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Physico-chemical properties of extrudates and their relation to lipid incorporation and lipid oxidation

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ABSTRACT

Extrusion cooking is a key technology in food processing used to produce a wide range of products and extrudates such as cereals, cornflakes and snacks. In addition, extrusion plays a central role in the production of animal food. The oxidation of lipids in extrudates is problematic as this is associated with considerable quality deteriorations. Most conspicuous is the rancid off-flavour. Lipids can interact differently with the matrix in an extrudate and be incorporated into the matrix to different degrees. The aim of this thesis is to understand the relationship between lipid oxidation and the structural properties of the extrudate and the interaction of lipids with the matrix in order to create a basis for reducing oxidation processes in extrudates.

A fractionated lipid extraction was developed, which enabled the characterization and investigation of oxidation processes in different regions of an extrudate. Three fractions were obtained which can be assigned to surface lipids, lipids adsorbing on the inner lamellas of the extrudate and matrix-incorporated lipids. Matrix-incorporated lipids are finely dispersed in the amylose-amylopectin matrix and can only be extracted after an amylase treatment which causes a degradation of the starch matrix. It was shown that the water content of the extrusion mass influences the microstructure and the expansion. The higher the proportion of lipids incorporated in the matrix, the higher is the oxidative stability of the extrudate. Furthermore, the effects of a lipid-based coating on lipid oxidation in extrudates with different microstructures were investigated. Coating with MCT oil inhibited lipid oxidation in corn extrudates beyond the effects of dilution. This effect was particularly pronounced in porous extrudates, as it could be shown that the coating adheres mainly to the surface, migrates only slightly into the core and closes micro cracks. The formation of radicals in model systems and extrudates was investigated by electron paramagnetic resonance spectroscopy. The extrusion process formed stable protein radicals and lipid radical concentration increased simultaneously with the formation of hydroperoxides in the model system. In addition, it was demonstrated that the reaction rate of lipid oxidation is influenced by the matrix. However, the logarithmic plot of the reaction rate, based on the increase of hydroperoxide formation after the lag phase, led to an overestimation of lipid oxidation at room temperature and requires the application of more complex models.

Within this thesis, different mechanisms and matrix effects could be identified that influence lipid oxidation during extrusion and storage. They provide a basis for the derivation of factors to increase the oxidative stability of extrudates.

KURZDARSTELLUNG

Die Kochextrusion ist eine Kerntechnologie in der Lebensmittelverarbeitung, mit der eine Vielzahl von Produkten bzw. Extrudaten wie z. B. Cerealien, Cornflakes, Snackprodukte hergestellt werden. Darüber hinaus spielt die Extrusion in der Herstellung von Tierfuttermitteln eine zentrale Rolle. Problematisch ist die Oxidation von Lipiden in Extrudaten, da dies mit erheblichen Qualitätsbeeinträchtigungen einhergeht. Am auffälligsten ist das ranzige Off-Flavour. Lipide können in einem Extrudat unterschiedlich stark mit der Matrix interagieren und in unterschiedlichem Maße in die Matrix inkorporiert werden. Das Ziel dieser Arbeit ist, die Zusammenhänge zwischen Lipidoxidation und den strukturellen Eigenschaften des Extrudates bzw. Wechselwirkungen von Lipiden mit der Matrix zu verstehen, um eine Grundlage zur Reduzierung von Oxidationsvorgängen in Extrudaten zu schaffen.

Es wurde eine fraktionierte Fettextraktion entwickelt, die die Charakterisierung und Untersuchung von Oxidationsvorgängen in verschiedenen Regionen eines Extrudates ermöglichte. Es wurden drei Fraktionen gebildet, die man den Oberflächenlipiden, den Lipiden, die an den inneren Lamellen des Extrudates adsorbieren, und den Matrix-inkorporierten Lipiden zuordnen kann. Matrix-inkorporierte Lipide liegen feinst dispergiert in der Amylose-Amylopektin-Matrix vor und sind erst nach einer Amylase-Behandlung, die einen Abbau der Stärkematrix bewirkt, einer Extraktion zugänglich. Es zeigte sich, dass der Wassergehalt der Extrusionsmasse die Mikrostruktur und die Expansion beeinflusst. Je höher der Anteil an Lipiden, die in der Matrix inkorporiert sind, desto höher ist die oxidative Stabilität des Extrudates. Des Weiteren wurden die Effekte eines lipid-basierten Coatings auf die Lipidoxidation in Extrudaten mit unterschiedlicher Mikrostruktur untersucht. Es konnte gezeigt werden, dass ein Coating mit MCT-Öl eine Inhibierung der Lipidoxidation in Maisextrudaten bewirkt, die die Effekte einer Verdünnung übersteigt. Dieser Effekt war besonders in porösen Extrudaten ausgeprägt, da gezeigt werden konnte, dass das Coating hauptsächlich auf der Oberfläche haftet, nur wenig ins Innere migriert und Mikrorisse verschließt. Die Bildung von Radikalen in Modellsystemen und Extrudaten wurde mittels Elektronenspinresonanz-Spektroskopie untersucht. Durch den Extrusionsprozess wurden stabile Proteinradikale gebildet und Lipidradikalkonzentration stieg parallel zur Bildung von Hydroperoxiden im Modellsystem an. Zusätzlich konnte gezeigt werden, dass die Reaktionsgeschwindigkeit der Lipidoxidation durch die Matrix beeinflusst wird. Die logarithmierte Darstellung der Reaktionsgeschwindigkeit, basierend auf dem Anstieg der Hydroperoxidbildung nach der lag-Phase, führte jedoch zu einer Überschätzung der Lipidoxidation bei Raumtemperatur und macht die Anwendung komplexerer Modelle notwendig.

Im Rahmen der Arbeit konnten verschiedene Mechanismen und Matrixeffekte aufgezeigt werden, die Einfluss auf die Lipidoxidation während der Extrusion und der Lagerung nehmen. Sie bilden eine Grundlage für die Ableitung von Faktoren zur Erhöhung der oxidativen Stabilität von Extrudaten.

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LIST OF ABBREVIATIONS

AUC	Area under the curve
CD	Conjugated dienes
CI	Complexing index
CWT	Cell wall thickness
DMPO	5,5-dimethyl-pyrroline N-oxide
DSC	Differential scanning calorimetry
E_a	Activation energy
EMS	Extrusion model system
EPR	Electron paramagnetic resonance spectroscopy
ESR	Electron spin resonance spectroscopy
FA	Fatty acid(s)
FAMEs	Fatty acid methyl esters
HS-GC	Headspace-gas chromatography
GC-MS	Gas chromatography mass spectrometry
k	Reaction rate constant
MCFA	Medium-chain fatty acids
MCT	Medium-chain triglycerides
MTBE	Methyl tert-butyl ether
PBN	N-tert-Butyl-α-phenylnitrone
PCA	Principal component analysis
PI	Peroxidability index
POBN	α-(4-Pyridyl N-oxide)-N-tert-butylnitrone
PUFA	Polyunsaturated fatty acids
SEI	Sectional expansion index
SME	Specific mechanical energy input
TMSH	Trimethylsulfonium hydroxide
TVP	Texturized vegetable protein
μCT	Computerized microtomography

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 MOTIVATION AND OBJECTIVES

Extrusion cooking is one of the key technologies in the industrial production of low moisture foods (Schuchmann 2008). Lipid-containing extrudates, e.g., cereals, snack products, and kibbles are very susceptible to lipid oxidation because they have a high surface area with increased oxygen access (Lin et al. 1998b). Lipid oxidation is one of the major reasons of food deterioration of low moisture lipid-containing foods (Barden and Decker 2016), generating hydroperoxides that decompose to volatile oxidation products such as hexanal (Choe and Min 2006; Frankel 2005). These compounds are responsible for a reduced shelf life and rancid off-flavors and consumers do not accept rancid products of poor quality. Therefore, enhancement of oxidative stability of extrudates is of growing interest to avoid food waste. Typically, artificial or natural antioxidants are added as stabilizers (Camire et al. 2005; Kong et al. 2011; Paradiso et al. 2009). In terms of clean labeling, consumers demand products that are as natural as possible without additives (Waraho et al. 2011).

During extrusion cooking, lipids are dispersed in and on the starchy matrix (Pilli et al. 2011). Because it is known that free fatty acids and monoglycerides can be incorporated into the amylose helix and triglycerides not (Bhatnagar and Hanna 1994), the added lipids, in particular, triglycerides are entrapped in the matrix between the amylose helices. Besides the matrix-incorporation, lipids are present on the inner-surface of extrudate cell walls (Yilmaz et al. 2001) or on the outer surface. For extraction of the incorporated lipids acid hydrolysis or pretreatment with α-amylase, resulting in a destruction of the structure and a release of entrapped lipids is necessary (Strange and Schaich 2000; Thachil et al. 2014). Therefore, a promising alternative to the use of antioxidants is the optimization of the extrusion process to produce extrudates with higher lipid incorporation into the product and reduced surface lipids.

Extrudates have a sponge-like microstructure with a porous surface resulting in an increased oxygen accessibility (Chanvrier et al. 2014; Chanvrier et al. 2015; Gondek et al. 2013). A coating can act as a shield against oxygen (Hassan et al. 2018; Ganiari et al. 2017) and could increase the oxidative stability of extrudates. Kibbles are coated with fats to apply a palatant on the extrudate (Sunvold and Corrigan 2010; Ye et al. 2019). The influence of a coating with a low-oxidizable MCT oil on the lipid oxidation has not been investigated yet.

In many foods, electron paramagnetic resonance spectroscopy (EPR) was used for monitoring oxidation (Roman et al. 2010; Thomsen et al. 2000; Zawada et al. 2015). However, there are no studies that have considered the entire oxidation processes of all compounds, i.e., lipids as well as protein and carbohydrate oxidation in extrudates using EPR. The establishment of suitable methods for oxidation analysis using EPR would be an important milestone for understanding oxidation processes in extrudates.

Lipid oxidation is influenced by various factors (e.g., heat (Crapiste et al. 1999), light (Manzocco et al. 2012), oxygen (Johnson and Decker 2015a), or presence of metal ions (Schaich 1992) and antioxidants). For acceleration of lipid oxidation, e.g., elevated storage temperatures and model systems can be used. However, high temperatures are accompanied by undesired side-reactions when using different matrices (Mancebo-Campos et al. 2007; Frankel 2005). Thus, it is sophisticated to compare the kinetic of lipid oxidation in extrudates and model systems and use the Arrhenius-like behavior to predict the shelf life of low moisture foods.

The main objective of the present thesis was to understand the effect of structural properties of extrudates on lipid oxidation.

Therefore, a complete approach was followed to investigate the lipid binding and lipid distribution in extrudates with a novel developed lipid extraction method. In addition, the oxidation status of the resulting lipid fractions (surface lipids, inner-surface lipids, and matrix-incorporated lipids) was analyzed. Simultaneously, the effect of the feed-water content of the extrusion mass on a lipid-containing corn extrudate and its relationship to the microstructure were investigated. (**Chapter 3**). In addition, the effect of a lipid-based MCT-oil coating on the lipid oxidation in extrudates was evaluated under consideration of its migration behavior (**Chapter 4**). For the measurement of early stages of oxidation reactions, the EPR was chosen to develop and implement a suitable method for measurement of radicals in extrudates and extrusion model systems (**Chapter 5**). The results of an oxidation kinetic study on the accelerated storage of extrudates and an extrusion model system under elevated temperatures complete this work (**Chapter 6**). The latter study was a pre-test for an international interlaboratory study, which is not part of the thesis and will be completed in 2020.

For this doctoral thesis, the following hypotheses were stated:

Hypothesis 1: The microstructure of extrudates influences lipid incorporation in the matrix of extrudates.

a) **Different microstructures can be realized by varying the feed-water content. (Manuscript 1)**

Background:

Extrusion cooking is a complex process with a variety of mutually influencing parameters, determining the final extrudate (Schuchmann 2008). During extrusion cooking proteins denature and the starch granules swell under water absorption and lose their crystalline structure due to the gelatinization (Bhattacharya and Hanna 1987). Therefore, an amorphous matrix is formed and lipids are finely dispersed in this matrix. One factor, affecting the viscosity of the melted dough in the extruder, and thus the energy input, is the feed-water content (Alvarez-Martinez et al. 1988; Ding et al. 2005; Ilo et al. 1996). Lower energy input affects the expansion behavior and in turn also the microstructure. In extrudates with thicker cell walls more lipids could be incorporated in the starchy matrix than in extrudates with thin cell walls.

Experimental approach:

Extrudates with varying microstructure will be produced using different feed-water contents in a cornmeal-based premix, which will be extruded in a twin-screw extruder. The microstructure of the extrudates will be characterized by physical parameters, such as expansion and density. Furthermore, computerized microtomography will be used as a noninvasive method to analyze morphology of the microstructure.

b) **The use of a fractionated lipid extraction protocol enables to determine the degree of incorporation of lipids in extrudates and the effect on lipid oxidation.**

Background:

Extrusion cooking leads to the formation of an amorphous matrix. At the same time, lipids are dispersed by the shear forces in and on this starchy matrix (Pilli et al. 2011). Free fatty acids and monoglycerides can be incorporated into the amylose helix. This interaction is called amylose-lipid-complex. Triglycerides cannot enter the helix because of steric hindrance

(Bhatnagar and Hanna 1994). Therefore, it must be considered that during extrusion the added lipids are entrapped in the matrix between the amylose helices and not in the amylose helix. In addition, lipids can be incorporated in the cells of extrudates forming the "inner-surface lipid fraction" (Yilmaz et al. 2001) or adhere to the outer surface of the extrudate particles. An indicator of lipid incorporation is a reduced extractability with conventional solvent extraction procedures. Methods for extraction of the complete lipids in a sample, such as Soxhlet method, can degrade lipids and complicate further analyses, e.g., measurement of oxidation (Strange and Schaich 2000). An alternative is the pretreatment with α-amylase, resulting in a destruction of the structure and a release of entrapped lipids (Strange and Schaich 2000; Thachil et al. 2014).

Experimental approach:

A fractionated lipid extraction protocol will be developed that enables the measurement of three different lipid fractions. The extracted lipids will be characteristic for specific regions of an extrudate and different degrees of entrapment (surface lipids, inner-surface lipids, and matrix-incorporated lipids). The extraction protocol will be designed to allow calculation of the distribution behavior and further analysis with the extracted lipids.

Hypothesis 2: Lipid incorporation in extrudates affects their oxidative stability. (Manuscript 1)

a) **Non-incorporated lipids adhering to the surface of extrudates are prone to oxidation.**
b) **Lipids incorporated in the extrudate matrix have a higher oxidative stability.**

Background:

Lipid oxidation is essentially influenced by the presence of oxygen and its access to susceptible lipids (Johnson and Decker 2015a). Because of expansion at the die, extrudates have a high specific surface, which results in increased oxygen exposure, favoring oxidation (Lin et al. 1998b). Binding of lipids into the matrix of extrudates was associated in studies with an increase in oxidative stability (Thachil et al. 2014; Guzman et al. 1992). However, such studies have so far only investigated total oxidation and attributed this effect to the degree of lipid binding

without direct proof. No differentiation was made between the oxidation status in different lipid fractions.

Experimental approach:

Extrudates will be stored under accelerated oxidation conditions at elevated temperatures. The oxidative stability of the three different lipid fractions, obtained by fractionated lipid extraction, will be evaluated using a spectrophotometric method for the determination of the hydroperoxide concentration. Furthermore, the hexanal formation, a marker for rancidity, will be analyzed of the whole extrudate to confirm the oxidation status by headspace-gas chromatography.

Hypothesis 3: The coating with a low oxidizable oil inhibit lipid oxidation of the sunflower/rapeseed oil in the extrudates. The coating provides a protective effect that exceeds the dilution effect by enhancing the lipid content of extrudates with low oxidizable oil. (Manuscript 2)

Background:

The expansion of the extrudates at the die results in a porous structure with a high specific surface. Therefore, lipids of extrudates have high accessibility to oxygen, which can promote lipid oxidation (Andersen et al. 2000). Coatings were applied on foods to protect a compound or the food against environmental influences such as oxygen, moisture, heat or light (Hassan et al. 2018; Ganiari et al. 2017). A lipid-based edible medium-chain triglycerides (MCT) coating may counteract the oxygen accessibility during storage and could improve the oxidative stability. In addition, sealed microcracks would affect the shelf life positively (Gray et al. 2008).

Experimental approach:

Corn extrudates coated with the low-oxidizable MCT oil will be examined and the effect of the coating on the lipid oxidation will be evaluated. The allocation behavior of the applied coating will be evaluated by measuring the fatty acid profile of the extracted lipid fractions and by visualization of the coating using fluorescence microscopy and computerized microtomography.

Hypothesis 4: The combination of high temperature, pressure, and shear stress during extrusion is considered to result in radical formation of different nature. The investigation of model systems varying in their composition (lipid, proteins, and carbohydrates) and varying sample preparation of the matrix enables the annotation of radicals measured by electron paramagnetic resonance spectroscopy. (Manuscript 3).

Background:

Extrusion cooking is a high-temperature short-time process that enables the formation of radicals, leading to oxidation in the extrudates (Schaich and Rebello 1999a). The electron paramagnetic resonance spectroscopy (EPR) measures paramagnetic species with an unpaired electron, e.g., free radicals. Lipid radicals are short-lived radicals with a half-life up to few nanoseconds. For detection, these unstable radicals must be captured with a spin trap (e.g. PBN or POBN) (Davies and Hawkins 2004; Cui et al. 2017). During the oxidation of proteins and carbohydrates derived stable radicals are formed, which can be measured directly (Schaich and Rebello 1999a). The oxidation of both, short-lived lipid radicals and stable protein and carbohydrate radicals in extrudates have not been investigated, yet.

Experimental approach:

For analysis of complex starch-protein-lipid model systems and extrudates by EPR, suitable methods will be developed. Short-lived lipid radicals will be analyzed using a spin trap. During method development, the spin traps POBN and PBN will be evaluated and suitable incubation conditions will be examined. In addition, the lipid extraction protocols, which use different solvents and extraction procedures will be optimized to maximize the concentration of extracted radicals. The assessment of the method will be performed with a stripped oil sample and detected lipid radicals in the oil samples and the extrudates will be identified by simulation of the EPR spectra. Stable radicals will be analyzed directly in the ground sample without extraction. During method development different glass capillaries will be examined to identify a suitable sample holder for the ground extrudate in the EPR. The origin of the detected stable radicals will be identified by comparing the hyperfine coupling constants of the radicals in extrudates with the measured values in model systems made with different corn flour constituents (cornstarch, cornmeal, zein, and cornstarch with zein).

To evaluate formation of radicals and development of lipid oxidation, both, complex starch-protein-lipid model systems as well as extrudates will be examined and the results will be compared with conventional markers for lipid oxidation.

Hypothesis 5: The sample matrix of extrudates, an extrusion model system, and bulk oil influences lipid oxidation and the Arrhenius-like behavior derived from different accelerated temperatures. (Manuscript 4)

Background:

As low-moisture foods, extrudates are relatively stable against oxidative deterioration (Labuza and Dugan 1971) and oxidation studies are time-consuming. Classically, lipid oxidation studies are performed at elevated temperatures to accelerate the oxidation rate (Ragnarsson and Labuza 1977). The combination of different storage temperatures can be used for prediction of the shelf life at room temperature based on the Arrhenius equation (Manzocco et al. 2012). Another approach is the use of purified oils, which are void of antioxidative tocopherols. With these oils, model systems can be prepared that imitate the real product but oxidize in less time (Bauer et al. 2013).

Experimental approach:

Extrudates, commercial oil used for the production of extrudates, purified vegetable oil and an extrusion model system prepared with this purified oil will be oxidized under accelerated shelf life testing conditions at elevated temperatures (20–120 °C). Lipid oxidation will be monitored during storage and side reactions, e.g., the non-enzymatic browning due to the Maillard reaction, will be examined. Furthermore, lipid oxidation kinetic parameters, such as the activation energy and the reaction rate constant will be calculated based on the Arrhenius equation. Finally, the suitability of the extrusion model systems as model system for lipid oxidation in extrudates will be discussed.

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CHAPTER 2

THEORETICAL BACKGROUND

2. THEORETICAL BACKGROUND

2.1. EXTRUSION

2.1.1. EXTRUSION COOKING

Extrusion technology (lat. *extrudere*) is a manufacturing process in which raw materials (the dough or premix) are continuously forced through a formative die. The products of this production stage are called extrudates. Extrusion cooking is a high-temperature short-time process where the starches gelatinize under formation of an amorphous matrix.

The first extruders were developed in the 19th century for the use in sausage- and rubber processing. Extrusion cooking as conducted today was applied for the first time in the 30s of the last century in the production of ready-to-eat-cereals (Riaz 2000). In the 1940s expanded corn snacks and in the 1950s the first extruded kibbles were launched (Schuchmann 2008). Since then, extrusion has become one of the most widely used technologies in the food industry (cereals, snack products, pasta, texturized vegetable protein (TVP), flat bread, kibbles, etc.) (Alam et al. 2016). This can be explained by the multitude of processes (e.g. mixing, kneading, dispersing, homogenization, gelatinizing, texturing, cooking, melting, roasting, caramelizing, sterilizing, forming and drying) that can be realized with a single device (Meuser et al. 2004; Riaz 2000).

In the present work, a twin-screw extruder with co-rotating intermeshing screws was used for the extrusion experiments. Twin-screw extruders are continuous process plants with two screws rotating in opposed (counter-rotating) or in the same direction (co-rotating).

Figure 2.1 illustrates the schematic structure of an extruder. The preconditioned raw materials (premix) are dosed by a weight feeder to the screws that convey the premix into the heatable barrel. Thereby a characteristic temperature profile can be applied to the extrudates. The consistent and stable material feed by a weight feeder is an important factor for the production of a homogeneous, reproducible and standardized product (Frame 1994).

The extruder can be divided in three sections. In the feeding zone, the screw elements had a high free volume that enables the transport of the raw material into the barrel. In the transition and kneading zone, where the screw pitch decreases, the product is compressed and the temperature and pressure increase. The last section, the cooking zone, excels by an increased compression of the product and an influx of mechanic (pressure, shear forces) and thermic

energy (Huber 2000). The friction between the extrusion material and the screw, influenced by water and lipid content in the recipe, results in shearing, heating and subsequently gelatinization of the starch. This process stage transforms the powders into amorphous fluids that are pressed under high pressure through a formative die. At the formative die orifice, the product expands and an automatic cutter device cuts the endless strand in the desired length. The extrudate contracts, cools down and cures. Finally, the extrudates are dried with a belt dryer or a drum dryer, for example.

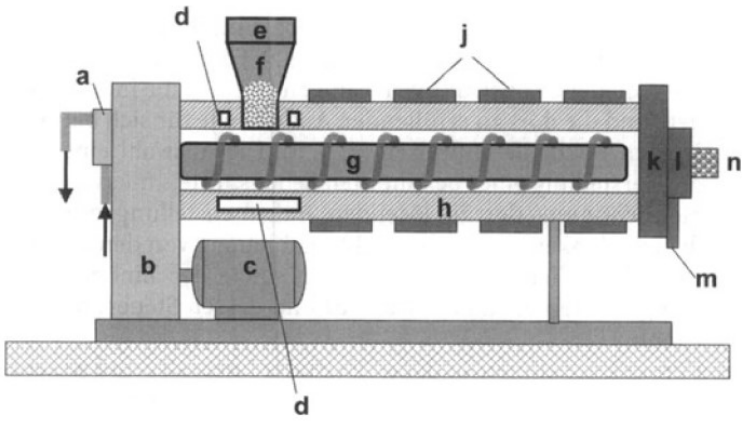

Figure 2.1: *Schematic structure of an extruder*
a) controller for the barrel heating; b) reducing gear for the screw; c) engine; d) cooling jacket; e) hopper; f) raw material; g) screw; h) barrel; j) thermocouples with heating jackets; k) + i) die; m) die heating; n) extrudate. (Meuser (2004) p. 523)

The expansion (**Figure 2.2**) is a characteristic of extrudates and is influenced by high pressure and the temperature-difference between the inside of the barrel and the periphery (Alvarez-Martinez et al. 1988). The extruder heats the water above 100°C, like in a pressure cooker, and at the die, the water can vaporize abruptly under formation of bubbles and product cavities (Abu-hardan et al. 2011). The expansion process begins at the die when the overheated water in the dough begins to evaporate. The steam-volume increases to a maximum where the steam pressure inside the expanded dough is in equilibrium with the external air pressure. As the dough cools down to room temperature, the vapor pressure inside decreases, the dough shrinks (volume decreases), loses moisture and hardens. Factors affecting the expansion are process parameters that had an influence on the temperature and the pressure in the extruder barrel. These include among others the water content, screw speed, and feed rate. Studies showed that a higher feed-moisture content resulted in extrudates with a higher density and a lower

expansion (Basediya et al. 2013; Ding et al. 2006; Gulati et al. 2016). In general, the density of extrudates is inversely related to the expansion ratio. The expansion of extrudate samples can be characterized by measuring the expansion index, a ratio between the diameter of the extrudate and that of the die, their bulk density or by analyzing the microstructure with imaging techniques such as the computed microtomography.

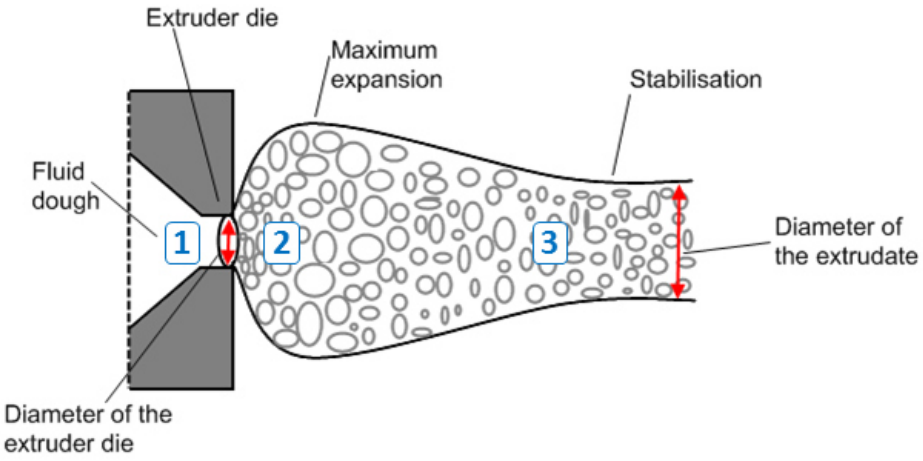

Figure 2.2: *Characteristic of the expansion process during extrusion: 1: In the extruder barrel, the premix undergoes compression, friction and melts to a fluid dough due to the rotating screws and the heating. At the same time, pressure and temperature rise. 2: At the die orifice an abrupt expansion occurs due to the pressure and temperature drop as well as evaporating water. Direct after the die the extrudate has its maximum expansion. 3: This is followed by contraction and stabilization of the porous structure through hardening, drying and recrystallization processes. (Own illustration)*

2.1.2. IMPACT OF PROCESS PARAMETERS AND OPERATING CONDITIONS ON THE PRODUCT RESPONSE

The extruder is a black box and homogenization, gelatinization, denaturation and heating are in a complex interrelationship, influenced by extrusion processing variables. These factors affect the extrudate quality and can be categorized in independent and dependent variables. Independent variables can be controlled directly (e.g. recipe formulation, barrel temperature, feed rate, extruder configuration, screw speed). Dependent variables respectively parameters (retention time, throughput, specific mechanical energy input (SME), product temperature and pressure) are controlled by changing independent variables (Huber 2000; Frame 1994; Basediya et al. 2013). The configuration of the screw elements influences the residence time (left handed

screw elements), the conveying and the efficiency of homogenization and dispersion of the dough and so the SME. For consistency of the extrusion process, a precise throughput is necessary. Discontinuous feeding can cause pressure fluctuations and changed residence time in the extruder. Therefore, accurate feeding of the premix to the extruder, with a high linearity and stability, is important for homogenous product. Increasing the screw speed results in an increase of SME, temperature and expansion and a decrease of the torque, pressure, and residence time (Yilmaz et al. 2001). Higher feed rates can cause lower temperature in the extruder and in turn reduced expansion ratios and higher density (Ding et al. 2006).

The specific mechanical energy is a scale-independent measure of the mechanical energy input that an extruder transfers to the extrudate. It enables the comparison of different extrusion systems producing the same product. The SME depends among others on the water content, screw speed, throughput and can be calculated according to the following equation that is in line with (Onwulata et al. 1994):

$$SME\ \left[\frac{kWh}{kg}\right] = \frac{\frac{n_{act}[rpm]}{n_{max}[rpm]} * torque\ [\%] * P_{max}\ [kW]}{feed\ rate\ [\frac{kg}{h}]}$$

where n_{act} is the screw speed in rpm used in the experiment, n_{max} is the maximum screw speed in rpm, torque is the used torque from the motor in %, P_{max} is the motor power of the extruder in kW and the feed rate is the amount of premix that is fed of the gravimetric weight feeder to the extruder in kg/h.

2.1.3. RAW MATERIALS AND PHYSICOCHEMICAL EFFECTS DURING EXTRUSION-COOKING

Raw materials for extrusion cooking are carbohydrates (starches, meals or grits from corn, rice, wheat, barley, oat, etc.), proteins, lipids, fibers, water, and possible flavoring or functional ingredients. Basic ingredients can be premixed in a preconditioner (advantage: better blending of the raw materials) or they were mixed directly in the extruder.

Carbohydrate components play an essential role because of their structure-forming properties. The starch loses under temperature and water influence its crystalline structure and turns into a fluid mass with increased water binding capacity (Ilo et al. 2000). This transition is called gelatinization. Extrusion cooking resulted in incomplete gelatinization and a part of the starch remains in its native state (Sotillo et al. 1994). This ratio, the gelatinization degree, can be

determined by various methods (enzymatic assay (Chiang and Johnson 1977), differential scanning calorimetry).

Besides fats and oils, water act in the extrusion process as a lubricant and plasticizer by reducing the friction and the viscosity of the extrusion mass. This affects the retention time, the SME and lowers the die pressure and expansion ratio (Ilo et al. 2000). Extrusion processes range in moisture content from 10 to 40 %. The total water content depends on the water content of the raw materials and on the added water. Lin et al. (1997) state, that the degree of starch gelatinization was significantly decreased with increased fat content of the extrudates as well as with higher water contents (Lin et al. 1997).

In special extrudates from high moisture extrusion, such as texturized vegetable protein (TVP), proteins from soy, wheat, pea, or lupine are used as main ingredient (Pietsch et al. 2017). These products with a meat-like structure (fibers, layers) are offered as vegan meat alternatives. Proteins added or as a part of other ingredients, such as flours, denaturize under the process conditions of extrusion cooking and become better digestible. Because of cross-linking, proteins contribute to structural characteristics of extrudates (Moisio et al. 2015b; Chanvrier et al. 2015) In addition, there is the formation of color and flavor products in the context of the Maillard reaction (Singh et al. 2007).

2.1.4. LIPID INCORPORATION DURING EXTRUSION COOKING

Cornstarch is composed of amylose and amylopectin in a ratio of approx. 23:77 (Liu et al. 2006). Extrusion cooking processes with its thermal impact leads to gelatinization of the starch (Chiang and Johnson 1977). The starch granules swell under water absorption and lose their crystalline structure due to the gelatinization (Bhattacharya and Hanna 1987). At the same time, proteins denature. Therefore, an amorphous matrix is formed and lipids are able to enter this matrix. Simultaneously, the lipid droplets were finely dispersed in the amorphous matrix because of the shear forces. Based on a literature review, different theories exist for the phenomenon of lipid binding and lipid entrapment during extrusion processes.

The conformation of amylose changes in the presence of possible ligands to the so-called V-amylose with a single helix (Putseys et al. 2010). The inside of the helix is hydrophobic and the outside is hydrophilic. Free fatty acids, monoglycerides, but also aroma compounds (e.g. decanoic acid, hexanoic acid or 1,5-decanolactone), could act as complexing ligand and can so be incorporated into the amylose helix (**Figure 2.3**) (Biais et al. 2006).

In this case, the polar hydrophilic head of the fatty acids is outside of the amylose helix because of steric hindrance. Furthermore, a high degree of unsaturation, double bonds, hinder the access into the helix. This kind of interaction is called amylose-lipid-complex.

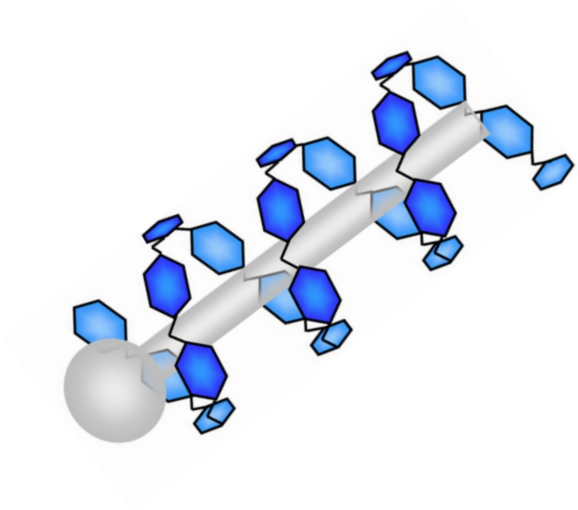

Figure 2.3: *Amylose-lipid complex: Amylose helix complexed with a ligand, e.g., a free fatty acid. modified from (Putseys et al. 2010)*

Bhatnagar and colleagues reported that the lipid type and the amylose-content strongly influence the extent of complex formation (Bhatnagar and Hanna 1994). Triglycerides cannot enter the helix because of steric hindrance. In corn starch, the amylose-lipid-complex formation could be observed with added fatty acids (myristic, stearic and behenic acid) but no binding could be measured with tristearin (Bhatnagar and Hanna 1994). A long retention time (e.g. by reduction of the screw speed) supports the complex formation between amylose and lipids. Furthermore, the formation of amylose lipid complexes is directly connected to the gelatinization of the starch and can be measured by DSC (differential scanning calorimetry) analysis (Genkina et al. 2015). Bhatnagar and Hanna (1994) showed in addition that with increased amylose content of the starch/meal, more complexes were formed in extrudates. Beside the DSC, also the complexing index (CI) can be used to determine the extent of amylose-lipid complex formation. The CI expresses the iodine-binding capacity of amylose. If amylose is complexed with lipids, the iodine-binding capacity is reduced (Putseys et al. 2010; Lau et al. 2016).

The objective of many studies was the observation of starch-lipid interactions and the formation of amylose-lipid complexes in artificial starch–free fatty acid suspensions. However, starch-triglyceride interactions as they occur in the extrusion cooking process were not investigated. Since free fatty acids occur only to a very small extent in commercially available products or extrudates, it can be assumed that amylose-lipid complexes play a subordinate role here. Rather, it must be considered that the added lipids are distributed in the matrix and not in the amylose helix. It can be assumed that lipids (triglycerides) are entrapped between the amylose helices and are thus incorporated in the amylose matrix.

Furthermore, it is possible that the lipid droplets are weakly bound on the inner-surface of extrudate cell walls. Yilmaz et al. (2001) showed that lipid droplets can be localized on the surface of starch-oil extrudates by scanning electron microscopy. Most sensitive to lipid oxidation are the surface lipids, which can be attacked by direct contact with oxygen. The different degrees of lipid inclusion are visualized in **Figure 2.4**. Based on the extractability the lipid fractions in extrudates could be divided into surface lipids, inner-surface lipids, and matrix-incorporated lipids. The oil allocation can be analyzed with the newly developed extraction protocol for lipids out of extrudates (Paper 1). The protocol offers the possibility to differentiate between the three different lipid fractions. The method is based on different solubilities of lipids in solvents and a final amylase treatment for matrix destruction and lipid release out of the matrix. The principle to use an amylase treatment for lipid release from the matrix based on studies from Thachil et al. (2014) and Strange and Schaich (2000).

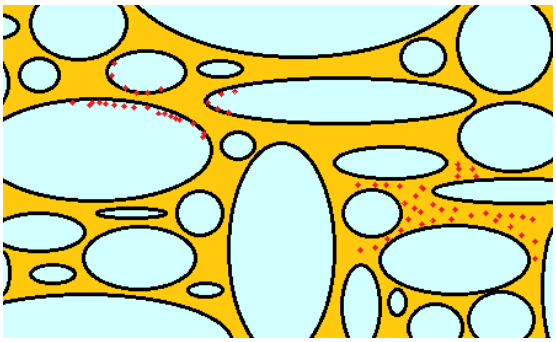

Figure 2.4: *Theory of lipid incorporation in extrudates (lipids are marked as red dots on the cell walls or inside the cell walls) (own illustration)*

2.2. COATING TECHNIQUES USED FOR EXTRUDATES

Encapsulation processes, e.g., liposomic entrapment (Frenzel et al. 2015), spray drying (Serfert et al. 2009), and fluidized bed coating (Berg et al. 2012), are used in the food industry to improve the product stability by protection of the encapsulated compound against environmental influences such as oxygen, moisture, heat or light. Further reasons for encapsulation are to mask undesirable taste and odor to manufacture a product with a good consumer acceptance. Another typical technique is the fluidized bed coating for microencapsulation of bioactive substances, which can be targeted released in the human gut (Theismann et al. 2019).

Edible coatings are defined as ingredients that interact between food and its environment. The most common hydrophobic barriers include oils, fats, and waxes (Morillon et al. 2002).

Many studies with lipids as coating layer deal with the function as a barrier against moisture, oxygen, or to protect against intense aroma (Mehyar et al. 2012; Ye et al. 2019; Garcia et al. 2000; Stuchell and Krochta 1995). Studies with medium chain triglycerides used as coating against oxidative deterioration are missing. Medium chain trigylcerides (MCT) are lipids with medium-chain fatty acids comprising 6–10 carbon atoms (Bach and Babayan 1982; Marten et al. 2006). Because of their fatty acid profile MCT have a high oxidative stability (Raudsepp et al. 2014). Based on the morphology of the coating different types can be distinguished (**Figure 2.5**). Coated extrudates can be classified as a simple respectively irregular encapsulation with a core (the extrudate) surrounded with a coating layer. Lipids that were encapsulated during extrusion cooking in the starchy-matrix can be classified as a matrix-incorporation.

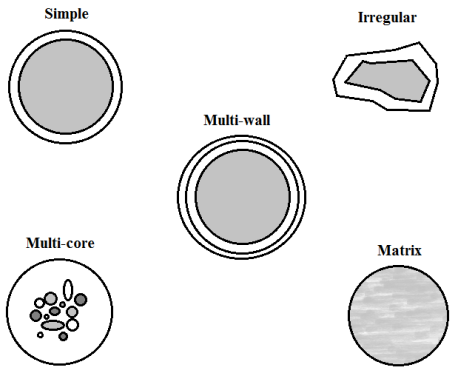

Figure 2.5: *Various forms for encapsulation of bioactive substances (own illustration)*

The coating of extrudates, in particular kibbles, is commonly applied in the pet food industry and based on the morphology it can be described as a simple monolayer encapsulation with an irregular shape. These fat coatings were made, for example, from of poultry fat (Ye et al. 2019).

Often, additional flavorings and appetizer are added to the coating to increase the palatability for the pets (Sunvold and Corrigan 2011). In addition, antioxidants can be added to the coating material (Ganiari et al. 2017; Ye et al. 2019). Lipid-based edible coatings may counteract the increased oxygen accessibility of extrudates during storage and block moisture and oxygen (Hassan et al. 2018; Kester and Fennema 1989). Studies revealed that acetylated-monoglyceride coating slow oxygen diffusion in frozen salmon (Stuchell and Krochta 1995) and whey-protein-coated peanuts oxidized slower than uncoated peanuts during shelf life testing (Maté and Krochta 1996; Maté et al. 1996; Lee and Krochta 2002). This data suggests that also in extrudates, a coating layer could contribute to an increased stability of the lipids. Corresponding studies have not yet been carried out.

2.3. LIPID OXIDATION

2.3.1. MECHANISM OF LIPID OXIDATION

Unsaturated lipids occurring in natural and processed foods play an important role in nutrition (Mozaffarian et al. 2010). However, lipid oxidation is the major cause of deterioration of the food quality. The field of lipid oxidation is an expanding research area that is still not fully explored. Especially the mechanisms of lipid oxidation in extrudates are complex. By comparison with emulsions or bulk oils, in extrudates, air-lipid-starch interfaces affect the autoxidation and the high temperatures during extrusion processing can contribute to radical formation (Lampi et al. 2015).

This chapter aims to inform about the fundamental mechanisms of lipid oxidation in extrudates. Lipids (solid fats and liquid oils) are important structural and functional components in foods that contribute largely with its high caloric density to the energy intake of human. Lipids occur naturally as triglycerides, but free fatty acids may also be present in small amounts in oils and fats. Triglycerides are esters of glycerol combined with three fatty acids that can be saturated, monounsaturated or polyunsaturated. Especially long-chain polyunsaturated fatty acids (linoleic and linolenic acid) play an important role in human nutrition because of their health benefits. The degree of oxidizability increases with increasing amounts of unsaturated lipids (Frankel 2005). The lipid oxidation is accompanied by the formation of secondary oxidation products that are detectable as rancid off-flavor. Lipid oxidation is therefore one of the most undesirable reactions in lipid chemistry and the industrial food process chain. The lipid

oxidation reactions lead to quality losses in taste, smell, and nutritional value. Affected foodstuffs can cause great economic loss, because they are not suitable for sale and consumption.

The main mechanism of lipid autoxidation is the oxidation of free radicals in a radical chain reaction. It is the reaction of molecular oxygen with organic compounds and its mechanism is extensively reviewed (Labuza and Dugan 1971; Choe and Min 2006). Briefly, the cycle of lipid oxidation can be differentiated into the three steps initiation, propagation, and termination.

In the initiation-phase, unsaturated fatty acids (LH) lose a hydrogen radical (H•) in the presence of initiators (I) and lipid alkyl radicals (L•) are formed. This phase is promoted by initiators such as light, heat and present metal ions (Frankel 1984; Belingheri et al. 2015; Schaich 1992).

Initiation:

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

This is followed by the propagation phase where the alkyl radicals (L•) react with atmospheric oxygen under formation of peroxyl radicals (LOO•). These peroxyl radicals can abstract a hydrogen from unsaturated lipids (LH) under formation of relatively stable and nonvolatile lipid hydroperoxides (LOOH) (Frankel 1984). The propagation reaction is the chain reaction in the true sense of the word. The propagation phase takes place as long as there are possible reactants, e.g. fatty acids with unsaturated double bonds.

Propagation:

$$L^{\bullet} + O2 \longrightarrow LOO^{\bullet}$$
$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$$

The decomposition of hydroperoxides into alkoxyl radicals (LO•) can be attributed to β-scission reactions, which were promoted by heat, light, and transition metals. Alkoxyl radicals are very reactive and can break the aliphatic chain of fatty acids under formation of low molecular weight volatiles with rancid smell (Barden and Decker 2016).

Thermal decomposition of hydroperoxides:

$$LOOH \longrightarrow LO^{\bullet} + OH^{\bullet}$$

In the presence of antioxidants (AH), the radical chain reaction can be interrupted. Antioxidants, e.g. α-tocopherol provide a hydrogen atom that can react with the peroxyl radical to stable radicals (A•) (Halliwell and Chirico 1993). These radicals react with other peroxyl radicals under formation of unreactive products in a termination reaction that stop the chain reaction (Porter et al. 1995).

Antioxidants:

$$LOO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$
$$A^{\bullet} + LOO^{\bullet} \longrightarrow non - radical\ products$$
$$A + A^{\bullet} \longrightarrow non - radical\ products$$

In the termination phase, peroxyl radicals accumulate and react with each other under formation of non-radical products, such as fatty acid dimers (Frankel 2005).

Termination:

$$LOO^{\bullet} + LOO^{\bullet} \longrightarrow non - radical\ products$$

2.3.2. KINETIC OF LIPID OXIDATION

Kinetics of lipid oxidation provide information about the course of lipid oxidation (Labuza and Dugan 1971). The kinetic of oxidation of unsaturated lipids is depicted in **Figure 2.6**. It illustrates that measuring just one parameter does not help to draw meaningful conclusions about status of lipid oxidation, because low hydroperoxide concentrations can be measured both in fresh oil and in a strongly oxidized lipid sample. Therefore, in lipid oxidation studies it is advisable to determine several oxidation products, primary hydroperoxides and secondary volatile aldehydes. Propagation and β-scission can be monitored and reliable statements about the oxidation status are possible.

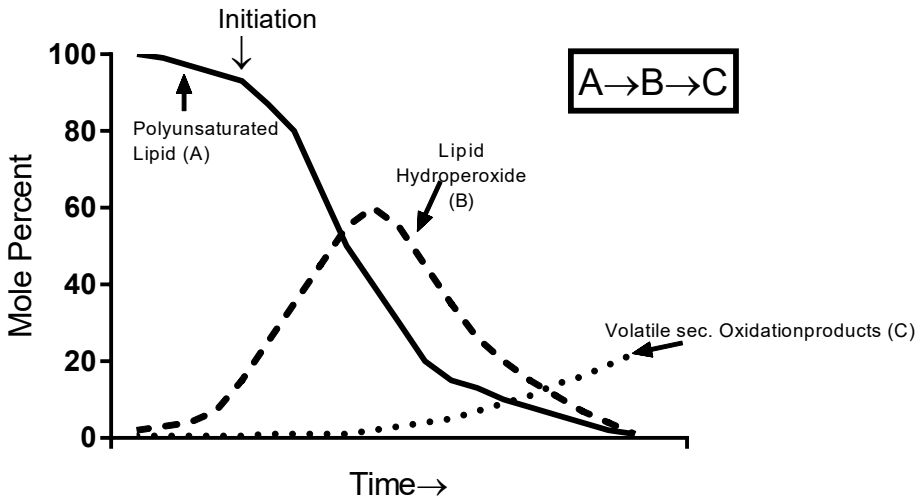

Figure 2.6: *Kinetic of the oxidation of unsaturated lipids and the formation of primary and secondary oxidation products (own illustration, modified from Gardner (1983))*

In accelerated shelf life testing, the rate of oxidation is of high interest (Ragnarsson and Labuza 1977). At high temperatures, the oxidation reactions are fastest, implementing short analyses for shelf life determination. In chemical kinetics, the temperature dependence of the reaction rate of a chemical reaction is represented by the following Arrhenius equation (Arrhenius 1889):

$$k = A * e^{-\frac{E_a}{RT}}$$

where *k* is the reaction rate coefficient, *A* is a pre-exponential factor, *Ea* is the activation energy, *R* is the universal gas constant (8.31446 $Jmol^{-1}$ K^{-1}), and *T* is the absolute temperature [K]. The logarithmic form of the Arrhenius equation can be used for calculation of E_a (Frankel 1993). It states that the logarithmic of the reaction rate (ln k) varies linearly with the reciprocal of the temperature (1/T) with the slope $-E_a/R$ and is shown as follows:

$$\ln k = A' - \frac{E_a}{R} * \frac{1}{T}$$

The Arrhenius equation is derived from a plot of log k vs. 1/T(K), called Arrhenius plot. For simplification of the lipid oxidation kinetics, a linear approach can be assumed. In fact, the oxidation cannot be described over the complete oxidation time with a linear equation because lipid oxidation reactions do not follow a first-order kinetic (Barden and Decker 2016). Rather, the lag phase is followed by the propagation phase, which is characterized by an exponential growth of the lipid oxidation products.

Resolved according to E_a, the activation energy can be calculated from the slope of the Arrhenius equation using the following formula:

$$E_a = -\frac{Ln\ k}{T} * R + A'$$

The activation energy (E_a) is an empirical variable that is measured in joules per mole (J/mol) or kilojoules per mole (kJ/mol). It is the energetic barrier that must be overcome by the reaction partners during a chemical reaction. In general, the lower the activation energy, the faster the rate of reaction and high activation energies inhibit chemical reactions (Labuza and Dugan 1971). The E_a depends on the presence of prooxidative metal catalysts (lower E_a) or antioxidants (higher E_a) (Tan et al. 2001; Golmakani et al. 2019; Frankel 2005)

2.3.3. FACTORS AFFECTING LIPID OXIDATION IN EXTRUDATES

Lipids are necessary for extrusion cooking and contribute as structural and functional components to the extrusion process (Ilo et al. 2000). The deterioration of lipid-containing extrudates is a big problem as rancidity is undesired from the consumer and the industry. Oxidative reactions occur in every lipid-containing matrix. High temperatures during extrusion cooking promote oxidation, i.e., decrease the lipid stability (Rao and Artz 1989). As shown by Rao and Artz (1989) increasing extrusion temperatures resulted in an increased transition metal content, too (Rao and Artz 1989).

