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Formation of Early and Advanced Maillard Reaction Products Correlates to the Ripening of Cheese

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ABSTRACT: The present study deals with the characterization of the ripening of cheese. A traditional German acid curd cheese was ripened under defined conditions at elevated temperature, and protein and amino acid modifications were investigated. Degree of proteolysis and analysis of early [Amadori compound furosine (**6**)] and advanced [N^{ε}-carboxymethyllysine (**4**), N^{ε}-carboxyethyllysine (**5**)] Maillard reaction products confirmed the maturation to proceed from the rind to the core of the cheese. Whereas **6** was decreased, **4** and **5** increased over time. Deeper insight into the Maillard reaction during the ripening of cheese was achieved by the determination of selected α-dicarbonyl compounds. Especially methylglyoxal (**2**) showed a characteristic behavior during storage of the acid curd cheese. Decrease of this reactive structure was directly correlated to the formation of **5**. To extend the results of experimental ripening to commercial cheeses, different aged Gouda types were investigated. Maturation times of the samples ranged from 6 to 8 weeks (young) to more than 1 year (aged). Again, increase of **5** and decrease of **2** were able to describe the ripening of this rennet coagulated cheese. Therefore, both chemical parameters are potent markers to characterize the degree of maturation, independent of coagulation.

KEYWORDS: *cheese ripening, Maillard reaction, N^{ε}-carboxymethyllysine, N^{ε}-carboxyethyllysine, furosine, proteolysis*

INTRODUCTION

The ripening of cheese is a complex process and involves microbiological and biochemical changes resulting in flavor formation and texture development.[1] The major chemical changes during cheese ripening are catabolism of residual lactose[2,3] and lactate,[4] lipolysis and metabolism of fatty acids,[5] and proteolysis and catabolism of amino acids.[6] The extent of these reactions as well as dehydration during maturation mainly determines the texture of ripened cheese. Reactive intermediates and stable end products resulting from the reactions mentioned above have an important impact on the specific flavor of the particular cheese variety.[7] Different enzymes from milk, coagulant, starter bacteria, and secondary microflora as well as exogenous enzymes for accelerated ripening are the most important catalysts leading to changes of the major ingredients.[1,8]

Because proteolysis is the major biochemical event during cheese production and maturation, it has been an important subject of research for decades.[9−13] Basically, caseins are cleaved to peptides by proteinases, which are further degraded to free amino acids by peptidases.[6] The extent of proteolysis is often expressed as a general nonspecific parameter as the percentage of the pH 4.6 soluble nitrogen related to total nitrogen analyzed by the Kjeldahl method. Further information on the ripening process can be obtained by the analysis of the pH 4.6 insoluble fraction of cheese with electrophoretic techniques[10] as well as by determination of the amino acid and peptide profile of the soluble fraction with chromatographic means.[14]

Parallel to the breakdown of the protein network, the modification of α-amino groups of proteins, peptides, and amino acid side chains occurs simultaneously.[15] Especially the nucleophilic ε-amino group of lysine reacts with carbonyl groups of reducing sugars and their degradation products. This complex series of reaction pathways is called the Maillard reaction or nonenzymatic browning and accompanies always the thermal treatment and storage of milk and dairy products.[16,17] As a more stable intermediate of the early Maillard reaction, the Amadori product was determined in several cheese varieties and was correlated to maturation times.[18,19] Furosine (**6**), the measurable form of Amadori compounds after acid hydrolysis, was increased during the ripening of Manchengo cheese and accompanied by the decrease of the reducing sugar galactose[20] (Figure 1). In the later course of the nonenzymatic browning, the formation of advanced glycation endproducts (AGEs) such as N^{ε}-carboxymethyllysine (**4**) and N^{ε}-carboxyethyllysine (**5**) can be observed.[21] Formation pathways of these AGEs include either the decomposition of Amadori compounds or the reaction of amino acids with reactive intermediates, for example, dicarbonyl compounds such as glyoxal (**1**) or methylglyoxal (**2**).[16] However, in the literature there is little or no information about **4** and **5** during the ripening of cheese. Besides the formation of **1** and **2** via Maillard reactions, these dicarbonyls were produced during the growth of starter bacteria and secondary microorganisms and were also detected in some cheese varieties.[22−25] Besides **1** and **2**, diacetyl (**3**) was also determined as an important precursor structure for flavor formation related to dicarbonyl compounds in milk products.[26,27]

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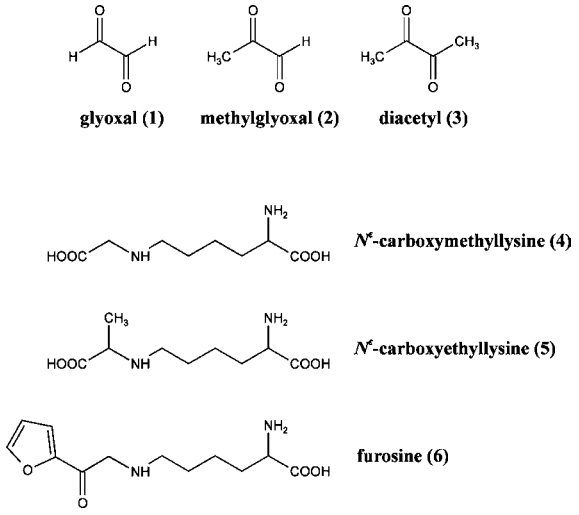

Figure 1. Structures of α-dicarbonyl compounds and amino acid modifications investigated in the present study.

