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List of Abbreviations

Table 1 List of abbreviations used in this thesis

MSG	Monosodium glutamate
TCA	Trichloroacetic acid
PB	Pyrophosphate buffer
DNPH	2,4-Dinitrophenylhydrazine
EGTA	Ethyle glycol-bis(aminoethyl ether)-N,N,N',N'-tetra-acetic acid
Tris-Maleate	Tris-(hydroxymethyl)-aminomethane-maleate
HPLC	High pressure liquid chromatography
LC	Liquid chromatography
GC	Gas chromatography
MS	Mass spectrometry
ROS	Reactive oxygen species
PUFA	Poly-unsaturated fatty acids
TBA/TBARS	2-thiobarbituric acid/2-thiobarbituric acid reactive species
MDA	Malondialdehyde
AAS	α-aminoadipic semialdehyde
GGS	γ-glutamic semialdehyde
AGEs	Advanced glycation end products
CML	N-ε-carboxymethyl-lysine
CEL	N-ε-carboxyethyl-lysine
MG-H1	Methylglyoxal-derived hydroimidazolone
G-H1	Glyoxal-derived hydroimidazolone
MOLD	Imidazolium cross-link derived from methylglyoxal-lysine dimer
GOLD	Imidazolium crosslink derived from glyoxal-lysine dimer
MG	Methylglyoxal
GO	Glyoxal
GOLA	Glyoxal lysine amide
MGLA	Methylglyoxal-lysine amidine
FLD	Fluorescence detector
DAD	Diode-array detector
NFPA	Nonafluoropentanoic acid
TFA	Trifluoracetic acid
UV	Ultraviolet
ELISA	Enzyme-linked immunosorbent assay
rpm	Revolutions per minute
NMR	Nuclear Magnetic Resonance
ESR/EPR	Electron spin resonance/Electron paramagnetic resonance
CID	Collision-induced dissociation
CE	Collision energy
MRM	Multiple reaction monitoring
RFU	Relative fluorescence units
SD	Standard deviation
c	Concentration
AUC	Area under the curve
TG	Triacylglycerol
rt	Retention time
S/N	Signal-to-noise-ratio

1 Introduction

Monosodium L-Glutamate (MSG) is a sodium salt of glutamic acid (IUPAC: Sodium 2-aminopentanedioate) globally used as an additive for a broad spectrum of foodstuffs [1]. In the European Union MSG is listed as an approved flavour enhancer under E621 [2].

MSG is used with the intention to increase the palatability of foodstuffs and to contribute the so-called “umami” taste [3]. This term, also referred to as the unique fifth taste, originates from Japanese, meaning "meaty, brothy, savoury", and was introduced in 1909 by Japanese scientist Kikunae Ikeda, who extracted and identified glutamic acid from a broth prepared from seaweed (*Laminaria japonica*) [4]. Among the various forms of glutamate mentioned above, MSG is considered to show the strongest flavour enhancing capacity and umami intensity [1].

In a report in the New England Journal of Medicine in 1968, the author describes an unspecific symptom complex in patients after dining in Chinese restaurants [5]. Subsequently, these symptoms were readily attributed to the “foreign” ingredient MSG and safety concerns around the use of this additive arose, leading to extensive research on the safety of MSG [6-8].

The Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded in their scientific opinion of 2017 that no adverse effects from MSG consumption, including short-term and chronic, could be observed in different animal studies [8]. Furthermore, the US-American Food and Drug Administration (FDA) also recognised this food additive as “generally recognized as safe” or GRAS [7].

Despite the considerable research that has been conducted and reviewed in the past decades to assess the sensory characteristics and organoleptic properties as well as the safety of monosodium glutamate as a food additive [9-12], studies specifically investigating its fate during household cooking and storage are lacking. Furthermore, there is a considerable gap in research focusing on the impact MSG fortification has on protein and lipid oxidation and which possibly harmful products could be formed during these processes. Consequently, these aspects of the use of MSG in foodstuffs has not been considered in the numerous safety evaluations so far.

2 Literature Review

2.1 Monosodium Glutamate and "The Fifth Basic Taste"

Glutamic acid was first isolated by H. Ritthausen in 1866 from wheat gluten, which consecutively gave the amino acid its name. In his publication, Ritthausen also noted an unexpected flavour when tasting the glutamic acid solutions, which reminded him of "the least amount of meat extract" [13].

More than 40 years later, in 1909, the Japanese scientist Kikunae Ikeda proposed the existence of a taste distinct from the four basic tastes sweet, sour, bitter, and salty. According to Ikeda, it is most abundantly present in broths prepared from dried bonito and seaweed (*Laminaria japonica*) and therefore he named this taste "umami", derived from Japanese "umai", meaning "meaty, brothy, savoury". He could prove that the compound responsible for this taste sensation in the aforementioned seaweed broth was the ionic form of monovalent glutamic acid, that can be found as salts in respective foodstuffs at neutral pH [4]. Subsequently, scientists also discovered other compounds eliciting the umami taste, such as 5'-inosinate (salt of inosine-5'-monophosphate) [14] and 5'-guanylate (salt of guanosine-5'-monophosphate) [15], and showing a synergism between glutamate and nucleotides in humans [16]. It should be noted that, although these first steps of the umami taste research were performed in Japan using traditional Japanese dishes, the same compounds are abundantly present in ingredients that have traditionally been used worldwide, including vegetables, like tomatoes or mushrooms, seafood, meat, and ripened cheese [17, 18].

It took more than a century and thorough interdisciplinary research for Ikeda's hypothesis to be confirmed and for umami to be internationally accepted as "the fifth basic taste". Of pivotal importance for this process was the identification of specific umami taste receptors in the early 2000s to provide evidence for the taste sensation "umami" being different and independent from the four basic tastes that had been known previously [19, 20]. Up to this day several receptors specific for umami compounds have been reported, such as taste-specific variants of the metabotropic glutamate receptors 1 and 4 (taste-mGluR1, taste-mGluR4) [21, 22] and the G-protein-coupled receptor TAS1R1 + TAS1R3 heterodimer [23, 24].

Long before the question of the fifth taste and mechanism for monosodium L-glutamate's flavour enhancing property were agreed on, it was already used as a food additive as Ikeda immediately patented a process for isolating monosodium glutamate, and started to produce it in 1909 together with the Suzuki Chemical Company [4]. Although the company first had problems selling their product with the trade name Ajinomoto ("essence of taste"), it gained more and more popularity in household consumers and restaurants likewise attracting other companies to produce and sell MSG as well. MSG's popularity also spread across the borders of Japan to other Asian countries and subsequently, in the

1930s and 1940s, to the USA, where it was not only used in the rising numbers of Chinese restaurants, but especially in factory-processed food and canned foods for the military-industrial complex. MSG was valued due to its ability to enhance and intensify flavours, creating more tasteful products [25].

2.1.1 The Monosodium Glutamate Symptom Complex and Safety Evaluations

Following the increasing popularity of Chinese restaurants in the USA, a report by Robert Ho Man Kwok describing an unspecific symptom complex in patients after dining in Chinese restaurants was published in the New England Journal of Medicine in 1968 [5]. The author describes the symptoms as numbness at the back of the head that soon wandered further to arms and back as well as general weakness and palpitations starting 15-20 min after the meal. Even though the author offered several possible explanations for the so-called "Chinese restaurant syndrome" (CRS) that would later be called "MSG symptom complex", including cooking wine and sodium content, MSG attracted the most attention, possibly due to its foreignness [5, 6]. This led to extensive research on the safety of MSG in the following decades and it has been reassessed regularly ever since by different expert bodies [7, 8].

Initial studies researching this symptom complex and demonstrating reactions to MSG showed methodological flaws and lacked robust study designs, which fellow researchers at that time already acknowledged [6, 26]. Some of the key aspects questioned by Tarasoff and Kelly [26] were the absence of food when administering the MSG, inadequate placebos or no placebos at all, lack of randomization and double-blinded design, inadequate sample sizes and statistical analysis as well as symptom suggestion (high placebo responses). Subsequent double-blind studies with a thorough design failed to prove clear, consistent, and reproducible adverse effects after administration of MSG with food [26, 27].

After oral administration, glutamate is resorbed in the intestine and extensive pre-systemic metabolism takes place in the gut-walls. Only a minor proportion of the ingested glutamate enters the portal vein and, therefore, the liver, where it undergoes hepatic metabolism [7, 28-31]. Stegink et al. [32] also describe the impact of carbohydrate rich foods to reduce peak plasma concentration of glutamate. Consequently, scientists argue that due to this extensive metabolism in the intestines and the liver a rise of glutamate concentration in the blood only occurs after oral administration of very high doses on an empty stomach or if it is administered by parenteral routes [7]. There is no conclusive evidence that a high plasma concentration of glutamate leads to an increase in glutamate concentration in the brain [8, 33-35] and there is evidence that none transits the placenta [33].

The impact of MSG on obesity, asthma, rhinitis, urticaria and/or angio-oedema, blood pressure, hormonal changes were also examined, but the individual studies rarely created a uniform picture.

Instead, they rather produced conflicting results or suffered from questionable study design. A brief overview is given below.

The first report of increased obesity in infant mice treated with either single or multiple subcutaneous injections of MSG was published in 1969 [36]. The following three epidemiological studies yielded contradictory results [37-39], while an interventional human study revealed no discernible correlation [40], which provided no evidence that MSG can be linked to obesity in humans [8, 33].

The first link between MSG consumption and asthma was drawn after a case report in 1981 [41], but the few subsequent experimental human studies led to contradictory results and were therefore inconclusive [42-47]. Furthermore, a mouse study suggested that MSG does not have a substantial role in the development of asthma or acute asthmatic responses [48].

There were two distinct case reports with double blind, placebo-controlled challenges relating chronic rhinitis symptoms to MSG intake [49, 50] but experimental studies as reviewed by Williams and Woessner [51] were lacking a robust study design or failed to reproduce their results in a double-blind challenge. The same holds true for cutaneous reactions after MSG consumption. In two reports a positive correlation was described [52, 53], but experimental studies were inconclusive once again [51].

A placebo-controlled crossover study with eight healthy men, gave evidence that administration of MSG as well as a high protein meal could significantly stimulate insulin secretion, whereas MSG had no impact on the plasma concentrations of prolactin, luteinizing hormone, follicle-stimulating hormone, growth hormone and cortisol [54, 55]. Another study with lower (typical dietary) MSG concentrations did not show an increased insulin secretion after administration [56]. Studies have delivered some evidence that a high intake of MSG (more than 3,000 mg/day) may lead to elevated systolic and diastolic blood pressure although these changes might not necessarily be clinically relevant. However, the results from different studies were conflicting as well [57-59].

The Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded in their scientific opinion of 2017 that acute, short-term and subchronic toxicity is low for MSG (and only appeared in very high doses of more than 5000 mg/kg body weight per day) and that there is no evidence of carcinogenicity, reproductive or developmental toxicity. From the data collected, the Panel derived an acceptable daily intake (ADI) of 32 mg MSG/kg bodyweight (bw) per day or 27.8 mg glutamate/kg bw per day [8].

2.1.2 The Use of MSG Today

In spite of the alleged health risks and consequent controversy, MSG is still globally used as an additive for a broad spectrum of foodstuffs [1]. The production of MSG nowadays relies on fermentation of sugar beet molasses or other carbohydrate sources by coryneform bacteria resulting in L-glutamic acid

and subsequent addition of sodium hydroxide [1, 8, 60]. In the European Union MSG (E621) is, as other glutamic acid–glutamates including monopotassium glutamate and calcium diglutamate, an authorised food additive for different food categories varying from seasonings and condiments to bakery wares and meat products according to Annex II of Regulation (EC) No 1333/2008 [2].

2.1.3 Research Status

While the safety and toxicological effect as well as the effect of MSG on sensory and organoleptic properties of meat and meat derived products has been extensively investigated and reviewed [8-12], only few studies are focussing on the potential interactions of MSG with other food components, especially regarding oxidative processes.

The only publication dealing with the impact of MSG on food oxidation, by Jacobson and Koehler [61], assessed the development of rancidity by the 2-thiobarbituric acid (TBA) test (expressed as mg of malondialdehyde per 1000 g sample) in cooked light and dark turkey meat with 0.3 % added MSG after refrigeration or frozen storage. The authors could not identify a significant difference in malondialdehyde concentrations between the samples with added MSG and the controls.

Other publications are mainly focussed on the quantification of MSG in several food matrices and with different analytical approaches [62, 63], possible reaction products and stability/reactivity of MSG [64] as well as the potential use of MSG to reduce NaCl for foodstuffs [10, 11, 65-69].

Different approaches for quantifying MSG in different food matrices have been developed, using inter alia HPLC combined with ultraviolet/diode array detection [62, 70, 71] or fluorescence detection after derivatization with dansyl chloride [72, 73], spectrophotometric determination by ligand exchange complexation [63], LC (-MS) [74, 75], High-Performance Thin Layer Chromatography [76], enzymatic methods [77], and biosensor based detection [78]. Furthermore, different studies about monitoring MSG (as free glutamic acid) in different foodstuffs and different countries have been published [79, 80].

A study conducted by Wu et al. [64] in 2000, investigated the volatile products formed, when different mixtures of soybean oil and/or water, sugar and/or monosodium glutamate were heated between 100 and 170°C in either water or oil for different time periods. They observed formation of 2,5-dimethyl pyrazine and methyl pyrazine after heating of MSG and sugar at or after 160 min heating, whereas 2-pyrrolidone was found in oil-heated samples of soybean oil with MSG. In the samples containing sugar, furfural and 5-hydroxy methyl furfural were found and in the mixtures with soybean oil, sugar, and MSG 23 pyrazines were detected.

Nguyen et al. [81] found MSG to be very stable during the canning process (124°C for 30 min, pH 5) with a recovery of 93–100 %. In a study conducted by De Castro et al. in 2014 [82], the effects of different packing conditions (glass bottles and plastic pouches), heat treatment, presence of potassium

sorbate as preservative and storage times on the stability of monosodium glutamate (MSG) in fermented Spanish-type green table olives and pickled cucumbers were elucidated. The authors concluded that MSG content of pickled cucumbers and pasteurized green olives was stable for up to 1 year of storage in all aforementioned conditions, but was degraded to variable extent in different conditions when used in unpasteurized green olives due to fermentation by lactic acid bacteria and yeasts [82].

The replacement of sodium chloride with monosodium glutamate is one of the strategies discussed to reduce salt in food stuffs, especially in processed meat products, which has been extensively studied and reviewed as for example by Inguglia et al. [65] in terms of acceptability, sensory characteristics, technological and safety aspects [10, 11, 65-69].

Although different aspects of the use of monosodium glutamate in foodstuffs have already been covered by research, studies focussing on the fate of this common additive upon household cooking and storage as well as thorough analysis aiming to elucidate the question whether it impacts protein and lipid oxidation are lacking.

2.2 Lipid Oxidation in Meat

Meat and meat derived products can be valuable sources of protein, fat-soluble vitamins, minerals, and bioactive compounds. The lipids in meat products contribute to many desirable characteristics, such as tenderness and succulence, and they have an influence on their flavour and consumer acceptance as reviewed by Font-i-Furnols and Guerrero [83].

Lipids in meat can be divided in two groups: intermuscular (depot) and intramuscular (tissue) lipids. The depot lipids are usually found in connective tissues, whereas tissue lipids are integrated into muscle tissues [84]. The latter can again be classified in two subclasses: the neutral lipids, such as cholesterol, free fatty acids, and (mono-, di-, or tri-)glycerides, and the polar fraction, which includes phospholipids and sphingolipids [85]. The free fatty acids are often considered a separate group [86]. However, lipids are prone to oxidation, causing lipid oxidation to be the main non-microbial cause of quality impairment in meat products [87, 88]. Within the aforementioned subclasses, phospholipids are the most susceptible to oxidation due to their high content of polyunsaturated fatty acids (PUFAs) [89]. The process of lipid oxidation leads to rancidity and off-flavours and impacts the organoleptic properties of the product as well as its nutritional value, which is detrimental to consumers' acceptance [90]. Lipid oxidation can proceed following three different mechanisms: autoxidation, enzymatic-catalysed oxidation, and photo-oxidation. In general, the mechanisms of lipid oxidation and its effect in meat products has been extensively studied and reviewed by several authors, including Choe and Min [91], Min and Boff [92], Amaral et al. [93], Min and Ahn [87], Ladikos and Lougovois [94], Domínguez et al. [95], Love and Pearson [96] and Baron and Andersen [97].

2.2.1 Autoxidation

Usually, the autoxidation process is divided in three phases: initiation, propagation, and termination. The direct reaction between the double bond of an unsaturated fatty acid (singlet electronic state) and an oxygen molecule (triplet electronic state) is a spin-restricted reaction. Therefore, activation of the oxygen by temperature or light is necessary and results in the formation of singlet oxygen ($^1O_2$) or reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HOO^{\bullet}$), or iron-oxygen complexes (ferryl- and perferryl radical) [87, 98-100]. Hereinafter, a hydrogen is abstracted from the unsaturated fatty acid resulting in an alkyl radical usually stabilized by a double-bond rearrangement forming conjugated dienes or trienes [87].

During the propagation phase lipid-lipid interactions lead to an accelerated radical formation. In a first step, the alkyl radical reacts with molecular oxygen to form a highly reactive peroxyl radical that generates a hydroperoxide and an alkyl radical by hydrogen abstraction from adjacent lipids. Those so-called primary oxidation products, the lipid hydroperoxides, are readily decomposed at elevated

temperatures and in the presence of metals, such as heme and non-heme iron resulting in the generation of new reactive hydroxyl, peroxyl and alkoxyl radicals [87, 91, 101]. Furthermore, lipid hydroperoxides can form dimers and polymers which again are prone to oxidise and decompose into volatile products. The decomposition of peroxyl and alkoxyl radicals gives rise to secondary lipid oxidation products such as alcohols, ketones, epoxides, aldehydes, and alkanes [93, 94, 102].

During the termination step, stable or slow-to-react products are being generated by radicals' reaction with either radical or non-radical compounds (antioxidants). If the process involves the reaction of two radicals, radical–radical coupling and disproportionation enables the formation of a non-radical adduct, but if the other reaction partner is an antioxidant, the antioxidant either transfers a hydrogen atom to the radical or accepts electrons from the latter to form a stable complex and/or produces a less reactive antioxidant radical [93, 102]. However, it should be noted that unstable compounds may also be formed and subsequently degenerate by radical formation [103].

2.2.2 Photo-Oxidation

The mechanism of initiation distinguishes photo-oxidation from autoxidation as it requires the presence of sensitizers (such as myoglobin or hemoglobin) along with light exposure [91, 100]. Initially, the singlet sensitizer absorbs light energy and transitions to an excited triplet state. Subsequently, the excited triplet sensitizer may react with molecular oxygen ($^3O_2$) giving rise to either singlet oxygen ($^1O_2$) or a superoxide radical anion ($O_2^{\bullet -}$). Singlet oxygen can attack the double bond of an unsaturated fatty acid giving rise to a hydroperoxide bypassing the formation of an alkyl radical. Conversely, the superoxide radical anion may either abstract hydrogen from unsaturated fatty acids and therefore initiate lipid autoxidation or react with hydrogen peroxide generating a hydroxyl radical and singlet oxygen ($H_2O_2 + O_2^{\bullet -} \rightarrow HO^{\bullet} + OH^- + {}^1O_2$). Transition metals serve as catalysts in this reaction [92, 104]. Lastly, the excited triplet sensitizer could also directly abstract hydrogen from an unsaturated fatty acid, giving rise to an alkyl radical that might undergo the free radical chain reaction mechanism mentioned in section 2.2.1 [91, 100, 104].

2.2.3 Enzyme-Catalysed Oxidation

The enzyme lipoxygenase, which contains ferrous iron in its active site, plays a significant role in this lipid oxidation pathway. It can generate a conjugated diene system prone to react with molecular oxygen after abstracting hydrogen from the methylene group of a PUFA [100]. Subsequently, the resulting peroxyl radical may abstract hydrogen from another unsaturated fatty acid generating a conjugated hydroperoxy diene and an alkyl radical. The process then follows the mechanisms described in the previous section (section 2.2.1).[93].

2.2.4 Factors Influencing Lipid Oxidation

In summary, it can be ascertained that lipid oxidation is a complex process involving multiple mechanisms and intertwined reactions influenced by different factors. In the following subsections, conditions influencing oxidative stability in meat products post-slaughter are explored. The impact of pre-slaughter conditions will not be elaborated but has also been studied and reviewed [93, 95, 105-108].

2.2.4.1 Sample composition and processing

The susceptibility to oxidation depends on the lipid composition and on the level of unsaturation of the fatty acids, especially the amount of phospholipids as they present a high degree of unsaturation making them more prone to oxidation. Lean meat contains a higher percentage of phospholipids that makes it sensitive to oxidation [89, 96, 109]. The total lipid content and the fatty acid composition vary among the animal species, and therefore sensitivity to oxidation is dependent on species, location and muscle type [87]. Nevertheless, the unsaturation of fat has been claimed to have more impact than the total amount of fat [95]. Other authors also suggested the species-dependent heme pigment content and catalase activity to be a key influence on lipid oxidation which is congruent with the findings that beef with the highest content in heme pigment is more susceptible to lipid oxidation than pork and chicken [110-112]. However, studies of Min et al. [110] indicate that the content of free ionic iron and myoglobin in raw meat and the ferric reducing ability of raw meat have the most significant impact on oxidative stability. Although they showed raw chicken meat (thigh and breast) to be more stable than raw beef, they proved chicken thigh meat to be as susceptible to oxidation as beef upon cooking, linking the amount of PUFAs to the degree of oxidation when sufficient free ionic iron was present. Furthermore, food preparation processes like grinding, chopping, and cooking accelerate lipid oxidation, because they disrupt muscle tissue and thereby release membrane-bound phospholipids, enzymes and metal containing pigments (such as heme) and increase the surface area of unsaturated fatty acids exposed to oxygen [93]. Furthermore, the temperature increase while cooking inactivates antioxidant enzymes, reduces the activation energy needed for lipid oxidation and leads to decomposition of hydroperoxides to free radicals [87].

2.2.4.2 Presence of Anti- or Pro-oxidants

Metal ions like iron and copper play a crucial role in initiation and catalysis of lipid oxidation processes due to their electron donating affinity. Iron, the most prevalent transition metal in biological systems is present in five different forms: transferrin, ferritin, heme pigments, iron-dependent enzymes, and small iron chelates (“free iron”) [93]. Upon cell disruption these iron containing compounds can be released. The relative importance and mechanism of the different species, especially for myoglobin, have not definitely been established, but it has been proposed that free iron and/or ferrylmyoglobin have key

roles for lipid peroxidation in meat. Ferrylmyoglobin is generated by the reaction of myoglobin with hydrogen peroxide (H_2O_2) or lipid hydroperoxides (LOOH), but myoglobin is also a source of free ionic iron and hematin. Furthermore, all of the compounds mentioned can initiate and/or catalyse lipid oxidation, for example, via the Fenton reaction [87, 97, 113-119].

Another pro-oxidative compound is sodium chloride, a widely used additive to improve preservation, flavour, and texture in meat products. Various mechanisms have been proposed to explain this effect: Firstly, sodium chloride could cause membrane disruption, leading to the release of catalysts and ionic iron from iron-containing molecules. Secondly, it could promote metmyoglobin formation, and lastly, the enzymatic activity of protective enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase could be decreased due to the impact of salt [87, 98, 120, 121].

