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FORMATION OF VALUABLE MAILLARD FLAVOUR COMPOUNDS BY MODEL REACTIONS AND FERMENTATION

VORMING VAN WAARDEVOLLE MAILLARD-AROMACOMPONENTEN DOOR MODELREACTIES EN FERMENTATIE

door

ir. An ADAMS

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The author,

ir. An Adams

The promoter,

Prof. dr. ir. Norbert De Kimpe

We dance round in a ring and suppose,

but the Secret sits in the middle and knows.

Robert Frost

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Bedankt!

An Adams

Gent, april 2005

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ABBREVIATIONS

2-AP	2-acetyl-1-pyrroline
6-ATHP	6-acetyl-1,2,3,4-tetrahydropyridine
ASA	L-(+)-ascorbic acid
ATCC	American Type Culture Collection
COST	European cooperation in the field of scientific and technical research
DHA	1,3-dihydroxyacetone
DSM(Z)	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DVB/Car/PDMS	divinylbenzene/carboxen/polydimethylsiloxane
Glc	D-glucose
Glu	L-glutamic acid
Gly	glycine
HMW	high molecular weight
IMW	intermediate molecular weight
IS	internal standard
LMW	low molecular weight
LN	Likens-Nickerson extraction
MWCO	molecular weight cut off
PC	principal component
PCA	standard plate count agar
Pro	L-proline
pyrrolizine	5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine
pyrrolizine-OH	5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine
RF	response factor
RI	retention index
RSD	relative standard deviation
SDSE	steam distillation – solvent extraction
SIM	selected ion monitoring
SPME	solid phase microextraction
TCA	trichloroacetic acid
YMPG	yeast extract malt extract peptone glucose broth

GENERAL INTRODUCTION

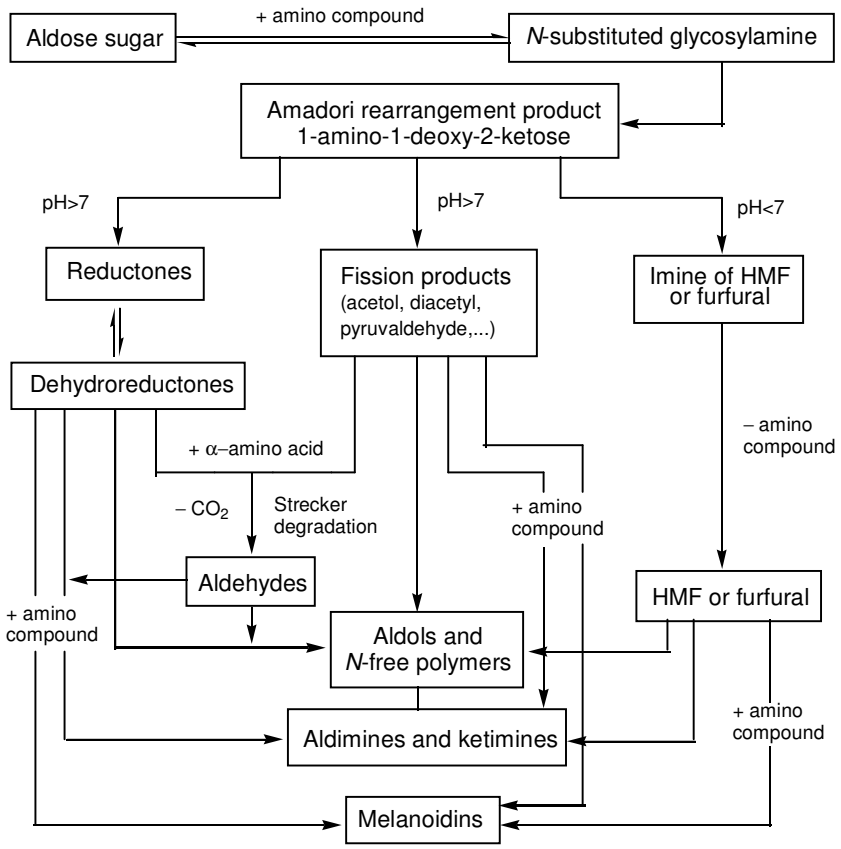
1 The Maillard reaction

Whenever food is cooked, baked, roasted or fried, the development of a unique aroma and an agreeable brown colour determines its value and consumer acceptability. Louis-Camille Maillard (1912) was the first to report on the development of a yellow-brown colour on gentle heating of sugars and amino acids in water.[1] In fact, this non-enzymatic browning comprises a complex network of reactions, initiated when a free amino group of an amino acid, a protein or an amine reacts with the carbonyl group of a reducing carbohydrate. The reaction was named the Maillard reaction, and it has since been studied in a large number of investigations, covering its many outcomes and implications.

The products of the Maillard reaction are numerous, and include high molecular weight brown pigments, known as melanoidins, and a whole range of potent aroma volatiles and flavouring substances. The development of colour and flavour in stored food products is not always desired and the Maillard reaction has been associated with losses of essential amino acids, with a decrease of digestibility and with the formation of toxic and potentially mutagenic compounds. Nevertheless, Maillard reaction products possess a well-established antioxidant activity, and some reports of antimutagenic activity have been published. Either way, the Maillard reaction is of vital importance for food quality, for which flavour is one of the most straightforward parameters.

The first coherent reaction scheme was put forward by Hodge in 1953 (Scheme 1).[2] It is initiated by the condensation reaction of a reducing sugar with a compound possessing a free amino group, leading to the formation of an *N*-substituted glycosylamine that rearranges to the corresponding 1-amino-1-deoxy-2-ketose (Amadori rearrangement). The subsequent degradation of the Amadori compound is highly dependent on the pH of the system. At low

pH, 1,2-enolization predominates, resulting in the formation of furfural (from pentoses) or 5-hydroxymethylfurfural (HMF) (from hexoses). At higher pH, 2,3-enolization occurs, with the formation of reductones and a variety of fission products such as highly reactive α-dicarbonyl compounds. In fact, all three pathways occur in food with the pH of the system influencing the ratio of products formed. Many side reactions lead to the formation of low molecular weight compounds, but due to the high reactivity of the intermediates, a complex polymerization starts, ending in brown high molecular weight melanoidins.



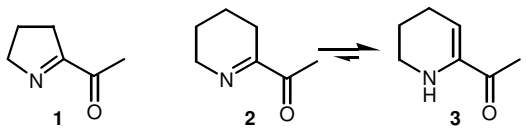
Scheme 1. Scheme of the Maillard reaction, adapted from Hodge [2]

This scheme is, of course, just a summary of the reactions that take place. Years of research have been devoted to the unravelling of the complexity and diversity of the Maillard reaction. New reaction pathways, not accounted for by Hodge, were discovered, and the relative

importance of the different intermediates was reconsidered.[3] For example, Namiki has shown that a free radical route operates, especially at high pH.[4] In the scheme presented by Hodge, the Amadori rearrangement is a key step in the reaction progress. Comparable to aldose sugars, ketose sugars undergo the Heyns rearrangement with the formation of 2-amino-2-deoxyaldoses. Later studies, however, questioned the central importance of the Amadori rearrangement product.[3] The deoxyhexosuloses (3-deoxyaldoketose, 1-deoxy-2,3-diketose and 4-deoxy-2,3-diketose) have also been established as key intermediates.[5] Despite the numerous studies devoted to it, the Maillard reaction remains a controversial issue.

2 Aim and outline of the thesis

Flavour is one of the most important characteristics of a food product. In processed food, the Maillard reaction is a major contributor to flavour formation. Carbohydrate fragments, formed mainly by retro-aldol reactions, react with amino acid residues resulting in the formation of a wide variety of heterocyclic flavour compounds. During years of research, a vast quantity of Maillard flavour compounds has been identified, and among these, some very potent flavours were discovered. 2-Acetyl-1-pyrroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** (which occurs in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine **2**) (Scheme 2) are the most important flavour compounds of cooked rice and bread, respectively, and their flavour significance has been shown in many other food products. *Chapter 1* of this thesis contains a literature overview of the chemistry of these important flavour compounds, of their occurrence and significance, the mechanisms of formation, their biological origin, the developed synthetic routes, and their applications in the food industry.



Scheme 2. 2-Acetyl-1-pyrroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** (in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine **2**)

Chapter 2 presents an extensive study of one of the most important model reactions for the formation of the bread flavour compound 6-acetyl-1,2,3,4-tetrahydropyridine **3**. The reaction of proline with 1,3-dihydroxyacetone, a carbohydrate degradation product, has been reported to yield an intense bread-like flavour, which was ascribed to 6-acetyl-1,2,3,4-tetrahydro-pyridine. This reaction is, however, poorly described in the literature and was therefore subjected to a thorough analysis. Side products were identified and the importance of the reagents and reaction conditions was investigated in order to realize an optimal and reproducible bread flavour formation. In the light of the results, the existing hypothetical reaction mechanisms were evaluated.

The biological formation of 2-acetyl-1-pyrroline **1** is the subject of *Chapter 3*. The production of flavour compounds by fermentation creates the possibility of developing a 'bioflavour', which is highly favoured over the synthetic counterpart in food products, mainly due to the consumer's preference for natural products. In the literature, the production of 2-acetyl-1-pyrroline by *Bacillus cereus* strains has been described. This fermentation was further investigated by evaluating the influence of different precursors on the production of 2-acetyl-1-pyrroline and of alkylpyrazine flavour compounds.

To complete this study on the flavour implications of the Maillard reaction, the impact of melanoidins on the aroma profile of food was evaluated. These intriguing brown polymeric Maillard reaction products are poorly defined in terms of structure and chemical properties, although they display a variety of functional properties in everyday food products. Heating of

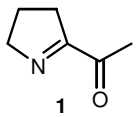
these dietary compounds leads to the formation of volatiles that contribute to aroma formation. In addition, the generation of low molecular compounds from the melanoidin structure yields information on the structural domains present in the original polymer, when carefully interpreted. *Chapter 4* therefore offers a systematic study and comparison of the thermal degradation of various model melanoidins and of melanoidins isolated from bread crust, coffee and tomato. To optimize the organoleptic qualities of a food system, both melanoidins and flavour compounds are important. The potential of melanoidins to selectively bind flavour compounds influences the aroma profile of food products. Accordingly, *Chapter 4* closes with a study of the interaction of food-derived melanoidins with some model flavour compounds.

Finally, in *Chapter 5*, the main findings of this research are summarized and a comprehensive conclusion is drawn.

1 2-ACETYL-1-PYRROLINE AND 6-ACETYL-1,2,3,4-TETRAHYDRO-PYRIDINE, TWO OF THE MOST IMPORTANT MAILLARD FLAVOUR COMPOUNDS

1.1 Occurrence and significance

1.1.1 Sources of 2-acetyl-1-pyrroline



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Scheme 1.1. 2-Acetyl-1-pyrroline **1**, the most important flavour compound of cooked rice

2-Acetyl-1-pyrroline **1** [2-AP; IUPAC name 5-acetyl-3,4-dihydro-2*H*-pyrrole; also 1-(3,4-dihydro-2*H*-pyrrol-5-yl)ethanone] was identified for the first time as the most important flavour compound of cooked rice (Scheme 1.1).[6] Since then, the flavour compound has been identified in a great variety of processed and cooked food products, especially in various rice varieties, both fragrant and non-fragrant varieties.[7] The content of 2-AP was shown to be positively correlated with desirable rice flavour characteristics.[8] 2-Acetyl-1-pyrroline appeared to be the most important discriminator, on quantitative and olfactive considerations, to differentiate between scented and non-scented rice.[9] Besides in cooked rice, 2-AP has also been identified among the volatiles of various other cooked cereals and cereal products: bread crust,[10] toasted bread,[11] corn tortillas,[12] popcorn,[13] cooked sweet corn products,[14] extrusion cooked maize flour,[15] rice cakes,[16] puff-pastries,[17] and cooked acha (a cereal indigenous to the Sahel region in Africa).[18] In all cases, 2-AP, though mostly present in low concentrations, contributes in great measure to the cereal odour-notes of the food products. 2-Acetyl-1-pyrroline was shown to be a potent odorant of boiled potatoes,[19] is one of the key aroma compounds in roasted wild mango seeds,[20] roasted sesame seeds,[21] pan-fired green teas,[22]

cured tobacco leaves,[23] boiled or fried mung bean,[24] taro (a tropical root crop),[25] and it is formed during the heating of bacuri and cupuaçu fruit pulp.[26,27] It was detected in pale lager beer,[28] in peanut and pumpkin seed oils,[29] honey,[30] and in several soy-based products.[31] Also products of non-vegetable origin contained 2-AP as a highly aroma-active constituent: dry and fresh milk, [32,33] Camembert and Swiss Gruyere cheese,[34,35] rennet casein,[36] liquid Cheddar whey,[37] Iberian dry-cured ham,[38,39] Mediterranean dried sausages,[40] cooked beef,[41] boiled carp fillet,[42] boiled salmon, cod and trout,[43,44] cooked blue crab claw meat,[45] cooked lobster tail meat,[46,47] boiled prawns,[48] ripened anchovy,[49] crayfish-processing by-products,[50] tuna sauce,[51] stored sardines,[52] and enzyme-hydrolysed oyster cooker effluent.[53]

These findings led to the assumption that 2-AP is a resultant of the Maillard reaction, and that it is formed during the cooking or processing of rice and other food products. This thermal formation, together with the instability of the compound, complicated the development of adequate analytical procedures. The determination of important, but low-concentrated volatiles depends greatly on the development of sensitive analytical techniques. In order to obtain truthful quantitative determinations of 2-AP concentrations in food products, care must be taken to avoid losses due to the instability of the compound on the one hand, and not to overestimate the 2-AP quantities by additional formation upon heat treatment of precursors on the other hand.

Buttery and co-workers determined the 2-AP concentration in 10 different rice varieties.[7a] This was done by steam distillation − continuous extraction of uncooked rice and resulted in concentrations of 6 ppb to 90 ppb, expressed relative to the dry weight of the rice. Within the same variety, brown rice contained somewhat higher 2-AP concentrations than white rice, indicating that the degree of milling influences the 2-AP content. Using an acid-phase solvent extraction, the effect of other interfering compounds could be lessened.[7b] Still, a recovery of only 28 % 2-AP was reached. Taking this into account and making use of an internal

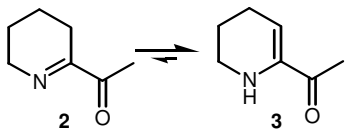
standard, 2-AP concentrations for brown aromatic rice varieties of 560-760 ppb were calculated. Different research groups refined the analytical method for the determination of 2-AP. Following extraction of 2-AP from rice by microscale steam distillation – solvent extraction, GC-MS analysis was performed in the more sensitive SIM (Selected Ion Monitoring) mode. This led to experimental concentrations of 76-156 ppb of 2-AP in rice.[7e] Solid Phase Microextraction (SPME) was used for the screening of several rice varieties for the presence of 2-AP. Solid Phase Microextraction followed by GC-MS was proven to be a very useful technique for the screening and qualitative differentiation among rice varieties, but not for the quantification of 2-AP.[7h] Schieberle and co-workers developed stable isotope dilution assays for the quantification of 2-AP in bread crust [10] and in freshly popped corn.[54] In wheat bread crust, the concentration of 2-AP was determined to be 78 µg/kg, while in freshly popped corn 24 µg 2-AP/kg was found. In other heated food products, 2-AP concentrations in the range of 1-10 ppb were found.[12,14,16,42] Calculation of the so-called flavour dilution factor by aroma extract dilution analysis in different food products revealed the extraordinary importance of 2-AP in the aroma of a variety of food products.[7i,42,46]

In later investigations, however, 2-acetyl-1-pyrroline also revealed its presence in raw plant material. Buttery and co-workers already noticed that the characteristic 2-AP odour was present in uncooked rice, but they could not confirm this by extraction.[7a] Mahatheeranont et al. quantified 2-AP in uncooked Thai rice, using a non-thermal solvent extraction method.[55] Concentrations of 2-AP in fresh uncooked brown aromatic rice were found to be as high as 3 ppm.[56] Yoshihashi used a stable isotope dilution assay to quantify 2-AP in aromatic rice under different conditions and in various parts of the plant.[57] The results revealed that 2-AP was not formed during the cooking or postharvest processes of aromatic rice, and confirmed the biological origin of 2-AP.

Also in Pandan leaves (*Pandanus amaryllifolius*),[58] raw Myabi muskmelon fruit,[59] and in chempedak fruit and jackfruit,[60] 2-AP was identified. 2-Acetyl-1-pyrroline was detected among the volatiles of dry spinach [61] and raw French beans.[62] In both cases, the concentration of 2-AP and as a result its importance in the overall aroma increased upon cooking of the vegetables. The dried flowers of the plant *Vallaris glabra* (so-called 'bread flowers') contain the highest reported concentrations of 2-acetyl-1-pyrroline, namely 26 ppm.[63] In Pandan leaves, which are traditionally added during cooking of common rice to impart a scented rice aroma, 2-AP is present at a concentration of 1 ppm, which is more than 10 times higher than the 2-AP concentration in scented rice varieties.[58]

In contrast to its mostly desired presence, 2-AP was shown to be responsible for a 'mousy' off-flavour that developed when raw pearl millet grits were wetted and slowly dried.[64] 2-Acetyl-1-pyrroline was also identified as one of the components responsible for the development of a 'mousy' off-flavour in wine.[65] Its presence there was shown to be of microbial origin and will be discussed later (Chapter 1.3).

1.1.2 Sources of 6-acetyl-1,2,3,4-tetrahydropyridine



Scheme 1.2. The bread flavour compound 6-acetyl-1,2,3,4-tetrahydropyridine **3** (occurring in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine **2**)

Already in 1963, Wiseblatt and Zoumut isolated a substance with a bread-like flavour after boiling of fermented liquid brews, containing only glucose and yeast in an inorganic aqueous buffer solution.[66] A structure identification was, however, only published in 1969, when

Hunter et al.[67] isolated the bread flavour compound from a modified Wiseblatt reaction of proline with 1,3-dihydroxyacetone in the presence of sodium bisulfite, and identified it as 6-acetyl-1,2,3,4-tetrahydropyridine **3** (occurring in tautomeric equilibrium with 6-acetyl-2,3,4,5-tetrahydropyridine **2**) (Scheme 1.2). Regular mistakes are found concerning the name of this compound. According to the IUPAC nomenclature rules, compound **2** is named 1-(3,4,5,6-tetrahydropyridin-2-yl)ethanone and compound **3** is named 1-(1,4,5,6-tetrahydropyridin-2-yl)ethanone.

6-Acetyl-1,2,3,4-tetrahydropyridine has a typical roasty odour, resembling the flavour of crackers and popcorn, and is regarded nowadays as a very important Maillard flavour compound. Spraying week-old bread with an aqueous solution containing only 6 ppm of the sodium bisulfite complex of this compound returned a desirable fresh-bread odour to the product.[67] 6-Acetyl-1,2,3,4-tetrahydropyridine (6-ATHP) contributes to the aroma of several baked products: potato chips,[68] bread crust,[69] popcorn,[70] corn tortillas,[71] toast,[11] and rice cakes.[72] Both 2-AP and 6-ATHP contribute significantly to the flavour of bread crust, although 2-AP has the highest odour unit in wheat bread crust, and 6-ATHP dominates in rye bread crust.[69] Both flavour compounds are found in breadcrumb in 30-fold lower concentrations than in the crust. This is due to the lower water activity in the outside crust, stimulating Maillard reactions. 6-Acetyl-1,2,3,4-tetrahydropyridine was also detected in wort and beer, food products containing high concentrations of proline.[73]

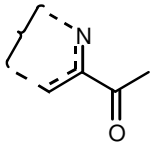
In Table 1.1, a comparison is presented of the concentrations and odour activity values of 2-AP and 6-ATHP in various food products.

Table 1.1. Concentrations (conc.) and odour activity values (OAV) of 2-acetyl-1-pyrroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** in different food products [10,11,13]

	2-AP		6-ATHP	
Food product	conc. (µg/kg)	OAV	conc. (µg/kg)	OAV
Wheat bread crust	19	2,602	53	981
Popcorn	24	3,288	437	8,092
Toasted wheat bread	8.8	1,205	1.5	28
Roasted sesame	30	4,110		
Basmati rice	610	83,516		
Cooked sweet corn	44	6,027		

1.1.3 Flavour properties

Based on their organoleptic properties, α-acetyl-*N*-heterocycles are considered a separate class of Maillard reaction products.[74] They generally have low to very low odour thresholds and a pleasant roasty cracker-like odour, which is attributed to the α-iminoketone or α-acyl-enamine structural element as part of a ring system (Scheme 1.3).[75,76]



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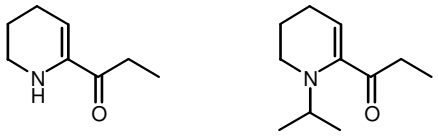
Scheme 1.3. Structural requirements for roasty, cracker-like flavour characteristics [75,76]

This structural requirement also applies to acyclic α-iminoketones but not to *N*-alkylated α-acetyl cyclic enamines, which do not show the roasty flavour characteristics at all.[77] For instance, 6-propionyl-1,2,3,4-tetrahydropyridine **5** displays Maillard flavour characteristics, while 1-isopropyl-6-propionyl-1,2,3,4-tetrahydropyridine **6** does not (Scheme 1.4).[77]



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Scheme 1.4. 6-Propionyl-1,2,3,4-tetrahydropyridine **5**, a compound with Maillard flavour characteristics, and 1-isopropyl-6-propionyl-1,2,3,4-tetrahydropyridine **6**, without Maillard flavour characteristics

Because of these properties, and since α-acetyl-*N*-heterocycles are generally formed in higher concentrations than other acetyl- or acyl-*N*-heterocycles, they are of utmost importance in the flavour of heated food products and in process flavourings.

Within this group of flavour compounds, many publications have focused on 2-AP **1** and 6-ATHP **3**, because of their significance and extremely low odour thresholds. These odour thresholds were determined in different media, as is reported in Table 1.2.

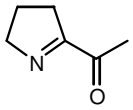
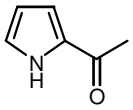
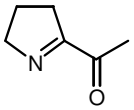
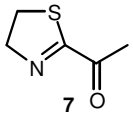
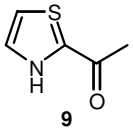
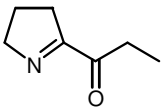
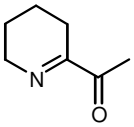
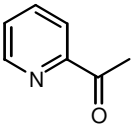
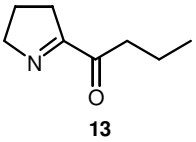
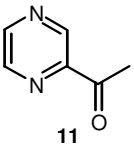
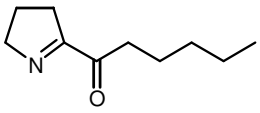
Table 1.2. Odour thresholds of 2-acetyl-1-pyrroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** in different media

	Odour threshold (μg/kg)	
	2-AP	6-ATHP
Air	0.02 ng/l [13]	0.06 ng/l [13]
Water	0.1 [7]	1.0 [71] / 1.6 [11]
Starch	0.0073 [11]	0.054 [11]
Sunflower oil	0.1 [34]	-

The presence of a longer alkyl chain, or an aromatic ring system, significantly increases the odour threshold of comparable α-acetyl-*N*-heterocycles (Table 1.3). This is clearly demonstrated in the odour threshold of 2-acetyl-1*H*-pyrrole **8**, which does not smell roasty and has an odour threshold of > 2,000 ng/l air. 2-Propionyl-1-pyrroline **12**, with an alkyl chain of one carbon atom more than 2-acetyl-1-pyrroline **1**, has a low odour threshold similar to

2-acetyl-1-pyrroline **1** and a roasty, popcorn-like odour. Higher homologues of 2-propionyl-1-pyrroline **12**, however, such as 2-butanoyl-1-pyrroline **13** and 2-hexanoyl-1-pyrroline **14**, do not smell roasty and possess very high odour thresholds.[13]

Table 1.3. Odour threshold values of different α-acetyl-*N*-heterocycles [13,78]

Flavour compound	Odour threshold (µg/l H_2O)	Aromatic flavour compound	Odour threshold (µg/l H_2O)	2-Alkanoyl-1-pyrroline homologues	Odour threshold (ng/l air)
 **1**	0.1	 **8**	170,000	 **1**	0.02
 **7**	1.3	 **9**	10	 **12**	0.02
 **3**	1.6	 **10**	19	 **13**	> 2,000
		 **11**	62	 **14**	> 2,000

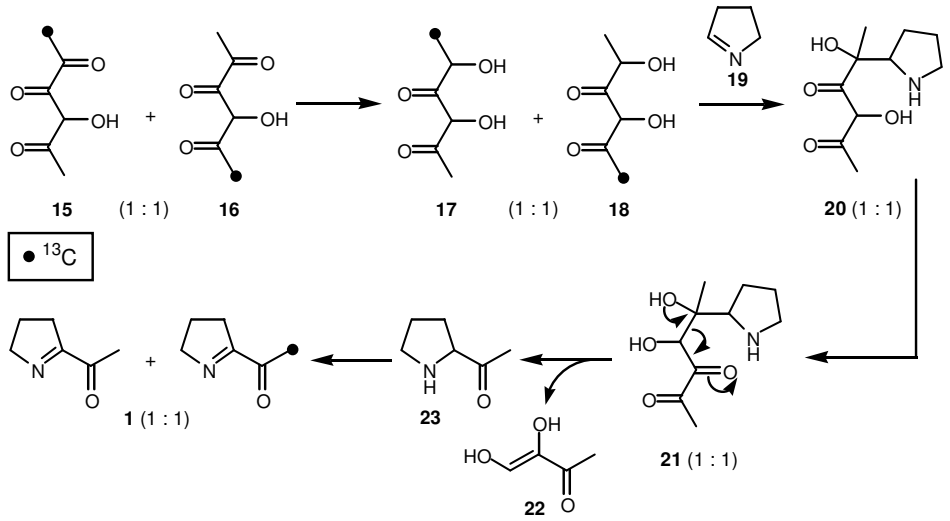
1.2 Mechanism of formation

1.2.1 Studies of the mechanism of formation of 2-acetyl-1-pyrroline

Tressl et al. reported that small amounts of 2-AP were formed when model mixtures of proline and monosacccharides were heated.[79] Schieberle showed that heat treatment of a ground yeast/sucrose mixture was an important source of 2-AP formed during the bread baking process.[80] Precursor studies showed the formation of 2-AP in heated model systems of proline with sugars, sugar degradation products and especially their phosphorylated

derivatives. Conversion of the latter activated derivatives into higher amounts of 2-AP holds a mechanistic rationale (*vide infra*).

