

Glycation compounds in peanuts

Anne Wellner · Lena Nußpickel · Thomas Henle

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Abstract In the present study, the extent of the Maillard reaction in peanuts was investigated, using *N*- ϵ -fructosyllysine (ϵ -Fru-Lys, determined via furosine) as an indicator for the early stage, and pyrraline and *N*- ϵ -carboxymethyllysine (CML) as representatives for advanced glycation. In commercial samples, ϵ -Fru-Lys ranged between 1.5 and 13.3 mmol/mol lysine. Pyrraline was found in amounts between not detectable (below 0.3 mmol/mol lysine) and 9.0 mmol/mol lysine, and CML between 0.8 and 2.7 mmol/mol lysine. Lysine modification by glycation products was very low in cooked and fried peanuts (below 1%). In laboratory-scale roasting experiments, the amount of ϵ -Fru-Lys reached maximum values of 12.0 mmol/mol lysine after 20 min at 160 °C, whereas pyrraline increased up to 38.5 mmol/mol lysine after 25 min at 170 °C. Amount of CML was up to 1.8 mmol/mol lysine in peanuts roasted for 25 min at 170 °C. Such high amounts of pyrraline have not yet been described for any other food item. Only about one tenth of the totally observed lysine modification of up to 50% can be explained by the three glycation compounds, indicating that currently unknown reactions occur during peanut roasting. Reactions between proteins and carbonyl compounds, most likely originating from oxidative degradation of lipids, may play an important role for lysine derivatization in peanuts and should be analyzed more in detail.

Keywords Peanuts · Maillard reaction · Furosine · *N*- ϵ -fructosyllysine · Pyrraline · *N*- ϵ -carboxymethyllysine (CML) · Lipid peroxidation

Abbreviations

MRP	Maillard reaction product
AP	Amadori product
AGE	Advanced glycation end product
CML	<i>N</i> - ϵ -carboxymethyllysine
ϵ -Fru-Lys	<i>N</i> - ϵ -fructosyllysine

Introduction

Peanuts (*Arachis hypogaea* L.) are a popular snack food and serve as ingredients for many different foods such as spreads or bakery products. Being a rich source of protein as well as phytosterols, tocopherols and monounsaturated fatty acids [1], peanuts are rarely consumed in raw form. In the US and Europe, peanuts generally are roasted, whereas in Asian countries, frying and cooking are the prevalent methods of preparing peanuts for consumption [2]. During heating, several chemical reactions occur. Besides lipid peroxidation, the Maillard reaction is of outstanding importance due to the high protein content of around 25% and the presence of reducing sugars [1, 3, 4]. Up to now, very little is known about the extent of the Maillard reaction in heat-treated peanuts. To the best of our knowledge, only Maleki et al. [5] and Chung et al. [6] had investigated the formation of Maillard reaction products (MRPs) during roasting of peanuts, using immunological methods, namely ELISA and Western Blot. Antibodies detecting the advanced glycation compound *N*- ϵ -carboxymethyllysine (CML) or “AGEs” were used. The term “AGE” (advanced glycation end product) stands for a large number of individual reaction products,

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A. Wellner · L. Nußpickel · T. Henle (✉)
Institute of Food Chemistry, Technische Universität Dresden,
01062 Dresden, Germany
e-mail: Thomas.Henle@chemie.tu-dresden.de

indicating unknown specificity of the used antibody. Increasing levels of MRPs correlating with intensity of heat treatment were found, but no information about the concentration of defined reaction products was given.

