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Role of proteins and phenolic compounds on structural properties and oxidative stability of oleogel systems

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M.Sc. Maria Wittmüss

aus Leipzig

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Dekan: Prof. Dr. Tim Diekötter

1. Berichterstatterin: Prof. Dr. Karin Schwarz

2. Berichterstatter: Prof. Dr. Stephan Drusch

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Abstract

Oleogels represent a sustainable innovation in the nutritional context due to their health and ecological advantages compared to conventional solid fats. By structuring unsaturated fatty acids with the help of gelators, they enable the processing of healthy, regional oils into a solid form. This provides a sustainable alternative to solid fats, which are often sourced from tropical regions and contain high levels of saturated fatty acids. However, to fully exploit this potential, various challenges arising from the composition and structure of oleogels must be addressed. The present work focuses on two of these key issues.

A major challenge is the development of suitable oleogelators. Protein represents a promising, healthy, and sustainable option, but their functionality in this context still requires extensive investigation. In this study, different proteins were evaluated and compared regarding their suitability as oleogelators. It was found that, in addition to whey protein, plant-based alternatives such as soy, rapeseed, or sunflower proteins can exhibit comparable oil-binding capacities. Furthermore, structural relationships influencing protein functionality as oleogelators were identified.

The high content of unsaturated fatty acids in oleogels increases their susceptibility to oxidative deterioration. As this aspect is crucial for food applications, it was given particular attention in this study. On the one hand, the effect of the proteins on lipid oxidation in the oleogel matrix was investigated; on the other hand, the potential for additional stabilization through phenolic compounds was explored. It was demonstrated that both proteins and phenolic compounds significantly reduce the formation of oxidation products in the matrix. The protein restructuring process proved to be a critical factor, depending on the protein source and the applied process parameters. The resulting protein structure influences oil-binding capacity, the antioxidative effect of the proteins, and their interaction with phenolic components – and thus the overall antioxidative potential of the oleogel.

Overall, it becomes evident that protein structuring plays a central role in understanding and controlling the properties of oleogels. This work contributes significantly to unlocking the full potential of protein-based oleogels and their application in sustainable, healthier food products.

Kurzdarstellung

Oleogele stellen auf Grund ihrer gesundheitlichen und ökologischen Vorteile gegenüber konventionellen festen Fetten eine nachhaltige Innovation im Ernährungskontext dar. Durch ihre Fähigkeit, ungesättigte Fettsäuren mithilfe von Gelatoren zu strukturieren, ermöglichen sie die Verarbeitung gesunder, regionaler Öle in fester Form. Damit bieten sie eine nachhaltige Alternative zu festen Fetten, die häufig aus tropischen Regionen stammen und einen hohen Gehalt an gesättigten Fettsäuren aufweisen. Um dieses Potenzial in vollem Umfang nutzen zu können, sind verschiedene Herausforderungen zu bewältigen, die sich aus der Zusammensetzung von Oleogelen ergeben. Die vorliegende Arbeit beschäftigt sich mit zwei dieser Themen eingehend.

Eine wesentliche Problemstellung ist die Herstellung geeigneter Oleogelatoren. Proteine stellen hier eine vielversprechende, gesunde und nachhaltige Option dar, müssen in dieser Funktion jedoch noch umfassend erforscht werden. In der vorliegenden Arbeit wurden daher unterschiedliche Proteine hinsichtlich ihrer Eignung als Oleogelatoren untersucht und miteinander verglichen. Dabei zeigte sich, dass neben Molkenprotein auch pflanzliche Alternativen, wie Soja-, Raps- oder Sonnenblumenprotein eine vergleichbare Ölbindekapazität aufweisen können. Es wurden strukturelle Zusammenhänge identifiziert, die die Eigenschaften von Proteinen als Oleogelatoren beeinflussen.

Der hohe Anteil ungesättigter Fettsäuren in Oleogelen führt zu einer verstärkten Anfälligkeit für oxidativen Verderb. Da dieser Aspekt für den Einsatz in Lebensmitteln von zentraler Bedeutung ist, wurde er in der Arbeit besonders berücksichtigt. Zum einen wurde die Wirkung der Proteine auf Lipidoxidation im Oleogel untersucht, zum anderen die Möglichkeit einer Stabilisierung durch den Einsatz von phenolischen Substanzen. Dabei konnte gezeigt werden, dass sowohl Proteine als auch phenolische Verbindungen die Bildung von Lipidoxidationsprodukten innerhalb der Oleogelmatrix reduzieren. Der Umstrukturierungsprozess der Proteine erwies sich, in Abhängigkeit von der Proteinquelle und den angewendeten Prozessparameter, als entscheidender Faktor. Die resultierende Proteinstruktur beeinflusst dabei das Ölbindevermögen sowie im Zusammenspiel mit phenolischen Verbindungen die stabilisierende Wirkung auf Lipide.

Es wird deutlich, dass die Proteinstrukturierung eine zentrale Rolle für das Verständnis der Zusammenhänge im Oleogel spielt. Die vorliegende Arbeit leistet einen wichtigen Beitrag das Potenzial proteinbasierter Oleogele in Zukunft vollständig auszuschöpfen und für nachhaltige, gesündere Lebensmittelanwendungen nutzbar machen zu können.

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List of Abbreviations

AA	Amino Acid
α-LA	α-Lactalbumin
ANS	8-Anilino-1-naphthalenesulfonic acid ammonium salt
ATR-FTIR	Attenuated total reflection Fourier-transform infrared spectroscopy
AUC	Area under the curve
β-LG	β-Lactoglobulin
BSA	Bovine serum albumin
C	Control
CA	Caffeic acid
CGA	Chlorogenic acid
GA	Gallic acid
HS-GC	Headspace-gas-chromatography
HPLC	High-performance-liquid-chromatography
LMWO	Low molecular weight oleogelator
LOx	Lipid oxidation
MPM	Mussel protein
NFK	N-formylkynurenine
OBC	Oil binding capacity
PC	Phenolic compounds
PreD	Before denaturation
PostD	After denaturation
PrOX	Protein oxidation
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RPM	Rapeseed protein
SA	Sinapic acid
SfPM	Sunflower protein
SPI	Soy protein Isolate
WPI	Whey protein isolate

1 General Introduction

1.1 Motivation and Objectives

The global demand for sustainable and health-oriented food products is increasing rapidly, driven by population growth and a growing awareness of environmental and health concerns (Fraser, 2020; Smith et al., 2024). This has led to a rising interest in regional, plant-based alternatives to conventional animal-derived ingredients. Particularly promising are side stream products from oil extraction processes, which offer an underutilized but valuable source of plant proteins (Cairone et al., 2023; J. Yang et al., 2022).

At the same time, health-conscious consumers are seeking food products with improved fat quality—specifically those rich in unsaturated and essential fatty acids, and with reduced levels of saturated fats. However, achieving desirable texture and functionality in such products remains a technological challenge. Oleogels present a promising strategy to structure liquid oils, replacing solid fats while maintaining functionality (Feichtinger & Scholten, 2020).

Despite growing interest, proteins are still among the least explored structuring agents (oleogelators) for oleogel systems (Kavya et al., 2024). Their inherent hydrophilicity, coupled with the complexity and inefficiency of current processing methods, limits their practical application. Moreover, protein functionality in oleogels is strongly influenced by the source and extraction process (Chandran et al., 2024), and the scientific understanding of their structuring, aggregation, and network formation is still limited. In addition, due to the high degree of unsaturation in the fatty acid profile, oleogels are highly prone to lipid oxidation (LOx) (Philipp M. Meissner et al., 2019). The role of proteins in oxidative stability in oleogel systems is not well understood, and little is known about how they may contribute to or inhibit oxidation in such systems. Investigations in similar systems resuggest, that the addition of phenolic compounds (PC) could have protective effects (Tian et al., 2021), but the mechanisms behind these interactions remain largely unexplored in protein based oleogels.

The main objectives of the present thesis is to gain a deeper understanding of protein-based oleogels by exploring both their structural and oxidative characteristics. First, it seeks to identify which physicochemical and functional properties of proteins influence their oil-binding capacity (OBC), and whether these characteristics can be used to predict the performance of proteins in oleogel systems. A second focus lies on comparing the effects of proteins from different sources—plant-based versus animal-derived—on the oxidative stability of oleogels. Finally, the study investigates whether the addition of specific phenolic compounds can enhance the oxidative stability of whey protein isolate (WPI)-based oleogels, by what mechanism and if such effects are transferable to soy protein-based systems.

1.2 Hypotheses

For this doctoral thesis, the following hypotheses were stated.

Hypothesis 1: Plant-based proteins such as soy, rapeseed, and sunflower exhibit oil-binding capacities comparable to whey protein isolate (WPI) when following the same modification process.

Previous studies have demonstrated that whey protein isolate (WPI) can function effectively as an oleogelator by forming protein aggregates during thermal denaturation, which are capable of immobilizing liquid oil (Vries, 2017). In the search for more sustainable and regionally sourced alternatives, plant-based proteins are being examined for their oil-binding capacity (OBC) under similar modification conditions as applied to WPI.

To assess the extent to which proteins from different plant sources compare with whey protein isolate (WPI) in terms of oil-binding capacity, soy, sunflower, and rapeseed proteins will be examined. All protein samples will be subjected to the same processing conditions previously applied to WPI to increase surface hydrophobicity. Their oil-binding capacity will then be determined and directly compared to that of WPI. Concurrently, relationships between oil-binding capacity and structural or physicochemical parameters are being examined, including alterations in secondary protein structure - resulting from heat-induced aggregation – measured by using attenuated total reflection Fourier-transform infrared

spectroscopy (ATR-FTIR), as well as solubility, particle size distribution, and sample composition.

Hypothesis 2: Protein gelators of different origins exhibit distinct oxidation rates within protein-based oleogels, thereby differentially influence lipid oxidation processes in the oleogel matrix.

Due to their molecular structure, proteins are known to inhibit oxidative processes in lipid-rich systems (Ryan J. Elias et al., 2008). It can therefore be assumed that proteins differing in structure and particle formation also vary in their antioxidant activity. To understand the mechanism behind these effects is of high relevance in order to optimize oxidative stability of protein-based oleogel systems.

To investigate this, lipid and protein oxidation will be studied in oleogels based on protein aggregates derived from various animal (mussel and whey) and plant sources (soy, rapeseed and sunflower). Oxidation markers—including hydroperoxides, hexanal, carbonyl compounds, and N'-formylkynurenine (NFK)—will be monitored and evaluated over a storage period of 22 days at +40 °C.

Hypothesis 3: Lipid oxidation in WPI-based oleogels can be reduced by the addition of phenolic compounds during the formation of protein aggregates.

Phenolic compounds are widely recognized for their antioxidant potential, primarily due to the number and position of hydroxyl groups on their aromatic structures (Alu'datt et al., 2017). However, their interactions with proteins can significantly influence this activity, either enhancing or inhibiting it, depending on the nature of the binding and resulting structural change (Q. Zhang et al., 2021b). In protein-based oleogels, such interactions may play a key role in determining the oxidative stability of the system.

To explore this, sinapic- gallic- chlorogenic- and caffeic acid will be incorporated into whey protein solutions during the aggregation phase. Whey protein isolate (WPI) will be used as a

model system, as it is devoid of secondary plant metabolites and will therefore allow the effects and mechanisms of the added compounds to be assessed without interference. The resulting protein aggregates will be separated from unaggregated protein material and subsequently be dispersed in oil to form oleogels. Oxidative stability will be evaluated by monitoring lipid oxidation markers (e.g., hydroperoxides and hexanal) over a storage period of eight days. In order to elucidate the underlying mechanisms, investigations will be conducted to assess differences in binding strength between phenolic compounds (PC) and proteins, as well as changes in protein concentration during the washing step in the presence of PCs. Additionally, the binding interactions between oxidation products and proteins, as well as alterations in the secondary structure of proteins, measured by using ATR-FTIR, will be examined.

Hypothesis 4: The effect of sinapic- and caffeic acid on oxidative stability in WPI-based oleogels can be transferred to soy-protein-based oleogels.

Both sinapic acid and caffeic acid have been shown to exhibit high antioxidant activity due to their specific structural features. These compounds are known to reduce lipid oxidation in lipid-rich systems effectively (Brewer, 2011; Zamora & Hidalgo, 2016). However, limited information is currently available regarding their behavior in protein-based oleogels and the underlying mechanisms by which they may enhance oxidative stability in such complex matrices. While interaction between SA and CA are shown to increase their antioxidant potential, it remains unclear whether these findings are transferable to oleogels structured with protein, specifically plant-derived proteins. Given the differences in protein structure, surface properties, and functional groups, the interaction and spatial arrangement of phenolic compounds, proteins, and lipids may differ substantially between systems. Consequently, further investigation is required to determine whether the antioxidative potential of these phenolic acids can be realized in plant protein-based oleogels and through which specific mechanisms this may occur.

Soy protein isolate-based oleogels will be prepared under the addition of sinapic acid and caffeic acid to examine how lipid and protein oxidation are affected during storage. Oxidative stability will be evaluated over a 22-day storage period at 40 °C. Lipid oxidation will be assessed by measuring hydroperoxides and hexanal, while protein oxidation will be determined by analyzing carbonyl content and N-formylkynurenine (NFK) levels.

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2 Theoretical background

2.1 Oleogels

The term oleogel combines oleo, referring to oil, and gel, which describes a three-dimensional structure formed from at least two components, creating a dispersed system immobilizing the fluid component (Nayak & Das, 2018). Oleogels are therefore defined as structured oil-based systems in which the liquid oil is entrapped within a network of self-assembled oleogelators (Dassanayake et al., 2009). This leads to the formation of a semi-solid material that no longer exhibits fluidity.

2.1.1 Relevance

Fats and oils have played a fundamental role in human nutrition throughout history. In terms of reducing health risks, the World Health Organization recommends a maximum total fat intake of 30 % of total daily energy consumption (Guideline Development Group on consumption of tropical oils, 2023). This recommendation has become increasingly relevant, given the rising global prevalence of overweight and obesity, which threaten the health of billions of people worldwide. Since 2000, the number of overweight children under the age of five has increased by nearly six million, reaching over 38 million in 2020 (World Health Organization et al., 2021). One contributing factor to this development is the excessive intake of dietary fats. In 2019, the average proportion of fat in daily energy intake in OECD countries was 37.6 %, significantly exceeding the recommended threshold (Csákvári et al., 2023). Although dietary fats are essential for normal physiological functions, they are energy-dense and can therefore promote energy imbalance and unhealthy weight gain when consumed in excess (Sherwood et al., 2000). Obesity, in turn, is associated with increased mortality risk and a higher incidence of non-communicable diseases such as cardiovascular diseases, type 2 diabetes, and certain types of cancer (Afshin et al., 2017). Interestingly, several studies have reported either no association or even a decreased risk of weight gain with higher-fat diets, provided these fats consist predominantly of unsaturated fatty acids (Beulen et al., 2018; X. Liu et al., 2018). These findings highlight the importance of fat quality in addition to fat

quantity. A shift toward increased consumption of unsaturated fatty acids at the expense of saturated fats may thus represent a promising strategy to improve population health outcomes on a global scale.

Fats not only serve important nutritional functions but also contributes to the technological properties in many food products. Numerous fat-containing foods depend on the structural characteristics of fats, such as their temperature-dependent solidity. This firmness is typically achieved through a higher proportion of saturated fatty acids, which increase the melting point of the fat. As a result, the desired structural properties of solid fats—critical for texture, stability, and mouthfeel in various food applications—are closely associated with an elevated content of saturated fatty acids (Vieira et al., 2015). However, as discussed above, saturated fats are linked to adverse health effects, creating a conflict between technological functionality and nutritional recommendations. Vegetable oils, which are naturally liquid at room temperature, can be transformed into solid fats through various processes, including partial hydrogenation, fractionation, and interesterification (Sivakanthan & Madhujith, 2020). These techniques are widely used to impart solid-like functional properties to oils, making them suitable for applications that require firmness, spreadability, or heat stability. However, these modifications can have adverse effects on health. Partially hydrogenated fats, in particular, contain process-derived trans fatty acids, which differ from the naturally occurring trans fats found in some plant and animal sources. These trans fats have been strongly associated with negative health outcomes, including increased risk of cardiovascular disease (Kummerow, 2009). As a result, the food industry has increasingly shifted to using naturally saturated fats, primarily derived from tropical oils such as palm oil, through fractionation processes (Pușcaș et al., 2020). While this strategy avoids the formation of harmful trans fats, it introduces new concerns. The use of palm oil as a major source of saturated fatty acids not only sustains health risks associated with high saturated fat intake but also poses significant environmental challenges. Palm oil production is closely linked to deforestation, loss of biodiversity, and elevated greenhouse gas emissions, particularly carbon dioxide (LAURANCE et al., 2010), making it a controversial and potentially unsustainable alternative.

Oleogels may offer a promising solution by combining a high content of polyunsaturated fatty acids with a semi-solid structure. This enables the development of healthier fat-based food products without compromising the desirable textural and functional properties typically provided by conventional solid fats.

2.1.2 Oleogelators

In order to entrap the liquid oil, oleogelators are needed to form a network and thereby replacing saturated fatty acids. Oleogels can be formed by a wide range of structuring agents by different gelation mechanisms. The final gelation process of the oleogel will be influenced by the structure of the oleogelator, the characteristics of the oil phase and the processing method, preparing the oleogel (L. Li et al., 2022). This building blocks can be classified into low-molecular-weight- (LMWO), polymeric- (PO), and Miscellaneous oleogelators (Patel, 2015). LMWOs are directly dispersible in oil, making them considerably easier to apply as oleogelators. Within the class of LMWOs, common representatives include waxes (Hwang et al., 2013; Toro-Vazquez et al., 2007), phytosterols (Bot & Agterof, 2006), and oligopeptides (Gong et al., 2013). A distinguishing feature of these compounds is their self-assembly behavior. Depending on their molecular structure and environmental conditions, LMWOs can organize into various supramolecular architectures such as fibers, tubes, or inverse micelles. These primary structures can further assemble into larger, hierarchical networks, often forming crystalline structures (Vries, 2017; Vries et al., 2015).

In contrast, among POs, ethyl cellulose is the only known polymer capable of structuring oil through direct addition (Scholten, 2019). Other POs, like proteins and carbohydrates, are generally less suitable for gelating hydrophobic liquids due to their predominantly hydrophilic nature, unless they undergo specific modification.

2.1.3 Proteins as oleogelators

An ideal oleogelator should meet several criteria: it must be food-grade, tasteless, cost-effective, and sustainable. Furthermore, it should withstand exposure to water and elevated temperatures, exhibit high gelling efficiency, and reproduce the characteristic mouthfeel of conventional fats, which it is intended to replace (Scholten, 2019). However, most

oleogelators currently available on the market do not fulfill all of these requirements. Therefore, there remains a strong need to identify and develop alternative oleogelator candidates that not only meet consumer expectations but also offer a clear advantage over traditional fat-based systems.

In this context, proteins are considered a promising alternative due to their high nutritional value, extensive consumer acceptance and the absence of any regulatory issues associated with their use (i.e. green labelling) (Vries, 2017). They are widely recognized as effective gelling agents in aqueous environments, primarily due to their good solubility in water. When the concentration of proteins exceeds a critical threshold—referred to as the critical gelling concentration—they begin to form a three-dimensional, cross-linked network, thereby immobilizing the solvent and forming a gel (Patel, 2015).

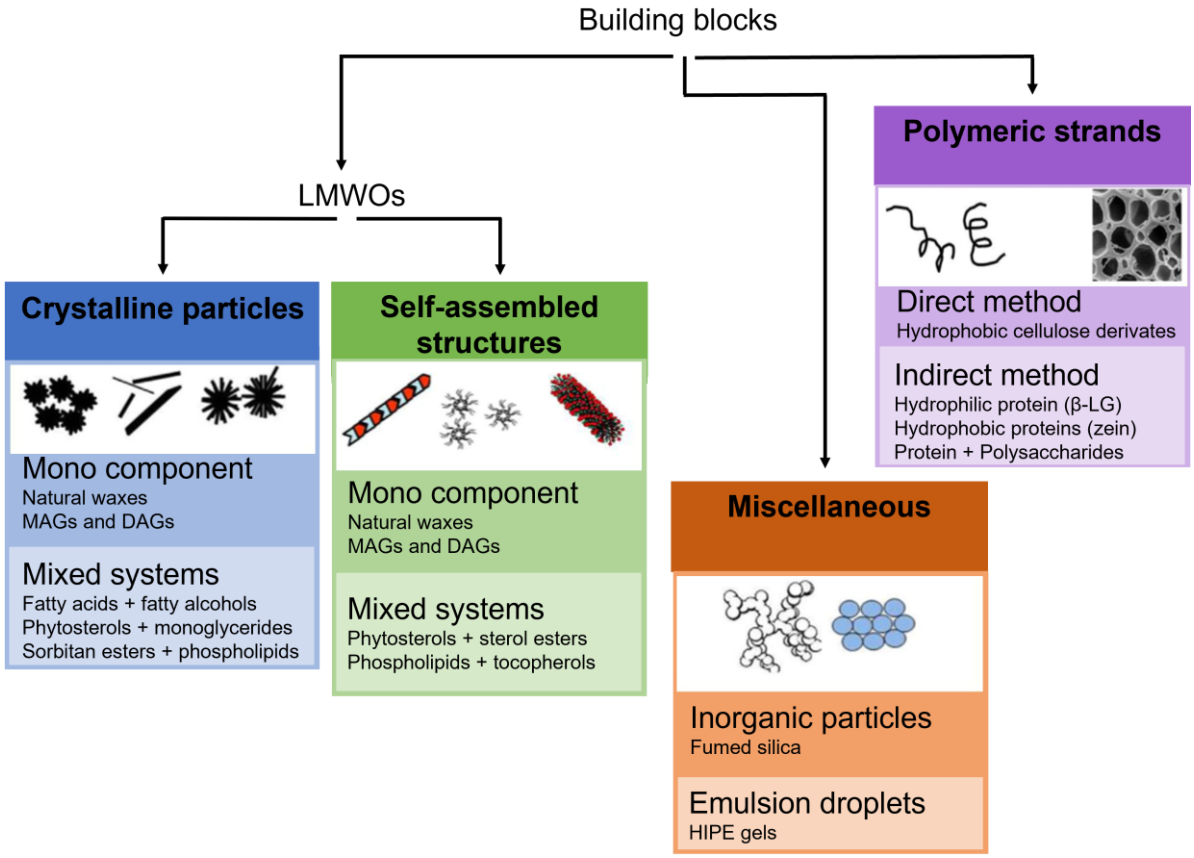

Figure 2.1: Schematic representation of oil structuring through alternative approach using non-TAG building blocks, categorized into different classes with suitable examples modified from(Patel & Dewettinck, 2015).

In nonpolar media such as oils, however, these hydrophilic biopolymers do not disperse efficiently and are therefore unable to establish the necessary network structures. Nevertheless, proteins possess hydrophobic groups within their molecular structure, which are typically buried inside the folded, globular conformation (Gosal & Ross-Murphy, 2000). To harness this potential for oleogelation, indirect methods are required to induce conformational changes in hydrated proteins. These modifications allow the formation of dried microstructures based on hydrophilic polymers, which can subsequently be used to structure liquid oils effectively.

In the case of whey protein, several strategies have been explored to enhance its oil-binding capacity. Romoscanu and Mezzenga (2006) described an emulsion-templated approach, resulting in a fine polyhedral network of protein films capable of entrapping a substantial amount of oil. Following this, mechanical shearing steps are applied, converting the oil phase into a continuous phase and forming a matrix that is defined as an oleogel (Feichtinger & Scholten, 2020).

Vries et al. (2017) described an alternative method to effectively disperse proteins in oil and establish a protein-based network within a hydrophobic environment. In this approach, protein aggregates are first formed by heating an aqueous protein solution above their denaturation temperature in a narrow pH range (~5.6), resulting into unfolding of the protein and forming disulfide-stabilized aggregates. These aggregates are then transferred into oil through a stepwise solvent exchange process or freeze-dried and directly dispersed into oil to form an oleogel by forming an interconnected network, entrapping the oil (Vries et al., 2015). To ensure effective gelation, further agglomeration should be avoided, as the presence of larger aggregates has been shown to result in weaker gel structures (Vries, Lopez Gomez, et al., 2017). In the following chapters, oleogels prepared by dispersion of freeze-dried protein aggregates were used sample matrix.

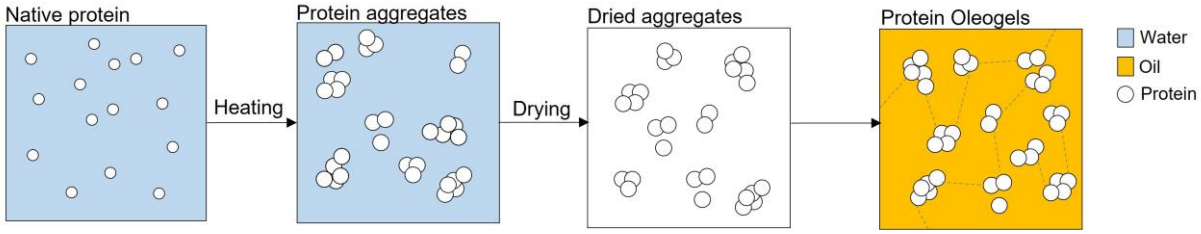

Figure 2.2: Processing of protein based oleogels via dispersion of freeze-dried protein aggregates in oil (modified from Vries et al. 2017).

2.2 Oxidation

Protein-based oleogels consist of proteins and oils—two components that are highly susceptible to oxidative degradation. Lipid and protein oxidation in such systems can lead to a range of undesirable effects, including changes in sensory properties, loss of nutritional value, reduced product quality, and significantly shortened shelf life. To ensure the long-term stability of protein-based oleogels and to develop targeted strategies for preventing oxidation, a comprehensive understanding of the underlying oxidative mechanisms within these complex matrices is essential.

2.2.1 Lipid oxidation

Oxidative deterioration basically means degradation of organic material due to their reaction with oxygen, involving the formation of radicals, which can result into changes in the materials properties. These changes can result in form of discoloration, off-flavor, formation of toxic compounds and decreases nutritional quality and safety (Falowo et al., 2014; Frankel, 1980) In lipid-rich systems, unsaturated—and particularly polyunsaturated—fatty acids are highly susceptible to autoxidation. This susceptibility depends on the availability of allylic hydrogen atoms, which can react with peroxyradicals to initiate oxidation (Frankel, 1980). The degree of oxidizability increases with the number of double bonds, meaning that lipids containing higher levels of polyunsaturated fatty acids are more prone to oxidation (Johnson & Decker, 2015). Given their high content of polyunsaturated fatty acids, lipid oxidation (LOx) is a key concern in systems such as oleogels.

The process of LOx, which follows a radical chain reaction mechanism, is generally divided into three stages: initiation, propagation, and termination.

Initiation:

Initiation occurs when a hydrogen atom (H) in an allylic position relative to a fatty acid double bound is abstracted from an unsaturated fatty acid molecule (LH), resulting in the formation of an alkyl radical ($L^{\bullet}$). This step can be catalyzed by various initiators, including thermal dissociation, hydroperoxide decomposition, metal ions, and exposure to light. (Berton-Carabin et al., 2014; Frankel, 1980).

$$LH \xrightarrow{Initiator} IH + L^{\bullet}$$

Propagation:

The propagation step follows initiation and describes how the alkyl radical (L•) reacts with atmospheric oxygen to form a lipid peroxyl radical (LOO•). This peroxyl radical can, in turn, abstract a hydrogen atom from another unsaturated lipid molecule (LH), resulting in the formation of a lipid hydroperoxide (LOOH)—the primary LOx product (Frankel, 1980; Johnson & Decker, 2015). The propagation phase continues as long as reactive substrates are available, such as unsaturated double bonds in fatty acids. Primary LOx products, such as hydroperoxides (LOOH), are inherently unstable and can decompose into a wide range of secondary oxidation products, including alkoxy (LO•), peroxyl (LOO•), and hydroxyl (•OH) radicals, as well as new lipid radicals (Shahidi & Zhong, 2010).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Among these, volatile compounds like hexanal and propanal are particularly significant, as they contribute to the off-flavors and quality deterioration of lipid-containing foods (Berton-Carabin et al., 2014). These decomposition processes are primarily driven by both radical and non-radical mechanisms, accelerated by the presence of metal catalysts and elevated

temperatures (Johnson & Decker, 2015). However, the chain reaction can be interrupted by the presence of antioxidants or by the depletion of susceptible lipids (Shahidi & Zhong, 2010). When this occurs, the final stage of lipid autoxidation—termination—takes place.

Termination:

Termination, the final step of the autocatalytic radical chain reaction, marks the end of the chain process by the recombination of radicals into non-radical, stable products (Johnson & Decker, 2015; Shahidi & Zhong, 2010). Due to the diverse decomposition pathways of hydroperoxides, various radicals are generated, leading to numerous possible combinations between them.

$$\left.\begin{array}{l} ROO^{\bullet} \\ RO^{\bullet} \\ R^{\bullet} \end{array}\right\} \rightarrow \textit{Non-radical products}$$

2.2.2 Protein oxidation

In contrast to LOx, protein oxidation often proceeds without visible indicators, yet it can profoundly impact protein structure and function (Xiong & Guo, 2020). Oxidative stress induces a variety of chemical modifications, affecting both amino acid side chains and the protein backbone. These alterations include thiol oxidation, carbonyl formation, and conformational changes, which may compromise protein stability, solubility, and biological activity (Hülsebusch et al., 2025).

In food systems, protein oxidation can be triggered either directly by reactive oxygen or nitrogen species (ROS/RNS) or indirectly via LOx products (Xiong & Guo, 2020). Particularly, lipid-derived radicals and hydroperoxides can interact with proteins through radical transfer or covalent bonding, resulting in significant structural and functional changes. The process often begins with ROS-induced formation of protein radicals, which can localize to aliphatic,

aromatic, heteroatom-containing, or backbone sites. These radicals are capable of migrating between sites and can even transfer from side chains to the protein backbone (Hellwig, 2020). The specific mechanism and the chemical structure of the final oxidation products depend on the site of attack, the oxidizing system, and the reaction conditions (Davies, 2005). However, no universal scheme of protein oxidation exists, since the course of the reaction is strongly influenced by the properties of the protein, the nature of the oxidizing system, and the molecular environment of the protein.

Under low oxygen conditions and high radical concentrations, radical dimerization may occur, especially involving cysteine and tyrosine residues, leading to the formation of disulfide cysteine and dityrosine, respectively (Hülsebusch et al., 2025). In the presence of molecular oxygen, carbon-centered protein radicals can react to form peroxyl radicals, which may stabilize through hydrogen abstraction, yielding relatively stable protein hydroperoxides at aliphatic or aromatic residues (Hellwig, 2020). These hydroperoxides can then promote cleavage of carbon–heteroatom bonds, particularly in residues like lysine and arginine. Specific amino acids are especially susceptible to oxidative modification. For instance, radical attacks on tyrosine and tryptophan lead to the formation of fluorescent compounds such as dityrosine and N-formylkynurenine (NFK). In the case of tryptophan, initial radical formation yields a stabilized tryptophyl radical. Upon oxygen addition, hydroperoxide intermediates are formed, which then undergo rearrangement and β-scission to yield NFK (P. Meissner, 2021).

Carbonyl formation can occur through various reaction pathways. Direct reactions of reactive oxygen species (ROS) with aliphatic amino acid side chains, such as lysine or arginine, result in the formation of an unstable amine intermediate, which is subsequently converted into a carbonyl group (Davies, 2005). Besides side-chain modifications, the protein backbone is also a major target of oxidative damage. Peroxyl radicals and side-chain-derived hydroperoxides may decompose into α-carbon alkoxyl radicals, which undergo β-scission, leading to peptide backbone cleavage. This process generates protein carbonyls such as allysine and can proceed via two pathways: α-amidation (towards the N-terminus) or the diamide pathway (towards the C-terminus) (Hülsebusch et al., 2025; P. Meissner, 2021). Moreover, secondary LOx products, especially epoxides and carbonyl compounds, can covalently bind to proteins via

Michael addition or Schiff base formation. These reactions not only introduce additional carbonyl groups into the protein but may also promote cross-linking and accelerate the degradation of protein functionality (Xiong & Guo, 2020).

Oxidation-induced modifications of proteins can also be observed at the level of secondary and tertiary structures, thereby exerting a significant impact on their overall functionality (Hülsebusch et al., 2025).

2.3 Phenolic compounds

Phenolic compounds (PC) are belonging into the class of secondary metabolites, ubiquitous in plants and plant derived foods and beverages (Cheynier, 2012). Structurally, this group includes a diverse range of molecules, from simple phenols to complex polyphenols such as flavonoids and stilbenes, along with numerous derivatives. Polyphenols are more narrowly defined as *"plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or polyketide pathways, containing more than one phenolic ring and lacking nitrogen-based functional groups in their simplest form"* (Quideau et al., 2011). In contrast, the broader category of phenolic compounds encompasses a wider variety of chemical structures and functionalities. Phenolic compounds are of particular interest due to their diverse bioactivities, which include antioxidant, anti-inflammatory, antimicrobial, and antiviral properties (Ignat et al., 2011). These properties make them promising candidates not only for promoting human health but also for enhancing food quality and shelf life, particularly through their potential role in inhibiting oxidative and microbial spoilage. As a result, phenolic compounds are increasingly being investigated for applications in functional foods and natural preservation strategies.

Phenolic compounds are classified into several subgroups, including phenolic acids, flavonoids, stilbenes, tannins, and lignans. This classification is based primarily on the number of phenolic rings they contain and the structural elements that connect these rings to one another (Ignat et al., 2011).

Phenolic acids represent a major class of PCs and are structurally characterized by a carboxylic acid group directly attached to an aromatic benzene ring (Durazzo et al., 2019). All phenolic acid derivatives are biosynthesized from the aromatic amino acid L-phenylalanine via the shikimate pathway (Robbins, 2003). They are commonly divided into two structural subgroups based on the length and structure of their side chains. The first subgroup, known as hydroxybenzoic acids, exhibits a C6–C1 structure, consisting of a six-carbon aromatic ring (C6) and a one-carbon side chain (C1). Typical representatives of this group include gallic acid and vanillic acid (Ignat et al., 2011). The second subgroup, hydroxycinnamic acids, is defined by a C6–C3 structure, in which a three-carbon aliphatic side chain is attached to the aromatic ring. Prominent examples of hydroxycinnamic acids are caffeic acid, ferulic acid, and sinapic acid (Ignat et al., 2011). Structural variations during synthesis influence not only the classification of phenolic acids but also their chemical reactivity, antioxidant capacity, and biological functions in plants and food systems. Despite their relatively simple molecular structure, phenolic acids are known to form strong associations with primary metabolites such as proteins and carbohydrates, thereby affecting the physicochemical properties and stability of food matrices (Alu'datt et al., 2017).

Flavonoids belong to the group of polyphenols and represent one of the most diverse and widespread classes of plant secondary metabolite(Alu'datt et al., 2017) They can be further subdivided into several major subgroups, including anthocyanins, flavones, isoflavones, flavanones, flavonols, and flavanols (Ignat et al., 2011). These compounds are biosynthetically derived from the aromatic amino acids phenylalanine and tyrosine, and are structurally defined by a common backbone of 15 carbon atoms arranged in a C6–C3–C6 configuration.

Tannins, stilbenes, and lignans are less abundant in plants compared to other phenolic compounds and are therefore less prominent in the human diet (M. Saxena et al., 2012). Nevertheless, they contribute significantly to human health and food stability due to their bioactive properties (Oluwole et al., 2022). Tannins, which represent the third major group of phenolic compounds, can be broadly classified into two main categories: hydrolysable tannins and condensed tannins (Durazzo et al., 2019; Ignat et al., 2011). Hydrolysable tannins are composed of a central core of polyols, typically glucose, esterified with gallic or ellagic acid

(Oluwole et al., 2022). In contrast, condensed tannins—also known as proanthocyanidins—are high molecular weight polymers consisting of two to more than 200 flavanol monomer units, such as catechin and epicatechin (Ignat et al., 2011). Their complex structure and polymeric nature contribute to their strong antioxidant capacity and their ability to interact with proteins, which is relevant in both nutritional and technological contexts.

Stilbenes are a small but significant group of non-flavonoid polyphenols, characterized by a structure consist of two benzene rings linked by a double bond (C6–C2–C6) (Ignat et al., 2011). They are primarily found in grapes, berries, and peanuts. The most well-known stilbene is resveratrol, which has attracted considerable scientific interest due to its antioxidant, anti-inflammatory, and potential cardioprotective effects (Oluwole et al., 2022). Despite their relatively low abundance in foods, stilbenes have been linked to several health-promoting properties, particularly in the context of the Mediterranean diet.

Lignans are another group of non-flavonoid phenolic compounds, derived from the combination of two phenylpropanoid units at β and β^1 carbons, that can be additional linked to ether lactone or carbon (Durazzo et al., 2019; Oluwole et al., 2022) They are widely distributed in seeds (especially flaxseed), whole grains, vegetables, and fruits (Oluwole et al., 2022). Lignans are known for their potential protective effects against hormone-related diseases, such as breast and prostate cancer (Durazzo et al., 2019; Oluwole et al., 2022).

2.3.1 Interaction with Protein

Due to their beneficial effects on human health and food stability, phenolic compounds are commonly found in foods—either through their natural occurrence in plant-derived ingredients, as they are secondary plant metabolites, or through intentional addition during food processing to exploit their functional properties. It is well established that phenolic compounds can interact with other components in food systems, particularly proteins. However, the underlying mechanisms of these interactions remain complex and not fully understood, largely due to the vast structural diversity of both phenolic compounds and food matrix components (Kieserling et al., 2024). Among these, proteins have been identified as primary interaction partners, as they offer a variety of structural features—such as functional groups, binding sites, and conformational flexibility—that facilitate diverse modes of

interaction with phenolic compounds (Kieserling et al., 2024). In addition to the chemical mechanisms underlying these interactions, it remains an open question how such interactions influence the functional- and structural properties of both the proteins and the phenolic compounds involved.

Interactions between proteins and phenolic compounds can occur through both reversible (non-covalent) and irreversible (covalent) mechanisms. Non-covalent interactions are primarily driven by a combination of hydrophilic and hydrophobic forces, including hydrogen bonding, electrostatic or ionic interactions, and hydrophobic stacking (Kieserling et al., 2024; Quan et al., 2019a). Due to their excellent hydrogen-donating capacity, phenolic compounds are capable of forming hydrogen bonds with the carbonyl groups of proteins. Additionally, interactions can occur between the hydroxyl groups of phenolic compounds and various functional groups of the protein, such as the carbonyl and amino groups of the peptide backbone, as well as the hydroxyl, thiol, or carbonyl groups present in certain side chains (Quan et al., 2019a). Van der Waals forces may also contribute to these interactions, particularly through non-aromatic hydrophobic amino acid side chains, including those of isoleucine, leucine, methionine, and cysteine, although their overall contribution is generally considered to be minor. Furthermore, ionic or electrostatic interactions can occur between deprotonated functional groups of phenolic compounds and positively charged amino acid side chains of proteins. The strength and occurrence of these interactions are highly dependent on the physicochemical conditions of the system, particularly the pH. Variations in pH can influence both the ionization state of the phenolic compounds and the protonation or deprotonation of protein side chains, thereby affecting their charge distribution and interaction potential (Le Bourvellec & Renard, 2012).

