extraction of the digestate some additional modifications had to be made. Since only 5 g of initial sample were used for digestion and the final volume of the digestate was 40 mL, it was inevitable to use the whole digestate juice for the lipid extraction to get enough yield for the different lipid measurements. The procedure of the lipid extraction was the same as for the milk and soy drink samples, but with the difference that the whole digestate sample was used. The 40 mL of digestate were split into four 50 mL tubes with screwed caps. In each tube 10 g of digestate were dissolved in a 1:3 ratio (w/v) with methanol. After the precipitation and centrifugation step, 240 mL of chloroform were added into the beaker with supernatant and precipitate, which equals to the ratio of 2:1 (chloroform: methanol). Similarly to the milk and soy drink samples, 120 mL of the KCl solution were then added into the separation funnel. After one hour of stirring under inert atmosphere, the mixture was transferred into the funnel. The organic phase was collected in the round flask with tip bottom. The flask was dried under rotatory evaporation until an oily liquid was left. The oily residue was transferred into a 2 mL Eppendorf tube and dried under nitrogen. The final fat extract was stored under argon atmosphere and -20°C.

The determination of the fat content was calculated through weight difference.

$$\text{fat yield [g]} = \text{ependorf tube with dried fat [g]} - \text{empty ependorf tube [g]} \quad \text{Equation 1}$$

The yield of each sample and their digestate from the lipid extraction according to Folch is summarized in Appendix Table 1.

4.4.3 Determination of the fatty acid profile by gas chromatography with flame ionization detection (GC-FID)

The fatty acid composition in undigested milk and soy drink was determined, in order to be able to hypothesize possible oxidized lipids structures within these samples or their digestates. With that purpose, sample preparation was taken from Lall et al. (2009), and modifications from Pignitter et al. (2014) were taken into account as well.^{68,69} A total of 100 mg of extracted fat was transferred into a 50 mL tube, and then a volume of 500 µL of 1% internal standard solution was added. In the case

of milk samples, nonadecanoic acid methyl ester (NME) was used as internal standard and for the soy drink sample heptadecanoic acid methyl ester (HME) was used. The solution with the extract was diluted with 2 mL of toluene and, as antioxidant, 100 mg of pyrogallol were subsequently added to the tubes. Then, 4 mL of sodium methoxide 0.5 M (in methanol) solution were added, and the tubes were flushed for 2 min under nitrogen. For derivatization, the tubes were put into a 50°C pre-heated water bath for 10 min. Afterwards, 200 µL glacial acetic acid were quickly added to stop the process of derivatization. The tubes were then cooled for approximately 30 min at 4°C in a fridge. Next, 5 mL of bidistilled water and 5 mL of n-hexane were added to the tubes, which were then vortexed for 2 min. The mixture was left standing still for 5 min to obtain a proper phase separation. The next step was collecting the upper organic phase in a 15 mL tube with screw cap. The extraction was repeated by adding again 5 mL bidistilled water and 5 mL of n-hexane into the lower polar phase. The tubes were again vortexed for 2 min and left to rest for 5 min to obtain a clear phase separation. The upper organic phase was collected and combined with the previous one. A small amount of sodium sulphate, roughly 250 mg, was added to the 15 mL tube to absorb any moisture, which was vortexed for 30 seconds. The samples were filtered through a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter directly into 1.5 mL GC-vials. The vials were closed with a special crimp cap with a double-layer silicon/polytetrafluoroethylene (PTFE) septum.

The determination of the fatty acid profile of the samples was performed by a GC-FID method according to Grüneis et al. (2019).⁷⁰ The parameters were set as stated in table 13.

Table 13: GC parameters for the determination of the fatty acid profile

GC type	Shimadzu GC-2010 Plus
Autosampler	AOC 20is autosampler
Injector volume	1 µL
Temperature of the injector	220°C
Split ratio	20
GC-Column	Phenomenex ZB-WAX Zebron™ (PEG) capillary column 30 m x 0.25 mm x 0.25 µm
Carrier gas	Nitrogen
Flow	3 mL/min
Detector	Hydrogen FID
Temperature of the detector	270°C

For the FID part, which was at a temperature of 270°C, hydrogen and synthetic air were used and had a flow rate of 30 mL/min and 400 mL/min, respectively.⁷⁰

In table 14 the temperature gradient is shown, which was used for the separation of the different fatty acid methyl esters.

Table 14: Temperature gradient for the determination of the fatty acid profile

Heating rate [°C/min]	Temperature [°C]	Hold time [min]
-	60	2
13	150	0
2	240	0

For identification and quantification, a Supelco 37-Component FAME mix was used. This mixture was diluted in different concentrations from 1:1 (not diluted) to 1:100 to obtain the external standard calibration curves. All different concentrations were filtered through a 0.2 µm syringe filter and directly transferred into a GC-vial. In total 18 fatty acid methyl esters were quantified, which were detected in the samples.

In table 15 the important information about the different fatty acid methyl esters was summarized.

Table 15: Summary of the retention time, equation and coefficient of determination (R^2) from all 18 fatty acid methyl esters

Methyl ester name	Retention time [min.]	Equation	R^2
Methyl butyrate	1.5	$y = 1096.9x - 4519.9$	0.999
Methyl hexanoate	3.1	$y = 1269.3x - 5099.7$	0.999
Methyl octanoate	5.5	$y = 1420.6x - 6984.0$	0.998
Methyl decanoate	7.6	$y = 1520.3x - 1439.0$	0.999
Methyl undecanoate	8.6	$y = 1481.5x - 1115.7$	0.999
Methyl laurate	9.5	$y = 1597.9x - 1959.1$	0.998
Methyl tridecanoate	10.7	$y = 1490.5x - 2626.5$	0.996
Methyl myristate	12.2	$y = 1560.7x - 5184.7$	0.996
Methyl myristoleate	12.8	$y = 1374.3x - 3383.2$	0.996
Methyl pentadecanoate	14.1	$y = 1453.9x - 4001.5$	0.994
Methyl cis-10-pentadecanoate	14.8	$y = 1447.1x - 3842.7$	0.994
Methyl palmitate	16.3	$y = 1558.7x - 10870.0$	0.994
Methyl palmitoleate	16.9	$y = 1423.1x - 4035.6$	0.994
Methyl heptadecanoate	18.9	$y = 1405.0x - 7244.7$	0.990
Methyl stearate	21.9	$y = 1459.1x - 9919.8$	0.989
Methyl cis-9-oleate	22.5	$y = 2237.7x - 13096.0$	0.995
Methyl linoleate	23.8	$y = 1437.2x - 4188.6$	0.995
Methyl linolenate	25.9	$y = 1379.0x - 4003.4$	0.994

To determine the recovery rate, a 1% NME and 1% HME solution was measured as well, to compare the same areas within the samples of these internal standards. The recovery of NME in LT milk, HT

milk and UHT milk was greater than or equal to 100% and the recovery of HME in soy drink was in an interval from 55%-84%. The calculation of the methyl ester content from the calibration curves was conducted with Equation 2.

$$FAME \text{ content } \left[\frac{g}{100g} \right] = \frac{\frac{area-d}{k} \cdot V_{ext}}{E \cdot rec} \cdot 100 \quad \text{Equation 2}$$

area: peak area of the methyl ester of the respective sample [mAU]; d: intercept of the standard curve; k: slope of the standard curve; E: initial weight of the sample [g] rec: recovery of NME or HME; V_{ext} : total volume of the extract [L]; 100: factor to calculate the amount in g/100g

4.4.4 Determination of lipid oxidation products by 1H NMR

To determine the presence of certain lipid oxidation groups in the samples and their occurrence during in vitro digestion via NMR, 100 mg of fat was weighed into a 1.5 mL Eppendorf tube. The sample fat was dissolved in 600 μ L of deuterated chloroform with 0.03% tetramethylsilane (TMS). Then the solutions were pipetted into an NMR tube. The NMR tubes were measured in a Bruker Avance 600 spectrometer operating at 600 MHz. In table 16 the NMR settings of the device are listed.

Table 16: NMR parameters for the determination of lipid oxidation products

Parameters	Setting
Device	Bruker Avance 600 spectrometer
Pulse width	90°
Relaxation delay	1 s
Spectral width	12500 Hz
Acquisition time	2.62144 s
Number of scans	32
Total acquisition time	2 min 3 s

The spectra were analysed using MestreNova (version 14.0) by Mestrelab Research, after Fourier transform, baseline correction, and phase correction, taking into consideration that the areas of the signals were proportional to the number of protons that generated them.

The relative quantification of the acyl groups and lipid oxidation products was expressed either as a molar percentage (molar%) or in millimoles per mole (mmol/mol) of total acyl groups (AG) and fatty acids (FA) and calculated according to equations 3 and 4.^{71,72}

$$x \text{ (molar \% AG + FA)} = \frac{\frac{A_x}{nH_x}}{\frac{A_{AG+FA}}{nH_{AG+FA}}} \cdot 100 \quad \text{Equation 3}$$

A_x : area of component x; nH_x : number of protons component x; A_{AG+FA} : area of the signal corresponding to acyl groups (AG) and fatty acids (FA); nH_{AG+FA} : number of protons contributing to the AG and FA signal; 100: percentage scaling factor

$$x \text{ (mmol/mol AG + FA)} = \frac{\frac{A_x}{nH_x}}{\frac{A_{AG+FA}}{nH_{AG+FA}}} \cdot 1000 \quad \text{Equation 4}$$

A_x : area of component x; nH_x : number of protons component x; A_{AG+FA} : area of the signal corresponding to acyl groups (AG) and fatty acids (FA); nH_{AG+FA} : number of protons contributing to the AG and FA signal; 1000: Molar scaling factor (mmol/mol)

4.4.5 Determination of oxidized polar lipids by LC-MS

Before determining the oxidized polar lipids from the fat samples, it was necessary to separate them from the non-polar lipids. The separation procedure was a solid-phase extraction (SPE) according to Grüneis et al. (2019).⁷⁰

Firstly, 50 mg of sample fat was weighed in a 2 mL Eppendorf tube and dissolved in 2mL petroleum ether via vortex. The SPE equipment was placed in a hood and one Strata SI-1 silica SPE cartridge per sample was plugged in. After the waste collector was put into the equipment, the vacuum pump was started. Before loading the samples into the cartridges, a conditioning of the cartridges was necessary. For this purpose, the cartridges were rinsed with 10 mL (2 times 5 mL) of a mixture of petroleum ether/diethyl ether (90/10). Afterwards, each dissolved sample from the Eppendorf tube was loaded into the cartridges. From this point on, the cartridges were not allowed to get dry, and it was ensured that the content came out drop by drop. Firstly, a washing step was performed to remove the non-polar lipid fraction by adding 15 mL (3 times 5 mL) petroleum ether/diethyl ether (90/10). The vacuum was stopped and the waste collector was replaced by a rack with 15 mL open tubes for collecting the polar lipid fractions. The equipment was closed and the vacuum started again. To elute the polar lipid fraction, 15 mL (3 times 5 mL) diethyl ether was added to the cartridges. After this step the collected samples were evaporated under nitrogen, until dryness. The dried samples were stored at -40°C until further use. The samples were then reconstituted with 1000 µL isopropanol. Lastly, the samples were filtered through a 0.22 µm PVDF syringe filter into HPLC vials with inserts, and analyzed via UHPLC-HRMS/MS.

The LC settings for the separation of oxidized triacylglycerides (oxTGs) and oxidized diacylglycerides (oxDGs) were summarized in table 17.

Table 17: Parameters of the LC-system for the determination of oxTGs and oxDGs

Parameters	Setting
Device	Thermofisher Vanquish Horizon UHPLC system
Mobile phase A	Water:acetonitrile 40:60 (v/v) with 0.1% formic acid and 10 mM ammonium acetate
Mobile phase B	Acetonitrile:isopropanol 20:80 (v/v) with 0.1% formic acid and 10 mM ammonium acetate
Flow rate	0.6 mL/min
Injection volume	10 µL
Precolumn	Zorbax Eclipse Plus C18 precolumn (5 x 2.1 mm, 1.8 µm)
Stationary phase (LC column)	Zorbax RRHT Eclipse Plus C18 column (2.1 x 150 mm, 1.8 µm)
Oven temperature	60°C

For these compounds a mobile phase gradient was used to get better separation of the different oxidized species. Table 18 shows the gradient over the time period set for these measurements.

Table 18: Mobile phase gradient, expressed as %B for the determination of oxTGs and oxDGs

Time [min.]	% B
0	5
1	5
4	95
11.5	100
12.5	5
14	5

Moreover, for the detection and identification of the oxidized TGs and DGs, the MS parameters applied are shown in table 19.

Table 19: MS parameters for oxTGs and oxDGs measurements

Parameters	Setting
Device	Agilent G6546 quadrupole-time of flight (Q-TOF) mass spectrometer
Ion source type	Electrospray (ESI)
Ionization mode	Positive
Nebulizer pressure	35 psi

Sheath gas	Nitrogen (N ₂)
Sheath gas flow	10 L/min
Sheath gas temperature	300 °C
Drying gas	Nitrogen (N ₂)
Drying gas flow	12 L/min
Drying gas temperature	200 °C
Nozzle voltage	1000 V
Capillary voltage	3500 V
MS mode	Auto MS/MS
Collision energies (for MS/MS fragmentation)	20-40 eV
Mass range	<i>m/z</i> 50-1500
Acquisition rate	4 spectra/s

In case of the determination of the oxidized monoacylglycerides (oxMGs) and oxidized free fatty acids (oxFAs), different parameters were used for the LC-system, including mobile phase gradient, and the MS-system. In table 20, the setup of the UHPLC system parameters is summarized.

Table 20: UHPLC parameters for the oxMGs and oxFAs determination

Parameters	Setting
Device	ThermoFisher Vanquish Horizon UHPLC system
Mobile phase A	Water:acetonitrile 95:5 (v/v) with 0.1% acetic acid and 10 mM ammonium acetate
Mobile phase B	Water:acetonitrile 5:95 (v/v) with 0.1% acetic acid and 10 mM ammonium acetate
Flow rate	0.25 mL/min
Injection volume	10 µL
Precolumn	Phenomenex Kinetex 100 Å C18 (2 × 2.1 mm, 2.6 µm)
Stationary phase (LC column)	Phenomenex Kinetex 100 Å C18 (150mm × 2.1 mm, 2.6 µm)
Oven temperature	40°C

Due to the different mobile phase composition, a different mobile phase gradient had to be used. The concentrations of mobile phase B are shown in table 21.

Table 21: UHPLC mobile phase gradient, expressed as %B, for the determination of oxMGs and oxFAs

Time [min.]	% B
0	5
2.4	5
16.8	70
28.8	95
34.8	95
35.4	5
40	5

For the MS-system, the same parameters were used as in table 18 but with the main difference of the ionization mode for the oxFAs. Thus, in table 22 the setup of the MS-parameters is shown.

Table 22: MS parameters for oxMGs and oxFAs measurements

Parameters	Setting
Device	Agilent G6546 quadrupole-time of flight (Q-TOF) mass spectrometer
Ion source type	Electrospray (ESI)
Ionization mode	Positive (for OxMGs) Negative (for OxFAs)
Nebulizer pressure	35 psi
Sheath gas	Nitrogen (N ₂)
Sheath gas flow	10 L/min
Sheath gas temperature	300 °C
Drying gas	Nitrogen (N ₂)
Drying gas flow	12 L/min
Drying gas temperature	200 °C
Nozzle voltage	1000 V
Capillary voltage	3500 V
MS mode	Auto MS/MS
Collision energies (for MS/MS fragmentation)	20-40 eV
Mass range	<i>m/z</i> 50-1500
Acquisition rate	4 spectra/s

The software MS-DIAL (version 5.5) and Agilent Mass Hunter were used for data processing, including data smoothing, peak detection and retention time alignment. Identification was performed by spectral matching against a native MS-DIAL database for oxidized TGs and FAs and an in-house

database for oxidized DGs and MGs. Comparisons between sample groups were made semiquantitatively in an area under the curve basis, after quality control (QC) normalization, which was obtained by including an injection of a representative pool of samples every 5-10 injections.

To obtain a comprehensive understanding of lipid oxidation products profile, both ^1H -NMR spectroscopy and LC-QTOF mass spectrometry were employed. NMR was utilized to gain a broad overview of the formation of different groups of lipid oxidation products during digestion. In contrast, the LC-QTOF method was applied to identify specific structures of oxidized lipids and their digestive behavior, since milk primarily consists of triacylglycerols.

4.4.6 Determination of protein content by Kjeldahl method

The determination of the protein content in the milk and soy drink samples was carried out by the Kjeldahl method according to Matissek & Fischer, (2021).⁷³ Thus, the weight of sample needed for the quantification depended on the expected protein content. In the case of milk and soy drink 5 g of liquid was needed. The homogenized samples were weighed into nitrogen-free parchment boats. The boats were put into 300 mL Kjeldahl flasks, and two more Kjeldahl flasks were used as blanks. In each flask two 5 g tablets of the Kjeldahl Missouri catalyst (4.98 g K_2SO_4 and 0.02 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were added. Afterwards, 20 ml of 98% sulfuric acid were added to each flask. Furthermore, one scoop of a spoon with antifoam was added to each flask, to avoid extreme bubbling during the heating procedure. From this point on, the start of the protein digestion was executed in the fume hood. The setting of the instrument for the digestion is shown in table 23. It shows the temperature gradient and the duration time used for this experiment.

Table 23: Setting of the Digest Automat K-438 by Büchi for milk and soy drink samples⁷³

	1	2	3	4	Cooling
Temperature [°C]	230	300	350	420	-
Duration [min]	15	40	40	60	30

After the digestion was completed, 60 ml of a 4 % boric acid solution with a pH of 4.65 were added to each titration flask. Both flasks (digestion and titration) were placed in the distillation device, where the distillate was collected in the titration flasks with 4% boric acid. Before starting the distillation, setting up the correct volumes was needed. Table 24 includes the settings for the distillation instrument.

Table 24: Instrument settings of the distillation⁷³

Water [mL]	1 M NaOH [mL]	Reaction time [s]	Distillation [s]	Stirrer speed
50	90	5	300	5

After the automatically adding of the correct amount of water and 1 M NaOH volumes the distillation was started. After the distillation was finished, 5 drops of Tashiro indicator were added to the titration

flasks and titration was performed, using a 25 ml burette. The titrant was a 0.2 M HCl solution. The titration was observed until a clear color change could be seen from green to “colorless” to pink. The consumed volume of the titrant was required for the determination of the nitrogen content and thereby the total protein content.

The equations 6-8 represent the calculation pathway of the protein content in the samples.

$$\omega(N) = \frac{(V_{sample} - V_{blank})[ml]}{m_{sample}[g] \cdot 1000 \left[\frac{ml}{L}\right]} \cdot z \cdot c \cdot f \cdot M(N) \quad \text{Equation 6}$$

$$\% N = \omega(N) \cdot 100\% \quad \text{Equation 7}$$

$$\% P = \omega(N) \cdot PF \cdot 100\% \quad \text{Equation 8}$$

$\omega(N)$: Mass fraction of nitrogen [gN/g sample]; V_{sample} : consumption of titrant for the sample [mL]; V_{blank} : consumption of titrant for the reagent blank [mL]; z : Molecular valence factor of 1 for HCl; c : concentration of the measuring solution (0.2N HCl) [M]; f : factor of the standard solution (usually 1 for commercial solutions); $M(N)$: molar mass of nitrogen (14.007 g/mol); m_{sample} : mass of the sample [g]; 1000: conversion factor [mL/L]; PF : protein factor (6.38 for milk); $\%(N)$: mass percent of nitrogen; $\%(P)$: mass percentage of protein

4.4.7 Determination of advanced glycation end products (AGEs) by LC-MS/MS

The following determination method was used for analysis of acid-resistant advanced glycation end products. To analyze both free and protein-bound AGEs the samples were processed according to Teerlink et al. (2004), incorporating modifications from Auñon-Lopez et al. (2025) and Bieck et al. (2025).^{65,74,75}

Firstly, 1 mL of milk or digestate was transferred into a 2 mL safety cap Eppendorf tube and was freeze-dried overnight. An amount of 20 mg of the lyophilized sample was then transferred to a new 2 mL Eppendorf tube with safety cap. The freeze-dried milk was dissolved in 100 μ L water and 500 μ L of a 200 mM sodium borate buffer containing 100 mM borohydride solution with a pH adjusted to 9.2. The tubes were vortexed for a few seconds and then left for incubation at room temperature for 2 hours. After incubation 1 mL of a 40% TCA (trichloroacetic acid) solution was added for protein precipitation. The tubes were vortexed again for a few seconds and then they were centrifuged with 16,000 x g for 10 minutes at room temperature. The supernatants were transferred into 5 mL Eppendorf tubes. 1 mL of 10% TCA solution was added in the tubes with the pellet. Then the tubes were vortexed and centrifuged just like the first time. Again, the supernatant was taken and combined with the previous one in the same 5 mL Eppendorf tube for each sample. This fraction was stored at -20°C, which was later used for the determination of the free AGEs. On the other hand, 500 μ L of 6 M HCl were added in the tubes with pellet, which represented the fraction containing the protein-bound AGEs. After mixing these tubes they were incubated for 20 hours at 110°C in the incubator,

for protein hydrolysis. The next day the Eppendorf tubes were dried on the speed-vac vacuum concentrator at 60°C in the AQ-V mode. Once the samples were dried, the residues were redissolved in 500 µL of a 5 mM perfluoropentanoic acid. Then the sample solutions were filtered through a 0.2 µm PVDF filter into HPLC vials with insert and were closed with a cap with septum. Moreover, a 5 mM perfluoropentanoic acid was as well used as a blank.

In the case of the free AGEs measurement, there was an additional step before filtering into the HPLC vial. A little amount of the supernatant was diluted with the 5 mM perfluoropentanoic acid in a ratio of 1:5 (v/v), that resulted in a total volume of 450 µL. Afterwards, the diluted sample was filtered as previously described for the protein-bound AGEs samples. This step was necessary to lower the concentration of TCA injected into the mass spectrometer. Table 25 indicates the parameters that were set on the LC-MS/MS device for the measurement of AGEs.

Table 25: LC parameters for the determination of advanced glycation end products (AGEs)

Parameters	Setting
Device	Shimadzu LCMS-8040 system
Pump	Shimadzu LC-20AD prominence Liquid Chromatograph
Mobile phase A	Water with 0.1% formic acid and 6 mM ammonium formate with pH=2.4
Mobile phase B	Acetonitrile:mobile phase A 80:20 (v/v) with 0.1% formic acid
Flow rate	0.4 mL/min
Autosampler	Shimadzu SIL-20AC HT prominence Auto sampler
Injection volume	10 µL
Column oven	Shimadzu CTO-20AC prominence column oven
Precolumn	Luna HILIC 200 Å (4mm × 2 mm, 3 µm; Phenomenex)
Stationary phase (LC column)	Luna HILIC 200 Å (100 mm × 3 mm, 3 µm; Phenomenex)
Oven temperature	25°C

For the measurements of the AGEs a mobile phase gradient was used, which is shown in table 26.

