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„Analysis of protein oxidation and Maillard reaction products in meat analogues "

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Elena Maria Theresa Hinterreiter-Kern

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Elena Hinterreiter-Kern

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LIST OF ABBREVIATIONS

AAMA	N-acetyl-S-(2-carbamoylethyl)-l-cysteine
AAS	α-aminoadipic semialdehyd
AGEs	Advanced Glycation Endproducts
ALEs	Advanced Lipoxidation Endproducts
$BMDL_{10}$	Benchmark Dose Lower Confidence Limit 10%
CML	N-ε-(carboxymethyl) lysine
ELISA	Enzyme-Linked Immunosorbent Assay
GAMA	N-acetyl-S-(2-hydroxy- 2-carbamoylethyl)-l-cysteine
GGS	γ-glutamic semialdehyde
HPLC	High Pressure Liquid Chromatography
kg	Kilogram
LC-ESI-MS	liquid chromatography with Electrospray-Interface and tandem mass spectrometry
LC-MS	liquid chromatography with tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of Quantification
µg	Microgram
mg	Milligram
mL	Millilitres
MOE	Margins of exposure
nm	Nanometres
PROTOX	Protein Oxidation
QqQ	Triple Quadrupol
ROS	reactive oxygen species
S/N	Signal to noise ratio
UV	Ultraviolet

1. Introduction and hypotheses

Meat analogues such as tofu, tempeh and seitan are plant-based foods that can be technologically produced from soy or wheat flour. For environmental, ethical and health reasons, the meat-free alternatives business is thriving. The demand for vegan and vegetarian alternatives is also expected to grow in the coming years and, consequently, the market share of such foods is likely to increase.[1] The reasons for a vegan lifestyle are manifold, because in addition to the health aspect, an ethical component also plays an essential role. The awareness of ethically acceptable consumer goods is growing for many product categories. Whether for food, cosmetics or clothing, the demand for products produced in a fair, humane and animal-friendly manner is high.[2]

Therefore, vegan diet does not consume any animal foods, although this is also often expanded to cosmetics and clothing. In contrast, Satija and Hu (2018) describe a plant-based diet as reducing the intake of animal products, but not avoiding them at all. Products used for these analysis are typically consumed vegan products, such as tofu, tempeh and seitan.[3]

Tofu and tempeh are produced out of soy, while seitan is manufactured out of wheat, and both are used similarly to meat and meat products. In supermarkets they can be found as sausages, steaks or bought unprocessed as blocks to be later on fried, baked or deep-fried at home. Depending on the processing grade, proteins in the plant source (soy and wheat) can denaturate and lose their biological functionality.[4] Oxidation of the backbone or side chains of proteins leads to changes in the chemical and physical properties of the protein and, thus, to changes in compatibility, taste and biological value.[5]

Heat exposure during preparation of food can cause numerous of these chemical processes. On the one hand, these give the dishes a desired pleasant aroma, but on the other hand, they influence the quality and digestibility of the food. During the production and processing of soy and wheat flour, proteins may denature, or proteins, peptides or amino acids may react with each other or with other food components. This can reduce the biological value, but also promote the formation of carcinogens, toxins or other harmful substances can be formed.[5]

Maillard Reaction takes place at temperatures higher than 100°C between reducing sugars and the amino group of amino acids, peptides, or proteins. The products generated from the reaction contribute to the flavor profile of the food and some of them have adverse health effects when consumed regularly. Such contaminants have been

detected in numerous publications on meat products, with some uncertain entry on vegan or vegetarian meat substitutes.[6]

However, there is still the need for regulation and monitorization of harmful Maillard reaction products in meat products. Vegetarians and vegans consume meat analogues as an equivalent, which should have the same claim in terms of safety and monitoring. The control and rapid detection of undesirable substances in these frequently consumed goods is needed to ensure the safety of meat-containing and meat-free products.[6]

2. Literature review

Although the proportion of the population following a vegan diet is only about 1% worldwide, the popularity of plant-based diets is steadily increasing. Veganism does not only consists in a trend, it is also considered a moral statement against animal abuse and expoloitation.[7] Additionally, the plant-based diet contributes to the reduction of water usage and helps to decrease the amount of gas emissions[8] while potentially increasing physiological health[9]. The market for meat substitutes is expected to continue growing. In 2021, the market value was 16 billion U.S. dollars, and is expected to reach 21 billion U.S. dollars by 2025.[1]

While tofu has a long history in Asia, soy-based foods have no such tradition in central Europe. Hence, it is no surprise that numerous studies focus extensively in examining meat and fish products in regards to heating procedures[10] and oxidation.[11]

While a vegetarian diet excludes animal products, the vegan diet additionally cuts foods that have their origin in animals, such as eggs, milk, dairy products or honey. As a result, compounds typically found in animal products are limited, although not excluded, in plant-based food and diets.[12] The vegetarian diet is low in cholesterol, total fat, sodium and multiple other not recommended compounds, but high in antioxidants, fiber, magnesium and polyunsaturated fatty acids. This is why this diet is considered to correlate with a lower risk of lifestyle-associated diseases.[9]

Apart from that, vegans have been proven to show a lower protein intake compared to omnivore people.[13] Depending of age, sex and health conditions, the Societies for Nutrition of Germany, Austria and Switzerland recommend an intake of about 15% of the daily energy requirement through protein.[14] According to Bakaloudi et al. (2021), protein intake in participants on a vegan diet ranged from 13 to 15% of the total caloric intake, so an increase was recommended, especially for the amino acids tyrosine, lysine, methionine and tryptophane, which showed the lowest plasma levels compared to the omnivore diet group.[13] To replace meat as a source of protein, analogues such as tofu, tempeh or seitan are typically consumed, even though the protein levels are usually higher in meat, depending on the species.[15]

However, this type of products has been reported to contain non-desirable compounds as well. Goerke et al. (2019) showed that vegetarian and vegan meals do have a higher amount of the contaminant acrylamide than their meat counterpart, leading to the assumption the acrylamide content in meat substitutes should not be disregarded. Likewise, it is reasonable to hypothesize that other processing-related contaminants could be formed during production or preparation of these analogues.[16]

2.1 Meat analogues

Food products always adapt to the lifestyle and values of the population. Thus, the Western diet is currently particularly focused on convenience products that are easy to prepare and rich in flavor. With vegetarianism came substitutes to meat for frying or cooking, for example, soy, peas, or wheat. These alternatives were intended to remind consumers of the familiar taste and texture experience of meat without consuming actual meat in the process.[17]

Commonly used meat substitutes include tofu, tempeh, and seitan, which can be purchased ready-made in grocery stores or produced by consumers in household kitchens. Tofu is probably the best known product and is made from soybeans. In addition to protein, it is rich in calcium and iron[17], although the nutrient composition depends also on the soy used.[18] Tempeh is also soy-based and originates from Indonesia. It consists of fermented soybeans and has a denser texture compared to tofu. Seitan is made from wheat gluten and can be made of flour. Washing starch out of a wheat flour dough produces seitan, which is then briefly blanched and fried.[17]

In the case of soy-based foods, their excessive consumption is often a topic of discussion, especially for baby and infant foods, due the presence of isoflavones in soy, being the main point of controversy the relationship between these estrogenic isoflavones and the occurrence of breast cancer. He and Chen (2013) reported lower breast cancer incidence and longer life expectancy in women for whom soy-based foods were part of the traditional diet than in women on a Western diet,[19] whereas Messina and Wood (2008) described a positive correlation between the excessive intake of isoflavones (which can hardly be achieved by diet) and the occurrence of breast cancer in postmenopausal women. The amount of isoflavones consumed only through diet is, therefore, not considered a risk for developing breast cancer. Amounts that show a detrimental effect must be supplied via appropriate dietary supplements or medications.[20]

2.2 Protein oxidation

Protein oxidation (PROTOX) is a phenomenon that involves the molecular changes in proteins which can cause irreversible damages to protein structure. During processing, producing and storage, molecular oxygen can interact with food and can be converted into mere reactive oxygen species (ROS).[21]

Protein oxidation causes changes in secondary and tertiary structure levels, leading to conformational changes in the proteins. In food products this can affect their quality and

taste, since changes on chemical and physical characteristics of proteins take place. In contrast to the modifications during the Maillard reaction, these changes usually do not have a positive effect on the sensory and optical properties of the food. For meat, fish and other muscle food, oxidation of proteins leads to reduced water-holding capacity and texture building capacity, essential properties to obtain the expected quality in food products.[22]

Proteins are targets for ROS, which can impact food quality during production and processing. Among ROS there can be hydroxyl radical (HO·), superoxide radical ion ($\cdot O_2^-$), hydroperoxyl radical (HOO·), hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$).[21] ROS generate a radical (R·), which is converted into a peroxyl radical (POO·).[22] A large part of these reactive species is formed by photooxidation or enzymatic reactions in the meat product.[23]

α-aminoadipic (AAS) (built by oxidation from lysine) and γ-glutamic (GGS) semialdehydes (built by oxidation from arginine) (see Figure 1) are considered as the main carbonyl products from metal-catalyzed oxidized proteins. They are often used as parameters to estimate the extent of protein oxidation for numerous food products.[11]

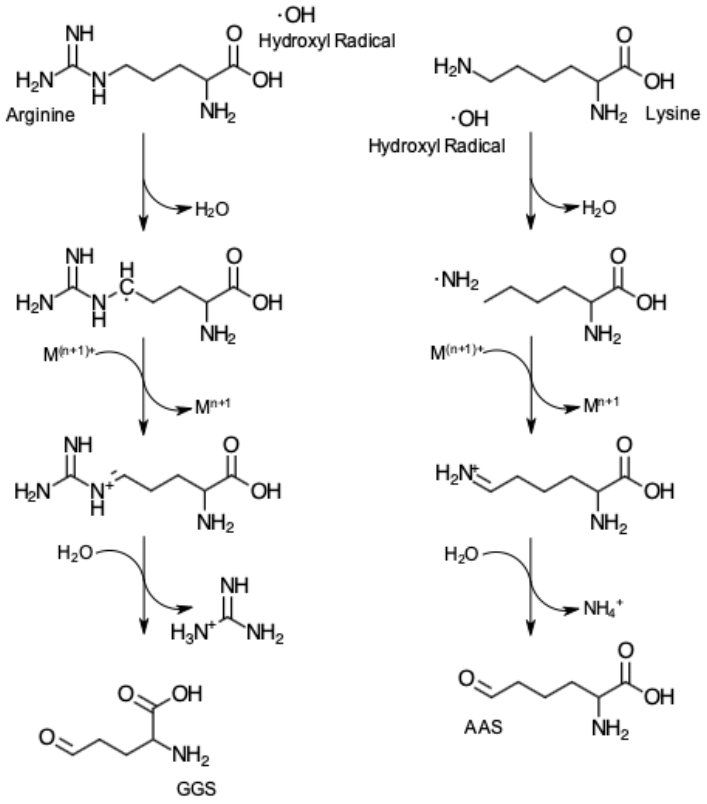

Figure 1: Formation of GGS from arginine and AAS from lysine after Armenteros et al. (2008); drawn with ChemDoodle

Villaverde and Estévez (2013) proved that AAS and GGS can even be formed in the presence of reducing sugars by the mechanism of the Maillard pathway.[24]

Protein oxidation occurs not only in the side chains of proteins, but also in the peptide backbone and can lead to protein cross linking, amino acid side chain modification or protein fragmentation.[23] Metal-catalyzed oxidation (MCO) is the most common way for oxidation of protein in food. This reaction leads to the formation of carbonyl derivates like AAS and GGS, which can be up to 70% of the total carbonyls resulting from meat protein oxidation.[24] While it is possible to monitor the formation of AAS and GGS semialdehydes specifically by HPLC-MS, to estimate the amount of protein oxidation in general, the determination of the overall protein carbonyl content is widely applied in food chemistry.[25]

2.2.1 Carbonyl content

The oxidation of some amino acids like lysine, arginine and proline leads to the formation of carbonyl derivatives. In addition, they can be formed by the cleavage of the peptide backbone or by secondary reactions between some amino acid side chains with lipid oxidation products, reducing sugars or their oxidation products.[23] There are three pathways for the generation of carbonyls out of proteins, taking the first one place in the side chains of amino acids such as lysine, threonine, arginine or proline. The other pathways involve the reaction of the δ-amino group of an alkynyl amino acid with their oxidation product, or the oxidation of the glutamyl side chains or peptide backbone.[26] Protein oxidation can lead through the Fenton reaction, which is considered a source of ROS. By abstracting a hydrogen atom from the neighboring carbon, the ε-amino moiety of the alkaline amino acid can be attacked by reactive species, resulting in the formation of an imino group as an unstable intermediate. Subsequently, this intermediate can undergo hydrolysis to produce the corresponding protein carbonyl. This reaction leads to the deamination of lysine, while γ-glutamic semialdehyde is formed.[27]

While processing food, carbonyls arise during preparation and storage, which have not only sensory and technological effects on the final product. Carbonyls are also associated *in vivo* with health disorders and oxidative stress.[26] Thus, adequate protein quality should be maintained, especially in processed foods with a high protein content.

Besides the formation of carbonyls, other chemical reactions such as the loss of sulfhydryl groups, loss of tryptophan fluorescence, or the formation of intra- and intermolecular cross-links can occur.[26]

The analysis of carbonyl content can be performed using the photometric DNPH (2,4-dinitrophenylhydrazine) method, which is a relatively simple way to detect carbonyls in a

target. This method is well established because of its easy implementation and is known as a routine analysis for determination the carbonyl content.[22] The method allows to evaluate quantitatively the amount of carbonyls in the sample via standard photometry. Alternative methods are mostly costly and time consuming in comparison.[28]

Detection is based on the reaction of DNPH with the protein carbonyl group (see Figure 2), which forms 2,4-dinitrophenylhydrazone derivates of these carbonyl compounds, which can be measured photometrically at 370 nm. [11]

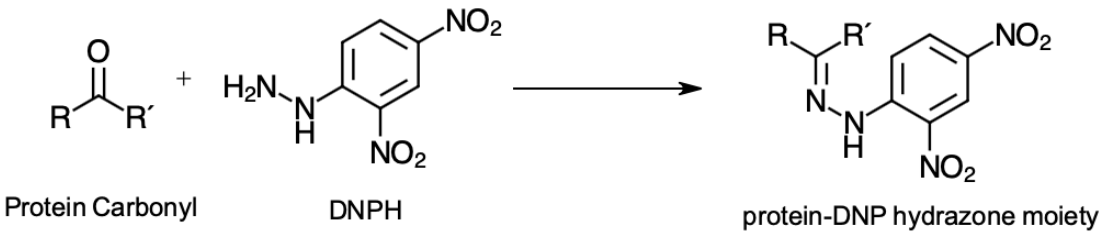

Figure 2: Reaction from DNPH with a carbonyl compound; drawn with ChemDoodle

Alternative methods for the determination of the carbonyl content are, for example HPLC, immunocytochemical detections (like ELISA or Western immunoblotting) or LC-ESI-MS.[11] However, the determination using the DNPH method has decisive advantages: by means of the photometric measurement, the carbonyl content per gram of protein in the sample can be measured. Additionally, the use of standards is not necessary, and the measurement takes place simultaneously with the protein determination. Whereas immunocytochemical methods are highly sensitive and specific, the DNPH method is more efficient in time and cost.[28] However, even though the method is cheap and easy to perform, a large sample amount is needed and, for samples with a limited protein content, it might not be the right method due to its lower sensitivity compared to the alternatives.[29]

2.3 Maillard reaction

The Maillard reaction has been named after the French chemist Louis Maillard, who described it in the beginning of the 20th century.[30] In 1953 the first coherent scheme was published by Hodge (see Figure 3), where he summarized the chemical reactions related to the Maillard process.[31]

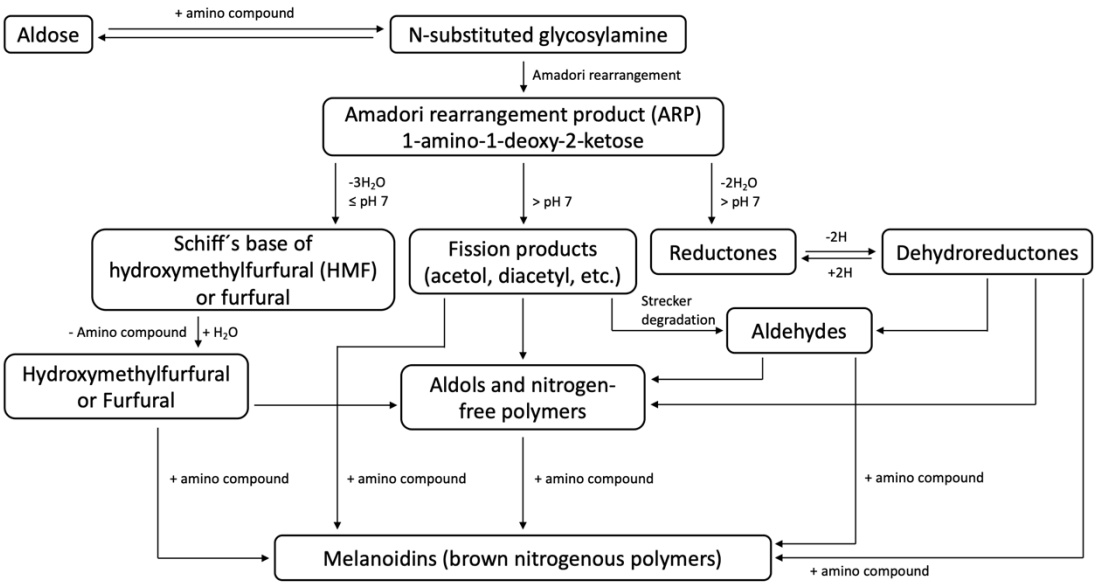

Figure 3: Maillard reaction scheme (based on Hodge, 1953).

It describes the aroma-forming, non-enzymatic reaction between reducing sugars and certain amino acids. Especially during the traditional preparation of certain food groups, such as roasting coffee beans, baking bread and cakes, cooking meat and fish or deep-frying potatoes, typical aroma-forming substances are produced.[32] The process not only leads to an change in color (browning) of the food, but also to an effect on nutritive value and flavor.[33] Although there is an interest for the building of some flavor, aroma color-compounds, products with health disadvantages are also formed during this reaction.[32]

The early phase of the reaction occurs in two steps. First, the lone pair electrons at the nitrogen of the amino acid reacts with the carbon atom of the sugar and subsequently forms a glycosylamine by ring closure (see Figure 4).[34]

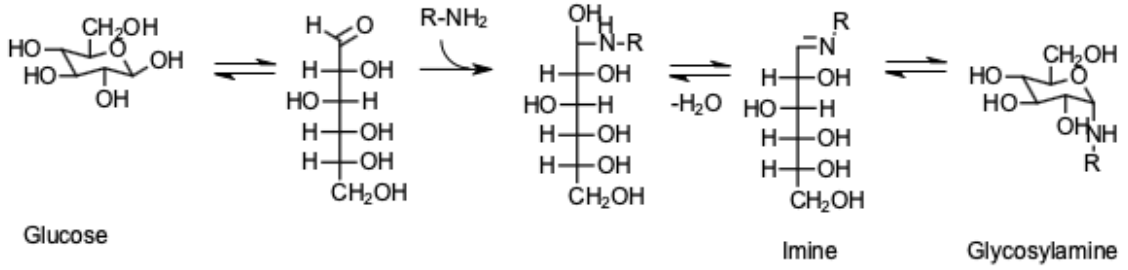

Figure 4: First step of the Maillard reaction. Formation of glycosylamine from glucose and an amino group modified from Parliment et al. (1989); drawn with ChemDoodle

The initially formed products are unstable and are subsequently reorganized by the Amadori rearrangement (see Figure 5) in the case of aldoses and by Heynes

rearrangement in the case of ketoses. Afterwards, they can react in the advanced stage to form Advanced Glycation End-Products (AGEs).