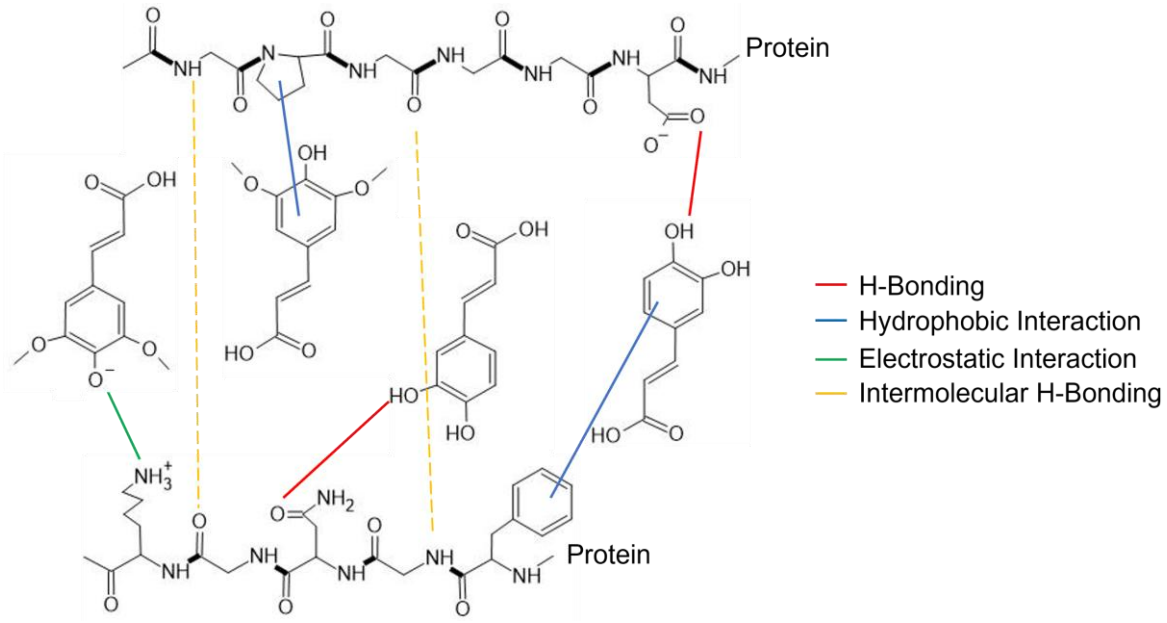

Figure 2.3: Non-covalent interactions between caffeic-, sinapic acid and proteins (modified from Shahidi et al. 2023).

Covalent interactions between phenolic compounds and proteins require oxidative conditions, in which phenolic compounds are transformed into electrophilic o- or p-quinones. These quinones can bind covalently to nucleophilic groups in protein side chains, either through enzymatic or non-enzymatic mechanisms (Kieserling et al., 2024; Quan et al., 2019a). Under non-enzymatic conditions, phenolic compounds are susceptible to oxidation in alkaline environments, forming semiquinone radicals that subsequently rearrange into reactive quinones (F. Liu et al., 2017). Enzymatically, oxidation occurs in the presence of oxygen and is catalyzed by polyphenol oxidases such as catecholoxidases and laccases (Le Bourvellec & Renard, 2012). The resulting quinones readily undergo nucleophilic addition reactions with amino acid residues in proteins, leading to covalent cross-links that can significantly alter the physicochemical and functional properties of both interaction partners (F. Liu et al., 2017).

The interactions between phenolic compounds (PCs) and proteins are significantly influenced by both extrinsic and intrinsic factors. Extrinsic factors include environmental conditions such as pH and temperature, whereas intrinsic factors involve the structural characteristics and types of both the phenolic compounds and the proteins.

Temperature, in particular, has a notable impact on PC–protein interactions. Kieserling et al. (2024) summarized, that moderate increases in temperature can promote protein unfolding, thereby enhancing the interaction with PCs by exposing additional binding sites (Quan et al., 2019b). However, if increases in temperature may lead to protein aggregation, which reduces the accessibility of these binding sites and consequently diminishes both non-covalent and covalent interactions. An exception must be noted in the case of enzymatically catalyzed covalent interactions. Here, the temperature must be optimized for the specific enzyme involved, as elevated temperatures may lead to enzyme denaturation or inactivation, thus impairing the reaction.

Similarly, the structures of both proteins and phenolic compounds (PCs) also influence these interactions by affecting the accessibility of functional groups involved in the binding process. Larger PCs, for instance, have been shown to interact more readily with proteins due to their greater surface area, which increases the likelihood of contact and binding site exposure. Protein structure likewise plays a crucial role, as variations in amino acid composition, surface hydrophobicity, and isoelectric point influence the distribution and accessibility of binding sites on the protein surface. These structural characteristics determine the extent and nature of the interactions with PCs (Quan et al., 2019b).

Conversely, interactions between proteins and polyphenols also affect the properties of resulting complexes, depending on the nature of the interaction. Understanding these changes is particularly important when aiming to use protein–polyphenol complexes as functional ingredients in biologically relevant matrices such as food systems. Properties such as antioxidant capacity, solubility, emulsifying ability, and sensory characteristics are key factors that must be thoroughly understood in order to utilize these complexes effectively. Moreover, due to the structural diversity of the reaction partners and the complexity of the reaction conditions, a wide range of reaction products can be formed—each with distinct functional properties. Fully unlocking the potential of these complexes requires a comprehensive understanding of these interactions and their outcomes.

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3 Oil binding capacity and related physicochemical properties of commercial plant protein products

Maria Wittmüss, Jonas Amft, Timon R. Heyn*, Karin Schwarz

Institute of Human Nutrition and Food Science, Division of Food Technology, Heinrich-Hecht Platz 10, D-24118 Kiel, Kiel University, Germany.

* Corresponding author (theyn@foodtech.uni-kiel.de)

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Highlights

- Plant proteins can lead to comparable oil binding capacities as shown for whey protein isolate.
- Oil binding capacity correlates positively with carbohydrate content, solubility, particle size, and α-helix structure.
- Purification methods affect oil binding and protein properties strongly.

Abstract

Providing sufficient food to a growing population while addressing the rise of food-related diseases requires innovative solutions. The use of plant proteins as structuring agents for oils has shown potential because of their healthier fatty acid profiles compared to solid fats. Thus, this study aims to investigate the potential correlations between the properties of proteins and their oil binding capacity (OBC) to provide insights into their applicability in structuring oil. Two samples each of sunflower, rapeseed, and soy protein products obtained from different manufacturers were denatured by heat and dispersed in oil after freeze-drying. Whey protein isolate (WPI) was used as a reference. The results indicate that, with some modifications, rapeseed and sunflower proteins can bind amounts of oil (3.7 oil [g]/protein [g]) comparable to that of WPI (4.04 oil [g]/protein [g]). However, the extraction process affects the final results and the texture of the protein-oil dispersion. Moreover, the protein solubility in NaCl, carbohydrate content, particle size, and the quantity of α-helical structures are positively correlated with the OBC, whereas an increase in the firmness of the dispersion is negatively associated with it. There was no correlation found between the changed β-sheet structure resulting from the heat treatment and the OBC. This study emphasizes the ability of plant proteins to structure oil and explains how to evaluate the physical and structural properties of plant proteins. Technical insights that may lead to the production of more sustainable and healthier food products are provided.

3.1 Introduction

The solid or semi-solid consistencies of fats have texturizing functions in food, but the saturated fatty acids responsible for this are undesirable from a nutritional point of view. If solid fats are replaced by liquid oils that are structured using proteins, waxes, or cellulose, the proportion of unsaturated fatty acids can be increased (DGE, 2023; Kostik et al., 2013). This could help reduce the incidence of coronary heart disease in the population (Wolfram et al., 2015) and may also improve the ecological footprint by using less tropical fats. Proteins, including whey protein, have the potential to adsorb fat after certain modifications (Feichtinger & Scholten, 2020). Using proteins for structuring oil could have numerous benefits. For instance, proteins contribute to the nutritional value and the structured oil could be sold as a clean-label product because there are no regulations prohibiting the use of proteins in food (Scholten, 2019; Vries et al., 2015). Considerable progress has been made in understanding the oil-binding mechanism and rheological properties of a protein-lipid matrix made using proteins from milk (Meissner et al., 2020b; Vries, Lopez Gomez, et al., 2017). From a sustainability perspective, it is necessary to consider to what extent these findings can be applied to plant-based sources. Furthermore, in the bioeconomic approach, it is preferable to use sources that provide proteins as a side-stream product. Rapeseed, sunflower, and soybean are economically important oilseeds that generate considerable amounts of protein-rich press cake after de-oiling, which represents an opportunity for the practical utilization of this byproduct (Pilorgé, 2020). The composition and protein content of the obtained oilseed meals differ depending on the plant source, pretreatment (e.g., dehulling and storage), and oil extraction method (Ramachandran et al., 2007). Harsh extraction conditions reduce the protein's nativity, thereby altering its technological properties (e.g., solubility and conformation), which affects the applicability of the protein in the end product (Ochoa-Rivas et al., 2017). For instance, proteins extracted via alkali or enzymatic processes can be used to manufacture emulsion-based food products (Tirgar et al., 2017). Therefore, the extraction method must be tailored to attain the desired properties of the protein for a particular application.

Furthermore, de Vries et al. (2017) demonstrated that the OBC of whey proteins can be increased by heating, shearing, and freeze-drying. The underlying principle is that hydrophobicity is enhanced through denaturation, whereby the hydrophobic core of the globular whey protein is exposed to the surface, culminating in superior dispersibility in oil.

Thus, we hypothesized that this approach could be transferred to plant proteins and that they may be applied to structured oils. To achieve an enhanced oil-binding capacity, various plant proteins were denatured and lyophilized. The obtained powder (composed of protein aggregates) was dispersed in oil following the method of Meissner et al. (2020a) and Vries et al. (2017) for whey protein isolate (WPI).

The objective of this study was to identify the properties of plant proteins obtained by different extraction procedures that correlate with the oil binding capacity (OBC) and the texture of the protein-lipid dispersion. Therefore, we characterized the untreated protein powders from different manufacturers regarding physico-chemical properties related to hydrogel preparation and parameters relevant for particles acting as oil structuring agents. We analyzed the protein product´s composition and functional properties, including solubility, surface hydrophobicity and charge as well as structural and morphological parameters (i.e., particle size and FTIR).

The use of plant proteins to structure oils could be a great advancement in developing novel and healthier foods. Combining the nutritional value of healthier oils and proteins with the stability that solid fats typically provide could contribute to a healthier version of many products. However, the use of plant proteins as structuring agents for the oil phase has received little attention (Martins et al., 2020). Therefore, this work could serve as a starting point for investigating plant-based oleogels.

3.2 Material and Methods

Whey protein isolate (WPI) with 93.4% protein and 75% β-lactoglobulin (dry matter basis) was obtained from Davisco Foods International (Le Sueur, MN, USA). SUPRO 313 IP and VEGACON 90 KK soy protein isolates (SPIs) with 89.9% and 89.3% protein (dry matter basis) were kindly provided by KUK (Ried im Innkreis, Austria) and EUROSOY GmbH (Hamburg, Germany), respectively. Rapeseed protein meal (RPM) with 49.4% and 30.2% protein (dry matter basis) was kindly provided by Vegetein (Canolein, Jõgeva, Estonia) and the Fraunhofer CBP Institute (Leuna, Germany), respectively. Organic Heliaflor 55 and Suntein sunflower protein meal (SfPM) with 55% and 51% protein (dry matter basis) were kindly provided by All Organic Treasures GmbH (Wiggensbach, Germany) and Georg Breuer Food Ingredients (Königstein, Germany), respectively. TRUPRO 2000 pea protein concentrate with 83% protein (dry matter basis) was kindly provided by KUK-Handels GmbH. VARIOPLANT HP 60 OW hemp protein concentrate with 60% protein (dry matter basis) was kindly provided by EUROSOY. Lupine protein isolate with 92.4% protein (dry matter basis) was kindly provided by ProLupin (Grimmen, Germany). Pumpkin Protein Concentrate was kindly provided by All organic treasures GmbH with 60% protein in dry matter. Fully refined sunflower and rapeseed oil were purchased in a local supermarket. Ultra-pure water (Milli Q) was used for all solutions (<18.2 MΩ cm-1).

All chemicals used were of analytical grade: hydrochloric acid (HCl) and β-mercaptoethanol (β-ME), purchased from Merck KGAA (Darmstadt, Germany); ethanol, ethyl acetate, isopropanol, methanol, phosphoric acid, sodium chloride, sodium dodecyl sulfate (SDS), and Tris, purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). A 30% acrylamide/Bis solution (37.5:1), ammonium persulfate (APS), bromophenol blue, Coomassie brilliant blue R-250 and G-250, glycine, Tris, Precision Plus Protein All Blue Prestained Protein Standard, a 20% SDS solution, and N,N,N',N'-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories GmbH (Feldkirchen, Germany).

3.2.1 Sample composition

AGROLAB LUFA GmbH (Kiel, Germany) analyzed the samples. The crude protein content was determined using the Kjeldahl (N × 6.25) method (BVL L 17.00-15:2013-08). The total fat content was determined after acid digestion, using extraction and gravimetry (§64 LFGB L 17.00-4:2017-10). The water content was determined as the drying loss (§64 LFGB L 17.00-1:2002-12). The sugar content was determined by measuring fructose, glucose, lactose, maltose, and sucrose using HPLC (DIN 10758:1997-05). Content of carbohydrates was calculated.

3.2.2 Sample preparation

3.2.2.1 Preparation of aggregates

Protein aggregates were prepared according to Meissner et al. (2020b) with some modifications. Briefly, protein powder (6%, w/w) was dissolved in Milli Q water under constant stirring. The desired pH (5.6 ± 0.6; isoelectric point, IEP) was adjusted using 2 M HCL before stirring the solution at 20°C room temperature (RT) for 2 h. Subsequently, the solution was denatured for 20 min at 85°C in a temperature-controlled water bath, using 50 mL plastic tubes. The resulting protein aggregate dispersion was homogenized using a rotor-stator homogenizer (Ultra-Turrax) at 13000 rpm for 3 min. The samples were then centrifuged at 4000 x g for 20 min at RT to collect the aggregates in a pellet. The supernatant was discarded and the aggregates were dispersed in fresh water using the Ultra-Turrax (13000 rpm, 3 min). After washing, the protein aggregates were frozen at -80°C for 2 days and then they were freeze-dried (Martin Christ, Osterode am Harz, Germany) for 72 h. The resulting aggregates were ground in a mortar until a fine powder was obtained. All samples were prepared in triplicate.

3.2.2.2 Preparation of dispersions

The lyophilized protein powders were mixed with a combination of sunflower and rapeseed oils (1:1, v/v) at a concentration of 6.8%. The mixture was stirred for 10 min and then centrifuged at 4000 x g for 20 min at RT. The resulting solidified phase was carefully separated from any unbound oil, which was then discarded. To ensure optimal drainage of excess oil,

the samples were left suspended upside down for 10 min. The remaining gel-like plug was analyzed in subsequent measurements.

3.2.3 Physicochemical properties

3.2.3.1 Solubility

To determine protein solubility, the samples were centrifuged at 4000 x g for 20 min at RT. The protein content was measured using the Lowry assay at 540 nm with bovine serum albumin (BSA) as a standard, as described by Lowry et al. (1951). Measurements were performed in 800 mg/mL concentrated protein solution in Milli Q water or 0.5 M sodium chloride (NaCl) at a neutral pH or pH 9.

3.2.3.2 Surface hydrophobicity

Surface hydrophobicity was determined using 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) as a fluorescence probe following Lam and Nickerson (2015). Protein was suspended in phosphate buffer (pH 7.4) at a concentration of 1 mg/mL. The samples were diluted to four concentrations between 0.6 and 0.3 mg/mL. After adding ANS (20 µL) to the protein solution (1.6 mL) the samples were incubated in the dark for 5 min. Fluorescence intensity was measured at 390 nm (excitation wavelength) and 490 nm (emission wavelength) with excitation and emission slit widths of 2.5 nm and 5 nm, respectively, on a fluorescence spectrophotometer (Varian Cary 154 Eclipse, Agilent Technologies, Santa Clara, CA, USA). All results were corrected by the fluorescence of water with ANS. All samples were measured at least in triplicate. A plot of the initial slope of fluorescence intensity according to the protein concentration was taken as an index of surface hydrophobicity.

3.2.3.3 Isoelectric point

The isoelectric point (IEP) of the protein was determined by the zeta potential, using a Zetasizer Nano ZS instrument (Malvern Instruments, Herrenberg, Germany). The protein was diluted in Milli Q water to a concentration of 1 mg/mL. Several pH values between 12 and 1 were adjusted using 2 and 6 mM HCl and NaOH to obtain a linear function and the pH value at which the zeta potential reaches 0. The temperature was set at 25°C with an initial

equilibration time of 90 s. For all measurements, a disposable folded capillary cuvette (Malvern Instruments, Malvern, Grovewood, UK) was used.

3.2.3.4 Protein composition

The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli under reducing (+ β-mercaptoethanol, β-ME) or non-reducing conditions (Bio-Rad, Laboratories, Inc.). Briefly, 20 µg of protein was adjusted to 50 µL with water and mixed with the same amount of sample buffer (62.5 mM Tris-HCl, pH 6.8; 25% glycerol, 0.01% bromophenol blue, 2% SDS, and 715 mM of the reducing agent β-ME). Samples were denatured in a thermoshaker (Thermal Shake Lite, VWR, Darmstadt, Germany) at 95°C for 5 min. A 4% stacking and a 12% resolving gel were prepared according to the manufacturer's instructions, using a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA) system. Then, 10 µL of the sample was loaded onto each well. SDS-PAGE was performed at 40 V for 10 min to collect the sample into the stacking gel and, subsequently, at 100 V for separation. The running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS. To determine the protein size, a marker (Precision Plus Protein All Blue Prestained Protein Standard) was used for each gel. After separation, the protein bands were fixed using a solution of 30% MeOH, 60% H2O, and 10% acetic acid (v/v) for 20 min under constant shaking at 40 rpm. Staining was performed using a solution of 0.2% Coomassie brilliant blue R-250 and 0.005% G-250 (both w/v) in 10% ethanol, 40% methanol, 2% glycerol, and 48% water (all v/v) for 25 min, followed by destaining in a solution of 10% acetic acid, 5% ethanol, and 85% water for several hours. These procedures were conducted under constant shaking. The resulting stained gels were then scanned to analyze the data.

3.2.3.5 Particle size

The particle size distributions of the solubilized protein and protein aggregates were measured by the static light scattering (SLS) method, using a particle size distribution analyzer (LA-950V2 Horiba, Retsch Technology GmbH, Haan, Germany). The sample was dispersed in distilled water in the wet chamber until the RPM and B transmittances were in the range of 70–90%. Measurements were performed using a refractive index of 1.45 for protein

aggregates and 1.33 for water. All samples were measured at least in triplicate. The median and the modal value of the particle size distribution were determined.

3.2.3.6 Secondary structure

To investigate the secondary structure of the proteins before and after denaturation, ATR-Fourier transform infrared spectroscopy (FTIR) was performed using a CONFOCHECK TENSOR II system (Bruker Optics, Ettlingen, Germany). A wavenumber range of 4000–900 cm−1 was considered. Measurements were conducted at 25°C against Milli Q water as the background. The protein solution (20 µL) was placed on the ATR crystal and 120 scans were recorded and averaged per analysis. The spectra were corrected and vector-normalized using OPUS version 7.5 (Bruker Optics GmbH, Ettlingen, Germany). The second derivation of the amide I band (1590–1700 cm-1) was calculated with nine smoothing points, using OPUS to determine the secondary structures of the protein samples. Moreover, we analyzed the normalized curves using Fourier self-deconvolution for quantitation. This entailed using a resolution enhancement factor of 3.0 cm-1 and a bandwidth of 10 cm-1 over a wavenumber range of 1700–1480 cm-1. We then integrated the resulting bands within the amide I range (1700–1590 cm-1) to calculate and quantify the structural components. All measurements were performed at least in triplicate.

3.2.3.7 Oil binding capacity

The oil binding capacity (OBC) was measured by stirring the freeze-dried and finely ground protein aggregate powder (10% w/w) for 10 min with a mixture of sunflower and rapeseed oils (1:1, v/v). After the dispersion was centrifuged (4,000 x g, 20 min, RT), the samples were stored overhead for 10 min so the excess oil could drain off. All samples were measured at least in triplicate. The final weight was recorded and the OBC was calculated using Equation (1), where W Oleogel is the weight of the final oleogel and W protein aggregate is the weight of the proportion of protein aggregates in the sample:

OBC [(g (oil))/(g (Protein aggregates))]= (w_(oleogel)-w_(protein aggregates))/w_(protein aggregates)

3.2.4 Texture of the protein-oil-dispersion

The texture of the obtained protein-oil dispersion was measured using a texture analyzer (AMETEK Brookfield CTX, Brookfield, Hadamar-Steinbach, Germany). A standardized probe (diameter = 8 mm) entered the sample at 1 mm/s with a trigger load of 0.5 g at a depth of 10 mm. The maximal load in g was used for the comparison. All samples were prepared in triplicate.

3.2.5 Statistical analysis

All measurements were performed in triplicate. The data were compiled and analyzed using GraphPad Prism (version 10.0.2, GraphPad Software, San Diego, CA, USA).

Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with $p < 0.05$. The Pearson R correlation was performed using normalized data.

3.3 Results and Discussion

Various commercial plant protein meals and isolates were analyzed to determine their functional properties before aggregate formation (Supplementary Table 1). The OBC, solubility, particle size, secondary structure, and information about the extraction method were analyzed. Plant proteins from rapeseed and sunflower were chosen for further measurements because they exhibited higher OBC values in the preliminary measurements compared to other plant proteins (pea, pumpkin, lupin, and hemp) (Table S3.1). Soy protein isolate was included as an additional reference protein alongside WPI. Protein isolates and meals derived from three oil-press cakes (rapeseed, soybean, and sunflower) were analyzed to determine their composition (Table 3.1) and were used to form aggregates. Whey protein isolate (WPI) served as a reference protein. Ethanol extraction was used by the manufacturers to purify rapeseed proteins. For soy protein, the key step was precipitation at the isoelectric point (SPI 1) or ethanol extraction (SPI 2). For sunflower protein meals, CO_2-extraction (SfPM 1) and cold pressing (SfPM 2) were used (Table 3.1). The OBC of the protein aggregates and textural properties of the protein-oil dispersions were investigated (Fig. 3.2).

3.3.1 Oil binding capacity

The OBC describes how many grams of oil per gram of protein remain trapped within the protein matrix after centrifugation. For aggregates from WPI, the OBC was 4.04 ± 0.11 oil [g]/protein [g] (Fig. 3.2a), which is in line with previous findings. For the vegetable protein aggregates SPI 2, SfPM 1, and RPM 1, the OBC was significantly lower (approximately 1.5 oil [g]/protein [g]), whereas SfPM 2, RPM 2, and SPI 1 showed OBCs similar to that of WPI. Furthermore, we investigated the effect of the pH during aggregate formation. The aggregation was forced at the IEPs of the plant proteins, which were markedly lower than the IEP of WPI (pH 5.6) (Table 3.1).

For RPM 2, the OBC was higher at higher pHs (IEP (pH 2.89) + 0.5 and pH 5.6). By contrast, for SPI 1, the highest OBC was observed for protein aggregates formed at the IEP (pH 3.12).

Therefore, the methods for isolating proteins from press cake had a greater effect on the OBC than the pH during protein aggregate formation. Isoelectric precipitation (SPI 1) yielded a higher OBC than ethanol extraction (SPI 2). For sunflower proteins, cold pressing (SfPM 2) yielded a higher OBC than pressing followed by de-oiling with CO_2. Ethanol extraction was used for all rapeseed protein samples; thus, the higher protein concentration of one of these samples suggests a more intensive processing that led to a higher OBC.

The influence of the extraction process on the technological properties of proteins was proven by Tirgar et al. (2017). Therefore, the extraction solvent, temperature, and pH may also affect the protein's ability to entrap oil. The extent of denaturation varies depending on the extraction method, which results in different unfolding stages of the protein. As a result, there are varying degrees of protein sidechain exposure, leading to a range of interactions and network formations (Jarpa-Parra et al. 2014).

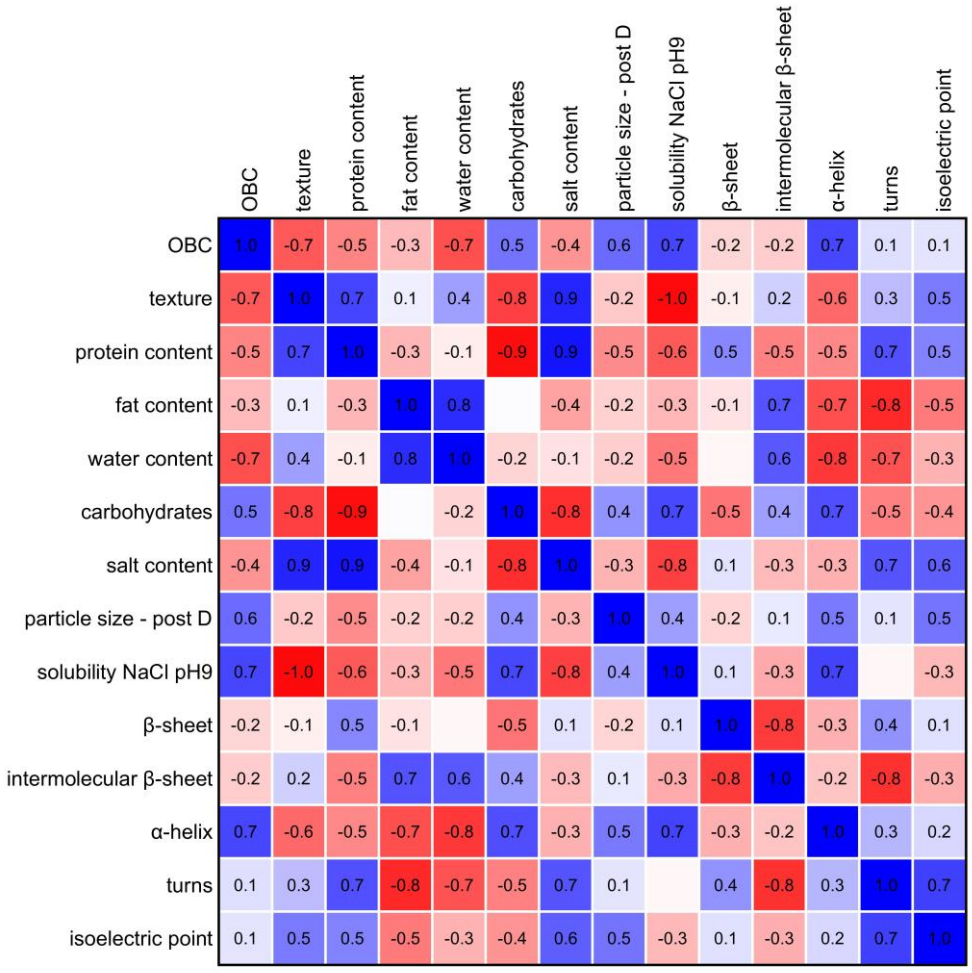

Figure 3.1: Pearson R Correlation of measured parameters in protein based oleogels. Physicochemical und functional parameters of rapeseed-, soy-, sunflower- and whey protein samples and protein aggregates formed at pH 5.6. Values are given as means. n=3. Normalization was conducted.

3.3.2 Correlation of parameters with OBC

To visualize the correlations between the different parameters of the protein samples and the techno-functional properties of the protein aggregates, a correlation matrix was generated. Figure 3.1 shows the correlation between OBC and various sample composition parameters corresponding to aggregates formed at pH 5.6. The carbohydrate content, particle size after denaturation, solubility, and α-helix fractions had a positive influence on the OBC (blue), whereas the protein content, water content, and firmness (texture) of the solid phase after centrifugation of the dispersion were correlated negatively with the OBC (red). The relationships between these parameters and the OBC are discussed below. Surface hydrophobicity was measured prior to aggregation due to the turbidity caused by heat treatment, which impeded measuring the hydrophobicity of aggregate (Table S3.1). Although significant differences were found there were no differences in the calculated ratio of hydrophobic to hydrophilic amino acids based on the manufacturer's specifications (Table S3.2). Also, no correlation was found between measured surface hydrophobicity and OBC, so it was not considered further.

3.3.2.1 OBC-sample composition

A negative correlation of -0.5 and p = 0.333 was found between the protein concentration and the OBC, indicating that a lower protein concentration led to a higher OBC.

The presence of other sample components next to the protein can explain this relationship. For sunflower and rapeseed, the protein content was only between 30% and 55% (Table 3.1) (i.e., up to 70% of the sample is composed of dietary fiber, carbohydrates, fat, and other constituents that affect the OBC). Thus, RPM 1, which had a protein content of approximately 30%, experienced a notable reduction in its OBC. This suggests that there is a range in which the proportions of other constituents—in addition to proteins—increased the OBC. Measuring the water content could provide further insights. For most of the analyzed proteins, the water content did not exceed 9% and the ones with a higher OBC always had a lower water content. By contrast, RPM 1 had a water content of 13%. The negative correlation between the water content and the OBC is illustrated in Figure 3.1. Among soy protein isolates, where the protein

content was close to 90%, SPI 1 had a higher OBC as a result of its lower water content. Numerous studies have demonstrated that polysaccharides combined with proteins enhance the oil-binding ability (Davidovich-Pinhas, 2019; Dickinson, 2008; Feichtinger & Scholten, 2020; Slavin & Carlson, 2014; Wijaya et al., 2019). This correlation has been mainly demonstrated in emulsion-based processes, as polysaccharides enhance the emulsion-stabilizing effect by increasing protein solubility through conjugation (Dickinson, 2008). In this work, however, we directly dispersed the freeze-dried protein aggregates after heat treatment instead of applying an emulsion-based process. Notwithstanding, we observed a noticeable correlation between the proportion of carbohydrates in the sample and its solubility, as well as a positive correlation between the enhanced solubility and the OBC (Fig.3.1). This correlation suggests that there may be another mechanism between carbohydrates and proteins that could lead to an improvement in OBC. Since it is not expected that increased polarization of proteins by conjugation with sugars causes an improved dispersibility in the oil phase. In summary, soy protein samples have a higher protein proportion and a lower carbohydrate content than SfPM and RPM, and thus, their OBCs are lower

Table 3.1: Information about the tested Proteins prior to aggregate formation.

Protein source	Sample lable	Protein content	Water content	Carbohydr. content	Fat content	Extraction method	Solubility				Surf. Hydrophob.	IEP
		[%]	[%]	[%]	[%]		[%]					
							MQ N	MQ 9	NaCl N	NaCl 9		
Rapeseed	**RPM 1**	30.20	13.10	19.9	35.8	Ethanol extraction	20.4	21.4	39.8	39.6	104.46	2.6
	RPM 2	49.41	3.65	37.1	0.8	Ethanol extraction	45.8	60.4	94.6	93.2	114.17	2.9[a]
Soy	**SPI 1**	89.86	5.97	<1.0	4.1	IE precipitation, spray dried	78.6	77.9	81.1	69.5	178.18[a]	3.1[ab]
	SPI 2	89.32	8.55	1.4	2.6	Ethanol extraction	50.6	49.6	6.9	3.91	177.65[a]	3.7
Sunflower	**SfPM 1**	56.96	8.66	29.8	2.1	Pressing, deoiling (CO_2)	49.3	92.4	98.5	87.4	148.86 [b]	2.9[ac]
	SfPM 2	52.83	7.26	26.7	9.9	Cold pressing, grinding	46.0	96.5	106.9	90.4	145.87 [b]	3.6
Whey	**WPI**	93.37	4.48	4.1	<0.3	-	111.2	111.3	98.9	108.5	533.93	5

Protein composition was specified by AGROLAB LUFA GmbH. Results are given as the mean, n=3, a-c are statistically significant differences between the sample within one measurement, $p<0.05$. IEP = isoelectric point, SfPM = sunflower protein meal, RPM = rapeseed protein meal, SPI = soy protein isolate, WPI = whey protein isolate, MQ = Milli Q water, NaCl = sodium chloride, N = neutral pH, 9 = pH 9.

3.3.2.2 OBC-Particle size

The particle sizes of the proteins before (Fig. 3.2b) and after (Fig. 3.2c) aggregate formation were determined. As observed in prior measurements, there was a notable variation in the particle sizes of rapeseed, soy, and sunflower proteins because of the different extraction procedures. The heat treatment of WPI led to an increase in its particle size (1.2–17.5 µm). However, for all plant-based proteins, except for RPM 2, heating resulted in smaller particle sizes.

Before aggregation, SfPM 2 exhibited the largest particle size. This suggests that there was no association or aggregation of plant proteins during the heat treatment. After the heat treatment, the protein solutions were subjected to homogenization using an Ultra-Turrax, which disrupts large particles. The larger cellular components present in the samples start to break down into smaller particles during heating and homogenization. This breakdown leads to the release of substances that aid oil binding in contrast to samples without heat treatment (data not shown). Furthermore, any protein aggregation may be concealed by the breakdown of larger cellular components. The minuscule particle size of SPI 1 (<1 µm) compared to SPI 2 after heat treatment and homogenization can be attributed to the extraction process. SDS-PAGE of SPI 1 (Fig. 3.3e–j) revealed a strong band of low-molecular-weight proteins, which contrasts with SPI 2. Hydrolysis may have occurred in SPI 1 during the extraction process because of the heating and extreme pH, resulting in smaller particles (Hill 1965). Moreover, different extraction techniques produce diverse aggregation behaviors and particle sizes because of the distinct compositions of the protein compartments (Tanger et al., 2022). A study reported that milled rapeseed concentrate had an average particle size ranging from 100 µm to 400 µm (W. Jia et al., 2021), while in the present study, the particle size of rapeseed meal was approximately 50 µm. Thus, the findings of Jia and colleagues deviate from ours. This observation reinforces the importance of the protein isolation procedure because even manufacturers using the same key step (ethanol extraction) attained dissimilar outcomes. In summary, for sunflower and rapeseed proteins, there is a relationship between particle size and OBC, as those that exhibited a larger particle size upon heat treatment also showed higher

OBCs, which are in line with those of WPI. Larger particles promote aggregation in dispersed systems, leading to the development of a network through intermolecular interactions that eventually entrap oil.

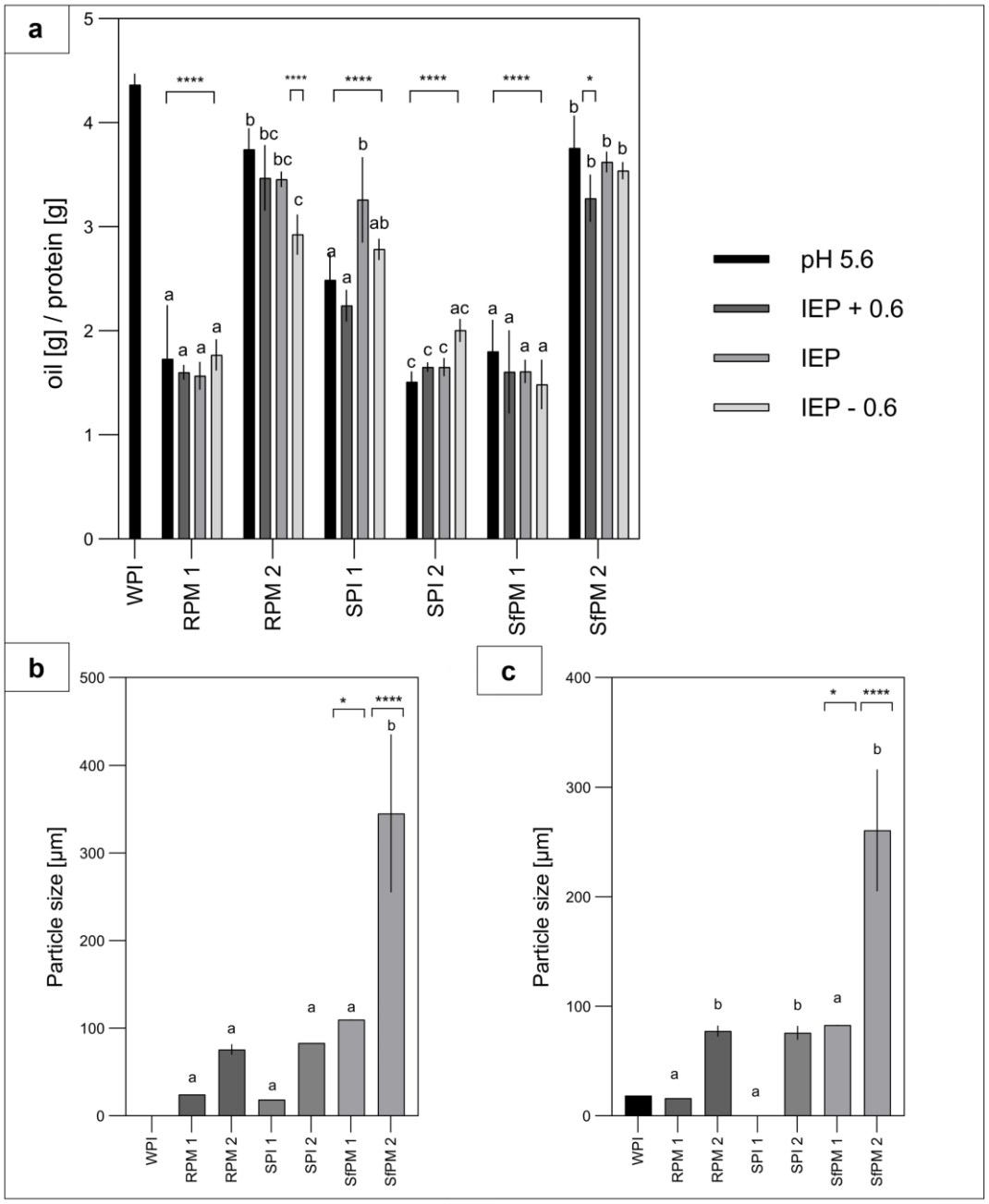

Figure 3.2: Oil binding capacity (OBC) of protein in g of bound oil per g protein (oil[g]/protein [g]) at different pH values (a) and Particle sizes of protein at pH 5.6, (b) before denaturation, (c) after denaturation. WPI= Whey protein isolate, RPM = rapeseed protein meal, SPI = soy protein isolate, SfPM = sunflower protein meal. Results are given as the mean ± SD, n=3, * (a) a-d are statistically significant differences within a plant source, (b-c) statistically significant differences between the control (WPI) and every sample, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3.2.3 OBC-Secondary structure

FTIR revealed that, for the reference protein (WPI), particle formation leading to an increase in the OBC was accompanied by a notable change in the secondary structure. Intermolecular interactions were particularly affected, as observed in the amide I band at a wavenumber of 1618 cm^{-1} (Byler & Purcell, 1989). Furthermore, structural elements such as the α-helix (1648–1658 cm^{-1}) and random coil (1640–1648 cm^{-1}) were also affected, all of which can influence the OBC by exposing hydrophobic regions of the molecule.