The aim of the present study was to characterize the ripening of cheese on the basis of the Maillard reaction. For this purpose, the ripening process of a traditional German acid curd cheese (Harzer cheese) was investigated. Early (**6**) and advanced (**4**, **5**) Maillard reaction products were analyzed and correlated to the degree of proteolysis and the content of α-dicarbonyl compounds. The results were compared with commercially available Gouda cheeses of different ages.

MATERIALS AND METHODS

Materials and Cheese Samples. Chemicals of the highest quality available were obtained from Aldrich (Taufkirchen, Germany), Fluka (Taufkirchen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma (Taufkirchen, Germany), unless otherwise indicated. Quinoxalines of glyoxal (**1**), methylglyoxal (**2**), and diacetyl (**3**) were purchased from Aldrich.

N^{ε}-Carboxymethyllysine (**4**) was synthesized according to the method of Glomb and Pfahler.[28]

N^{ε}-Carboxyethyllysine (**5**) was synthesized according to the method of Fujioka and Tanaka.[29]

Furosine (**6**) was synthesized according to the method of Henle et al.[30]

2,3-Pentanedione quinoxaline (IST) was synthesized according to the method of Glomb and Tschirnich.[31]

Different Gouda varieties and unripened acid curd cheese were purchased from several local supermarkets.

Ripening of Acid Curd Cheese. To characterize the ripening of cheese, a commercial acid curd cheese (local varieties are called Handkäse or Harzer cheese) was ripened under defined conditions. For this several unripened cheeses from the same batch were stored at 16 ± 1 °C and 93% relative humidity. At the beginning of ripening, cheeses were differentiated into core and rind, and both fractions were analyzed separately. During the first 7 days, samples were taken every 24 h. After that, samples were taken every 48 h until 21 days.

Determination of Total Protein of Cheese Samples. The protein contents of acid curd cheese samples and Gouda samples were determined by using the Kjeldhal method.[32] Protein content was calculated using the factor 6.38 for milk and dairy products. All samples were determined in triplicate. Total protein content showed a coefficient of variation of <5%.

Determination of Dry Matter of Cheese Samples. Cheese samples (1−2 g) were weighed into an aluminum cup, mixed with sea sand, and dried for 4 h at 105 °C. After cooling, the samples were weighed and then dried again to constant mass. Dry matter was calculated from the mass loss after drying. In the case of acid curd cheese three cheeses were pooled. Both acid curd cheese samples and Gouda samples were determined in triplicate. Dry matter showed a coefficient of variation of <3%.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Caseins. For casein extraction, 2.5 g of cheese was homogenized with 30 mL of acetic acid water (pH 5−6). Subsequently, the pH was adjusted to 4.6 with 25 wt % acetic acid. After centrifugation (4500 rpm, 10 min), the supernatant was decanted and the residue rehomogenized with 20 mL of acetic acid water and 10 mL of methylene chloride. After phase separation by centrifugation, caseins generated a pellet at the phase boundary. The liquids were removed, and the process was repeated twice. The pellet was washed with acetone, dried, and rubbed to a powder. Caseins were dissolved in sample buffer (pH 8.6, 100 mM Tris, 2 mM 1,4-dithiothreitol, 8 mM urea) to a concentration of 10 mg/mL.

The accurate protein content of this solution was determined by using a method modified from that of Bradford.[33] Calibration was performed with commercial casein in the range of 0.01−0.03 mg/mL. Subsequently, the protein concentration was adjusted to 1 μg/μL with Laemmli buffer [pH 6.8, 125 mM Tris-HCl, 0.1 M 1,4-dithiothreitol, 1 mM EDTA, 4.8% (w/v) SDS, 20% (w/v) glycerol, 0.5% (w/v) bromophenol blue]. Caseins were analyzed on 15 and 16% acrylamide separation gels with a cross-linking of 3.3% using a running buffer [0.1 M Tris, 1 M glycine, 0.5% (w/v) SDS]. Twenty microliters of sample solutions, molecular weight marker, and standard substances were applied to the wells of a 5% acrylamide stacking gel with a cross-linking of 3.3%. The running condition was 10 min at 100 V, 40 min at 200 V, and 15 min at 100 V. After separation, gels were soaked in 20% (w/v) trichloroacetic acid for 30 min, washed [methanol/distilled water/acetic acid 25:65:10 (v/v/v)] for 30 min and stained [0.1% (w/v) Coomassie Brilliant Blue G250, methanol/distilled water/acetic acid 25:65:10 (v/v/v)] for 30 min. After decoloration, gels were scanned and analyzed with a gel documentation system (ChemiDoc XRS, Bio-Rad, Munich, Germany) and QuantityOne software.