On the contrary, antioxidants can stabilize free radicals and therefore retard lipid oxidation and rancidity. They can exert this effect via different pathways including donating a hydrogen atom ($H^{\bullet}$) (primary antioxidants), binding metal catalysts, scavenging oxygen, absorbing UV radiation, inhibiting enzymes, or decomposing hydroperoxides (secondary antioxidants) [122]. While animals possess protective mechanisms against oxidation, they are mostly lost upon meat preparation. For this reason, synthetic antioxidants, such as BHA (butylated hydroxyanisole), BHT (butyl hydroxytoluene), PG (propyl gallate), and TBHQ (tert-butylhydroquinone) used to be applied in the food industry but have fallen into disrepute as some studies found evidence of their toxicity and carcinogenicity [123-126]. However, this concern led to the extensive study of the antioxidative effect and potential use of natural antioxidants in meat products as reviewed by Kumar et al. [103]. Several authors described the positive influence of different plant, herb and spice extracts on the oxidative stability of meat of different species at various storage conditions, such as combinations of sage, oregano and honey [127], black current extracts [128], rosemary extracts [129], olive leaf extracts [130, 131], extracts of different greens such as bok choy, garlic chives, *Chrysanthemum coronarium L.*, *Aralia elata*, pumpkin and broccoli [132], *Clitoria ternatea* extract (blue pea flower petal) [133] and extracts of clove, cinnamon, oregano and black mustard [134]. Despite the application potential of those extracts their use to date is still very limited due to the fact that their production is costly, their safety is not yet fully established and they can also change other attributes of the end product, such as flavour and colour, which could reduce the consumers' acceptance of the product [103].

2.2.4.3 Storage (Temperature, Time, Light, Atmosphere)

The rate of oxidation processes such as hydroperoxide decomposition increases with higher temperatures, which renders storage temperature an important parameter to control [98, 135]. Considering this, it has also been shown that freezing delays (albeit does not stop) lipid peroxidation and enzymatic lipid oxidation, but upon thawing the peroxidase system is active again [87, 136].

Furthermore, accelerated lipid oxidation is observed after thawing as the formation of extracellular ice crystals disrupts cells and releases prooxidant compounds [137, 138]. Therefore, novel methods for freezing and thawing are being developed as reviewed by Leygonie et al. [139].

However, light, especially of shorter wavelengths, has been stated to have a bigger impact than temperature as it stimulates the production of $^1O_2$ and promotes the initiation of photo-oxidation [95, 140, 141]. This is problematic, because meat and meat products are usually directly exposed to light to be more appealing for consumers [88]. Therefore, active packaging strategies involving the use of films containing antioxidants or UV-absorbent packaging materials, allowing an increased shelf life of meat products during storage, are being proposed as an alternative to traditional methods [142, 143]. Furthermore, long storage periods promote the release of iron from heme-proteins [95, 135, 144].

As oxygen is the main reaction partner in lipid oxidation processes, the gas composition surrounding the product has a vital impact. It has been shown that pro-oxidants such as ionic iron, hemoglobin and NaCl as well as the fat content and fatty acid composition did exert little to no effect on the oxidation process, when meat was stored in an atmosphere deprived of oxygen [145]. Various studies have confirmed a decrease in lipid oxidation products when meat is packaged in low O_2 concentration (for example, in nitrogen-based atmosphere or vacuum) compared to storage in an O_2 rich atmosphere [145-149]. Nevertheless, fresh meat, especially red meat, is usually stored in an O_2 rich atmosphere to maintain the red colour which accelerates oxidation [88, 139].

2.2.5 Analytic Techniques to Assess Lipid Oxidation

In general, as outlined in the previous sections, lipid oxidation includes the formation of two groups of products. Primary products (hydroperoxides and conjugated dienes/trienes), which due to their instability, decompose easily and give rise to secondary lipid oxidation products (carbonyls, volatiles, aldehydes) [150]. Both should be quantified as complementary indicators to gain a full picture of lipid oxidation with methods reviewed by several authors including Domínguez et al. [95], Barriuso et al. [150], Ross et al. [151] and Gray et al. [95, 150-152].

Concerning the early stages of lipid oxidation, hydroperoxides are the most significant primary products. The so-called peroxide value is traditionally measured making use of the redox activity of peroxides with iodometry or alternatively the ferric-xylenol orange (FOX) assay [95, 150, 152-154]. Alternatively, conjugated compounds serve as an oxidation indicator in this early stage. After lipid extraction with only small amounts of organic solvent, the absorbance of the organic extracts can be measured at 234 and 268 nm. This method has also been applied for meat products [154, 155]. Nonetheless, the signals of interest can be interfered by compounds such as carotenoids [150].

However, the quantity of hydroperoxides and conjugated dienes/trienes is only representative of the degree of oxidation in the early stages, which underscores the importance of measuring secondary oxidation products, such as rancid aroma provoking volatile compounds [151, 152]. The entirety of volatile substances is usually assessed with headspace GC-MS [151, 156], whereas unsaturated aldehydes are usually quantified by the p-anisidine method [150] and carbonyls by the DNPH assay (elaborated in section 2.3.2) [95]. Moreover, malondialdehyde (MDA) is a common marker of lipid oxidation, which has traditionally been quantified with the colorimetric thiobarbituric acid test (TBA) [152]. This test makes use of the fact that TBA forms a coloured complex with MDA. However, the method is now referred to as thiobarbituric acid reactive substances (TBARS) method as it is not specific for MDA because other compounds may also contribute [150]. Still, it has commonly been used to assess lipid oxidation in meat products [155, 156].

Other methods including HPLC, fluorescence emission, Raman spectroscopy, infrared spectroscopy or magnetic resonance have also been proposed and reviewed [150].

2.3 Protein Oxidation in Meat

Oxidation is not solely confined to lipids but can also occur in proteins. These processes can result in various alterations, including modification of the amino acid sidechains, as well as the protein backbone and the protein's constitution, including conformational changes, unfolding, formation of protein–protein cross-linkages, protein carbonyl formation and fragmentation. These modifications alter properties on the protein level (solubility and hydrophobicity) as well as on the final (meat) product level (water-holding capacity, tenderness, and gelation) [157-163]. Additionally, those modifications can have negative effects on bioavailability, digestibility of proteins and therefore the nutritional values of meat proteins [164]. The principles of protein oxidation and its implications for meat products have been thoroughly summarised by several authors, including Zhang et al. [157] and Lund et al. [164], Poojary and Lund [165], Bao and Ertbjerg [166], Domínguez et al. [167] and Estévez [168], Estévez and Luna [169], Bao et al. [170] and Soladoye et al. [171].

In general, oxidation can be induced by ROS in the backbone of proteins or the side chains of the amino acids [165]. These ROS can be generated via different pathways including photo-oxidation, directly by absorption of ultraviolet radiation by chromophoric groups or indirectly by activation of oxygen [172]; metal-catalysed oxidation, where alkaline amino acids such as lysine, histidine, arginine, threonine, and proline are particularly prone [173, 174]; and enzyme catalysed oxidation [175, 176].

Although the selectivity of radical attacks on free amino acids is well-understood, the processes in peptides and proteins are an ongoing area of research as reviewed by Hawkins et al. [177] and summarised in the following passages: In free amino acids, the radical has low affinity to the protonated

amine group at the α-carbon as it has strong electron-withdrawing and deactivating properties. Therefore, in free amino acids, attack of radicals on side chains are common as they may contain groups that can stabilize radicals through electron delocalisation (hydroxy-, carboxyl-, amide-groups). While electron-rich radical-stabilizing groups seem to influence radical formation in amino acids, their impact is less pronounced when the amine group on the α-carbon forms a peptide bond as electron delocalisation to both the neighbouring amide group and the carbonyl function can stabilize the radical. Thus, backbone oxidation is not as unlikely in peptides as it is in free amino acids. The situation is increasingly complex and unclear with larger structures where secondary and tertiary structures are present [177].

A substantial part of fundamental research on radical–mediated protein modification was performed by Garrison et al. [178, 179], Swallow [180], and Schuessler and Schilling [181], who carried out studies with proteins under $HO^{\bullet}$ and/or $O_2^{\bullet-}$ formation. The major pathway suggested by these studies was summarised by Stadtman [182] and Davies et al. [183, 184] as follows and is depicted in Figure 1:

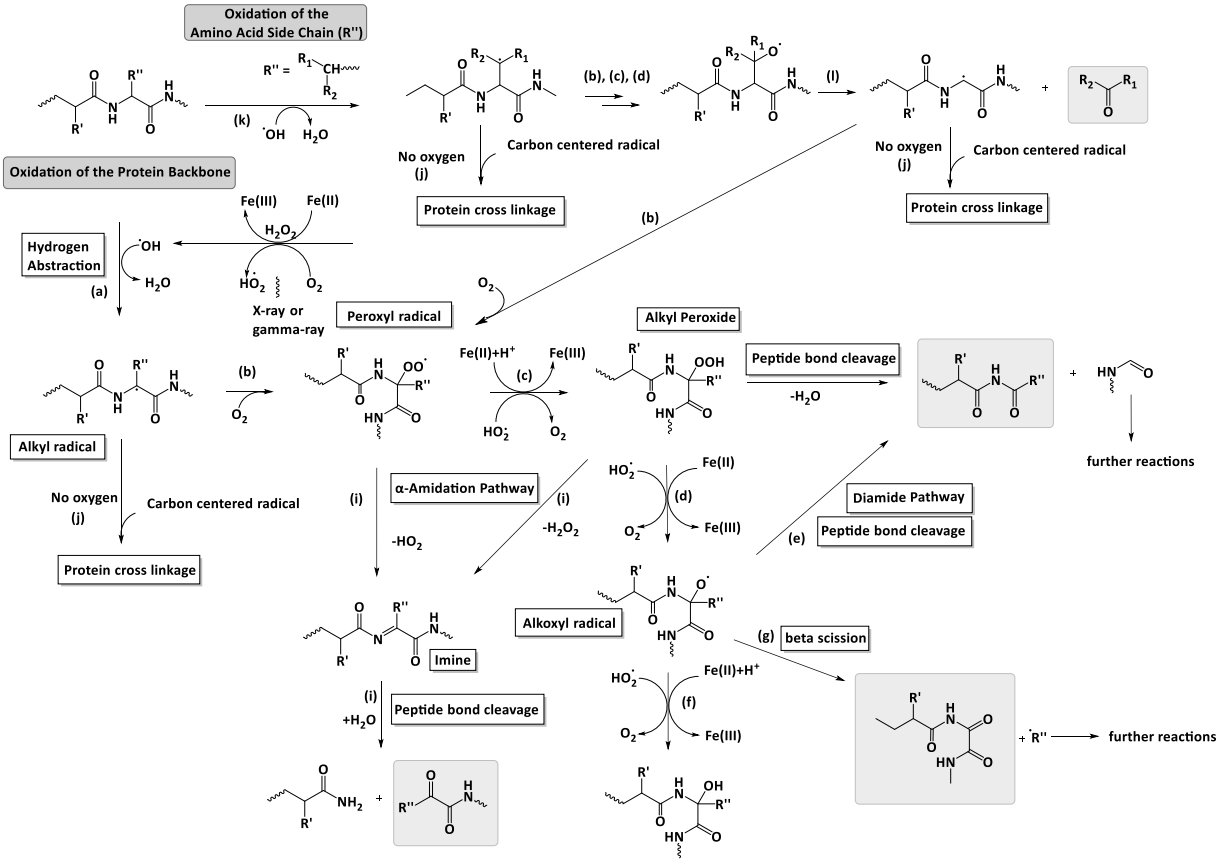

Figure 1 Schematic overview of protein oxidation pathways. *This figure incorporates and links suggested protein oxidation pathways from various authors, including Garrison et al. [178, 179], Swallow [180], and Schuessler and Schilling [181], Stadtman [182] and Davies et al. [175, 183, 184].*

The first step involves hydrogen abstraction by a hydroxyl radical from either α-CH group of the peptide backbone (a) or the amino acid side chain (k), forming a carbon-centred radical. The alkyl radical formed on the backbone may either generate an alkyl-peroxyl radical upon addition of O_2 (b) or undergo cross-

linkage in the absence of oxygen (j). The further reactions of the alkyl-peroxyl radical can then follow different pathways [182]. On the one hand, these peroxyl radicals may be eliminated generating imines, followed by backbone fragmentation upon hydrolysis (i) [185]. On the other hand, the next step could involve hydrogen abstraction forming an alkyl peroxide (c) that upon decomposition to an alkoxyl radical (d) can result in backbone fragmentation following the Diamide pathway (e) [182, 186]. The alkoxyl radical formed could also either recombine with hydroperoxide and form a protein alcohol (f) [182] or undergo beta scission and give rise to protein carbonyls (g) [165].

Amino acids with aliphatic sidechains have low susceptibility to oxidation and thus primarily react with the reactive radicals undergoing hydrogen atom abstraction and forming aforementioned carbon-centred radicals (k). These radicals can dimerise, which is unlikely in the presence of O_2 and low radical flux [177] or react with O_2 generating a peroxyl radical (b) and following the pathway described before (c,d) resulting in an alkoxyl radical [187]. Apart from the mechanisms mentioned above, the alkoxyl radical can also undergo beta scission and give rise to a carbon radical on the protein backbone (which can undergo the reaction cycle again) and a carbonyl formed from the amino acid's side chain (l) [177, 178, 182, 188].

It is noteworthy that the different protein radicals generated during these reactions have the ability to abstract hydrogen atoms from amino acid residues from the same or a different protein and restart the cycle [182]. These reactions could not only result in fragmentation but could also induce conformational changes in the secondary and tertiary structure of the protein [183, 184].

Other common oxidative modifications of side chains of amino acids include thiol oxidation and aromatic hydroxylation [189].

The sulphur containing amino acids cysteine and methionine are very susceptible to oxidation due to their sulphur content [175, 178]. The oxidation of cysteine side chains can occur by one-electron or two-electron mediated pathways, both yielding similar end products. In radical-induced oxidation, formed thiyl radicals can either build disulfide bonds with thiols/thiolates, or generate thiyl peroxyl radicals after reaction with O_2 [190, 191]. The two-electron oxidation yields different unstable acids (sulfenic acid, sulfinic acid, sulfonic acid) [192] which subsequently form oxyacids upon hydrolysis or disulfide bonds with another thiol group [193]. Methionine oxidation primarily forms sulfoxide, which can be further oxidized to sulfone [194, 195].

For amino acids with aromatic sidechains, addition is the most common reaction upon oxidation, although substitutions are observed when reacting with heteroatom substituents [177, 196]. The predominant oxidation product of tyrosine is a tyrosine phenoxyl radical, which can self-dimerise and form the cross-linked di-tyrosine, resulting in intra- or intermolecular crosslinks in proteins [177]. Other

classic protein oxidation products for aromatic amino acids are asparagines, aspartic acid residues, and oxo-histidine formed from histidine, hydroxy phenylalanine derivatives from phenylalanine and kynurenines from tryptophan [157].

Intermolecular bridges, most commonly formed by disulfide or dityrosine linkage, result in protein aggregation and polymerization [197, 198], due to an oxidation-induced unfolding process increasing the surface hydrophobicity of the oxidized protein [183, 184].

Furthermore oxidation of side chains can result in specific products, such as N-pyruvyl derivatives and oxalic acid intermediates formed upon hydrogen abstraction from γ-carbon atom of a glutamyl residue, [178] and formation of 2-pyrrolidone, which later is hydrolysed to 4-aminobutyric acid, by proline oxidation [199].

2.3.1 Impact on Proteins and Meat Quality

Oxidation can alter the primary structure of the protein, which subsequently, influences the secondary, tertiary, and quaternary structure, leading to unfolding [183]. Even though amino acid residues exposed on the surface are more readily oxidized compared to those on the inside of the protein structure, the latter has far more impact on unfolding, surface hydrophobicity and conformation [200, 201]. Post-mortem proteolysis, facilitated by proteolytic enzymes such as calpains, is a crucial process in meat tenderization [166, 202]. Generally, heavily oxidized proteins may be less susceptible to enzymatic degradation due to protein aggregation upon cross-linkage, whereas moderately oxidized proteins showing decreased stability due to unfolding processes may be more susceptible to decomposition [184, 203-205]. These alterations impact the physicochemical characteristics of the meat products, leading to a change in meat texture and tenderness [166, 206-208].

Other affected physicochemical characteristics might include colour, flavour, texture, solubility, precipitation and gelation, as well as nutritional aspects [167]. Another factor related to the texture of meat products is the water-holding capacity. The predominant part of water in muscle foods is stored within the myofibrillar matrix (composed of actin and myosin) [168]. Oxidative alterations lead to changes in proteins charge state and structure, which subsequently leads to modifications in the myofibrils volume [166, 206]. The modification in protein charges affect amino acids with positively charged side chains, such as histidine, lysine, and arginine, as they lose their positive charge upon carbonyl formation. The increase in net negative charges leads to higher electrostatic repulsion and myofibrils volume, which is positively linked to water holding capacity [166, 168, 173, 209]. In addition, formation of cross-linked structures upon oxidation poses constraints and limits the ability of myofibrils to swell [206]. Moderate protein oxidation has a detrimental effect on protein solubility due to

denaturation and precipitation, whereas it positively influences gelation, and emulsification due to the formation of stable crosslinks [157, 158, 164, 209, 210].

Moreover, the flavour of meat products can be influenced upon oxidation by two main processes. Firstly, protein oxidation products, including protein carbonyls, such as α-aminoadipic -, and γ-glutamic semialdehydes may undergo the Maillard reaction, forming aroma-active Strecker aldehydes. These Strecker aldehydes can give rise to additional aroma-active compounds in the later stages of the Maillard reaction [168]. On the other hand, oxidation of sulphur-containing amino acids plays a role in the generation of off-flavours [211].

Overall, protein oxidation significantly impacts the nutritional value of meat products by altering the amino acid profile and causing a loss of essential amino acids as well as protein polymerization and aggregation which limits their digestibility and bioavailability, especially upon cooking [162, 212-214]. Although the impact of protein oxidation and its resulting products has been well-established in a medical context and in terms of disease development [182, 215-221], it is only in recent years that potentially detrimental health effects of the intake of oxidised food proteins gained attention [169, 222].

Protein oxidation in meat products is, similarly to lipid oxidation, influenced by several different factors, some of them are related to processing, as reviewed by Domínguez et al. [167]. In general, the principles promoting lipid oxidation (temperature and light [223], aerobic packaging [224], freezing and thawing [170, 225], salt content [226, 227]) and their mechanisms also hold true for protein oxidation and, likewise, incorporation of antioxidant plant extracts have been shown to delay protein oxidation [128, 133, 167, 228, 229]. Therefore, these will not be elaborated further. Regardless, it is noteworthy, that the effect of NaCl alternatives on protein oxidation has not yet been comprehensively studied as only few studies have focused on it to date [167, 230, 231].

2.3.2 Analytic Techniques to Assess Protein Oxidation

A variety of analytical methods can be employed to measure oxidative changes on protein components including the loss of tryptophan and sulfhydryl groups, and the formation of protein carbonyls and cross-links as reviewed by Domínguez et al. [167] and Estévez et al. [168].

The generation of carbonyls is the predominant damage upon oxidation of proteins and can follow one of three pathways, highlighted in Figure 1: peptide bond cleavage through the α-amidation pathway (i), β-scission (g) or oxidation of amino acid side chains (arginine, lysine, proline, and threonine) (k) [232]. Carbonyl derivates can also be a direct result of the reaction of lipid oxidation products (4-hydroxy-2-nonenal (HNE) and malondialdehyde) with amino acid side chains (histidine, cysteine and lysine) through Michael addition [178, 182, 188, 221, 233, 234].

The most common approach to quantify protein carbonylation is the 2,4-Dinitrophenylhydrazine (DNPH) assay, which is characterised by the reaction of DNPH with the carbonyl group of proteins to form a protein-bound 2,4-dinitrophenylhydrazone, absorbing at 370 nm [235, 236]. The derivatised samples are measured at 280 nm to assess to protein content and at 370 nm for the protein-hydrazone content, which enables the result to be expressed as nanomole carbonyl per mg of protein without separate protein quantification. The formula includes a correction factor to account for the fact that DNPH itself absorbs light at 280 nm to the extent of 43 % of its absorption at 370 nm [236]. This method has been used to measure the carbonyl content in a variety of meat products, including ground meat, dry-cured ham, dry cured loin, dry cured sausage, cooked sausage, liver paté [237], chicken breast, pork, beef [235], as well as fish [238]. Although other methods have been reported, they are rarely applied [167, 239].

Apart from the quantification of the total carbonyl content, the measurement of specific carbonyl compounds such as α-aminoadipic- (AAS) and γ-glutamic semialdehyde (GGS) can serve as an alternative [167]. Several procedures have been proposed, but all of them involve a derivatization step with either $NaBH_4$, fluoresceinamine ($FINH_2$) [240, 241] or p-aminobenzoic acid (ABA) [242], chromatographic separation (GC or HPLC) and detection by a diode array detector (DAD), mass spectrometer or fluorescence detector [229, 240-244].

Protein carbonyls, mostly AAS and GGS, may also lead to the formation of Schiff bases, after reaction with the ε-amino group of (protein-bound) lysine/arginine, or other AAS residues [245, 246]. The carbonyls reacting with the amino groups may also be formed upon lipid oxidation [247]. The resulting cross-linkage can be quantified using fluorescence spectroscopy and has been applied for meat products as well [229, 245, 248, 249].

The oxidation of cysteine leads to the formation disulfide cross-links which can be assessed with photometric methods after derivatisation using 5’5-dithiobis (2-nitrobenzoate) (DTNB, Ellman reagent) or 4,4’-dithiodipyridine (4-DPS) [222]. On the other hand, the aromatic amino acid tryptophan exhibits fluorescence at 350 nm, when excited at 280 nm. The loss of this natural fluorescence accounts for oxidative degradation of tryptophan and radical formation [245, 250].

The alteration of proteins due to oxidation can, as mentioned before in section 2.3 lead to cross linking and fragmentation. The resulting changes in molecular weight patterns can be observed by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) [167, 171].

2.4 Maillard Reaction and Advanced Glycation End Products in Meat

In 1912 Louis-Camille Maillard published a novel non-enzymatic browning reaction of amino acids with sugars upon heating, which would consecutively be named the Maillard reaction or glycation/glycoxidation in medical sciences [251-253]. The principles of Maillard reaction, the products formed and its implications for food have been reviewed by several authors, including Echavarría et al. [254], Zamora and Hidalgo [255], O'Brien et al. [256], Huang et al. [257].

The Maillard reaction is a complex process involving a vast range of reactions, such as cyclisation, dehydrations, retroaldolisations, rearrangements, isomerisations and condensations [258]. A schematic overview of the process can be found in Figure 2.