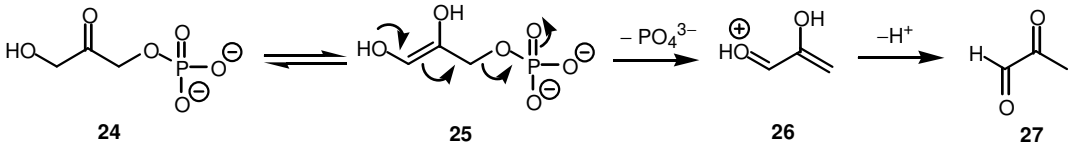
Experiments with labelled carbohydrates indicated that in 2-AP **1**, generated from proline and U-^{13}C-glucose, the label was present only in both carbons of the acetyl group.[79] From the reaction of proline and 1-^{13}C-glucose, Rewicki et al. detected a 1:1 mixture of unlabelled and singly labelled 2-AP (with the label on the methyl group).[81] They proposed a mechanism as is displayed in Scheme 1.5. From 1-deoxy-2,3-glucosone, two isomers of 'diacetyl-formoin' (**15** and **16**) are derived with two equivalent sites of reactivity. Aldol addition of 1-pyrroline **19**, a degradation product of proline, to the dihydro-form of diacetyl-formoin (**17** and **18**) is followed by retro-aldol cleavage, yielding 2-acetylpyrrolidine **23** that oxidizes to 2-acetyl-1-pyrroline **1** with the expected extent and position of labelling.



Scheme 1.5. Formation of a 1:1 mixture of 2-acetyl-1-pyrroline/2-[2-^{13}C]acetyl-1-pyrroline from [1-^{13}C]-glucose and L-proline [81]

Labelling of proline demonstrated that the carbon of the carboxyl group of proline was absent in 2-AP.[79] These findings also support the hypothesis that 2-AP in thermally degraded proline/glucose mixtures is formed by 'acylation' of 1-pyrroline by a two-carbon sugar fragment, among which 2-oxoaldehydes seemed to be the most effective.

Various model experiments revealed that phosphate ions are essential in the realization of high yields, since the replacement of a phosphate buffer by a malonate buffer decreased the yields of model reactions to about one-third.[54] Triose phosphates occurring in yeast have been identified as 2-AP precursors.[80] The high yield obtained from the reaction of 1,3-dihydroxyacetone phosphate **24** with proline [80] can be explained by the generation of 1,2-propanedione **27**, which is the actual active sugar fragment in the formation of 2-AP **1** (Scheme 1.6). The enol 1,3-dihydroxyacetone phosphate, i.e. compound **25**, is able to expel phosphate as a leaving group much better than the analogous hydroxyl group of 1,3-dihydroxyacetone. In this way, the protonated enol structure **26** is generated which affords the reactive sugar degradation compound **27**.

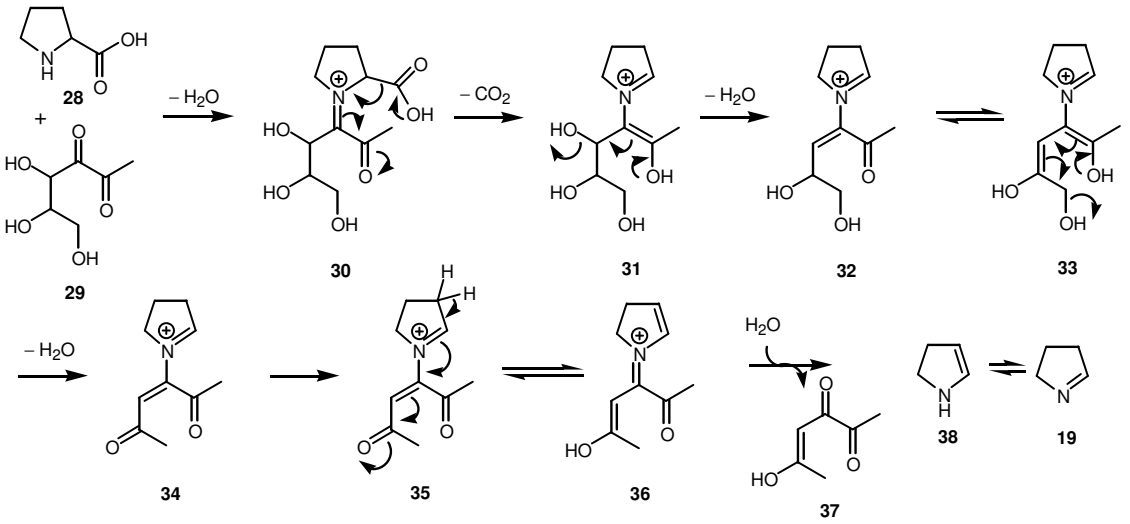


Scheme 1.6. Generation of 1,2-propanedione **27** from dihydroxyacetone phosphate **24**

When reacting equimolar amounts of 1-pyrroline **19** and 1,2-propanedione **27** in an aqueous buffer solution, yields of 2-AP **1** of 5 mol % were accomplished.[84] 2-Acetyl-1-pyrroline constituted 72 % of the volatile fraction in 1-pyrroline/1,2-propanedione model reactions.[79] The reaction of 1-pyrroline **19** with two other α-oxoaldehydes, namely 1,2-butanedione and phenylglyoxal, yielded 2-propionyl-1-pyrroline **12** and 2-benzoyl-1-pyrroline in yields comparable with the formation of 2-AP **1** from 1,2-propanedione **27**.[54] These results confirm that 1-pyrroline **19** and 1,2-propanedione **27** are most probably the active reagents in the formation of 2-AP **1**.

To determine the origin of 1-pyrroline in bread crust, Schieberle and co-workers separately reacted the most important amino acids of yeast with 1,2-propanedione in model experiments.[82] The results revealed that 2-AP was formed from proline and from ornithine in

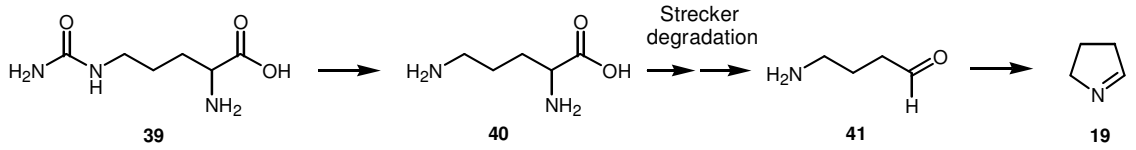
comparable amounts.[82] Both amino acids are able to form 1-pyrroline **19**, the key intermediate in the formation of 2-AP **1**. 1-Pyrroline **19** results from the Strecker degradation of proline **28**, catalyzed by α-dicarbonyl compounds, such as 1,2-propanedione **27** or a deoxyosone, e.g. 1-deoxyosone **29**, an intermediate generated from the dehydration of fructose (Scheme 1.7).[54] The reaction starts with the formation of iminium ion **30**, followed by decarboxylation and water elimination leading to intermediate **32**. A second water molecule is eliminated in a retro-Michael reaction yielding intermediate **34**, from which 1-pyrroline **19** can be generated via hydrolysis of iminium ion **36**.



Scheme 1.7. Hypothetical reaction pathway leading from proline **28** and 1-deoxyosone **29** to 1-pyrroline **19** [54]

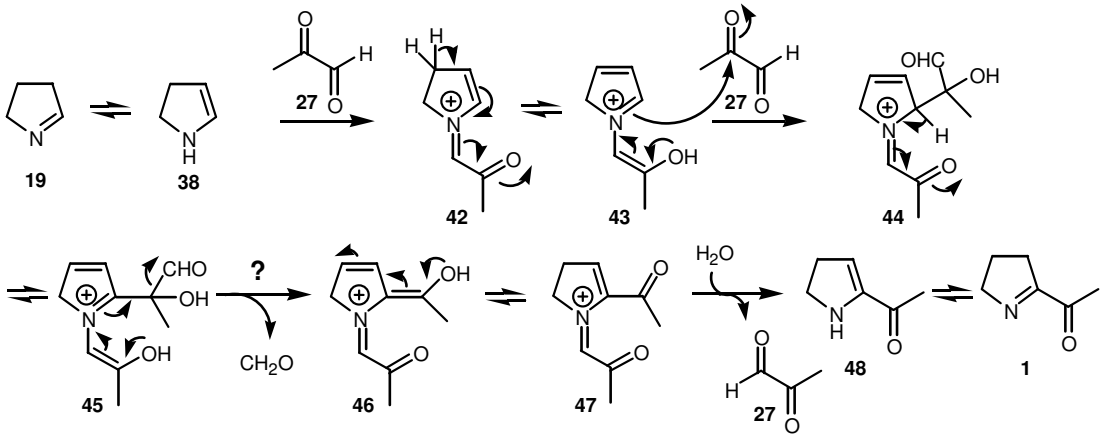
When the nucleophilic attack of proline **28** is aimed at the carbonyl group of carbon-2 of 1-deoxyosone **29** instead of carbon-3, a similar pathway can be constructed for the formation of 1-pyrroline **19**.

Citrulline **39** and ornithine **40**, however, are also possible precursors of 1-pyrroline **19**, through cyclization of 4-aminobutanal **41** via a Strecker degradation protocol (Scheme 1.8).[82]



Scheme 1.8. Formation of 1-pyrroline **19** from citrulline **39** and ornithine **40** [82]

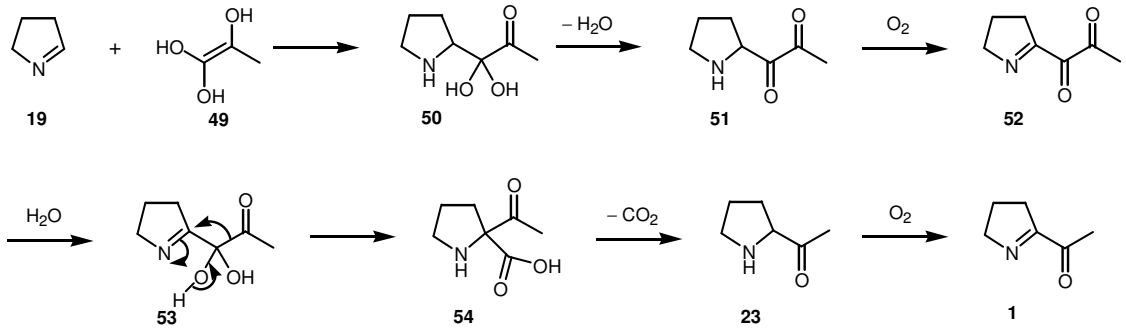
From these results, a reaction mechanism was proposed for the formation of 2-acetyl-1-pyrroline **1**, starting with the formation of an iminium species between the tautomer 2-pyrroline **38** and 1,2-propanedione **27** (Scheme 1.9). Tautomerism and subsequent nucleophilic attack of intermediate **43** to a second molecule of 1,2-propanedione **27** provides iminium ion **44**, which, upon deformylation according to the authors, is hydrolysed into 1,2-propanedione **27** and 2-acetyl-2-pyrroline **48** that tautomerizes into 2-acetyl-1-pyrroline **1**. Several questions arise concerning this reaction pathway. At first, the nucleophilic attack of the intermediate **43** may as well be aimed at carbon-1 of 1,2-propanedione **27** and the products of this pathway are not described. Secondly, elimination of the formyl group cannot occur as described because the formyl group is not a leaving group.



Scheme 1.9. Hypothetical mechanism of formation of 2-acetyl-1-pyrroline **1** from 1-pyrroline **19** and 1,2-propanedione **27**, according to Schieberle [54]

Since higher amounts of 2-AP **1** were formed from the reaction of 1,2-propanedione **27** and 1-pyrroline **19** under aqueous conditions, the hydrated 1,2-propanedione **49** was proposed as the reactive species. On the other hand, it was shown that 2-acetylpyrrolidine **23** could be

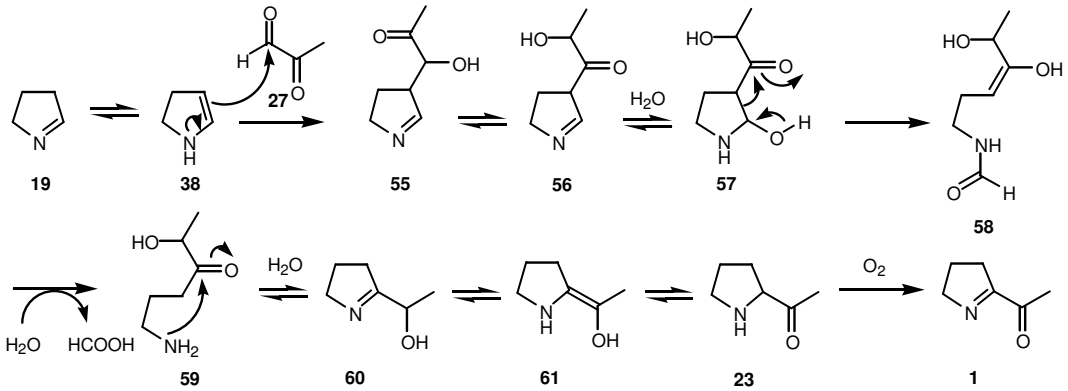
easily oxidized to 2-AP **1** in high yields.[83] From these findings, a new reaction mechanism was proposed (Scheme 1.10).[84] 1-Pyrroline **19** condenses with hydrated 1,2-propanedione **49** to generate 2-(1,2-dioxopropyl)-pyrrolidine **51**, which spontaneously oxidizes with air oxygen to the corresponding 1-pyrroline **52**, the latter undergoing an addition of water to the central reactive carbonyl function (hydrate formation) and subsequent semibenzilic rearrangement. The β-ketoacid **54** thus formed decarboxylates to 2-acetylpyrrolidine **23**, affording 2-acetyl-1-pyrroline **1** upon spontaneous air oxidation.



Scheme 1.10. Proposed reaction mechanism for the formation of 2-acetyl-1-pyrroline **1** from 1-pyrroline **19** and 1,2-propanedione hydrate **49** [84]

Labelling experiments showed that 2-AP mainly incorporated two carbon atoms from glucose, as was discussed above, but a minor amount of 2-AP incorporated three carbon atoms from glucose. The finding that 2-AP was equally formed from 2-methyl-1-pyrroline, though in somewhat lower amounts, suggests that the carbon-2 of 1-pyrroline is lost during the reaction with 1,2-propanedione. This could be explained in an alternative reaction pathway for the formation of 2-AP **1** from the reaction of 1-pyrroline **19** with 1,2-propanedione **27** (Scheme 1.11). This pathway starts with a nucleophilic attack of the tautomeric 2-pyrroline **38** at carbon-1 of 1,2-propanedione **27**. Addition of water to the imine **56** formed, followed by ring opening and subsequent hydrolysis of the generated *N*-substituted formamide **58**, yields 6-amino-2-hydroxy-3-hexanone **59**. Upon cyclization

2-(1-hydroxyethyl)-1-pyrroline **60** is formed. This compound tautomerizes to 2-acetyl-pyrrolidine **23** via 1-(2-pyrrolidinylidene)ethanol **61**.[84] Spontaneous air oxidation yields 2-acetyl-1-pyrroline **1**.



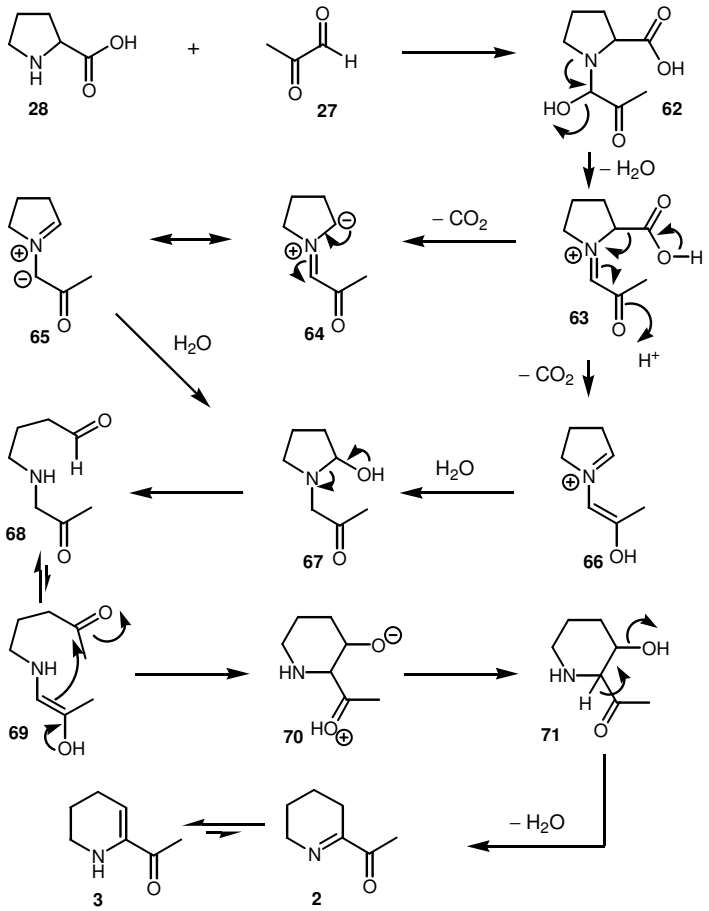
Scheme 1.11. Alternative reaction pathway leading from 1-pyrroline **19** and 1,2-propanedione **27** to 2-acetyl-1-pyrroline **1** [84]

Both pathways establish 2-acetylpyrrolidine **23** as the direct precursor of 2-acetyl-1-pyrroline **1** that is formed by spontaneous oxidation. The air oxidation of 2-acetylpyrrolidine **23** to 2-acetyl-1-pyrroline **1** is similar to the known oxidation of α-aminoketones and α-aminoimines to α-iminoketones and α-diimines, respectively.[85]

1.2.2 Studies of the mechanism of formation of 6-acetyl-1,2,3,4-tetrahydropyridine

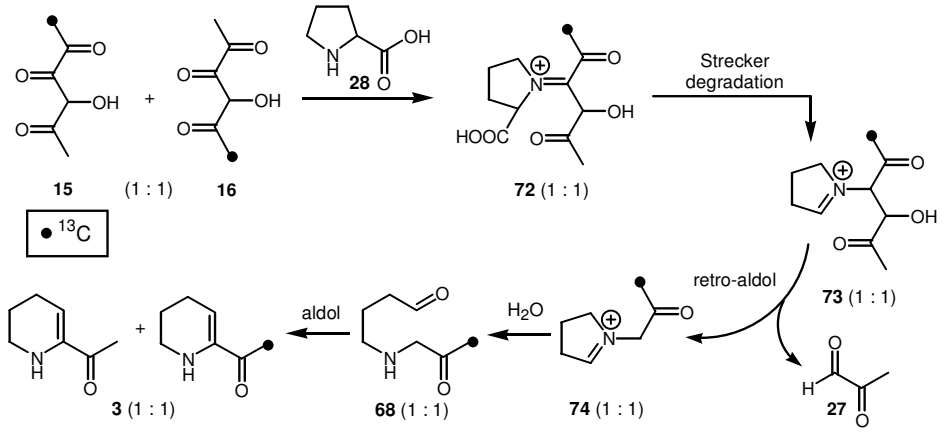
6-Acetyl-1,2,3,4-tetrahydropyridine **3** was first isolated from the model reaction of proline with 1,3-dihydroxyacetone in dry reaction conditions.[67] Various other model experiments of proline with sugars or their degradation products yielded 6-ATHP.[73] Unlike 2-acetyl-1-pyrroline, which is formed from proline or ornithine when reacted with the sugar degradation product 1,2-propanedione, 6-ATHP is formed exclusively from proline and preferentially from fructose as compared to glucose or 1,2-propanedione.[54]

For the formation of 6-acetyl-1,2,3,4-tetrahydropyridine **3**, the so-called Hodge mechanism has long served as the standard mechanism.[76] This mechanism is described in numerous textbooks in which it remained unquestioned for several decades. It was proposed in 1972 and starts with the nucleophilic addition of the proline **28** nitrogen atom at carbon-1 of 1,2-propanedione **27** as is displayed in Scheme 1.12. Elimination of water from adduct **62** yields iminium species **63**. Decarboxylation results into the azomethin ylide **64**, which is in mesomeric equilibrium with the resonance form **65**. Addition of water to **65** affords unstable adduct **67** that ring opens to *N*-2-oxopropyl-4-aminobutanal **68**. These last steps may also be represented as a concerted process for the conversion of **63** into **67** via **66**. Intramolecular aldol-type condensation of ketoaldehyde **68** affords intermediate **71**, from which 6-acetyl-1,2,3,4-tetrahydropyridine **3** is formed by elimination of water.



Scheme 1.12. Hodge mechanism for the formation of 6-acetyl-1,2,3,4-tetrahydropyridine **3** [76]

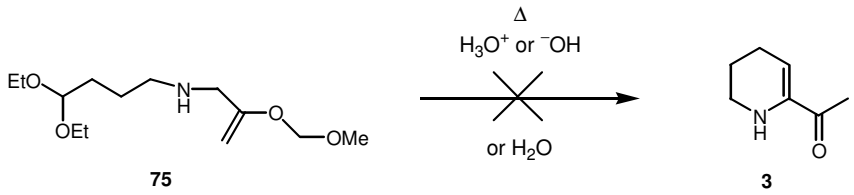
Experiments performed by Rewicki and co-workers with labelled U-^{13}C-glucose and proline indicated the presence of three carbon labels in 6-ATHP, two in the acetyl group and one labelled carbon atom in the ring.[86] When proline was reacted with 1-^{13}C-glucose, a mixture of unlabelled 6-ATHP (60 %) and singly labelled 6-ATHP with the label on the methyl group was obtained.[81] The authors hypothesized a reaction pathway similar to the pathway for 2-AP described in Scheme 1.5, starting from two equivalent isomers of 'diacetyl-formoin' (**15** and **16**), which react with proline **28** with the formation of compound **72** (Scheme 1.13). Strecker degradation and retro-aldol cleavage lead to compound **74**, which undergoes hydrolytic ring opening to *N*-2-oxopropyl-4-aminobutanal **68**, yielding 6-ATHP **3** upon cyclization. This mechanism is similar to the Hodge mechanism presented in Scheme 1.12 and both mechanisms proceed via the common intermediate *N*-2-oxopropyl-4-aminobutanal **68**.



Scheme 1.13. Formation of a 1:1 mixture of 6-acetyl/6-[6-^{13}C]acetyl-1,2,3,4-tetrahydro-pyridine from [1-^{13}C]-glucose and proline [81]

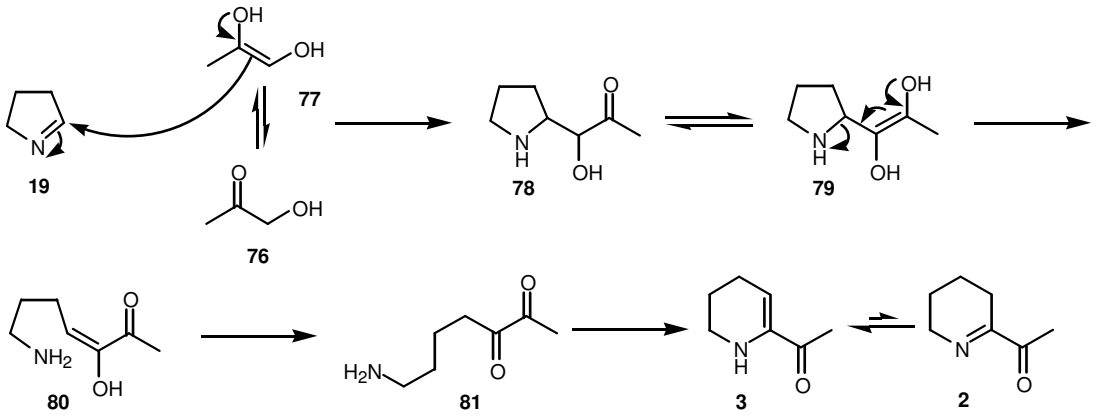
To investigate the intermediacy of this labile *N*-2-oxopropyl-4-aminobutanal **68**, a doubly protected form of this compound **75** was synthesized.[87] This diprotected *N*-2-oxopropyl-4-aminobutanal **75** was then subjected to a broad variety of hydrolytic conditions (acidic, basic, neutral, and combinations thereof), which are known to yield the free aldehyde and amine, but

in the complex reaction mixtures obtained, there was no formation of even the slightest trace of 6-ATHP **3** (Scheme 1.14).[87] Accordingly, it was concluded that the so-called Hodge mechanism is most probably not operative.