Extrudates are prone to oxidation because of their high specific surface (high surface / volume ratio) with an increased oxygen accessibility (Lin et al. 1998; Gray et al. 2008). The lipids in extrudates underlie the lipid autoxidation during all stages of production and storage and their stability against oxidation is determined by numerous factors (e.g. oxygen contact, light, a_w-value and moisture, temperature, presence of prooxidants such as metal ions, and use of antioxidants). The highest impact has the composition of the lipids and their fatty acid profile. On the one hand are triglycerides build of fatty acids with a high degree of unsaturation (high number of double bonds) which are prone to oxidation. Peroxyl radicals can attack these double bonds under formation of lipid hydroperoxides (Frankel 1984). On the other hand, there are lipids with saturated short or medium-chain fatty acids. These fatty acids with no double bonds have a very high oxidative stability.

The total lipids in a sample can be attributed to the lipid content in the extruded premix and a possible lipid amount that is added when a coating is applied. The lipids were incorporated in different lipid fractions (surface lipids, inner surface lipids, and matrix-incorporated lipids)

based on their allocation behavior (**Chapter 2.1.4**). Because of their different oxygen accessibility, the oxidative stability is affected by the degree of lipid binding. Extrusion processing parameters such as added fat content, feed moisture content, temperature profile, screw speed, shear forces, and the expansion affect the lipids, their distribution in the extrudate and certainly the extent of lipid oxidation (Lin et al. 1998). With increasing water contents, the extrusion mass is "liquefied" and the viscosity lowers. Water acts as a plasticizer, causing a lower pressure in the extruder. Simultaneously, the increase of the temperature is lower because the friction is reduced. These changes lead to a lower energy input, which results in a reduced SME (Ilo et al. 1996; Kaisangsri et al. 2016). At the die orifice, the extrudate expands less vigorously (Moisio et al. 2015a; Ding et al. 2005).

Classically, antioxidants are added to improve the oxidative stability of extrudates. In the recent years, enormous research has been conducted to replace artificial antioxidants with natural antioxidants. For example, phenolic compounds (Viscidi et al. 2004) as well as food extracts such as rosemary extract (Kong et al. 2011) were added to extrudates resulting in an increased shelf life. Beside rosemary extract, ascorbic acid and tocopherol were investigated in extruded cornflakes, with the result of a reduced amount of volatile oxidation compounds (Paradiso et al. 2009). But also the addition of antioxidant-rich foods and by-products, e.g., Ginkgo, potato peel, onion, wheat bran, could be an option to improve the oxidative stability, of extrudates (Camire et al. 2005; Brennan et al. 2011). Bauer et al. reported that ground corn fiber, with an high amount of ferulic acid and polyaminoconjugates, is an effective antioxidant in fish oil-containing emulsions (Bauer et al. 2013). The addition of corn fibers could also be easily implemented in extrudates but appropriate studies are still outstanding.

Additional antioxidant acting substances could be produced by Maillard reactions during extrusion cooking (Camire 2000). Maillard reaction, also called non-enzymatic browning, is the reaction of amino groups from proteins with carbonyl groups from carbohydrates under high temperatures. Both compounds are common in foods and the reaction leads to browning of the product and formation of volatiles (Singh et al. 2007). Therefore, Maillard reactions can be measured by change of color or by the formation of (volatile) Maillard reaction products. From Maillard reaction products it is known that they increase the antioxidative activity with increasing browning of a sample (Mastrocola and Munari 2000). For example, extruded rye bran had higher concentrations in Maillard reaction products in extrudates produced with lower water contents (13 and 16% water) and these extrudates had in turn a higher oxidative stability (Moisio et al. 2015a).

To avoid lipid oxidation and rancidity in extrudates during storage, the inherent enzymes of the raw materials must be inactivated. Extrusion cooking denatures and, therefore, inactivates these enzymes, in particular lipases, lipoxygenase, and peroxidase that can promote lipid oxidation. In a study with extruded oat, even low extrusion temperatures (70 °C) inactivated the endogenous lipases of oat (Moisio et al. 2015b). Further study showed that inherent enzymes of cereal brans (wheat, rye, oat, and maize) were inactivated at mild extrusion cooking conditions (< 120 °C, 20% moisture, and low shear stress) (Meister et al. 1994).

Another factor that affects the lipid oxidation in extrudates, is the presence of metal ions, e.g., iron or copper, which are added as a nutritional supplement to fulfill legal requirements (Schaich 1992). Metal ions such as Fe and Cu are known to promote lipid oxidation by catalyze radical formation in the initiation reaction. Metal ions can donate electrons, resulting in increased rates of free radical production. In the context of extrusion cooking, a higher temperature is associated with increased transition metal content in cornmeal/starch-based extrudates (Rao and Artz 1989). In addition the iron-wear of the screw can contribute to a small increase of the iron content with an acceleration of lipid oxidation (Paradiso et al. 2009). An opposing effect is shown under presence of antioxidants (e.g. tocopherol), which interrupt the radical chain reaction. In addition, oxidation processes are influenced by temperature. Thus, thermal treatment during production has as much influence as storage temperature. Besides the lowest possible temperature during storage and storage in the dark, oxygen contact, oxygen concentration and the exclusion of oxygen also play a decisive role (Johnson and Decker 2015). Furthermore, extrudates have after drying a low water activity (approx. 0.4–0.5) with low moisture content. As shown by Labuza et al. (1972) the water content and the a_w-value of the food product influences strongly the lipid oxidation. Their findings (**Figure 2.7**) show that samples with a relatively high or relatively low a_w-value show lower oxidative stability of the lipids. Otherwise, a_w-values from 0.2 to 0.5, which also includes extrudates, are characterized by a high oxidative stability.

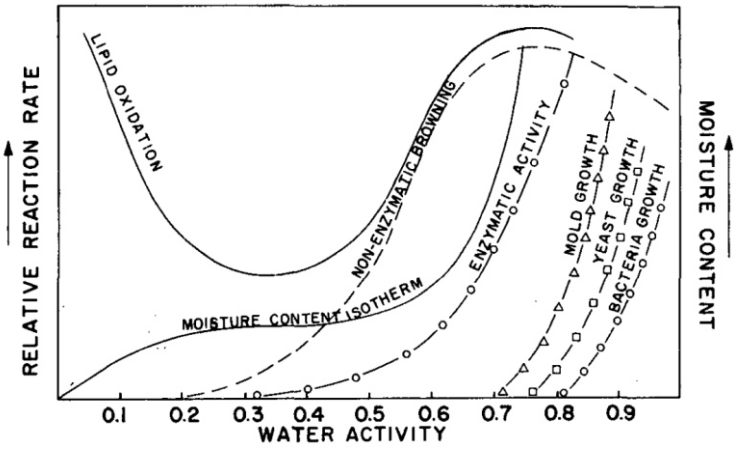

Figure 2.7: *Stability of foods with different water activities (Labuza et al. 1972)*

2.3.4. MEASUREMENT OF LIPID OXIDATION

2.3.4.1. ACCELERATED STORAGE TESTS FOR ANALYSIS OF LIPID OXIDATION

Lipid oxidation reactions are time consuming, because the speed of oxidation at room temperature is quite slowly. For prediction of shelf life and for analysis of lipid oxidation in a shorter time, accelerated storage tests are commonly used. As presented in the previous chapter, the rate of lipid oxidation depends among others on the a_w-value, temperature, oxygen access, and light (Choe and Min 2006). Accelerated oxidation tests, where the samples were stored at constant elevated temperatures (40–60°C) are the most popular methods to accelerate the lipid oxidation (Ragnarsson and Labuza 1977). These tests were also called "Schaal oven" tests and based on the reaction-rate-temperature rule based on the Arrhenius equation (**Chapter 2.3.2**). It states that the rate of a reaction increases exponentially with the temperature, i.e., a 10 °C rise in temperature doubles the reaction rate (Ragnarsson and Labuza 1977). Advantages of these simple assays are that they require no specific equipment and that they can be used for oils, fats, and foods. Methods, such as the Rancimat method, using high temperatures (>100 °C) must be considered with prudence (Mancebo-Campos et al. 2007; Labuza and Dugan 1971). Under high temperatures the lipid oxidation kinetics are modified and side reactions accompany that lead e.g., to formation of Maillard reaction products.

Besides the temperature, iron-catalyzed lipid oxidation is a further method to accelerate oxidation in storage tests. Added metal ions, e.g., iron and copper, act as radical initiators and prooxidants and increase the radical formation in the induction period of lipid oxidation (Schaich 1992). Naturally, metal ions were added to foods to fulfill statutory requirements or

they were generated involuntary during food processing. A typical example for the latter is the abrasion during the extrusion process. Another mechanism is used, when oxygen atmosphere is controlled to accelerate lipid oxidation or to enhance the oxidative stability by decrease the oxygen concentration in the headspace (Johnson and Decker 2015). However, these methods often based on changed reaction mechanisms that do not reflect oxidation at room temperature. For this reason, the gold standard for accelerated storage tests is the storage at elevated temperatures (conclusion of the roundtable discussion at the "2nd International Symposium on Lipid Oxidation and Antioxidants" in Graz, 2018).

Another possibility to accelerate lipid oxidation studies is the use of purified, so-called stripped oils (Bowen et al. 2006; Cui et al. 2017; Orlien et al. 2000; Roman et al. 2013). This means the elimination of all antioxidant substances such as tocopherols in the oil. In addition, the oils are void of prooxidants, e.g., trace metals and existing hydroperoxides. Oil stripping can be performed by adsorption chromatography (Lampi and Kamal-Eldin 1998). Therefore, a glass column is packed with activated aluminum oxide as stationary phase. The oil to be purified is mixed in the equal volume of n-hexane (as elution solvent) and this mixture is passed through the column. Finally, hexane is removed and success of oil-purification can be tested using HPLC. In addition to the accelerated oxidation time, stripped oils have the further advantage that specific antioxidants can be added and their effects can be considered alone.

2.3.4.2. ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Lipid oxidation is characterized by the formation of alkyl, peroxyl and alkoxyl radicals (Zamora and Hidalgo 2016). The formation of these free radicals can be used as early markers of oxidation. An innovative approach for the non-destructive determination of these oxidation markers is the electron paramagnetic resonance spectroscopy (EPR) (Thomsen et al. 2000; Velasco et al. 2005; Jensen et al. 2005). Synonymous name for the EPR is electron spin resonance (ESR) spectroscopy. Today, EPR is used to investigate free radicals in chemical systems, biological tissues as well as in foods (Roman et al. 2010). A continuous X-band (9.5 GHz) EPR spectrometer consists of a magnet, a microwave bridge, a cavity and a computer for data processing.

The principle of EPR is similar to the magnetic resonance technique NMR. The EPR is a spectroscopic tool that measures the absorption of electromagnetic irradiation by unpaired

electrons, i.e., paramagnetic substances, in an applied external magnetic field (Roman et al. 2010). Unpaired electrons have a spin, which gives them a magnetic moment. In the external magnetic field, the paramagnetic electrons can orient in a parallel or antiparallel direction to the magnetic field leading to two energy levels (**Figure 2.8**) (He et al. 2014). Without microwave irradiation more radicals will be oriented parallel to the magnetic field, i.e., in the lower energy level. Microwave irradiation with a constant frequency excites some of these lower-level electrons to the higher energy level and a transition occurs. An EPR spectrum is obtained when the magnetic field is varied at a fixed microwave frequency. From the right external magnetic field strengths at which the microwave frequency is absorbed, an EPR resonance spectrum can be recorded (He et al. 2014). From the first-derivative of the absorption spectrum, finally the characteristic EPR spectrum is obtained.

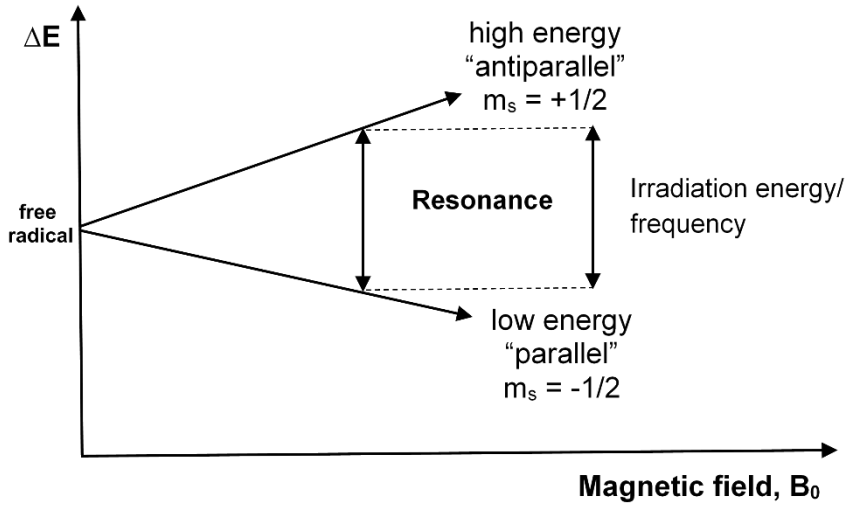

Figure 2.8: *Energy diagram showing the antiparallel state and the parallel state and the resulting energy difference leading to an EPR signal (modified from He et al. 2014).*

Besides free radicals, also transition metal ions can be captured by EPR. Free paramagnetic radicals, e.g. hydroxyl radicals are molecules with a half-life time of about 1 ns (Kivelä et al. 2012). Short-lived free lipid radicals were generated during lipid oxidation in the radical chain reaction. These radicals react within nanoseconds, too. Unlike stable protein and carbohydrate radicals, short-lived lipid radicals cannot be detected directly by EPR. For analysis, lipid-derived radicals must be captured by spin traps (Velasco et al. 2005). Spin trapping was first introduced in late 1960´s (Janzen and Blackburn 1969). Spin traps such as N-*tert*-butyl-α-phenylnitrone (PBN) (**Figure 2.9**), α-(4-Pyridyl N-oxide)-N-*tert*-butylnitrone (POBN) or 5,5-

dimethyl-pyrroline N-oxide (DMPO) are usually nitrone or nitroso compounds, which reacts with a free radical under formation of a more stable detectable paramagnetic spin adduct (Cui et al. 2017; Davies and Hawkins 2004).

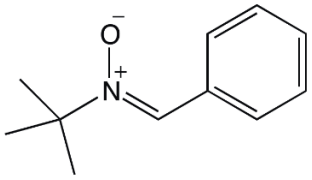

Figure 2.9: *Structure of the spin trap PBN*

According to Velasco et al. (2005) the EPR signal intensity is directly proportional to the radical concentration. However, in the presence of antioxidants, EPR measurements are hindered. For example tocopherols compete with the added spin trap and decrease their ability to interact with free radicals (Cui et al. 2017). In this case, the EPR do not allow prediction of lag phase of oxidation.

A change in the line shape of the EPR spectra giving insight into the detected paramagnetic species. In addition, the number of lines in the EPR spectrum, called hyperfine structure, is characteristic for different free radicals. The trapped radical species can be identified by the EPR-spectra, the line shape of the spectra, its g-value, and the hyperfine couplings. Radicals can be carbon-centered, oxygen-centered, and nitrogen-centered. The EPR spectra from nitroxide-based spin traps shows a triplet (Lagercrantz 1971). However, with nonspecific spin traps such as PBN and POBN the EPR spectrum is often similar, regardless of the trapped radical, because these nitrone spin traps capture the radical at the carbon atom next to the nitroxide group (Damian et al. 2007). In general, traps are not radical-specific, but some traps are more or less useful for catching certain radicals (Szterk et al. 2011).

Both, POBN and PBN, have been shown in studies to be suitable spin traps to study lipid derived radicals (Park et al. 2011; Novakov et al. 2001; Velasco et al. 2004; Dikalov and Mason 2001). In cases of PBN, alkyl as well as peroxyl and alkoxyl radicals could be trapped. Detection of alkyl radicals is unlikely, because they have a low steady-state concentration (Novakov et al. 2001) and it was found that alkyl radicals react faster with oxygen than with the spin trap (Velasco et al. 2004). Peroxyl radicals trapped by PBN are relatively unstable and decompose at room temperature under formation of alkoxyl radicals. Therefore, the authors

suggest that alkoxyl radicals are, an indirect evidence for peroxyl radicals (Dikalov and Mason 1999, 2001). Davison et al. reported lipid-derived alkoxyl radicals, trapped with PBN, from linoleic acid (decomposed of lipid hydroperoxides) in human blood samples (Davison et al. 2008). This is in line with the statement from Frankel, that alkoxyl radicals can be seen as precursors of most stable oxidation products (Frankel 1983).

2.3.4.3. PRIMARY LIPID OXIDATION PRODUCTS

Lipid hydroperoxides, the main initial products of autoxidation are also called primary oxidation products. Because peroxide values reach a maximum and then decrease, it is advisable to measure secondary oxidation products, too. Primary oxidation products do not cause flavor deterioration and can be analyzed by measuring the peroxide value. The peroxide value states the milliequivalents of peroxide oxygen combined per kilogram of fat and is determined by titration (Wheeler 1932). This method based on the ability of hydroperoxides to liberate iodine from potassium iodide (Barriuso et al. 2013). I_2 is then titrated with sodium thiosulfate. Peroxide value determinations are time and solvents consuming and relatively large sample amounts are needed (1–5 g).

An alternative is the determination of the hydroperoxide concentration by the spectrophotometric ferric thiocyanate method (Drusch et al. 2007; Serfert et al. 2009; Mihaljević et al. 1996). This method based on the ability of hydroperoxides to oxidize ferrous to ferric ions ($2Fe^{2+} + ROOH + 2H^+ \rightarrow 2Fe^{3+} + ROH + H_2O$). In the next reaction step with thiocyanate, a red iron complex is formed, which can be measured photometrically at 485 nm after incubation at 60 °C for 30 min ($[Fe(H_2O)_6]^{3+} + 3\ SCN^- \rightarrow [Fe(SCN)_3(H_2O)_3] + 3\ H_2O$). The hydroperoxide concentration can be calculated with the calibration function (c) according to the following equation:

$$Hydroperoxides\ concentration\ \left[\frac{\text{mmol}}{\text{kg}}\text{oil}\right] = \frac{c\ (Fe(III)[\frac{\mu g}{5ml}]}{sample\ weight\ [g] * 55.84} * Dilution$$

The thiocyanate method has the advantages of a high sensitivity combined with easy, fast, safe, and cheap sample handling (Chapman and Mackay 1949). By comparison with peroxide value according to Wheeler, a small sample amount (5–50 mg) is needed for the analyses. The determination of the hydroperoxides by the thiocyanate method provides comparable results to the reference method peroxide value, independent of the fatty acid composition (Mihaljević et

al. 1996). However, it must be noted that hydroperoxides, measured using the thiocyanate method, are approx. 2.0 - 3.5 times higher than according to Wheeler and a calibration is needed (Lips et al. 1943).

Conjugated dienes (CD) were formed after peroxides formation during lipid oxidation. Natural present non-conjugated double bonds are converted to conjugated double bonds. These CD (two conjugated double bonds separated by a single bond) are primary oxidation products and can be measured in the photometer due to their absorption of ultraviolet light at 234 nm. Therefore, oil is solved in isopropanol (Stöckmann et al. 2000) and absorption is measured at 234 nm. Hydroperoxide concentration can be calculated using the molar extinction coefficient for methyl linoleate hydroperoxides (in methanol) of 26,000 (Chan and Levett 1977). The advantage of using conjugated dienes is that no further sample preparation is required and only small amounts of oil are required for the analysis. Whereas, the absorption can also be falsified by other components with similar UV absorption.

2.3.4.4. VOLATILE SECONDARY LIPID OXIDATION PRODUCTS

In the ongoing oxidation reactions, the hydroperoxides can break down in volatile compounds (secondary oxidation products), because of β-scission reactions (Halliwell and Chirico 1993; Frankel 1983). The decomposition is induced by thermal treatment but can also be metal-catalyzed (Frankel 1983). These aldehydes and ketones or alcohols can be measured by headspace-gas chromatography (HS-GC) and are responsible for the off flavors (“rancidity”) of oxidized products. Flavor deterioration is important, because the volatiles already have an impact on flavor at low concentrations. Typical compounds are hexanal and propanal (Lampi et al. 2015). Hexanal is an important volatile of the oxidation of omega-6 polyunsaturated fatty acids (e.g. linoleic acid) and propanal is originated by the oxidation of omega-3 acids (e.g. alpha-linolenic acid) (Frankel 1991; Choe and Min 2006). In addition to measuring volatiles via headspace-gas chromatography and identification using a standard, measurement with a GC-MS and simultaneous identification via the detected masses of volatile compounds is also possible.

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CHAPTER 3

EFFECT OF WATER ADDITION ON THE MICROSTRUCTURE, LIPID INCORPORATION, AND LIPID OXIDATION OF CORN EXTRUDATES

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3.1. ABSTRACT

Lipid oxidation is a major cause of quality deterioration during the production and storage of fat containing extrudates, which is a result of the high specific surface of the matrix with increased oxygen access. During the extrusion process, corn starch gelatinizes and forms an amorphous matrix that incorporates lipids. To estimate the effect of incorporation on oxidative stability of lipids a model extrudate of corn meal, water and a mixture of sunflower- and rapeseed oil was developed. The extrusion process was conducted on a laboratory twin screw extruder with varying moisture contents, affecting in turn process parameters such as the specific mechanical energy. A fractionated extraction protocol served to obtain surface lipids, inner surface lipids and lipids incorporated in the starch matrix. Oxidation was analyzed at different points in time during storage at 40 °C in the three lipid fractions. Furthermore, the microstructure of the extrudates was analyzed by computerized microtomography (μCT). The results showed that increasing fat inclusion decreased the rate of lipid oxidation. A compact structure with low expansion was achieved by high water contents. These extrudates in turn exhibited an increased amount of incorporated lipids less susceptible to oxidation.

Practical applications: The presented work investigates extrusion technology, widely used by industry. Lipid oxidation covers an important topic, as stable products are necessary in order to avoid waste. The stabilization of lipids in extrudates against oxidation is therefore of great interest. With the model extrudate used in this study and a newly developed lipid fraction extraction protocol, it was possible to define the regions in terms oxidation status. The results of this study provide the basis for products with an enhanced stability against lipid oxidation because of their structural characteristics considering the principles of green chemistry.

3.2. INTRODUCTION

Extrusion cooking is an interesting, economical and widely used key technology in industry in the recent years. Extrusion technology combines many operation steps in a continuous process. This high-temperature short-time process is employed for food products, such as snack foods, RTE-cereals or kibbles for animal nutrition. A mixture of raw materials is continuously forced under pressure and temperature through a formative die. During this process, various reactions occur in the extrudates. For example, lipids are distributed in the matrix by the shear forces. Starch gelatinizes and proteins denaturize under formation of an amorphous matrix, which incorporates lipids (Ilo et al. 2000). High gelatinization degrees are required if an increased lipid inclusion is aimed for (Pilli et al. 2011). The product expands after releasing from the die by water evaporation and the autoxidation of lipids is promoted by high temperatures in the extruder. Extrudates containing unsaturated lipids are susceptible to lipid oxidation because of high specific surface with an increased oxygen access (Ilo et al. 2000). Furthermore, heat and possible metal abrasion in the extruder could contribute to accelerated oxidation (Lampi et al. 2015; Schaich 1992).

An indicator for the lipid binding and incorporation is a reduced oil extractability. The structure of an extrudate can be considered as a solid foam or a sponge-like structure. The lipids could be adhered on the inner surface of included air bubbles (Yilmaz et al. 2001). The lipid droplets could also be incorporated into the lamella (Gray et al. 2008). Another possible reaction during extrusion cooking is the complex formation of lipids with amylose (Abu-hardan et al. 2011; Genkina et al. 2015; Thachil et al. 2014). However, these complexes can only be formed with free fatty acids or monoglycerides (Mercier et al. 1980; Bhatnagar and Hanna 1994). The complex formation depends, among other things, on the kind of fat and the process parameters (Pilli et al. 2008; Pilli et al. 2012).

For the determination of lipid oxidation products in extrudates, it is necessary to extract all lipid fractions, including the matrix incorporated lipids. Conventional methods for the complete fat extraction (e.g. Soxhlet) can degrade the lipids and complicate the measurement of lipid oxidation (Strange and Schaich 2000). A possible method for extracting entrapped lipids that are bound to the starch is the extraction after pretreatment with α-amylase (Strange and Schaich 2000; Thachil et al. 2014). Studies reported that extrudates showed higher oxidative stability when it was assumed that lipids were bound into the matrix (Thachil et al. 2014; Guzman et al. 1992). But previously described methods for the quantification of the fat content and the

measurement of the oxidation status lack information on different fat fractions (surface lipids, incorporated lipids). This study examined for the first time the lipid oxidation status of corn extrudates in three lipid fractions differentiated in the level of incorporation in the extrudate (**Figure 3.1**). This gives improved knowledge about where lipids are present in the product and how the degree of lipid inclusion affects oxidation in the regions concerned. Often only the overall oxidation of extruded samples is measured and assumptions are made that a reduced oxidation could be attributed to the complexation of lipids with the starch.

The aim of the present study is therefore to understand the relationship between the microstructure and lipid oxidation in extrudates. Therefore, it is of importance to determine the region, where lipids are incorporated and quantify the degree of lipid incorporation. The impact of water addition on microstructure, lipid incorporations and lipid oxidation is of practical relevance.

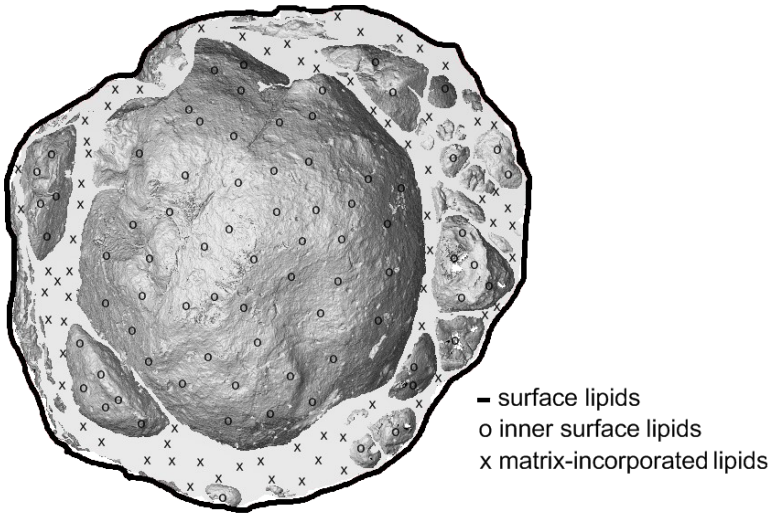


Figure 3.1: *Lipid fractions in Extrudates extractable with the new developed fractionated lipid extraction protocol*

3.3. MATERIALS AND METHODS

3.3.1. EXTRUSION PROCESS

The extrusion process was conducted on a laboratory twin-screw extruder with contrarotating intermeshing screws (Brabender Mod. DSE 35/7D). The basic recipe for the homogenous premix, which was produced before extrusion, is based on native (Molino Merano; Italy) and pregelatinized corn meal (Interquell; Germany) (50:50 w/w). After adding 10% of a commercial rapeseed / sunflower oil mixture (50:50 v/v) and water to the corn meal, the ingredients were mixed with a Stephan Universal Machine (table cutter) to the homogenous premix. The extrusion process was performed with added water contents from 10 – 22%. The premix was fed by a gravimetric weight feeder (Coperion Ktron Model K2-ML-D5-T35-QC) to the extruder with a constant feed rate of 18 kg/h. The temperature controller of the extruder were set to 60/125/125 °C and the screw speed was set to 333 rpm. The extruder was operated by one open die and an automated cutting device. The produced extrudates were dried in air permeable plastic bags in a climate cabinet (ThermoTEC TCS-501) at 30 °C and 20% humidity for 48 hours. After drying, the samples were stored in triplicate in 1000 mL glass bottles at 40 °C in a climate cabinet in the dark.

The extruder was equipped with a digital measuring instrument, which permanently monitors the engine parameters (**Table 3.1**). The specific mechanical energy (SME) was calculated by the following equation that is in line with Onwulata et al. (1994):

$$SME\ \left[\frac{kWh}{kg}\right] = \frac{\frac{n_{act}[rpm]}{n_{max}[rpm]} * torque\ [\%] * P_{max}\ [kW]}{feed\ rate\ [\frac{kg}{h}]}$$

where n_{act} is the screw speed in rpm used in the experiment, n_{max} is the maximum screw speed in rpm, torque is the used torque from the motor in %, P_{max} is the motor power of the extruder in kW and the feed rate is the amount of premix that is fed of the gravimetric weight feeder to the extruder in kg/h.

3.3.2. FRACTIONATED LIPID EXTRACTION

The principle of the method, using amylase for lipid release from extruded matrix, is derived from Thachil et al. (2014). A protocol for fractionated extraction of lipids that allows determination of the fat content and of the oxidation status in the different lipid fractions was developed in this study. For method development see supplementary material (**Supplementary Table 3.2**) in the online version of this article.

Lipids removed by the first extraction step are defined as surface lipids. For quantification of surface lipids, 35 mL cyclohexane were added to 5 g of intact extrudates and shaken for 1 h at 190 rpm. After filtration the solvent of the extract was evaporated at 65 °C and 250 mbar and solvent residues were removed with nitrogen. The amount of surface fat was determined gravimetrically.

Lipids removed by the second extraction step are defined as inner surface lipids. For extraction, the dried extrudates obtained from surface lipid extraction were milled for 10 seconds with an analytical mill (IKA A11 basic, Germany) and placed into 50 mL falcon tubes. 15 mL cyclohexane was added and after sonication for 20 seconds (cycle 9, 50% power, Bandelin sonoplus, sonication probe VS 70) the tubes were centrifuged at 4500 rpm for 4 minutes. The extraction procedure was repeated twice using each time 10 mL solvent. After filtration the solvent was evaporated (65 °C at 250 mbar) and solvent residues were removed with nitrogen. The amount of inner surface fat was determined gravimetrically.

Lipids removed by the third extraction step are defined as matrix-incorporated lipids. For the determination of the matrix-incorporated lipids, the extruded samples were subjected to an amylase treatment after inner surface lipid-extraction (Thachil et al. 2014). 20 mL of a phosphate buffer (pH 5.9) were mixed with α-amylase from *Bacillus licheniformis* (Sigma Aldrich) in a concentration of 17.6 U/mL and added to the extruded sample. Closed falcon tubes were incubated at 60 °C for 2 h at 300 rpm, followed by addition of 10 mL cyclohexane, sonication and centrifugation. An aliquote of 5 mL supernatant was subjected to vacuum centrifugation for solvent removal. The amount of matrix-incorporated fat was determined gravimetrically.

3.3.3. DEGREE OF GELATINIZATION

The degree of gelatinization was determined according to the methods of Chiang and Johnson (1977) and Lin et al. (1997). The degree of gelatinization is the ratio of gelatinized starch to total starch of a sample. The total starch content was measured after a treatment of 20 mg freeze dried sample with 1 N NaOH for total gelatinization. After solubilization in an acetate buffer (80 mmol, pH 4,5) and incubation with amyloglucosidase the released glucose was determined. The glucose reacted with o-toluidine in a boiling water bath and finally the absorbance of the green chromophore was measured at 630 nm in a photometer against acetic acid. The amount of gelatinized starch was measured in the same way without the alkali treatment. The degree of gelatinization was calculated by following equation:

$$\text{Degree of gelatinization } [\%] = \frac{(\text{GS} - \text{K}) \text{ x } 100}{\text{TS} - \text{K}}$$

where GS is the amount of gelatinized starch (g/100 g), TS is the total starch content (g/100 g) and K is the percentage of native starch attacked by amyloglucosidase.

3.3.4. HYDROPEROXIDE CONTENT

Hydroperoxides were determined by the ferrous thiocyanate method (Drusch et al. 2007). 10-50 mg (depending on the oxidation status) of the oil samples obtained from fractionated lipid extraction were solved in 5 mL 2-propanol. Then 50 µL iron(II) chloride solution as well as 50 µL ammonium thiocyanate solution were added. After vortexing, samples were incubated in a water bath at 60 °C for 30 min. Directly after cooling down to room temperature the extinction was measured against 2-propanol at a wavelength of 485 nm. All analyses of hydroperoxides were performed with triplicates. The total hydroperoxide concentration was calculated by multiplying the different hydroperoxide concentrations of the three different lipid fractions with their proportion of the total fat content.

3.3.5. VOLATILE OXIDATION PRODUCTS

Volatile oxidation products (specifically hexanal) were analyzed by an Agilent 6890 series gas chromatograph with headspace autosampler (HS-GC). Therefor 1 g of milled extrudate was weighed in 20 mL airtight HS-GC-glass vials. After equilibration at 70 °C for 15 min an aliquot

of the headspace was injected into the gas chromatograph. The HS-GC was running in split mode (4:1) and was equipped with a J&W DB-1701 column (60 m x 0.32 mm x 3 µm). The injection port was operated at 220 °C. Initially oven temperature was set to 45 °C where it was held for 2 minutes, then raised up to 85 °C at 15 °C min^{-1} and maintained for 4 minutes and finally raised up to 220 °C at 15 °C min^{-1} and maintained for 3 minutes. Detection was done by a flame ionization detector (FID). Hexanal was identified using the retention time of an external standard. Results were displayed as area under the curve (AUC).

3.3.6. DENSITY

The density of the extrudates was determined by filling a slim 250 mL measuring cylinder up to the 150 mL marking with extrudates. After weighing the extrudates, 0.75-1.00 mm glass beads were added, compacted and filled up to 200 mL marking. After removing the extrudates, the volume of the glass beads was measured. The density was calculated by following equation:

$$\text{Density } [\text{g/cm}^3] = \frac{\text{W}}{\text{V}}$$

W is the weight of the extrudates in grams and V is the volume of extrudates in mL (computation: 200 mL – volume of the glass beads).

3.3.7. SECTIONAL EXPANSION INDEX (SEI)

The expansion of the extrudates, a parameter for the size of the extrudates, was measured using a vernier caliper. The diameter of 30 randomly selected samples was determined in millimeter. The diameter of the extruder die is 8 mm (≙100%). The expansion was calculated by following equation:

$$Expansion\ [\%] = \frac{diameter\ of\ the\ sample\ [mm]}{diameter\ of\ the\ die\ [mm]} \text{x}100$$

3.3.8. COMPUTERIZED MICROTOMOGRAPHY

The intact dried extrudates were scanned with a phoenix nanotom® 180NF (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany). Each scan was optimized individually in order to find the most suitable scan parameters for good contrast between solid phase and air phase as well as to achieve the maximum resolution for the different sized samples. The acceleration voltage ranged from 46 kV to 50 kV and the current ranged from 390 µA to 740 µA. This results into tube power / target power ranging from 17.9 to 37.0 W / 4.25 to 8.76 W. The x-ray-beam was generated with a molybdenum target and was filtered with 0.1 mm Cu. Due to the different diameters of the extrudates, the achieved voxel length differed between 14.45 µm and 28.81 µm. A µ-CT scan consists of 1440 projections while the sample was full rotated in 0.25° steps. The projections were recorded with a CCD detector at effective 1146 by 1152 pixel in 2x2 binning mode. The reconstruction was done with the software datos|x reconstruction 1.5 (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany). Further image processing was done with VG StudioMax 2.0 (Volume Graphics GmbH, Heidelberg, Germany). The data sets were imported into myVGL 3.1 (Volume Graphics GmbH, Heidelberg, Germany), a viewer for data processed by volume graphics software. In myVGL, the mean cell wall thickness (CWT) of 50 randomly selected lamellas was measured and the 3D-visualization was performed.

3.3.9. STATISTICAL ANALYSIS

Statistical significance was analyzed by using Graph Pad Prism 6. The experiments were performed in triplicate and all values are expressed as means ± SE. The statistical significance was determined by a one-way ANOVA with $p < 0.05$.

3.4. RESULTS AND DISCUSSION

The correlation of the extrudate microstructure, the incorporation of lipids in the extrudate matrix and the lipid oxidation was studied with different water contents in the premix.

3.4.1. EXTRUSION PROCESSES

The motor power, the motor torque and the specific mechanical energy (SME) during the extrusion process are presented in **Table 3.1**. With increasing water content, motor torque and SME were reduced. This is related to the added amount of water acting as a plasticizer and thereby reducing the viscosity (Ilo and Berghofer 1999). I.e., extrudates undergo less energy input with increasing feed water contents. The present findings are in agreement with Kaisangsri et al. (2016).

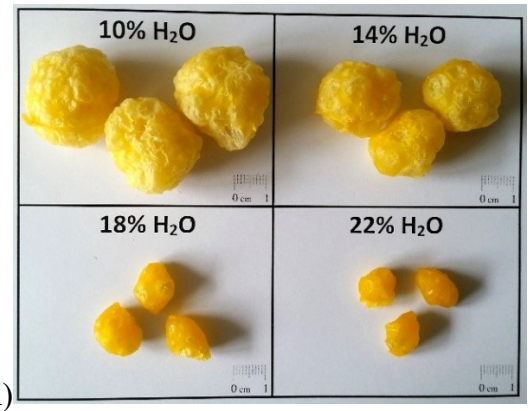
Table 3.1: *Motor power, motor torque and SME during the extrusion process*

Sample	Motor power [A]	Motor torque [%]	SME [kWh/kg]
10% H_2O	6.6	41.6	0.104
14% H_2O	5.9	30.0	0.075
18% H_2O	5.7	26.9	0.067
22% H_2O	5.4	24.8	0.062

3.4.2. IMPACT OF THE WATER CONTENT ON THE EXTRUDATE

Figure 3.2 A demonstrates that with increasing water content the size of extrudates decreased and the surface appeared smoother. For the investigation of different feed-water contents on the physicochemical properties of the extrudates the expansion and the density were analyzed (**Figure 3.2 B**).

(A)



(B)

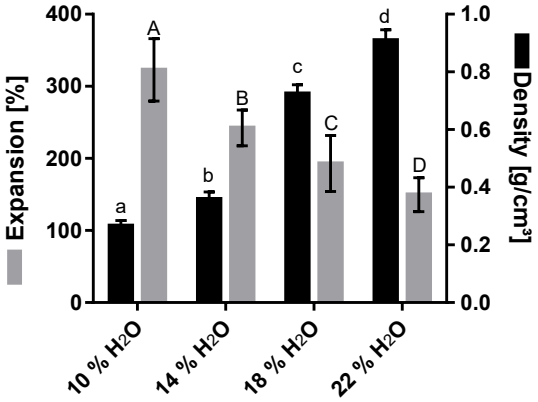


Figure 3.2: *(A) Photographs of the produced extrudates and (B) Comparison of expansion and density of the extrudates (expansion: n=30; density: n=6; p=0.05, Tuckey)*

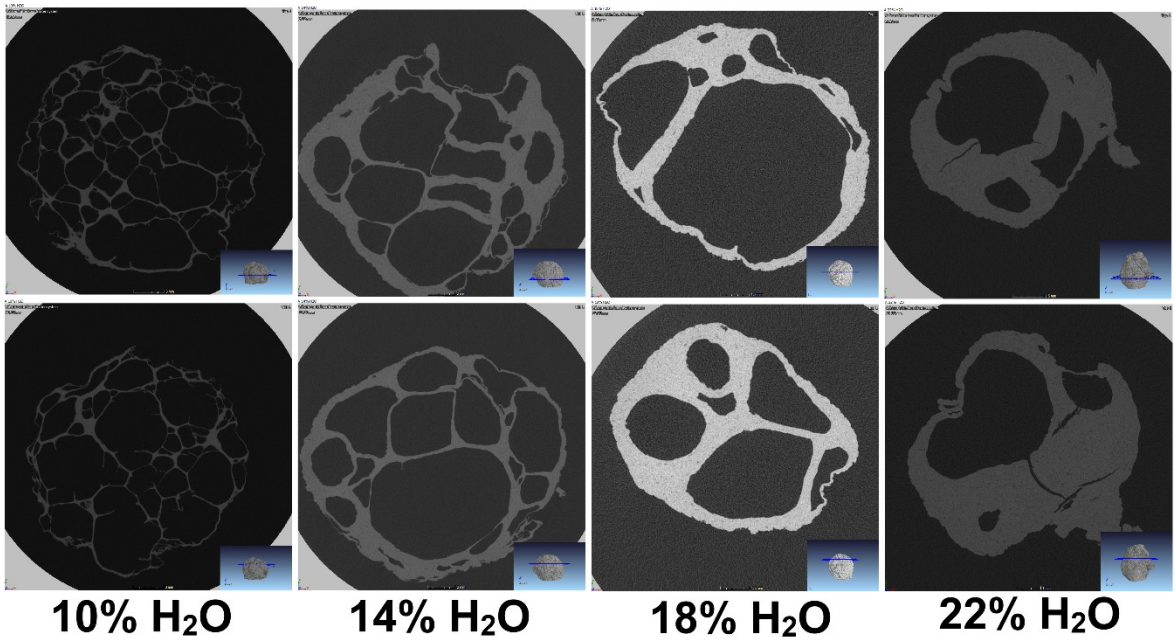
In accordance with Alvarez-Martinez et al. (1988) the density of extrudates was inversely related to the sectional expansion of extruded products. In contrast to samples with an increased feed water content, the expansion degree of samples with low water content was significantly higher. In addition, these samples then showed a decreased density. The gelatinization degrees may be found as supplementary material (**Supplementary Figure 3.8**) in the online version of this article. The values amounted to over 90% for all samples and showed no significant differences. Also Lue et al. (1991) reported gelatinization degrees > 100%; this indicate that the starch was completely gelatinized during extrusion.

The expansion of extrudates mostly depends on moisture content in the premix (Ilo et al. 1996; Ding et al. 2005). Rye bran extrusion experiments done by Moisio et al. (2015a) showed the highest expansion ratios of 250% in samples produced with the lowest water contents. The observed increase in expansion at lower feed water contents was attributed to a higher dough viscosity and vice versa (Kaisangsri et al. 2016). With low water contents, the sample was exposed to a higher friction between dough, screw elements and barrel. Simultaneously the shear forces were increased with a higher motor torque (**Table 3.1**) and increased product temperature and pressure (Lin et al. 1998a). A larger pressure and temperature drop at the die leads to an abrupt evaporation of superheated water vapor, which promotes expansion and bubble formation. Consequently the product expands intensively at the die (Wang et al. 2005). The findings of our experiment are in accordance with the results from Ding et al. (2005) for extruded rice-based snacks.

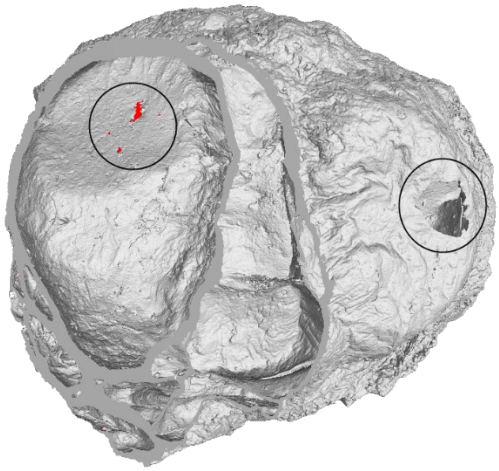
3.4.3. IMPACT OF THE WATER CONTENT ON THE MICROSTRUCTURE

Figure 3.3 A shows micro-CT images of the investigated samples. The extrudates with 10% added water in the premix have large cavities filled with air. In these extrudates thin and filigree cell walls are visible. The surface is rough and torn (**Figure 3.3 B**). For example the samples with 18% added water had fewer and smaller cavities inside, a smoother surface and the scans show significantly thicker cell walls. This observation was confirmed by measuring the cell wall thickness of the inner lamellas (**Figure 3.3 C**). The mean cell wall thickness of the extrudates with 10% water was 0.18 mm in contrast to the samples with 22% water where the values were significantly higher (about 1 mm). These findings are in accordance with measurements of the microstructure of extruded rice pellets with X-ray tomography. In this study a higher moisture content (23%) resulted in an increased mean wall thickness (Chanvrier et al. 2015). Also stereomicroscopic pictures of twin screw extruded rye bran depicted the most porous structure in extrudates with low water contents during the extrusion process (Moisio et al. 2015a).

(A)



(B)



(C)

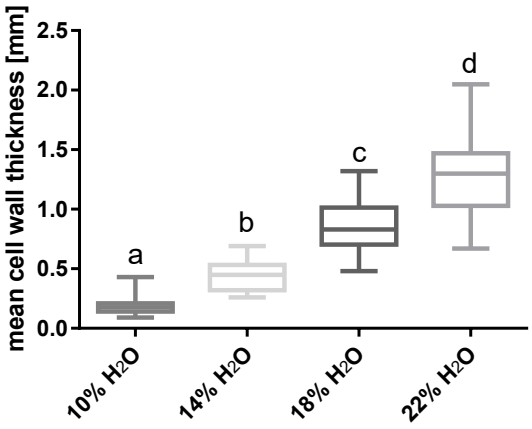


Figure 3.3: *(A) micro-CT scans of the extrudates, (B) micro-cracks on the surface of extrudates and (C) mean cell wall thickness of the extrudates measured in the micro-CT-scans. Bars with the same letter are not significantly different from each other ($p \leq 0.05$, n=50)*

3.4.4. LIPID BINDING IN EXTRUDATES WITH DIFFERENT FEED WATER CONTENTS

Figure 3.4 demonstrates the impact of increasing feed water content on the fat inclusion degree. On the one hand, it is possible to produce extrudates with a significantly reduced amount of surface lipids by increasing the water content. On the other hand, the amount of matrix-incorporated lipids was increased in these extrudates. The extraction protocol provided three

lipid fractions differing in their degree of incorporation in the extrudate and binding in the matrix: lipids located on the surface and close to the surface (surface lipids), lipids that are incorporated and accessible after grinding (inner surface lipids) and lipids which are incorporated in the matrix and only extractable after degradation of the starch-based matrix using α-amylase (matrix-incorporated lipids). It is suggested that matrix-incorporated lipids are stored between the amylose helices or dispersed in the amorphous regions but do not form complexes with the amylose helices. Amylose helices have crystalline regions in form of ordered structures intersected by amorphous regions. In this amorphous regions ligands, such as aroma compounds can be placed within the helices, between the helices, or dispersed in the amorphous region (Biais et al. 2006). The high degree of gelatinization achieved in extrudates in the present study in combination with the high degree of lipid incorporation in the matrix supports the importance of the role of amorphous region for lipid incorporation (Pilli et al. 2011). Thachil et al. (2014) stated that there are different locations for fat in the starch matrix of extruded products next to direct complexation of fatty acids with amylose and amylose-lipid-complexes are only formed with free fatty acids and monoglycerides (Bhatnagar and Hanna 1994; Putseys et al. 2010; Chao et al. 2018)

The encapsulation of lipids in the molten starch matrix during extrusion cooking depends on the kind of lipid and on the extrusion parameters. With confocal laser scanning microscopy Moisio et al. (2015b) localized fine dispersed lipids in the matrix. They found that the amount of free lipids, unbound to the starch depends on the extrusion temperature and the SME. At higher SME a separation of the lipids from the extrudate structure was observed (Moisio et al. 2015b). In addition, it must be considered that the ability of amylose to form complexes with lipids may have been reduced by the formation of lipid-protein complexes (Pilli et al. 2015). Since in our experiments edible oils were used, containing mainly triglycerides, it can be assumed that amylose complex formation plays a subordinate role and that lipid binding between the helices in the matrix is more likely. Bhatnagar and Hanna (1994) used the same line of reasoning. They tested extrudates of corn starches and various lipids and the highest complex formation was observed in the samples with the highest amylose content. These authors only prove the complex formation with fatty acids and a monoglycerol whereas no interaction was found with the triacylglycerol “tristearin”. Starch extruded with triglyceride show the same V-type pattern in the X-ray diffractogram as the starch alone (Bhatnagar and Hanna 1994).

The different expansion ratios can explain varying fat-amounts in the surface lipid fraction. Extrudates with higher expansion show a larger surface area (Włodarczyk-Stasiak and Jamroz 2009) on which more oil can accumulate. In the case of extrudates with high expansion and porous structures, it is also possible that the solvent used to extract the surface lipids not only extract fat from the surface, but also lipids, which were located just below the surface in air cavities with micro-cracks. Furthermore, there was no discernible difference in the extracted fat contents of the samples at high feed water (18% and 22%). Both were compact with a smooth surface and thick lamellas compared to the samples with 10% or 14% feed water. The total moisture content in the A-sample with 18% added water was 23.2% and in the A-sample with 22% it was 26.2%. Such high water contents indicate that the energy input was reduced (**Table 3.1**). It is reasonable that both melted doughs are very similar and there is no difference in the lipid binding. Based on the results of this study, it can be assumed that a high level of lipid incorporation into the matrix of the extrudates can be achieved at high (≥ 18%) water contents. Pilli et al. (2011) also reported a positive association between water content and complex formation between starch and oleic acid and pistachio nut flour as lipid source. Water contents of 16% and 21% were tested. Due to a higher water content, the starch grains swell completely and make amylose more accessible for diffuse and binding of ligands between the helices. Furthermore, under gelatinization conditions, at high water contents, the water may disorganize the structure and release amylopectin from the starch grains in addition to amylose (Matignon and Tecante 2017) which may enhance lipid incorporation between the amylose helices.