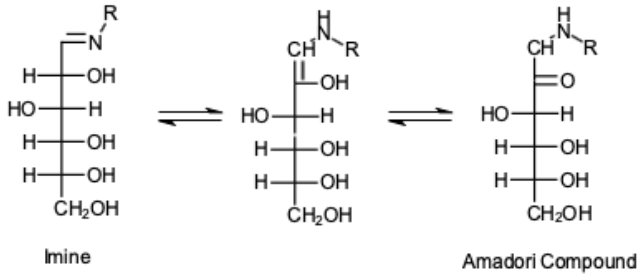

Figure 5: Amadori rearragement. Modified from Parliment et al. (1989); drawn with ChemDoodle

Via 1,2- or the 2,3-enolisation, dicarbonyl-compounds are formed, which are highly reactive towards nucleophile molecules such as amino acids, as shown in Figure *6*. As a further consequence, so-called Strecker aldehydes are formed during Strecker degradation, which represent important aroma components. The flavor impression is specific depending on the side chain of the amino acid.[34]

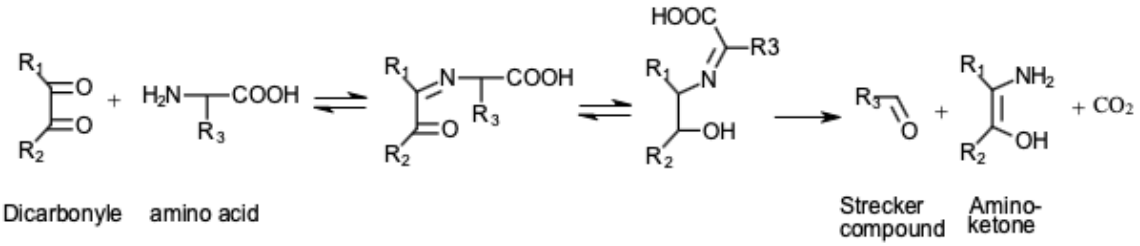

Figure 6: Strecker degradation; drawn with ChemDoodle

Because of the free amino group present in the side chain of the amino acid lysine, it is likely to react via the Maillard reaction. As a result of that, the loss of lysine in diverse heated food products depends, among other things on water activity, temperature conditions and pH-value.[35]

In the advanced stage of the Maillard reaction, products with health disadvantages (AGEs) like carboxymethyllysine (CML), pentosidine or acrylamide are formed. These substances have been described to be pro-oxidative and pro-inflammatory and have a correlation with the emergence of food-related diseases like diabetes, Alzheimer´s disease, atherosclerosis, and several other chronic diseases.[36] The modern western diet is rich in heat treated food, hence the reason why the level of consumed AGEs should be considered.[37]

The challenge for the food industry is to reduce these products in order to minimize their formation and, thus, the risk of their associated diseases.[32] The Maillard reaction can be inhibited by lowering the pH, maintaining the lowest possible temperature, using non-

reducing sugars, avoiding critical water contents during processing and storage, and using sulfite.[38]

2.3.1 N-ε-(carboxymethyl) lysine

The structure of CML (Figure *7*) is characterized by a lysine residue that has been modified by the addition of a carboxymethyl group. This modification can lead to the formation of crosslinks and other structural changes of proteins and lipids.[39]

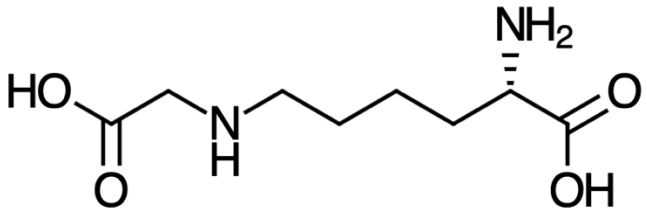

Figure 7: Structure of N-*ε*-(carboxymethyl) lysine, drawn with ChemDoodle

N-*ε*-(carboxymethyl) lysine (CML) is used as a common of AGEs or advanced lipoxidation endproducts (ALEs) because it is very well characterized. It is formed by a Maillard reaction between a reducing sugar and the *ε*-amino group of lysine. In the Amadori rearrangement lysine is transformed into fructoselysine, which can be converted into CML.[40] The content of formed CML in food is affected from diverse factors like the composition of the food or the processing conditions applied. Products high in proteins and fat leads to a higher amount on CML than food rich in carbohydrates. In addition to that, the method of food preparation has an effect on the CML content: cooking with dry heat (grilling, roasting,…) leads to higher contents than moisture heating (boiling, stewing,…).[41] Therefore, highly processed food which is a large part of the western diet, might contain large amounts of CML. This can be of special concern in the case of infants, whose heat treated infant formulas might contain a considerable amount of CML intake.[42]

Moreover, since CML is formed from lysine, the amino acid pattern of the cooked product is crucial in its formation. The composition of amino acids depends not only on the product or raw material, but also on the species of the plant and the products derived from it. Accordingly, Shoup et al. (1966) showed considerable differences in the amino acid pattern between wheat species, which influence the reactivity of the proteins they contain.[43]

CML is formed *in vivo* as well, as a result of normal aging and disease, which causes the same effects as if consumed in food.[44] *In vivo* studies in rats showed that it can accumulate in certain tissues such as kidney, liver, and brain.[45] Health issues like

arteriosclerosis, diabetes and Alzheimer's diseases have been linked to the consumption of CML. It has also been reported to promote oxidative stress, inflammation, and aging.[41]

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is a widely used tool for the screening of a wide range of pharmaceutical, chemical, and biological compounds. Because of its high sensitivity, selectivity, speed of analyses and effectivity, it is a common tool in many routine laboratories.[46]

Small molecules can be detected and quantified, but peptides and amino acids can also be analyzed by LC-MS. Moreover, selected amino acids can be determined simultaneously by LC coupled to a tandem mass spectrometer equipped with Electrospray-Interface (ESI) and a triple quadrupole (QqQ). By use of an LC column, the components of the injected sample are separated based on their polarity (which determines their affinity to the column stationary phase) and eluted to the mass spectrometer. Once there, the molecules are first charged (ionized) and analyzed by the first quadrupol (Q1) on one mass-to-charge ratio (m/z). The ions falling into this range pass into the collision cell (Q2) and convert into fragments, highly specific to the original ions. In the second analyzer (Q3), m/z fragments typical for the ions of interest are detected.[47]

Methods for the CML detection include liquid chromatography coupled to mass spectrometry (LC-MS/MS), gas chromatography coupled to mass spectrometry (GC-MS/MS) or high-pressure liquid chromatography (HPLC) coupled with fluorescence detection. By using a tandem MSsystem the sensitivity of the method improves but the equipment costs are higher. Additionally, it is possible to measure CML, N-ε-(Carboxyethyl)lysine, pyrraline, pentosidine and lysine simultaneously, which saves the amount of sample needed and time.[10] Multiple reaction monitoring (MRM) allows to distinguish targeted molecules even when the separation by LC is not optimal. Therefore, it is used for specific analytes with known fragments in complex matrix.[47] On the other hand, the determination by GC-MS/MS requires a derivatization of carboxylic acids and amino groups prior to analysis, which is quite time-consuming.[10]

2.3.2 N-ε-(carboxyethyl)lysine

N-ε-(Carboxyethyl)lysine (CEL) is formed during the reaction of methylglyoxal and lysine in the Maillard reaction. Like CML, it belongs to the AGEs category, which are potentially harmful compounds generated during the heating of food. CEL, just like CML, is a well-known marker for AGEs, and research has used as such in meat products in the past.[48]

As shown in Figure *8*, it differs from CML in its ethyl group side chain. It is expected that this difference in structure may contribute to its biological function.[49]

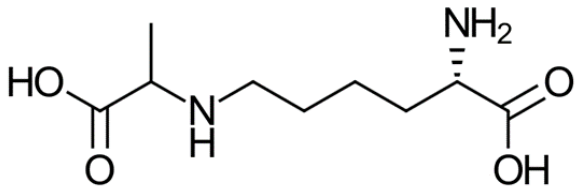

Figure 8: Structure of N-*ε*-(carboxyethyl) lysine; drawn with ChemDoodle

Meat products, which are high in protein and fat, can generate CEL during processing, which may increase oxidative stress, and promote the emergence of diabetes and other chronic diseases. The cooking method and the type of used oil (if present) influences the CEL level as well as the amount of contained salt. The formation of CEL was reported several times in meat and meat products and can variate depending on the composition of the tissue.[50] As also reported by Chen and Scott Smith (2015), red meat shows higher contents in CML and CEL than white meat and fish under the same preparing conditions.[51]

2.3.3 Pyrraline

Pyrraline is another AGE, formed during the Maillard reaction where amino acids react in the presence of reducing sugars.[52] Its chemical structure, shown in Figure 9, is known to by highly stable and resistant to enzymes in the human organism. The substance is nearly completely released and resorbed during digestion and its excretion takes place almost completely in urine. This contrasts with other AGEs, since only a small proportion of them are excreted again via the kidneys.[52,53]

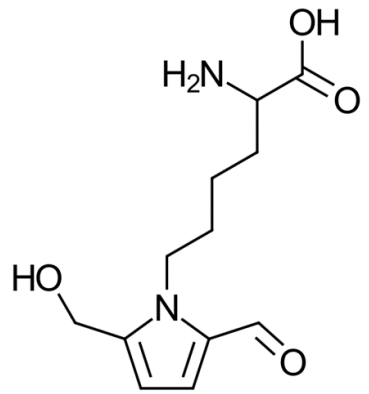

Figure 9: Structure of pyrraline; drawn with ChemDoodle

Pyrraline can be detected by ELISA, HPLC combined with various detectors such as diode array detector (DAD) or LC-MS. While ELISA is often used for analysis, LC-MS has significant advantages. On the one hand, no monoclonal antibodies are required. On the other hand, several AGEs can be measured simultaneously by LC-MS, while ELISA kits usually target a single molecule. This makes LC-MS a more cost-efficient technique compared to ELISA.[54]

2.3.4 Pentosidine

The structure (see Figure 10) of pentosidine was first described by Sell and Monnier (1989), who isolated the substance, now known to be detectable as a result of the Maillard reaction in foods like chicken, beef or salmon. The structure is a cross-linked adduct between the pentose sugar ribose and the amino acids lysine and arginine.[55]

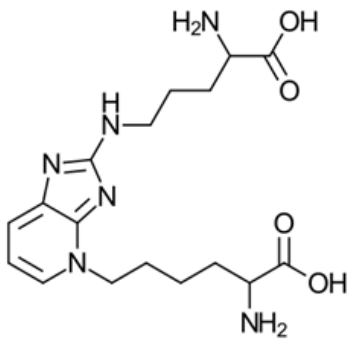

Figure 10: Structure of pentosidine; drawn with ChemDoodle

Via Schiff base formation, reducing sugars and amino acids react to form the Amadori product and subsequently produce AGEs, such as pentosidine. Pentosidine can be used as an indicator for the non-enzymatic browning reaction and is a typical processing contaminant when heating food. The western modern diet is rich in highly-processed foods and thus also in AGEs, which may have negative effects on age-related disorders.[52]

2.3.5 Acrylamide

Acrylamide is a processing-related contaminant, which is formed during Maillard-like reaction between certain amino acids (asparagine and methionine) and reducing sugars.[56] Therefore, a carbonyl and the α-amino group of the asparagine react forming a Schiff base, which decarboxylates and subsequently forms a product that can react further in two different ways. Either it hydrates and forms a 3-aminopropionamide, which subsequently forms acrylamide under the influence of heat, or the Schiff base

decomposes directly to acrylamide by elimination of an imine. The compound is low in molecular weight, and highly water soluble and organic solvents (see Figure 11).[57]

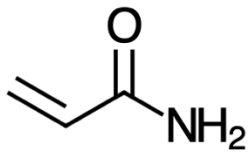

Figure 11: Structure of acrylamide

The amount of acrylamide formed depends on the sample matrix, temperature, duration of frying, and whether frying or baking are performed. Especially potato chips (average medium bound level 389 µg/kg), processed cereal based food (average medium bound level 73 µg/kg) or coffee substitutes (average medium bound level 1 499 µg/kg) are rich in acrylamide,[16,57] although its formation usually only occurs during heat treatments above 120°C and more prominently in dry foods.[58] The estimated dietary intake for adults is between 0.4 and 3.4 µg/kg body weight/day acrylamide for adults, although this can be much higher for children due to corresponding food preferences and lower body weight. A guideline for reducing the acrylamide content of industrially manufactured foodstuffs has been drawn, and levels are also regularly monitored. However, as home-prepared food is not subject to any monitoring, it can sometimes unknowingly contribute to a high acrylamide intake. Likewise, tobacco smoke is also known to contribute to a significant intake.[57]

Acrylamide is regarded as a carcinogenic, neurotoxic, and genotoxic substance, which means it can interact with the human gene-bases. As a result, a margins of exposure (MOE) value can be determined to estimate the risk on a too high intake. Therefore the estimated daily intake is set in relation to the Benchmark Dose Lower Confidence Limit 10% ($BMDL_{10}$). $BMDL_{10}$ indicates the concentration of the carcinogen that caused a tumor in 10% of the animals in animal experiments. MOE for carcinogenic and genotoxic substances of 10,000 or higher is considered to pose a low risk to public health. For acrylamide, this value is much lower for all age groups (factor 23-200), which raises general concerns about the intake level of acrylamide.[57]

The amount of consumed acrylamide can be assessed using short-term biomarkers of exposure like N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) and N-acetyl-S-(2-hydroxy- 2-carbamoylethyl)-L-cysteine (GAMA). These acrylamide-related substances can be measured in urine within 3 days following exposure.[59] Based on this exposure biomarker, vegans and omnivore people were compared by Goerke et al. (2019). While vegan/vegetarian dishes were mostly lower in acrylamide level, compared to meat dishes, the values of the urine biomarkers for vegetarians or vegans were higher than in

the omnivores group. This may correlate with the individual nutritional preferences of the participants, as the consumption of bread or roasted potato dishes might be preferred by vegans.[16]

Various methods for determination the acrylamide content have been described. Colorimetric methods are based on the measurement of the Maillard reaction-induced brown color. Some studies have shown a correlation between the brown color or redness parameter and acrylamide content, but this only allows food products to be compared with each other rather than to identify the chemical moiety responsible for the color effect.[60]

The amount of acrylamide in food can be determined by using, for instance NP-HPLC (normal phase high performance liquid chromatography) coupled with UV detection,[61] GC-MS, LC-MS and ELISA. The advantages of ELISA include low cost, ease of use and mobile application.[60]

Mass spectrometry methods are, on the one hand, considered standard methods, as they meet EU standard guidelines with low LOD and LOQ. They are considered to be particularly sensitive, selective and versatile.[60]

Determination with HPLC is a widely used method, where the food sample should be extracted and filtered to remove any solids. With the coupling to a tandem mass spectrometer the sample preparation can be simplified, but instruments are expensive. For the measurement with GC-MS the sample can be analyzed after derivatization by bromation or other sample preparation steps.[62]

2.4 Goals of the thesis

This work aims to quantitatively evaluate the presence of naturally occurring processing contaminants in meat analogue food products, which has already been carried out for meat and meat products in numerous studies. Firstly, the study focuses on tofu, tempeh and seitan as meat analogs, which are produced under household conditions and fried in vegetable oil. Secondly, protein oxidation products are to be investigated. Products are always fried under the same conditions and then measured directly to exclude oxidation processes after processing. For this purpose, the carbonyl content is used as a marker and analyzed using the DNPH assay. This photometric method is also a commonly used tool in the study of other food groups to estimate the degree of protein oxidation.

2.4.1 Impact of which plat based raw material is used for manufacturing meat analogues regarding protein oxidation and Maillard reaction products

To measure the influence of protein oxidation or heat-induced contaminants on the raw materials used to produce the meat analogs, two products from soy (tofu and tempeh) and one product from wheat (seitan) were used. The aim of the work is to investigate out what influence the agricultural product has on the processed product. Since soy and flour differ in protein content and amino acid pattern, it can be assumed that reactions in the food differ during preparation.

2.4.2 Extent of increase in protein oxidation and Maillard reaction products from raw plant product (soybean or flour) to edible meat substitute

To understand the progress of protein oxidation and the formation of heat-induced Maillard products throughout each step of the preparation process, measurements are taken at three points in the preparation process. As shown in Figure 12, all samples are treated differently in the first step, but fried under the same conditions and measured in the same steps of the process. This work aims to find out which preparation steps have an influence on the protein quality of the sample.

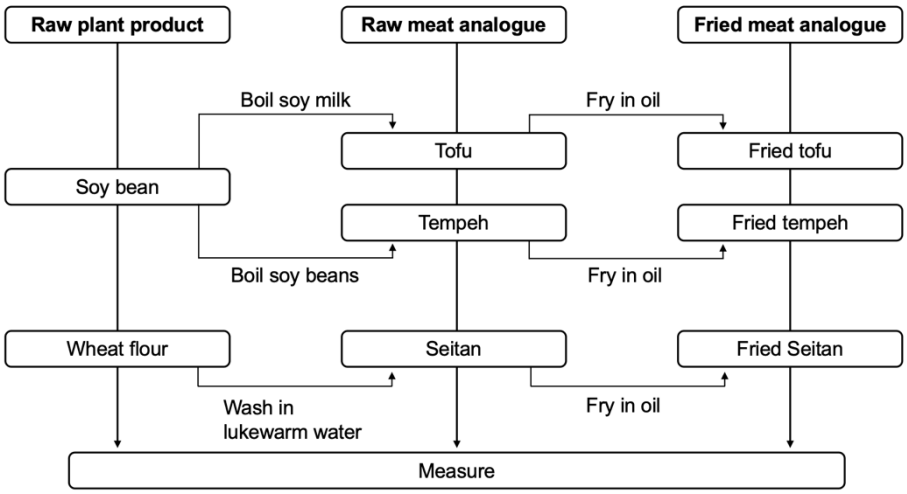

Figure 12: Overview from the raw plant products to the fried edible meat analogue

Furthermore, it is intended to assess the effects the fermentation (for tempeh) in contrast to the mixing and cooking (for tofu) of the soybeans has on the possible formation of undesirable by-products. Cooking of soymilk during tofu production is expected to result in higher lysine losses and higher levels of protein oxidation products or Maillard reaction associated products. It is expected that after each step the amount of protein oxidation products and that of Maillard induced products will increase, so that the lowest amounts

are detectable in the raw vegetable product, whereas the highest levels are contained in the fried samples.

2.4.3 Study design

To investigate these research questions, routine laboratory analyses are aimed to be carried out. To detect protein oxidation products, carbonyls will be measured photometrically using the DNPH method. These should give an indication of the progress of protein oxidation. Furthermore, LC-MS will be used to measure N ε-(carboxymethyl)lysines (CML), N ε-(carboxyethyl)lysines (CEL), pyrraline, pentosidine as well as acrylamide, which can be formed due to Maillard or Maillard-like reactions.

3. Methods and materials

3.1 Samples

The raw soybeans were bought on the internet, produced in Canada, there was no further information about genotype or producer (Art. Nr: 15301, LOT: 82A0922, MHD:01.03.2024). The flour was bought at a regional shop from the brand “Ja Natürlich”. All further samples were produced under household conditions.

3.2 List of chemicals, devices, software and consumables

The sample preparations were done under household conditions with household machines. For mixing the soybeans a household Mixer (Philips HR2195, 900W) was used. The mass was passed through a passing cloth (AIEVE, 50x50 cm, 100% cotton) to remove plant residues. For heating up the soymilk a gas stove was used. Nigari was purchased from Natrue & Partage (Magnesium Chloride, Lot: 2021.05.13-PO3907, BBD: 13.05.2024). The tofu was pressed in a mechanic tofu press (Tofuture). Tempeh starter culture was received from Tempehtation (*Rhizopus oligosporus*, *Rizophus oryzae*) and apple cider vinegar (Clever) was purchased from a local grocery store. The kneading of the seitanmass was done in a Kitchen Machine (KitchenAid Artisan, 300W). Samples were fried in a pan (induction pot, 200 mm) coated with 25 mL of oil (rapeseed oil from “Krone-Öl”) on an induction hob.

The determination of the protein content was performed with the Kjeldahl-method in the Faculty of Food Chemistry and Toxicology. The machines were distillation apparatus (K-355, Büchi) and Digestion machine (K-432/438, Büchi). Nitrogen-free weighing boats (Whatman). Chemicals for the experiment are listed in Table 1.

The following tables contains all chemicals, devices, software and consumables used for the experiments of this thesis.