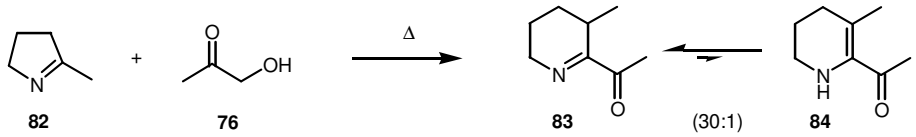
Scheme 1.14. Failure of the diprotected *N*-2-oxopropyl-4-aminobutanal **75** to yield 6-ATHP **3** under diverse hydrolytic conditions [87]

Reaction of 1-pyrroline **19** and 1-hydroxy-2-propanone **76**, both Strecker degradation products of the reaction of proline **28** with an α-dicarbonyl compound, yielded significant amounts of 6-ATHP under basic conditions.[84] From these findings, a different mechanism was proposed for the formation of 6-acetyl-1,2,3,4-tetrahydropyridine **3**, starting from 1-pyrroline **19** and 1-hydroxy-2-propanone **76** (Scheme 1.15).[84] The pathway starts with the attack of carbon-1 of enolized 1-hydroxy-2-propanone **77** at carbon-2 of 1-pyrroline **19**. This step might explain the preferred formation of 6-ATHP at higher pH values. The intermediate 2-(1-hydroxy-2-oxopropyl)pyrrolidine **78** thus formed was synthesized and was shown to yield 6-ATHP **3** upon heating.[84] The ring enlargement proceeds via ring opening to 7-aminoheptane-2,3-dione **81**, which finally cyclizes to 6-ATHP **3**. Such cyclizations have been used later in syntheses of this Maillard compound (cf. Chapter 1.4).



Scheme 1.15. Proposed reaction mechanism for the formation of 6-ATHP **3** from the condensation of 1-pyrroline **19** and 1-hydroxy-2-propanone **76** [84]

Performing the reaction of 1-hydroxy-2-propanone **76** with 2-methyl-1-pyrroline **82** instead of 1-pyrroline **19** confirmed this pathway, since ring enlargement yielded 2-acetyl-3-methyl-3,4,5,6-tetrahydropyridine **83** (Scheme 1.16). This compound showed the same popcorn-like odour at a similar low odour threshold as 6-ATHP, but the tautomeric equilibrium was shifted strongly to the imine tautomer **83**, stabilized by the electron-donating effect of the methyl group.

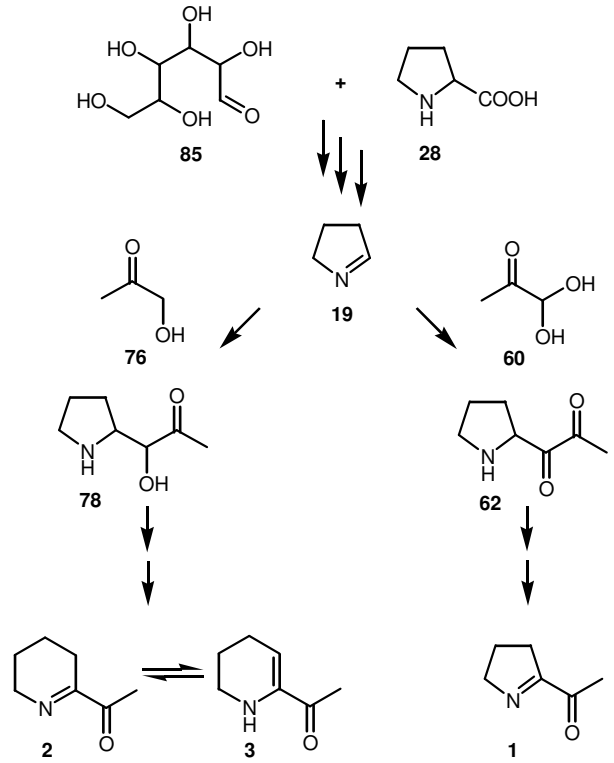


Scheme 1.16. Formation of 2-acetyl-3-methyl-3,4,5,6-tetrahydropyridine **83** from the reaction of 2-methyl-1-pyrroline **82** with 1-hydroxy-2-propanone **76** [83]

A pathway for the formation of 6-ATHP from lysine via 2,3,4,5-tetrahydropyridine, analogous to the formation of 2-AP from ornithine via 1-pyrroline could not be established.[82] Possibly, the intermediate 2,3,4,5-tetrahydropyridine is not stable, and rapidly trimerizes.[108]

1.2.3 Common reaction pathway for the formation of 2-acetyl-1-pyrroline and 6-acetyl-1,2,3,4-tetrahydropyridine

Both flavour compounds 2-acetyl-1-pyroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** are essentially formed from the same precursors: 1-pyrroline and carbohydrate fragments. The relative concentrations of both odorants in proline model systems and thermally processed proline-rich food products depend on the predominant carbohydrate cleavage product present. If high amounts of 1,2-propanedione **27** are present, 2-AP **1** will be preferably formed from the hydrated compound **60**, whereas in the presence of the reduction product 1-hydroxy-2-propanone **76**, the formation of 6-ATHP **3** is favoured (Scheme 1.17).



Scheme 1.17. Formation of 2-AP **1** and 6-ATHP **3** from 1-pyrroline **19** and carbohydrate fragments [88]

When 1,2-propanedione **27** in low concentrations is reacted with proline, the formation of 6-ATHP **3** dominates the formation of 2-AP **1**. This is explained by the formation of

1-hydroxy-2-propanone **76** by Strecker reaction of 1,2-propanedione **27** in the presence of high amounts of free amino acids. When, on the contrary, high amounts of 1,2-propanedione **27** are present, 1-pyrroline **19** formed by Strecker degradation will preferably react with the excess 1,2-propanedione **27** present in the reaction mixture, with the formation of 2-AP **1** as a consequence.[84]

In reaction mixtures of carbohydrates and proline 6-ATHP mostly predominates. Model reactions of proline with different monosaccharides, performed by Tressl et al.,[86] yielded 6-ATHP concentrations that were 7 to 40 (for glyceraldehyde) times higher than the corresponding 2-AP concentrations.

In the reaction with 1,2-propanedione **27**, ornithine **40** was shown to be an efficient precursor of 2-AP **1**, but not of 6-ATHP **3**. The formation of 1-pyrroline **19** from this amino acid does not occur via Strecker degradation. Therefore, no 1-hydroxy-2-propanone **76**, a necessary precursor of 6-ATHP **3**, is formed as a Strecker product. Since baker's yeast contains 3.5 times more ornithine **40** than proline **28**, this explains the higher concentrations of 2-AP **1**, as compared to 6-ATHP **3**, found in bread crust.[11] In popcorn, where no ornithine is present, mainly 6-ATHP **3** is formed.[54]

The formation of these odorants in Maillard model systems consisting of glucose and proline was compared with model systems of the corresponding Amadori compound fructosyl-proline. In contrast to other odorants such as 4-hydroxy-2,5-dimethyl-3-(2*H*)-furanone, 2-AP **1** and 6-ATHP **3** were found to be formed preferentially from glucose/proline model systems as compared to the degradation of the Amadori product.[88] This is another indication that the formation of these flavour compounds does not imply the major Maillard reaction pathways through the Amadori compound and subsequent enolization reactions, but that 2-AP and 6-ATHP are instead formed by side reactions of sugar degradation products and 1-pyrroline.

1.3 Biological origin

1.3.1 Biological formation of 2-acetyl-1-pyrroline in rice plants

Following reports of the formation of 2-AP in the aerial parts of rice plants grown in paddy fields,[57] the biological formation of 2-AP was studied in callus and seedlings of aromatic rice.[89] 2-Acetyl-1-pyrroline **1** was formed in the aromatic rice at temperatures below that of thermal generation, for instance, in bread baking. Precursor studies indicated an increase in 2-AP concentration when proline (× 3), ornithine (× 1.5) and glutamic acid (× 1.5) were present in the solution. Labelling of proline indicated that proline was the nitrogen source for 2-AP, but the carbon source of the acetyl group of 2-AP was not the carboxyl group of proline. It was proposed that 2-AP is formed by acetylation of 1-pyrroline. Glutamic acid is the biosynthetic precursor of proline and ornithine, which both may lead to 1-pyrroline as the immediate precursor of 2-acetyl-1-pyrroline.

Different studies on the genetic control of the typical aroma of rice have been reported. The concentration of 2-AP in rice seems to be regulated by at least two chromosomal regions in the plant.[90] Among the volatiles of the rice flavour produced by plant cell cultures of Basmati rice, 2-AP could, however, not be detected.[91]

The content of 2-acetyl-1-pyrroline in rice might be controlled not only by the genetic background, but also by other factors, such as ecological or cultivation factors. An examination of the 2-acetyl-1-pyrroline content of various rice samples in Thailand showed that the samples from irrigated areas had lower 2-AP contents than rain-fed areas.[92] Drought conditions during cultivation seemed to have an important contribution to aromatic rice quality. Proline accumulation is a common metabolic response of higher plants to water deficits,[93] and may therefore be responsible for the higher 2-AP production.

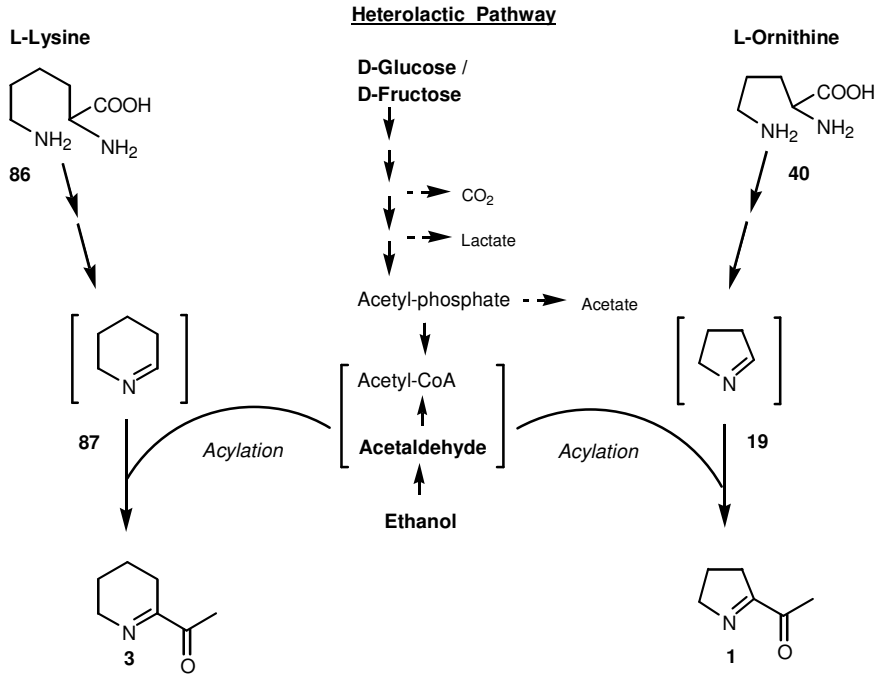
Another study describes the variation of 2-AP concentration in aromatic rice in Japan over a period of three years.[94] Most samples showed similar 2-AP concentrations with standard deviations of about 30 %. However, a few samples showed extremely high or low 2-AP concentrations as compared to the year average. During grain development, the 2-AP concentration in brown rice reached a maximum at four or five weeks after heading, decreasing rapidly afterwards. The 2-AP concentration was higher in brown rice ripened at lower temperature. On the basis of these results, it is recommended to cultivate aromatic rice at cool temperature and high altitude to optimize the scented rice flavour, and it should be harvested earlier than other cultivars. Also the application of nitrogen fertilizer influenced 2-AP concentrations.[94]

1.3.2 Detection of 2-acetyl-1-pyrroline and 6-acetyl-1,2,3,4-tetrahydropyridine as microbial metabolites

2-Acetyl-1-pyrroline was shown to be responsible for a 'popcorn, corn chip' aroma formation, which was observed from several *Bacillus cereus* strains isolated from cocoa fermentation boxes in Brazil.[95] Upon further investigations, 2-AP was detected among the volatiles produced by specific strains of *Bacillus cereus*, under specific growth conditions. 2-Acetyl-1-pyrroline was detected from *B. cereus* cultures grown on solid standard plate count agar at a temperature of 35 °C, i.e. well below the temperatures required for its thermal formation. Labelling experiments established proline and glutamic acid as nitrogen sources and glucose as carbon source required for the formation of 2-AP by these *B. cereus* strains. The highest production was noted when *B. cereus* was grown on plate count agar supplemented with 1 % of glucose (or amylose), and amounted to 11.5 µg (or 12.8 µg, respectively) 2-AP for 25 g of medium during two days. These are very low yields, but due to the low odour threshold, a pleasant flavour is noticeable. The results of these precursor studies are in agreement with the

results of Yoshihashi et al.[89] concerning 2-AP formation in rice, indicating a common pathway for the biological formation of 2-AP.

Microbiologically induced spoilage of wine is characterized by the development of an offensive 'mousy-like' off-flavour. This mousy off-flavour is caused by 6-ethyl-2,3,4,5-tetrahydropyridine, 6-acetyl-1,2,3,4-tetrahydropyridine and 2-acetyl-1-pyrroline.[65,96] All known type strains of the spoilage yeasts *Brettanomyces* and *Dekkera* and many heterofermentative wine lactic acid bacteria are capable of producing this mousy off-flavour and the mousy *N*-heterocycles.[97,98] The formation of the mousy heterocycles 2-AP and 6-ATHP by *Lactobacillus hilgardii* was investigated in detail.[99] These studies demonstrated that the biosynthesis of 2-AP and 6-ATHP is concomitantly dependent upon the metabolic pathways involved in the lactic acid fermentation of sugars, the metabolism of ethanol, and of L-ornithine and L-lysine. The catabolism of L-lysine **86** and L-ornithine **40** via the cadaverine and putrescine pathways, respectively, leads to the formation of 2,3,4,5-tetrahydropyridine **87** and 1-pyrroline **19**, respectively. In the presence of a carbohydrate source, such as fructose, and ethanol, acetyl-CoA may accumulate and induce acylation of the 2,3,4,5-tetrahydro-pyridine **87** and 1-pyrroline **19** intermediates, thus yielding 6-ATHP **3** and 2-AP **1**, respectively (Scheme 1.18).



Scheme 1.18. Proposed pathway for the formation of 2-AP **1** and 6-ATHP **3** by *Lactobacillus hilgardii* DSM 20176 [99]

Another major factor affecting the production of mousy *N*-heterocycles was the presence of metal ions, particularly Fe^{2+}.[99] The reason for this remains unclear.

Microorganisms, such as *Lactobacillus pontis*, can also increase the development of roasty notes in food products by proteolysis, yielding free amino acids such as ornithine as precursors for flavour formation.[100]

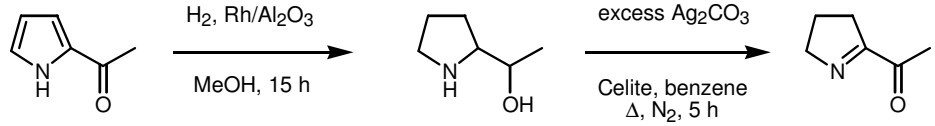
2-Acetyl-1-pyrroline has been identified as a characteristic flavour compound in Mediterranean dried sausages covered with mould. Since the surface of the sausages contained higher amounts of 2-AP than the core, it was suggested that the mould growing on the surface of the sausages, produced 2-AP. The dominating mould species, *Penicillium nalgiovense*, was grown on media with and without various supplements but the popcorn odour only developed in media where the sausage product itself was added.[40]

Considering the results of the different studies on the thermal as well as biological origin of 2-acetyl-1-pyrroline, a common mechanism of formation can be presumed, since in all cases the acylation of 1-pyrroline **19** is described as the key step.

1.4 Synthesis

1.4.1 Synthetic procedures developed for 2-acetyl-1-pyrroline

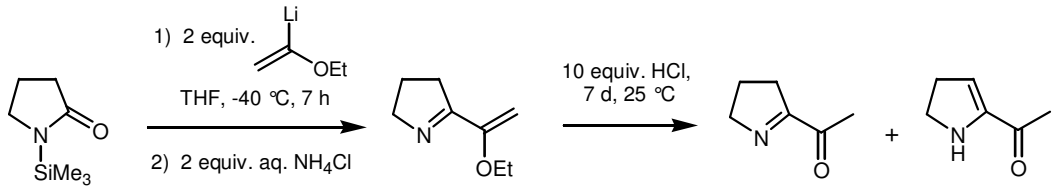
Various syntheses for 2-acetyl-1-pyrroline have been described in the literature. The first synthesis (Scheme 1.19) of the rice flavour compound **1** consisted of an oxidation of 2-(1-hydroxyethyl)pyrrolidine **88** with a large excess of silver carbonate on Celite in benzene. The reaction mixture consisted of a large number of products, of which only 10 % (by GC) appeared to be 2-acetyl-1-pyrroline **1**.[7a] Drawbacks, such as high cost, the use of benzene, and a low yield, disable the application of this method on a large scale.



Scheme 1.19. First reported synthesis of 2-acetyl-1-pyrroline **1**, starting from 2-acetyl-1*H*-pyrrole **8** [7a]

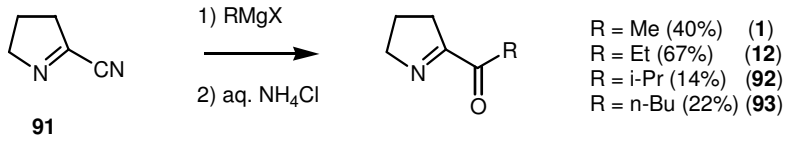
It should be pointed out that 2-acetyl-1-pyrroline **1** occurs exclusively as the imino tautomer. Contrary to 6-acetyl-2,3,4,5-tetrahydropyridine **2**, 2-acetyl-1-pyrroline **1** does not show any tendency to tautomerize to the enamine form **48**. Even deprotonation by base with subsequent reprotonation produces the imino tautomer **1**, a phenomenon that can be expected from the viewpoint of the stability of unsaturated five-membered ring compounds.

Due to the instability of 2-AP **1**, several synthetic efforts focused on more stable, carbonyl-protected analogues, such as the 1-ethoxyethenyl derivative **90** generated from the addition of 1-ethoxyvinyllithium to 1-(trimethylsilyl)butyrolactam **89** (Scheme 1.20).[101] Acid hydrolysis under severe conditions (100 equiv. HCl during 2 days, or 10 equiv. HCl during 7 days), of enol ether **90** yielded 97 % of 2-AP **1**. The presence of the enamino tautomer **48**, however, does not fit any other literature reports, and is therefore questionable. In fact, it concerns the isomeric compound **99**, as proven later (*vide infra* Scheme 1.23).[104]



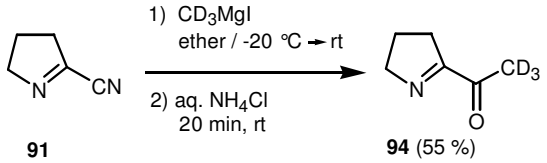
Scheme 1.20. Synthesis of 2-acetyl-1-pyrroline **1** through a stable precursor [101]

A flexible method for the synthesis of not only 2-acetyl-1-pyrroline **1**, but also of other 2-acyl-1-pyrrolines, entailed the addition of a Grignard reagent to imidoyl cyanide **91** and subsequent mild hydrolysis (Scheme 1.21).[102]



Scheme 1.21. Syntheses of 2-acetyl-1-pyrroline **1** and other 2-alkanoyl-1-pyrrolines [102]

The method described above also allows the synthesis of specifically deuterated 2-acetyl-1-pyrroline **94**, which is in this way available for the quantitative analysis of the rice flavour compound **1** using the stable isotope dilution assay.[103] The deuterium incorporation was performed by addition of trideuteriomethylmagnesium iodide to 2-cyano-1-pyrroline **91** (Scheme 1.22).



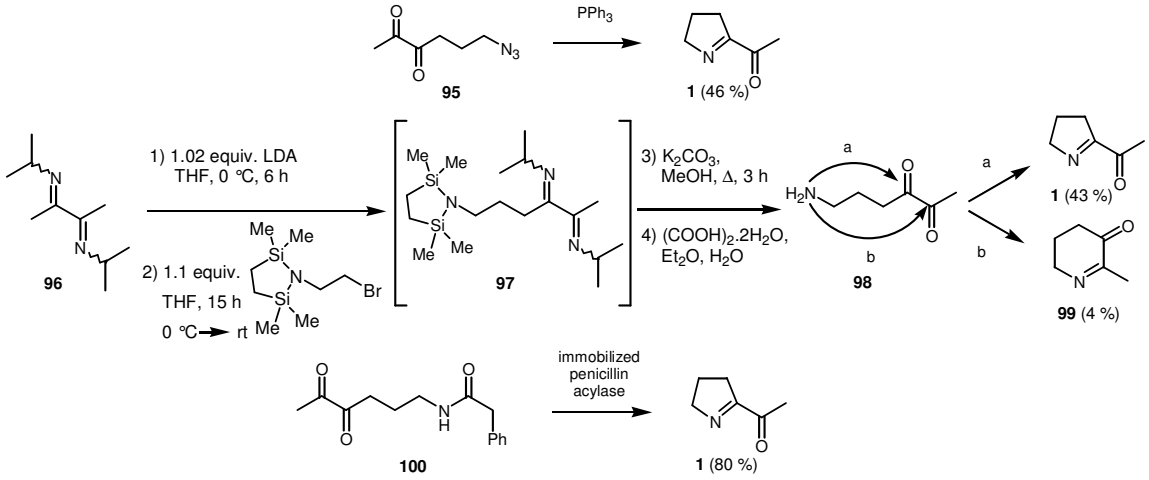
91 **94** (55 %)

Scheme 1.22. Synthesis of deuterated 2-acetyl-1-pyrroline **94**, with deuteration exclusively at the methyl group [102]

Three methods entailing the cyclization of 6-amino- or 6-azidohexane-2,3-diones to form 2-acetyl-1-pyrroline **1** have been published and are presented in short in Scheme 1.23. Rewicki et al.[81] created access to monocyclic and bicyclic α-enaminoketones by intra-molecular aza-Wittig reaction from the corresponding azidodiketone, such as **95**. The yields obtained are lowered by thermal decomposition during the isolation procedure (yield of 2-AP from **95**: 46 %).

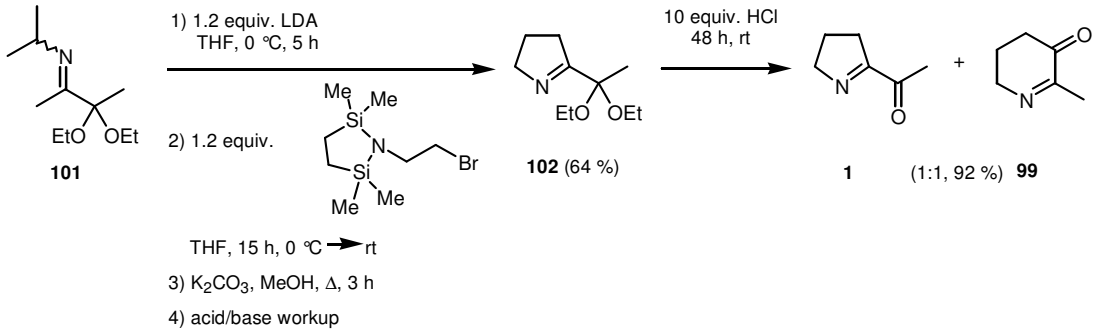
A short synthetic strategy was developed depending on an amino-protected functionalized α-diimine **97** as the key intermediate.[104] This labile key intermediate was produced by α-deprotonation of α-diimine **96** and subsequent alkylation with 'stabase'-protected 2-bromoethylamine. After deprotection, and hydrolysis of the α-diimine into the α-diketone **98**, spontaneous cyclization led to the desired azaheterocycle (2-AP **1** in 43 % yield). The extent of the formation of structural isomer **99** depended on the excess of acid applied. It is proposed that it concerns this isomer **99**, which is formed by acid hydrolysis of compound **90** (Scheme 1.20), instead of the stated enamine-tautomer of 2-acetyl-1-pyrroline **48**, which has never been identified elsewhere.[101]

Favino et al.[105] developed a synthesis of the *N*-protected acyclic 6-amino-2,3-diketone **100**, which is enzymatically hydrolysed into 6-amino-2,3-hexanedione **98** using immobilized penicillin acylase. Spontaneous ring closure yields 2-AP **1** (yield 80 %) (Scheme 1.23).