Table 26: LC mobile phase gradient, expressed as concentration of B (%) for the determination of AGEs

Time [min.]	% B
0-2	90
3-5	87
9-15	30
20-28	90

The following table 27 presents the settings of the mass spectrometer.

Table 27: MS parameter setting for the determination of AGEs

Parameter	Setting
Device	Shimadzu triple quadrupole LCMS-8040 mass spectrometer
Ion source type	Electrospray (ESI)
Ionization mode	Positive
Nebulizing gas	Nitrogen (N ₂)
Nebulizing gas flow rate	3 L/min
Desolvation line temperature	170°C
Drying gas	Nitrogen (N ₂)
Drying gas flow rate	13 L/min
Heat block temperature	350 °C
MS mode	Multiple reaction monitoring (MRM)
Collision-induced dissociation (CID) gas	Argon (Ar)
CID pressure	230 kPa

The transition list of the targeted AGEs, as well as precursor amino acids, is presented in table 28.

Table 28: Multiple reaction monitoring (MRM) mode transitions used for the determination of CML, CEL, GOLD, MOLD, MG-H1, G-H1, furosine, pentosidine, argpyrimidine, L-lysine and L-arginine

Analyte	Precursor ion (m/z)	Product ions			
		Quantifier (m/z)	CE (V)	Qualifier (m/z)	CE (V)
CML	205.0	84.0	-19	130.0	-14
CEL	219.0	84.0	-18	130.0	-12
GOLD	327.7	84.0	-34	-	-
MOLD	341.3	84.0	-42	-	-
MG-H1	229.0	114.0	-30	211.0	-10
G-H1	215.2	70.0	-30	-	-

Furosine	255.0	130.0	-18	84.0	-28
Pentosidine	379.0	250.0	-22	187.0	-30
Argpyrimidine	255.0	192.0	-15	140.0	-15
L-Lysine	147.0	130.0	-10	84.0	-20
L-Arginine	175.0	116.0	-20	70.0	-30
				60.0	-30

To determine the concentration of each AGE in the samples the standard addition method was used. The standards used were CML, GOLD, MG-H1 and furosine, which were utilized as AGE class-representative standards. To obtain the standard curves for the protein-bound AGEs, the standards were added at the same time as the 6 M HCl solution. For the free AGEs standard curves, the addition was done at the beginning before the first precipitation step. In the case of pentosidine, argpyrimidine, L-lysine and L-arginine only a semi-quantitative measurement was made in a QC-normalized peak area basis.

The data analysis was carried out with MacCoss Lab Software Skyline (version 26.1). The concentration of the protein-bound AGEs in milk and soy drink was calculated after equations 9 and 10, which can be seen below. In addition, the standard calibration curves and their equations for protein-bound and free AGEs were summarized in Appendix figure 1-15.

$$conc. \left[\frac{\mu g \text{ AGE}}{g \text{ milk}} \right] = conc. \left[\frac{\mu g \text{ AGE}}{mL} \right] \cdot \frac{V [mL]}{E [g]} \cdot \frac{m (FD \text{ milk}) [g]}{g \text{ of fresh milk}} \quad \text{Equation 9}$$

E: initial weight of freeze-dried sample [g]; V: volume in which dried sample got redissolved [mL]; m (FD milk): freeze-dried milk weight; conc. $\mu g \text{ AGE}/mL$: concentration of the AGEs in the final HPLC vial.

$$conc. \left[\frac{\mu g \text{ AGE}}{g \text{ protein}} \right] = conc. \left[\frac{\mu g \text{ AGE}}{g \text{ milk}} \right] \cdot \frac{100 g \text{ milk}}{\text{amount of protein in milk [g]}} \quad \text{Equation 10}$$

amount of protein in milk: according to Kjeldahl determination [g]

For free AGEs in undigested samples, Equation 11 was applied, incorporating a larger solvent volume ($V = 2.6 \text{ mL}$) and a 1:5 dilution factor.

$$conc. \left[\frac{\mu g \text{ AGE}}{g \text{ milk}} \right] = conc. \left[\frac{\mu g \text{ AGE}}{mL} \right] \cdot \frac{V [mL]}{E [g]} \cdot \frac{m (FD \text{ milk}) [g]}{g \text{ of fresh milk}} \cdot 5 \quad \text{Equation 11}$$

E: initial weight of freeze-dried sample [g]; V: volume in which dried sample got redissolved [mL]; m (FD milk): freeze-dried milk weight; conc. $\mu g \text{ AGE}/mL$: concentration of the AGEs component calculated from the standard curve equation

The calculation of the digestate samples was different. For digestate samples (Equation 12), the results were divided by 0.125 to convert the concentration to µg AGE per g milk, based on the digestion ratio of 5 g milk in 40 mL digestate (1 mL = 0.125 g).

$$conc. \left[\frac{\mu g \text{ AGE}}{g \text{ milk}} \right] = conc. \left[\frac{\mu g \text{ AGE}}{mL} \right] \cdot \frac{V [mL]}{m (FD \text{ digestate}) [g]} \cdot \frac{m (FD \text{ digestate}) [g]}{mL \text{ of digestate}} \cdot \frac{mL \text{ of digestate}}{0.125 [g]} \quad \text{Equation 12}$$

V: volume in which dried sample got redissolved [mL]; m (FD digestate): freeze-dried digestate weight; conc. µg AGE/mL: concentration of the AGEs component calculated from the standard curve equation

To express concentrations relative to protein content, Equation 10 was applied to the digestate samples.

Equation 13 includes an additional step for CEL, MOLD, and G-H1 to adjust for molecular weight differences from their respective standards (CML, GOLD, and MG-H1), because these structural analogues behave similarly in MS on a molar rather than a mass basis.

$$conc. \left[\frac{\mu g \text{ AGE}}{mL} \right] = \frac{\left(\frac{(normalized \text{ area} - y)}{k} \right) \left[\frac{\mu g}{mL} \right]}{M_1 \left[\frac{g}{mol} \right]} \cdot M_2 \left[\frac{g}{mol} \right] \quad \text{Equation 13}$$

k: slope of the linear equation; y: y-intercept of the linear equation; M₁: molecular weight of standard compound (e.g. CML, GOLD and MG-H1); M₂: molecular weight of determined compound (e.g. CEL, MOLD and G-H1)

4.5 Statistical analysis

All experiments were carried out with four independent replicates per group (n=4). GraphPad Prism 10.5.2 was used to conduct the relevant statistical analyses. All data was tested for normal distribution by the Shapiro-Wilk normality test. The presence of significant outliers was evaluated with a Grubb's test. To discriminate statistically significant differences (p < 0.05) between the samples due to drink type and state of digestion, a two-way analysis of variance (ANOVA) was used. If there were significant differences, the two-way ANOVA was followed by Tukey's post-hoc test, which allowed to perform pairwise comparisons between the specific groups. In the case of a statistical comparison of only milk samples or only digestate samples being relevant, a one-way ANOVA test was carried out, followed by Tukey's post-hoc test. If non-equal variances between the different groups were found via Brown-Forsythe test, a Welch ANOVA test was performed instead. This was followed by a Dunnett T3 test. For a statistical comparison between only one sample type before and after digestion, an unpaired t- test was used. In the event of heterogeneous standard deviations, an unpaired t- test with Welch's correction was carried out.

The results with a p-value lower than 0.05 (p < 0.05) were considered as significant and marked with different lower-case letters in the results graphics. Values below the limit of quantification (LOQ,

signal-to-noise ratio < 10) were not considered for statistical comparisons and therefore labeled as < LOQ. All (semi)-quantitative results were expressed as mean $\pm$ standard deviation (SD). Moreover, to further visualize the influence of variables on certain parameters, especially in the case of oxidized lipids, a principal component analysis (PCA) was used. In addition, correlations between the different parameters were performed by two-tailed Pearson's correlation tests.

5 Results and discussion

This thesis aimed to evaluate differences between the various types of processed milk and soy drink in progression of lipid oxidation and the Maillard reaction, as well as the impact that digestion had on these products. To accomplish this purpose, this study focused on analyzing the beverages' fatty acid profile and protein content, as well as their lipid oxidation products before and after digestion and a series of different AGEs. These results helped to examine the oxidative status of TGs, DGs, MGs and FAs within the samples and assessed how far new MRPs were built during digestion, and how this was reflected on the precursor amino acids contents.

5.1 Lipids

5.1.1 Fatty acid profile

Milk fat can contain more than 400 different fatty acids.⁷⁶ However, the vast majority are minor components that contribute minimally to the total profile. Consequently, only the predominant fatty acids were detected in the analyzed milk and soy drink samples.

The results for LT and HT-milk of the fatty acid composition are presented in figure 15 and figure 16.

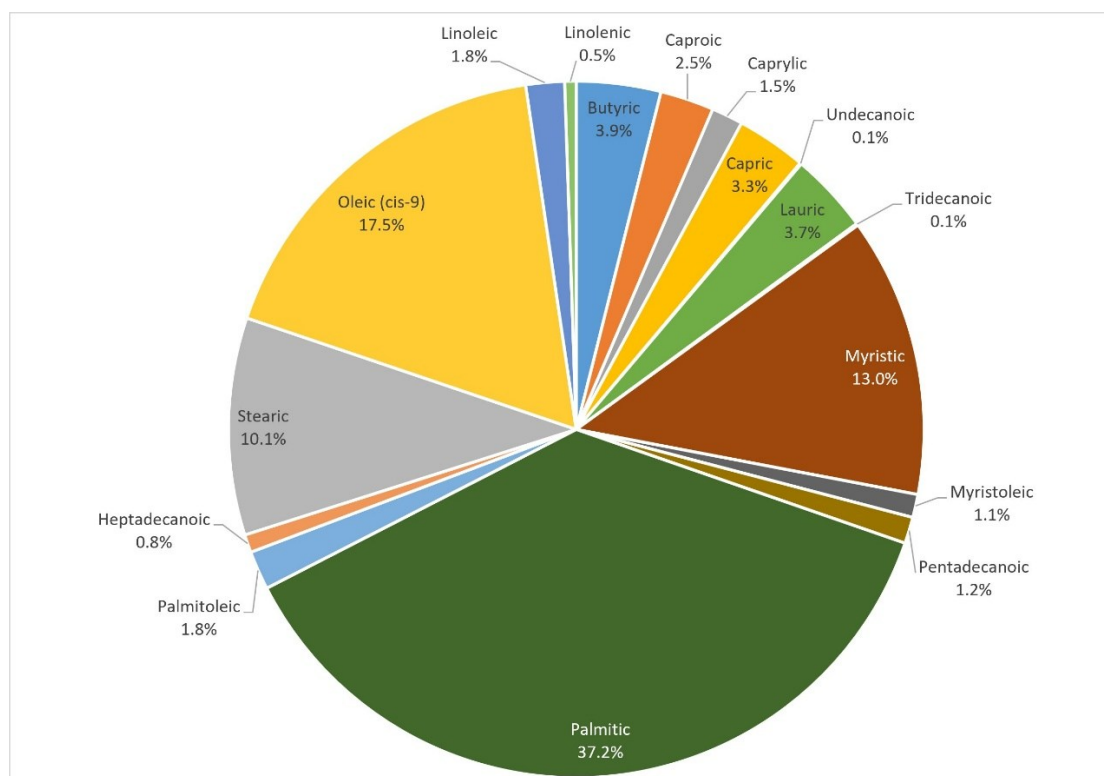


Figure 15: LT-milk fatty acid composition expressed as percent by weight of total fatty acids. Mean values are shown (n=4).

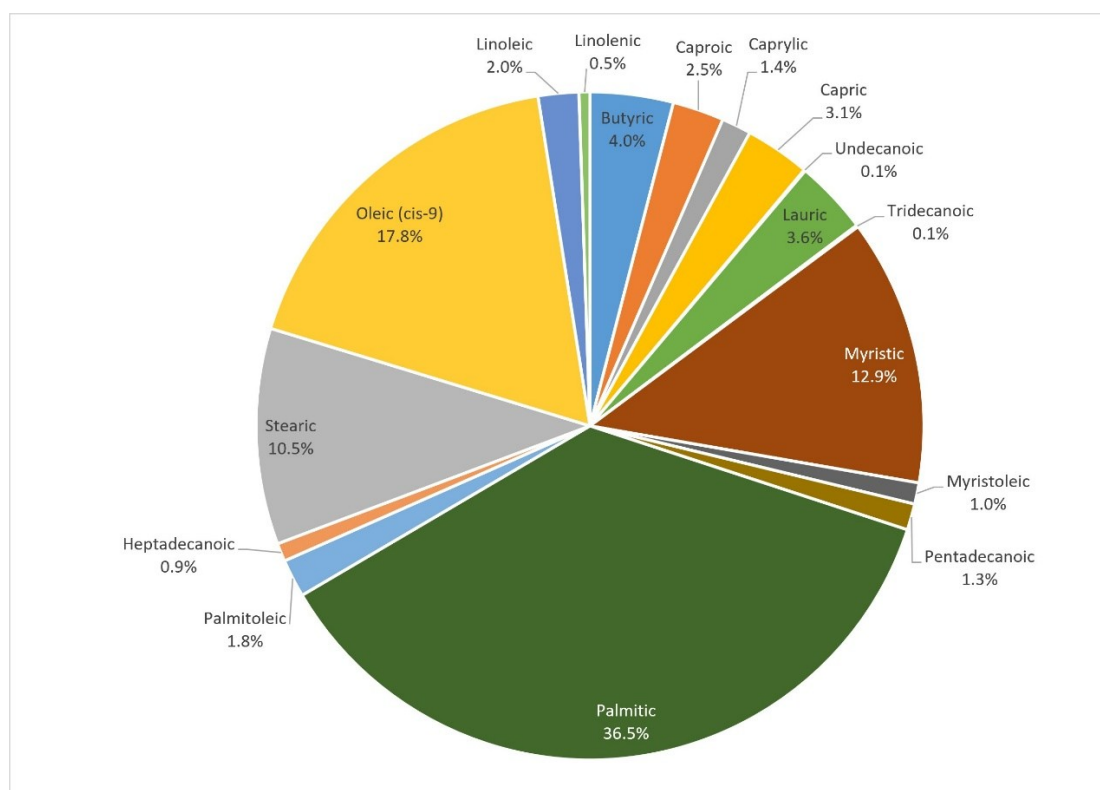


Figure 16: HT-milk fatty acid composition expressed as percent by weight of total fatty acids. Mean values are shown (n=4)

Prior research on the milk fatty acid composition from milk by Gómez-Cortés & Amigo (2022) presents a similar picture.⁷⁷ The results obtained for the LT- and HT-milks analyzed were also in line with the results reported on bovine milk by Lindmark Månsson (2008), with certain minor differences.⁷⁶ Regarding the saturated fatty acids, the percentages of palmitic acid and myristic acid were lower in her study (30.6% for palmitic acid and 10.9% for myristic acid). Furthermore, tridecanoic acid was not detected by Lindmark Mansson (2008), but Gómez-Cortés & Amigo (2022) reported its presence with low percentage values (0.2%), comparable to the results displayed in figure 15 and 16.^{76,77} On the contrary, oleic acid, a monounsaturated fatty acid, showed values of 22.5% by Lindmark Mansson (2008) and 21.8% by Gómez-Cortés & Amigo (2022), notably higher than the milks studied in this thesis.^{76,77}

Milk fatty acid composition is significantly influenced by seasonal diet shifts, metabolism, and ambient temperature. While summer grazing and heat alter specific lipid profiles, winter feeding typically increases the proportion of saturated fatty acids.^{76,78} Therefore, the exact fatty acid composition can greatly differ across different milk products.

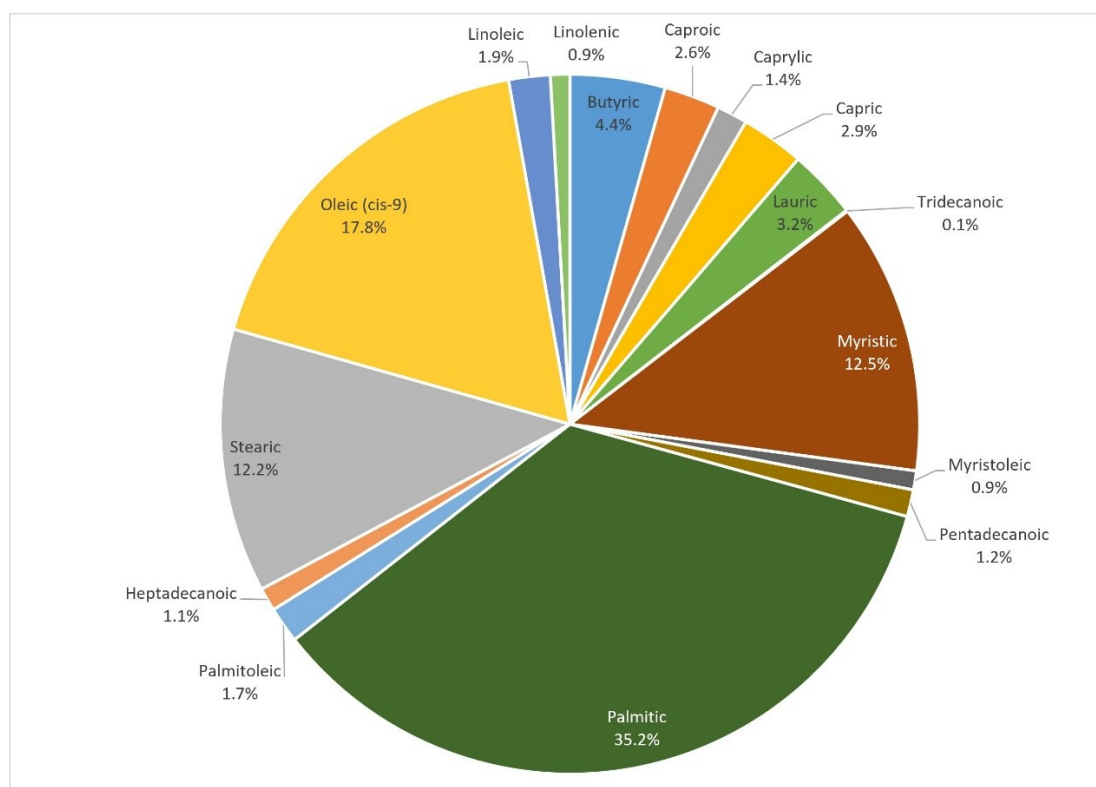


Figure 17: UHT-milk fatty acid composition expressed as percent by weight of total fatty acids. Mean values are shown (n=4)

The fatty acid profile of the UHT-milk (figure 17) also corresponds to the values of the aforementioned study.⁷⁶ Comparing the fatty acid composition of UHT-milk with LT-milk and HT-milk reveals a higher content of stearic acid in the UHT-milk. Likewise, the percentage of linolenic acid is almost twice as high as in the other two types of milk. The latter piece of information may indicate a higher future susceptibility to oxidation. It is known that the milk fatty acid profile undergoes distinct shifts in response to seasonal dietary changes, metabolic variations, and environmental temperatures, which may lead to differences across commercial products.⁷⁸

On the other hand, soy drink (figure 18) showed a clearly distinct fatty acid profile, compared to cow milk. The data largely aligned with the results of Peñalvo et al. (2004) with the exceptions that linoleic acid displayed a lower percentage (54.8%) and oleic acid showed higher values (20.4%).⁷⁹

Similarly, the lipid profile of soy drinks fluctuates based on cultivation and processing variables. Growth factors like climate and soil quality alter the bean's fatty acid synthesis, while storage and thermal extraction determine the final composition and its susceptibility to oxidation.⁸⁰

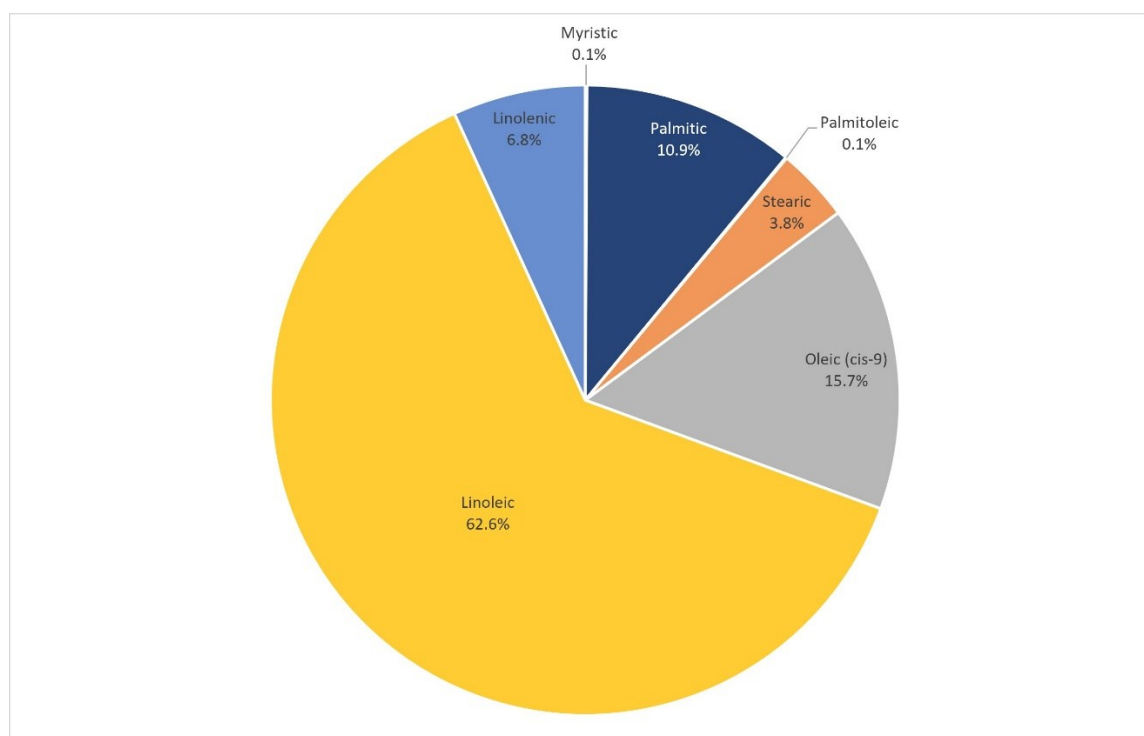


Figure 18: UHT-soy drink fatty acid composition expressed as percent by weight of total fatty acids. Mean values are shown (n=3).

The most notable difference in cow milk and soy drink lied on the ratio in the between the saturated and unsaturated fatty acids. Cow milk contained a higher amount of saturated fatty acids, in agreement with previous research from Gómez-Cortés & Amigo (2022), while it was evident that soy drinks were markedly richer in unsaturated fatty acids.⁷⁷ This was to be expected, as plant-based greens, such as soy, are known to contain a significantly higher amount of unsaturated fatty acids.⁷⁹ Consequently, in line with the previously discussed milk variants, the soy drink appears inherently more susceptible to lipid oxidation.