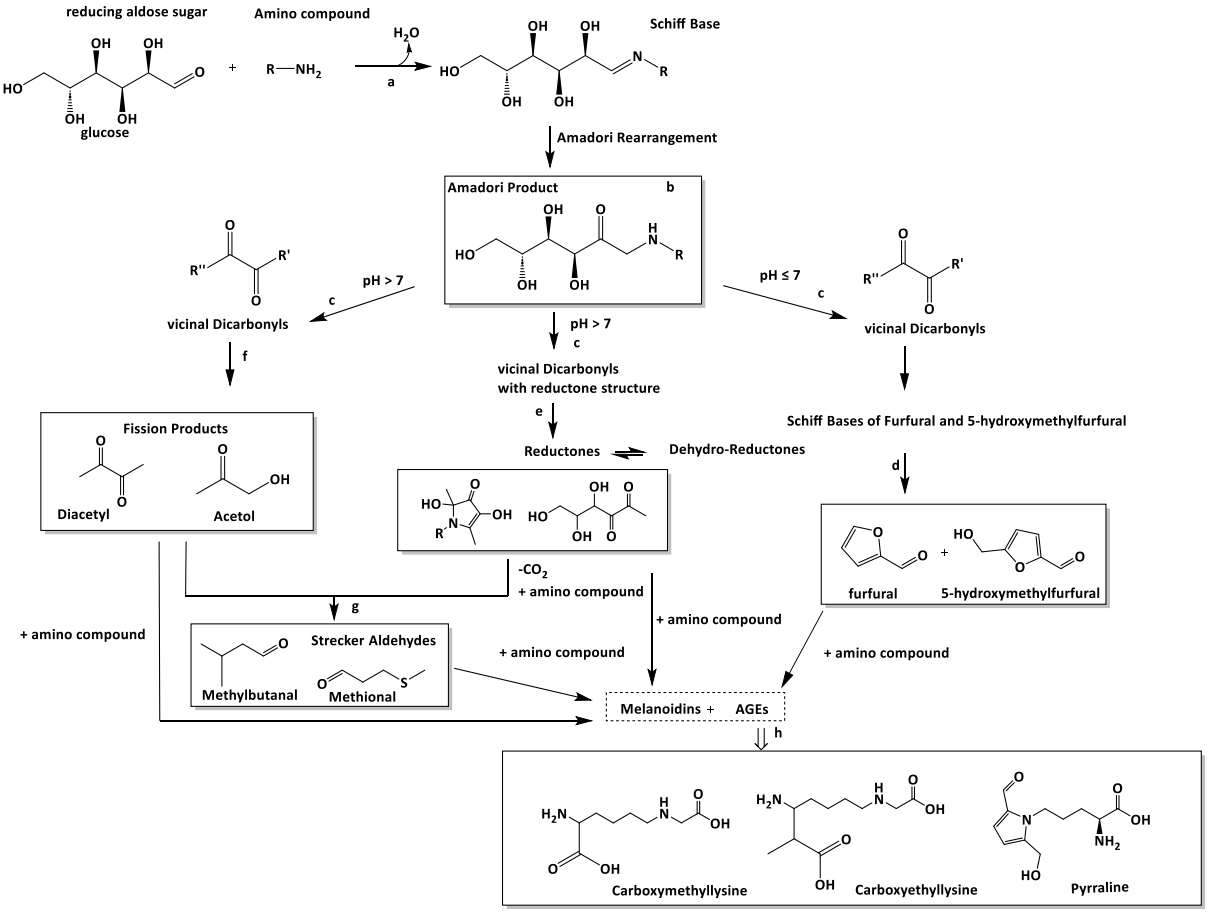

Figure 2 Schematic pathway of the "classic" ionic Maillard reaction and (intermediate) products. *This figure incorporates and links pathways suggested by various authors, including Hodge [259], Lund et al. [253], Hellwig et al. [252] and Martins et al. [260].*

The pathway of the Maillard reaction, as summarised by Lund et al. [253], Martins et al. [260] and Hellwig et al. [252] is explained in the following: In a first step, the protein amine group reacts with the carbonyl moiety of a reducing sugar and a Schiff base is formed (a). This Schiff base undergoes rearrangement to Amadori products (or Heyns products for ketoses) (b). The further steps yield α-dicarbonyl compounds (deoxyosones) (c), which are prone to be attacked by nucleophiles. The exact

pathway of the further reactions and formed intermediate products depend largely on the pH as depicted in Figure 2. While furfural and 5-hydroxymethylfurfural (d) are generated in an acidic environment, reductones (compounds with α-ketoenediol structure) (e) and fission products such as diacetyl and acetol (f) are formed if the pH is above 7. Eventually, upon further reactions and rearrangement processes these highly reactive intermediates form Strecker aldehydes (g) and advanced glycation products (AGEs), such as N-ε-(carboxymethyl)lysine (CML), N-ε-(carboxyethyl)lysine (CEL) and pyrraline (h), as well as melanoidins, brown nitrogen rich polymers and co-polymers [252, 253, 260]. In general, the product spectrum of the Maillard reaction, especially of AGEs, is very heterogeneous and complex [261].

The dicarbonyls formed in the subsequent steps after Amadori/Heyns rearrangement, such as 2-hexosulose, 1- and 3-deoxysone, methylglyoxal (MG), and glyoxal (GO) are usually formed by degradation of the sugar via retro-aldol cleavage, but they could also be derived from Amadori products or Schiff bases via the Namiki pathway [262, 263]. The dicarbonyls undergo further reactions including the Strecker degradation upon reaction with an amino compound, which characteristically, releases CO_2 [260, 264]. Furthermore, characteristic compounds influencing aroma, the so-called Strecker aldehydes are generated and these aldehydes can give rise to other aroma active compounds, such as furans, furanones and pyrazines [265]. They can also contribute to the characteristic browning observed upon cooking by generation of brown pigments and polymers, commonly referred to as "melanoidins", upon reaction with amino compounds [254]. Thus, the Maillard reaction impacts several facets of food quality such as colour and aroma either in a positive or, especially when occurring in excess, in an undesirable way, leading to bitter and burnt notes [253, 258]. Furthermore, toxic compounds such as acrylamide can be formed [265].

The aforementioned scheme serves as a comprehensive overview of the reaction mechanisms in the "classic" ionic pathway [259], but is worth noting that oxidative (autoxidation of sugars) [266] or free radical (Namiki) pathways [262] may also contribute [267]. The free radical pathway involves stable N,N'-dialkylpyrazine-cation-radicals generated from the Schiff base generation in the early Maillard reaction steps preceding the Amadori rearrangement [262, 263, 268, 269]. Which of the specific pathways dominates can be influenced by the pH level. The ionic pathway described above predominates at pH 5, but at higher pH, the oxidative pathway and the involvement of radicals increases [259, 270]. The proportion of the radical mechanisms is primarily determined by capability of the sugar to form fragments, whereas the impact of the amino acid is negligible [270]. However, apart from those stable radicals, the effects of short-lived radicals are not very well researched [267].

As mentioned before, the amine moiety, reacts with a carbonyl group that is in most cases derived from reducing monosaccharides, but di-, oligo-/poly-saccharides or pentoses (which are present in meat)

could as well be the substrate [255]. However, it is the reacting amino acid that will determine the structure of the final products, including AGEs [271]. Due to the high reactivity of primary amines, the sidechain of lysine as well as the guanidine group of arginine are the most reactive precursors among the different amino acids [272]. Thus, AGEs are predominantly formed from lysine and arginine, but cysteine-derived products have also been reported [273]. One of the most common markers for AGEs are N-ε-carboxymethyl-lysine (CML), first identified by Ahmed in 1986 [274] and the homologue N-ε-carboxyethyl-lysine (CEL) [275-278]. These AGEs can be generated in multiple ways, but the formation mechanisms of the two homologues are similar. Firstly, fructoselysine, an unstable Amadori rearrangement product formed upon condensation of glucose with the ε-amino group of lysine, can be oxidised to CML. The second mechanism involves the reaction of glyoxal (GO) or methylglyoxal (MG), which can be formed via lipid peroxidation and auto-oxidation of reducing sugars [267, 279]. The reaction of GO with lysine's ε-amino group produces CML, whereas MG yields N-ε-carboxyethyl-lysine (CEL) [274, 280, 281]. It has been shown that about half of the produced CML in a glucose/lysine system originates from oxidation of Amadori products, the rest being most likely generated by the Namiki pathway of the Maillard reaction [282].

However, the reacting amine group could also be bound to a protein, which could result in permanent alterations or cross-linking and therefore impact the functionality of the protein. An example of a compound with crosslinking properties would be pentosidine, derived from pentose with lysine and arginine residues of proteins [267, 283].

2.4.1 Influential Factors of Maillard Reaction and AGEs formation

The Maillard reaction as well as the kinetics and the product range of AGEs in foodstuffs depend on various internal and external factors. The internal factors include the product composition (presence of precursors, transition metals or pro- and antioxidants), while pH, time, temperature, and water content are among the external factors [267].

The initial pH does not only have impact on the pathway of the reaction as mentioned above but also on its kinetics [267]. The reaction turnover was shown to be low at acidic pH and to increase proportionally to the pH, until a pH value around 10 is reached, where the amount of protons necessary to catalyse reactions such as the rearrangements is too low [284]. The water content is of similar importance to the Maillard reaction as it influences the mobility of reactants, but a high water content could lead to strong dilution of the reactants and therefore decrease the rate of the reaction [256].

2.4.2 Maillard Reaction and Advanced Glycation End products in Meat

Several studies investigated the content of selected AGEs (CML, CEL, MOLD, MOLA, and MGLA) in different muscle foods such as fish, pork, beef, and chicken and the influence of heat treatment processes, the type of meat and the presence of food additives, as reviewed by Li et al. [280].

Studies have shown that heat processing has an enormous impact on the advanced glycation products formed. Eggen and Glomb [285] reported CML in raw pork to be predominantly GO-derived, whereas upon grilling, MGO-derived AGEs (CEL, MOLD, and MOLA) showed a much stronger increase than GO-derived AGEs (CML, GOLD, GOLA). However, other studies could not confirm these results, but rather produced contradicting results, as they presented higher concentrations of CML than CEL in processed pork [286]. It has been published that the internal temperature while processing has an impact on the products formed. A significant increase in the amount of fluorescent compounds was already observed when the internal temperature of roast beef reached 90 °C, whereas it had to reach 300 °C to detect CML [287]. Apart from the temperature, the type of heat treatment also has an impact on the formation of AGEs. Studies showed CML was predominantly generated by grilling and frying, but only to a lesser extent while roasting [288]. In terms of the conditions of heating, it has been shown that cooking under high moisture conditions compared to dry heat cooking seems to reduce the formation of AGEs. Furthermore, the reduction of time and temperatures and the addition of acidic ingredients may decrease the concentration of AGEs formed as well [289].

2.4.3 Analytic Techniques to Assess AGEs

Generally, as summarised by Li et al. [280], analytical approaches to quantify AGEs can be divided into two categories: instrumental and immunoassay methods. Instrumental analysis mostly includes (ultra-) HPLC and GC coupled with either diode array, fluorescence, ultraviolet or tandem mass spectrometry detection, whereas the most common immunoassay method is the enzyme-linked immunosorbent assay (ELISA).

The spectrum of AGEs structures is broad and so are the properties these compounds hold, and therefore also the possibilities for their detection [280]. Whereas pyrraline absorbs in the ultraviolet spectrum and hence can be measured using HPLC-DAD [290], fluorescent and crosslinking AGEs, such as pentosidine can be quantified via HPLC-FLD [291]. If the compounds are not inherently fluorescent, they can be derivatised to be analysed with beforementioned method [288]. CML and CEL are most commonly quantified with MS methods, but they are prone to be influenced by matrix interference and ion suppression effects [292]. Different approaches have been tested, such as incorporation of nonafluoropentanoic acid (NFPA) as an ion pair reagent to achieve better retention on reversed-phase columns [280, 293].When GC is selected, pretreatment of samples, involving derivatization of both the carboxyl group with methanol at a low pH and the amine group with TFA anhydride is usually necessary [280, 294].

On the one hand, immunochemical methods are fast and convenient and therefore these methods allow to upscale the sample number easily. Moreover, commercial kits are available, but these are only recommended in the medical field up to date. On the other hand, the results are prone to be affected by matrix effects [280]. The methods of instrumental analysis excel in sensitivity, selectivity, repeatability and reproducibility, but require extensive sample preparation as summarised by Zhu et al. [295]. As of now, there is a lack of standardized methods for the quantification of AGEs [257].

3 Goals and Aims

As outlined in detail in section 2.1.3 extensive research has been undertaken to evaluate the safety of monosodium glutamate as a food additive as well as to assess its sensory characteristics and technological aspects [9-12]. However, research regarding the reactivity of this common additive upon household cooking and storage covering its impact on protein and lipid oxidation is lacking.

Therefore, our group recently studied the impact of monosodium glutamate on lipid oxidation in pork burger patties (unpublished). The results indicated lipid peroxidation promoting effects after addition of 1.2 % MSG to the meat as evidenced by the significant decrease in the content of linoleic acyl groups and the increase in the alkanals content that were observed via ^{1}H-NMR and SPME-GC/MS. Nevertheless, neither the mechanisms nor the intermediary products contributing to those effects in the meat products, nor the impact of MSG on protein oxidation are known.

The aims of the present thesis are firstly to determine the impact of MSG fortification on protein oxidation and the (non-)volatile profile of meat, along with storage and cooking and, secondly, to elucidate the role of MSG in the promotion of lipid and protein oxidation. Furthermore, mechanisms and intermediary molecules involved in these pathways should be identified. These findings will contribute to the full picture of the potential drawbacks of MSG commonly used as a food additive and may point towards the re-evaluation of its safety if needed.

4 Material and Methods

4.1 Materials

Table 2 Materials used for the present thesis

Device, consumable, or software	Supplier
Arium pro, Ultrapure Water System	Sartorius Stedium Biotech GmbH
Centrifuge 5418 R	Eppendorf AG
Centrifuge 5810 R	Eppendorf AG
MR Hei-Tec magnetic stirrer	Heidolph Instruments GmbH & Co.KG
PioneerTM-series Balance, PA114	OHAUS Europe GmbH
PioneerTM-series Balance, PA2102	OHAUS Europe GmbH
Sartorius Entris 224I-1S	Sartorius Lab Instruments GmbH & Co. KG.
Pipettes 2.5 µL, 10 µL, 100 µL, 1000 µL, 5000 µL	Eppendorf AG
Filter paper 1001-125, pore size 11 µm	Whatman International Ltd.
Syringe filter Rotilabo, PVDF, pore size 0.22 µm, ⌀ 13 mm	Carl Roth GmbH & Co. KG
Syringe filter Rotilabo, PVDF, pore size 0.20 µm, ⌀ 13 mm	Carl Roth GmbH & Co. KG
Syringe filter Rotilabo, PVDF, pore size 0.45 µm, ⌀ 13 mm	Carl Roth GmbH & Co. KG
1000 ml, 2000 mL Bottles	Duran Group GmbH
Screw-cap tube, 15 mL	Sarstedt Inc.
Screw-cap tube, 50 mL	Sarstedt Inc.
Microtube Rotilabo®	Carl Roth GmbH & Co. KG
Round-bottom flask 100 mL	Duran Group GmbH
Syringe Injekt 10 mL	B. Braun Melsungen AG
Syringe Injekt 2 mL	B. Braun Melsungen AG
Sterican® needle Gr. 2, G 21 x 1 1/2""/ ø 0,80 x 40 mm	B. Braun Melsungen AG
T18 brushless digital ULTRA-TURRAX	IKA®-Werke GmbH & Co. KG
S18N-19G dispersing tool	IKA®-Werke GmbH & Co. KG
Phenomenex Strata® Si-1 silica columns 500 mg/6 mL	Phenomenex Inc.
Vacuum manifold Chromabond SPE 730150 N	Phenomenex Inc.
Septa, ⌀ 8 mm, thickn. 1.3 mm	Carl Roth GmbH & Co. KG
Micro-Insert, 0.2 mL	VWR International GmbH
HPLC vial, 1.5 mL	Agilent Technologies
Screw caps, PP, black	Carl Roth GmbH & Co. KG
Ultrasonic cleaner USC500 TH	VWR International GmbH
Freeze-drier VaCo 5-II	ZIRBUS technology GmbH
Incubator	VWR International GmbH
Heating block	VWR International GmbH
Rotavapor, R-210	BÜCHI Labortechnik AG
Concentrator plus	Eppendorf Austria GmbH
Vortex RS-VA 10	Phoenix Instrument GmbH
pH meter pHenomenal® 1000L	VWR International GmbH

FlexStation 3 Multi-Mode Microplate-Reader	Molecular Devices, LLC.
SoftMax® Pro Software	Molecular Devices, LLC.
Microplate reader Spark®	Tecan Group Ltd.
SparkControl ™	Tecan Group Ltd
Quartz cuvette	Hellma GmbH & Co. KG
96-well plate	Greiner Bio-One GmbH
Digest Automat K-438	BÜCHI Labortechnik AG
Distillation Unit K-355	BÜCHI Labortechnik AG
Burette	Paul Marienfeld GmbH & Co. KG
Kjeldahl flasks 300 mL	BÜCHI Labortechnik AG
Volumetric flasks	Paul Marienfeld GmbH & Co. KG
Kjeldahl weighing boats	Whatman International Ltd.
Beakers	Duran Group GmbH
Titration flasks	Duran Group GmbH
IKA IKAMAG RCT Heated Magnetic Stirrer	IKA®-Werke GmbH & Co. KG
pH meter Lab 845	SI Analytics GmbH
Volumetric pipette 20 mL, 30 mL	Paul Marienfeld GmbH & Co. KG
Fisherbrand™ Analytical Balance, 220g x 0.1mg	Fisher Scientific Company
LCMS-8040 with LC-20 AD	Shimadzu Europa GmbH
LabSolutions v. 5.99 SP2	Shimadzu Europa GmbH
Skyline v.23.1	MacCoss Lab, Department of Genome Sciences, University of Washington
Luna HILIC 200 Å column, 100 × 3 mm, 3 µm	Phenomenex Inc.
HILIC 4 x 2.00 mm SecurityGuard	Phenomenex Inc.
Dual-Pressure Linear Trap-Quadrupole-Orbitrap mass spectrometer	Thermo Fisher Scientific
Xcalibur 4.1.50	Thermo Fisher Scientific Inc.
GCMS-QP 2010 Ultra mit AOC 5000 Plus	Shimadzu Europa GmbH
GC/MS-Solution Version 4.52	Shimadzu Europa GmbH
Zebron ZB-5ms column (30 m x 0.25 mm x 0.25 µm)	Phenomenex Inc.
X-band Bruker Elexsys-II E500 CW-EPR spectrometer	Bruker Biospin GmbH
Bruker Xepr 2.6b.160	Bruker Biospin GmbH
Sample tubes E102215 (10x ø 4mm)	Bruker Biospin GmbH
NMR Bruker Avance 400	Bruker Biospin GmbH, Rheinstetten, Germany
NMR Tubes	Norell Inc.
MestReNova 14.3.3-33362	Mestrelab Research S.L.
GraphPad Prism®	GraphPad Software, Inc.
Office 365	Microsoft

4.1.1 Chemicals

Table 3 Reagents used for the preparation of the pork patties

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Monosodium Glutamate	$C_5H_8NO_4Na$	≥ 99.0 %, food grade	Ajinomoto Foods Europe SAS	-	-	-

Table 4 Reagents used for extraction according to Bligh and Dyer

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Chloroform	$CHCl_3$	≥99 %	Carl Roth GmbH & Co KG	Danger	06, 08	H302 H315 H319 H331 H336 H351 H361d H372
Methanol	CH_3OH	LC-MS grade	VWR International GmbH	Danger	02, 06, 08	H225 H301 H331 H311 H370
Sodium sulfate (anhydrous)	Na_2SO_4	≥99.0 %	Sigma-Aldrich	-	-	-
Diethylether	$C_4H_{10}O$	≥99.5 %	Carl Roth GmbH & Co KG	Danger	02, 07	H224 H302 H336
Petrolether	C_6H_{14}	p.a.	Carl Roth GmbH & Co KG	Danger	02, 07, 08, 09	H225 H304 H315 H336 H411
2-Propanol	C_3H_8O	≥99.5 %, LC-MS grade	Carl Roth GmbH & Co KG	Danger	02, 07	H225 H319 H336

Table 5 Reagents used for NMR analysis

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Methanol-[D4] (98 % D) with 0.03 % TMS	D_3COD	99.80 %	Carl Roth GmbH & Co KG	Danger	02, 06, 08	H225 H301H 311H3 31 H370
Deuterium oxide	D_2O	99.90 %	Eurisotop Cambridge Isotope Laboratories, Inc.	-	-	-

Table 6 Reagents used for monitoring of Schiff bases content

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Sodium phosphate monobasic	NaH_2PO_4	≥ 99,0 %	Sigma-Aldrich Chemie GmbH	-	-	

Table 7 Reagents used for quantification of the carbonyl content with the DNPH method

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Sodium Pyro-phosphate tetrabasic decahydrate	$Na_4P_2O_7 \cdot 10\ H_2O$		Sigma-Aldrich	Danger	05, 07	H302 H318
Tris-(hydroxy-methyl)-aminomethan-maleat (Tris-Maleate)	$C_8H_{15}NO_7$	≥99.5 %	Sigma-Aldrich	-	-	-
Potassium chloride	KCl	≥99.5 %	Carl Roth GmbH & Co KG	-	-	-
Magnesium chloride hexahydrate	$MgCl_2 \cdot 6\ H_2O$	≥99 %	Carl Roth GmbH & Co KG	-	-	-
Ethylen-glycol-bis(aminoethyl -ether)-N,N,N',N'-tetraacetic acid (EGTA)	$[CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$	≥97.0 %	Sigma-Aldrich	-	-	-
Trichloro-acetic acid (TCA)	CCl_3COOH	≥99 %	Carl Roth GmbH & Co KG	Danger	05, 07, 09	H314 H335 H400 H410
Hydrochloric acid 36 %	HCl		Merck KGaA	Danger	05, 07	H290 H314 H335
2,4-Dinitrophenyl hydrazine (DNPH)	$C_6H_6N_4O_4$	99 %	AppliChem GmbH	Danger	07	H302 H319 H315
Ethanol 96 %	C_2H_5OH	p.a.	VWR International GmbH	Danger	02, 07	H225
Ethylacetate	$C_4H_8O_2$	≥99.5 %	Carl Roth GmbH & Co KG	Danger	02, 07	H225 H319 H336
Guanidine hydrochloride	$CH_5N_3 \cdot HCl$	≥ 98 %	Sigma-Aldrich	Warning	07	H302

						H332 H315 H319
Sodium Phosphate monobasic	H_2NaO_4P	≥99.0 %	Sigma-Aldrich	-	-	-

Table 8 Reagents used for the simultaneous detection of different AGEs

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Sodium borohydride	$NaBH_4$	≥97 %	Carl Roth GmbH & Co KG	Danger	02, 05, 06, 08	H260H 301H3 14H36 0FD
Sodium borate buffer	H_3BO_3 / $Na_2B_4O_7 \cdot 10H_2O$	Ultra Pure	VWR chemicals	Danger	08	H360
Trichloro-acetic acid (TCA)	CCl_3COOH	≥99 %	Carl Roth GmbH & Co KG	Danger	05, 07, 09	H314 H335 H400 H410
Hydrochloric acid 36 %	HCl		Merck KGaA	Danger	05, 07	H290 H314 H335
Perfluoro-pentanoic acid	$CF_3(CF_2)_3COOH$	97 %	Sigma-Aldrich Chemie GmbH	Danger	05, 08	H318H 361d
Ammonium formate	NH_4HCO_2	≥99.0 %, LC-MS grade	Sigma-Aldrich	Warning	07	H319
Formic acid	HCO_2H	≥99 %, LC-MS grade	VWR chemicals	Danger	02, 05, 06	H226 H302 H314 H331
Acetonitrile	H_3CCN	LC-MS grade	VWR chemicals	Danger	02, 07	H225 H302 H312 H332 H319
Water	H_2O	LC-MS grade	VWR chemicals	-	-	-
L-Arginine	$C_6H_{14}N_4O_2$		Sigma-Aldrich	-	-	-
L-Lysine	$C_6H_{14}N_2O_2$	≥98 %	Sigma-Aldrich	-	-	-
CML	$C_8H_{16}N_2O_4$	p.a.	Iris Biotech GmbH	-	-	-

Table 9 Reagents used for measuring protein content using the Kjeldahl method

Chemical	Chemical formula	Purity	Supplier	Signal Word	GHS	Hazard
Sulfuric acid	H_2SO_4	95 %	VWR International GmbH	Danger	05	H290 H314 H318

Kjeldahl tablets Missouri	$K_2SO_4/CuSO_4 \cdot 5H_2O$	-	BÜCHI Labortechnik AG	-	09	H412
Boric acid	H_3BO_3	≥ 99,8 %	Carl Roth GmbH & Co KG	Danger	08	H360FD
Sodium Hydroxide ≥ 32 %	NaOH	Extra pure	Carl Roth GmbH & Co KG	Danger	05	H290 H314 H318
Sodium Hydroxide 20 %	NaOH	Extra pure	Carl Roth GmbH & Co KG	Danger	05	H290H 314 H318
HCl 0,2 M, volumetric standard solution	HCl	p.a.	Carl Roth GmbH & Co KG	Warning	05	H290
Tashiro's indicator solution in Ethanol	$C_{15}H_{14}N_3NaO_2/C_{16}H_{18}ClN_3S/C_2H_6O$	-	Carl Roth GmbH & Co KG	Warning	02	H226

4.2 Methods

4.2.1 Preparation of the Meat Patties

Grinded pork meat was acquired from a commercial supplier (13.02.2023, neck). The burger patties were prepared using a similar method to Botsoglou et al. [131]. Three groups of patties with different concentrations of monosodium glutamate were prepared, 0 % (w/w) (the control group, A), 0.4 % (w/w) (group C) and 1.2 % (w/w) (group E) MSG. These concentrations were chosen following recommendations for the optimal concentrations for the use of MSG for European customers [296]. Immediately after addition of MSG (160 mg for group C and 480 mg for group E), the samples were thoroughly mixed manually and formed into patties (40 g/patty) with average dimensions of 5 cm diameter and 1 cm thickness. An overview of the sample groups is shown in Table 10. The raw samples without storage time at 4 °C were directly frozen at -80 °C until sample preparation. The samples to be cooked without storage time at 4 °C were heated in an incubator at 180 °C for 15 min before freezing at -80 °C. The other samples were stored in a fridge at 4 °C for 2-4 days. They were subsequently cooked in an oven at 180 °C for 15 min and stored at -80 °C until sample preparation. Prior to sample preparation and analysis, they were thawed at room temperature.