Scheme 1.23. Synthesis of 2-acetyl-1-pyrroline **1** via cyclization of 6-amino- or 6-azidohexane-2,3-diones [81,104,105]

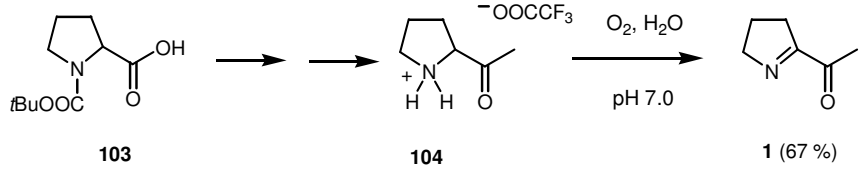
The second pathway shown in Scheme 1.23 was also applied to the preparation of the acetal-protected 2-AP analogue **102**, starting from α,α-diethoxyketimine **101**.[104] Using the procedure shown in Scheme 1.24, the diethyl acetal **102** could be obtained in pure form after column chromatography. These types of stabler compounds show great potential for use as flavouring agent in food products, since gradual hydrolysis yields 2-AP **1** and ethanol.



Scheme 1.24. Synthesis of the stabler diethyl acetal of 2-acetyl-1-pyrroline **102** [104]

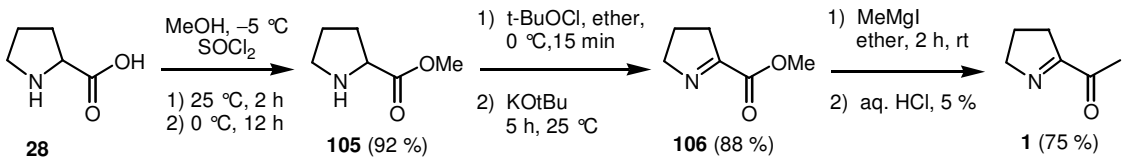
The finding that 2-acetylpyrrolidine **23** is oxidized spontaneously in aqueous medium at neutral pH under the influence of oxygen (air) to afford 2-acetyl-1-pyrroline **1** is of fundamental importance.[84] One of the most recent synthetic procedures developed for 2-AP **1**,

starting from *N*-Boc-protected proline **103**, is based on this oxidation step and was developed to prove this oxidation (Scheme 1.25). 2-Acetylpyrrolidine trifluoroacetate **104** is spontaneously oxidized by air oxygen, upon increasing the pH of the aqueous solution (overall reaction yield: 43 %, yield based on L-proline).



Scheme 1.25. Synthesis of 2-acetyl-1-pyrroline **1** from proline, based on the oxidation of 2-acetylpyrrolidine [84]

Recently, another synthetic procedure for the preparation of 2-acetyl-1-pyrroline **1** from proline **28** appeared in the patent literature.[106] The procedure starts with the esterification of proline **28** via the acid chloride prepared with thionyl chloride (Scheme 1.26). The resulting methyl ester of proline **105** is then subjected to an oxidation and a Grignard reaction, in a procedure identical to what was described earlier for the preparation of 2-acetyl-1-pyrroline **1** via 2-cyano-1-pyrroline **91**.[102] The described process claims to provide access to 2-acetyl-1-pyrroline **1** in gram scale and 60 % overall yield, starting from proline **28**, in four steps.[106] However, addition of a Grignard reagent to the oxidized methyl ester of proline **106** was described before.[102] The authors reported then that the Grignard reagent adds twice onto the ester to form the corresponding tertiary alcohol, which then needs to be separated from the labile Maillard compound **1**. This problem was circumvented by the addition of Grignard reagents to 2-cyano-1-pyrroline **91** (Scheme 1.21).[102]

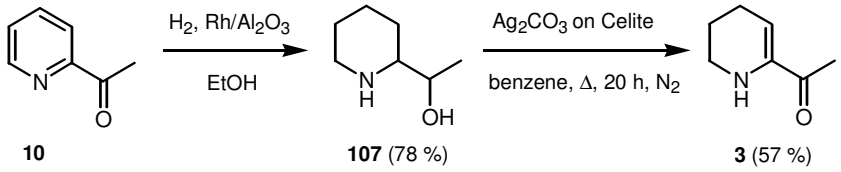


Scheme 1.26. Synthesis procedure of 2-acetyl-1-pyrroline **1** from proline **28** [106]

1.4.2 Synthetic procedures developed for 6-acetyl-1,2,3,4-tetrahydropyridine

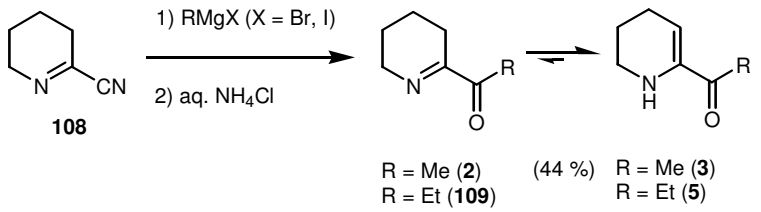
Many synthetic strategies developed for 2-AP **1** can be applied for the synthesis of 6-ATHP **3**, and *vice versa*, although with varying yields.

The first rational synthesis of 6-ATHP **3** was reported in 1971 and included the oxidation of 2-(1-hydroxyethyl)piperidine **107** with a large excess of silver carbonate.[107] It concerns a well-established procedure starting from the relatively inexpensive 2-acetylpyridine **10** with an overall yield of 44 % (Scheme 1.27). On lab-scale, this procedure is still widely used, although it is less attractive for industrial purposes because of the use of a large excess of silver reagent.



Scheme 1.27. First reported synthesis of 6-acetyl-1,2,3,4-tetrahydropyridine **3** [107]

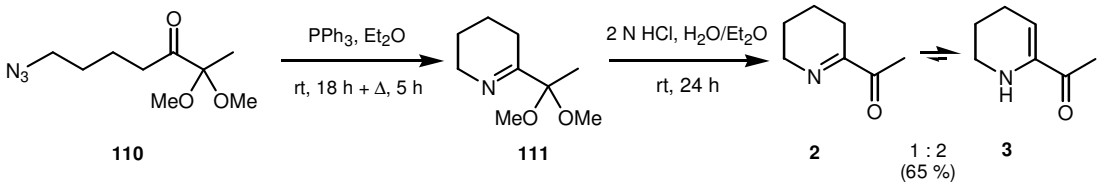
The addition of Grignard reagents to 6-cyano-2,3,4,5-tetrahydropyridine **108** opened the door to the bread flavour component **3** (44 % yield) (Scheme 1.28), but also to the higher analogues 6-propionyl-1,2,3,4-tetrahydropyridine **5** and 2-propionyl-3,4,5,6-tetrahydro-pyridine **109**,[108] which show the same popcorn-like odour note and a similar low odour threshold as 6-ATHP **3**.[109] This process provided access to a large-scale production of the bread flavour compound.



Scheme 1.28. Addition of Grignard reagents to 6-cyano-2,3,4,5-tetrahydropyridine **108** to form important bread-like flavour compounds [108]

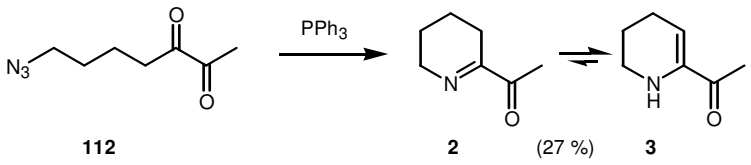
Using this synthetic procedure, the freshly prepared flavour compound occurred as a 4:1 mixture of the imino form **2** and the enamine form **3**, as was shown by ^{1}H-NMR (in $CDCl_3$). On standing, however, this ratio gradually changed to a ratio in favour of the enamine form (up to 1:2). Both tautomers can be isolated, but GC-analysis of imino tautomer **2** already shows an equilibration to the enamine tautomer **3**.

Another synthetic approach towards 6-ATHP and some of its more stable acetal and enol ether derivatives was accomplished by elaboration and ring closure of appropriately functionalized imines. Aza-Wittig type cyclization of functionalized δ-azidoketones, carrying an acetal function in α'-position, such as **110**, as is shown in Scheme 1.29, proved to be the most successful route (yield of 6-ATHP **3** from δ-azidoketone **110**: 47 %).[110]



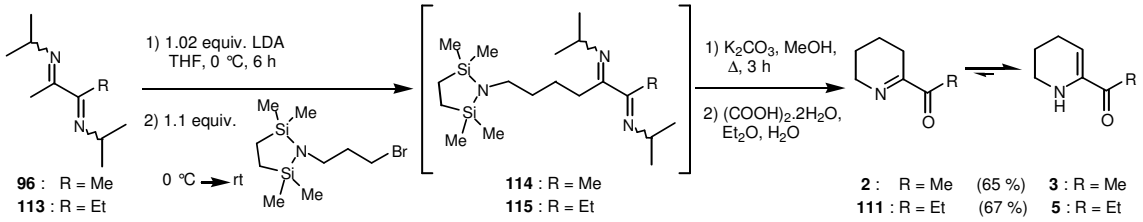
Scheme 1.29. Synthesis of 6-acetyl-1,2,3,4-tetrahydropyridine **3** through cyclization of functionalized δ-azidoketones **110** [110]

Rewicki et al.[81] accomplished a synthesis for 6-ATHP **3** from the δ-azidodiketone **112**, in analogy with the pathway described for 2-AP **1** in Scheme 1.30.



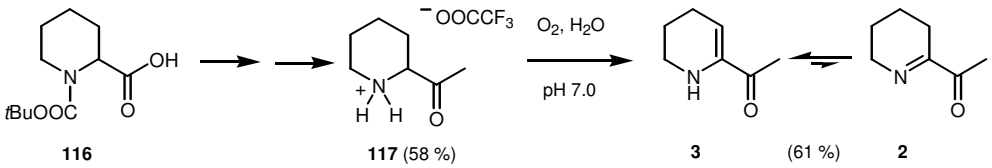
Scheme 1.30. Schematic synthetic route developed for 6-ATHP **3** by intramolecular aza-Wittig reaction of the δ-azidodiketone **112** [81]

Cyclization of the appropriate amino-protected functionalized α-diimine (**114** and **115**), similar to the synthesis of 2-AP **1** described in Scheme 1.23, gave good results for the synthesis of 6-ATHP **3** (65 % yield from **96**) and for the synthesis of higher homologue 6-propionyl-1,2,3,4-tetrahydropyridine **5** (67 % yield from **113**) (Scheme 1.31).[104]



Scheme 1.31. Synthesis of 6-ATHP **3** from the functionalized α-diimine **114** [104]

The most recent synthetic pathway involves the formation of 2-acetylpiperidine from the *N*-protected cyclic α-amino acid pipecolinic acid **116** (Scheme 1.32). 2-Acetylpiperidine oxidizes spontaneously with oxygen at pH 7 to provide 6-ATHP **3** in 35 % overall yield (based on pipecolinic acid).[84]



Scheme 1.32. Recent synthetic pathway for 6-acetyl-1,2,3,4-tetrahydropyridine **3** from the corresponding α-amino acid [84]

1.5 Stability and applications

The maintenance of their particular flavour characteristics is of crucial importance in the marketing of fragrant rice varieties. However, during storage, the flavour of rice can deteriorate as a result of different mechanisms: breakdown, diffusion into the environment, and generation of undesirable volatiles. To investigate the loss of 2-AP in rice, rice was stored

at 30 °C in three different stages, namely as paddy, brown (dehulled) and white (milled) rice, and in two different conditions, namely in air and under reduced pressure (at 84 % relative humidity).[111] In all cases, the 2-AP content of the rice diminished by 40 to 50 % after three months. Neither reduced oxygen tension, nor the way of storage could preserve the flavour compound as a contributor to the desirable characteristics of stored fragrant rice. Another study compared the effect of different drying methods and storage time on the losses of 2-AP from aromatic rice.[56] The average 2-AP concentration of the rice samples subjected to the different drying methods had decreased more than four times after 10 months of storage as compared to after one month of storage, the highest rates of decrease occurring in the beginning of storage. In general, slightly higher concentrations of 2-AP were found from the drying methods that employed lower temperatures.

2-Acetyl-1-pyrroline and 6-acetyl-1,2,3,4-tetrahydropyridine are among the most important flavour compounds of freshly popped corn. From the observation that the flavour of popcorn is not stable, the stability of four of the most important popcorn flavour compounds was investigated, being 6-ATHP (437 µg/kg popcorn), 2-AP (24 µg/kg), 2-propionyl-1-pyrroline (17 µg/kg) and acetylpyrazine (8 µg/kg).[54] Storage in polyethylene bags at room temperature led to a significant decrease of the presence of the flavour compounds. After one week of storage, only 25 to 30 % of the amounts of the flavour compounds remained, except for acetylpyrazine, which remained present in constant amounts. The presence of an aromatic ring system seems to increase the stability of the flavour compound, but also involves weaker flavour characteristics, as is indicated by significantly higher odour thresholds (cf. Table 1.3). The results of these different investigations lead to the conclusion that in order to apply these flavour compounds in food products, their stability has to be increased.

In the flavour industry, encapsulation is a popular modern technique for converting a volatile aroma concentrate into a stable powder form. Maltodextrin and gum acacia were tested as

wall materials for microencapsulation of 2-AP by spray drying.[112] Whereas the concentration of 2-AP in basic solution decreased by 63 % after seven days, and by 30 % after 35 days in acidic solution (formation of salts), microencapsulation with 70:30 acacia-maltodextrin resulted in only 28 % loss after 72 days of storage. Favino et al.[105] enhanced the stability of 2-AP in the presence of β-cyclodextrin, due to the formation of inclusion complexes formed in aqueous solution and in the gas phase, as was proven by NMR and mass spectrometry experiments. The same applied to 6-ATHP. Duby and Huynh-Ba synthesized a stable precursor of 2-AP (Scheme 1.20), and encapsulated 2-AP immediately after hydrolytic conversion on carbohydrate matrices. With 1 % β-cyclodextrine, the flavouring agent remained stable during a period of 110 days at –20 °C.[101]

Similar procedures can be applied to other carbonyl-protected derivatives of 2-AP that have been developed,[104] yielding an encapsulated powder of 2-AP applicable as a flavour enhancer in foodstuffs. A different process has been described for the stabilization of 2-acetyl-1-pyrroline in an easily dispersible powder form.[113] Starch, or gum acacia, is dissolved in water containing an emulsifier, after which a solution of 2-acetyl-1-pyrroline in ethanol is added and homogenized. Subsequently, the mixture is dried by vacuum shelf drying or spray drying to obtain the flavour compound in a dry powder form that can be used for the flavouring of rice and other food products. Sensory analysis of sweetened basmati rice revealed that the rice with the added flavour was preferred to natural basmati rice.[113]

The use of 2-AP as a food flavouring is patented, and the authors describe the preparation of stable salts by physiologically acceptable acids such as citric acid.[114] Also 6-ATHP, as such or as its bisulfite complex or its salts, was claimed to be useful for flavouring bread and other bakery products.[115,116]

Many applications of 2-acetyl-1-pyrroline as a food flavouring have been patented: for instance, starch foods containing 2-acetyl-1-pyrroline, useful in low-protein diets,[117] a food

coating composition manufactured from scented rice, containing at least 40 ppb 2-acetyl-1-pyrroline,[118] distilled alcoholic beverages containing 0.2-200 ppb of 2-acetyl-1-pyrroline,[119] and a 2-acetyl-1-pyrroline containing flavour composition for tea beverages.[120]

1.6 Summary and outlook

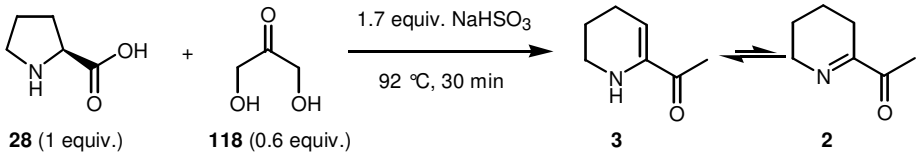
Since its discovery as the most important flavour compound of cooked rice, 2-acetyl-1-pyrroline **1** has not ceased to reveal its presence in a vast variety of food products, always being one of the most flavour-significant compounds present. 6-Acetyl-1,2,3,4-tetrahydropyridine **3** is another important α-acetyl-*N*-heterocycle often detected in cereal products. Both potent flavour compounds are closely related in formation and occurrence. Understanding the mechanisms of their formation, however, is a difficult task, due to the low yields and the high reactivity of intermediates and end products, which hinder the isolation and identification. Due to the instability and volatility of these flavour compounds, the search for efficient synthetic strategies and the development of stable precursors remain a great challenge for many organic chemists. The extremely low odour thresholds and pleasant cracker-like flavour properties create a large interest for these compounds in the food industry, and many attempts have been undertaken to facilitate their application, for instance, by encapsulation.

A lot of research has been conducted to unravel the chemistry of the most important flavour compounds of bread and cooked rice, but many questions still have to be answered before their formation can be controlled and their extraordinary flavour properties can be optimally applied to enhance the flavour of food products. It is amazing to discover that such simple compounds, which occur in daily foodstuffs, have such a remarkable and fascinating chemical rearrangement behaviour in terms of their formation.

2 DETAILED INVESTIGATION OF THE PRODUCTION OF THE BREAD FLAVOUR COMPONENT 6-ACETYL-1,2,3,4-TETRAHYDROPYRIDINE IN PROLINE/1,3-DIHYDROXYACETONE MODEL SYSTEMS

2.1 Introduction

The importance of 6-acetyl-1,2,3,4-tetrahydropyridine, a key odorant of bread crust and popcorn, has been thoroughly discussed in Chapter 1. In this context, the so-called 'Hunter reaction' for the production of 6-acetyl-1,2,3,4-tetrahydropyridine **3** (6-ATHP) has been referred to many times in the literature, but it always remained more or less obscure.[67] Hunter and co-workers reacted L-proline **28** and 1,3-dihydroxyacetone **118** in the presence of an excess sodium bisulfite at 92 °C for 30 min, without solvent (Scheme 2.1).[67] This condensation reaction gave rise to low yields (not quantified) of 6-ATHP, in the presence of some unidentified side products. The crucial role of sodium bisulfite in the development of the bread flavour component was never clarified. Therefore, a detailed study of the model reaction of L-proline and 1,3-dihydroxyacetone was undertaken. Proline is one of the most important amino acids in bread, and 1,3-dihydroxyacetone is a common sugar degradation product, identified, among others, in caramelization mixtures and in the crust of bakery products.[121] In this work, the importance of the different reagents in the model reaction, the influence of the reaction conditions and the evolution of the reaction products were systematically studied to realize an optimal bread flavour formation. Reaction products were identified and the reaction mechanisms involved were reconsidered.



28 (1 equiv.) **118** (0.6 equiv.) **3** **2**

Scheme 2.1. Model reaction performed by Hunter et al.[67] for the production of the bread flavour component

2.2 Results and discussion

2.2.1 Identification and characterization of the reaction products

Model reactions with L-proline **28** and 1,3-dihydroxyacetone **118** were performed, applying different reaction conditions. Upon heating of the dry mixture, a brown-coloured foamy mass was formed that rose to fill the flask and then collapsed to a dark brown sticky mixture. This reaction mixture was dissolved in an aqueous sodium hydroxide solution and was extracted with diethyl ether. Gas chromatographic analysis of most extracts revealed the presence of the two stable tautomeric forms of the bread flavour compound 6-ATHP (**2** and **3**), and of two other main reaction products. A typical gas chromatogram is shown in Figure 2.1.

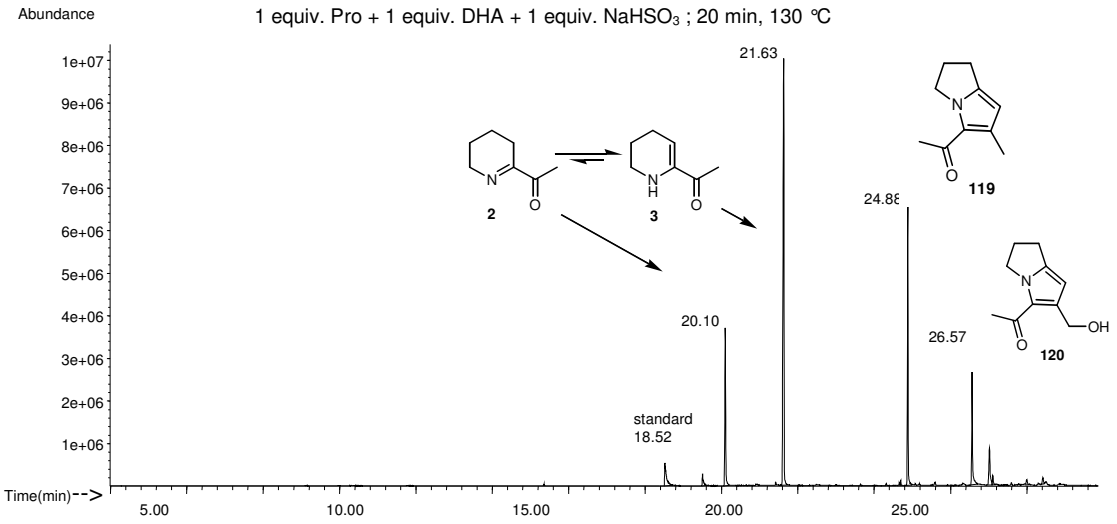
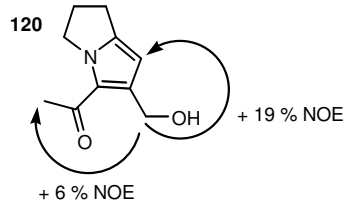


Figure 2.1. Gas chromatogram of the reaction products from the reaction of L-proline **28** with equimolar amounts of 1,3-dihydroxyacetone **118** and sodium bisulfite (20 min, 130 °C)

Two side products occurred in relatively high amounts, but were previously not identified in the so-called Hunter reaction. After separation by column chromatography, their structure was determined by a combination of mass spectrometry, IR spectroscopy and NMR spectroscopy.

A first side product was identified as 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** by comparison with literature data. Different 2,3-dihydro-1*H*-pyrrolizines have been identified in model reactions of proline with monosaccharides, i.e. glucose, rhamnose, arabinose, erythrose and glyceraldehyde,[122,123] in a heated xylose/lysine model system,[124] and in a threonine/ sucrose model reaction.[125] Concerning their presence in food, only the identification of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine and of 6-methyl-2,3-dihydro-1*H*-pyrrolizine-5-carbaldehyde in malt and beer has been reported.[79] L-Proline is the major free amino acid in malt, and beer flavour is formed during kilning, wort boiling, pasteurization and elevated storage conditions. 6-Methyl-2,3-dihydro-1*H*-pyrrolizine-5-carbaldehyde has also been characterized in cooked asparagus.[126]

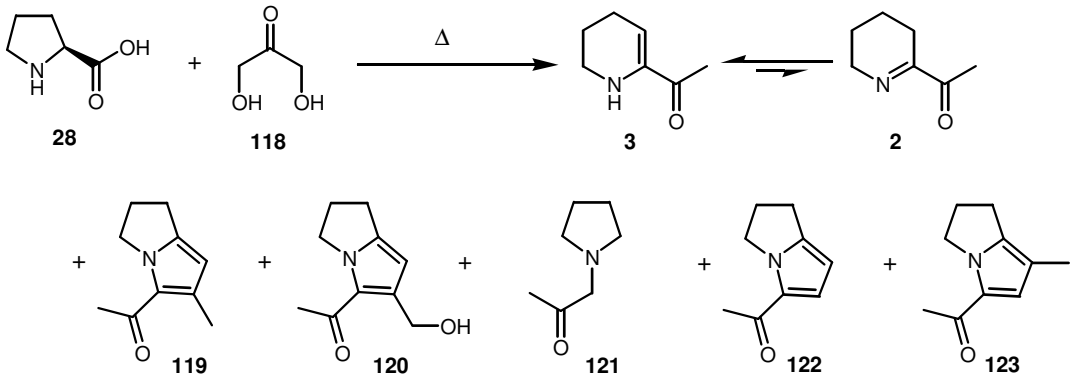
The second side product showed similar spectra, but did not exactly match any of the previously described 2,3-dihydro-1*H*-pyrrolizines.[122,123] By detailed NMR spectroscopy, it was identified as 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120**, a compound which is described here for the first time. In order to confirm the 5,6-substitution pattern of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** a NOE difference NMR experiment was carried out, in which the CH_2-signal of the hydroxymethyl group was irradiated. The fact that a substantial NOE effect on the nearby acetyl group was observed, confirms the proposed substitution pattern (Scheme 2.2).



Scheme 2.2. NOE difference NMR experiment of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120**, performed to confirm the substitution pattern

Although the overall odour of the extract remains roasty cracker-like in the presence of low amounts of these 2,3-dihydro-1*H*-pyrrolizine side products, especially 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** has an acrid and unpleasant odour. The aroma of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** has been described in the literature as smoky, bitter and medicine-like.[122] In order to describe the odour of the new compound 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120**, an (untrained) odour panel evaluated both 2,3-dihydro-1*H*-pyrrolizine side products. To describe the odour of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119**, the panellists used stale, musty, stuffy and woody as main descriptors. The odour of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** was mainly described as stale, unpleasant and acidic. The odour thresholds of both compounds were estimated by the odour panel by sniffing dilute aqueous solutions containing either compound **119** or **120** as compared to blank aqueous solutions in a series of triangle tests. The odour threshold of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** was determined as 24 ppb in water, while the threshold of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** was significantly higher, namely 1070 ppb in water. These values need to be considered as estimates, since significant variations in odour threshold values have been shown, due to individual differences of panel members, to the use of different analytical methods, etc.[127]

Other minor compounds in the reaction mixture were identified and are displayed in Table 2.1. The condensation reaction of L-proline **28** and 1,3-dihydroxyacetone **118** with the different reaction products is shown in Scheme 2.3.