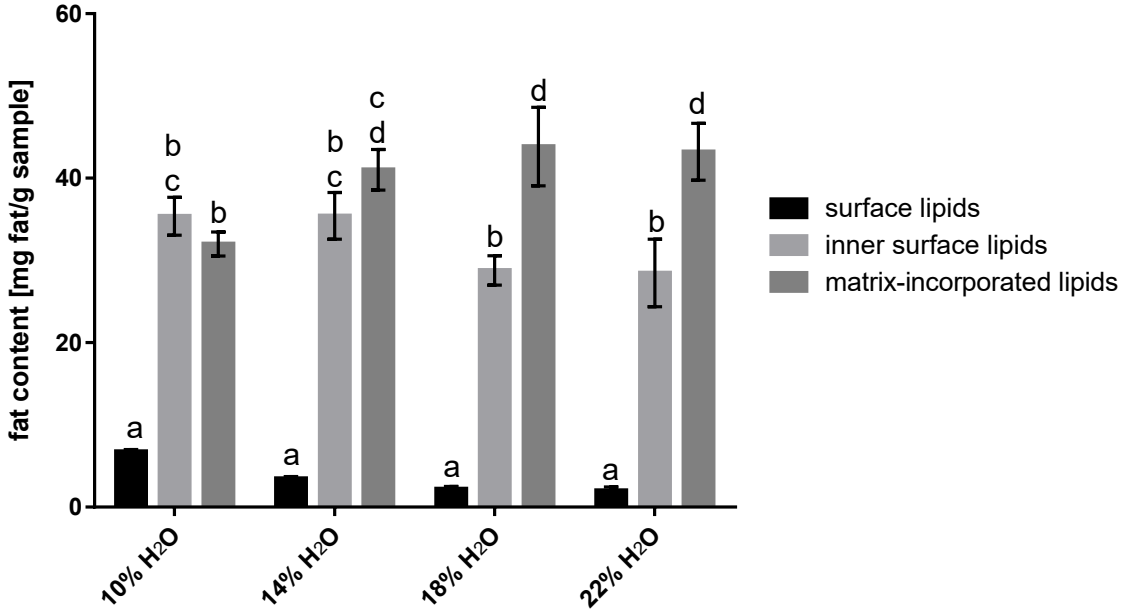


Figure 3.4: *Fat content in the different lipid fractions in mg fat / g sample. Bars with the same letter are not significantly different from each other ($p \leq 0.05$, n=3).*

3.4.5. IMPACT OF THE FAT INCLUSION DEGREE ON THE LIPID OXIDATION IN THE EXTRUDATES DURING STORAGE

Figure 3.5 and **Figure 3.6** show the impact of water content on formation of hydroperoxides of the extrudates after 20 days storage. In all extrudates oxidation of the inner surface lipids and the matrix-incorporated lipids was markedly lower compared to the surface lipids. Extrudates with higher water content oxidized less, which was accompanied with decreased expansion and increased amounts of incorporated lipids (**Figure 3.4**). I.e., the starch matrix seemed to counteract oxidation-promoting factors such as accessibility of oxygen whereas surface lipids are attached at or near the extrudate surface and thus exposed directly to oxygen. Figure 3.4 shows that these inner lipid fractions (inner surface lipids and matrix-incorporated lipids) have a large proportion of the total lipids in the extrudates. **Figure 3.6 A** shows the impact of the different lipid fractions on the calculated total hydroperoxides content (**Figure 3.6 B**). Samples with a higher water content and a reduced expansion also have a significantly reduced overall oxidation compared to the samples with low feed water and a high sectional expansion index. Extrudates with low feed water possessed a larger surface area, larger cells and thinner walls (**Figure 3.3 A**) which would favor oxygen penetration and explains higher formation of

hydroperoxides. The formation of hexanal (**Figure 3.7**) showed the same tendencies as the calculated total amounts of hydroperoxides. Over the storage period of 34 days at 40 °C hexanal raised from values below 10 up to 60.4 AUC-units in the samples with 10% water. In the samples with high feed water the hexanal content did not increase during the entire storage period.

This leads to the conclusion, that the matrix acts as a shield against lipid oxidation which could be demonstrated for the first time evaluating the degree of oxidation status in three lipid fractions differing in their degree of incorporation in corn extrudate matrix. This is supported by studies of Guzman et al. (1992) and Thachil et al. (2014) describing reduced oxidation of lipids by complexation with starch. Guzman et al. (1992) noted that lipids bound to amylose in cornmeal extrudates showed no peroxide formation whereas in the free lipids peroxides were formed. According to Zadernowski et al. (1997) the same trends can be observed in extruded oat flours without fat addition. In both studies a distinction between free and bound fat was made by changing the extraction solvent. The free lipids, extracted with hexane or petroleum ether out of the ground extrudate, showed lower peroxide values as the bound lipids, extracted afterwards with chloroform/methanol mixture or with water-saturated butanol (Zadernowski et al. 1997; Guzman et al. 1992). In-house preliminary tests showed that a solvent-based fat extraction cannot dissolve all lipids. The fractionated extraction protocol with amylase pre-treatment, used in in this study, is required for complete lipid release from the starch matrix. Especially the matrix-incorporated lipids are not accessible for solvent-extraction. Furthermore, these studies do not make any statement about the oxidation of the surface lipids. Also Lalush et al. (2005) showed that complexes of amylose with conjugated linoleic acid, which were prepared by water/DMSO and KOH/HCl complexation methods, protects fatty acids against oxidation. Further Gray et al. (2008) pointed out that linoleic acid oxidized less in an extruded starch matrix, when surface lipids were removed, and micro-cracks were eliminated. Fatty acids are better protected if they are in a glassy-state starch matrix compared to extrudates that were held in the rubbery state. One explanation is a reduced diffusion rate with limited oxygen permeability (Gray et al. 2008).

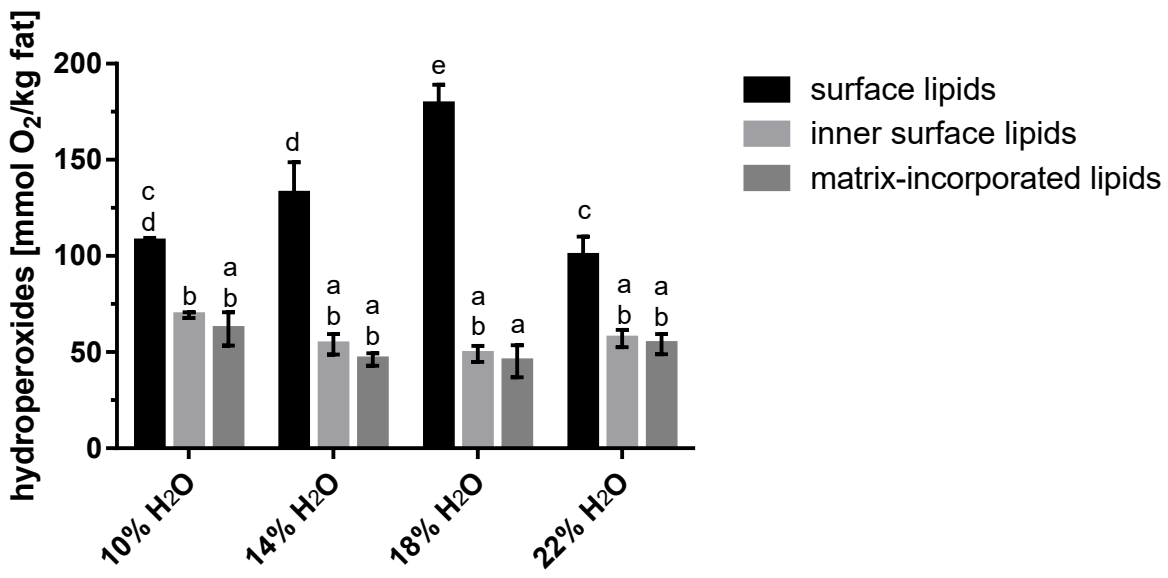
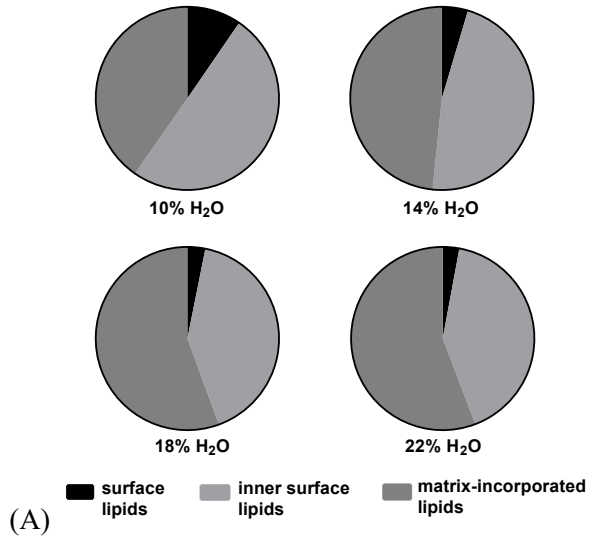
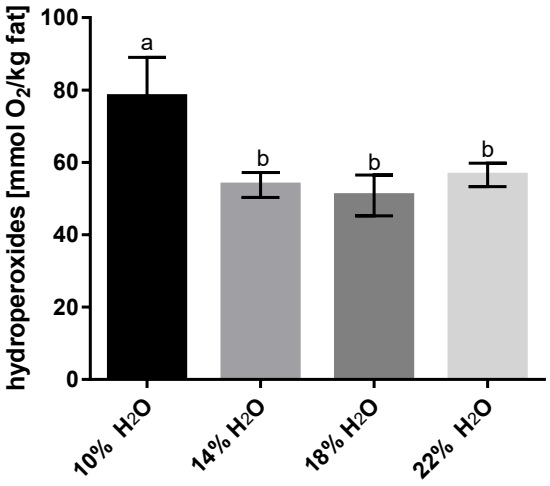


Figure 3.5: *Effect of feed water content on the hydroperoxide concentration in the different lipid fractions after storage at 40 °C at day 20. Bars with the same letter are not significantly different from each other ($p \leq 0.05$, n=3).*



(A)



(B)

Figure 3.6: *(A) Lipid distribution of the different lipid fractions and its impact on (B) the calculated total hydroperoxides at day 20. Bars with the same letter are not significantly different from each other ($p \leq 0.05$, n=3).*

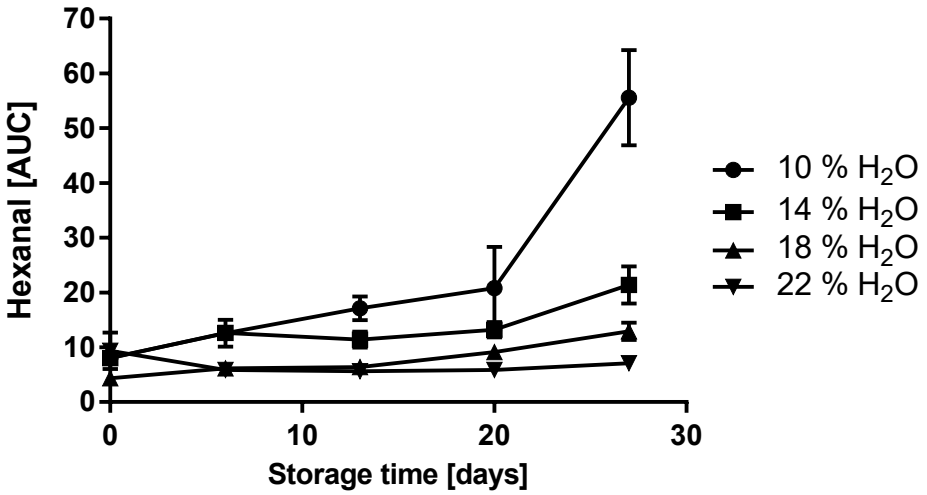


Figure 3.7: *Hexanal formation in the extrudates during storage at 40 °C (n=3)*

3.5. CONCLUSIONS

Due to the porous nature of extrudates, they are susceptible to oxidation as there is large surface for contact with oxygen. The impact of the water content on the extrudate structure resulted in reduced expansion and increased density when increasing the feed water content. The computed tomographic scans highlighted that the extrudates produced with a low feed water content showed not only an increased expansion, they also showed distinct thin cell walls (< 0.3 mm), whereas the products produced with higher water contents had thicker lamellas (up to 1 mm) inside of the extrudate. At the same time, more incorporated lipids could be stored in these thick cell walls. Less surface lipids were on the outer surface of the extrudates produced with high feed water content, which led to increased lipid inclusion and reduced lipid oxidation. Furthermore, the overall oxidation as well as the hexanal formation were reduced with increasing water content. On the whole, higher water content in the extrudate premix leads to structural features such as increased lipid incorporation and lower porosity which causes lower lipid oxidation in extrudates.

Acknowledgements

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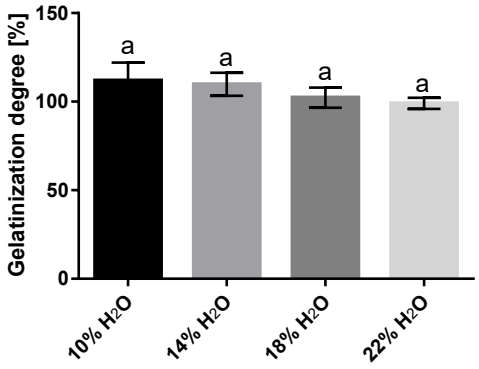
3.7. SUPPLEMENTARY MATERIAL:

In the **Supplementary Table 3.2** more details for the method development are shown. The extraction protocol consists of three extraction steps. In the first step we removed 3.53 ±0.51 mg fat / g sample of the lipids, which are considered as surface lipids. The amounts of surface lipids are not significantly different. Further, we investigated the number of extraction cycles to be conducted to sufficiently remove all lipids (inner surface lipids, step 2) prior to amylase treatment. Therefore, we repeated the ultrasound-assisted extraction cycles up to 5 times. An increase of the ultrasound-assisted extraction cycles did not lead to a significant increase of the yield. At the same time, this method leaves matrix-incorporated lipids in the sample that are only extractable after amylase treatment (step 3). Therefore, a threefold repetition of the ultrasound-assisted extraction was considered sufficient to investigate the inner surface lipids.

Supplementary Table 3.2: *Extraction yields [mg fat / g sample] depending on the number of ultrasound-assisted extraction cycles (1 x – 5 x) in the extraction of the inner surface lipids (step 2). Surface lipids removed in step 1 amounted to 3.53 ±0.51 mg fat / g sample and are not significantly different.*

Lipid fraction	Number of ultrasound-assisted extraction cycles				
	1x	2x	3x	4x	5x
Inner surface lipids	27.64 ±1.73^{a}	34.03 ±1.23^{ab}	39.90 ±4.53^{b}	37.85 ±3.28^{b}	39.83 ±0.37^{b}
Matrix-incorporated lipids	48.30 ±2.80^{a}	43.71 ±1.39^{a}	41.61 ±1.70^{a}	41.65 ±1.99^{a}	41.74 ±5.75^{a}

Means with different lowercase letters in the same horizontal line are significantly different (n=3; p=0.05, Tuckey).



Supplementary Figure 3.8: *Gelatinization degrees of the extrudates. Bars with the same letter are not significantly different from each other (n=3; p=0.05, Tuckey).*

CHAPTER 4

MCT OIL COATING IMPROVES THE OXIDATIVE STABILITY OF SURFACE LIPIDS IN CORN EXTRUDATES

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4. MCT OIL COATING IMPROVES THE OXIDATIVE STABILITY OF SURFACE LIPIDS IN CORN EXTRUDATES

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4.1. ABSTRACT

Lipid-containing extrudates are highly susceptible to lipid oxidation because of their porous structure and high specific surface area. The objective of this study is to investigate the effect of a low-oxidizable medium-chain triglycerides (MCT) coating on the oxidative stability. Therefore, cornmeal was extrusion cooked with commercial vegetable oil and various water contents (10–18% on dry matter) and partially coated with 5% (w/w) MCT oil. Lipid oxidation in the extrudates is examined during storage at +40 °C by measuring hydroperoxide concentrations in different lipid fractions (surface, inner-surface, and matrix-incorporated lipids). Hexanal was analyzed using headspace-gas chromatography. Coating allocation was studied by fatty acid profiles of the lipid fractions, by fluorescence microscopy and computerized-microtomography. Application of an MCT coating led to lower hydroperoxide concentrations and significantly reduced hexanal formation during storage. In particular, the surface lipids are stabilized. MCT oil diffuse only to small extent inside the extrudates and its effect on lipid oxidation can be attributed to differenct effects: filling surface microcracks, reducing the release of volatiles, and dilution of oxidizable lipids. This effect was strongest in porous extrudates with low feed-water content.

Practical applications: Inhibition of lipid oxidation of fatty extrudates is of great importance for producers as well as consumers. In this study, we examine whether it is possible to improve the oxidative stability of extrudates by application of a lipid-based coating. The results of this study suggest that it will be possible in the future to produce extrudates that are protected from oxidation by a thin natural oil film. We expect that especially products such as kibbles could benefit from treatment with a low-oxidizable lipid-based coating. In these products, fats are already used in combination with other functional ingredients. The combination of an MCT coating with a palatant or antioxidant is, therefore, an obvious alternative and of high relevance for manufacturers.

4.2. INTRODUCTION

In recent decades, extrusion has become a key technology in the industrial processing of agricultural commodities and has formed the technological basis for the production of numerous low-moisture products such as cereals, snacks and animal feed (Riaz 2000). Although lipid oxidation is one of the most important spoilage mechanisms in foods with low moisture content (Barden and Decker 2016), there have been so far only a few studies on lipid oxidation in extruded products. Lipid oxidation is favored by high levels of unsaturated fatty acids and can be accelerated by various factors such as temperature, light, oxygen, and catalysts such as metal ions (Choe and Min 2006). The protection of unsaturated fatty acids by encapsulation, inclusion, and coating is therefore particularly important.

Because of expansion at the die, extrudates have a high specific surface area, which results in increased oxygen exposure. Because the expansion of the extrudates results in a porous structure, a lipid-based edible coating may counteract the increased oxygen accessibility during storage and block moisture and air (Hassan et al. 2018). Lipid-based coatings are used in the food industry to protect against moisture, loss of flavor and to protect food against oxygen (Ganiari et al. 2017; Hassan et al. 2018; Kester and Fennema 1989). A common process step in the production of pet food kibbles is the coating with fats or oils including a palatant (Sunvold and Corrigan 2010). In the literature, there are several examples of coatings that were used as a barrier for oxygen to prevent lipid oxidation. Acetylated monoglycerides slow oxygen diffusion in frozen salmon (Stuchell and Krochta 1995) and studies showed that lipids of whey-protein-coated peanuts oxidized slower than lipids of uncoated peanuts during shelf life testing at 40–60 °C (Maté et al. 1996; Maté and Krochta 1996; Lee and Krochta 2002). Investigations concerning walnuts and pine nuts concluded that edible coatings of pea starch and whey protein isolate in combination with carnauba wax inhibited lipid oxidation effectively (Mehyar et al. 2012).

Computed tomographic scans in our previous study highlighted that the extrudates produced with low feed-water contents showed not only an increased expansion and a reduced density, they also showed distinct thin cell walls, whereas the products produced with higher water contents had thicker lamellas inside the extrudate. In thicker lamellas, more lipids could be incorporated, which are better protected against lipid oxidation. Furthermore, we have shown that surface lipids oxidize more than incorporated lipids. The previous study concluded that higher water content in the extrudate premix protects the extrudates better against lipid

oxidation by structural characteristics, increased lipid inclusion, lower porosity and a better encapsulation in the matrix (Amft et al. 2019). Andersen et al. (2000) stated that oxygen that permeates through glassy matrices could affect the storage of dehydrated foods (Andersen et al. 2000). Barden and Decker (2016) reviewed that reduction of oxygen accessibility during storage helps to improve the shelf life of foods (Barden and Decker 2016). Gray et al. (2008) showed that glassy starch extrudates with linoleic acid that have micro-cracks on their surface had a shorter shelf life than extrudates in a rubbery-state without micro-cracks (Gray et al. 2008).

One possibility to protect corn extrudates better from oxidation could therefore be a coating that fills microcracks in the surface. At present, there are no studies that deal with coatings on extrudates and its influence on lipid oxidation. MCTs are lipids with medium-chain fatty acids (MCFA), comprising 6–10 carbon atoms, which are saturated and not oxidizable. In addition, MCT oil shows low viscosity, which would ensure its homogeneous distribution on the surface of extrudates (Marten et al. 2006). The use of MCT oil for prevention of lipid oxidation in extrudates has not yet been investigated. To close this knowledge gap, the present study investigated the effect of a coating with a medium-chain triglycerides oil (MCT oil), a low-oxidizable oil, on lipid oxidation in corn extrudates.

The aim of this study was to understand the effect of a coating on lipid oxidation in corn extrudates. Therefore, lipids of different regions in the extrudate were extracted (**Figure 4.1**) (Amft et al. 2019) and lipid oxidation markers were measured during storage at 40 °C. In addition, the allocation of lipids in extrudates was investigated using different imaging approaches. This paper proposes a new approach to inhibit lipid oxidation in extrudates by application of a lipid-based coating.

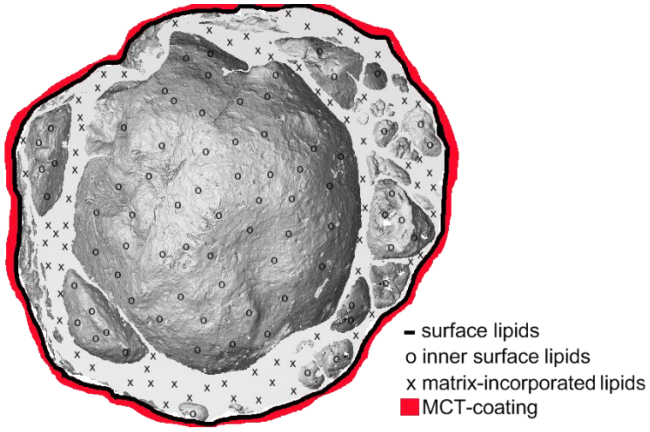

Figure 4.1:. *Schematic illustration of an extrudate based on a microtomography image and theoretical distribution of lipids in the extrudate. The investigated coating is applied to the extrudate surface but can also migrate in the outer regions of the extrudate. Modified from Amft et al. (2019)*

4.3. MATERIALS AND METHODS

4.3.1. EXTRUSION PROCESS

The extrusion process was conducted on a laboratory twin-screw extruder with contrarotating intermeshing screws (Brabender Mod. DSE 35/7D). The homogeneous premix, which was produced before, is based on native (Molino Merano; Italy) and pregelatinized cornmeal (Interquell; Germany) (50:50 w/w). After adding 10% of a commercial rapeseed/sunflower oil mixture (50:50 v/v) and water to the cornmeal, the ingredients were mixed with a Stephan Universal Machine (table cutter) to obtain the premix. The added water contents ranged from 10 to 18%, based on the dry matter of the ingredients. The premix was fed by a gravimetric weight feeder (Coperion Ktron Model K2-ML-D5-T35-QC) to the extruder with a constant feed rate of 18 kg/h. The temperature controllers of the extruder were set to 60/125/125 °C and the screw speed was set to 333 rpm. The extruder was operated with one open die (8 mm diameter) and an automated cutting device. After equilibration of the extruder, the extrudates were collected and dried in air-permeable plastic bags in a climate cabinet (ThermoTEC TCS-501) at 30 °C and 20% humidity for 48 h. After drying, one part of the extrudates was coated and the other remained untreated. Samples were sampled in 3 1000 mL glass bottles and stored in triplicate at +40 °C in a dark climate cabinet for 27 days. The physicochemical properties (specific mechanical energy (SME), sectional expansion index, density, and microstructure) of the extrudates were described previously (Amft et al. 2019). With increasing feed-water content, the SME and the expansion were decreased and the density, as well as the cell wall

thickness, were increased. In addition, the extrudates with a high feed-water content exhibited an increased amount of incorporated lipids that are better protected against lipid oxidation (Amft et al. 2019).

4.3.2. COATING OF EXTRUDATES WITH MCT-OIL:

The dried extrudates were coated with 5% (w/w) MCT oil ("MCT oil Type V Ph. Eur." from Gustav Heess, Leonberg, Germany) in a self-constructed tumble system. The coater consists of a motor, which rotates an inclined stainless-steel bowl (27 cm diameter) with 70 rpm for 3 minutes.

4.3.3. FRACTIONATED LIPID EXTRACTION

The determination of the proportions of the different lipid fractions to the total lipid content (**Table 4.2**), as well as their oxidation status, was performed after a fractionated lipid extraction, as described previously in detail (Amft et al. 2019). Briefly, surface lipids were extracted from intact extrudates by adding 35 mL cyclohexane to 5 g extrudates and shake them for 1 h at 190 rpm on a shaking plate. After filtration, the solvent of the extract was removed using rotary evaporator and the amount was determined gravimetrically. Inner surface lipids were quantified in the ground extrudates from the previous extraction step in an ultrasonic-assisted extraction procedure with cyclohexane, which was repeated twice. The solvent was removed using rotary evaporator and the amount was determined gravimetrically. For gravimetrical determination of the matrix-incorporated lipids the ground extrudates from the previous extraction step were subjected to an amylase treatment according to Amft et al. (2019).

4.3.4. ANALYSIS OF LIPID OXIDATION

Lipid oxidation parameters were determined as described by Amft et al. (2019). Hydroperoxides were determined using the ferrous thiocyanate method (Amft et al. 2019), which was performed according to Stöckmann et al. (2000). The total hydroperoxide concentration was calculated by multiplying the different hydroperoxide concentrations of the three different lipid fractions with their proportion of the total fat content. Volatile oxidation products (specifically hexanal) were analyzed in 1 g ground extrudate that was weighed in 20

mL airtight HS-GC-glass vials and equilibrated at 70 °C for 15 min. An Agilent 6890 series gas chromatograph equipped with a J&W DB-1701 column (60 m × 0.32 mm × 3 µm) with headspace autosampler (HS-GC) was used for analyses (Bauer et al. 2013; Amft et al. 2019). Hexanal was identified using an external reference standard (Sigma-Aldrich, Germany) and results were displayed as the area under the curve (AUC).

4.3.5. FATTY ACID PROFILE OF THE EXTRACTED LIPIDS

The fatty acids were methylated with trimethylsulfonium hydroxide (TMSH) (Arens et al. 1994; Schulte and Weber 1989; Butte 1983; Haas et al. 2016). 50 mg of extracted oil was dissolved in 2.5 mL methyl tert-butyl ether (Carl Roth, Germany). Then, 25 µL TMSH reagent (0.2504 M in methanol, Sigma-Aldrich, Germany) were added to 50 µL of this solution, vortexed and the mixture was injected directly into an Agilent 6890 series gas chromatograph equipped with a J&W DB-23 column (60 m × 0.25 mm × 0.25 µm). The GC was run in split mode (100:1) and the temperature of the injector was 250 °C. The carrier gas was hydrogen. The initial oven temperature was set to 80 °C, raised to 180 °C at 25 °C min^{-1} and maintained for 2 min, raised to 182 °C and maintained for 16 min. Then the temperature was raised to 197 °C at 6 °C min^{-1}, maintained for 15 min and finally raised to 240 °C at 10 °C min^{-1} maintained for 3 min. Detection was done by a flame ionization detector and Supelco 37 Component FAME Mix (Sigma-Aldrich, Germany) served as the standard. The relative content of fatty acid methyl esters (FAMEs) was calculated by comparing the AUC of single FAMEs with the sum of all FAMEs using Agilent Chem Station software version B.04.03 and "R" for an automated readout of the data.

4.3.6. PEROXIDABILITY INDEX AND CALCULATION OF THE DILUTION EFFECT

The peroxidability index (PI) was calculated according to Witting and Horwitt (1964) (Witting and Horwitt 1964) who used numerical values from Holman (1954) (Holman 1954) with the fatty acid (FA) content as follows:

$$\begin{aligned} PI = {} & (\%\ \text{monoenoic FA} \times 0.025) + (\%\ \text{dienoic FA} \times 1) + (\%\ \text{trienoic FA} \times 2) \\ & + (\%\ \text{tetraenoic FA} \times 4) + (\%\ \text{pentaenoic FA} \times 6) \\ & + (\%\ \text{hexaenoic FA} \times 8) \end{aligned}$$

To eliminate the dilution effect of the MCT oil, correction factors for the individual lipid fractions and the total lipids were calculated. These factors indicate how much the amount of C_{18} in the uncoated samples changed in relation to the amount of C_{18} in the coated samples. Based on these factors, "MCT-corrected" values of lipid oxidation markers were calculated that exclude the MCT-dilution effects.

The factors for "MCT-corrected" values for the individual lipid fractions were calculated as follows and can be found in the supplementary material (**Supplementary Table 4.3**) in the online version of this article.

$$\text{Factor "MCT} - \text{corrected"} = \frac{\Sigma\ \%\ C_{18}\ of\ NC - samples\ (T0 + T27)}{\Sigma\ \%\ C_{18}\ of\ C - samples\ (T0 + T27)}$$

The factors for "MCT-corrected" values for the hexanal contents were calculated as follows:

$$\text{Factor "MCT} - \text{corrected"} = \frac{\Sigma\ \%\ C_{18}\ of\ three\ lipid\ fractions\ of\ NC - samples\ (T0 + T27)}{\Sigma\ \%\ C_{18}\ of\ three\ lipid\ fractions\ of\ C - samples\ (T0 + T27)}$$

The factors for hexanal contents were for extrudates with 10% feed-water 1.467, for extrudates with 14% feed-water 1.413, and for extrudates with 18% feed-water: 1.479.

The factors for "MCT-corrected" values for the total lipids (T0 + T27) were calculated as follows:

$$Factor\ "MCT - corrected" = \frac{\begin{matrix}[\%\ C_{18}\ of\ NC\ in\ SL\ \times\ \%\ SL\ in\ TL] \\ +\ [\%\ C_{18}\ of\ NC\ in\ ISL\ \times\ \%\ ISL\ in\ TL] \\ +\ [\%\ C_{18}\ of\ NC\ in\ MIL\ \times\ \%\ MIL\ in\ TL]\end{matrix}}{\begin{matrix}[\%\ C_{18}\ of\ C\ in\ SL\ \times\ \%\ SL\ in\ TL] \\ +\ [\%\ C_{18}\ of\ C\ in\ ISL\ \times\ \%\ ISL\ in\ TL] \\ +\ [\%\ C_{18}\ of\ C\ in\ MIL\ \times\ \%\ MIL\ in\ TL]\end{matrix}}$$

where SL are surface lipids, TL are total lipids, ISL are inner-surface lipids, and MIL are matrix-incorporated lipids.

The factors for total lipids were for extrudates with 10% feed-water 1.393, for extrudates with 14% feed-water 1.227, and for extrudates with 18% feed-water: 1.299.

4.3.7. FLUORESCENCE MICROSCOPY

For visualization of the oil-allocation of the MCT coating, Nile red was dissolved in MCT oil (0.0625 mg / g oil). The coating with the stained MCT oil was performed as described above.

The Nile red fluorescence on the lamellas was visualized using a Zeiss Axio Zoom V16 microscope equipped with a 38 HE filter (excitation: BP 470/40, beam splitter: FT 495, emission: BP525/50). The illuminator HXP 200C (Zeiss, Germany) was used for fluorescence excitation. Lipids on the extrudate lamellas were stained with 0.01% (w/v) Nile red dissolved in acetone, which was applied on a piece of the extrudate and excess dye was rinsed with distilled water (Moisio et al. 2015b). The microscopic images were analyzed with AxioVision 4.8 software (Zeiss, Germany).

4.3.8. COMPUTERIZED MICROTOMOGRAPHY

The migration of the coating into the extrudate was analyzed with computerized microtomography (µCT). Coated extrudates (with Nile red stained-MCT oil) were scanned with a phoenix nanotom® 180NF (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany). Because of individual optimization of the scans, the acceleration voltage ranged from 47 kV to 48 kV and the current ranged from 350 µA to 365 µA. This results in tube power/target power ranging from 16.8 to 17.3 W/3.98 to 4.09 W. The x-ray beam was generated with a molybdenum target and was filtered with 0.1 mm Cu. The achieved voxel length was 16.0 µm for all samples. A computerized µCT scan consists of 1440 projections while the sample was full rotated in 0.25° steps. The projections were recorded with a CCD detector at effective 1146 by 1152 pixels in 2 × 2 binning mode. The reconstruction was done with the software datos|x reconstruction 1.5 (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany). Further image processing was done with VG StudioMax 2.0 (Volume Graphics GmbH, Heidelberg, Germany) and the µCT scans were evaluated using the myVGL 3.1 software (Volume Graphics GmbH, Heidelberg, Germany).

4.3.9. STATISTICAL ANALYSIS

The extrusion and coating procedure was performed once and these extrudates were sampled in 3 sub-samples. Further experiments and analyses were performed in triplicate and all values are expressed as means ± SD. Statistical significance was analyzed using Graph Pad Prism 6 (GraphPad Software, San Diego, USA) performing a two-way analysis of variance with $p < 0.05$ combined with a Tukey multiple comparison test and an unpaired parametric t test with $p < 0.05$. The rate of hydroperoxide formation, i.e., the slopes of oxidation curves were determined by linear regression analyses with curve fitting (see supplementary materials, Supplementary Figure 4.8).

4.4. RESULTS AND DISCUSSION

4.4.1. EFFECT OF AN MCT COATING ON LIPID OXIDATION

Primary (hydroperoxides) and volatile secondary (hexanal) oxidation products were monitored in the coated and uncoated extrudates during storage at +40 °C. The coated samples showed only a marginal increase of hydroperoxide contents whereas the hydroperoxide value of samples without a coating increased over the storage time up to a hydroperoxide concentration of about 200 mmol/kg oil. As expected, the coating resulted in an overall lower lipid oxidation, especially in surface lipids (**Figure 4.2 A**). This can be explained with the high amount of saturated MCFA in the coating (**Table 4.2**), which is not oxidizable (Bhatnagar et al. 2009). Furthermore, MCT coating caused significantly lower hydroperoxide concentrations in the inner-surface lipids on day 27 (**Figure 4.2 B**). The inner-surface lipids exhibited higher hydroperoxide concentrations after 27 days of storage when no coating was applied on the extrudates (59.4–86.7 mmol/kg oil) than samples with a coating layer (51.4–53.1 mmol/kg oil). Furthermore, the coating also had a slight inhibiting effect on the oxidation of entrapped lipids. For matrix-incorporated lipids, this effect depended on the feed-water content of the sample. Only in samples with a high expansion (10% H_2O), the coating had a reducing effect on the matrix-incorporated lipid hydroperoxides. A higher expansion, which is associated with an increase in porosity, was obtained by a decrease of the feed-water content (Amft et al. 2019). In the samples with higher water contents and a reduced expansion, i.e., compact structure, no difference between the hydroperoxide-concentrations was found. (),

To determine further the effect of a coating on the extruded particle, the total hydroperoxide concentrations (**Figure 4.3 A & B**) and the formation of hexanal (**Figure 4.3 C & D**) were investigated. During storage, the coated samples showed lower hydroperoxide formation, visible in a lower slope of the curves (**Figure 4.3 A** and **Table 4.1**). These findings were statistically analyzed by the calculated total hydroperoxides concentrations after 27 days of storage (**Figure 4.3 B**), which showed that the MCT coating significantly reduced ($p < 0.05$) the hydroperoxide formation in all extrudates (regardless of the different water contents and the microstructure). Nevertheless, this effect was strongest in the porous samples and decreased with increasing water content. The hexanal formation (**Figure 4.3 C**) showed the same trends over the storage period but more clearly than the hydroperoxides. Samples with lower water content and increased expansion had higher hexanal values, which is in accordance with the

formation of hydroperoxides. In samples without coating, hexanal increased from 5 up to 15–60 AUC, whereas in the samples with an MCT coating the hexanal content did not increase during the entire storage period (hexanal values below 5 AUC units). The significant reduction of the hexanal formation in the extrudates with a coating after 27 days of storage (**Figure 4.3 D**) allows the conclusion that hydroperoxides are formed but react only very slowly further to secondary oxidation products. Frankel (1984) stated that under oxygen, lipid hydroperoxides decompose to nonvolatile secondary products, which form volatile breakdown products. In addition, masking or dilution have occurred due to the addition of MCT. An increase in the lipid content by a factor of 1.4 through the coating decrease the concentration of oxidizable lipids that can contribute to the hexanal formation. This dilution leads to reduced concentrations of volatile hexanal by a factor of 1.4, too. Addition of the same amounts of premix lead to negligible reductions of hexanal (data shown in the supplementary materials, **Supplementary Figure 4.6**). Therefore, we assume that the partitioning behavior contributes to reduced volatile hexanal values. The lipophilic nature of hexanal accounts to a decreased release of hexanal from the lipid phase into the gas phase, which is analyzed in headspace-gas chromatography.

Sunvold and Corrigan (2010) reported in their patent reduced oxidation rates with reduced aldehyde formation and less rancidity in coated kibbles. Their claimed coating comprises a protein component among others chicken by-product meal and a binder and can be applied in the range of 0.1 to 75%. The inventors attribute the positive effect of their coating on lipid oxidation especially to the protein component, which prevents contact between fat and the iron, but they did not demonstrate the effect of a lipid-containing coating (Sunvold and Corrigan 2010).

The bound and incorporated lipids had on their own lower hydroperoxide concentrations than the surface lipids. We conclude based on our findings that with increased expansion more oxygen can migrate into the matrix, where it acts oxidatively. Our results are consistent with Marmesat et al.'s findings in commercial fried nuts, where oxygen mainly affects the outer layer of the nuts and leads to higher surface oxidation compared with internal lipids (Marmesat et al. 2006). The oxygen contact with the unsaturated lipids in the extrudate could be hindered by the coating, which seems to serve as an additional barrier against oxygen migration. Indeed, next to the protective effect of a coating, a dilution effect must be taken into consideration. MCT oil mixed with other oils, which naturally have a high content of unsaturated fatty acids, have a positive effect on stabilization of lipids against oxidation by the dilution of oxidizable

lipids. For example, mayonnaise made with a proportion of MCT oil showed low peroxide values compared with mayonnaise made with linseed oil (Raudsepp et al. 2014). This is consistent with the observations made in this study. To eliminate this dilution effect of the MCT oil, which results in reduced oxidizability of the remaining lipids, correction factors for the individual lipid fractions and the total lipids were calculated.

A protective effect of the coating, which goes beyond a diluting effect of the oxidizable lipids, can be seen in the 10%-feed-water extrudates. Values of the total hydroperoxide concentrations, corrected for the dilution-effect of the MCT oil (**Figure 4.3 B**), showed that these extrudates had significantly reduced hydroperoxide concentrations compared with the uncoated extrudates. Significantly reduced hexanal values were observed in all extrudates, but the effect was reduced with increasing feed-water content, i.e., in extrudates with 10% water the protection effect of the coating is higher as the dilution effect because of the added MCT oil. The extrudates with low feed-water are naturally more porous and here, in particular, it can be assumed that the low-viscosity MCT coating will seal and eliminate microcracks. Gray pointed out that the oxidation rate was significantly reduced in starch extrudates devoid of microcracks (Gray et al. 2008). In the denser extrudates, produced with higher water contents, the results only indicate a dilution effect by the MCT.

The slopes of the hydroperoxide and hexanal curves (**Table 4.1**) indicate a protective effect on the lipids. Especially in the surface lipids, the hexanal formation is inhibited. These effects go beyond the diluting effect of the MCT. The inner-surface lipids exhibit a significant protective effect of the coating only for the 10% samples. In the extrudates with higher feed-water contents, the lower slopes can be justified by a dilution of the oxidizable lipids. Furthermore, the matrix-incorporated lipids do not experience any measurable protection by the coating. With regard to the total hydroperoxides and the hexanal formation, a protective effect of the coating that goes beyond a diluting effect can be clearly observed. This protective effect decreases with increasing water contents and thus decreasing porosity of the extrudates. For the total hydroperoxides, however, this effect was only significant for the 10% samples. The hexanal formation was significantly reduced at all water contents.

Table 4.1: *Slopes of the curves corrected by the factor for MCT dilution*

	hydroperoxides of the surface lipids	hydroperoxides of the inner-surface lipids	hydroperoxides of the matrix-incorporated lipids	total hydroperoxides	hexanal
10%_NC	4.76 ±0.30	2.47 ±0.02	2.69 ±0.15	2.88 ±0.24	1.13 ±0.78
10%_C	0.32 ±0.08	1.52 ±0.11	2.29 ±0.27	1.49 ±0.14	-0.01 ±0.01
10%_C_MCT-corrected[a]	1.34* ±0.31	1.89* ±0.13	[#]	2.07* ±0.19	-0.01 ±0.01
14%_NC	4.72 ±0.73	1.54 ±0.18	1.67 ±0.23	1.92 ±0.19	0.40 ±0.08
14%_C	0.1 ±0.05	1.38 ±0.19	1.48 ±0.22	1.22 ±0.21	-0.01 ±0.02
14%_C_MCT-corrected[a]	0.50* ±0.25	1.49 ±0.21	[#]	1.49 ±0.25	-0.01* ±0.01
18%_NC	5.85 ±0.18	1.61 ±0.19	1.41 ±0.25	1.61 ±0.22	0.296 ±0.04
18%_C	-0.06 ±0.02	1.40 ±0.02	1.68 ±0.21	1.12 ±0.07	-0.036 ±0.01
18%_C_MCT-corrected[a]	-0.01* ±0.00	1.53 ±0.02	[#]	1.46 ±0.08	-0.02* ±0.00

[a]"MCT-corrected" represent extrapolated values corrected for dilution by the MCT oil (Supplemental materials Supplementary Table 4.3). [#]Because no change in the fatty acid profile was observed in the matrix-incorporated lipids due to the MCT coating, no MCT-corrected values were calculated for these lipids. MCT-corrected slopes with a star (*) in the same column within the same water content are significantly different from the slopes of the uncoated samples ($p \leq 0.05$, n=3)

A study showed that lipid-containing coatings made from cassava starch, glycerol, carnauba wax, and stearic acid increased the barrier against oxygen and water vapor on apple slices. The film properties depended on their formulation and the resulting film matrix (Chiumarelli and Hubinger 2014). Drusch et al. postulated that the oxidative stability of lipids microencapsulated into glassy carbohydrate matrices depended on the oxygen diffusivity of the wall materials (Drusch et al. 2009). In addition, Boerekamp et al. stated that the oxidative stability of electrosprayed fish-oil-loaded capsules depends on the oxygen barrier properties of the encapsulating material. Lower oxygen permeability of the tested biopolymers was associated with increased oxidative stability (Boerekamp et al. 2019). This could explain the reduced lipid oxidation rates in extrudates when a coating was applied.

The effect of an MCT coating on lipid oxidation over a storage period of 14 weeks under accelerated shelf-life conditions can be found in the supplementary material (**Supplementary Figure 4.7**) in the online version of this article. This long-time study confirmed the results of the present study and showed that also over a longer period the coating had an inhibiting effect on the oxidation of lipids and the formation of hydroperoxides and hexanal.

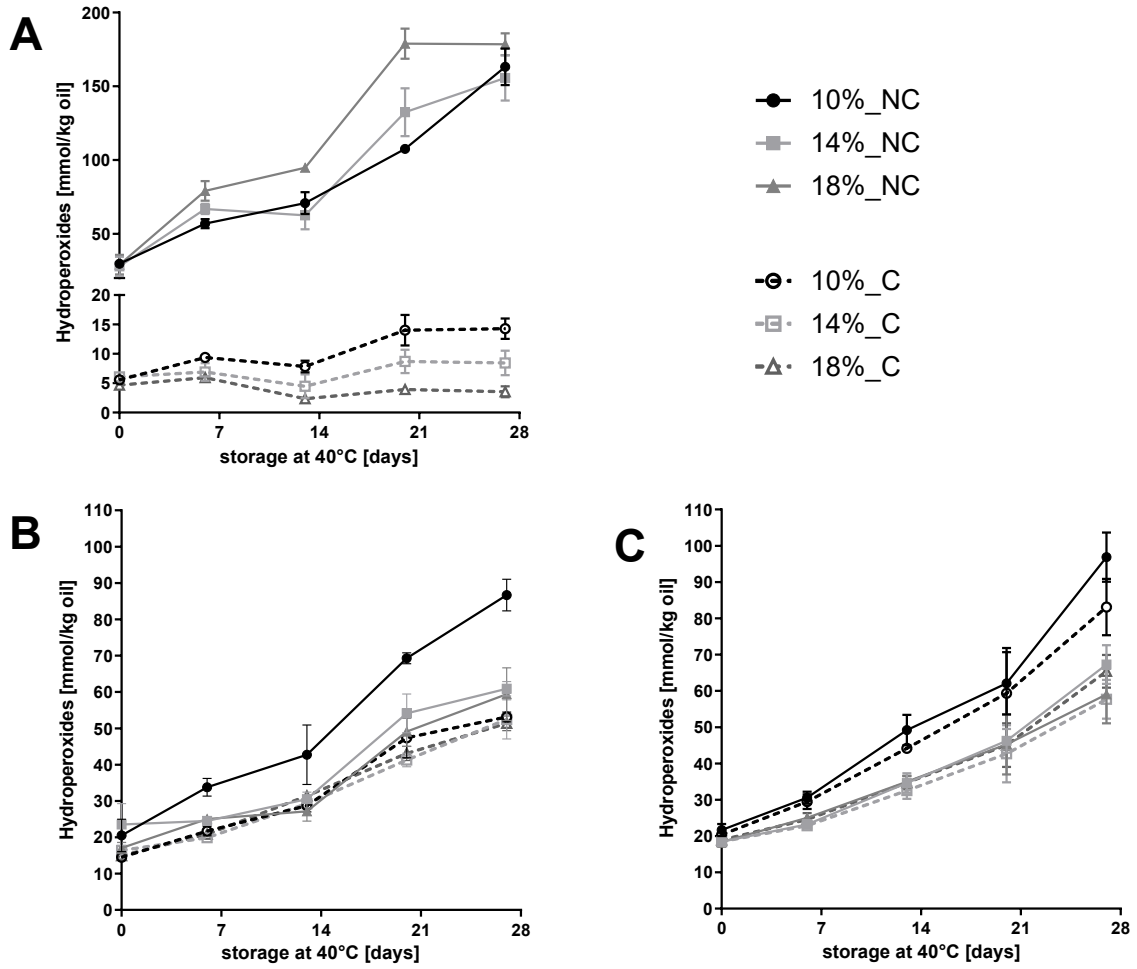

Figure 4.2: *Formation of hydroperoxides in the three different lipid fractions (A: surface lipids, B: inner-surface lipids, C: matrix-incorporated lipids) in the coated (C) and uncoated (NC) extrudates during storage at 40 °C (n=3).*

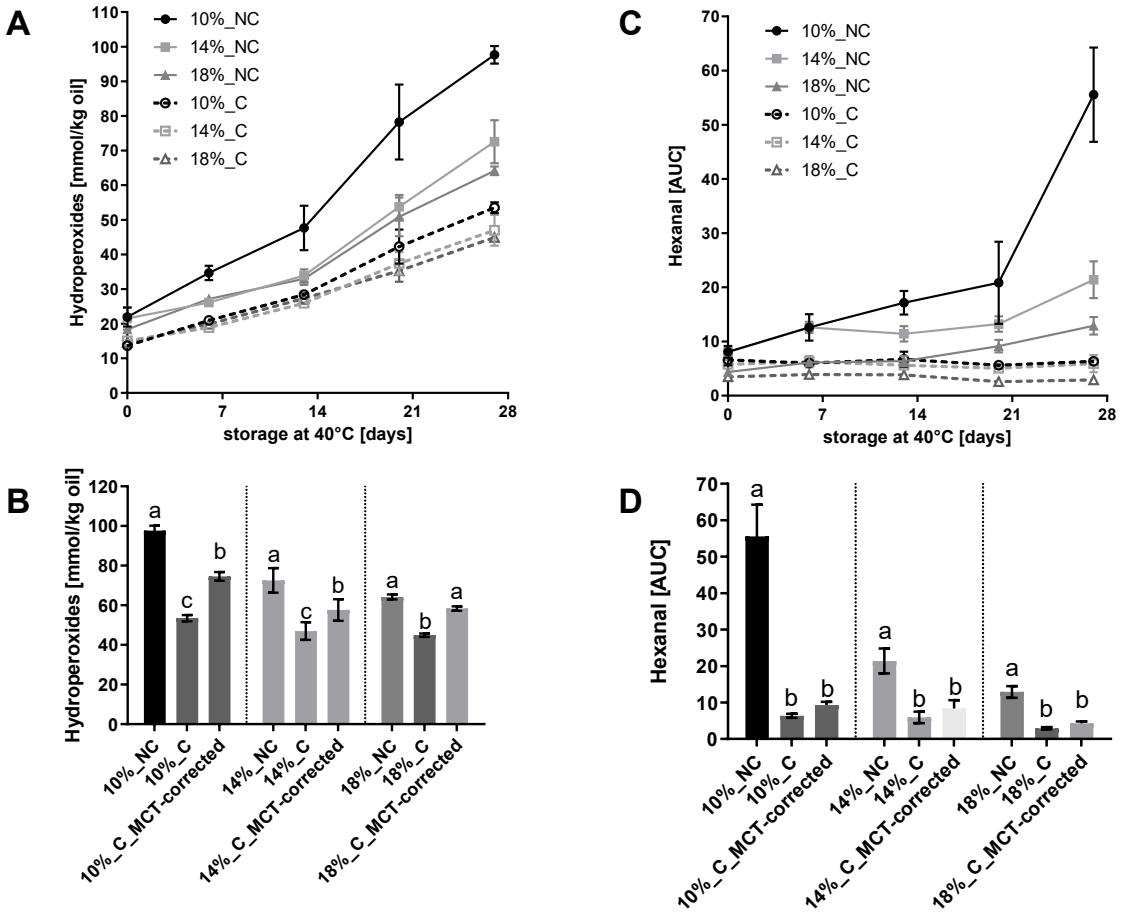

Figure 4.3: *Effect of MCT coating on formation of lipid oxidation markers during storage of the extrudates at 40°C (A: hydroperoxides; C: hexanal) and after 27 days storage (B: hydroperoxides; D: hexanal). The bars in (B) and (D) labeled "MCT-corrected" represent values corrected for dilution by the MCT oil (Supplemental materials Table S1). Bars in (B) and (D) within the same water contents with the same letter are not significantly different from each other $p \leq 0.05$, n=3).*

4.4.2. DISTRIBUTION OF MCFA IN THE EXTRUDATE DEPENDING ON THE COATING

The FA profile in the different fat fractions of the extrudates at day 0 and after 27 days of storage and their PI are reported in **Table 4.2**. The PI provides information about the oxidizability of the different extrudates, depending on their different fatty acid profiles (Cortinas et al. 2003). A differentiation of lipids from MCT coating and sunflower/rapeseed is possible due to their characteristic fatty acid profiles. For example, C_8 and C_{10} are only present in MCT oil whereas C_{18} are only present in sunflower/rapeseed oil mixture.