Table 10 Overview of the total sample groups

		Raw	Cooked			
		Day 0	**Day 0**	**Day 2**	**Day 3**	**Day 4**
0 % MSG (A)	m(Patty) [g] m(MSG) [mg]	40.21 -	40.00 -	40.10 -	40.04 -	40.00 -
	m(Patty) [g] m(MSG) [mg]	39.95 -	40.05 -	40.10 -	40.05 -	40.10 -
	m(Patty) [g] m(MSG) [mg]	39.99 -	39.95 -	40.15 -	39.98 -	40.07 -
0.4 % MSG (C)	m(Patty) [g] m(MSG) [mg]	39.98 160.7	39.99 160.9	40.08 160.1	40.05 159.4	40.07 161.6
	m(Patty) [g] m(MSG) [mg]	40.02 162.5	40.03 160.6	40.04 161.5	40.04 159.5	40.01 160.6
	m(Patty) [g] m(MSG) [mg]	40.02 160.2	40.04 161.7	40.07 160.3	39.92 160.8	40.00 160.8
1.2 % MSG (E)	m(Patty) [g] m(MSG) [mg]	40.03 481.0	40.08 481.1	40.05 479.5	39.98 480.0	40.09 481.1
	m(Patty) [g] m(MSG) [mg]	40.04 480.8	40.09 480.1	40.09 480.7	40.04 479.5	39.98 480.2
	m(Patty) [g] m(MSG) [mg]	40.07 480.2	40.07 479.1	39.96 479.4	39.92 480.0	40.01 481.0
	Total: 45 samples					

4.2.2 Bligh and Dyer Extraction

The extraction of lipids was performed using the Bligh and Dyer method [297] following a protocol outlined by Pebriana et al. [298] and Pérez-Palacios et al. [299] with modifications.

A centrifuge tube was filled with 5 g meat and after the addition of 15 mL chloroform-methanol mixture (1:2 v/v) the mixture was homogenized using an Ultra Turrax homogenizer for 2 min at 8000 rpm, centrifuged (10 min, 1250 g), and filtered using filter paper. The filtrate was collected in a separate tube.

15 mL chloroform was added to the residue remaining in the tubes, homogenized for 2 min at 8000 rpm, centrifuged (10 min, 1250 g), and filtered using filter paper. The filtrate was added to the previous filtrate and mixed with 5 mL distilled water.

After shaking vigorously, the mixture was allowed to stand until the biphasic system separated. The aqueous (upper) phase was transferred to a round bottom flask and, after removing the residual methanol in the aqueous phase using a rotary evaporator, the extract was frozen at -80 °C.

Subsequently, the aqueous phase was freeze-dried, dissolved in 1 mL methanol, filtered with a 0.20 µm syringe filter and flushed with argon prior to further use.

The organic phase was dried with anhydrous sodium sulphate (approx. 1-2 g) and centrifuged at room temperature for 1 min with 1250 g. If an intermediate phase was formed, it was removed by gentle aspiration with a pipette. The solvents were evaporated using a vacuum rotary evaporator at room temperature and in order to separate polar lipids from non-polar lipids, solid phase extraction according to Márquez-Ruiz [300] with some modifications was performed. The whole lipid fraction was dissolved in 2 mL petroleum ether and added to the Phenomenex Strata® SI-1 silica cartridges (500 mg/6 mL), which had been conditioned beforehand with 10 ml petroleum ether/diethyl ether (90:10 v/v). The solvent was passed through while the yellow-coloured sample was retained on the column.

Subsequently, the non-polar fraction was eluted with 15 ml petroleum ether/diethyl ether (90:10 v/v) and a second fraction containing polar compounds was eluted with 20 ml diethyl ether. The solvent of the polar fractions was evaporated using a rotary evaporator. The polar lipid extracts were dissolved in 1 mL 2-propanol, filtered with a 0.20 µm filter and stored under argon atmosphere at -80 °C until further use.

4.2.3 Analysis of the Non-Volatile Profile of Aqueous Extracts by Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

150 µL aqueous extract in methanol was dried in a concentrator for 15 min, the residue was dissolved in 600 µL deuterated methanol which contained 0.03 % tetramethylsilane as internal reference. Additionally, a 100 mmol/L MSG standard was prepared in deuterated water. ^{1}H-NMR spectra of the samples were acquired using a Bruker Avance 400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 400 MHz. The following acquisition parameters were used as in previous studies [301]: spectral width 6410 Hz, relaxation delay 3 s, number of scans 64, acquisition time 4.8190 s, and pulse width 90°, with a total acquisition time of 8 min 38 s. The analysis of the spectra was performed using MestReNova 14.3.3, following Fourier transformation (FT), baseline correction and phase correction.

4.2.4 Analysis of the Volatile Profile of Polar Lipid Extracts by GC-MS

The polar lipid fractions underwent GC-MS analysis with the following conditions (adjusted parameters from a previous study [302]). The GC/MS (GC-QP2010 Ultra, Shimadzu, Korneuburg, Austria) was equipped with a Zebron ZB-5ms column (30 m x 0.25 mm x 0.25 µm Phenomenex, Aschaffenburg, Germany) and helium as a carrier gas with a constant pressure at 53 kPa, a total flow of 4.0 mL/min and a column flow of 1 mL/min. Liquid injection of 1 µL sample was performed in splitless mode with a 10 µL syringe (Shimadzu, Korneuburg, Austria). The injector temperature was 250 °C. The initial

temperature of the oven was kept at 50 °C for 5 min, increased to 300 °C at a rate of 4 °C/min and was kept there for another 30 min. Ion source and quadrupole mass analyzer temperatures were held at 230 °C and 150 °C respectively. The temperature of the interface was set at 305 °C. An Ionization energy of 70 eV was used. A scan event was performed for compounds between m/z 40 and m/z 600. The compounds were identified by matching their mass spectra with spectra from a commercial library by more than 85 % (NIST, ver. 11.0 library).

4.2.5 Analysis of the Non-Volatile Profile of Polar Lipid Extracts by Untargeted HPLC-High-Resolution-MS/MS

The polar lipid fractions were analysed with an HPLC coupled to a Dual-Pressure Linear Trap-Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Germering, Germany). The parameters of the method were used following a protocol outlined by Grüneis et al [303].

The samples were injected (3 µL) into an UltiMate 3000 series HPLC system (Dionex/Thermo Fisher Scientific, Germering, Germany) and separated on a C18 column (Atlantis 2.1 mm × 150 mm, 3 µm, Waters) at 25 °C. The mobile phase was acetonitrile/H_2O (60/40) with 0.1 % formic acid and 10 mM ammonium formate (A) and acetonitrile/ isopropanol (20/80) with 0.1 % formic acid and 10 mM ammonium formate (B). Formic acid was added to facilitate ionization in positive mode. Ammonium formate induced the formation of ammonium adducts, which led to an increase in sensitivity. The HPLC gradient elution was programmed as follows: 0−15 min with 40−100 % B, 15−25 min with 100 % B, and 25−27 min with 40 % B. The flow rate was set at 0.3 mL/min.

The following ESI ion source settings were applied: source voltage: 3.5 kV, capillary voltage: 25 V, capillary temperature: 300 °C, sheath gas flow: 45 AU (N_2) and aux gas flow: 10 AU (N_2).

Each sample was analysed in positive mode in the range of m/z 50–2000. Calibration was performed using a lock mass (m/z 226.9515, $((HCOONa)_3+Na)^+$. Furthermore, the LC-MS/MS experiments were performed in top 6 mode, where the highest 6 ions at each time point were selected for fragmentation. The normalized collision energy (CID) was set to 35 eV. The analysis of the results was performed using Xcalibur 4.1.50, for data visualization (Thermo Fisher Scientific, Germering, Germany) and Skyline v. 23.1, for peak integration (MacCoss Lab, Department of Genome Sciences, University of Washington). The chromatograms were manually compared, evaluating possibly significant peak changes within different sample groups. The compounds of interest were identified using the ms2 spectra, with the assistance of databases (MassBank Europe, MassBank of North America) and previous literature, including Grüneis et al [303].

4.2.6 Simultaneous (Semi-) Quantification of Free Lysine, Arginine and Glutamate

The aqueous extracts were 1:2 diluted with LC-MS grade water for the semi-quantification of lysine and arginine for all the sample groups (A, C, E). For the quantification of glutamate, the samples of group A were 1:2 diluted and of group C and E 1:10 diluted with LC-MS grade water. MSG standards for quantifying glutamate in the concentration range of 5-1000 µmol/L were also prepared (R^2=0.9926; y=169664x+4E+06). The samples were injected directly into the mass spectrometer (LCMS 8040, Shimadzu, Korneuburg, Austria) and measured in Multiple Reaction Monitoring (MRM) mode with the parameters shown below in Table 11. All measurements were carried out in positive mode. The transitions for glutamate were optimized by performing product ion scans and precursor ion scans with the glutamate standard. The limit of quantitation for MSG was 5 µmol/L. Protein-bound lysine and arginine was determined according to section 4.2.9.

Table 11 MRM qualifier and quantifier ions and collision energies (CE) used for the determination of lysine, arginine and glutamate

Product	**Precursor ion (m/z)**	**Product ions**				**Reference**
		Quantifier (m/z)	**CE (V)**	**Qualifier (m/z)**	**CE (V)**	
Lysine	147	130	-10	84	-20	Liu et al., 2019 [304] Gómez-Ariza et al., 2005 [305]
Arginine	175	116	-20	70	-30	Liu et al., 2019 [304] Gómez-Ariza et al., 2005 [305]
				60	-30	Martens-Lobenhoffer et al., 2012 [306]
Glutamate	189	84	-20	56	-20	-

The analysis of the chromatograms was performed using Skyline v.23.1 and LabSolutions v. 5.99 SP2.

4.2.7 Schiff Bases Monitoring and Tryptophan Loss

The natural fluorescence of tryptophan and the emission of fluorescence by protein oxidation products (Schiff base structures) were assessed by using fluorescence spectroscopy according to Utrera et al. [245].

Patties (1 g) were homogenized in 10 mL 20 mM sodium phosphate buffer (pH 6.5) using an Ultra-Turrax homogenizer for at 8000 rpm for 30 s. The water homogenates were filtered to remove insoluble particles. A 500 µL aliquot of the homogenates was redissolved in 10 mL 20 mM sodium phosphate buffer. 200 µL of these dilutions were dispensed in a 96-well-plate. Emission spectra of tryptophan were recorded from 300 to 400 nm in 5 nm steps with the excitation wavelength established at 283 nm. The concentration of tryptophan was expressed as fluorescence intensity units emitted at 345 nm.

Emission spectra of Schiff bases were recorded from 370 to 500 nm in 5 nm steps with the excitation wavelength set at 350 nm. The content of Schiff base structures was expressed as fluorescence intensity units emitted at 460 nm.

4.2.8 Quantification of Carbonyl Content by DNPH Method

Protein oxidation, as measured by the total carbonyl content, was evaluated by derivatisation with DNPH according to the method described by Armenteros et al. [237] and Levine et al. [236] with modifications.

Meat products were thawed, minced, and then homogenized 1:10 (w/v: 0.5 g/5 mL) in pyrophosphate buffer (pH 7.4) (PB) consisting of 2.0 mM $Na_4P_2O_7$, 10 mM tris–maleate, 100 mM KCl, 2.0 mM $MgCl_2$ and 2.0 mM EGTA using an Ultra Turrax homogenizer at 16800 rpm for 30 s.

The proteins of a 50 µL aliquot were precipitated by adding 0.5 mL 10 % TCA and centrifuged for 5 min at 5000 g at room temperature. The supernatant was removed, and the pellet was treated with 0.5 mL 0.2 % (w/v) DNPH in 2 N HCl. After incubation for 1 h at room temperature (shaken every 15 min) the proteins were again precipitated with 0.5 mL 10 % TCA. The pellet was washed three times with 1 mL ethanol/ethyl acetate (1:1 v/v), shaken and vortexed, and centrifuged for 5 min at 10000 g.

After drying for 30 min the pellet was dissolved in 0.75 mL 20 mM sodium phosphate buffer pH 6.5 containing 6 M guanidine hydrochloride, shaken, and centrifuged for 2 min at 5000 g to remove insoluble fragments.

The absorption of the samples at 280 and 370 nm was measured in a quartz glass cuvette. The concentration of carbonyls in the sample was calculated applying Equation 1, which includes a correction factor to account for the fact that DNPH itself absorbs light at 280 nm to the extent of 43 % of its absorption at 370 nm.

Equation 1 Calculation of carbonyl content according to Levine et al., 1994 [236]

$$\frac{c\,(carbonyl)}{c\,(protein)} = \frac{\left(\varepsilon_{protein\,280}\right)\cdot(Absorbance_{370})}{\varepsilon_{carbonyl\,370}\cdot(Absorbance_{280} - 0.43\cdot Absorbance_{370})} = \frac{mol\,(carbonyl)}{mol\,(protein)}$$

$\varepsilon_{protein\,280}$ … molar extinction coefficient of proteins at 280 nm

$\varepsilon_{carbonyl\,370}$ … molar extinction coefficient of carbonyls at 370 nm

$Absorbance_{280}$ … absorbance at 280 nm

$Absorbance_{370}$ … absorbance at 370 nm

4.2.9 Simultaneous Detection of Different AGEs

The sample preparation procedure adhered to the protocol outlined by Teerlink et al. [293] with modifications. 20 mg freeze-dried meat sample was suspended in 100 µL distilled water and 500 µL

100 mmol/L sodium borohydride in 200 mmol borate buffer (pH 9.2) were added. After 2 hours of incubation at room temperature, 1 mL 40 % TCA was added to precipitate proteins. The samples were vortexed for 2 seconds, centrifuged (16000 g, 10 minutes) and upon removal of the supernatant, the protein pellet was washed with 1 mL 10 % TCA and centrifuged (16000 g, 10 minutes). The supernatant was removed and 500 µL 6 mol/L HCl were added to the protein pellet. To hydrolyse the proteins, the samples were incubated for 20 hours at 110 °C and subsequently dried in a vacuum concentrator at 60 °C. The residue was dissolved in 500 µL 5 mmol/L perfluoropentanoic acid and vortexed for 2 seconds. The samples were filtered through a 0.20 µm nylon syringe filter prior to analysis with LC-MS (LCMS 8040, Shimadzu, Korneuburg, Austria).

The analytes were separated using a column (Luna HILIC 200 Å column (100 × 3 mm, 3 µm, Phenomenex, Aschaffenburg, Germany), equipped with a HILIC precolumn (4 × 2.00 mm, 3 µm, Phenomenex) and 6 mmol/L ammonium formate and 0.1 % (v/v) formic acid in water (pH 2.4) as solvent A and 80 % (v/v) acetonitrile and 20 % (v/v) of mobile phase A with 0.1 % formic acid as solvent B with the following gradient: 0-2 min 90 % B, 3-5 min 87 % B, 9-15 min 30 % B, 20-30 min 90 % B (injection volume 10 µL, flow rate 0.4 mL/min). Subsequently external standards were used to quantify CML (range: 0.02-50 mg/L; R^2=0.9994; y=331723x+1781.8), lysine (range: 50-500 mg/L; R^2=0.9988; y=198127x+3E+06) and arginine (range: 50-1000 mg/L; R^2=0.9602; y=436562x+4E+07), whereas the other analytes were semi-quantified using the area under the curve values. The multiple-reaction monitoring (MRM) parameters detailed in Table 12, in positive mode.

Table 12 MRM qualifier and quantifier ions and collision energies (CE) used for the determination of AGEs, lysine and arginine

Product	Precursor ion (m/z)	Product ions				Reference
		Quantifier (m/z)	CE (V)	Qualifier (m/z)	CE (V)	
CML	205	84	-19	130	-14	Zhang et al., 2011 [307]
CEL	219	84	-18	130	-12	Zhang et al., 2011 [307]
Furosine	255	130	-18	84	-28	Troise et al., 2014 [308]
MOLD	341.3	84	-42	-	-	Lin et al., 2023 [309]
GOLD	327.3	84	-34	-	-	Lin et al., 2023 [309]
MG-H1	229	114	-30	211	-10	Baye et al., 2019 [310], Lin et al. 2023 [309]
				166	-20	
				70	-20	
G-H1	215.2	70	-30	-	-	Lin et al., 2023 [309]

Argpyrimidine	255	192	-15	140	-15	Zhang et al., 2011 [307]
Pentosidine	379	250	-22	187	-30	Zhang et al., 2011 [307]
Lysine	147	130	-10	84	-20	Liu et al., 2019 [304] Gómez-Ariza et al., 2005 [305]
Arginine	175	116	-20	70	-30	Liu et al., 2019 [304] Gómez-Ariza et al., 2005 [305]
				60	-30	Martens-Lobenhoffer et al., 2012 [306]

4.2.10 Electron Spin Resonance Spectroscopy (ESR)

Portions of the meat patties of the different groups were freeze-dried, placed in ESR quartz tubes (10x ø 4mm, Bruker Biospin GmbH) and directly measured following the protocol outlined by Yu et al. [311] with alterations.

The electron spin resonance was measured using an X-band Bruker Elexsys-II E500 CW-EPR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) with following parameters: center field 3505 G, modulation frequency 100 kHz, modulation amplitude 10 G, receiver gain 60 dB, attenuation 10 dB, sweep width 200 G, microwave power 20 mW, microwave frequency 9.43 GHz. The Software XEPR 2.6b.160 by Bruker Biospin GmbH was used to analyse the spectra and calculate the spin count of the samples.

4.2.11 Determination of Moisture Content by the Loss on Drying (LOD) Method

The determination of moisture content was performed according to the standardised reference method [312].

3 g of each sample was weighed in a beaker and subsequently heated at 103 °C for 3 h before cooling down in a desiccator and subsequent weighing. The difference in weight was determined as the moisture content.

4.2.12 Determination of the Loss on Cooking

Another set of patties was prepared, each with triplicates of each MSG concentration A, C and E. These patties were weighed and subsequently cooked at 180 °C for 15 minutes before cooling down and weighing. The difference in weight before and after cooking between the different groups was analysed.

4.2.13 Determination of Protein Content by Kjeldahl Method

The protein content was determined using the Kjeldahl method described by Mihaljev et al. [313].

1 g homogenized sample was weighed in with nitrogen-free weighing boats, transferred to a 300 mL Kjeldahl flask and 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 additional 30 minutes cooldown.

50 mL water and 90 mL 32 % NaOH were subsequently added to the digested solution following steam distillation for 240 s per sample. The distillate was collected in a titration flask containing 60 mL 4 % (w:w) boric acid.

15 drops Tashiro indicator were added to the distillate and the solution was titrated with 0.2 mol/L HCl until the colour change from green to grey, marking the equivalence point, appeared.

The nitrogen and protein content were calculated according to Equation 2 and Equation 3.

Equation 2 Calculation of weight percentage of nitrogen in the samples

$$w\,\%\,N = \frac{(V_{sample} - V_{Blank})}{m_{sample} \cdot 1000} \cdot z \cdot c \cdot f \cdot M \cdot 100$$

Equation 3 Calculation of weight percentage of protein in the samples

$$w\,\%\,P = \frac{(V_{sample} - V_{Blank})}{m_{sample} \cdot 1000} \cdot z \cdot c \cdot f \cdot M \cdot PF \cdot 100$$

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 [mol/L]

f … factor of the standard solution (in this case 1)

M … molar mass of nitrogen (14.007 g/mol)

PF … protein factor (in this case 6.25)

4.3 Statistical Analysis

At least three independent replicates were tested per MSG concentration in the respective sample group. The results were expressed as mean ± standard deviation. Statistical analyses were performed with GraphPad Prism® 9.5 (Boston, MA, USA). Normal distribution of the results was tested with the D'Agostino-Pearson test ($\alpha > 0.05$) and equal variances were tested with the Brown-Forsythe test ($p > 0.05$). Significance of differences was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test. Statistical significance was accepted for $p < 0.05$. Pearson's correlation coefficients were calculated to correlate the results of the MSG (semi-)quantification with LC-MS and ^{1}H-NMR ($p < 0.05$).

Limit of detection (LOD) and Limit of quantification (LOQ) of the different quantified analytes were determined using the signal-to-noise-ratio (S/N) of the measured standards, where the LOD corresponds to a S/N of 3 and LOQ to a S/N of 10 [314]. In instances where the lowest concentration of a standard curve showed a S/N ratio above 10, that concentration was claimed as LOQ.

5 Results

5.1 Quantification of Glutamate

Despite initially tripling the MSG content in group E compared to group C, the LC-MS results demonstrated that the actual concentration of MSG in the samples of group E did not exhibit a threefold increase in comparison to group C (as depicted in Figure 3).

For group C the recovered MSG concentration ranged from 4.527-12.095 mg/100 g sample, whereas group E exhibited 10.916-24.313 mg MSG/100 g sample. These findings remained consistent throughout the different storage durations and the raw and cooked samples.