Plant protein analyses also revealed changes in the secondary structure after heating (Fig. 3.3a-d). The denaturation-induced changes in the four proteins tested at pH 5.6 were mainly reflected in the α-helix and β-sheet structures (Fig. 3.3a-d). The intramolecular β-sheet structures (~1630 cm^{-1}) decreased (from 35.6% to 22%), while a peak at 1615 cm^{-1}—associated with intermolecular β-sheets—appeared in WPI and RPM. This finding agrees with a previous study (O'Loughlin et al., 2015). Similarly, sunflower protein samples exhibited a new peak associated with intermolecular β-sheets, but no decrease in intramolecular β-sheets was observed in these samples. SPI 2 exhibited similar changes, however, SPI 1 showed a contrasting result. In the SPI 1 spectrum, there was only one large peak at 1625 cm^{-1} associated with intra β-sheets that increased in intensity. In fact, SPI 1 did not show any protein bands in the range between 250 and 5 kDa in SDS-PAGE (Fig. 3.3f, i). Moreover, unhydrolyzed glycinin was present at the top border of the gel but was too large to pass through. This suggests that the proteins in this sample contain small particles resulting from hydrolysis and large particles that have remained unhydrolyzed and aggregated. These results were confirmed by SEC measurements (data not shown).

The α-helix shifted in the direction of the random coil after heat treatment with a slight increase in intensity for WPI (Fig. 3.3d). This shift can be attributed to the change in the relative intensity of the overlapping bands, specifically, the α-helix and random coil, as described by Ryu et al. (2024) as a result of differences in the strength of the molecular interactions. While RPM also showed a shift accompanied by a small increase in intensity, sunflower protein showed a decrease without any shift. Similar to the observations regarding the β-sheets, SPI 1

exhibited a divergent pattern, with a peak at 1650 cm^{-1} before denaturation, followed by a strong decrease in intensity and a new peak at 1658 cm^{-1} thereafter. By contrast, SPI 2 showed no changes in the α-helical structure during denaturation.

When comparing RPM samples at pH 5.6, RPM 1 showed a more pronounced peak for intermolecular β-sheets. This contradicts the assumption that a greater number of intermolecular β-sheets leads to a higher OBC, as the measured OBC was lower for RPM 1 than for RPM 2. The total AUC for the α-helix after the heat treatment was significantly higher for RPM 2 as was the OBC. However, for RPM 2, there was only a slight increase in these parameters at pH 5.6 after heating. Yang et al. (2014) investigated the changes in the secondary structure of rapeseed protein upon heating by performing FTIR measurements. As a result, they found a decreased peak at 1631 cm^{-1}, representing an unfolding process. By contrast, an increase in peak intensity was observed at 1691, 1678, 1659, and 1618 cm^{-1}, suggesting that some aggregation happened via exposed reactive groups. This was partially confirmed by our results. For RPM 2, there was a decrease in the β-sheet structure at 1635 cm^{-1} and an increase in intermolecular β-sheets at 1618 cm^{-1} and in the α-helix structure at 1657 cm^{-1}. Yang et al. (2014) also found that 80°C is insufficient to achieve the complete denaturation of napin at an acidic pH. Nevertheless, according to the analysis of the secondary structure, aggregates were formed to some extent in our study. Moreover, a substantial amount of lipids remained in the RPM 1 sample (Table 3.1). Previous research has shown that the presence of lipids can induce and influence protein structuring (Schestkowa et al., 2020). Therefore, some of the differences between RPM 1 and RPM 2 may be due to variations in the sample composition and lipid content.

Aside from the changes in the β-sheet structure resulting from heat exposure, the secondary structure of the α-helix and random coil of SPI 2 remained unaffected by variations in heat or pH. Two constant peaks appeared around 1655 cm^{-1} and 1642 cm^{-1}, and were assigned to the α-helix and random coil. SPI 1 exhibits a clear secondary structure that is also altered by heat, although the absence of bands in the SDS page (Fig. 3.3f, i) indicates protein degradation during extraction. No intermolecular β-sheet structure was observed for SPI 1, either before

or after denaturation. However, a fairly high OBC was observed at the end, which contradicts the assumed relationship between the inter β-sheet and OBC for WPI and RPM.

At pH 5.6, SPI 1 shows only one peak before denaturation (1650 cm^{-1}), which cannot be assigned to the α-helix or random coil. However, after denaturation, a pattern with two peaks at 1651 cm^{-1} and 1658 cm^{-1} is evident, indicating an increase in the α-helical structure due to denaturation. Thermally denatured glycinin (soy protein) was reported to show an increase in the α-helical structure, associated with protein aggregation (R.-H. Chen et al., 1990). This observation was partially confirmed by our results because the amount of the α-helical structure did not increase after heating for SPI 2, but it did for SPI 1. A correlation with OBC was found, which was notably greater for SPI 1 compared to SPI 2. Nevertheless, the total amount of the α-helical structure after denaturation was higher in SPI 2, indicating that the increase rather than the total amount of the α-helical structure is the crucial factor for an optimal OBC.

The increase in the intermolecular β-sheet structure for SfPM after denaturation exhibited a more pronounced effect for SfPM 2. This can be attributed to the type of processing, as SfPM 2 was derived from cold-pressed oil, which preserved the nativity of proteins to a larger extent. However, the pattern appears slightly different because there was no decrease in the intramolecular β-sheets after denaturation at pH 5.6, even though a new peak appeared at 1613 cm^{-1}. The analysis of SfPM revealed an α-helical peak at 1556 cm^{-1}. After denaturation, there was a slight decrease in peak intensity but no changes in peak position.

Moreover, the α-helical structures of both SfPM samples did not change according to the pre- and post-denaturation comparison and the total amount comparison between the post-denatured samples. This outcome suggests that the expression of the α-helical structure did not affect the OBC in this case. Only minor changes in protein conformation were noted for the SfPM samples, which were mostly unaffected by the extraction procedure. However, sunflower protein exhibited noticeable effects as the pH varied after the heat treatment (Fig. S3.1c). Under acidic conditions, both SfPM proteins showed a pronounced peak around 1624 cm^{-1}, which could not be classified as inter- or intramolecular β-sheets. However, an

acidic pH caused an increase in the β-sheet structure of sunflower protein after the heat treatment. This observation was confirmed by a previous study that also detected this peak close to the IEP after heat treatment (Malik & Saini, 2019). The onset temperature for sunflower protein concentrate is approximately 100°C (Salgado et al., 2011), surpassing the temperature used in the current study and that used by Malik et al. (2019). It is possible that a shift in the onset temperature occurred due to a change in pH toward higher acidity, as evidenced by the increase in the β-sheet structure. The increased intermolecular β-sheet structure was attributed to the aggregation of the denatured protein (Rui et al., 2011). Nevertheless, the OBC did not exhibit any pH-dependent changes, suggesting that, for the SfPM samples, there is no positive correlation between the β-sheet structure and the OBC. On the other hand, correlations were observed between secondary structural elements, for example, a significant negative correlation (-0.8, $p < 0.076$) between the intra- and inter-β-sheet structures. This suggests that native proteins have a higher proportion of intramolecular β-sheet structure, whereas denatured proteins have more intermolecular β-sheet structure.

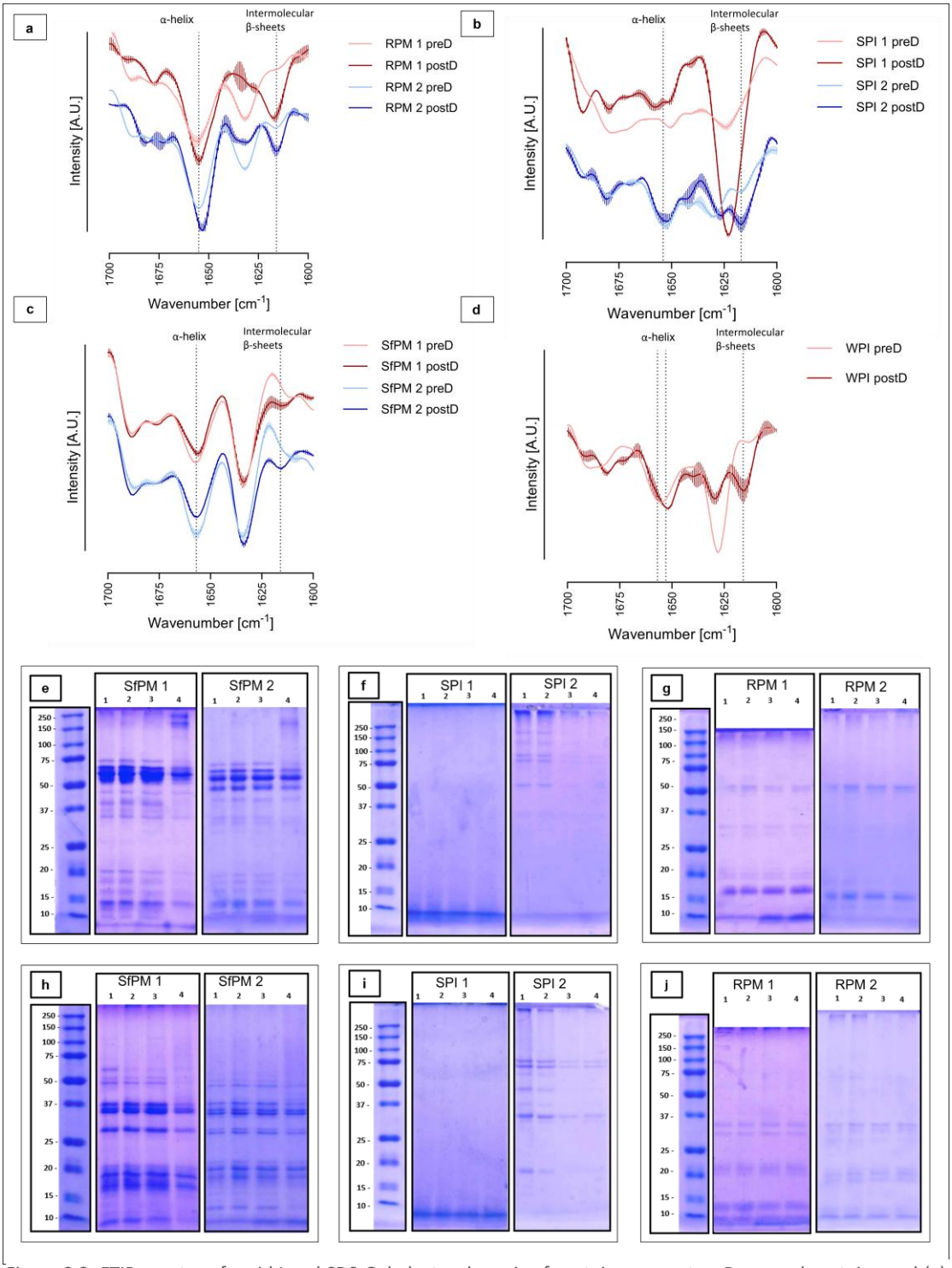

Figure 3.3: FTIR spectra of amid I and SDS-Gel electrophoresis of protein aggregates. Rapeseed protein meal (a), soy protein isolate (b), sunflower protein meal (c) and Whey protein isolate (d) were analyzed at pH 5.6. Samples are shown pre and post denatured (pre D; post D) in second derivation. Results are given as the mean ± SD, n=3. SDS-Gel electrophoresis of plant protein under non-reducing (-β-ME) (e-g) and reducing (+β-ME) conditions (h-j). Sunflower (e,h), soy (f,i) and rapeseed (g,j) protein were solubilize in Milli Q Water at neutral pH (lane 1), at Milli Q water at pH 9 (lane 2), in NaCl at neutral pH (lane 3) or in NaCl at pH 9 (lane 4) 2 µg of protein were applied on each well.

3.3.2.4 OBC-Solubility and protein profile

To investigate the protein solubility before aggregate formation, the proteins were dissolved in water or a 0.5 M NaCl solution at neutral pH or pH 9. Both solvents and pHs yielded 100% solubility for WPI. However, distinct outcomes were found for plant proteins.

For instance, while RPM 2 showed solubility above 90% in 0.5 M NaCl (Table 3.1), its solubility was much lower in pure water. The same solubility pattern, but at a substantially lower level was found for RPM 1. Interestingly, the solubility of soy protein samples was barely influenced by the pH but exhibited notable differences depending on the extraction method, the manufacturer, and the solvent (Table 3.1). Both SPI 2 and SPI 1 showed high solubility values around 50% and 75% in water, respectively, but a contrasting pattern was observed in salt. SPI 1 retained a high solubility above 60% but that of SPI 2 decreased to approximately 5%.

For both sunflower protein samples, the solubility in water at a neutral pH was below 50%. The addition of salt or a higher pH resulted in a substantial increase in solubility up to 100%. No differences in solubility were found for the SfPM samples, which contrasted with the differences in their OBCs. SDS-PAGE was conducted revealing differences between the protein patterns by different extracting procedures (Fig. 3.3e-j). For SfPM 2 and SfPM 1 that may be related to the differences in their OBCs. The band above 250 kDa is associated with the uncleaved globulin helianthinin (Kabirullah und Wills 1983), which exhibits greater prominence in SfPM 1 and is linked with a lower OBC. This protein is potentially stabilized by disulfide bonds as the bands disappear under reducing conditions. Helianthinin is an 11S globulin with a hexameric structure (González-Pérez & Vereijken, 2007). The higher the quantity of the uncleaved helianthinin, the lower the number of peptides because helianthinin is composed of six subunits, each containing an acidic and a basic component. Therefore, one helianthinin molecule can be split into up to 12 units. Having a higher number of units increases the likelihood of interaction, resulting in a higher OBC because of the enhanced protein network. This finding emphasizes the importance of the protein composition for the OBC and is in line with a study that reported that albumin possesses a higher ability to bind oil compared to globulin (LAWAL et al., 2005; Mundi & Aluko, 2012).

Solubility varied across plant protein types, according to their profiles. For instance, although albumins are soluble in water, globulins necessitate salt for solubilization (Gazzola et al., 2014; Osborne, 1924). The solubility of samples is affected by differences in protein composition resulting from variations in protein sources and extraction methods. Thus, it appears that the extraction method is a crucial factor because different processing steps lead to differences in the composition and nativity of the proteins (Fig. 3.3a-j). The correlation analysis between the solubility of plant proteins and their OBCs revealed that higher solubility in 0.5 M NaCl resulted in a higher OBC for soy and rapeseed proteins.

3.3.3 Texture of the protein – oil dispersion

Texture analyses were carried out to elucidate the properties of protein aggregates on a macroscopic scale. The maximal load to penetrate the samples within a defined distance was measured to establish whether the protein source and extraction method affected the texture of the formed dispersions. The samples containing WPI aggregates had the lowest load at only 242.2 g when compared to all plant-based samples (Fig. S3.2). SPI 2 displayed the highest load, requiring a force of 1919 g to penetrate the IEP sample at a 10 mm depth. A decrease of 0.6 pH units significantly reduced the sample firmness, whereas higher pHs seemed to have little effect. However, SPI 2 consistently showed a greater load than SPI 1 for all pHs. Other samples exhibited a similar texture and stability. The maximum load for these dispersions ranged from 500 to 1000 g. The dispersion containing SfPM and SPI 1 exhibited lower values (500–600 g). By contrast, RPM 1 exhibited higher loads, whereas samples with RPM 2 showed a wide range of loads (480–1100 g) for various pHs. Overall, there was an inverse correlation of -0.63 ($p = 0.16$) between the OBC and the texture of the plant protein dispersion, meaning that a higher OBC results in softer matrices. Accordingly, a higher OBC means that more oil is trapped in the solid phase after centrifugation. This observation is consistent with a higher carbohydrate content being correlated with a softer texture (-0.753, $p = 0.084$). Thus, a higher carbohydrate content resulted in a higher OBC (Fig. 3.1). In contrast with the OBC, a positive correlation (0.73, $p = 0.101$) was found between the texture and the protein content, showing that a higher amount of protein leads to a firmer texture of the solid phase after the dispersion

is centrifuged. Likewise, a significant negative correlation of -0.96 ($p = 0.002$) was observed between the solubility of the proteins in NaCl and the firmness of the resulting solid phase after the dispersion is centrifuged.

This outcome suggests that less soluble proteins can form a more stable solid phase because insoluble components in protein powders (e.g., large aggregates, fibers, and cell compartments) hinder mobility. Thus, an optimal protein environment is crucial for binding oil and attaining a structured dispersion.

3.4 Conclusion

This study proved that specific protein properties are required to achieve a high OBC and structured protein-oil-dispersions. Functionalizing whey protein isolate (WPI) for structured oil applies to plant proteins such as those from rapeseed, sunflower, and soybean, and can result in comparable high OBCs and texture properties (firmness) depending on the protein composition, extraction method, structure, and physicochemical properties. The OBC of WPI was 4.04 oil [g]/protein [g] and was similar to that of some rapeseed and sunflower protein samples (3.7 oil [g]/protein [g]). Moreover, the manufacturing process and the composition of the protein meals and isolates affect the OBC. The carbohydrate content, protein solubility in NaCl, particle size, and the quantity of α-helical structures were positively associated with the OBC, whereas the firmness of the dispersion was negatively associated with it. A shift in the β-sheet structures towards a more intermolecular β-sheet structure was observed in most samples. However, this change in β-sheets did not correlate with the OBC across groups. In conclusion, this study demonstrates the potential of plant proteins in oil binding and targeted modification of the textural properties of plant oils. Nonetheless, further research is required to better understand the effects of processing factors during the extraction, isolation, and concentration of the protein on their OBCs and textural properties.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used “DeepL Write” and “ChatGPT” in order to improve readability and language of the work. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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3.6 Supplementary Material

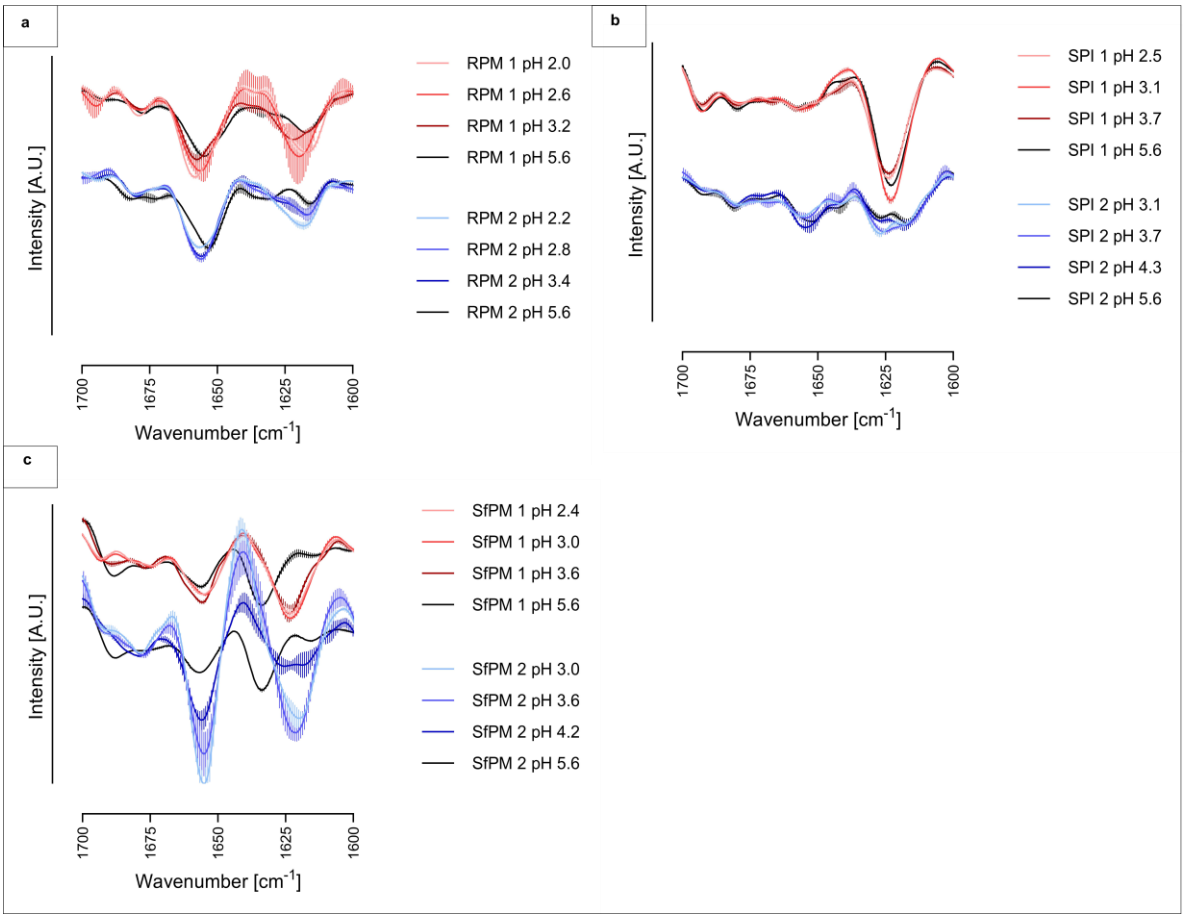

Figure S3.1: Second deviation of amid I spectra analyzed by FTIR. RPM = Rapeseed protein meal (a), SPI = Soy protein isolate (b), SfPM = Sunflower protein meal (c) at different pH-values. Samples are shown post denatured. Results are given as the mean ± SD, n=3.

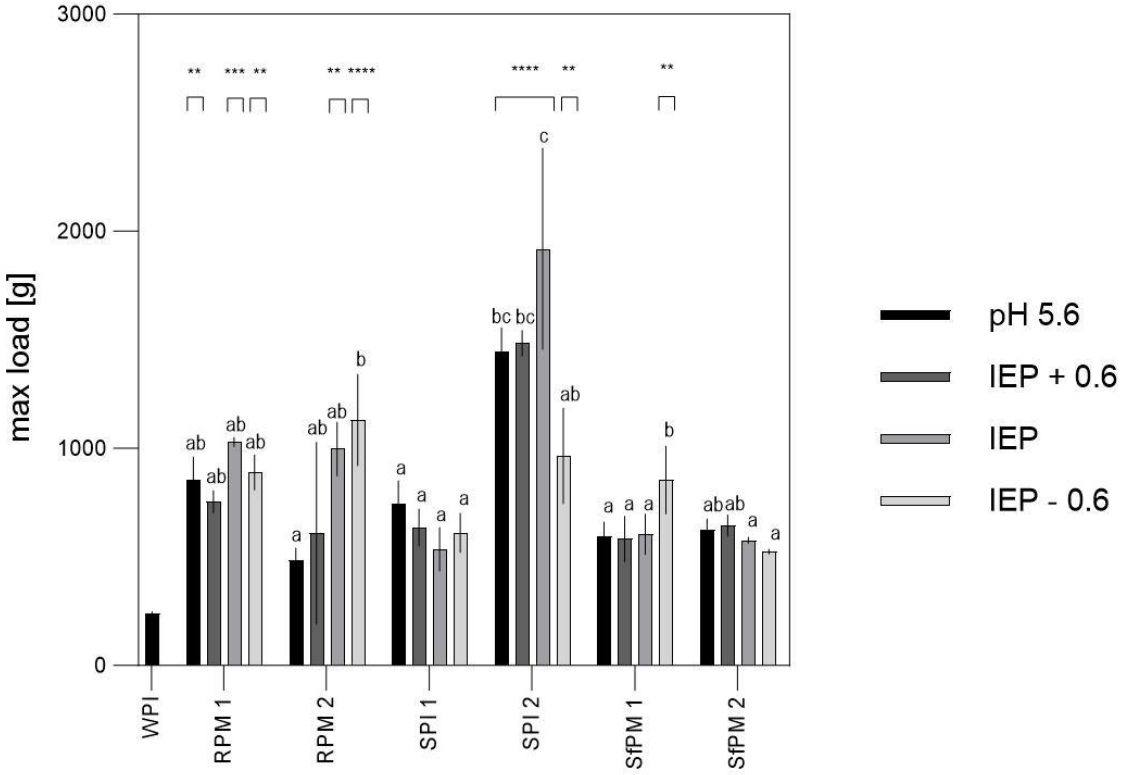

Figure S3.2: Gel strength measured in max load [g] at different pH values. WPI= Whey protein isolate, RPM = Rapeseed protein meal, SPI = Soy protein isolate, SfPM = sunflower protein meal. Results are given as the mean ± SD, n=3, * statistically significant differences between the control (WPI) and every sample, $p<0.05$, a-c are statistically significant differences within a plant source, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p>0.0001$.

Table S3.1: Supplemental Characteristic Information about the tested Proteins prior to aggregate formation.

Protein source	Sample label	Protein content	Extraction method	OBC	Solubility			Surface hydrophobicity	IEP	Zeta potential
		[%]		[$g_{Oil}/g_{Protein}$]	[%]				pH	[mV]
					MQ N	MQ 9	NaCl N	NaCl 9		
Rapeseed	RPM 1	30.2	Ethanol extraction	1.73	20.38	21.43	39.80	39.61	104.46^{b}	2.6^{a}	
	RPM 2	49.4	Ethanol extraction	3.74	45.80	60.43	94.56	93.18	114.17^{c}	2.9	-11.97^{a}
Soy	SPI 1	89.8	IE precipitation. spray dried	2.49	78.61	77.88	81.09	69.53	178.18^{g}	3.1	-27.30^{ef}
	SPI 2	89.3	Ethano lextraction	1.53	50.56	49.60	6.99	3.91	177.65^{g}	3.7	
Sunflower	SfPM 1	56.9	Pressing, deoiling (CO_2)	1.80	49.27	92.44	98.49	87.43	148.86^{e}	2.9	-25.80^{cd}
	SfPM 2	52.8	Cold pressing, grinding	3.75	45.99	96.48	106.93	90.38	145.87^{e}	3.5	-27.53^{cdf}
Pea		87.2		1.75	32.14	38.30	0.14	1.29	134.57^{d}	3.9	-29.80^{cef}
Hemp		60		2.06	4.29	14.71	74.03	88.96	157.60^{f}	2.6^{a}	-20.03^{b}
Pumpkin		60	Pressing, magnetic separation	2.60	-4.26	-1.20	46.16	46.35	98.08^{a}	1.87	-24.07^{cd}
Lupine		93.5		1.83	72.74	75.02	53.97	55.83	300.58^{h}	4.1	-25.37^{cd}
Whey	WPI	97.7		4.36	111.15	111.26	98.89	108.49	533.93^{i}	5	-29.73^{f}

Protein content was specified by the manufacturer.Results are given as the mean ± SD, n=3, a-g are statistically significant differences between the sample within one measurement, p<0.05. IEP = isoelectric point, OBC = Oil binding capacity, SfPM = sunflower protein meal, RPM = Rapeseed protein meal, SPI = Soy protein isolate, WPI = Whey protein isolate, MQ = Milli Q Water, NaCl = sodium chloride.

Table S3.2: Distribution [%] of amino acids .

		WPI	RPM 2	SPI 1	SPI 2	SfPM 1	SfPM 1
		[%]	[%]	[%]	[%]	[%]	[%]
Hydrophilic	Arginine	2.9	8.5	7.6	6.7	8.9	9.3
	Aspartic acid	11.1	7	11.6	10.1	9.7	9.8
	Glutamic acid	17.8	20.7	17	18	20.8	21.9
	Histidine	2.2	3.2	2.3	2.8	2.7	2.8
	Lysine	8.5	5.7	6.3	6.1	3.8	3.6
	Serine	4.7	4	5.2	3.6	4.1	4.7
	Threonine	7.6	4.8	3.7	3	3.8	4.1
	Tyrosine	3.5	3.7	3.3	2.3	2.6	2.5
Hydrophobic	Alanine	4.5	4.8	4.3	3.7	4.8	4.7
	Cysteine	1.3	0.3	1.2	1.0	1.3	1.7
	Glycine	2.2	4.5	3.7	3.9	6.6	6.4
	Isoleucine	6.3	4.6	4.9	4.6	4.8	4.1
	Leucine	10.4	7.9	8.2	7	6.9	6.7
	Methionine	1.7	1.9	1.3	1.1	2.2	2.2
	Phenylalanine	3.6	4.5	5.2	4.8	5.2	4.9
	Proline	3.2	6.4	5.1	4.2	4.3	4.3
	Tryptophan	1.9	1.3	1.4	1.6	1.6	1.3
	Valine	6.8	5.9	5	4.8	5.8	4.9
Ratio hydrophobic: hydrophilic		0.72:1	0.73:1	0.71:1	0.70:1	0.77:1	0.72:1

Provided by the respective manufacturers of RPM, SPI and SfPM as well as described in literature for WPI (Deutsche Forschungsanstalt für Lebensmittelchemie, 2000). The ratio of hydrophobic to hydrophilic amino acids was calculated as a value for hydrophobicity. WPI= Whey protein isolate, RPM = Rapeseed protein meal, SPI = Soy protein isolate, SfPM = sunflower protein meal.

4 Alternative protein sources as oleogelators affect the oxidative stability of oleogels

Maria Wittmüss, Karin Schwarz*, Jonas Amft, Timon R. Heyn

Institute of Human Nutrition and Food Science, Division of Food Technology, Heinrich-Hecht Platz 10, D-24118 Kiel, Kiel University, Germany.

* Corresponding author (kschwarz-2@foodtech.uni-kiel.de)

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Highlights

- Proteins contribute to a reduced formation of lipid oxidation products.
- The antioxidative capacity of proteins is strongly dependent on protein structure.
- No distinct differences were observed between animal- and plant-derived proteins in their impact on the oxidative stability of protein-based oleogels.
- ATR-FTIR enables time-resolved monitoring of oxidation in protein-based oleogel matrices.
- The applicability of ATR-FTIR for assessing oxidation in protein-based oleogels is limited across different protein sources.

Abstract

Protein-based oleogels represent a promising alternative to conventional solid fats, offering healthier and more sustainable options. Using proteins from rapeseed, sunflower, or mussel may further enhance ecological benefits. This study investigated the influence of protein structure on LOx in oleogel systems. While no significant differences were observed between animal- and plant-based proteins, the protein structure—affected by the source and processing methods such as isolation—strongly modulated their antioxidant potential. Fourier-transform infrared spectroscopy (FTIR) provided insights into structural changes without time-consuming sample preparation. Although no universal FTIR markers for oxidation were identified across all protein sources, detailed analyses suggest FTIR could help monitor oxidative processes, warranting further investigation into the relationship between protein structure and oxidative stability in oleogels.

4.1 Introduction

Proteins are capable of functioning as oleogelators by inducing the structuring of liquid oils through the formation of protein interactions within the oil (Vries, Wesseling, et al., 2017). The utilization of a gelator to stabilize liquid oil, which is characterized by a high polyunsaturated fatty acids (PUFA) content, has the potential to yield a solid structure analogous to a solid fat with higher nutritional value. This solid structure could be employed as a substitute for natural solid fats, such as butter or palm oil (Feichtinger & Scholten, 2020), which are rich in saturated FAs and thereby associated with a higher risk in cardiovascular disease (Souza et al., 2015).

From a nutritional perspective, the use of proteins as oleogelators is a growing relevance. This issue is further complicated by the predominantly hydrophilic nature of proteins, which results in poor dispersibility in oil. It is therefore evident that a specific preparation of the oleogel is necessary in order to ensure the successful function of the proteins as a gelator (Vries, 2017). A variety of methodologies have been described in the existing literature for the purpose of enhancing the oil-binding capacity of proteins (Scholten, 2019). One such methodology involves the augmentation of the hydrophobicity of proteins. The procedure entails the application of heat to unfold the protein structure, thereby exposing the hydrophobic core and inducing the formation of aggregates (Vries, Wesseling, et al., 2017).

In addition to arguments relating to health, there are also ecological aspects that have led to research focusing on protein based oleogels as a substitute for conventional solid fats. The utilization of protein as oleogelators has the potential to enhance the efficacy of alternative protein sources, such as whey- and mussel protein, in addition to plant-based alternatives, including rapeseed and sunflower protein derived from oil production. These protein sources have the potential to function as valuable nutritional components. Despite their present underutilization, due to processing difficulties and the presence of components that affect taste and color, these products nevertheless possess the capacity to address the global demand for protein (Wittmüss et al., 2024), a necessity for the sustenance of a growing population (Janitha P.D. Wanasundara et al., 2016). However, their current application remains limited, primarily due to technological challenges in processing and the presence of

compounds that negatively influence taste and color. Moreover, these alternative proteins exhibit diverse structural and functional properties, necessitating further research to fully exploit their potential in the development of novel food structures.

Oleogels are susceptible to LOx, a phenomenon that is particularly pronounced in the presence of a high proportion of PUFAs (Shahidi & Zhong, 2010). Given the finding that the process of LOx results in the deterioration of oleogel-containing food products, this issue must be addressed. Furthermore, the presence of protein in the system suggests the possibility of protein oxidation, as well as the occurrence of co-oxidation processes between lipids and proteins, a phenomenon that has been documented already (Yi et al., 2019). The addition of proteins is frequently implemented to inhibit LOx (Faraji et al., 2004), yet this process can result in increased protein oxidation. Because plant proteins differ from animal-based proteins in their structure—and thus in their functionality—it is assumed that different protein sources will have varying effects on LOx in protein-based oleogel systems.

There are many well-established methods for evaluating oxidative status in different sample matrices, either through the quantitative determination of oxidation products or by assessing oxidative stability, for example with the Active Oxygen Method (AOM) or the Oil Stability Index (OSI) (Russin et al., 2003). However, these approaches usually require extensive sample preparation, making them both time- and cost-intensive (Russin et al., 2003). A more straightforward assessment of oxidative stability without elaborate pretreatment steps could be achieved by measuring spectral signals in defined regions using ATR-FTIR. This method could prove particularly advantageous if the isolation or extraction of the relevant molecules proves difficult due to their poor solubility. In several studies, this technique has already been applied to oils in order to capture spectral changes associated with oxidation processes over time (Guilln & Cabo, 2000a, 2000b; Tena et al., 2017). In these cases, samples were placed in the ATR-System under controlled conditions and repeatedly measured, enabling the detection of characteristic spectral changes that reflect the degree of oxidation. Specific spectral regions have been identified that correspond to the absorption of oxidation-related structures and can thus serve as indicators of LOx, such as peaks at 3430, 1750 or 988 cm^{-1}, corresponding to

stretching vibrations od hydroperoxides, carbonyl groups and trans double bonds (Daoud et al., 2019; Tena et al., 2017).

Because protein-based oleogels contain not only lipids but also proteins, the question arises as to whether changes in FTIR spectra might capture not only LOx, but also protein oxidation and potentially even co-oxidation processes. If this is the case, FTIR could represent a promising tool for the rapid assessment of oxidative status in complex systems such as oleogels, allowing the simultaneous monitoring of oxidative changes in more than one component without the need for extensive sample preparation.

The present study therefore has two objectives: first, to investigate whether ATR-FTIR is a suitable method for analyzing the oxidative status of food metabolites that are difficult to degrade, such as protein-rich oleogels. To this end, a statistical comparison will be made between the change in various bands identified and classified as relevant by FTIR and the change in conventional oxidation markers - such as LOx markers (hydroperoxides and hexanal) and protein oxidation markers (carbonyls, NFK). These studies were carried out on oleogels stabilized by protein-based oleogelators derived from different animal- and plant sources. Therefore, it is also possible to determine whether the oxidation status of lipids and proteins before and during storage depends on the protein source.

4.2 Material and Methods

4.2.1 Materials

WPI was obtained from Davisco Foods International with 97.7 % protein and 75 % β-lactoglobulin in dry matter. Soy Protein Isolate was kindly provided by EUROSOY GmbH (Hamburg, Germany). Rapeseed Protein was kindly provided by the Fraunhofer CBP Institute (Leuna, Germany). Sunflower protein was kindly provided by Georg Breuer Food Ingredients (Königstein, Germany). Blue mussels (*Mytilus edulis*) were provided by Kiel Marine Farm (Kiel, Germany) fresh after their harvest in 2023 and directly frozen at −20 °C for further use. Fully refined sunflower and rapeseed oil were purchased in a local supermarket. Ultra-pure water (MilliQ) was used for all applied solutions (<18.2 MΩ cm-1). All used chemicals were of analytical grade: aluminum oxide, ammonium thiocyanate, barium chloride, guanidine hydrochloride, hexane, hydrochloric acid (HCl), iron (II) sulfate, ortho-phosphoric acid and trichloroacetic acid (TCA) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Dinitrophenylhydrazine (DNPH) and Trypsin from bovine pancreas was purchased from Merck KGA (Darmstadt, Germany). Isopropanol, cyclohexane, Ethanol (98%) and Ethanol acetate was procured from VWR International GmbH (Darmstadt, Germany).

4.2.2 Sample preparation

4.2.2.1 Extracting mussel protein

The extraction of myofibrillar protein from the mussel was conducted as follows: A quantity of 350 g of frozen mussels was weighed and thawed in tap water. Any byssus threads, barnacles, and dirt were meticulously removed during the cleaning process. Subsequent to the collection of the meat, the residual dirt and liquid were eliminated by rinsing with distilled water using a sieve. Subsequent to draining, the weight of the cleaned meat was determined, followed by the addition of distilled water at a 1:2 ratio. To achieve a preliminary breakdown, the mixture was processed in a Thermomix® TM6 at level 7 for 30 seconds. Further homogenization was then performed using an Ultra-Turrax rotor-stator homogenizer at 15,000 rpm for 30 seconds, ensuring a uniform consistency. Subsequently, the homogeneous mixture underwent centrifugation at 3,000 x g and 17°C for 10 minutes. Following the removal of the liquid layer, the remaining pellet was mixed with distilled water (1:2 ratio) and dispersed

using a vortexer. A second centrifugation step (3,000 x g, 17°C, 10 minutes) was conducted to further wash the pellet. For the purpose of protein extraction, the washed pellet was resuspended in a 0.6 M NaCl solution (1:2 ratio) and dispersed using a vortexer. The resulting suspension was then subjected to shaking at 200 rpm for 50 minutes at 17°C. Finally, a further centrifugation step (3,000 x g, 17°C, 10 minutes) was performed in order to separate the myofibrillar proteins from the remaining material. The collected material was then freeze-dried in order to obtain the final protein powder.

4.2.2.2 Preparation of protein aggregates

Protein aggregates were prepared according to Meissner et al. (2020b) with some modifications. In brief, WPI powder (7.5% w/w) was dissolved in MilliQ water under constant stirring. Polyphenol stock solution (55.55 mM) and Protein solution (7.5%) were mixed with MilliQ water, so the desired concentration of WPI (6%). pH was adjusted at 5.6 using 2 M HCL before stirring the solution at 20°C room temperature (RT) for 2 h. Subsequently, the solution was denatured for 20 min at 85°C in a temperature-controlled water bath, using 50 mL plastic tubes. The resulting protein aggregate dispersion was homogenized using a rotor-stator homogenizer (Ultra-Turrax) at 13000 rpm for 3 min. The samples were then centrifuged at 4000 x g for 20 min at RT to collect the aggregates in a pellet. The supernatant was discarded and the aggregates were dispersed in fresh water using the Ultra-Turrax (13000 rpm, 3 min). After washing, the protein aggregates were frozen at -80°C for 2 days and then they were freeze-dried (LSC Plus, Martin Christ, Osterode am Harz, Germany) for 72 h. The resulting aggregates were ground in a mortar until a fine powder was obtained. All samples were prepared in triplicate.

The protein used in this study were whey protein isolate (WPI), soy protein isolate (SPI), mussel protein (MPM), sunflower protein concentrate (SfPM) and rapeseed protein concentrate (RPM).