Besides the fact that the Maillard reaction contributes to the sensory quality of the final product, either negative as well as positive nutritional consequences should be taken into account [7]. Modification of essential amino acids such as lysine by carbohydrates in the course of the Maillard reaction can lead to a significant decrease in the nutritional value of proteins [8]. Furthermore, some MRPs are discussed to be mutagenic, carcinogenic or cytotoxic, while others show antioxidant properties [9]. In this context, an important fact is that glycation can affect allergenicity [9]. In principle, the allergenic potential can be decreased as well as increased due to heat treatment. Most food allergens, such as birch pollen-associated allergens in apples and carrots, are heat-labile. Due to protein denaturation, IgE-binding capacity is strongly reduced or even completely lost [10]. On the other hand, certain food allergens are extremely heat-stable, such as peanut allergens [11]. While cooking does not enhance the allergenic potential of peanuts [2, 12], an increase of IgE reactivity in roasted peanuts caused by formation of “neoepitopes” due to Maillard reaction is discussed [5], but underlying mechanisms are unclear. The formation of “neoallergens” has also been shown for roasted pecan nuts [13]. For glycated ovalbumin, an enhanced T-cell activation due to glycation has been demonstrated [14].

Thus, an analysis of the extent of the Maillard reaction and related amino acid modification in heat-treated peanuts is of particular importance. Here, we measured the amount of selected amino acid derivatives as indicators of various stages of the Maillard reaction, using chromatographic techniques. The Amadori compound *N*- ϵ -fructosyllysine was analyzed as furosine together with *N*- ϵ -carboxymethyllysine after acid hydrolysis. Pyrroline was quantified after enzymatic hydrolysis. For all samples, the extent of lysine derivatization was assessed. Following the analysis of a number of commercially available samples, model studies in order to obtain insights into the impact of heat treatment to the formation of MRPs in peanuts were performed.

Materials and methods

Chemicals and reference substances

Pepsin (EC 3.4.23.1), leucine aminopeptidase (EC 3.4.11.2), prolidase (EC 3.4.13.9), and trifluoroacetic anhydride were purchased from Sigma-Aldrich (Taufkirchen, Germany). Pronase E was obtained from Merck (Darmstadt, Germany). Sodium pentanesulfonate was from Chromatographie Handel

Müller (Fridolfing, Germany). Hydrochloric acid, 1 mol L⁻¹ in methanol, was obtained from Grüssing (Filsum, Germany). All chemicals were of the highest purity available.

Furosine dihydrochloride was purchased from PolyPeptide Laboratories (Strasbourg, France). Pyrroline was synthesized according to Henle and Bachmann [15]. Reference material for CML and CMO was synthesized according to the method of Delatour et al. [16].

Samples

Thirteen commercial peanut products were purchased from local retail stores. They consisted of two raw peanut samples, five roasted peanut samples, four samples of peanut butter, and two peanut puffs.

Heating experiments were performed as follows. Raw peanuts were shelled and boiled in water (100 °C) for 20 min, fried in peanut oil at 120 °C for 5 min (approximately 20 g peanuts in 500 mL oil), or dry-roasted at 170 °C for 20 min, respectively.

For roasting experiments, peanuts were roasted with shell at 150, 160 and 170 °C for 5, 10, 15, 20, and 25 min.

Sample preparation and general methods

Before analysis, all samples were ground and defatted by Soxhlet extraction with petroleum ether at 78 °C for 6 h. The protein content of the defatted peanut powders was determined by Kjeldahl method, using the specific factor 5.3 for oilseeds [3].

Quantification of furosine

Furosine was determined according to Resmini et al. [17] after acid hydrolysis with 8 mol L⁻¹ hydrochloric acid at 110 °C for 23 h, using 40 mg of protein in 2 mL hydrochloric acid. The hydrolyzates were subjected to solid-phase extraction with C-18 material (C18-Max, Grace Davison Discovery Sciences, delivered by Alltech Grom, Rottenburg-Hailfingen, Germany) according to [12]. Aliquots were dried under vacuum, dissolved in 0.2 mol L⁻¹ hydrochloric acid, and filtered (0.45 μ m). For quantification, an HPLC system (Knauer, Berlin, Germany) was used, consisting of a WellChrom pump K-1001, a gradient mixing system (solvent organizer K-1500 and dynamic mixing chamber K-5001), an automatic injector Basic Marathon, a column oven and a diode array detector WellChrom K-2700. For separation, an RP-8 column “furosine dedicated” (Grace Davison Discovery Sciences/Alltech, Sedriano MI, Italy) at 33 °C was used, and a gradient with 0.4% acetic acid (solvent A) and 0.3% KCl in 0.4% acetic acid (solvent B). Flow rate was 1.2 mL/min, and the following gradient was applied: 0% solvent B for 15.5 min, to 50% B in 7 min,

held at 50% B for 2.5 min, to 0% B in 2 min, held at 0% B for 7 min. For external calibration, furosine dihydrochloride was used.