Table 1: Chemicals for determination of the protein content

Meanings of pictograms:  = corrosive;  = serious health hazard;  = flammable

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Picto-gram
Kjeldahl tablets 5g	4.98 g K_2SO_4 0.02 g $CuSO_4 + 5$ H_2O		Büchi		
Sulfuric acid	H_2SO_4	98 %	Roth	Danger	
Sodium hydroxide	NaOH	32 %	Roth	Danger	
Hydrochloric acid standard solution	HCl	0.1 N	Roth	Warning	
Boric acid	H_3BO_3	99.8 %	Roth	Danger	
Tashiro indicator			Roth	Warning	

The carbonyl content was measured according to the DNPH method, with a UV-VIS 1800 spectrophotometer (Shimadzu UV-1800).

Table 2: Chemicals for determination of the carbonyl content using the DNPH method

Meanings of pictograms:  = corrosive;  = health hazard/hazardous to the ozone layer;  = hazardous to the environment;  = flammable

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Trichloroacetic acid	CCl_3COOH	≥ 99 %	Roth	Danger	
Sodium dodecyl sulphate	$C_{12}H_{25}NaO_4S$	≥ 99.3 %	Roth	Danger	
Guanidin-hydrochloride	$CH_5N_3 \cdot HCl$	≥ 99 %	Merck	Warning	
Ethanol	C_2H_6O	≥ 99.8 %	VWR	Danger	
Ethylacetate	$C_4H_8O_2$	≥ 99.5 %	Roth	Danger	
Potassium chloride	KCl	≥ 99.5 %	Roth	-	-

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
2,4-Dinitro-phenylhydrazine	$C_6H_6N_4O_4$	99% moistened with ~ 33% of H_2O	PanReac AppliChem	Warning	
Hydrochloric acid	HCl	36 %	Merck	Warning	 

N$^{\varepsilon}$-(Carboxyethyl)lysine and lysine content were measured simultaneously with a LC-MS system. The chemicals listed in **Table *3***.

Table 3: Chemicals for the determination of Nε-(Carboxyethyl)lysine and lysine.

Meanings of pictograms:  = serious health hazard;  = health hazard/hazardous to the ozone layer;  = flammable;  = acute toxicity;  = corrosive;  = hazardous to the environment;

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Borate buffer			VWR chemicals	Danger	 

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Sodium borohydride			Roth		
Trichloroacetic acid	CCl_3COOH	≥ 99 %	Carl Roth	Danger	
Hydrochloric acid	HCl	36 %	Merck	Warning	
Perfluoropentanoic acid	$C_5HF_9O_2$	97%	Sigma	Danger	
Lysine	$C_6H_{14}N_2O_2$	>98%	Sigma	-	-
CML	$C_9H_{18}N_2O_4$	>98%	St. Iris Biotech	-	-
Ammonium formate	CH_5NO_2	100%	Sigma	Warning	

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Formic acid	HCO_2H	LC-MS Grade	VWR chemicals	Danger	
Acetonitrile	H_3CCN	LC-MS Grade	VWR chemicals	Danger	
Water	H_2O	LC-MS grade	VWR chemicals	-	-

For the determination of acrylamide the chemicals in **Table 4** were used.

Table 4: Chemicals for the determination of the acrylamide content.

Meanings of pictograms:  = corrosive;  = flammable;  = acute toxicity;  = serious health hazard;

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Carrez solution I		n.a.	Carl Roth	Danger	
Carrez solution II		n.a.	Carl Roth	-	-
Acetic acid	CH_3COOH		Sigma Aldrich	Danger	

Chemical	Chemical Formula	Purity	Supplier	Signal Word	GHS Hazard Pictogram
Acrylamide standard	C_3H_5NO	Analytical standard	Sigma Aldrich	Danger	 
Formic acid	HCO_2H	LC-MS Grade	VWR	Danger	  
Acetic acid	CH_3COOH	100%, LC-MS grade	Sigma Aldrich	Danger	 

Other instruments used during sample preparation and method performance are listed in Table 5.

Table 5: Other devices, tools and consumables used

Name of Consumable, tool or device	Supplier
Analytical Balance: Pioneer-series Balance, max. capacity: 110 g, readability 0.0001 g	Ohaus
Vortex mixer: RS-VA 10	Phoenix Instruments
Centrifuge 5418R	Eppendorf
Centrifuge 5810R	Eppendorf
Incubator	Heraeus Instruments

Name of Consumable, tool or device	Supplier
Vakuum centrifuge: Concentrator plus	Eppendorf
Freeze dryer: VaCo 5-II	Zirbus
Heating block	VWR
Induction hob:	Zepter International
Thermometer: MR Hei-Tec	Heidolph
Pot: 200mm	Zepter
Measuring cylinder 500 mL, 1000 mL	VWR
Eppendorf tubes 1.5 mL, 2.0 mL	Starlab
pH-meter	VWR
quartz cuvette	
Homogenizer T 10 basic ULTRA-TURRAX Disperser 220...240 50/60 Hz	IKA
Heating oven	
Duran bottle 500 mL, 1000 mL	Fisher Scientific
Ultrasonic cleaner	VWR
Pipettes 100 µL, 200 µL, 1000 µL, 5000 µL	Eppendorf
Syringe filters ROTILABO 0.45 µm	Roth
Mirco inserts 0.2 mL, 31x5mm	VWR
Screw top vials ROTILABO ND8, Brown Glass, with labelling space, 1.5 mL	Carl Roth
Septum: ⦸8mm, 1.3mm thickness	Roth

Name of Consumable, tool or device	Supplier
Syringe: 2mL	Braun
Water distillation system	Sartorius
Zotero	Corporation for Digital Scholarship
Office 365	Microsoft
SigmaPlot 14.5	Systat
ChemDoodle	iChemLabs

3.3 Methods

3.3.1 Production conditions

All products were made in triplicates and stored separately in 50 mL Falcon tubes at -20°C.

3.3.1.1 Production of tofu

Tofu was produced according to Li et al. (2015) with some adjustments[63]. 250 g soybeans were soaked in water for 24 hours. Soybeans were separated in two equal sized portions and pureed with double amount of hot water in a high-powered blender. The puree was squeezed in a straining cloth and the resulting "soy milk" was collected. The liquid was boiled for 10 minutes with constant stirring to remove bacteria and reach the desired taste and was cooled for 4 minutes afterwards. For each liter of soymilk 45 g magnesium chloride were mixed with 45 mL water and added to the cooled down soy milk. The warm mass was allowed to stand for 10 minutes, resulting in a pudding-like consistency. This was scooped out of the pot into a pressing mold lined with the straining cloth and pressed at tightest stage for 8 minutes. The resulting tofu was taken out of the press and cut in 1 cm cubes and frozen at -20°C until use.

3.3.1.2 Production of tempeh

Tempeh was produced based after the procedure described in Esaki et al. (1996).[64] 350 g soybeans were soaked in water for 24 hours and afterward cooked at medium heat in 2 L water for 60 minutes. The water was removed, and the soybeans were pealed. Beans were dried at room temperature with a kitchen towel and separated in 3 equal parts. 17 g apple cider vinegar and 0.5-0.7 g tempeh starter culture (*Rhizopus bacteria*) were added. The mass was filled in 1 L plastic bags, formed to compact loaves, and closed by folding over. The plastic bag was pierced with a scalpel every 2 cm over the entire surface.

The tempeh was ripened at 34°C in an incubator until the mass is evenly coated with white mold. After 48 hours the tempeh was taken out of the plastic bag and cut in 1 cm^3 cubes and frozen at -20°C until use.

3.3.1.3 Production of seitan

Seitan was produced out of wheat flour (type W 480). For this, 250 g flour was kneaded with 150 mL lukewarm water for 20 minutes using a household stand mixer. The dough was left covered at room temperature for 20 minutes and then washed out through a sieve under running water and the water was collected in a pot. This washing process was repeated six times until the water in the pot was clear. Afterwards, the seitan was blanched for 10 minutes, cut in 1 cm cubes and frozen at -20°C until use.

3.3.2 Preparation conditions

All the samples were stored at -20°C until use. Frying steps were performed right before usage.

3.3.2.1 Conditions of frying

The samples were defrosted at room temperature and fried in sunflower oil (Krone-Öl) at 130°C for 3.5 minutes. For the determination of the acrylamide by LC-MS (see page 31) another triplicate was fried at 190°C for 1.5 minutes. The process was performed with an induction cooking field and temperature was monitored with a thermometer.

All measurements were performed with the raw material (flour or soybean), the raw product (tofu, tempeh or seitan) and the fried product.

3.3.2.2 Conditions of freeze-drying

Samples were fried (see Conditions of frying) and 1-3 g were weight in a 50 mL falcon tube. 10 mL distilled water were added and homogenized at 22.000 rcf for 2 minutes. The samples were frozen at -20 °C over night and afterwards freeze-dried for 48 hours. The dried samples were stored at room temperature until use.

3.3.3 Determination of the protein content by Kjeldahl method

Protein content was determinate by Kjeldahl according to Marcó et al. (2009)[65] with the following procedure.

1 g of homogenized sample was weighed in in a nitrogen-free weighing boats in a 300 mL Kjeldahl flask. 2 Kjeldahl tablets (5 g/ tablet) and 20 mL concentrated sulfuric acid were added. Blanks containing all these reagents were simultaneously processed. The flasks were placed in the preheated digestion block at 420 °C for 150 minutes with 30 minutes cooldown. 60 mL 4% (v:v) boric acid were added and introduced into a titration flask and transferred under the collecting tube of the distillation apparatus. 90 mL 32% (w:v) sodium hydroxide solution was subsequently added, and the solutions were distilled for 240 seconds. Finally, 5 drops of Tashiro indicator were added to the distillate.

The concentration of total protein content was calculated with Equation 1.

$$\%P = \frac{(v_{sample} - v_{blank})}{m_{sample} \times 1000} \times z \times c \times f \times M(N) \times PF \times 100\% \qquad \textbf{Equation 1}$$

%P: mass percentage of protein

v_{sample}: consumption of titrant for the sample [mL]

v_{blank}: consumption of titrant for the blank [mL

m_{Sample}: mass of the sample [g].

z: molar valence factor (1 for HCl)

c: concentration of the measuring solution [M]

f: factor of the standard solution (usually 1 for commercial solutions)

M(N): molar mass of nitrogen (14.007 g/mol)

PF: protein factor (in this case 6.25)

3.3.4 Determination of the carbonyl content by DNPH-based method

For the determination of the carbonyl content the method described by Soglia et al. (2015)[66] was applied: 4 aliquots for each sample group and 2 aliquots for blank were made. Then, 0.45 - 2.25 g sample was weighted in, mixed with 0.15 M potassium chloride and homogenized (22 000 rcf, 1 minute). Depending on the sample matrix, a different mixing ratio (sample:KCl) was selected:

- Tofu: 1:2 (w:v)
- Tempeh 1:5 (w:v)
- Seitan 1:3 (w:v)
- Flour and soybeans 1:4 (w:v)

100 µL of the homogenized sample were pipetted into a 2 mL Eppendorf tube and 1 mL 10% TCA was added. The mixture was then centrifuged (5000 rcf, 5 minutes) and the supernatant was removed. 400 µL 5% SDS were added and the tubes were heated in a heating block at 100 °C for 10 minutes and ultrasonicated at 40 °C for 30 minutes. The samples were then treated with 0.8 mL of 0.3% (w:v) DNPH in 3 M HCl and incubated for 30 minutes. 0.8 mL of 3 M HCl was added to the blanks.

400 µL of 40% trichloroacetic acid was added to precipitate the proteins and centrifuged (5000 rcf, 5 minutes). The pellet was washed three times with 1 mL ethanol-ethyl acetate (1:1, v:v) solution with a vortex step and a centrifugation step (5000 rcf, 5 minutes) between every washing step.

Pellets were dried under the hub for 30 minutes and dissolved in 1.5 mL 6 M guanidine hydrochloride in 20 mM sodium hydrogen phosphate (pH 6.5).

After incubation overnight at 4 °C and centrifugation at 5000 rcf for 5 minutes the absorbance at 280 and 370 nm was measured with a spectrophotometer and a quartz cuvette at 25 °C in order to quantify protein concentration and carbonylcontent.

LOD and LOQ were determined from the blank using *Equation 2* and *Equation 3* (n_{blank} = 10).

$$LOD = mean(blank) + standard\ deviation\ (blank) \times 3$$ **Equation 2**

$$LOQ = mean\ (blank) + standard\ deviation(blank) \times 10$$ **Equation 3**

To determine the carbonyl content (nmol carbonyl/mg protein), Equation 4 was used. For the molar absorptivity of the hydrazone, ε_{M370nm} = 22,000 and ε_{M276nm} = 9460, which equals 43% (factor 0.43) of that at 370nm.

$$\text{carbonyl content [nmol/mg protein]} = \frac{\text{Abs}_{370} - \text{Abs}_{370}(\text{blank})}{22{,}000 \times [\text{Abs}_{280} - (\text{Abs}_{370} - \text{Abs}_{370}(\text{blank})) \times 0.43]} \times 10^6$$

Equation 4

3.3.5 Simultaneous determination of N ε-(carboxymethyl) lysine, N ε-(carboxyethyl) lysine, pyrraline, pentosidine and lysine by LC-MS

The method applied was done according to Teerlink et al. (2004).[67] Samples were freeze-dried (see "3.3.2.2 Conditions of freeze-drying") and 20 mg of sample was weighed in 2 mL Eppendorf plastic vials with safety cap.

The samples were dissolved in 100µL distilled water and 500µL 100 mmol/L sodium borohydride dissolved in 200 mmol/L borate buffer (pH 9.2) were added, prior to incubation for 2 hours at room temperature.

1 mL 40% TCA were added for precipitation of proteins and vortexed for 2 seconds. The samples were centrifuged (16,000 rcf, 10 minutes) afterwards and the supernatant was removed. The pellet was washed with 1 mL 10% TCA afterwards and centrifuged again (16,000 rcf, 10 minutes). The supernatant was removed and 500 µL 6 mol/L HCl were added.

Sample proteins were hydrolized for 20 hours at 110 °C in a heating oven and afterwards dried in a vacuum concentrator at 60 °C.

The residue was dissolved in 500 µL 5 mmol/L perfluoropentanoic acid and vortexed briefly. The samples were pressed through a 0.20 µm nylon syringe filter prior to HPLS coupled to a triple quadrupole MS with an ESI source.

Targeted analyses were performed on a LC-MS system (LCMS-8040; Shimadzu, Korneuburg, Austria) and separated on a column (TMS endcapped HILIC with a carbon loading of 17.5%, 150 × 2.1 mm, 3 µm, Phenomenex), with a precolumn (HILIC 4 x 2.00mm SecurityGuard)

10 µL sample were injected. Mobile phase A consisted of 6mmol/L ammonium formate and 0.1% (v/v) formic acid in water (pH 2.4). Mobile phase B consisted of 80% (v/v) acetonitrile and 20% (v/v) of mobile phase A, with 0.1% (v/v) of formic acid. The following gradient was set: 0-2 min 90% B; 3-5 min 87% B; 9-15 min 30% B; 20-30 min 90% B. The flow rate was set to 0.4 mL/min.

Multiple reaction monitoring mode [MRM(+)] was used for quantification. The transitions from precusor ion to product ion for CML, CEL, pyrraline, pentosidine and lysine and were chosen as shown in Table 6. Following MS settings were set: nebulizing gas flow (N_2), 3 L/min; drying gas flow (N_2), 13 L/min; desolvation line temperature, 170 °C; heat block temperature, 350 °C; and argon as collision-induced dissociation (CID) gas.

Table 6: Precusor ions and product ions (m/z) from CML, CEL, pyrraline, pentosidine and lysine

	Precusor ion m/z	**Product ions m/z**			
		Quantifier ion	**CE (V)**	**Qualifier ion**	**CE (V)**
CML	205	83.6	-19	130.1	-14
CEL	219	84	-18	130	-12
Pyrraline	255	237	-8	175	-12
Pentosidine	379	250	-22	187	-30
Lysine	147	130	-10	84	-20

Calibration curves for CML (6 calibration standards, 0.02 – 1.10 mg/L) and Lysine (6 calibration standards, 98 – 390 mg/L) (see Figure Appendix 1 and Figure Appendix 2) obtained by linear regression were used to quantify their concentrations in the samples (R^2 = 0.994). CEL, pyrraline and pentosidine were determined semi-quantitatively, expressing its content in area units.

To analyze the sensitivity of this method, the limit of detection (S/N = 3) and the limit of quantitation (S/N = 10) were determined using the signal-to-noise ratio method.[68] Areas with S/N were above 10 were considered as above LOQ and S/N above 3 were considered as above LOD.

3.3.6 Determination of acrylamide by LC-MS

Based on the method described in Gökmen et al. (2006),[69] the samples were measured with LC-MS. Briefly, 1 g of homogenized sample was weighed in 50 mL screw cap tubes. 500 µL Carrez I and 500 µL Carrez II solution were added in order to coagulate proteins. 0.2 mM acetic acid was added to adjust to 6 mL total volume. After mixing in a vortex mixer for 2 minutes, the mixture was centrifuged at 2540 rcf for 10 minutes at -5°C. The clear supernatant (avoiding the oily top layer) was pressed threw a 0.20 µm nylon syringe filter prior to LC-MS analysis.

For determination, targeted analyses was performed: 10 µL of the sample was injected into a LC-MS system (LCMS-8040; Shimadzu, Korneuburg, Austria). For separation a reversed phase column (Shimadzu, Shim-pack, VP-ODS, 150 x 4.6 mm, particle size: 5 µm) with a precolumn (Shimadzu, Shim-pack, GVP-ODS, 10 x 4.6 mm) and an isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid in water was used, at a flow rate of 0.4 mL/min at 25°C.

Multiple reaction monitoring mode [MRM(+)] was applied with the following MS settings: nebulizing gas flow (N_2), 3 L/min; drying gas flow (N_2), 12 L/min; desolvation line temperature, 250 °C; heat block temperature, 350 °C; collision-induced dissociation (CID) gas argon; and collision energy, 20 eV. Multiple reaction monitoring (“MRM”) is used for quantitation of analytes by LC-MS/MS, where an ion transition from precursor/parent ions to product/daughter ions takes place through fragmentation. The precusor ion at m/z 72 and the product ion at m/z 55 were measured in positive mode.

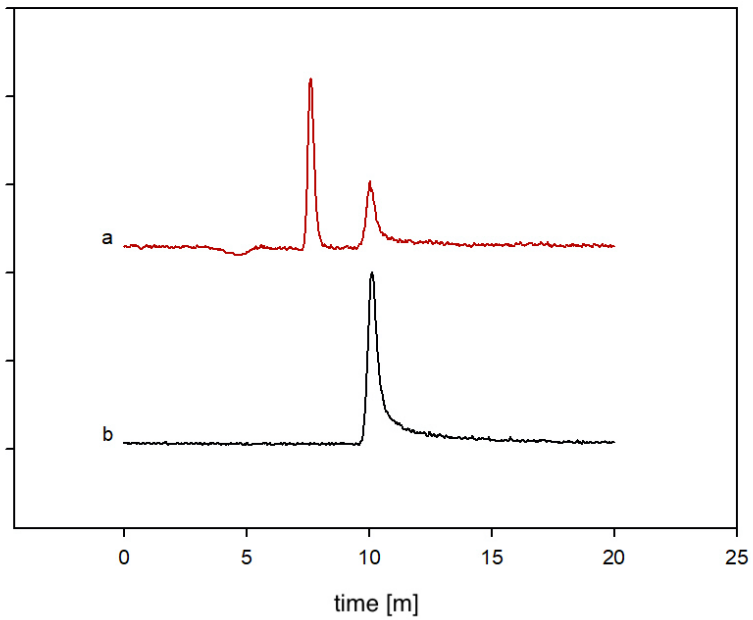

Figure 13: Chromatograms of a tofu sample (a) and standard (b)
The red chromatogram (a) shows the signal intensity of the fried tofu sample (fried at 190°C for 1.5 minutes), which clearly shows an interference peak after 7.5 minutes in addition to the analyte peak after 10 minutes. The chromatogram in black (b) shows only one peak, as it was prepared only from the pure standard and distilled water and thus shows no interference.