4.2.2.3 Purification of oil

The purification of the 50:50 (v/v) sunflower and rapeseed oil from natural antioxidants and trace metals was conducted according to Amft et al. (2020). Therefore, activated aluminiumoxid was used to prepare a filtering column, which the oil mixed 50:50 (v/v) with n-

hexane has to pass through. The oil was tested for residual tocopherols by using HPLC afterwards.

4.2.2.4 Preparation of oleogels

Thereafter, the aggregates were dispersed in an oil mixture composed of stripped sunflower and rapeseed oil (1:1 v/v) (10 wt.%). While dispersing the protein aggregates in the oil phase, 2.8% MilliQ water was added to the system. Following a stirring period of 10 minutes, the samples were subjected to homogenization with a rotor-stator homogenizer (Ultra-Turrax) at 10,000 rpm for 30 sec for two times, to ensure uniform distribution of the phases.

4.2.2.5 Storage Experiment

The accelerated storage experiments was performed over a period of 22 days at 40°C in the dark. Therefore, Protein in oil dispersion were stored in a climatic chamber (Memmert GmbH + Co. KG, Schwabach,Deutschland) and analyzed at day 0, 2, 4, 8, 15 and 22 days. The control group (C) consisted of bulk oil without added water and was stored under the same conditions as the other samples. Water was excluded from the control to avoid phase separation between the oil and water, which would result in differing interfacial effects. For analysis of lipid oxidation, lipid phase was separated by centrifuging the sample at 20,000 x g for 10 min.

4.2.2.6 Defatting

For defatting the protein phase of the sample after decanting the unbound oil, 500 mg of the protein phase were mixed with 5 mL of cyclohexane. The samples were sonicated for 20 seconds, at 50% power, cycle 9 (*Sonopuls VS 70, Bandelin electronic GmbH & Co. KG, Berlin, Germany).* Afterwards, the samples were centrifuged for 4 min at 40000g at room temperature. The supernatant was discarded and the procedure was repeated two more times. The resulting protein pellet was dried overnight.

4.2.2.7 Protein Hydrolysis

Enzymatic hydrolysis of the defatted Proteins were performed based on the method described by Deng et al. (2018). Therefore 50 mg of the defatted Protein powder were mixed in 5 mL Milli Q water and adjusted to a pH value of 8. The sample was stirred for 30 min at 37°C in a water bath. Trypsin (10,000 units/mg) was dissolved in Milli Q water (5mg/500µL) and 50 µL

were added to each sample of 5 mL. The samples were stirred for 2 h at 37°C while correcting the pH regularly by using 0.2M NaOH if needed. To inactivate the enzyme after 2 h of incubating the samples were put in a water bath at 90°C for 10 min right before centrifuging them for 30 min at 4000 x g at 4°C. The supernatant was stored at 4°C overnight before measuring further parameters. Protein concentration was determined according to Lowry (1951) as previously described in **chapter 3.2.2.1**

4.2.3 Lipid oxidation parameters

4.2.3.1 Hydroperoxides

Primary LOx products were measured by determining the amount of hydroperoxides using the ferric thiocyanate method according to Amft et al. (2019). Therefore 10 mg of the oil phase were mixed with 5 mL Isopropanol. 50 µl of each, ferrous solution and thiocyanate solution were added before incubation of the sample for 30 minutes at 60°C in a water bath. Afterwards the samples were measured at 485 nm against a blank with no oil. Results were expressed as mmol hydroperoxides/kg of oil.

4.2.3.2 Volatile lipid oxidation compounds

Volatile oxidation products hexanal was analyzed in 900 mg oil weighed into 20 mL airtight HS-GC vials using a HS-20 headspace sampler (Shimadzu, Kyoto, Japan) and a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-1701 column (60 m x 320 µm x 1 µm, Agilent Technologies, Santa Clara, CA, USA). The following parameters were utilized for the extraction of volatiles using the HS-20 system: sample line temperature 85 °C; transfer line temperature 100 °C; GC cycle time 30 min; shaking level 3. The following oven temperature program was utilized: an initial 45 °C hold for 2 min, a 25 °C/min increase to 70 °C for 3 min, a 20 °C/min increase to 182 °C for 3 min, and a 30 °C/min increase to 240 °C for 3 min. The experimental setup involved the utilization of a flame ionization detector, with hydrogen and air flow rates set at 30 and 200 mL/min, respectively. Nitrogen was utilized as carrier gas at a linear velocity of 26 cm/s, with a split ratio of 1:4. The acquisition of data was conducted utilizing the device-related software LabSolutions (Shimadzu). The identification of hexanal was achieved through the analysis of the retention time of an external standard, with the peak area being quantified by the area under the curve (AUC).

4.2.4 Protein oxidation parameters

4.2.4.1 Carbonyl Content

Protein bound carbonyls were spectrophotometrically determined at 370 nm after derivatization with DNPH to quantify protein oxidation. The method, based on Levin et al. (1990) with slight modifications according to Scheidegger et al. (2016). Hydrolyzed protein samples were diluted to a final concentration of 3.33 mg/mL using MilliQ water. 0.6 mL of the sample were precipitated by the addition of 1 mL 20% TCA. After centrifuging at 20°C and 10.000 x g for 5 min the sample and discarding the supernatant the protein pellet was either mixed with 1 mL of 2M HCL as a control, or with 1mL DNPH. After 1 h of incubation time in the dark, 0.8 mL of TCA were added and a centrifugation step (5 min; 10,000 x g) was performed. The Pellet was washed three times, using 1mL of ethanol/etylacetate (1:1; v/v), and finally redissolved in 2 mL 6M Guanidine-HCL (pH 2.3). The measured Carbonyl content was corrected by the protein concentration.

4.2.4.2 N-formylkynurenine

N-formylkynurenine (NFK) was determined as a measure for protein oxidation. Therefore, hydrolyzed protein solution was diluted to a concentration of 0.5 mg/mL using sodium phosphate buffer (pH 7.8). The samples were measured by fluorescence spectroscopy *(Cary Eclipse Varian Agilent, Palo Alto, CA, USA)* at an excitation wavelength of 325 nm and emission wavelengths of 410 and 435 nm. The excitation and emission slit widths were set to 5 mm each.

4.2.5 Infrared spectrum

Secondary structure of protein was analyzed by Attenuated total reflection Fourier-transform infrared spectroscopy (FTIR), using a CONFOCHECK TENSOR II system (Bruker Optics, Ettlingen, Germany) as previously described in Manuscript 2. Therefore 20 µL of the oleogel were placed onto the ATR-crystal in order to investigate structural changes over the storage process. The spectra were corrected and vector-normalized using OPUS version 7.5 (Bruker Optics GmbH, Ettlingen, Germany). The identification of oxidation markers by ATR-FTIR analysis was achieved through the normalization of the full spectrum (800-4000 cm-1) and the subsequent analysis of individual peaks in terms of their intensity.

4.2.6 Statistical analysis

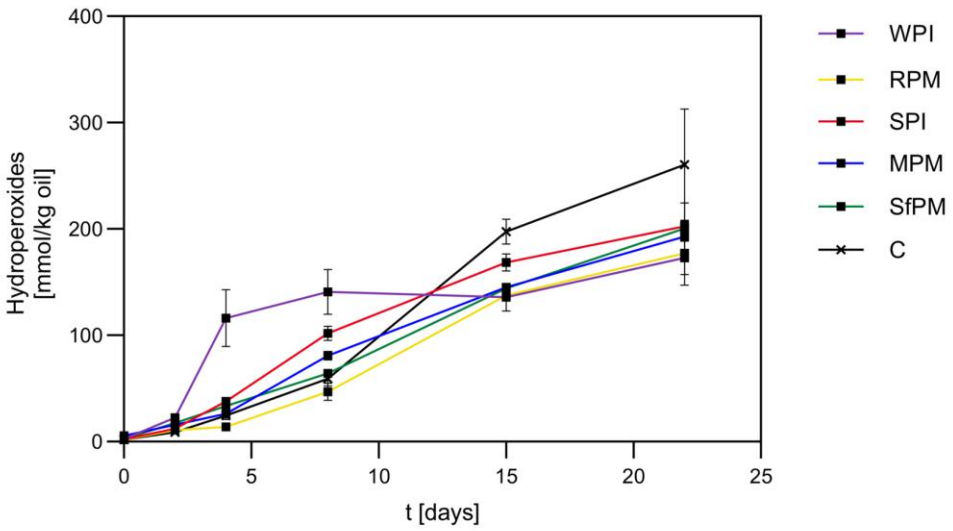
All experiments were at least conducted in triplicates. The data were compiled and analyzed using GraphPad Prism (version 10.0.2, GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with $p < 0.05$. The Pearson R correlation was performed using normalized data.

4.3 Results

4.3.1 Lipid oxidation

4.3.1.1 Hydroperoxide

The formation of hydroperoxides, as primary LOx products, was monitored to evaluate LOx in the oil phase of protein-in-oil dispersions during a 22-day storage period. In the control group containing bulked oil (C), a steady increase in hydroperoxide levels was observed, reaching a maximum of 260 mmol/kg oil on day 22 (Fig. 4.1). The addition of protein resulted into a significant lower hydroperoxide amount at day 22, for WPI as well as for SfPM. As shown in



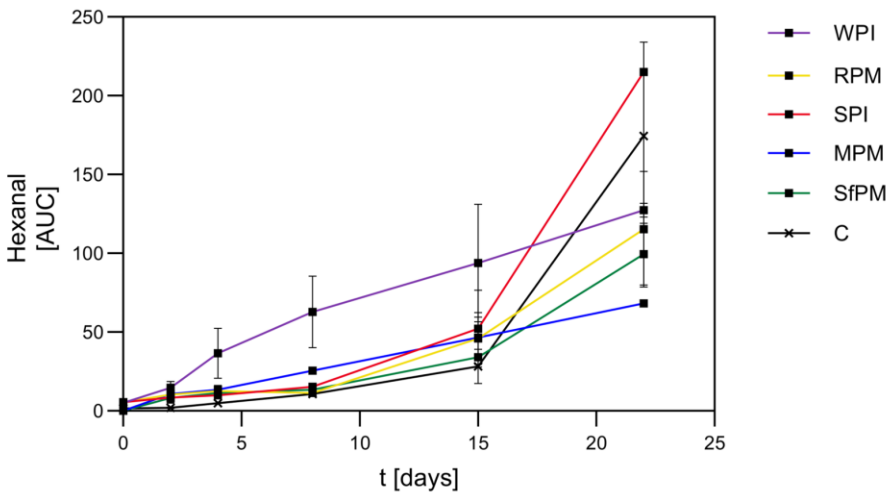
t [days]	WPI	SPI	MPM	RPM	SfPM	C
0	$2.50^{c;B}$	$2.09^{e;BC}$	$1.94^{f;A}$	$5.56^{d;BC}$	$2.54^{f;B}$	$1.83^{e;C}$
2	$22.42^{c;A}$	$12.11^{de;C}$	$10.84^{e;B}$	$15.80^{d;CD}$	$17.46^{e;B}$	$8.88^{de;D}$
4	$116.10^{b;A}$	$37.84^{d;B}$	$13.86^{d;B}$	$26.10^{cd;B}$	$33.35^{d;B}$	$24.39^{d;BC}$
8	$140.67^{ab;A}$	$101.97^{c;B}$	$46.93^{c;BC}$	$80.91^{c;D}$	$64.13^{c;CD}$	$59.20^{c;CD}$
15	$135.94^{ab;C}$	$168.46^{b;B}$	$137.57^{b;C}$	$145.24^{b;C}$	$144.02^{b;C}$	$197.49^{b;A}$
22	$172.68^{a;B}$	$202.48^{a;AB}$	$177.06^{a;AB}$	$192.86^{a;B}$	$200.39^{a;AB}$	$260.36^{a;A}$

Figure 4.1: Formation of hydroperoxides [mmol/Kg oil] in the oil phase of protein-based oleogels. 10% of whey protein Isolate (WPI), soy protein isolate (SPI)-, mussel protein (MPM)- , rapeseed protein (RPM)- and sunflower protein (SfPM) aggregates were dispersed into oil with 2.8% added water, stored over 22 days at 40 °C and investigated in comparison to bulked oil without added protein and water (C). Results are given as the mean ± SD, n=3; a-f are statistically significant differences ($p<0.05$) within a row, A-D are statistically significant differences within a timepoint over the different groups.

Figure 4.1, rapeseed (RPM), mussel (MPM), and sunflower protein (SfPM) exhibited oxidation patterns similar to soy, with hydroperoxide levels rising steadily from 0 on day 0 to approximately 200 mmol/kg oil (in SfPM) on day 22. In contrast, the WPI system displayed a distinct oxidation profile, characterized by a rapid increase in hydroperoxides during the first 8 days, followed by a plateau phase reaching around 150 mmol/kg oil.

4.3.1.2 Hexanal

Hexanal, a key secondary product of LOx, was quantified using headspace gas chromatography (HS-GC) (Fig. 4.2). In the control group containing bulk oil (C), hexanal levels increased exponentially over the 22-day storage period, reaching a maximum of 174 AUC on day 22. In WPI system, a sharp increase in hexanal content was observed during the first 8 days, followed by a plateau phase. By day 22, the hexanal level in this group reached 130 AUC. In the SPI system, the onset of hexanal formation was slightly delayed compared to WPI. Nevertheless, a substantial increase was observed over time, with hexanal reaching 215 AUC by day 22. For the RPM, MPM, and SfPM protein groups, a consistent increase in hexanal was observed over time, with rapeseed samples showing the highest concentration at 115 AUC by day 22 (Fig. 4.2). Notably, the hexanal progression pattern varied among the systems: while SPI, RPM, and SfPM exhibited an exponential increase, WPI and MPM followed a more linear trajectory.



t [days]	WPI	SPI	MPM	RPM	SfPM	C
0	$5.18^{c;AB}$	$5.60^{c;A}$	$0.00^{d;C}$	$5.55^{b;AB}$	$0.00^{c;C}$	$1.48^{b;BC}$
2	$14.63^{c;A}$	$8.43^{c;AB}$	$10.64^{cd;AB}$	$10.35^{b;AB}$	$8.13^{bc;AB}$	$1.90^{b;B}$
4	$36.66^{bc;A}$	$9.89^{c;B}$	$13.38^{cd;AB}$	$12.60^{b;AB}$	$11.38^{bc;B}$	$4.99^{b;B}$
8	$62.83^{b;A}$	$15.23^{c;B}$	$25.45^{bd;B}$	$11.28^{b;B}$	$13.51^{bc;B}$	$10.74^{b;B}$
15	$72.54^{b;A}$	$52.20^{b;A}$	$46.58^{ab;A}$	$45.97^{b;A}$	$34.14^{b;A}$	$28.22^{b;A}$
22	$130.38^{a;AB}$	$215.17^{a;A}$	$68.27^{a;B}$	$115.33^{a;AB}$	$99.57^{a;AB}$	$174.41^{a;AB}$

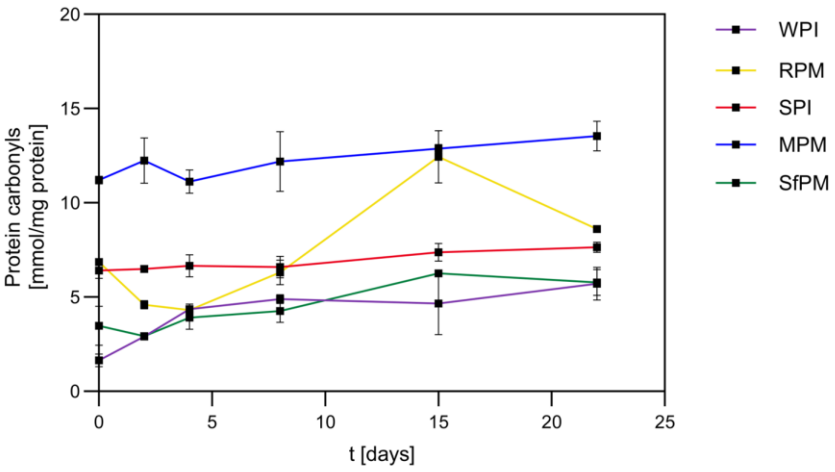
Figure 4.2: Formation of hexanal [AUC] in the oil phase of protein-based oleogels. 10% of whey protein Isolate (WPI)- , soy protein isolate (SPI)- , mussel protein (MPM)- , rapeseed protein (RPM)- and sunflower protein (SfPM) aggregates were dispersed into oil with 2.8% added water, stored over 22 days at 40 °C and investigated in comparison to bulked oil without added protein and water (C). Results are given as the mean ± SD, n=3; a-f are statistically significant differences ($p<0.05$) within a row, A-D are statistically significant differences within a timepoint over the different groups.

4.3.2 Protein oxidation

4.3.2.1 Protein-Carbonyls

Protein carbonyl formation was used as a marker to assess the progression of protein oxidation during a 22-day storage period (Fig. 4.3). Similar to the trends observed for LOx markers (Figs. 4.1 and 4.2), a substantial increase in protein carbonyl content was detected in the WPI sample, reaching up to 6.0 mmol/mg protein by day 22.

In the SPI sample, no increase in protein carbonyls was observed over the storage period. Interestingly, the initial carbonyl level in this sample was already high at day 0 (6.58 mmol/mg protein), exceeding the final value measured in WPI at day 22.MPM showed even higher levels of protein carbonyls compared to the other samples; however, these levels remained stable throughout the storage period, indicating no further oxidative progression. In contrast, the alternative plant proteins (SfPM and RPM) exhibited substantially lower carbonyl levels compared to mussel protein and were more comparable to those found in soy and WPI samples. No consistent trend of increase was observed in these plant proteins. For instance, the RPM sample showed a marked increase in carbonyl content up to day 15, followed by a sharp decline by day 22.

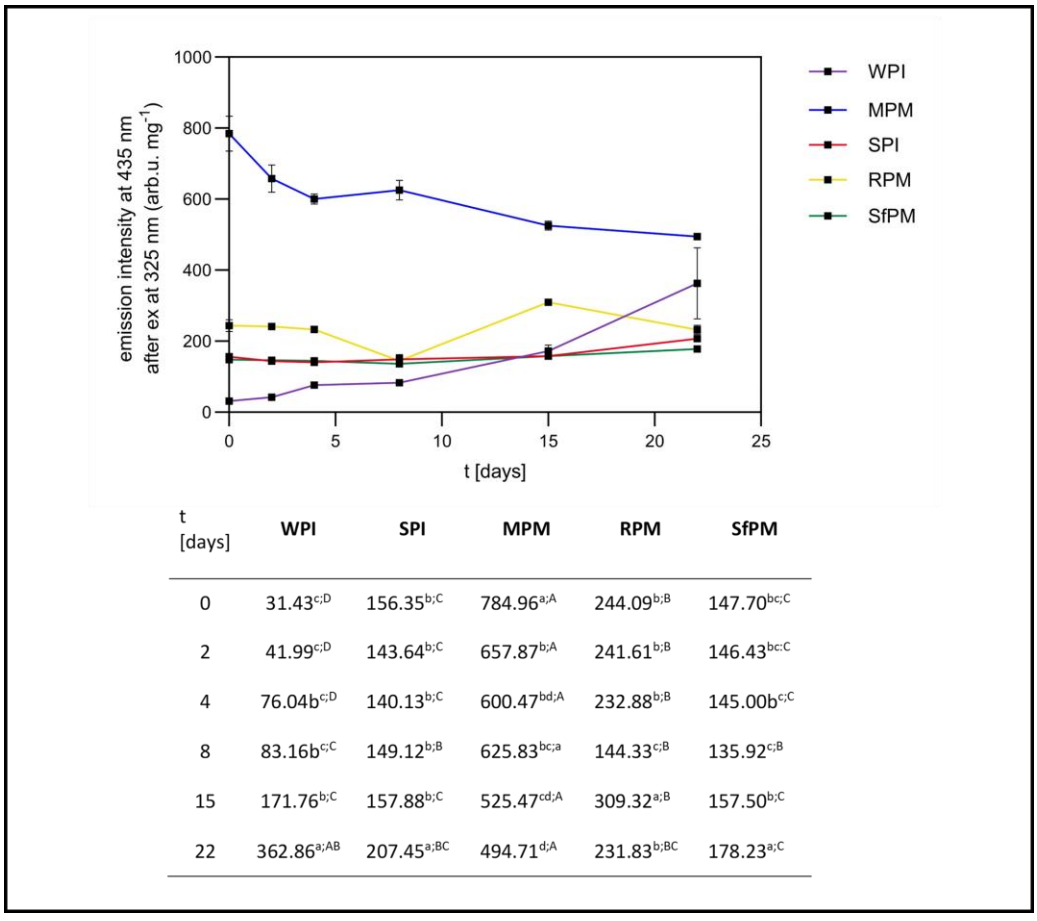


t [days]	WPI	SPI	MPM	RPM	SfPM
0	$1.69^{c;D}$	$6.58^{b;B}$	$11.21^{a;A}$	$6.86^{bc;B}$	$3.48^{bc;C}$
2	$2.97^{bc;C}$	$6.71^{ab;B}$	$12.24^{a;A}$	$4.58^{cd;C}$	$2.93^{c;C}$
4	$4.46^{ab;C}$	$6.93^{ab;B}$	$11.13^{a;A}$	$4.31^{d;C}$	$3.90^{ac;C}$
8	$5.06^{ab;B}$	$6.83^{ab;B}$	$12.19^{a;A}$	$6.30^{bd;B}$	$4.25^{ac;B}$
15	$4.85^{ab;B}$	$7.65^{ab;B}$	$12.87^{a;A}$	$12.44^{a;A}$	$6.26^{a;B}$
22	$6.00^{a;D}$	$7.85^{a;BC}$	$13.54^{a;A}$	$8.60^{b;B}$	$5.78^{ab;CD}$

Figure 4.3: Formation of protein carbonyls [mmol/mg protein] in the protein phase of protein-based oleogels. 10% of whey protein Isolate (WPI)-, soy protein isolate (SPI)-, mussel protein (MPM)- , rapeseed protein (RPM)- and sunflower protein (SfPM) aggregates were dispersed into oil with 2.8% added water, stored over 22 days at 40 °C and investigated after extracted and hydrolyzed. Results are given as the mean ± SD, n=3; a-f are statistically significant differences ($p<0.05$) within a row, A-D are statistically significant differences within a timepoint over the different groups.

4.3.2.2 N-formylkynurenine

Fluorescence of N-formylkynurenine (NFK) was measured to quantify tryptophan oxidation as an indicator of protein oxidation over the 22-day storage period (Fig. 4.4). Similar to the trend observed for protein carbonyl formation, WPI sample showed a significant increase in NFK fluorescence, reaching an emission intensity of 363 arb. units by day 22. In contrast, the SPI and SfPM samples did not exhibit a comparable increase, with only a modest rise observed on day 22. The RPM sample showed a trend consistent with its carbonyl content: fluorescence peaked at day 15 (309 arb. units/mg) and then sharply decreased to 231 arb. units/mg by day 22. Mussel protein displayed a markedly elevated NFK fluorescence at day 0 (785 arb. units/mg protein), followed by a pronounced decline of approximately 37% over the storage period.



t [days]	WPI	SPI	MPM	RPM	SfPM
0	31.43$^{c;D}$	156.35$^{b;C}$	784.96$^{a;A}$	244.09$^{b;B}$	147.70$^{bc;C}$
2	41.99$^{c;D}$	143.64$^{b;C}$	657.87$^{b;A}$	241.61$^{b;B}$	146.43$^{bc;C}$
4	76.04b$^{c;D}$	140.13$^{b;C}$	600.47$^{bd;A}$	232.88$^{b;B}$	145.00b$^{c;C}$
8	83.16b$^{c;C}$	149.12$^{b;B}$	625.83$^{bc;a}$	144.33$^{c;B}$	135.92$^{c;B}$
15	171.76$^{b;C}$	157.88$^{b;C}$	525.47$^{cd;A}$	309.32$^{a;B}$	157.50$^{b;C}$
22	362.86$^{a;AB}$	207.45$^{a;BC}$	494.71$^{d;A}$	231.83$^{b;BC}$	178.23$^{a;C}$

Figure 4.4: Formation of N-formylkynurenine (NFK) [arb.u.mg-1] in the protein phase of protein-based oleogels. 10% of whey protein Isolate (WPI)-, soy protein isolate (SPI)-, mussel protein (MPM)-, rapeseed protein (RPM)- and sunflower protein (SfPM) aggregates were dispersed into oil with 2.8% added water, stored over 22 days at 40 °C and investigated after extracted and hydrolyzed. Results are given as the mean ± SD, n=3; a-f are statistically significant differences ($p<0.05$) within a row, A-D are statistically significant differences within a timepoint over the different groups.

4.3.3 Oxidation marker– FTIR

FTIR spectra of the oil and protein phases of the oleogels were recorded across the full spectral range (800–4000 cm^{-1}) over a storage period of 22 days (Fig. 4.5). This analysis aimed to identify markers of oxidation through structural changes in the samples. To achieve this, peaks that exhibited spectral shifts or intensity changes over time were selected for further analysis.

Eight characteristic peaks were identified at 912, 988, 1163, 1375, 1650, 1744, 2923 and 3430 cm^{-1}, corresponding to structural features associated with oxidation processes described in literature before. Their intensities were measured and are presented in Table. S4.1.

The peaks at 988 and 1163 cm^{-1} showed a continuous increase in intensity during storage for WPI, SPI, and MPM, whereas for RPM and SfPM this increase was less pronounced. When comparing peak intensities across all protein groups, it became evident that the bands at 912 and 988 cm^{-1} were noticeably higher in RPM and SfPM compared to the other proteins. Interestingly, the 912 cm^{-1} peak did not exhibit a consistent pattern within the individual protein groups over time. Only minor changes in intensity were observed at 1375 cm^{-1}

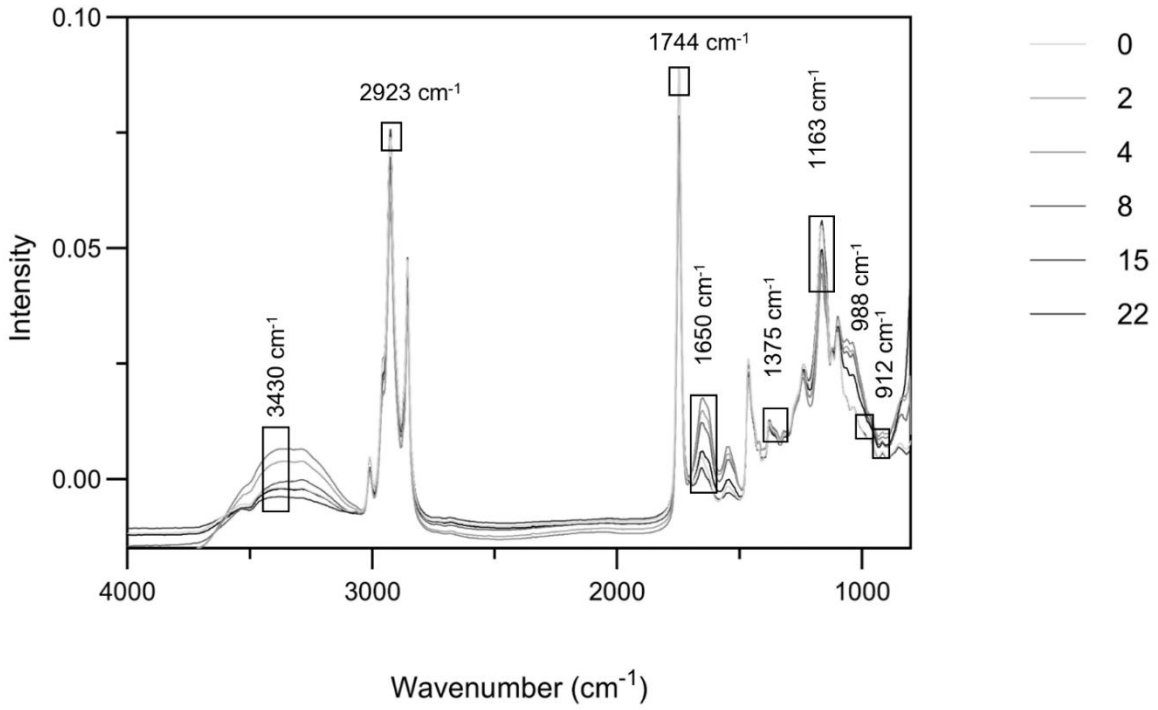


Figure 4.5: Intensity of Attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) in the oleogel stored over 22 days at 40 °C were investigated. Results are given as the mean, n=3.

throughout the storage period. The peak at 1650 cm^{-1} decreased in intensity during storage for all samples, with SPI showing the lowest final intensity. In contrast, the 1744 cm^{-1} peak displayed distinct trends depending on protein source: WPI and SPI showed relatively high initial values with only slight changes over time, while MPM, RPM, and SfPM exhibited a marked increase in intensity during storage. At 2923 cm^{-1}, clear differences were observed between WPI and the other protein sources (SPI, RPM, MPM, and SfPM), with WPI consistently showing lower intensities over the entire storage period. RPM, MPM and SfPM were showing a noticeable increase in peak intensity over storage time. At 3430 cm^{-1}, only minor changes were observed in WPI and soy protein samples, whereas stronger variations were detected for MPM, RPM, and SfPM.

4.3.4 Correlation

To facilitate the visualization of the relationships between oxidation parameters and FTIR spectral changes, a correlation matrix was created over all protein sources, labeled as average, as well as individual correlations for each protein source oleogel between day 2 and 8 to cover propagation phase properly (Fig. 4.6). As expected, correlation on average were much less intensive in their expression compared to the individual correlations. Nevertheless, a strong positive correlation was observed among the LOx markers across all protein sources, particularly between hydroperoxide and hexanal content. This correlation was displayed by all other individual correlations, except RPM, with no correlation at all, due to the missing increase in Hexanal levels during the first 8 days of storage (Fig. 4.2). In contrast, the correlation of carbonyl content—a marker of protein oxidation—was positive correlated to the LOx parameters for all protein sources individually, whereas in average the correlation between these two parameters were remarkably low. For NFK a positive correlation to LOx parameters was observed for WPI, SPI but strongly negatively correlated for MPM, RPM and SfPM, which in average only resulted in no correlation at all.

Bands at 1160, 1744, and 2923 cm^{-1} showed consistent positive associations with LOx markers in average. However, these correlations were not uniform across protein sources. While MPM, SfPM, and RPM confirmed the positive relationships, SPI and WPI instead exhibited negative associations between these peaks and LOx parameters, such as hydroperoxides and hexanal.

It was found only weak correlation between carbonyl content and FTIR peaks over average, whereas in RPM, MPM, WPI, SPI and partly in SfPM stronger correlations were observed, while no uniform pattern was observed across all protein sources. Positive correlations to carbonyl content were shown by peaks at 1744 and 2923 cm^{-1} in RPM, whereas in SPI and WPI these correlations were strongly negative and no correlation was found for MPM and SfPM.

Next to correlations between FTIR marker and oxidation parameters, correlations were found between FTIR markers itself. A strong negative correlation between 2923 and 3430 cm^{-1}, as

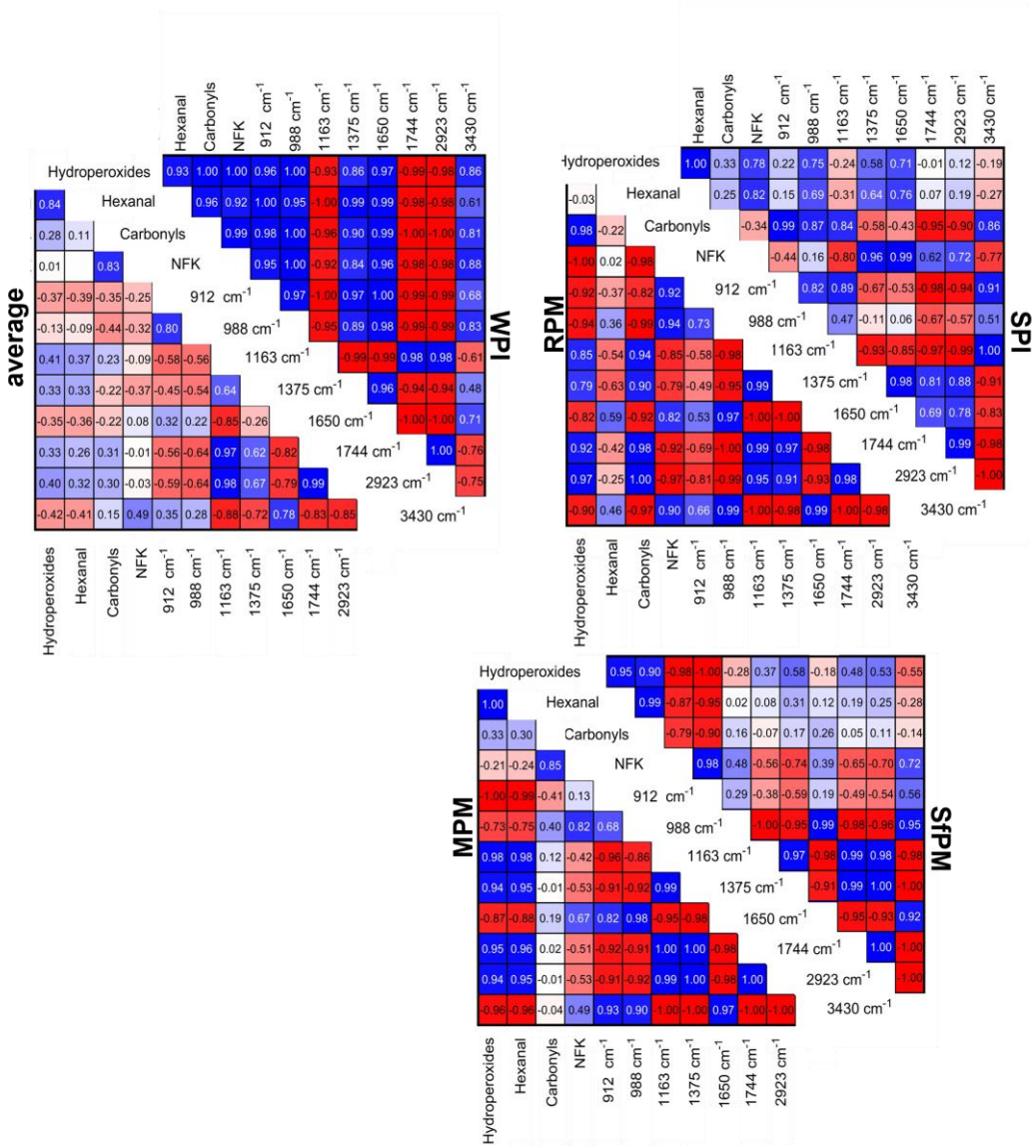


Figure 4.6: Pearson R correlation between measured oxidation parameters and FTIR intensity at different wavenumbers whey protein isolate (WPI), soy protein isolate (SPI), mussel protein (MPM), rapeseed protein (RPM) and sunflower protein (SfPM) based oleogels between day 2-8 and overall protein sources in average over the hole storage period of22 days. Results are given as the mean, n=3.

well as 1744 and 3430 cm^{-1} was found in all samples and was therefore also clearly reflected in the averaged correlation matrix. In contrast, strong positive correlations across all protein sources were detected between 1744 and 2923 cm^{-1} as well as between 1650 and 3430 cm^{-1}. The same phenomenon was observed for the correlations between 1163 and 1744 cm^{-1} and between 1163 and 2923 cm^{-1}, where all protein sources showed strong positive correlations except soy, which revealed the opposite trend.

4.4 Discussion

The importance of chemically and physically stable oil with a superior nutritional profile has resulted in a significant increase in research interest in oleogels. Proteins have been shown to exhibit antioxidant activity in lipid–protein systems. However, their structural and functional differences—particularly between plant- and animal-based sources—may lead to varying effects on lipid and protein oxidation. This study focused on ascertaining the potential for FTIR measurement to detect a correlation between oxidative changes and changes in FTIR spectrum, in order to assess the potential of this method for the most accurate estimation of the oxidative status in protein-based oleogels. Therefore, the effect of different protein sources on oxidative stability in protein-based oleogels over a 22-day storage period was examined and correlated with changes in FTIR spectrum over time.

4.4.1 Proteins as antioxidants in protein-based Oleogels.

Due to their structural characteristics, proteins possess inherent antioxidant properties that can contribute to the inhibition of oxidation processes in systems susceptible to oxidative degradation through a variety of mechanisms (R. J. Elias & Decker, 2010b). As illustrated in Figure 4.1 and 4.2, different protein sources exhibit distinct kinetic behaviors in their ability to inhibit LOx. This highlights the need to elucidate the underlying molecular mechanisms responsible for these differences. The fact, that WPI shows a different curve progression in formation of hydroperoxides, compared to SPI, RPM and SfPM could be due to the animal-based nature of whey, which results in differences in amino acid composition and secondary protein structure. Animal-based proteins present a complete complement of essential amino

acids, whereas plant-based proteins—especially from legumes and grains—are typically deficient in one or more essential amino acids, like methionine, lysin or others (Gorissen et al., 2018). Several antioxidant mechanisms, including free radical scavenging, metal ion chelation, peroxide decomposition, and aldehyde quenching are largely influenced by the amino acid composition and the protein's tertiary structure, which determine the accessibility and surface exposure of reactive amino acid side chains. Therefore AA composition, next to changes in secondary protein structure by processes like denaturation can have noticeable effect on the antioxidant potential of protein (Kut et al., 2023). In literature it was shown, that some AA, including tryptophan, methionine, histidine, lysine, cysteine, arginine and tyrosine, are more potent in their antioxidant effectiveness with cysteine and tryptophan often showing the highest activity in various assays due to their ability to scavenge radicals and chelate metals (Guidea et al., 2020; Xu et al., 2017b). Since the kinetics of hydroperoxide formation in mussels—as a representative animal protein source—do not differ significantly from those observed in plant-based proteins (Fig. 4.1), a general distinction between animal and plant protein sources in this context appears unwarranted. However, when analyzing oxidation kinetics using headspace gas chromatography (HS-GC) (Fig. 4.2), the hexanal formation curves for WPI and MPM exhibit notable similarities, characterized by a more linear progression that tends to flatten over time. In contrast, plant-based protein sources display distinctly different profiles, typically showing an exponential increase in hexanal concentration as oxidation progresses. These findings suggest that underlying mechanistic differences between animal- and plant-derived proteins may influence secondary LOx pathways. Therefore, the involvement of protein source-specific mechanisms cannot be entirely ruled out.

In addition to the intrinsic protein structure, interactions between proteins and surrounding matrix components — including other proteins — may influence antioxidant potential through physical or shielding effects. For example, Hu et al (2003) demonstrated that in emulsions, droplet size — and thus the total interfacial surface area — significantly affects antioxidant efficiency. Smaller droplets provide a larger surface area, resulting in more extensive interface exposure and thus a greater possibility for prooxidant interactions. Furthermore, the thickness of the interfacial protein layer in emulsions may influence the interaction between lipids and

aqueous-phase prooxidants. A thicker layer can enhance the interaction of protein components with LOx intermediates, allowing them to scavenge free radicals or chelate prooxidant metal ions more effectively (M. Hu et al., 2003). A similar effect may occur in oleogel systems, where protein network formation can contribute to oxidative stability. The immobilization of the lipid phase within the network may limit the diffusion of radicals and reduce the propagation of oxidation reactions, thereby inhibiting LOx through physical confinement (Shang et al., 2021; Wei et al., 2021). These protective effects are likely to depend strongly on the structural properties of the protein matrix, including the degree of aggregation, particle size distribution, and the ability to form cohesive networks.