Scheme 2.3. Reaction products of the condensation reaction of L-proline **28** and 1,3-dihydroxyacetone **118**

Table 2.1. Identified reaction products of the condensation reaction of L-proline **28** and 1,3-dihydroxyacetone **118** with their retention index (on HP5 column)

Compound	Kovats Index	Literature data
1-(1-pyrrolidinyl)-2-propanone **121**	1019	proline/sugar model experiments [128]
5-acetyl-2,3-dihydro-1*H*-pyrrolizine **122**	1391	proline/sugar model experiments [123]
5-acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine **123**	1496	proline/sugar model experiments [123]
5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119**	1530	proline/sugar model experiments [123]
5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120**	1754	-

Nitrogen-containing heterocycles in food have been correlated, not only with flavour development, but also in several cases with toxicity.[129] Therefore, fundamental knowledge on their formation is essential in the search for food quality control.

2.2.2 Influence of the reaction conditions

The reaction of L-proline **28** and 1,3-dihydroxyacetone **118** in equimolar amounts was performed at different temperatures varying from 100 to 200 °C (shown in Figure 2.2).

The amounts of 6-ATHP (**2** and **3**) formed were very low (0.01-0.03 mol %) and remained more or less constant with increasing temperatures. The production of the 2,3-dihydro-1*H*-pyrrolizines, i.e. 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** ('pyrrolizine') and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** ('pyrrolizine-OH'), increased with increasing temperatures. In order to obtain an optimal bread flavour, involving a maximal formation of the bread crust flavour compound 6-ATHP and a limited formation of the unpleasantly smelling 2,3-dihydro-1*H*-pyrrolizines, heating at moderate temperatures appears to serve best.

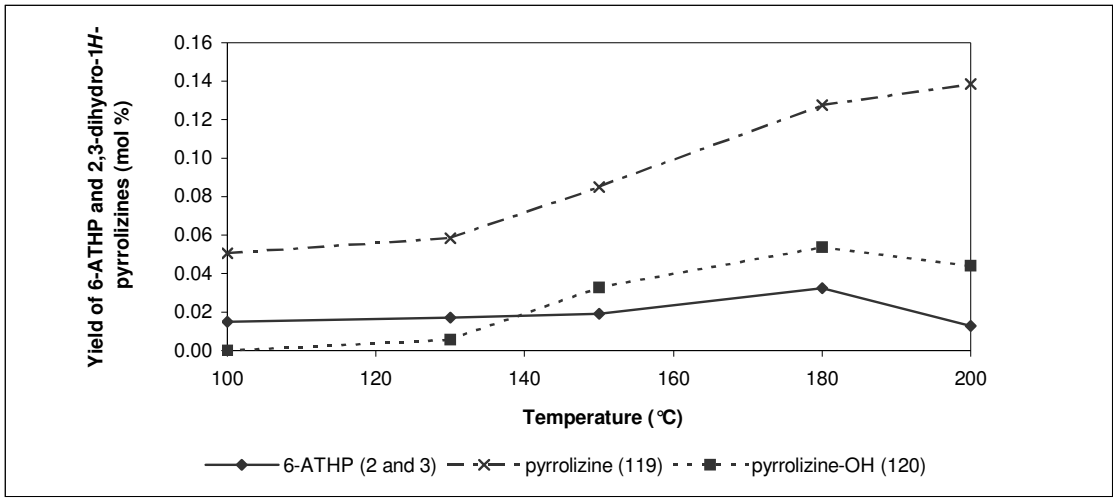


Figure 2.2. Yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**) from the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** (1 equiv., 20 min), as a function of reaction temperature

In the reaction described by Hunter and co-workers,[67] the addition of bisulfite was critical: the formation of the bread flavour compound 6-ATHP was promoted and the isolation thereof from the reaction mixture was facilitated. Therefore, the influence of the addition of sodium bisulfite to the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** was investigated at a moderate temperature of 90 °C (Figure 2.3). From this graph, it can be concluded that the addition of one equivalent of sodium bisulfite caused a substantial increase (about 100-fold)

of the amount of 6-ATHP (**2** and **3**) recovered. When an excess of bisulfite was used (in particular more than two equivalents) the yield of 6-ATHP was not improved significantly, as compared to the reaction without the addition of bisulfite. Concerning the yield of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119**, a slight increase resulted from the addition of one and two equivalents of sodium bisulfite. The yields of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** were somewhat higher when one equivalent of sodium bisulfite was added to the reaction mixture.

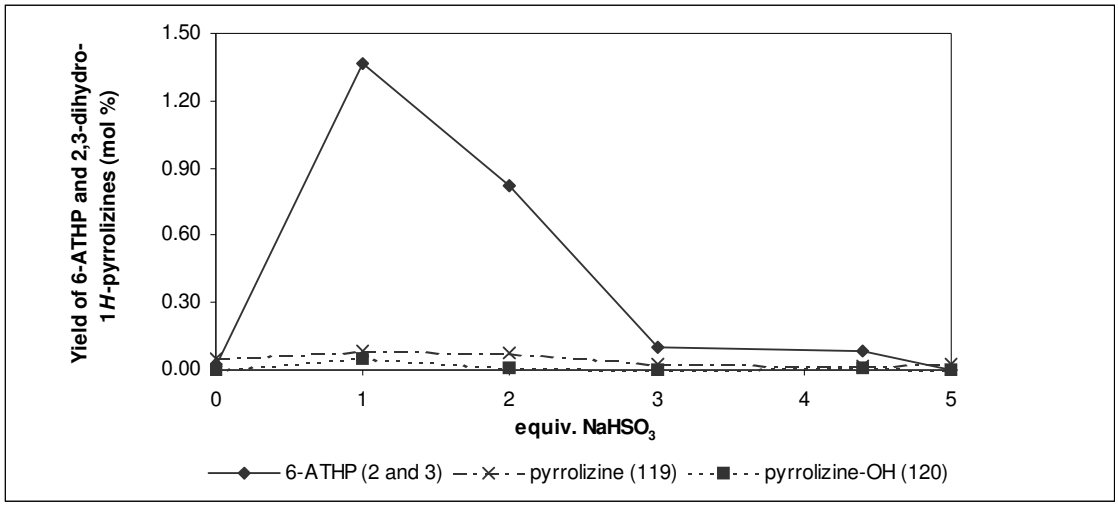


Figure 2.3. Influence of the addition of different amounts of sodium bisulfite in the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** (1 equiv., 90 °C, 20 min) on the yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**)

Sodium bisulfite is a reducing species. Other reducing agents tested in the reaction, such as $FeSO_4$, KI and sodium thiosulfate $Na_2S_2O_3$, resulted in a black mass of reaction products and a very difficult extraction afterwards. Traces of 2,3-dihydro-1*H*-pyrrolizines (**119** and **120**) and of 6-ATHP (**2** and **3**) could be detected, but quantification was not relevant, due to the low recovery. Good yields of the bread flavour compound resulted from the reaction of L-proline and 1,3-dihydroxyacetone in the presence of the reducing agent sodium dithionite ($Na_2S_2O_4$), as is shown in Figure 2.4. In this graph a comparison is made between the yields

of the reaction of equimolar amounts of L-proline and 1,3-dihydroxyacetone performed at 130 °C without additives, with one equivalent of sodium bisulfite $NaHSO_3$ and with one equivalent of sodium dithionite $Na_2S_2O_4$. In comparison with the addition of $NaHSO_3$, less 6-ATHP (**2** and **3**) was formed when $Na_2S_2O_4$ was added, but a significant increase of 6-ATHP was realized as compared to the neat reaction of proline **28** and 1,3-dihydroxyacetone **118**. Comparable amounts of 2,3-dihydro-1*H*-pyrrolizines (**119** and **120**) were recovered from these model reactions.

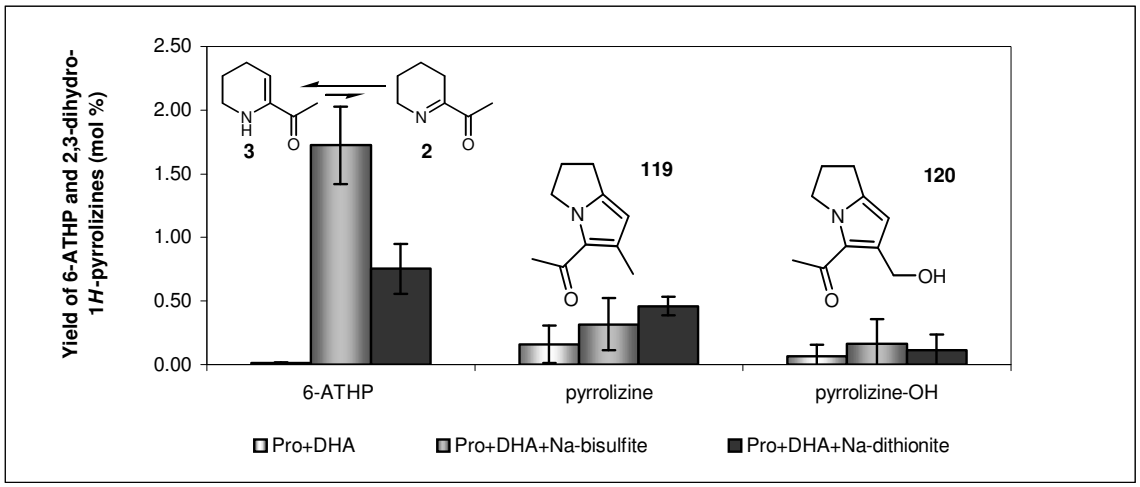


Figure 2.4. Influence of the addition of one equivalent of $NaHSO_3$ (sodium bisulfite) and of one equivalent of $Na_2S_2O_4$ (sodium dithionite) in the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** (130 °C, 20 min) on the yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**)

In addition to its reducing activity, bisulfite is known to stabilize imines by nucleophilic addition, thus preventing hydrolytic cleavage.[130] Such a stabilizing effect might explain the facilitated isolation and improved recovery of the unstable 6-ATHP in the presence of sodium bisulfite. To release 6-ATHP from its stable bisulfite addition product, alkali was added to the reaction mixture prior to extraction. When dithionite is used as the reducing agent, oxidation

forms bisulfite, which can then stabilize the 6-ATHP formed. This might explain the positive effect of $Na_2S_2O_4$ in contrast with the other reducing species tested.

The influence of the use of a different number of equivalents of 1,3-dihydroxyacetone in the reaction with proline was investigated. In Figure 2.5 is shown that, in the presence of bisulfite (1 equiv.), maximum yields were accomplished when one or half an equivalent of 1,3-dihydroxyacetone was used. This indicates that 1,3-dihydroxyacetone is not a limiting reagent in the reaction. However, too much 1,3-dihydroxyacetone reduced the yield of 6-ATHP substantially, since side reactions increasingly occurred. Especially the formation of 2,3-dihydro-1*H*-pyrrolizine **120** increased with increasing 1,3-dihydroxyacetone concentrations.

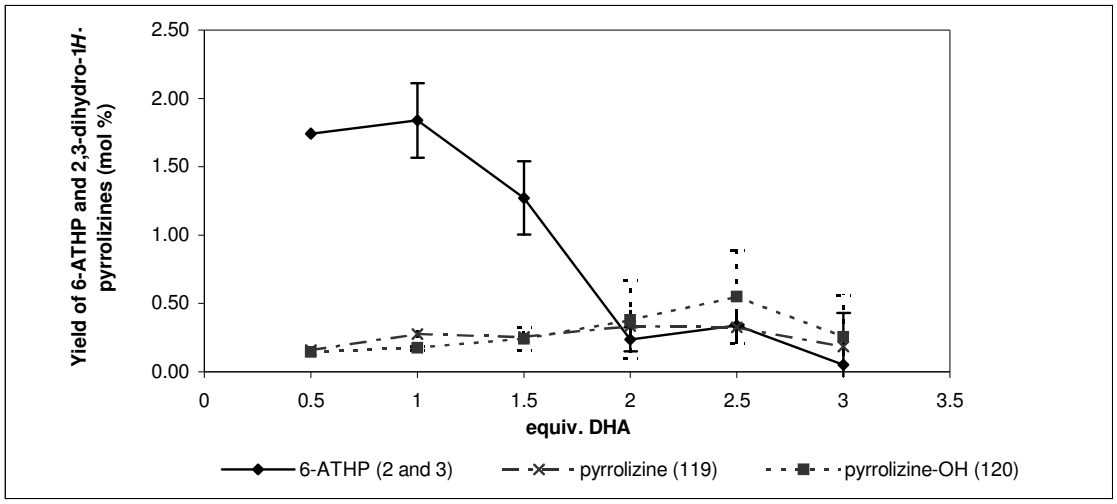


Figure 2.5. Influence of the amount of 1,3-dihydroxyacetone **118** (DHA) used in the reaction with L-proline **28** (1 equiv. $NaHSO_3$, 120 °C, 20 min) on the yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**)

The reaction of equimolar amounts of L-proline and 1,3-dihydroxyacetone with one equivalent of $NaHSO_3$ was performed at temperatures ranging from 60 °C to 200 °C. The graph, shown in Figure 2.6, displays the average of two or three replications with the standard deviations as error bars.

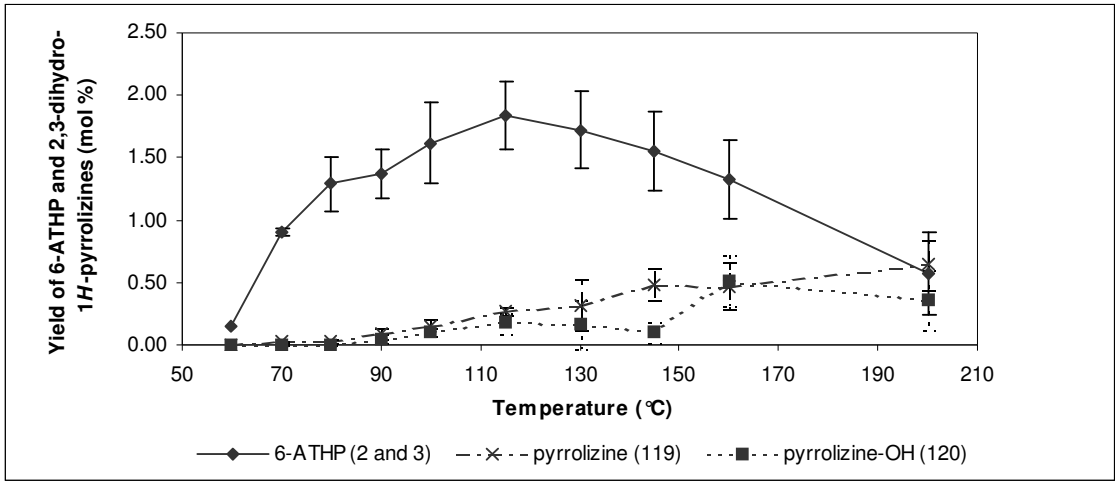


Figure 2.6. Influence of temperature on the yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**) in the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** (1 equiv. $NaHSO_3$, 20 min)

It is clear that the production of 6-ATHP reaches a maximum around 120 °C. At higher temperatures, the unstable 6-ATHP formed is probably degraded again, since less of the principal bread flavour compound was recovered from the reaction. The amount of 2,3-dihydro-1*H*-pyrrolizines (**119** and **120**) produced, however, increased with increasing temperatures between 60 and 200 °C. These results are in agreement with the influence of temperature on the reaction of L-proline and 1,3-dihydroxyacetone without the addition of $NaHSO_3$ (Figure 2.2). This allows the conclusion that an optimal heating temperature can be found, where the formation of the bread flavour compound 6-ATHP is maximal, but where the production of undesirable side products remains limited. Although extrapolation from this model system to a real food system is not straightforward, this should be taken into account, since baking at higher temperatures can have negative consequences for the flavour, and possibly safety, of the resulting food product.

In the reaction of L-proline and 1,3-dihydroxyacetone in the presence of one equivalent of $NaHSO_3$, optimal yields of 1.8 ± 0.3 mol % 6-ATHP were found at 115 °C. The addition of two equivalents of sodium bisulfite also improved the formation of 6-ATHP, and at 130 °C yields of 6-ATHP of 1.7 ± 1.0 mol % were noted. Thus, the maximum yield of

6-ATHP was accomplished from the neat reaction of L-proline and 1,3-dihydroxyacetone in the presence of two equivalents of $NaHSO_3$ at 130 °C and amounted to 2.7 mol % of 6-ATHP (3.3 mg 6-ATHP per mmol of L-proline). It must be noted, however, that the reproducibility of the reaction decreased when more equivalents of sodium bisulfite were used, which is probably due to a concentration effect. The yields reported here are moderate, but establish, as compared to literature data, where 45 µg of 6-ATHP per mmol of proline were obtained in the reaction with glucose,[88] a very significant increase. Due to the very low odour threshold of 6-ATHP (1.6 ppb in water), the aroma developed in the reaction is strong, despite the relatively low yields.

A reaction time of 20 minutes was chosen because it was visually observed that in most cases the rising and most significant browning of the reaction mixture were terminated after 15-20 minutes. To investigate the influence of the reaction time, the reaction of L-proline and 1,3-dihydroxyacetone (two equivalents of $NaHSO_3$, 130 °C) was stopped after five minutes, on the one hand, and the reagents were allowed to react for one hour, on the other hand. The results of these experiments are shown in Figure 2.7.

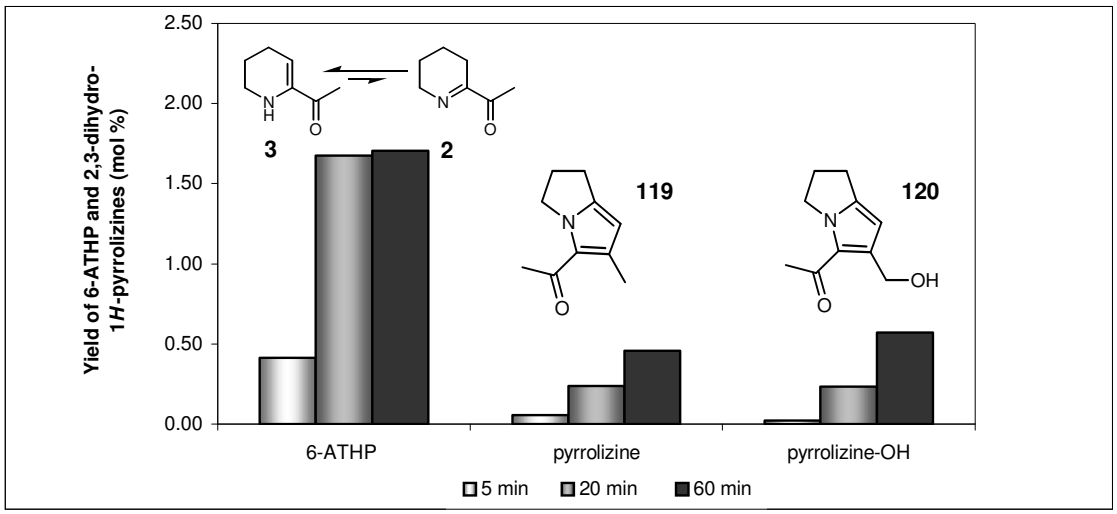
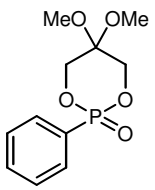


Figure 2.7. Influence of reaction time on the yield (mol %) of 6-ATHP (**2** and **3**) and 2,3-dihydro-1*H*-pyrrolizine side products (**119** and **120**) in the reaction of L-proline **28** and 1,3-dihydroxyacetone **118** (2 equiv. $NaHSO_3$, 130 °C)

As is the case for the influence of the reaction temperature, an optimal reaction time (around 20 min) can be found, where the yield of 6-ATHP is maximal, and where the production of 2,3-dihydro-1*H*-pyrrolizines remains limited. After this time, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** are increasingly formed, while the recovery of the unstable 6-ATHP (**2** and **3**) does not increase. Since almost no 6-ATHP (0.03 %) was recovered after heating of L-proline and 1,3-dihydroxyacetone without sodium bisulfite for one hour (data not shown), the formation of a stabilized salt of 6-ATHP with bisulfite is probable.

The reaction of proline and 1,3-dihydroxyacetone was also performed in the presence of phosphates. Aim of the addition of Na_3PO_4 and NaH_2PO_4 was to stimulate *in situ* the formation of 1,3-dihydroxyacetone phosphate, and hence increase bread flavour formation by an improved formation of 1,2-propanedione, the actual active species in the reaction (cf. Chapter 1 - Scheme 1.6). The catalytic role of phosphates and their influence on the course of the Maillard reaction has been shown.[131,132] When Maillard reactions are performed in phosphate buffer, the presence of phosphate may have a more pronounced effect than the pH itself. Under the evaluated conditions of phosphate additions to the dry model system, however, extraction became very difficult, and few flavour compounds were recovered. A positive effect could not be demonstrated. The reaction of the 1,3-dihydroxyacetone phosphate precursor **124** [133] (Scheme 2.4) with L-proline also did not generate any flavour compounds.



124

Scheme 2.4. 5,5-Dimethoxy-2-phenyl-1,3,2-dioxaphosphinane 2-oxide **124**, a precursor of 1,3-dihydroxyacetone phosphate, which was reacted with L-proline [133]

Blank et al. studied in detail Maillard model reactions of glucose and L-proline in phosphate buffer solutions.[134] The highest yields of Maillard reaction products were obtained at pH 7 and 8. Acetic acid was the most important product of the reaction; up to 0.04 mol % of 6-ATHP was obtained together with very low yields (up to 0.004 mol %) of 2-acetyl-1-pyrroline.

Performing the Hunter reaction in phosphate buffer solutions (pH 7) requires a dilute reaction system. Extracts obtained from the reaction of L-proline, 1,3-dihydroxyacetone and sodium bisulfite in phosphate buffer showed a high impurity, and low amounts of 6-ATHP were formed. When the reaction was performed by reflux in a small amount of water (5 ml) without pH adjustment, the yield of 6-ATHP was only one fourth of the reaction yield under dry conditions. The presence of water seems to inhibit the formation of 6-ATHP and of 2,3-dihydro-1*H*-pyrrolizines. This is in agreement with the results of an investigation of the effect of the moisture content on flavour formation in a microwave-heated propylene glycol-based proline/xylose model system (10 min, 130 °C). The content of 2,3-dihydro-1*H*-pyrrolizines decreased strongly with increasing moisture content (from 0 to 5 % H_2O).[135] In general, a low water activity is known to induce the Maillard reaction.[136]

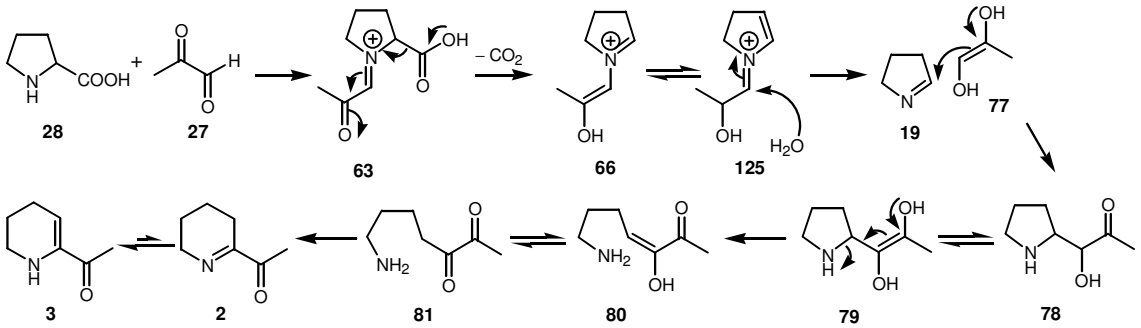
The negative influence of water in the reaction makes it difficult to evaluate the influence of the pH. An experiment was performed in which L-proline was reacted with 1,3-dihydroxyacetone in the presence of concentrated HCl (1 equiv.) as an acid on the one hand, and in the presence of $NaHCO_3$ (1 equiv.) as a base on the other hand. The yields of 6-ATHP diminished slightly (with 22 %, in the case of addition of $NaHSO_3$ and $NaHCO_3$) to drastically (with 90 %, in the case of addition of $NaHSO_3$ and HCl). Sodium bisulfite may react with HCl with the formation of sulfur dioxide gas. Therefore, the positive effect of

bisulfite is probably lost in the presence of acid, and yields cannot be compared. In addition, the negative influence of the water added when concentrated HCl was used, lowered the yield. Addition of $NaHCO_3$ alone gave very low yields, comparable with the yields without additives.

As is demonstrated by the different experiments performed, many factors influence the formation of these Maillard flavour compounds in a simple model system. The monitoring of some isolated parameters of this model reaction illustrates the extremely complex pattern of these Maillard reactions in real food systems.