Signals at 10 minutes were used for quantitative evaluation. Figure 13 compares a typical chromatogram of a standard with that of a sample. In the chromatogram of the sample, it is seen that besides the analyte peak, another peak, which is not suitable for quantification, is shown. The retention times were compared with those of the standards and evaluated accordingly.

External calibration curves with acrylamide standards were used for quantification. The standards were subjected to triplicate measurements, and subsequently, the calibration curve was constructed based on the means, as illustrated in **Fehler! Verweisquelle konnte nicht gefunden werden.**. Standards between 10 µg/L and 200 µg/L were measured and LOD and LOQ were calculated by using the signal to noise ratio (S/N) method.[68] Values lower than 7.0 µg/L were considered as below the LOD, while values between 7.0 and 23.5 µg/L were considered as below the LOQ.

3.4 Statistical analysis

All experiments were performed in three independent replicates and two technical replicates for each of them. A Nalimov outlier-test was carried out and outliers were not considered for further calculations. For statistical analysis, SigmaPlot 14.5 (Systat) was used, and all data was tested for normal distribution by the Shapiro-Wilk test. For normally distributed data, an One-Way ANOVA (analysis of variance) was performed, whereas for data without a normal distribution, an ANOVA on ranks was performed. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups.

Results with a p-value lower than 0.05 ($p < 0.050$) were considered as significant and marked with different lower-case letters in the graphs. Results below the LOQ were not considered for calculations. All results are given in mean ± standard deviation.

4. Results

4.1 Protein content

Initially, the protein content of the samples studied was determined using the Kjeldahl method. Soybean, flour, and each of the raw meat analogs (tofu, tempeh, seitan) were measured. Later results were referred to the protein content of the raw meat analogues.

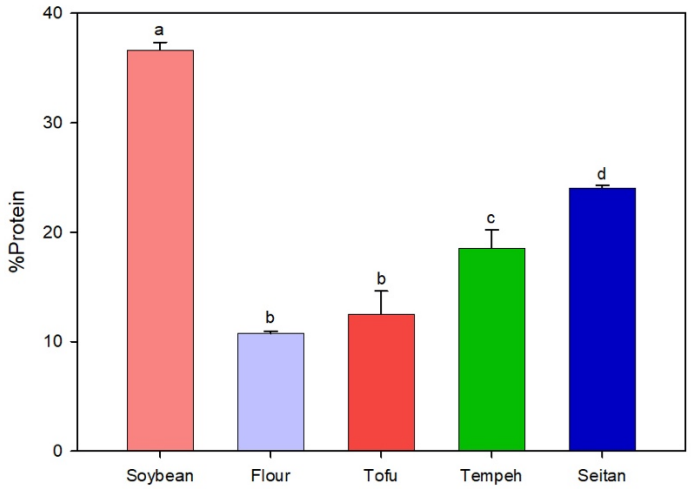

Figure 14: Protein content of the studied plant-based products in %Protein (=g/100g), determined with the Kjeldahl method.

The y-axis shows the protein contents in % (g/100g food). The products used are indicated on the x-axis. Statistical differences were tested with the ANOVA on Ranks test. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different lowercase letters (a, b, c, d) indicate significant differences ($p<0.05$).

As shown in Figure 14, the protein content is the highest in the raw dried soybean, and it decreases when processed into tofu and tempeh, while the protein content increases when flour is processed into seitan. This increase can be explained by the leaching of starch and the retention of gluten proteins during the manufacturing process. The decrease in protein content due to the production of tofu and tempeh results from the increased water content in the meat analogs compared to the dried soybean.

4.2 Protein oxidation

To determine the degree of protein oxidation in the three meat analogs, the carbonyl content was determined by the DNPH method. This is used as a marker in numerous studies.[27]

4.2.1 Determination of the carbonyl content

Tofu was prepared according to Li et al. (2015): the first step consists of soaking in the soybeans in water and obtaining soy milk by pressing out the emulsion. Subsequently, the soy milk is heated for several minutes with stirring and adding magnesium chloride. After pressing the mass to a cube to lose some water, tofu was ready to be examined.[63] As shown in Figure 15, the degree of protein oxidation increases during the preparation of tofu. A significant increase in carbonyls is noticeable both during the production of raw tofu and during frying at 130°C in oil.

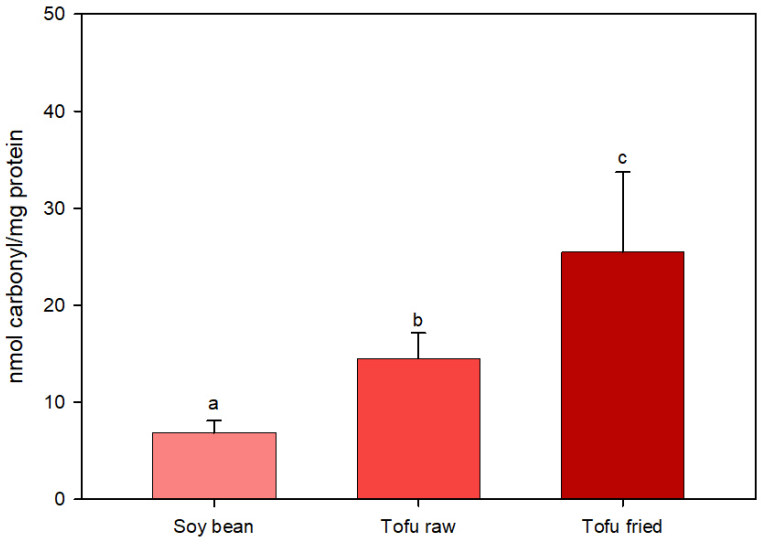

Figure 15: Carbonylcontent in the soybean, raw tofu and fried tofu.
The graph shows the comparison between dry soybeans, raw tofu and fried tofu. Differences were determined by ANOVA on ranks and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data are shown as mean ± standard deviation ($n_{soybean}=9$; $n_{tofu\ raw} = 25$; $n_{tofu\ fried} = 25$).

The results shown in Figure 16 indicate a significant decrease of carbonyl groups from the soybean to the raw tempeh, but an increase from raw tempeh to fried tempeh. The differences shows that the state of protein oxidation after the manufacturing process of tempeh is less advanced compared to tofu, even though the soybeans are heated as well heated to prepare the tempeh. As shown in Figure 14, protein content is decreasing from soybean to tempeh as well, which could mean, that most of this protein loss corresponds to protein in an oxidized state. However, in tempeh, a significant increase in carbonyl content can also be observed after heating the product.

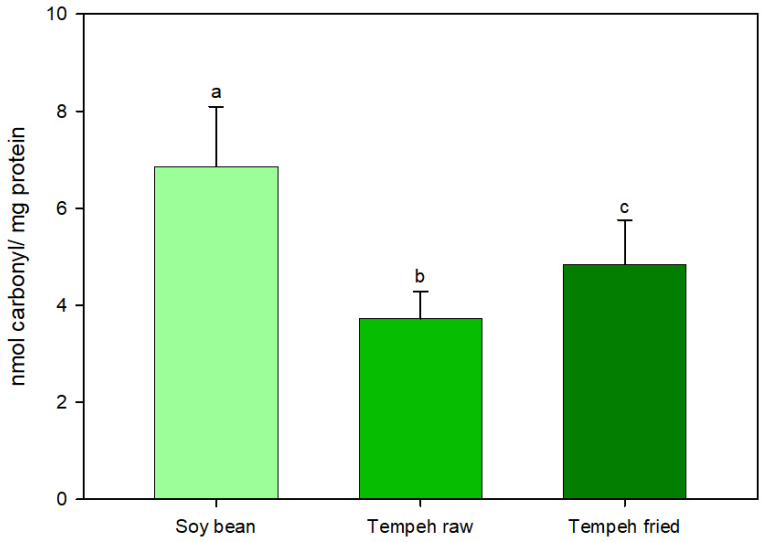

Figure 16: Carbonylcontent in the soybean, raw tempeh and fried tempeh
The graph shows the comparison between dry soybean, raw tempeh and fried tempeh. Differences were tested by ANOVA on ranks and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data are shown as mean ± standard deviation ($n_{soybean}=9$; $n_{tempeh\ raw} = 24$; $n_{tempeh\ fried} = 25$).

As it can be seen in Figure 17, in contrast to the results from the raw products showed above, there is no increase in carbonyl content after the seitan is prepared out of the flour. The most likely reason for this is that, during the manufacturing process, the flour is less heat treated and less processed than soybeans. While the protein content from flour to seitan is increasing, no significant difference between flour and raw seitan can be detected. Oxidized protein might be lost while washing out the starch during the manufacturing step. However, during the high temperature heating to fry the seitan, more carbonyls are produced, an increase that can be detected, although not significant compared to the carbonyl content in flour.

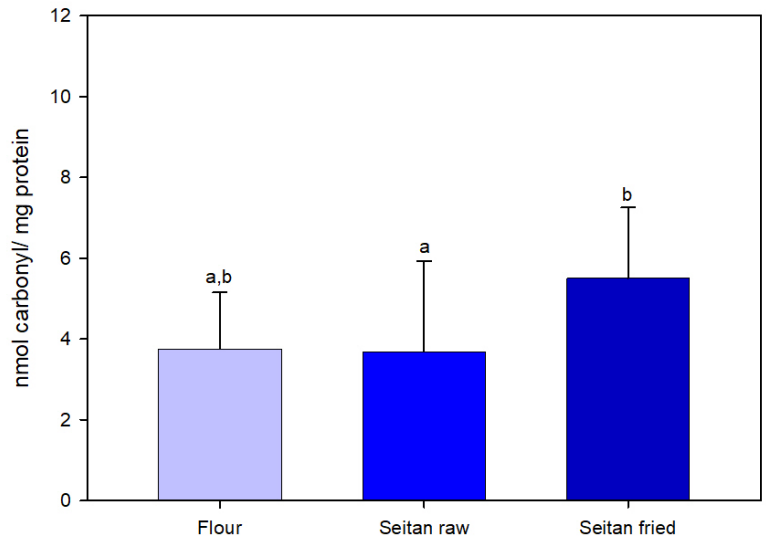

Figure 17: Carbonyl content in flour, raw seitan and fried seitan.
The graph shows the comparison between flour, raw seitan and fried seitan. Differences were determined by ANOVA on Ranks and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 9$; $n_{seitan\ raw} = 23$; $n_{seitan\ fried} = 25$).

Combining the results shown in Figure 15, Figure 16 and Figure 17, there is a tendency of a significant increase during the high temperature processing of raw products (frying in sunflower oil at 130°C for 3.5 minutes). Meanwhile, an increase of carbonyl during the manufacturing steps (from the raw material to the raw meat analogue) can only be found for tofu.

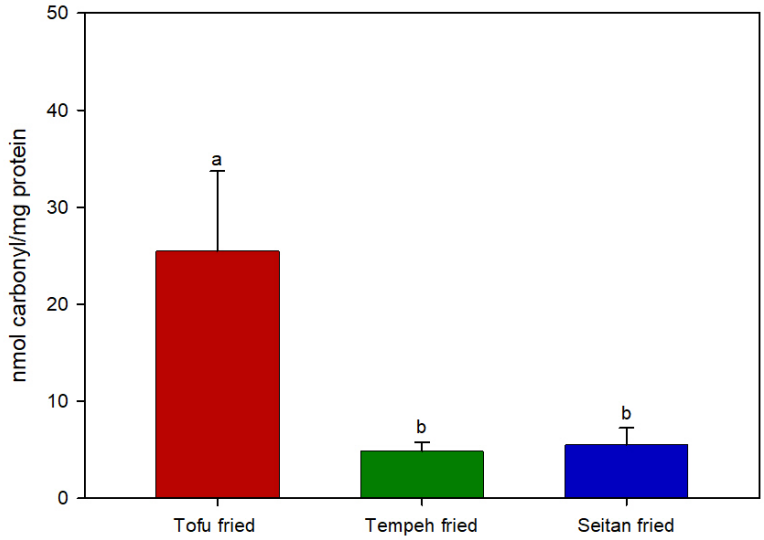

Figure 18: Carbonyl content in fried meat analogues.
The graph shows the comparison between the carbonyl contents of the three different meat analogues. Differences were tested with an ANOVA on Ranks and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups.

Different letters (a, b, c) indicate significant differences. Significant outliers were excluded for the calculations. Data is shown as mean ± standard deviation ($n_{tofu\ raw}$ = 25; $n_{tempeh\ raw}$ = 25; $n_{seitan\ raw}$ = 25).

As shown in Figure 18, the carbonyl content of tofu is significantly higher than that of tempeh or seitan, whereas the content in tempeh and seitan do not differ significantly. This trend is also evident when comparing the raw products (see Figure Appendix 4). On basis of the obtained results, it can be assumed, that mixing the soybeans and the heat treatment of the resulting soy milk might have an effect of the level of protein oxidation for the product. The results show that this processing of the beans has a great effect on the protein oxidation of the final product, as the content in tempeh is significantly lower. Although the soybeans were also cooked for 60 minutes during tempeh production, as these are not suitable for raw consumption by humans, it appears that the prior grinding and pressing of the bean also has an influence, since, unlike tofu, tempeh is prepared directly from whole soybeans.

4.3 Advanced glycation end products (AGEs)

For the determination of AGEs, semi-quantitative and quantitative methods were chosen. While standards for quantitative elucidation were used for CML and acrylamide, peakareas were compared for CEL, pyrralin, and pentosidine, allowing only a comparative indication of the increase in these processing contaminants.

4.3.1 Determination of N-ε-(carboxymethyl)lysine (CML)

For the analysis of CML, tofu, tempeh and seitan were fried and thereafter each raw and fried sample was freeze-dried. For the comparison of the data from the raw product to the fried meat analogs, the values referred to the dried samples were used for better comparability. For the analysis of the soybeans and the flour, the samples were simply crushed, without freeze-drying them beforehand.

The comparison of the meat analogs to each other was used with the values referred to the fresh commercially consumed samples. More information about the CML content in the fresh samples can be found in the Appendix.

In Figure 19 the CML content of soybean and raw and fried tofu are compared. An increase of CML concentration from soybean to tofu is detectable, most likely due to the manufacturing steps that involve heat in the tofu preparation. According to Figure 19, the CML concentration is not increasing while frying in oil, since no significant difference was

found, compared to the raw tofu samples. Further Information to the content in the fresh samples is available in Figure Appendix 5.

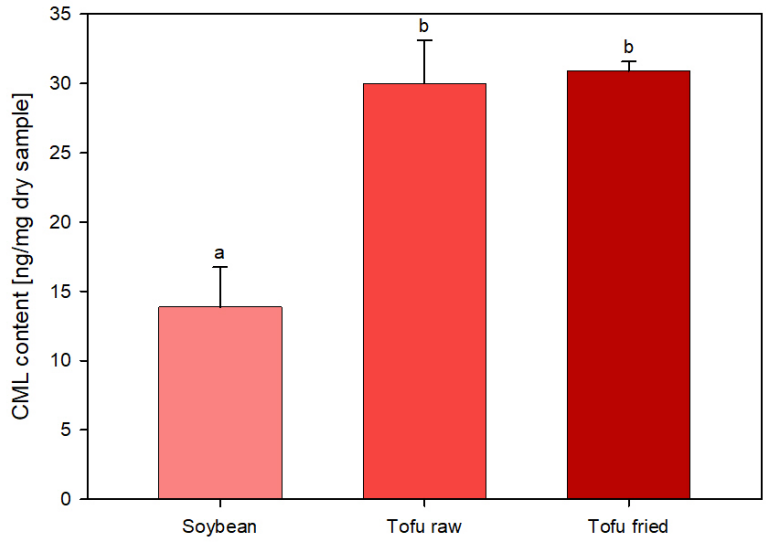

Figure 19: CML content of soybean and in raw and fried tofu.
The graph compares the CML content of soybeans with raw and fried tofu. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a,b) indicate significant difference. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tofu\ raw} = 3$; $n_{tofu\ fried} = 3$).

The significant difference in the CML content between the soybean and the raw and fried tempeh are described in Figure 20. During the process of cooking the soybeans in water for 60 minutes and fermenting them, the CML content does not increase significantly, but during frying in oil (130°C, 3.5 minutes), a significant increase in CML concentration can be reported. Further information to the content in the fresh samples is provided in Figure Appendix 6.

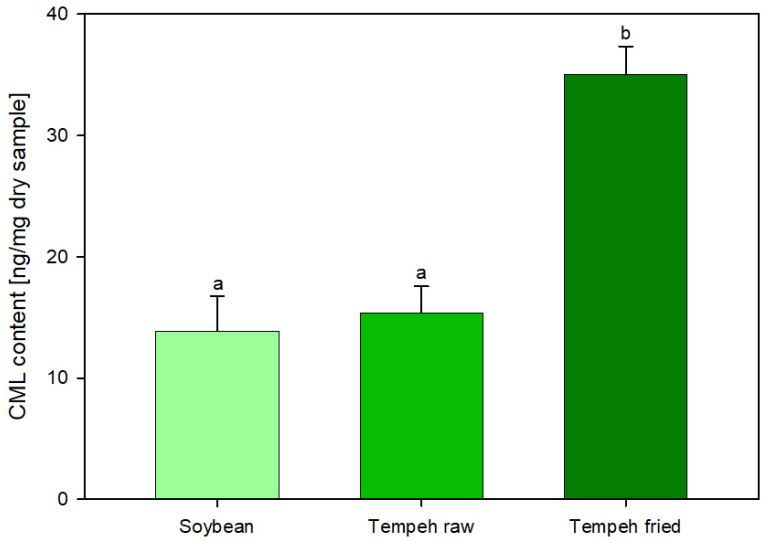

Figure 20: CML contents in soybean and in raw and fried tempeh

The graph shows the comparison of the CML contents of soybeans raw tempeh and fried tempeh. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tempeh\ raw} = 3$; $n_{tempeh\ fried} = 3$).

In Figure 21, the significant change in CML concentration for seitan is illustrated. It is shown that although the CML concentration decreases when the seitan is blanched, there is no significant difference between the fried seitan and raw seitan. The fried seitan also shows no difference from the flour as the initial product.

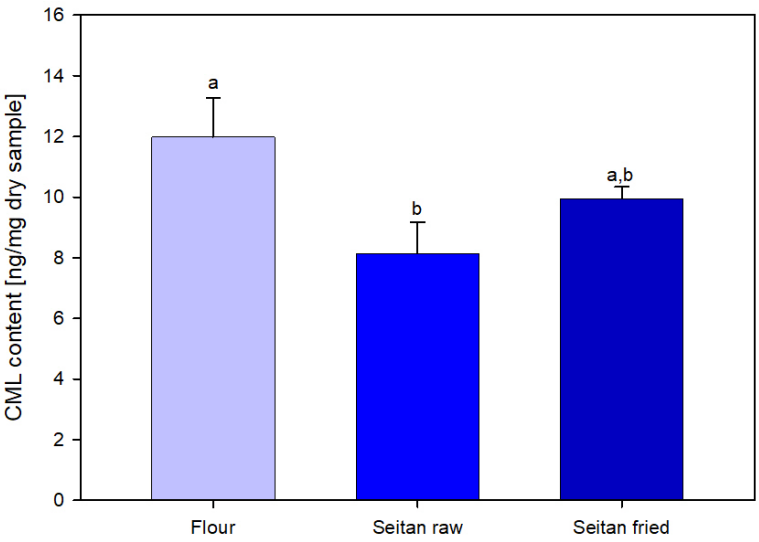

Figure 21: CML Concentration of flour and raw and fried seitan.

The graph shows the comparison of the CML contents of flour, raw seitan and fried seitan. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc

test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation (n_{flour} = 3; $n_{seitan\ raw}$ = 2; $n_{seitan\ fried}$ = 2).

While tofu shows a significant change in CML concentration in the conversion from soybean to the raw meat analogue, tempeh only shows an increase by frying in oil at 130°C. While manufacturing the tempeh, soybeans are cooked in water for 60 minutes, while the soybeans get mixed and cooked for 30 minutes to prepare tofu. However, in both samples, the CML content increases from soybean to the fried edible meat analogue. The increase in CML concentration in seitan is, however, not significantly detectable from flour to fried product. While the decrease of CML is significantly detectable from flour to raw seitan, the increase from the raw seitan to the fried seitan does not show a significant change. The production of the three meat analogs differs from each other in key steps, with the raw materials being heated in different ways. The effect of the different processing of soy can be seen in Figure 19 and Figure 20. It appears that blending the soybeans and boiling the squeezed soymilk for 30 minutes has a greater impact on the formation of CML than boiling the whole soybeans for 60 minutes, but it seems that the final CML concentrations in tempeh increases significantly after frying at 130°C. This is also evident in Figure 22 and Figure 23.