5.1.2 Lipid oxidation products via NMR

The initial approach to determine the lipid oxidation in milk and soy drink involved measuring the various acyl groups using ¹H-NMR. For this purpose, the bis-allylic proton signals of the polyunsaturated acyl groups (omega-3 triunsaturated and omega-6 diunsaturated) were evaluated. The results of the different acyl groups are being presented in figure 19 to 23.

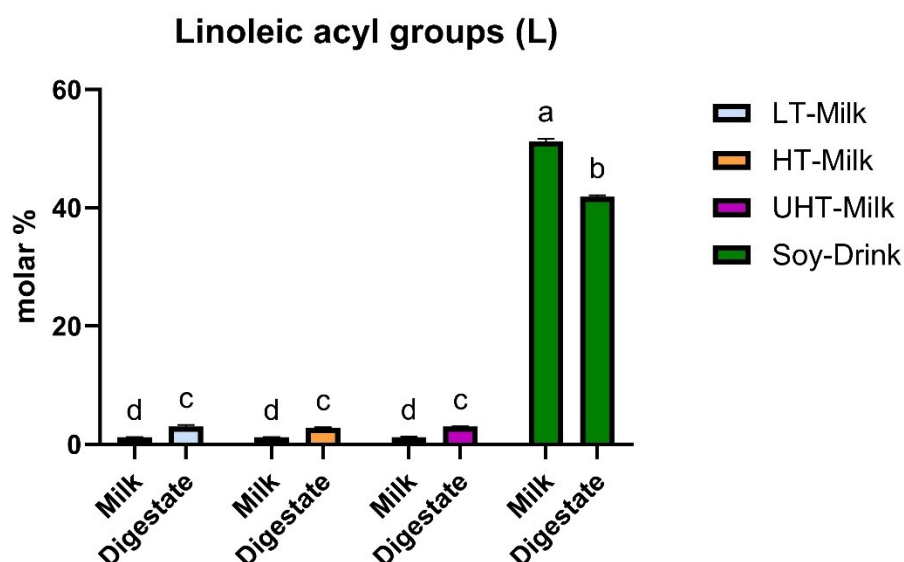


Figure 19: Comparison of linoleic acyl groups of different milk types and soy drink before and after in vitro digestion, in molar % related to AG + FA. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids

In figure 19 the linoleic acyl groups content in molar % is presented. Comparing the amount of linoleic acyl groups before and after digestion, it can be said that in all cow milks the concentration increased after digestion. Furthermore, there were no significant differences in figure 19 between the different processed milk types, a trend that was maintained for their digestates.

In contrast, the amount of linoleic acyl groups in the soy drink decreased during digestion. Martin-Rubio et al. (2021) reported similar results, with the content of linoleic acid decreasing after digestion in virgin and refined soybean oil. This may relate to the fact that linoleic acyl groups are vulnerable to autoxidation during in vitro digestion.⁸¹

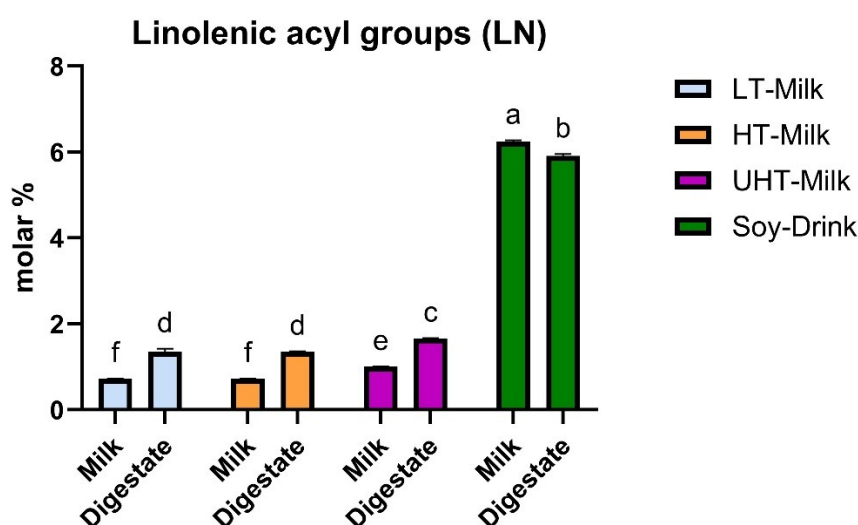


Figure 20: Comparison of linolenic acyl groups of different milk types and soy drink before and after in vitro digestion, in molar % related to AG + FA. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids

The results of linolenic acyl groups, presented in figure 20, showed similar trends as those of linoleic acyl groups. However, compared to the amount of linoleic acyl groups (figure 19), the content of linolenic acyl groups was significantly lower across the samples ($p < 0.05$).

In contrast to the linoleic acyl profiles, significant differences emerged in the linolenic acyl profile between UHT milk and the other two milk types, as well as their respective digestates. This aligns consistently with the FAME analysis, which reveals a higher proportion of linolenic acid in the UHT milk profile.

Although linolenic acyl groups were less abundant than linoleic acyl groups, they followed the same pattern in soy milk, with their molar percentage decreasing after digestion. This decline aligns with findings by Martin-Rubio et al. (2021) for virgin and refined soybean oil and can be attributed to the same underlying mechanisms as the linoleic acyl groups.⁸¹

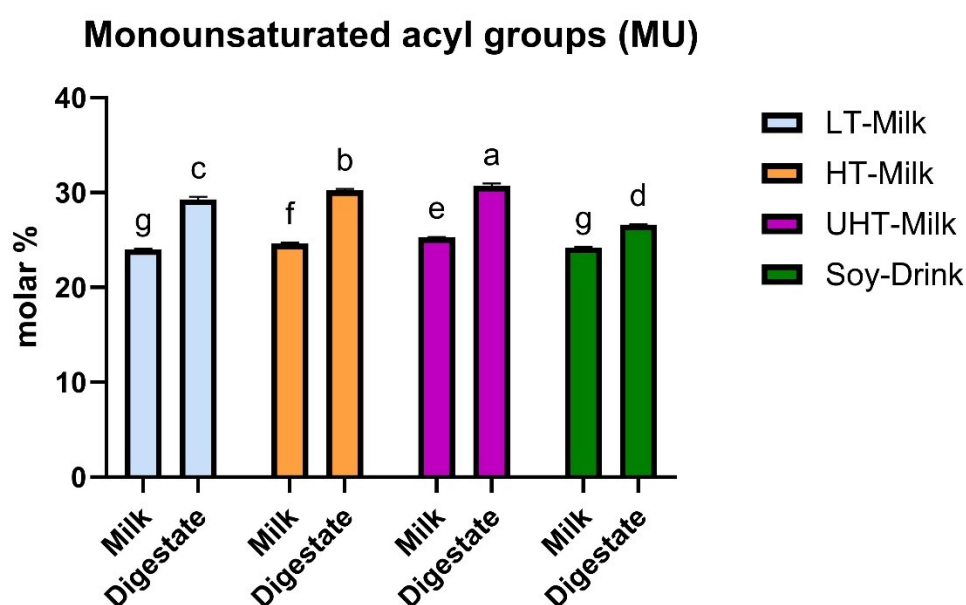


Figure 21: Comparison of monounsaturated acyl groups of different milk types and soy drink before and after in vitro digestion, in molar % related to AG + FA. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids

The results for monounsaturated acyl groups are illustrated in figure 21. In the case of all samples the same pattern could be observed. After digestion the amount of monounsaturated acyl groups increased. An increasing proportion of monounsaturated acyl groups in soy beverages is plausible. Since these groups exhibit higher oxidative stability than polyunsaturated acyl groups, the latter may contribute to this increase through the oxidative shift and/or the loss of double bonds.

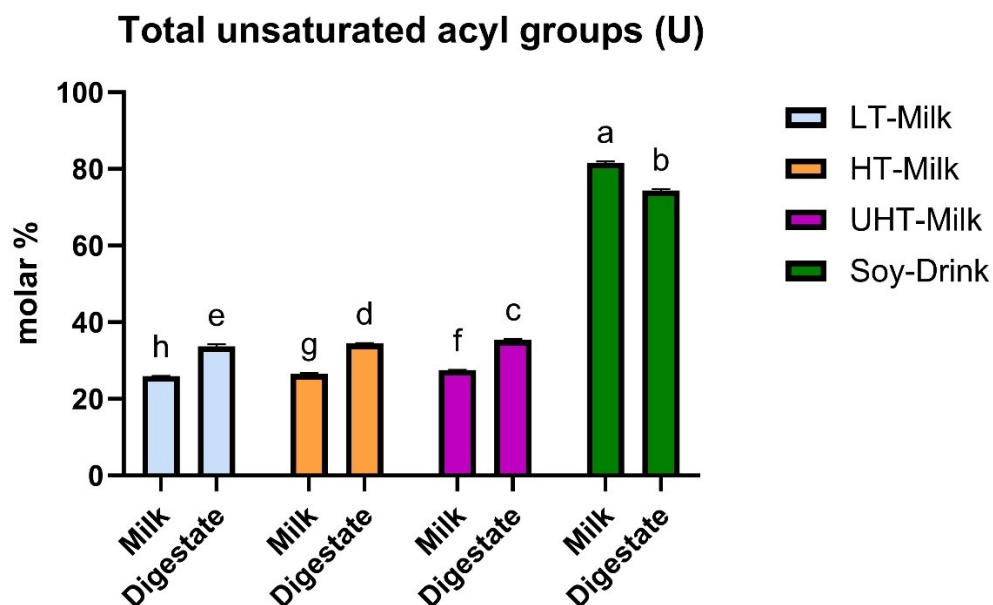


Figure 22: Comparison of total unsaturated acyl groups of different milk types and soy drink before and after in vitro digestion, in molar % related to AG +FA. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids

Figure 22 displays the total unsaturated acyl content. Across all three differently processed milk types, the concentration of total unsaturated acyl groups was lower before digestion than after. It is evident that unsaturated acyl groups behave differently from their saturated counterparts, exhibiting an opposite trend (figure 23).

Conversely, the soy drink exhibited the opposite trend, showing a significant decrease in total unsaturated acyl groups after digestion, clearly linked to the decrease of polyunsaturated acyl groups. This aligns with the findings of Martin-Rubio et al. (2021) for soybean oil.⁸¹

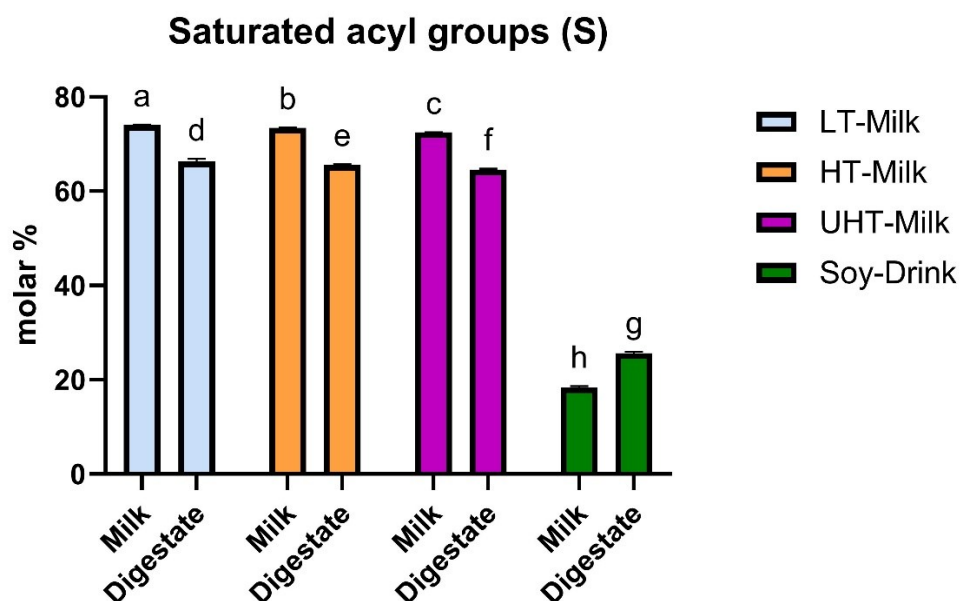


Figure 23: Comparison of saturated acyl groups of different milk types and soy drink before and after in vitro digestion, in molar % related to AG + FA. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

As shown in figure 23, the level of saturated acyl groups decreased during digestion in processed milk types, whereas the exact opposite behavior was observed for soy drink.

Contrary to the expected decline in unsaturated and rise in saturated acyl groups, the dairy milk samples showed the opposite trend. This deviation may stem from the loss of less lipophilic, short-chain lipids during Folch extraction, which are not present in soy drink. As enzymatic hydrolysis during milk digestion may release short-chain saturated acyl groups (e.g. butyric acyl groups) as fatty acids, their recovery from the digestate samples may have been hindered, thereby decreasing the total content of saturated acyl groups. This apparent discrepancy between the milk fatty acid composition by GC-FID (3.9-4.4% butyric acid) and the decrease of saturated acyl groups (mean difference of 8% pre and post digestion) can be primarily attributed to the different units of measurement. While the initial fatty acid profile is expressed in weight percentage (wt%), the subsequent digestate analysis is based on molar percentage (molar % related to AG and FA) and therefore not completely comparable, as a certain molar % for butyric acyl groups is bound to contribute less to the total fat weight than the same molar % for longer-chain acyl groups.

Apart from the evaluation of different acyl groups, primary and secondary oxidation products were also analyzed using NMR. Primary lipid oxidation products like hydroperoxy-conjugated dienes (CD-OOH) and secondary lipid oxidation products like hydroxy-conjugated dienes (CD-OH) were determined. In addition, the thesis focused on another secondary oxidation product group, which are the *n*-alkanals.⁸²

Primary conjugated diene hydroperoxides exhibit characteristic stereoisomerism involving *cis* and *trans* double bonds. The primary kinetic product is an unstable conjugated *cis,trans* isomer ((*Z,E*)-

CD-OOH). The thermodynamically later-forming product is a *trans,trans* isomer ((*E,E*)-CD-OOH) and is more stable.⁸³ Figure 24 illustrates the results of the hydroperoxy-conjugated dienes isomer (*E,E*)-CD-OOH in the different processed milk types.

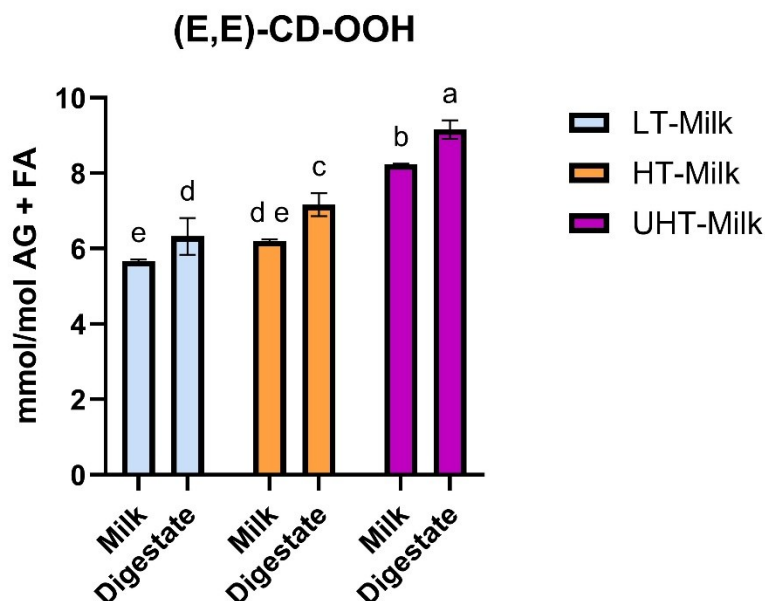


Figure 24: Comparison of *trans,trans*-conjugated dienes associated to hydroperoxides ((*E,E*)-CD-OOH) before and after digestion in milk samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

It can be observed that with a higher temperature treatment during milk processing the concentration of (*E,E*)-CD-OOHs significantly increased. Despite distinct differences among the milk types and their digestates, (*E,E*)-CD-OOH concentrations increased across all samples after digestion.

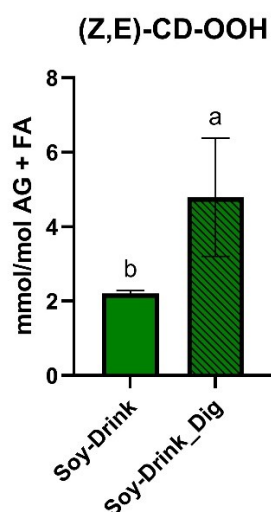


Figure 25: Comparison of *cis,trans*-conjugated dienes associated to hydroperoxides ((*Z,E*)-CD-OOH) in the soy drink sample and its digestate. Statistical analysis was performed using an unpaired, two-tailed Welch's t-test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

In the case of the soy drink it can be observed an increase of (Z,E)-CD-OOHs after digestion (figure 25). In the literature it is confirmed that this kind of hydroperoxide product can be present in soy drinks.⁸⁴

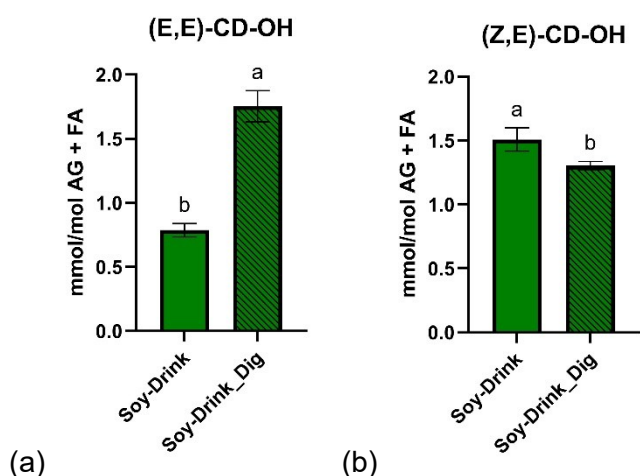


Figure 26: Comparison of (a) *trans,trans*-conjugated dienes associated to hydroxides ((E,E)-CD-OH) and (b) *cis,trans*-conjugated dienes associated to hydroxides ((Z,E)-CD-OH) in the soy drink sample and its digestate. Statistical analysis was performed using an unpaired, two-tailed t-test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

As oxidation continues, hydroxy conjugated dienes can be formed out of the previous hydroperoxy conjugated dienes. In figure 26a the results of (E,E)-CD-OH for soy drink are presented.

The increase in (E,E)-CD-OHs during digestion can be explained by the fact that this isomer can originate from the preceding hydroperoxides, (E,E)-CD-OOHs and (Z,E)-CD-OOHs. In addition, it can be formed through the isomerization of (Z,E)-CD-OHs. This mechanism would also account for the decrease in the (Z,E)-CD-OH isomer observed in Figure 26b.⁸¹

It should be acknowledged that this type of hydroxides was detected and quantified in this master thesis as opposed to Martin-Rubio et al. (2021), who did not detect these oxidized conjugated dienes in soybean oil.⁸¹ This leads to the conclusion that different processing can lead to the generation of a different oxidation products profile. So far, there is no evidence in the scientific literature of the presence of these specific oxidation products in bovine milk.

On the other hand, figure 26b presents the results of the (Z,E)-CD-OH isomers. Similar to the (E,E)-CD-OHs, these products were only found in the soy drink and its digestate. Nevertheless, they displayed a contrasting trend during digestion, as it can be seen that the concentration of (Z,E)-CD-OH was slightly higher in the soy drink than in the digestate.

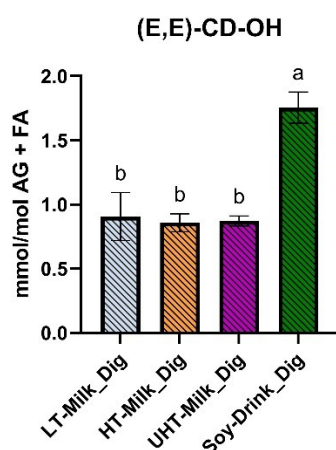


Figure 27: Comparison of *trans,trans*-conjugated dienes associated to hydroxides ((*E,E*)-CD-OH) in the different digestate samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

Figure 27 represents the results for the (*E,E*)-CD-OH in the digestates of the different samples previously displayed for the soy drink and its digestate (figure 26). As shown, the milk digestates contained this kind of hydroxide as well, among which there were no significant differences. In contrast, distinct differences were evident when comparing the cow's milk digestates to the soy drink digestate. However, the formation of (*E,E*)-CD-OH derived from the preceding hydroperoxide remained observable throughout the digestion process.

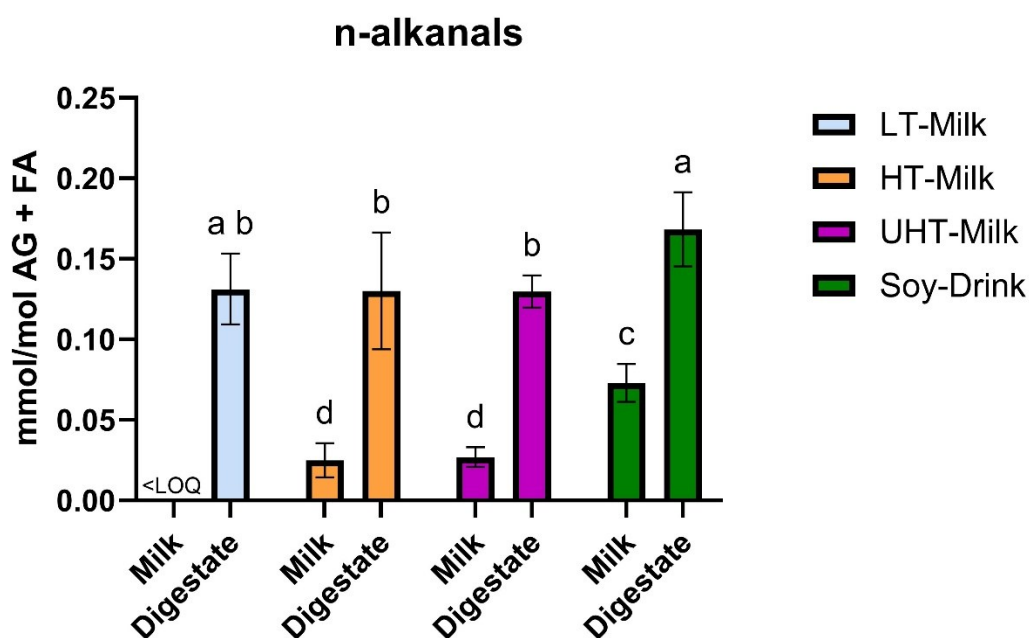


Figure 28: Comparison of n-alkanals in the different samples before and after in vitro digestion. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$. AG + FA: acyl groups + fatty acids.

As a further example of secondary lipid oxidation products, n-alkanals were detected and the results are shown in figure 28. Even if the amount of n-alkanals was very low, compared to the conjugated diene groups, it was still quantifiable in almost all samples.