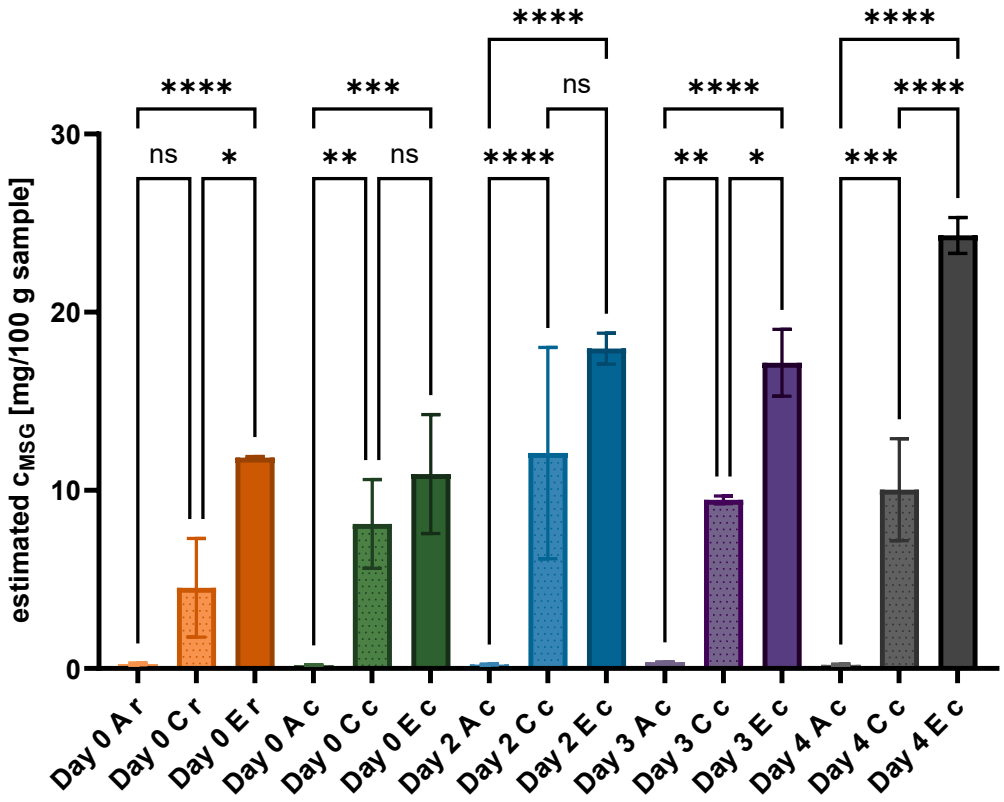

Figure 3 Quantification of glutamate with LC-MS. *Shown is the MSG content for the samples with the three MSG concentrations used (A, C, E), after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

Exemplary ^{1}H-NMR spectra of raw samples without additional MSG (concentration A) and with added MSG in different concentrations (C and E) as well as the spectrum of a pure MSG standard are displayed in Figure 4. The identification of the β-CH_2 and γ-CH_2 group of glutamate could be accomplished through review of corresponding literature and the comparison with the MSG standard spectrum [315, 316]. The addition of MSG to the pork patties led to more complex peak shapes for the MSG signals, to

the appearance of new prominent peaks in the region of interest and slight changes of the chemical shift due to matrix effects and coupling of protons in close vicinity to the β-CH_2 and γ-CH_2 group of glutamate. Nevertheless, the persistence of the distinct peak shapes and the peak range facilitated the identification process.

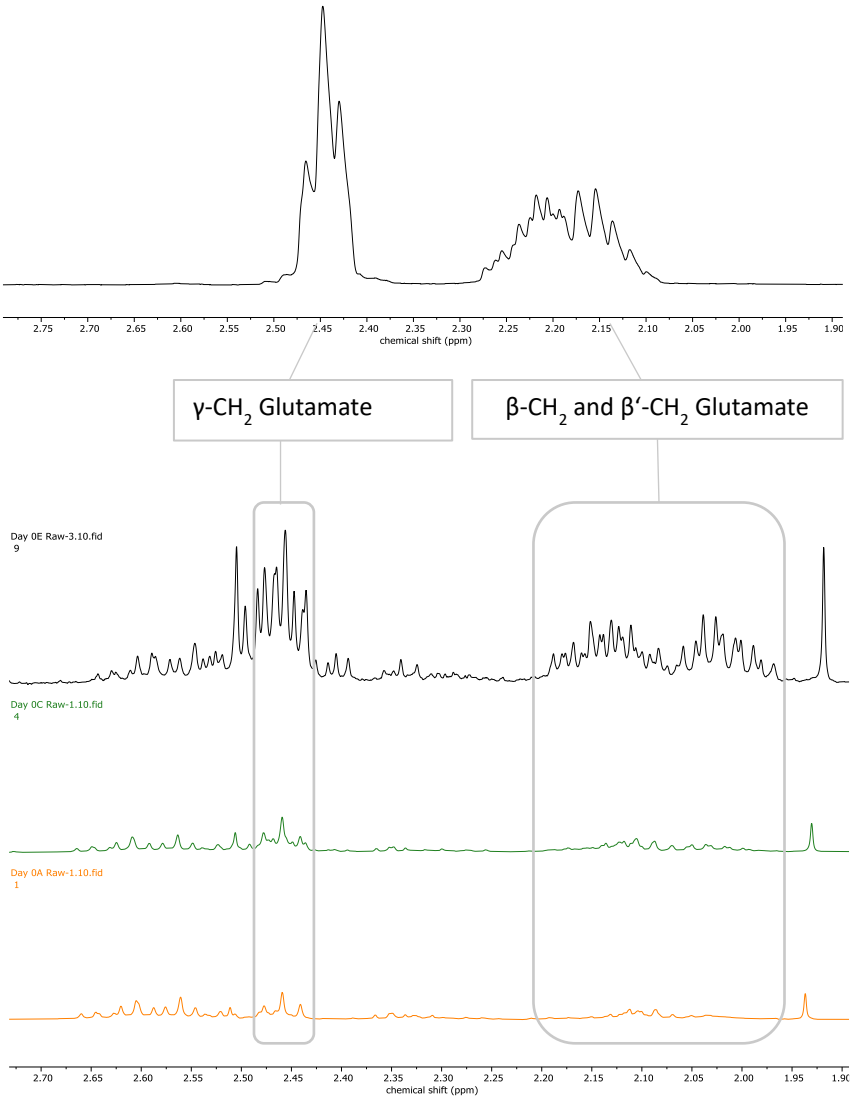

Figure 4 Exemplary ^{1}H-NMR spectra of raw samples with different MSG concentrations. *MSG standard on top and below the zoomed in spectra of raw samples with MSG concentration E (black), C (green) and without MSG (yellow) as well as the assignment of the functional groups to the corresponding peaks in standard and sample spectra.*

Moreover, it should be noted that not only the samples spiked with MSG exhibited the characteristic glutamate peaks, but the samples without added MSG also showed the natural occurrence of glutamate in low concentrations, as presented in Figure 5 [317].

Furthermore, the NMR measurements showed that the area under the curve (AUC) values for the proton signals of both beta- and gamma-methylene groups, in the case of concentration E, did also not show a threefold increase, which was consistent during storage and cooking (as shown in Figure 5). Hence, the NMR results corroborated the results of quantification of MSG obtained through LC-MS,

revealing a linear correlation between the results from both analytical methods as depicted in Figure 6.

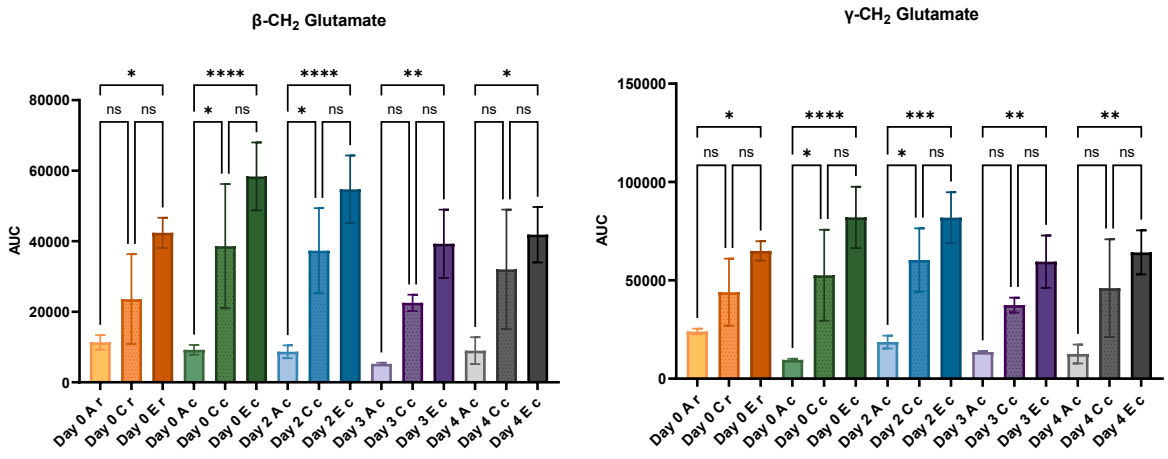

Figure 5 Proton signal areas of -$C_\beta H_2$- and -$C_\gamma H_2$- groups of glutamate with ^{1}H-NMR. *Shown are the different values of the AUC for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage. AUC: area under the curve*

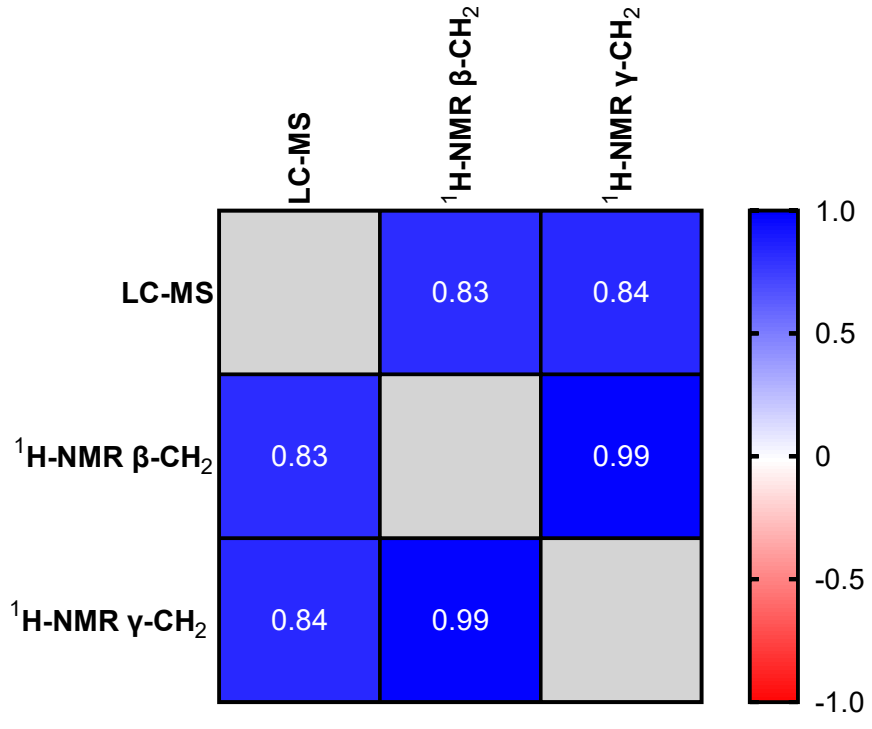

Figure 6 Heatmap of the correlation matrix generated by the Pearson correlation coefficient. *Shown are the results for the correlation analysis for MSG quantification results of LC-MS and $C_\beta H_2$- and -$C_\gamma H_2$- groups of glutamate with ^{1}H-NMR for all sample groups. The scale is set from -1 (red) to 1 (blue), $p < 0.05$.*

5.2 Moisture content by Loss on Drying and Loss on Cooking Methods

The meat patties contained between 62.19 ± 1.84 % and 66.98 ± 1.11 % water determined by the loss on drying at 103 °C for 3 h (as displayed in Table 13).

The patties lost between 18.07 ± 0.84 % and 19.41 ± 2.29 % water during the 15 min of cooking at 180 °C during sample treatment (as shown in Table 13).

There was no significant difference in the loss on drying or the loss on cooking between the MSG concentrations, showing that there is no significant influence of MSG on the water retention behaviour.

Table 13 Results of the Loss on Drying and Loss on Cooking for the samples with the three MSG concentrations (A, C, E) used. *Results depicted as mean ± standard deviation [% weight loss], n=3.*

	Loss on Drying	**Loss on Cooking**
MSG concentration	mean ± SD [% weight loss]	mean ± SD [% weight loss]
conc. A	63.49 ± 1.68	18.07 ± 0.84
conc. C	66.98 ± 1.11	18.26 ± 0.69
conc. E	62.19 ± 1.84	19.41 ± 2.29

5.3 Analysis of the Volatile Profile of Polar Lipid Extracts by GC-MS

The results of the GC-MS measurements lacked a consistent trend, which could warrant a statistically significant difference in the volatile profile between the samples with different MSG concentrations. A set of exemplary chromatograms is shown in Figure 7.

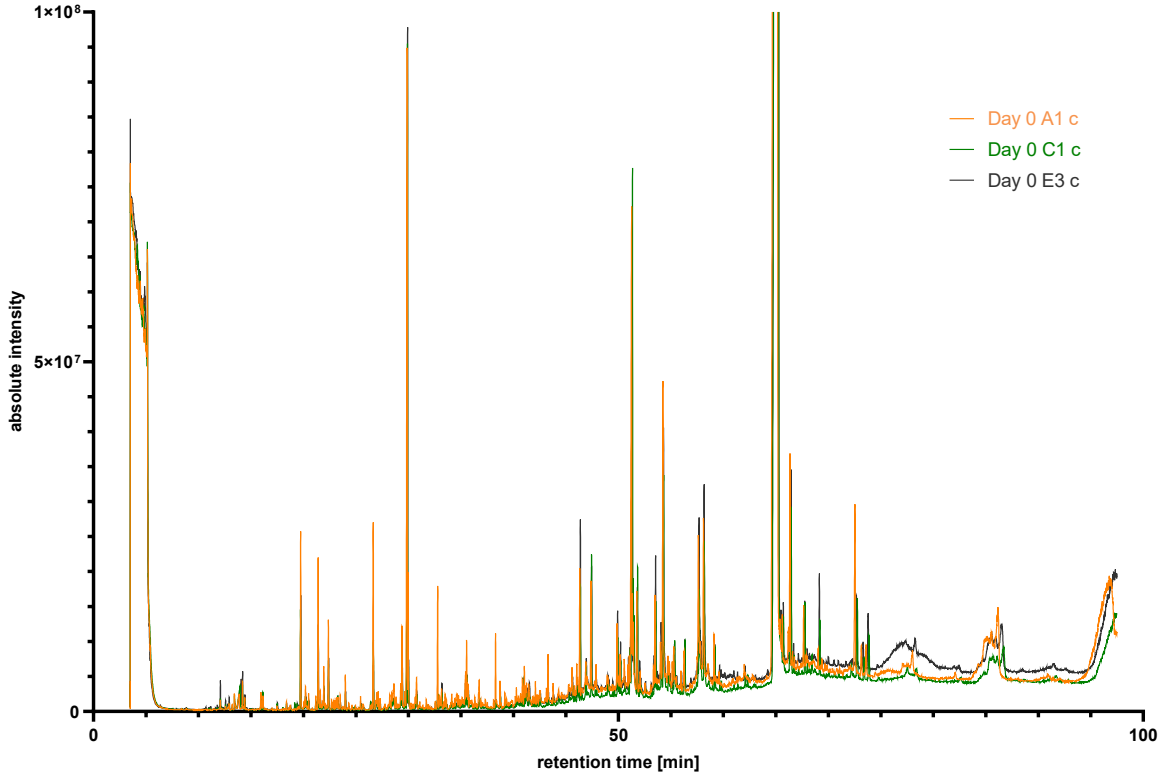

Figure 7 Exemplary GC-MS chromatograms of cooked samples with different MSG concentrations. *The zoomed in spectra of cooked samples with MSG concentration E (black), C (green) and without MSG (yellow).*

5.4 Analysis of the Non-Volatile Profile of Polar Lipid Extracts by Untargeted HPLC-High-Resolution-MS/MS

Table 14 Overview of the identified compounds by HPLC-HRMS/MS.

Name	Formula	rt [min]	Precursor mass [m/z]	Adduct	Theoretical mass [m/z]	Mass error [ppm]	Product masses [m/z]		
Dibutyl-adipate	$C_{14}H_{26}O_4$	8.49	259.190	$[M+H]^+$	259.191	6.416	129.055	147.065	111.044
Di(2-ethyl-hexyl)-adipate	$C_{22}H_{42}O_4$	8.48	371.315	$[M+H]^+$	371.317	4.856	259.190	129.055	147.065
TG 18:1/18:2/18:2	$C_{57}H_{100}O_6$	16.74	898.783	$[M+NH_4]^+$	898.787	4.399	599.506	603.536	881.763
TG 18:0/18:0/18:0 [O]	$C_{57}H_{108}O_7$	16.54	922.841	$[M+NH_4]^+$	922.844	3.282	621.541	603.536	
TG 18:0/18:0/18:1 [O]	$C_{57}H_{106}O_7$	16.09	920.826	$[M+NH_4]^+$	920.829	3.343	603.537	601.522	621.540
TG 18:1/18:1/18:1 [O]	$C_{57}H_{102}O_7$	15.70	916.794	$[M+NH_4]^+$	916.797	4.012	617.516	603.537	631.493

TG: Triacylglycerol; TGs names are followed by their fatty acid composition, where the first number represents the carbon chain length, and the second number represents the number of double bonds.

In the polar lipid extracts six compounds could be identified (as presented in Table 14): dibutyl-adipate, Di(2-ethyl-hexyl)-adipate, TG (18:1/18:2/18:2), as well the epoxidized triacylglycerols 18:0/18:0/18:0 (54:0), 18:0/18:0/18:1 (54:1) and 18:1/18:1/18:1 (54:3). In general, the formation of lipid hydroperoxides could not be observed. The concentration of epoxidized TGs, especially 54:1 and 54:3 showed the tendency to increase for the highest MSG concentration. However, due to the high standard deviations within the same sample subgroups, these tendencies proved not to be significant. For the formation of di(2-ethyl-hexyl)-adipate and one of its fragments dibutyl-adipate, as well as TG 18:1/18:2/18:2, a consistent trend regarding the MSG concentration was lacking. Although distinct subgroups showed an MSG dependent increase or decrease, these remained localised effects, that did not translate to all sample groups. This can partly be attributed, once again, to the high standard deviation observed. Therefore, the results of the HPLC-MS/MS did not suggest a statistically significant difference in the polar lipid extracts between the different MSG concentrations. A set of exemplary chromatograms is shown in Figure 8.

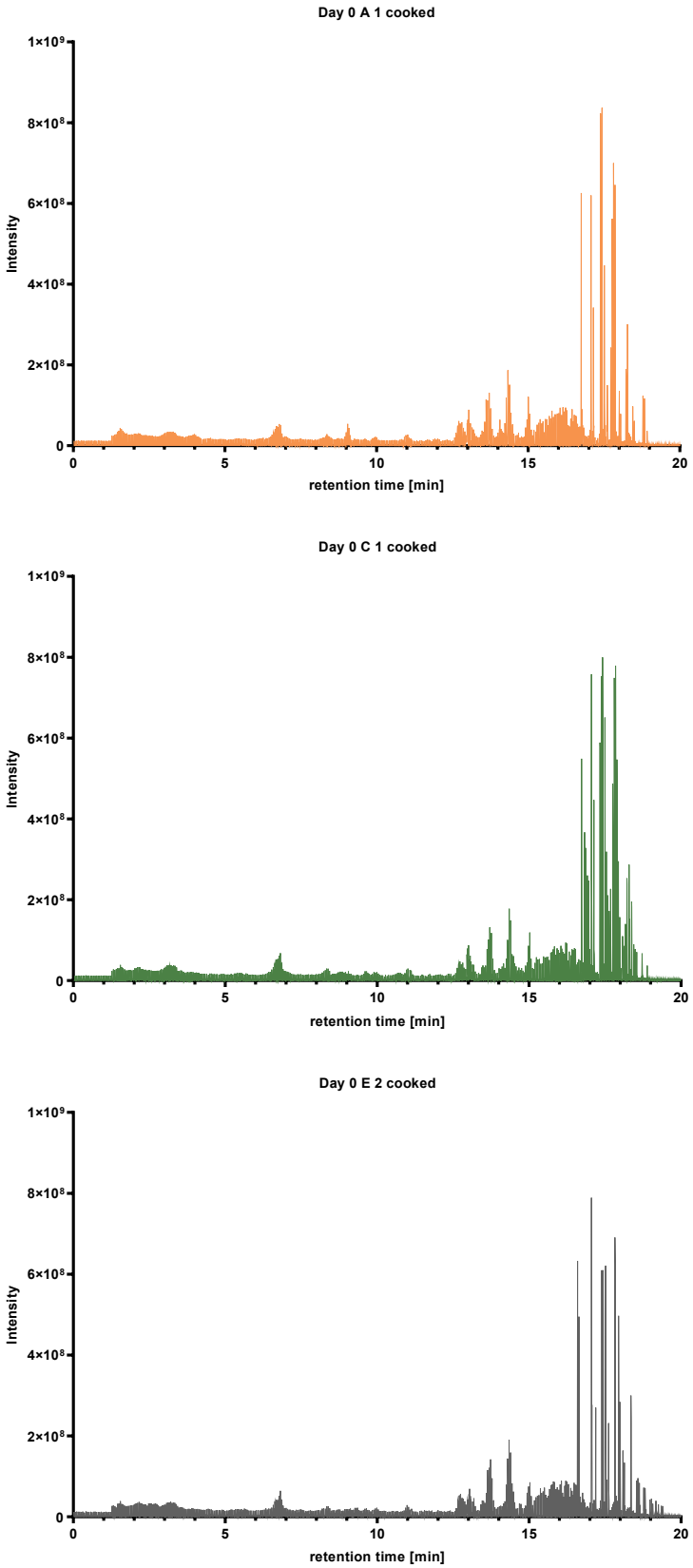

Figure 8 Exemplary total ion chromatograms of cooked samples with different MSG concentrations. *The zoomed in chromatograms of cooked samples without storage with MSG concentration E (black), C (green) and without MSG (yellow).*

5.5 Amino Acid Modifications

5.5.1 Tryptophan Loss

There was no significant difference in the tryptophan concentration between the samples with different MSG concentrations, but there was substantial loss of tryptophan upon cooking, as can be seen in Figure 9.

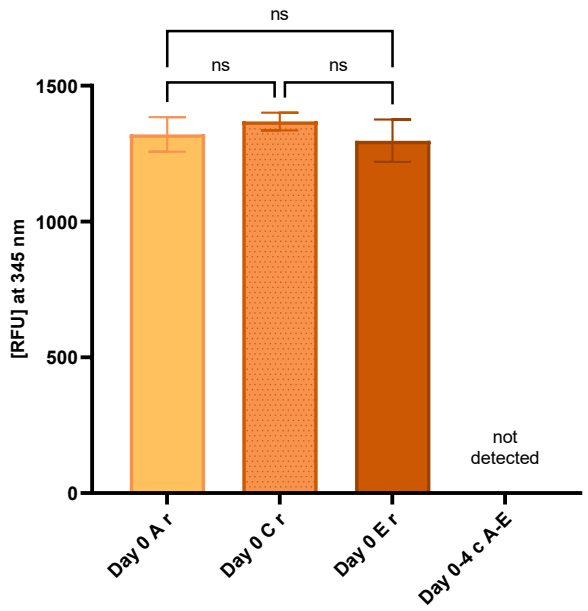

Figure 9 Results for the tryptophan loss for the samples of different MSG concentrations. *Shown are the relative fluorescence units (RFU) at 345 nm for the samples with the three different MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test. Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

5.5.2 Loss of Free Lysine and Arginine

There was no observable loss of free lysine dependent on the concentration of MSG as depicted in Figure 10. However, a significant decrease in lysine concentration could be noted after cooking and the trend persisted during storage as shown in Figure 11. After two days of storage and subsequent cooking, the loss of lysine was particularly pronounced, but the concentration of free lysine remained stable for the rest of the storage days.