In this context, structural differences between animal- and plant-based proteins are likely to play a role. Plant proteins typically exhibit lower solubility compared to animal proteins, largely due to their more hydrophobic nature—an evolutionary adaptation for storage stability in seeds (Day et al., 2022b). Furthermore, commercially extracted plant proteins often show even further reduced solubility as a result of protein denaturation, aggregation, and loss of native structure caused by harsh extraction and processing conditions (J. Yang et al., 2024). These factors may compromise their ability to physically entrap lipids and limit oxidation, compared to more soluble and structurally flexible animal-derived proteins.

Interestingly, animal-based proteins initially appeared less effective in suppressing LOx compared to plant-based proteins. However, over time, their oxidative stability improved, as evidenced by a flattening of the hexanal and hydroperoxide-formation curves. In contrast, plant-based protein systems showed a more linear—and in some cases even exponential—increase throughout the storage period. This behavior may be partially explained by the better dispersibility of animal proteins such as WPI, which forms smaller particles due to its higher solubility. Smaller particle sizes increase the available interfacial area, potentially leading to more effective interaction between water, protein phase and lipid phase in the early stages. This may initially enhance oxidation, as shown by the significantly accelerated propagation phase of LOx in WPI- compared to the other protein oleogels (Fig. 4.1). After prolonged storage (e.g., after 22 days), oxidation levels in both systems tended to change. This could be attributed to the superior amino acid composition of animal proteins, which may provide

more effective intrinsic antioxidant capacity over time, in contrast to certain plant proteins such as soy, which may be less effective due to lower solubility, larger aggregates, and a less favorable amino acid profile.

Co-oxidation processes between lipids and proteins are highly probable in complex food systems, particularly due to the close interaction of these components and the presence of water, which facilitates radical mobility. Protein oxidation can occur not only through direct attack by reactive oxygen species (ROS), but also indirectly via secondary LOx products(W. Zhang et al., 2013). Amino acid residues such as histidine, cysteine, methionine, lysine, tyrosine, and tryptophan are especially susceptible to oxidative modification. These oxidative changes can lead to structural alterations in the protein, including aggregation and the formation of covalent cross-links (Wu et al., 2009). The susceptibility of a given protein to such modifications is therefore highly dependent on its amino acid composition and structural conformation. This may also explain observed differences in oxidative behavior between animal- and plant-based proteins, which differ not only in their amino acid profiles but also in their tertiary and quaternary structures, solubility, and aggregation tendencies.

The progressive increase in both carbonyl compounds and NFK observed in WPI aligns with expectations, considering that protein oxidation is generally a secondary process following LOx. The absence of a sharp initial rise in protein oxidation markers, despite rapid hydroperoxide formation, supports this sequence of events. Interestingly, a flattening of the oxidation curve in later storage stages suggests that protein oxidation in WPI may reach a plateau, potentially due to structural changes or depletion of reactive amino acid residues (Ismail et al., 2020).

In contrast, SPI exhibited no notable increase in carbonyls or NFKs during storage. This likely reflects a pre-existing high oxidation state, attributable to the harsh conditions commonly used in commercial protein extraction, which are known to induce protein denaturation and oxidative modification. The lack of further increase suggests that the oxidative potential of the soy protein may have already been largely exhausted prior to the start of storage.

Mussel protein showed a markedly higher level of protein oxidation—both in carbonyls and NFKs—compared to the plant and dairy proteins. Notably, this elevated oxidation state was already present at day 0, suggesting that oxidative modification had occurred before the start of storage. This may result either from oxidative stress during purification or from biological processes occurring in the living animal. Several studies have linked exposure to trace metals such as copper and cadmium to increased protein carbonylation in mussels supporting the idea that environmental factors could contribute to the initial oxidation state (Dailianis et al., 2009; Kirchin et al., 1992). While direct comparisons between organisms as fundamentally different as soy and mussel remain problematic, such findings highlight the importance of considering both biochemical composition and processing history when evaluating protein oxidation.

What is particularly surprising, however, is the lack of any further increase in carbonyl content during storage in mussel protein, and even more so, the observed decrease in NFK levels over the 22-day storage period. This trend runs counter to expectations and raises questions about possible degradation, conversion, or interactions of oxidized residues over time.

Despite its high initial oxidation state, MPM proved to be the most effective in inhibiting LOx, as evidenced by the lowest hexanal levels in the lipid phase. This finding is consistent with previous studies showing antioxidant properties of MPM, even after processing steps such as enzymatic hydrolysis (Dong et al., 2017). Such effects may stem from bioactive peptides or specific AA compositions that confer radical scavenging or metal-chelating abilities.

4.4.2 Oxidation marker found in the infrared spectra - Correlation

The aim of this study was to evaluate whether FTIR spectroscopy can be used to assess oxidation processes in protein-based oleogels and whether changes in peak intensity represent reliable markers for lipid and protein oxidation. For this purpose, classical oxidation parameters (hydroperoxides, hexanal, carbonyls, and NFK) were measured in parallel with FTIR spectra over a 22-day storage period, and the results were correlated.

The data show that FTIR is in principle capable of capturing changes in the oleogel systems during storage (Fig. 4.5). Several peaks that have previously been reported as relevant for

oxidation monitoring also displayed time-dependent changes in the present dataset. These include the OH-stretching vibration around 3430 cm^{-1}, the carbonyl and ester region at 1744 cm^{-1}, as well as bands at 1375 cm^{-1}, 1163 cm^{-1} and 988 cm^{-1}. However, it became clear that these changes were not consistently correlated with the chemically determined oxidation markers.

When comparing the correlation across all protein sources over the entire storage period of 22 days with the correlations of the individual protein sources in their propagation phase (days 2–8) (Fig. 4.6) – it becomes evident that an overall correlation cannot reflect the individual correlations, as the differences between the various protein sources are simply too great.

A central example is the band at 3430 cm^{-1}, which has been associated with hydroperoxides in the literature described to increase over storage time of lipids (Guilln & Cabo, 2000b; Tena et al., 2017). In the samples analyzed here, the expected pattern of an initial increase in hydroperoxides followed by a decrease due to decomposition into secondary products could be observed (Fig. 1). At the same time, correlations with changes in measured peak intensities at 3430 cm^{-1} were negative in most protein matrices (RPM, SPI, SfPM and MPM). This demonstrates that FTIR-signals do not exclusively reflect the concentration of intact hydroperoxides but are also influenced by interactions with the protein matrix or by intermediate oxidation structures that are not detected by conventional chemical assays.

Comparable limitations were also observed in In C=O stretching region (1850–1550 cm^{-1}) (Tena et al., 2017). While the band at 1744 cm^{-1} , corresponding to R-O-C=O carbonyl absorption of the triglyceride ester linkage, should theoretically increase with progressing oxidation (H. S. Lam et al., 2004), overlap with neighboring bands (1725 and 1705 cm^{-1}, corresponding to aldehydic and ketonic structures) led to inconsistent results (Daoud et al., 2019; Guilln & Cabo, 2000a). The band at 1650 cm^{-1}, as already pointed out by Guillen and Cabo (2000a)and Daoud et al. (2019), is equally difficult to interpret, since different vibrations overlap in this spectral range, probable differ between protein sources.

More reliable markers were identified in the regions around 1375 cm^{-1} and 1163 cm^{-1}, corresponding to the symmetric bending vibration of methyl groups (Asemani & Rabbani,

2020) and C-O stretching vibration and CH_2 bending vibration (Rohman & Che Man, 2013). The increase of the 1375 cm^{-1} band can be attributed to methyl-containing secondary products such as short-chain aldehydes and alcohols, but may also indicate protein–lipid interactions, e.g. Schiff base formation. This suggests that this band could serve as an indicator not only for lipid but also for co-oxidation processes. Thereby this peak is the only correlating positively with LOx parameters over all protein sources. The 1163 cm^{-1} band, on the other hand, followed the expected mechanism of hydroperoxide decomposition via β-scission and thus proved to be a robust marker of LOx, reported to increase with progressing LOx by Hayati et al. (2005). Still in the present study, this correlation cold only be confirmed in MPM and RPM over prolongation period.

A central finding of this study is that correlations between FTIR signals and conventional oxidation parameters were strongly dependent on the protein source. While MPM, RPM and SfPM showed comparable patterns, WPI consistently deviated. This is in line with its distinct oxidation profile, which is most likely linked to structural differences. SPI showed an intermediate behavior compared to the others (Fig. 4.6). These results underline, that proteins do not only passively undergo oxidation but actively influence oxidation kinetics depending on their structure, composition, and processing history.

The protein oxidation markers provided additional evidence for this protein dependency: in animal proteins (WPI, MPM), a positive correlation between carbonyls and NFK was observed, whereas plant proteins (RPM, SPI, SfPM) showed a negative relationship. This points to different mechanisms of protein oxidation that again affect how FTIR-signals need to be interpreted.

In summary, the findings clearly demonstrate that FTIR spectroscopy provides valuable insights into oxidation processes in protein-based oleogels, but that a universal application of FTIR peaks as direct indicators of oxidation status is not feasible. Differences in protein composition, in the duration and extent of the individual oxidation phases, as well as in protein–lipid interactions result in highly protein-dependent patterns. FTIR is therefore best suited as a complementary screening method, allowing structural changes to be tracked rapidly and without complex sample preparation. For a reliable assessment of the oxidative

status, however, the method must be combined with conventional chemical analyses. Future research should aim at establishing protein source-specific FTIR markers in order to use this approach more effectively in product development.

4.5 Conclusion

This study highlights the complex relationship between different protein sources and the oxidative stability of protein-based oleogels. The findings demonstrate that the ability of proteins to inhibit both lipid and protein oxidation depends strongly on the protein source and, very likely, on processing conditions. Such conditions may alter protein nativity and thereby influence structural properties through unfolding or aggregation processes. In addition to the intrinsic antioxidative capacity of the proteins, these factors may affect the distribution of sample components within the matrix, particularly through their arrangement at interfacial areas, ultimately impacting the oxidative stability of the oleogel.

Furthermore, it can be concluded that assessing the oxidative status in protein-based oleogels using FTIR is indeed feasible, as already shown on literature in other sample matrices. However, comparisons across different protein sources are considerably more complex and less conclusive, as variations in protein structure lead to distinct signal changes during measurement. Based on the current state of knowledge, no single marker for protein or LOx can yet be identified that provides sufficient explanatory power across all investigated protein sources.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used “DeepL Write” and “ChatGPT” in order to improve readability and language of the work. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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4.7 Supplementary Material

Table S4.1: FTIR-peak intensity in whey- (WPI), Soy- (SPI), Mussel- (MPM), Rapeseed- (RPM) and sunflower- (SfPM) based oleogels over storage period of 22 days. n=3.

cm^{-1}	[days]	**WPI**	**SPI**	**MPM**	**RPM**	**SfPM**
912	2	0.0051	0.0063	0.0064	0.0102	0.0086
	4	0.0058	0.0073	0.0064	0.0092	0.0083
	8	0.0063	0.0067	0.0056	0.0081	0.0077
	15	0.0065	0.0079	0.0050	0.0056	0.0065
988	2	0.0061	0.0080	0.0074	0.0174	0.0159
	4	0.0069	0.0085	0.0071	0.0179	0.0166
	8	0.0072	0.0086	0.0070	0.0163	0.0158
	15	0.0089	0.0110	0.0076	0.0100	0.0133
	22	0.0093	0.0090	0.0092	0.0150	0.0171
1163	2	0.0546	0.0538	0.0420	0.0467	0.0390
	4	0.0516	0.0551	0.0437	0.0445	0.0336
	8	0.0481	0.0538	0.0466	0.0494	0.0404
	15	0.0513	0.0539	0.0560	0.0560	0.0505
	22	0.0564	0.0565	0.0491	0.0499	0.0420
1375	2	0.0141	0.0135	0.0110	0.0125	0.0113
	4	0.0145	0.0131	0.0117	0.0119	0.0107
	8	0.0154	0.0138	0.0124	0.0129	0.0118
	15	0.0152	0.0130	0.0126	0.0122	0.0123
	22	0.0138	0.0136	0.0130	0.0116	0.0123
1650	2	0.0133	0.0039	0.0258	0.0150	0.0259
	4	0.0217	0.0036	0.0236	0.0177	0.0290
	8	0.0280	0.0041	0.0224	0.0124	0.0259
	15	0.0184	-0.0030	0.0030	0.0024	0.0144
	22	-0.0006	-0.0014	0.0180	0.0063	0.0241
1744	2	0.0872	0.0922	0.0649	0.0704	0.0523
	4	0.0809	0.0899	0.0695	0.0667	0.0442
	8	0.0772	0.0916	0.0751	0.0780	0.0561
	15	0.0793	0.0889	0.0917	0.0890	0.0752
	22	0.0876	0.0882	0.0788	0.0786	0.0571
2923	2	0.0133	0.0767	0.0558	0.0599	0.0467
	4	0.0217	0.0750	0.0602	0.0586	0.0410
	8	0.0280	0.0765	0.0650	0.0668	0.0500
	15	0.0184	0.0756	0.0779	0.0757	0.0666
	22	-0.0006	0.0749	0.0696	0.0699	0.0527
3430	2	-0.0056	-0.0072	0.0179	0.0023	0.0112
	4	-0.0026	-0.0052	0.0152	0.0049	0.0140
	8	-0.0036	-0.0071	0.0116	-0.0020	0.0095
	15	-0.0044	-0.0066	-0.0042	-0.0042	0.0024
	22	-0.0078	-0.0076	0.0052	-0.0029	0.0060

5 Phenolic compounds inhibit lipid oxidation in whey protein-based oleogels beyond their antioxidant potential

Maria Wittmüss, Jonas Amft, Erik Schledermann, Karin Schwarz, Timon R. Heyn*

Institute of Human Nutrition and Food Science, Division of Food Technology, Heinrich-Hecht Platz 10, D-24118 Kiel, Kiel University, Germany.

* Corresponding author (theyn@foodtech.uni-kiel.de)

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Highlights

- LOx was inhibited in WPI oleogels processed with sinapic- and caffeic acid.
- SA and CA inhibited LOx in WPI oleogels, beyond their antioxidant activity.
- Addition of PCs changes protein composition in WPI aggregates.
- LOxPs correlated with WPI secondary structure changes.

Abstract

Protein-based oleogels offer a promising solution to nutritional and ecological challenges by combining plant-based oils with proteins as oleogelators to substitute conventional solid fats. However, the oxidative stability of oleogels remains a concern, particularly because of the high amount of polyunsaturated fatty acids. Phenolic compounds (PCs) may influence the oxidative stability of protein-based oleogels, but their contribution and the underlying mechanisms remain unclear. Therefore, this study investigated whether the addition of individual PCs could improve the oxidative stability of whey protein isolate (WPI)-based oleogels. WPI aggregates were prepared in the presence of gallic acid (GA), caffeic acid (CA), sinapic acid (SA), or chlorogenic acid (CGA). Lipid oxidation was monitored during eight days of storage under accelerated conditions by measuring hydroperoxide and hexanal formation. Protein composition, secondary structure, and PC–protein binding were additionally analyzed to investigate potential mechanisms underlying the observed effects. The results indicate that SA and CA inhibit lipid oxidation during storage by 31% and 38% respectively, while GA and CGA do not result in further effects beyond those provided by the protein alone. This effect does not appear to correspond to the reported antioxidant potential of the PCs, as well as the binding affinity between PC and protein. Rather, shifts in protein structure and composition were associated with improved oxidative stability. These findings suggest that protein structural modulation may contribute to the effects of specific PCs on lipid oxidation, although further mechanistic studies are required to confirm this relationship.

5.1 Introduction

Oleogels could provide an improvement in both health and sustainability aspects of food by structuring unsaturated fatty acids with different kinds of oleogelators (Feichtinger & Scholten, 2020; Scholten, 2019) and thereby replacing saturated fatty acids with unsaturated ones. In this way, oleogels could be used in food applications to replace conventional fats while maintaining their structural and sensory functions and simultaneously reducing the intake of saturated fatty acids (Chen et al., 2022a). Protein-based oleogels are of particular interest because proteins provide additional nutritional value and may be sourced regionally, while potentially supporting clean-label approaches and increasing consumer acceptance. Because proteins are not readily dispersible in oil due to their hydrophilic nature, it is necessary to implement specific modification steps to prepare a protein-based oleogel (Scholten, 2019). A heating step could be used to form a three-dimensional network through aggregates and cross-links, thereby enhancing oil-binding capacity by increasing the accessibility of lipophilic regions in proteins (Gosal & Ross-Murphy, 2000; Vries, 2017).

Oleogels are highly susceptible to lipid oxidation (LOx) in comparison to conventional plastic or solid fats, containing a high degree of saturated fatty acids. The process of LOx contributes significantly to the deterioration of food products (Grosso et al., 2026). During this autocatalytic chain reaction process, lipid hydroperoxides undergo decomposition, resulting in the formation of reactive hydroxyl radicals and other reactive oxygen species (Meissner et al., 2023). Moreover, the presence of proteins in a protein-based oleogel also results in protein oxidation. There is evidence indicating that the presence of proteins in the system slows down LOx (Berton-Carabin et al., 2014; Chen et al., 2022b; Zhu et al., 2018). However, the precise manner in which these two oxidation processes influence each other in an oleogel remains unclear.

Given that oxidation processes have the potential to significantly impair the value of the product over time, it is imperative to identify a solution that can reduce this risk. One potential approach is the utilization of natural antioxidants, such as phenolic compounds (PCs), to increase the product's quality and shelf life. PCs are biologically active compounds found in

plants, including fruits, vegetables, cereals, and coffee (Abbas et al., 2017). They contain one or more phenolic rings and possess antioxidant properties that are mediated by various mechanisms such as quenching of reactive oxygen species and inhibition of autoxidative chain reactions, chelation of metal ions and reduction of localized O_2 concentrations (Brewer, 2011; Zamora & Hidalgo, 2016). The antioxidant capacity of PCs is contingent upon their structural configuration. Based on previously reported literature data, the antioxidant potential of the PCs used in this study can be ranked as follows: gallic acid (GA) > caffeic acid (CA) > chlorogenic acid (CGA) > sinapic acid (SA). This ranking is based on their reported antioxidant activity, with the superior antioxidant capacity of GA compared to CA specifically observed in the ABTS assay (Biskup et al., 2013; Cos et al., 2002; Olszowy et al., 2019; Sousa et al., 2004). PCs have the capacity to interact with proteins via covalent or non-covalent binding. Covalent binding is dominated by the formation of disulfide bonds between quinones produced from the oxidation of polyphenols and the nucleophilic residues of the protein (Quan et al., 2019). This binding exhibits higher thermal and oxidative stability compared to non-covalent binding (Zhou et al., 2020). In contrast, non-covalent interactions between milk proteins and PCs are primarily characterized by hydrophobic interactions and hydrogen bonding. These interactions are relatively common and lead to the formation of reversible, weak complexes that are susceptible to alterations in environmental conditions (Mao et al., 2023; Schild et al., 2023).

Beta lactoglobulin (β-LG),the main component of whey protein isolate (WPI), contains a variety of hydrophobic residues, including leucine, isoleucine, valine, and tryptophan, which are capable of forming hydrophobic interactions with non-polar aromatic rings present in PCs (Kontopidis et al., 2004; Li et al., 2020). Furthermore, hydrogen bonds can be formed between the hydroxyl groups on PCs and various polar groups on the side chains of β-LG. In the case of charged PCs, electrostatic interactions are also possible with oppositely charged groups on the protein surfaces (Baba et al., 2021). Furthermore, it is assumed that the interaction between a protein and a PC may result in an unfolding process that could potentially lead to modifications in the secondary protein structure (Meng & Li, 2021). Moreover, experiments

have demonstrated that varying process conditions affect the protein structure in different ways (Wittmüss et al., 2024). It can be hypothesized that the denaturation process, which produces protein aggregates, is influenced by the presence of both bound and unbound PCs, which may exert either an enhancing or a weakening effect. This may also impact the oxidation of the oleogels at a later stage. The nature of the interaction between a PC and the protein is likely to be a determining factor in the antioxidant effect, which may ultimately lead to a reduction of LOx in oleogels. However, the precise mechanism by which this occurs remains unclear.

This approach will investigate the effect of incorporating PCs into oleogels as a strategy to extend their shelf life. The purpose of this study was firstly to ascertain the extent to which different PCs, when incorporated into the preparation of protein oleogelators, are capable of mitigating LOx in the protein-in-oil-dispersion. The second objective was to determine whether the incorporation of PC during denaturation of proteins resulted in structural and compositional changes of the resulting aggregates. Therefore, the effects of PCs with different molecular sizes and numbers of hydroxyl groups (GA, CA, SA, and CGA) on LOx, protein composition, and the secondary structure of protein aggregates were investigated over an eight-day storage period under accelerated conditions. This study aimed to identify the mode of action of PCs and establish relationships between structural changes in the proteins and oxidation processes.

5.2 Material and Methods

5.2.1 Materials

Whey protein isolate (WPI) was obtained from Davisco Foods International with 97.7% protein and 75% β-lactoglobulin in dry matter. Fully refined sunflower and rapeseed oil were purchased in a local supermarket. Ultra-pure water (Milli Q) was used for all applied solutions (<18.2 MΩ cm^{-1}). All used chemicals were of analytical grade: aluminium oxide, ammonium thiocyanate, barium chloride, hexane, hydrochloric acid (HCl), iron (II) sulfate and sinapic acid

was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). β-mercaptoethanol, caffeic acid, chlorogenic acid, gallic acid were purchased from Merck KGaA (Darmstadt, Germany). Isopropanol was procured from VWR International GmbH (Darmstadt, Germany). A 30% acrylamide/Bis solution (37.5:1), ammonium persulfate (APS), bromophenol blue, Coomassie brilliant blue R-250 and G-250, glycine, Tris, Precision Plus Protein All Blue Prestained Protein Standard, a 20% SDS solution, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories GmbH (Feldkirchen, Germany).

5.2.2 Sample preparation

5.2.2.1 Aggregate preparation

Protein aggregates were prepared according to Meissner et al. (2020) with some modifications. In brief, WPI powder (7.5% w/v) was dissolved in Milli Q water under constant stirring. Polyphenol stock solution (55.55 mM) and protein solution (7.5% w/v) were mixed so the desired concentration of WPI (6% w/v) and PC (10 mM) was reached. The protein solution was adjusted to pH 5.6 ± 0.06 using 2 M HCl before stirring the solution at 20°C room temperature (RT) for 2 h. Subsequently, the solution was denatured for 20 min at 85°C in a temperature-controlled water bath, using 50 mL plastic tubes. The resulting protein aggregate dispersion was homogenized using a rotor-stator homogenizer (Ultra-Turrax) at 13000 rpm for 3 min. The samples were then centrifuged at 4000 x g for 20 min at RT to collect the aggregates in a pellet. The supernatant was discarded and the aggregates were dispersed in fresh water using the Ultra-Turrax (13000 rpm, 3 min). After washing, the protein aggregates were frozen at -80°C for 2 days and then they were freeze-dried (LSC Plus, Martin Christ, Osterode am Harz, Germany) for 72 h. The resulting aggregates were ground in a mortar until a fine powder was obtained. All samples were prepared in triplicate.

5.2.2.2 Purification of oil

The purification of the 50:50 (v/v) sunflower and rapeseed oil from natural antioxidants and trace metals was conducted according to Amft et al. (2020). Therefore, activated aluminium oxide was used to prepare a filtering column, which the oil mixed 50:50 (v/v) with n-hexane has to pass through. Thereafter, hexane removal was performed under nitrogen using a rotary

evaporator. The oil was tested for residual tocopherols by using HPLC (<0.5 ppm tocopherols, according to DGF method F-II 4a with minor modifications. Briefly, 50 mg of fresh oil was dissolved in 1 mL n-heptane and analyzed by HPLC (Agilent 1100) using a Nucleosil-OH column (125 × 2 mm, 5 µm) at a flow rate of 0.4 mL/min. Isocratic elution was performed with n-heptane/isopropanol (0.12% isopropanol). Tocopherol isomers were detected by fluorescence with excitation at 295 nm and emission at 332 nm. Identification and quantification were based on retention times and peak areas using calibration curves for each tocopherol isomer. Total tocopherol content was calculated as the sum of the individual isomer concentrations. Samples were analyzed in triplicate.

5.2.2.3 Preparation of oleogels

The lyophilized protein powders were mixed with a combination of stripped sunflower and rapeseed oils (1:1, v/v) at a concentration of 6.8%. The mixture was stirred for 10 min. The unbound oil was not removed in order to not interfere with the quantification of LOx products.

5.2.2.4 Storage experiment

To induce oxidation all samples were stored at 40°C, in the dark for 8 days under constant conditions. The 8-day period was selected as the measurement time point because preliminary experiments had repeatedly confirmed that, under the experimental conditions used, a measurable degree of lipid oxidation was expected to be detectable at this time point as described by Amft et al. (2020). Therefore, WPI aggregates with either no polyphenol – acting as control group (C), or 10 mM GA, SA, CGA and CA were dispersed in purified oil and analyzed after 0, 2, 4, and 8 days. Next, the lipid phase was separated by centrifuging the sample at 20,000 x g for 10 min.

5.2.3 Lipid oxidation parameters

5.2.3.1 Hydroperoxides

Primary LOxP were measured by determining the amount of hydroperoxides using the ferric thiocyanate method as described by Amft et al. (2019). First, 10 mg of the oil phase were mixed with 5 mL Isopropanol. 50 µl of each iron(II) chloride solution and ammonium thiocyanate solution were added before incubation of the sample for 30 min at 60°C in a water bath. Afterwards the samples were measured at 485 nm against a blank without oil. Results were expressed as mmol hydroperoxides/kg of oil. Methods are described more in detail in **chapter 4.2.3**.

5.2.3.2 Hexanal

Hexanal was analyzed as a marker for volatile oxidation products. Therefore, 900 mg of oil were transferred from the original storage vials and weighed into 20 mL airtight HS-GC vials. Static headspace sampling was performed using the HS-20 headspace (Shimadzu, Kyoto, Japan) and a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a DB-1701 column (60 m x 320 µm x 1 µm, Agilent Technologies, Santa Clara, CA, USA). The parameters for the extraction of volatiles using the HS-20 system were utilized according to Hülsebusch et al. (2026): The samples were incubated at 70 °C for 30 min at shaking level 3. Sample line temperature 85 °C; transfer line temperature 100 °C; GC cycle time 30 min; shaking level 3. The following oven temperature program was utilized: an initial 45 °C hold for 2 min, a 25°C/min increase to 70 °C for 3 min, a 20 °C/min increase to 182 °C for 3 min, and a 30 °C/min increase to 240 °C for 3 min. The experimental setup involved the utilization of a flame ionization detector, with hydrogen and air flow rates set at 30 and 200 mL/min, respectively. Nitrogen was utilized as the carrier gas at a linear velocity of 26 cm/s, with a split ratio of 1:4. The acquisition of data was conducted utilizing the device-related software LabSolutions (Shimadzu). The identification of hexanal was achieved through the analysis of the retention time of an external standard, with the peak area being quantified by the area under the curve (AUC).

5.2.4 Interaction between hydroperoxides and WPI-aggregates

To ascertain whether the decline in hydroperoxides is a genuine reduction in LOx or an interaction between LOxP and protein aggregates, the concentration of hydroperoxides in oil, which had been stored for eight days at 40 °C and then mixed with WPI aggregates for 2 and 24 h, was quantified according to Amft et al. (2019). Accordingly, a dispersion of WPI aggregates with the addition of 10 mM PC during production and a previously oxidized stripped oil was prepared in accordance with the aforementioned methodology (**chapter 4.2.2.4**). After a mixing time of 2 h the amount of Hydroperoxides in the oil phase was determined like previously described (**chapter 4.2.3**). After another 22 h the measurement was repeated.

5.2.5 Protein loss during washing step

Protein concentration after the washing step (see **2.2.1**) depending on the addition of different phenolic compounds was analyzed using Lowry assay at 540 nm with bovine serum albumin (BSA) as a standard, as described by Lowry et al. (1951). Therefore, 1 mL of the sample, diluted in Milli-Q water, was mixed with 1 mL of freshly prepared reagent A, consisting of 1 part copper–tartrate complex solution, 2 parts of 5% SDS solution, and 1 part of 0.8 M NaOH. After 10 min, 0.5 mL of 2 M Folin–Ciocalteu phenol reagent was added. The samples were incubated at room temperature for 30 min before measurement.

5.2.6 Changes in Protein composition during washing step

In order to take e closer look at changes in protein composition, since WPI is a protein mixture, depending on the addition of different PCs, the supernatant fraction obtained after aggregate preparation (**2.2.1**) was analyzed by using HPLC. The measurement was conducted using an UltiMate 3000 system (Fisher scientific GmbH, Schwerte, Germany) with a diode array detector at a wavelength of 205 nm and a PLRP-S column (PLRP-S, 300 Å, 8 µm, 150 x 4.6 mm; Agilent Technologies, Santa Clara, USA) equipped with a guard column. A 0.1% (w/v) protein solution was prepared in Milli Q water. Analytical separation was conducted following Keppler et al. (2021). In contrast, the protein aggregates collected as the pellet after aggregate preparation were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) according to Laemmli under reducing (+ β-mercaptoethanol, β-ME) conditions (Bio-Rad, Laboratories, Inc.). This method was conducted according to Wittmüss et al. (2024).

5.2.7 Binding behavior between the aggregates and phenolic compounds

The binding between WPI and the four PCs was investigated by using HPLC. Because of the insolubility of the protein after denaturation a measurement of the unbound PCs in the supernatant of aggregate preparation (**2.2.1**) was conducted. The HPLC analysis was conducted using an UltiMate 3000 system (Fisher scientific GmbH, Schwerte, Germany) with a diode array detector at a wavelength of 280 nm and a PLRP-S column (PLRP-S, 300 Å, 8 µm, 150 x 4.6 mm; Agilent Technologies, Santa Clara, USA) equipped with a guard column. The used method according to Ali et al. (2018) allowed a conclusion to be drawn as to how many of the PCs had bound to the protein and thereby remained in the sample.

5.2.8 Secondary structure of the protein

To investigate the secondary structure of the proteins before and after denaturation, ATR-Fourier transform infrared spectroscopy (FTIR) was performed using a CONFOCHECK TENSOR II system (Bruker Optics, Ettlingen, Germany) according to Wittmüss et al (2024). The spectra were corrected and vector-normalized using OPUS version 7.5 (Bruker Optics GmbH, Ettlingen, Germany). The second derivation of the amide I band (1590–1700 cm^{-1}) was calculated with nine smoothing points, using OPUS to determine the secondary structures of the protein samples. Moreover, we analyzed the normalized curves using Fourier self-deconvolution for quantitation. This entailed using a resolution enhancement factor of 3.0 cm^{-1} and a bandwidth of 10 cm^{-1} over a wavenumber range of 1700–1480 cm^{-1}. The resulting bands within the amide I range (1700–1590 cm^{-1}) were then integrated to calculate and quantify the structural components.

5.2.9 Statistics

All experiments were conducted in triplicates. The data were compiled and analyzed using GraphPad Prism (version 10.0.2, GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with $p < 0.05$.

5.3 Results

5.3.1 Lipid Oxidation

5.3.1.1 Hydroperoxides

To characterize the influence of GA, SA, CA and CGA on LOx in oil, primary LOxP (hydroperoxides) were measured over a period of eight days in a dispersion of WPI aggregates and stripped oil. Hydroperoxide formation increased to 95.7 mmol/kg oil in the control sample after eight days at 40 °C (Fig. 1). CGA and GA showed no inhibitory effect, while SA and CA reduced hydroperoxide levels in a concentration-dependent manner. Following a period of eight days, the quantity of hydroperoxides was significantly diminished by 31.3% through CA

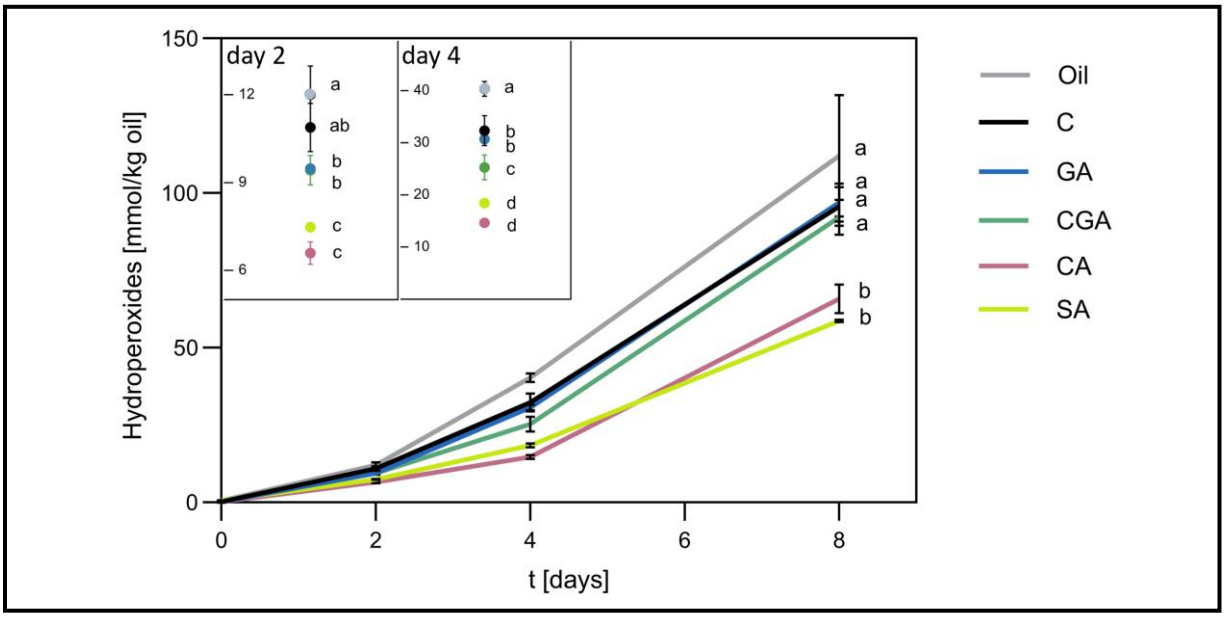

Figure 5.1: Formation of hydroperoxides [mmol/kg oil] in bulk oil (oil) and oleogels containing 6.8% protein aggregates, stored for 8 days at 40°C. 10 mM of gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) were added prior denaturation. C represents the control group containing 6.8% protein aggregates without addition of PCs. Results are given as the mean ± SD, n = 3. a-d are statistically significant differences ($p < 0.05$) between treatments within each storage time point.

and 38.6% through SA in comparison to the control group, upon the addition of 10 mM of the PC. This result indicated a significant effect of these PCs in reducing LOx. Pre-tests showed that the addition of WPI alone reduced LOx, i.e., hydroperoxide concentration, by 14.6% (Fig. 1). The addition of CA and SA significantly increased this effect. A difference between the control and PC added groups was already seen at day 2 of storage (Fig. 5.1).

5.3.1.2 Volatile lipid oxidation compounds

A significant increase in hexanal levels was observed in the stripped oil sample in comparison to the control sample, even after a storage period of 4 days (Fig. 2). The influence of PC on secondary LOxP was then examined, revealing that GA and CGA exhibited a profile analogous to that of the control group, thereby indicating that the addition of GA and CGA was not effective in reducing hexanal levels during the storage period of eight days. In contrast, the presence of SA and CA resulted in a substantial decline in hexanal levels as early as 2 days post-storage.

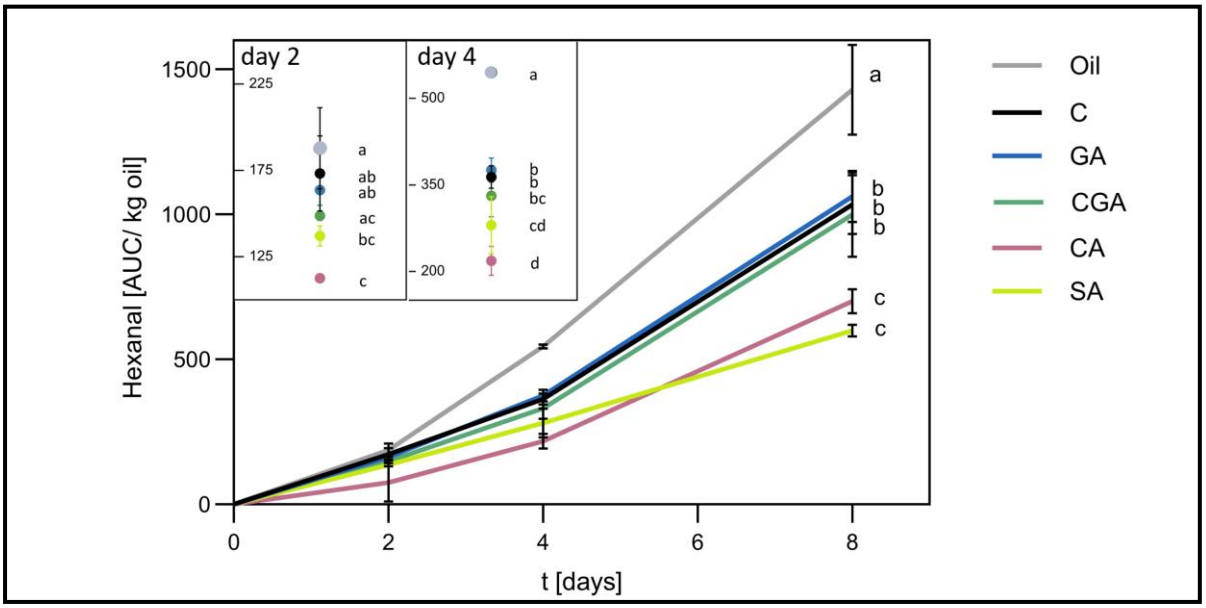

Figure 5.2: Formation of hexanal [AUC/kg oil] in bulk oil (oil) and oleogels containing 6.8% protein aggregates, stored for 8 days at 40°C. 10 mM of gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) were added prior denaturation. C represents the control group containing 6.8% protein aggregates without addition of PCs. Results are given as the mean ± SD, n = 3. a- d are statistically significant differences ($p < 0.05$) between treatments within each storage time point.

5.3.2 Yield of protein aggregates depending on the addition of PC

In order to separate WPI aggregates from the non-aggregated protein, a washing step was carried out during the sample preparation. This resulted in a reduction in protein concentration. In order to quantify the protein loss and to ascertain whether there was a dependence on the different PCs used, the protein content in the supernatant was determined using Lowry assay. The incorporation of all PCs, with the exception of GA, resulted in an elevated loss of protein compared to the control group (C). The most pronounced impact, and thus the lowest protein concentration in the aggregates, was observed by the addition of 10 mM CGA and SA, while CA exhibited only a marginally reduced effect.

Table 5.1: Yield of protein aggregates after the washing step depending on the PCs added.

	C	**CA**	**SA**	**GA**	**CGA**
[mg/mL]	85.6 ± 0.7^{a}	75.5 ± 1.7^{b}	69.8 ± 2.7^{c}	82.1 ± 2.2^{c}	66.8 ± 1.8^{c}

Shown is the remaining protein yield in WPI aggregates in % under the influence of 10 mM added gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) prior denaturation based on an original protein concentration of 60 mg/mL. C represents the control group containing 6.8% protein aggregates without addition of PCs. Results are given as the mean ± SD, n = 3. a- c are statistically significant differences ($p < 0.05$) between the treatment groups.