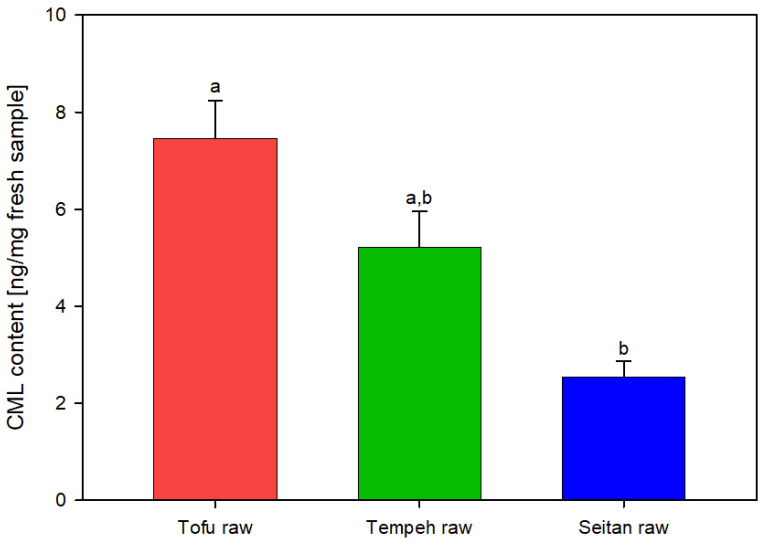

Figure 22: CML concentration of raw tofu, tempeh and seitan.
The graph shows the comparison of the CML contents between the three raw meat analogues. Contents are referred to the fresh samples weight, prior to freeze-drying. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{tofu\ raw}$ = 3; $n_{tempeh\ raw\ raw}$ = 3; $n_{seitan\ raw}$ = 2).

For a better representation of the content in meat analogue products, values from the fresh products considering the water content were used. While Figure 22 shows significant difference in CML content between tofu and seitan, this is not the case for tempeh. As already mentioned above, the CML concentration from soybean to raw tempeh, does not seem to increase as it does in the preparation of tofu. But after frying tempeh, the CML concentration increases significantly and is even higher than in fried tofu, as shown in Figure 22 and Figure 23. The results indicate that there are significant differences in CML among the three fried meat analogues tested. Additionally, the levels of CML were found to be more than twice as high in fried tofu and fried tempeh compared to fried seitan, despite the fact that all three were fried in oil under identical conditions.

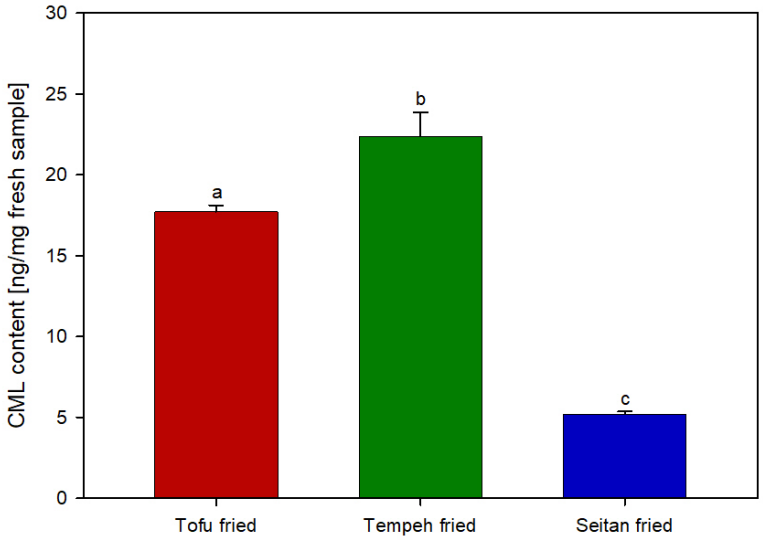

Figure 23: CML content of fried and fresh meat analogues.
The graph shows the comparison of the CML contents between the three fried meat analogues. Contents are referred to the fresh samples weight, prior to freeze-drying. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{tofu\ fried} = 3$; $n_{tempeh\ fried} = 3$; $n_{seitan\ fried} = 2$).

4.3.2 Determination of N-ε-(carboxyethyl)lysine (CEL)

For the analysis of CEL, tofu, tempeh and seitan were fried and thereafter each raw and fried sample was freeze-dried. Soybeans and flour were analyzed without freeze-drying due to their low water content. Analysis were done semi-quantitatively by comparing the areas under the curve (AUC).

As shown in **Fehler! Verweisquelle konnte nicht gefunden werden.**, there is no significant change in the area values between soybeans, raw tofu and fried tofu. Since these results refer to the dry mass of raw and fried tofu, the proportions might differ after

taking account of the fresh masses. However, the results indicate no changes in the CEL concentrations from soybean to the edible tofu.

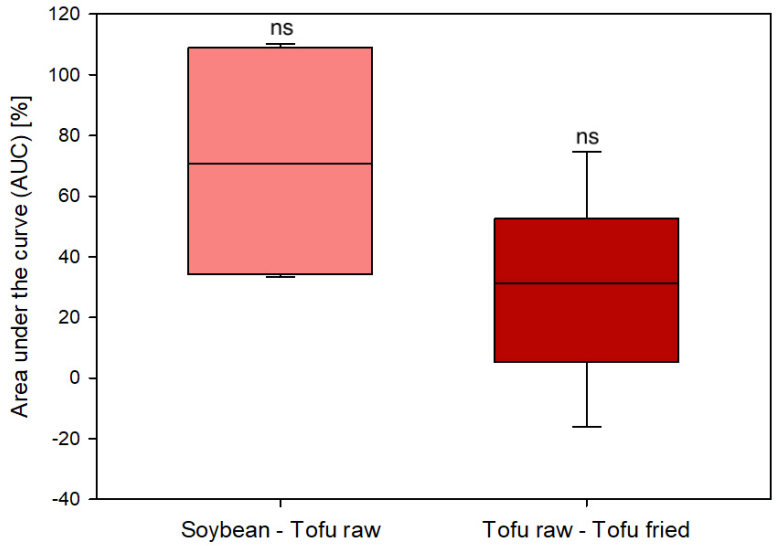

Figure 24: Change in AUC in the determination of CEL content in soybean to raw tofu and from raw tofu to fried tofu.

The graph shows the change in the values of area under the curve from soybean to raw tofu and from raw tofu to fried tofu. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA, ns indicates a no statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 2. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 2$; $n_{tofu\ raw} = 3$; $n_{tofu\ fried} = 3$).

Figure 25 describes the change in the area under the curve between soybean and fried tempeh. Both the change of AUC from soybean to raw tempeh and from raw tempeh to fried tempeh show a significant increase. The signals, therefore, indicate a significant rise of the CEL content from the raw product to the edible product.

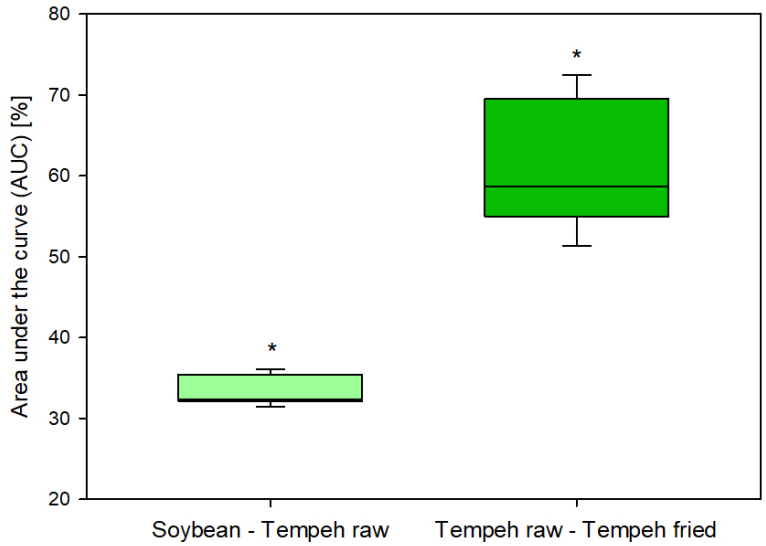

Figure 25: Change in AUC in the determination of CEL content from soybean to raw tempeh and from raw tempeh to fried tempeh.

The graph shows the change in the values of the area under the curve from soybean and raw and fried tempeh. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, * indicates a statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 2. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 2$; $n_{tempeh\ raw} = 3$; $n_{tempeh\ fried} = 3$).

As depicted in **Fehler! Verweisquelle konnte nicht gefunden werden.**, the percent increase in the area under the curve (AUC) from flour to raw seitan is considerably high, although the observed range of variability is also considerably wide. There is no statistically significance, that there is an increase or decrease of CEL in seitan during production.

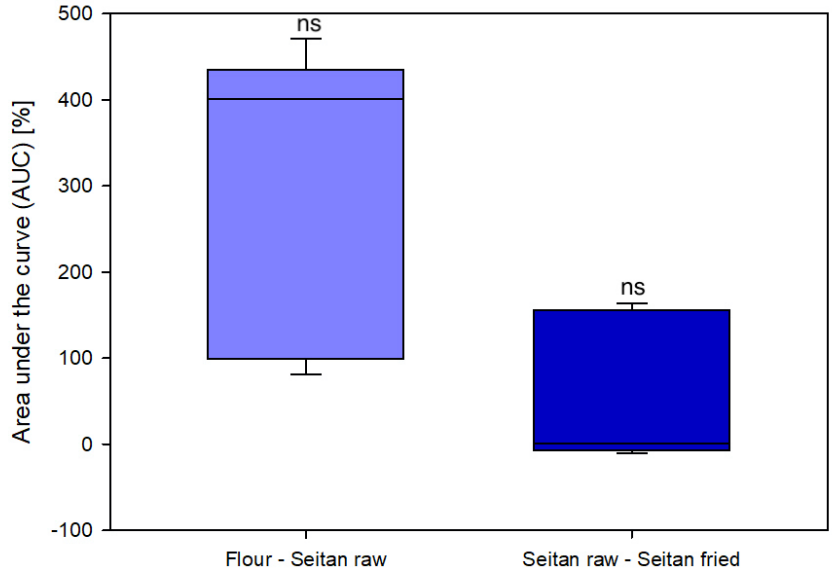

Figure 26: Change in AUC in the determination of CEL content from flour to raw seitan and from raw seitan to fried seitan.

The graph shows the change in the values of the Area under the curve from flour and raw and fried seitan. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, * indicates a statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 2. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 3$; $n_{seitan\ raw} = 3$; $n_{seitan\ fried} = 3$).

Among the three products tested, only an increase in integrated peak area can be detected for tempeh, taking into account that the dry mass of the samples enables a more accurate comparison between them. The obtained results provide valuable information on the expected increases in CEL concentration, even in the absence of external calibration curves. However, for making quantitative statements and for

comparison with values reported in previous literature, it is imperative to establish a linear regression.

4.3.3 Determination of pyrraline

For the analysis of pyrraline, tofu, tempeh and seitan were fried and thereafter each raw and fried sample was freeze-dried. Soybeans and flour were analyzed without freeze-drying due to their low water content. Analysis were done semi-quantitatively by comparing the areas under the curve (AUC).

No change in the area under the curve can be verified for any from the tested products (see Figure 27, Figure 28 and Figure 29). None of the three meat analogs showed an increase during either preparation or frying in oil. Furthermore, no calibration curves were measured, which only allows semiquantitative conclusions.

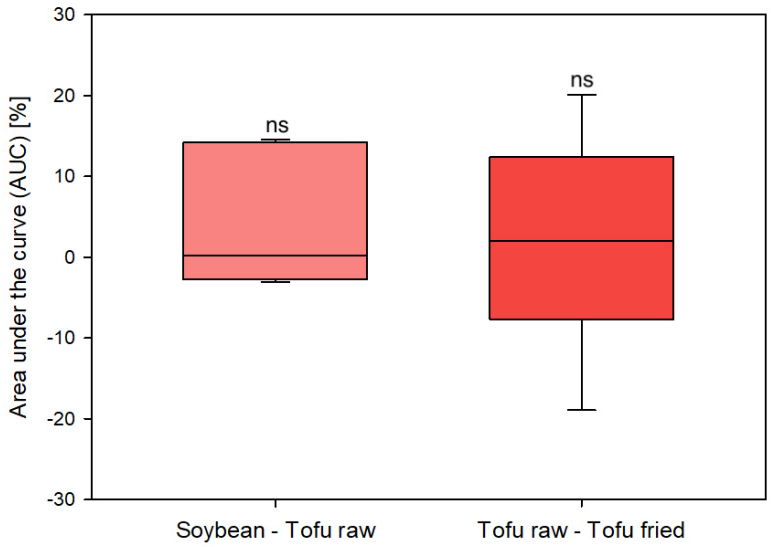

Figure 27: Change in AUC in the determination of pyrraline content from soybean to raw tofu and from raw tofu to fried tofu.

Shown is the change in the values of the area under the curve from soybean and raw and fried tofu. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, ns indicates no statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 3. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 2$; $n_{tofu\ raw} = 3$; $n_{tofu\ fried} = 3$).

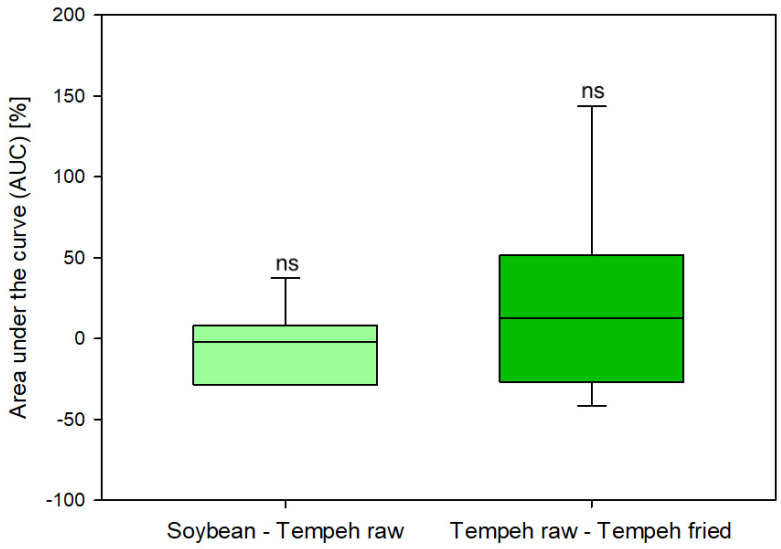

Figure 28: Change in AUC in the determination of pyrraline content from soybean to raw tempeh and from raw tempeh to fried tempeh.

Shown is the change in the values of the area under the curve from soybean and raw and fried tempeh. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, ns indicates no statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 3. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 2$; $n_{tempeh\ raw} = 3$; $n_{tempeh\ fried} = 3$).

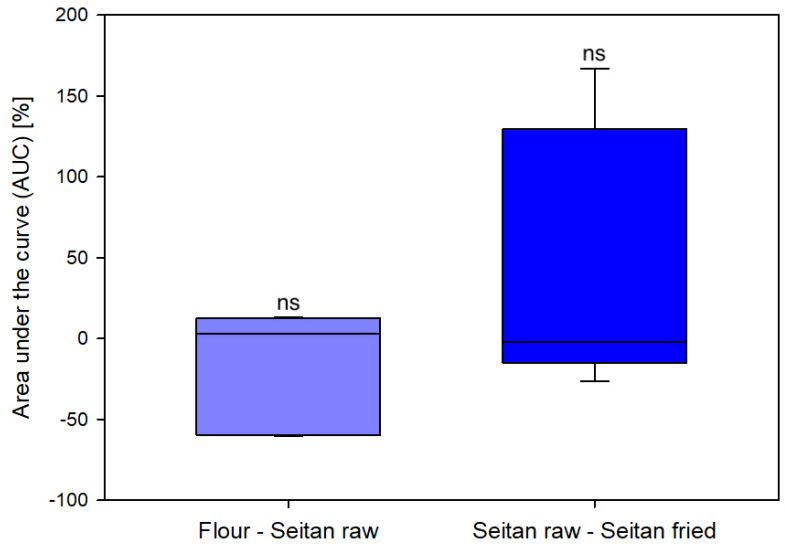

Figure 29: Change in AUC in the determination of pyrraline content from flour to raw seitan and from raw seitan to fried seitan.

Shown is the change in the values of the area under the curve from flour and raw and fried seitan. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, ns indicates no statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 3. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 2$; $n_{seitan\ raw} = 3$; $n_{seitan\ fried} = 3$).

All means of the differences between the AUC values resulting from the measurement of pyrraline are close to zero, which implies, that neither an increase nor a decrease in its concentration is taking place.

4.3.4 Determination of pentosidine

For the analysis of pentosidine, tofu, tempeh and seitan were fried and thereafter each raw and fried sample was freeze-dried. Soybeans and flour were analyzed without freeze-drying due to their low water content. Analysis were done semi-quantitatively by comparing the areas under the curve (AUC).

As shown in Figure 30, no significant change of the integrated peak area from soybean to raw tofu can be detected. Yet, an increase in pentosidine concentration can be observed due to the change in peak area from raw tofu to fried tofu.

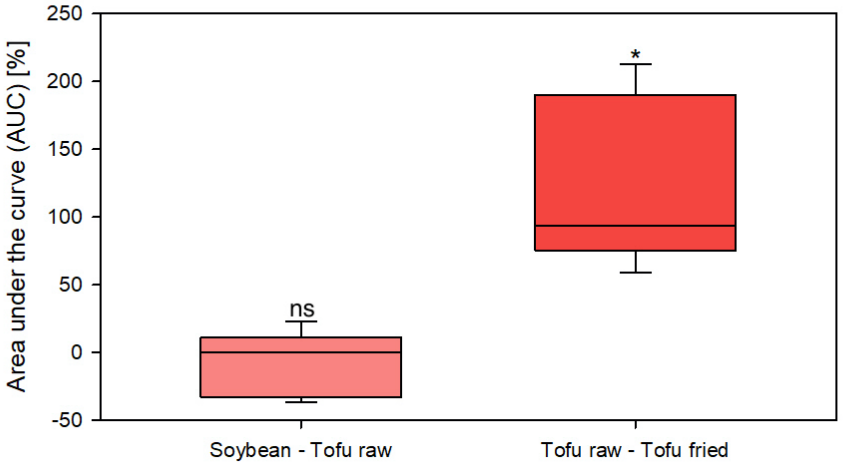

Figure 30: Change in AUC in the determination of pentosidine content from soybean to raw tofu and from raw tofu to fried tofu.

The graph shows the change in the values of the area under the curve from soybean and raw and fried tofu. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, ns indicates no statistical difference, * indicates a statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 4. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tofu\ raw} = 3$; $n_{tofu\ fried} = 3$).

The integrated peak area from soybean to raw tempeh shows a significant decrease, which indicates, that the pentosidine concentration in the sample has declined. By including the dry mass in the calculation, this trend might be contrary. As shown in Figure 31, the pentosidine concentration increases significantly while frying in oil.

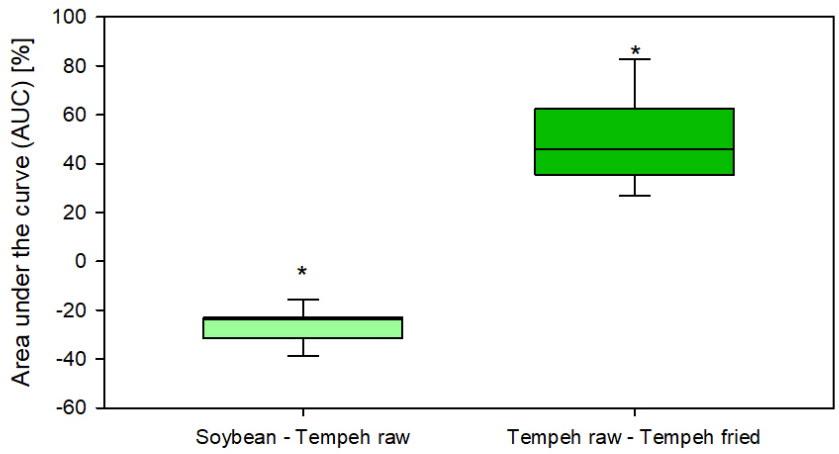

Figure 31: Change in AUC in the determination of pentosidine content from soybean to raw tempeh and from raw tempeh to fried tempeh.