Determination of Glyoxal (1), Methylglyoxal (2), and Diacetyl (3). Free and reversibly bound α-dicarbonyl compounds in cheese samples were determined as quinoxalines after derivatization with *o*-phenylenediamine (*o*-PD).[31] Five grams of cheese was freeze-dried. Gouda cheeses were defatted with 3 × 6 mL of petroleum ether. Subsequently, 100 μL of 50 μM internal standard, 2,3-pentandione quinoxaline (IST), 500 μL of 11 mM *o*-PD solution, and 0.6 M trifluoroacetic acid were added and homogenized to give a total volume of 10 mL. The suspension was kept in the dark and shaken at room temperature for 24 h. After centrifugation and filtration, an aliquot of the filtrate was diluted with H_2O for HPLC analysis with mass spectrometric quantification. In the case of acid curd cheese, three cheeses were pooled. Both acid curd cheese samples and Gouda samples were determined in triplicate.

Quantitative results were obtained from matrix-adjusted external calibration (coefficient of determination > 0.99) with commercially available quinoxalines. For this, selected cheese samples were homogenized with 10 mL of 0.6 M trifluoroacetic acid and were shaken for 24 h at room temperature. Calibrations of **1**, **2**, and **3** ranged between 2.5 and 1000 pmol/mL, between 75 and 2500 pmol/mL, and between 75 and 2500 pmol/mL, respectively. Contents of **1**, **2** and **3** showed coefficients of variation of <20, <10, and <5%, respectively.

Determination of N^{ε}-Carboxymethyllysine (4), N^{ε}-Carboxyethyllysine (5), and Furosin (6). The content of the amino acid modifications of cheese samples was obtained after acid hydrolysis. Forty milligrams of Gouda and 250 mg of acid curd cheese, respectively, were weighed in threaded culture tubes. In the case of analysis of **4** and **5** samples were reduced by the addition of $NaBH_4$. Gouda samples were defatted with 3 × 1 mL of petroleum ether. Subsequently, samples were dissolved in 3 mL of 6 N HCl and heated for 20 h at 110 °C after removal of oxygen by degassing with argon. After acid hydrolysis, volatiles were removed in a vacuum concentrator and the residues were diluted with 0.1 N HCl to concentrations appropriate for HPLC analysis with mass spectrometric quantification.

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Table 1. Mass Spectrometer Parameters for Quantification of Amino Acid Modifications and α-Dicarbonyl Compounds (MRM Mode)[a]

	Q1 mass (amu)	Q3 mass (amu)	dwell time (ms)	DP (V)	CE (V)	CXP (V)
4	205.20	130.10	100.00	80.00	19.00	11.00
5	219.10	84.10	100.00	54.00	33.00	7.00
6	255.10	192.10	100.00	55.00	22.00	16.00
quinoxaline of **1**	131.10	77.00	100.00	32.00	40.00	6.00
quinoxaline of **2**	145.00	77.00	100.00	50.00	41.00	5.00
quinoxaline of **3**	159.20	118.10	100.00	75.00	33.00	10.00
quinoxaline of IST	173.00	158.10	100.00	30.00	33.00	11.00

[a]Ionization was performed in ESI-positive mode.

In the case of acid curd cheese three cheeses were pooled. Both acid curd cheese samples and Gouda samples were determined in triplicate.

Quantitative results were obtained from calibration using the standard addition method (coefficient of determination > 0.99) with synthesized reference material. Calibrations of **4**, **5**, and **6** ranged between 2 and 40 pmol/mL, between 2.5 and 70 pmol/mL, and between 16 and 450 pmol/mL, respectively. **4** showed coefficients of variation of <13%. **5** showed coefficients of variation of <25%. **6** showed coefficients of variation of <20%.

High-Performance Liquid Chromatography (HPLC) and Mass Spectrometric Quantification of α-Dicarbonyl Compounds and Protein Modifications. For determination of the amino acid modifications and the α-dicarbonyl quinoxalines, a Jasco (Groß-Umstadt, Germany) quaternary gradient unit LG-2080-04 and pump PU-2080Plus with degasser DG-2080-54, autosampler AS-2057Plus, column oven set at 15 °C, and a mass spectrometer were used. Chromatographic separations of amino acid modifications were performed on stainless steel columns (VYDAC 218TP54, 250 × 4.6 mm, RP18, 5 μm, Hesperia, CA) using a flow rate of 1.0 mL/min. The mobile phase used was water (solvent A) and MeOH/water (7:3 v/v; solvent B). To both solvents (A and B) was added 1.2 mL/L heptafluorobutyric acid (HFBA). Samples were injected at 2% B and run isocratically for 13 min, the gradient then changed to 40% B in 17 min, then changed to 100% B in 5 min, and was held for 10 min. Subsequently, the gradient was changed again to 2% B and was held for 15 min.