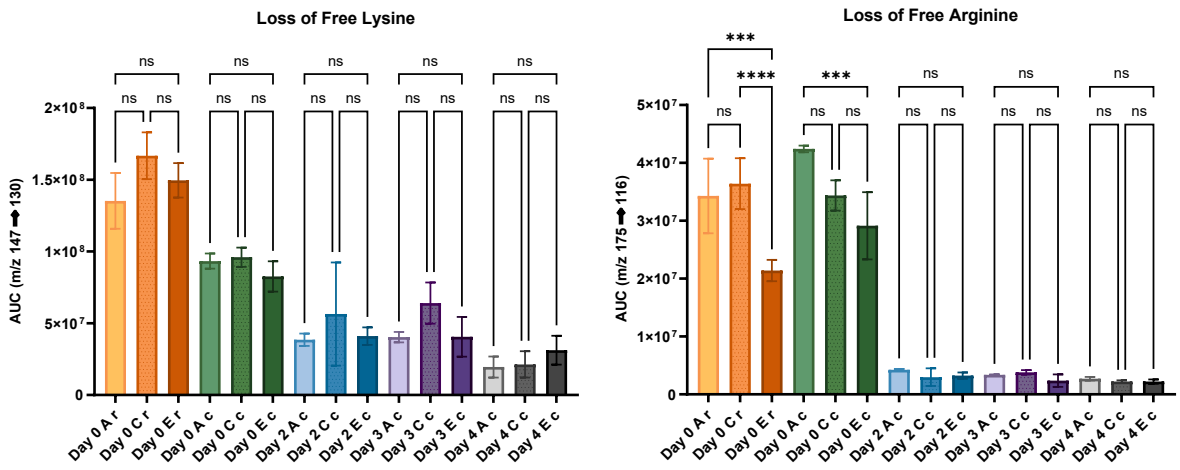

Figure 10 Evaluation of free lysine and arginine content by LC-MS. *Shown is the free lysine and arginine content for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage AUC: area under the curve*

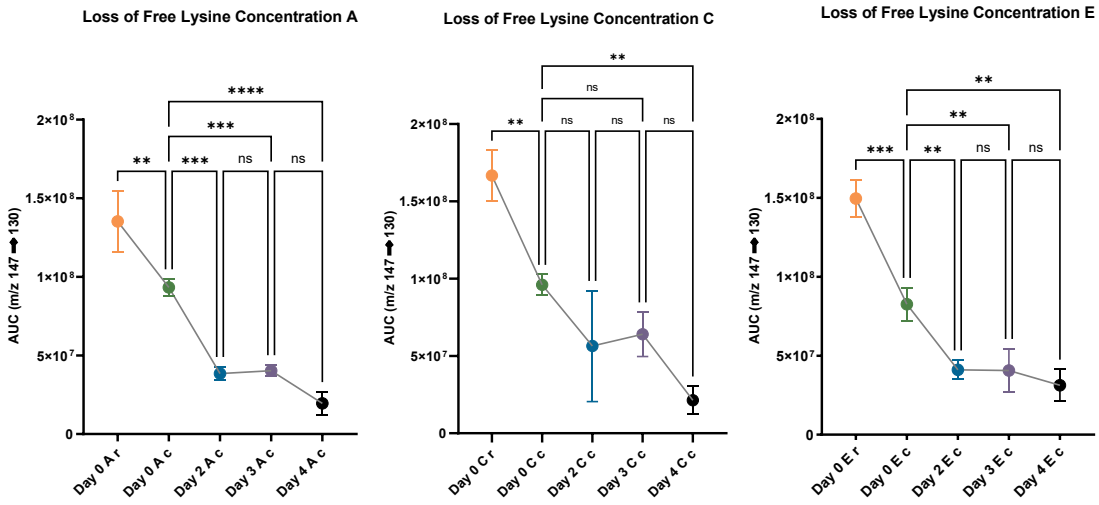

Figure 11 Storage- and cooking dependent loss of free lysine, as determined by LC-MS. *Shown are the different values of the AUC after storage up to 4 days and before (indicated with r) and after cooking (indicated with c) within the same MSG concentration group. Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported for the consecutive storage days and relative to the Day 0 cooked group within the same storage stage as well as between raw and cooked samples within the same MSG concentration group. AUC: area under the curve.*

In comparison, there was a significant difference in the free arginine content for the highest MSG concentration for the Day 0 raw and Day 0 cooked groups, but not for the other storage days as presented in Figure 10. Figure 12 depicts the impact of cooking and storage on the loss of free arginine.

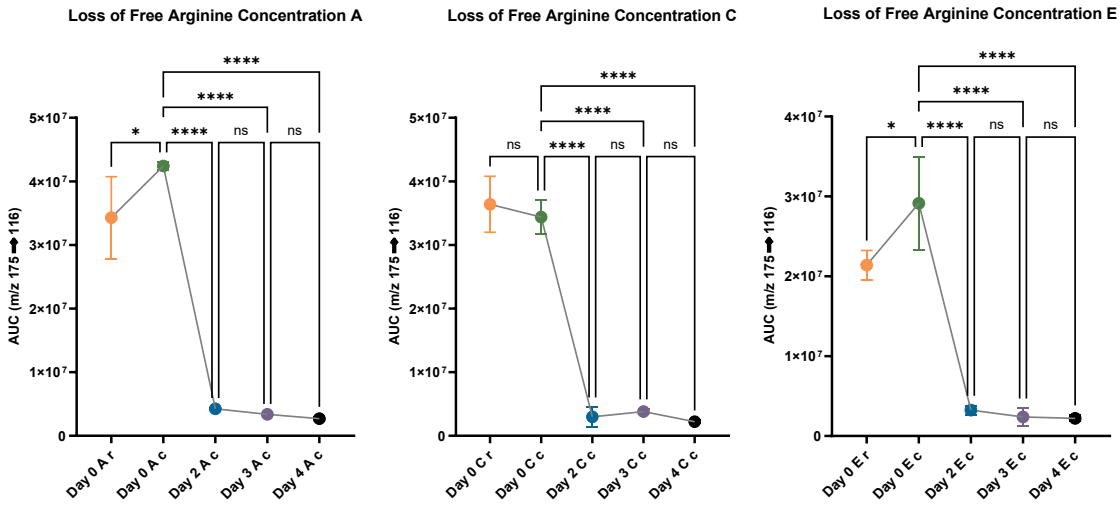

Figure 12 Storage- and cooking dependent loss of free arginine, as determined by LC-MS. *Shown are the different values of the AUC after storage up to 4 days and before (indicated with r) and after cooking (indicated with c) within the same MSG concentration group. Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported for the consecutive storage days and relative to the Day 0 cooked group within the same cooking stage as well as between raw and cooked samples within the same MSG concentration group. AUC: area under the curve.*

There is a significant increase in free arginine after cooking for concentration groups A and E, whereas cooking had no significant impact on the samples of group C. After two days of storage and subsequent cooking the decrease in the content of free arginine was most pronounced and then the level of free arginine remained constant for the rest of the storage days (as can be seen in Figure 12)

5.5.3 Protein-bound Lysine and Arginine

The MSG concentration did not have a significant impact on the concentration of neither protein-bound lysine nor protein-bound arginine. The duration of storage as well as cooking did not influence the level of protein-bound lysine or arginine as depicted in Figure 13.

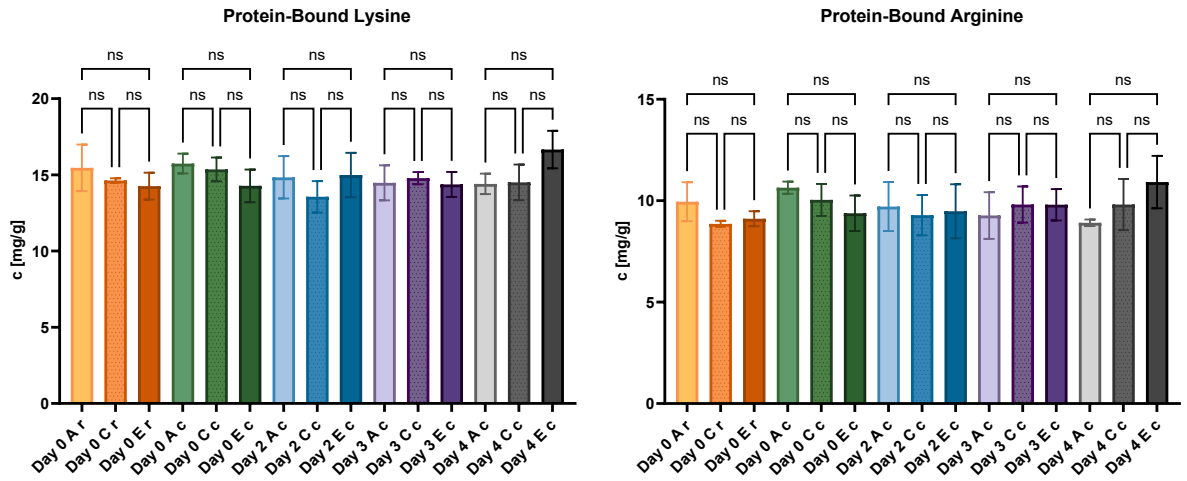

Figure 13 Quantification of protein-bound lysine and arginine with LC-MS. *Shown are the different concentrations of protein-bound lysine and arginine for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

5.6 Advanced Glycation End Products

A variety of different AGEs has been (semi-)quantified using LC-MS. N-ε-carboxymethyl-lysine (CML) was quantified using a standard, whereas N-ε-carboxyethyl-lysine (CEL), pentosidine, imidazolium crosslink derived from glyoxal-lysine dimer (GOLD), imidazolium cross-link derived from methylglyoxal-lysine dimer (MOLD), methylglyoxal-derived hydroimidazolone (MG-H1) and glyoxal-derived hydroimidazolone (G-H1) were only determined semi-quantitatively.

No significant difference, neither MSG concentration dependent, nor storage or cooking dependent was observed for CML (as displayed in Figure 14), CEL and pentosidine (as can be seen in Figure 15), MOLD and GOLD (as shown in Figure 16) and G-H1, whereas MG-H1 showed an increase for the Day 4 E cooked group compared to concentration A and C (as can be seen in Figure 17).

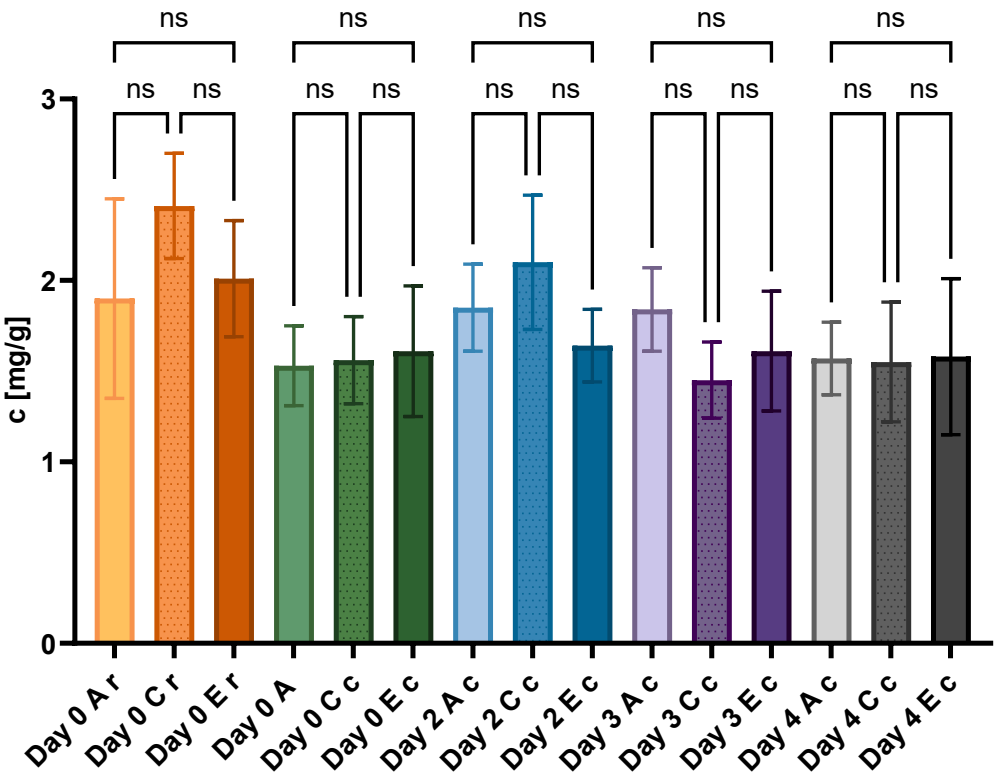

Figure 14 Quantification of N-ε-carboxymethyl-lysine (CML) with LC-MS. *Shown are the different concentrations of CML for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

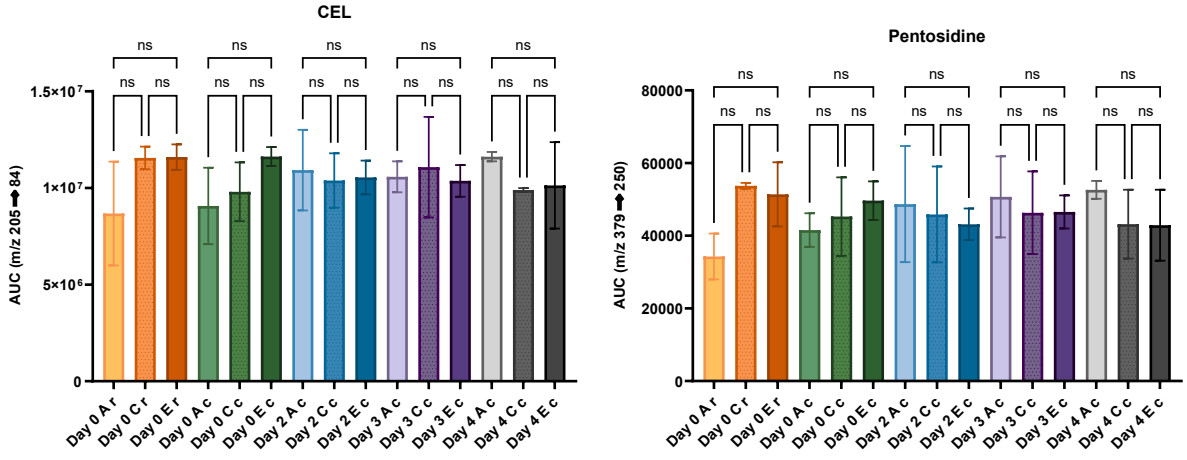

Figure 15 Semi-quantification of N-ε-carboxyethyl-lysine (CEL) and pentosidine with LC-MS. *Shown are the different values of the AUC for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage. AUC: Area under the curve*

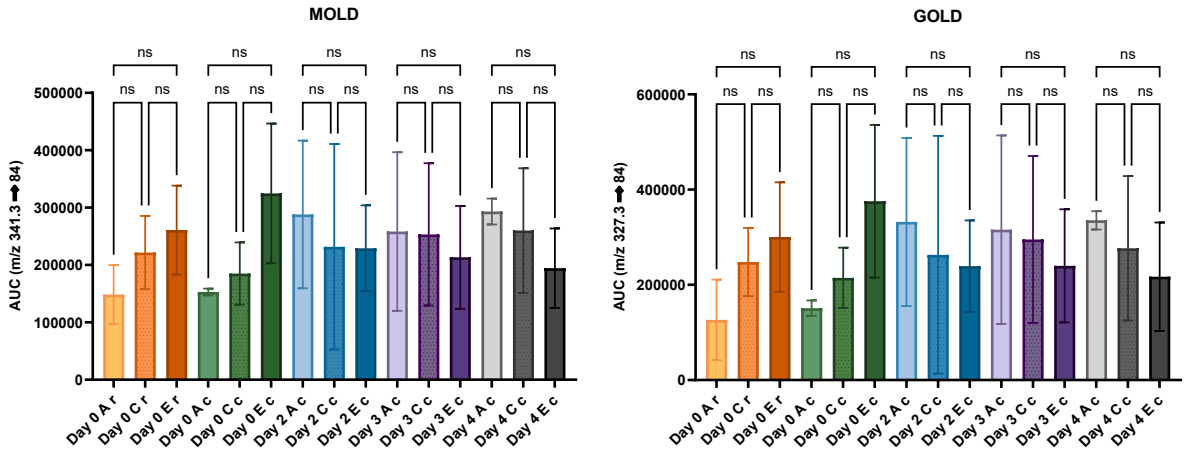

Figure 16 Semi-quantification of imidazolium cross-link derived from methylglyoxal-lysine dimer (MOLD) and imidazolium crosslink derived from glyoxal-lysine dimer (GOLD) with LC-MS . *Shown are the different values of the AUC for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage. AUC: Area under the curve*

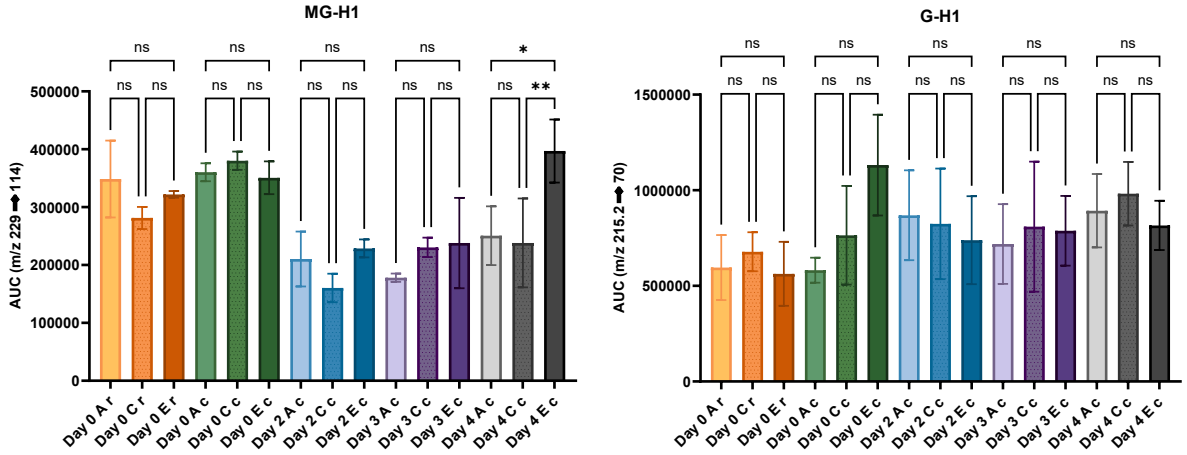

Figure 17 Semi-quantification of methylglyoxal-derived hydroimidazolone (MG-H1) and glyoxal-derived hydroimidazolone (G-H1) with LC-MS. *Shown are the different values of the AUC for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * (p < 0.05), ** (p < 0.01), *** (p < 0.001), and **** (p < 0.0001). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage. AUC: Area under the curve*

5.7 Protein Modifications

5.7.1 Determination of the Protein Content by Kjeldahl Method

The protein content within the meat samples was found to range between 18.61 ± 0.80 % and 22.78 ± 0.58 %, as displayed in Table 15. There was no significant difference in protein content between the samples with different MSG concentrations.

Table 15 Protein content for the samples with three different MSG concentrations. *Results depicted as mean ± SD [w% protein].*

Protein Content	
sample	Mean ± SD [w% protein]
Day 0 A raw	20.15 ± 0.68
Day 0 C raw	18.82 ± 0.50
Day 0 E raw	18.61 ± 0.80
Day 3 A cooked	21.99 ± 0.72
Day 3 C cooked	22.78 ± 0.58
Day 3 E cooked	21.21 ± 1.13
mean	**20.60 ± 1.73**

5.7.2 Electron Spin Resonance Spectroscopy (ESR)

The results of ESR measurements demonstrated that neither the MSG concentration (as displayed in Figure 18), nor the storage time (as shown in Figure 19) had a significant impact on the quantity of stable unpaired electrons. However, the cooking process significantly decreased the number of radicals in the samples (as shown in Figure 19).

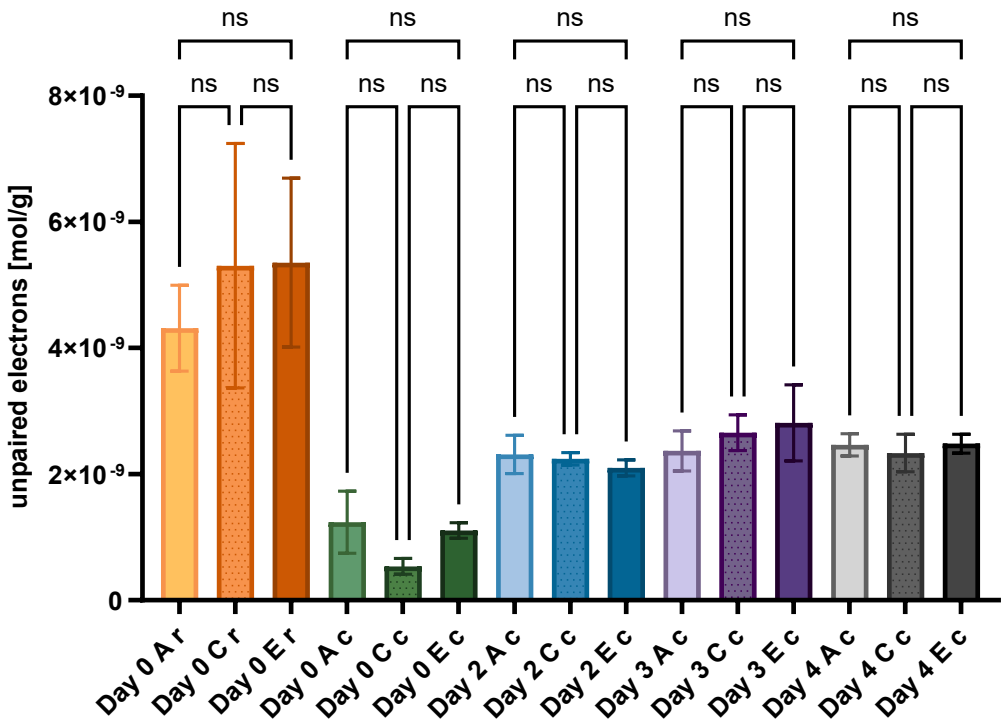

Figure 18 Quantification of stable unpaired electrons [mol/g] with ESR. *Shown are the different concentrations of unpaired electrons [mol/g] for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

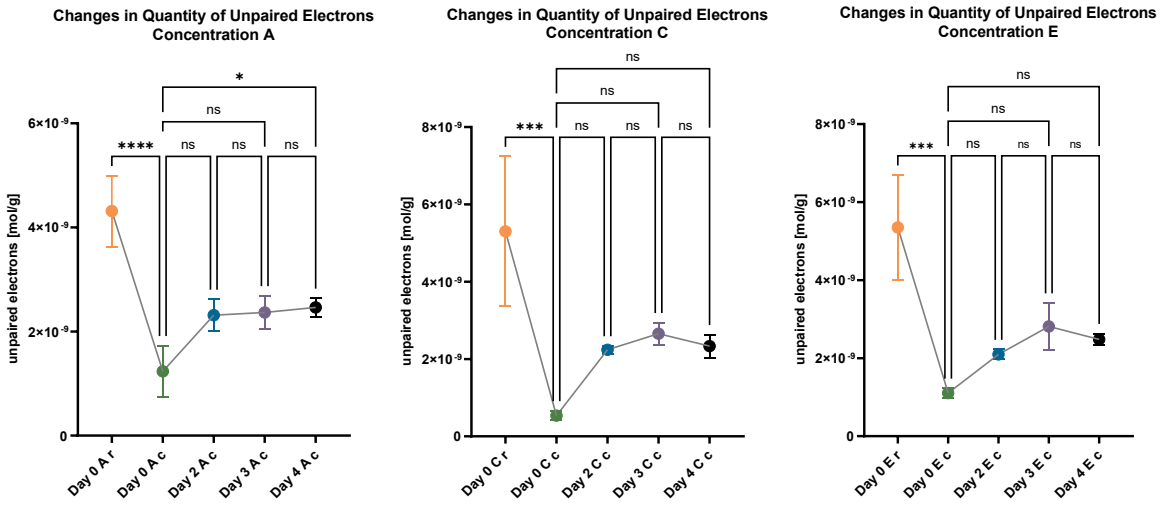

Figure 19 Quantitative determination of storage- and cooking dependent changes in stable radicals with ESR. *Shown are the unpaired electrons [mol/g] after storage up to 4 days and before (indicated with r) and after cooking (indicated with c) within the same MSG concentration group. Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported for the consecutive storage days and relative to the Day 0 cooked group within the same cooking stage as well as between raw and cooked samples within the same MSG concentration group.*

5.7.3 Quantification of Carbonyl Content by DNPH Method

The protein carbonyl content was determined using the DNPH assay. The results (depicted in Figure 20) showed a notable increase in the carbonyl content for the raw samples with the highest MSG concentration (3.66 ± 0.50, 3.47 ± 0.37 and 5.19 ± 1.53 nmol/mg protein for A, C and E, respectively). However, this trend, which was observed in the raw samples, did not persist for the cooked samples across various storage days as illustrated in Figure 20.