Substantial amounts of MCFA, up to 51.8% C_8 and lower proportions of the remaining FA were only present in the surface lipids of the coated samples. The fatty acid composition in the surface lipids on day 0 and 27 was almost unchanged. Furthermore, the proportion of MCFA increased and the unsaturated FA decreased with increasing feed-water content and reduced porosity of the extrudate. This can be explained with a lower surface area, where the coating accumulates as a thicker film and in turn reduces the release of lipids from the surface of the extrudates. The coated extrudates had significantly lower PI than the reference samples due to the changed FA profiles with the high amount of MCFA without double bonds. It can be concluded that a coating dilutes the fatty acids present on the surface, resulting in a proportion of less unsaturated fatty acids, which are particularly susceptible to oxidation.

Without the coating, the inner-surface lipids had an identical FA profile and PI as the surface and matrix-incorporated lipids. Coated extrudates had reduced MCFA amounts below 10% compared with the surface lipids. This suggests that the coating not only adheres to the surface of the extrudates but could also migrate in small amounts into the fraction of internal lipids. In the inner-surface lipids, the MCT amount was increased with decreased feed-water contents. In extrudates with 10% water addition, this was particularly noticeable, accompanied by reduced PI values. This can be explained by increased expansion and porosity. Extrudates produced with an increased feed-water content have fewer cavities and thicker cell walls due to lower expansion, which inhibit the MCT oil from penetrating into the interior of the samples (Moisio et al. 2015a; Chanvrier et al. 2015; Amft et al. 2019). The MCT contents in the inner-surface lipids of the coated samples increased by a factor of approx. 1.7 during storage regardless of the water content. This internal migration may be related to the long time the extrudates have been exposed to elevated temperatures during storage. The results indicate, confirmed by the imaging techniques, that a large part of the lipid coating adheres to the surface and only a small amount migrates into the extrudate.

In the matrix-incorporated lipids, no significant differences could be observed in the FA profile and the PI between coated and uncoated samples. The oxidizabilities of matrix-incorporated lipids are at the same level regardless of the application of a coating. Furthermore, no MCFA were measurable in this fraction, which is why no dilution effects can be assumed here from the MCT. This shows that matrix-incorporated lipids can be assumed to contain lipids, which are spatially separated from the inner-surface lipids.

Table 4.2: *Percentage of the main fatty acids in the lipid fractions of the coated and uncoated extrudates at day 0 and 27 [%] and the peroxidability index (PI) of the lipids in the samples (n=3)*

	sample	proportion of total lipids [%]	C8:0	C10:0	C16:0	C18:0	C18:1 n9 c	C18:2 n6c	C18:3 n3	C20:1 n9	PI
Surface lipids	10%_NC_t0*	9.5	0.0	0.0	6.4	2.7	43.9	40.5	3.5	0.7	48.7
	14%_NC_t0*	4.3	0.3	0.1	6.6	2.9	44.5	39.6	3.3	0.6	47.4
	18%_NC_t0*	5.2	0.4	0.0	7.0	3.2	45.5	40.0	2.6	0.5	46.3
	10%_C_t0*	28.3	44.9	31.9	1.5	0.6	9.9	9.4	0.8	0.2	11.3
	14%_C_t0*	20.6	45.7	32.3	1.4	0.6	9.5	8.9	0.8	0.1	10.7
	18%_C_t0*	20.0	51.8	36.5	0.8	0.3	5.0	4.8	0.4	0.0	5.8
	10%_NC_t27*	8.1	0.3	0.4	6.5	2.7	44.2	39.9	3.4	0.7	47.9
	14%_NC_t27*	5.3	0.3	0.3	6.5	2.7	44.5	40.0	3.4	0.8	47.9
	18%_NC_t27*	3.5	0.8	0.7	7.1	3.1	45.6	40.3	2.3	0.0	46.1
	10%_C_t27*	21.8	43.3	30.8	1.7	0.7	11.2	10.5	0.9	0.2	12.6
	14%_C_t27*	17.2	48.2	34.2	1.2	0.5	7.7	7.3	0.6	0.0	8.7
	18%_C_t27*	25.3	52.0	37.1	0.7	0.3	4.5	4.4	0.4	0.1	5.3
Inner-surface lipids	10%_NC_t0*	46.3	1.4	1.0	6.3	2.6	42.9	40.1	3.5	0.7	48.2
	14%_NC_t0	48.8	0.5	0.5	6.1	2.6	43.7	40.3	3.5	0.7	48.5
	18%_NC_t0	52.1	0.1	0.0	6.2	2.7	44.2	41.2	2.9	0.7	48.0
	10%_C_t0	43.2	9.1	6.5	5.4	2.2	37.0	34.7	3.0	0.6	41.6
	14%_C_t0	42.2	3.2	2.3	6.0	2.5	41.7	38.7	3.3	0.7	46.4
	18%_C_t0	38.2	3.1	2.2	6.0	2.5	41.7	39.1	3.3	0.7	46.8
	10%_NC_t27	45.8	0.6	0.3	6.6	2.7	45.1	39.1	3.0	0.7	46.3
	14%_NC_t27	42.3	0.4	0.0	6.4	2.6	44.6	40.2	3.3	0.7	47.9
	18%_NC_t27	43.1	0.2	0.1	6.3	2.6	44.5	40.8	3.3	0.7	48.5
	10%_C_t27	48.4	15.6	10.9	4.8	1.9	32.9	30.1	2.4	0.5	35.8
	14%_C_t27	44.6	6.7	4.7	5.6	2.3	39.6	36.4	3.0	0.6	43.3
	18%_C_t27	34.6	5.9	6.4	5.6	2.3	39.2	36.0	2.9	0.6	42.9
matrix-incorporated lipids	10%_NC_t0	44.2	0.2	0.2	6.7	2.6	42.8	41.8	3.5	0.7	50.0
	14%_NC_t0	46.9	0.1	0.1	6.4	2.6	43.3	41.2	3.6	0.7	49.4
	18%_NC_t0	42.6	0.1	0.0	6.5	2.6	43.1	41.6	3.5	0.7	49.8
	10%_C_t0	28.5	0.1	0.1	6.7	2.6	42.8	41.8	3.5	0.7	49.9
	14%_C_t0	37.2	0.0	0.0	6.5	2.6	43.6	41.5	3.6	0.8	49.8
	18%_C_t0	41.8	0.0	0.0	6.4	2.6	43.4	41.7	3.5	0.7	49.8
	10%_NC_t27	46.1	0.0	0.0	6.6	2.6	43.5	41.4	3.5	0.7	49.5
	14%_NC_t27	52.4	0.0	0.0	6.5	2.6	44.6	41.1	3.4	0.7	49.0
	18%_NC_t27	53.4	0.0	0.0	6.4	2.6	43.8	41.3	3.5	0.7	49.4
	10%_C_t27	29.8	0.3	0.2	6.7	2.5	42.9	41.6	3.4	0.7	49.5
	14%_C_t27	38.2	0.1	0.1	6.5	2.6	43.3	41.4	3.5	0.7	49.4
	18%_C_t27	40.1	0.0	0.0	6.4	2.6	43.5	41.5	3.5	0.7	49.6

Abbreviations: 10, 14, 18% = feed-water content of the extrudates; NC = no coating; C = MCT coating; t0 = storage day 0; t27 = storage day 27; n=1*

4.4.3. OIL ALLOCATION ON THE LAMELLAS AND MIGRATION BEHAVIOR OF THE COATING

The lipid distribution in the extrudates was investigated using fluorescence microscopy with the lipophilic fluorescent dye Nile red (**Figure 4.4**). In images (**Figure 4.4 A**) of extrudate lamellas where the lipids were stained with Nile red, red fluorescence was visible, whereas the matrix exhibited autofluorescence caused by other compounds (Moisio et al. 2015b). It could be assumed that the added oil of the extrudate recipe is dispersed on the surface of the extrudate cell walls, forming a thin lipid film. Because of their localization, these lipids can be classified as inner-surface lipids. Separated lipid droplets were not visible, as shown by Yilmaz et al. in an extruded starch/sunflower oil matrix with a lower oil amount (5% weight on a dry starch basis) and a higher magnification using a scanning electron microscope (Yilmaz et al. 2001). The present findings are in accordance with Barden et al., who investigated the lipid distribution in low-moisture crackers by confocal microscopy. The researchers pointed out that lipids formed a phase that surrounded the starch without formation of dispersed lipid droplets. Furthermore, protein bands were visible that did not provide a physical protection for lipids regarding lipid oxidation (Barden et al. 2015). Moisio et al. extruded oat flour (without added lipids) and investigated the effect of extrusion process parameters on the starch, protein, and lipid reorganization by visualization with confocal laser scanning microscopy and Nile red (Moisio et al. 2015b). They pointed out that an extrusion temperature of 130 °C could be too high for encapsulating lipids inside the extruded oat matrix. Furthermore, they found that at a screw speed of 100 rpm more lipids seemed to be bound than at 400 rpm. The authors explained this with a longer residence time. At 400 rpm, the lipids were separated from the structure and lipid droplets are visible (Moisio et al. 2015b). The extrusion temperature used in this study was 125 °C at a screw speed of 333 rpm with an oil content of 10%. Higher temperatures and distinct higher amounts of oil could explain why the whole lamella is more or less colored on the surface by the lipophilic Nile red and individual lipid droplets are not visible. It seems that due to coalescence a thin film is formed, rather than individual droplets.

The surface of the extrudates without a coating and with MCT coating, both dyed with Nile red, is displayed in **Figure 4.4 B**. Without a coating, only the autofluorescence from the matrix was visible. The coated extrudate resembled a hilly landscape on the surface. The images showed that the coating layer fills the “valleys” and microcracks and forms a thin and smooth film on the surface of the extrudates. These findings contribute to explain the inhibitory effects of the

MCT coating in this study. The technique of fluorescence microscopy can provide important information about the allocation of the lipids inside an extrudate or on their surface. However, fluorescence microscopy is not suitable for an analysis of the MCT coating and its migration behavior because the stained coating is carried over into the interior of the extrudate during sample preparation. Preliminary trials showed also that methods such as the cryomicrotome were unsuitable for extrudates. To preserve the filigree structure of an extrudate, the μCT, a noninvasive imaging method, was used for this purpose. Nile red was used as a contrasting agent based on the findings of Contardo and Bouchon (2018) in fried starchy-gluten matrices. (Contardo and Bouchon 2018).

The allocation of the MCT coating on the extrudates' surface in the cross section and the migration into the extrudate is shown in the μCT pictures (**Figure 4.5**). An analysis of the extrudates with a coating in μCT revealed clear differences in the scans compared with the uncoated extrudates. In the samples with coating, dark gray areas could be observed on the surface and in cavities, which did not appear in the control sample without coating (Amft et al. 2019). In addition, these areas have a typical appearance for liquids. In narrow cells of the extrudate, for example, the formation of a concave meniscus can be seen, indicating capillary effects of liquids. These gray spots correspond to the MCT coating stained with Nile red. In the μCT scans (**Figure 4.5**) it can be observed that the coating layer was more highly concentrated in some surface areas, e.g., in grooves and outer cavities of the extrudate surface. These results agree with the observations made in fluorescence microscopy (**Figure 4.4 B**).

In extrudates with a low water content (10%), a porous structure could be observed with clear incorporations of the coating in the outer few millimeters. Because of the thin and partly not intact cell walls, the coating can migrate more easily into the interior. In extrudates with higher water contents, oil incorporations can only be recognized in external cavities. In the case of a channel from the surface to the interior of the extrudate having been formed, oils migrate into the inner cavities. The reduced migration in extrudates with high feed-water contents can be attributed to the significantly thicker cell walls and the more compact structure, which has been shown in previous work (Amft et al. 2019). In conclusion, migration of the dyed MCT oil from the surface to the extrudate core was only visible in the outer layer of the extrudate. Measurements of the μCT scans with the caliper-tool of the myVGL software indicate that the coating is limited to the outer 2–3 mm of the extrudates. This indicates that the majority of the coating remained on the surface and did not migrate into the extrudate.

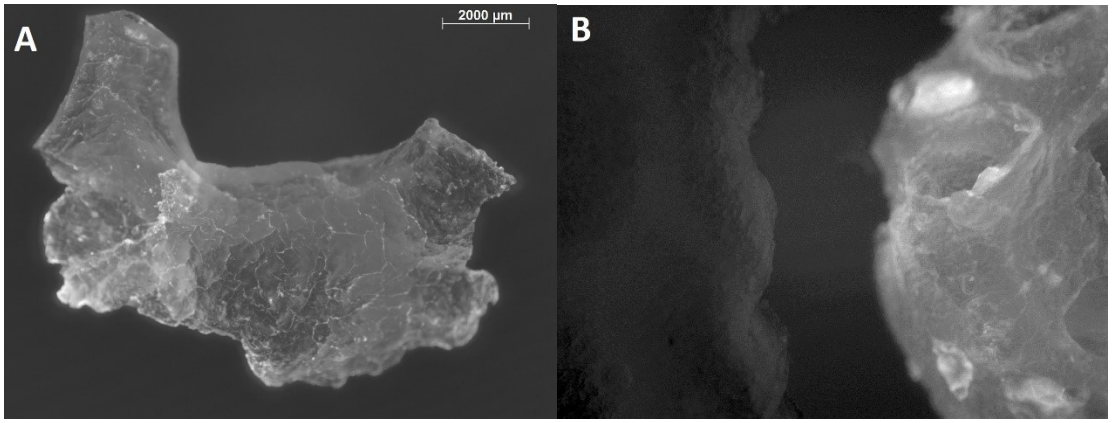

Figure 4.4: *A) Microscopic image of an extrudate lamella dyed with Nile red and B) Surface of the extrudates without a coating on the left side and with an MCT-coating dyed with Nile red on the right side*

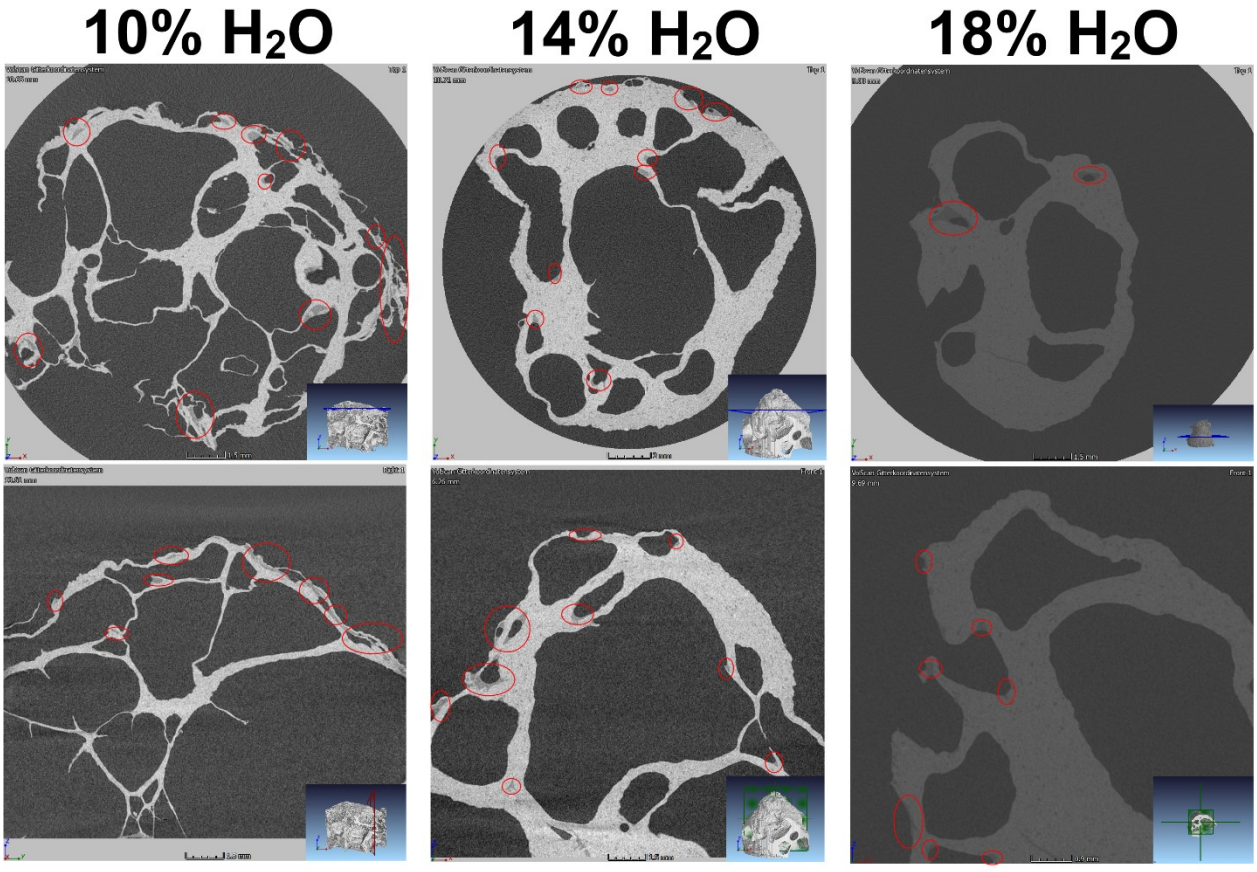

Figure 4.5: *µCT scans for visualization of the MCT coating allocation (red ellipses around the gray regions which denote the MCT coating) on the extrudate and the migration behavior in the different feed-water contents (10, 14 and 18% H_2O). The light regions within the scan correspond to the extrudate matrix, the black regions represent air and the dark gray regions denote the MCT coating*

4.5. CONCLUSIONS

Lipid-containing extrudates are susceptible to oxidation because of their large surface area. A coating seems to be a suitable way to minimize oxygen accessibility and to delay the lipid oxidation. Our study presented the potential of MCT oil as an edible coating for extruded low moisture foods to delay lipid oxidation. In measurements of lipid oxidation rates in different lipid fractions, it was found that mainly the outer regions of an extrudate particle are protected and showed, in turn, a decreased lipid oxidation. The concentration of total hydroperoxides and the formation of volatile aldehydes could be significantly reduced by the application of a coating, too. Because of the reduced hexanal contents, the coated extrudates were also sensory inconspicuous. We observed that the coating appeared to have no effect on the stabilization of the lipids in the core of the extrudate particle and that here a dilution effect can be assumed. In summary, the above results indicated that the stabilization of lipids against oxidation by the application of an edible lipid-based coating is possible. Especially porous extrudates can benefit from the protective effect of the coating.

Acknowledgments

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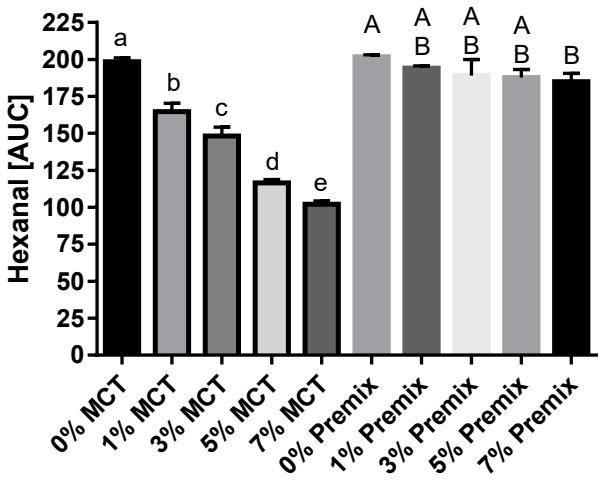
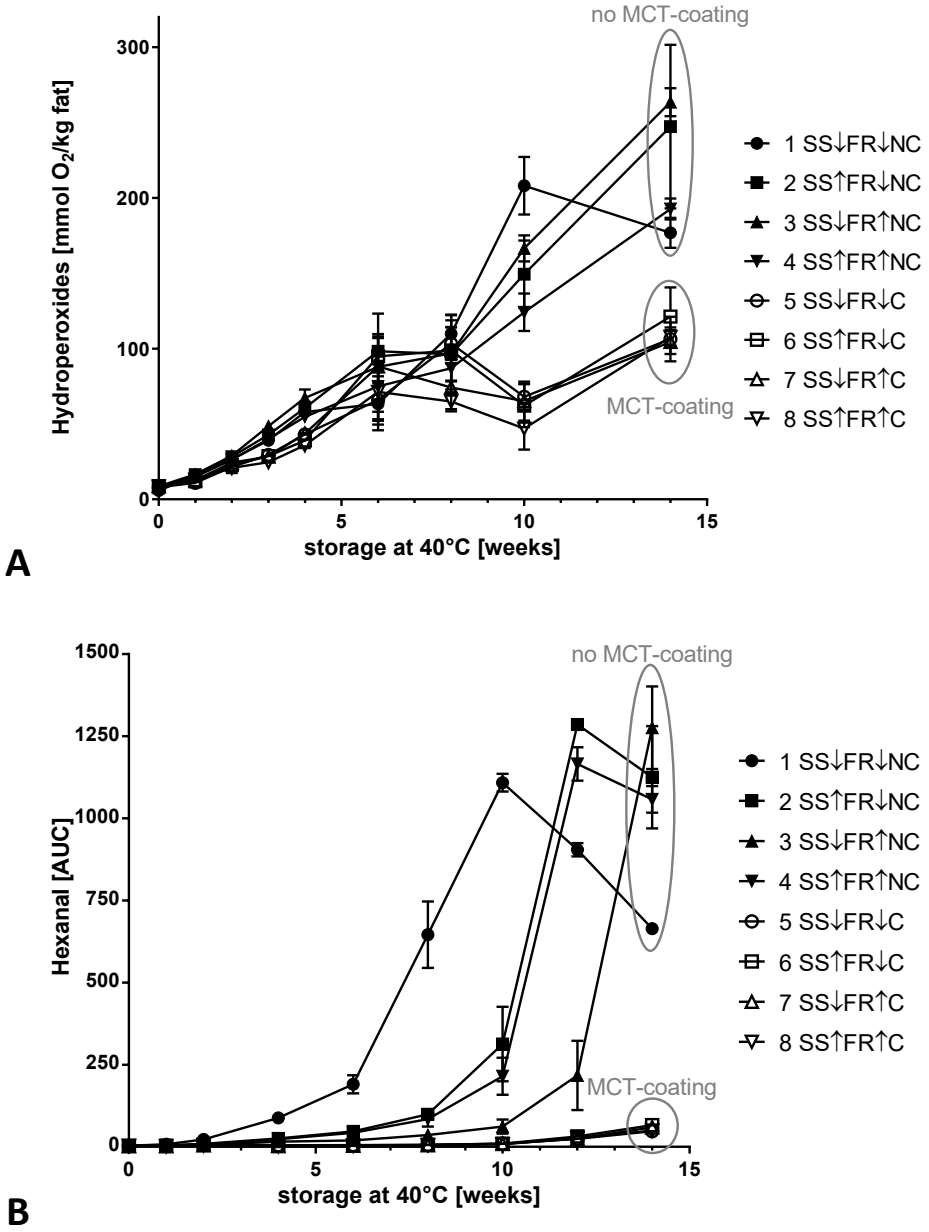
4.7. SUPPLEMENTARY MATERIAL



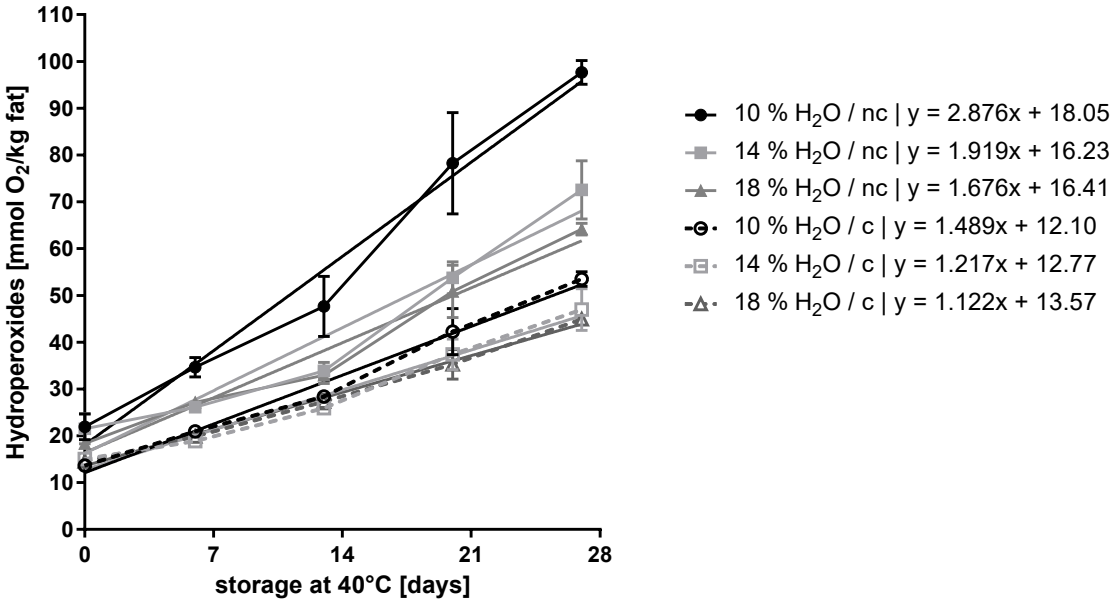
Supplementary Figure 4.6: *Hexanal masking by coating: Effect of various amounts of MCT oil addition vs. various amounts of premix (same water content as the extrudate) addition on the measureable hexanal concentration in oxidized corn extrudates. Bars with the same letter are not significantly different from each other ($p \leq 0.05$, n=3)*

Long-term study: Effect of a MCT coating on lipid oxidation in extrudates

In a second experiment with the varying process-parameters screw speed and feed rate, the effect of the MCT coating on lipid oxidation was investigated. The water content of the premix was constant at 14% and the screw speed (SS) varied from 286 to 382 rpm. The feed rate (FR) was 12 and 24 kg/h. One half was not treated (NC) and the other half was finished with an MCT coating (C) on the surface as described in the materials and methods part. The long-term study (**Supplementary Figure 4.7**) showed that also over a longer period of time (14 weeks) at 40 °C the coating had an inhibiting effect on the oxidation reactions. Both hydroperoxides and hexanal formation showed significantly lower values over the entire storage period when a coating was applied on the surface of the extrudates. A design of experiments showed that the different process parameters that were originally focused on (SS and FR) showed no noteworthy effect whereas the coating (C) showed distinct effects. The results of this long-term study have been presented as a poster at the 15th Euro Fed Lipid Congress in Uppsala, Sweden (2017).



Supplementary Figure 4.7: *Formation of (A) hydroperoxides and (B) hexanal in extrudates with/without MCT coating during storage at 40 °C over 14 weeks (SS↓ = screw speed 286 rpm; SS↑ = screw speed 382 rpm; FR↓ = feed rate 12 kg/h; FR↑ = feed rate 24 kg/h; NC = no coating; C = MCT coating).*



Supplementary Figure 4.8: *Linear regression analysis and curve fitting of the formation of total hydroperoxides during storage (Original figure was Figure 4.3 A)*

Supplementary Table 4.3: *Factor calculation to extrapolate the effects of lipid dilution by MCT oil used for calculation of "MCT-corrected"-values:*

	sample	C18:0	C18:1 n9 c	C18:2 n6c	C18:3 n3	sum of C18 FA	**Mean Factor of T0+T27 ±SD**
Surface lipids	10%_NC_t0*	2.7	43.9	40.5	3.5	90.6	
	14%_NC_t0*	2.9	44.5	39.6	3.3	90.4	
	18%_NC_t0*	3.2	45.5	40.0	2.6	91.2	
	10%_C_t0*	0.6	9.9	9.4	0.8	20.7	**4.12 ±0.36**
	14%_C_t0*	0.6	9.5	8.9	0.8	19.8	**5.11 ±0.75**
	18%_C_t0*	0.3	5.0	4.8	0.4	10.5	**9.10 ±0.61**
	10%_NC_t27*	2.7	44.2	39.9	3.4	90.2	
	14%_NC_t27*	2.7	44.5	40.0	3.4	90.7	
	18%_NC_t27*	3.1	45.6	40.3	2.3	91.4	
	10%_C_t27*	0.7	11.2	10.5	0.9	23.3	**4.12 ±0.36**
	14%_C_t27*	0.5	7.7	7.3	0.6	16.1	**5.11 ±0.75**
	18%_C_t27*	0.3	4.5	4.4	0.4	9.6	**9.10 ±0.61**
Inner-surface lipids	10%_NC_t0*	2.6	42.9	40.1	3.5	89.1	
	14%_NC_t0	2.6	43.7	40.3	3.5	90.2	
	18%_NC_t0	2.7	44.2	41.2	2.9	90.9	
	10%_C_t0	2.2	37.0	34.7	3.0	76.9	**1.25 ±0.12**
	14%_C_t0	2.5	41.7	38.7	3.3	86.2	**1.08 ±0.05**
	18%_C_t0	2.5	41.7	39.1	3.3	86.7	**1.09 ±0.06**
	10%_NC_t27	2.7	45.1	39.1	3.0	89.9	
	14%_NC_t27	2.6	44.6	40.2	3.3	90.7	
	18%_NC_t27	2.6	44.5	40.8	3.3	91.1	
	10%_C_t27	1.9	32.9	30.1	2.4	67.3	**1.25 ±0.12**
	14%_C_t27	2.3	39.6	36.4	3.0	81.2	**1.08 ±0.05**
	18%_C_t27	2.3	39.2	36.0	2.9	80.5	**1.09 ±0.06**
matrix-incorporated lipids	10%_NC_t0	2.6	42.8	41.8	3.5	90.8	
	14%_NC_t0	2.6	43.3	41.2	3.6	90.7	
	18%_NC_t0	2.6	43.1	41.6	3.5	90.9	
	10%_C_t0	2.6	42.8	41.8	3.5	90.7	**1.00 ±0.00**
	14%_C_t0	2.6	43.6	41.5	3.6	91.3	**1.00 ±0.01**
	18%_C_t0	2.6	43.4	41.7	3.5	91.3	**1.00 ±0.00**
	10%_NC_t27	2.6	43.5	41.4	3.5	91.0	
	14%_NC_t27	2.6	44.6	41.1	3.4	91.7	
	18%_NC_t27	2.6	43.8	41.3	3.5	91.2	
	10%_C_t27	2.5	42.9	41.6	3.4	90.4	**1.00 ±0.00**
	14%_C_t27	2.6	43.3	41.4	3.5	90.7	**1.00 ±0.01**
	18%_C_t27	2.6	43.5	41.5	3.5	91.1	**1.00 ±0.00**

CHAPTER 5

ANALYSIS OF RADICAL FORMATION BY EPR IN COMPLEX STARCH-PROTEIN-LIPID MODEL SYSTEMS AND CORN EXTRUDATES

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5. ANALYSIS OF RADICAL FORMATION BY EPR IN COMPLEX STARCH-PROTEIN-LIPID MODEL SYSTEMS AND CORN EXTRUDATES

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Published previously in Food Chemistry (2020): Amft, J., Steffen-Heins, A. and Schwarz, K. (2020), Analysis of radical formation by EPR in complex starch-protein-lipid model systems and corn extrudates. Food Chem., 331: 127314. doi: 10.1016/j.foodchem.2020.127314.

5.1. ABSTRACT

The formation of short-lived and stable radicals was investigated using electron paramagnetic resonance (EPR) spectroscopy and compared with hydroperoxides and hexanal in complex starch-protein-lipid model systems, as well as in corn extrudates. Stable radicals were detected directly in ground samples. Short-lived lipid radicals were measured ex situ in ethyl acetate extracts of model systems and extrudates by the use of the spin trap PBN. Significant adduct formation was found after 30 min at 50 °C. During storage, lipid radicals (PBN adducts) increased in model systems. Simulation of EPR spectra from bulk oil demonstrated that mainly alkoxyl radical adducts were detected, to which rapidly decomposing peroxyl radical adducts also contributed. Stable radicals in extrudates were attributed to protein radicals based on g-value of 2.00467 compared with 2.00474 found in model system prepared with zein. The signal intensity of the stable radical remained constant during storage, but increased during extrusion.

5.2. INTRODUCTION

Low-moisture foods such as cereals, snack products, as well as kibbles are processed by extrusion technology. Lipids improve and normalize the extrusion process, play an important role as plasticizers, and affect as lubricants the shearing and the mechanical energy input and in turn the texture and expansion of the final product. Oils containing a high content of polyunsaturated fatty acids (PUFA) are used in extrudates to replace saturated fatty acids in order to improve the nutritional profile. Especially unsaturated lipids are prone to oxidation, a radical chain reaction that leads to undesired oxidation products that are correlated with an increased risk for cancer. It is generally established to measure primary and secondary processes of lipid oxidation based on the formation of lipid hydroperoxides and aldehydes. A promising method to predict the oxidation states of low moisture foods is the electron paramagnetic resonance (EPR) spectroscopy by detecting paramagnetic unpaired electrons in an external magnetic field. This allows the measurement of free radicals that are formed during early stages of oxidation prior to hydroperoxide formation (Velasco et al. 2005). In extrudates the presence of lipid alkyl, peroxyl, and alkoxyl radicals can be expected (Zamora and Hidalgo 2016). For detection of radicals by EPR, their concentration must be high enough and a sufficient spin relaxation time is required (Buettner 1987). Radicals derived from carbohydrates and proteins are predominantly carbon- or nitrogen-centered radicals with a relatively high molecular weight. Steric hindrance and substituent effects lead to a strong delocalization of the free electron, so that such radicals are quite unreactive and therefore have a long half-life. They can be directly detected by EPR and are referred to as stable radicals in the following. The knowledge about the detection of both, short-lived and stable radicals, in low moisture foods is of great significance because it allows insights into radical formation mechanisms and helps to derive suitable measures for protection against oxidation.

Schaich and Rebello (1999) studied the free radical production in wheat flour extrudates with EPR. In the spectra, the authors found peaks from stable nitrogen-centered radicals, which were formed due to heat-induced peptide decomposition during extrusion and reactions between lipid radicals and amino groups. Factors affecting the radical formation were e.g., heat, shear stress, lipid oxidation reactions, and the protein content (Schaich and Rebello 1999). A limiting factor for the direct EPR spectroscopy of solid substances such as extrudates is their water content.

Free oxygen-centered radicals produced during lipid oxidation are short-lived, which makes it impossible to detect them directly at room temperature (Buettner 1987). For example, hydroxyl

and alkoxyl radicals exhibit a half-life of $1x10^{-9}$ s and $1x10^{-6}$ s, respectively (Sies and Stahl 1995). For that reason, spin traps, e.g., 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), α-(4-Pyridyl N-oxide)-N-*tert*-butylnitrone (POBN) or N-*tert*-butyl-α-phenylnitrone (PBN) are used, which capture radicals present and form a relatively stable nitroxide radical, the spin adduct (Cui et al. 2017). Studies by Velasco et al. (2005) and Novakov et al. (2001) demonstrated that both spin traps are suitable for detection of lipid-derived radicals. Using a spin trap for assaying the free radical concentration in foods implies that the spin trap is used in an appropriate solvent in a suitable concentration with convenient incubation conditions. There is still considerable ambiguity with regard to the incubation conditions of spin traps. PBN was dissolved in former studies in ethanol, toluene, heptane or directly in the oil. Incubation times differed from a few seconds up to hours and the temperature is in the range from room temperature to 70 °C (Cui et al. 2017; Szterk et al. 2011; Velasco et al. 2004). Therefore, we identified suitable conditions for PBN adduct formation in our present study using a Fenton system that enable the measurement of PBN-radical adducts in complex starch-lipid-protein matrices using the proposed lipid extraction of this study.

So far, several methods, e.g., accelerated solvent extraction (Lampi et al. 2015) or petroleum ether extraction (Rao and Artz 1989) have been developed to extract lipids and analyze lipid oxidation in extrudates. However, there is a lack of studies investigating both lipid and protein/carbohydrate oxidation in complex systems using EPR. The need for a rapid and simple procedure to measure stable radicals derived from carbohydrates or proteins and to measure short-lived lipid radicals using EPR was our driving force.

To evaluate the formation of both, short-lived and stable radicals, formed in complex systems we aimed at developing a protocol that enables the measurement of lipid radicals by spin trapping technique and stable radicals by direct measurement in complex mixtures containing starch, protein and lipids and corn based extrudates after grinding. For comparison, the oxidation of lipids was evaluated by quantification of hydroperoxides using photometry and hexanal via gas chromatography. In addition, we aimed to differentiate the nature of stable radicals that may be formed in complex systems by proteins and/or carbohydrates.

5.3. MATERIALS AND METHODS

5.3.1. MATERIALS

N-*tert*-Butyl-α-phenylnitrone (PBN) was purchased from Enzo Life Science (Germany). Native corn starch was from Cargill (Germany), native cornmeal was purchased from Paul's Mühle (Germany) and pregelatinized cornmeal was from Interquell (Germany). Rapeseed oil and sunflower oil were purchased from a local supermarket. Zein and linoleic acid (technical grade 62%) were obtained from Sigma Aldrich (Germany). EDTA, iron(II) sulfate heptahydrate, hydrogen peroxide 30% and the reagents ethanol, acetone, MTBE, diethyl ether, and dichloromethane were purchased from Carl Roth (Germany). Petroleum ether, ethyl acetate, 2-propanol, and hexane were from VWR (Germany). The borosilicate glass capillaries were purchased from Hilgenberg GmbH (Germany).

5.3.2. METHODS

5.3.2.1. PURIFICATION OF SUNFLOWER/RAPESEED OIL (50:50)

A mixture of sunflower and rapeseed oil (50:50) was purified and all antioxidants were removed by adsorption chromatography as described by Lampi and Kamal-Eldin (1998) with some modifications. In brief, a glass column was packed with 250 g activated aluminum oxide (8 h at 100 °C and 12 h at 200 °C) and filled with n-hexane. Commercial rapeseed and sunflower oil were mixed (50:50 v:v) in the equal volume of n-hexane (as elution solvent). This mixture was passed through the column that was wrapped with aluminum foil to prevent photooxidation. The purified oil-hexane-mixture was collected in a brown ice-cooled glass bottle. The solvent was removed using rotary evaporation under nitrogen atmosphere and the success of oil-purification was tested using HPLC (<0.5 ppm tocopherols; method DGF F-II 4a).

5.3.2.2. MODEL SYSTEM BASED ON CORNFLOUR-CONSTITUENTS

A model system was selected for fast measurements of lipid oxidation in a low-moisture matrix. The model system was prepared by mixing the dry component (85%) with the lipid component (10%) and distilled water (5%) for 10 s in an analytical mill (IKA A11 basic, Germany). For method development (part 5.4.2. and 5.4.3.), the mixture was prepared with purified

rapeseed/sunflower oil and for the final tests of the method and radical identification (part 5.4.4.), pure linoleic acid was used as lipid component. The dry ingredients were native cornmeal, native cornstarch, corn zein or native cornstarch mixed with 8% zein. For oxidation studies, the samples were stored in 500 mL glass bottles at +40 °C in the dark.

5.3.2.3. EXTRUSION PROCESS

The extrusion process was conducted on a laboratory twin-screw extruder with contrarotating intermeshing screws (Brabender Mod. DSE 35/7D). The premix was prepared by mixing native (Paul`s Mühle, Germany) and pregelatinized cornmeal (Interquell; Germany) (50:50 w/w) with 10% of a commercial rapeseed/sunflower oil mixture (50:50 v/v) and 14% tap water in a Stephan Universal Machine. The premix was fed to the extruder by a gravimetric weight feeder (Coperion Ktron Model K2-ML-D5-T35-QC) with a constant feed rate of 12 kg/h. The temperature controllers of the extruder were set to 60/125/125 °C and the screw speed was set to 286 rpm. The extruder was operated with one open die (8mm diameter) and an automated cutting device. After equilibration of the extruder, the extrudates were collected and dried in air-permeable plastic bags in a climate cabinet (ThermoTEC TCS-501) at 30 °C and 20% humidity for 48 h. The dried extrudates were stored, in triplicate, in 1000 mL glass bottles at 40 °C in the dark.

5.3.2.4. ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR)

The EPR measurements were performed on a Bruker Elexsys II E500-CW-EPR spectrometer (Rheinstetten, Germany) operating with X-band at 9.85 GHz and a modulation frequency of 100 kHz at room temperature. The evaluation was performed with the Bruker-software Xepr 2.6b.52. For measurements 1 mM EDTA-water was prepared with distilled water. Hydrogen peroxide solution was prepared by adding 50 µL hydrogen peroxide 30% to 25 mL EDTA-water. A 1 mM iron solution was prepared with iron(II) sulfate heptahydrate solved in EDTA-water. PBN solutions were prepared in a 200/600 mM concentration with ethanol.

5.3.2.5. EPR FOR MEASUREMENT OF STABLE RADICALS

Ground samples (model systems or extrudates) were filled into glass capillaries and subjected to EPR. Glass capillaries with different qualities (soda, borosilicate, quartz) were tested and the diameter was optimized with regard to the water content (see supplementary materials, Fig. S1). Borosilicate and quartz glass were used previously by Andersen et al. (2011), Bidzińska et al. (2012), Labanowska et al. (2014) and Yordanov and Mladenova (2004). All capillaries had a length of 10 cm, an outer diameter of 4 mm, and an inner diameter of 3 mm. Borosilicate glass capillary was used for the present study because noise signals were lowest. After filling the capillary with the ground sample (IKA A11 basic) and sealing the end with putty (Haematocrit sealing compound from Brand; Germany), the capillary with the sample was put into an EPR-sample tube (10 mm o.d.) for measurement. The following EPR settings for measurement of stable radicals were used: microwave attenuation = 10 dB; modulation amplitude = 8.5 G; receiver gain = 60 db; sweep width = 80 G; microwave power = 20 mW; time constant = 327.68 ms; conversion time = 80.01 ms and center field = 3511.45 G. Spin fitting via Bruker software was applied to determine the g-value of the measured radicals. Peak picking was used to determine peak height and peak width of the radical peaks.

5.3.2.6. EPR FOR MEASUREMENT OF SHORT-LIVED RADICALS

Lipids were extracted from model systems and extrudates and short-lived radicals were trapped with PBN. The extraction efficiency of radicals from the model system was tested with the solvents dichloromethane, diethyl ether, ethyl acetate, acetone, MTBE, petroleum ether, and cyclohexane to identify the most appropriate solvent. Ethyl acetate offers the best extraction efficiencies and was used as extraction solvent for further studies with the model systems and extrudates. Therefore, 10 g of dried and ground sample were weighed into 50 mL centrifuge tubes and 15 mL ethyl acetate were added. After sonication for 20 seconds (cycle 9, 50% power, Bandelin sonoplus, sonication probe VS 70) the tubes were centrifuged at 3000 rpm for 3 minutes. The extracts were filtered and the extraction procedure was repeated with 10 mL of solvent. Then, 5 mL extract were transferred in 15 mL centrifuge tubes and the solvent was removed in a centrifugal evaporator (90 min.).

For identification of suitable incubation conditions by Fenton-reaction generated hydroxyl radicals, 300 µL hydrogen peroxide solution were mixed with 150 µL Fe^{2+} solution and 50 µL PBN solution. After vortexing for 3 s, the microreaction tubes were incubated at 30 and 50 °C

in a water bath up to 160 min and regularly samples were taken. For further studies with extracted lipid samples and stripped oil, most suitable PBN (25 µL 200 mM) were pipetted to 45 mg weighed oil in PCR-tubes. Only in the case of extrudates, higher PBN concentrations (600 mM) were used from storage day 3 because a high radical formation was expected. After vortexing, samples were incubated for 30 min at 50 °C. The analyses were performed in triplicates. Generally, the double integral of the middle field doublet (A_0) was calculated for the area of PBN adducts. For experiments identifying suitable incubation conditions and for experiments with the model system, PBN adducts were expressed as intensity (I_0), calculated by peak-to-peak-amplitude of the first peak of the middle field doublet. The EPR settings for measurement of short-lived lipid radicals were as follows: microwave attenuation = 10 dB; modulation amplitude = 1.0 G; receiver gain = 80 db; sweep width = 80 G; microwave power = 20 mW; time constant = 163.84 ms; conversion time = 100.05 ms and center field = 3511.45 G. Spectral analyses of individual PBN-spectra were carried out by simulation using EPRsimC software developed by Strancar et al. (2005). To simulate experimental spectra, a spin trap simulations model was used. The tensors for PBN-OH radical were identified using EasySpin software and were as follows: g_{xx} = 2.0055, g_{yy} = 2.0059, g_{zz} = 2.0059 and the A tensors were set to, $A_{xx} = A_{yy}$ = 19.8 MHz and A_{zz} = 96.1 MHz. The radicals were identified in approximation to the hyperfine constants of Xepr software from Bruker: alkoxyl radical (A_N=13.5 G; A_H=2 G), peroxyl radical (A_N=15 G; A_H=3.2 G), hydroxyl radical (A_N=15.6 G; A_H=3.2 G), and hydroxyethyl radical (A_N=16.2 G; A_H=3.34 G).

5.3.2.7. ANALYSIS OF HYDROPEROXIDE CONCENTRATION AND VOLATILE SECONDARY OXIDATION PRODUCTS

Hydroperoxides were analyzed in triplicates by the ferrous thiocyanate method (Amft et al. 2019). 10-50 mg of the oil sample were solved in 5 mL 2-propanol. 50 µL iron(II) chloride solution and 50 µL ammonium thiocyanate solution were added, vortexed and for 30 min. incubated at 60 °C in a water bath. After cooling down to room temperature the extinction of the sample was measured at a wavelength of 485 nm against 2-propanol. Hexanal, as the main aldehyde from linoleic acid, was analyzed with an Agilent 6890 series gas chromatograph, equipped with a J&W DB-1701 column, with headspace autosampler (HS-GC). 1 g sample was weighed in 20 mL glass vials and sealed airtight. The method used for HS-GC analysis is

described in detail by Amft et al. (2019). Hexanal was identified using an external standard and results were displayed as area under the curve (AUC).