Quantification of pyrraline

Pyrraline was determined after a three-step enzymatic hydrolysis using pepsin, pronase E, leucine aminopeptidase, and prolidase according to Förster et al. [18]. The HPLC system was as described above. The column used was a Eurospher 100-5 C18 column (125 × 4.6 mm, 5 μm, Knauer, Germany), eluted isocratically with 7.5 mol L⁻¹ sodium pentanesulfonate (pH 3.0) in 10% ethyl alcohol. For external calibration, pyrraline reference material synthesized according to Henle and Bachmann [15] was used in a concentration range from 1 to 12 nmol/mL.

Quantification of *N*-ε-carboxymethyllysine (CML)

CML was quantified by GC/MS according to Dunn et al. [19] with some modifications, as recently described [20]. Acid hydrolysis was accomplished with 6 mol L⁻¹ hydrochloric acid for 23 h at 110 °C under nitrogen to avoid degradation of *N*-ε-fructosyllsine to CML. CMO was used as internal standard.

Amino acid analysis

Amino acid composition was determined after acid hydrolysis with 6 mol L⁻¹ hydrochloric acid as described for CML analysis. Additionally, the enzymatically hydrolyzed samples, used for pyrraline analysis, were analyzed in comparison to the corresponding acid hydrolyzate to assess the efficiency of enzymatic digestion. Measurement was

accomplished by ion-exchange chromatography with post-column ninhydrin derivatization according to Henle et al. [21]. An amino acid analyzer S4300 (SYKAM, Fürstfeldbruck, Germany) was used.

Free amino acids were quantified after centrifugal ultrafiltration. For this purpose, aqueous extracts (10 g defatted peanut sample in 90 g water) were treated with Vivaspin 4 concentrators (MWCO 5,000 Da, Sartorius, Göttingen, Germany).

Results and discussion

Identification and quantification of MRPs

Amadori products of lysine were analyzed indirectly as furosine, which is formed during acid hydrolysis from *N*-ε-fructosyllsine. Figure 1 shows the chromatogram of a roasted peanut sample, which illustrates that furosine was identified in heat-treated peanuts by comparison of retention time with a commercially available reference substance, the characteristic UV spectrum (absorption maximum at 280 nm) and standard addition. The rate of conversion from *N*-ε-fructosyllsine to furosine depends on the conditions of acid hydrolysis, in particular concentration of hydrochloric acid, temperature, and duration. For hydrolysis with 8 mol L⁻¹ hydrochloric acid at 110 °C for 23 h, Krause et al. [22] had determined that 46.1% of the Amadori compound is transformed to furosine, which results in a conversion factor of 2.2. Using this conversion factor, the molar concentration of the Amadori product present in the sample before hydrolysis can be calculated from furosine data.

For calculation of the lysine modification, the respective lysine amounts were determined by amino acid analysis

Fig. 1 RP-HPLC with UV-detection ($\lambda = 280$ nm) for the determination of furosine in an acid hydrolyzate of a roasted peanut sample, containing 170 mg furosine/kg protein (black), compared with furosine reference material (gray). UV spectrum shows specific UV absorption