Moreover, storage did not seem to influence the formation of protein carbonyls. Conversely, cooking contributed to a significant increase in carbonyl content in the samples throughout different duration of storage, except for the samples with two days of storage.

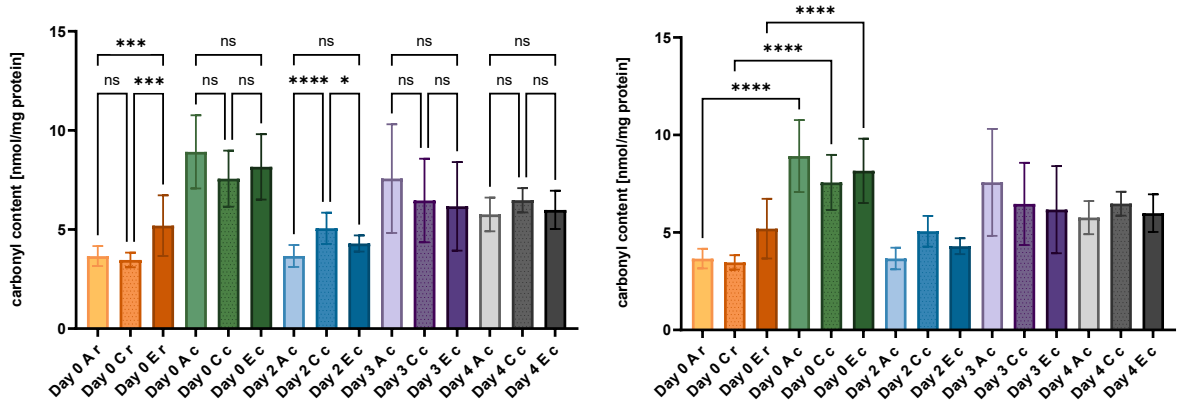

Figure 20 Quantification of protein carbonyls with the DNPH method. *Shown are the different concentrations of protein carbonyls for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n ≥ 10). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns.*

5.7.4 Schiff Bases Monitoring

The formation of Schiff bases as a protein modification was established using a fluorometric method. There was a significant increase in Schiff bases formation due to MSG addition as presented in Figure 21. The concentration of Schiff bases in group A was significantly lower than in group E across all the sample groups, including raw and cooked samples, independent of storage time. The quantity of Schiff bases in the samples of group C did not show a significant increase compared to group A and to group E for the samples of the Day 0 raw, Day 3 cooked and Day 4 cooked groups. In addition, there was no observable difference in Schiff bases formation between the various raw and cooked samples or between different storage days.

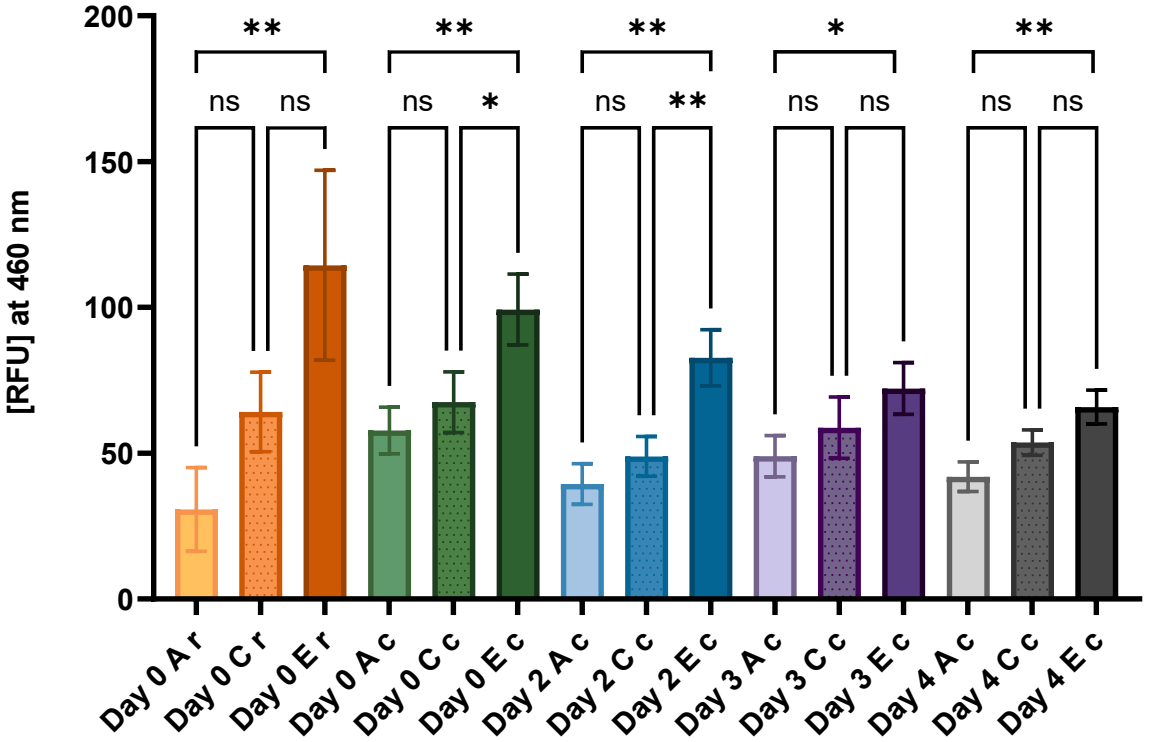

Figure 21 Schiff bases content determined by fluorimetry. *Shown are the different relative fluorescence units (RFU) at 460 nm for the samples with the three MSG concentrations (A, C, E) used, after storage up to 4 days and before (indicated with r) and after cooking (indicated with c). Data are shown as mean ± SD (n=3). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Tukey test and is indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Non-statistical significance is indicated with ns. Statistical significance is reported exclusively for the different monosodium glutamate (MSG) concentrations within the same storage and cooking stage.*

6 Discussion

6.1 Quantification of MSG

The concentration of MSG in the raw samples, as well as the cooked samples with and without storage time was determined quantitatively with LC-MS and semi-quantitatively with ^{1}H-NMR to determine the reactivity of the food additive in the pork patties.

The results of the quantification of MSG with these two analytical techniques demonstrated a loss of the initially added MSG. Although triple the concentration of MSG was added to the samples of group E compared to the samples of group C, it could not be recovered to this extent. This confirms the reactivity of MSG in the pork meat samples.

It should be noted that the recovery of MSG during the sample preparation, which included Bligh and Dyer extraction, drying under nitrogen and in a concentrator for NMR as well as redissolution in the respecting solvent was not determined. Therefore, the quantity of MSG in the different sample groups A, C, E should only be seen relatively to each other to assess its reactivity, rather than focussing solely on absolute values.

6.2 Moisture Content by Loss on Drying and Loss on Cooking Methods

The water-holding capacity of raw meat is one of the most important quality parameters and can be influenced by various factors including the salt content [318, 319]. Meat products typically contain high concentrations of NaCl due to its advantageous impact on the functional properties of meat products, such as improved texture and succulence through water-binding [10]. Conversely, sodium chloride has also been identified as a pro-oxidant facilitating lipid and protein oxidation through various mechanisms [87, 98, 120, 121, 226, 227].

Different strategies have been investigated to replace sodium chloride with other salts, such as potassium-, calcium- and magnesium salts as well as monosodium glutamate. The latter has been extensively studied in terms of acceptability, sensory characteristics, technological and safety aspects [10, 11, 65-69]. The present study did not find any impact of MSG on water binding properties of the pork patties. There was no significant difference in cooking loss or loss on drying for the pork patties regardless of the added MSG concentration. Therefore, there is no indication that MSG has a similar positive impact on texture and succulence due to water-binding as sodium chloride has, which is in accordance with previous research [10]. The effect of monosodium glutamate on food oxidation and the underlying mechanisms will be discussed in section 6.6.

Furthermore, the possible differences in water loss between samples of varying MSG concentration do not need to be taken into consideration for the present study. Consequently, the levels of analytes studied can be considered consistent across the different sample groups, indicating no significant differences attributable to variations in concentration due to varying water content.

6.3 Analysis of the (Non-)Volatile Profile of Polar Lipid Extracts by Untargeted HPLC-High-Resolution-MS/MS and GC-MS

Although localised tendencies in specific subgroups were observed in the untargeted analysis of the polar lipid extracts, these trends, on the one hand, did not predominantly translate to the entirety of sample groups tested and, on the other hand, did not prove to be statistically significant due to high standard deviations. Therefore, no clear, consistent effects of MSG on the polar lipid extracts could be elucidated. The aforementioned high standard deviation could be attributed to heterogeneity of the samples. Especially for the cooked samples it may be due to the uneven heat exposure of the patties during the sample preparation process. As the patties were directly put in the oven after refrigerated storage, the surface of the patties experienced greater heat, which facilitated water loss and the progression of reactions such as oxidation due to the increased energy input. However, the core was consequently exposed to less heat, which could explain the uneven distribution of reaction products and therefore the high standard deviation observed, despite sampling being as consistent as possible within replicates. Even though the differences in formation of epoxides in the samples with varying MSG concentrations were not significant, it is noteworthy that the generation of hydroperoxides could not be observed, which suggests that MSG addition does not translate to an increase on primary lipid oxidation products content, indicating its effect on lipid oxidation occurs at a rapid rate, as previous results from our group showed an increase on secondary lipid oxidation products such as aldehydes (unpublished). To conclude, no intermediates in the MSG oxidative promoting effect could be identified in the polar lipid phase with GC-MS and HPLC-MS/MS.

6.4 Amino Acid Modifications

Previous research has attributed the decrease of fluorescence upon cooking to the oxidative degradation of tryptophan [245, 250]. The increased temperatures facilitate the cleavage of existing hydroperoxides to ROS, initiating oxidative processes. Notably, hydroxyl radicals and superoxide radicals oxidise aromatic rings. The elevated temperatures also lead to the destruction of muscle tissue, releasing catalytic iron [214, 245].

The results of the present study showed that indeed cooking leads to a considerable decrease in the concentration of tryptophan. In connection with the previous research mentioned, it can be concluded that cooking leads to oxidative damage of tryptophan and therefore a decrease in fluorescence.

This evidence is supported by the results of ESR measurements of the present study showing a profound decrease in the quantity of radicals upon cooking. This indicates that the radicals react with biomolecules, such as proteins and amino acids leading to the loss of radicals and increased oxidative damage. Conversely, MSG does not seem to have an impact on the oxidation of tryptophan, which is corroborated by the results of ESR measurements that did not show a difference in radical formation depending on MSG concentration.

Furthermore, cooking also resulted in noticeable loss of free lysine, which is in accordance with the results mentioned above. On the contrary, there is an increase in free arginine after cooking, which may be explained by a release of arginine from proteins/peptides upon cooking.

The loss of free lysine and arginine is likely attributed to amino acid modifications occurring during various processes. The high reactivity of the side chains of both these amino acids leads to their participation in different processes such as AGEs formation and generation of α-aminoadipic and γ-glutamic semialdehydes [243, 272]. Generally, the level of free lysine and arginine was the lowest after two days of storage and subsequent cooking and remained stable for the rest of the storage days. Notably, the loss of lysine was mostly influenced by the cooking process, whereas the loss of arginine was affected strongest by storage. The MSG concentration had no impact on the loss of free lysine, whereas it seemed to have an influence on the loss of free arginine for the Day 0 raw and Day 0 cooked groups. After two or more days of storage and subsequent cooking, these changes were no longer observed, pointing towards the need of MSG for this effect. For the samples of the Day 0 raw group MSG might contribute to the degradation of the free arginine through carbonyl- or Schiff bases formation. For the samples of Day 0 cooked, no impact of MSG concentration on protein carbonyl formation could be observed, suggesting potential involvement in Schiff bases formation.

Interestingly, the modifications described in this section only seem to occur in free lysine and arginine as the concentration of protein-bound lysine and arginine is neither influenced by MSG concentration, nor cooking or storage time. This might be due to the greater accessibility of free amino acids compared to proteins with complex secondary, tertiary and quaternary structures [320].

6.5 Advanced Glycation End Products

An array of different AGEs was (semi-)quantified using a LC-MS method. Except MG-H1, which showed an increase for the highest MSG concentration after 4 days of storage and subsequent cooking, no significant differences in AGEs formation could be observed, neither dependent on duration of storage nor cooking or MSG concentration.

The high standard deviation in this study for cooked samples may be attributed to the uneven heat exposure of the patties during the cooking process. While the surface of the patties is exposed to

greater heat, which facilitates water loss and browning, the core experiences less heat. Consequently, the surface is expected to contain more AGEs than the core. The exact core temperature was not measured, thus, temperature differences between surface and core could not be assessed. Although the sample portions for the AGEs measurements were drawn from the surface and the core, naturally a lower ratio of surface to core will favour the core. The unequal distribution of AGEs and possibility of inadequate heating of the patty might contribute to the high standard deviation and the absence of a significant difference for cooked samples. Additionally, the results of protein-bound lysine and arginine confirm that there is no consumption of those amino acids by the Maillard reaction in later stages.

Furthermore, this sample preparation procedure might not be optimal for this specific sample type. Other authors suggest an extra step to completely remove lipids before proteins are precipitated, when analysing meat products [311]. The last step of the sample procedure involves a high reaction temperature of 110°C. Therefore, it is possible that a proportion of the AGEs detected is generated during the last step of sample preparation [267, 311]. This offers an explanation for the absence of a significant difference between raw and cooked samples and the high concentration of CML detected, which is significantly higher than values stated in literature [257].

6.6 Protein Modifications

The protein content of a sample aliquot was determined using the Kjeldahl method to determine whether the addition of MSG has a significant impact on this parameter. The protein level found is in accordance with results published in literature, such as by Barbin et al. [321], who quantified the protein content of different pork muscles, including *longissimus dorsi* (LD), *semimembranosus* (SM), *semitendinosus* (ST) and *biceps femoris* (BF) and found concentrations ranging from 20.88 % to 25.23 %. Furthermore, the precision of the results found is within the range of 1.54-1.87 %, postulated by Mihaljev et al. [313] for determination of the protein content using the Kjeldahl method for meat samples. Therefore, the Kjeldahl method delivered solid proof that the addition of MSG did not lead to a significant change in the protein concentration in the meat samples.

The generation of carbonyls is the predominant damage upon oxidation of proteins and is most commonly assessed with the 2,4-Dinitrophenylhydrazine (DNPH) assay [232, 235, 236]. However, as mentioned in 2.3, protein oxidation is a very complex process that does not always involve carbonyl formation.

The results of the carbonyl content for the raw samples are in accordance with values found in literature, which are usually below 5 nmol/mg protein for raw meat depending on the species and used method [235, 237, 322]. The carbonyl content was higher than reported previously for cooked pork patties with/without refrigerated storage (1.57 ± 0.29 - 3.24 ± 0.19 nmol/mg protein for cooked pork

patties without/with 6 days of refrigerated storage) [133]. Alternatively, the results presented in this thesis are similar to the carbonyl content published for processed meat products like sausages [237]. There is no continuous trend regarding the carbonyl formation during storage, but cooking seems to increase carbonyl formation. The cooking process could enhance protein oxidation through various mechanisms. For instance, the increased temperature and therefore the energy input might inactivate antioxidant enzymes and accelerate decomposition of hydroperoxides to free radicals which can initiate protein oxidation [87]. Moreover, the elevated temperatures can lead to cell disruption and the release of iron, which can catalyse oxidation processes [245]. This is in accordance with the results from the ESR measurements, which showed a profound decrease in the quantity of radicals after cooking and a subsequent mild increase after two days of storage. The reduced quantity of radicals after cooking suggests, as aforementioned, that the radicals reacted with biomolecules present in the meat samples and induced alterations. The inverse proportionality of results from DNPH and ESR measurements can be seen a reflexion of these processes taking place during cooking.

Protein modifications can also involve the formation of Schiff bases. These compounds can be formed following different pathways and as a product of various, partially interlinked, processes. On the one hand, Schiff bases are formed in the first step of the Maillard reaction upon reaction of the protein amine group reacts with the carbonyl moiety of a reducing sugar [252, 253, 260]. On the other hand, Schiff bases could be generated upon reaction of carbonyls, which could be derived from proteins, such as AAS and GGS, or from lipid oxidation, reacting with the ε-amino group of (protein-bound) lysine/arginine, or other AAS residues [214, 245-247].

The contribution of each route to the formation of these fluorescent products cannot solely be determined from the assay used in this thesis. Therefore, the Schiff bases formation needs to be seen in connection with the results from other experiments to create a uniform picture. The impact of MSG on the formation of Schiff bases as intermediate products of the Maillard Reaction is unlikely (although not impossible) as no MSG dependent increase in AGEs formation could be determined. Furthermore, neither the level of protein-bound nor free lysine decreased in an MSG concentration dependent way. Only the loss of free arginine for Day 0 raw and Day 0 cooked groups showed a trend dependent on the concentration of MSG. There is no evidence, except for the Day 0 raw group, that the generation of Schiff bases follows the AAS/GGS pathway, as no increase in protein carbonyls could be demonstrated, and AAS and GGS have not been quantified specifically. Therefore, the predominant pathway of MSG dependent Schiff bases formation seems to be upon the reaction of lipid oxidation derived carbonyls with an amino acid which would be in accordance with previous findings by our group that showed that the formation of lipid oxidation derived carbonyls is influenced by the MSG concentration (unpublished). The formation of Schiff bases was not promoted by cooking or refrigerated storage as opposed to previous studies [245].

Previous studies hypothesised that Schiff bases formed upon Maillard reaction undergo the Amadori rearrangement rapidly, whereas those generated upon the interaction of malondialdehyde with proteins could be more stabilized due to protein dimerization. The formation of protein cross-links might have negative implications for the meat product, involving loss of water-holding capacity [323] and nutritional value due to decreased susceptibility to digestive proteases as well as changes in meat texture and tenderness [166, 206-208, 214].

Schiff bases are not commonly measured as a marker for oxidative protein modifications [245]. Therefore, protein alterations that do not solely involve protein oxidation processes, such as protein carbonylation, which would be seen in traditional assays such as the DNPH assay, could easily be overseen. Thus, fluorescence spectroscopy could prove to be a useful tool to assess protein modifications provoked by lipid oxidations processes (protein-lipid-oxidation). Furthermore, this method requires minimal sample preparation and equipment whilst delivering highly sensitive results.

7 Conclusion

The present thesis investigated the reactivity of MSG in pork meat and its impact on protein oxidation, as well as on the progression of the Maillard reaction. To do so, the volatile and non-volatile profile of extracts obtained via Bligh and Dyer extraction were analysed with GC-MS, ^{1}H-NMR and HPLC-high-resolution-MS/MS. Additionally, relevant substrates for common chemical processes in food, such as the Maillard reaction and oxidative processes were quantified. This included tryptophan, lysine and arginine. Specific products of these processes, including protein carbonyls, Schiff bases, stable radicals and selected advanced glycation end products were (semi-)quantified.

The aim of the study was to determine the impact of MSG fortification on protein oxidation and the (non-)volatile profile of meat, along with storage and cooking. The results of the (semi-)quantification of MSG with LC-MS and ^{1}H-NMR demonstrated a loss of the initially added MSG, which confirms the reactivity of MSG in the pork meat samples. However, the number of MSG dependent alterations observed were limited. The only modification consistent throughout all the sample groups was the increased formation of Schiff bases for the highest MSG concentration (1.2 %), sharing this trend with the lipid oxidation promoting effect that was previously described by our group (unpublished). This suggests the potential existence of a link between the Schiff bases formation and lipid oxidation promoting roles of MSG. Moreover, MSG dependent increase in the loss of free arginine for the Day 0 raw/cooked group and protein carbonyl formation for the Day 0 raw group were found, pointing towards a more complex relationship between MSG and its effects on pork meat.

No MSG dependent impact in any of the groups tested was observed on the formation of stable radicals, free lysine and protein-bound lysine content, protein-bound arginine content, AGEs formation, protein content and on the (non-)volatile profile of the polar lipid extracts. Nevertheless, for some of these parameters cooking or storage had a significant impact: Cooking led to a profound decrease in the quantity of radicals, the concentration of tryptophan and free lysine as well as an increased formation of protein carbonyls. On the contrary, there was an increase in free arginine after cooking, which could be explained by a release of arginine from proteins/peptides upon cooking. On the other hand, storage up to 2 days had a decreasing effect on the level of free lysine and arginine, which remained stable afterwards. After reviewing the results of the untargeted analysis of polar lipid extracts with HPLC-MS/MS and GC-MS, no intermediates in the pathway of oxidation-promoting effect of MSG could be identified.

In conclusion, this study showed that MSG has a concentration dependent effect on the protein-moieties of raw and cooked pork meat patties, upon Schiff bases formation, which might exert influence of the quality of the meat product. Therefore, this thesis presents novel perspectives on the

interactions between MSG and meat products and provides valuable evidence for future re-evaluations of the risks imposed by this food additive. Hence, the aim of the thesis to elucidate the impact of MSG fortification on protein moieties, especially regarding oxidative stability, of pork meat along with storage and cooking was achieved.

8 Abstract

Monosodium glutamate (MSG) is a common flavour enhancer for foodstuffs that contributes to the "umami" taste. While its sensory properties and safety aspects have been extensively evaluated, the reactivity upon household cooking and storage and its impact on oxidative stability of foodstuffs are less explored.

Therefore, this study focused on the impact of MSG on meat quality, regarding non-enzymatic oxidation and the progression of the Maillard reaction as well as underlying mechanisms. Pork meat patties with 0.0 %-1.2 % MSG (w/w) were prepared, stored up to 4 days at 4°C and cooked in an oven at 180°C for 15 minutes. After Bligh and Dyer extraction, subsequent untargeted analysis of the (non-)volatile profile of the extracts with GC-MS, ^{1}H-NMR and HPLC-high-resolution-MS/MS were performed. Moreover, relevant substrates and selected products of the Maillard reaction and oxidative processes were (semi-)quantified.

LC-MS and ^{1}H-NMR results proved the reactivity of MSG by partial decrease in its concentration. However, no significant impact of MSG addition could be observed for the (non-)volatile profile of polar lipid extracts, the formation of stable radicals and the progression of late stages of the Maillard reaction. For the raw samples, MSG promoted protein carbonyl formation. Furthermore, a significant increase ($p<0.05$) in Schiff bases formation could be observed throughout all groups tested. This effect could be associated with the progression of lipid-protein co-oxidation.