5.3.2.8. STATISTICAL ANALYSIS

All experiments were conducted in triplicate and data are presented as the mean values ± standard deviations. Statistical analysis was performed with the software GraphPad PRISM 6 (GraphPad Software, San Diego, USA).

5.4. RESULTS AND DISCUSSION

5.4.1. SUITABLE PBN-INCUBATION CONDITIONS FOR ANALYSIS OF SHORT-LIVED RADICALS

To receive the characteristic PBN adduct signal a sample incubation with PBN at elevated temperatures (e.g., at 60 °C) is common to accelerate the induction period, in which endogenous antioxidants of the sample matrix are depleted (Kocherginsky et al. 2005). Because we have used an antioxidant-free system to identify suitable incubation conditions, we suspect that the temperature increase is also necessary to lower the activation energy for the reaction of PBN with a radical. PBN was selected because it is commonly used to measure lipid oxidation, thus enabling comparability with other studies.

The initial experiments focused on identification of proper incubation conditions for PBN in an antioxidant-free system. Hydroxyl radicals (•OH) were generated immediately by Fenton reaction, which is considered to be the main source of hydroxyl radical formation in biological systems (Southworth and Voelker 2003). Since iron is to be expected in extrudates, e.g., from raw materials or by abrasion, we worked with the EDTA as a chelator. It has been reported that chelated iron can also produce radicals from nitrones (Finkelstein et al. 1980). Our preliminary experiments with extracts from model systems showed that the samples containing additional iron exhibited further peaks interfering with the peaks attributed to POBN-adducts in the EPR spectrum (**Supplementary Figure *5.7***).

Two different temperatures were tested for PBN and the spin trapping capacity was assessed by formation of PBN adducts (**Figure 5.1**). After 60 minutes of incubation only small intensities of PBN adducts could be measured at 30 °C. At 50 °C samples showed significantly higher concentrations of PBN adducts than after incubation at 30 °C. A maximum was observed after 30 minutes, which indicates that 30 minutes are an optimal incubation time for spin trapping with PBN. After this steady-state level the PBN adducts decrease because of a higher degradation of spin adducts compared with their formation (Kocherginsky et al. 2005). Based on these results, all further experiments were performed with the spin trap PBN and an incubation temperature of 50 °C for 30 min.

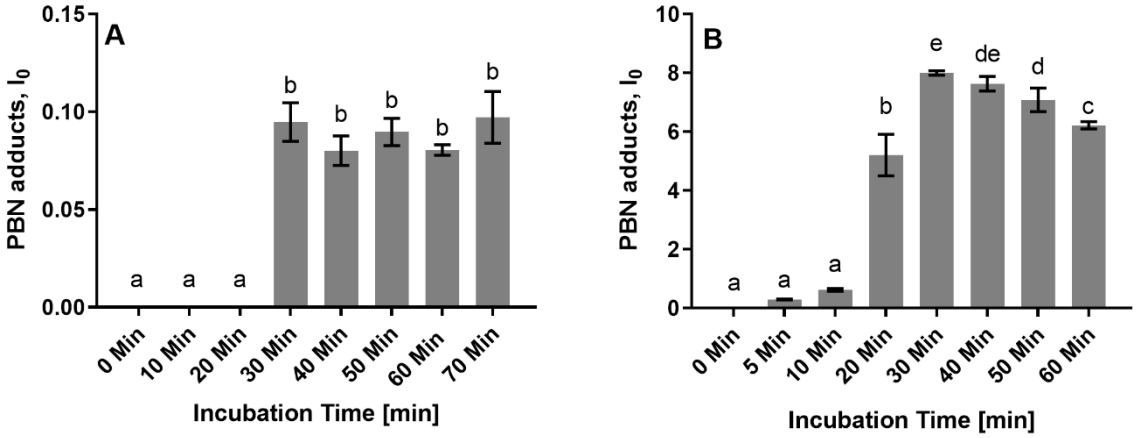

Figure 5.1: *Measurement of spin trapping capacity of PBN at various incubation conditions (A: PBN 30 °C, B: PBN 50 °C).Trapped hydroxyl radicals were generated by Fenton reaction. Bars with the same letter are not significantly different from each other $p \leq 0.05$, n=3.*

5.4.2. EVALUATION OF THE LIPID-RADICAL EXTRACTION PROTOCOL

The extraction efficiency of radicals from the model system prepared with purified rapeseed/sunflower oil was tested with the solvents dichloromethane, diethyl ether, ethyl acetate, acetone, MTBE, petroleum ether, and cyclohexane to identify the most appropriate solvent (**Figure 5.2**). Centrifugal evaporation was used for solvent removal. Except for diethyl ether, all EPR spectra showed the same pattern (**Figure 5.2 A**). Our findings showed that the highest concentration of PBN adducts was detected in the model system extracted with dichloromethane, diethyl ether, and ethyl acetate (**Figure 5.2 B**). These solvents had a polarity index between 3.1 and 5.1. Samples extracted with diethyl ether showed a different peak-pattern in the EPR-spectrum. Freeze-drying after the centrifugal evaporation revealed that the changed spectrum was attributable to solvent residues, which formed peroxides. Although diethyl ether was stabilized with BHT it is conceivable that ether peroxide radicals were present. Dichloromethane was excluded for further experiments because of safety issues and a quite long time for solvent removal (>3 h). The long time for solvent removal was not expected due to the low boiling point of approx. 40 °C. MTBE, petroleum ether and cyclohexane extracted lower amounts of PBN-adducts as the other solvents and had in comparison lower polarities. This indicates that solvents with polarity indices below 2.5 are less suitable for extraction of radicals (**Figure 5.2 B**). Although acetone has a polarity index of 5.1, which should be suitable

for extraction of radicals, it was excluded for further experiments. The centrifugal evaporation of the solvent was problematic as small amounts of water were solved in acetone during the extraction resulting in a higher density than the oil that covers the acetone phase in the centrifuge tube and hinders its evaporation. For these reasons, the preferred solvent for the lipid radical extraction was ethyl acetate, which was removed by centrifugal evaporation within 60 min and has a polarity, which seems to be suitable for extracting lipid radicals. Bösterling and Trudell (1981) and Trudell (1987) showed that ethyl acetate prevents hydrolysis of PBN-hydroxyl and PBN-superoxide radicals and increases their stability.

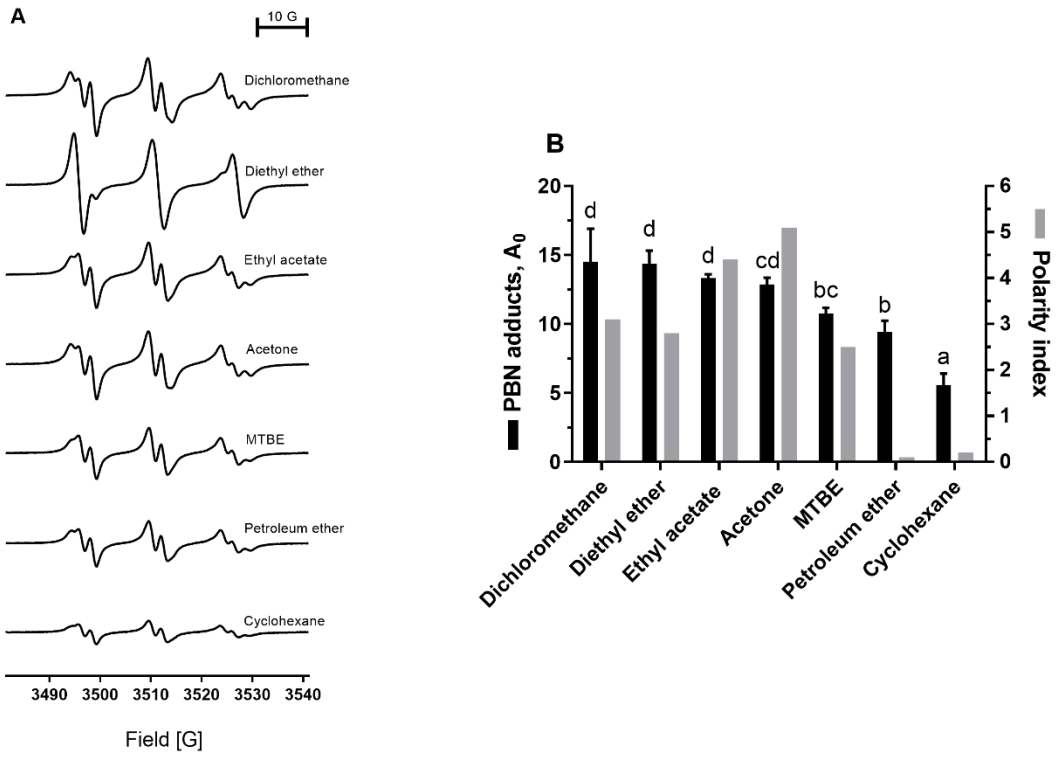

Figure 5.2: *Model system extracted with dichloromethane, diethyl ether, ethyl acetate, acetone, MTBE, petroleum ether, or cyclohexane. Solvents were removed by centrifugal evaporation; A) EPR spectra, spectra are means of four spectra B) PBN adducts (A_0) of the model system extracted with different solvents and the polarity index of the used solvents according to (Snyder 1978; Lalman and Bagley 2004; Ramluckan et al. 2014), extracted by double determination and each extract was measured twice. Bars with the same letter are not significantly different from each other $p \leq 0.05$, $n=3$.*

5.4.3. ASSESSMENT OF THE METHOD FOR SHORT-LIVED RADICALS IN STRIPPED BULK OIL WITH CLASSICAL OXIDATION PARAMETERS AND IDENTIFICATION OF THE RADICALS

Short-lived radicals formed in the lipid phase were trapped after extraction ex-situ and simulation of the spectra enabled assignment of different radical species. Monitoring lipid oxidation in purified (i.e., antioxidant were removed) sunflower/rapeseed oil during storage showed an increase in oxidation parameters (**Figure 5.3 D**). PBN-adducts rose from 0.65 up to 10.48 after 2 weeks of storage and over the time a clear increase in peaks in the EPR spectra was observed (**Figure 5.3 A**). In the same period, without lag-phase, the hydroperoxide content in oil rose from 7.17 to almost 500 mmol O_2/kg oil and the volatiles hexanal and propanal increased sharply after seven days (**Figure 5.3 D**). The radical concentration exhibited a higher increase than the hydroperoxide concentration until storage day 4, after which this ratio changed. Overall, the formation of PBN adducts correlated well with the formation of hydroperoxides. This is consistent with previous results from Cui et al. (2017) in stripped soybean oil. The rapid oxidation process without time-delay can be explained by the absence of antioxidants and the accelerated storage conditions.

According to Zamora and Hidalgo (2016) lipid alkyl (R•), alkoxyl (RO•), and peroxyl radicals (ROO•) can be expected in our samples. Indeed, several peaks in the EPR-spectra indicated a superposition of different radicals and the simultaneous detection of multiple radicals is likely. Simulation of PBN-radical spectra confirmed that hydroxyethyl, alkoxyl, peroxyl, and hydroxyl radicals were trapped in the oil sample (**Figure 5.3 B & C**). The proportion of hydroxyethyl radicals was stable over storage (30.9% ± 2.3%) and related to the increasing PBN adduct concentration an increase over time was observed. It can be assumed that they are formed due to ethanol oxidation, in which the PBN is dissolved (Andersen and Skibsted 1998). It is known that hydroxyl radicals react faster with ethanol than with PBN, so that trapping of 1-hydroxyethyl radicals predominates (Pou et al. 1994; Kocherginsky et al. 2005). Hydroxyl radicals are ubiquitously occurring radicals, which theoretically can also originate in the context of the thermally induced dissociation of hydroperoxides to hydroxyl and alkoxyl radicals (Zamora and Hidalgo 2016). In the present study, the percentage of hydroxyl radicals and peroxyl radicals decreased slightly during storage. The main lipid radicals identified were the alkoxyl radical, which raised from 28 to over 62% during storage. The alkoxyl radicals contributed in a high extent to the detected PBN adducts (**Figure 5.3 C**). The data from

simulated EPR spectra (**Figure 5.3 B**) clarified that alkoxyl radicals dominated the overall spectrum.

An in-vivo study of Novakov et al. (2001) tried to measure alkyl radicals (R•) in liver and lung extracts of rats. The authors observed no PBN-detectable alkyl radicals and explained this with their low steady-state concentrations and only low β-scission from alkoxyl radicals to alkyl radicals. The hypothesis that alkoxyl radicals were trapped agreed with studies of Dikalov and Mason, who used the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Dikalov and Mason 1999). It can be assumed that PBN-peroxyl radical adducts decompose under formation of PBN-alkoxyl radical adducts and that these alkoxyl radicals were an indirect evidence for peroxyl radical generation (Dikalov and Mason 1999). Davison and colleagues supported this assumption, as they reported lipid-derived alkoxyl radicals from linoleic acid (decomposed of lipid hydroperoxides) in human blood samples that were trapped with PBN (Davison et al. 2008).

Also, temperature-induced decomposition of the hydroperoxides to hydroxyl and alkoxyl radicals can be neglected during the 30-minute incubation. This effect is described to play significant role for distinct higher temperatures (100–110 °C) (Roman et al. 2012) or longer decomposition times (48 h at 60 °C) (Hopia et al. 1996).

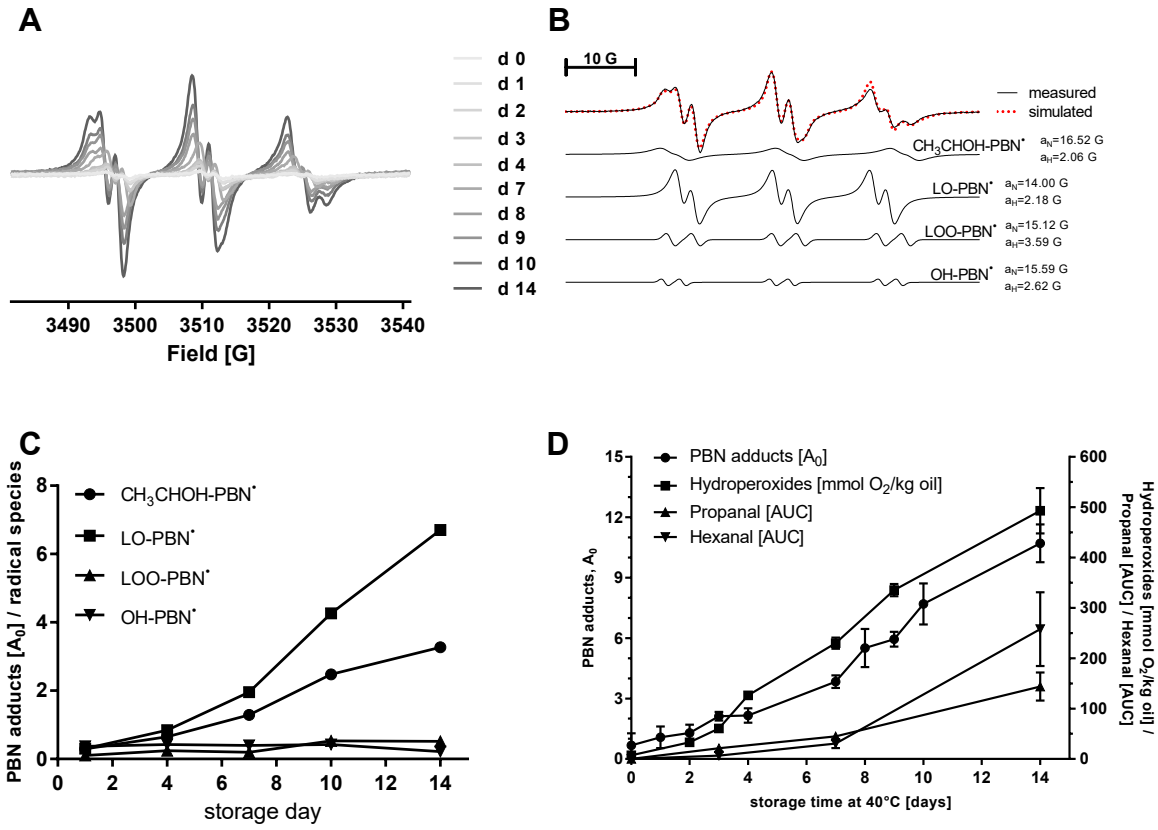

Figure 5.3: *Lipid oxidation in stripped sunflower/rapeseed oil during storage at 40 °C. n=3. A) EPR-spectra of the oil during storage. B) Measured and simulated EPR spectra of PBN adducts at day 14. In the measured EPR spectrum PBN-OH, PBN-CH_3CHOH, PBN-LOO, and PBN-LO are superimposed. C) Formation of the different simulated radical species related to the total PBN adduct concentration (A_0) during storage based on repeated spectra simulation (n=6). Day 0 was not simulated because of a low signal-to-noise ratio. D) Formation of PBN adducts [A_0], hydroperoxides, and the volatiles propanal and hexanal during storage. Data points represent means ± SD.*

5.4.4. CO-FORMATION OF SHORT-LIVED AND STABLE RADICALS IN MIXTURES OF CORN FLOUR CONSTITUENTS AND LIPID

This section of this study uses a mixture of corn flour constituents and linoleic acid as lipid constituent to investigate the co-formation of stable and short-lived radicals in an antioxidant-free model system, which allows to make conclusion for an extrusion matrix based on a similar composition. In order to characterize the model system precisely and to focus on the stable radicals, linoleic acid was chosen as lipid compound. The formation of different oxidation

markers in the model systems made with linoleic acid during storage at 40 °C is depicted in **Figure 5.4**. The EPR spectra of stable radicals show a singlet spectrum (**Figure 5.4 A & B**), the formation PBN adducts (**Figure 5.4 C**) reflects the formation of lipid radicals and the peak intensity and peak width (**Figure 5.4 D**) are markers for stable radicals in the powder sample. During storage, only a small increase in lipid radicals could be observed in all systems, except for the cornstarch-lipid mixture that showed a strong increase in the first 24 h by a factor of 6.5 and a slight increase during further storage. In contrast, in mixture containing cornstarch with zein, cornmeal or pure zein in addition to linoleic acid no to marginal increases could be observed. During 7 days of storage, the protein-containing samples showed a small increase. Before storage, the mixture based on zein had 4.5 times higher concentrations of stable radicals than mixture made with cornmeal. Furthermore, the peak intensity of zein (**Figure 5.4 D**) was reduced in the first 24 h compared with the starting values and remained stable during storage. The highest levels could be measured in the model system made with pure zein followed by the system produced with cornmeal. Overall, both, peak intensity and peak width, showed no changes over the storage period (**Figure 5.4 B**).

The EPR spectra of stable radicals in model systems containing cornmeal or corn starch indicated the superposition of different radical species (**Figure 5.4 A & B** and **Supplementary Figure 5.8**). The evidence of at least two different C-centered radicals in starch-based samples was also described in the work of Dyrek et al. (2007). In the pure zein system, a superposition of several radical species could not be clearly deduced from the single very broad peak, but would indeed be very likely (**Figure 5.4 A** and **Supplementary Figure 5.8**). The obtained averaged g-values including all superimposed radical species in each sample differed significantly with regard to the presence and absence of zein (**Table 5.1**). After 7 days of storage, the averaged g-value of radical species in the cornstarch-based model system was 2.00597 (average over the complete storage time: 2.00547). This is in line with results from Łabanowska et al. (2013), who reported a g-value of 2.0054 for cornstarch from Cargill, too. Slightly higher g-values were found for potato starch (native: 2.0059 and oxidized: 2.0065) (Łabanowska et al. 2008). Mixtures containing proteins had averaged g-values of 2.00443 (cornmeal) and 2.00474 (pure zein). The addition of zein to the cornstarch reduced the averaged g-value to 2.0051. Zein is the major protein of corn (Shukla and Cheryan 2001), for which no reported g-values of the resulting radicals have been found in the literature. For a general comparison of radicals generated by proteins and amino acids, for example, those of fish are

used, which are given from 2.0021 to 2.0049 (Saeed et al. 1999). In wheat flour singlet radicals with g = 2.003–2.0046 and a peak width of 10 G is reported (Andersen et al. 2011) and in corn flour an average g-values = 2.0049 was found (Labanowska et al. 2014). This corresponds to the radicals in the cornmeal with a mean peak width of 10.5 G and an averaged g-value of 2.00453 over storage time. Because the averaged g values of the model system with cornmeal and zein are very similar (**Table 5.1**) and significantly more radicals can be measured in the presence of zein (**Figure 5.4 A, B and D**), we assume that the stable radicals detected originate mainly from corn proteins. From the available results, it is not possible to explicitly conclude the type of stable radical species. Schaich and Rebello (1999) extruded wheat flour and reported after the extrusion process the formation of nitrogen-centered radicals with g values in the range of 2.0053–2.0059, whose origin they attributed to the proteins. In addition, it is also described that carbon-centered radicals were measured in heated gluten (g =2.0057), the protein fraction from wheat flours (Labanowska et al. 2014). Hence, for the present spectra of stable radicals for corn model systems, it is very likely that carbon- and nitrogen-centered radical species from proteins are present in combination. In addition, Schaich and Rebello (1999) showed that gluten heated at 180 °C had higher EPR signal levels than heated starch and Andersen et al. (2011) proposed that wheat bran is the main source of radicals in heated whole wheat flour. Nevertheless, studies with the EPR showed that carbohydrate radicals are formed in heat treated cornstarches (Łabanowska et al. 2013; Bidzińska et al. 2012) which could lead to a superposition of the spectra, too.

In the formation of primary and secondary oxidation products, the same trends as on the level of lipid radical formation could be observed (**Figure 5.4 C & D**). The samples with cornstarch showed the highest concentrations of oxidation products by far followed by corn starch samples with added zein. The model system made with zein had the highest starting values for hydroperoxides and hexanal and their concentrations decreased during storage. We suspect that the zein contained traces of lipids that were already oxidized to hydroperoxides, which decomposed during storage at elevated temperatures. In samples made with cornmeal, no increase of oxidation products could be measured. The results let assume that with increasing protein content the formation of radicals as well as of primary and secondary lipid oxidation products was reduced. It is possible that the protein fractions will interfere lipid oxidation. Studies from Wang et al. (1991) found that zein showed antioxidative effects especially at high aw values (0.9) in protein (zein)-methyl linoleate powder model systems. These effects were

explained with synergistic effects of traces of phenolic compounds and by physical shielding of the lipids by the protein (Wang et al. 1991). The PBN adduct concentration in mixtures with cornstarch and zein was higher compared with the mixtures with cornmeal or pure zein. Zein and starch may not be as homogenously in the mixtures distributed as in cornmeal, so that some lipids are not protected by zein.

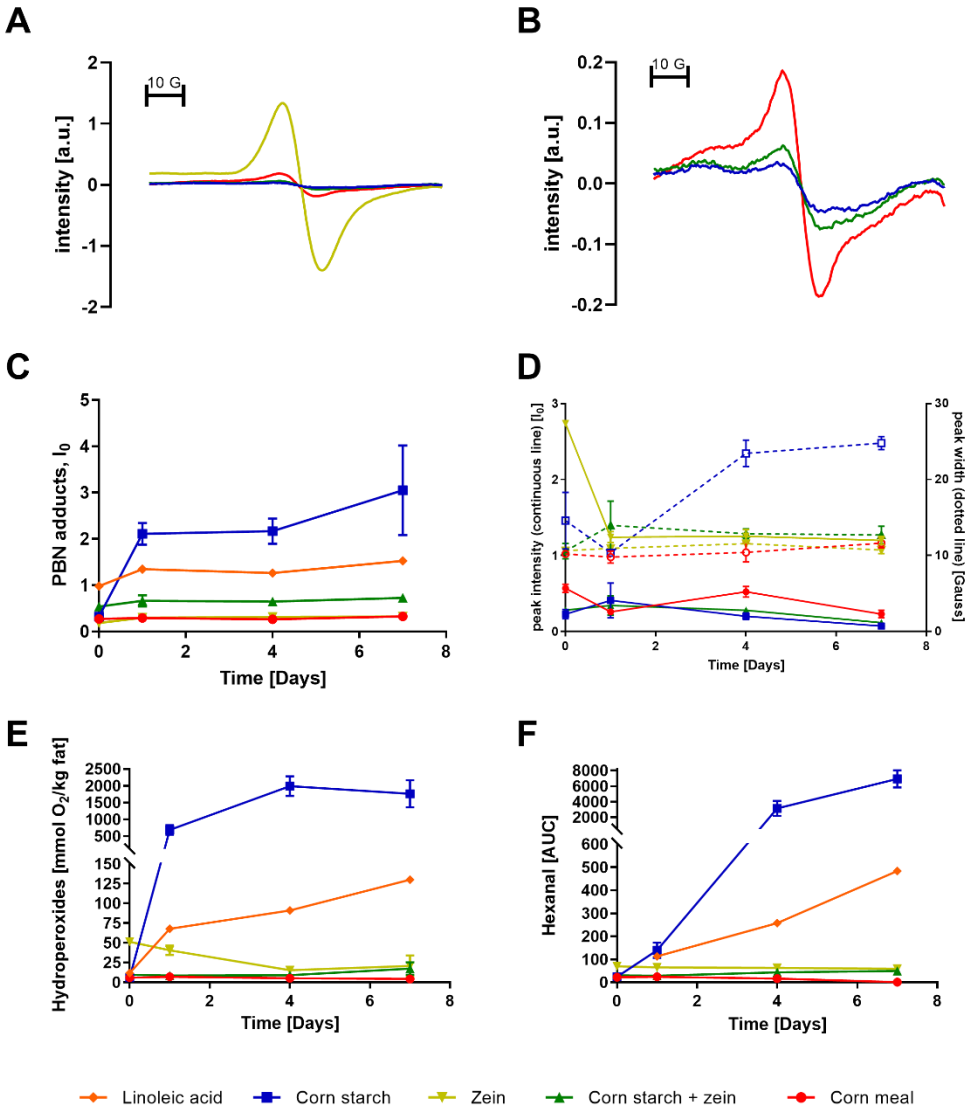

Figure 5.4: *Measurement of oxidation markers in the model systems and pure linoleic acid during storage at 40 °C. A & B: EPR-spectra of the stable radicals detected in the model systems (B with a higher resolution and without zein), C: PBN adducts in the extracted lipid sample, D: Formation of stable radicals in the model system (peak intensity = continuous line, peak width = dotted line), E: Hydroperoxides formation and F: Hexanal formation.*

5.4.5. RADICAL FORMATION AND LIPID OXIDATION IN CORN EXTRUDATES

The formation of radicals and the oxidation in extrudates was analyzed in the premix prior to extrusion and in the final extrudate (**Figure 5.5**). The premix had a mean hydroperoxide concentration of 3.0 mmol O_2/kg fat. Its hexanal concentration was 3.13 AUC and the mean score for the PBN adducts was 0.0435 A0. After extrusion and drying, all parameters increased, e.g., hydroperoxides had a concentration of 6.97 mmol O_2/kg fat. The effects of extrusion on oxidation are also clearly visible in the formation of hydroperoxides, where an almost linear increase was observed from storage day 4. Hexanal, as an important volatile secondary oxidation product of the linoleic acid, showed also a linear increase as the hydroperoxides. In the control, the premix, no primary and secondary oxidation products were formed during storage (data not shown).

At the beginning of the storage, the PBN adduct concentrations in extrudates were on a higher level than in the premix. During the storage test, we receive an EPR signal and saw a slight increase in PBN adducts in the first days of storage (**Figure 5.5 A**). However, the signal-to-noise ratio is very low. When evaluating the PBN adduct content, it must be considered that the PBN adduct concentrations in the extrudates (**Figure 5.5**), prepared with commercial unstripped oil, are distinctly reduced (the scale was reduced to a tenth) compared with the stripped oil, void of tocopherols (**Figure 5.3**). We tested the effect of extrusion cooking on tocopherol degradation and observed no significant difference between the premix and the extrudate (data not shown). According to Cui et al. (2017) no PBN adducts were measurable in stripped oils with added tocopherols. Velasco et al. (2004a) reported decreased EPR signals and in turn a reduced amount of spin adducts in the presence of α-tocopherol. It must be assumed that reaction kinetic leads to a competition between PBN and antioxidant resulting in low PBN-adduct formation (Jerzykiewicz et al. 2013). The distinct difference in the tocopherol contents must be considered in the present study and the low PBN adduct concentrations in the extrudate can be attributed to tocopherols interfering with the ability of PBN to trap lipid radicals. According to Kocherginsky et al. (2005) higher temperatures or longer incubation times could overcome this problem because the antioxidants were depleted during incubation and subsequently detection of EPR signal is possible. To avoid further oxidation of lipids, this was not carried out. In conclusion, it can be suggested that extrusion acts as an impulse, resulting in the slight increase in PBN adducts in the extract. This indicates that heat, pressure, and shear

forces during extrusion process lead to the formation of lipid radicals and increase the oxidation rate.

Only selected EPR spectra were simulated because of low peak intensities. Simulations of spectra from storage day 3 confirmed alkoxyl radicals (29.7% ±0.9%) as the main lipid-derived radicals. In addition, hydroxyl radicals and peroxyl radicals contributed to the superimposed spectra of lipids extracted from extrudates. Overall, the lipid radicals increased slightly over storage and extrudates had higher PBN adduct concentration compared with the premix before extrusion.

The analysis of the g-values of the detected stable radicals in the extrudates (average over time: 2.00471) led to the conclusion that these were N-centered protein radicals (**Table 5.1**). Spectra of the stable radicals are provided in the supplementary materials (**Supplementary Figure 5.9**). Comparing the g-values from the extrudates with the g-values from the model systems prepared with different kinds of corn-constituents confirmed this assumption.

It is noteworthy that compared with the tocopherol-free model systems the stable radical concentration was approximately doubled in the extrudates. As was to be anticipated, tocopherols reduced the measurability of lipid radicals. However, no inhibitory effect of tocopherols was observed on the ability to measure stable radicals. The peak intensity, an indicator for concentration of stable radicals, was increased in the extrudates compared with the premix. This indicates formation of stable radicals during extrusion processing. However, over the storage period no change in peak intensity and peak width was observed, suggesting that there was no further increase in protein radicals during storage. Our findings were confirmed with various studies that have shown that heat treatment leads to the formation of radicals in cereals (Schaich and Rebello 1999b; Labanowska et al. 2014; Andersen et al. 2011). However, Andersen et al. (2011) reported that radicals also occur naturally in native unheated flours. Krupska et al. (2012) and Bidzińska et al. (2012) reported that unlike the influence of heat, a pressure treatment does not seem to have any effect on radical formation in starches. In addition, shear forces during extrusion correlate strong with the formation of sulfur oxyl radicals (g-value: 2.01–2.02) (Schaich and Rebello 1999b). It is likely that synergistic effects of heat, pressure, and shear forces contributed to the formation of stable radicals during extrusion. That is, the extrusion process exerted an impulse, which shortened the lag-phase for lipid oxidation but did not initiate a long lasting continuous increase in the formation of stable radicals.

Table 5.1: *g-values of the detected stable radicals in the extrudates (incl. premix) and in the model systems (n=3)*

sample	storage day	averaged g-value
premix	0	$2.00413 \pm 1.34 \times 10^{-4}$
extrudate	0	$2.00467 \pm 2.52 \times 10^{-5}$
extrudate	14	$2.00475 \pm 4.51 \times 10^{-5}$
cornmeal-based model system	7	$2.00443 \pm 9.54 \times 10^{-5}$
cornstarch-based model system	7	$2.00597 \pm 2.56 \times 10^{-4}$
cornstarch + zein -based model system	7	$2.00510 \pm 1.80 \times 10^{-4}$
zein-based model system	7	$2.00474 \pm 4.36 \times 10^{-5}$

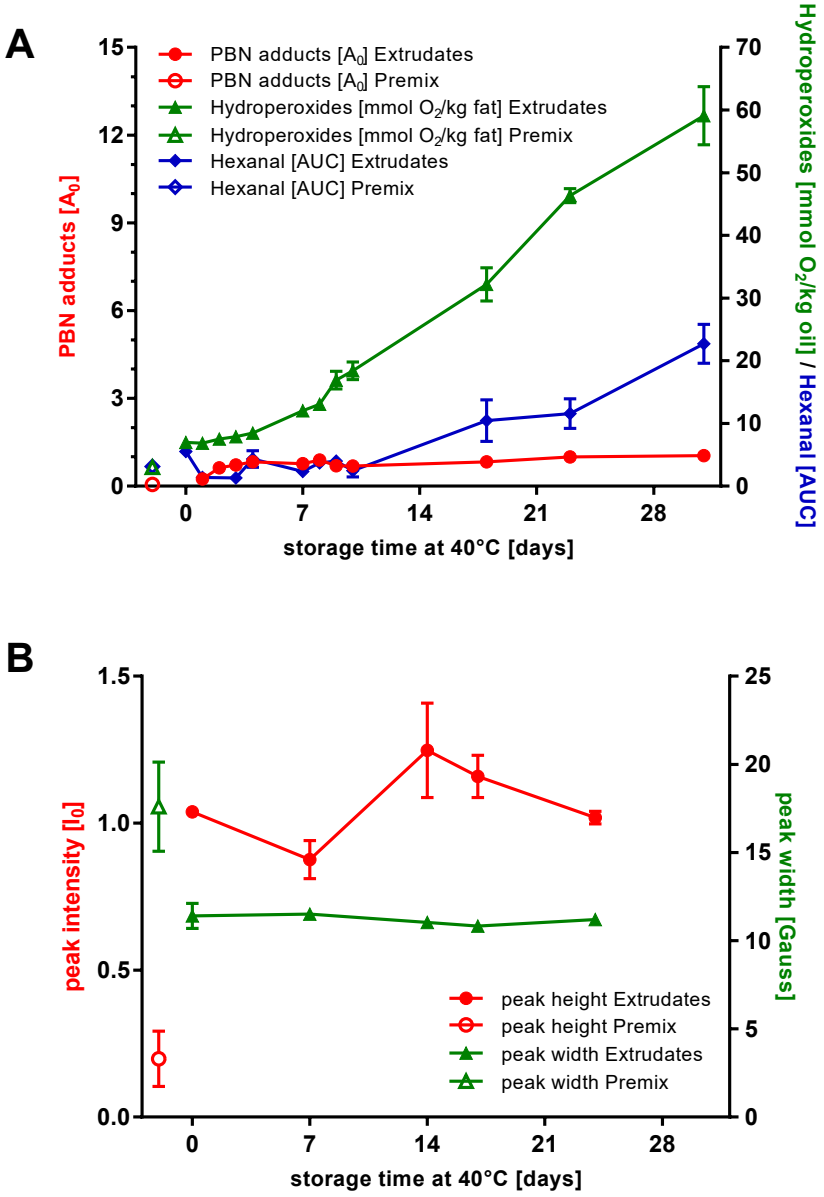

Figure 5.5: *Measurement of oxidation markers in the premix as control and in the extrudates during storage at 40 °C. A: Formation of PBN adducts [A_0] and hydroperoxides in the extracted oil sample and formation of hexanal in the ground extrudate, B: Formation of stable radicals in the ground extrudate samples [peak intensity and peak width].*

5.5. CONCLUSIONS

Oxidation is one of the main reactions that reduces the quality of foods with low moisture content. Because of a lack of suitable methods for analyzing complex starch-protein-lipid matrices with EPR, this work provides approaches and presents their development. For measurement of lipid oxidation products, an extraction step is necessary due to the nature of these samples (solid starch-protein matrix with dispersed lipids). Prior to EPR measurement, an incubation (50 °C / 30 min) of the sample containing the spin-trap PBN is required. The results of this study clarified that these incubation conditions are suitable for antioxidant-free model systems, e.g., Fenton-systems and complex systems using stripped oils or pure fatty acids. In extrudates prepared with commercial oils, PBN and the oil-originating antioxidants, i.e., tocopherols compete for the interaction with the radical. That explains the low PBN adduct formation. However, the formation of stable radicals was not affected by tocopherols and can be measured in situ in starch-protein-lipid model systems as well as in extrudates in 3 mm glass capillaries.

Based on the findings, we assume that the extrusion conditions initiate the radical chain reaction, so that a slightly higher formation of short-lived radicals can be observed during storage in the extruded sample compared with the premix. Spectra simulations of model systems reveal that mainly alkoxyl radical adducts were detected as lipid-derived radicals in lipid-containing systems, including extrudates. In addition, constant hydroxyethyl radical contents and small amounts of peroxyl and hydroxyl radicals were trapped with PBN. The findings from the investigated model systems demonstrate that the presence of proteins (zein), lowers the formation of short-lived radicals. Based on the very similar averaged g values of the model system with cornmeal and zein and a considerably higher content of radicals in the presence of zein, we assume that the stable radicals detected originate mainly from corn proteins. The insights of this study may provide the basis for a targeted use of antioxidants to inhibit radical formation in a complex extruded matrix.

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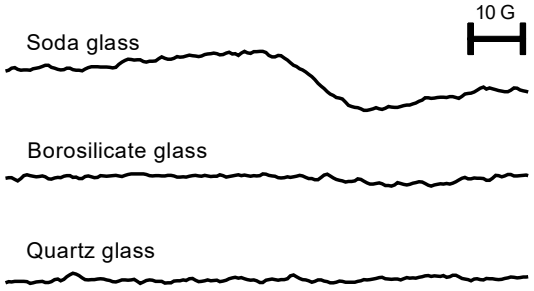
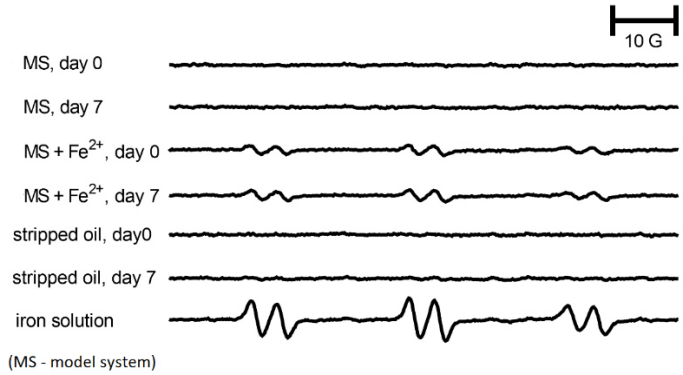
5.7. SUPPLEMENTARY MATERIAL

Method development for analysis of stable radicals in powdery samples

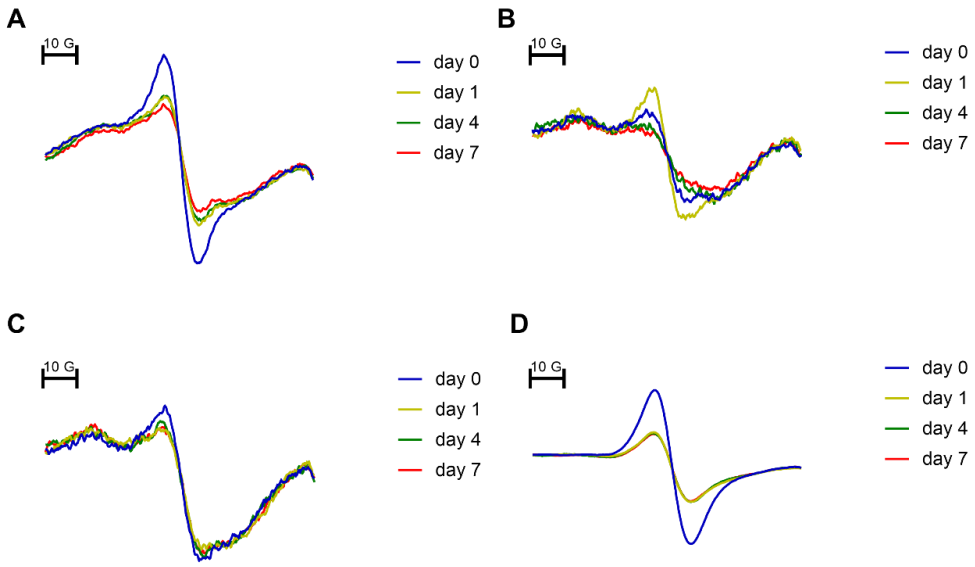
For the analysis of stable radicals in extrudates and EMS, methods were developed because no suitable exist. Stable radicals, e.g., originating from proteins, show a so-called "powder spectrum" with no hyperfine splitting. Therefore, a spectrum with a flat baseline is of high relevance for the identification of peaks that come from a stable radical. Testing an EPR-sample tube (10 mm diameter) with a sample inside no signal was measurable. It is likely that the water content was too high leading to reduced irradiation by the microwaves in the resonator. Therefore, thinner glass capillaries of 3 mm inner diameter were tested, which allows the EPR-analysis of powdery samples with water contents up to 14%. This limit is justified by the fact that tuning the ESR was impossible with higher water contents. During method development, empty glass capillaries of different glass qualities without a sample were tested to identify the best capillaries for EPR (Supplementary Figure 5.6). Empty glass capillaries made of soda glass show a clear EPR-signal with a peak in the region where the radicals are expected. The purity is, therefore, not appropriate for EPR measurements. Capillaries of borosilicate and quartz show a very similar spectrum, whereas borosilicate has a more regular noise. Indeed, both capillaries show no signals within the field of 3450 to 3550 G, which makes them suitable for the measurement of stable radicals.



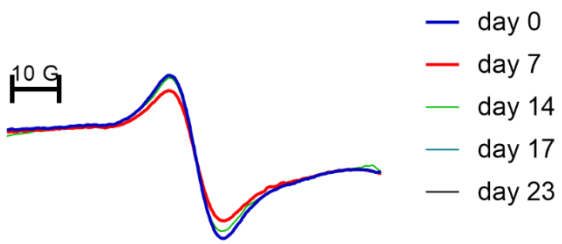
Supplementary Figure 5.6: *EPR-spectra of empty glass capillaries with different glass qualities to identify a suitable capillary for measurement of stable radicals in the dry sample*



Supplementary Figure 5.7: *EPR-spectra model system prepared with and without iron, the stripped oil compound (day 0-7) and iron solution (day 0) with the spin trap POBN; spectra are means of three spectra.*



Supplementary Figure 5.8: *EPR-spectra of stable radicals in the model system during storage (day 0, 1, 4, 7) A) cornmeal B) cornstarch C) cornstarch + zein D) zein; n=3*



Supplementary Figure 5.9: *EPR-spectra of stable radicals in extrudates during storage, n=3*

CHAPTER 6

ACCELERATED SHELF LIFE TESTING OF LOW MOISTURE FOODS: INFLUENCE OF STORAGE TEMPERATURE ON LIPID OXIDATION IN CORN EXTRUDATES, AN EXTRUSION MODEL SYSTEM, AND BULK OIL

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6. ACCELERATED SHELF LIFE TESTING OF LOW MOISTURE FOODS: INFLUENCE OF STORAGE TEMPERATURE ON LIPID OXIDATION IN CORN EXTRUDATES, AN EXTRUSION MODEL SYSTEM, AND BULK OIL

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6.1. ABSTRACT

Lipid oxidation is a major cause of oxidative deterioration of lipid-containing foods. Accelerated shelf life tests at elevated temperatures are commonly performed in lipid oxidation studies. The aim of the present study was to evaluate the influence of storage temperature on lipid oxidation in extrudates, an extrusion model system and bulk oil. In addition, both systems were compared using the oxidation kinetic parameters activation energy (E_a) and reaction rate (k). Therefore, corn meal was extrusion cooked together with commercial vegetable oil and water. These extrudates and the used oil were stored for several weeks at temperatures from 20 to 100 °C. By comparison, an extrusion model system (EMS) prepared with cornstarch, purified vegetable oil, and water was stored at temperatures from 20 to 120 °C. Lipid oxidation was assessed by hydroperoxide contents and by volatile secondary oxidation products. Furthermore, effect of non-enzymatic browning was investigated. Increasing the storage temperature accelerated the lipid oxidation (increased reaction rate) resulting in increased concentrations of oxidation markers in the extrudates and the EMS. GC-MS analysis of the EMS and the browning of the extrudates revealed that Maillard reaction products were formed at high storage temperatures. For shelf life prediction attention must be paid to the used storage temperatures, as these affect the kinetic parameters.

6.2. INTRODUCTION

Lipid oxidation is a major cause of oxidative deterioration of lipid-containing foods. The speed of oxidation is quite slowly at room temperature, which makes its monitoring a tedious procedure. The most popular methods to accelerate lipid oxidation are accelerated oxidation tests, in which samples were stored at constant elevated temperatures (40–60 °C) (Ragnarsson and Labuza 1977). Other factors affecting the speed of oxidation in storage tests are oxygen, light, pH value, the addition of prooxidants such as iron and copper, and the use of radical initiators. However, these methods are often based on changed reaction mechanisms that do not reflect oxidation at room temperature (Frankel 2005). For this reason, the gold standard for accelerated storage tests is the storage at elevated temperatures. Another possibility for the prediction of lipid oxidation in extrudates is the use of model systems. For lipid containing products, a suitable option is the use of purified, i.e., stripped oils, in which all antioxidative acting substances like tocopherols are removed by adsorption chromatography.

Accelerated shelf life tests with elevated temperatures based on the chemical kinetic that the rate of a reaction increases exponentially with the temperature, i.e., a 10 °C rise in temperature doubles the reaction rate. Advantages of these simple assays are that they require no specific equipment and that they can be used for oils, fats, and foods. The temperature dependence of the reaction rate of a chemical reaction is represented by the Arrhenius equation (Arrhenius 1889). The logarithmic form of the Arrhenius equation can be used for calculation of the activation energy (E_a) (Frankel 1993). The plot of the logarithmic form of the reaction rate constant (ln k) vs. 1/T [K] is called Arrhenius plot and can be used to approximate shelf life of foods. In this study, on the one hand a simplified linear approach was assumed, which is consistent with previous studies that used a linear regression for determination of the oxidation kinetic parameters (Manzocco et al. 2012; Mancebo-Campos et al. 2008; Huang and Sathivel 2008). Lipid oxidation reactions often do not follow a first-order kinetic. Rather, the lag phase is followed by the propagation phase, which is characterized by an exponential growth of the lipid oxidation products (Barden and Decker 2016). On the other hand, a nonlinear regression analysis with a fitting of the whole oxidation curve using a differential equation was performed according to Meissner at al. (Meissner et al. 2019). The use of nonlinear approaches is in line with Mancebo-Campos et al. (2008) and Crapiste et al. (1999).

Previous studies focused on the measurement of lipid oxidation in various foods and oils. Little work has been done to investigate the influence of storage temperature on oxidation in

accelerated storage tests of extrudates. In particular, oxidation studies on extrudates are often only carried out at a certain temperature. In addition, food manufacturers prefer to use high temperature methods to determine shelf life due to time constraints. Fast methods, such as the Rancimat or Oxipress, commonly use temperatures above 100 °C for acceleration of lipid oxidation. Such methods must be considered with prudence (Mancebo-Campos et al. 2007; Labuza and Dugan 1971), because high temperatures accompany with increased decomposition of the hydroperoxides and especially with extrudates side reactions and Maillard reactions must be taken into account (Frankel 2005).

The objective of the present study is to understand the effect of the storage temperature on lipid oxidation and to evaluate the effect of the matrix on the kinetic of lipid oxidation. In addition, the influence of storage temperature on side reactions, e.g. of Maillard reaction is studied by measuring color formation and formation of volatiles. Parameters of lipid oxidation kinetics are analyzed in a linear and a non-linear approach and effect of including different temperatures for calculation of E_a is presented.

6.3. MATERIALS AND METHODS

6.3.1. EXTRUSION PROCESS

The extrusion process was conducted on a laboratory twin-screw extruder with contrarotating intermeshing screws (Brabender Mod. DSE 35/7D) as described previously (Amft et al. 2019a). In brief, a premix was produced made of native (Molino Merano; Italy) and pregelatinized corn meal (Interquell; Germany) (50:50) 10% of a mixture of commercial rapeseed oil and sunflower oil (50:50 v/v) and 14% water. The premix was fed by a gravimetric weight feeder (Coperion Ktron Model K2-ML-D5-T35-QC) to the extruder with a constant feed rate of 18 kg/h. The temperature controller of the extruder were set to 60/125/125 °C and the screw speed was set to 333 rpm. The extruder was operated by one open die and an automated cutting device. The produced extrudates were dried in air permeable plastic bags in a climate cabinet (ThermoTEC TCS-501) at 30 °C and 20% humidity for 48 hours. 100 g extrudate was stored in 1000 mL glass bottles (Schott Duran) in triplicate per storage temperature. The mixture of commercial rapeseed/sunflower oil was stored in triplicate in glass bottles (40 g/100 mL glass bottle). The

extrudates and the oil were stored in heating ovens (Memmert, Germany) at 20 °C, 40 °C, 60 °C, 80 °C, and 100 °C in the dark.