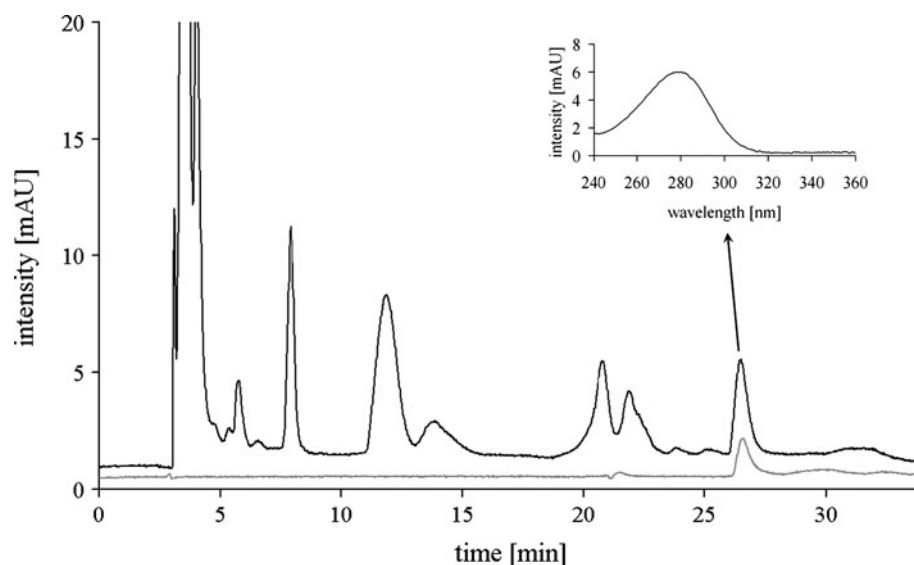
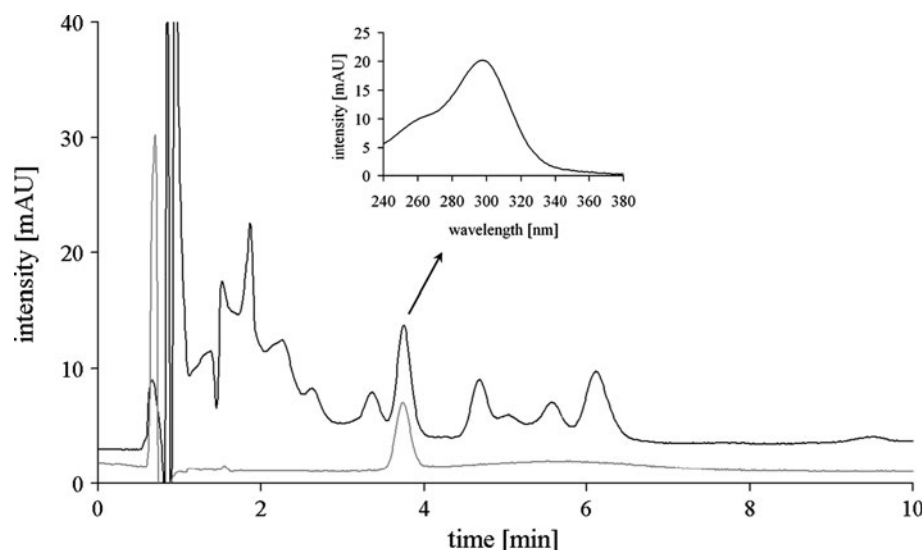


Fig. 2 RP-HPLC with UV-detection ($\lambda = 297$ nm) for the determination of pyrraline in an enzymatically hydrolyzed roasted peanut sample, containing 270 mg pyrraline/kg protein (black), compared with pyrraline reference material (gray). UV spectrum shows specific UV absorption of pyrraline



after acid hydrolysis. For this, it was taken into account that significant amounts of lysine are released from the Amadori product during acid hydrolysis. According to Krause et al. [22], 56.2% of *N*- ϵ -fructosyllysine is converted to lysine under the applied conditions. For calculation of unmodified lysine, the total lysine contents obtained after acid hydrolysis were corrected by the calculated amount of lysine formed from degradation of *N*- ϵ -fructosyllysine.

Pyrraline was analyzed after enzymatic hydrolysis and could be detected in roasted peanut samples (Fig. 2). Identification was done by UV spectroscopy (characteristic absorption maximum at 297 nm) as well as by comparison of retention time and standard addition using synthesized reference material. In order to assess completeness of the applied enzymatic hydrolysis, selected samples were submitted to acid and enzymatic hydrolysis, respectively. Compared with the complete acid hydrolysis, between 90 and 100% of aliphatic and hydrophobic amino acids such as leucine, isoleucine, and valine were measured after enzymatic hydrolysis (data see supplementary material). This indicates that the 3-step hydrolysis procedure, originally developed for milk proteins [23], is a suitable sample preparation method also for the determination of acid-labile glycation compounds in peanut proteins.