In conclusion, the present thesis has offered insights into the interplay of MSG with pork meat constituents, contributing to a better understanding of the potential drawbacks of MSG usage.

9 Zusammenfassung

Mononatriumglutamat (Monosodium glutamate, MSG) ist ein weit verbreiteter Lebensmittelzusatzstoff, der zum sogenannten “umami” Geschmack beiträgt und als Geschmacksverstärker dient. Obwohl die sensorischen Eigenschaften und die Sicherheit von MSG umfassend untersucht wurden, ist die Reaktivität während haushaltsüblicher Lagerung und Lebensmittelzubereitung sowie der Einfluss auf die oxidative Stabilität weitgehend unerforscht.

Diese Studie hat sich daher auf den Einfluss von MSG auf Fleischqualität, insbesondere in Bezug auf nicht-enzymatische Oxidation und Maillard Reaktion, sowie die zugrundeliegenden Mechanismen fokussiert. Es wurden Bratlinge aus faschiertem Schweinefleisch mit 0.0 %-1.2 % MSG (w/w) zubereitet, zwischen 0-4 Tage bei 4°C gelagert und anschließend bei 180°C für 15 min in einem Ofen gebraten. Nach erfolgter Bligh and Dyer Extraktion wurden sowohl das flüchtige als auch das nicht-flüchtige Profil der Extrakte mit GC-MS, ^{1}H-NMR und HPLC-high-resolution-MS/MS qualitativ untersucht. Außerdem wurden sowohl Substrate als auch Produkte lebensmittelchemischer Reaktionen, einschließlich der Maillard Reaktion und oxidativer Prozesse, (semi-)quantifiziert.

Die Ergebnisse von LC-MS und ^{1}H-NMR Analysen zeigten eine partielle Abnahme der MSG-Konzentration und damit eine Reaktivität von MSG in den Proben. Es konnte allerdings kein Einfluss auf das (nicht-)volatile Profil der polaren Lipidextrakte, die Bildung stabiler Radikale und die Bildung von AGEs gefunden werden. Für die ungekochten Proben, trug MSG zur Proteincarbonyl Bildung bei. Außerdem zeigte sich eine signifikante Steigerung ($p<0.05$) des Gehalts an Schiff Basen in allen untersuchten Gruppen. Dieser Effekt entsteht vermutlich über Lipid-Protein Co-Oxidation.

Die vorliegende Masterarbeit liefert neue Erkenntnisse über den Einfluss von MSG auf Schweinefleisch und geht damit potentiellen Nachteilen der Verwendung von MSG in Lebensmitteln nach.

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11 Appendix

11.1 Quantification of MSG

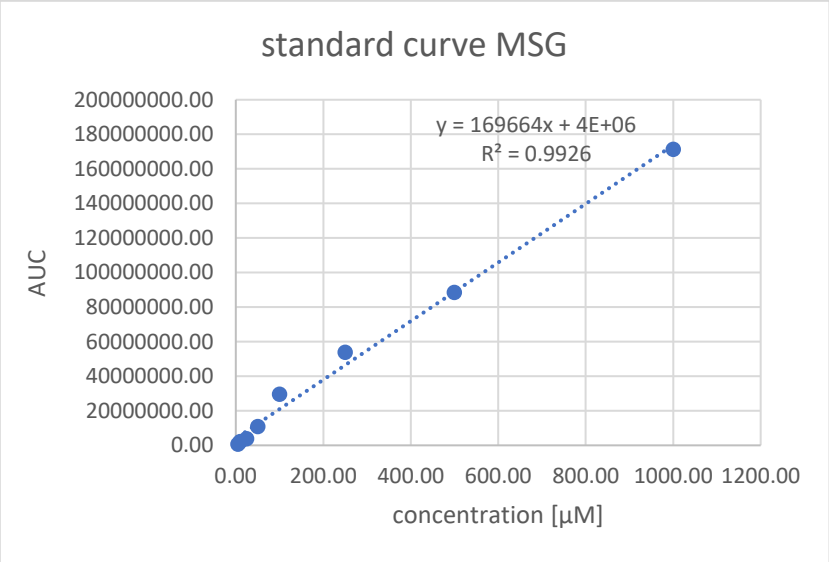

Figure 22 External standard curve used for quantification of MSG with LC-MS

Table 16 External standard curve used for quantification of MSG with LC-MS

c [µM]	AUC
5.00	833186.67
10.00	2275571.33
25.00	3921063.67
50.00	10848105.67
100.00	29593552.67
250.00	53831485.33
500.00	88555162.67
1000.00	171342144.00

Table 17 Raw data for the quantification of MSG with LC-MS as displayed in Figure 3

concentration of MSG			
sample	mean c [mg]/100g sample	SD c [mg]/100g sample	n
Day 0 A raw	0.258	0.053	3
Day 0 C raw	4.527	2.770	3
Day 0 E raw	11.838	0.057	3
Day 0 A cooked	0.192	0.013	3
Day 0 C cooked	8.116	2.496	3
Day 0 E cooked	10.916	3.336	3
Day 2 A cooked	0.219	0.028	3
Day 2 C cooked	12.095	5.933	3
Day 2 E cooked	17.956	0.865	3
Day 3 A cooked	0.356	0.023	3
Day 3 C cooked	9.473	0.216	3
Day 3 E cooked	17.162	1.877	3
Day 4A cooked	0.211	0.042	3
Day 4 C cooked	10.039	2.856	3
Day 4 E cooked	24.313	1.006	3

Table 18 Raw data for the semi-quantification of beta-CH_2 Glutamate with ^{1}H-NMR as displayed in Figure 5

beta-CH_2 Glutamate (2.02m)								
Day 0 A raw			**Day 0 C raw**			**Day 0 E raw**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
11323.68	2068.21	3	23586.28	12722.44	3	42394.04	4261.28	3

Day 0 A cooked			**Day 0 C cooked**			**Day 0 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
9196.27	1390.73	3	38602.12	17628.02	3	58378.36	9636.33	3

Day 2 A cooked			**Day 2 C cooked**			**Day 2 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
8662.18	1813.48	3	37298.32	12065.96	3	54711.75	9579.92	3

Day 3 A cooked			**Day 3 C cooked**			**Day 3 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
5217.5	321.33	3	22561.29	2296.96	3	39283.79	9686.06	3

Day 4A cooked			**Day 4 C cooked**			**Day 4 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
8967.48	3804.897	3	32023.75	16952.83	3	41851.74	7870.845	3

Table 19 Raw data for the semi-quantification of gamma-CH_2 Glutamate with ^{1}H-NMR as displayed in Figure 5

gamma-CH_2 Glutamate (2.46m)								
Day 0 A raw			**Day 0 C raw**			**Day 0 E raw**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
23988.91	1428.46	3	43997.84	17039.02	3	64950.55	4896.44	3

Day 0 A cooked			**Day 0 C cooked**			**Day 0 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
9559.29	590.7973	3	52573.94	23128.65	3	82002.71	15579.97	3

Day 2 A cooked			**Day 2 C cooked**			**Day 2 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
18641.08	3272.43	3	60353.21	16104.57	3	81867.11	12956.56	3

Day 3 A cooked			**Day 3 C cooked**			**Day 3 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
13570.49	470.11	3	37404.63	3765.79	3	59512.55	13297.04	3

Day 4A cooked			**Day 4 C cooked**			**Day 4 E cooked**		
mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n	mean [AUC]	SD [AUC]	n
12566.33	4806.771	3	46024.24	24842.31	3	64213.6	11178.65	3

11.2 Tryptophan Loss

Table 20 Raw data for the semi-quantification of the tryptophan loss as displayed in Figure 9

Tryptophan loss									
Day 0 A raw			Day 0 C raw			Day 0 E raw			Day 0-4 cooked
mean [RFU at 345 nm]	SD [RFU at 345 nm].	n	mean [RFU at 345 nm]	SD [RFU at 345 nm].	n	mean [RFU at 345 nm]	SD [RFU at 345 nm].	n	mean [RFU at 345 nm]
1320.48	63.65	3	1368.26	32.75	3	1297.51	78.28	3	Not detected

11.3 Loss of Free Lysine and Arginine

Table 21 Raw data for the semi-quantification of free lysine with LC-MS as displayed in Figure 10 and Figure 11

Loss of Free Lysine			
sample	mean [AUC]	SD [AUC]	n
Day 0 A raw	135270442.7	19426845.35	3
Day 0 C raw	166710693.3	16275425.81	3
Day 0 E raw	149589378.7	11982113.26	3
Day 0 A cooked	93310896	5259077.783	3
Day 0 C cooked	96010506.67	6697064.735	3
Day 0 E cooked	82614856	10522522.69	3
Day 2 A cooked	38484431.33	4320756.747	3
Day 2 C cooked	56426085.67	35927501.32	3
Day 2 E cooked	41071050.67	6099460.476	3
Day 3 A cooked	40327581.33	3617729.273	3
Day 3 C cooked	64051942.67	14387073.08	3
Day 3 E cooked	40600870.67	13844677.68	3
Day 4 A cooked	19495567	7312954.252	3
Day 4 C cooked	21310121	9170294.985	3
Day 4 E cooked	31253652.67	10043307.39	3

Table 22 Raw data for the semi-quantification of free arginine with LC-MS as displayed in Figure 10 and Figure 12

Loss of Free Arginine			
sample	mean [AUC]	SD [AUC]	n
Day 0 A raw	34288828	6446766	3
Day 0 C raw	36399517	4398527	3
Day 0 E raw	21394928	1840455	3
Day 0 A cooked	42418777	575631	3
Day 0 C cooked	34383649	2614836	3
Day 0 E cooked	29133064	5804021	3
Day 2 A cooked	4236171	156985	3
Day 2 C cooked	2969140	1521126	3
Day 2 E cooked	3236752	555851	3
Day 3 A cooked	3364311	95749	3
Day 3 C cooked	3809208	378990	3
Day 3 E cooked	2375573	1080778	3
Day 4 A cooked	2705929	301865	3
Day 4 C cooked	2207434	224183	3

Day 4 E cooked	2208303	375824	3

11.4 Protein-bound Lysine and Arginine

Table 23 Raw data for the quantification of protein-bound lysine with LC-MS as displayed in Figure 13

Lysine [mg/g]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
15.46	1.53	3	14.63	0.14	3	14.26	0.88	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
15.74	0.65	3	15.36	0.78	3	14.28	1.07	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
14.84	1.39	3	13.56	1.03	3	14.99	1.46	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
14.48	1.15	3	14.78	0.40	3	14.37	0.82	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
14.41	0.67	3	14.51	1.17	3	16.66	1.23	3

Table 24 Raw data for the quantification of protein-bound arginine with LC-MS as displayed in Figure 13

Arginine [mg/g]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
9.95	0.96	3	8.86	0.15	3	9.11	0.37	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
10.64	0.30	3	10.04	0.79	3	9.38	0.88	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
9.71	1.21	3	9.29	0.99	3	9.48	1.33	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
9.27	1.15	3	9.81	0.89	3	9.80	0.77	3

Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
8.91	0.16	3	9.81	1.26	3	10.92	1.29	3

11.5 AGEs

Table 25 Raw data for the quantification of CML with LC-MS as displayed in Figure 14

CML [mg/g]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
1.90	0.55	3	2.41	0.29	3	2.01	0.32	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
1.53	0.22	3	1.56	0.24	3	1.61	0.36	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
1.85	0.24	3	2.10	0.37	3	1.64	0.20	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
1.84	0.23	3	1.45	0.21	3	1.61	0.33	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
1.57	0.20	3	1.55	0.33	3	1.58	0.43	3

Table 26 Raw data for the semi-quantification of CEL with LC-MS as displayed in Figure 15

CEL [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
8669550.67	2684802.11	3	11550805.00	587448.45	3	11593444.67	655007.04	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
9063554.33	1976834.94	3	9798047.00	1520436.10	3	11621490.00	487674.76	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
10919504.00	2082009.58	3	10380493.00	1409752.96	3	10541877.67	873353.67	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
10574381.00	798479.15	3	11073404.67	2600300.56	3	10363155.67	820111.58	3

Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
11614299.00	241111.07	3	9889433.00	97369.17	3	10130500.33	2231699.43	3

Table 27 Raw data for the semi-quantification of pentosidine with LC-MS as displayed in Figure 15

Pentosidine [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
34284.00	6324.59	3	53724.33	814.88	3	51383.33	8823.30	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
41557.67	4630.87	3	45252.00	10817.89	3	49647.00	5311.13	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
48694.33	15987.19	3	45859.67	13198.25	3	43126.67	4353.96	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
50685.33	11163.26	3	46302.00	11383.59	3	46555.67	4553.93	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
52613.67	2465.87	3	43188.67	9466.94	3	42875.67	9763.85	3

Table 28 Raw data for the semi-quantification of GOLD with LC-MS as displayed in Figure 16

GOLD [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
126204.00	84618.65	3	247823.33	71710.94	3	300448.33	114916.16	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
151032.33	16116.78	3	214734.33	63230.20	3	375443.67	160261.73	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
332002.00	176355.36	3	263039.33	249694.22	3	239169.67	96017.09	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
315807.00	197949.47	3	295287.00	175439.56	3	239781.00	118783.44	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		

mean	SD	n	mean	SD	n	mean	SD	n
335408.67	19161.06	3	276788.33	151664.83	3	217126.67	113858.56	3

Table 29 Raw data for the semi-quantification of MOLD with LC-MS as displayed in Figure 16

MOLD [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
148324.00	51572.35	3	221669.67	63876.94	3	260868.67	77493.96	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
153012.67	5960.27	3	185103.00	54197.50	3	324817.33	121797.02	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
288013.67	128689.11	3	231746.67	179079.83	3	229088.00	74786.19	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
258353.33	138141.21	3	253238.33	123976.23	3	213350.00	89522.33	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
293114.67	22559.73	3	260002.00	108627.58	3	194468.00	69319.35	3

Table 30 Raw data for the semi-quantification of MG-H1 with LC-MS as displayed in Figure 17

MG-H1 [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
348549.00	66478.70	3	281368.00	19075.54	3	322017.33	5777.41	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
360272.33	15512.91	3	380239.33	15708.49	3	350734.00	28387.48	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
210324.33	47421.16	3	160334.67	24658.08	3	228701.67	15483.61	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
178143.00	7160.54	3	230441.00	16831.52	3	237954.00	77959.28	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
250682.33	50724.08	3	238164.00	76868.85	3	396955.33	54560.84	3

Table 31 Raw data for the semi-quantification of G-H1 with LC-MS as displayed in Figure 17

G-H1 [AUC]								
Day 0 Raw								
Day 0 A raw			Day 0 C raw			Day 0 E raw		
mean	SD	n	mean	SD	n	mean	SD	n
595827.00	169708.88	3	678433.33	101845.28	3	563014.33	167356.17	3
Day 0 Cooked								
Day 0 A cooked			Day 0 C cooked			Day 0 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
581970.67	65681.14	3	764393.00	258105.05	3	1131638.00	263432.30	3
Day 2 Cooked								
Day 2 A cooked			Day 2 C cooked			Day 2 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
868974.33	234974.44	3	824174.00	289139.73	3	738875.00	230160.34	3
Day 3 Cooked								
Day 3 A cooked			Day 3 C cooked			Day 3 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
718798.33	208580.76	3	809470.67	340218.01	3	788351.33	182287.50	3
Day 4 Cooked								
Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean	SD	n	mean	SD	n	mean	SD	n
893056.00	192052.43	3	981733.67	166159.96	3	815981.67	128848.35	3

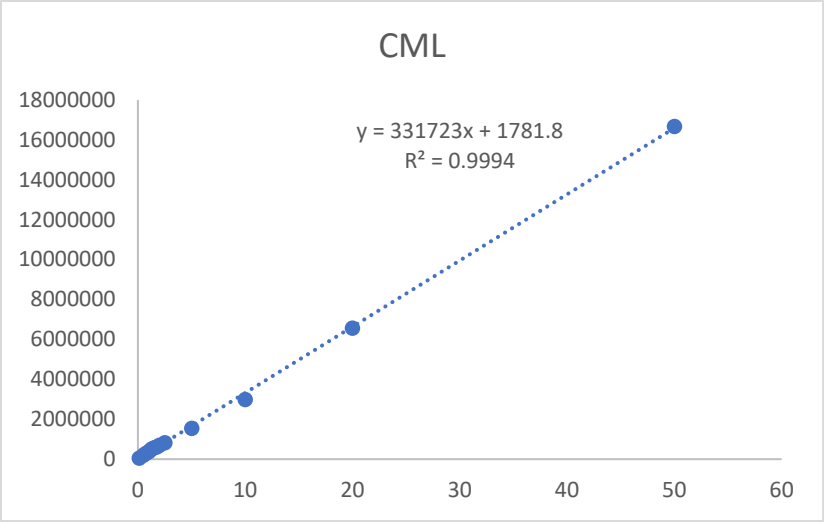

Figure 23 External standard curve used for quantification of CML with LC-MS

Table 32 External standard curve used for quantification of CML with

CML (mg/L)	Area
0.02	29185
0.05	23359
0.1	54453
0.5	201741
0.75	303093
1	380398
1.25	508395
1.5	576725
1.75	620247
2	702990
2.5	834830
5	1558204
10	2997015
20	6565460
50	16681093

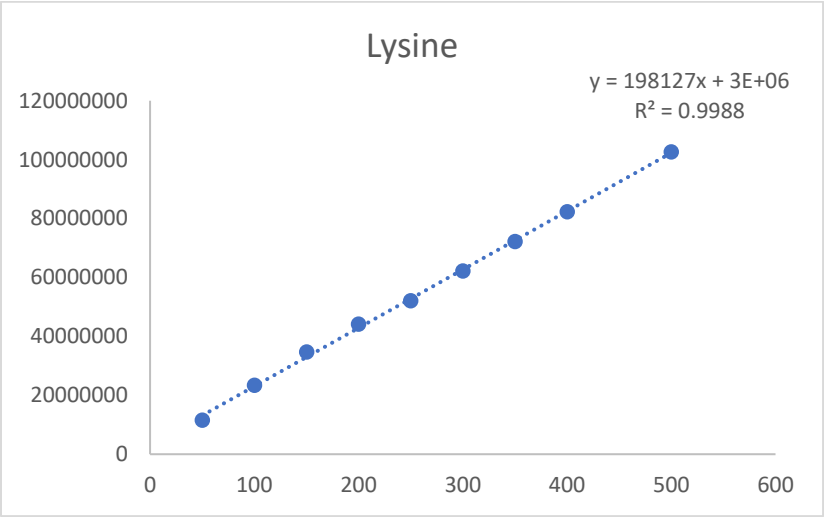

Figure 24 External standard curve used for quantification of lysine with LC-MS

Table 33 External standard curve used for quantification of lysine with LC-MS

Lys (mg/L)	Area
50	11489033
100	23403040
150	34650584
200	44122052
250	52017136
300	62134748
350	72162232
400	82269680
500	102524000

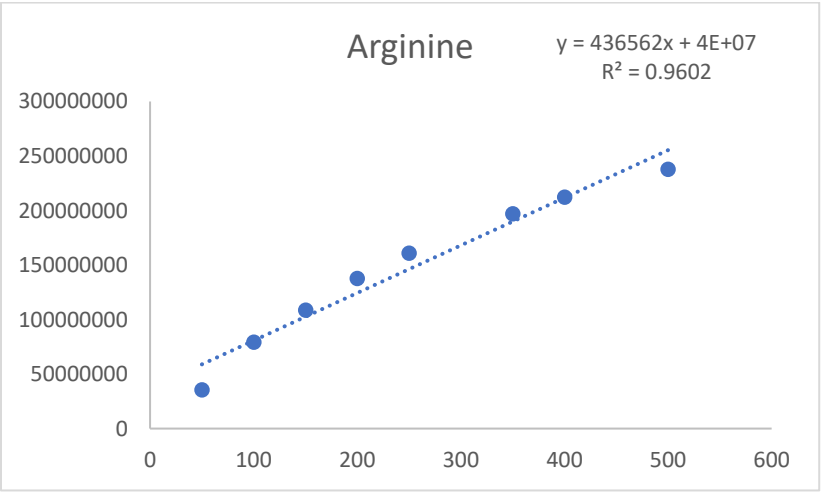

Figure 25 External standard curve used for quantification of arginine with LC-MS

Table 34 External standard curve used for quantification of arginine with LC-MS

Arginine (mg/L)	Area
50	35635084
100	79230304
150	108797264
200	137847104
250	161059040
350	197111552
400	212211808
500	237824752
1000	193054992

11.6 ESR

Table 35 Raw data for the quantification of unpaired electrons as displayed in Figure 18 and Figure 19

ESR			
	unpaired e- [mol/g]		
	mean	SD	n
Day 0 A raw	4.31381E-09	6.79438E-10	3
Day 0 C raw	5.3034E-09	1.93853E-09	3
Day 0 E raw	5.35258E-09	1.33939E-09	3
Day 0 A cooked	1.23543E-09	4.92313E-10	3
Day 0 C cooked	5.36072E-10	1.24938E-10	3
Day 0 E cooked	1.10497E-09	1.22735E-10	3
Day 2 A cooked	2.31329E-09	3.03487E-10	3
Day 2 C cooked	2.24154E-09	9.90917E-11	3
Day 2 E cooked	2.09665E-09	1.27127E-10	3
Day 3 A cooked	2.36727E-09	3.17901E-10	3
Day 3 C cooked	2.65641E-09	2.83873E-10	3
Day 3 E cooked	2.81207E-09	6.02966E-10	3
Day 4 A cooked	2.46266E-09	1.76193E-10	3
Day 4 C cooked	2.33317E-09	2.97145E-10	3
Day 4 E cooked	2.48217E-09	1.48871E-10	3

11.7 Carbonyl Content

Table 36 Raw data for the quantification of protein carbonyls as displayed in Figure 20

Carbonyl Content								
Day 0 A raw			**Day 0 C raw**			**Day 0 E raw**		
mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n
3.66	0.50	14	3.47	0.37	12	5.19	1.53	12

Day 0 A cooked			**Day 0 C cooked**			**Day 0 E cooked**		
mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n
8.92	1.85	20	7.57	1.42	16	8.16	1.65	10

Day 2 A cooked			**Day 2 C cooked**			**Day 2 E cooked**		
mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n
3.67	0.55	14	5.06	0.79	18	4.30	0.41	10

Day 3 A cooked			**Day 3 C cooked**			**Day 3 E cooked**		
mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n

7.57	2.74	18	6.46	2.11	18	6.17	2.23	10

Day 4 A cooked			Day 4 C cooked			Day 4 E cooked		
mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n	mean [nmol/mg protein]	SD [nmol/mg protein]	n
5.76	0.85	15	6.48	0.61	11	5.99	0.97	14

11.8 Schiff Bases

Table 37 Raw data for the semi-quantification of Schiff bases as displayed in Figure 21

Schiff bases [RFU at 460 nm]			
	Day 0 A raw	**Day 0 C raw**	**Day 0 E raw**
mean	30.74	64.16	114.43
SD	14.37	13.62	32.58
n	3	3	3

	Day 0 A cooked	**Day 0 C cooked**	**Day 0 E cooked**
mean	57.77	67.50	99.26
SD	8.00	10.41	12.14
n	3	3	3

	Day 2 A cooked	**Day 2 C cooked**	**Day 2 E cooked**
mean	39.44	48.90	82.69
SD	6.97	6.81	9.63
n	3	3	3

	Day 3 A cooked	**Day 3 C cooked**	**Day 3 E cooked**
mean	48.98	58.75	72.20
SD	7.04	10.50	8.88
n	3	3	3

	Day 4 A cooked	**Day 4 C cooked**	**Day 4 E cooked**
mean	41.94	53.68	65.81
SD	5.05	4.37	5.79
n	3	3	3