Chromatographic separations of α-dicarbonyl quinoxalines were carried out on a stainless steel column (Knauer Eurospher 100-5 C18, 250 × 4.6 mm Berlin, Germany) using a flow rate of 1.0 mL/min. The mobile phase used was water/MeOH 3:7 (v/v), with 0.8 mL/L HCOOH. Quinoxalines were separated within 15 min in an isocratic run.

Mass spectrometric quantification was performed using an Applied Biosystem API 4000 quadrupole instrument (Applied Biosystem, Foster City, CA) equipped with an API source using an electrospray ionization (ESI) interface in positive mode. The HPLC system was connected directly to the probe of the mass spectrometer. Nitrogen was used as sheath and auxiliary gas. To measure the α-dicarbonyl compounds and protein modifications, the multiple reaction monitoring (MRM) mode of HPLC-MS/MS was used. The optimized parameters for mass spectrometry are given in Table 1.

RESULTS

To investigate Maillard reaction products during the ripening of cheese, unripened acid curd cheeses (Harzer cheese) were stored under defined conditions. This cheese variety was chosen for the present study because of the simple matrix (fat content ≤ 1%) and the expeditious changes during maturation. At the beginning of storage the cheeses can be divided into a white, quark-like, crumbled core and a yellowish, semitransparent, elastic rind. After 7 days of ripening, the core vanished and the whole cheese became like the rind at the beginning of the experiment. To characterize the course of ripening, cheese samples were investigated for the degree of proteolysis, amino acid modifications, and α-dicarbonyl content. To transfer the parameters of ripening to rennet coagulated cheese varieties, commercially available Gouda cheeses were analyzed. The different types of Gouda were classified into young (6−8 weeks, $N = 12$), middle-aged (2−6 month, $N = 10$), and old (>1 year, $N = 11$) grades.

Proteolysis of Caseins. Changes of the protein composition of the pH 4.6 insoluble fraction of cheeses were investigated by SDS-PAGE. This method allows insights into the breakdown of milk caseins to smaller proteins and peptides. Prior to electrophoretic separation, the extracts were adjusted to the same protein content, which was determined according to the method of Bradford. Because of this, the intensity of protein bands and the degree of proteolysis can be compared to each other. Molecular weights of proteins were determined by a molecular weight standard, and identification of caseins was performed by comparing bands with standard reference material.

Breakdown of caseins and formation of respective decomposition products of the acid curd cheese during the ripening are depicted in Figure 2. The molecular weight of milk proteins

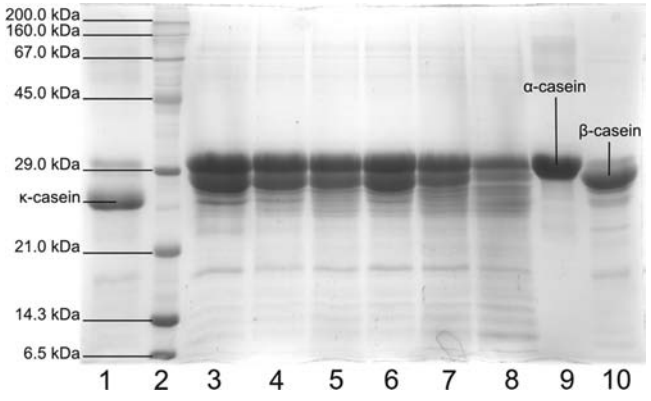

Figure 2. SDS-PAGE ($T = 15\%$, $C = 3.3\%$) of the ripening of acid curd cheese (Harzer cheese). Lanes: 1, κ-casein standard; 2, molecular weight standard; 3, core, 0 days; 4, rind, 0 days; 5, rind, 5 days; 6, core, 5 days; 7, 12 days; 8, 21 days; 9, α-casein standard; 10, β-casein standard.

was 29.5 kDa for α-casein, 27.5 kDa for β-casein, and 25.5 kDa for κ-casein. These anomalously high M_r values are in accordance with their atypical SDS-binding capacity and their resulting reduced electrophoretical mobility.[34] Because of the method of coagulation during production, κ-casein was detected in every sample of this acid curd cheese variety. During the ripening, the intensity of α- and β-casein bands decreased, whereas the intensity of decomposition products

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(M_r 10−16 kDa) increased. Additionally, β-casein (−76%) was degraded to a greater extent than α-casein (−54%). Furthermore, proteolysis on the outside of cheeses was more advanced than in the core (e.g., Figure 2, lanes 3 and 4). This is in line with the observed changes in color and texture of this cheese variety during maturation.

In Figure 3 the casein patterns of selected rennet coagulated Gouda samples are shown. In contrast to the acid curd cheese, the proteolysis was more pronounced and α-casein was degraded to a greater extent than β-casein. Simultaneously, peptides between 10 and 20 kDa showed an increase with prolonged maturation.