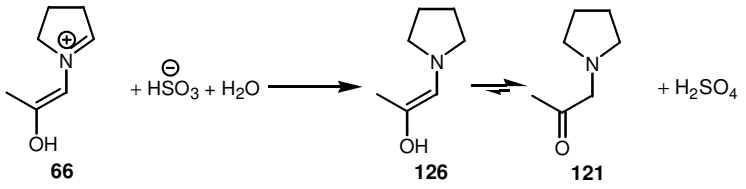
2.2.3 Formation pathways of the reaction products

As is discussed in Chapter 1, the most recent mechanistic proposal for the formation of 6-acetyl-1,2,3,4-tetrahydropyridine **3**, starts from 1-pyrroline **19** and 1-hydroxy-2-propanone **76** (*vide supra* Scheme 1.15).[137,138] These two compounds are formed by Strecker degradation of proline **28**, initiated by an α-dicarbonyl compound, in this case 1,2-propanedione **27**. Starting from proline **28** and 1,2-propanedione **27**, the reaction proceeds as in Scheme 2.5. The intermediate 2-(1-hydroxy-2-oxopropyl)pyrrolidine **78** in the reaction pathway was synthesized by Hofmann and Schieberle and yielded 6-acetyl-1,2,3,4-tetrahydropyridine **3** upon heating.[138]



Scheme 2.5. Mechanism of formation of 6-ATHP (**2** and **3**), according to Hofmann & Schieberle [138]

In the Hunter model reaction under investigation, 1,2-propanedione **27** results from the dehydration of 1,3-dihydroxyacetone **118**. The negative influence of water as a solvent in the reaction can be explained by its inhibiting effect on this dehydration. Sodium bisulfite (or dithionite as a substitute) reduces 1,2-propanedione **27** to 1-hydroxy-2-propanone **76**, which is a reagent in the reaction as well (as the enolized form **77**). When 1,3-dihydroxyacetone **118** was heated (130 °C, 10 min) in the presence of $NaHSO_3$ (one and two equivalents), traces of 1-hydroxy-2-propanone **76** were detected. Nevertheless, 1-hydroxy-2-propanone may also be present in the reaction mixture as a product of the Strecker degradation of proline. Use of excess $NaHSO_3$ reduces the yield, since too much reductive activity eliminates essential reagents and the presence of several carbonyl groups remains essential in the reaction sequence. The presence of 1-(1-pyrrolidinyl)-2-propanone **121** in the extracts of the experiments performed confirms the pathway presented in Scheme 2.5, since it can be formed by reduction of intermediate **66** (Scheme 2.6). This is another indication that too much reductive activity eliminates important intermediates in the reaction sequence, and thus reduces the yield.

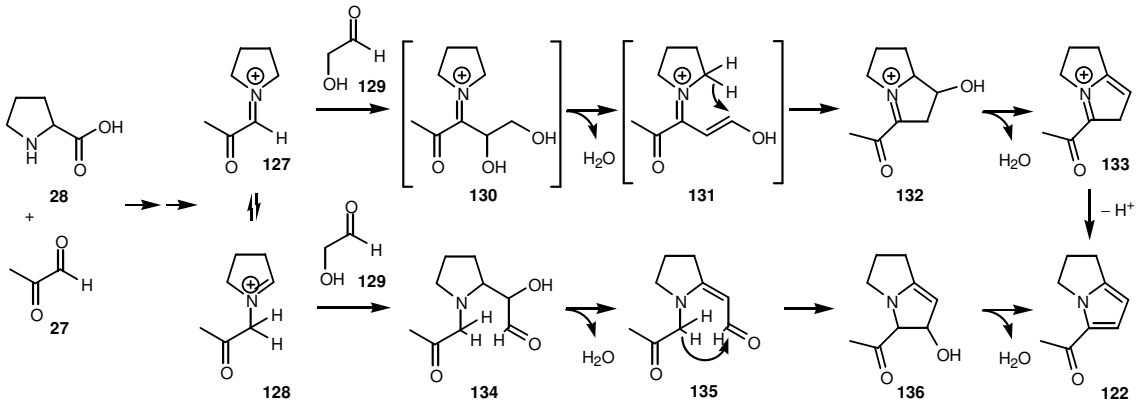


Scheme 2.6. Possible reduction of intermediate **66** by bisulfite, leading to the formation of reaction product **121**

Under the given reaction conditions and with the analytical method described, 2-acetyl-1-pyrroline **1** was never detected, not even when the extracts were analysed by GC-MS in SIM

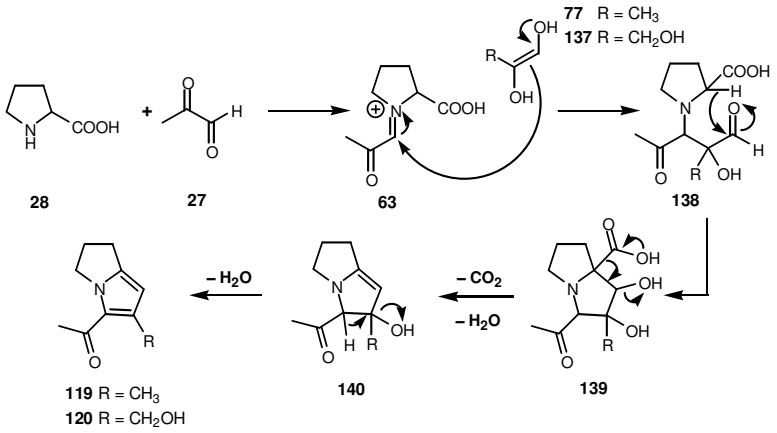
mode (selecting only those ions specific for 2-acetyl-1-pyrroline). However, both important flavour compounds, 6-acetyl-1,2,3,4-tetrahydropyridine **3** and 2-acetyl-1-pyrroline **1**, are essentially formed from the same intermediates, as is discussed extensively in Chapter 1. 2-Acetyl-1-pyrroline **1** is formed when 1,2-propanedione **27** is present in high amounts, whereas in the presence of the reduction product 1-hydroxy-2-propanone **76**, the formation of 6-ATHP **3** is favoured, as is confirmed here. Furthermore, the formation of 2-acetyl-1-pyrroline was suggested to result from 1-pyrroline **19** and 1,2-propanedione hydrate **60**, since the amounts of 2-acetyl-1-pyrroline **1** formed were much lower under dry-heating conditions. In the reaction described here, both the presence of bisulfite and the absence of water lead to the exclusive formation of 6-ATHP.

For the formation of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine **122** in proline/monosaccharide model systems, a reaction mechanism has been proposed by Tressl et al. (Scheme 2.7).[123] This mechanism involves the formation of an iminium ion in two tautomeric forms **127** and **128** from the reaction of proline **28** with 1,2-propanedione **27**. After this step, two possibles routes are given. According to the first route, iminium ion **127** undergoes an aldol condensation reaction with hydroxyacetaldehyde **129** to form compound **130**, followed by water elimination and intramolecular Michael addition. Alternatively, iminium ion **128** undergoes a nucleophilic attack of hydroxyacetaldehyde **129** to form compound **134**, which is followed by the elimination of water and an intramolecular aldol condensation. Elimination of a second water molecule results in 5-acetyl-2,3-dihydro-1*H*-pyrrolizine **122**.



Scheme 2.7. Reaction mechanism for the formation of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine **122** in proline/monosaccharide model systems, as proposed by Tressl et al.[123]

A variation of this proposed mechanism, applied to the formation of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** identified in proline/1,3-dihydroxyacetone model systems, is displayed in Scheme 2.8. Iminium ion **63** undergoes a nucleophilic attack of enolized 1-hydroxy-2-propanone **77** (or enolized 1,3-dihydroxyacetone **137**) (Mannich reaction). Intramolecular aldol condensation forms the bicyclic transient compound **139**, after which decarboxylation and elimination of two molecules of water yield 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** when 1-hydroxy-2-propanone **76** is a reagent, while 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** is formed from 1,3-dihydroxyacetone **118** as the reagent. 5-Acetyl-7-methyl-2,3-dihydro-1*H*-pyrrolizine **125** is formed when the nucleophilic attack of the enolized 1-hydroxy-2-propanone **77** in Scheme 2.8 originates from carbon-1 instead of from carbon-2.

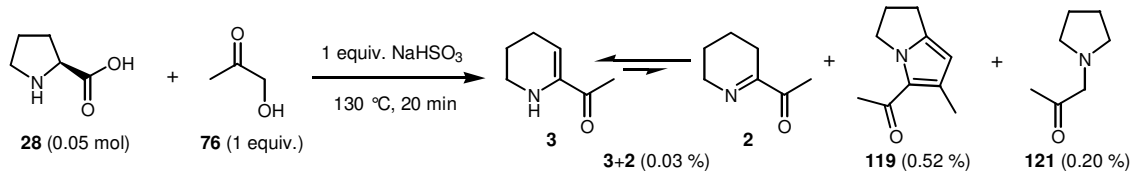


Scheme 2.8. Proposed mechanism of formation of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** (alternative to Tressl et al.[123])

Since 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** is formed from 1,3-dihydroxyacetone **118**, the presence of bisulfite or dithionite has no influence on its production, as can be deduced from Figure 2.4, but the more 1,3-dihydroxyacetone **118** is added, the more 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** is formed (Figure 2.5). Thus, the information obtained from the investigation of the reaction conditions confirms the reaction mechanism proposed.

The 'Hunter'-reaction was also performed with 1-hydroxy-2-propanone **76** as a reagent instead of 1,3-dihydroxyacetone **118** (Scheme 2.9). At 130 °C, with one equivalent of $NaHSO_3$, 1-hydroxy-2-propanone **76** and L-proline **28** reacted with the formation of 0.03 % of 6-ATHP **3** (cf. 1.72 % from the model reaction proline/1,3-dihydroxyacetone in the same circumstances) and 0.5 % of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119** (cf. 0.32 % from the model reaction proline/1,3-dihydroxyacetone). No 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** was detected, in contrast with the relatively large amounts of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine **119**. This is in agreement with the mechanism of formation proposed, since the formation of 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine

119 requires 1-hydroxy-2-propanone **76**, while 1,3-dihydroxyacetone **118** is needed for the formation of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120**.



Scheme 2.9. Yields of reaction products of the reaction of L-proline **28** and 1-hydroxy-2-propanone **76**

Sodium bisulfite or other inorganic reducing agents are in general not present in food products. However, as a result of the Maillard reaction, several reductones are formed in heated food products. These compounds possess an enediol structural element in α-position to a keto function, and are therefore reductive.[139]

In order to collect some more mechanistic information, halogenated acetone derivatives, i.e. chloroacetone, 1,3-dichloroacetone and 1,3-dibromoacetone, were reacted with L-proline in water instead of 1,3-dihydroxyacetone. Because halogens are much better leaving groups than the hydroxyl group, these reactions were expected to yield *in situ* 1,2-propanedione and produce 6-ATHP, although possible side effects of the resulting HCl or HBr must be taken into account. Different reaction conditions were applied: with and without $NaHSO_3$, in water and in basic solution. However, no 6-ATHP could be detected, nor any 2,3-dihydro-1*H*-pyrrolizines. Only condensation products of the carbonyl derivatives, containing no nitrogen, as can be deduced from the mass spectra, were detected in the extracts.

2.3 Conclusion

The reaction of L-proline and 1,3-dihydroxyacetone gave rise to a maximal yield of 2.7 % 6-acetyl-1,2,3,4-tetrahydropyridine, when heated at 130 °C in the presence of two equivalents of sodium bisulfite. The positive influence of sodium bisulfite is most probably due to a

combination of its reductive activity, providing necessary reagents for the reaction, and of its stabilizing effect on 6-acetyl-1,2,3,4-tetrahydropyridine, improving the recovery of this unstable compound from the reaction mixture. Side reactions yielded 2,3-dihydro-1*H*-pyrrolizines, among which 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine was quantitatively the most important one. 5-Acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine was described as a new compound, identified here for the first time.

2.4 Materials and Methods

2.4.1 Chemicals

L-Proline **28** (Pro, 99+ %), 1,3-dihydroxyacetone **118** (DHA, dimer 98 %), sodium bisulfite ($NaHSO_3$, powder p.a.), 1,3-dibromoacetone (tech., 70 %), chloroacetone (96 %), 1,3-dichloroacetone (99 %), silica gel (0.035-0.070 mm, pore diameter ca 6 nm), chloroform-d (0.03 *v/v* % TMS, 99.8+ atom % D), ethyl acetate, hexane and diethyl ether (c.p., stabilized with BHT) were from *Acros Organics*. Internal standard collidine (99 %), 1-hydroxy-2-propanone **76** (acetol, tech. 90 %), sodium hydrosulfite ($Na_2S_2O_4$, tech. 85 %) and sodium thiosulfate were from *Sigma-Aldrich*.

2.4.2 Model reactions

L-Proline **28** (0.05 mol), 1,3-dihydroxyacetone **118** and $NaHSO_3$ (both in varying amounts) were mixed and ground in a mortar. The powder was put in a two-necked 250-ml round-bottom flask equipped with a condenser and a mechanical stirrer (*Heidolph*) and placed in a

preheated oil bath for 20 min at constant temperature (± 5 °C). During the heating period the mixture fused, gradually rose as a foamy mass to fill the flask, collapsed and finally dried up. An intense browning occurred during the course of the reaction. After 20 minutes, the oil bath was replaced by an ice bath, and the mixture was allowed to cool down. The reaction products were dissolved in 100 ml of 2 N NaOH and extracted three times with 50 ml of diethyl ether. The yellow-coloured extract was boiled for 10 minutes with decolourizing charcoal and was dried with $MgSO_4$. After filtration, the extract was concentrated by evaporation and analysed by GC-MS. Quantification was accomplished with collidine as an internal standard. In these quantifications, a response factor of one was assumed, since pure and stable standards of the evaluated compounds are difficult to obtain. Most experiments were performed in duplicate. In this case, error bars are included in the graphs representing twice the standard deviation.

For the different variations of the reaction described, all reagents were carefully mixed before the reaction was started, or were dissolved in water or an aqueous buffer solution, and the same reaction procedure was applied.

2.4.3 Mass spectrometry

For the analysis of the extracts a Hewlett-Packard 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector-Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) Injector (*Gerstel*), and a HP5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 µm) was used. Working conditions were: injector 250 °C; transfer line to MSD 250 °C; oven temperature start 35 °C, hold 5 min, programmed from 35 to 60 °C at 2 °C min^{-1} and from 60 to 250 °C at 20 °C min^{-1}, hold 5 min; carrier gas (He) 1 ml min^{-1}; split 1/10; ionization EI 70 eV; acquisition parameters: scanned m/z 40-200 (0-20 min), 40-400 (> 20 min). When the MS was operated in SIM mode for the detection of 2-acetyl-1-pyrroline ions 111, 83 and 69 were monitored.

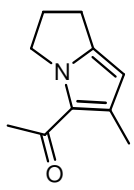
2.4.4 Column chromatography

The different compounds in the extracts were separated by chromatography over a short silica column (15 cm, i.d. 3 cm) using a solvent mixture of hexane and ethyl acetate (7:3). Spots were visualized on TLC (silica gel) by iodine vapours.

2.4.5 NMR spectroscopy

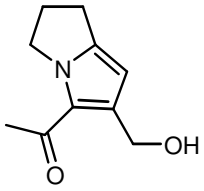
High-resolution ^{1}H-NMR (270 MHz) and ^{13}C-NMR (68 MHz) spectra were taken in $CDCl_3$ as solvent (using tetramethylsilane as the internal standard) with a JEOL EX 270 NMR-spectrometer.

5-Acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine **119** (referred to as 'pyrrolizine')



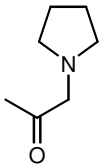
^{1}H-NMR (270 MHz, $CDCl_3$): δ(ppm) 2.38 (3H, s, CH_3), 2.39 (3H, s, CH_3CO), 2.43 (2H, quintet, J=7.4Hz, $C\underline{H}_2CH_2N$), 2.77 (2H, t, J=7.6Hz, CH_2CN), 4.29 (2H, t, J=7.2Hz, CH_2N), 5.74 (1H, s, =CH); ^{13}C-NMR (67 MHz, $CDCl_3$): δ(ppm) 15.9 (CH_3), 24.1 ($\underline{C}H_2CN$), 26.8 ($\underline{C}H_2CH_2N$), 29.2 ($\underline{C}H_3CO$), 49.2 (CH_2N), 104.5 (=CH), 125.5 (HC=$\underline{C}$N), 132.9 ($\underline{C}CH_3$), 143.7 ($\underline{C}COCH_3$), 187.2 (C=O); IR (KBr, cm^{-1}) $\nu_{C=O}$=1630; mp 47 °C; MS (70 eV) *m/z* (%) 148 (100), 163 (81), 120 (27), 149 (26), 65 (13), 91 (13), 77 (11), 93 (9), 92 (8), 43 (7) (data in agreement with reference 123).

5-Acetyl-6-hydroxymethyl-2,3-dihydro-1H-pyrrolizine **120** ('pyrrolizine-OH')



^{1}H-NMR (270 MHz, $CDCl_3$): δ(ppm) 2.47 (3H, s, CH_3CO), 2.50 (2H, quintet, J=7.4Hz, $C\underline{H}_2CH_2N$), 2.83 (2H, t, J=7.6Hz, CH_2CN), 4.27 (2H, t, J=7.2Hz, CH_2N), 4.63 (2H, s, CH_2OH), 5.94 (1H, s, =CH); ^{13}C-NMR (67 MHz, $CDCl_3$): δ(ppm) 24.2 ($\underline{C}H_2CN$), 26.8 ($\underline{C}H_2CH_2N$), 28.4 ($\underline{C}H_3CO$), 49.2 (CH_2N), 59.2 (CH_2OH), 103.4 (=CH), 126.0 (HC=$\underline{C}$N), 138.5 ($\underline{C}CH_2OH$), 148.8 ($\underline{C}COCH_3$), 187.1 (C=O); IR (KBr, cm^{-1}) ν_{OH}=3450, $\nu_{C=O}$=1610; mp 86 °C; MS (70 eV) *m/z* (%) 179 (100), 164 (48), 148 (38), 133 (36), 132 (32), 134 (32), 108 (32), 163 (19), 43 (17), 106 (14).

1-(1-Pyrrolidinyl)-2-propanone **121** (data in agreement with reference 128)



MS (70 eV) *m/z* (%) 84 (100), 42 (26), 55 (16), 85 (7), 43 (4), 41(4), 127 (3), 83 (2), 81 (2).

2.4.6 Sensory evaluation

The odour evaluation and determination of the odour threshold of 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **119** and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine **120** were performed by 14 and 17 untrained panellists, respectively. Determination of the odour threshold was accomplished according to the method of 'triangular-forced-choice' (cf. ASTM E679-91 odour standard). Solutions of the pure compound in odorous-free distilled water were presented to the panellists in colourless and odourless glass erlenmeyers. Five ascending concentration steps were used, with a concentration factor of three between each step. Each step comprised two blanks and one dilute aqueous solution of the 2,3-dihydro-1*H*-pyrrolizine; the panellists were asked to identify the differing sample by smelling at the top of the open erlenmeyers.

3 FORMATION OF PYRAZINES AND 2-ACETYL-1-PYRROLINE BY *BACILLUS CEREUS*

3.1 Introduction

The production of flavour compounds by plant cell cultures or microbial fermentation is being developed in reply to the consumer's demand for 'natural' products. Microbial *de novo* production or bioconversion of natural precursors leads to aroma compounds that can be labelled as 'natural flavours', and represents as such a promising area in the field of food science.

Pyrazines comprise a group of heterocylic nitrogen-containing compounds which have been shown to contribute significantly to the unique roasted aroma of many heated food products.[140,141] Alkylpyrazines are important products of the Maillard reaction, formed usually at temperatures above 100 °C.[142] Trialkylated pyrazines such as 2-ethyl-3,5-dimethyl-pyrazine and 2,3-diethyl-5-methylpyrazine were shown to be impact flavour compounds of coffee,[143] roasted sesame seeds,[144] and roasted beef.[145] A whole range of flavour-significant alkylpyrazines was detected in maple syrup,[146] roasted peanuts,[147] and in doughs and breads.[148]

Various reports are made in the literature of the microbial origin of pyrazines in fermented food products, i.e. in fermented soybeans, cocoa, and cheese.[149] Tetramethylpyrazine was found to be responsible for the characteristic odour of natto, a Japanese fermented soybean product, which is usually inoculated with *Bacillus subtilis*, also known as *Bacillus natto*.[150] Sugawara et al.[151] identified nine pyrazine compounds in laboratory-made and commercial natto; the most important pyrazines were tetramethylpyrazine, trimethylpyrazine and 2,5-dimethylpyrazine. Sugawara et al.[152] and Yamaguchi et al.[153] identified a wide range of pyrazines in *Bacillus subtilis* cultures, i.e. pyrazine, methylpyrazine, 2,3-dimethylpyrazine,

2,5-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine, tetramethylpyrazine, ethylpyrazine, 2-ethyl-6-methylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2,6-diethyl-3-methylpyrazine and 2-methyl-5-propenylpyrazine. When *Bacillus natto* was cultivated in basal medium containing cooked soybeans, the type and amount of pyrazine produced depended on the addition of amino acids.[154] *Bacillus subtilis* produced trimethylpyrazine and tetramethylpyrazine when grown on coconut,[158] and 2,5-dimethylpyrazine and tetramethypyrazine when grown on soybeans in solid-state [155] and solid-substrate [156] fermentation. Owens et al.[157] reported the formation of 2,5-dimethylpyrazine, trimethylpyrazine and 3,6-dimethyl-2-ethylpyrazine by *Bacillus subtilis* fermentation of soybeans.

Various alkylated 2-methoxypyrazines were found to be responsible for the development of musty potato-like odours, and can be produced by *Penicillium caseicolum* in cheese,[158] by *Pseudomonas taetrolens* in milk,[159] by *Pseudomonas perolens* in fish,[160] and in synthetic medium,[161,162] and by *Serratia* and *Cedecea*.[163] *Aspergillus oryzae* was shown to be responsible for the production of 19 substituted pyrazines, when it was grown on soybeans and wheat flour in solid-state fermentation.[164] Several alkylated 2-hydroxypyrazines have been reported in cultures of *Aspergillus sojae* and *Aspergillus paraciticus*.[149]

During the fermentation of cocoa beans, the production of alkylpyrazines by *Bacillus subtilis* and *Bacillus megaterium* was shown to play a role in flavour development.[165] In an investigation of the contribution of microbial metabolites to cocoa flavour, Romanczyk et al. observed the production of 2-acetyl-1-pyrroline, together with some alkylpyrazines, by *Bacillus cereus* strains isolated from cocoa fermentation boxes in Brazil.[95] 2-Acetyl-1-pyrroline is one of the most important Maillard flavour compounds, with very potent cracker-like flavour characteristics, and contributes significantly to the flavour of a large number of heated food products, in particular of rice and cereal products (cf. Chapter 1). Of the 21

Bacillus strains tested, only *B. cereus* strains ATCC 10702, 27522, 33019, 14737 and five *B. cereus* strains isolated from the cocoa boxes, produced 2-acetyl-1-pyrroline when grown on solid plate count agar (PCA) enriched with certain precursors (proline, ornithine, glutamic acid, glucose, amylose, amylopectin, lactose, maltose, ribose, sucrose and *N*-acetyl-glucosamine). A series of ^{13}C- and ^{15}N-labelling experiments showed *B. cereus* to utilize glucose as carbon source, and glutamic acid or proline as nitrogen source for the formation of 2-acetyl-1-pyrroline. Sampling was done by simultaneous steam distillation – solvent extraction of two-day old surface cultures. The highest yields were obtained when *B. cereus* was grown on PCA enriched with 1 % of glucose (i.e. 458 µg 2-AP/kg) and 1 % of amylose (i.e. 514 µg 2-AP/kg).

The main purpose of this work was to investigate the biological origin of pyrazines and 2-acetyl-1-pyrroline in *Bacillus cereus* cultures and to exclude any heat-induced flavour formation. Thus, the possibility to use these *B. cereus* strains for the biological production of roasted flavour compounds was evaluated. Several detection methods were compared, and the influence of different precursors was studied.

3.2 Results and discussion

3.2.1 Thermal generation of Maillard flavour compounds by sterilization of growth media

In order to prove the biological origin of pyrazines and 2-acetyl-1-pyrroline, the possibility of the formation of these compounds as artefacts, for instance, from the autoclaved culture media, had to be ruled out.

Therefore, blank culture media (plate count agar PCA and YMPG, a common liquid culture medium) were autoclaved for 20 min at 1.2 bar, and the volatiles released from the sterilized

media were sampled, using Solid Phase Microextraction (SPME) and solvent extraction (of the liquid medium). No flavour compounds were detected in the extracts using these procedures.

Investigations of the intensity of Maillard browning after heating of amino acids and sugars in an autoclave apparatus for 10 minutes have shown that browning was strongly dependent on the pH, with maximum browning obtained at pH 10.[166] The initial pH of PCA before heating was measured as 6.7, similar to the initial pH of YMPG (6.2). Adjustment of the pH before autoclaving clearly showed an increased browning with increasing pH values (Figure 3.1). Browning is often used as an indicator for Maillard reactivity, although sugar caramelization also contributes to browning.