A higher content of n-alkanals was observed in the more intensively heat-treated milk types (HT and UHT), whereas they remained undetected in the low-temperature (LT) milk. Although subsequent in vitro digestion triggered a general increase in secondary lipid oxidation products ($p < 0.05$), it simultaneously minimized inter-sample variations, resulting in a lack of significant differences ($p > 0.05$) between the milk types post-digestion. Comparing the soy drink to the cow milk samples, significant differences were observed in both the undigested and digested samples. The study of Martin-Rubio et al. (2021) showed a matching trend, namely the increase in n-alkanals during digestion in soybean oil, only with higher concentrations. This leads to the conclusion, that digestion promotes overall lipid oxidation across all samples.⁸¹

The soy drink was the only sample that fully exhibited the expected behavior regarding lipid oxidation. Following in vitro digestion, a significant decrease in unsaturated acyl groups was observed, accompanied by a simultaneous increase in lipid oxidation products. Furthermore, a measurable increase in oxidized lipid products post-digestion confirmed that oxidation also took place within the bovine milk. Before digestion, the milk samples had been subjected to different industrial processing treatments (LT, HT, and UHT), which typically alter the initial oxidative status and the milk fat profile. Oxidation variations among the processed milks suggest that both composition and heating temperature influence the outcome. The high linolenic acid content in UHT milk likely drove its pronounced oxidation, supporting the idea that initial fat composition dictates oxidative stability. Nevertheless, because LT and HT milks share the same fatty acid profile, their differences cannot be composition driven. Since HT milk showed a higher accumulation of the same markers than LT milk, despite their digestates, temperature for pasteurization remains a key factor for advancing lipid oxidation.

5.1.3 Lipid oxidation products via LC-QTOF

The results of the lipid oxidation products, analyzed via LC-QTOF, were visualized using heat maps and a principal component analysis (PCA) plot.

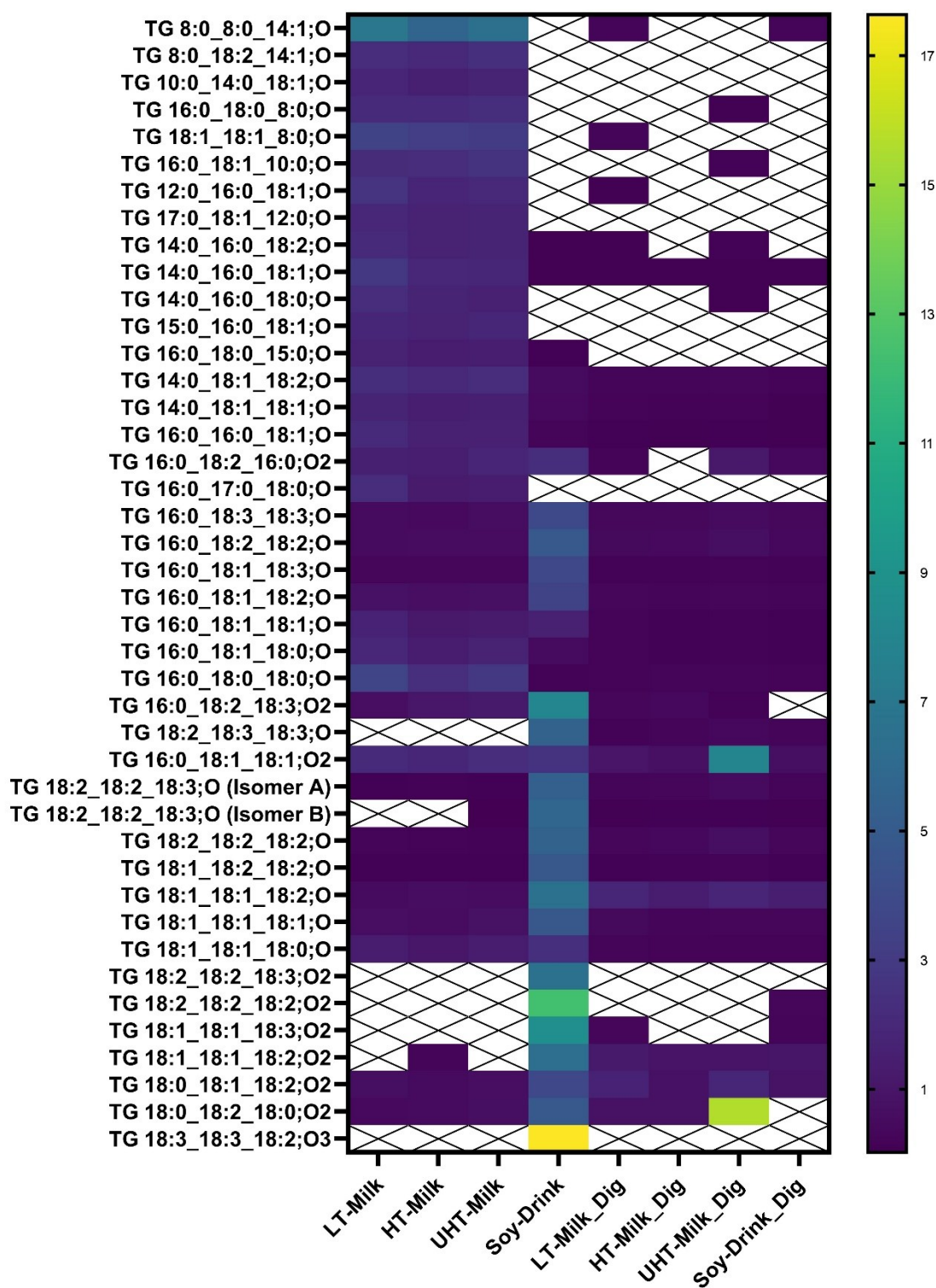


Figure 29: Overview of the oxidized triacylglycerols (TG) within the samples and their digestates. This heat map shows all semi-quantified oxidized TG with their area values, normalized to a quality control (QC) sample. The color intensity corresponds to the normalized area of each TG. Data is expressed as mean (n=4)

The heat map in figure 29 illustrates the profile of the 42 oxidized triacylglycerols (TGs) identified within the samples and their respective digestates. Notably, oxidized TGs rich in medium-chain saturated fatty acids appeared to be concentrated predominantly in the undigested cow milk samples. This trend is indicated by the first eight TGs, for example TG 8:0_8:0_14:1;O, TG 10:0_14:0_18:1;O and TG 16:0_18:0_8:0;O, shown in figure 29. This is in agreement with the fatty acid profiles determined in this thesis (figure 15-18), as these TGs were only detected in the cow milk samples and remained undetected in the soy beverage. Fittingly, triacylglycerols (TG 18:2_18:2_18:3;O₂, TG 18:2_18:2_18:2;O₂ and TG 18:3_18:3_18:2;O₃) with higher acyl chain lengths and number of unsaturations could be detected and semi-quantified in the soy drink. Moreover, oxidized TGs (TG 16:0_17:0_18:0;O and TG 16:0_18:0_18:0;O) that contain saturated fatty acids were significantly more abundant in milk compared to the soy drink. Occasionally, some of these oxidized TGs were detected in the digestate samples, but with very low peak areas. A similar trend can be spotted in the study of Zhao et al. (2024) investigating native TGs in milk formulars. While their study monitored the decrease of non-oxidized TGs, the present thesis specifically tracks the oxidized TGs, adding a new layer of information on the fate of milk and soy drink lipids under gastrointestinal conditions. Thus, both studies demonstrate that a fraction of TGs, regardless of their oxidative state, escapes complete hydrolysis during gastrointestinal digestion and remains detectable in the digestates.⁸⁵ This behavior after digestion was also observed in other food, such as the study by Martin et al. (2010), where TGs remained in fish oil after in vitro digestion.⁸⁶ Therefore, the persistence of residual TGs in the final digestate constitutes a standard behavior in food lipidomics.

Another noteworthy trend relates to the degree of oxidation. TGs with multiple oxidation groups, such as TG 18:2_18:2_18:3;O₂, TG 18:2_18:2_18:2;O₂, and TG 18:3_18:3_18:2;O₃, exhibited a higher relative abundance in the soy drink, driven by its high content of easily oxidizable polyunsaturated fatty acids (figure 18). Notably, TG 18:3_18:3_18:2;O₃ was detected in the soy beverage and showed the highest overall signal intensity, which aligns with the soy drink's initial fatty acid profile. Moreover, the exceptionally high abundance of certain species like TG 16:0_18:1_18:1;O₂ and TG 18:0_18:2_18:0;O₂ (figure 29) in the UHT milk digestate requires further investigation. These molecules potentially represent hydroperoxides formed during early oxidation stages, or dihydroxides generated during advanced oxidation where acyl chain double bonds are lost. However, other pathways cannot be ruled out, as these signals could also stem from alternative structures such as two epoxides, hydroxy-epoxides, or keto-hydroxy derivatives.

Therefore, future research using retentions time discrimination is necessary to fully characterize the identity of these oxidation groups, since there are no commercially available standards for oxTGs, oxDGs and oxMGs.

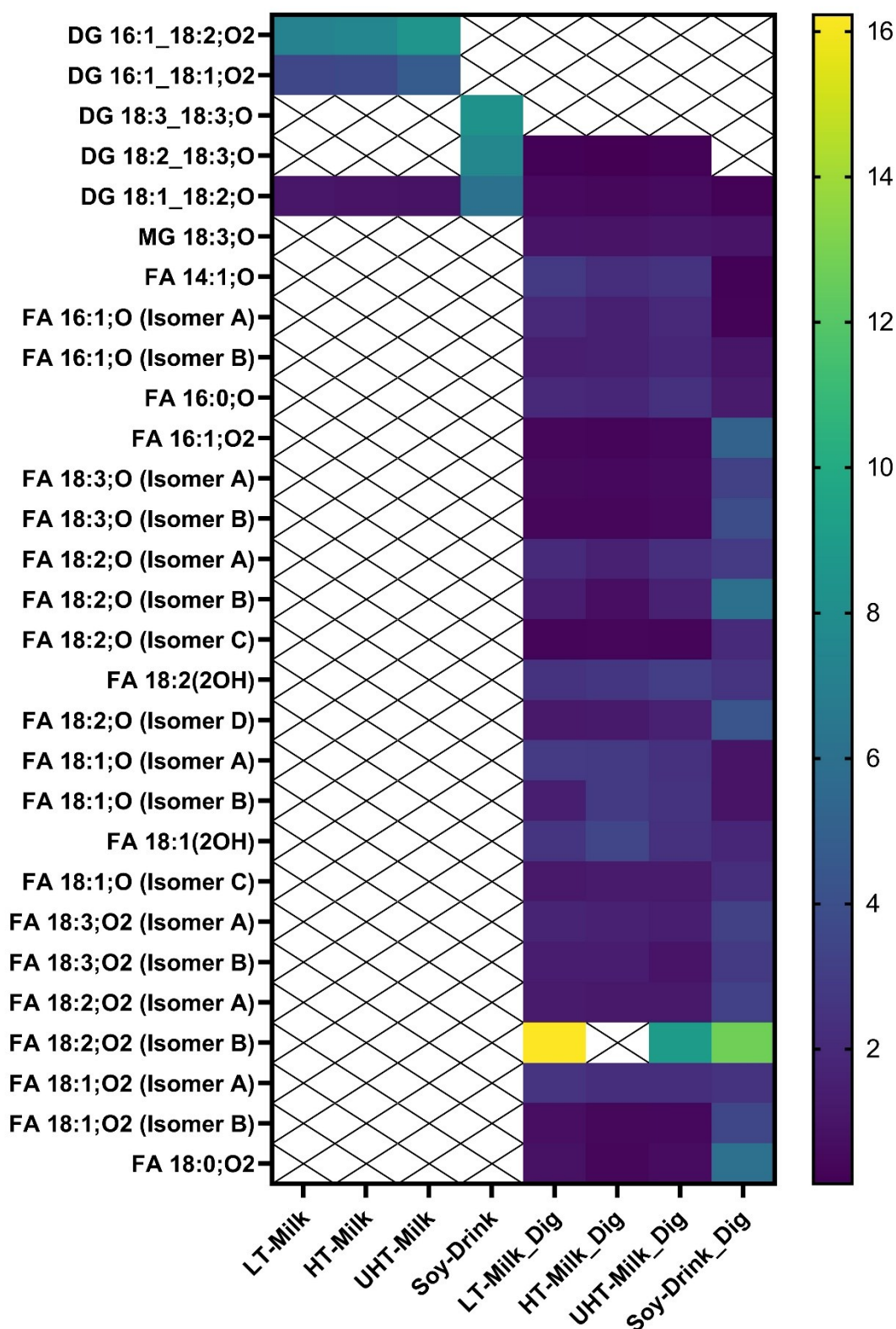


Figure 30: Overview of the oxidized diacylglycerols (DG), monoacylglycerols (MG) and free fatty acids (FA) within the samples and their digestates. This heat map shows all semi-quantified DG, MG and FA with their normalized area values, normalized to a quality control (QC) sample. The color intensity corresponds to the normalized area of each lipid. Data is expressed as mean (n=4).

In the heat map of figure 30 the focus is placed on other oxidized acylglycerols and free fatty acids. A matching distribution pattern was evident for DG 16:1_18:2;O₂ and DG 16:1_18:1;O₂. These two oxidized DGs were semi-quantified with higher areas in the cow milk samples. This finding is strongly

supported by the corresponding fatty acid profiles (figures 15-18), which demonstrate a significantly higher abundance of palmitoleic acid (16:1) in cow milk compared to the soy drink, thereby providing the necessary substrate for the formation of these specific DGs species.

Conversely, DG 18:3_18:3;O, DG 18:2_18:3;O, and DG 18:1_18:2;O were predominantly found in the soy drink sample. This abundance reflects the fatty acid profile of the soy drink (figure 18), which is characteristically rich in linoleic and linolenic acids.

Taking a closer look into the behavior of DG 18:2_18:3;O and DG 18:1_18:2;O before and after digestion, some similarities have been described in the literature for their non-oxidized counterparts. The decrease of the oxidized species DG 18:2_18:3;O and DG 18:1_18:2;O in the soy drink after digestion aligns with the digestive behavior described by Xu et al. (2023), who focused on monitoring non-oxidized DGs in soybean oil.⁸⁷ The detection and tracking of these specific oxidized DG species during digestion represents a novel finding of this thesis, as these oxidized forms have not been previously identified in milk.

Only one compound was identified among oxidized monoacylglycerols (MGs): MG 18:3;O. Regarding its areas before digestion, levels were found to be undetectable. However, following digestion, the increased formation of oxidized MGs due to lipolysis was plausible. This trend was previously described by Zhao et al. (2024) for infant formulas, albeit limited to non-oxidized species.⁸⁵ This is, therefore, the first time that such an oxidized species has been detected in milk and soy drink digestates.

Lastly, the heat map in figure 30 lists numerous different oxidized free fatty acids. As expected, the content of oxidized FA was semi-quantified in the digestates, but not in the undigested samples.

However, a few distinct exceptions were observed. For instance, one of the oxidized isomers corresponding to FA 18:3 can be highlighted, as the statistical analysis reveals a significant difference in its content between UHT and both LT and HT milk ($p < 0.05$), which is documented in Appendix figure 16. Due to the higher initial concentration of linolenic acid in UHT milk (figure 17), the proportion of oxidized linolenic acid in its digestate was significantly higher than in the other two processed milk digestates ($p < 0.05$).

An extraordinary behavior is visible for FA 18:2;O₂. Compared to other oxidized FAs, an extremely high proportion of FA 18:2;O₂ is evident in the digestate from LT milk, followed by soy drink and UHT milk. It seems like that free linoleic acid in LT milk is more susceptible to double oxidation than the UHT processed products. Interestingly, no statistically significant differences were observed among these three digestates ($p > 0.05$; see Appendix figure 17). The study from Zhao et al. (2024) shows an increase in non-oxidized FA 18:2 after gastrointestinal digestion, making it more susceptible to oxidation, similar to other unsaturated fatty acids.⁸⁵ Overall, the different milk processing types did not lead to statistically significant differences in the majority of the oxidized fatty acid species found ($p > 0.05$). This contrasts with the NMR data showing the content of (*E,E*)-CD-OOHs (figure 24), where significant differences ($p < 0.05$) between the different thermally processed milk types after digestion were observed. However, NMR also displayed the absence of processing related

significant differences ($p > 0.05$) after digestion for (*E,E*)-CD-OHs (figure 27) and n-alkanals (figure 28), a trend shared with the most oxidized fatty acids detected by LC-QTOF. Therefore, the overall results showed a clear downplay on the influence of milk processing once digestion took place.

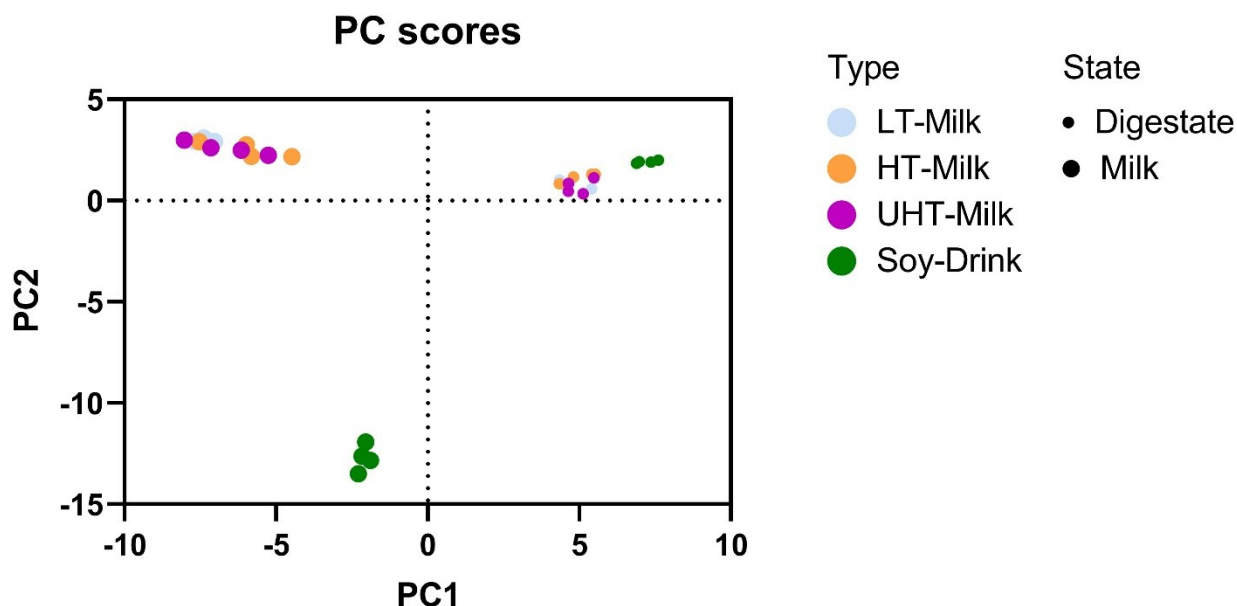


Figure 31: Principal Component Analysis (PCA) score plot of sample profiles using all oxidized lipid species that have been detected. Samples and their digestates are projected onto the first two principal components (PC1 and PC2), which explain 83.4% of the total variance, respectively. Colors denote the different processed cow milk and soy drink, and the size of the dots represents the samples before and after digestion, revealing distinct clustering of the milk samples to the soy drink samples.

In figure 31 the score plot of the principal component analysis is shown. The variables used for this PCA plot were all oxidized lipid species, previously listed in figure 29 and 30. The application of PCA effectively simplifies the complex lipidomic data, highlighting the main variances and directional trends between the cow milk and soy drink samples before and after in vitro digestion.

Clear differences were observed between cow milk and soy drinks, as well as before and after digestion. However, it was not possible to distinguish between the different LT, HT and UHT milk individually. The results of the principal component analysis (PCA) carry significant implications for physiological relevance. During digestion, lipids previously oxidized by thermal treatment undergo subsequent oxidative degradation. This metabolized lipid matrix leads to a substantial convergence of the initially distinct profiles of milk and soy beverages. Consequently, the marketed attributes, namely the 'freshness' of milk and the 'healthier fatty acid profile' of soy drinks, do not necessarily reflect the biochemical reality encountered by intestinal cells. While consumers typically evaluate food products based on their initial state and retail marketing claims, these PCA data demonstrate that gastrointestinal processing is the critical determinant for physiological impact. The perceived health benefits driving consumer purchasing decisions are ultimately could be potentially compromised by the generation of lipid oxidation products within the gastrointestinal tract. Regardless, other chemical reactions, such as the Maillard reaction during heat treatment and digestion, must also be taken into consideration.

5.1 Advanced glycation end products (AGEs)

For the determination of AGEs and furosine, both quantitative and semi-quantitative approaches were taken. While standards were used for the quantitative determination of CML, CEL, GOLD, MOLD, MG-H1, G-H1 and furosine, the normalized peak areas of pentosidine, argpyrimidine, L-arginine and L-lysine were compared, which provided only a comparative indication of the increase in these processing-related compounds. Furthermore, the results examine the occurrence of the different Maillard reaction products in the protein-bound and free form.

5.1.1 Protein-bound furosine and AGEs

The results shown in the following figures are expressed in $\mu\text{g/g}$ protein with the corresponding results in $\mu\text{g/g}$ milk shown in the Appendix. The protein content used to calculate the values in $\mu\text{g/g}$ protein was determined by the Kjeldahl method and is available in Appendix table 2.

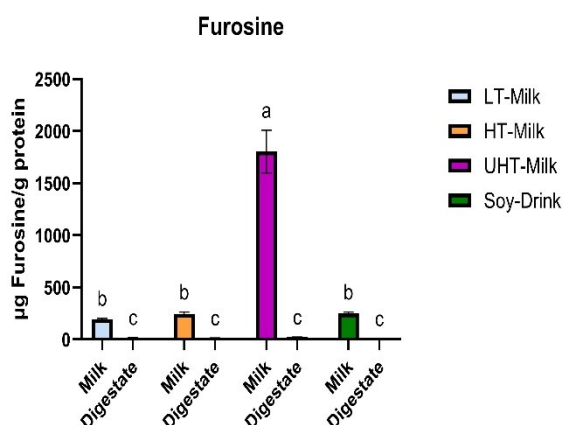


Figure 32: Concentration of protein-bound furosine in $\mu\text{g furosine/g protein}$ before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

While not an AGE, furosine was determined in this thesis. It is one of the best-known Amadori products and serves as a representative of early stage Maillard reaction.^{7,88} Taking a look into the results of furosine in figure 32, it can be seen that the highest concentration was clearly in the UHT-milk. The rest of the undigested samples shared similar concentrations, with no significant differences between them. This notable furosine concentration gap between UHT-milk and soy drink was illustrated as well in the study by Pucci et al. (2024).⁷ One logical observation that can be drawn from Figure 32 is that the heat treatment during milk processing had an impact on the promotion of furosine. As the formation of Maillard reaction products is promoted by higher temperature, harsher processing conditions, in this case UHT treatment, likely resulted in higher furosine concentrations compared to LT and HT milk processing.⁸⁸

After digestion a notably marked decrease of the furosine concentration could be observed in all samples, although its content was still detectable and quantifiable. The main reason for such behavior, which is mentioned as well in the study by Wei et al. (2026) can be explained as follows: Amadori products do not represent the endpoint of the reaction. Furosine accumulation is affected by follow-

up reactions that produce intermediates such as α -dicarbonyls (α -DCs) and 5-hydroxymethylfurfural (5-HMF).⁸⁹ Since furosine is an early-stage glycation product and not the final product of the Maillard reaction, tracking the progression toward advanced glycation end-products (AGEs) becomes necessary to better understand the influence of milk processing and digestion.