Shown is the change in the values of the Area under the curve from soybean and raw and fried tempeh. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, * indicates a statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 4. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tempeh\ raw} = 3$; $n_{tempeh\ fried} = 3$).

An increase in pentosidine concentration can be described in Figure 32. The statistical analysis using One-way ANOVA indicates that there is a significant overall increase in the integrated peak area when transitioning from flour to raw seitan and from raw seitan to fried seitan. Although the increase in the integrated peak area from flour to raw seitan is more pronounced, a significant increase in the integrated peak area resulting from the frying process in sunflower oil can also be observed in seitan.

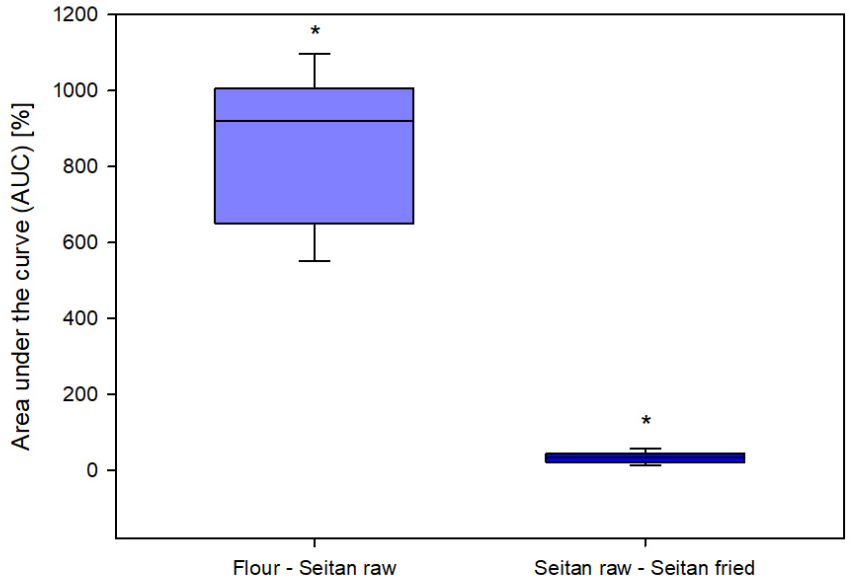

Figure 32: Change in AUC in the determination of pentosidine content from flour to raw seitan and from raw seitan to fried seitan.

Shown is the change in the values of the area under the curve from flour and raw and fried seitan. The y-axis shows AUC decrease (negative) or increase (positive) in %, the x-axis shows the analyzed samples. Differences were determined by One-way ANOVA, * indicates a statistical significance ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Further information can be found in Table Appendix 4. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 3$; $n_{seitan\ raw} = 3$; $n_{seitan\ fried} = 3$).

Due to the heating in oil of the tested meat analogs, it can be assumed that an increase in pentosidine concentration is measurable throughout. A significant change during the manufacturing step starting from the raw material (soybean or flour) can be observed significantly only for seitan and tempeh. The increase of the AUC, while an indicator of a total increase in pentosidine, does not provide any absolute quantification of its content, which should be considered for future studies.

4.3.5 Determination of lysine

For the analysis of lysine, tofu, tempeh and seitan were fried and thereafter each raw and fried sample was freeze-dried. The results were referred to the mass of the freeze-dried samples, for more precise comparison between the results. Soybeans and flour were analyzed without freeze-drying due to their low water content. To compare between the three meat analogues, values referred to the fresh samples were compared. Analysis was performed quantitatively using lysine standards.

As shown in Figure 33, lysine concentration significantly decreases in tofu while manufacturing and frying in oil. Lysine content seems to decrease consistently, which is also in line with the literature.[70]

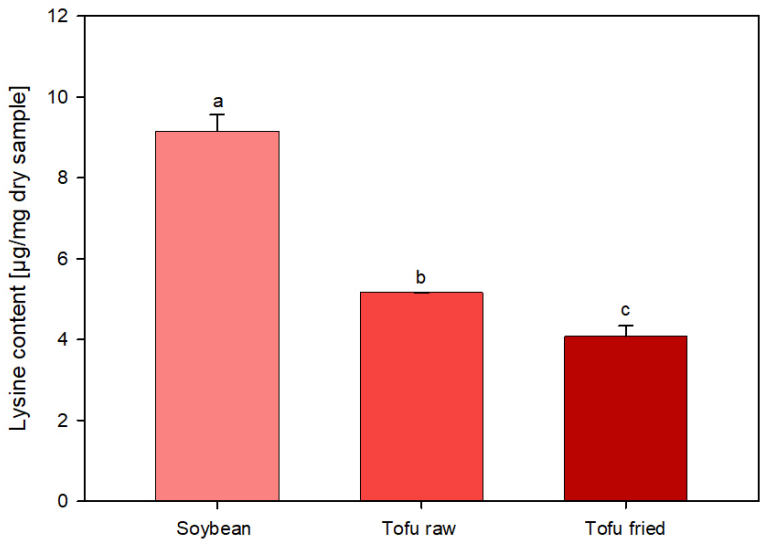

Figure 33: Lysine content in soybean, raw and fried tofu.

The graph shows the different lysine concentrations from soybean to fried tofu. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed meat analogs. Differences were determined by One-way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tofu\ raw} = 2$; $n_{tofu\ fried} = 3$)

The lysine content in soybean, raw tempeh and fried tempeh differ significantly from each other as shown in Figure 34. Cooking and fermentation of the soybean decreases the lysine concentration, as it does frying in oil. The trend of decrease in lysine content behaves similarly to tofu, as it can be noticed when comparing Figure 33 and Figure 34.

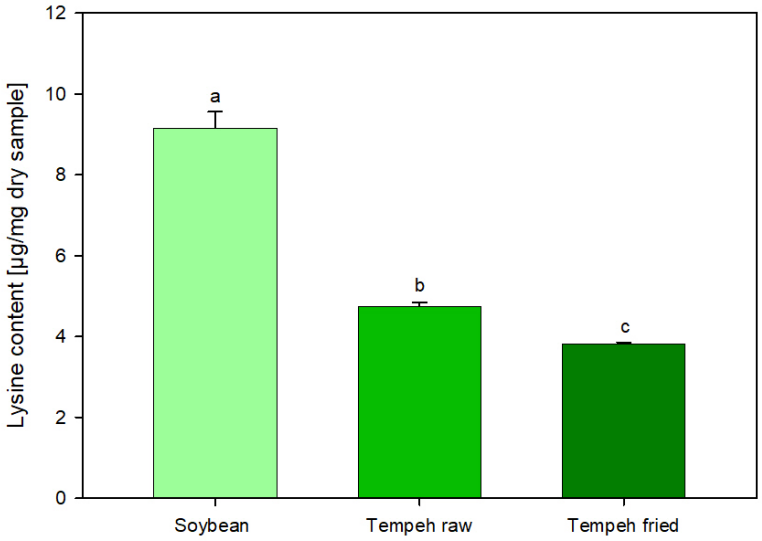

Figure 34: Lysine content of soybean, raw tempeh, and fried tempeh.

The graph shows the decrease in lysine concentration of soybean, tempeh raw and fried. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed meat analogs. Differences were determined by One-way ANOVA and indicated at a significance at ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tempeh\ raw} = 3$; $n_{tempeh\ fried} = 3$)

As illustrated in Figure 35, a steady increase in lysine concentration is detectable from preparing seitan out of flour and from frying seitan in oil. Therefore, it is clear that the leaching of the starch and cooking during production have a significant influence on the lysine content. Opposite to tofu and tempeh, the lysine concentration of seitan is increasing significantly. This correlates with the fact, that protein concentration is decreasing while preparation from tofu and tempeh out of soybean but increasing while preparation seitan out of flour. Both parameters show significantly higher values for soybeans compared to flour.

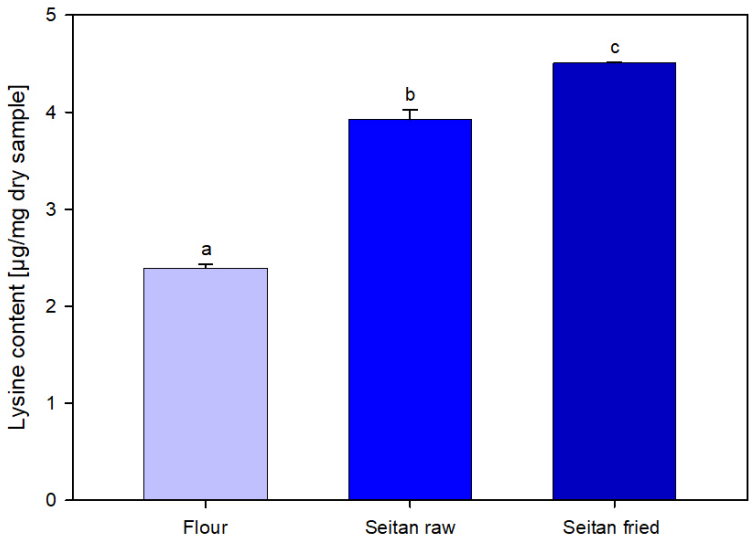

Figure 35: Lysine concentration on flour, raw seitan and fried seitan.

The graph shows the lysine concentration in the flour and raw and fried seitan. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed samples. The values are statistically compared using One-way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 2$; $n_{seitan\ raw} = 3$; $n_{seitan\ fried} = 2$)

As lysine concentration decreases because of the Maillard related reaction during the heating process, a decrease of the lysine content in all three meat analogues was expected. This effect is also suggested by Figure 33 and Figure 34. The nucleophilic nitrogen on the side chain of lysine is particularly reactive, reacts with reducing sugars, and is subsequently prone to form AGEs.[33] On the one hand, a decrease in lysine is detected for tofu and tempeh from soybean to raw meat analogues and from the raw to the fried meat analogues. On the other hand, the change in lysine content while manufacturing and preparing seitan is completely different.

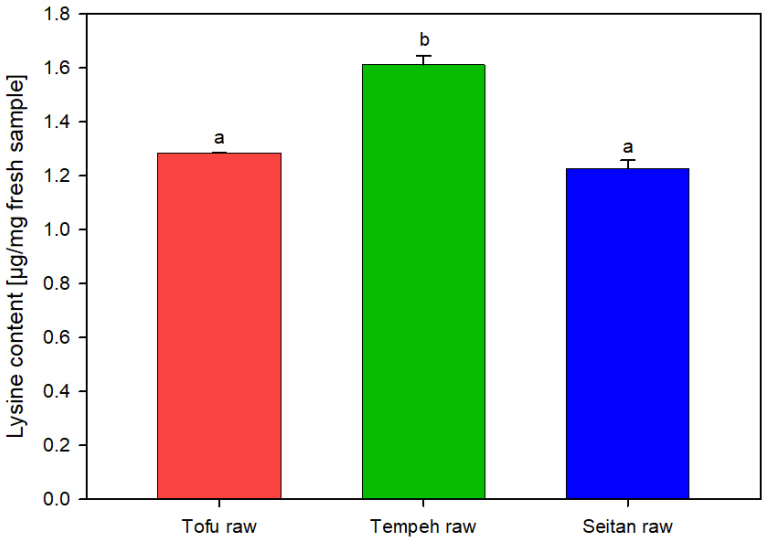

Figure 36: Lysine content in raw tofu, tempeh and seitan.

The graph shows the comparison of the lysine contents of the three raw meat analogues. Contents are referred to the fresh samples weight, prior to freeze-drying. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed meat analogs. The values are statistically compared using One-way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Significant outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{tofu\ raw} = 2$; $n_{tempeh\ raw} = 3$; $n_{seitan\ raw} = 3$)

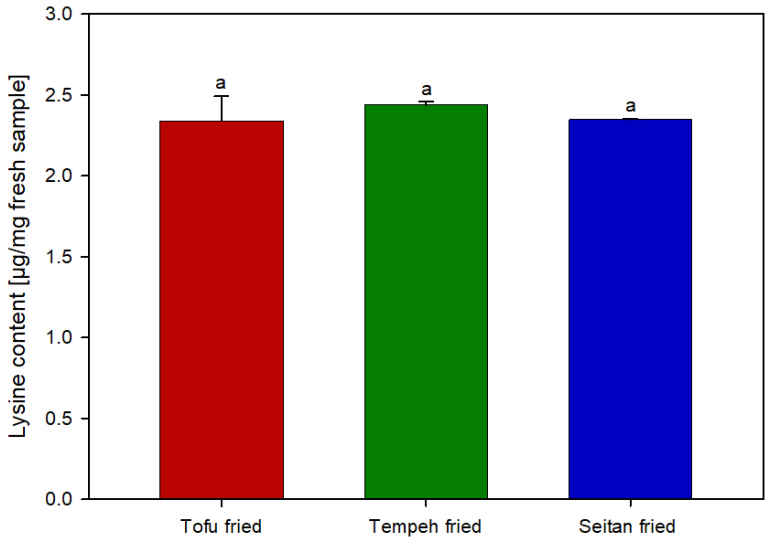

Figure 37: Lysine content in fried tofu, tempeh and seitan.

The graph shows the comparison of the lysine contents of the three meat analogues. Contents are referred to the fresh samples weight, prior to freeze-drying. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed meat analogs. The values are statistically compared using One-way ANOVA and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Different letters (a, b, c) indicate significant differences. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{tofu\ fried} = 3$; $n_{tempeh\ fried} = 3$; $n_{seitan\ fried} = 2$)

Comparing the three raw meat analogues, tempeh has the highest amount of lysine, whereas tofu and seitan do not differ from each other. As shown in Figure 36, the difference between the amount of lysine in raw tofu compared to raw seitan is not significantly higher, but tempeh shows a higher amount of lysine. Regarding the lysine content in all fried meat analogues, the contents are not significant different from each other. While the raw tempeh still has higher contents in contrast to seitan and tofu, no difference can be found within the fried samples, as shown in Figure 37.

4.3.6 Determination of acrylamide

For the determination of the acrylamide content, the meat analogues were each treated with two different conditions. Each of them was treated with frying at 130°C for 3.5 minutes and frying at 190°C for 1.5 minutes. According to the relevant literature, acrylamide is formed at particularly high temperatures.[57] Measurements were also carried out with the dry and raw soybeans, flour, and raw meat analogs. Determination by LC-MS failed to detect acrylamide (< LOD) in the soybean, flour, tempeh and seitan. Non-quantifiable but detectable results were shown by raw tofu and tofu treated at 130°C. Only tofu fried at 190°C showed contents above the LOQ (see Table 7).

Table 7: Acrylamide content
The table shows the data of the acrylamide measurement via LC-MS. Outliers were excluded from the calculations. LOD and LOQ were calculated by using the standard deviation of the response and the slope using the calibration curve described in **Fehler! Verweisquelle konnte nicht gefunden werden.**. Further information can be found in Table Appendix 5.

Sample	Mean [µg/kg]	Standard deviation [µg/kg]	Relative standard deviation [%]
Soybean	< LOD	-	-
Flour	< LOD	-	-
Tofu raw	< LOQ	-	-
Tofu 130°C, 3.5 min.	< LOQ	-	-
Tofu 190°C, 1.5 min.	190.336	14.950	7.85
Tempeh raw	< LOD	-	-
Tempeh 130°C, 3.5 min.	< LOD	-	-
Tempeh 190°C, 1.5 min.	< LOD	-	-
Seitan raw	< LOD	-	-

Sample	Mean [µg/kg]	Standard deviation [µg/kg]	Relative standard deviation [%]
Seitan 130°C, 3.5 min.	< LOD	-	-
Seitan 190°C, 1.5 min.	< LOD	-	-

However, there is evidence, that during the process of preparing the tofu out of soybeans, the acrylamide content is increasing, because of the change from LOD in the soybeans LOQ in the raw or the at 130°C fried product. To assess the statistical significance of potential differences between the reading obtained from tofu fried at 190°C and the determined limit of quantification (LOQ), a paired t-test was conducted. A statistically significant (α=0.01) elevation of the acrylamide content in tofu that was subjected to heating at 190°C was detected. This finding means that the process of frying tofu is associated with a significant increase in acrylamide content.

5. Discussion

The experiments were conducted to evaluate the protein quality of different meat analogues like tofu, tempeh and seitan. All three products showed differences in their constitution and the influence of their manufacturing steps. Protein contents of flour, soybean, raw tofu, raw tempeh and raw seitan were determined, in order to provide a better comparison between the products, since protein is the starting product of all contaminants analyzed. On the other hand, lysine is an reactive amino acid that easily reacts on with its alkaline side chain, preferentially with reducing sugars, in the course of the Maillard reaction.

5.1 Protein content

According to Bakaloudi et al. (2021),[13] there is a significant deficit in protein intake on a vegan diet compared to an omnivorous lifestyle. Where the total energy intake is lower for vegans, the consumption of protein is slightly below the recommended limit of 15% of total energy intake. Food groups like vegetables, fruits or cereals are known to have a lower protein concentration than milk, dairy products or meat.[13] Meat analogues consisting of soy or wheat are regarded as a good alternative to meat and meat products. The protein content of tofu (12.49 ± 2.11g/100 g food) was the lowest of the three analyzed meat analogues. Protein content may vary depending on the specific type of tofu (silken tofu, regular tofu, firm tofu,…). Wang (1983) reported a protein content of 7.5 g/100g of food for a very soft type of tofu.[15] Considering that the analyzed tofu is a firmer variety, the observed higher protein content is a justifiable finding. The content is comparable with the amount of protein in Frankfurters (sausage made from pork) (12.4 g/100 g food). Compared to beef steaks (21.2 g/100 g food) or other meats, the protein content in tofu is lower. Tempeh with a total protein concentration of 18.53 ± 1.71g/100 g food has, however, a higher protein content which can be compared to the total amount of protein in chicken drumsticks (18.2 g protein/100 g food). Compared to the numerous of meat dishes like meat of hare, lamb or duck, the protein concentration in tempeh is similar. The highest protein meat substitute analyzed is seitan, with 24.05 ± 0.26 g/100 g food, comparable to the protein concentration in turkey breast (24.1 g protein/100 g food). [71]

It is reasonable to assume, that the consumption of meat analogues improves the supply of protein, due to the similar protein concentrations between meat and its substitutes. Seitan has an even higher protein content than numerous meat products and can therefore significantly improve the protein supply. Additionally, with a vegan diet, the total fat intake is described as lower, and significant differences have been found involving a

reduced intake of monounsaturated and saturated fatty acids in vegan diets, which is beneficial against numerous lifestyle-associated diseases.[13]

5.2 Protein oxidation

To determine the degree of protein oxidation, the DNPH method was used to monitor the carbonyl content. The photometric method used is a quick and easy tool to estimate the degree of protein oxidation in food.[25]

While results from the carbonyl content showed a steadily increase in tofu from soybean to fried meat analogue, tempeh and seitan did not show a consistent trend. Soglia et al. also described an increase in protein oxidation while cooking meat at 100°C for 10 minutes.[66] Thus, it is obvious that protein oxidation happens during the production of tofu by cooking of the soy milk. All the three examined samples have in common that a significant increase could be observed from raw to fried meat substitute. Therefore, the impact of frying in oil at 130°C has an effect on protein oxidation, whereas the influence of the manufacturing of the meat analogues depends on the individual characteristics of said manufacturing. Additionally, tempeh showed a decrease of carbonyls from soybean to the raw meat analogue, but no significant difference between flour and seitan was found, which deems the seitan manufacturing process as the mildest of the three evaluated meat analogues in regards to protein oxidation, as it showed the least impact.