5.3.3 Binding phenolic compounds to WPI

After the formation of protein aggregates a washing step was conducted which may lead to the removal of PCs. To quantify the portion of PC remaining in the supernatant, the relative amount was determined by using HPLC and compared with the initial PC concentration. The difference between both concentrations was used to estimate the proportion of PCs that was no longer detected in the supernatant and may therefore have remained associated with the protein aggregates. At an initial PC concentration of 10 mM, the estimated proportions were 25% for CGA, 18% for GA, 14% for CA, and 0% for SA (Fig. 5.3). The apparent association behavior of SA differed from that of GA, CA, and CGA. While the proportion of GA, CA, and CGA potentially associated with the protein increased with increasing concentration, only a

minor proportion of SA appeared to remain associated with the protein, with the majority remaining in the supernatant irrespective of the amount added.

To evaluate the influence of homogenization on the binding behavior between PC and WPI, samples were subjected to Ultra-Turrax for 3 min at 13,000 rpm. It was found that this process resulted in a slight increase in the amount of PC bound to WPI without affecting the concentration-dependent patterns (Data not shown).

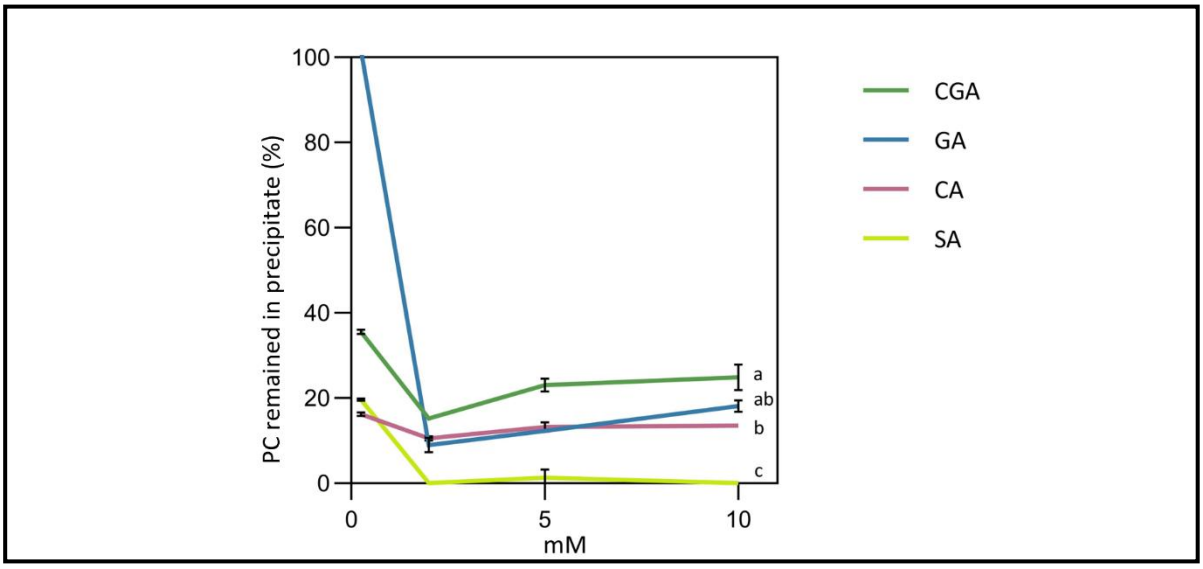

Figure 5.3: Proportion of remaining gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) in 6% whey protein isolate (WPI) in water after heating at 85°C for 20 min, expressed as a percentage of the initial concentration. Results are given as the mean ± SD, n=3. a- c are statistically significant differences ($p < 0.05$) between treatment groups at different concentrations within a timepoint.

5.3.4 Changes in protein composition depending on the addition of PC

Protein composition of the whey protein isolate (WPI) aggregates formed in the presence of different phenolic compounds—caffeic acid (CA), gallic acid (GA), chlorogenic acid (CGA), and sinapic acid (SA)—was first evaluated by SDS-PAGE (Suppl Fig 5.1). The analysis focused on the protein fraction incorporated into the aggregates. Distinct bands corresponding to bovine serum albumin (BSA) and β-LG were identified in all aggregate samples.

The β-LG band intensity was more pronounced in the control and CGA-treated samples compared to aggregates formed in the presence of SA, CA, and GA (Table 5.2). In contrast, aggregates formed in the presence of SA and CA appear to contain a lower proportion of α-lactalbumin (α-LA) visible as a distinct α-LA band in the SDS-PAGE profiles.

BSA band intensity differed between treatments and was significantly higher in aggregates formed with SA and CA compared to the control and CGA, indicating a phenolic-dependent redistribution of BSA into the aggregated fraction.

Table 5.2: SDS Gel electrophoresis of 6% whey protein isolate (WPI) aggregates with added gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) performed under reducing conditions (+ β-ME).

	BSA 72 kDa	**β-LG** 18 kDa	**α-LA** 14 kDa
C	0.005 ± 0.01^{c}	0.424 ± 0.04^{ab}	0.144 ± 0.08^{a}
GA	0.043 ± 0.01^{ac}	0.316 ± 0.02^{b}	0.090 ± 0.01^{bc}
CA	0.052 ± 0.02^{ab}	0.322 ± 0.04^{b}	0.080 ± 0.01^{c}
SA	0.058 ± 0.01^{a}	0.320 ± 0.04^{b}	0.075 ± 0.01^{c}
CGA	0.016 ± 0.02^{ac}	0.442 ± 0.06^{a}	0.133 ± 0.03^{ab}

C represents the control group containing 6.8% protein aggregates without addition of PCs. The proportions of bovine serum albumin (BSA), β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) are given as the relative band intensities normalized to the total intensity of the respective lane and are given as the mean ± SD, n = 3. Significant differences within a row are marked by different letters ($p < 0.05$).

To complement the SDS-analysis, the corresponding supernatants obtained during aggregate formation were analyzed by HPLC (Table 3). In these supernatants, only α-LA was detected and quantified, representing the protein fraction not incorporated into the aggregates. The α-LA concentration in the supernatant was slightly higher for the control and GA samples, indicating a lower degree of α-LA incorporation into the aggregates under these conditions. Conversely, lower α-LA levels in the supernatants of SA, CA, and CGA treatments suggest enhanced incorporation of α-LA into the aggregates in the presence of these phenolic compounds.

Table 5.3: Proportion of α-LA by performing High-performance liquid chromatography measurement (HPLC) of the washing residue of WPI aggregates under the influence of 10 mM added gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) after denaturation

	C	CA	SA	GA	CGA
[AUC]	259.7 ± 15.9 [a]	200.6 ± 5.2 [b]	167.8 ± 1.4 [b]	282.4 ± 14.1 [a]	179.1 ± 21.1 [b]

C represents the control group containing 6.8% protein aggregates without addition of PCs. Results are given as the mean ± SD, n = 3. a- c are statistically significant differences ($p < 0.05$).

5.3.5 Changes in secondary protein structure - FTIR

GA, CA, SA, and CGA induced only minor changes in the FT-IR spectra of WPI prior to denaturation (data unshown). GA increased the parallel β-sheet structure (1618 cm^{-1}) (Hülsebusch et al., 2026; Zou et al., 2013) while reducing the non-planar β-sheet component (1693 cm^{-1}) (Haris & Severcan, 1999; Hülsebusch et al., 2026), with similar but weaker effects observed for SA, CA, and CGA (Table 5.4). No concentration-dependent changes were detected. Additionally, SA, CA, and CGA increased α-helical content (1652 cm^{-1}) (Haris & Severcan, 1999; Timon R. Heyn et al., 2021). No peak shifts occurred, but changes in band intensities were evident upon PC addition.

Marked changes were found in the FTIR spectra after denaturation. All phenolic compounds influenced the secondary structure of WPI during heating. An increase in intermolecular β-sheet structures (1618 cm^{-1}) compared to the control was observed for all samples except those containing sinapic acid (SA). In contrast, the addition of SA resulted in a decrease in intermolecular parallel β-sheet structures. An increase in intramolecular β-sheet structures (1625–1630 cm^{-1}) (Haris & Severcan, 1999; Timon R. Heyn et al., 2021) was observed upon addition of chlorogenic acid (CGA), whereas no differences compared to the control were detected for SA and caffeic acid (CA).

With respect to the α-helix content (1660 cm^{-1}), no changes were observed for samples containing SA and CGA. In contrast, a slight increase in the proportion of α-helix structures was observed after addition of gallic acid (GA) and CA. An opposite trend was observed for intramolecular non-planar β-sheet structures (1690 cm^{-1}). The presence of SA led to an increase in this structural component, whereas GA, CA and CGA showed no differences

compared to the control. No effects of any of the four phenolic compounds on the turn structures were detected, even at the highest concentration applied.

Table 5.4: Proportion in secondary structural components in the WPI aggregate (C) due to the addition of gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) post denaturation (postD).

	Interm. β-sheet 1612-20 cm^{-1}	**Intram. β-sheet 1625-30 cm^{-1}**	**Intram. β-sheet 1627-36 cm^{-1}**	**α-helix 1655-59 cm^{-1}**	**non-planar β -sheet 1691 cm^{-1}**
	1614	**1626**	**1636**	**1651**	**1692**
C	15.1 ± 6.2^{b}	22.8 ± 5.3^{ab}	16.5 ± 1.8^{a}	24.2 ± 4.2^{a}	3.7 ± 0.4^{b}
	1621	**1628**	**1636**	**1650**	**1692**
GA	29.9 ± 6.4^{a}	27.3 ^{ab}	15.9 ± 1.7 ^{a}	30.5 ± 2.6^{a}	3.9 ± 1.5^{ab}
	1619	**1630**	**1636**	**1651**	**1694**
CA	23.0 ± 5.6^{ab}	18.3 ^{ab}	21.9 ± 4.7 ^{a}	31.3 ± 0.4^{a}	2.8 ± 0.5^{b}
	1617	**1628**	**1638**	**1652**	**1696**
SA	13.2 ± 0.8^{b}	17.2 ± 0.3^{b}	15.3 ± 1.6^{a}	24.3 ± 4.4^{a}	7.5 ± 1.7^{a}
	1618	**1629**		**1650**	**1694**
CGA	24.5 ± 4.2^{ab}	32.3 ± 1.5^{a}		24.6 ± 1.1^{a}	4.6 ± 1.6^{ab}

The proportions represent the area of each deconvoluted peak relative to the total integrated area of the amide I band (1700–1590 cm^{-1}) for each sample. Bold values indicate the wavenumber at which the maximum of the respective deconvoluted peak was observed. Results are given as the mean ± SD, n = 3. Significant differences within a row are marked by different letters ($p < 0.05$).

5.3.6 Interaction between WPI aggregates and hydroperoxides

In order to evaluate whether the observed reduction in hydroperoxides was attributable to a decrease in LOx, or rather to an interaction between LOxP and protein aggregates, an experiment was conducted. To this end, the quantity of hydroperoxides present in the oil that had previously undergone oxidation was measured 24 hours after the addition of WPI aggregates, with and without the presence of PC (Fig. 4). The levels of hydroperoxides remained largely unchanged across the various groups. As anticipated, an increase in hydroperoxide concentration was evident over the 24-hour period, attaining a concentration of approximately 50 mmol/kg oil. No significant differences were observed between the test groups.

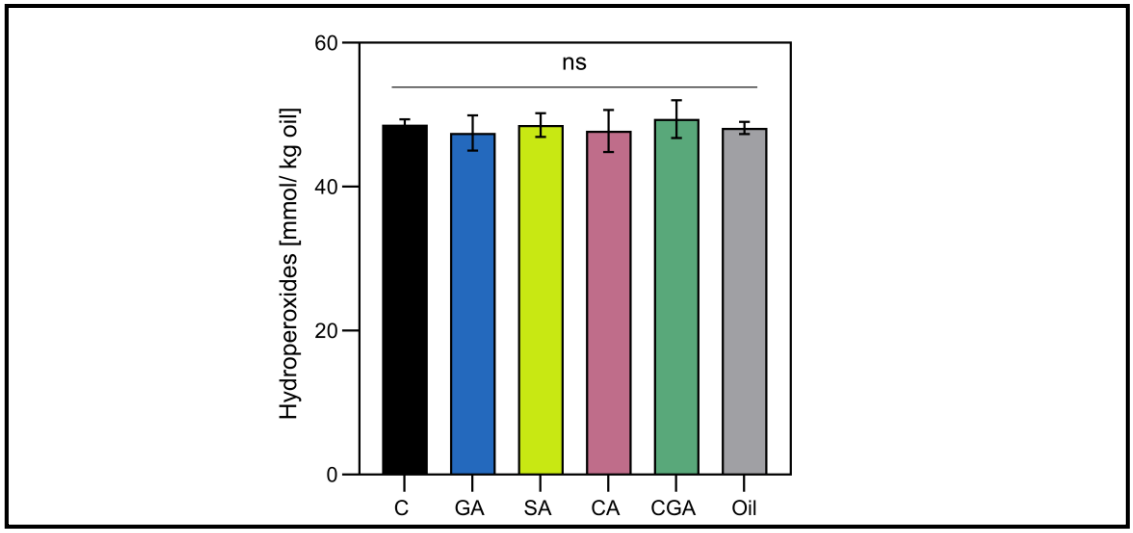

Figure 5.4: Amount of Hydroperoxides [mmol /kg oil] after mixing oxidized oil (8 days at 40°C) with 6.8% protein aggregates, treated with gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) after 24 h. C represents the control group containing 6.8% protein aggregates without addition of PCs. Results are given as the mean ± SD, n = 3. No significant differences within the time groups were observed.

5.4 Discussion

The objective of this study was to ascertain whether the incorporation of antioxidant PC into an oleogel would enhance the oxidative state within the system. The results obtained demonstrated that the addition of CA and SA during the formation of protein particles led to a considerable inhibition in LOxP in oleogels, whereas GA and CGA did not demonstrate any impact (Fig. 5.1 and 5.2).

5.4.1 Antioxidative effects occur independently of phenolic antioxidant potential

The antioxidant capacity of PCs depends on their structural configuration. In accordance with the extant literature, the observed effect of oxidation-reduction should have been in the order GA>CA>CGA>SA (Biskup et al., 2013; Cos et al., 2002; Olszowy et al., 2019; Sousa et al., 2004). This expectation is primarily related to the structural differences between the antioxidants (e.g. the number of hydroxyl groups on the aromatic ring) but does not consider interactions with the system in which they were investigated. Therefore, the reported order cannot be expected to apply directly to the present oleogel system. Unexpectedly, however, the order of antioxidant effects was found to be opposite to that reported.

Consequently, measurements were conducted to elucidate their binding behavior with WPI to identify concentration-dependent effects. The quantity of PC present in the supernatant was measured, enabling the calculation of the residual PC in the aggregates. In consideration of the washing phase, it is evident that only the PC that was bound to the aggregates could be effective in the oleogel.

The ratio of each PC and protein in the resulting aggregates was calculated by taking into account the differences in protein concentration caused by the washing step. No correlation could be observed between the amounts of PCs transferred to the final oleogel and the antioxidant effect observed during the storage period.

Given that the pH was maintained below 8 throughout the experiment, it is likely that the interactions between PC and protein are attributable to non-covalent bonds (Lund, 2021),

particularly hydrogen bonds between the PC hydroxyl and the protein carboxyl groups (Baba et al., 2021). Sun et al. (2022) have demonstrated that the binding affinity of phenolic acids to proteins depends on the number of hydroxyl groups. Qi et al. (2023) found, that the binding affinity of GA was weaker than that of CA. However, no significantly higher binding behavior could be observed for CA compared to GA in our study. In a study conducted by Riihimäki et al. (2008), it was demonstrated that there are approximately 2 non-covalent binding sites between native β-LG and CA/SA . Conversely, no binding sites could be identified between GA and β-LG (Schild et al., 2023). Nevertheless, we were able to demonstrate that a portion of the GA remained associated with the protein aggregates (Fig. 5.3), which indicates that GA was incorporated in WPI aggregates, possibly reflecting the use of aggregated rather than native β-LG. In contrast, the addition of SA did not result in the observed phenomenon, which is in contradiction to the results reported by Riihimäki et al. (2008). The authors found that the PCs are primarily associated with the external regions of the β-LG, rather than the central calyx (Riihimäki et al., 2008). This makes them highly susceptible to pH fluctuations. Consequently, it is plausible that the four PCs, which were tested, may exhibit varying sensitivities to pH 5.6, leading to disparate impacts on their binding affinities with β-LG.

Furthermore, the impact of heat application during the preparation of protein aggregates on the binding and subsequent antioxidant effect of PCs and protein aggregates warrants investigation. He et al. (2015) demonstrated that the non-covalent interaction between β-LG and epigallochatechin was enhanced due to the increased exposure of hydrophobic residues on the protein after unfolding by heat. It can be hypothesized that the previously rather weak interaction is enhanced by the effect of heat, as the hydrophobic regions on the protein surface are more exposed. While heat treatment of PCs itself has no significant reducing effect on Ferric-ion reducing antioxidant power or total phenolic content (Kılıç Bayraktar et al., 2019), numerous studies have shown that the antioxidant activity of PC-protein complexes decreases at temperatures above 85 °C (Kılıç Bayraktar et al., 2019; Qie et al., 2020). These findings suggest a stronger interaction between PCs and proteins, leading to a reduced accessibility of reactive groups in both PCs and proteins (Sun et al., 2022). Consequently, one

explanation for the diminished protection against LOx in the oleogel system by the addition of CGA could be the highest degree of interaction between protein and CGA. This in turn may lead to an incorporation of CGA in the WPI aggregates and a reduced accessibility of the antioxidative groups.

The present study demonstrated that while GA and CGA exhibited no antioxidant effect in the oleogel, SA and CA led to a substantial inhibition of LOx. This outcome was unexpected and could not be ascribed to the antioxidative capacity of the individual PCs, nor the binding behavior of SA and CA with protein, which was considerably lower in comparison to GA and CGA. (Fig. 3). Nevertheless, the observation of enhanced LOx supports the hypothesis that the PCs influence oxidation processes via mechanisms other than their antioxidant potential alone or the binding affinity to the protein.

To exclude that the effect is due to the binding of LOxP, WPI was incubated with bulk oil rich in hydroperoxides. It was found that the amount of hydroperoxides present in the oil is not affected by adsorption to the protein phase (Fig. 5.4). Consequently, it can be deduced that the protective factors present contribute to the reduced formation of oxidation products, though the mechanisms by which this occur require further clarification, e.g. by influencing the secondary protein structure and protein composition.

5.4.2 Changes in protein composition and structure by adding PC influences lipid oxidation

The alteration in the protein composition and secondary structure upon the addition of the PCs was subjected to more detailed analysis, with the aim of drawing inferences from these differences that could explain the development of an improved LOx process. PC not only modulate antioxidative environment but also affect aggregation of proteins, as well as their structural organization and thereby protein composition, significantly influencing oxidative stability in oleogels.

It can be assumed that the antioxidant capacity of the aggregates is strongly influenced by changes in protein composition induced by the addition of phenolic compounds (PCs). SDS-

PAGE and HPLC analyses revealed pronounced differences in the relative proportions of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA) between the treatments. SDS-Page revealed that aggregates from the control and CGA groups contained higher levels of β-LG (Table 5.2) indicating a lower proportion of α-LA in turn. Analysis of the supernatants obtained during aggregate formation via HPLC further revealed differences in α-LA incorporation. Higher α-LA concentrations were detected in the supernatants of the control and GA samples, suggesting a lower degree of α-LA incorporation into aggregates under these conditions. In contrast, aggregates formed in the presence of SA and CA appeared to retain more α-LA due to HPLC results. Numerous studies have demonstrated that α-LA possesses a remarkably high antioxidant capacity, which exceeds that of β-LG by far (Corrochano et al., 2019; Sadat et al., 2011). Given the relatively high antioxidant capacity reported for α-LA, its increased incorporation into the aggregate structure may contribute to the enhanced oxidative stability observed in these systems. However, aggregates formed with CGA also exhibited elevated α-LA levels without a corresponding reduction in lipid oxidation, indicating that protein composition alone cannot fully explain the observed antioxidant effects. The fact that SDS-Page revealed a lower α-LA content in SA and CA treated aggregates contradicts these findings. This discrepancy may be related to the different fractions analyzed: HPLC assessed the supernatant, whereas SDS-PAGE assessed the isolated aggregates. Differences in the sensitivity of both methods to protein fractions and aggregation states may further contribute to this discrepancy. Nevertheless, it is clear that the WPI aggregates supplemented with SA and CA are more similar in composition compared to those supplemented with CGA or the control.

Given that protein composition alone does not provide a sufficient nor entirely clear explanation for the improved oxidative stability in the oleogel systems supplemented with SA and CA, further investigations were conducted to determine the extent to which protein denaturation may have contributed to this effect. Protein denaturation and unfolding represents a key factor influencing antioxidant capacity. FTIR analysis demonstrated that the presence of PCs affected the secondary structure of WPI.

Intramolecular β-sheet structures (≈1630 cm^{-1}) are reported to decrease during protein denaturation, indicating a loss of native structure (Panick et al., 1999). Notably, the lowest intramolecular β-sheet values were observed in the SA and CA samples (**chapter 5.3.5**), suggesting a higher degree of protein denaturation induced by these phenolic compounds. Increased denaturation and unfolding likely result in greater exposure of antioxidant amino acid residues that were previously buried within the native protein structure, thereby enhancing the overall antioxidant capacity (R. J. Elias & Decker, 2010).

The increased incorporation of α-LA in SA and CA aggregates was not reflected by an increase in α-helical structures in the FTIR measurements. However, FTIR provides information on the overall secondary structure of the protein mixture and does not allow differentiation between individual protein species. Consequently, changes in protein composition may be masked by denaturation effects. The absence of an increase in α-helical content despite higher α-LA levels may therefore indicate partial unfolding of α-LA, further supporting the role of protein denaturation in enhancing antioxidant capacity.

The third major factor affecting antioxidant capacity is the extent of aggregation of the denatured proteins. While protein unfolding promotes the exposure of antioxidant amino acid residues, excessive aggregation may counteract this effect by re-burying reactive sites within dense protein networks (Feng et al., 2022).

Intermolecular β-sheet structures (≈1620 cm^{-1}), which are commonly associated with strong protein–protein interactions and irreversible aggregation (Andreas Barth, 2007; T. R. Heyn et al., 2022; Shivu et al., 2013), were most pronounced in aggregates formed in the presence of gallic acid (**5.3.5**). In contrast, significantly lower levels were observed in the control and SA samples, while CA and CGA showed intermediate values. Strong intermolecular β-sheet formation is associated with reduced molecular flexibility and limited accessibility of reactive amino acid side chains (Ryan J. Elias et al., 2008), which may impair the functional antioxidant capacity of protein-based systems.

The reduced intermolecular β-sheet content observed in the SA and CA groups may therefore indicate weaker aggregation of the denatured proteins, favoring higher antioxidant activity. This interpretation is further supported by the increased protein loss observed during the washing step for aggregates formed in the presence of SA (**5.3.2**). Increased protein loss suggests weaker aggregation or less densely crosslinked structures, making proteins more susceptible to removal during washing. In this context, the lowest protein loss observed for CA indicates a balance between sufficient aggregation for structural stability and limited aggregation that preserves antioxidant functionality.

However, similarly low levels of intermolecular β-sheets were also observed in the control group, likely due to the higher protein concentration in this system, due to less protein loss during the washing step. This demonstrates that aggregation behavior alone is insufficient to explain the differences in lipid oxidation, and that antioxidant capacity results from the combined effects of protein composition, denaturation, and aggregation.

The behavior of CA is particularly notable, as aggregates formed in its presence showed both low protein loss and strong oxidative protection. This suggests that neither maximal protein retention nor minimal aggregation alone determines functional performance and can fully explain the observed differences in lipid oxidation, but rather an intermediate aggregate state that balances structural stability and molecular accessibility.

Therefore, the oxidative stability of the oleogels appears to depend on the combined effects of protein composition, structural organization, and aggregate stability. SA and CA, in particular, promote aggregates enriched in proteins with higher antioxidant potential while simultaneously limiting excessive intermolecular crosslinking and promoting structural reorganization.

Several limitations should be considered when interpreting these findings. Protein composition was assessed using complementary but indirect approaches, and discrepancies between SDS-PAGE and HPLC results, particularly for gallic and chlorogenic acids, highlight the complexity of the system. Furthermore, FTIR analysis provides only average structural

information and cannot resolve protein-specific conformational changes. Despite these limitations, the consistent trends observed across multiple analytical approaches support the conclusion that phenolic compounds modulate the protein matrix in a manner that contributes to the improved oxidative stability of WPI-based oleogels.

5.5 Conclusion

This study highlights the complex relationship between protein structure and LOx in oleogels (protein-in-oil dispersions). The findings demonstrate that individual PC, particularly SA and CA, significantly inhibit LOx by decreasing the formation of primary (hydroperoxides) and secondary LOxP (hexanal) by up to 40% compared to the control. By contrast, GA and CGA did not exhibit similar antioxidative effects. Unexpectedly, the antioxidant effects could not be directly attributed to the binding interaction between PC and protein, as no consistent pattern were found from the binding analyses. Instead, the results suggest that alteration in protein composition induced by PCs may contribute to the observed effects. The addition of SA and CA appears to result in a comparable protein composition of the aggregates; however, neither the protein composition nor the degree of denaturation and aggregation alone seems to fully explain the observed phenomenon. We therefore propose an intermediate aggregate state that balances structural stability and molecular accessibility as a possible explanation. Nevertheless, further mechanistic research is required to deepen the understanding.

In conclusion, this study proposes a mechanism, whereby structural modulation of proteins by specific PCs may contribute to their observed effects on lipid oxidation. This may contribute to the development of more oxidation-stable protein-based oleogels for potential food applications.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used “DeepL Write” and “ChatGPT” in order to improve readability and language of the work. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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5.7 Supplementary Material

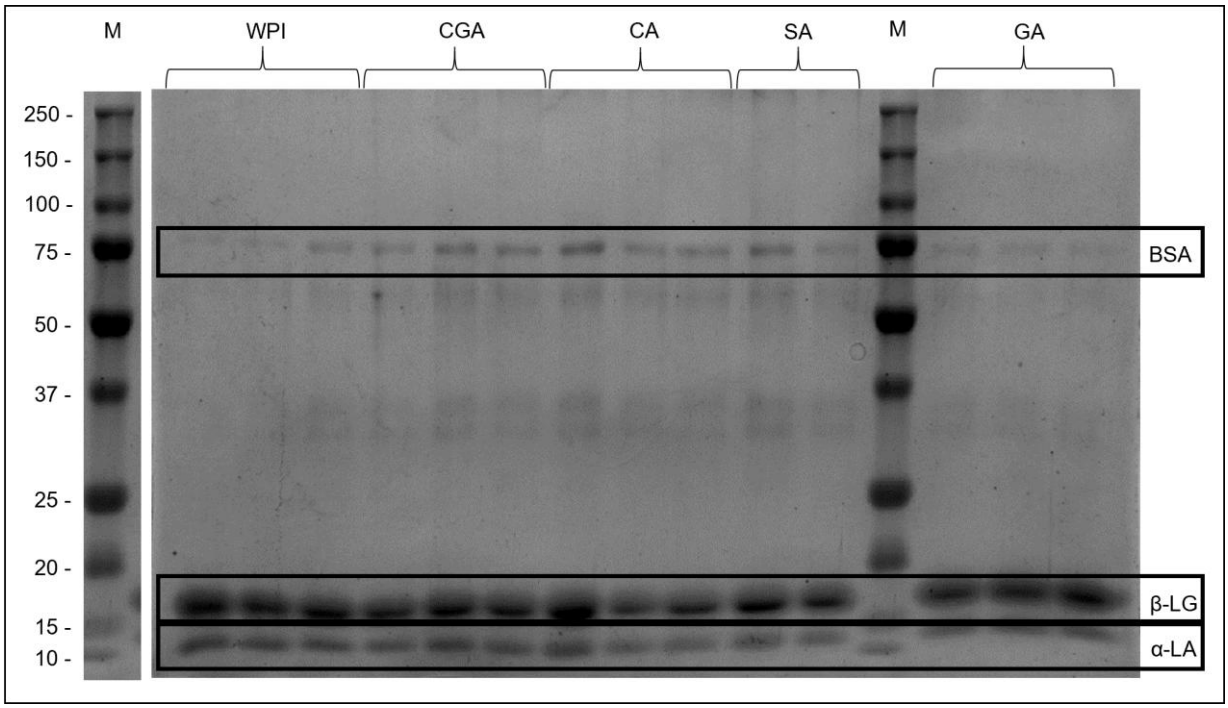


Figure S5.1: SDS Gel electrophoresis of 6% whey protein isolate (WPI) aggregates with added gallic acid (GA), sinapic acid (SA), caffeic acid (CA) and chlorogenic acid (CGA) performed under reducing conditions (+ β-ME). The lane labeled with M is the protein standard. The marked boxes are referring to bovine serum albumin (BSA), β-lactoglobulin (β-LG) and α-lactalbumin (α-LA).

6 Whey vs. Soy Protein: Phenolic compounds as modulators of lipid and protein oxidation in oleogels

Maria Wittmüss, Karin Schwarz*, Jonas Amft, Timon R. Heyn

Institute of Human Nutrition and Food Science, Division of Food Technology, Heinrich-Hecht Platz 10, D-24118 Kiel, Kiel University, Germany.

* Corresponding author (kschwarz-2@foodtech.uni-kiel.de)

Manuscript

Highlights

- Formation of lipid oxidation products can be inhibited by addition of CA and SA in soy protein-based Oleogels.
- SA suppressed protein carbonyl formation in WPI-based, but not in SPI-based oleogels.
- The addition of water in protein based oleogels system increases the effect of PCs significantly.

Abstract

Oxidative stability remains a major challenge for oleogels due to their high content of unsaturated fatty acids, which limits their application in food products. Previous studies have shown that incorporation of phenolic compounds (PCs) into whey protein isolate (WPI)-based oleogels reduces the formation of lipid oxidation products, such as hydroperoxides and hexanal. In this study, we investigated whether these antioxidative effects can be transferred to plant protein-based oleogels. Specifically, the influence of caffeic and sinapic acids on the oxidative stability of soy protein-based oleogels was compared to that in WPI-based systems to gain insight into the underlying mechanisms. The results showed that both phenolic compounds effectively inhibited lipid oxidation, whereas protein oxidation remained largely unaffected. These changes were also reflected in protein solubility, which was influenced by PC addition. Overall, the findings indicate that the antioxidative benefits of phenolic compounds observed in WPI oleogels can be transferred to plant-based oleogels. Nevertheless, the mechanisms and structural interactions involved are complex, highlighting the need for further investigation.

6.1 Introduction

Oleogels have emerged as promising systems for structuring liquid oils into semi-solid materials without compromising the nutritional quality of their fatty acid profile. Unlike traditional solid fats, oleogels can deliver high levels of unsaturated fatty acids while maintaining desirable textural properties (Scholten, 2019). One strategy for structuring oils involves the use of proteins as oleogelators. Although proteins are inherently hydrophilic and not intuitively suited for oleogelation, it has been shown that, upon appropriate modification and aggregation, they can exhibit effective oil-binding capabilities (Vries, 2017).

This approach is not limited to animal-derived proteins such as whey protein isolate (WPI); plant-based proteins like soy, rapeseed and sunflower protein can also form aggregates that significantly enhance oil-binding capacity, achieving levels comparable to WPI-based systems (Wittmüss et al., 2024). However, a major challenge associated with these systems is their susceptibility to oxidation due to the high content of unsaturated fatty acids. In addition to LOx, protein oxidation and co-oxidation processes can occur, further compromising the stability and functionality of the oleogel (Meissner et al., 2020b).

Proteins themselves can exert some antioxidant activity through various mechanisms, including radical scavenging and metal ion chelation (Ryan J. Elias et al., 2008), but these effects are often insufficient to fully prevent oxidative degradation. Therefore, the incorporation of phenolic compounds (PCs) as natural antioxidants has attracted increasing interest. The efficacy of PCs in such complex matrices is highly dependent on their interactions with both proteins and lipids (Kieserling et al., 2024). While the underlying mechanisms remain largely unclear, previous studies using WPI-based oleogels have demonstrated that the addition of selected phenolic compounds can significantly reduce LOx (**chapter 5**).

The central aim of this study is to investigate whether these protective effects observed in WPI-based systems can be translated to oleogels based on soy protein as a more sustainable,

plant-derived alternative. Additionally, the study seeks to explore whether phenolic compounds can also influence protein oxidation within the oleogel matrix.

6.2 Material and Methods

6.2.1 Material

WPI was obtained from Davisco Foods International with 97.7% protein and 75 % β-lactoglobulin in dry matter. Soy Protein Isolate was kindly provided by Eurosoy (Hamburg, Germany). Fully refined sunflower and rapeseed oil were purchased in a local supermarket. Ultra-pure water (MilliQ) was used for all applied solutions (<18.2 MΩ cm-1). All used chemicals were of analytical grade: aluminum oxide, ammonium thiocyanate, barium chloride, guanidine hydrochloride, hexane, hydrochloric acid (HCl), iron (II) sulfate, ortho-phosphoric acid, sinapic acid and trichloroacetic acid (TCA) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Caffeic acid, Dinitrophenylhydrazine (DNPH) and Trypsin from bovine pancreas was purchased from Merck KGA (Darmstadt, Germany). Isopropanol, cyclohexane, Ethanol (98%) and Ethanol acetate were procured from VWR International GmbH (Darmstadt, Germany).

6.2.2 Sample preparation

6.2.2.1 Preparation of protein aggregates

Sample preparation, preparation of the protein-in-oil dispersion and purification of the oil were carried out as previously described in manuscript 2Protein isolates were first dissolved in Milli-Q water at a concentration of 6 wt%, with the pH adjusted to 5.6. The dispersions were then subjected to thermal treatment by incubation in a water bath at 85 °C for 20 minutes to induce protein aggregation. Whey protein isolate (WPI) and soy protein isolate (SPI) served as the protein sources in this study. As phenolic compounds, sinapic acid (SA) and caffeic acid (CA) were incorporated into selected WPI and SPI dispersions at a final concentration of 10 mM during the heating step. After aggregation, the protein suspensions underwent a washing step with ultrapure water. The washed aggregates were then freeze-dried for 72 h to obtain dry protein materials suitable for further use.

For oleogel preparation, the dried aggregates were dispersed at 10 wt% into an oil phase composed of stripped sunflower oil and rapeseed oil (1:1 v/v). Milli-Q water (2.8 wt%) was added to facilitate dispersion of the aggregates. The mixtures were stirred for 10 minutes, followed by high-speed homogenization using a rotor-stator device (Ultra-Turrax) at 10,000 rpm for 30 seconds, performed twice, to achieve homogeneous dispersion of the protein aggregates within the oil matrix. The process was described more in detail in **chapter 4.2.2.4**

6.2.2.2 Storage experiment

The storage experiments was performed over a period of 22 days at 40°C. Therefore, Protein in oil dispersion were stored in a climatic chamber (Memmert GmbH + Co. KG, Schwabach, Deutschland) and analyzed after 0, 2, 4, 8, 15 and 22 days. Therefore, lipid phase was separated by centrifuging the sample at 20,000 x g for 10 min. The process was described more in detail in **chapter 4.2.2.5.**

6.2.2.3 Defatting

To eliminate oil from the protein phase, 500 mg of the previously separated protein-phase material was defatted using cyclohexane. The process was described more in detail in **chapter 4.2.2.6.**

6.2.2.4 Protein hydrolysis

Enzymatic hydrolysis of the defatted protein material was conducted following the procedure outlined by Deng et al. (2018), with slight modifications. The Process was described more in detail in **chapter 4.2.2.7.** Protein concentration in the hydrolysates was quantified using the Lowry method (1951) as previously described.

6.2.3 Lipid oxidation parameters

Primary (Hydroperoxides) – as well as secondary (Hexanal) LOx products were analyzed by using the ferric thiocyanate method according to Amft et al. (2019) as well as a HS-20 headspace sampler (Shimadzu, Kyoto, Japan) and a GC-2010 gas chromatograph (Shimadzu,

Kyoto, Japan) equipped with a DB-1701 column (60 m x 320 µm x 1 µm, Agilent Technologies, Santa Clara, CA, USA) . Both methods were previously described in **chapter 4.2.3**.

6.2.4 Protein oxidation parameters

Protein oxidation was quantified by measuring protein-bound carbonyl groups after derivatization with 2,4-dinitrophenylhydrazine (DNPH) using a spectrophotometric assay at 370 nm. The procedure was described more in detail in **chapter 4.3.2**.

6.2.5 Statistics

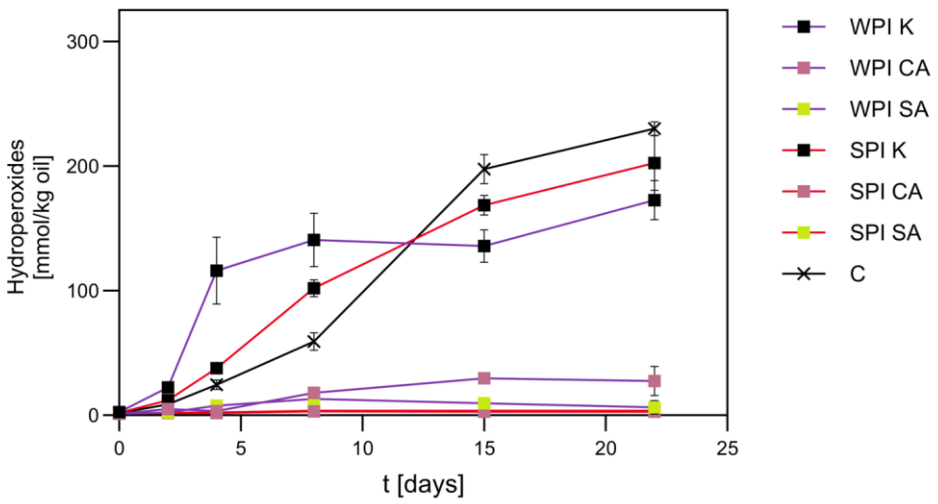
All experiments were at least conducted in triplicates. The data were compiled and analyzed using GraphPad Prism (version 10.0.2, GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was performed using Tukey's multiple comparison test with $p < 0.05$.