CML was determined by GC/MS after acid hydrolysis and derivatization. A reduction of the Amadori product before hydrolysis with sodium borohydride, as suggested by Dunn et al. [19], was not performed. Samples were hydrolyzed under nitrogen to avoid oxidative cleavage of the Amadori products and formation of CML caused by atmospheric oxygen. Hydrolyzates obtained after acid and enzymatic hydrolysis were compared, showing identical results. Since CML contents in the investigated peanut samples were near the detection limit of 40 mg/kg protein,

Table 1 Glycation compounds in acid hydrolyzates of commercial peanut samples

	<i>n</i>	Furosine (ϵ -Fru-Lys)	Pyrraline	CML
Peanut puffs	2	166–256 (8.1–13.3)	340–382 (7.6–9.0)	61–63 (1.7–1.8)
Peanut butters	4	73–91 (1.5–2.0)	268–357 (2.4–3.8)	63–203 (0.8–2.7)
Roasted peanuts	5	129–267 (4.6–9.7)	ND–361 (ND–6.8)	50–77 (1.0–1.6)
Unroasted peanuts	2	ND–24 (ND–0.8)	ND (ND)	ND (ND)

Data are given in mg/kg protein. In brackets (italicized) are the respective values given in mmol/mol lysine. For calculation of the content of *N*- ϵ -fructosyllysine (ϵ -Fru-Lys) from furosine, a conversion factor of 2.2 according to Krause et al. [22] was used. ND not detectable (below 20 mg/kg protein for furosine and pyrraline, and 40 mg/kg protein for CML)

possible formation of CML during acid hydrolysis was found to be insignificant.

MRPs in commercial peanut samples

The results obtained for MRPs in thirteen commercially available peanut samples are shown in Table 1. Data given for lysine modification are only a rough estimation, as the initial lysine content of the individual samples was unknown. Therefore, measured amount of unmodified lysine and of MRPs were summed up and taken as “total lysine”.

In the unroasted peanuts, the glycation compounds were not detectable or near the detection limit in case of *N*- ϵ -fructosyllysine. Compared to this, considerably higher amounts of MRPs were found in the roasted and processed

peanut samples, ranging from 73 to 267 mg/kg protein for *N*- ϵ -fructosyllysine, up to 382 mg/kg protein for pyrrole and up to 203 mg/kg protein for CML. Amounts were similar for all product groups. It is obvious that remarkably high amounts of pyrrole were measured, which were comparable or even higher compared with furosine. This is an observation that has not been reported for heated foods up to now. As a matter of principle, Amadori products such as *N*- ϵ -fructosyllysine were generally found as the predominant MRP [8]. Even in foods with high pyrrole contents, such as milk powders and bakery products, the concentrations of furosine were about tenfold higher [24, 25]. That phenomenon might be due to the composition of peanuts. Content of reducing sugars is very low (around 1%), compared with a large amount of protein [4]. Additionally, the relatively severe heating conditions with temperatures around 140–180 °C might induce degradation of Amadori products to advanced glycation compounds. In roasted peanuts, therefore, the advanced stages of the Maillard reaction predominate.

Heat treatment of peanut samples

To study the impact of various heat treatments on the formation of glycation compounds, raw peanuts were subjected to boiling, frying in peanut oil, and dry roasting. For calculation of lysine modification, the sum of lysine and measured lysine derivatives in the raw sample after acid hydrolysis was taken as initial lysine content. Lysine modification in all heated samples was calculated taking the amount of lysine into account, which is formed from *N*- ϵ -fructosyllysine during acid hydrolysis [22].

Small amounts of *N*- ϵ -fructosyllysine of around 4.7 mmol/mol lysine were detectable in the raw peanut sample, increasing to 6.5 mmol/mol lysine during boiling, 7.4 mmol/mol lysine during frying, and 9.8 mmol/mol lysine during dry roasting. It is obvious that the heated samples differed only marginally in their AP contents. CML was not detectable in raw and boiled peanuts, and with more drastic heating conditions up to 1.4 mmol/mol lysine were found. Pyrrole was only formed after intense heating, and resulting lysine blockage was up to 0.7%. Taken together, lysine modification due to the measured MRPs was the highest in dry-roasted peanuts with 1.8% in total, which was expectable since the highest temperature was applied.