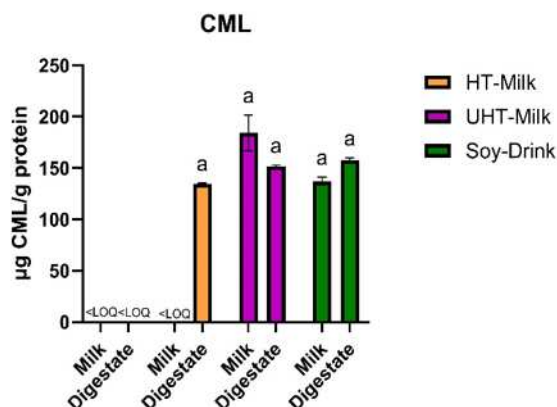


Figure 33: Concentration of protein-bound Nε-Carboxymethyllysine (CML) in µg CML/g protein before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

A key AGE that is often measured in dairy products and soy beverages is CML.^{7,88} Figure 33 illustrates the CML content before and after in vitro digestion. It can be highlighted that CML was detectable in LT and HT milk but could not be quantified. CML was, however, quantifiable in the digestate of HT milk. Regarding the UHT milk and soy drink, CML was found in quantifiable amounts before as well as after digestion. However, the changes in concentration for both UHT milk and the soy drink after digestion were not statistically significant, indicating that the levels remained essentially unchanged. Compared to the findings of Li et al. (2023), who reported values between 200 and 400 µg/100 g of milk, the undigested samples in the present study exhibited a higher mean concentration of 580 µg/100 g milk of UHT milk. However, CML values may vary due to storage conditions and the sterilization process.⁹⁰

A concentration of 100 µg/100 g milk for undigested soy drink has been stated in the literature by Pucci et al. (2024), whereas a mean level of 390 µg/100 g milk was determined in the present thesis.⁷ This variation can probably be attributed to differences in the soybean varieties as well as the exact processing techniques utilized by the manufacturer.⁸⁰

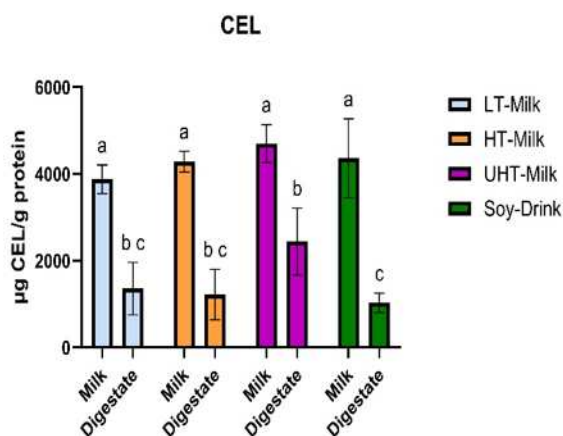


Figure 34: Concentration of protein-bound Nε-Carboxyethyllysine (CEL) in µg CEL/g protein before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Nε-Carboxyethyllysine (CEL), which is structurally similar to CML with the difference of holding an ethyl group instead of a methyl one, is also frequently analyzed in the determination of AGEs.^{7,88} As it can be seen in figure 34, there was a clear decrease in protein-bound CEL after digestion in all three types of milk and in the soy drink. The same digestive pattern for CEL is evident in the study by Bieck et al. (2025), although it was examined only in the case of soy drinks.⁶⁵ Moreover, the bar chart shows that the CEL concentrations in all samples did not differ significantly before digestion. However, significant differences were observed after digestion, but only between the digestates of UHT and soy drink, the latter displaying a lower protein-bound CEL concentration. There were no significant differences between the digestates of LT-, HT- and UHT-milk.

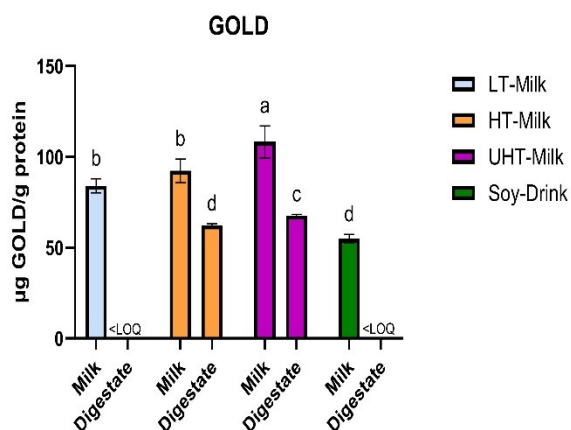


Figure 35: Concentration of protein-bound glyoxal-derived lysine dimer (GOLD) in µg GOLD/g protein before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Furthermore, glyoxal-derived lysine dimer (GOLD) was quantified in a protein-bound state in most of the samples. In figure 35 it can be seen that GOLD was more likely to be protein-bound before digestion. Thus, a significant decrease in GOLD concentration after digestion could be clearly observed in all samples. The study by Zhang et al. (2023) highlights a similar pattern following

digestion. They used whey protein instead of milk, and a decrease in GOLD was detected and even the addition of glyoxal (GO) had no impact.⁹¹ This kind of similar decline was already seen in CEL (figure 34).

In LT milk and the soy drink, nearly all protein-bound GOLD was depleted after digestion. Distinct variations in the GOLD content were evident across the different heat treatments. In the bar chart of figure 35 significant higher concentrations of GOLD were noted in UHT milk compared to LT and HT milk. This confirms that harsher thermal processing intensifies the formation of this specific compound.

Regarding the soy drink, the GOLD concentration was clearly lower than in cow milks. This difference in concentration can be explained by the fact that cow milk contains more L-lysine than soy drink. This fact is described as well in Pucci et al. (2024).⁷

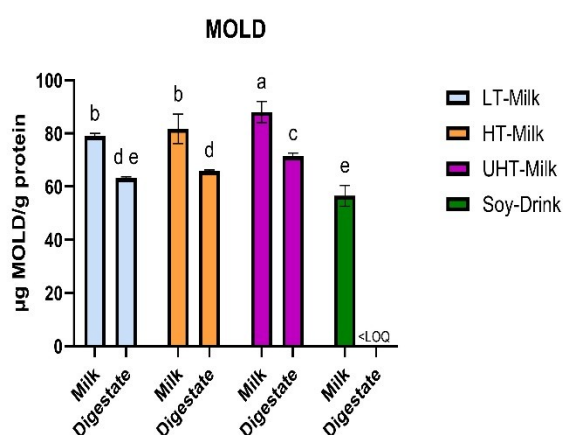


Figure 36: Concentration of protein-bound methylglyoxal-derived lysine dimer (MOLD) in µg MOLD/g protein before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Figure 36 represents the concentration of MOLD in the different samples before and after digestion. The behavior was almost identical to that of GOLD in figure 35, albeit with slightly lower concentrations. Specifically, UHT milk again exhibited the highest concentration compared to LT and HT milk, when expressed in relation to protein content. Furthermore, after digestion, protein-bound MOLD was detected in soy milk, but with values below LOQ.

In this case the same reasons as for GOLD can be attributed to these results, due to the similar chemical structures of MOLD and GOLD.

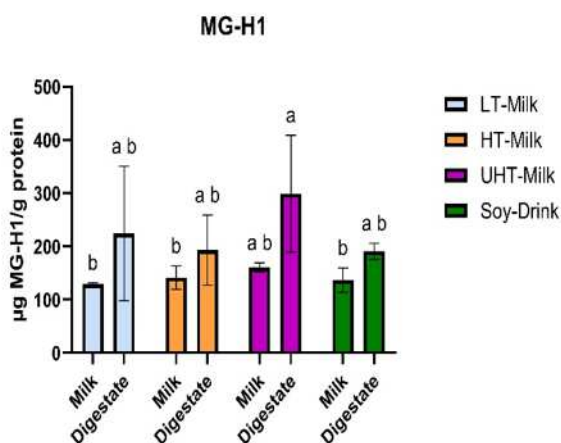


Figure 37: Concentration of protein-bound methylglyoxal-derived hydroimidazolone (MG-H1) in $\mu\text{g MG-H1/g protein}$ before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Another advanced glycation end-product that is commonly formed in foods and analyzed in many studies, is MG-H1.⁹⁰ Figure 37 compares the MG-H1 concentration before and after digestion in the analyzed samples. No statistically significant differences in protein-bound MG-H1 concentrations were observed before and after digestion across all samples in the bar chart. Regarding the MG-H1 values of the undigested cow milk samples, this thesis determined a mean concentration of 490 $\mu\text{g}/100\text{ g milk}$. In contrast, the values of MG-H1 in cow milk (690 $\mu\text{g}/100\text{ g milk}$) reported by Bieck et al. (2025) were higher than the finding in this thesis.⁶⁵ This difference could be explained by storing conditions applied.⁹⁰

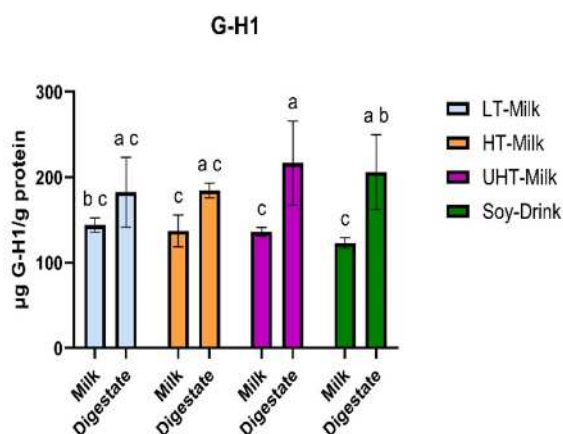


Figure 38: Concentration of protein-bound glyoxal-hydroimidazolone (G-H1) in $\mu\text{g G-H1/g protein}$ before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

As already mentioned in the results from MOLD and GOLD, where similar structural properties resulted in the same behavior, the same applied for MG-H1 and G-H1. However, the concentrations of protein-bound G-H1 significantly rose after digestion of UHT milk and soy drink, a key difference compared to the MG-H1 results. A possible explanation for these results could be that protein-bound MG-H1 was more efficiently digested.

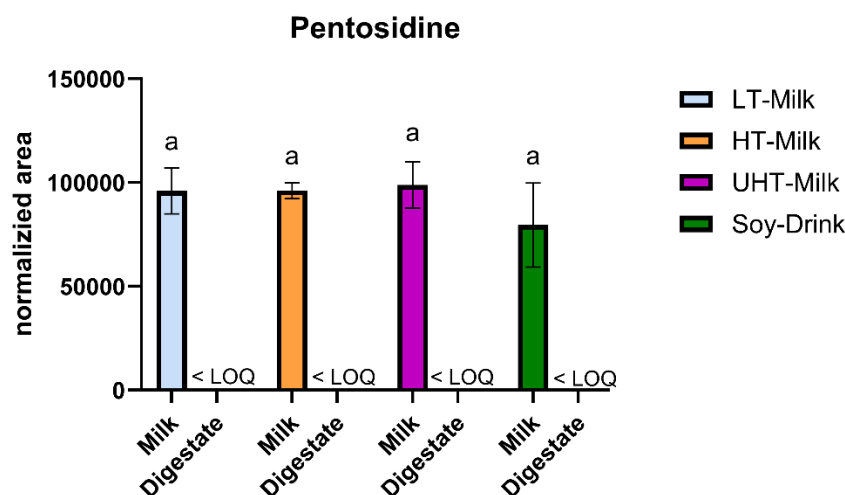


Figure 39: Semi-quantitative comparison of normalized areas of protein-bound pentosidine before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Continuing with the results of those substances that were only analyzed semi-quantitatively, the results from pentosidine are presented in figure 39. In this case, there were no significant differences between the normalized areas of the undigested milk and soy drink samples. In addition, this AGE was not detected in protein-bound form in the digestates of each sample type. A study by Zenker et al. (2020), where heat treatments different as those pasteurization were applied to milk powder, pointed out that pentosidine is more likely to be protein-bound.⁹² This evidence supports the results of this thesis in figure 39, where pentosidine was only found in protein-bound form before digestion.

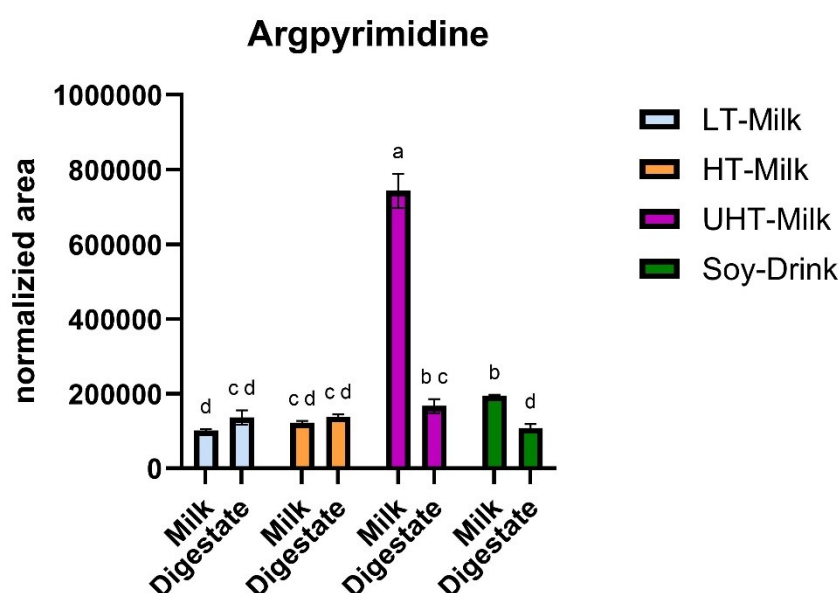


Figure 40: Semi-quantitative comparison of the normalized areas of protein-bound argpyrimidine before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

In figure 40 the normalized areas of the milk samples and soy drink before and after digestion are presented for argpyrimidine. The normalized peak area of argpyrimidine was initially higher in UHT milk than in the other milk types and the plant-based drink. However, digestion led to a distinct reduction within the UHT group, resulting in an alignment of values across all milk types. Semi-quantitative assessment revealed that the areas for argpyrimidine remained statistically unchanged in LT and HT milk during in vitro digestion. In contrast, the soy beverage exhibited a significant reduction in the argpyrimidine area after digestion. This kind of behavior has not been reported in current literature.

The concentration of pentosidine and argpyrimidine depends heavily on the concentrations of the reaction substances, like methylglyoxal (MGO) or glyoxal (GO) and the amino acids like lysine and arginine, as shown in the study of Zhang et al. (2023).⁹¹ An increase could be possible after gastrointestinal digestion as Zhang et al. (2023) showed in whey protein, but also a decrease is conceivable due to the fact that protein-bound AGEs can be released after digestion and remained in a free form state. This was already found in the study of Bieck et al. (2025) in soydrink for other AGEs substances like CML or CEL.⁶⁵

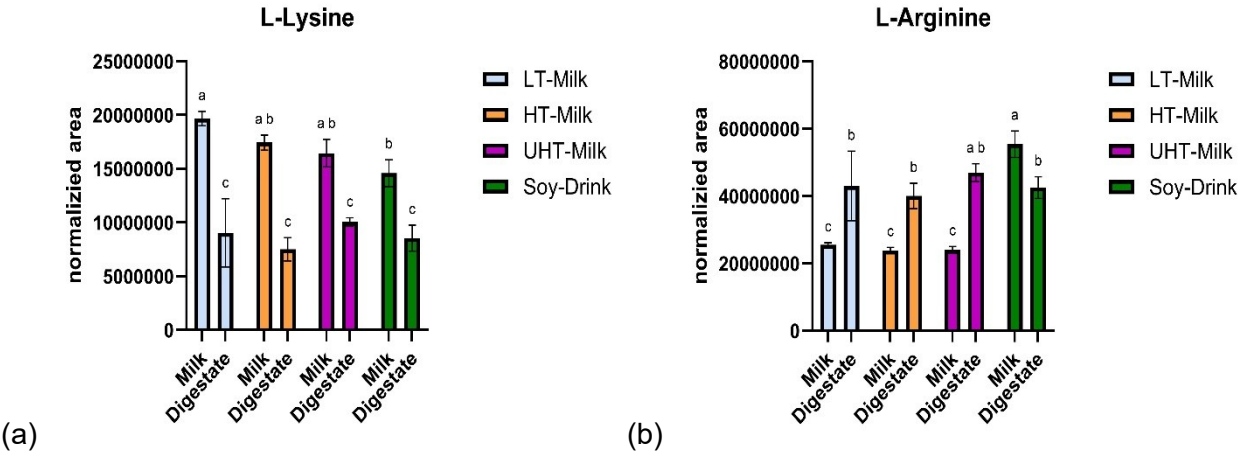


Figure 41: Semi-quantitative comparison of the normalized areas of protein-bound (a) L-lysine and (b) L-arginine before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Precursor amino acids were semi-quantified to help contextualize AGE formation during digestion. The results of protein-bound L-lysine are presented in figure 41a, where the values are normalized areas before and after digestion. The statistical analysis revealed that only the lysine content of LT-milk was significantly higher than the soy drink. Fittingly, the study by Pucci et al. (2024) demonstrated that lysine concentration in soy drink was significantly lower than in cow milk.⁷ It can be observed in figure 41a that in all 4 different samples the lysine concentration significantly decreased after digestion, to similar levels for all drink types.

It is well known that during digestion amino acid release as well as the Maillard reaction can take place and therefore a decrease in protein-bound L-lysine is expected. It can be found as free L-lysine or react with other substances like reducing sugars to form advanced glycation products such as

CML. This kind of behavior was already reported in the literature where an increase in free L-lysine during digestion was observed.⁹³

On the other hand, the results of the protein-bound L-arginine are visualized in figure 41b. One noteworthy finding was that the normalized area of protein-bound L-arginine after digestion increased in all three cow milk samples.

The opposite behavior could be seen in soy drink, which followed the expected trend after digestion, namely a decrease due to protein breakdown and amino acid release and possible participation in the Maillard reaction. This trend has been well reported in previous studies, such as the one from Serena-Romero et al. (2023), which investigated the release of L-arginine during digestion.⁹⁴

5.1.2 Free AGEs

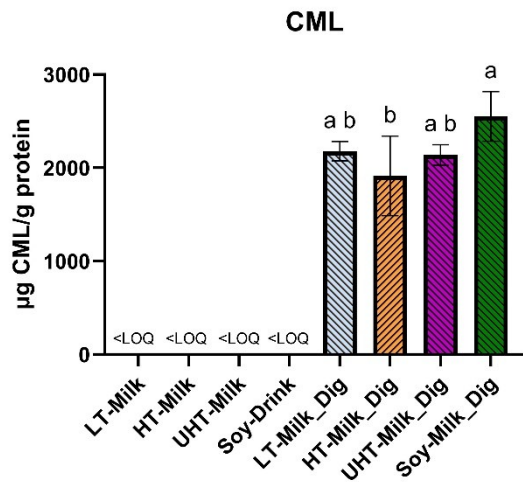


Figure 42: Concentration of free Nε-Carboxymethyllysine (CML) in µg CML/g protein before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Figure 42 shows the results of free CML in the different samples. Free CML was only quantified in the digestates, but still detectable before the digestion. A significant difference in free CML concentration was only apparent between HT milk and the soy drink, when expressed in terms of protein content. In the study by Bieck et al. (2025), where the occurrence of free CML in plant-based dairy alternatives was monitored, the amount of free CML showed an increase after digestion too.⁶⁵

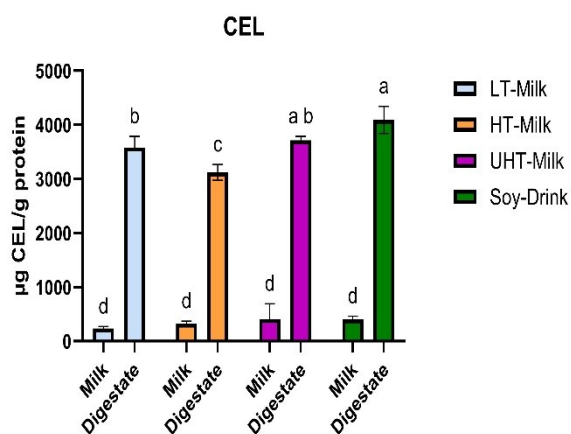


Figure 43: Concentration of free Nε-Carboxyethyllysine (CEL) in µg CEL/g protein before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

In the case of free CEL, the results were shown in the bar chart of figure 43. Unlike free CML, free CEL was detected before digestion in milk at quantifiable concentrations. However, no significant differences were observed across all beverage samples. Similarly, a highly marked increase in free CEL was observed after in vitro digestion in all samples, reaching higher concentrations than those of free CML. This is likely due to the release of protein-bound CEL during digestion. In the corresponding digestates, a significant difference was uniquely observed between HT milk and all remaining samples and between LT and soy drink digestate when expressed in terms of protein content. While Bieck et al. (2025) reported only a minor increase in free CEL after digestion, the higher amounts observed in this study could potentially be attributed to a higher initial lysine content in the analyzed soy drink.⁶⁵

It can be highlighted that the behavior of free CML and CEL closely mirrors the trends observed for their respective protein-bound counterparts (figure 33 and 34) during digestion. There, a decline was observed after digestion, but an increase was detected in the free form of these two substances.