6.3 Results

6.3.1 Lipid Oxidation

6.3.1.1 Hydroperoxides

LOx of protein-in-oil dispersions was assessed by tracking the formation of hydroperoxides in the oil phase over a 22-day storage period. In the control sample containing bulk oil (C), a continuous rise in hydroperoxide content was observed, with a strong increase after 8 days and a final concentration of 260 mmol/kg oil after 22 days (Fig. 6.1). The incorporation of



t [days]	WPI K	WPI CA	WPI SA	SPI K	SPI CA	SPI SA	C
0	$2.50^{c;A}$	$1.26^{b;C}$	$1.30^{a;C}$	$2.09^{e;AB}$	$1.51^{c;C}$	$1.35^{c;C}$	$1.83^{e;BC}$
2	$22.42^{c;A}$	$4.93^{b;D}$	$2.42^{a;E}$	$12.11^{de;B}$	$1.82^{b;E}$	$1.95^{b;E}$	$8.88^{de;C}$
4	$116.10^{b;A}$	$3.44^{b;C}$	$7.66^{a;C}$	$37.84^{d;B}$	$1.89^{b;C}$	$2.29^{b;C}$	$24.39^{d;BC}$
8	$140.67^{ab;A}$	$12.08^{ab;D}$	$13.17^{a;D}$	$101.97^{c;B}$	$3.02^{a;D}$	$3.55^{a;D}$	$59.20^{c;C}$
15	$135.94^{ab;C}$	$29.72^{a;D}$	$11.87^{a;DE}$	$168.46^{b;B}$	$2.82^{ab;E}$	$3.70^{a;E}$	$197.49^{b;A}$
22	$172.68^{a;B}$	$27.47^{a;C}$	$9.54^{a;C}$	$202.48^{a;AB}$	$2.86^{ab;C}$	$3.54^{a.C}$	$260.36^{a;A}$

Figure 6.1: Formation of hydroperoxides [mmol/Kg oil] in bulk oil (C) and oleogels containing 10% whey protein aggregates (WPI) or soy protein aggregates (SPI) with 2.8% added water. Oleogels are prepared without (K) or with 10 mM of sinapic acid (SA) or caffeic acid (CA) added prior denaturation and stored for 22 days at 40°C. Results are given as the mean ± SD, n = 3. a-d are statistically significant differences ($p<0.05$) within a row, A-D are statistically significant differences within a timepoint over the different groups.

protein notably changed hydroperoxide formation in oleogel systems over the storage period. WPI based oleogels were showing a much stronger propagation phase (day 0-2) compared to SPI K and C, whereas a plateau forms from day 8 onwards, and the hydroperoxide level in the WPI oleogels no longer rises rapidly. As of day 12, the C and SPI K groups have surpassed the WPI oleogel with regard to hydroperoxide formation. On day 22, WPI K shows a significantly lower amount of hydroperoxides formed than in the C group. In samples containing soy protein, a similar trend of reduced oxidation was observed when structured as oleogels, in comparison to the bulk oil, however this change was not significant. Interestingly, the oxidation behavior of the WPI system differed from that of soy: it exhibited a sharp increase in hydroperoxides during the initial 8 days, followed by a plateau at around 150 mmol/kg oil.

The addition of chlorogenic acid (CA) and sinapic acid (SA) significantly reduced hydroperoxide formation in WPI-, as well as in SPI-based Oleogels. Although an increase was still observed over time, the levels remained significantly lower compared to the WPI K. When SA or CA were added to SPI the same course was found. No statistically significant difference was found between the antioxidant effects of CA and SA neither in WPI nor in SPI systems.

6.3.1.2 influence of water on hydroperoxide formation

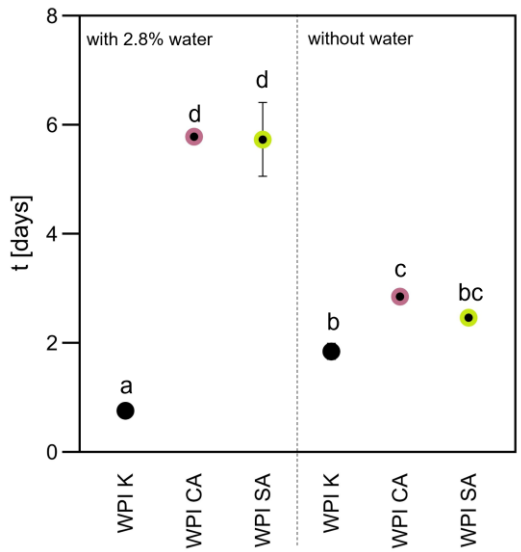
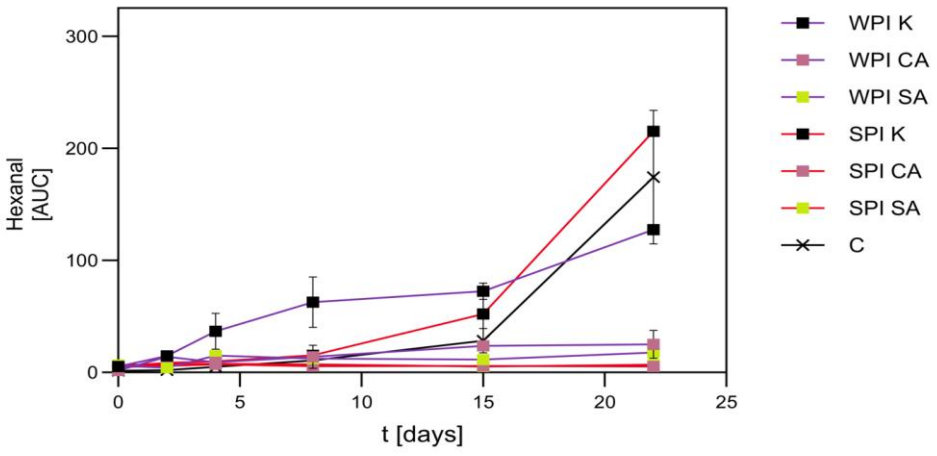


Figure 6.2: Timespan of Oleogels to reach 10 mmol/Kg oil hydroperoxide formation in whey (WPI) protein based oleogels without (K) or with 10 mM of sinapic acid (SA) or caffeic acid (CA) added prior denaturation. without or with 2.8% added water. Results are given as the mean ± SD, n = 3. a-d are statistically significant differences ($p<0.05$) .

In order to facilitate a more comprehensive evaluation of the influence of water on the antioxidant effect of proteins and phenolic compounds, the time at which oleogels, with and without the addition of water, reached a concentration of 10 mmol/kg oil hydroperoxides was observed. It was observed that WPI-based oleogel without any phenolic compounds added (WPI K) reached this hydroperoxide concentration after 1.8 days of storage. The addition of water shortened this duration to 0.75 days. In contrast, the addition of PCs extended the time required to reach this hydroperoxide level from 2.9 or 2.5 days (for CA and SA, respectively, without water) to 5.75 days when water was present.

6.3.1.3 volatile lipid oxidation compounds



t [days]	WPI K	WPI CA	WPI SA	SPI K	SPI CA	SPI SA	C
0	$5.18^{c;AB}$	$1.75^{a;AB}$	$5.72b^{c;AB}$	$5.60^{c;AB}$	$4.72^{a;AB}$	$6.63^{a;A}$	$1.48^{b;B}$
2	$14.63^{c;A}$	$14.01^{a;A}$	$4.27^{c;B}$	$8.43^{c;AB}$	$5.70^{a;B}$	$7.28^{a;Ab}$	$1.90^{b;B}$
4	$36.66^{bc;A}$	$9.12^{a;B}$	$14.90^{a;B}$	$9.89^{c;B}$	$7.06^{a;B}$	$7.59^{a;B}$	$4.99^{b;B}$
8	$62.83^{b;A}$	$13.91^{a;B}$	$12.31^{ab;B}$	$15.23^{c;B}$	$5.57^{a;B}$	$7.10^{a;B}$	$10.74^{b;B}$
15	$72.54^{b;A}$	$23.56^{a;B}$	$11.44^{ac;B}$	$52.20^{b;AB}$	$5.95^{a;B}$	$5.35^{a;B}$	$28.22^{b;B}$
22	$130.38^{a;B}$	$24.93^{a;C}$	$17.79^{a;C}$	$215.17^{a;A}$	$5.18^{a;C}$	$7.04^{a;C}$	$174.41^{a;AB}$

Figure 6.3: Formation of hexanal [AUC] in bulk oil (Oil) and oleogels containing 10% whey protein aggregates (WPI) or soy protein aggregates (SPI) with 2.8% added water, without (K) or with 10 mM of sinapic acid (SA) or caffeic acid (CA) added prior denaturation were stored for 22 days at 40°C. Results are given as the mean ± SD, n = 3. a-f are statistically significant differences ($p<0.05$) within a row, A-F are statistically significant differences within a timepoint over the different groups.

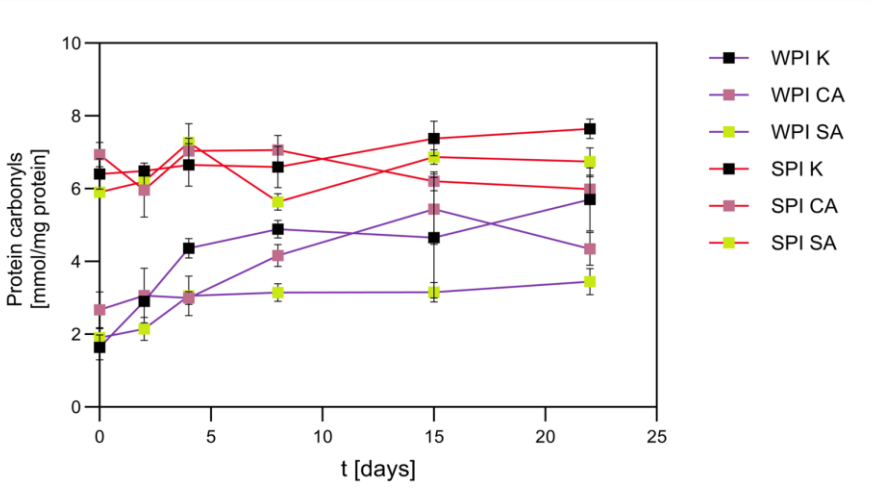
To assess secondary LOx, hexanal was quantified using headspace gas chromatography (HS-GC) (Fig. 6.3). In the control group containing bulk oil (C), hexanal levels showed an exponential rise over the 22-day storage period, culminating at 174 AUC on day 22. The whey protein isolate (WPI) Oleogels exhibited a pronounced increase in hexanal within the first 8 days, after which levels reached a plateau. By day 22, hexanal concentration in this system reached 130 AUC. In comparison, the SPI system demonstrated a slightly slower initiation of hexanal formation than WPI, but showed a strong increase over time, with values climbing to 215 AUC by the end of the storage period.

The incorporation of the phenolic compounds sinapic acid (SA) and chlorogenic acid (CA) into WPI aggregates effectively suppressed hexanal formation, with levels remaining stable at approximately 20 AUC—comparable to the WPI K group at day 2. In contrast, soy dispersions containing SA or CA showed minimal hexanal formation throughout the storage period, not exceeding 5 AUC.

6.3.2 Protein oxidation

Protein carbonyl formation was used as a marker to assess the progression of protein oxidation during a 22-day storage period (Fig. 6.4). Similar to the trends observed for lipid oxidation markers (Figs. 6.1 and 6.3), a substantial increase in protein carbonyl content was detected in the WPI K sample, reaching up to 6.0 mmol/mg protein by day 22.

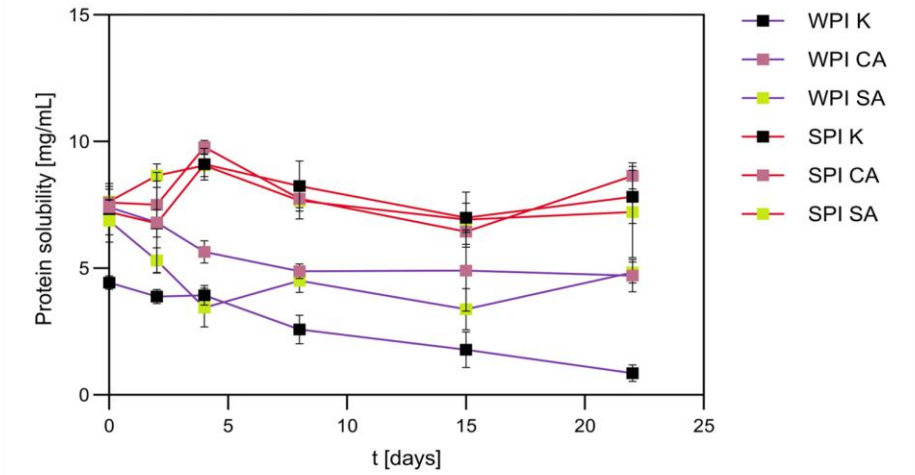
However, the addition of SA significantly inhibited this increase. In SPI sample, no clear increase in protein carbonyls was observed over the storage period. Interestingly, the initial carbonyl level in this sample was already high at day 0 (6.58 mmol/mg protein), exceeding the final value measured in WPI K at day 22. This high initial level could not be reduced by the addition of either CA or SA.



t [days]	WPI K	WPI CA	WPI SA	SPI K	SPI CA	SPI SA
0	$1.69^{c;B}$	$2.67^{b;B}$	$0.97^{ab;B}$	$6.58^{b;A}$	$6.59^{a;A}$	$3.92^{bc;A}$
2	$2.97^{bc;B}$	$1.39^{b;B}$	$2.99^{b;B}$	$6.71^{ab;A}$	$6.14^{a;A}$	$6.19^{bc;A}$
4	$4.46^{ab;B}$	$2.99^{b;C}$	$3.53^{ab;C}$	$6.93^{ab;A}$	$7.41^{a;A}$	$7.27^{a;A}$
8	$5.06^{ab;CD}$	$4.16^{ab;D}$	$2.98^{ab;E}$	$6.83^{ab;AB}$	$6.82^{a;A}$	$5.63^{c;BC}$
15	$4.85^{ab;BC}$	$5.43^{a;AC}$	$2.84^{ab;C}$	$7.65^{ab;A}$	$6.21^{a;AB}$	$6.87^{ab;AB}$
22	$6.00^{a;B}$	$4.35^{ab;C}$	$3.45^{a;C}$	$7.85^{a;A}$	$5.99^{a;B}$	$6.93^{ab;AB}$

Figure 6.4: Formation of Protein carbonyls [mmol/mg protein] in the protein phase of 10% whey protein aggregates (WPI) or soy protein aggregates (SPI) in oil dispersion. Samples with 2.8% added water. without (K) or with 10 mM of sinapic acid (SA) or caffeic acid (CA) added prior denaturation were stored over 22 days at 40 °C Results are given as the mean ± SD, n = 3. a-f are statistically significant differences ($p<0.05$) within a row, A-F are statistically significant differences within a timepoint over the different groups.

6.3.3 Protein solubility following enzymatic hydrolysis



t [days]	WPI K	WPI CA	WPI SA	SPI K	SPI CA	SPI SA
0	$4.43^{a;B}$	$7.40^{a;A}$	$6.87^{a;A}$	$7.22^{ab;A}$	$7.59^{bc;A}$	$7.63^{a;A}$
2	$3.88^{ab;C}$	$6.81^{a;AC}$	$5.30^{ab;BC}$	$6.76^{b;AC}$	$7.50^{bc;AB}$	$8.65^{a;A}$
4	$3.93^{a;C}$	$5.64^{a;B}$	$3.44^{b;C}$	$9.10^{ab;A}$	$9.78^{a;A}$	$9.06^{a;A}$
8	$2.58^{bc;C}$	$4.88^{a;B}$	$4.51^{b;BC}$	$8.25^{ab;A}$	$7.74^{bc;A}$	$7.67^{a;A}$
15	$1.78^{cd;C}$	$4.90^{a;AB}$	$3.38^{b;BC}$	$6.99^{ab;A}$	$6.45^{c;A}$	$6.92^{a;A}$
22	$0.85^{d;C}$	$4.71^{a;B}$	$4.83^{ab;BC}$	$7.82^{ab;A}$	$8.64^{ab;A}$	$7.21^{a;AB}$

Figure 6.5: Solubility of protein aggregates [mg/mL] in Oleogels after hydrolyzation with trypsin. Whey protein isolate (WPI) and soy protein isolate (SPI) 10% in oil dispersion with 2.8% added water, without (K) or with 10 mM of sinapic acid (SA) or caffeic acid (CA) added prior denaturation were stored for 22 days at 40°C. Results are given as the mean ± SD, n = 3. a-d are statistically significant differences ($p<0.05$) within a row, A-C are statistically significant differences within a timepoint over the different groups.

Solubility of protein aggregates isolated from stored oleogels were measured post-storage in order to observe the disparities between protein source and the influence of SA and CA (Fig. 6.5). It is interesting to note that no alterations in protein solubility were detected in the SPI experiment, neither in the context of storage nor in response to the incorporation of CA and SA. In contrast, the solubility of WPI exhibits a substantial decline over time, with CA and SA proving to be a highly effective inhibitor of this decline. At an initial concentration of 10 mg/mL, SPI solubility was approximately 70%, irrespective of the presence of SA or CA. For

WPI, solubility was already significantly lower on day zero at approximately 45%, and decreased to approximately 10% over the storage period. The incorporation of CA and SA resulted in a final WPI solubility of approximately 47% on day 22, thereby stabilizing solubility at the level of the initial situation on day 0.

6.4 Discussion

Oleogels, particularly those based on proteins, have attracted increasing interest due to their potential in health-related, technological, and ecological aspects. However, their high content of unsaturated fatty acids makes them highly susceptible to oxidative degradation, underlining the need to understand oxidation mechanisms and develop strategies to improve oxidative stability and shelf life. Previous studies have demonstrated that the incorporation of phenolic compounds such as sinapic acid (SA) and caffeic acid (CA) during the formation of whey protein isolate (WPI) aggregates can enhance oxidative stability in WPI-based oleogels. The present study investigates whether these antioxidative effects are transferable to oleogels based on more sustainable plant-derived proteins, such as soy protein isolate (SPI).

6.4.1 Influence of sample matrix composition on oxidative stability in protein based oleogels

The oxidative stability of oleogels is not solely determined by their chemical composition but is also strongly influenced by the structural characteristics of the sample matrix. In particular, the incorporation of water into the oleogel system introduces a complex set of physical and chemical changes that significantly affect oxidation behavior. In literature the formation of water-oil interfaces was often described to create new reactive sites and facilitates the incorporation of prooxidant species, such as transition metals, thereby accelerating LOx compared to bulk oil systems (Bao & Pignitter, 2023). Furthermore McClements et al. (2000) described, that by establishing new interphases, amphilic oxidation products are concentrated in this area and due to the fact, that LOx is an autocatalyzed reaction, strongly affects reaction rate an thereby accelerates LOx. This statement was confirmed by the results shown in Figure

6.2, whereby the addition of water in a WPI-based oleogel without PCs led to a significantly faster increase in hydroperoxides compared to the WPI K sample without water. This outcome aligns with the LOx phenomena documented in the extant literature, attributable to the incorporation of water into the matrix (Philipp M. Meissner et al., 2019).

Beyond the direct prooxidative effect of water, its presence alters the spatial distribution and structural organization of proteins within the matrix. It is not well studied yet, how protein structuring really occurs under the presence of water and how these may influence their antioxidant potential. Meissner et al. (2019) observed that the addition of approximately 3% water to oleogels resulted in the formation of water droplets. In contrast, lower amounts of added water were strongly bound to the freeze-dried protein and therefore did not lead to the formation of distinct water droplets. This observation was supported by measurements of water activity conducted by Meissner et al. (2019), which showed a marked increase in water activity for oleogels with around 3% added water compared to those with lower water content. The higher water activity indicated the presence of non-bound water, such as dispersed water droplets. Therefore, we hypothesize that the same phenomenon regarding the amount of added water also occurred in our study, as our samples contained a similar water content (2.8%) to that reported by Meissner et al. (2019). Since the formation of water droplets within the continuous oil phase resembles a water-in-oil emulsion, it was assumed that the distribution of proteins in the oleogel upon water addition follows a pattern comparable to that observed in emulsions. Proteins may localize at the newly formed interfaces of water droplets (Jorgensen et al., 2004), where they can exert antioxidative functions depending on their surface activity, molecular structure, and ability to form cohesive interfacial layers. The characteristics of these protein layers—such as thickness, continuity, and charge— may play a crucial role in modulating interfacial oxidation (Berton-Carabin et al., 2014).

The protein source and its physicochemical properties are therefore essential determinants of oxidative stability in such systems. Whey protein isolate (WPI) is known for its high solubility and globular structure, allowing for better dispersion and more efficient interfacial

adsorption. In contrast, soy protein isolate (SPI), primarily composed of glycinin and β-conglycinin, typically exhibits lower solubility and a higher degree of structural unfolding due to processing (Abirached et al., 2012). This may result in less efficient aggregation and weaker interfacial organization, potentially reducing its capacity to stabilize the system oxidatively. This is in line with the results shown in Figure 6.1-2, representing a significantly lowered formation of primary and secondary LOx products by WPI compared to SPI. Additionally, unbound proteins dispersed within the oil phase may contribute to radical scavenging, but their efficacy is likewise dependent on the structural integrity and molecular mobility of the protein species involved. When considering the solubility data obtained in this study, it became evident that protein aggregates in SPI exhibited a markedly higher solubility compared to those in WPI. These findings are consistent with the explanation that WPI, due to its higher solubility in the native state (prior to aggregation), forms more stable aggregates. While these aggregates display stronger antioxidant activity, they are also less soluble than those formed from SPI.

Interestingly, the solubility of WPI aggregates decreased drastically over storage time, whereas that of SPI aggregates remained unchanged. This phenomenon can be linked to protein oxidation. Previous studies have demonstrated that protein solubility is strongly influenced by the oxidation status, with increased oxidation leading to a significant reduction in solubility (BOATRIGHT & HEITlARACHCHY, 1995; Estrada et al., 2018; Hülsebusch et al., 2025; Shen et al., 2023). Accordingly, the progressive decrease in solubility observed for WPI-K oleogels can be explained by the advancing protein oxidation during storage (Fig. 6.4). In contrast, no increase in protein oxidation was detected in SPI-K oleogels, which is in line with the unchanged solubility observed (Fig. 6.5).

The influence of the phenolic compounds (PCs) further supports this interpretation. Both CA and SA were able to slow down protein oxidation over time in WPI oleogels, whereas no such effect was evident in the SPI systems (Fig. 6.4). A similar trend was observed for protein solubility. Taken together, these results indicate a clear relationship between protein solubility and the extent of protein oxidation during storage.

An increase in protein oxidation was observed in WPI-based oleogels, in contrast to SPI-based oleogels, which were showing a high degree in protein oxidation since day 0 with no further increase observed (Fig. 6.3). The absence of any increase in SPI may be explained by extensive oxidation already occurring during protein isolation, due to harsh processing conditions such as high temperature, exposure to solvents, pH shifts, and mechanical forces. Several studies have confirmed that thermal and mechanical processing can induce protein oxidation (N. Chen et al., 2013; Duque-Estrada et al., 2019; Duque-Estrada et al., 2020). which may also apply to the preparation of protein aggregates. The elevated oxidation prior to storage could explain the lack of further oxidation during storage, as the process had already advanced.

Protein oxidation should also be considered in the context of its impact on the antioxidative capacity of proteins, which can, in turn, influence LOx. Liu et al. (2016) demonstrated that excessive alkali treatment during rice protein extraction impaired antioxidative activity, indicating that the degree of modification is critical. This is consistent with the present findings, as SPI, which exhibited higher protein oxidation, also promoted higher LOx rates compared with WPI-based oleogels, which showed lower degrees of protein oxidation.

Taken together, these findings underscore the importance of matrix design in protein-based oleogels. Water content, protein source, and structural characteristics must be considered collectively, as they jointly define the system's oxidative response.

6.4.2 Influence on phenolic compounds (PCs) on oxidative stability in protein based Oleogels

The structural characteristics of the sample matrix appear to play a decisive role in the interaction behavior with phenolic compounds (PCs) too. In the present study, the addition of CA and SA resulted in a significant reduction in the formation of LOx products in both cases (WPI and SPI), which indicates an increased antioxidant effect. It is therefore hypothesized that a bond between protein and PC has been established, resulting in enhanced antioxidant capacity within the system. The enhanced effect of the addition of the two phenolic

components in soy may be attributable to both the improved interaction and the distribution of the PCs, which are a consequence of the divergent protein structures.

The antioxidant potential of CA and SA is well studied, with CA showing higher potential compared to SA due to their chemical structure (Cos et al., 2002; Sousa et al., 2004). It is described in literature, that in emulsion systems the ability of effectively inhibiting the formation of hydroperoxides of phenolic compound was in a different order compared to bulked oil. Based on the fact, that specific interactions between components, such as proteins or other emulsifier, may play an important role in antioxidant activity, by having an effect on partitioning of the antioxidant (Pekkarinen et al., 1999).

Interactions between PCs and proteins are expected (Budryn & Rachwal-Rosiak, 2013) . These may contribute to an improved antioxidative capacity of protein-PC conjugates (Y. Li et al., 2024). In the present study, the exact mechanism remains unclear, particularly regarding the binding mode between PCs and proteins. Still, it seems that interaction between proteins and PCs are primarily non-covalent, since the conditions required for a covalent bond between the components, such as alkaline pH, enzymes or ultrasound are not present. However, it can be hypothesized that these interactions influence the antioxidant capacity of the individual components. For both proteins, interactions with PCs likely occur primarily via hydrophobic interactions and hydrogen bonding, due to their mainly non-covalent interaction (Mao et al., 2023). This capacity is also contingent on the structure of the proteins, and consequently the availability of potential binding sites. Heat treatment is generally reported to strengthen protein–PC interactions due to better binding affinity based on protein unfolding (Song et al., 2023). However, such binding can also reduce the antioxidant efficiency of PCs if critical active sites become blocked. Differences in the antioxidant effect were observed between WPI- and SPI-based oleogels. In WPI, stronger binding of PCs may have reduced their antioxidant activity, which could explain the weaker protective effect compared with SPI (Fig. 6.1 & 6.2). Li et al. (2024) described that SA at higher concentrations exhibited a significantly lower binding rate to SPI at a pH of 7 in comparison to WPI. In light of the fact that the present study revealed a higher concentration than was the case in Li´s study, the assumption of lower

binding between SA and SPI than with WPI may be the case. Furthermore, disparities in binding rates may be attributable to variations in protein structures arising from the protein source and alterations during prior processing. Since no binding between these components was measured in the present study, no precise statement about binding behavior can be made. Upon addition of water, it remains unclear whether proteins carrying bound PCs are preferentially located at the interface or exist as unbound aggregates in the oil phase. Such structural arrangements could influence the overall effectiveness of the PCs, suggesting that the spatial distribution of protein–PC complexes is an important factor in their antioxidant activity (Schwarz et al., 2000). Regarding the results in Figure 6.2 the addition of water significantly increases the effect of PCs to inhibit LOx in protein based oleogels. This could not be attributed to better binding, since the water was added a few steps later and therefore had no effect on initial binding activity. Still the effect could be attributed to some changes in PC distribution and probably location of PCs, being more focused on the interfaces and therefore are more effective by being able to act directly at the scene of the action. Studies have shown that membrane-inserted or interfacially active phenolic compounds are more effective at inhibiting LOx than those that remain in the bulk oil or water phases, highlighting the importance of their physical distribution and interaction with lipid membranes or emulsion droplets (Costa et al., 2021; Socrier et al., 2019). How PCs arrange themselves in a water-containing system strongly depends on their molecular structure, particularly on whether they are more hydrophilic or hydrophobic. In our case, it must also be considered that the PCs interacted with the proteins, which ultimately determines their localization. Since proteins are amphiphilic, they preferentially migrate to the oil–water interface, and the bound PCs are carried along. For caffeic acid, which is rather hydrophilic, one would not expect an accumulation at the interface without protein binding. Through its interaction with proteins, however, it is transported to the interface, where it can unfold its antioxidant effect most efficiently.

In terms of protein oxidation, variations in the antioxidant efficacy of WPI added with CA and SA highlight that distinct phenolic compounds exert different effects through their specific

interactions with proteins. The fact that CA contributes to a significantly smaller improvement in protein oxidation compared to SA, while no differences in the effectiveness of the two phenolic compounds were observed in soy, can likely be attributed to the binding affinity between the proteins and the phenolic compounds. Since the binding interactions were not directly investigated in this study, this explanation remains speculative and is based on literature reports. It has been shown that WPI exhibits a stronger non-covalent binding to CA than to SA (**chapter 5.3.3**). This could explain why SA, with fewer antioxidant-active hydroxyl groups blocked by protein interactions, exerts a more pronounced protective effect against protein oxidation in WPI.

The literature provides only limited insight into protein oxidation in comparable systems, and the mechanisms remain largely unresolved. Nevertheless, some similarities between WPI and SPI behavior suggest that certain aspects may be transferable, although it remains unclear whether the same mechanisms are responsible in both systems.

6.5 Conclusion

This study shows that the antioxidative effects of phenolic compounds are not limited to whey protein systems but can also be observed in soy protein oleogels, supporting the hypothesis that effects previously identified in WPI-based gels are transferable to plant-based alternatives. These effects are, however, strongly matrix-dependent, with water playing a decisive role by altering protein structuring and thereby influencing the antioxidative activity of both proteins and PCs. In WPI-based systems, PCs not only inhibited LOx but also reduced protein oxidation, which in turn affected the solubility of protein aggregates after trypsin digestion. This may point to a potential improvement in protein digestibility when such oleogels are used in food-related contexts. Overall, the findings highlight that plant-based protein systems have considerable potential for functional food applications, but successful implementation will require targeted optimization in terms of protein source and formulation.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used “DeepL Write” and “ChatGPT” in order to improve readability and language of the work. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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7 General discussion

The aim of this study was to evaluate the properties of different proteins as including the oxidative stability of the resulting oleogels. In addition, the study examined to what extent the incorporation of phenolic compounds could enhance oxidative stability. Extensive investigations were conducted using proteins and polyphenols from various sources in order to elucidate both the effects and the underlying mechanisms. By addressing these aspects, the study aimed to fill existing knowledge gaps and contribute to the development of optimized oleogels, which could in the near future improve food products from a health, technological, and ecological perspective. Furthermore, aspects related to the assessment of oleogels in terms of their sustainability and ecological value were discussed, in order to enable a holistic evaluation of oleogels across different dimensions.

The hypotheses of the present thesis were graphically linked to the respective manuscripts and discussed in the context of their key findings (Fig. 7.1). Both the hypotheses and the main highlights from each manuscript were considered in the following discussion.

The first manuscript evaluates the oil binding properties of plant proteins (**chapter 3**), while the second manuscript explored the antioxidative efficacy of proteins in oleogels and considered differences between various protein sources (**chapter 4**). The third manuscript focused on the additional effects of different phenolic compounds, investigating which compounds could provide further antioxidative benefits and through which mechanisms these effects occur (**chapter 5**). Building on these findings, the fourth manuscript provided an overall comparison, assessing the transferability of phenolic compound effects (**chapter 6**) observed with WPI to soy protein, whose oil-binding capacity and antioxidative potential had been previously characterized. WPI served as a model protein for this comparative analysis.

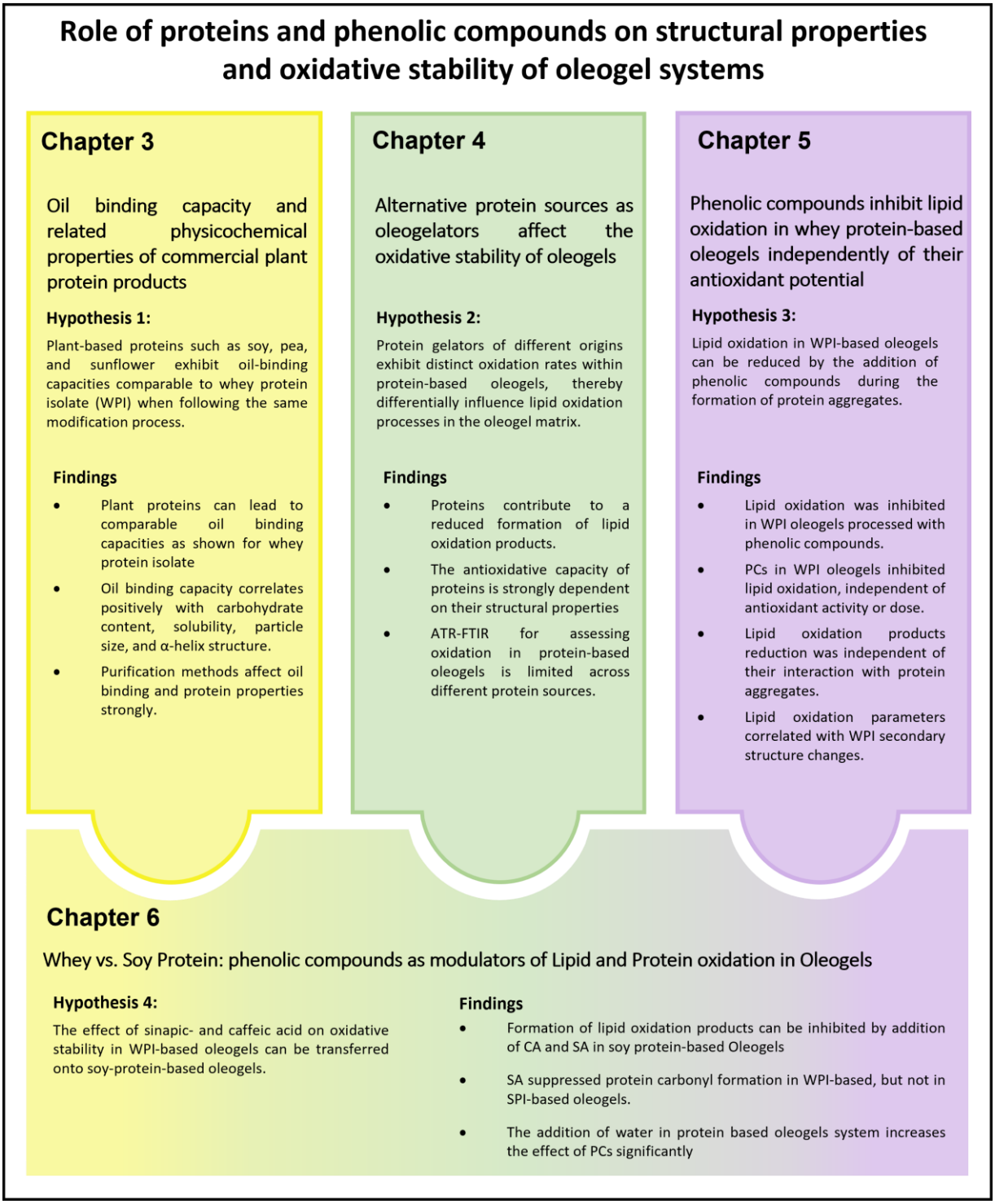

Figure 7.1: Schematic structure, hypotheses under investigation and main results as highlights of the present thesis.

7.1 Impact of protein conformation on oleogelator performance

7.1.1 Alterations of protein secondary structure as a function of source and extraction

Proteins from different sources naturally vary in their structural organization, from the primary sequence up to the quaternary level. Differences in amino acid composition and sequence arrangement are well known to influence techno-functional properties (Langendörfer et al., 2023).

Beyond the inherent properties of the protein source, processing conditions represent another decisive factor in shaping protein techno-functionality. Isolation and downstream treatments—including heating, pH shifts, high pressure, ultrasound, or solvent exposure—can induce conformational changes, partial unfolding, or aggregation, thereby altering the physicochemical and functional behavior of proteins (Di Wu et al., 2020; Han et al., 2024). It is written in literature, that these factors could result in disruption of hydrogen bonding and molecular interaction, thereby making protein structure looser and more flexible and even causes unfolding due to denaturation. Consequently, when comparing proteins from different sources, it is crucial to recognize that they may already have undergone distinct processing steps depending on the supplier, which are often not fully disclosed.

The results presented in **chapter 3** clearly highlighted, that differences between the same protein source obtained from different manufacturers—likely due to variations in extraction and processing methods—had an effect on protein functionality that was as large as, if not larger than, the differences observed between distinct protein sources. For example, changes in the secondary structure were evident for soy protein isolates. A comparison between SPI1 and SPI2 revealed a pronounced difference in β-sheet content: SPI1 exhibited a substantial increase in β-sheet structures after the heating step, whereas no such increase was observed for SPI2 under identical treatment conditions. Analysis of SDS-PAGE gels further confirmed a clear distinction between the two isolates. SPI1 showed marked signs of hydrolysis, which were not evident in SPI2. Interestingly, according to the manufacturers, both samples had been isolated using ethanol extraction. Regarding SPI in **chapter 4**, the significantly higher

content in protein carbonyls compared to other protein sources, may also be attributable to differences into processing steps. It indicated differences in protein structure, thereby leading into differences in their vulnerability to oxidation. Taken together, these findings suggest that processing history plays a decisive role in determining the structural and functional properties of proteins, and thereby their suitability as oleogelators.

7.1.2 Protein structural modifications and their effect on oil-binding capacity

The strong link between protein structure and functionality has been established previously (**chapter 7.1.1**). In the present study, this principle was confirmed and further elaborated, as clear correlations were found between protein structure and oil-binding capacity (OBC) (**chapter 3**). The ability of proteins to act as oleogelators appears to depend on their structural organization across multiple levels, from primary to quaternary structure, and on their susceptibility to denaturation and aggregation. These findings align with earlier reports that denaturation is the decisive step in the formation of protein-based oleogelators by increasing the exposure of hydrophobic core areas of whey protein (Vries et al., 2015). This leads to **Hypothesis 1**, which states that plant-based proteins such as soy, rapeseed, and sunflower exhibit oil-binding capacities comparable to WPI following the same modification process, examined in detail in the following chapter.

Heat-induced denaturation disrupts intramolecular interactions and promoting aggregation, which in turn enhances OBC (Vries et al., 2015). However, the extent of denaturation and aggregation was shown to be highly protein-specific, depending not only on process parameters (e.g., temperature, pH, duration) but also on intrinsic protein features such as initial conformation and extraction history. Indeed, different isolation methods resulted in divergent aggregation behaviors and particle size distributions, as reported in the literature (W. Jia et al., 2021; Tanger et al., 2022). Jia et al. (2021), for example, observed particle sizes between 100 µm and 400 µm for milled rapeseed concentrate, whereas in the present study rapeseed meal showed particle size between 25 and 80 µm. Such discrepancies underline the importance of extraction methodology, since even processes using similar key steps (e.g., ethanol extraction) may yield different structural and functional outcomes.

Spectroscopic analysis further substantiated the structural basis of these functional changes. FTIR revealed that, for WPI, particle formation and the associated OBC increase were accompanied by pronounced changes in secondary structure, particularly in α-helical (1648–1658 cm^{-1}) and β-sheet regions, both of which are linked to the exposure of hydrophobic residues. Comparable denaturation-induced changes were detected for plant proteins (**Fig. 3.3 a–d**), with α-helical and β-sheet content showing the strongest variation. Previous work on soy protein glycinin demonstrated that thermal denaturation increased α-helical structures in parallel with aggregation (R.-H. Chen et al., 1990). This observation was only partially confirmed here: for SPI 1, the α-helical fraction increased upon heating and correlated with a markedly higher OBC, whereas SPI 2 showed no such increase despite having a higher total α-helical content after denaturation. These results suggest that the relative change in α-helical structures, rather than their absolute abundance, is the critical determinant for OBC. In contrast, SfPM samples exhibited only minor conformational shifts, with α-helical content remaining unchanged before and after denaturation, regardless of extraction conditions. Correspondingly, OBC values did not appear to be governed by secondary structure in these samples. This stability indicates that SfPM may be structurally less responsive to denaturation under the tested conditions.

Taken together, these findings demonstrate that molecular-level features, such as particle size and secondary structure changes, particularly α-helices, govern the oleogelation potential of proteins. Importantly, the extraction method not only affects compositional purity but also determines structural responsiveness, which in turn modulates functionality. In **chapter 3**, **Hypothesis 1** was confirmed, showing that plant proteins can achieve OBC values comparable to WPI when subjected to similar modification processes. Moreover, significant correlations between specific structural markers and OBC were established, suggesting that these parameters may serve as predictive indicators for the screening and rational selection of potential protein-based oleogelators.