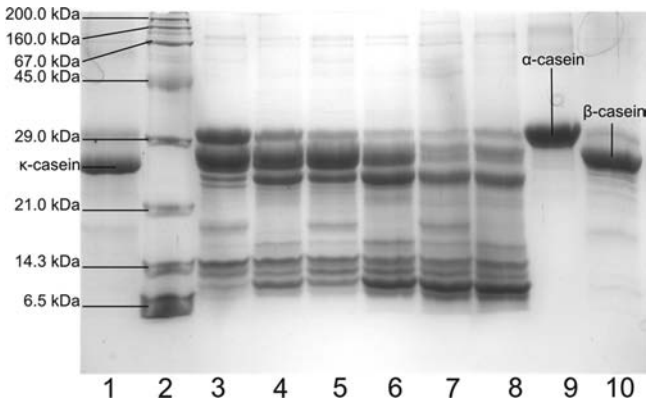

Figure 3. SDS-PAGE (T = 16%, C = 3.3%) of different Gouda cheeses. Lanes: 1, κ-casein standard; 2, molecular weight standard; 3 and 4 (raw milk Gouda), young; 5 and 6 (raw milk Gouda), middle-aged; 7 and 8, old; 9, α-casein standard; 10, β-casein standard.

Analysis of Selected α-Dicarbonyls. The contents of **1**, **2**, and **3** in cheese were analyzed as their respective quinoxalines after derivatization with *o*-PD. Quantification was performed with HPLC-MS/MS and matrix-assisted calibration. For this, the workup of selected cheese samples was performed without *o*-PD and spiked with the respective quinoxalines to give an external calibration. Because of the enzymatic and nonenzymatic elimination of phosphate from glycerine-3-phosphate or dihydroxyacetonphosphate and the resultant formation of **2**, acidic conditions were chosen to avoid artifact formation during workup. The Gouda cheeses were additionally defatted prior to derivatization. For better comparison, the α-dicarbonyl concentrations of samples were related to the respective dry matter.

In Figure 4 the contents of **1**, **2**, and **3** of the acid curd cheese (Harzer cheese) during ripening are shown. The core and the rind of the cheeses were also analyzed separately during the first 6 days of storage. Results differed only slightly (after 22 h; **1**, core, 1.8 ± 0.1 nmol/g dry matter, and rind, 2.4 ± 0.5 nmol/g dry matter; **2**, core, 27.6 ± 1.2 nmol/g dry matter, and rind, 26.0 ± 0.1 nmol/g dry matter; **3**, core, 7.2 ± 0.2 nmol/g dry matter, and rind, 7.2 ± 0.5 nmol/g dry matter). Thus, for better comparison, the concentrations were calculated for the whole cheese. As shown in Figure 4 **3** was decreased rapidly by 54% during the first 3 days and did not change until the end of storage. In contrast, **2** was slightly increased at the beginning of ripening. After 7 days, this dicarbonyl was linearly degraded to 11.6 nmol/g dry matter. **1** was increased by 80% and degraded afterward to the concentration of the unripened cheese.

The determination of **1** and **3** (Figure 5) in rennet coagulated Gouda cheeses gave similar concentrations as in acid curd Harzer cheese. However, **2** was determined at lower contents compared to Harzer cheese. When Gouda samples between different ripening grades were compared, only **2** showed a significant correlation to increased ripening time. Whereas average values of **1** and **3** revealed no differences in relation to age, the concentrations of **2** in Gouda cheeses decreased with increasing maturation. Statistical analyses were not performed because of large age inhomogeneity within the ripening grades: young commercially available Gouda cheese covered a short period (6−8 weeks), and old varieties were stored for 1−5 years before sale.

Analysis of Amino Acid Modifications. Early and advanced Maillard reaction products were determined by HPLC-MS/MS after acid hydrolyses. Samples of the ripening of acid curd cheese were directly treated with 6 M HCl, whereas Gouda samples were defatted prior to hydrolysis. For comparison between different cheese varieties, concentrations were related to the respective protein content.

As depicted in Figure 6, the amino acid modifications **4** and **5** increased during the maturation of Harzer cheese by 38 and 115%, respectively. In contrast, **6** was degraded only by approximately 15%. In contrast to the α-dicarbonyls