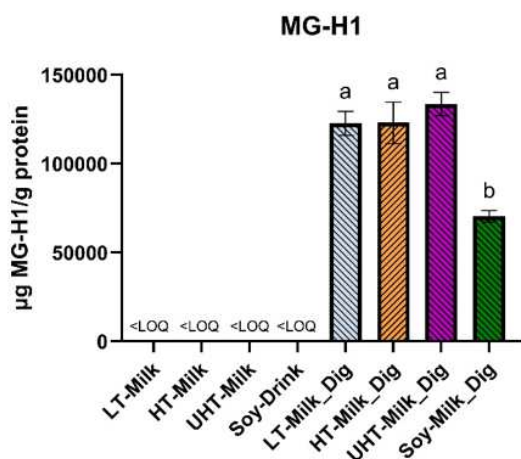


Figure 44: Concentration of free methylglyoxal-derived hydroimidazolone (MG-H1) in µg MG-H1/g protein before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Another free advanced glycation end product that could only be quantified in the digestates of the different samples was free MG-H1, as shown by the bar chart in figure 44. Compared to the soy drink digestate, the digestates of all three cow milk samples exhibited significantly higher MG-H1 concentrations. No significant differences were found between the different types of processed milk digestates. While the study by Bieck et al. (2025) found no increase in MG-H1 in soy milk, this master's thesis provided new evidence that free MG-H1 can indeed be present after digestion.⁶⁵

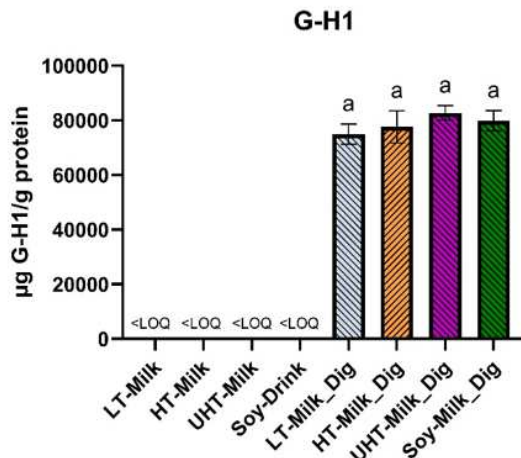


Figure 45: Concentration of free glyoxal-hydroimidazolone (G-H1) in µg G-H1/g protein before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

In figure 45 the results of free G-H1 are illustrated. After digestion, a strong release of the free form was observed for G-H1, with a profile nearly identical to that of MG-H1. Remarkably, free G-H1 exhibited a hundredfold higher concentration compared to its protein-bound counterpart (figure 38). The exact same trend was observed for MG-H1 (figure 37 and 44).

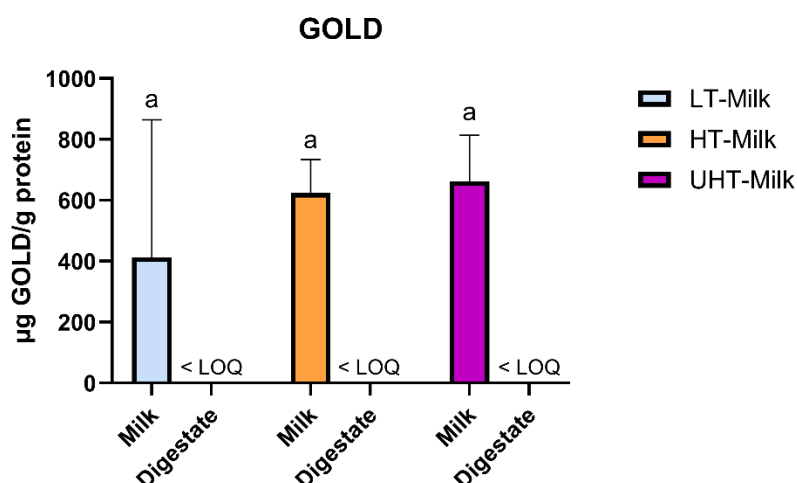


Figure 46: Concentration of free glyoxal-derived lysine dimer (GOLD) in $\mu\text{g GOLD/g protein}$ before and after digestion in the different milk samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Free GOLD, for which the results are presented in figure 46, shows an opposite behavior than the previous AGEs (figure 42-45), which has never been reported before in any literature dealing with AGEs formation in dairy products. It represents a striking exception in the spectrum of free AGEs examined, as it was found exclusively in the milk samples before digestion. Contrary to the increase, that would be expected due to its release from protein-bound form, free GOLD was no longer detectable in the digestates. Further research is needed to clarify the causes of this atypical pattern.

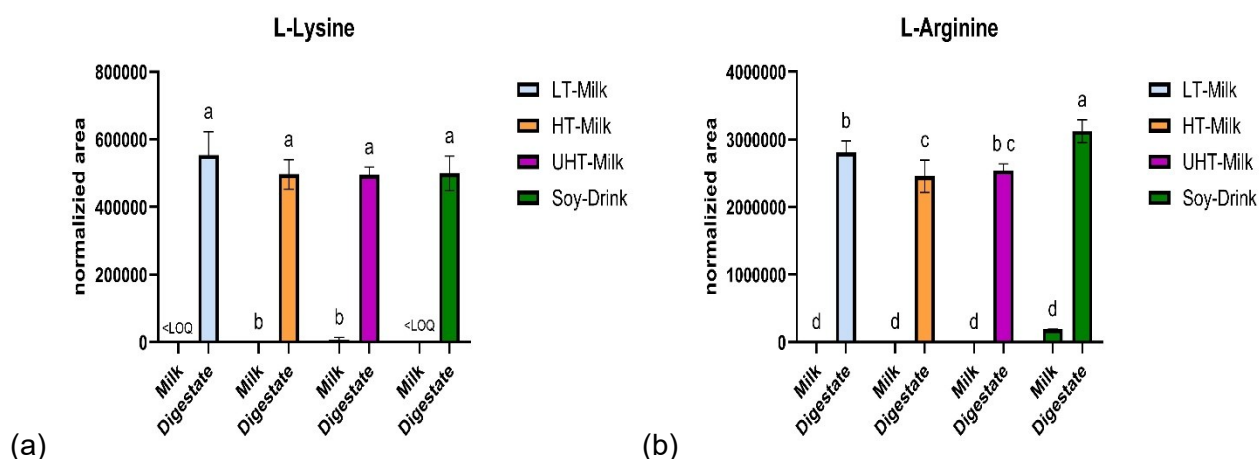


Figure 47: Semi-quantitative comparison of the normalized areas of free (a) L-lysine and (b) L-arginine before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

The bar chart in figure 47a compares the normalized areas of L-lysine before and after digestion. In LT milk and soy drink, undigested free lysine could be detected but not quantified. It was semi-quantified in the HT- and UHT-milk before digestion, which is supported by the findings of Arena et al. (2025), where it was determined in pasteurized bovine milk as well.⁹⁵ As expected,

gastrointestinal digestion led to a rise in L-lysine levels across all tested samples, reaching non significantly different levels between them.

In the bar chart of figure 47b, the values of free L-arginine before and after digestion are presented. In contrast to free L-lysine, free L-arginine could be semi-quantified in all samples prior to digestion. Fittingly, Arena et al. (2025) also determined that L-arginine can be present in free form in pasteurized bovine milk.⁹⁵ As it was already seen in the case of free L-lysine, the same increase trend after digestion was observed for free L-arginine. However, in the bar chart of L-arginine (figure 47b) there were significant differences to be seen between the cow milk samples and the soy drink. A more pronounced increase in free L-arginine occurred after digestion of soy drink. This is likely due to the fact that L-arginine is naturally more abundant in soy beverages.⁷

Protein-bound lysine-derived AGEs (CML, CEL, GOLD and MOLD), as well as their precursor amino acid L-lysine, displayed a significant decrease in concentration following digestion. In the case of MOLD and GOLD, significant processing-induced differences were observed. Specifically, ultra-high-temperature (UHT) milk displayed marked deviations compared to the low-temperature (LT) and high-temperature (HT) milk, showing higher contents when expressed in relation to protein content. These results confirmed the hypothesis of this master's thesis, which states that different milk processing promotes the formation of specific AGEs, a finding that is further supported by the measurement data obtained during in vitro digestion. An opposite trend was observed for MG-H1, G-H1, and their precursor amino acid L-arginine. For the majority of the samples, no statistically significant differences in MG-H1 and G-H1 concentrations were detected, regarding both the comparison before and after digestion and the differentiation between individual samples and their respective digestates. Consequently, these two AGEs remained largely unaffected by milk processing and digestion. Exceptionally, G-H1 demonstrated significant differences before and after digestion specifically within the UHT milk and soy drink. An increase in G-H1 can be explained by the fact that their precursor substances originate not only from carbohydrate intake but also directly from lipid oxidation.⁶¹

Overall, a general trend could be described for the detected free AGEs in the samples, as their concentration increased after vitro digestion with the exception of GOLD. The formation of free AGEs after digestion is physiologically plausible. Under the influence of digestive enzymes, the AGEs are released from the protein complex.⁶⁵

However, not all AGEs present in protein-bound form, such as MOLD, pentosidine, and argpyrimidine, could be detected. Additionally, furosine, an early-stage glycation product, could not be quantified. This might be due to the higher limit of detection in the free AGEs analyses, as a result of the higher dilution factor needed to bypass its technical limitations, possibly preventing the accurate quantification of these missing substances. However, in the case of furosine, the further progression of the Maillard reaction might have contributed to its decrease during digestion.

In order to compare the overall Maillard reaction progress, Table 29 focuses on the total advanced glycation end product content (total AGEs) quantified before and after digestion.

Table 29: Total content of specific AGEs (CML, CEL, GOLD, MG-H1, and G-H1) in different types of milk and soy drink before and after in vitro digestion. Values represent the sum of protein-bound and free AGEs fractions (mean $\pm$ SD, $\mu\text{g/g}$ protein; (n = 3–4)). Statistical analysis was performed for each type of AGEs separately using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$).

type of AGEs	STATE	sample types			
		LT milk	HT milk	UHT milk	soy drink
CML	before digestion	< LOQ	< LOQ	184 $\pm$ 15 ^c	137 $\pm$ 3 ^c
	after digestion	2180 $\pm$ 89 ^b	2049 $\pm$ 368 ^b	2289 $\pm$ 96 ^a	2710 $\pm$ 230 ^a
CEL	before digestion	4111 $\pm$ 286 ^b	4608 $\pm$ 210 ^b	5109 $\pm$ 453 ^a	4768 $\pm$ 790 ^a
	after digestion	4939 $\pm$ 555 ^a	4341 $\pm$ 520 ^b	6156 $\pm$ 672 ^a	5124 $\pm$ 290 ^a
GOLD	before digestion	496 $\pm$ 391 ^a	716 $\pm$ 90 ^a	770 $\pm$ 107 ^a	55 $\pm$ 2 ^b
	after digestion	< LOQ	62 $\pm$ 1 ^b	68 $\pm$ 1 ^b	< LOQ
MG-H1	before digestion	141 $\pm$ 21 ^c	141 $\pm$ 19 ^c	160 $\pm$ 8 ^c	137 $\pm$ 20 ^c
	after digestion	123024 $\pm$ 5860 ^a	123367 $\pm$ 10112 ^a	134011 $\pm$ 5665 ^a	70655 $\pm$ 2789 ^b
G-H1	before digestion	144 $\pm$ 7 ^c	137 $\pm$ 16 ^c	136 $\pm$ 4 ^c	123 $\pm$ 5 ^c
	after digestion	75137 $\pm$ 3144 ^b	77799 $\pm$ 5088 ^a	82899 $\pm$ 2296 ^a	80099 $\pm$ 3175 ^a

The expected increase in total AGEs was clearly observed for CML, MG-H1, and G-H1 after digestion. Significant differences were found between highly heated products (UHT milk, soy drink) and lower temperature milk types (LT, HT milk). Regarding the MG-H1 content in the digestates, significant differences were observed exclusively between the cow milk and the soy drink, whereas the different thermal processing levels of the cow milk (LT, HT and UHT) did not differ statistically from one another. For total G-H1, significant differences ($p < 0.05$) were uniquely observed between LT milk and all other samples analyzed, whereas no statistical divergence ($p > 0.05$) was found among HT milk, UHT milk, and the soy drink. From this data, it could be concluded that the Maillard reaction progressed during digestion.

In order to evaluate the interrelationship between MRPs and LOPs, a comprehensive correlation analysis between the content of the most prominent AGEs in the samples and the most representative lipid oxidation products detected after digestion was additionally carried out (Appendix Figure 30). Thus, positive significant correlations ($p < 0.05$) were found between the total concentration of the AGEs that displayed the higher increase during digestion (CML, MG-H1 and G-H1) and several oxidized lipid species. This included the oxidized monoacylglycerol MG 18:3;O ($r = 0.9862$ for CML, $r = 0.9594$ for MG-H1, $r = 0.9992$ for G-H1) and the majority of the oxidized fatty acids detected (e.g. FA 14:1;O, $r = 0.7061$ for CML, $r = 0.9334$ for MG-H1, $r = 0.7885$ for G-H1), as well as (E,E)-CD-OHs ($r = 0.8468$ for CML, $r = 0.7843$ for G-H1), and n-alkanals ($r = 0.9572$ for CML, $r = 0.8584$ for MG-H1, $r = 0.9409$ for G-H1). These findings suggest potential links between the progression of the Maillard reaction and lipid oxidation during the in vitro digestion of milk and soydrink, although they

also point towards a high complexity in their possible interactions, as not all oxidized lipids and AGEs (e.g. CEL) showed a significant correlation between them.

Elevated levels of dietary advanced glycation end products (AGEs) are associated with health risk. Both free and protein-bound AGEs bind to the RAGE receptor, where they induce chronic inflammatory processes and oxidative stress through the activation of NF- κ B. Their continuous accumulation of AGEs in the body leads to progressive damage to tissues and organs. The pathological consequences of this accumulation are far-reaching, ranging from endothelial dysfunction and accelerated atherosclerosis to the promotion of insulin resistance and the progression of nephropathies and retinopathies.^{96 97}

Regarding the pathological aspects of dietary AGEs, minimizing their burden remains a key nutritional goal. However, this thesis indicates that varying milk processing methods do not result in a straightforward or consistently lower AGE content post-digestion. Consequently, lower-temperature processing does not automatically guarantee a reduced total AGE load after gastrointestinal transit. This inconsistency in trends highlights the complexity of thermal treatment in milk during digestion. Interestingly, the plant-based alternative beverage (soy drink) also exhibited a similarly pronounced increase in AGEs during gastrointestinal transit.

To resolve these ambiguities, future research must investigate the gastrointestinal dynamics of AGEs during digestion, as well as the proportion of the post-digestion AGE pool that is actually bioavailable and contributes to endogenous pools.

6 Conclusion

The differently processed cow milk samples and soy drink sample were evaluated in terms of lipid oxidation products and Maillard reaction products before and after *in vitro* digestion. To characterize the occurrence of these products during digestion, the parameters related to the lipid content were first examined.

In the case of lipid oxidation, different acyl groups were analyzed and contrasted with that of different oxidized lipid groups. A decrease in unsaturated acyl groups was observed for the soy drink, accompanied by a confirmed increase in oxidized lipids during digestion. In addition, all digested milk types exhibited a trend ($p < 0.05$) where primary oxidized lipids (conjugated diene hydroperoxides) increased with the temperature of the heat treatment. These findings support the initial hypothesis, that higher thermally treated milk will generate more LOPs in the digestive process. Regarding the undigested samples, a higher content of *n*-alkanals was observed in the more intensively heat-treated milk types (HT and UHT), whereas no detection was achieved in the low-temperature (LT) milk. The subsequent *in vitro* digestion led to a general increase in concentration of secondary oxidized lipids ($p < 0.05$), but it minimized the differences between the milk types, so that no significant differences ($p > 0.05$) could be detected. Furthermore, lipid oxidation products were further characterized via LC-QTOF. In the undigested state the results showed the prominence of oxidized TGs and DGs, while post-digestion analyses revealed the rise of oxidized FAs and a MG. The identification of these novel oxidized lipids (especially oxDGs and oxMGs) in milk, soy drink, and their digestates revealed clear pre-digestive differences between cow milk and soy drink samples. Post-digestion, these differences were less pronounced, further suggesting that the digestive process minimizes the impact of processing on the occurrence of lipid oxidation.

Another part of this master's thesis investigated Maillard reaction products (MRPs) before and after *in vitro* digestion. In addition to various AGEs, the early-stage glycation product furosine was examined. In the undigested state, the results demonstrated that high-temperature thermal milk processing had a significant impact on the formation of this product. Following digestion, however, furosine was no longer detectable, suggesting that it underwent further reactions during the digestive process. Protein-bound and free AGEs exhibited complementary behavioral patterns upon completion of digestion. While protein-bound AGEs decreased, free AGEs accumulated. Overall, a clear trend emerged showing that the total AGE concentration increased after digestion in milk and soy drink.

In conclusion, the data demonstrated a post-digestion rise in both MRP and LOP levels. The impact of digestion proved to be a central factor, as it effectively leveled out the original differences in AGEs and LOPs between the thermally treated milk types and the soy drink. For consumers, these findings require a shift in perspective, due to the rarely considered complexity of digestion in milk and soy drink. They tend to focus exclusively on the product itself at the time of purchase, assuming its health benefits. The fact that the digestive process actively promotes the formation of MRPs and LOPs is often overlooked and should be taken into account.

7 Abstract

In the food industry, milk and soy beverages undergo thermal processing for product safety reasons. This process can be carried out at various temperatures. During this process, Maillard reaction products (MRPs) and lipid oxidation products (LOPs) can be formed. The digestive process promotes their further formation in the body. However, it is unclear how the different heating methods used for milk and soy beverages affect these compounds during digestion. Therefore, in this master's thesis, three different heat-treated cow's milk samples (LT, HT, and UHT milk) and one soy beverage were selected and subjected to in vitro digestion. Consequently, the objective was to determine the influence of different thermal processing methods on formation of MRPs and LOPs during digestion.

Lipid oxidation, as detected by $^1\text{H-NMR}$, showed that undigested HT and UHT milk (0.02 ± 0.01 mmol/mol acyl groups + fatty acids (AG+FA)) exhibited higher n-alkanal levels compared to LT milk. Digestion promoted oxidation in all milk types ($p < 0.05$) but minimized the differences between them (0.14 ± 0.04 mmol/mol AG+FA). In general, an increase in lipid oxidation products during digestion was monitored. Other LOPs determined by LC-QTOF, including oxidized triacylglycerols, diacylglycerols, monoacylglycerols and fatty acids, were identified in cow milk, soy drink and their digestates. Although lipid oxidation products increased significantly after digestion, for most oxidized lipid species, no significant differences were observed among the various digested milk types. Furthermore, novel oxidized lipids, especially oxidized DGs (e.g. DG 16:1_18:2;O₂ and DG 18:1_18:2;O) and one oxidized MG (MG 18:3;O), were identified, representing one of the main novelties of this thesis.

Regarding the Maillard reaction, furosine, an early-stage Maillard product, showed elevated levels in non-digested UHT milk. A significant increase in the total AGE concentration was observed in all milk types and the soy beverage after digestion. Of particular note is the increase in MG-H1 (123367 ± 10112 $\mu\text{g/g}$ protein in HT milk and 134011 ± 5665 $\mu\text{g/g}$ protein in UHT milk) and G-H1 (82899 ± 2296 $\mu\text{g/g}$ protein in UHT milk and 80099 ± 3175 $\mu\text{g/g}$ protein in soy drink) after digestion. As precursors for these MRPs can be generated via oxidative pathways, these findings indicate that simultaneous lipid oxidation might promote the formation of AGEs during digestion.

In line with the aim of this thesis, it was clearly demonstrated that the digestion process caused an increase in MRPs and LOPs. In particular, the summed increase in free MRPs as well as free oxidized fatty acids was reliably characterized and (semi)quantified in the digestate samples. Thus, these results highlight the importance of assessing digestive dynamics of MRPs and LOPs after food intake.

8 Zusammenfassung

In der Lebensmittelindustrie werden Milch und Sojagetränke aus Gründen der Produktsicherheit thermisch behandelt. Dieser Prozess kann bei verschiedenen Temperaturen durchgeführt werden. Dabei können Maillard-Reaktionsprodukte (MRPs) und Lipidoxidaionsprodukte (LOPs) entstehen. Die Verdauung fördert deren weitere Bildung im Körper. Es ist jedoch unklar, wie sich die verschiedenen Erhitzungsmethoden für Milch und Sojagetränke auf diese Verbindungen während der Verdauung auswirken. Daher wurden in dieser Masterarbeit drei verschiedene wärmebehandelte Kuhmilchproben (LT-, HT- und UHT-Milch) sowie ein Sojagetränk ausgewählt und einer In-vitro-Verdauung unterzogen. Ziel war es, den Einfluss verschiedener thermischer Behandlungsmethoden auf die Bildung von MRPs und LOPs während der Verdauung zu bestimmen.

Die mittels $^1\text{H-NMR}$ nachgewiesene Lipidoxidation zeigte, dass unverdaute HT- und UHT-Milch ($0,02 \pm 0,01$ mmol/mol Acylgruppen + Fettsäuren (AG+FA)) höhere n-Alkanal-Werte aufwiesen als LT-Milch. Die Verdauung förderte die Oxidation in allen Milchttypen ($p < 0,05$), verringerte jedoch die Unterschiede zwischen ihnen ($0,14 \pm 0,04$ mmol/mol AG+FA). Generell wurde ein Anstieg der Lipidoxidaionsprodukte während der Verdauung beobachtet. Weitere mittels LC-QTOF bestimmte Lipidoxidaionsprodukte, darunter oxidierte Triacylglycerine, Diacylglycerine, Monoacylglycerine und Fettsäuren, wurden in Kuhmilch, Sojamilch und deren Verdauungsprodukten identifiziert. Obwohl die Lipidoxidaionsprodukte nach der Verdauung signifikant anstiegen, wurden für die meisten oxidierten Lipidarten keine signifikanten Unterschiede zwischen den verschiedenen verdauten Milchttypen festgestellt. Des Weiteren wurden neuartige oxidierte Lipide, insbesondere oxidierte DGs (z. B. DG 16:1_18:2;O₂ und DG 18:1_18:2;O) sowie ein oxidiertes MG (MG 18:3;O), identifiziert, was eine der wichtigsten Neuerungen dieser Arbeit darstellt.

Im Hinblick auf die Maillard-Reaktion zeigte Furosin, ein frühes Maillard-Produkt, erhöhte Konzentrationen in unverdauter H-Milch. Nach der Verdauung wurde in allen Milchsorfen und dem Sojagetränk ein signifikanter Anstieg der Gesamtkonzentration an AGEs beobachtet. Besonders hervorzuheben ist der Anstieg von MG-H1 (123367 ± 10112 µg/g Protein in HT-Milch und 134011 ± 5665 µg/g Protein in UHT-Milch) und G-H1 (82899 ± 2296 µg/g Protein in UHT-Milch und 80099 ± 3175 µg/g Protein in Sojadrink) nach der Verdauung. Da Vorstufen dieser MRPs über oxidative Wege gebildet werden können, deuten diese Ergebnisse darauf hin, dass eine gleichzeitige Lipidoxidation die Bildung von AGEs während der Verdauung fördern könnte.

Im Einklang mit dem Ziel dieser Arbeit konnte eindeutig gezeigt werden, dass der Verdauungsprozess einen Anstieg von MRPs und LOPs verursacht. Insbesondere wurde der Gesamtanstieg freier MRPs sowie freier oxidierter Fettsäuren in den verdauten Proben zuverlässig charakterisiert und (semi-)quantifiziert. Diese Ergebnisse unterstreichen somit die Bedeutung der Untersuchung der Verdauungsdynamik von MRPs und LOPs nach Nahrungsaufnahme.