6.3.2. PURIFICATION OF SUNFLOWER/RAPESEED OIL (50:50)

A mixture of sunflower and rapeseed oil (50:50) was purified and freed from all antioxidants by adsorption chromatography as described by Lampi and Kamal-Eldin (1998) with slight modifications. In brief, a glass column was packed with 250 g activated aluminum oxide (8 h at 100 °C and 12 h at 200 °C) as stationary phase and filled with n-hexane. Commercial rapeseed and sunflower oil were mixed (50:50 v:v) in the equal volume of n-hexane (as elution solvent). This mixture was passed through the column that was wrapped with aluminum foil to prevent oxidation. The purified oil-hexane-mixture was collected in a brown ice-cooled glass bottle. The solvent was removed using rotary evaporation under nitrogen atmosphere. After flushing with nitrogen, the oil was stored at -20 °C until usage and the success of oil-purification was tested using HPLC (<0.5 ppm tocopherols; method DGF F-II 4a).

6.3.3. EXTRUSION MODEL SYSTEM (EMS)

The EMS was prepared by mixing 85% cornstarch with 10% of a mixture of purified sunflower and rapeseed oil (50:50 v/v). Then 5 % distilled water were added and the model system was homogenized with a Stephan Universal Machine (UMC 5) for 60 s. Finally, the dough was rolled out in 3 mm thickness on a baking tray and then baked at 125 °C in a convection oven (Zanussi Model FCV/E 10L6) for 8 min up to a moisture content of approx. 8 % (similar range as the extrudates after drying). 50 g of the dried EMS was stored in 500 ml glass bottles (Schott Duran) in triplicate per storage temperature. The mixture of stripped rapeseed/sunflower oil was stored in triplicate in glass bottles (30 g/250 mL glass bottle). The EMS and the stripped oil were stored in heating ovens (Memmert, Germany) at 20 °C, 40 °C, 60 °C, 80 °C, 100 °C and 120 °C in the dark.

6.3.4. LIPID EXTRACTION

For lipid extraction from extrudates and EMS 5 g of dried and ground sample were weighed into 50 mL falcon tubes and 15 mL cyclohexane were added. After sonication for 20 seconds (cycle 9, 50 % power, Bandelin sonoplus, sonication probe VS 70) the tubes were centrifuged at 4500 rpm for 4 minutes. The extracts were filtered through filter paper in Erlenmeyer flasks (100 mL) and the extraction procedure was repeated twice with 10 mL of solvent. The solvent was evaporated using a rotary evaporator (65 °C, 250 mbar) and solvent residues were removed with nitrogen.

6.3.5. DETERMINATION OF HYDROPEROXIDES BY FERROUS THIOCYANATE METHOD

Hydroperoxides were determined by the ferrous thiocyanate method (Amft et al. 2019a). In brief, 20 mg of the oil were solved in 5 mL 2-propanol and diluted if necessary. 50 µL iron(II) chloride solution and 50 µL ammonium thiocyanate solution were added. After vortexing, samples were incubated in a water bath (60 °C for 30 min) and cooled down to room temperature. The extinction was measured directly against 2-propanol at a wavelength of 485 nm.

6.3.6. DETERMINATION OF PROPANAL AND HEXANAL BY STATIC HEADSPACE-GAS CHROMATOGRAPHY

Volatile oxidation products were analyzed according to Amft et al. (2019a) using an Agilent 6890 series headspace-gas chromatograph (HS-GC). In brief, 1 g of sample was weighed in 20 mL airtight HS-GC-glass vials that were equilibrated at 70 °C for 15 min. The gas chromatograph was equipped with a J&W DB-1701 column (60 m × 0.32 mm × 3 µm). Detection was done with a flame ionization detector. Propanal and hexanal were identified using the retention time of an external standard (Sigma-Aldrich, Germany) and their peaks were analyzed by their area under the curve.

6.3.7. GC-MS FOR IDENTIFICATION OF VOLATILES AT DIFFERENT TEMPERATURES

Volatiles formed at elevated temperatures in the EMS and in the stripped oil were analyzed using a GC-MS. 1 g of the EMS-sample and 0.5 g of the oil were weighed in GC-MS vials and closed airtight. The sample was injecting using a Gerstel MPS 2XL - DHS dynamic headspace autosampler after equilibration for 15 min at 75 °C. The gas chromatograph (Agilent 6890N) was equipped with a J&W DB-5MS column (60 m × 0.32 mm × 0.25 µm), carrier gas was helium, and the GC was running in split mode (3:1). The initial oven temperature was set to 30 °C, raised to 50 °C at 5 °C min^{-1} and finally raised to 230 °C at 10 °C min^{-1}. The injector was operated at 250 °C. Subsequently an Agilent 5975 inert mass selective detector was used for mass spectrometric analyses. Volatiles from 27 to 200 mass/charge ratio (m/z) were detected and then identified using AMDIS software and the NIST mass spectral library.

6.3.8. SPECTROPHOTOMETRIC ANALYSIS OF THE EXTRUDATES

The color changes in the extrudates due to the storage at elevated temperatures were measured after 14 days of storage with a spectrophotometer (X-Rite, Model SP62, USA) using the CIE L*a*b* color system, where L* defines the lightness, a* denotes the red to green value and b* represents the yellow to blue value. Each sample was analyzed 9-fold.

6.3.9. OXIDATION KINETICS IN EXTRUDATES AND EMS

In chemical kinetics the temperature dependence of the reaction rate of a chemical reaction is represented by the following well-known Arrhenius equation (*Equation 1*) (Arrhenius 1889):

$$\textit{Equation 1:} \quad k = A * e^{-\frac{E_a}{RT}}$$

where k is the reaction rate coefficient, A is a pre-exponential factor, E_a is the activation energy, R is the universal gas constant (8.31446 $Jmol^{-1} K^{-1}$), and T is the absolute temperature [K]. The logarithmic form of the Arrhenius equation (*Equation 2*) was used for calculation of E_a (Frankel 1993). It states that the logarithmic of the reaction rate (ln k) varies linearly with the reciprocal of the temperature (1/T) with the slope $-E_a/R$ and is shown as follows:

$$\textit{Equation 2:} \quad \ln k = A' - \frac{E_a}{R} * \frac{1}{T}$$

The Arrhenius equation is derived from a plot of log k vs. 1/T (K). These Arrhenius plots of extrudates, EMS, and the stored oils can be found in **Figure 6.5**. Resolved according to E_A, the activation energy was calculated from the slope of the Arrhenius equation using the following formula (*Equation 3*):

$$\textit{Equation 3:} \quad E_a = -\frac{Ln\, k}{T} * R + A'$$

For calculation of parameters describing oxidation kinetic, a linear approach was followed. Therefore, the linear regression was fitted to the initial part of the curves excluding a lag phase, when present (Manzocco et al. 2012). Furthermore, a non-linear approach (*Equation 4*) (Crapiste et al. 1999) was tested using "R" (v.3.6.1) for fitting of the entire oxidation curves. Thereby, the concentration of lipid hydroperoxides (in brackets for simplifying the equation) by time is described semi empirical by a monomolecular autocatalytic formation rate k_1 and a bimolecular degradation rate k_2 up to the maximum of lipid hydroperoxides. According to Meissner et al. (2019), the R package deSolve (v.1.24) was used for calculations with differential equation and minpack.lm (v.1.2.1) for fitting of the oxidation kinetic parameters with a nonlinear least square regression using the Levenberg-Marquardt algorithm. Thereby, the formation rates were further calculated using *Equation 1*, which allows the direct determination of the activation energy by fitting different temperature samples at once. For reasons of plausibility, the regression model was first calculated internally and then optimized with restrictions ($A_{1,min}$ = 5, $A_{2,min}$ = 0,0001, $E_{a1,\,min}$ = 1, $E_{a2,\,min}$ = 1, $LOOH_{t=0,\,min}$ = 0.1, $A_{1,max}$ = Inf, $A_{2,max}$ = Inf, $E_{a1,\,max}$ = Inf, $E_{a2,\,max}$ = Inf, $LOOH_{t=0,\,max}$ = 15). Initial borders for A and E_a were estimated by their ability to provide well suited fits and the initial concentration range was estimated using the chemical determinations ($[LOOH]_{t=0}$ = 10 mmol/kg oil). Standard deviation was estimated using the jackknife resampling technique in R (Meissner et al. 2019). In the following, the activation energy of the formation rate (k_1) was compared to those in the linear approach.

$$\textit{Equation 4:} \quad \frac{d[\mathrm{LOOH}]}{d\mathrm{t}} = [\mathrm{LOOH}] * \mathrm{k}_1 - [\mathrm{LOOH}]^2 * \mathrm{k}_2$$

6.3.10. STATISTICAL ANALYSIS

The experiments were performed in triplicate and all values are expressed as means ± SD. A principal component analysis (PCA) was performed in "R" using the package ggplot2 (v.3.2.1). Statistical analysis and graph preparation were performed using Graph Pad Prism 6 (GraphPad Software, San Diego, USA).

6.4. RESULTS

6.4.1. LIPID OXIDATION IN EXTRUDATES AND ITS OIL STORED AT ELEVATED TEMPERATURES

Hydroperoxide formation in the extrudates and in the oil used for extrusion of these extrudates was analyzed during storage at elevated temperatures from 20 to 100 °C (**Figure 6.1 A & B**). First, it can be observed that the extrusion process increased the hydroperoxide contents compared with the unextruded oil. Extrudates and oil, which were stored at 20 °C, showed only a marginal increase in hydroperoxides over the storage period. Extrudates stored at 40 °C had a decreased slope compared with the course of the oil oxidation, i.e., the oil oxidized slower until storage day 21. After this induction phase, the oxidation rate increased. In extrudates and oil stored at higher temperatures (60–100 °C) a maximum hydroperoxide concentration can be observed followed by a decrease of the hydroperoxides. Furthermore, it can be observed that the maximum hydroperoxide concentration decreased with increasing storage temperature between 60 °C and 100 °C. Extrudates and oil exhibit the same course of hydroperoxide formation but the extrudates had lower hydroperoxide concentrations than the oil. At 20 °C and 40 °C the hydroperoxide concentration increased continuously. Extrudates stored at 40 °C exhibited a hydroperoxide concentration of 131.6 mmol O_2/kg fat at the end of the storage period, whereas the oil had a concentration of 278.5 mmol O_2/kg fat. The analysis of hexanal formation (**Figure 6.1 C & D**) revealed that lower hexanal concentrations were measured in the oil samples than in the extrudates. Furthermore, oil stored at 20 and 40 °C showed no hexanal formation and compared with extrudates the hexanal formation began with a time delay.

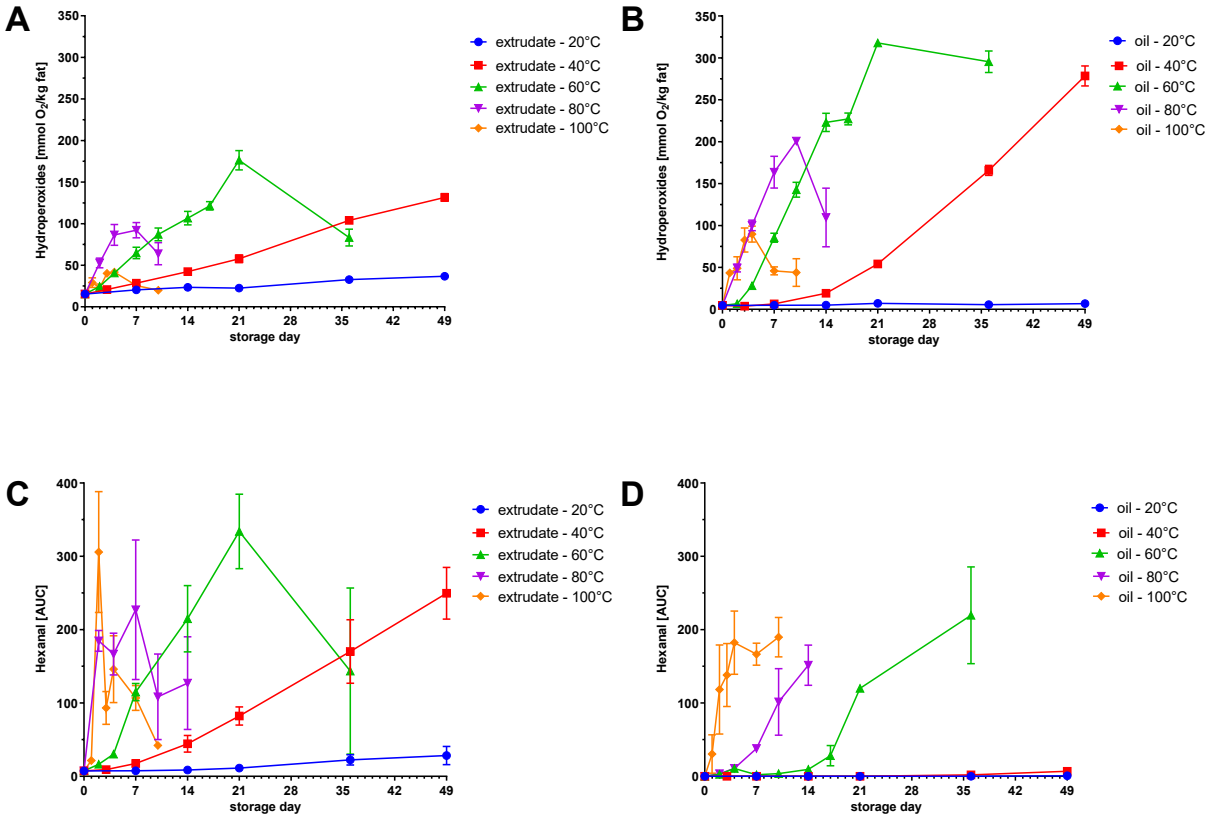

Figure 6.1: *Formation of hydroperoxides and hexanal in the extrudates and in the oil used for extrusion during storage at accelerated storage conditions (n=3).*

6.4.2. COLOR CHANGES IN THE EXTRUDATES BECAUSE OF HIGH TEMPERATURES

Color changes in the extrudates because of high storage temperatures are expressed as CIE L*a*b*-values (**Table 6.1**). Up to 60 °C all extrudates showed nearly the same L*a*b*-values without color changes due to the storage temperature. Above a storage temperature of 80 °C, a change in color was noticed and extrudates were darker. L*-values decreased and a*-values increased. Increased a*-values denote to increased red values and thus contribute to the shift from a dark yellow color to the light red-brown color of the extrudates. The change in color was even more pronounced in the extrudates stored at 100 °C. The L*-values, of extrudates stored at such high temperatures, further decreased and a drop in the b*-values was noticed, leading to a reduced yellow color of the extrudates. Extrudates stored at 100 °C had adopted a clear brown shade as shown in **Table 6.1**.

Table 6.1: *Temperature-dependent color changes in the extrudates expressed as CIE L*a*b*-values (n=9)*

storage temperature [°C]	L*-value	a*-value	b*-value	color
20	55.00 ±3.14	2.94 ±1.03	35.81 ±2.94	
40	59.59 ±2.24	2.99 ±0.50	37.48 ±1.68	
60	59.86 ±3.21	3.40 ±0.97	35.84 ±3.07	
80	49.50 ±4.99	10.61 ±2.14	32.09 ±3.38	
100	36.40 ±4.51	13.60 ±1.91	20.54 ±4.90	

6.4.3. LIPID OXIDATION IN THE EMS AND ITS OIL STORED AT ELEVATED TEMPERATURES

Lipid oxidation in the EMS was monitored by hydroperoxide and hexanal formation for max. 10 days (**Figure 6.2**). The EMS stored at room temperature (20 °C) showed the same marginal increase of the hydroperoxide concentration as the extrudates at this temperature (**Figure 6.1**). The same trend could also be observed at 40 °C. However, during the 7 days of storage a much higher hydroperoxides concentration was measured (**Figure 6.2 A**). EMS stored at 60 and 80 °C showed the same oxidation process within the 7 days as the extrudates with a significantly longer storage time. EMS stored from 100 to 120 °C oxidized within the first 6 hours, reaching a maximum hydroperoxide concentration. Subsequently, the lipid hydroperoxides began to decrease because of their decomposition to secondary lipid oxidation products. This was noticeable in increasing concentrations of hexanal.

In the EMS, the hexanal formation showed the same course as the hydroperoxide formation independently from the observed storage temperature (**Figure 6.2 C**). Indeed, the AUC-values for hexanal of the EMS are significantly higher than for the stripped oil. This can be explained by the circumstance that hexanal formation was normalized on the lipid amount in the sample. By comparison with the extrudates and the unstripped oil, in the EMS higher concentrations of lipid oxidation products were detectable than in the stripped oil sample (**Figure 6.2**).

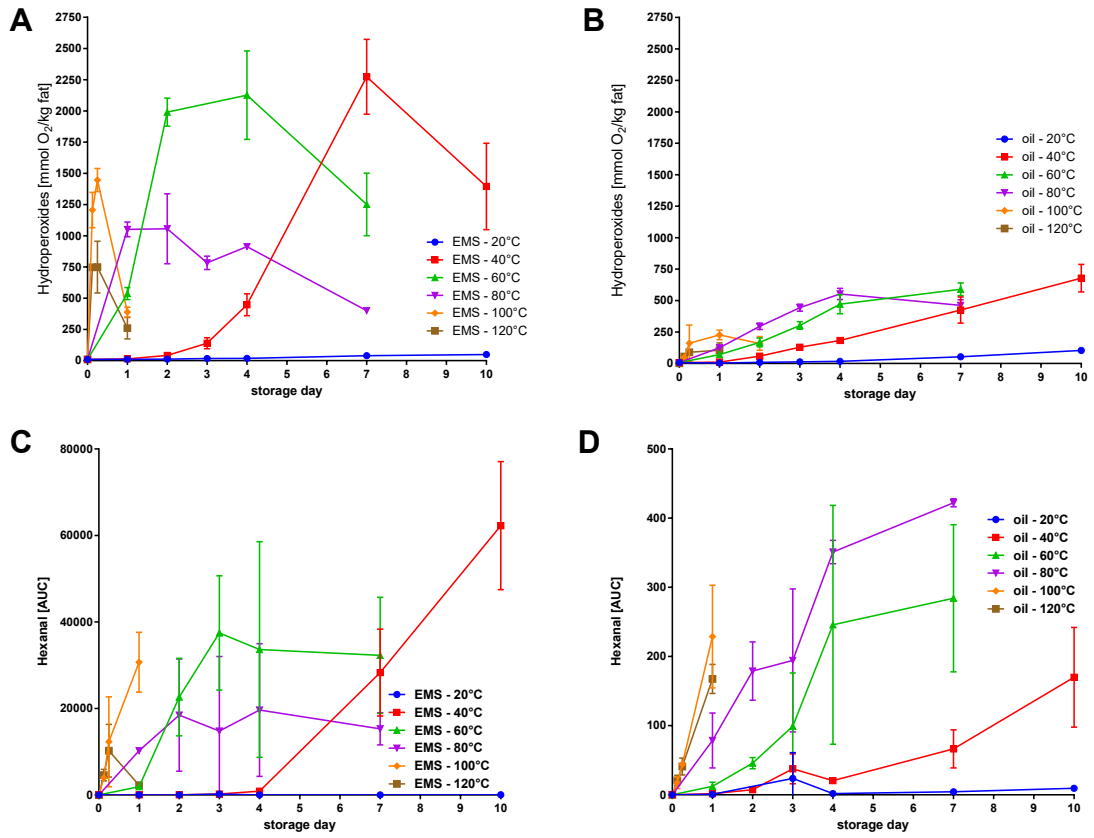

Figure 6.2: *Formation of hydroperoxides and hexanal in the EMS and in the oil used for EMS during storage at accelerated storage conditions (n=3).*

6.4.4. INFLUENCE OF TEMPERATURE ON THE PROFILE OF VOLATILES IN THE EMS

The EMS was analyzed during storage using the GC-MS to identify temperature specific compounds of lipid oxidation and Maillard reactions (**Figure 6.3**). It was noticeable that with increasing temperature the number of detected volatile oxidation products increased. While no volatile oxidation products were detected at 20 °C and 40 °C within the 24 h, more volatile substances were measured up to a storage temperature of 100 °C. In addition to hexanal and propanal, these also included other aldehydes. Hexanal and propanal could be detected in low concentrations in the EMS stored at 60 °C and in much higher concentrations in the 80 °C samples. Furthermore, pentanal, heptanal, 2-heptenal, octanal, 2-octenal and nonanal were formed during storage at 80 °C. The profile of volatile decomposition products of the samples stored at 100 °C was particularly noteworthy. It showed the highest concentrations of the above-

mentioned substances. In addition, 1-pentanol and 2-heptanone were measured for the first time. A raised temperature to 120 °C showed no further changes in the volatile compounds, except for octanal and 2-pentylfuran, which continued to rise and hexanal which had a reduced content. In the spectra of the EMS, 2-ethylfuran, 2-pentylfuran, 2-butylfuran, and 2,6 pyrazine diamine were detectable and their concentrations increase with increasing temperature.

A PCA was performed to clearly assign volatile compounds to the various systems investigated and to assign the effect of temperature-dependent formation. The PCA revealed that based on the volatiles a clear differentiation can be made between the stripped oil and the EMS (**Figure 6.4 A**). Generally, EMS was characterized by the volatiles hexanal, pentanal and 2-pentylfuran. In addition, in **Figure 6.4 B** it can be observed that with increasing temperature no effect on volatiles was observable in the oil, whereas in the EMS mainly the volatiles hexanal and 2-pentylfuran increase with increasing temperature.

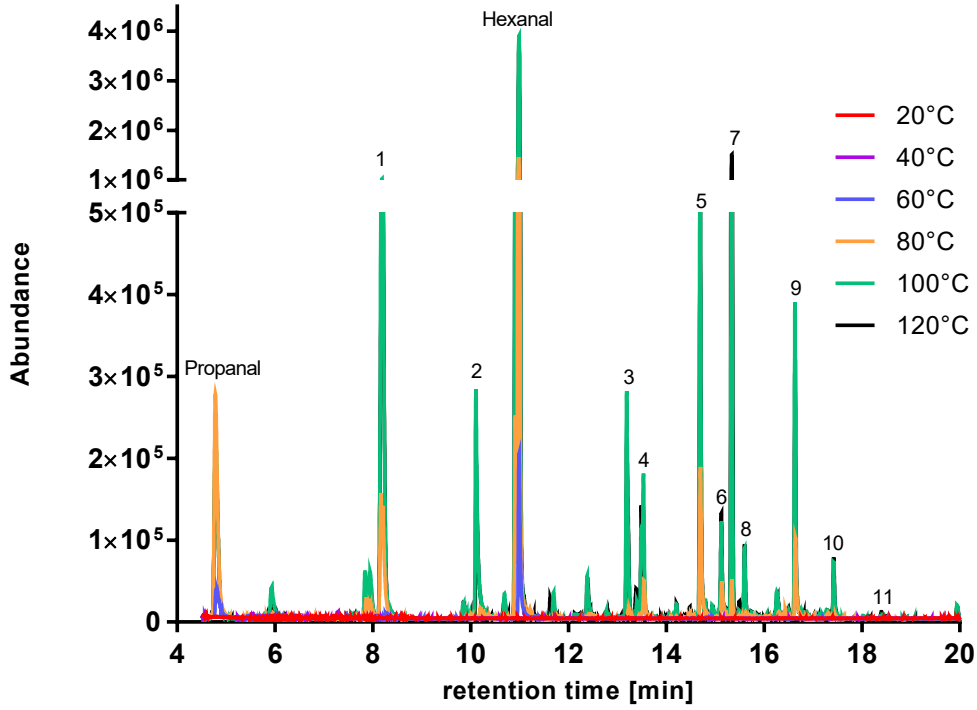

Figure 6.3: *Temperature-dependent formation of volatile oxidation products in the EMS after 24 h of storage (1) Pentanal/2-ethylfuran, 2) 1-pentanol, 3) 2-heptanon/2-butylfuran, 4) heptanal, 5) 2-heptenal, 6) octenol, 7) 2-pentylfuran, 8) octanal, 9) 2-octenal, 10) nonanal, 11) 2,6 Pyrazine diamine).*

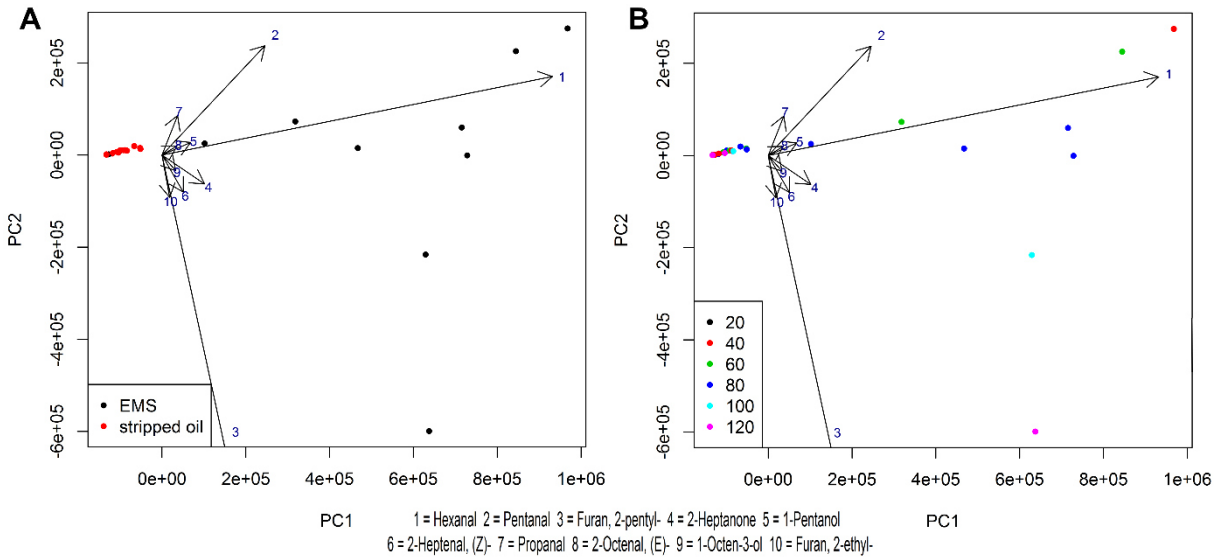

Figure 6.4: *PCA of the main volatiles A) in the oil and the EMS and B) differentiated between the different storage temperatures.*

6.4.5. KINETIC MODEL OF OXIDATION IN EXTRUDATES AND EMS

Arrhenius plots of the linear approach (**Figure 6.5**) were used to obtain the E_a for the oxidation reactions. Depending on the selected temperatures used for the Arrhenius plot, the plots indicate a temperature dependence of the Arrhenius model (Supplementary Figure 6.8). The reaction rate constant k and E_a calculated from the hydroperoxide concentration, compare the oxidation kinetics of the systems used in this study (Table 6.2).

According to the linear approach, the E_a of the EMS samples (61 kJ/mol) were higher than the E_a of the extrudates (42 kJ/mol). In comparison, the E_a of stripped oil (36 kJ/mol) were below the E_a of commercial oils (64 kJ/mol), containing tocopherols. By comparison, the results of the nonlinear approach showed comparable E_a in all samples except for extrudates, where a lower E_a was calculated. Compared with the oil, the reaction rate increases in the extrudates at higher temperatures. **Figure 6.5** shows that the 20 °C samples in particular behave differently, as indicated by low reaction rate constants (Table 6.2).

It was observed that, regardless of the used approach, the reaction rate increase for hydroperoxide formation (**Table 6.2**) and for hydroperoxide decomposition respectively hexanal formation (**Table 6.3**), with increasing temperature. Furthermore, the tocopherol-free systems, EMS and stripped oil, had higher reaction rates for hydroperoxide formation and

decomposition than the extrudates and commercial oil. Extrudates exhibited a higher reaction rate for hydroperoxide decomposition to hexanal (k_2) compared with commercial oil. In the antioxidant-free systems, the EMS had a distinct higher reaction rate with a lower E_a than the stripped oil.

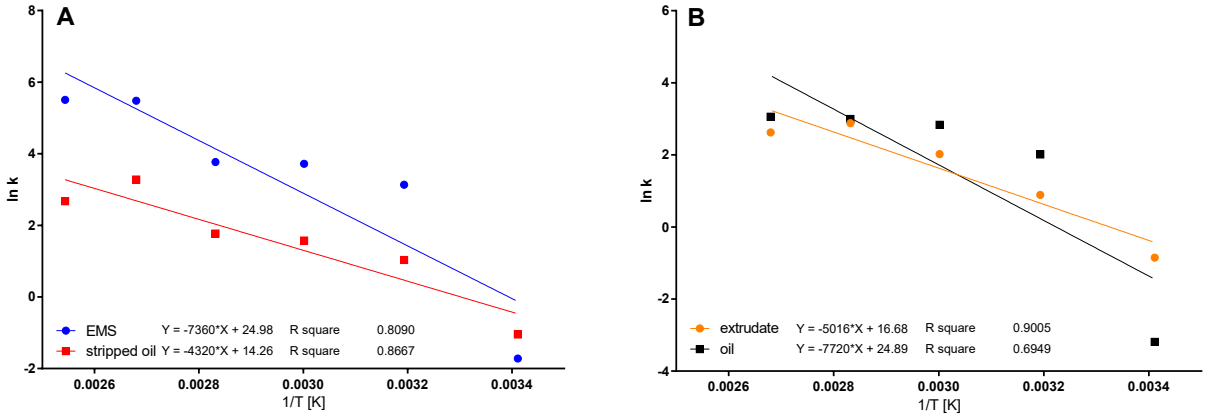

Figure 6.5: *Arrhenius plot based on the linear approach for the rate of lipid oxidation in A) the EMS and stripped oil and B) the extrudates and commercial oil.*

Table 6.2: *Oxidation kinetic parameters for hydroperoxide formation of the extrudates, EMS and the oils expresses as oxidation rate constants (k_1) and activation energies (E_a) at the experimental temperatures calculated using a linear and a nonlinear approach (± Jackknife SE)*

		reaction rate constant (k_1) [meq O_2 kg oil^{-1} days^{-1}]						E_a [kJ mol^{-1}]
		20 °C	40 °C	60 °C	80 °C	100 °C	120 °C	
linear approach	extrudate	0.43	2.44	7.57	17.82	13.79	X	41.70
	commercial oil	0.04	7.48	17.03	20.05	21.27	X	64.19
	EMS	0.18	22.95	41.27	43.40	239.68	246.15	61.20
	stripped oil	0.35	2.81	4.84	5.88	26.59	14.52	35.92
non-linear approach	extrudate	0.01	0.07	0.30	1.18	3.96	X	66.40 ±14.2
	commercial oil	0.02	0.09	0.37	1.27	3.81	X	60.13 ±14.9
	EMS	0.87	3.60	12.53	37.89	101.75	247.14	54.12 ±16.5
	stripped oil	0.16	0.49	1.28	3.02	6.49	12.90	41.95 ±9.1

Table 6.3: *Oxidation kinetic parameters for hydroperoxide decomposition (non-linear approach) and hexanal formation (linear approach) of the extrudates, EMS and the oils expresses as oxidation rate constants (k_2) and activation energies (E_a) at the experimental temperatures calculated using a linear and a nonlinear approach (± Jackknife SE)*

		reaction rate constant (k_2) [linear approach: AUC days^{-1} / non-linear approach: meq O_2 kg oil^{-1} days^{-1}]						E_a [kJ mol^{-1}]
		20 °C	40 °C	60 °C	80 °C	100 °C	120 °C	
linear approach	extrudate	0.59	5.62	17.14	31.86	149.21	X	58.56
	commercial oil	-	0.38	9.74	14.69	51.06	X	74.29
	EMS	-	426.37	741.16	384.92	1246.7	1704.5	16.42
	stripped oil	-	1.04	4.84	5.88	14.84	26.59	39.20
non-linear approach	extrudate	0.0000	0.0003	0.0026	0.0174	0.0968	X	93.95 ±21.5
	commercial oil	0.0000	0.0002	0.0015	0.0097	0.0511	X	90.96 ±25.0
	EMS	0.0022	0.0071	0.0199	0.0499	0.1133	0.2366	44.93 ±36.5
	stripped oil	0.0001	0.0005	0.0021	0.0083	0.0281	0.0837	66.68 ±16.6

6.5. DISCUSSION

6.5.1. IMPACT OF STORAGE TEMPERATURE ON LIPID OXIDATION IN LOW MOISTURE FOODS

Generally, it could be observed that an increase of the storage temperature was accompanied with an increase of the oxidation rate, resulting in a faster hydroperoxide formation. The present findings are in agreement with the results previously reported by other authors (Crapiste et al. 1999; Mancebo-Campos et al. 2008; Gómez-Alonso et al. 2004). Furthermore, we have shown that the maximum hydroperoxide concentration decrease with increasing storage temperature. We suggest that the decomposition of hydroperoxides exceed the hydroperoxide formation with increasing temperature. This was shown, for example, in the non-linear approach, where, for selected samples, the increase with temperature was greater for the hydroperoxide degradation (**Table 6.3**) than for the hydroperoxide formation (**Table 6.2**).

According to Reynhout the free radical chain reaction reaches a maximum reaction time at high temperatures that cannot be further increased by rising temperature (Reynhout 1991). Crapiste et al (1999) suggest that E_a for decomposition reactions of oxidation products is higher than the E_a for the hydroperoxide formation (Crapiste et al. 1999). Therefore, it can be assumed that at higher temperatures more hydroperoxides were decomposed than formed, while the formation rate predominates at low temperatures. This assumption is confirmed by the measurements of hexanal formation. In the extrudates, the EMS, and the oils higher hexanal contents were

measurable with increasing storage temperatures. This is in line with previous studies that reported increased amount of volatiles with increasing storage time and with increasing temperatures (Beltran et al. 2005; Roman et al. 2013a; Jeleń et al. 2000).

Overall, higher hydroperoxide concentrations were measurable in the EMS compared with the extrudates and in the stripped oil compared with the unstripped oil. This observation can be explained with the absence of antioxidative tocopherols, which inhibit continuously the oxidation reactions in unstripped oils. Without tocopherols in the EMS and stripped oil unhindered oxidation can take place. The analysis of hexanal confirmed this effect, because tocopherols inhibit the decomposition of hydroperoxides to volatile compounds (Cui et al. 2017). The aldehydes measured in the EMS stored at temperatures above 80 °C (e.g. pentanal or octanal), as volatile cleavage products of the hydroperoxides, were also confirmed by Jeleń et al. (2000) in vegetable oils stored at elevated temperatures, e.g., rapeseed oils. The increasing content of octanal and nonanal is justified by the dominant oleic acid of rapeseed oil. The content of 1-pentanol and 2-heptanone in the samples stored at 100 °C could possibly assigned to significant side reactions, which occur at higher temperatures (Frankel 1983; Frankel 2005).

The reduced oxidation of the oil compared with the oxidation of the extrudates during the first days of storage can be explained with the extrusion process leading to the formation of lipid radicals (Amft et al. 2019b). These initiators of lipid oxidation promote the induction phase leading to accelerated oxidation and an increase in hydroperoxides. This prooxidative effect of extrusion cooking is described by Schaich and Rebello (Schaich and Rebello 1999a), too.

6.5.2. INFLUENCE OF HIGH STORAGE TEMPERATURE ON FORMATION OF MAILLARD REACTION PRODUCTS

In contrast to pure oil, the storage of extrudates at high temperatures seems to lead to side reactions. These include the Maillard reaction, for example. This also explains the dark color of the extrudates (Purlis 2010). Maillard reaction products are known to have an antioxidant effect (Alfawaz et al. 1994). This partly explains the relatively low hydroperoxide and hexanal concentrations in the extrudates compared to the oil samples stored under the same conditions.

Because the perception of color is highly subjective, the analysis with a measuring device is the most reliable method to specify a color and to communicate that color. Browning of extrudates and a decrease of the L*-value was observable at temperatures above 80 °C. This can be

attributed to non-enzymatic browning reactions taking place under storage conditions with high temperatures. The Maillard reaction, a typical browning reaction, is the heat catalyzed reaction of amino acids with carbohydrates (Friedman 1996). Browning over time is accompanied with a decrease auf the L*-value, as shown during toasting of bread (Capuano et al. 2008) or during bread baking (Purlis and Salvadori 2009). In a preheated model system, containing pregelatinized starch, glucose, lysine, and soybean oil, a decrease of the L*-values was reported during heating at 100 °C for 90 min. In the subsequent storage for up to 180 days, the L*-values further decreased (Mastrocola and Munari 2000). Incidentally, a typical process leading to Maillard reactions is the extrusion process itself (Singh et al. 2007b; Ilo and Berghofer 1999).

In this study, GC–MS was used to identify volatile compounds of the EMS (**Figure 6.3**). The PCA identified that volatile compounds belonging to aldehydes from lipid oxidation but also volatiles from the Maillard reaction contributed to the volatile profile of the EMS. Maillard reaction products were observed because of the browning at higher storage temperatures (Purlis 2010; Fors 1983). The detected 2,6 pyrazine diamine at 100 and 120 °C belongs to the heterocyclic aromatic compounds of pyrazines, which were aromatics formed via Maillard reaction and during roasting (Starowicz et al. 2019). Simple sugar fragmentation products formed by Maillard reaction are volatile compounds such as furans (Fors 1983). In this study we detect e.g., 2-ethylfuran and 2-butylfuran. Furans from coffee, produced by Maillard reaction, showed an inhibitory effect on hexanal oxidation during storage for 40 days (Yanagimoto et al. 2002). However, it must be considered that furans, e.g., 2-pentylfuran can also be attributed to pathways involved in lipid oxidation, in particular from aldehyde decomposition (Crews and Castle 2007). This could also explain the distinct degradation of the hexanal and the increase of 2-pentylfuran in EMS samples stored at 120 °C.

The fact that the supposed Maillard reaction products were only detectable in the EMS samples and not in the oil samples (**Figure 6.4 A**) suggests that they were products of the non-enzymatic browning reaction. This would also explain the lower concentrations of oxidation products in the EMS samples stored at 80 °C compared with samples stored at 60 °C (**Figure 6.2**).

6.5.3. LIPID OXIDATION KINETICS OF EXTRUDATES, EMS, AND OILS

The selected matrix in which the oil was present or the bulk oil without a matrix had a crucial influence on lipid oxidation. In general, Frankel (2005) suggested E_a in foods ranging from 15 to 25 kcal/mol (≙ 62.8–104.7 kJ/mol). We calculated an E_a for the sunflower/rapeseed oil mixture from 60.1 to 64.2 kJ/mol, depending on the used approach. For sunflower oil an E_a of 63.8 kJ/mol is reported and rapeseed oil tend to have higher values (88.5) (Adhvaryu et al. 2000). These approximate reference values depend in turn on the presence of prooxidative metal catalysts (lower E_a) or antioxidants (higher E_a) (Frankel 2005; Tan et al. 2001; Golmakani et al. 2019).

We showed that oil purification increases the reaction rate constant (k_1 and k_2) and lowers the E_a of the stripped sunflower/rapeseed oil mixture, i.e., the stripped oil had a lower E_a than the commercial oil (**Table 6.2** and **Table 6.3**). This difference can be explained by the absence of tocopherols. Antioxidants delay the propagation phase, which results in a higher energy need for the formation and decomposition of the hydroperoxides (Mancebo-Campos et al. 2008). Golmakani et al. (2019) reported that antioxidants and antioxidative pomegranate by-products reduced the E_a of linseed oil. Our assumption is in line with results from Gómez-Alonso et al., who investigated oxidations kinetics of purified olive oil and reported an E_a value of 32.1 kJ/mol, which is within the range of our measurements (Gómez-Alonso et al. 2004). Mancebo-Campos et al. (2008), from the same research group, performed a kinetic study of accelerated storage tests (40–60 °C) with virgin olive oil and reported higher E_a (64.8 kJ/mol) for unpurified olive oil.

Depending on the used approach, the calculated E_a of the extrudates varied between 42 and 66 kJ/mol. Following the linear approach, we showed that lipids in the extrudates had lower E_a than the commercial oil. We assume that the protective effect of the extrudate matrix is reduced at higher temperatures because the oxygen permeation through glassy matrices increased with the temperature (Andersen et al. 2000). This observation is in line with Orlien et al. (2006), who reported lower E_a for oil encapsulated in a glassy food matrix (60 kJ/mol) compared with bulk oil (80 kJ/mol). The authors attributed this effect to the oxygen diffusion, which is the limiting factor in the glassy matrix at higher temperatures (60 °C), reducing the E_a. A lower E_a for oxidation of the extrudate compared with the commercial oil was also expressed in the significantly reduced hydroperoxide concentrations in the extrudate compared with the oil (**Figure 6.1**). For example, extrudate stored at 80 and 100 °C had hydroperoxide concentrations

reduced by half. We assume that more amino acids were available for the Maillard reaction in the cornmeal-based extrudates than in the cornstarch-based EMS. Mastrocola and Munari (2000) investigated the influence of Maillard reaction products on lipid oxidation in starch-based model systems. The authors found that the antioxidant activity of the Maillard reaction products developed with increased browning of the samples. Extruded rye bran had higher concentrations in Maillard reaction products in extrudates produced with lower water contents (13 and 16% water) and these extrudates had in turn a higher oxidative stability (Moisio et al. 2015a). In our study, a distinct browning of the extrudates was observed at storage temperatures from 80 °C to 100 °C. Especially these samples showed a lower E_a than the samples were no browning and in turn no Maillard reaction occurred. In EMS, a steady-state of k_1 was observed at 100 and 120 °C, where Maillard reaction products were detected by GC-MS (**Figure 6.3** and **Figure 6.4**).

Because the question of E_a has not yet been investigated on extrudates, corresponding reference data are missing. In comparison, E_a measured for oxidized lipids in cookies for infants, based on oxidation analysis with differential scanning calorimetry, ranged from 86 to 113 kJ/mol (Wirkowska et al. 2012). The analysis of the oxidation kinetics based on the linear approach, revealed that extrudates had a higher reaction rate and for oxidation a lower E_a is necessary compared with the commercial oil. We suggest that this observation is related to the different energy input of the samples, a possible increase of transition metals, e.g., iron, due to abrasion of the screw (Rao and Artz 1989), and a possible decomposition of tocopherols during the extrusion process originating from the oil. We tested the effect of extrusion cooking on tocopherol degradation and observed no significant difference between the premix and the extrudate (**Supplementary Figure 6.9**). Zielinski et al. (2001) reported that extrusion cooking significantly decrease the tocopherol and tocotrienol content in extruded cereals, however, they used extrusion temperatures up to 200 °C.

The reaction rate of extrudates and commercial oil depends, especially in the linear approach, on the temperature. For example, the extrudates stored at 60 °C have the same reaction rate as the oils at 40 °C. This effect could be attributed to the incorporation of lipids in the matrix of extrudates leading to an increased oxidative stability (Amft et al. 2019a). The deviating values for the non-linear approach can be explained methodically, because this approach considers the total hydroperoxide formation, including the lag phase, which is not considered in the linear approach. Nevertheless, following both approaches for oxidation kinetic calculation, with

increasing storage temperatures the reaction rate constants increase (Orlien et al. 2006). This was accompanied by a reduction of the E_a. Furthermore, extrudates exhibited a higher reaction rate for hydroperoxide decomposition (hexanal formation) (k_2) compared with commercial oil. This explained the higher hexanal concentrations in the extrudates compared with commercial oil stored at the same temperatures (**Figure 6.1 C & D**). The antioxidant-free systems, EMS and stripped oil, had a distinct higher reaction rate with a lower E_a than the extrudates and commercial oil, explaining the significantly higher hydroperoxide decomposition and hexanal formation than the extrudates and commercial oil (**Figure 6.2 C&D** vs. **Figure 6.1 C&D**).

The slope of the Arrhenius plot and thus indirectly the reaction rate (k_1 and k_2) for lipid oxidation were strongly temperature-dependent. On the one hand, in the Arrhenius plot the oil samples showed a plateau at a storage temperature of 60 °C and above. For example, in commercial oil, higher temperatures did not lead to a further increase in the reaction rate for hydroperoxide formation (k_1) (**Table 6.2**). At the same time, the reaction rate for the degradation of the hydroperoxides increased with increasing temperature (k_2) (**Table 6.3**). If only k_1 is considered, it could therefore be assumed that a maximum reaction rate exists. As mentioned above, the free radical chain reaction reaches at distinct higher temperatures (180 °C) a maximum reaction time that cannot be further increased by rising temperature (Reynhout 1991). On the other hand, extrudates exhibited a similar reaction rate as oils at temperatures of 30/40 °C and above but the ratio changes at room temperature. Thus, the reaction rate at room temperature (20 °C) deviated strongly from the theoretical value predicted using the linear regression in the Arrhenius plot. This leads to an underestimation of lipid oxidation in the extrudates, when performing shelf life predictions. Evaluation of the calculated k and E_a revealed that the selection of temperatures to be included in the calculation had a strong effect on the calculated values.

6.5.4. COMPARISON: EXTRUDATE VS. EMS

In the present study, the EMS was prepared with purified oil to shorten the oxidation time. The corresponding oil for the systems was also subjected to the storage study in order to better understand the matrix effects. The studied low moisture foods, extrudate and EMS are both complex systems with interfaces between the ingredients. The difference to bulk oil is that the oil is finely dispersed in a starch matrix. The criteria for the suitability of the EMS as a model

system for extrudates are that the lipids are distributed in the starchy matrix like in extrudates. To imitate the energy input and thermal treatment of the extrusion process on the oil, the EMS was mixed and afterwards heated in an oven.

Both the extrudate and the EMS use the same matrix components and the oil is dispersed and partially enclosed by the matrix. In addition, the starch gelatinizes due to the high process temperatures, which were set up for both systems at 125 °C. However, the extrusion cooking process is characterized by a melt of the raw materials under simultaneous high pressure, shear forces and temperature. This cannot be achieved in the EMS, because shearing and heating occur at different times. Furthermore, the resulting shear rate in the system is lower due to the geometry of mixer compared with the extruder. To compensate the lower impact of shearing the heating time was increased to 8 min in the EMS (extrusion time approximately 20–30 s) in order to achieve a comparable water content.

Despite the differences in the production process, the curves of oxidation had a similar course but the EMS showed the same trends in hydroperoxide and hexanal formation as the extrudates in a fifth of the time. Therefore, the EMS enables the simulation of oxidation in corn extrudates in a short test period.

6.6. CONCLUSIONS

Analysis of lipid oxidation and the prediction of shelf life with accelerated storage tests is of high interest. In this work, the influence of the storage temperature on lipid oxidation in extrudates and an EMS was investigated. In conclusion, our results showed that the reaction rate increases with increasing storage temperature. Furthermore, the maximum hydroperoxide concentration decreases with increasing storage temperature, indicating a maximum reaction rate at temperature above 60 °C. Analysis of volatiles suggested that mainly hexanal increased with higher temperatures. Furthermore, furans were detectable at temperatures above 80 °C, indicating Maillard reaction. This was confirmed by the browning of extrudates and reduced L*-values. The results of this study demonstrate that rapid methods, using high temperatures, must be critically evaluated. This is because side reactions (e.g. Maillard reaction) occur in the sample within a very short time, which have an effect on the oxidation process. In addition, it was shown that temperatures above 60 °C do not lead to a linear increase in the reaction rate. This can result in incorrect shelf life predictions because the lipid oxidation is underestimated.

Based on the results of the present study, the EMS can be used for prediction of oxidation reactions in corn extrudates. The curves of oxidation had a similar course and especially the hydroperoxide and hexanal formation showed the same trends. The tocopherol content in the EMS was reduced more than during the extrusion process. This has the advantage that antioxidants in different concentrations and combinations may be added to the EMS in further studies. Thus, detailed statements are possible about the effects or synergisms of different antioxidants (Bauer et al. 2013).