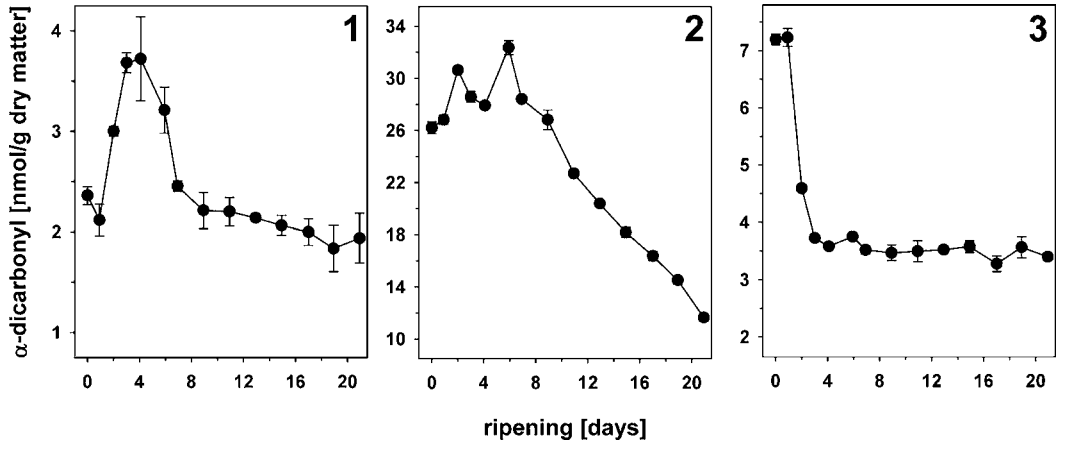

Figure 4. Formation of glyoxal (**1**), methylglyoxal (**2**), and diacetyl (**3**) during the ripening of acid curd cheese (Harzer cheese). Three cheeses were unified and determined in triplicate. Error bars denote standard deviation.

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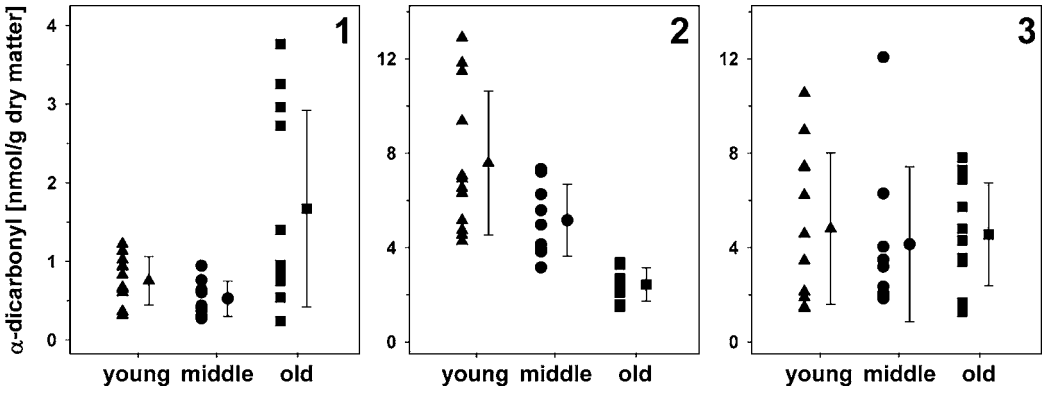

Figure 5. Contents of glyoxal (**1**), methylglyoxal (**2**), and diacetyl (**3**) in different grades of Gouda cheese (young, 6−8 weeks, $N = 12$; middle-aged, 2−6 months, $N = 10$; old, >1 year, $N = 11$). Samples were determined in triplicate. Error bars denote standard deviation within grades.

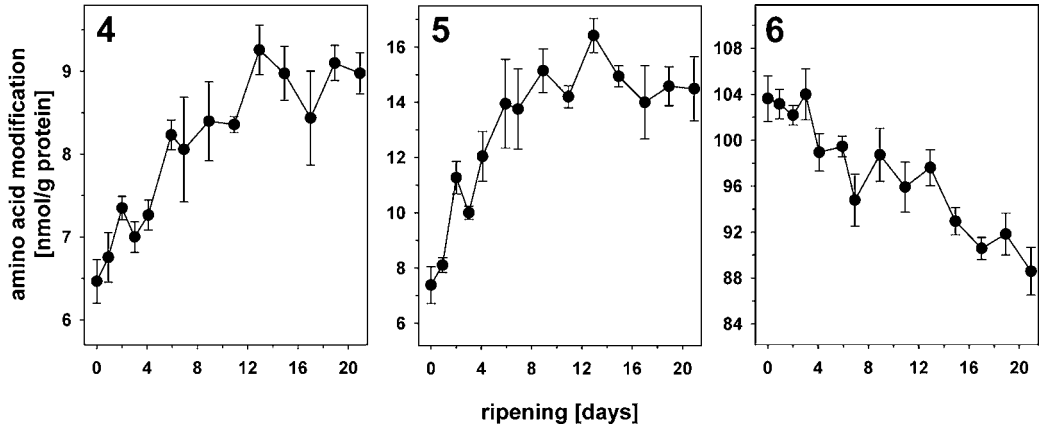

Figure 6. Formation of Maillard reaction products [N^{ε}-carboxymethyllysine (**4**), N^{ε}-carboxyethyllysine (**5**), furosine (**6**)] during the ripening of acid curd cheese (Harzer cheese). Error bars denote standard deviation.