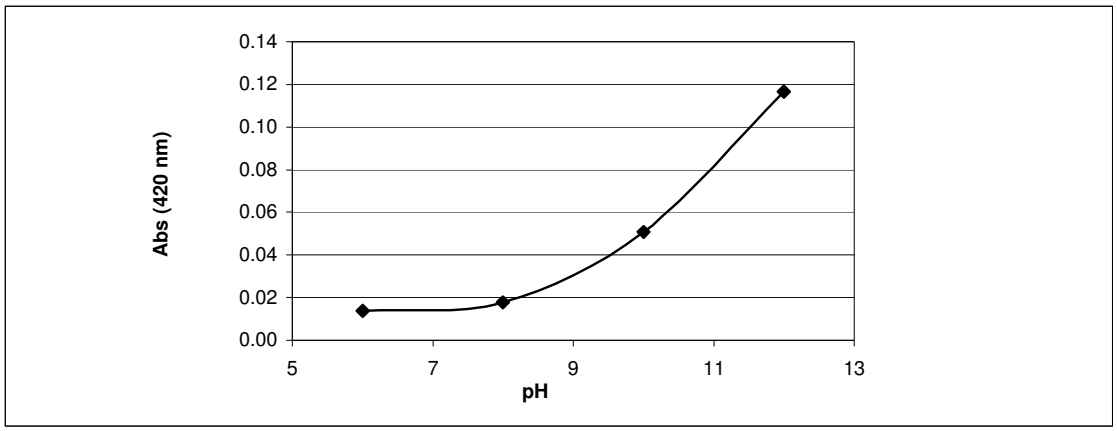
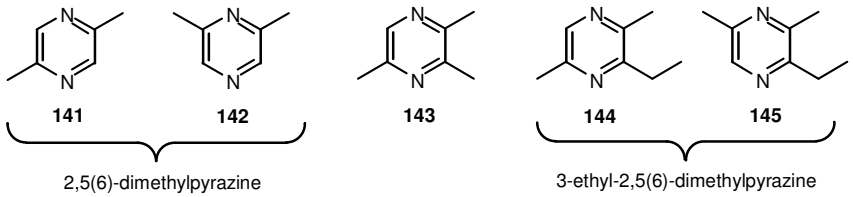


Figure 3.1. Maillard browning (measured as absorbance at 420 nm) after autoclaving (20 min, 1.2 bar) of YMPG medium, in function of the pH

Monitoring of the volatiles released from the autoclaved culture media showed that, from pH 8 upwards, the formation of some Maillard reaction compounds was detected. It concerned 2,5-dimethylpyrazine **141** (or 2,6-dimethylpyrazine **142**), trimethylpyrazine **143**, and 3-ethyl-2,5-dimethylpyrazine **144** (or 3-ethyl-2,6-dimethylpyrazine **145**) (Scheme 3.1).

It must be noted that under the given analytical conditions (HP-5 capillary column), 2,5-dimethylpyrazine **141** and 2,6-dimethylpyrazine **142** cannot be completely separated from

each other. Both compounds elute with a difference in retention index of one unit and their mass spectra are identical.[200] Unambiguous identification of a single chromatographic peak at this specific retention and with this specific mass spectrum is impossible. Both compounds will therefore be discussed 'together' as '2,5(6)-dimethylpyrazine' (Scheme 3.1). The same applies to 3-ethyl-2,5-dimethylpyrazine **144** and 3-ethyl-2,6-dimethylpyrazine **145** (or 2-ethyl-3,5-dimethylpyrazine), which will be named as '3-ethyl-2,5(6)-dimethylpyrazine' in later discussions.



Scheme 3.1. 2,5-Dimethylpyrazine **141** and 2,6-dimethylpyrazine **142**, not separable on HP-5 capillary column, trimethylpyrazine **143**, and 3-ethyl-2,5-dimethylpyrazine **144** and 3-ethyl-2,6-dimethylpyrazine **145**, also not separable on HP-5 capillary column

Prolonged heating (40 minutes instead of 20 minutes) did not increase browning. When the culture media were heated at atmospheric pressure, browning decreased significantly as compared to the autoclaving conditions (1.2 bar).

These results demonstrate that the formation of alkylated pyrazines as artefacts of autoclaving culture media is possible. Although pyrazine formation becomes detectable only after alkalinization of the media, care must be taken to exclude the formation of pyrazines as artefacts in all cases. Therefore, blank non-inoculated media will be included as controls in all the experiments performed.

3.2.2 Development of an appropriate sampling technique

Romanczyk and co-workers used simultaneous steam distillation – solvent extraction of two-day old petri dish cultures for the sampling of *Bacillus cereus* cultures.[95] This is a sensitive technique, but it is time-consuming and all cultures can be sampled only once. In addition, the possibility exists of inducing changes in the volatiles due to the thermal treatment applied. In order to monitor the production of volatiles in time, and to be able to screen the influence of a wide range of parameters, a faster and non-destructive sampling technique would be preferred. Headspace sampling with adsorption on Tenax can be applied to *B. cereus* surface cultures, when grown in appropriate erlenmeyers. Tenax is a general purpose sorbent, especially suitable for aromatics, and with a high stability.[167] As compared to conventional headspace sampling, the use of Solid Phase Microextraction (SPME) would allow an extensive and fast screening of the influence of different parameters. Solid Phase Microextraction combines sample preparation, extraction, concentration and sample introduction in one step, and it is known as a very sensitive technique.[168] Standard tests indicated that the three-component SPME-fiber DVB/Car/PDMS (divinylbenzene and carboxen on polydimethylsiloxane) gave higher recoveries for the analytes under study than an apolar PDMS fiber. In a comparison of different SPME-fibers for the analysis of coffee flavour compounds, Bicchi et al. found the same fiber DVB/Car/PDMS to give the highest recoveries for most flavour compounds, in particular for pyrazines.[169] Therefore, this three-component fiber was selected for use in the experiments.

In Figure 3.2, sampling of *B. cereus* cultures is shown by dynamic headspace sampling with adsorption on Tenax on the one hand, and by SPME on the other hand.

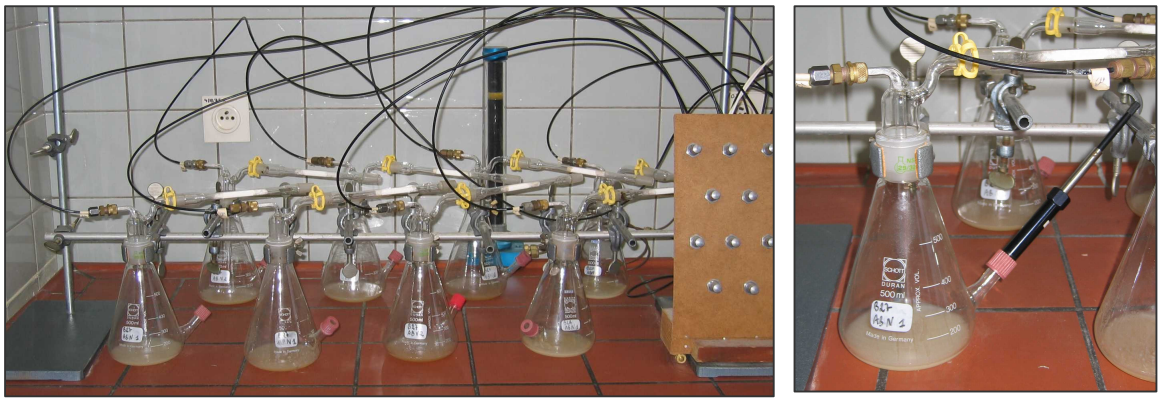


Figure 3.2. Dynamic headspace sampling of 50-ml *B. cereus* surface cultures (left-hand side) and SPME-extraction through a septum-sealed side inlet (right-hand side)

In a first experiment, the production of volatiles by two *B. cereus* strains (DSM 487 and DSM 2896) on 10-ml plate count agar (PCA) cultures was studied, using SPME. Proline, ornithine and starch (concentrations 2 %) were used as precursors since they increased flavour formation in previous investigations.[170] No flavour compounds could be detected, however, by 30-min SPME extractions in the period from day 2 until day 11 after inoculation.

In a following experiment, four 50-ml erlenmeyers were inoculated with *B. cereus* ATCC 27522, in parallel with eight 10-ml SPME-vials, on the same supplemented media (proline, ornithine, starch). No flavour compounds could be detected from SPME extractions of the 10-ml cultures, but after 72 h of headspace sampling of the 50-ml cultures, pyrazines were detected in the concentrated diethyl ether extracts. Detection of trace amounts of flavour compounds by SPME was possible by sampling of the 50-ml erlenmeyer cultures through a septum-sealed side inlet. The resulting chromatographic peaks, however, remained small and difficult to integrate.

These results indicate that SPME is not suitable for an extensive screening of the flavour production, due to the very low yields, which cause even the high sensitivity of SPME to be insufficient. Due to the numerous advantages of the technique, enabling a fast qualitative sampling of a high amount of cultures, SPME was used in some cases, where high flavour production was noted, to monitor the flavour production.

In most experiments, dynamic headspace sampling of the surface cultures with concentration of the volatiles on Tenax and subsequent desorption with diethyl ether was used to study the production of pyrazines and 2-acetyl-1-pyrroline by *B. cereus* solid-state cultures. Likens-Nickerson extraction (simultaneous steam distillation – solvent extraction) of petri dish and erlenmeyer cultures was performed in the final state of the experiments to determine the remaining concentrations in the agar.

Analysis by GC-MS of the extracts was performed in full scan mode when the yields were sufficient, or in SIM mode (Selected Ion Monitoring) when a higher sensitivity was required, scanning only those ions specific for the target analytes. For the quantification of the amounts of flavour compounds recovered, precise amounts of 2,4,6-trimethylpyridine were added to the samples as an internal standard prior to GC analysis.

3.2.3 Influence of different precursors

In various experiments performed, no flavour volatiles were detectable using the techniques described. The very low amounts of flavour compounds produced hamper an extensive comparison of precursors and culture conditions. In the following, a selection of experiments is presented, each one providing specific information on the origin of the flavour compounds.

In a first stage of the research, *B. cereus* strains DSM 487 en DSM 2896 were tested for the production of cracker-like flavour compounds on PCA medium. These strains proved, however, to be incapable of producing these compounds at detectable amounts when grown on plate count agar supplemented with proline, ornithine or starch. Only traces of pyrazines could be detected, but quantification was not relevant. Different experiments confirmed these results. Therefore, only *B. cereus* strains ATCC 27522 and 14737 were used in further investigations. *B. cereus* strain ATCC 14737 and DSM 2896 are actually catalogued as being equivalent strains, but a higher activity of *B. cereus* ATCC 14737 was found.

An experiment was set up to evaluate the influence of different precursors on the amount of flavour compounds produced by *B. cereus* ATCC 27522 on plate count agar (PCA). All supplements (glucose, starch, lysine, ornithine, glutamic acid) were added to 10 ml PCA to a concentration of 2 %, before medium sterilization. After autoclaving of the medium, the pH values of the culture media were measured. All cultures had a neutral pH (pH 7), except for those with lysine, which alkalinized the medium up to a pH of 11. *B. cereus* growth on lysine-containing medium was limited and developed later than on other media. Supplementation of PCA with glutamic acid yielded an acid medium (pH 3) that did not solidify. Further incubation and sampling was continued as in liquid shaking cultures, although here as well, bacterial growth was limited.

All cultures were sampled by SPME during 30 min at regular intervals during growth. 2,5-Dimethylpyrazine (or 2,6-dimethylpyrazine) was in all cases the most abundant flavour compound detected. A comparison of the amount of 2,5(6)-dimethylpyrazine produced by *B. cereus* ATCC 27522 from different precursors, and as a function of time, is displayed in Figure 3.3. Non-inoculated supplemented media were sampled after 3 days of incubation at 30 °C. No controls were included for media supplemented with glutamic acid or ornithine.

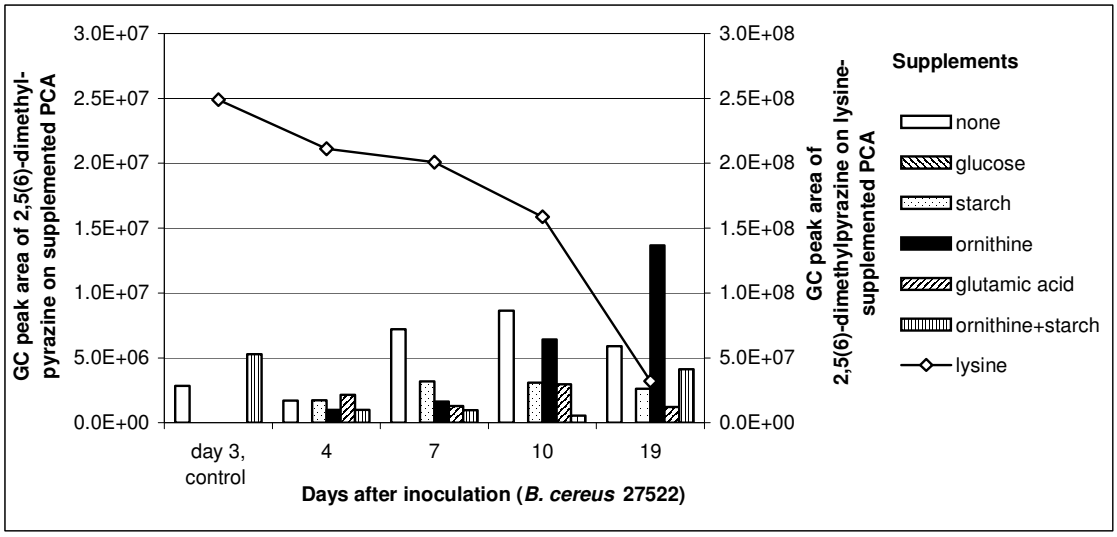
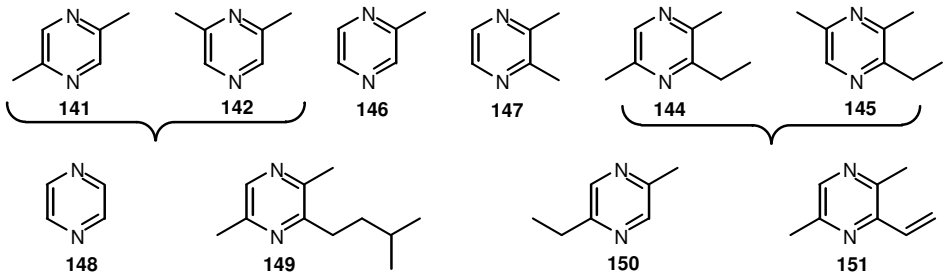


Figure 3.3. Production of 2,5(6)-dimethylpyrazine (**141** and **142**) by *Bacillus cereus* ATCC 27522 on 10 ml PCA, supplemented with different carbohydrates and amino acids, as measured by SPME during 20 days of growth. Controls were sampled on day 3.

The amounts of pyrazines detected from lysine-containing medium are more than 10 times higher than from media with other supplements, and are therefore presented in a different scale (right-hand axis) in Figure 3.3. Besides 2,5(6)-dimethylpyrazine (**141** and **142**), methylpyrazine **146**, 2,3-dimethylpyrazine **147**, 3-ethyl-2,5(6)-dimethylpyrazine (**144** and **145**), pyrazine **148**, 2,5-dimethyl-3-(3-methylbutyl)pyrazine **149**, 2-ethyl-5-methylpyrazine **150** and 2,5-dimethyl-3-vinylpyrazine **151** were detected from lysine-supplemented media, presented in decreasing order of concentration (as measured by GC peak area) (Scheme 3.2).



Scheme 3.2. Pyrazines tentatively identified in the headspace of lysine-supplemented *B. cereus* ATCC 27522 cultures

Different factors, such as the limited growth of *B. cereus* on lysine-containing agar, the high pyrazine release from the controls, and the high pH of the medium, indicate that the formation of these pyrazines on lysine-supplemented plate count agar does most probably not involve an enzymatic action of *Bacillus cereus*. Lysine is a very reactive amino acid in the Maillard reaction, due to the presence of the ε-amino group, and may thus enhance chemical flavour formation. Ornithine is the lower homologue of lysine and is expected to have a similar alkaline effect and a comparable reactivity. Contrary to lysine, ornithine is only commercially available as the monohydrochloride salt and was added as such to the microbial cultures. Therefore, the δ-amino group is in the beginning not available. Proton exchange with other components in the culture medium may cause a 'slow release' of the free δ-amino group and this might explain that the effect of free ornithine can only be measured after a certain period of time. Consequences are an increasing production of 2,5(6)-dimethylpyrazine as a function of time and no direct inhibition of bacterial growth. Previous investigations of Romanczyk et al.[95] also used ornithine hydrochloride in precursor experiments. The high reactivity of lysine and ornithine in Maillard reactions probably leads to the formation of various precursors, which are then converted to pyrazines by chemical reactions. The other precursors tested did not increase the production of pyrazines, as compared to non-supplemented culture media.

In a following experiment, the influence of different precursors on the amount of pyrazines detected in the headspace of *B. cereus* surface cultures was studied by Likens-Nickerson extractions of *B. cereus* ATCC 27522 inoculated petri dish cultures, after incubation for two days at 30 °C, in order to evaluate the production of pyrazines quantitatively. The tested precursors were starch (1 %), lysine (1 %), ornithine (2 %), and proline (2 %) added after medium sterilization, and 1,3-dihydroxyacetone, a sugar degradation product (DHA – 0.1 %), added before and after medium sterilization. Quantification was realized with 2,4,6-

trimethylpyridine as internal standard, and the resulting amounts of methylpyrazine **146**, 2,5(6)-dimethylpyrazine (**141** and **142**), trimethylpyrazine **143** and 3-ethyl-2,5(6)-dimethyl-pyrazine (**144** and **145**) are shown in Figure 3.4.

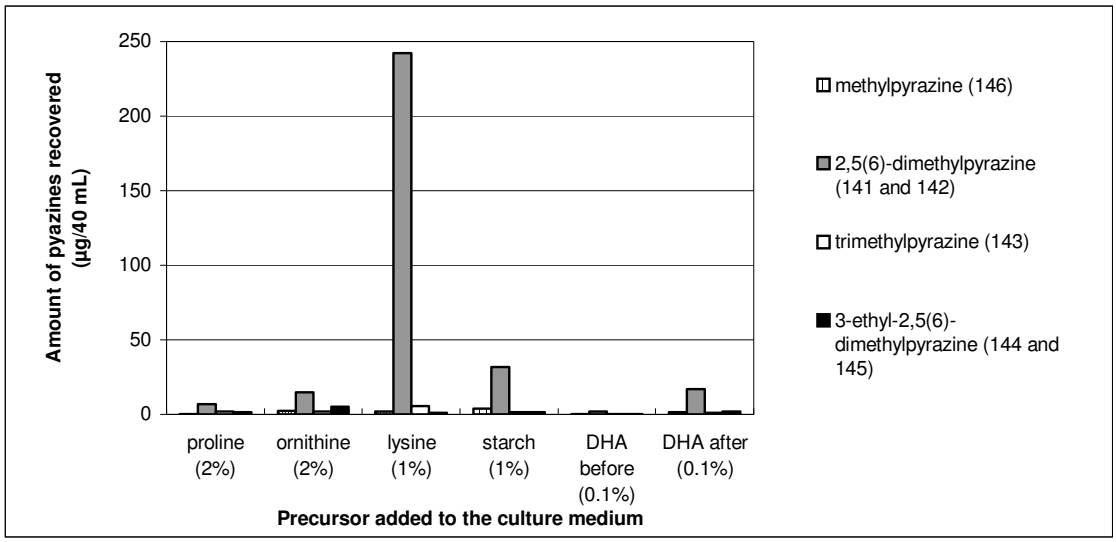


Figure 3.4. Production of different pyrazines by *Bacillus cereus* ATCC 27522 on different supplemented PCA cultures, as measured by Likens-Nickerson extraction of petri dish cultures (2 d, 30 °C). All precursors were added after sterilization, unless otherwise indicated.

Again, the high amounts of especially 2,5(6)-dimethylpyrazine (**141** and **142**) from lysine-containing media are obvious. Ornithine was a better precursor than proline for the different pyrazines, while the presence of starch increased especially the production of 2,5(6)-dimethyl-pyrazine. 1,3-Dihydroxyacetone inhibited bacterial growth when added before medium sterilization, and almost no pyrazines were formed from these cultures. 2-Acetyl-1-pyrroline was not detected among the volatiles of these bacterial cultures.

The combination of ornithine and starch has been shown to enhance flavour production by *B. cereus* cultures.[170] Therefore, *Bacillus cereus* ATCC 27522 was grown on plate count agar (50 ml) supplemented with 2 % ornithine and 2 % starch. The production of volatiles was monitored as a function of time, using SPME (1 h, rt). The average of two replicate bacterial

cultures was compared with a non-inoculated control. The results are shown in Figure 3.5 (*B. cereus* cultures) and Figure 3.6 (control).

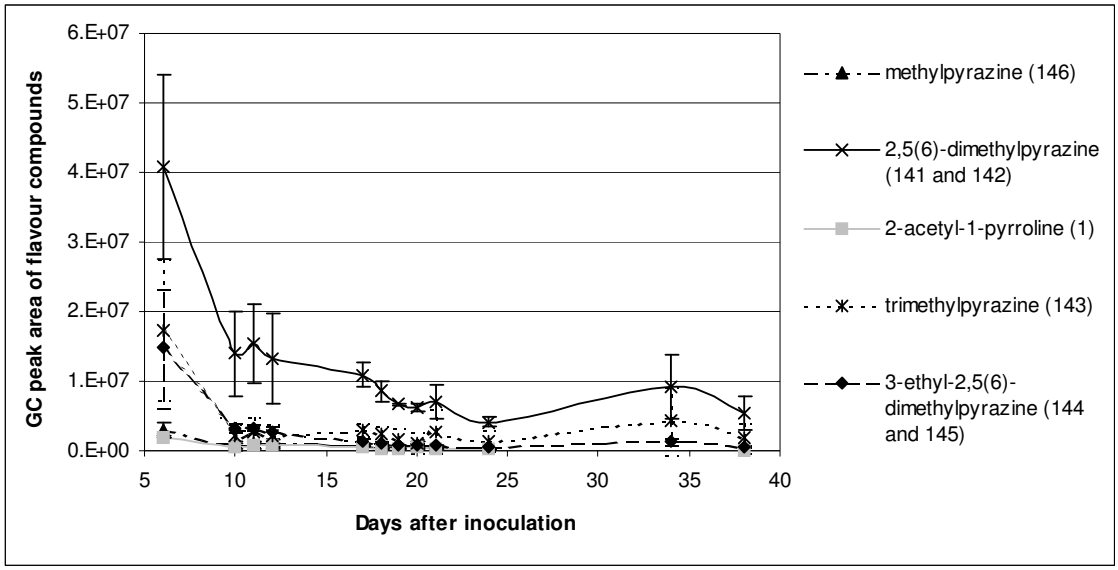


Figure 3.5. Headspace volatiles as a function of time, measured by SPME (1 h, rt), of 50-ml PCA cultures of *B. cereus* ATCC 27522, supplemented with 2 % ornithine and 2 % starch (added after medium sterilization). Average of two replications, with standard deviations.

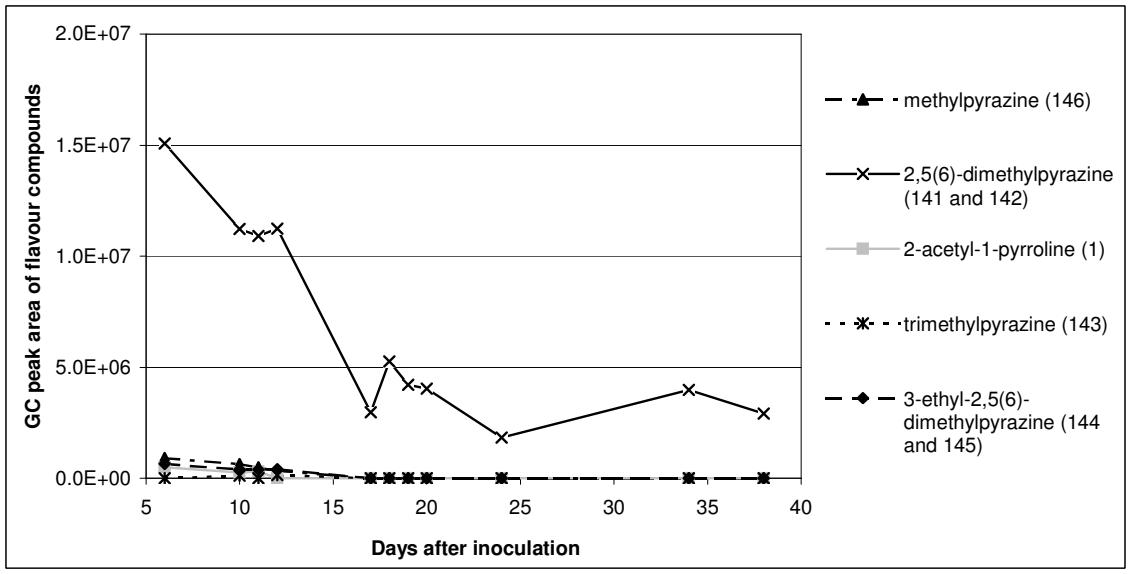


Figure 3.6. Headspace volatiles as a function of time, measured by SPME (1 h, rt), of 50-ml blank PCA, supplemented with 2 % ornithine and 2 % starch (added after medium sterilization).

In the headspace of the control flask as well as of the bacterial cultures, 2,5(6)-dimethyl-pyrazine (**141** and **142**) was the most abundant flavour compound, and the amounts of all pyrazines mainly decreased as a function of time. A similar time course was found for the bacterial cultures and for the control. The amounts of pyrazines detected in the headspace of *B. cereus* cultures were somewhat higher than in the control flask, but the deviation between the two replicate cultures was considerable. A similar deviation is possible for control flasks, but only one control flask was included in this experiment. The results do not allow the conclusion of an enzymatic pyrazine formation. Low amounts of 2-acetyl-1-pyrroline **1** were detected in the bacterial cultures and in the control flask.