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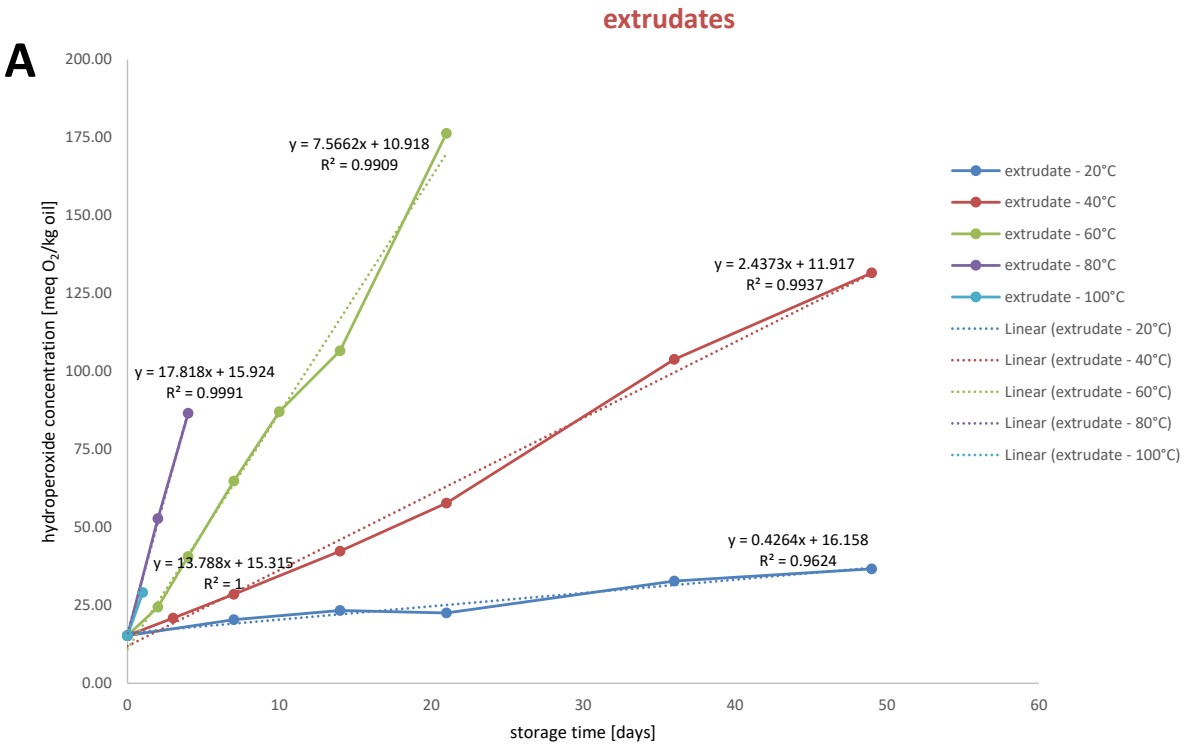
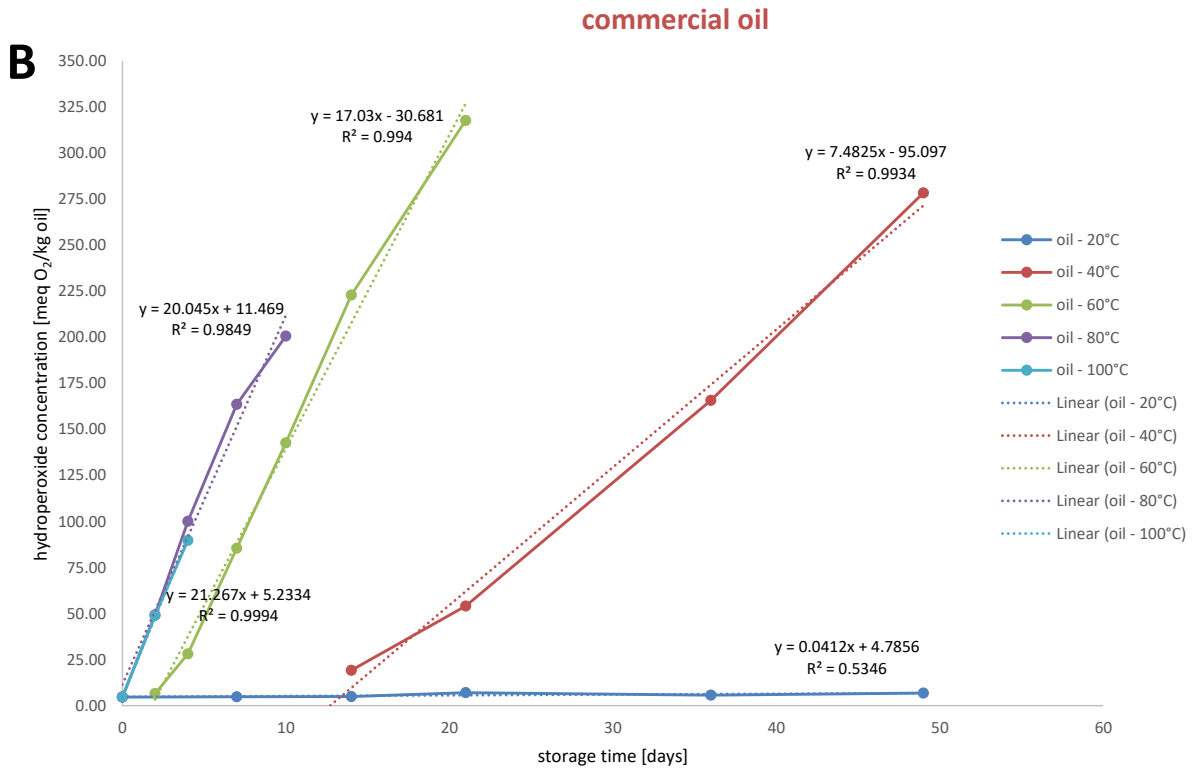
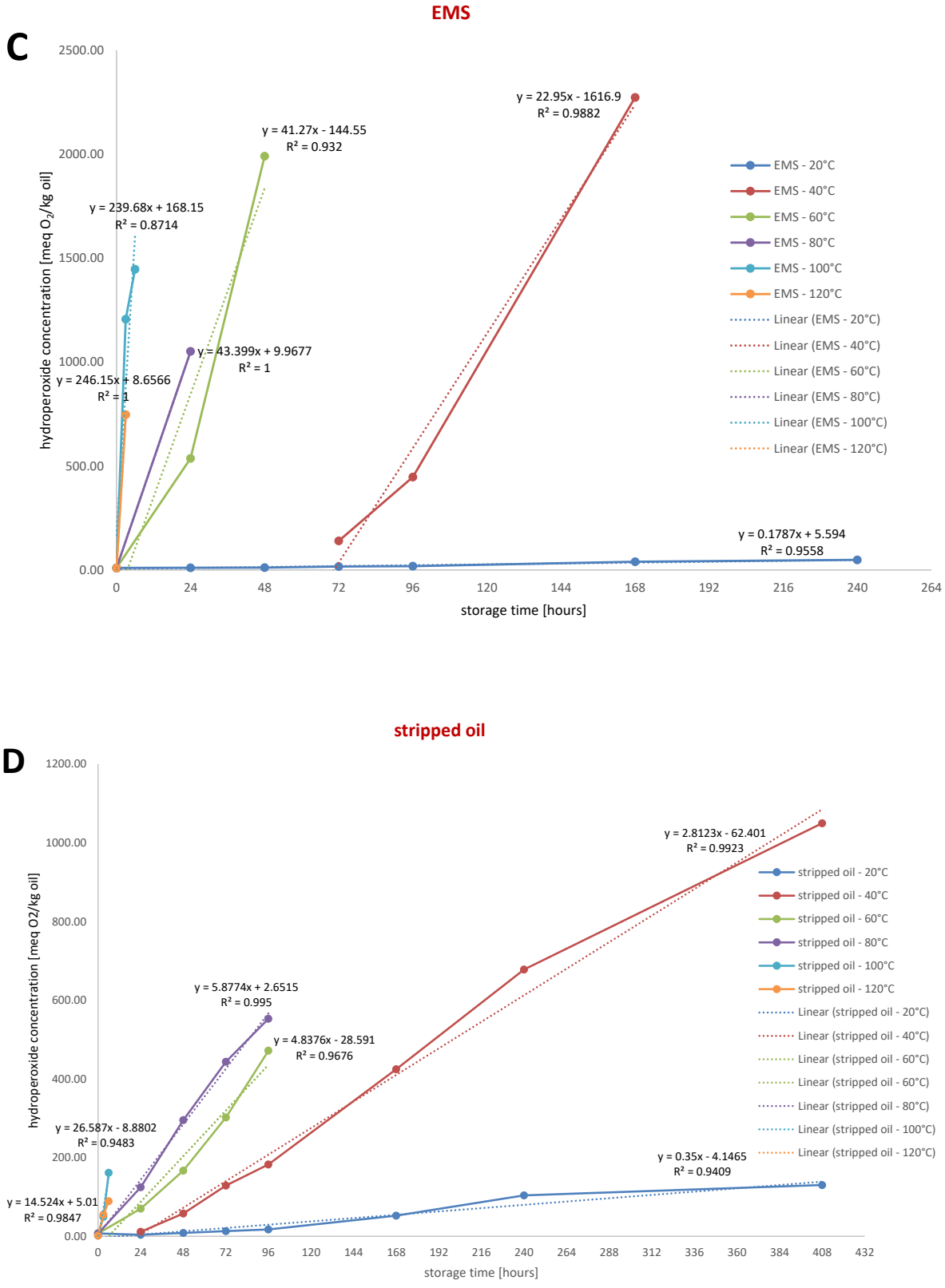
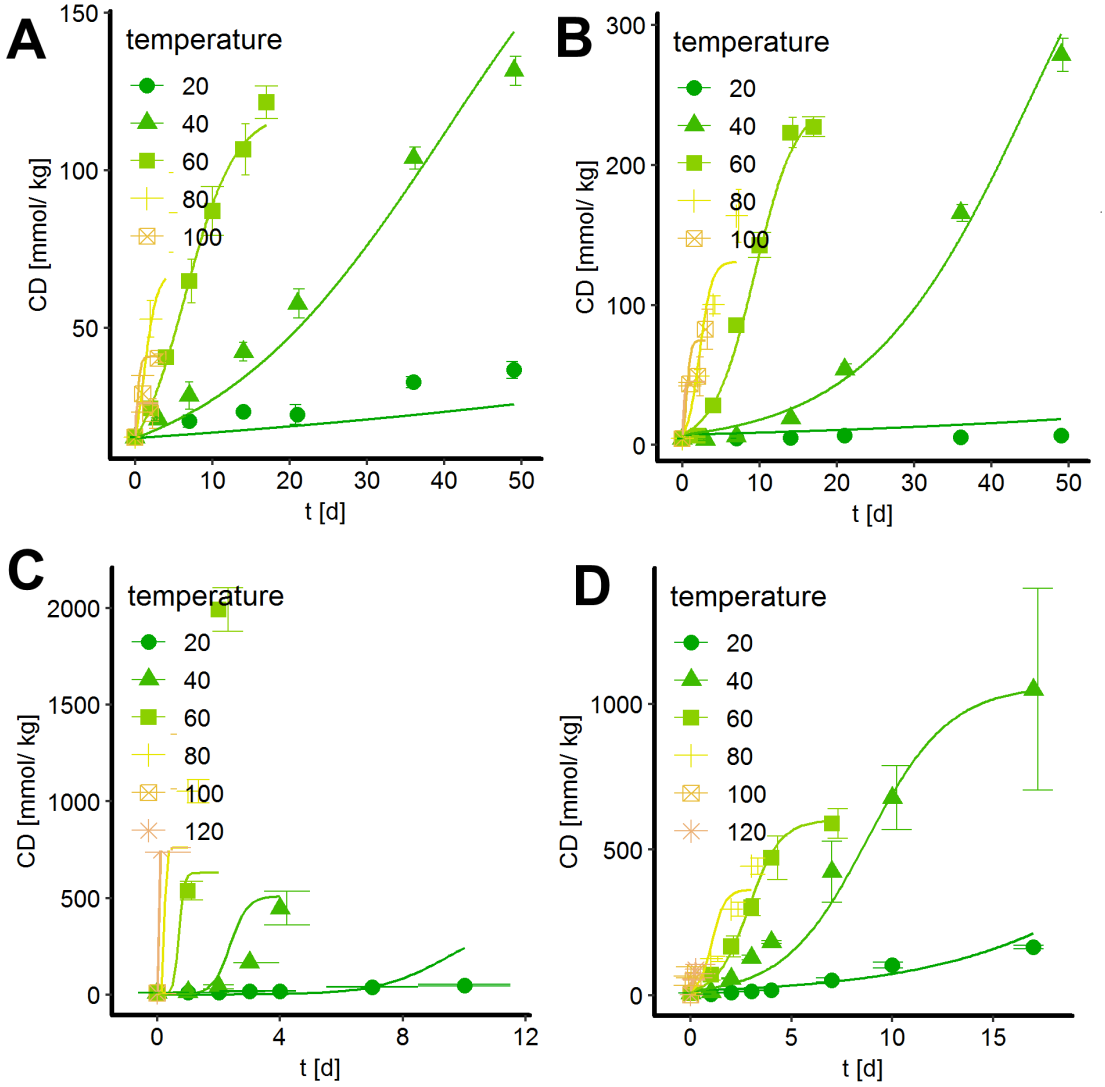
6.8. SUPPLEMENTARY MATERIALS



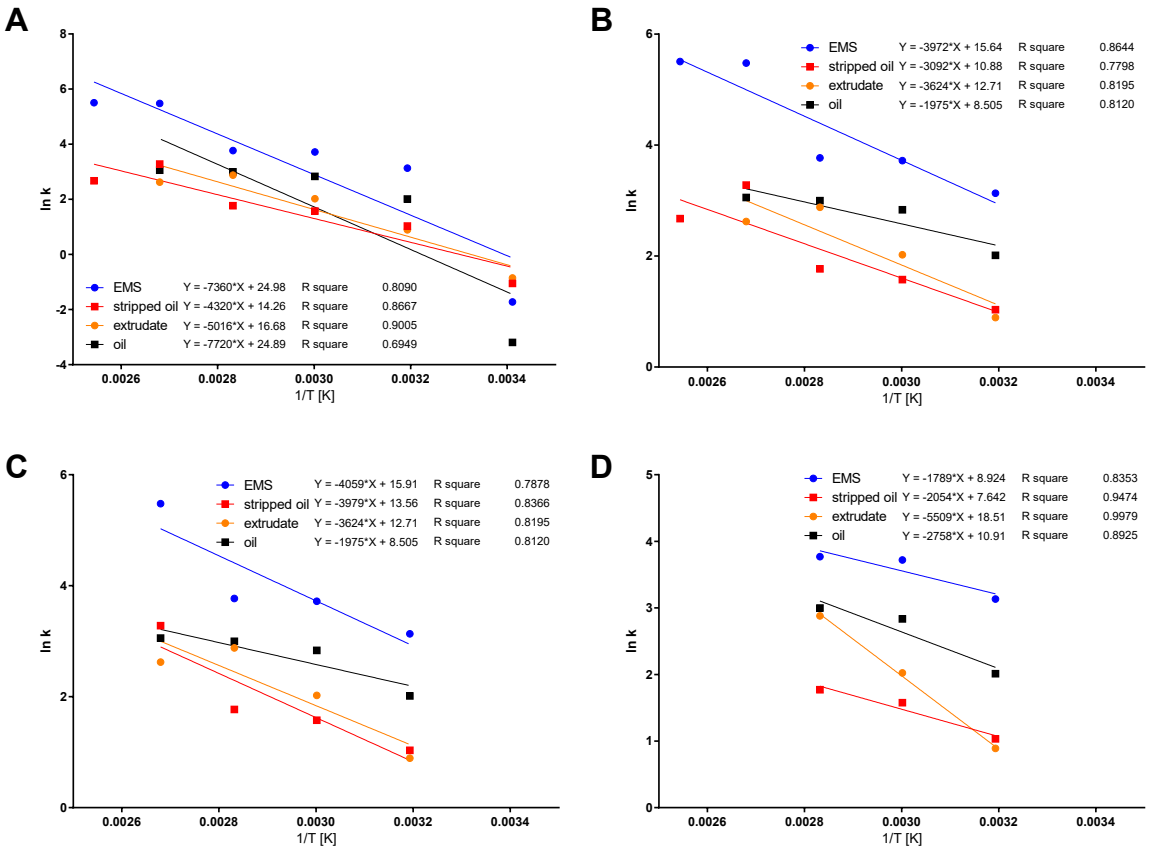




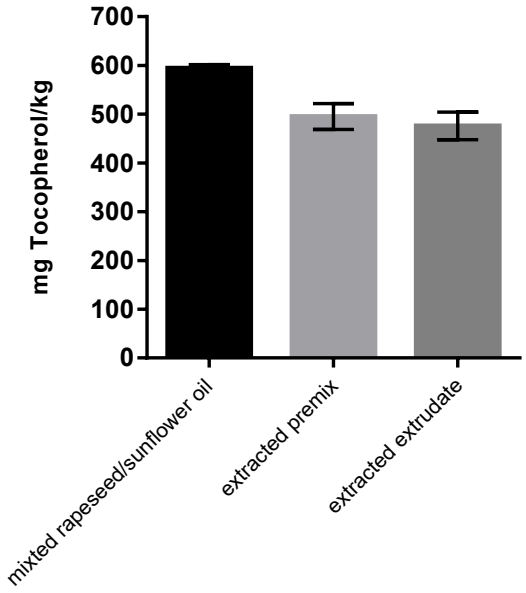
Supplementary Figure 6.6: *Linear approach for calculation of lipid oxidation kinetic parameters: A) extrudate, B) commercial oil used for extrudates, C) EMS, D) stripped oil used for EMS.*



Supplementary Figure 6.7: *Nonlinear approach for calculation of lipid oxidation kinetic parameters: A) extrudate, B) commercial oil used for extrudates, C) EMS, D) stripped oil used for EMS*



Supplementary Figure 6.8: *Arrhenius plots based on the linear approach: A) all temperatures, B) excluding 20 °C, C) excluding 20, 100 and 120 °C, D) including 40, 60 and 80 °C*



Supplementary Figure 6.9: *Tocopherol content in the rapeseed/sunflower oil mixture and in the cyclohexan-extracted lipid samples from premix and extrudate. Lipid extraction and its analysis of the tocopherol content according to the DGF F-II 4a method were performed as described in the material & methods part.*

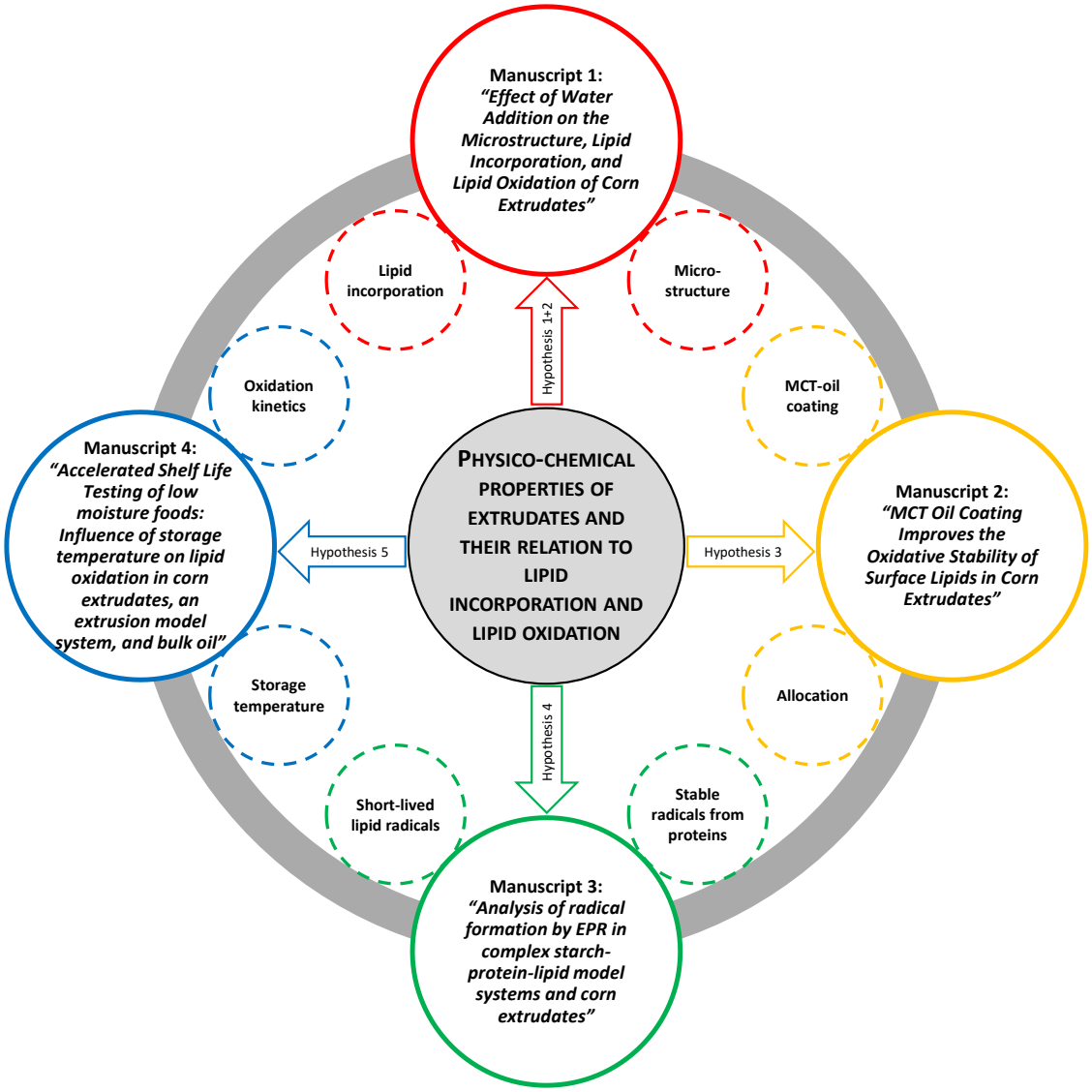
CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

The objective of the present thesis was to understand the effect of structural properties of extrudates on lipid oxidation and to gain knowledge about the oxidation mechanism in extrudates.

For the investigation of lipid incorporation in extrudates microstructure, a fundamental approach was followed (**Figure 7.1**) including a lipid extraction protocol, obtaining three fractions, i.e., surface lipids, inner-surface lipids, and matrix-incorporated lipids **(Hypothesis 1 / Chapter 3)**. The analysis of lipid oxidation in the resulting lipid fractions allows making profound statements about the influence of the microstructure and lipid incorporation on lipid oxidation **(Hypothesis 2 / Chapter 3)**. It was demonstrated in **manuscript 1** that in particular surface lipids are susceptible to lipid oxidation. For this reason, a study based on manuscript 1 dedicated to the influence of a lipid-based MCT-oil coating on lipid oxidation in corn extrudates. **Chapter 4** evaluates the stabilizing effect of this low oxidizable coating against lipid oxidation under consideration of its migration behavior **(Hypothesis 3)**. The combination of high temperature, pressure, and shear stress during extrusion is considered to result in radical formation. To fundamentally expand the knowledge of radical formation and oxidation in complex starch-protein-lipid model systems and extrudates, suitable analytical methods using electron paramagnetic resonance spectroscopy (EPR) were established. These include the use of spin trapping techniques for the measurement of lipid radicals and the direct measurement of stable radicals in the ground sample **(Hypothesis 4 / Chapter 5)**. The results of an accelerated shelf life study conclude this thesis with basic findings on the lipid oxidation kinetics in extrudates, and derived model systems. The study provides essential insights into the comparability of lipid oxidation experiments of low moisture foods at room temperature and accelerated storage tests at elevated temperatures **(Hypothesis 5 / Chapter 6)**.



1. **Hypothesis 1: The microstructure of extrudates influences lipid incorporation in the matrix of extrudates.**
   a. Different microstructures can be realized by varying the feed-water content.
   b. The use of a fractionated lipid extraction protocol enables to determine the degree of incorporation of lipids in extrudates and the effect on lipid oxidation.
2. **Hypothesis 2: Lipid incorporation in extrudates affects their oxidative stability.**
   a. Non-incorporated lipids adhering to the surface of extrudates are prone to oxidation.
   b. Lipids incorporated in the extrudate matrix have a higher oxidative stability.
3. **Hypothesis 3: The coating with a low oxidizable oil inhibit lipid oxidation of the sunflower/rapeseed oil in the extrudates. The coating provides a protective effect that exceeds the dilution effect by enhancing the lipid content of extrudates with low oxidizable oil.**
4. **Hypothesis 4: The combination of high temperature, pressure, and shear stress during extrusion is considered to result in radical formation of different nature. The investigation of model systems varying in their composition (lipid, proteins, and carbohydrates) and varying sample preparation of the matrix enables the annotation of radicals measured by electron paramagnetic resonance spectroscopy.**
5. **Hypothesis 5: The sample matrix of extrudates, an extrusion model system, and bulk oil influences lipid oxidation and the Arrhenius-like behavior derived from different accelerated temperatures.**

Figure 7.1: *Schematic structure and the investigated hypotheses (Chapter 1.1) of the present thesis*

7.1. INFLUENCE OF THE FEED-WATER CONTENT ON THE EXTRUDATE MICROSTRUCTURE

Extrusion processing parameters, e.g., screw speed (Moisio et al. 2015b), temperature (Chinnaswamy and Hanna 1988), feed rate (Ding et al. 2005), or feed-water content (Ding et al. 2006) influence the extrusion process and the properties of the produced extrudate. Referring to **hypothesis 1a**, it was demonstrated that the microstructure of corn extrudates can be influenced by varying the feed-water content. It was shown that an increase of the feed-water content resulted in a reduced expansion of the extrudates, a higher density (**Figure 3.2 B**), thicker cell walls (**Figure 3.3 C**), and a smoother surface (**Figure 3.2 A**). These changes can be explained by a reduced viscosity of the extrusion mass (Ilo and Berghofer 1999) and a reduced energy input (Kaisangsri et al. 2016). This was also confirmed in the present study by the reduced specific mechanical energy input (SME) and a reduced torque in extrudates produced with a high feed-water content (**Table 3.1**).

With an unchanged throughput and expansion, the density of the extrudates changes automatically. These changes are also reflected in the microstructure of the extrudates. Imaging of extrudates and their microstructure is important to understand the effects on lipid incorporation. The search for a suitable method to obtain independent evidence and detailed spatially resolved information for the physical parameters of the extrudate was of high interest for this study. Various cutting techniques, e.g. cryomicrotome or razor blade, have been tested to examine cross-sections of an extrudate under the fluorescence microscope. These invasive methods, which were intended to depict the microstructure of an extrudate, proven unsuitable due to the sample preparation. The latter led to the destruction of the structure. At the same time, invasive methods are not suitable for investigating the distribution of a coating (**Manuscript 2**), as a carry-over into the product occurs. A suitable noninvasive imaging method that simultaneously generates an 3D image of the extrudates is the computerized microtomography (μCT) (Schoeman et al. 2016). It was demonstrated that with low feed water contents (10%) the extrudates exhibited a rough and torn surface with micro-cracks and large cavities with thin and filigree cell walls. In contrast, the extrudates produced with higher feed-water contents exhibited a smoother surface and fewer cavities with thick lamellas (**Figure 3.3 A**). Increased cell wall thicknesses at higher moisture contents were also documented for extruded rice pellets (Chanvrier et al. 2015). A smoother surface at higher feed-water contents

could be explained with the lower energy input (**Table 3.1**), less pressure and in turn, a reduced expansion and contraction through the drying process (**Figure 2.2**).

7.2. INFLUENCE OF THE FEED-WATER CONTENT ON LIPID INCORPORATION

Most food extrudates are composed of starch-derived compounds, lipids, water, proteins as the main ingredients. The extrusion cooking process is characterized by melting of the dough under high temperatures, pressures, and shear forces. These circumstances lead to the formation of an amorphous matrix, a fine dispersion of lipids and gelatinization of starch (**Chapter 2.1.4**). A high degree of gelatinization is desired because the lipids can interact with the amylose helix (Genkina et al. 2015). The gelatinization degrees of the corn extrudates showed no significant differences between the various feed-water contents and amounted to over 90% (**Supplementary Figure 3.8**). Such high gelatinization degrees indicate a total gelatinization of the starch (Lue et al. 1991).

Many studies differentiate only between bound and unbound lipids in extrudates (Strange and Schaich 2000; Zadernowski et al. 1997; Moisio et al. 2015b). This differentiation is not very precise and does not fully consider the different binding mechanisms. Furthermore, literature reports of so-called amylose-lipid complexes, where the lipids are incorporated in the amylose helix. These can be formed during the extrusion process (Pilli et al. 2011). However, commercial products are composed of triglycerides, which cannot form this kind of inclusion complexes because of steric hindrance (Chao et al. 2018). Only free fatty acids and monoglycerides can enter the amylose helix (Bhatnagar and Hanna 1994). Rather, it must be assumed that the lipids, in particular triglycerides, are bound in different ways in the matrix of the extrudates and adhere to the surface.

Therefore, a fractionation method has been developed in this thesis that distinguishes the lipids based on their degree of incorporation in the extrudate and its matrix (**Chapter 3.3.2**). The extraction protocol provides three lipid fractions (**Figure 3.1**): lipids located on the surface and close to the surface are classified as “surface lipids”. Weakly incorporated lipids that are present on the cell walls of the extrudates are accessible after grinding and are classified as “inner-surface lipids”. Lipids that are incorporated in the amylose matrix and are, therefore, only extractable after degradation of the starch-based matrix using α-amylase are classified as “matrix-incorporated lipids”. It is suggested that, such aroma compounds (Biais et al. 2006),

matrix-incorporated lipids are stored between the amylose helices or dispersed in the amorphous regions of the starch but do not form complexes with the amylose helices.

In **manuscript 1**, the **hypothesis 1b** was confirmed that the fractionation protocol for lipid extraction – developed and established in this thesis – enables the analysis of lipid incorporation in extrudates and its effect on lipid oxidation. As discussed previously, the feed-water content influences the expansion and the microstructure of extrudates. For example, an increase in the feed-water content was associated with reduced porosity and significantly thicker cell walls (**Figure 3.3**). This observation is in accordance with the reduced expansion ratio at higher feed-water contents (**Figure 3.2 B**), causing a reduced surface area (Włodarczyk-Stasiak and Jamroz 2009). These structural changes explain the decrease in the proportion of surface lipids, whereas the matrix-incorporated lipids increase (**Figure 3.4**). Overall, a high level of lipid incorporation into the extrudate matrix can be achieved at high feed-water contents (≥18%). It can be proposed that a high degree of dispersion of lipids (micro droplets / molecular dispersion) in the carbohydrate matrix. i.e., the lipid incorporation is enhanced at higher water contents (**Figure 3.4**) because of complete swelling of the starch granules, the disruption of molecular structure and disassembly of the quasi-crystalline structure during gelatinization (Wang and Copeland 2013), and the formation of a colloidal solution. Furthermore, the water may disorganize the structure and release amylopectin from the starch granules additionally to amylose (Matignon and Tecante 2017). It can be assumed that this structural rearrangement of the carbohydrate fraction is a prerequisite for lipid incorporation between amylose helices that are only released after enzymatic treatment.

7.3. RELATIONSHIP BETWEEN LIPID INCORPORATION AND LIPID OXIDATION IN EXTRUDATES

In **manuscript 1**, the **hypothesis 2** was confirmed that the degree of lipid incorporation affects oxidative stability. The extent of lipid oxidation strongly depends on the available oxygen (Johnson and Decker 2015b). Lipid binding and incorporation can be seen as an encapsulation of the lipids in the starch-based matrix, reducing their oxygen accessibility. The extent of lipid oxidation in the different lipid fractions was monitored during storage at accelerated storage conditions (+40 °C) in the extracted lipids. An increase in the degree of lipid incorporation resulted in a reduced hydroperoxide concentration (surface lipids > inner surface

lipids > matrix-incorporated lipids) (**Figure 3.5**). That is, lipids exposed to oxygen, e.g., surface lipids, showed the highest oxidation rate. Comparable results were also reported for fried nuts where surface oxidation was higher than internal oxidation (Marmesat et al. 2006). In extrudates, this connection can also be explained by the extrudate microstructure that depends in turn on the feed-water content (**Figure 3.3**). An increase in the feed-water content resulted in lower oxidation of the lipids, i.e., the hydroperoxide and hexanal concentrations were reduced (**Figure 3.6** and **Figure 3.7**). This can be related to the reduced expansion and an increase of incorporated lipids, which in turn can be explained by the thicker cell walls and a reduced surface area. High expanded extrudates exhibit thin cell walls, which facilitates oxygen access and thus the formation of hydroperoxides (Lin et al. 1998b). It is suggested that the starch-based matrix acts as a shield against lipid oxidation. Studies, making a distinction between free and bound respectively complexed lipids, reported reduced oxidation of bound lipids (Guzman et al. 1992; Thachil et al. 2014). However, these studies lack differentiation for lipid oxidation in the different fractions (free and bound lipids) and the causal relationship between total oxidation of the extrudates and lipid binding was missing. In **manuscript 1,** it was possible to prove the relation between structural effect and lipid oxidation.

7.4. INFLUENCE OF A LIPID-BASED COATING ON LIPID OXIDATION IN EXTRUDATES

In **manuscript 2**, **hypothesis 3** was confirmed that a coating with the low oxidizable MCT oil enables an increase in oxidative stability of the extrudates. Based on the results of the fractionated lipid extraction protocol and the measurement of hydroperoxides it was proven that the coating prevents surface lipids from oxidation (**Figure 4.2**). The effect of the coating was reduced, with an increasing degree of lipid inclusion (**Table 4.1**). That is, the coating inhibited lipid oxidation of inner-surface and matrix-incorporated lipids only in case of porous and expanded extrudates. To understand the mechanisms of the lipid stabilizing coating, its distribution behavior was investigated using fatty acid profiles of the lipid fractions and the imaging techniques fluorescence microscopy and μCT.

The addition of MCT oil to food, in the case of this study to the extrudate, resulted in an increase of the lipid amount of the sample and a dilution of the oxidizable lipids (Raudsepp et al. 2014). MCT oil consists of saturated medium-chain fatty acids (C_8 and C_{10}), which do not oxidize

naturally (Bhatnagar et al. 2009). Therefore, the protective effect of the coating must be evaluated under the premise of a possible dilution effect. It was found that in particular the 10% feed-water extrudates benefit from the coating because total hydroperoxides were significantly reduced (**Figure 4.3 B**). **Figure 4.5** exhibits that the coating covers the surface and fills microcracks. Therefore, it can be suggested that the coating contributes to the inhibitory effects by sealing the porous surface of extrudates and the elimination of microcracks (Gray et al. 2008). MCT oil is predestined for this purpose as it has a low viscosity and can, therefore, be easily distributed on the surface. This assumption was confirmed in fluorescence microscopic analyses with Nile red (**Figure 4.4**). As the oxidative stability of microcapsules depends on the oxygen barrier properties (Drusch et al. 2009), it can be assumed that the protective effect of the MCT coating is attributable to an increased oxygen barrier (Chiumarelli and Hubinger 2014).

Based on the lipid fractions, the verification showed that the surface lipids, in particular, were protected, whereas the inner-surface lipids only benefited from the protective effect of the coating in the case of the more expanded extrudates (**Table 4.1**). The matrix-incorporated lipids did not benefit from the protective effect of the topical coating. It seems that these lipids are protected by the matrix and that this oxygen barrier cannot be strengthened by an external coating. In extrudates with a compact structure (high feed-water contents), only dilution of the oxidizable lipids must be assumed, which leads to lower oxidation. These findings agree with the observations made with µCT, where Nile red was used as a contrasting agent for the coating (Contardo and Bouchon 2018). The coating was limited to the outer 2–3 mm of the extrudate (**Figure 4.5**) and high amounts of medium-chain fatty acids (MCFA) were only detectable in the surface lipids of coated extrudates (**Table 4.2**). The dark gray areas in the µCT scans, which were not visible in the uncoated extrudates (**Figure 3.3**), can be attributed to the MCT coating and were only visible in the outer cavities indicating a reduced migration into the extrudate. These dark gray regions exhibited an appearance of liquids with formation of a meniscus because of capillary effects. The reduced migration behavior is in line with the absence of MCFA in the matrix-incorporated lipids. The inner-surface lipids were visible in fluorescence microscopy as a thin red fluorescent film on the surfaces of the lamellas (**Figure 4.4**) (Yilmaz et al. 2001; Barden et al. 2015). A fatty acid analysis of the inner-surface lipids showed that with increasing feed-water contents and in turn reduced expansion ratios and lower porosity, the amount of MCT in the inner-surface lipid fraction decrease (**Table 4.2**). The thicker cell

walls and the compact structure seem to hinder migration of the coating. Thus, more MCFA and less unsaturated fatty acids were present on the surface of the coated extrudates. This dilution of oxidizable lipids, explains the significantly reduced peroxidability index of the surface lipids. Over the storage period, small amounts of the coating migrated into the extrudates, measurable by a slight increase of the MCFA portion in the lipid profile of the inner-surface lipid fraction. This effect could be attributed to the relatively high storage temperature, which lowers the oil viscosity, thus increasing migration.

Especially noteworthy was the fact that no rancid off-flavors were perceptible and no increase in hexanal during storage was detected in coated extrudates (**Figure 4.3 C**). This was also evident in significantly reduced hexanal concentrations, independent of the water content and the microstructure (**Figure 4.3 D**). The effect decreased with increasing water content. Hexanal is an aldehyde from the decomposition of hydroperoxides, mainly originating from the oxidation of linoleic acid (Frankel 1993). Because of the lipophilic character of hexanal, it could be assumed that the hexanal has a changed partitioning behavior, i.e., that less hexanal passes from the lipid phase into the gas phase. The verification of this presumption revealed that it is more likely that the lipid oxidation is inhibited because increasing the lipid content with MCT oil has a lower effect on the measured hexanal content than would be expected due to dilution and increase of the total lipid content (**Supplementary Figure 4.6**).

In conclusion, the **hypothesis 3** can be confirmed because the protective effect of the edible coating outweighs the dilution effect, the coating shows only a low migration behavior into the extrudate and the coating inhibits lipid oxidation of the sunflower/rapeseed oil in the extrudates.

7.5. RADICAL FORMATION IN COMPLEX STARCH-PROTEIN-LIPID MODEL SYSTEMS AND EXTRUDATES

The suitability of EPR for the measurement of unpaired electrons, like free radicals, in various food products was reported in different studies (**Chapter 2.3.4.2**). However, the analysis of radicals in corn-based model systems and extrudates, using EPR, and the comparison with common lipid oxidation markers has not been studied before.

In **manuscript 3**, it was hypothesized (**Hypothesis 4**) that the EPR enables the investigation of radical formation in complex starch-protein-lipid systems. Spin trapping was used as suitable technique for studying radical formation of lipids and the protein as well as carbohydrate radical

formation was measured directly in the model systems and extrudates. The knowledge about the formation of short-lived lipid radicals and stable protein or carbohydrate radicals is of great interest because it allows insights into radical formation mechanisms in extrudates and helps to derive suitable measures for protection against oxidation. In the future, a combination of hydrophilic and lipophilic antioxidants could be used to inhibit the oxidation of lipids as well as proteins and carbohydrates.

Lipid radicals, such as alkoxyl and peroxyl radicals are short-lived radicals with a half-life up to few nanoseconds. Therefore, spin trapping is necessary to form stable spin trap adducts, which can be measured using EPR (Buettner 1987). Suitable incubation conditions for the spin traps POBN (data not shown) and PBN were evaluated by generation of hydroxyl radicals in the Fenton reaction (**Figure 5.1**). Incubation with PBN at elevated temperatures is recommended to accelerate the induction period, in which endogenous antioxidants of the sample matrix are depleted (Kocherginsky et al. 2005). Furthermore, it can be suspected that the temperature increase is also necessary to lower the activation energy (E_a) for the reaction of PBN with a radical.

The incubation conditions were systematically tested. It was demonstrated that incubation at 50 °C produced significantly higher PBN adduct concentrations than the lower incubation temperature of 30 °C (**Figure 5.1**). The spin trap POBN exhibited significantly lower POBN adduct concentrations. Thus, POBN was not considered for further experiments. In addition, after 30 min a maximum could be detected (**Figure 5.1 B**). The decrease of spin trap adducts in the ongoing process of incubation after a steady-state level can be attributed to the degradation of spin adducts that exceed their formation (Kocherginsky et al. 2005).

For lipid radical extraction, various solvents were evaluated (**Chapter 5.4.2**). Solvents with a polarity index, according to Snyder (1978), from 3 to 5 proved to be suitable because the extracts obtained from these solvents revealed a high PBN adduct intensity (**Figure 5.2 B**). However, with BHT stabilized diethyl ether was not suitable because of changed EPR spectra (**Figure 5.2 A**) due to ether peroxyl radicals present in the solvent. Ethyl acetate has proven to be the most suitable solvent because it could be completely removed from the extracted oil within a short time (60 min) and also has a stabilizing effect on the extracted radicals (Trudell 1987).

The comparison of the lipid radical measurement using EPR with common oxidation parameters in purified oil showed that the formation of PBN adducts and hydroperoxides correlated well during storage (**Figure 5.3 D**) (Cui et al. 2017). In the first storage days, the lipid radical formation was higher whereas in the further storage experiment the hydroperoxides seem to increase faster. This corresponds to the mechanism of lipid oxidation (**Chapter 2.3.1**). An expected lag phase in hydroperoxide formation was not observed and can be explained with the absence of antioxidants and the accelerated storage conditions. Simulation of the EPR spectra using EPRSIM (Strancar et al. 2005) indicated a superposition of different radical species (**Figure 5.3 B**). In oxidized lipid samples, lipid-derived alkyl (R•), alkoxyl (RO•), and peroxyl (ROO•) radicals could be expected (Zamora and Hidalgo 2016). The PBN adduct spectra simulation demonstrated that 1-hydroxyethyl, alkoxyl, peroxyl, and hydroxyl radicals were trapped in the purified oil (**Figure 5.3 B**) and in the extrudates. The data of the purified oil storage experiment reveal that during storage the amount of alkoxyl radicals and 1-hydroxyethyl radicals increase, whereas the PBN adduct concentrations of the remaining radicals decreased slightly (**Figure 5.3 C**). This is in line with other studies identifying alkoxyl radicals as the main lipid-derived radicals with the spin trap DMPO (Dikalov and Mason 2001) or in human blood samples (Davison et al. 2008). Alkyl radicals are not detectable because low steady-state concentrations and fast reaction of alkyl radicals with oxygen (Novakov et al. 2001). The low amount of peroxyl radicals is attributable to the decomposition of PBN-peroxyl adducts to PBN-alkoxyl radical adducts, which are indirect evidence for peroxyl radical generation (Dikalov and Mason 1999). The 1-hydroxyethyl radicals can be attributed to ethanol oxidation, which is induced by incubation conditions (Andersen and Skibsted 1998). In addition, hydroxyl radicals, from hydroperoxide decomposition, could react with ethanol to 1-hydroxylethyl radicals, which were trapped instead of hydroxyl radicals (Pou et al. 1994). Depending on the matrix, hydroperoxide decomposition to hexanal was observed at temperatures above 80 °C (**manuscript 4, Figure 6.1**). Decomposition of hydroperoxides to lipid radicals during incubation can be neglected because this is reported at distinct higher temperatures (Roman et al. 2012) than the incubation temperature used in **manuscript 3**. The extract contains a balance of alkoxyl radicals, peroxyl radicals and hydroperoxides, which correspond to the model systems and extrudates. Only the peroxyl radicals could be trapped, which decompose to alkoxyl radical adducts that were measured.

In **manuscript 3**, it was demonstrated that extrusion cooking process promotes the formation of stable radicals (**Figure 5.5 B**). Furthermore, it can be suggested that extrusion acts as an impulse, resulting in a slight increase in PBN adducts in the extract. In addition, the extrusion process increased the hydroperoxides and the hexanal concentration in the extrudate compared with the unextruded premix (**Figure 5.5 A**). This observation was confirmed in **manuscript 4**, where extrudates had higher hydroperoxide concentrations than the unextruded oil at beginning of the storage (**Figure 6.1 A & B**). During the accelerated storage test, the lipid radicals increase slightly and hydroperoxides and hexanal showed the same oxidation pattern in extrudates with an increase from storage day 4. By comparison, the premix did not oxidize during storage, indicating the oxidation-promoting effect of extrusion cooking. It can be suggested that extrusion supports the emergence of lipid radicals during storage because heat, pressure and shear forces leading to an onset of hydroperoxide formation.

However, the low PBN adduct concentrations in the extrudates (**Figure 5.5**) must be considered when evaluating the results. During extrusion cooking no distinct tocopherol degradation was measurable (**Supplementary Figure 6.9**), indicating that the presence of tocopherols decreased the EPR signal intensity and lowers the amount of PBN adducts (Cui et al. 2017; Velasco et al. 2004b). It must be assumed that tocopherols interfering with the ability of PBN to trap lipid radicals in a kinetic competition (Jerzykiewicz et al. 2013).

The concentration of stable radicals was increased after extrusion but did not change during storage (**Figure 5.5 B**). The increase in stable radicals is attributable to synergistic effects of temperature, pressure, and shear (Schaich and Rebello 1999a). For identification of the stable radicals in the extrudates, a simplified model system was prepared with linoleic acid and various corn flour constituents (cornmeal, cornstarch, zein, and cornstarch with zein). Cornmeal used for extrusion is a mixture of starch and a protein (zein) component. In the model systems, these compounds were evaluated separately to identify their potential contribution to the extrudate spectra (**Supplementary Figure 5.8** vs. **Supplementary Figure 5.9**). The measured hyperfine constants (**Table 5.1**) of the stable extrudate radicals identified them as nitrogen-centered protein radicals. The peaks exhibited similar g-values as the model systems produced with protein-rich matrices (cornmeal and zein) (Labanowska et al. 2014). The mixture of cornstarch with the lipid compound had higher g-values and zein is the main storage protein in corn (Shukla and Cheryan 2001). Therefore, the assumption can be confirmed that radicals with

similar g-values in both, cornmeal model system and extrudates produced with cornmeal, are radicals from the protein fraction (**Table 5.1**).

As could already be observed in the extrudates, the stable radicals did not increase in the complex starch-protein-lipid model systems during storage (**Figure 5.4 B**). Lipid radicals increased depending on the used raw materials with different intensity. Samples with proteins showed a small increase of lipid radicals compared with cornstarch-model system, which increased strongly (**Figure 5.4 A**). This phenomenon was also observed in the formation of hydroperoxides (**Figure 5.4 C**). Proteins are known to inhibit lipid oxidation, through an antioxidative-like mechanism that is still not fully explored (Hu et al. 2003; Sakanaka et al. 2004). It is probable that lipid oxidation products are masked so that they cannot be measured using conventional lipid oxidation markers.

In conclusion, **hypothesis 4** can be confirmed. The model systems and their extracts enable the annotation of the short-lived lipid radicals and of the stable radicals by EPR. It was demonstrated that extrusion cooking promotes radical formation and stable radicals, which can be attributed to proteins and can be measured directly in the ground model systems and extrudates. Spin trapping of lipid radicals would require tocopherol-free systems. However, the formation of stable radicals was clearly demonstrated, even in the presence of tocopherols.

7.6. ACCELERATED STORAGE TESTS FOR ANALYSIS OF LIPID OXIDATION IN LOW MOISTURE FOODS

Studies on lipid oxidation at room temperature are time-consuming. Therefore, accelerated shelf life tests are an obvious alternative. In **manuscript 4**, it was hypothesized (**Hypothesis 5**) that the sample matrix of extrudates, an extrusion model system (EMS), and bulk oil influences lipid oxidation and the Arrhenius-like behavior in accelerated oxidation studies at elevated temperatures.

In extrudates, EMS and bulk oil, an increase of the storage temperature was associated with an increase of the oxidation rate (**Table 6.2**). This was measurable by increased hydroperoxide and hexanal concentrations. Furthermore, samples stored at high storage temperatures (>60 °C) reached a maximum hydroperoxide concentration, followed by a decrease of hydroperoxides and an increase of hexanal (**Figure 6.1**). The maximum hydroperoxide concentration decreased with increasing storage temperature. In extrudates and commercial oil, this effect could be

attributed to the reaction rate constant, which was constant or decreased at storage temperatures above 80 °C (**Table 6.2**). Furthermore, the decomposition rate of hydroperoxides increases with an increase of the temperature (**Table 6.3**) and exceed the hydroperoxide formation (Crapiste et al. 1999).

Within the different systems investigated, there were clear differences in the oxidation behavior. The extrudates had lower hydroperoxide concentrations than the commercial oil (**Figure 6.1**). In contrast, the EMS had higher concentrations of oxidation markers than the stripped oil (**Figure 6.2**). It was also noticeable that the samples with stripped oil oxidized much more strongly than those produced with commercial oil. This observation can be attributed to the absence of tocopherols because there were no antioxidant effects in the system (Frankel 1996). This assumption is in accordance with an enhanced decomposition of hydroperoxides resulting in an increase of hexanal in the tocopherol-free systems (**Figure 6.2**) (Cui et al. 2017).