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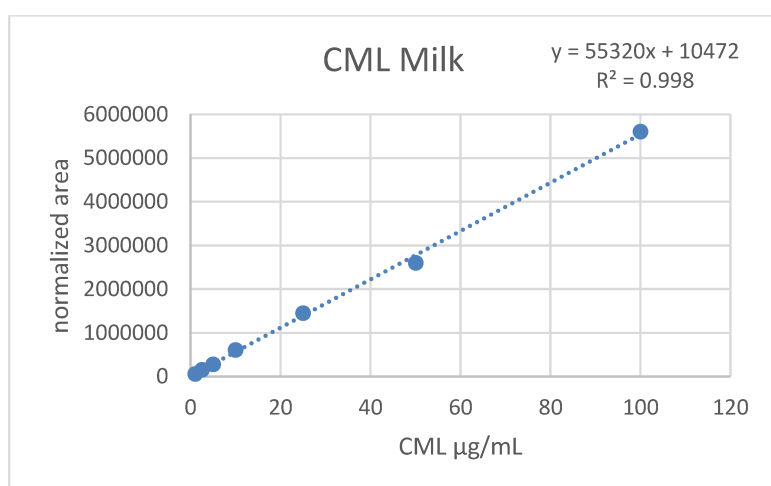
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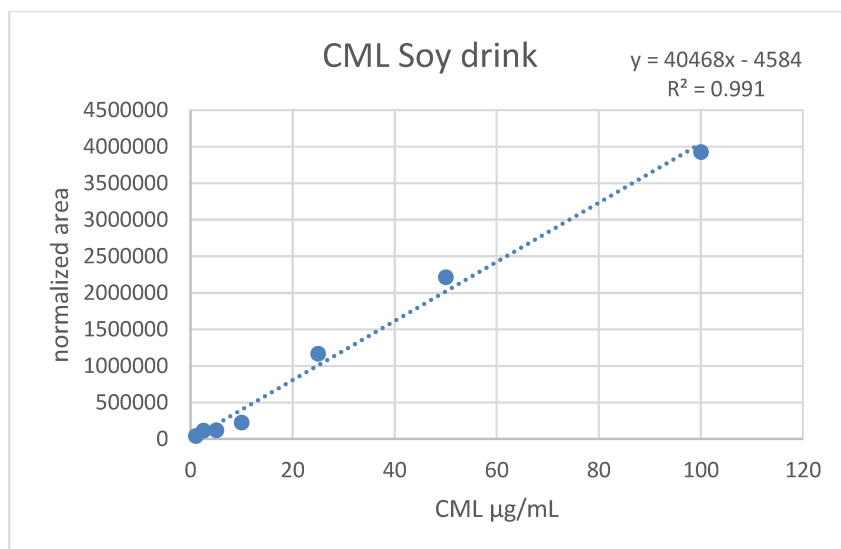
10 Appendix

Appendix Table 1: Lipid content of the three cow milk samples and soy drink and their digestates after Folch extraction

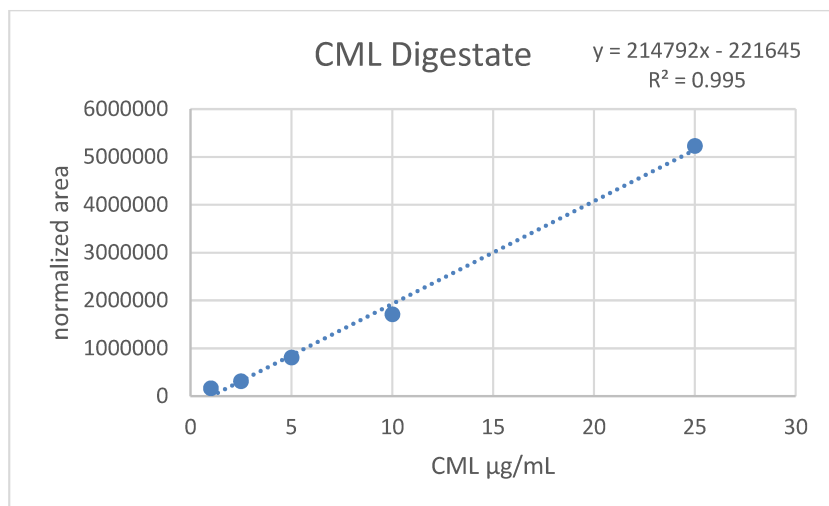
sample (state)	Replicate 1	Replicate 2	Replicate 3	Replicate 4
LT-milk (undigested)	0.694	0.656	0.574	0.670
LT-milk (digested)	0.180	0.162	0.191	0.201
HT-milk (undigested)	0.596	0.505	0.447	0.410
HT-milk (digested)	0.223	0.237	0.225	0.242
UHT-milk (undigested)	0.527	0.406	0.646	0.560
UHT-milk (digested)	0.189	0.153	0.179	0.191
soy drink(undigested)	0.223	0.287	0.280	0.300
soy drink (digested)	0.170	0.163	0.189	0.182



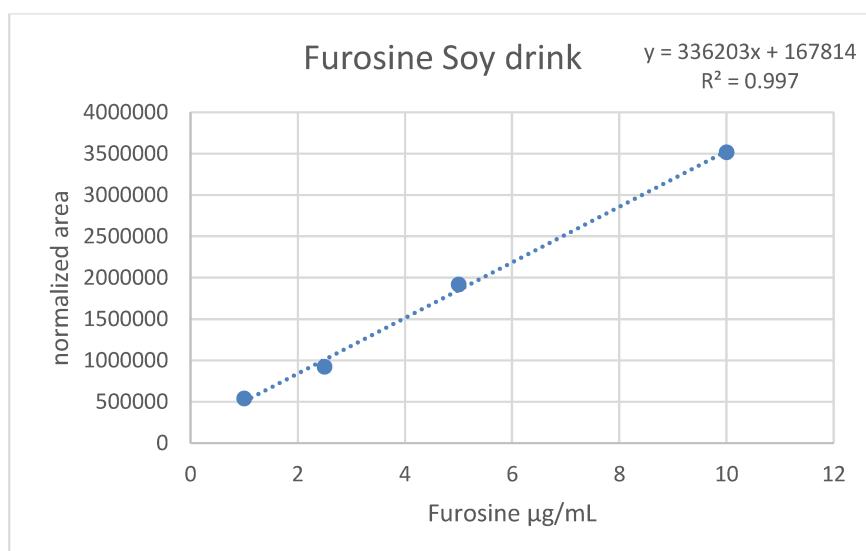
Appendix Figure 1: Calibration curve protein-bound CML from milk. For the calibration curve, 7 standards with the concentration 1 – 100 µg/mL were used. The resulting linear regression ($R^2 = 0.998$) was used for the quantification of the protein-bound CML concentration in the milk samples.



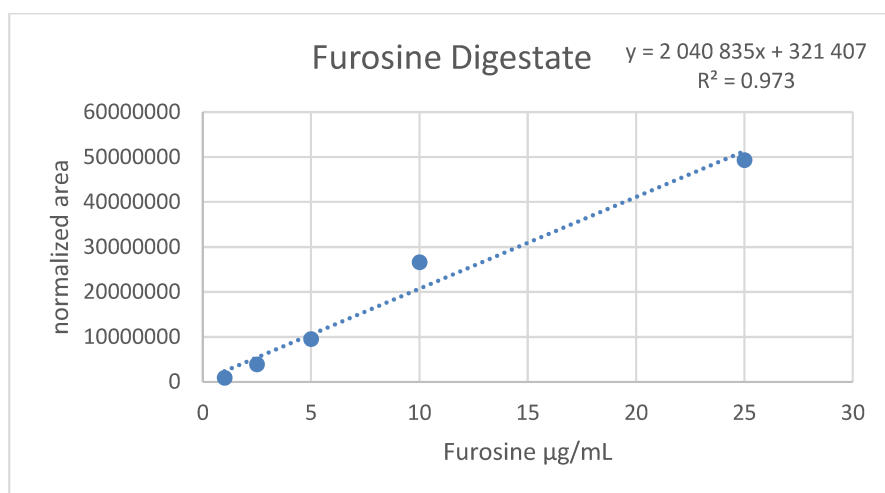
Appendix Figure 2: Calibration curve protein-bound CML from soy drink. For the calibration curve, 7 standards with the concentration 1 – 100 µg/mL were used. The resulting linear regression ($R^2 = 0.991$) was used for the quantification of the protein-bound CML concentration in the soy drink sample.



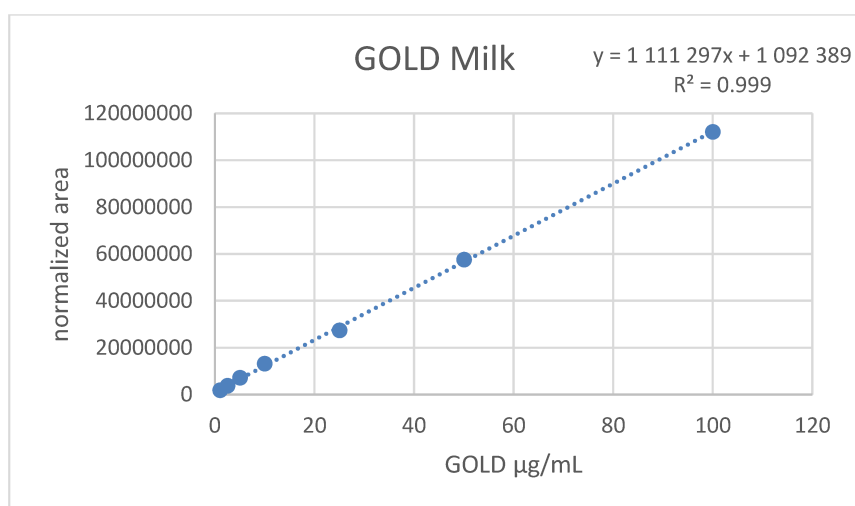
Appendix Figure 3: Calibration curve protein-bound CML from digestate . For the calibration curve, 5 standards with the concentration 1 – 25 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.995$) was used for the quantification of the protein-bound CML concentration in all milk and soy drink digestate samples.



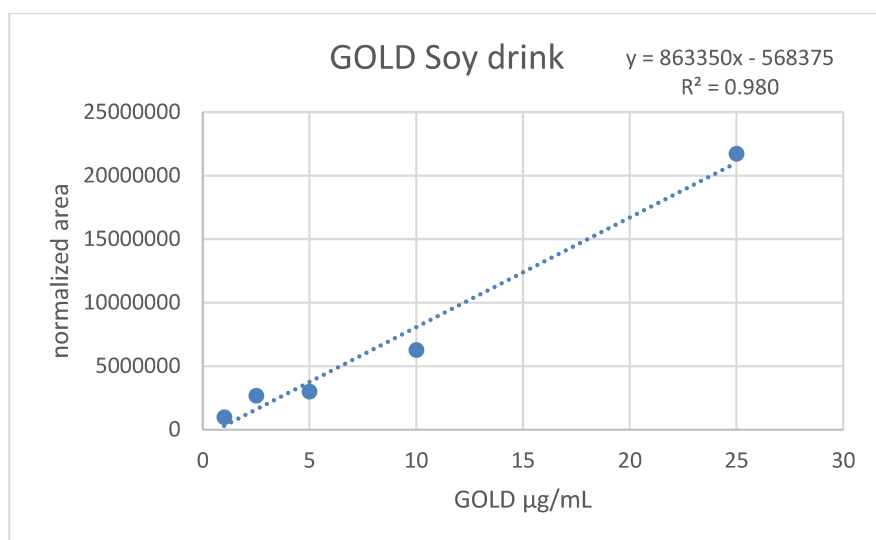
Appendix Figure 4: Calibration curve protein-bound furosine from soy drink. For the calibration curve, 4 standards with the concentration 1 – 10 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.950$) was used for the quantification of the protein-bound furosine concentration in the milk and soy drink samples.



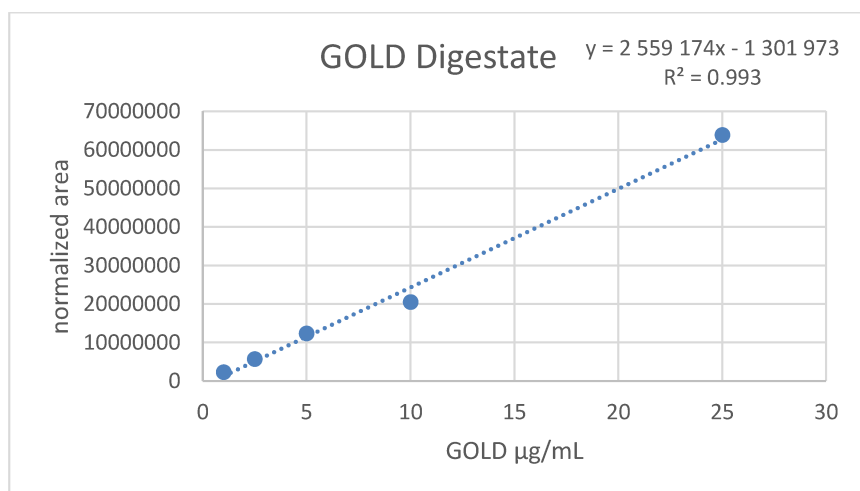
Appendix Figure 5: Calibration curve protein-bound furosine from digestate. For the calibration curve, 5 standards with the concentration 1 – 25 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.973$) was used for the quantification of the protein-bound furosine concentration in all milk and soy drink digestate samples.



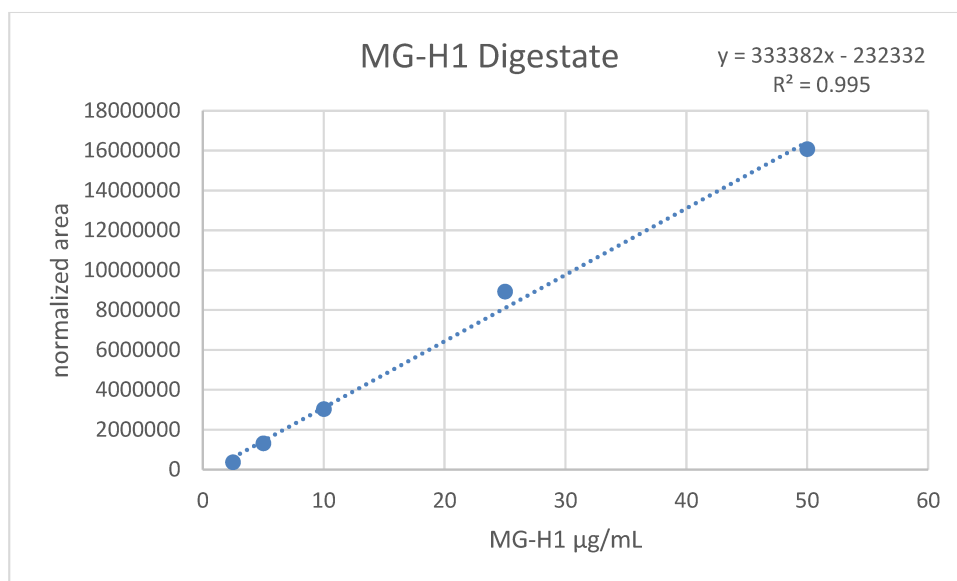
Appendix Figure 6: Calibration curve protein-bound GOLD from milk. For the calibration curve, 7 standards with the concentration 1 – 100 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.999$) was used for the quantification of the protein-bound GOLD concentration in the milk samples.



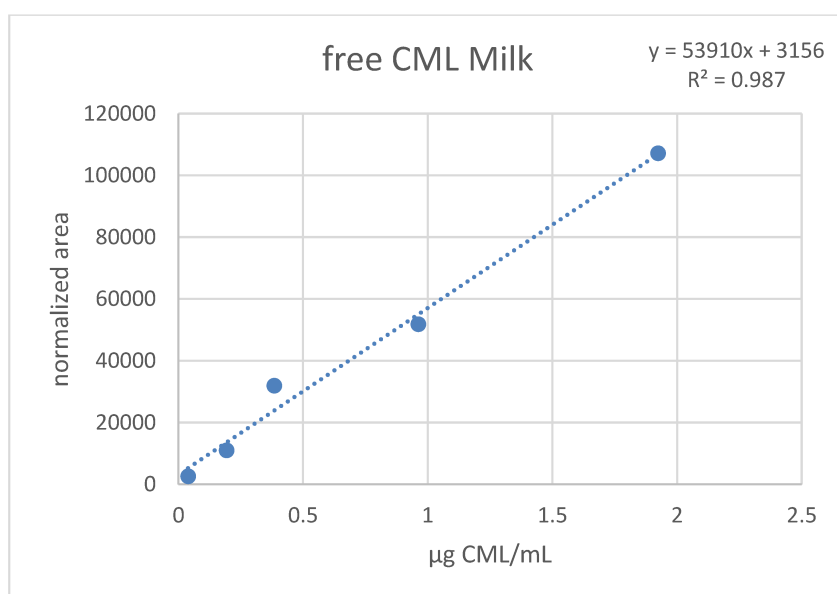
Appendix Figure 7: Calibration curve protein-bound GOLD from soy drink. For the calibration curve, 5 standards with the concentration 1 – 25 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.980$) was used for the quantification of the protein-bound GOLD concentration in the soy drink sample.



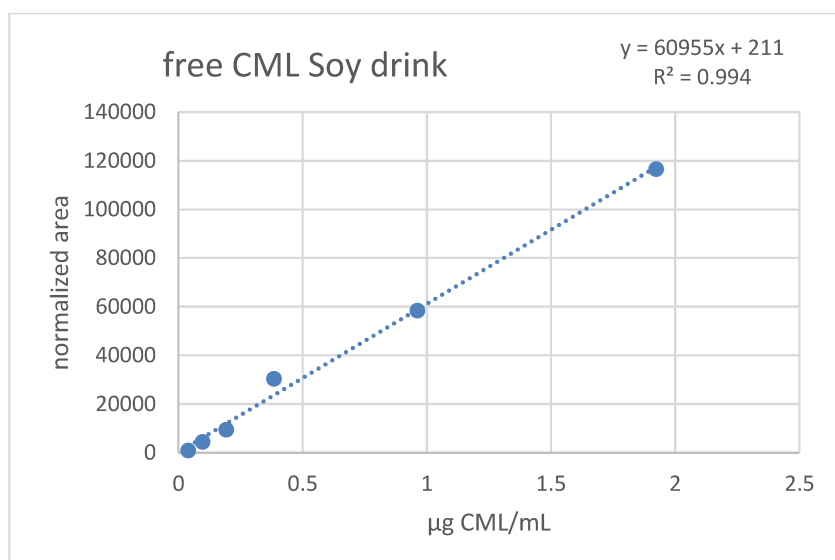
Appendix Figure 8: Calibration curve protein-bound GOLD from digestate . For the calibration curve, 5 standards with the concentration 1 – 25 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.993$) was used for the quantification of the protein-bound GOLD concentration in all milk and soy drink digestate samples.



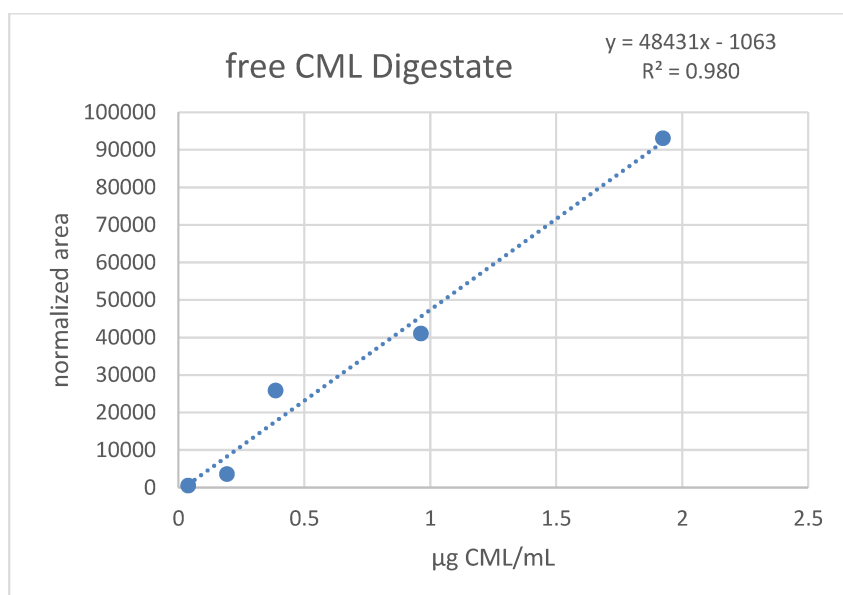
Appendix Figure 9: Calibration curve protein-bound MG-H1 from digestate. For the calibration curve, 5 standards with the concentration 2.5 – 50 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.995$) was used for the quantification of the protein-bound MG-H1 concentration in all milk and soy drink samples and their digestates.



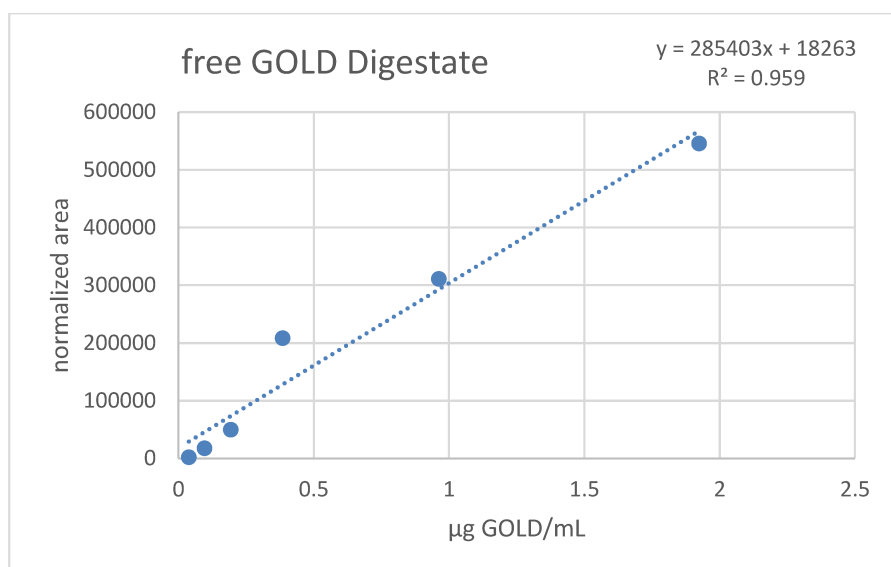
Appendix Figure 10: Calibration curve free CML from milk. For the calibration curve, 5 standards with the concentration 0.0385 – 1.9231 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.987$) was used for the quantification of the free CML concentration in the milk samples.