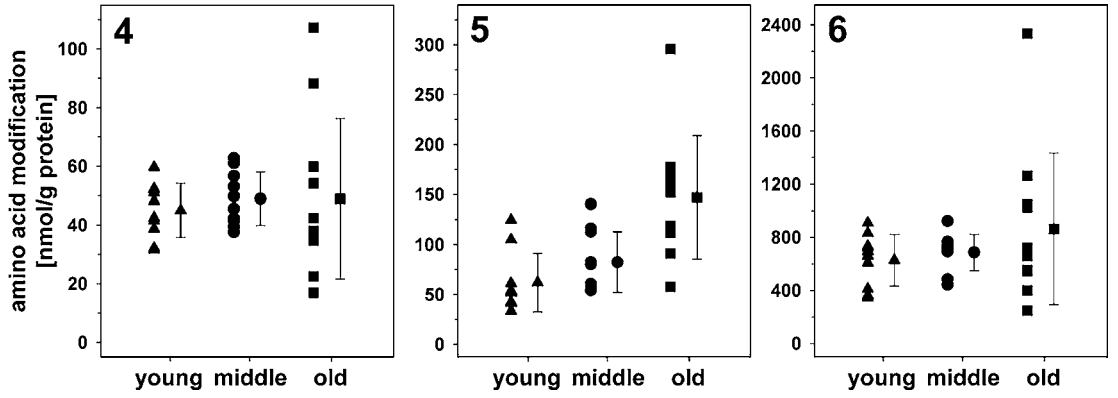

Figure 7. Formation of Maillard reaction products [(N^{ε}-carboxymethyllysine (**4**), N^{ε}-carboxyethyllysine (**5**), furosine (**6**)] in different grades of Gouda cheese (young, 6−8 weeks, $N = 12$; middle-aged, 2−6 month, $N = 10$; old, >1 year, $N = 11$). Samples were determined in triplicate. Error bars denote standard deviation within grades.

measured, there were differences between the core and the rind of the cheeses. For instance, after 1 day of ripening, the concentration of **4** at the edge (7.2 ± 0.9 nmol/g protein) exceeded that at the core (6.3 ± 0.5 nmol/g protein) by approximately 12%. The difference for **5** at the same time amounted to 45% (core, 6.6 ± 0.6 nmol/g protein; edge, 9.6 ± 0.6 nmol/g protein). In contrast, concentrations of **6** in the core (106.3 ± 3.3 nmol/g protein) were higher than those in the rind (100.0 ± 3.1 nmol/g protein). However, for a comprehensive view, the values in Figure 6 are again depicted for the whole cheese.

The concentrations of the amino acid modifications of the different Gouda cheeses were a magnitude of order higher than those of the acid curd cheese (Figure 7). For **4** and **6** there were no significant trends between the different ripening grades observable. However, the average values of **5** increased with increasing maturation by 135%. Interestingly, variation within an aging grade group increased for every compound.

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DISCUSSION

In food chemistry, the Maillard reaction has been a subject of interest for decades. However, this complex series of reactions was rarely investigated in the context of cheese ripening. Additionally, no study was published that combined the concepts of analyzing markers of early and advanced Maillard reaction products and determination of possible reaction partners for amino acids leading to these compounds.

The most important process during the maturation of cheese is proteolysis. As shown in Figures 2 and 3, the breakdown of the protein networks occurs in both acid curd cheese (Harzer cheese) and rennet coagulated Gouda cheese. However, the decomposition of α- and β-caseins differed in both cheese types, which confirms the findings of Krause et al.[10] They verified the preferential hydrolysis of α-casein in Gouda caused by rennets and bacterial proteases and the enhanced β-casein breakdown in acid curd cheese. Obviously, the type of coagulation has a significant influence on the protein pattern of the resulting cheese. The extensive decomposition of β-casein and the occurrence of γ-caseins in raw milk cheese were also used before to characterize the ripening process.[9,13] Furthermore, differences between the protein compositions result also from cheese-producing technologies such as ultrafiltration and heat treatment.[35] In the present study, electrophoretical analysis confirmed the maturation to proceed from the rind to the core of acid curd cheeses. Therefore, analysis of the protein breakdown allowed the degree of ripening within a cheese variety to be characterized but hinders comparison between different cheese types.

Besides hydrolyses of peptide bonds, modification of proteins, peptides, and free amino acids takes place during the maturation of cheese. Especially primary amino groups are capable of the reaction with residual lactose and the monosaccharides galactose and glucose. The content of **6** of the Gouda cheeses (Figure 7) was in line with the findings of Schwietzke et al.[15] and exceeded the concentrations of the Amadori product within the acid curd cheese (Figure 6). The reasons can be related to different processing conditions such as enhanced whey drainage and heat treatment or to differences in the composition of the raw material. Nevertheless, **6** has been used as a ripening parameter in Grana Padano[18] and Manchengo cheeses.[20] In the present study, because **6** of acid curd cheese decreased steadily, the Amadori product was also able to describe the maturation status for this kind of cheese. The higher concentration in the core of the cheese additionally confirmed the ripening to proceed from the outside. In contrast, Corzo et al. observed an increase of **6** accompanied by the loss of galactose during the ripening of Manchengo cheese.[20] Obviously, the early Maillard reaction in cheese, characterized by Amadori product formation, strongly depends on the cheese variety. Because of this and because the Amadori product represents an intermediate leading to quantitative important followup structures, the formation of advanced Maillard reaction products of lysine was analyzed herein. The average concentration of **4** in Gouda was, independent of age, 50 nmol/g protein (10 mg/kg protein), which was determined in the same order of magnitude by Assar et al.[36] for Cheddar cheese (23.2 mg/kg protein). In general, contents of **4** and **5** in Gouda were higher than in Harzer cheese. In addition to smaller concentrations of **6** in acid curd cheese, overall protein modifications via the Maillard reaction in Gouda cheeses occurred to a higher extent. With the enhanced proteolysis even in marginal matured Gouda types, one reason must be the comparably much longer ripening before marketing. Whereas Harzer cheese is sold after 2 weeks, the production of young Gouda takes at least 6 weeks. As mentioned above, also processing and raw material have strong impacts on markers of nonenzymatic browning. Nevertheless, **5** was identified as a robust marker for the ripening of cheese, regardless if acid or rennet coagulated. In contrast to **4**, **5** was not only increased during the ripening of Harzer cheese (Figure 6) but was also determined at higher contents in aged Gouda cheeses (Figure 7).