Fried tofu showed the highest amount of total carbonyls/protein: fried tofu had 24.378 ± 6.980 nmol/mg protein. Prior examination has documented up to 9.14 ± 0.11 µmol/mg protein in soy protein isolates (by storing if for 30 days)[72] and 3.10 ± 0.54 nmol/mg protein in bovine.[66] Even though soy protein isolate undergoes a more extensive processing compared to tofu, the higher carbonyl values detected in tofu suggest a greater degree of protein oxidation. This is because the production of protein isolate might involve the removal of oxidized protein. 4.845 ± 0.532 nmol carbonyl/mg protein, and 5.508 ± 0.838 nmol carbonyl/mg protein were found for fried tempeh and fried seitan, respectively. In contrast to tofu, these amounts were significantly lower, which is considered to be more beneficial for health. Compared to other foods consumed at similar levels (from fresh pork: 4.5 nmol carbonyls/mg protein to cooked bacon: 80 nmol carbonyls/mg protein), the carbonyl content in tofu, tempeh or seitan is depending on the meat average or lower.[27]

Guan et al. (2021) described the influence of the growing conditions and species of the soybeans to the tofu constitution and its reactivity while cooking. Because of the variety of possible soybeans and manufacturing processes of tofu, the levels of micro and

macronutrients may vary.[73] Future work should therefore consider this information, including the genotype of raw material used for the meat analogues as well, which allows a comparison between the types of tofu, tempeh and seitan. While there are currently no established regulatory limits for carbonyl levels in food, it is advisable to limit its consumption due to potential health concerns. Additionally, tofu, tempeh and seitan were prepared freshly and stored at -20°C in this study. However, storage has a decisive impact on protein oxidation, it should be noted that ready-to-eat products in stores might be stored for weeks or even months. Moreover, the conventionally products on the market might have a further degree of processing, what can lead to a stronger protein oxidation.[72]

Limitations for determination of the carbonyl content with photometric DNPH-method are described in Christos et al. (2018). Firstly, it is possible that a high percentage of protein may be lost due to the washing steps of the protein pellet. Recovery rates of 24% are described, which can lead to a change in the final results. Secondly, a high variability in the DNPH assay is expected, since the proteins are present as pellets. Due to the protein structure created by the addition of TCA, carbonyl groups might not be freely accessible for DNPH and might therefore react worse than carbonyl compounds free in solution.[28] Nonetheless, the photometric method using 2,4-dinitrophenylhydrazine (DNPH) is still a widely utilized and straightforward approach for assessing the degree of protein oxidation in food products, highly effective in the quantification of carbonyl content in food samples, which is crucial for determining the level of oxidized protein present, as excessive consumption of oxidized proteins has been associated with a range of adverse health effects.[27]

5.3 Glycation end products (AGEs)

AGEs are a result from the Maillard associated Amadori compounds. To estimate the amount of heat-induced Maillard products, CML, CEL, pentosidine, pyrraline and acrylamide were analyzed. Since lysine is very likely to react during heating due to its amino-containing side chain, forming most of the above-mentioned compounds, lysine content was also determined. All measurements were carried out using LC-MS.[74]

Lysine is an essential amino acid, and its low intake can cause kidney stones, physiological disorders, or slow growth. As it reacts during food processing, the structural changes related to lysine might cause different issues. Firstly, the modified lysine is not available for metabolism anymore, contributing to the health issues as mentioned above. Secondly, proteolytic enzymes might not access properly to protein, therefore hindering protein digestion in the body. However, this work is mainly focused on the issue that the

loss of lysine is usually accompanied by an increase in Maillard associated products.[75] On the one hand, the deficit of lysine brings the above mentioned health disadvantages, on the other hand, products resulting from the Maillard reaction can have an influence on the development of metabolic diseases like Alzheimer, diabetes and renal disorders.[51] Since can interact with numerous of compounds, such as reducing sugars, fats and their oxidation products, polyphenols, vitamins, food additives and another amino acids. During heat induced Maillard reaction, lysine is mostly reacting with reducing sugars resulting in the formation of a Schiff base.[76]

While tofu and tempeh showed a steady decrease in lysine during manufacturing and producing step, the lysine content in seitan was steadily increasing. Between all three meat analogues, no significant difference could be described when referred to the fresh weigh to the products, although their water content is different between each other. Results showed that there is a similar amount of lysine in all three fried products, although values in soybeans compared to flour and raw meat analogues are not the same: dry soybeans showed a lysine content of 9.158 ± 0.407 µg/mg, whereas flour only consisted out of 2.392 ± 0.041 µg/mg. These results are consistent with the decreasing protein content from soybean to tofu or tempeh and the increasing protein content from flour to seitan. The lysine content of the fried fresh analyzed samples did not show any significant differences between them, with an available lysine concentration of 2.341 ± 0.152 µg/mg for tofu, 2.439 ± 0.022 µg/mg for tempeh and 2.129 ± 0.314 µg/mg for seitan. These contents are five to ten times lower than the lysine contents in conventional meat and meat products, whereas the protein concentrations in all three meat analogues are comparable to the content of some meat products. Given the distinct origins of meat and plant-based meat analogs, it is anticipated that there will be differences in their amino acid composition, particularly in regards to lysine content.[71] As discussed above, different genotype and conditions of growing of flour or soy would maybe change the results and may impact the amino acid composition.[73]

There is a variation in the concentrations of CML, CEL, pyrraline, and pentosidine, across the stages of raw material, raw products, and fried products. The observed heterogeneity in the samples can be explained by the various factors affecting their production, including the manufacturing process and the impact of heat treatment. Furthermore, it is important to note that the formation of pentosidine, a compound known to contribute to protein crosslinking, can not only occur via the reaction involving lysine but also requires arginine for its formation.[52] However, the exact loss in the levels of arginine were not characterized in this study. As CEL, pyrraline and pentosidine were measured semi quantitatively, exact values for concentrations could not be determined. Nonetheless, a

trend in the concentration for the lysine associated AGEs in tofu could be described. CML and pentosidine showed a significant increase from the soybean to the fried tofu. While an increase in CML could be detected from the soybeans to the raw tofu, an increase in pentosidine was only noticeable after frying in oil at 130°C. In tempeh, the concentration in CML and CEL experienced an increase, but pentosidine in the beginning decreased from soybean to raw tempeh, but then increased during frying. From soybean to the fried tempeh there was no significant change in the pyrraline content, but the overall content of total AGEs, however, was increasing during the frying process. Based on the results, it is reasonable to hypothesize that the levels of CML in fried tempeh can potentially be lowered by utilizing lower cooking temperatures during preparation. However, it is noteworthy that the levels of CML in tofu tended to increase after the manufacturing process involving soybeans, and while frying did not appear to have a significant effect on CML levels in tofu, it may still be prudent to employ gentler cooking methods, particularly when preparing tempeh. The values for seitan showed a different effect: while pentosidine content was significantly increased from flour to raw seitan and from raw to fried seitan, CEL and pyrraline contents did not show any significant changes. CML content firstly decreased from flour to raw seitan, but the concentration of the fried seitan did not show any significant difference compared to flour or raw seitan. In the group of fried samples, tempeh displayed the highest concentration of CML, an extensively researched AGEs implicated in the pathogenesis of cardiovascular diseases, sarcopenia, and renal disorders. Given the well-established health risks associated with dietary CML, efforts should be made to limit its intake, making it imperative to pay attention to CML levels in food products.[10]

A value of 24.2 ± 91.6 µg CML/g food was described in meat dishes in previous literature.[40] Regarding the results from this study, CML content in all three meat analogues are slightly lower compared to values described in meat and fish. Fried products, taking into account their water content, showed following CML contents: 17.735 ± 0.391 µg/g food for tofu, 22.372 ± 1.486 µg/g food for tempeh and 5.187 ± 0.128 µg/g food for seitan. While the lysine content from the literature is 5 to 10 times higher in meat,[71] the concentration of CML shows a smaller difference in content between meat and plant-based meat analogues. However, these findings suggest that substituting meat with plant-based meat analogues, particularly seitan, can result in a significant reduction in CML consumption, with seitan containing only about one fifth of the CML content compared to meat. Overall, these observations provide scientific evidence supporting the benefits of replacing meat with plant-based alternatives in terms of lower CML consumption.[40]

Since a change in CEL and pentosidine concentration in tempeh and pentosidine in tofu and seitan could be described in this study, future work should therefore include a quantitative detection of pentosidine and CEL in the meat analogues. Quantitative analysis is needed to confirm if a much higher amount of AGEs in meat is truly present compared to the meatless analogues.

Where lysine, CML, CEL, pentosidine and pyrraline were determined simultaneously, the acrylamide content was measured using a different method. Acrylamide is a genotoxic carcinogen which is formed during the heat processing of food, especially potatoes chips, bread and bakery goods.[16] Prior work has documented a higher acrylamide consumption for vegans compared to people with omnivore diet. The same work has described meat substitutes like tofu possess an acrylamide content between 15 and 100 µg/kg food, whereas meals with meats show a content between 29 and 202 µg/kg food.[16] If compared to the acrylamide content determined in this work for tofu (fried at 190°C) with 190.336 ± 14.950 µg/kg food, the measured acrylamide concentration is in the range of the paper described.

Results are limited by the LOD and LOQ, since the amount of acrylamide in raw and fried (at 130°C) meat surrogates are not measurable at all. Furthermore, acrylamide content in tempeh and seitan fried at 190 °C was not detectable either. By adapting the method, the LOD could be decreased and therefore the acrylamide content could be determined for tempeh and seitan as well. Nonetheless, the present results indicated that, when subjected to frying at 190°C, tempeh and seitan did not evince any levels of acrylamide. Although the concentration of acrylamide in tofu (190.336 ± 14.950 µg/kg food) should not be overlooked, the absence of formation of this potentially harmful compound in tempeh and seitan renders them more favorable alternatives to meat and tofu.

To summarize, both protein oxidation products and Maillard products can be influenced by manufacturing and preparation of meat analogues. The increase of substances unfavorable to health due to heat treatment and food processing was suspected and confirmed. Fried tofu reported the highest carbonyl content, which indicates the highest degree of protein oxidation, accompanied by the highest level of acrylamide. Regarding CML, tempeh showed the highest concentrations, which leads to the assumption that Maillard reaction related products are higher in soy-based meat surrogates compared to wheat-based ones. With respect to CML content, it is worth noting that CML is produced during the manufacturing of tofu, whereas in tempeh, it is generated during the frying process at a temperature of 130°C. Therefore, it is possible that lowering the frying temperature or utilizing gentler frying conditions could potentially affect the levels of CML

in fried tempeh. Consequently, raw tempeh tends to have lower levels of AGEs, which may confer certain health benefits in comparison to tofu. In addition to that, this study showed that the most common meat analogue, tofu, is more prone to protein oxidation and generation of heat related compounds than other alternative plant-based protein source. Given the continuously expanding market for meat alternatives and the growing consumer demand for plant-based products with high protein content, it is imperative to explore sources other than soy for the development of meat analogues. The findings presented in this study provide compelling evidence in support of whey as a potentially superior alternative to soy for this purpose. Further studies are needed to confirm the lower AGEs content in seitan and other wheat-based meat analogues. Moreover, other analogues out of other raw materials like peas or jack fruits could be also analyzed and compared to soy- and wheat-based products. In addition, future work should compare freshly made meat substitutes to these provided in supermarkets, which are highly processed and stored at 5°C for weeks or months.

6. Conclusion

Tofu, tempeh and seitan were evaluated in terms of protein oxidation and Maillard related products during food processing. The aim of the study was to compare the raw material (flour or soybean) and to monitor the change in concentration of protein and Maillard products from raw material to raw product (tofu, tempeh, seitan) and from raw product to fried product. Even though the heating process during manufacturing of these three meat analogues out of soybean or flour differ from each other, all products were fried in oil with the same conditions. Typical frying conditions (130°C, 3.5 minutes) were chosen to imitate typical frying conditions at home.

Carbonyl content was determined using the DNPH method to estimate the extent of protein oxidation.

Since lysine is a reactive amino acid and is likely to interact with reducing sugars during heat-induced Maillard reactions, the lysine content was determined as well. An LC-MS method was chosen which could monitor its content with CML, CEL, pentosidine and pyrraline at the same time. While CML and lysine were determined quantitatively, CEL, pentosidine and pyrraline were determined semi-quantitatively and the evolution of the components was compared between the samples.

Moreover, acrylamide was measured via LC-MS as well, for which, in addition to the usual conditions, the samples were also fried in oil at 190°C for 1.5 minutes.

Carbonyl content in all three meat analogues showed a significant increase from the raw to the fried product in oil at 130°C. Similar results could be observed for pentosidine in all three products. A significant increase in CEL could only be seen in tempeh, whereas in tofu and seitan no significant change could be found. Pyrraline did not show any change in concentration in none of the tested products. In conclusion, it can be assumed that the heating process during manufacturing and preparation of the meat analogues leads to an increase in protein oxidation and Maillard reaction products but further studies are needed to include meat substitutes out of other sources such as peas or jack fruit. Moreover, the impact of highly processed plant-based products and the storage in supermarkets should be considered as well.

7. Abstract

A vegan or vegetarian lifestyle can lead to a deficit in protein, which is why the market for meat substitutes is booming. Since, meat is an important source for protein, vegans show an overall deficit in protein intake. Therefore, protein-rich meat analogues such as tofu, tempeh or seitan are consumed as a supplement in vegan diets.

Heating foods has an effect on both the oxidation of proteins and the formation of heat-induced Maillard products, such as the so-called advanced glycation end-products (AGEs). Both chemical processes in food have an effect on both food quality health when consumed. Therefore, the meat substitutes tofu, tempeh and seitan were analyzed photometrically for carbonyls determination as an indicator of protein oxidation and by LC-MS for determination of different AGEs. Due to the different methods and the different raw materials (flour or soy) used to produce the meat analogues, it was expected that the content of the analytes investigated would not show the same tendency as a result of the processing steps and that an overall increase in the content between the fried meat analogues could be detected at the end.

All three meat analogues were shown to change through the process from raw material to meat analogue and during frying in terms of protein oxidation and levels of heat-induced Maillard products. Protein oxidation appeared to be most advanced in tofu, which may be due to the manufacturing process needed for its production. The carbonyl content in the fried tempeh was significantly different from that of the fried tofu, leading to the assumption that cooking the whole soybeans in water has a lower input on protein oxidation than cooking a grinded soybeans emulsion. In addition, the highest CML contents could be found in tempeh and the semi-quantitative analysis results also suggested the formation of CEL from soybean to raw tempeh and from raw to fried tempeh. Seitan turned out to be the product with the tendency to have the lowest protein oxidation and AGEs formation, as well as the highest protein content. This fact leads to the suggestion that soy might not be the best material for manufacturing meat analogues. Therefore, this work arises the question whether there are other raw materials that are more suitable for the production of meat analogues compared to soy, in regards to health and food technology aspects.

8. Zusammenfassung

Eine vegane oder vegetarische Lebensweise kann zu einem Defizit an Eiweiß führen, weshalb der Markt für Fleischersatzprodukte boomt. Da Fleisch bei omnivorer Ernährung eine wichtige Eiweißquelle ist, weisen Veganer insgesamt ein Defizit bei der Eiweißaufnahme auf. Aus diesem Grund werden eiweißreiche Fleischersatzprodukte wie Tofu, Tempeh oder Seitan als Ergänzung zu einer veganen Ernährung verzehrt.

Das Erhitzen von Lebensmitteln wirkt sich sowohl auf die Oxidation von Proteinen als auch auf die Bildung von hitzebedingten Maillard-Produkten, wie den so genannten advanced glycation end-products (AGEs), aus. Beide chemischen Prozesse in Lebensmitteln wirken sich sowohl auf die Lebensmittelqualität als auch bei Verzehr auf die Gesundheit aus. Fleischersatzprodukte wie Tofu, Tempeh und Seitan wurden daher photometrisch zur Bestimmung von Carbonylen als Indikator für die Proteinoxidation und mittels LC-MS zur Bestimmung verschiedener AGEs analysiert. Aufgrund der unterschiedlichen Methoden und der verschiedenen Rohstoffe (Mehl oder Soja), die zur Herstellung der Fleischanaloga verwendet wurden, wurde erwartet, dass der Gehalt der untersuchten Analyten infolge der Verarbeitungsschritte nicht die gleiche Tendenz aufweisen würde und dass am Ende eine allgemeine Zunahme des Gehalts zwischen den gebratenen Fleischanaloga festgestellt werden könnte.

Es zeigte sich, dass sich alle drei Fleischanaloge während des Prozesses vom Rohmaterial zum Fleischanalog und während des Bratens in Bezug auf die Proteinoxidation und den Gehalt an hitzeinduzierten Maillard-Produkten verändern. Die Proteinoxidation schien bei Tofu am weitesten fortgeschritten zu sein, was auf den für seine Herstellung erforderlichen Prozess zurückzuführen sein könnte. Der Carbonylgehalt in gebratenem Tempeh unterschied sich signifikant von dem des gebratenen Tofus, was zu der Annahme führt, dass das Kochen der ganzen Sojabohnen in Wasser einen geringeren Einfluss auf die Proteinoxidation hat als das Kochen der Emulsion. Darüber hinaus wurden die höchsten CML-Gehalte in Tempeh gefunden, und die Ergebnisse der semiquantitativen Analyse deuten ebenfalls auf die Bildung von CEL von Sojabohnen zu rohem Tempeh und von rohem zu gebratenem Tempeh hin. Seitan erwies sich als das Produkt mit der geringsten Tendenz zur Proteinoxidation und AGE-Bildung sowie mit dem höchsten Proteingehalt. Diese Tatsache nahe, dass Soja möglicherweise nicht optimal für die Herstellung von Fleischanalogen ist. Das führt zu der Annahme, dass weitere Rohstoffe, neben Weizen (im Gegensatz zu Soja), gesundheitliche und lebensmitteltechnische Vorteile für die Herstellung von Fleischanaloge aufweisen.

9. List of literature

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Appendix

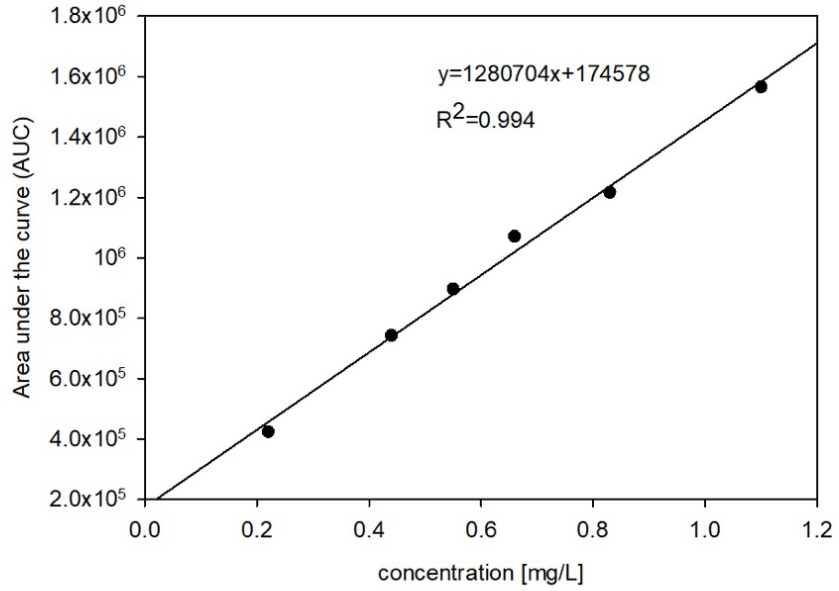

Figure Appendix 1: Calibration curve CML
For the calibration curve, 6 standards with the concentrations 0.02 - 1.10 mg/L were used. The resulting linear regression (R^2=0.994) was used for the quantification of the CML concentration in the samples.