Roasting experiments

Raw peanuts were dry-roasted without shell at temperatures between 150 and 170 °C for 5–25 min and were investigated for the formation of *N*- ϵ -fructosyllysine, pyrrole and CML, and the total lysine modification (Fig. 3).

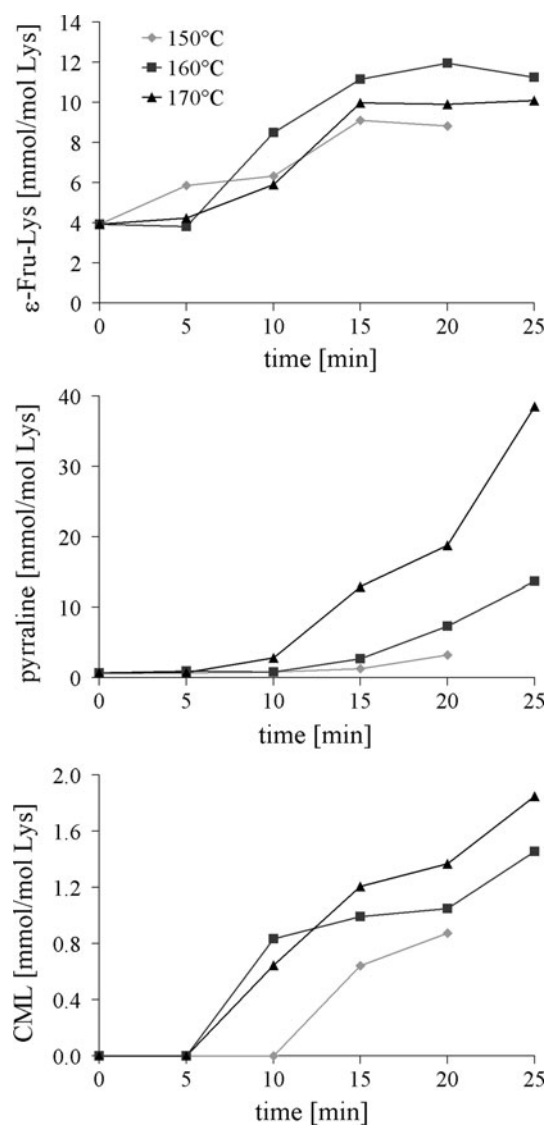


Fig. 3 Formation of MRPs during roasting of peanuts

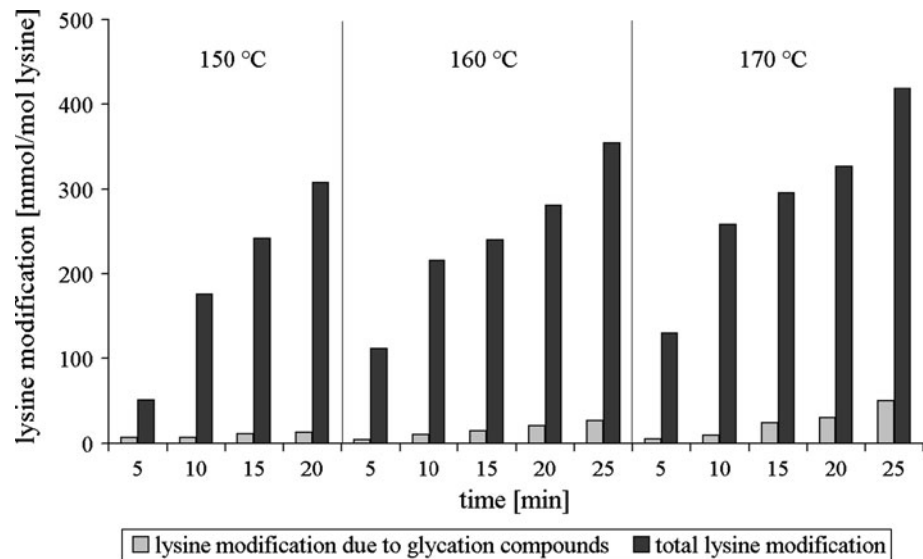
During roasting, a maximum lysine modification by *N*- ϵ -fructosyllysine of 1.2% was already obtained after 20 min at 160 °C, which did not increase further with higher temperature or longer heating time. Comparable AP contents were determined in the commercial peanut samples.