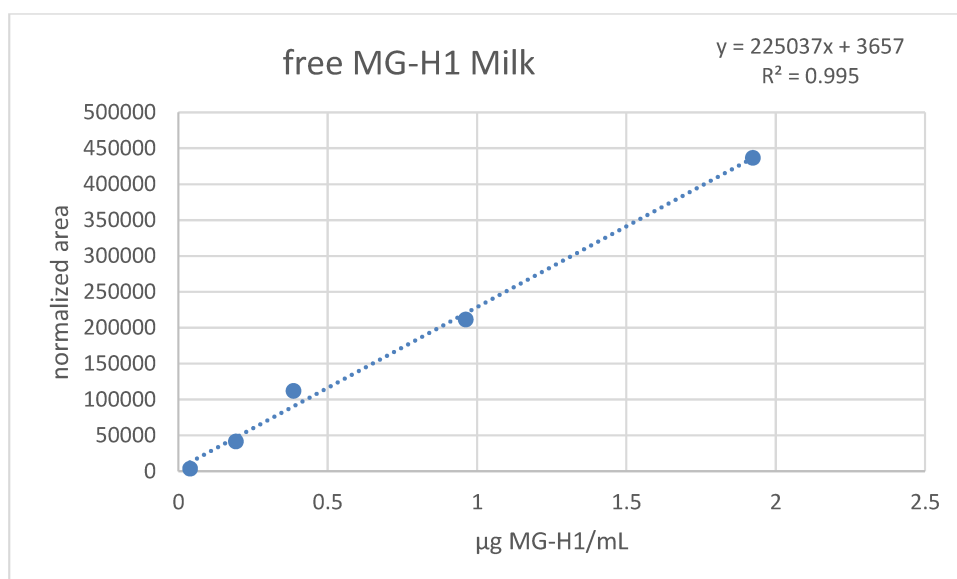
Appendix Figure 11: Calibration curve free CML from soy drink. For the calibration curve, 6 standards with the concentration 0.0385 – 1.9231 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.994$) was used for the quantification of the free CML concentration in the soy drink samples.



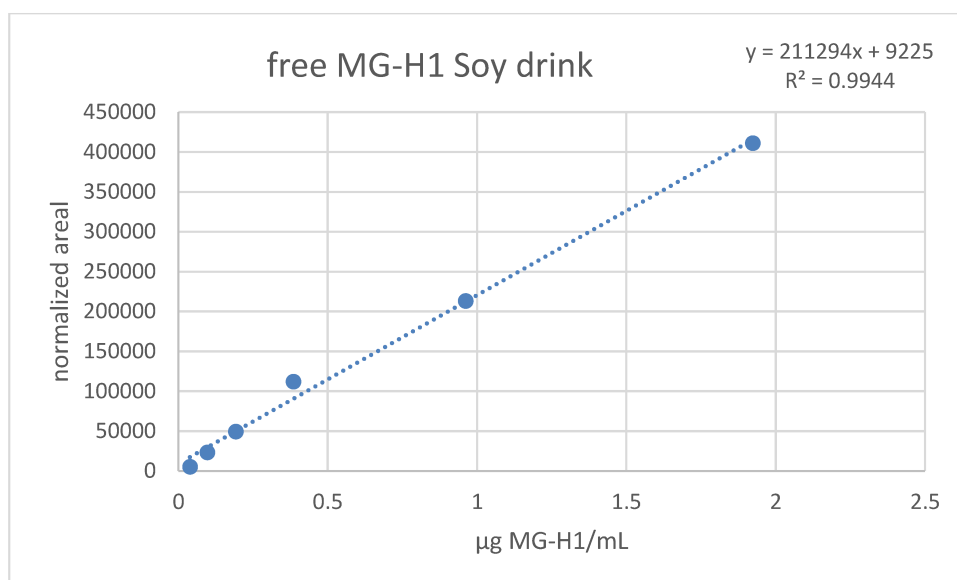
Appendix Figure 12: Calibration curve free CML from digestate. For the calibration curve, 5 standards with the concentration 0.0385 – 1.9231 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.980$) was used for the quantification of the free CML concentration in the milk and soy drink digestate samples.



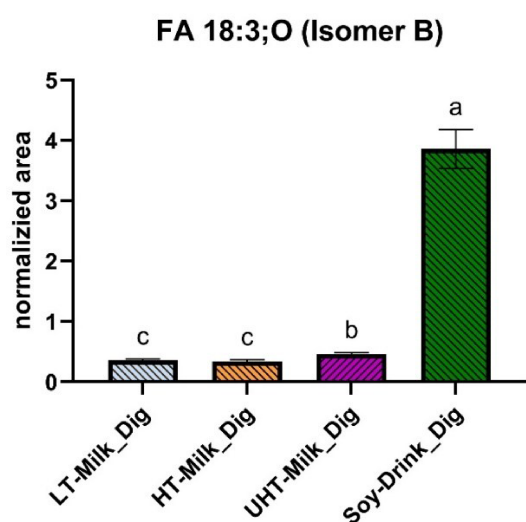
Appendix Figure 13: Calibration curve free GOLD from digestate. For the calibration curve, 6 standards with the concentration 0.0385 – 1.9231 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.959$) was used for the quantification of the free GOLD concentration in the milk and soy drink and their digestate samples.



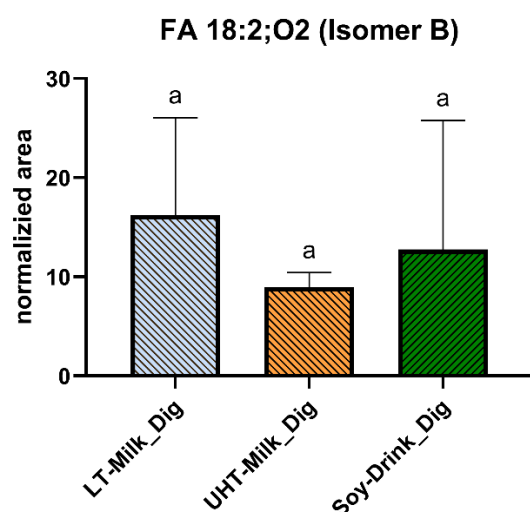
Appendix Figure 14: Calibration curve free MG-H1 from milk. For the calibration curve, 5 standards with the concentration 0.0385 – 1.9231 $\mu\text{g/mL}$ were used. The resulting linear regression ($R^2 = 0.995$) was used for the quantification of the free MG-H1 concentration in the milk samples and their digestate samples.



Appendix Figure 15: Calibration curve free MG-H1 from soy drink. For the calibration curve, 6 standards with the concentration 0.0385 – 1.9231 µg/mL were used. The resulting linear regression ($R^2 = 0.994$) was used for the quantification of the free MG-H1 concentration in the soy drink sample and its digestate sample.



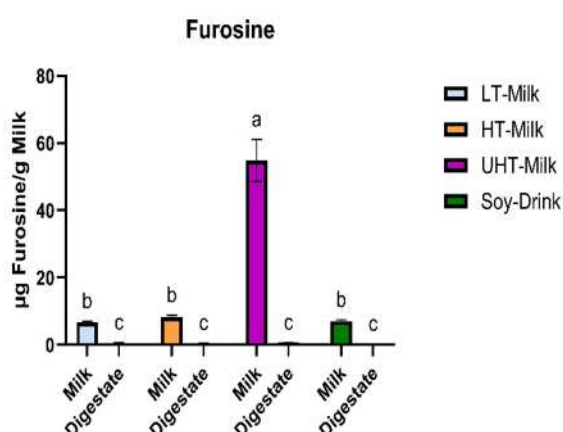
Appendix Figure 16: Bar chart of the oxidized free fatty acid (FA) 18:3;O (Isomer B) and for the digestates of the milk samples and soy drink. Statistical analysis was performed using a Welch ANOVA test with a Dunnett T3 test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



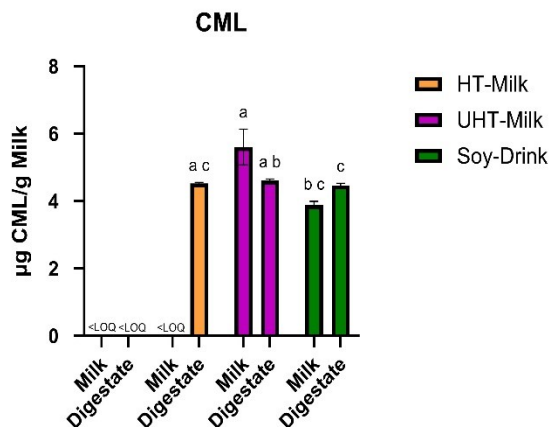
Appendix Figure 17: Bar chart of the oxidized free fatty acid (FA) 18:2;O₂ (Isomer B) and for the digestates of the LT milk and UHT milk and the soy drink. Statistical analysis was performed using a Welch ANOVA test with a Dunnett T3 test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.

Appendix Table 2: Protein content of the different milk products and soy drink in mass percentage of protein (%Protein=g/100g), determined with Kjeldahl method, as well as reference values for these products. The experimental values obtained via Kjeldahl are presented as mean $\pm$ SD ($n=4$)

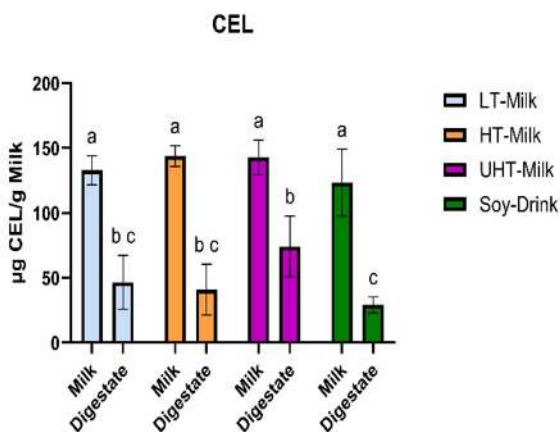
type	Kjeldahl (g/100g)	Package value (g/100mL)	According to Souci (g/100g) 98
LT-milk	3.43 $\pm$ 0.04	3.3	3.4
HT-milk	3.36 $\pm$ 0.03	3.3	3.4
UHT-milk	3.04 $\pm$ 0.03	3.4	3.2
Soy drink	2.83 $\pm$ 0.10	3.0	3.2



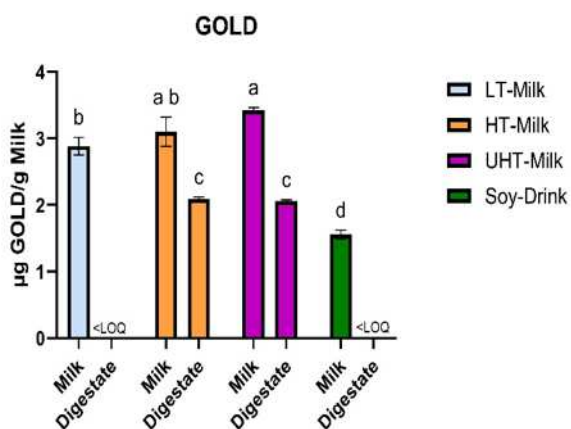
Appendix Figure 18: Concentration of protein-bound furosine in μg furosine/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



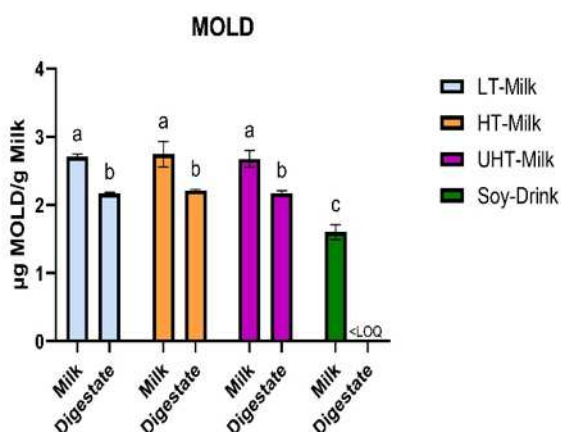
Appendix Figure 19: Concentration of protein-bound Nε-Carboxymethyllysine (CML) in µg CML/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



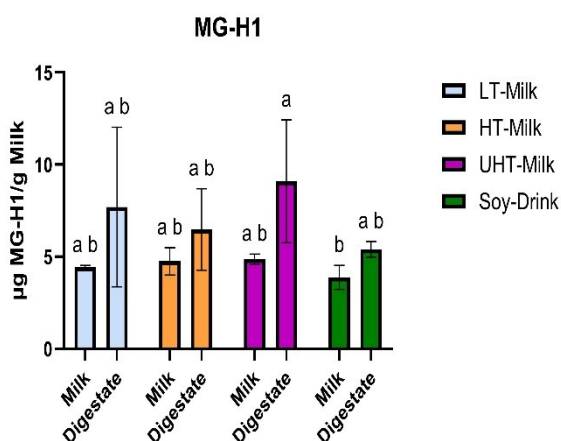
Appendix Figure 20: Concentration of protein-bound Nε-Carboxyethyllysine (CEL) in µg CEL/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



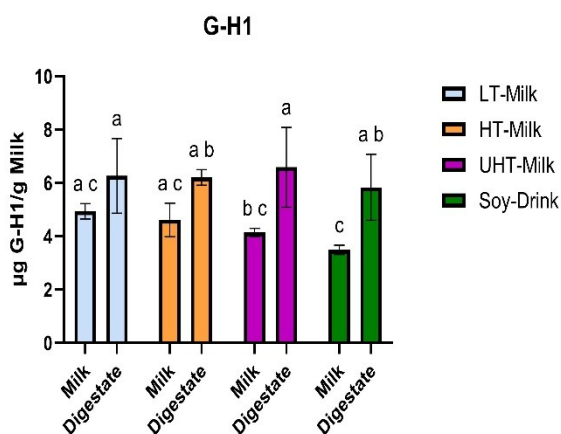
Appendix Figure 21: Concentration of protein-bound glyoxal-derived lysine dimer (GOLD) in µg GOLD/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



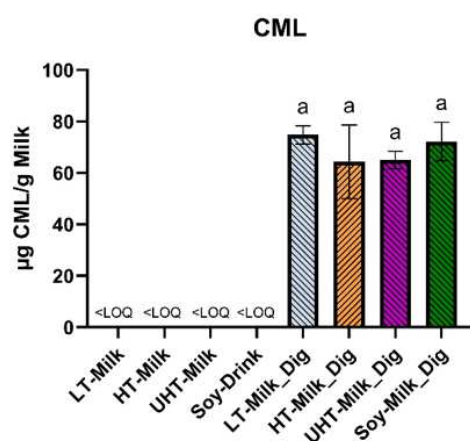
Appendix Figure 22: Concentration of protein-bound methylglyoxal-derived lysine dimer (MOLD) in µg MOLD/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



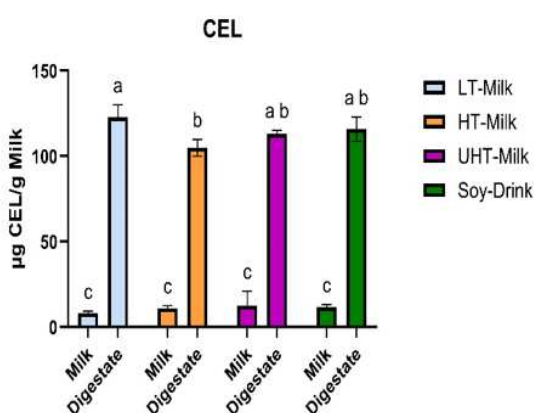
Appendix Figure 23: Concentration of protein-bound methylglyoxal-derived hydroimidazolone (MG-H1) in µg MG-H1/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



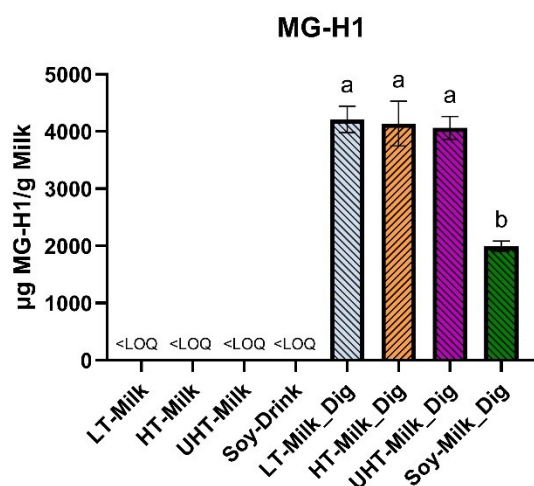
Appendix Figure 24: Concentration of protein-bound glyoxal-hydroimidazolone (G-H1) in µg G-H1/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



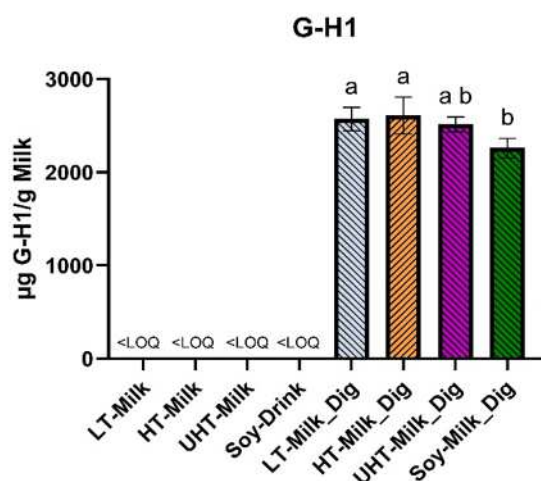
Appendix Figure 25: Concentration of free Nε-Carboxymethyllysine (CML) in µg CML/g milk before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



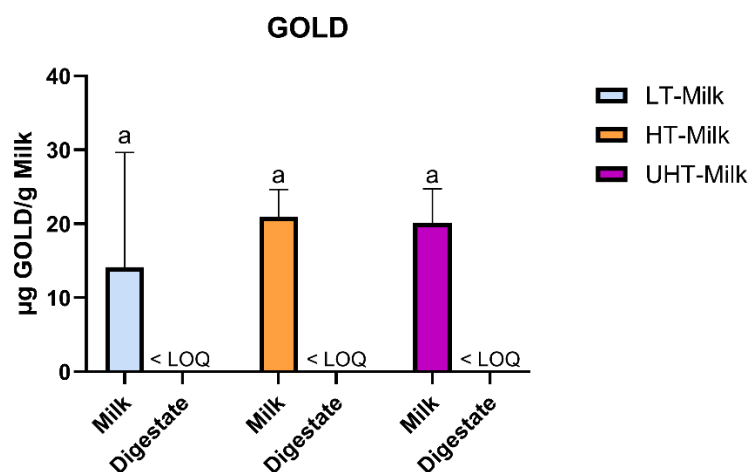
Appendix Figure 26: Concentration of free Nε-Carboxyethyllysine (CEL) in µg CEL/g milk before and after digestion in the different samples. Statistical analysis was performed using a two-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



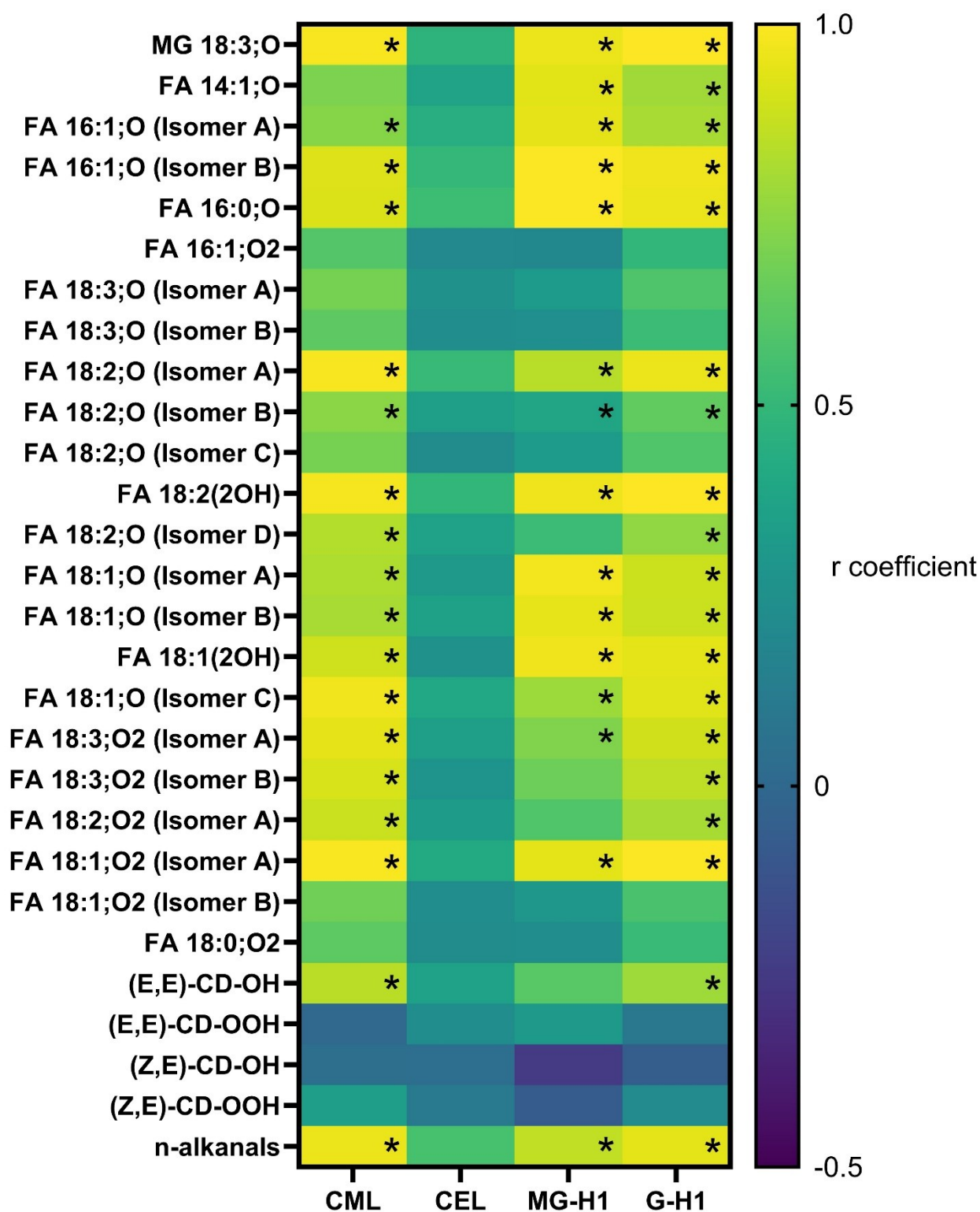
Appendix Figure 27: Concentration of free methylglyoxal-derived hydroimidazolone (MG-H1) in µg MG-H1/g milk before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



Appendix Figure 28: Comparison of the concentration of free glyoxal-hydroimidazolone (G-H1) in µg G-H1/g milk before and after digestion in the different samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



Appendix Figure 29: Concentration of free glyoxal-derived lysine dimer (GOLD) in µg GOLD/g milk before and after digestion in the different milk samples. Statistical analysis was performed using a one-way ANOVA with a Tukey post-hoc test. Different lower-case letters indicate significant differences ($p < 0.05$). Values are given as mean $\pm$ SD with $n=4$.



Appendix Figure 30: Correlation analyses between the content of representative lipid oxidation products after digestion and the total content of the most prominent AGEs. Data is presented as Pearson r coefficients obtained after two-tailed Pearson's correlation test (n = 4). Statistically significant correlations are indicated with * (p < 0.05)