Because α-dicarbonyl compounds are able to form Maillard reaction products and are involved in the production of cheese flavors, these highly reactive substances were determined within this study. Little information about α-dicarbonyl contents of cheese is given in the literature. Bednarski et al. investigated Cheddar, mozzarella, and Swiss cheeses for their concentrations of **1**, **2**, and **3**.[22] **3** was determined with the lowest content, independent of cheese variety. Because of the acidic derivatization conditions in the present study, concentrations of **1**, **2**, and **3** were analyzed at lower amounts. **3** was rapidly decreased during the ripening of Harzer cheese, reaching concentrations similar to those in Gouda cheeses. The death of starter bacteria, which are responsible for lactose conversion into **3**, might be the reason for this decrease. Additionally, in matured cheeses **3** reacts quickly with amino acids to form other important flavor compounds.[27] **1** and **2** also possess the ability to react with amino acids, but in this case advanced Maillard reaction products will be formed. The increase of **4** and **5** was accompanied by the decrease of **1** and **2** (Figure 4), respectively, which supports advanced Maillard reactions as important processes during the ripening of acid curd cheese. From the mechanistic point of view, **4** can be formed both from the reaction of lysine with **1** and from the oxidative fragmentation of the Amadori product. The simultaneous decrease of the dicarbonyl compound and the Amadori product paralleled by the increase of **4** suggests that both pathways are valid only for acid curd cheese, but not for Gouda. It can only be assumed that this correlation is weak during Gouda maturation and in the present investigation thus is undermined by the great inhomogeneity of the commercial samples due to unknown factors such as exact ripening conditions and times or storage and handling conditions. In Gouda, only **2** decreased during ripening (Figure 5). In contrast to **4**, **5** originates from the single reaction of lysine with the dicarbonyl **2**. Obviously, the decrease in **2** with simultaneously enhanced contents of **5** also in aged Goudas, both chemical parameters characterize the degree of maturation very well. The correlation between the formation of **5** and the degradation of **2** during storage of Harzer cheese is shown in Figure 8. The excellent linear relationship of the ratio between both chemical parameters to storage times confirms the unique ability to characterize the ripening of acid curd cheese via advanced Maillard reaction.

In conclusion, our investigations present the concept of analyzing protein modifications as a valuable tool of characterization of the ripening of cheese. Within a batch of an acid curd cheese proteolysis and markers of the Maillard reaction were able to describe the maturation. The decrease of early Maillard reaction products accompanying an increase of stable protein modifications is in line with the general course of nonenzymatic browning reactions. With the degradation of **2** and the simultaneous increase of **5**, the formation pathway of this lysine modification was identified in acid curd cheeses. The

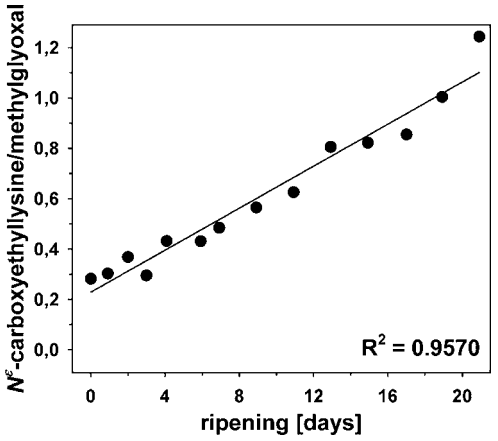

Figure 8. Ratio of N^{ε}-carboxyethyllysine (**5**) to methylglyoxal (**2**) during the ripening of acid curd cheese.

analysis of commercial Gouda cheeses of different maturation grades confirmed the robust character of both chemical parameters to describe the ripening of cheese in general. For classification of cheeses based on this concept, further cheese varieties should be investigated. In addition, it would be interesting to expand the range of **2**-derived amino acid modifications to arginine adducts. Quantitatively important structures might be imidazolinones in addition to argpyrimidine.[37] Furthermore, the origin of **2** in dairy products is another point of interest.

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