At higher storage temperatures (≥80 °C) color changes of the extrudates (**Table 6.1**) and changes in the volatiles of the EMS (**Figure 6.3**) could be observed. In detail, the results revealed that an increasing temperature was accompanied by reduced L*-values indicating a browning of the extrudates. In bread, the connection between browning and decreased L*-values is established (Purlis and Salvadori 2009; Capuano et al. 2008). Furthermore, the GC-MS measurements identified aldehydes from lipid oxidation, e.g., hexanal, propanal, pentanal, etc. as well as in the EMS stored at temperatures above 80 °C furans, e.g., 2-pentylfuran. Generally, the concentration of volatiles increases with the storage temperature. The principal component analysis of the volatiles, depicted in **Figure 6.4**, revealed a clear difference between stripped oil and EMS and that an increase of the storage temperature had no effect on the volatile profile of the oil. In addition, the furans and hexanal increased in the EMS with the temperature.

To compare the different low-moisture systems with the bulk oils, oxidation kinetic parameters were calculated (**Table 6.2** and **Table 6.3**) following two different approaches (Crapiste et al. 1999; Meissner et al. 2019; Manzocco et al. 2012). Following the linear approach, regarding the formation of hydroperoxides (k_1), the extrudates had a significantly lower E_a than the commercial oil (**Table 6.2**). This is attributable to the different energy inputs in the samples, which leads to pre-damage of the lipids in the extrusion process. According to the findings of **manuscript 1**, the energy input (SME) can be reduced by raising the feed-water content (Amft et al. 2019a). On the one hand, it was demonstrated that prooxidants such as free radicals were formed during extrusion cooking (**Manuscript 3**, **Figure 5.5**). Furthermore it is reported that

the transition metal content increase (Rao and Artz 1989). On the other hand, extrusion cooking could decompose tocopherols (Zielinski et al. 2001) even if this was not measurable in the extrudates used in this work (**Supplementary Figure 6.9**). Both, browning and specific volatiles indicated that Maillard reaction takes place under high storage temperatures. Maillard reaction products had an antioxidative activity in low moisture foods (Mastrocola and Munari 2000; Moisio et al. 2015a), which could explain the lower hydroperoxide concentrations in the extrudates compared with the commercial oil (**Figure 6.1**). By comparison, it can be assumed that the protective effect of the extrudate matrix is reduced at higher temperatures because the oxygen permeation increased with the temperature (Andersen et al. 2000). Overall, all these factors contribute to the reduced E_a (Tan et al. 2001; Golmakani et al. 2019).

For the practical application of accelerated shelf life tests, this study demonstrated that the reaction rate (k_1) is strongly temperature and matrix dependent. That is, especially samples stored at 20 °C differ in the Arrhenius plot (**Figure 6.5**). Furthermore, particular attention must be paid to rapid methods, e.g., Rancimat, using high temperatures to accelerate lipid oxidation because these can lead to altered reaction mechanisms. This thesis proved that, depending on the sample matrix, compounds, indicating Maillard reaction, were already formed within six hours. In addition, in the Arrhenius plots of the commercial oil samples and extrudates, a plateau was observed at temperatures above 60 °C (**Figure 6.5 B**). If only considering the reaction rate k_1 it can be assumed that a maximum reaction rate exists. However, this is reported for distinct higher temperatures (180 °C) (Reynhout 1991). It is more likely that the degradation of hydroperoxides compete with the hydroperoxide formation, resulting in a steady-state level. Therefore, the data generated at high temperatures can only be used to draw limited conclusions about oxidation at room temperature. This could lead to an overestimation of the lipid oxidation, which would question specifications on the shelf life at room temperature.

In conclusion, for prediction models of lipid oxidation reactions in extrudates, model systems are still a challenge. This is because shear forces, pressure and simultaneous temperature treatment are difficult to model. Therefore, the name “extrusion model system” may be misleading, since the processing steps of this model system are rather similar to the fabrication of a cookie. However, **manuscript 4** demonstrated that extrudates and EMS have a similar course of hydroperoxide formation but the EMS provides results in a fifth of the time.

In conclusion, **hypothesis 5** could be confirmed, because the sample matrix lead to side-reactions at accelerated temperatures (> 60 °C), which could influence the oxidation. Thus, the

Arrhenius-like behavior differs and complicate predictions for oxidation at ambient temperature.

7.7. CONCLUSIONS AND OUTLOOK

The present thesis confirmed that the process parameter water content, modifies the microstructure and lipid binding in the matrix of extrudates (**Hypothesis 1a**), resulting in extrudates with higher oxidative stability. This effect was due to a high degree of lipid dispersion (micro droplets / molecular dispersion) in the carbohydrate matrix, which was measurable with the developed enzyme-assisted fractionated lipid extraction protocol (**Hypothesis 1b**). These incorporated lipids exhibited in turn a higher stability against oxidation compared with non-incorporated lipids adhering to the surface of extrudates (**Hypothesis 2**). It must be assumed that reduced oxygen exposure in the starchy matrix contributes to this effect. This assumption is confirmed in **Chapter 4**, where the application of a coating with a low oxidizable MCT oil enables the stabilization of lipids, in particular, low incorporated lipids with a high oxygen exposure (**Hypothesis 3**). It was shown that the coating mainly adheres to the surface of the extrudates, where it fills micro cracks. Furthermore, the study showed that in particular, porous extrudates benefited from the protective effect of the coating and the effect decreased with increasing lipid inclusion. However, in the case of low expanded extrudates, the lower hydroperoxide contents can only be explained by dilution of the oxidizable lipids. To get a better efficiency of the coating and to improve the lipid stability, in further studies, the proposed MCT coating could be optimized by addition of antioxidants. Especially the oxidative stability of the non-matrix-incorporated lipids could benefit from additional antioxidants that can migrate with the coating in the outer areas of the extrudates.

In this thesis, the relationship between microstructure and lipid oxidation was investigated. This work focused on the reduction of lipid oxidation and not on the sensory properties or texture of the extrudates. With a change of the microstructure the product properties change at the same time. Low expanded extrudates with a high oxidative stability, may have a low consumer acceptance, because of sensory and textural disadvantages (Brennan et al. 2013). This must be considered when transferring the knowledge into practice.

The EPR study in this thesis demonstrated that lipid-derived radical formation can be measured using the spin trapping technique. The results correlate with commonly used markers for lipid

oxidation, e.g., hydroperoxide concentration. Spectra simulation revealed that mainly alkoxyl radicals were detected in oxidizing oils (**Hypothesis 4**). However, tocopherols present in extrudates hindered the detection of PBN adducts.

The direct measurement of ground starch-protein lipid model systems and extrudates enable the annotation of stable radicals in extrudates to proteins (**Hypothesis 4**). The EPR analysis revealed that extrusion processing let increase stable radicals. In the future, it would be interesting to combine the fractionated lipid extraction with the EPR analysis to improve the knowledge of lipid radical formation in different regions of extrudates. Furthermore, the direct addition of spin traps to the extrudate premix could provide further insights into the effect of extrusion on radical formation, provided that the extrusion conditions substitute the incubation time for adduct formation with the spin trap.

Accelerated storage tests are considered as the most suitable method for making oxidation tests and statements about the shelf life of a product in a short time. It could be shown that an EMS may be suitable for prediction of lipid oxidation in extrudates because both systems exhibited a similar course of hydroperoxide formation under accelerated storage conditions. However, the process condition during extrusion cannot be fully imitated. Further, attention must be paid to high-temperature oxidation experiments because of side-reactions like the Maillard reaction, which influence the oxidation mechanism. Also, oxidation rates at high temperatures (>60 °C) differ from that at room temperature, leading to an overestimation of lipid oxidation (**Hypothesis 5**).

Overall, it can be concluded that by modification of the extrusion process it is possible to influence the lipid incorporation and thus improve the oxidative stability. A coating with a low-oxidizable oil has positive effects on the stability. Modern analytical methods, like µCT and EPR, can be used to extend the knowledge of lipids and their behavior in complex foods such as extrudates. These insights in lipid incorporation and lipid oxidation in extrudates pave the way for increased use of polyunsaturated lipids to improve the nutritional quality without compromising lipid stability and product quality.

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8. SUMMARY

Extrusion cooking is a key technology in food industry, which is used to produce a large number of so-called *extrudates* (e.g. cereals, cornflakes, snack products and dry animal food). Extrudates reveal a high-specific surface and are prone to oxidation due to the increased oxygen access. Lipid oxidation is a major cause of quality deterioration during the production and storage of lipid-containing extrudates. It is accompanied by rancid off-flavors, which is the major reason for a reduced shelf life. During the extrusion process, starch gelatinizes and forms an amorphous matrix that incorporates lipids. The objective of this thesis is to understand the relationship between lipid oxidation and the structural properties or interactions of lipids with the matrix in order to create a basis for reducing or delaying oxidation processes in extrudates.

Chapter 3 describes the development of a fractionated lipid extraction. It allows the characterization and investigation of lipid oxidation processes in different regions of an extrudate. The extraction process distinguishes between surface lipids, inner surface lipids (lipids adhere on the inner surface of the lamellae in the extrudate) and matrix-incorporated lipids. Matrix-incorporated lipids are bound between amylose helices and cannot be extracted using mild solvent extraction conditions. Only an amylase treatment causes a sufficient structural destruction, which makes the lipids accessible for extraction.

It was shown that the feed-water content (10–22%) influences the microstructure and the expansion of an extrudate and results in differences in lipid distribution. An increase of the feed-water content lowers the viscosity of the dough during extrusion resulting in a reduced mechanical energy input. As a consequence, extrudates with a reduced expansion and a higher density were produced. Computerized microtomography (μCT) revealed that these extrudates had thicker cell walls and a smoother surface. Thus, the amount of surface lipids was reduced and the amount of matrix-incorporated lipids was increased. The better the lipids are incorporated in the matrix, the higher was their oxidative stability. This means that extrudates with a high feed-water content oxidized less than extrudates with a lower feed-water content.

Chapter 4 examines the influence of a lipid-based MCT (medium chain triglycerides) coating on lipid oxidation in extrudates with different microstructures. Coating with MCT oil has been shown to inhibit lipid oxidation in corn extrudates beyond dilution effects. In the extrudates with 10% feed-water, a protective effect was observable because the total hydroperoxides were significantly reduced. At higher feed-water contents, the reduced oxidation rate was attributable

to the dilution of oxidizable lipids. Especially the surface lipids benefited from the coating. The effect decreased with increasing lipid inclusion. Coating allocation was analyzed using fluorescence microscopy, µCT and MCT fatty acid distribution. The use of Nile Red increased the contrast in the µCT images and revealed that the MCT oil forms a thin layer on the surface and closed microcracks. The coating layer was limited to the outer 2–3 mm. Fatty acids from the MCT oil were mainly detected in the surface lipids and small amounts migrated into the inner-surface lipid fraction. This migration increased with an increase of the expansion and porosity of the extrudates (lower feed-water contents).

In **chapter 5**, analytical methods for the measurement of radicals in complex starch-protein-lipid model systems and corn extrudates using electron paramagnetic resonance spectroscopy have been developed. Spin trapping with PBN was used for the detection of lipid-derived radicals *ex-situ.* By simulating the spectra, mainly alkoxyl radical adducts were identified in purified oil and extrudates, to which rapidly decomposing peroxyl radical adducts contribute. Stable radicals were identified using a model system, which was prepared with corn flour constituents. Based on the g-values of the detected radicals (2.0047), they can be attributed to nitrogen-centered protein radicals. It was demonstrated that the extrusion promotes the formation of stable protein radicals. During storage of extrudates, hydroperoxides, and hexanal increased, whereas the intensity of stable radicals did not further increase.

In **chapter 6**, the influence of storage temperature on lipid oxidation in different systems, i.e., in extrudates, in an extrusion model system (EMS), and in oils was analyzed. With increasing storage temperature, an increase of the oxidation rate and higher hydroperoxide and hexanal concentrations were observed. Furthermore, browning of the extrudates and formation of volatiles occurred at storage temperatures above 80 °C that can be attributed to Maillard reaction. The analysis of the oxidation kinetics showed that the reaction rate is influenced by the matrix. The logarithmic plot of the reaction rate, based on the increase of hydroperoxide formation after the lag phase, does not allow a linear plot when room temperature is included and leads to an overestimation of the oxidation rate at ambient storage temperatures. Therefore, further factors (Maillard reaction) and models (non-linear model, degradation reaction) for accelerated storage conditions were considered.

In summary, it can be stated that different mechanisms and matrix effects could be derived that influence lipid oxidation during extrusion and storage of extrudates.

9. ZUSAMMENFASSUNG

Die Kochextrusion ist eine Schlüsseltechnologie der Lebensmittelindustrie, die zur Herstellung einer Vielzahl von sogenannten Extrudaten (z.B. Getreide, Cornflakes, Snackprodukte und Trockenfutter) eingesetzt wird. Extrudate zeigen eine hohe spezifische Oberfläche und sind aufgrund des erhöhten Sauerstoffkontaktes oxidationsanfällig. Die Lipidoxidation ist eine der Hauptursachen für Qualitätseinbußen bei der Herstellung und Lagerung von lipidhaltigen Extrudaten. Sie wird von ranzigen Off-Flavours begleitet, was der Hauptgrund für eine reduzierte Haltbarkeit ist. Während des Extrusionsprozesses verkleistert die Stärke und bildet eine amorphe Matrix aus, welche Lipide enthält. Das Ziel dieser Arbeit ist, die Zusammenhänge zwischen Lipidoxidation und den strukturellen Eigenschaften bzw. Wechselwirkungen von Lipiden mit der Matrix zu verstehen, um eine Grundlage zur Reduzierung bzw. Verzögerung von Oxidationsvorgängen in Extrudaten zu schaffen.

Kapitel 3 beschreibt die Entwicklung einer fraktionierten Lipidextraktion. Sie ermöglicht die Charakterisierung und Untersuchung von Lipidoxidationsprozessen in verschiedenen Bereichen eines Extrudates. Bei der Extraktion wird zwischen Oberflächenlipiden, innenliegenden Lipiden (Lipide, die auf der inneren Oberfläche der Lamellen im Extrudat haften) und Matrix-inkorporierten Lipiden unterschieden. Matrix-inkorporierte Lipide sind zwischen Amylose-Helices gebunden und können nicht mithilfe von Lösungsmitteln unter milden Extraktionsbedingungen extrahiert werden. Nur eine Amylase-Behandlung bewirkt eine ausreichende strukturelle Zerstörung, die die Lipide der Extraktion zugänglich macht.

Dabei konnte gezeigt werden, dass der Wassergehalt der Extrusionsmasse (10-22%) die Mikrostruktur und die Expansion eines Extrudates beeinflusst und so zu Unterschieden in der Lipidverteilung führt. Eine Erhöhung des Wassergehalts senkt die Viskosität der Extrusionsmasse während der Extrusion, was zu einem reduzierten mechanischen Energieeintrag führt. Infolgedessen entstanden Extrudate mit reduzierter Expansion und höherer Dichte. Die computergestützte Mikrotomographie (μCT) hat gezeigt, dass diese Extrudate dickere Zellwände und eine glattere Oberfläche aufweisen. Dadurch wurde die Menge der Oberflächenlipide reduziert und die Menge der Matrix-inkorporierten Lipide erhöht. Je besser die Lipide in die Matrix eingebunden sind, desto höher war ihre oxidative Stabilität. Das bedeutet, dass Extrudate mit einem hohen Wassergehalt weniger stark oxidiert sind als Extrudate mit einem niedrigeren Wassergehalt.

In **Kapitel 4** wird der Einfluss eines lipidbasierten MCT (mittelkettige Triglyceride) Coatings auf die Lipidoxidation in Extrudaten mit unterschiedlicher Mikrostruktur untersucht. Das Coating mit MCT-Öl hemmt die Lipidoxidation in Maisextrudaten über den Verdünnungseffekt hinaus. In Extrudaten mit 10% zugesetztem Wasser war eine Schutzwirkung zu beobachten, ersichtlich durch deutlich reduzierte Gesamtkonzentrationen der Hydroperoxide. Bei höheren Wassergehalten der Extrusionsmasse war die reduzierte Oxidationsrate auf die Verdünnung von oxidierbaren Lipiden zurückzuführen. Vor allem die Oberflächenlipide profitierten von dem Coating. Die Wirkung nahm mit zunehmendem Lipideinschluss ab. Die Verteilung des Coatings wurde mittels Fluoreszenzmikroskopie, µCT- und MCT-Fettsäureverteilung analysiert. Die Verwendung von Nil Rot erhöhte die Kontraste in den µCT-Aufnahmen und ließ erkennen, dass das MCT-Öl auf der Oberfläche einen dünnen Film bildet und Mikrorisse verschließt. Die Coatingschicht war auf die äußeren 2-3 mm beschränkt. Fettsäuren aus dem MCT-Öl wurden hauptsächlich in den Oberflächenlipiden nachgewiesen und nur geringe Mengen migrierten in die innere Oberflächenlipidfraktion. Die Migration nahm mit zunehmender Expansion und Porosität der Extrudate zu (geringerer Wassergehalt der Extrusionsmasse).

In **Kapitel 5** wurden analytische Methoden zur Messung von Radikalen mit Hilfe der Elektronenspinresonanz-Spektroskopie in komplexen Stärke-Protein-Lipid Modellsystemen und Maisextrudaten entwickelt. Die Spinfalle PBN wurde für den Nachweis von lipidbasierten Radikalen *ex-situ* verwendet. Mit Hilfe der Simulation der Spektren wurden hauptsächlich Alkoxylradikal-Addukte in antioxidantien-freiem Öl und Extrudaten identifiziert, die auch aus schnell zerfallenden Peroxylradikaladdukten entstehen können. Stabile Radikale wurden mittels eines Modellsystems identifiziert, das aus Maismehlbestandteilen hergestellt wurde. Anhand der g-Werte der nachgewiesenen Radikale (2,0047) konnten sie auf stickstoffzentrierte Proteinradikale zurückgeführt werden. Es konnte gezeigt werden, dass die Extrusion die Bildung von stabilen Proteinradikalen fördert. In den Extrudaten nahmen während der Lagerung die Konzentrationen an Hydroperoxiden und Hexanal zu, während die Intensität der stabilen Radikale nicht weiter anstieg.

In **Kapitel 6** wurde der Einfluss der Lagertemperatur auf die Lipidoxidation in verschiedenen Systemen, d.h. in Extrudaten, in einem Extrusionsmodellsystem (EMS) und in Ölen analysiert. Mit steigender Lagertemperatur wurde ein Anstieg der Oxidationsrate und höhere Hydroperoxid- und Hexanalkonzentrationen beobachtet. Darüber hinaus kam es bei

Lagertemperaturen über 80 °C zu einer Bräunung der Extrudate und zur Bildung von flüchtigen Verbindungen, die auf die Maillard-Reaktion zurückzuführen sind. Die Analyse der Oxidationskinetik zeigte, dass die Reaktionsgeschwindigkeit durch die Matrix beeinflusst wird. Die logarithmierte Darstellung der Reaktionsgeschwindigkeit, basierend auf dem Anstieg der Hydroperoxidbildung nach der lag-Phase, lässt bei Einbeziehung der Raumtemperatur keine lineare Darstellung zu und führt zu einer Überschätzung der Oxidationsgeschwindigkeit bei realen Lagerungstemperaturen. Es wurden deshalb weitere Faktoren (Maillardreaktion) und Modelle (nicht lineares Modell, Abbaureaktion) für akzelerierte Lagerungsbedingungen betrachtet.

Zusammenfassend lässt sich sagen, dass verschiedene Mechanismen und Matrixeffekte abgeleitet werden konnten, die Einfluss auf die Lipidoxidation während der Extrusion und der Lagerung von Extrudaten nehmen.

10. SUPPLEMENTAL

10.1. EXPERIMENTAL AND ANALYTICAL APPROACH FOR UNPUBLISHED MANUSCRIPTS

Table 10.1 provides an overview of used methods and the publication status of the manuscripts.

Table 10.1: *Overview of used methods in the present thesis*

Manuscript 1 (Chapter 3) *(accepted and published) doi:10.1002/ejlt.201800433*	**Manuscript 2 (Chapter 4)** *(accepted and published) doi:10.1002/ejlt.201900350*	**Manuscript 3 (Chapter 5)** *(accepted and published) doi:10.1016/j.foodchem.2020.127314*	**Manuscript 4 (Chapter 6)** *(manuscript)*
Extrusion processing with SME	Extrusion processing	Extrusion processing	Extrusion processing
Fractionated lipid extraction	Fractionated lipid extraction		
Degree of gelatinization			
Hydroperoxide Content (thiocyanate method)	Hydroperoxide Content (thiocyanate method)	Hydroperoxide Content (thiocyanate method)	Hydroperoxide Content (thiocyanate method)
Volatile Oxidation products (HS-GC)	Volatile Oxidation products (HS-GC)	Volatile Oxidation products (HS-GC)	Volatile Oxidation products (HS-GC)
Density			
Expansion			
µCT	µCT		
	MCT coating		
	Fatty acid profile		
	Peroxidability index		
	Fluorescence Microscopy		
		Oil purification	Oil purification
		Extrusion Model System	Extrusion Model System
		Lipid extraction	Lipid extraction
		EPR	
			GC-MS
			Spectrophotometric analysis
			Oxidation kinetics

10.1.1. EXTRUSION COOKING

The extruder used in this thesis was a laboratory twin-screw extruder with contrarotating intermeshing screws (Brabender Mod. DSE 35/7D, Duisburg, Germany). The theoretical background of extrusion cooking with its influencing process parameters is provided in detail in **Chapter 2.1**.

The basic recipe for the homogenous premix, which was produced before extrusion, based on native (Molino Merano; Italy) and pregelatinized corn meal (Interquell; Germany) (50:50 w/w). All experiments were performed with an addition of 10% of a commercial rapeseed / sunflower oil mixture (50:50 v/v). The extrusion processes of this thesis were performed with added water contents from 10 – 22%. The lipids and water were subsequently added to the corn meal. The ingredients were mixed with a Stephan Universal Machine (UMC 5, Hameln, Germany) (table cutter) to a homogenous premix. The premix was fed by a gravimetric weight feeder (Coperion Ktron Model K2-ML-D5-T35-QC, Niederlenz, Switzerland) to the extruder with constant feed rates from 12 to 18 kg/h. The temperature controller of the extruder were set to 60/125/125 °C in all studies and the screw speed was varied between 286 and 333 rpm. The extruder was operated by one open die and an automated cutting device. The produced extrudates were dried in air permeable plastic bags in a climate cabinet (ThermoTEC TCS-501, Weilburg, Germany) at 30 °C and 20% humidity for 48 hours. After drying, the extrudates were stored in triplicate in glass bottles at elevated temperatures in a climate cabinet in the dark.

The extruder was equipped with a digital measuring instrument, which permanently monitors the engine parameters. The specific mechanical energy (SME) was calculated in **manuscript 1** by the following equation that is in line with Onwulata et al. (1994):

$$SME\ \left[\frac{kWh}{kg}\right] = \frac{\frac{n_{act}[rpm]}{n_{max}[rpm]} * torque\ [\%] * P_{max}\ [kW]}{feed\ rate\ [\frac{kg}{h}]}$$

where n_{act} is the screw speed in rpm, n_{max} is the maximum screw speed in rpm, torque is the used torque from the motor in %, P_{max} is the motor power of the extruder in kW and the feed rate is the amount of premix that is fed of the gravimetric weight feeder to the extruder in kg/h.

10.1.2. FRACTIONATED LIPID EXTRACTION

The new developed and established protocol for fractionated extraction of lipids that allows determination of the fat content and of the oxidation status in the different lipid fractions was used in **manuscript 1 and 2**. The principle of the method, using amylase for lipid release from extruded matrix, is derived from Thachil et al. (2014) and Strange and Schaich (2000). The extraction protocol consists of three extraction steps that provide the in **chapter 2.1.4** proposed lipid fractions in extrudates.

Lipids removed by the first extraction step are defined as surface lipids. For quantification of surface lipids, 35 mL cyclohexane were added to 5 g of intact extrudates and shaken for 1 h at 190 rpm. After filtration the solvent of the extract was evaporated at 65 °C and 250 mbar and solvent residues were removed with nitrogen. The amount of surface fat was determined gravimetrically.

Lipids removed by the second extraction step are defined as inner surface lipids. For extraction, the dried extrudates obtained from surface lipid extraction were milled for 10 seconds with an analytical mill (IKA A11 basic, Germany) and placed into 50 mL falcon tubes. 15 mL cyclohexane was added and after sonication for 20 seconds (cycle 9, 50% power, Bandelin sonoplus, sonication probe VS 70) the tubes were centrifuged at 4500 rpm for 4 minutes. The extraction procedure was repeated twice using 10 mL solvent each time. After filtration the solvent was evaporated (65 °C at 250 mbar) and solvent residues were removed with nitrogen. The amount of inner surface fat was determined gravimetrically. The second extraction step was validated by repeating the ultrasound-assisted extraction cycle up to 5 times to confirm that three extraction steps are sufficient to remove all inner-surface lipids. An increase of the ultrasound-assisted extraction cycles did not lead to a significant increase of the yield compared with three extraction cycles. At the same time, this method leaves matrix-incorporated lipids in the sample that are only extractable after amylase treatment.

Lipids removed by the third extraction step are defined as matrix-incorporated lipids. For the determination of the matrix-incorporated lipids, the extruded samples were subjected to an amylase treatment after inner surface lipid-extraction. 20 mL of a phosphate buffer (pH 5.9) were mixed with α-amylase from *Bacillus licheniformis* (Sigma Aldrich) in a concentration of 17.6 U/mL and added to the extruded sample. Closed falcon tubes were incubated at 60 °C for

2 h at 300 rpm, followed by addition of 10 mL cyclohexane, sonication and centrifugation. An aliquot of 5 mL supernatant was subjected to vacuum centrifugation for solvent removal. The amount of matrix-incorporated fat was determined gravimetrically.

10.1.3. DEGREE OF GELATINIZATION

The degree of gelatinization was determined according to the methods of Chiang and Johnson (1977) and Lin et al. (1997). The degree of gelatinization is the ratio of gelatinized starch to total starch of a sample.

Freeze dried extrudates were milled with a ball mill (Retsch MM 301, Haan, Germany) at 30 1/s for 3 minutes and 20 mg were weighed into a 25 ml glass bottle. Acetate buffer (80 mmol, pH 4.5) was prepared by dissolving 5.25 g sodium acetate and 5.55 mL acetic acid in 2 L deionized water. An amyloglucosidase solution was prepared by dissolving 1.25 units of amyloglucosidase from aspergillus niger (Sigma-Aldrich, Taufkirchen, Germany) per milliliter acetate buffer (49 U/mg protein, 95% protein content). O-Toluidine reagent was prepared by dissolving 400 mg thiourea in 250 mL acetic acid and adding 16 mL o-toluidine.

Native samples with partially gelatinized starch were prepared by adding 5 mL of distilled water, 20 mL acetate buffer and 5 mL of enzyme solution to the weighed freeze-dried sample. The procedure for total starch content based on a gelatinization of the samples. Therefore, 3 mL distilled water and 1 mL 1 N NaOH were added and mixed. After 5 minutes 1 N HCl, 20 mL acetate buffer and 5 mL of enzyme solution were added. Both samples were heated to 55 °C for 60 minutes in a water bath under constant stirring. Then 2 mL of 25% trifluoroacetic acid were added to stop reaction. After cooling, an aliquot of 5 mL was transferred into a centrifugation tube and centrifuged at 4000 rpm for 5 minutes. Subsequently, 0.5 mL of the supernatant were transferred into a glass tube and 4.5 mL toluidine reagent were added. After heating in a boiling water bath for 10 minutes, the glass tubes were cooled down in an ice water bath for 10 minutes. Finally, 5 mL of acetic acid are added, and the absorbance was measured in a photometer at 630 nm against acetic acid.

The degree of gelatinization was calculated by following equation:

$$\text{Degree of gelatinization } [\%] = \frac{(\text{GS} - \text{K}) \text{ x } 100}{\text{TS} - \text{K}}$$

where GS is the amount of gelatinized starch (g/100g), TS is the total starch content (g/100g) and K is the percentage of native starch attacked by amyloglucosidase. Results were based on an average of three measurements.

10.1.4. HYDROPEROXIDE CONTENT USING THE THIOCYANATE METHOD

Lipid hydroperoxides are the main primary oxidation products (**Chapter 2.3.4.3**). They can be determined using the peroxide value method (Wheeler 1932). The hydroperoxides were determined using the ferrous thiocyanate method (Drusch et al. 2007; Mihaljević et al. 1996). This method based on the ability of hydroperoxides to oxidize ferrous to ferric ions. In a reaction with thiocyanate, a red iron complex is formed, which can be measured photometrically. Further details are provided in **Chapter 2.3.4.3**.

For determination of the hydroperoxide concentration 10-50 mg (depending on the oxidation status) of a lipid sample were solved in 5 mL 2-propanol. Then 50 µL iron(II) chloride solution as well as 50 µL ammonium thiocyanate solution were added. After vortexing, samples were incubated in a water bath at 60 °C for 30 min. Directly after cooling down to room temperature the extinction was measured against 2-propanol at a wavelength of 485 nm. All analyses of hydroperoxides were performed in triplicate. The hydroperoxide concentration was calculated according to the following equation:

$$Hydroperoxides\ concentration \left[\frac{\text{mmol}}{\text{kg}}\text{oil}\right] = \frac{c\ from\ calibration\ (Fe(III)[\frac{\mu g}{5ml}]}{sample\ weight\ [g] * 55.84} * Dilution$$

10.1.5. VOLATILE OXIDATION PRODUCTS USING HEADSPACE-GAS CHROMATOGRAPHY

In the ongoing oxidation reactions, the hydroperoxides can break down in volatile compounds (secondary oxidation products) because of β-scission reactions (Halliwell and Chirico 1993; Frankel 1983). Volatile oxidation products were detected using headspace-gas chromatography (HSGC) with an Agilent 6890 series gas chromatograph. For determination of hexanal and

propanal, 1 g of ground sample was weighed in HSGC-vials and closed airtight. The HSGC was running in split mode (4:1) equipped with a J&W DB-1701 column (60 m x 0.32 mm x 3 µm) and the injection port was operated at 220 °C. The initially oven temperature was set to 45 °C where it was held for 2 minutes, then raised to 85 °C at 15 °C min^{-1} and maintained for 4 minutes and finally raised to 220 °C at 15 °C min^{-1} and maintained for 3 minutes. Detection was done by a flame ionization detector (FID). Hexanal and propanal were identified using the retention time of external standards. With described chromatographic parameters, the retention time of propanal was 5.66 min. and of hexanal was 12.37 min. The peaks were analyzed by the area under the curve (AUC).

10.1.6. DENSITY OF THE EXTRUDATES

In **manuscript 1**, the density of the extrudates was determined by filling a slim 250 mL measuring cylinder up to the 150 mL marking with extrudates. After weighing the extrudates, 0.75-1.00 mm glass beads were added, compacted and filled up to 200 mL marking. After removing the extrudates, the volume of the glass beads was measured. The density was calculated by following equation:

$$\text{Density } [\text{g/cm}^3] = \frac{\text{W}}{\text{V}}$$

W is the weight of the extrudates in grams and V is the volume of extrudates in mL (computation: 200 mL – volume of the glass beads). This approach is in line with Singh et al. (2007).

10.1.7. EXPANSION OF THE EXTRUDATES

The expansion of the extrudates, a parameter for the size of the extrudates (Alvarez-Martinez et al. 1988), was measured in the present thesis using a vernier caliper (Singh et al. 2007). The diameter of 30 randomly selected samples was determined in millimeter. The diameter of the extruder die is 8 mm (≙100%). The expansion was calculated by following equation:

$$Expansion\ [\%] = \frac{diameter\ of\ the\ sample\ [mm]}{diameter\ of\ the\ die\ [mm]} \text{x}100$$

10.1.8. COMPUTERIZED MICROTOMOGRAPHY

Extrudates have a filigree porous structure. This is why all invasive methods led to a destruction of the microstructure. To preserve the microstructure, the μCT, a noninvasive imaging method was used in the present thesis (Schulze et al. 2012; Schoeman et al. 2016; Horvat et al. 2014). The intact dried extrudates (without and with a coating) were scanned with a phoenix nanotom® 180NF (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany) computerized microtomograph (μCT).

Each scan was optimized individually in order to find the most suitable scan parameters for good contrast between solid phase and air phase as well as to achieve the maximum resolution for the different sized samples. The x-ray-beam was generated with a molybdenum target and was filtered with 0.1 mm Cu. Due to the different diameters of the extrudates, the achieved voxel length differed. The detailed measuring parameters can be found in **manuscript 1 and manuscript 2**. A μ-CT scan consists of 1440 projections while the sample was full rotated in 0.25° steps. The projections were recorded with a CCD detector at effective 1146 by 1152 pixel in 2x2 binning mode. The reconstruction was done with the software datos|x reconstruction 1.5 (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany). Further image processing was done with VG StudioMax 2.0 (Volume Graphics GmbH, Heidelberg, Germany). The data sets were imported into myVGL 3.1 (Volume Graphics GmbH, Heidelberg, Germany), a viewer for data processed by volume graphics software. In myVGL, the cell wall thickness of randomly selected lamellas was measured, the migration of the coating layer was measured using the caliper tool, and the 3D-visualization was performed.

10.1.9. MCT-OIL COATING OF EXTRUDATES:

Coating of extrudates is, especially in the pet food industry, a common process step in kibble production. The theoretical background of coating of extrudates can be found in **Chapter 2.2** MCT oil was selected in the present thesis as coating material in **Chapter 4** because it is stable to oxidation due to its fatty acid composition (Raudsepp et al. 2014) and its low viscosity, which enables homogeneous coating of the extrudate surface (Marten et al. 2006). The coating of the dried extrudates was performed in a self-constructed tumble system, which imitates the functional principle of conventional drum coaters. It consists of a motor, which rotates an

inclined stainless-steel bowl (27 cm diameter) with 70 rpm for 3 minutes. The coating (MCT oil) was applied in a concentration of 5% (w/w) with a syringe.

10.1.10. FATTY ACID PROFILE:

For the fatty acid analysis of lipids, using a gas chromatograph with flame ionization detector (GC-FID), it is necessary to derivatize the fatty acids to fatty acid methyl esters (FAME), which increases their volatility. The standard method for lipid derivatization uses the catalyst boron trifluoride (Orsavova et al. 2015). The used method in this thesis is a rapid method, with a high derivatization efficiency. This enables high throughput analysis of the fatty acid profile of the high amount of lipid samples from fractionated lipid extraction. The fatty acids were methylated with trimethylsulfonium hydroxide (TMSH) (Arens et al. 1994; Schulte and Weber 1989; Haas et al. 2016). TMSH is a pyrolytic alkylation reagent for transesterification which need an injection port temperature of 250 °C (Butte 1983). The reaction already takes place at room temperature but can be promoted by heating the sample. Sample preparation consisted of dissolving the oil sample (20–50 mg) in methyl *tert*-butyl ether (2.5 mL) (MTBE) and adding TMSH (25 µL) to an aliquot (50 µL) of this mixture. The prepared sample was injected directly in an Agilent 6890 series gas chromatograph (Santa Clara, USA) equipped with a J&W DB-23 column. A detailed description of the gas chromatographic method including the used temperature profile of the column oven can be found in **Chapter 4**.

10.1.11. PEROXIDABILITY INDEX

The peroxidability index provides information about the oxidation equivalents of the different extrudates, depending on the oxidizability of the different fatty acids in a sample. It is a measure of the susceptibility of a lipid to lipid peroxidation (Hulbert et al. 2006). The PI was calculated as follows (Cortinas et al. 2003):

$$\begin{aligned} \mathrm{PI} = {} & (\%\ \text{monoenoic FA} \times 0.025) + (\%\ \text{dienoic FA} \times 1) + (\%\ \text{trienoic FA} \times 2) \\ & + (\%\ \text{tetraenoic FA} \times 4) + (\%\ \text{pentaenoic FA} \times 6) \\ & + (\%\ \text{hexaenoic FA} \times 8) \end{aligned}$$

10.1.12. FLUORESCENCE MICROSCOPY

Imaging by fluorescence microscopy or confocal microscopy is an appropriate technique for lipid visualization in extrudates and low moisture foods (Barden et al. 2015). The lipid distribution in extrudates and of the MCT-oil coating on the surface of the extrudates was investigated using fluorescence microscopy with the lipophilic fluorescent dye Nile red. Nile red is a lipid specific fluorescent dye used for lipid staining (Drusch and Berg 2008). For the visualization of the coating-allocation, Nile red was dissolved in MCT oil (0.0625 mg/g oil) and applied as previously described. The fluorescence of lipids present on the lamellas was visualized after staining the lamellas with Nile red dissolved in acetone, as described by Moisio et al. (Moisio et al. 2015b). For fluorescence microscopy, a Zeiss Axio Zoom V16 microscope (Jena, Germany) equipped with a 38 HE filter and the illuminator HXP 200C was used. The microscopic images were analyzed with AxioVision 4.8 software (Zeiss, Jena, Germany).

For analysis of the coating and its migration behavior, the fluorescence microscopy was not suitable. Microscopic analyses of complex 3D-foods are difficult because a preparation of the sample is necessary for a good image quality. Extrudates must be cut or sliced to examine the sample under the microscope and to analyze the internal structures. During sample preparation the stained coating is carried over into the interior of the extrudate. To avoid the carry over, different preparation techniques were tested. All tested methods, including the preparation using a cryomicrotome were unsuitable for extrudates. Because of the porous structure, all invasive cutting experiments led to a destruction of the microstructure of the extrudates. To preserve the filigree structure of an extrudate, the μCT, a noninvasive imaging method (Schulze et al. 2012) was used in the present thesis. The analyses were performed as described in **Chapter 3** with a phoenix nanotom® 180NF μCT (GE Sensing and Inspection Technologies, Wunstorf, Germany) (Amft et al. 2019a). In addition, Nile red (0.0625 mg/g oil) was used to improve the contrast of the coating (Contardo and Bouchon 2018).

10.1.13. MODEL SYSTEM OF CORN FLOUR CONSTITUENTS PREPARED WITH PURIFIED OIL

The Model system in the **Chapter 5** and the extrusion model system (EMS) in **Chapter 6** aimed to imitate parts of the extrusion process and to provide a model system with corn-based constituents that were mixed with a lipid component. The model systems are used to measure

oxidation processes that correspond to those in the extrudate but require less measuring time. Both systems were prepared with a sunflower/rapeseed oil mixture (50:50 w/w) that was previously purified from all antioxidants (e.g. tocopherols). The oils were purchased from a local store. The purification process removes trace metals and hydroperoxides in addition and was performed by column chromatography as described by Lampi and Kamal-Eldin (1998). In brief, a glass column was packed with activated aluminum oxide as stationary phase and the mixture of oil with n-hexane was passed through the column. Afterwards, the solvent was removed using rotary evaporation under nitrogen atmosphere. Oil purification is a common tool to accelerate lipid oxidation in experiments (Raudsepp et al. 2014; Velasco et al. 2004b; Roman et al. 2013b; Cui et al. 2017). Another advantage is the possibility of testing specific antioxidants in a defined concentration without interaction effects between different antioxidants (Bauer et al. 2013).

In this thesis two versions of the EMS were prepared. The EMS used for the EPR study (**Chapter 5**) were prepared with four different cereal-based ingredients (corn starch (Cargill, Krefeld, Germany), corn meal (Molino Merano, Lana, Italy), zein (Sigma-Aldrich, Taufkirchen, Germany), and corn starch with added 8% zein). 85% of a dry ingredient were premixed with purified oil (10%) and tap water (5%) und subsequently homogenized with an analytical mill for 10 seconds (IKA A11 basic, Staufen, Germany). The second version of the EMS, used in the storage temperature study (**Chapter 6**), was prepared with corn starch (85%), purified oil (10%) and tap water (5%). The ingredients were premixed and subsequently homogenized for 60 seconds with a Stephan Universal Machine (UMC 5, Hameln, Germany). The dough was then baked in a convection oven (Zanussi Model FCV/E 10L6) to a final water content comparable to extrudates after drying (~6-8%). Thus, shearing and influence of heat were mimicked.

10.1.14. LIPID EXTRACTION

For the analysis of hydroperoxides from extrudates and EMS 5 g of dried and ground sample were weighed into 50 mL falcon tubes and 15 mL cyclohexane were added. After sonication for 20 seconds (cycle 9, 50% power, Bandelin sonoplus, sonication probe VS 70, Berlin, Germany) the tubes were centrifuged at 4500 rpm for 4 minutes. The extracts were filtered through filter paper in Erlenmeyer flasks (100 mL) and the extraction procedure was repeated

twice with 10 mL of solvent. The solvent was evaporated using a rotary evaporator (65 °C, 250 mbar) and solvent residues were removed with nitrogen. Internal pre-tests showed that the chosen combination of solvent and sonication extracts lipids from the ground sample to a high degree. Compared to unassisted lipid extraction the ultrasound-assisted extraction reduces the time and allows a high throughput (Metherel et al. 2009).

The lipid extracts used for analysis of lipid radicals by EPR and the hydroperoxide concentration were made using a modified version of the above described standard protocol. Ethyl acetate was used instead of cyclohexane, which enabled improved radical extraction. To minimize the formation of radicals during ultrasound-assisted extraction, the third extraction step was omitted. In addition, a higher sample quantity (10 g) was used to extract sufficient amounts of lipids for further analysis. The results, presented in the supplemental of **Chapter 5**, showed that solvent residues falsified the ESR measurement. For this reason, a vacuum concentrator (Thermo Scientific Savant SC250EXP SpeedVac, Schwerte, Germany, 90 min. at 45 °C) was used instead of the rotary evaporator because this method does not leave any disturbing solvent residues in the sample.

10.1.15. ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR)

An innovative approach for the non-destructive determination of early oxidation markers is the electron paramagnetic resonance spectroscopy (EPR) (Thomsen et al. 2000; Velasco et al. 2005; Jensen et al. 2005). The EPR is a spectroscopic method for analysis of paramagnetic substances, e.g., free radicals with an unpaired electron, in an external magnetic field (Roman et al. 2010). The theoretical background of EPR with its physical measurement theory is provided in detail in **Chapter 2.3.4.2** For analysis of short-lived lipid radicals the spin trapping technique must be applied (Velasco et al. 2005). Commonly used spin traps are N-tert-butyl-α-phenylnitrone (PBN) and α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN). For PBN most likely peroxyl and alkoxyl radicals could be trapped (Dikalov and Mason 2001; Davison et al. 2008). By comparison, stable radicals derived from proteins or carbohydrates can be measured directly in the ground sample without spin trapping (Schaich and Rebello 1999b).

EPR measurements in this thesis were performed on a Bruker Elexsys II E500-CW-EPR spectrometer (Rheinstetten, Germany) operating with X-band at 9.85 GHz and a modulation frequency of 100 kHz at room temperature. The data evaluation was performed with the Bruker-

software Xepr 2.6b.52 and simulation of EPR spectra of lipid radicals was performed with EPRSIM (Strancar et al. 2005).

Stable radicals were measured directly in the ground sample filled in borosilicate glass capillaries (10 cm length, inner diameter 3 mm, outer diameter 4 mm, Hilgenberg GmbH, Malsfeld, Germany). EPR settings for measurement of stable radicals: microwave attenuation = 10 dB; modulation amplitude = 8.5 G; receiver gain = 60 db; sweep width = 80 G; microwave power = 20 mW; time constant = 327.68 ms; conversion time = 80.01 ms and center field = 3511.45 G. Spin fitting via Bruker software was applied to determine the g-value of the measured radicals. The g-value enables the identification of the detected radicals and its nature (protein or carbohydrate radicals).

For spin trapping of short-lived lipid radicals, 25 µL PBN (200/600 mM) were pipetted to 45 mg oil in PCR-tubes. The tubes were vortexed, incubated for 30 min. at 50 °C and measured directly. PBN adducts, were calculated by peak-to-peak-amplitude of the first peak of the middle doublet. EPR settings for measurement of short-lived lipid radicals: microwave attenuation = 10 dB; modulation amplitude = 1.0 G; receiver gain = 80 db; sweep width = 80 G; microwave power = 20 mW; time constant = 163.84 ms; conversion time = 100.05 ms and center field = 3511.45 G. Based on spectra simulation and identification in approximation to the hyperfine constants of Xepr software from Bruker, alkoxyl, 1-hydroxyethyl, peroxyl, and hydroxyl radicals were measured in the lipid samples.

10.1.16. GC-MS FOR IDENTIFICATION OF VOLATILES IN EMS

Static headspace-gas chromatography (HS-GC) was used in this thesis for analysis of volatile lipid oxidation products, e.g., hexanal and propanal, as described in **Chapter 3** (Amft et al. 2019a). The HS-GC analysis provides a chromatogram, which displays the detected compounds plotted over the retention time. The assignment to a chemical compound, e.g., a lipid oxidation product or a Maillard reaction product, is only possible by using external standards. This makes it difficult to identify unknown peaks, especially if it is unknown which reaction products are expected in the sample. In this case, gas chromatography mass spectrometry (GC-MS) can be used to monitor the whole volatile profile and identify unknown compounds by their molecular mass (Lampi et al. 2015). In the present thesis, an Agilent 6890N gas chromatograph (Santa Clara, USA) coupled with a Gerstel MPS 2XL - DHS dynamic headspace autosampler

(Mülheim an der Ruhr, Germany) was used. The gas chromatographic system was connected with an Agilent 5975 inert mass selective detector (Santa Clara, USA), which was used for mass spectrometric analyses. For analysis, 1 g of ground sample was weighed in GC-MS-vials with a screw cap and closed airtight. Volatiles from 27 to 200 m/z were detected and identified by spectra comparison (Starowicz et al. 2019) using the AMDIS software and the NIST Mass Spectral Library.

10.1.17. SPECTROPHOTOMETRIC ANALYSIS OF EXTRUDATES

The perception of color is highly subjective. Therefore, the analysis with a measuring device is the most reliable method to specify a color and to communicate that color. Storage of extrudates at elevated temperatures, above 80 °C, leads to non-enzymatic browning of the extrudates. The color changes were measured with a spectrophotometer (X-Rite, Model SP62, Grand Rapids, USA) using the CIE L*a*b* color system, where L* defines the lightness, a* denotes the red to green value and b* represents the yellow to blue value. In brief, the extrudates (n=9) were placed under the sensor of the spectrophotometer and the measurement was performed as specified by the manufacturer of the spectrophotometer. The approach to measure CIE L*a*b* - values, in particular the lightness (L*-value), was also used in further studies to investigate browning of foods (Capuano et al. 2008; Purlis and Salvadori 2009; Mastrocola and Munari 2000).

10.1.18. LIPID OXIDATION KINETICS IN EXTRUDATES, EMS AND OILS

Lipid oxidation kinetics provide information about reaction rate constants as well as the activation energies needed for oxidation reactions (Labuza and Dugan 1971). In accelerated shelf life testing, the rate of oxidation is of high interest (Ragnarsson and Labuza 1977). The temperature dependence of the reaction rate of a chemical reaction is represented by the Arrhenius equation (Arrhenius 1889), which is described in detail in **chapter 2.3.2** In food industry, the so-called Arrhenius plot, is used to create predictive models based on storage tests at elevated temperatures. In this way, the shelf life of many foods is calculated (Mancebo-Campos et al. 2008; Lee and Krochta 2002; Manzocco et al. 2012).

In this study, on the one hand a simplified linear approach was assumed. Therefore, the linear regression was fitted to the initial part of the curves excluding a lag phase, when present (Manzocco et al. 2012). The calculation was performed using Excel (Microsoft Office) .This approach is consistent with previous studies that used a linear regression for determination of oxidation kinetic parameters (Mancebo-Campos et al. 2008; Huang and Sathivel 2008). Barden and Decker (2016) stated that lipid oxidation reactions do not follow a first-order kinetic. Rather, the lag phase is followed by the propagation phase, which is characterized by an exponential growth of lipid oxidation products. Therefore, the oxidation theoretically cannot be described over the complete oxidation time with a linear equation. As a consequence, a non-linear regression analysis with a fitting of the entire oxidation curve was performed using "R"-version 3.6.1. According to Meissner et al. (2019), the R package deSolve (v.1.24) was used for calculations with differential equation and the package minpack.lm (v.1.2.1) for fitting of the oxidation kinetic parameters with a nonlinear least square regression using the Levenberg-Marquardt algorithm. Standard deviation was estimated using the jackknife resampling technique in R. The use of a nonlinear approach, in which the best-fitting equations were selected for calculation of oxidation kinetic parameters using the Arrhenius equation is in line with Mancebo-Campos et al. (2008) and Crapiste et al. (1999).

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