The amount of pyrrole increased continuously up to 3,330 mg/kg protein with increasing temperature and longer heating time, corresponding to 3.9% lysine modification. As already noted above, significantly higher amounts of pyrrole compared with *N*- ϵ -fructosyllysine were induced by roasting. It should be noted that certain temperature/time combinations performed during the roasting experiments led to products comparable in subjectively assessed browning with commercial peanut products (10 and 15 min at 170 °C, Fig. 4). For these samples, 0.1–0.4% lysine modification due to pyrrole was measured, indicat-

Fig. 4 Peanuts roasted at 170 °C for 5, 10, 15, 20, and 25 min



Fig. 5 Total lysine modification in roasted peanuts compared with the lysine modification due to the glycation compounds ϵ -Fru-Lys, pyrroline, and CML



ing that the roasting experiments gave a good simulation of technologically relevant conditions. Too harsh conditions caused strong browning (20 and 25 min at 170 °C, Fig. 4), which was associated with a bitter taste.

CML amounts also increased with higher temperature and longer roasting time, while the maximum lysine derivatization due to CML was only 0.2% after 25 min at 170 °C. In the commercial samples of roasted peanuts, comparable CML concentrations were measured. Compared with pyrroline, CML seems to play a minor role during glycation in peanuts.

In addition to the individual glycation compounds, the total lysine modification, related to the initial lysine content in raw peanuts, was determined. This total lysine modification was compared with the lysine modification originating in summation of *N*- ϵ -fructosyllysine, pyrroline, and CML. As can be seen from Fig. 5, the total lysine derivatization is up to tenfold higher compared with the lysine modification due to the three glycation compounds. This clearly points to the fact that further lysine-derived glycation compounds must have been formed to a very large extent, such as crosslink amino acids or high-molecular melanoidins. Furthermore, besides the relatively small amount of reducing sugars, carbonyl compounds resulting from lipid peroxidation reactions might play a key role for lysine modification during roasting of peanuts. The content of fat is very high (around 50%) in peanut seeds, and 85% of the fatty acids are unsaturated [1]. At present, there is only very limited knowledge about the role of lipid peroxidation products as

reaction partners for peptide-bound lysine. Studies using model systems have shown considerable losses of several amino acids due to modification by fatty acid-derived carbonyl compounds. As an example, dry incubation of casein with peroxidized ethyl linoleate induced derivatization of several amino acids such as lysine, arginine, glycine, isoleucine, and alanine of up to 50% [26]. A significant decrease in lysine, tryptophan, methionine, and cysteine content in whey proteins, caused by peroxidized methyl linolenate, was observed by Nielsen et al. [27]. Although individual lysine derivatives resulting from the reaction of the ϵ -amino group and fatty acid-derived carbonyl compounds have been identified in model systems [26, 28], up to now nothing is known about their formation in roasted peanuts or in any other food item. In addition, derivatization of lysine (and other amino acids) may also occur due to nucleophilic addition of the epsilon-amino group to dehydroalanine or dehydromethylalanine, which is formed via beta-elimination from serine and cysteine or threonine, respectively. From a quantitative point of view, however, corresponding reactions should be of minor importance [29, 30].

In conclusion, roasting of peanuts induces substantial lysine modification, which can only partially be explained by known glycation compounds. We assume that reactions between proteins and carbonyl compounds originating from oxidative degradation of lipids may play an important role for lysine derivatization in peanuts and should be analyzed more in detail. Such currently unknown pathways of protein modification may play a vital role for the physiological

properties of the final product, in particular with respect to peanut allergy.

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