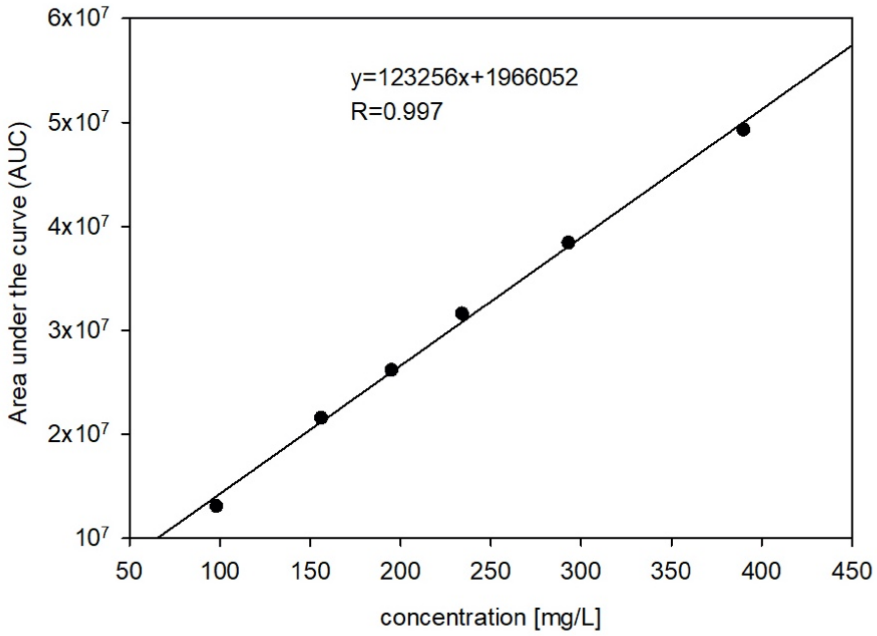

Figure Appendix 2: Calibration curve lysine
For the calibration curve, 6 standards with the concentrations 98 - 390 mg/L were used. The resulting linear regression (R^2=0.997) was used for the quantification of the lysine concentration in the samples.

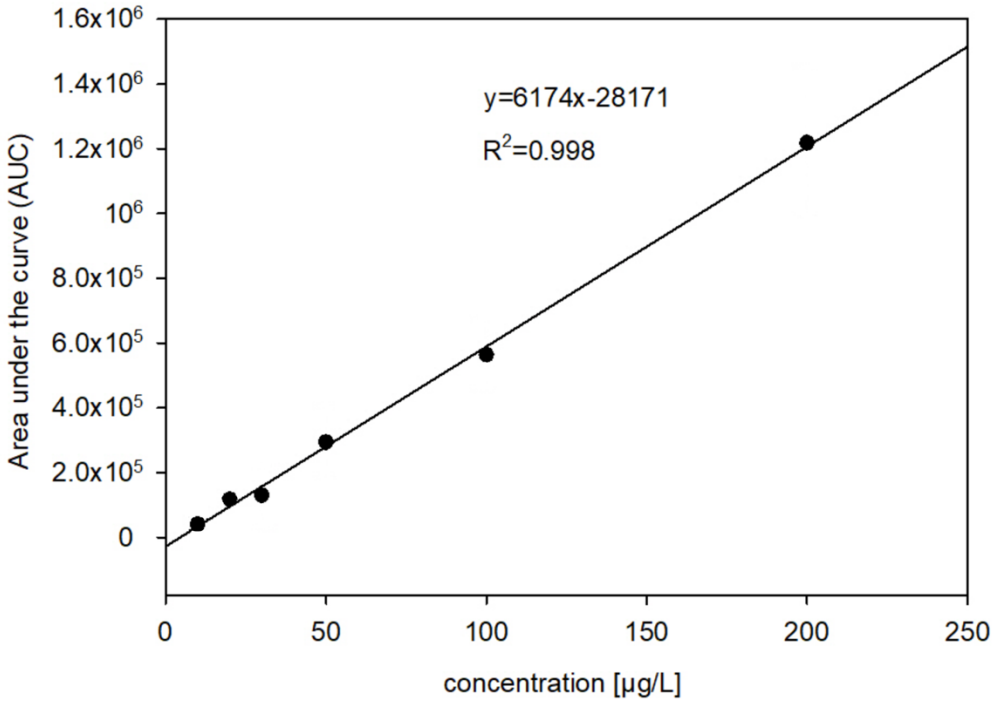

Figure Appendix 3: Calibration curve acrylamide
For the calibration curve, 6 standards with the concentrations 10 – 200 µg/L were used. The resulting linear regression (R^2=0.998) was used for the quantification of the acrylamide concentration in the samples.

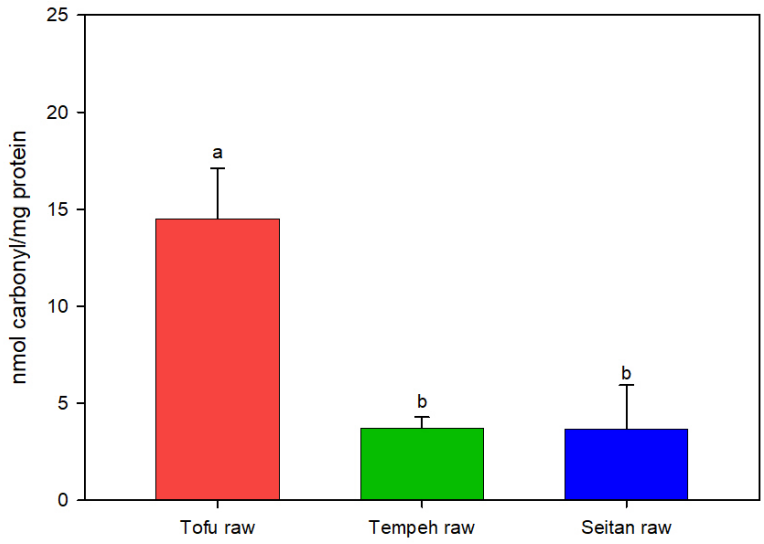

***Figure Appendix 4*: Comparison of the carbonyl content in raw meat analogues**
Shown is the comparison between raw tofu, tempeh and seitan before frying. Differences were determined by ANOVA on ranks and indicated at a significance of ($p<0.05$). This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data are shown as mean ± standard deviation ($n_{tofu\ raw}$ = 25; $n_{tempeh\ raw}$ = 24; $n_{seitan\ raw}$ = 23).

Table Appendix 1: Dry mass by gravimetric determination after freeze-drying.
The table summarizes all dry masses of raw and roasted meat analogues. Outliers were excluded from the calculation. Data are shown as mean ± standard deviation ($n_{tofu\ raw}$ = 3; $n_{tofu\ fried}$ = 2; $n_{tempeh\ raw}$ = 3; $n_{tempeh\ fried}$ = 3; $n_{seitan\ raw}$ = 3; $n_{seitan\ fried}$ = 2).

Sample	Mean [%]	Standard Deviation [%]
Tofu raw	24.9	2.8
Tofu fried	57.4	0.1
Tempeh raw	33.9	5.7
Tempeh fried	63.9	8.7
Seitan raw	31.3	0.8
Seitan fried	52.1	0.3

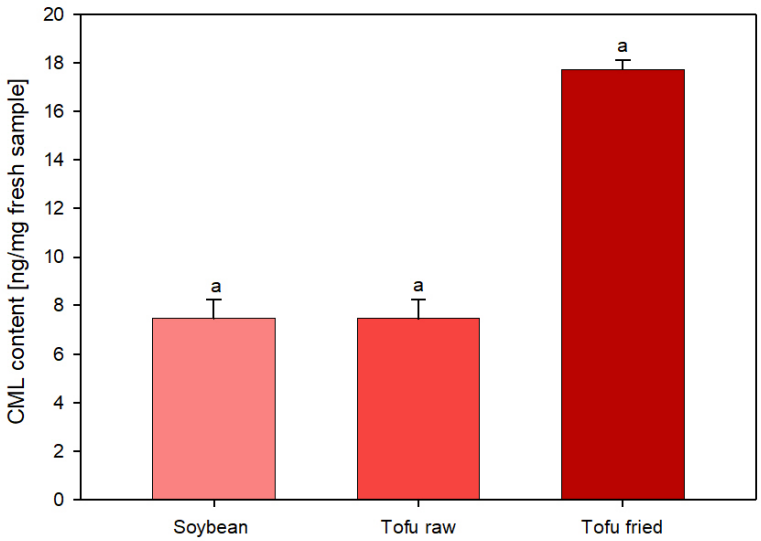

Figure Appendix 5: CML content in soybean, fresh raw tofu and fresh fried tofu
The graph shows the increase of CML concentration during the manufacturing and processing of tofu. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by ANOVA on Ranks and indicated at a significance of ($p<0.05$) with lower-case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean}$ = 3; $n_{tofu\ raw}$ = 3; $n_{tofu\ fried}$ = 3).

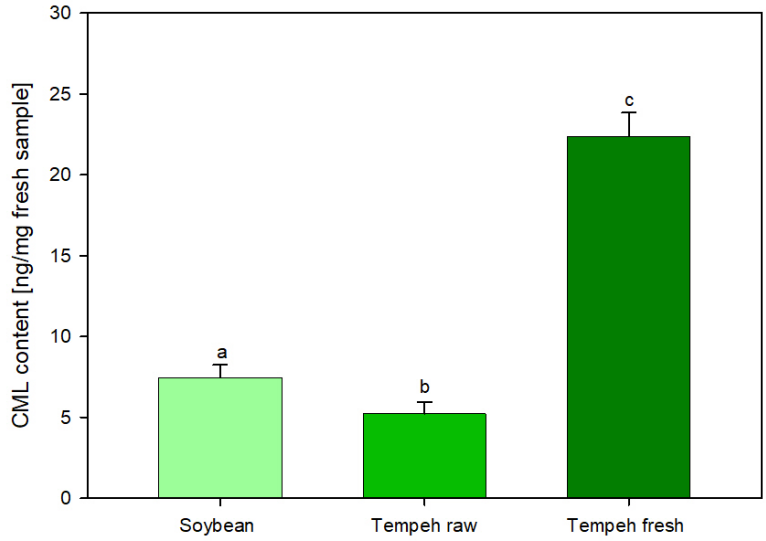

Figure Appendix 6: CML content in soybean, fresh raw tempeh and fresh fried tempeh.
Shown is the change of CML concentration during the manufacturing and processing of tempeh. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$) with lower case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean}$ = 3; $n_{tempeh\ raw}$ = 3; $n_{tempeh\ fried}$ = 3).

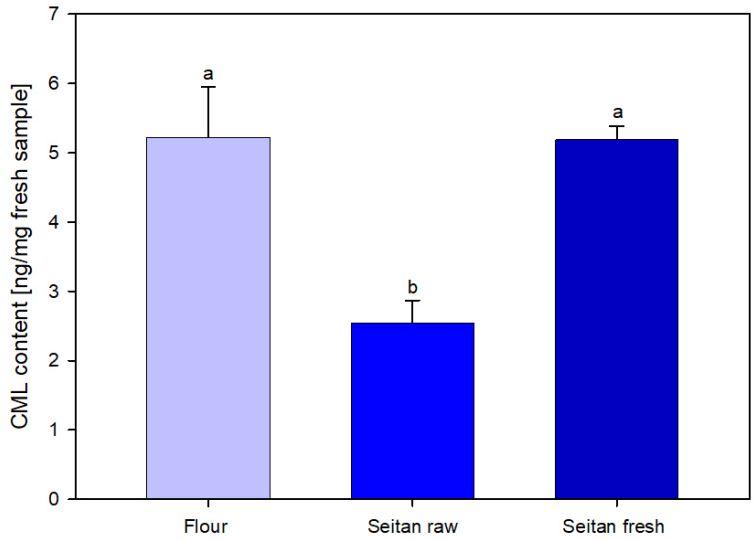

Figure Appendix 7: CML content in flour, fresh raw seitan and fresh fried seitan.
Shown is the change in CML concentration during the manufacturing and processing of tempeh. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$) with lower case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation (n_{flour} = 3; $n_{seitan\ raw}$ = 2; $n_{seitan\ fried}$ = 2).

Table Appendix 2: Area under the curve (AUC) for the determination of the CEL content.
Shown is the integrated Peak area for the determination of the CEL content. Outliers were excluded from the calculation ($n_{soybean}$ = 2; n_{flour} = 3; $n_{tofu\ raw}$ = 3; $n_{tofu\ fried}$ = 3; $n_{tempeh\ raw}$ = 3; $n_{tempeh\ fried}$ = 3; $n_{seitan\ raw}$ = 3; $n_{seitan\ fried}$ = 3).

Sample	Mean (AUC)	Standard deviation (AUC)
Soybean	9955976	47949
Tofu raw	17060055	3758958
Tofu fried	21058898	3125269
Tempeh raw	13288279	193781
Tempeh fried	21360711	1149138
Flour	611006	34298
Seitan raw	2508168	1160957
Seitan fried	3042816	65592

Table Appendix 3: Area under the curve (AUC) for the determination of the pyrraline content.
Shown is the integrated Peak area for the determination of the pyrroline content. Outliers were excluded from the calculation ($n_{soybean}$ = 2; n_{flour} = 2; $n_{tofu\ raw}$ = 3; $n_{tofu\ fried}$ = 3; $n_{tempeh\ raw}$ = 3; $n_{tempeh\ fried}$ = 3; $n_{seitan\ raw}$ = 3; $n_{seitan\ fried}$ = 3).

Sample	Mean (AUC)	Standard deviation (AUC)
Soybean	2287482	6315
Tofu raw	2376250	209256
Tofu fried	2398959	274697
Tempeh raw	2339366	762921
Tempeh fried	2610346	1180725
Flour	2937676	13512
Seitan raw	2508168	1160957
Seitan fried	2848405	370971

Table Appendix 4: Area under the curve (AUC) for the determination of the pentosidine content.
Shown is the integrated Peak area for the determination of the pyrroline content. Outliers were excluded from the calculation ($n_{soybean}$ = 3; n_{flour} = 3; $n_{tofu\ raw}$ = 3; $n_{tofu\ fried}$ = 3; $n_{tempeh\ raw}$ = 3; $n_{tempeh\ fried}$ = 3; $n_{seitan\ raw}$ = 3; $n_{seitan\ fried}$ = 3).

Sample	Mean (AUC)	Standard deviation (AUC)
Soybean	83991	4841
Tofu raw	79200	21418
Tofu fried	164135	9979
Tempeh raw	59861	6717
Tempeh fried	89370	6473
Flour	7382	1778
Seitan raw	67325	5417
Seitan fried	89387	7494

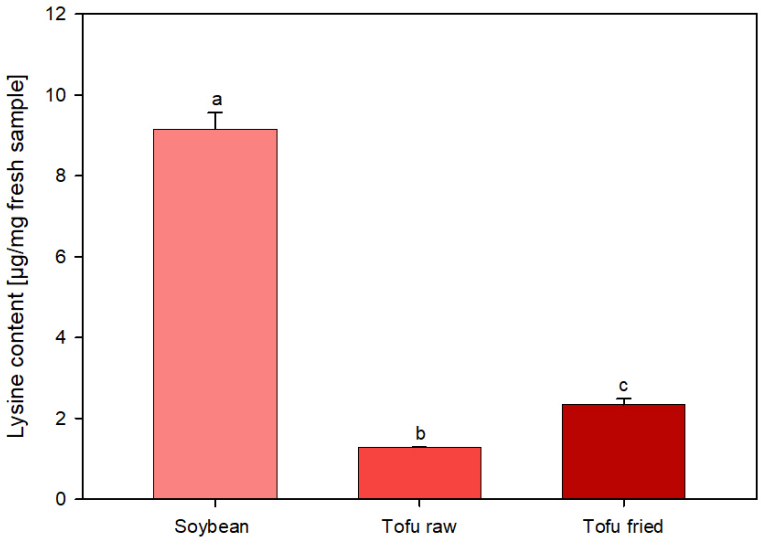

Figure Appendix 8: Lysine content in soybean, fresh raw tofu and fresh fried tofu
Shown is the change of lysine concentration during the manufacturing and processing of tofu. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$) with lower case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean}$ = 3; $n_{tofu\ raw}$ = 2; $n_{tofu\ fried}$ = 3).

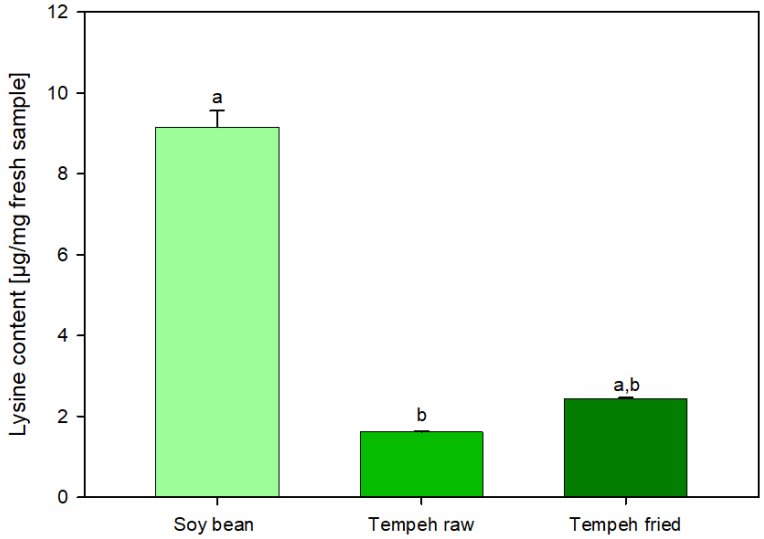

Figure Appendix 9: Lysine content in soybean, fresh raw tempeh and fresh fried tempeh
Shown is the change of lysine concentration during the manufacturing and processing of tempeh. The y-axis shows the lysine concentrations in µg/mg food, the x-axis shows the analyzed samples. Differences were determined by Kruskal-Wallis One Way Analysis of Variance on Ranks and indicated at a significance of ($p<0.05$) with lower case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{soybean} = 3$; $n_{tofu\ raw} = 2$; $n_{tofu\ fried} = 3$).

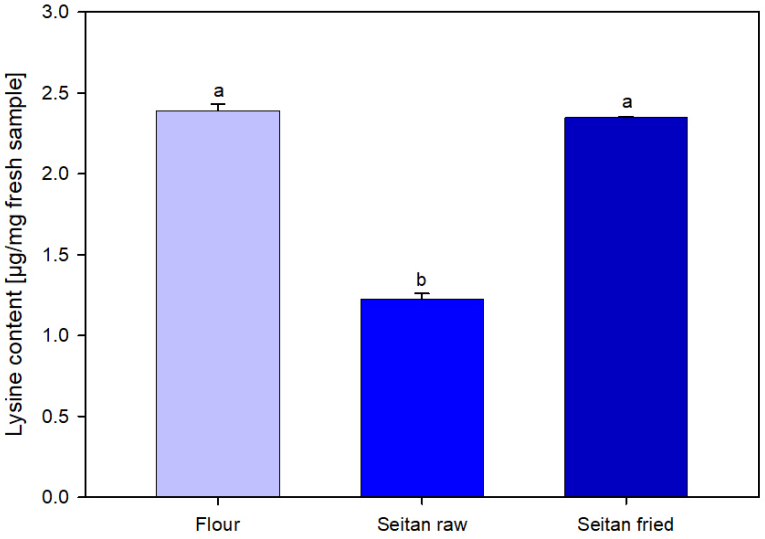

Figure Appendix 10: Lysine content in flour, fresh raw seitan and fresh fried seitan.
Shown is the change in lysine concentration during the manufacturing and processing of tempeh. The y-axis shows the CML concentrations in ng/mg food, the x-axis shows the analyzed samples. Differences were determined by One Way ANOVA and indicated at a significance of ($p<0.05$) with lower case letters. This was followed by Holm-Sidak post-hoc test to evaluate the statistical significance of differences between groups. Outliers were excluded from the calculation. Data is shown as mean ± standard deviation ($n_{flour} = 3$; $n_{seitan\ raw} = 3$; $n_{seitan\ fried} = 2$).

Table Appendix 5: Acrylamide content, LOD and LOQ
The results listed here show the calculated acrylamide contents. LOD and LOQ were calculated with the signal to noise ratio (s/n). Outliers were not considered in the calculation. Samples were measured in triplicates with one technical replicate each (n=6).

LOD	7.0 µg/L	
LOQ	23 µg/L	

Sample	**Mean [µg/L]**	**Standard deviation [µg/L]**
Soybean	5.7	0.43
Flour	6.8	1.1
Tofu raw	14	6.4
Tofu fried 130°C, 3.5 min.	20	2.3
Tofu fried 190°C, 1.5 min.	33	2.7
Tempeh raw	6.0	0.50
Tempeh fried 130°C, 3.5 min.	12	2.2
Tempeh fried 190°C, 1.5 min.	10	1.8
Seitan raw	8.4	1.2
Seitan fried 130°C, 3.5 min.	8.0	1.4
Seitan fried 190°C, 1.5 min.	6.6	0.8