7.1.3 Influence of polyphenol–protein interactions on protein structure and their effects on oil-binding capacity

Another parameter that has been shown to markedly alter protein secondary structure is the interaction of proteins with PCs (**chapter 5**). Previous studies have reported that PC–protein interactions can induce structural rearrangements, including unfolding, a reduction in α-helix and β-sheet content, and an increase in random coil and β-turn structures (Meng & Li, 2021). These changes are associated with modifications in protein functionality and aggregation behavior and are driven by factors such as the type and concentration of PCs, intrinsic protein structure, environmental conditions (pH, temperature, ionic strength), and the binding mode (covalent vs. non-covalent) (Shahidi & Dissanayaka, 2023; Q. Zhang et al., 2021a). Based on the relationships between protein structure and functionality discussed in **chapters 7.1.1 and 7.1.2**, it was hypothesized that interactions between proteins and PCs could also affect the OBC of the resulting oleogelators.

Structural analyses of WPI in the presence of PCs revealed measurable changes in secondary structure that depended on both the PC source and its concentration (**Table 5.4**). The detailed structural alterations are presented in **chapter 5.3.5**. FTIR analyses showed that the addition of PCs prior to protein denaturation caused almost no detectable changes in secondary structure compared to the control (**Table 5.4**). However, after heating for 20 min at 85 °C, the four tested PCs exerted a markedly stronger influence on secondary structure. These findings suggest that thermal treatment significantly enhances PC–protein interactions. This observation aligns with previous reports showing that elevated temperatures facilitate protein–polyphenol interactions and result in structural modifications of WPI (Budryn et al., 2015; Mao et al., 2023). The underlying mechanism of this phenomenon, however, remains unclear. It is not evident whether the stronger effects observed at elevated temperatures are primarily due to an increased interaction rate between proteins and PCs, or whether the modified environment caused by thermally activated PCs plays a decisive role. Interestingly, no increased retention of PCs in the protein phase was observed (**Fig. 5.3**), which would have indicated stable binding. This suggests that the observed structural changes may not come directly from stable PC–protein binding. Instead, they might result from reversible non-

covalent interactions that are subsequently disrupted during sample processing (e.g., ultraturrax treatment or centrifugation), or even from indirect environmental effects that induce conformational changes without covalent binding (J. Jia et al., 2017; Lund, 2021).

In order to evaluate the influence of PCs on OBC of protein, no effect of PC addition on the OBC of WPI was observed. In this context, a clear inconsistency emerges when comparing the findings from **chapter 3** and **chapter 5**. In **chapter 3**, a positive correlation between α-helix content and OBC was established, suggesting that higher α-helix levels are associated with improved oil-binding. However, in **chapter 5**, despite pronounced differences in secondary structure — specifically, lower α-helix content in the GA and CGA groups compared to CA, SA, and the control—no differences in OBC were observed. One possible explanation could be that the structural changes caused by PCs are rather small compared to those triggered by heat or pH shifts and therefore not strong enough to have an effect on OBC at the functional level. Another explanation might be that PCs influence the way the protein network forms in the oleogel, in such a way that the expected link between α-helix content and oil-binding is balanced out or masked. There is evidence from the literature showing that polyphenols not only affect protein secondary structure but also interfere with higher-order aggregation and network formation. For example, tannins and EGCG were reported to promote protein aggregation via hydrophobic interactions and hydrogen bonding, leading to insoluble complexes (Shahidi & Dissanayaka, 2023). Polyphenols can also self-associate through π–π stacking, forming micelles or colloidal particles, which then interact with proteins and build cooperative “polyphenol bridges” between molecules (Pianet et al., 2008). These processes can change protein–protein interactions that are key for gel network formation and may therefore mask the expected effect of secondary structure on OBC.

Altogether, these findings suggest that the way PCs modulate protein–protein interactions and network formation could be more decisive for OBC than changes in secondary structure alone. This offers a reasonable explanation for the discrepancy observed between **chapter 3** and **chapter 5**.

7.2 Control of oxidative stability in protein-based oleogel systems

In general, oleogels are highly susceptible to oxidative processes which results quality deterioration. This susceptibility is primarily related to their high content of unsaturated fatty acids but can be further influenced by additional factors (Gunstone, 1984). With regard to composition, three components are of particular importance, as they contribute to oxidative processes both individually and through their interactions: lipids, proteins, and—in some cases, for structural or experimental purposes—added water. In addition, the influence of added phenolic compounds is evaluated with regard to their potential to protect the oleogel system from oxidative stress. In the following sections, the oxidative processes within this complex sample system will be examined and discussed in order to provide a detailed understanding of the various influencing factors and their overall impact. **Hypotheses 2, 3, and 4** are addressed in detail in the following discussion, drawing on the data presented in **chapters 4–6**.

7.2.1 Role of proteins in modulating oxidative stability of oleogels

LOx is known to impair quality attributes such as flavor, aroma, texture, and color, and is therefore undesirable in food products (Ryan J. Elias et al., 2008). Since protein oxidation on the other hand did not affect food quality to the same extent, protein oxidation is still far less thoroughly researched. Nevertheless, it is obvious that radicals can negatively affect both lipids and proteins. However, the ability of proteins to interact with free radicals in food could also lead to the development of new antioxidant technologies if proteins could be used to inhibit oxidative reactions and thus protect oxidatively unstable fatty acids depending on structural properties (Ryan J. Elias et al., 2008). In the following chapter, **Hypothesis 2** of this study will be examined in greater detail. For this purpose, different protein sources were used as a basis for oleogel preparation in **chapter 4** and compared with respect to their influence on oxidative behavior. Particular attention was given to whether differences in protein structure and composition affect oxidative stability. WPI-based oleogels showed reduced LOx compared to bulk oil (**chapter 5**), supporting the idea that proteins act antioxidative through mechanisms such as radical scavenging, metal chelation, or physical shielding (R. J. Elias & Decker, 2010a; Ryan J. Elias et al., 2008). Elias et al. (2008) demonstrated that proteins

function as antioxidants if they are more easily oxidized than lipids, while the resulting protein radicals remain relatively unreactive. In β-lactoglobulin, tryptophan and cysteine residues contributed strongly to antioxidant capacity, whereas methionine did not, likely due to its buried location in the hydrophobic core. Structural modifications such as unfolding, which expose buried residues, may thus enhance protein antioxidative potential—an aspect relevant during oleogel formation where protein aggregation and partial unfolding occur (Ryan J. Elias et al., 2008). This interpretation is in line with other studies highlighting that protein conformation and accessibility of specific amino acid residues critically determine their antioxidative activity (Ryan J. Elias et al., 2008; Y. Ma et al., 2018).

Overall, these insights emphasize that both the amino acid composition and the spatial arrangement of amino acid side chains within the protein matrix play a decisive role in antioxidative effectiveness. This mechanistic understanding provides a plausible explanation for the results presented in **chapter 4**, where different protein sources displayed varying degrees of influence on LOx in the oleogel system.

The protein sources examined in **chapter 4** – WPI, soy, rapeseed, sunflower, and mussel – exhibited varying effects on LOx (**Section 4.3.1**). Overall, no clear distinction in the mode of action between animal and plant proteins could be established. While WPI, as an animal protein, showed distinctly different hydroperoxide formation kinetics compared to the plant proteins, mussel protein, as a second animal source, did not differ significantly from soy, rapeseed, or sunflower proteins (**Fig. 4.1**). In contrast, when examining hexanal as a secondary LOx marker, WPI and mussel proteins showed noticeable differences compared to the plant proteins (**Fig. 4.2**).

These differences may be attributed to variations in subunit composition between animal and plant proteins, reflecting their distinct sources, structures, and amino acid compositions (Day et al., 2022a; Xiao et al., 2023). Plant proteins tend to form larger aggregates, such as hexamers, with molecular weights of up to 360 kDa in the case of globulins from soy, sunflower, and rapeseed (Chmielewska et al., 2021; Janitha P.D. Wanasundara et al., 2016; Nishinari et al., 2014; Zilic et al., 2010). Animal proteins, in comparison, generally exhibit smaller sizes, although they also contain globulin and albumin fractions. According to the

surface-to-volume principle (Berg's law), smaller proteins have a relatively larger surface area, which allows more antioxidative amino acid residues to be exposed. This provides a plausible explanation for the potentially higher antioxidant activity of smaller, surface-active proteins.

In addition to the size of the protein units, the AA composition and, more specifically, the exposure of certain residues on the protein surface were considered with respect to their antioxidant properties. Aromatic residues (tryptophan, tyrosine), sulfur-containing residues (cysteine, methionine), imidazole-containing residues (histidine), and basic residues (lysine and arginine) have been reported to exhibit antioxidant potential ranging from moderate to very high (Nwachukwu & Aluko, 2019; Xu et al., 2017a). Clear differences between protein sources in their AA profile can also help explain their varying effects on oxidation processes in the oleogel system. While WPI and mussel proteins, as animal-derived proteins, possess a complete amino acid profile in terms of biological value (Herreman et al., 2020; Yaghubi et al., 2021), plant proteins often exhibit limitations in certain amino acids. In particular, rapeseed and sunflower proteins are relatively low in lysine, whereas soy protein contains somewhat fewer sulfur-containing amino acids (Arrutia et al., 2020; Herreman et al., 2020), thereby lacking in AA with previously described antioxidant properties. Overall, these observations suggest that plant proteins generally have lower antioxidant potential compared to animal proteins. Interestingly, this observation does not fully align with the findings from **chapter 4**. Neither LOx nor protein oxidation showed a clearly reduced rate in samples containing WPI or mussel protein compared to soy, rapeseed, or sunflower proteins. Rather, the actual effect of the proteins appears to be considerably more complex and seems to depend on additional factors, such as modifications of protein structure during processing.

Another factor that has been discussed as strongly influencing the antioxidant potential of proteins is their secondary structure. In general, a shift from α-helical structures toward an increased proportion of β-sheets, and random coils has been associated with improved antioxidant potential, as such structural rearrangements may enhance the exposure of antioxidative amino acid residues (J. Wang et al., 2023). For SPI, it was shown in **chapter 3** that at least partial hydrolysis of the proteins is likely **(Fig. 3.4)**. In literature, such hydrolysis has been described as enhancing the antioxidant activity of proteins (Aguilar, Jessika Gonçalves

dos Santos et al., 2019). Whether this effect also plays a role in the other protein sources, thereby influencing their antioxidant potential, cannot be excluded. This aspect should therefore be considered when evaluating the potential of different protein sources as oxidation-inhibiting components in protein-based oleogels. Nevertheless, structural changes were observed as a result of denaturation during the formation of protein aggregates. As demonstrated in **chapter 3**, such changes were clearly dependent on the protein source. Following heat treatment, WPI showed the highest proportion of β-sheet structures, which is consistent with its characterization as the protein with the strongest antioxidative potential. However, WPI also exhibited considerably fewer β-turn structures compared to the plant proteins, and significantly lower amounts of random coil structures than rapeseed protein. Thus, based on secondary structure alone, WPI cannot be unequivocally confirmed as the protein with the strongest antioxidative effect.

Proteins as oleogelators not only modulate LOx but are also susceptible to oxidation themselves, largely under the same conditions discussed above. The results presented in **chapter 4** indicate that soy protein, and to an even greater extent mussel protein, had already undergone oxidation prior to the start of the storage trial and did not show further increases during storage (**Fig. 4.3**). This observation is consistent with previous reports, which describe that protein extraction methods can promote protein oxidation and therefore no further progression was detectable. In contrast, an increase in protein oxidation markers was observed in WPI over the course of storage. Despite these differences, proteins clearly exerted an influence on LOx within the system, depending on its location and degree of oxidation. While strongly oxidized proteins tend to increase LOx, mild oxidation of proteins leads to increased protein flexibility and interfacial packing, resulting in reduced lipid oxidation in certain systems (J. Chen et al., 2022; Huang et al., 2025; Zhu et al., 2018). This could explain the strong LOx –inhibiting effect observed for mussel protein (**Fig. 4.2**).

Overall, the findings support **Hypothesis 2** by showing that proteins can modulate the oxidative stability of oleogels, though the extent of their antioxidative effect depends not only on amino acid composition but also on structural properties and processing-induced

modifications. Nevertheless, a definitive assertion regarding the optimal protein source for enhancing oxidative stability remains challenging, given the inconsistent outcomes observed.

7.2.2 Influence of water incorporation on oxidative stability

Lipid oxidation does not proceed identically in water-free and water-containing systems. In **chapters 4 and 6**, oxidation parameters were tracked under water addition and compared to dry systems (**chapter 5**). Based on the observed changes in the formation of LOx markers over time, the influence of water on the protein-based oleogel system, as well as the underlying mechanisms, are discussed.

The incorporation of water into protein-based oleogels induces structural rearrangements that affect both the physical and chemical stability of the system. As in water-in-oil emulsions, water addition to a hydrophobic phase leads to droplet formation (Philipp M. Meissner et al., 2019). These droplets enlarge the interfacial area and may promote reorganization of protein aggregates, which support the gel network. Literature suggests that water can strengthen protein gels through cohesive forces, thereby stabilizing the structure. Although aggregate distribution was not directly measured in this study, Meissner et al. described the formation of water droplets for Oleogels systems containing >2% water (Meissner et al., 2019). Therefore, the present system assumes the formation of water droplets. Since limited literature is available on water incorporation in protein-based oleogels, the explanation of the underlying mechanistic processes was derived from emulsion systems, with the understanding that the structural organization of proteins in oleogels under water incorporation is likely different from that observed in emulsions.

Previous work has demonstrated that the addition of water to whey protein–based oleogels markedly influences LOx, primarily through increased water activity and the formation of water droplets, which in turn generate additional water–lipid interfaces (Philipp M. Meissner et al., 2019). At these interfaces, solubilized metals may catalyze oxidation, while amphiphilic conjugated dienes accumulate and further promote it (Gómez & Fernández-Salguero, 1992). Interestingly, elevated diene formation does not always correspond to higher aldehyde levels. Water may stabilize hydroperoxides via hydrogen bonding, delaying their breakdown into secondary product (Philipp M. Meissner et al., 2019). These findings suggest that the

formation of secondary LOx markers may be masked in protein-containing systems, depending on the amount of water present (Meissner et al., 2020b).

This behavior was also evident in the present study. In water-containing protein oleogels, hydroperoxide levels stayed higher than in dry gels until day 8 and even exceeded bulk oil (**chapter 4-6**). This suggests that under increased interfacial area and water activity, pro-oxidative effects can outweigh the inherent antioxidative capacity of proteins. For hexanal, no consistent increase over bulk oil was observed, except with soy protein (**Fig. 4.2**), making it unclear whether the effect reflects protein antioxidant activity or hydroperoxide stabilization by water.

Proteins contribute further to the regulation of LOx through their adsorption at oil–water interfaces. Proteins are known to adsorb at oil–water interfaces in emulsions due to their amphiphilic nature (Norde & Lyklema, 1991), thereby stabilizing the interface and potentially protecting lipids from oxidation (Leman & Kinsella, 1989). Moreover, proteins themselves are susceptible to oxidation, particularly in highly interfacial systems. In water-containing oleogels, larger protein aggregates that are no longer bound to interfaces (J. Chen et al., 2022), are distributed between the gel network and water droplets, likely depending on their structural characteristics. This structural organization may enhance the antioxidative effect through physical mechanisms, such as shielding or compartmentalization, in addition to any intrinsic chemical antioxidant activity. Regarding hexanal, no increase relative to bulk oil was observed in most water-containing protein-based oleogels, with the exception of WPI (**Fig 4.2**). Whether this effect reflects the inherent antioxidant capacity of protein or the stabilization of hydroperoxides against fragmentation by water remains unclear.

Taken together, these findings suggest that in protein-based oleogels, the pro-oxidative influence of increased interfaces and water activity may, in certain contexts, outweigh the intrinsic antioxidative potential of proteins, while physical stabilization by protein aggregates can provide a secondary protective mechanism.

7.2.3 Improvement of oxidative status through phenolic compound incorporation

Phenolic compounds (PCs) are generally known for their antioxidative properties, which arise from their chemical structure (Durazzo et al., 2019; Ignat et al., 2011). Based on this, it was hypothesized that the incorporation of PCs into protein-based oleogels could improve oxidative stability, irrespective of their intrinsic antioxidative potential (**Hypothesis 3**). To investigate this, experiments were carried out in **chapter 5,** aiming to unravel both their effects and the underlying mechanisms. Furthermore, **Hypothesis 4** proposed that the addition of PCs would also enhance oxidative stability in soy protein-based oleogels. This aspect was addressed in detail in **chapter 6**.

The antioxidative capacity of PCs depends strongly on their chemical structure as well as on their interactions with proteins (Kieserling et al., 2024). These interactions can occur covalently or non-covalently; however, under the experimental conditions used here (neutral pH, absence of enzymatic activity), covalent interactions are unlikely to occur. Instead, non-covalent interactions are expected, which are influenced by protein structure, the availability of binding sites, the conformation of the PCs, and environmental factors such as pH and ionic strength.

The results presented in **chapter 5** demonstrated that SA and CA markedly reduced the formation of both primary and secondary LOx products. In contrast, no antioxidative enhancement was observed with GA and CGA, despite their much higher antioxidative potential reported in the literature (Cos et al., 2002; Sousa et al., 2004). Previous studies have suggested that PCs may improve the antioxidative capacity of proteins but can also lose part of their own activity upon binding, as reactive sites become unavailable (Morais et al., 2020b). Since no correlation between binding behavior of PCs and proteins and their antioxidative effect was found, this explanation seems to be insufficient in the present case. Moreover, unbound PCs are unlikely to contribute significantly, as they are washed out during processing. This raises the key question of which mechanisms are responsible for the improved oxidative stability if neither strong binding nor free PCs can fully explain the effect.

One possible explanation lies in structural modifications of the proteins during denaturation in the presence of PCs, potentially leading to an increased antioxidative capacity. For instance, higher levels of α-helical structures were preserved in CA- and SA-containing samples. This, however, contradicts previous findings where β-sheet and random coil structures were associated with enhanced antioxidative capacity (J. Wang et al., 2023). Considering that WPI is a protein mixture, it should also be noted that the presence of SA and CA may have induced a shift in the protein composition toward a higher proportion of α-LA. Such a shift could result from selective structural interactions, as β-LG likely retained a more native conformation in the presence of SA and CA and was therefore more extensively removed during the washing step. Since α-LA exhibits a higher antioxidative capacity than β-LG (Corrochano et al., 2019; Sadat et al., 2011), this compositional change could contribute to the enhanced antioxidant potential observed in the protein aggregates containing SA and CA. However, as this explanatory approach does not account for the improvement in oxidative stability observed in SPI-based oleogels, it cannot be regarded as a definitive explanation. Thus, no conclusive structural mechanism can be derived. It is worth considering that the relationship between secondary structure elements and antioxidative capacity may not follow a simple linear correlation. Instead, the effect might be context-dependent, influenced by factors such as the spatial arrangement of structural motifs and the surface accessibility of reactive amino acid residues (e.g., tyrosine, tryptophan, cysteine).

Changes in protein aggregate size may provide another explanation. Although not measured in this study, literature reports highlight a correlation between aggregate size, interfacial arrangement, and antioxidative capacity (Y. Zhang et al., 2018). Even though the oleogel systems studied here lacked water (**chapter 5**), and therefore classical interfacial effects cannot be expected, aggregate size, surface charge, and exposure of amino acid residues could still influence network formation and thereby affect oxidative stability on a physical rather than chemical level.

It should also be emphasized that findings from WPI-based oleogels cannot be directly transferred to plant protein-based systems (**Hypothesis 4**). The structural differences and distinct AA compositions of WPI and SPI inevitably lead to altered binding behavior with PCs

(Day et al., 2022a; Xiao et al., 2023). Previous studies have reported that the binding regions of SPI and WPI differ with respect to phenolic compounds such as SA and CA. In WPI, binding is mainly associated with hydrophobic patches and reactive side chains, whereas in SPI, the interaction sites are generally more non polar amino acids playing a key role (Morais et al., 2020a; Pessato et al., 2018; X. Wang et al., 2024). The binding mechanisms also vary: in SPI, van der Waals forces and hydrogen bonding appear to be more important, while hydrophobic interactions are less dominant compared to WPI (X. Wang et al., 2024). An additional factor that needs to be considered is the pretreatment history of the proteins and the extent of structural unfolding associated with it. Several studies have shown that unfolded proteins provide greater accessibility of binding sites and thus exhibit an enhanced propensity to interact with phenolic compounds (Cao et al., 2018; C.-M. Ma & Zhao, 2019). In this context, SPI is already partially unfolded due to the isolation process, whereas WPI largely retains its native conformation and undergoes unfolding mainly during the heating step that promotes aggregate formation. This structural difference may explain why SPI displayed stronger interactions with phenolic compounds in the present system, even though a lower binding affinity with CA has been predicted compared to WPI (Budryn et al., 2015).

While no direct binding measurements were performed in this study, the reduction of LOx products upon addition of SA and CA, previously established in WPI-based systems, was also evident in SPI-based oleogels. This observation suggests a certain degree of transferability of the antioxidative effect across protein matrices. However, the exact molecular mechanisms underlying these interactions remain unresolved. Future research should therefore aim to disentangle the relative contributions of protein structure, processing-induced unfolding, and the specific binding forces involved, in order to better understand and ultimately tailor protein–phenolic interactions in complex food systems.

Overall, **Hypothesis 3** can be confirmed, as certain phenolic compounds effectively reduced LOx in WPI-based oleogels. Consistent with **Hypothesis 4** and the findings in **chapter 6**, the inhibitory effects of SA and CA observed in WPI systems were also found in SPI-based oleogels.

7.3 Protein-based oleogels as emerging functional ingredients: importance and potential

7.3.1 Health-related and nutritional importance

In the context of nutritional improvement, several factors are particularly important, including adequate protein intake with a sufficient amino acid profile and a healthy supply of essential and polyunsaturated fatty acids (Cena & Calder, 2020). The attainment of both objectives is possible through the utilization of protein-based oleogels, with the optimization of oleogel components being instrumental in this regard. For instance, rapeseed protein constitutes a highly valuable source of plant-based protein. Rapeseed protein is notable for its elevated proportion of sulfur-containing amino acids, a feature that distinguishes it from other plant proteins (Wanasundara et al., 2023). This observation is further substantiated by an OBC comparison with WPI (**Fig. 3.2**), which lends credence to its potential as an oleogelator. Moreover, combining proteins from different sources may provide additional benefits. Literature reports have consistently shown that blends of proteins from various sources can outperform individual proteins in certain functional properties, such as solubility and gelling capacity (Coutinho et al., 2025; Grasberger et al., 2021; Guidi et al., 2022). From a nutritional perspective, protein mixtures could also offer an improved amino acid balance and overall dietary quality (Durga Lakshmi et al., 2025).

Further research is needed to identify the optimal combinations of proteins that provide both the best oil-binding capacity and the highest nutritional quality, particularly with respect to the AA profile. This becomes even more important when considering that protein functionality is highly dependent on interactions with other components in the matrix, as discussed earlier. Introducing additional compounds into the system, for example by blending different proteins, increases the level of complexity and makes it even more challenging to fully understand the underlying mechanisms.

The incorporation of PCs into oleogels not only improves their oxidative stability but may also add direct health benefits. PCs such as CA and SA, abundant in coffee and wine, have been linked to reduced risks of cancer, diabetes, metabolic syndrome, atherosclerosis, and

Alzheimer's disease (Muhammad Abdul Kadar, Nellysha Namela et al., 2021; Pavlíková, 2022). Daily intake from heavy coffee consumption can reach several hundred milligrams, indicating that relevant doses are achievable in a normal diet (Nićiforović & Abramovič, 2014; Olthof et al., 2001; Trandafir et al., 2013). Although up to 95% of CA can be absorbed in the small intestine, chlorogenic acid shows much lower uptake (Olthof et al., 2001) . Sinapic acid likely follows a similar pathway, but data are scarce. Animal trials consistently show beneficial effects already at concentration of 60-200 mg/kg diet per day CA and CGA (Cho et al., 2010; El-Seedi et al., 2012; Shimoda et al., 2006; Yeh et al., 2010). Our findings in **chapter 6** suggest that PC enrichment of oleogels can reach physiologically relevant levels. For example, the 10 mM CA–loaded oleogels in this study contained ~352 mg caffeic acid/L oleogel. Based on reported effective doses mentioned above, consumption of about 85 g oleogel per kilogram of food could already contribute to health-promoting effects. PC-enriched oleogels could thereby act as more than just solid fat replacers—they could serve as carriers for the delivery of bioactive substances with a noticeable effect on nutrition. This dual role strengthens their relevance for functional food development and warrants further in vivo validation.

7.3.2 Relevance for food technology applications

When evaluating the technological relevance of oleogels in food systems, it is important to note that their potential extends beyond health-related benefits. If oleogels are to serve as functional replacements for conventional solid fats, they must not only provide structural stability but also reproduce more complex technological functions, particularly melting behavior.

Different strategies have been explored to tackle the challenge of reproducing the melting behavior of solid fats. One option is the use of small quantities of high-melting fats as oleogelators, which has already been approved for use in food applications (Oishi et al., 2023). The crystallization behavior of waxes can in this way be exploited to mimic the melting profile of conventional fats, leading to thermo-reversible oleogels even at very low concentrations (as low as 0.5% w/w) (Tavernier et al., 2017). A drawback of such systems, however, is the typical waxy mouthfeel, which limits their acceptance (Oishi et al., 2023). Other oleogels with

thermo-responsive properties have also been reported, for instance those based on shellac (Patel et al., 2013).

Proteins, on the other hand, are more difficult to employ as oleogelators since they do not naturally undergo melting transitions. Promising results have nevertheless been obtained in combination with thermo-active components. Keshanidokht et al. (2023), for example, observed thermo-responsive behavior in glycerol-plasticized zein oleogels between 25–100 °C, with reversibility depending on the glycerol/zein ratio. De Vries et al. (2014) similarly described zein and oil droplets acting as hydrophobic nucleation sites, which enabled the growth of fibrils and resulted in thermo-responsive gelation. Wang et al. (2022) more recently reported that quercetin-loaded, zein-stabilized oleogels prepared via an emulsion-templated method also exhibited thermo-reversible properties.

Overall, these findings indicate that proteins alone are not sufficient to reproduce the melting characteristics of conventional fats. However, by combining them with thermo-active molecules or other oleogelators, their limitations may be overcome. Approaches based on multiple components therefore seem particularly promising, as they allow different functional properties to complement one another, opening the way towards oleogels with improved melting profiles, texture and sensory properties. In this context, the results of this study add to the existing body of knowledge by highlighting the functional potential of plant proteins as part of such multi-component systems. By examining their oleogelation capacity and their influence on lipid stability (**chapters 3 and 4**), this work provides insights into how protein-based structuring agents can be integrated with other oleogelators to address the challenge of melting behavior and to design more sustainable, plant-based alternatives to conventional fats.

7.3.3 Sustainability and ecological relevance

Recent studies have indicated a slow but steady rise in the importance of both the development and application of oleogels in food products. This increase can be attributed to oleogels' potential to serve as a substitute for conventional fats, predominantly animal-based, or sourced from tropical regions (Feichtinger & Scholten, 2020). It is evident that there are significant advantages to be gained from the utilization of regional resources and the

incorporation of sidestream-products in the food industry, which often exhibit comparable or even superior characteristics.

By-products arising from the processing of oilseeds or other agricultural raw materials contain valuable proteins (Östbring et al., 2019). However, to date, these proteins have only been utilized to a limited extent. Attributed to the harsh processing steps employed in industrial settings, which frequently result in diminished protein functionality (Fetzer et al., 2018; Östbring et al., 2019). Research findings of the present thesis indicate that plant proteins possess functional properties that are analogous to those of well-established oleogelators, such as WPI. Notably, their OBC and their favorable impact on the oxidative stability of lipid phases underscore the potential of these proteins for the development of innovative, plant-based food systems (**chapter 3 and 4**). The design of the industrial processing methods is therefore a key factor in ensuring the sustainable use of these resources. Whilst the focus of oil extraction has historically been on achieving maximum yield, it is imperative to consider the necessity of process adjustments in the future to ensure the functionality of by-products, such as protein fractions. Although such adjustments may reduce the overall oil yield, this could be counterbalanced by the valuable utilization of by-products. It is therefore imperative that further research will conducted to unravel the complex relationships between protein functionalities and their structural changes, with a view to creating the most optimal product for specific applications. Basic research, such as that carried out in this study (**chapter 3 and 4**), provides important insights in this regard.

Another sustainability aspect concerns the replacement of tropical fats (e.g., palm oil) with regional oils and their derivatives. Oleogels are a technique that allows for the functional modification of regional vegetable oils, thereby ensuring they exhibit comparable technological functionality (i.e. texture, stability, and melting behavior) in food products (Pușcaș et al., 2020). This approach has the dual benefits of reducing transport distances and mitigating ecological problems, such as deforestation in rainforests and the expansion of monocultures.

Nevertheless, it is important to critically discuss the extent to which the higher processing costs involved in the production of functional oleogels are ecologically justifiable. A

comprehensive assessment necessitates a holistic sustainability analysis encompassing the carbon footprint, water consumption, biodiversity aspects, and economic factors. Such an analysis could reveal that more intensive processing of regional resources may ultimately be ecologically and socially advantageous, as it reduces import dependency and mitigates environmental impacts. At the same time, the overall process is rather intricate, which results in oleogels could be classified as highly processed products. Processing steps such as spray drying, freeze drying, or solvent exchange processes are time-consuming, costly, and energy-intensive. Furthermore, oleogels are not currently manufactured on an industrial scale, which significantly increases costs. Therefore, the efficacy of the reduction in CO_2 emissions achieved by using fewer transport crates is debatable, given the resources expended on processing. It is also important to consider that the increased complexity of the production process will be reflected in the final product costs, which will be passed on to the end consumer. In this instance, too, it is uncertain whether acceptance by the end consumer will be achieved.

Oleogels represent a technological innovation that has the potential to make a significant contribution to more sustainable food production. However, this approach entails the targeted development of processes and a systemic evaluation of the ecological and economic consequences. Such an evaluation is necessary to ascertain that the replacement of conventional fats with oleogels results in a net enhancement of sustainability.

7.4 Closing remarks and outlook

In conclusion, this study demonstrates that protein-based oleogels represent a highly promising matrix for food applications, offering advantages in terms of sustainability, health, and functional performance compared to conventional solid fats. In addition to WPI, for which other studies have previously demonstrated an increased OBC in connection with modification processes, **chapter 3** also identified other plant proteins that exhibited corresponding OBC. Using plant proteins instead of proteins derived from terrestrial animals as oleogelators provide the basis to increase the ecological and nutritional benefits of oleogels. This finding serves to validate **Hypothesis 1**, thereby demonstrating the potential of these plant proteins to function as oleogelators. This chapter emphasized the necessity of a

structural understanding of proteins, given that, in addition to the protein source, modifications to the protein structure through processing during isolation or other procedures can also have a decisive influence on the OBC and other functions of the proteins.

The importance of understanding the structural properties of proteins in greater detail is also evident in **chapters 4 and 5**, where **hypothesis 2** could be confirmed by showing that proteins led to a reduction in LOx parameters in the oleogel, but no clear underlying mechanisms could be identified. Nevertheless, differences in oxidation kinetics among the various protein sources were observed, clearly indicating that they function differently in stabilizing LOx in protein-based oleogel system.

A further improvement in LOx was also observed through the additional introduction of PCs into the oleogel systems (**chapter 5**), which led to the assumption of **Hypothesis 3**. The most promising PCs in this case, SA and CA, were also effective in SPI-based oleogels, where they led to improved oxidative stability (**chapter 6**), thus confirming **Hypothesis 4**.

Overall, it must be noted that the central element is the interrelationship between protein composition, structuring and the resulting functionalities in the oleogel system. Future research should be much more targeted at specific applications. In this way, the relevant requirements for an individual product could be considered more specifically based on knowledge gained about structural influences on interaction and functionality. This work provides a foundation for understanding the complexity of this matrix in greater depth. These results should be built upon and this complex problem should be further approached step by step in order to ultimately achieve a healthy, sustainable, and technologically appealing product that makes the world a little bit better.

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8 Summary

The aim of the present study was to investigate the influence of different protein sources and phenolic components on the physical and chemical stability of oleogels. Particular attention was paid to the relationships between structural modifications of the proteins and their functional properties, including oil-binding capacity (OBC) the oxidative stability of the resulting oleogels.

In **chapter 3**, various plant proteins (SPI, RPM and SfPM) were examined and compared to whey protein isolate (WPI) as a model protein with respect to their functional properties. It was shown that all three plant proteins were able to bind comparable amounts of oil as WPI. The analysis of the secondary structure revealed that, in addition to the protein source, processing steps such as protein extraction have a decisive influence on the protein structure and, consequently, on their functionality. These results emphasize that a fundamental understanding of protein structure and its modifications is essential to elucidate the complex mechanisms that determine the suitability of proteins as oleogelators.

In **chapter 4**, the oxidative stabilization of lipids by proteins was investigated. Proteins inherently possess antioxidative properties that are strongly dependent on their structure, including amino acid composition and conformation, which determine surface exposure. It was demonstrated that proteins contribute to the reduction of lipid oxidation products in oleogels, with differences observed depending on the protein source. Once again, the central importance of understanding protein structure for functional properties became evident.

In **chapter 5**, the potential of various phenolic compounds to influence the oxidative susceptibility of WPI-based oleogels was investigated. The addition of sinapic acid (SA) and caffeic acid (CA) resulted in a significant reduction in the formation of lipid oxidation products, whereas gallic acid (GA) and chlorogenic acid (CGA) showed little or no effect. Analysis of the protein structure suggested that an increased proportion of α-helical structures correlated with the improved oxidative stability observed in SA- and CA-containing samples. However, no direct relationship between the binding affinity of the phenolic compounds to the protein

and their antioxidative effect could be established, indicating that the protective mechanism is not solely based on direct protein–phenol interactions.

Finally, **chapter 6** examined whether the positive effects of SA and CA could also be transferred to plant protein-based oleogels. In an SPI-based oleogel, SA and CA likewise significantly reduced the formation of lipid oxidation products, while no changes in protein oxidation markers were detected. This demonstrates that the protective effect of the phenolic components is not limited to WPI-based systems but can also be extended to oleogels with plant proteins, underscoring the practical relevance of this approach.

In summary, the study demonstrates that proteins are generally suitable as oleogelators, with particular emphasis on the potential of plant proteins. Furthermore, the incorporation of phenolic components represents a promising strategy to enhance oxidative stability within oleogel systems. Overall, the findings underline that a comprehensive understanding of protein structure is crucial for ensuring the stability and functionality of protein-based oleogels. Despite the high complexity of these systems, the results reveal the great potential of protein-based oleogels for sustainable and functional food applications.

9 Zusammenfassung

Ziel der vorliegenden Arbeit war es, den Einfluss verschiedener Proteinquellen und phenolischer Komponenten auf die physikalische und chemische Stabilität von Oleogelen zu untersuchen. Besonderes Augenmerk lag dabei auf den Zusammenhängen zwischen strukturellen Veränderungen der Proteine und deren funktionellen Eigenschaften, einschließlich Ölbindekapazität sowie der Oxidationsstabilität der resultierenden Oleogele.

In **Kapitel 3** wurden verschiedene pflanzliche Proteine (RPM, SPI und SfPM) im Vergleich zu Whey-Protein-Isolat (Whey-Protein-Isolate) als Modellprotein auf ihre funktionellen Eigenschaften hin untersucht. Es zeigte sich, dass alle drei pflanzlichen Proteine vergleichbare Mengen wie WPI an Öl binden konnten. Die Analyse der Sekundärstruktur der Proteine verdeutlichte, dass neben der Proteinquelle auch Prozessschritte wie die Proteinextraktion einen entscheidenden Einfluss auf die Proteinstruktur und damit auf deren Funktionalität haben. Die Ergebnisse unterstreichen, dass ein grundlegendes Verständnis der Proteinstruktur und ihrer Modifikationen notwendig ist, um die komplexen Mechanismen zu verstehen, die für den Einsatz von Proteinen als Oleogelatoren entscheidend sind.

In **Kapitel 4** wurde die oxidative Stabilisierung von Lipiden durch Proteine untersucht. Proteine besitzen von Natur aus antioxidative Eigenschaften, die stark von ihrer Struktur abhängen, einschließlich Aminosäurezusammensetzung und Konformation, die die Oberflächenexposition bestimmen. Es konnte gezeigt werden, dass Proteine im Oleogel zur Reduktion der Bildung von Lipidoxidationsprodukten beitragen, wobei Unterschiede aufgrund der Proteinquelle festgestellt werden konnten. Auch hier wurde die zentrale Bedeutung des Verständnisses von Proteinstruktur für die funktionellen Eigenschaften deutlich.

Kapitel 5 untersuchte, inwiefern die oxidative Anfälligkeit von WPI-basierten Oleogelen durch die Zugabe verschiedener phenolischer Komponenten beeinflusst werden kann. Die Zugabe von Sinapinsäure (SA) und Kaffeesäure (CA) führte zu einer signifikanten Reduktion der Bildung von Lipidoxidationsprodukten, während Gallussäure (GA) und Chlorogensäure (CGA) nur geringe oder keine Effekte zeigten. Die Analyse der Proteinstruktur deutete darauf hin,

dass ein erhöhter α-Helix-Anteil mit der durch SA und CA beobachteten oxidativen Stabilität korreliert. Es konnte jedoch kein direkter Zusammenhang zwischen der Bindungsaffinität der phenolischen Komponenten an das Protein und ihrem antioxidativen Effekt festgestellt werden, was darauf hinweist, dass der Schutzmechanismus nicht ausschließlich auf direkten Protein–Phenol-Interaktionen beruht.

Abschließend wurde in **Kapitel 6** untersucht, ob die positiven Effekte von SA und CA auch auf pflanzenprotein-basierte Oleogele übertragbar sind. In einem SPI-Oleogel konnten SA und CA ebenfalls die Bildung von Lipidoxidationsprodukten signifikant reduzieren, während keine Veränderungen der Proteinoxidationsmarker festgestellt wurden. Dies zeigt, dass der protektive Effekt der phenolischen Komponenten nicht auf WPI-basierte Systeme beschränkt ist, sondern auch auf Oleogele mit pflanzlichen Proteinen übertragbar ist, was die praktische Relevanz dieses Ansatzes unterstreicht.

Zusammenfassend lässt sich sagen, dass Proteine grundsätzlich gute Oleogelatoren darstellen, wobei insbesondere das Potenzial pflanzlicher Proteine hervorgehoben werden muss. Weiterhin ist auch das Einbringen von phenolischen Komponenten eine sinnvolle Erweiterung des System Oleogel, mit dem bestätigten Effekt einer oxidativen Stabilisierung im Oleogel. Die Arbeit zeigt, dass das Verständnis der Proteinstruktur entscheidend für die Stabilität und Funktionalität von proteinbasierten Oleogelen ist. Trotz der hohen Komplexität dieser Systeme verdeutlichen die Ergebnisse das große Potenzial von proteinbasierten Oleogelen für nachhaltige und funktionelle Lebensmittelanwendungen.