3.2.4 Quantification and reproducibility of pyrazine production

Supplementation with ornithine increased the formation of pyrazines from *Bacillus cereus* cultures. However, the catalytic activity of the microorganisms is not completely clear. To be able to evaluate the reproducibility and quantitative production of pyrazines on ornithine-supplemented medium, a new experiment was constructed. The headspace pyrazine concentration of non-inoculated, ornithine-supplemented plate count agar was compared with the production of pyrazines by *B. cereus* ATCC 14737 and *B. cereus* ATCC 27522 on the same ornithine-supplemented medium. Each culture was grown in triplicate and three similar control flasks were sampled. The production of pyrazines on ornithine-supplemented medium increased with time, as was found before (Figure 3.3). The results of the 5th and the 6th headspace sample, which yielded the highest amounts of pyrazines, are displayed in Figure 3.7 and Figure 3.8, respectively.

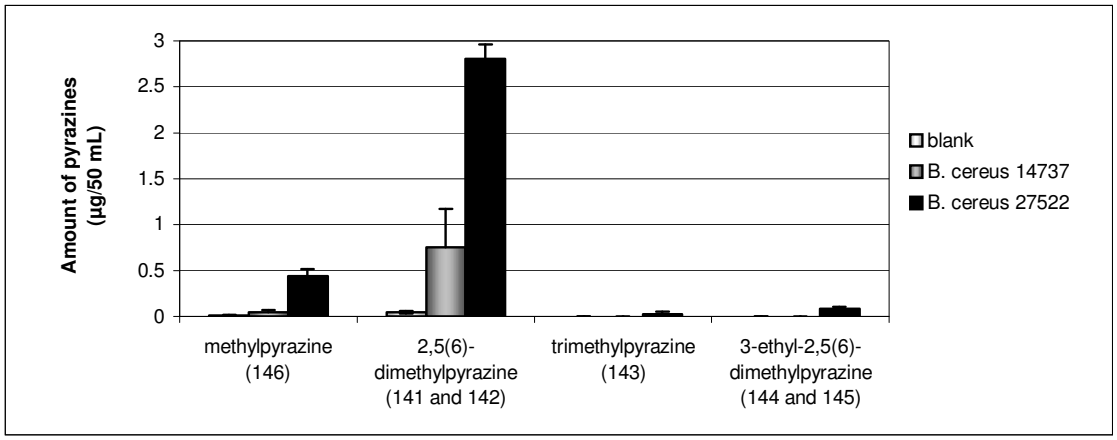


Figure 3.7. Amounts of pyrazines (µg/50 ml) detected in the headspace of blank and *B. cereus* inoculated plate count agar, supplemented with 2 % ornithine (added after medium sterilization). Results of the 5th headspace sample (6-day dynamic headspace started on the 10th day after inoculation). Error bars represent twice the standard deviation (n = 3).

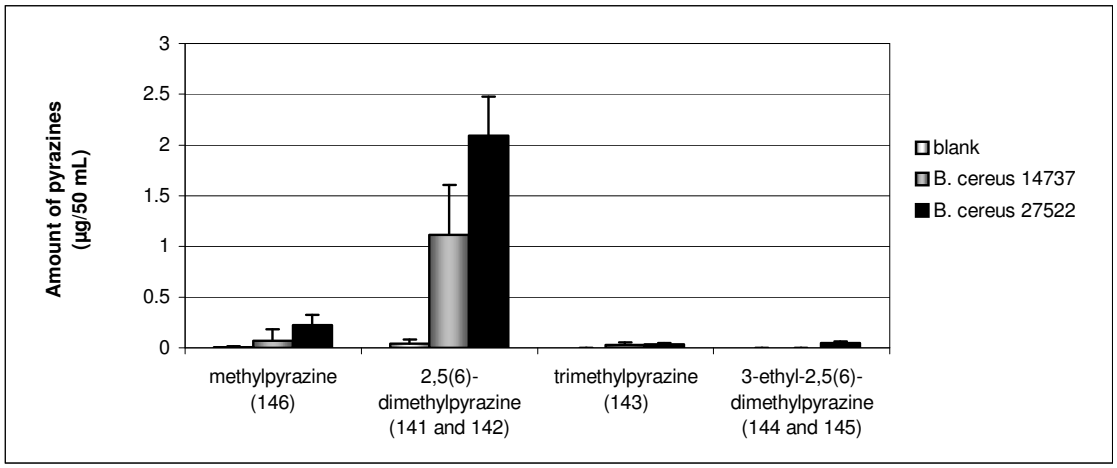


Figure 3.8. Amounts of pyrazines (µg/50 ml) detected in the headspace of blank and *B. cereus* inoculated plate count agar, supplemented with 2 % ornithine (added after medium sterilization). Results of the 6th headspace sample (6-day dynamic headspace started on the 16nd day after inoculation). Error bars represent twice the standard deviation (n = 3).

From these results it can be concluded that the tested *Bacillus* strains significantly enhanced the formation of pyrazines, especially of 2,5(6)-dimethylpyrazine (**141** and **142**), on ornithine-containing medium. *B. cereus* ATCC 27522 was a more efficient strain for pyrazine production than *B. cereus* ATCC 14737, and there was a significant difference with the

control. 2-Acetyl-1-pyrroline **1** was not detected in the headspace samples from this experiment.

It is generally believed that most pyrazines associated with microbial fermentations are not formed by enzyme-catalyzed reactions. The metabolic activities of the microorganisms rather generate various precursors, such as α-acetolactate, acetoin, free amino acids, ammonia, etc., which are converted to pyrazines by non-enzymatic chemical reactions.[149] The formation of efficient precursors from ornithine for the chemical formation of pyrazines is probably enhanced by the activities of *Bacillus cereus*.

A similar experiment was conducted to study the influence of starch supplementation (1 %). The yields of methylpyrazine **146** and 2,5(6)-dimethylpyrazine (**141** and **142**) for three consecutive headspace samples are displayed in Figure 3.9. In this graph, high standard deviations are found, showing a poor reproducibility. This may be due to the heterogeneous distribution of starch in the medium. In later stages of the experiment (headspace sample 2), significant amounts of pyrazines were detected in the headspace of non-inoculated medium. No significant difference between *B. cereus* strain ATCC 27522 and 14737 was found. Therefore, also in this case, an enzymatic pyrazine formation by the microorganisms is not probable.

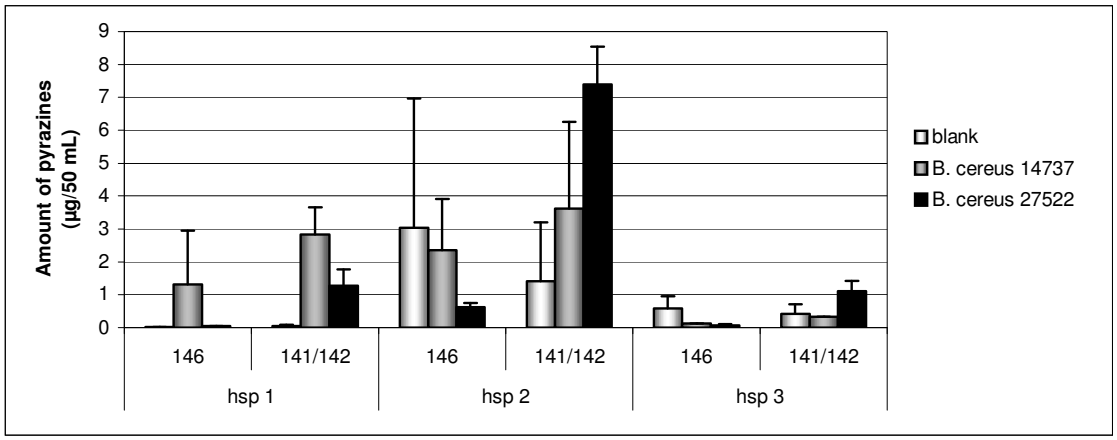


Figure 3.9. Amounts of methylpyrazine (**146**) and 2,5(6)-dimethylpyrazine (**141** and **142**) (µg/50 ml) detected in the headspace of blank and *B. cereus* inoculated PCA, supplemented with 1 % starch (added after medium sterilization). Results of the 1st (6-day dynamic headspace started on the 1st day after inoculation), 2nd (7 days started on the 7th day) and 3rd (7 days started on the 14nd day) headspace samples. Error bars represent twice the standard deviation (n = 3).

3.2.5 Investigation of the mechanism of 2-acetyl-1-pyrroline formation

The production of 2-acetyl-1-pyrroline **1** was studied by Likens-Nickerson extraction of PCA medium in petri dishes, inoculated with *Bacillus cereus* ATCC 27522 and ATCC 14737, in order to compare the results with the results obtained by Romanczyk and co-workers, using the same experimental procedure.[95] All the culture media were inoculated at the same time and incubated at 32 °C. Each day, the contents of two plates of each strain were combined and subjected to Likens-Nickerson extraction. The results are displayed in Figure 3.10. In the extracts of blank plate count agar petri dishes, no 2-acetyl-1-pyrroline was detected. The production of 2-acetyl-1-pyrroline increased, starting from day 1 until day 3 after inoculation, and decreased afterwards. *B. cereus* strain 27522 showed the highest production of the rice flavour compound. These results are in agreement with the findings of Romanczyk et al.,

although significantly higher amounts of 2-acetyl-1-pyrroline were reported (i.e. 87 µg/kg by *B. cereus* ATCC 27522 as compared to 26 µg/kg).[95]

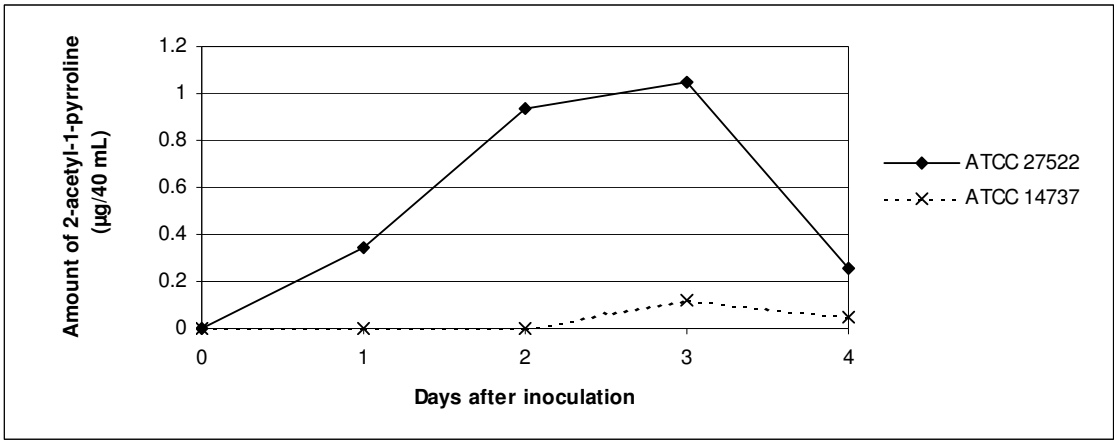
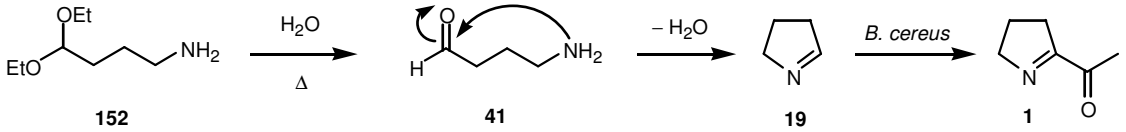


Figure 3.10. Production of 2-acetyl-1-pyrroline **1** by two *B. cereus* strains grown on plate count agar petri dishes (at 32 °C), as measured by Likens-Nickerson extraction

As compared to pyrazines, the production of the rice flavour compound 2-acetyl-1-pyrroline **1** is of special interest because of the extraordinary flavour properties of this compound (cf. Chapter 1). In most experiments, this compound was only detected in trace amounts and only in some cultures, which hampers a detailed study of its production. Previous experiments showed an enhancement of the production of 2-acetyl-1-pyrroline by *B. cereus* by the addition of proline and ornithine to the growth medium.[170]

In-depth studies of the mechanism of formation of 2-acetyl-1-pyrroline in model Maillard reactions showed that proline and ornithine are precursors of the thermal formation of 2-acetyl-1-pyrroline **1**, through the formation of 1-pyrroline (Chapter 1.2.1). Also glutamic acid, the biosynthetic precursor of ornithine and proline, enhanced 2-AP-production.[95] The active degradation product of ornithine is 4-aminobutanal **41**, which cyclizes to 1-pyrroline **19**. Therefore, a study of the influence of 4-aminobutanal **41** on the production of cracker-like flavour compounds by *B. cereus* was undertaken since 1-pyrroline itself is difficult to obtain

in pure and stable form. 4-Aminobutanal **41** is commercially available as the diethyl acetal **152**, from which the free aldehyde can be liberated by hydrolysis (Scheme 3.3).



Scheme 3.3. Hypothesis of the formation of 2-acetyl-1-pyrroline **1**, catalyzed by *B. cereus* strains, from 4-aminobutanal diethyl acetal **152** as precursor

Although plate count agar has a neutral pH (7.0) and hydrolysis usually requires acid catalysis, a limited hydrolysis is possible when 4-aminobutanal diethyl acetal **152** is autoclaved, dissolved in the hot medium, or by a possible hydrolytic action of the bacterial strains. To verify this, the hydrolysis of 4-aminobutanal diethyl acetal **152** in aqueous solutions was investigated. Dilute solutions of 4-aminobutanal diethyl acetal **152** in distilled water (0.5 %), without pH adjustment, were extracted with diethyl ether and analysed by GC-MS. Without heating, no 1-pyrroline was detected in the extract. Upon reflux for 20 minutes, about 0.3 % of 4-aminobutanal diethyl acetal **152** was converted to 1-pyrroline **19**. Autoclaving the same solution (121 °C, 1.2 bar, 20 min) resulted in a conversion of about 0.5 %. This indicates a low formation of 1-pyrroline **19** in heated aqueous solutions, which may be able to catalyze the formation of 2-acetyl-1-pyrroline **1** by the bacteria *in situ*.

In a first experiment, 4-aminobutanal diethyl acetal **152** was added to the culture medium prior to sterilization in different concentrations, namely 0.1, 0.2 and 0.4 %. At 0.4 % of 4-aminobutanal diethyl acetal, an intense browning of the PCA medium was noted after sterilization and bacterial growth was inhibited. One control flask, containing 0.1 % of 4-aminobutanal diethyl acetal, was included in the experiment, and the other flasks were inoculated with *B. cereus* ATCC 27522 (two flasks contained 0.1 % 4-aminobutanal diethyl

acetal, one was supplemented with 0.2 % and one with 0.4 % 4-aminobutanal diethyl acetal). Dynamic headspace analysis of the volatiles revealed no significant formation of pyrazines, but 2-acetyl-1-pyrroline was the most important compound in the headspace profile of all cultures. The amounts of 2-acetyl-1-pyrroline detected in the different headspace samples are displayed in Figure 3.11.

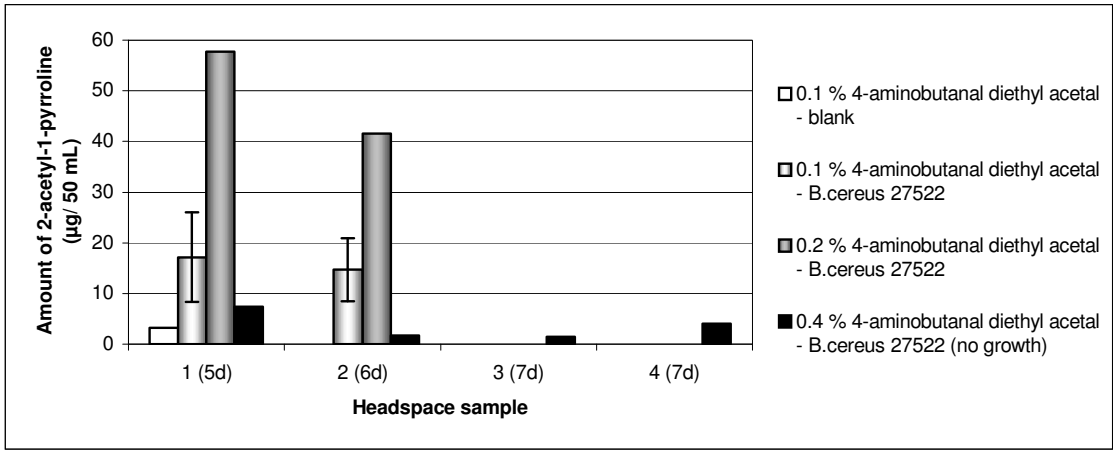
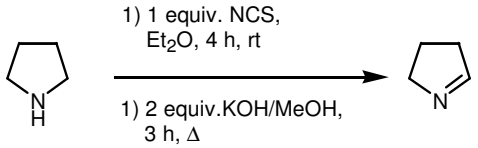


Figure 3.11. Amounts of 2-acetyl-1-pyrroline **1** (µg/50 ml) detected in the headspace of blank and *B. cereus* ATCC 27522 inoculated PCA, supplemented with 0.1, 0.2 or 0.4 % 4-aminobutanal diethyl acetal **152** (added prior to medium sterilization). Results of four consecutive headspace samples of different duration.

These results lead to the conclusion that the addition of 4-aminobutanal diethyl acetal **152** to the plate count agar (prior to sterilization) substantially increased the formation of 2-acetyl-1-pyrroline **1** by the bacterial cultures. The production of 2-acetyl-1-pyrroline increased with higher amounts of the added precursor, as long as bacterial growth was not inhibited (which was the case when 0.4 % 4-aminobutanal diethyl acetal was added). The production was the highest the first days after inoculation. In the control flasks (0.1 % and 0.4 % 4-aminobutanal diethyl acetal) 2-acetyl-1-pyrroline was present in the headspace and was detected during the complete experiment, but in much lower amounts than in the bacterial cultures.

To further investigate the influence of 1-pyrroline as a precursor, the production of 2-acetyl-1-pyrroline **1** by *B. cereus* ATCC 27522 grown on plate count agar was compared, when supplemented with 4-aminobutanal diethyl acetal **152** (0.2 %) added before sterilization, with 4-aminobutanal diethylacetal **152** (0.2 %) added after medium sterilization and with 1-pyrroline **19** (0.1 %) added after medium sterilization. 1-Pyrroline **19** is an unstable compound, since a trimer is formed. Therefore, it was synthesized from pyrrolidine **153** (Scheme 3.4),[171] distilled and used immediately at a low concentration of 0.1 %.



153 **19**

Scheme 3.4. Synthesis of 1-pyrroline **19** from pyrrolidine **153**, according to a procedure developed for the synthesis of 2,3,4,5-tetrahydropyridine [173]

All cultures were grown and analysed in duplicate, and duplicate controls were included. Three consecutive headspace samples of five days were collected, and after this, all cultures were subjected to a Likens-Nickerson extraction. 2-Acetyl-1-pyrroline **1** was the most important headspace constituent in all the cultures and control flasks. Methylpyrazine **146** and 2,5(6)-dimethyl-pyrazine (**141** and **142**) were detected in trace amounts, especially in those cultures where the production of 2-acetyl-1-pyrroline **1** was lower. The results of the production of 2-acetyl-1-pyrroline **1** are shown in Figure 3.12. The results of the first headspace samples showed a significant deviation, but the second and the third headspace samples yielded very reproducible amounts of 2-acetyl-1-pyrroline **1**.

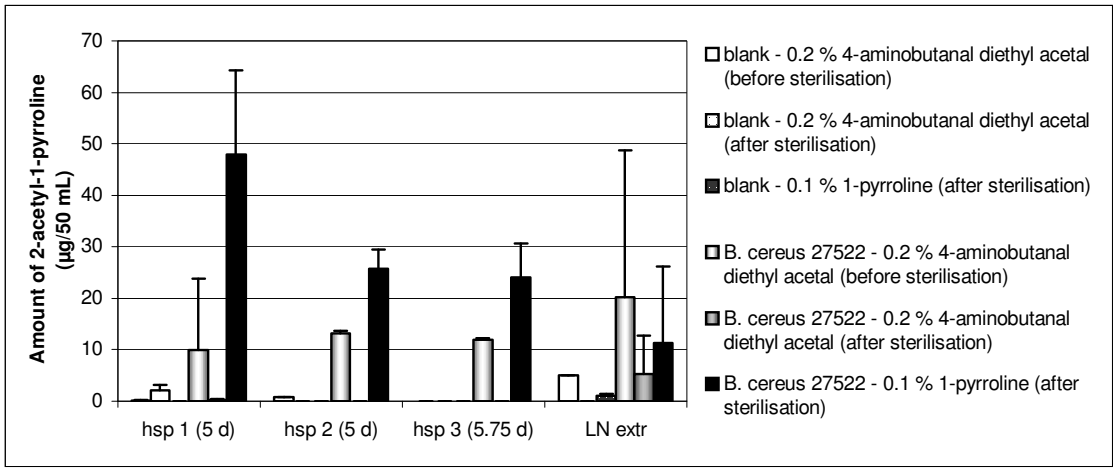
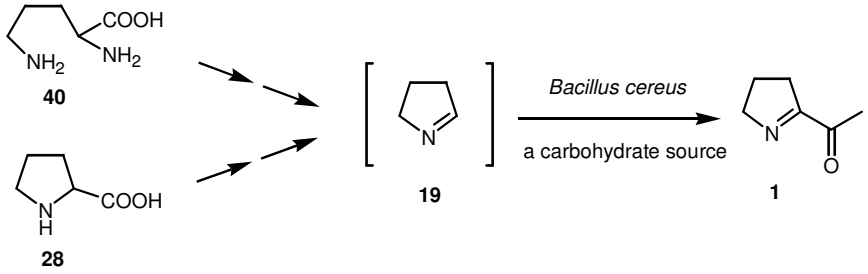


Figure 3.12. Amounts of 2-acetyl-1-pyrroline **1** (µg/50 ml) detected in the headspace of blank and *B. cereus* ATCC 27522 inoculated PCA, supplemented with 0.2 % 4-aminobutanal diethyl acetal **152** (added before or after medium sterilization) or with 0.1 % 1-pyrroline **19** (added after medium sterilization). Results of three consecutive headspace samples and of final Likens-Nickerson (LN) extraction. Error bars represent twice the standard deviation (n = 2).

A significant production of 2-acetyl-1-pyrroline **1** was measured in the *Bacillus cereus* ATCC 27522 cultures supplemented with 4-aminobutanal diethyl acetal **152** prior to medium sterilization, and especially in the cultures supplemented with 1-pyrroline **19**. No 2-acetyl-1-pyrroline was formed in the cultures supplemented with 4-aminobutanal diethyl acetal **152** after medium sterilization. Only traces of 2-acetyl-1-pyrroline **1** were detected in the blank media.

These results indicate that a heat-induced transformation of 4-aminobutanal diethyl acetal **152** is necessary before it can be efficiently converted to 2-acetyl-1-pyrroline **1** by the bacteria. As is shown in Scheme 3.3, the heat treatment is necessary to bring about the hydrolysis of 4-aminobutanal diethyl acetal **152** followed by the cyclization of 4-aminobutanal **41** to 1-pyrroline **19**. Without heat treatment, 4-aminobutanal diethyl acetal **152** was not converted to 1-pyrroline **19**, and no 2-acetyl-1-pyrroline **1** was formed (as is the case for the bacterial

cultures with addition of 4-aminobutanal diethyl acetal after medium sterilization). With heat treatment, but without bacterial activity (i.e. in the control flasks), 1-pyrroline **19** was formed and was detected in the gas chromatograms, but no 2-acetyl-1-pyrroline **1** was formed. Only those bacterial cultures where 4-aminobutanal diethyl acetal was converted to 1-pyrroline by heat-induced hydrolysis, or where 1-pyrroline was added as such, produced 2-acetyl-1-pyrroline. These findings strongly suggest that the production of 2-acetyl-1-pyrroline **1** occurs via the acetylation of 1-pyrroline **19** (which can be deduced from ornithine **40** or proline **28** in common culture media) (Scheme 3.5). This pathway is similar to the one described for the production of 2-acetyl-1-pyrroline **1** and 6-acetyl-1,2,3,4-tetrahydropyridine **3** by *Lactobacillus* sp. (cf. Chapter 1 - Scheme 1.18). Although the actual acylating C-2 intermediate is not known, a potential candidate is the acylating cofactor acetyl-CoA.



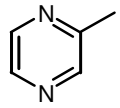
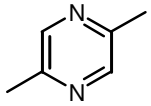
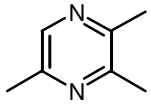
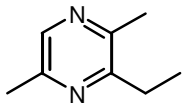
Scheme 3.5. Proposed pathway for the formation of 2-acetyl-1-pyrroline **1** by *Bacillus cereus* ATCC 27522 and ATCC 14737

The results of the Likens-Nickerson extraction suggest different conclusions and are rather irreproducible. During this simultaneous steam distillation – solvent extraction, the cultures are boiled in water for more than one hour. Under these conditions, additional reactions are probable, including additional hydrolysis of 4-aminobutanal diethyl acetal **152** and additional flavour formation, and the results of these extractions are therefore unreliable. The low reproducibility is probably due to variations in boiling time, since excessive foaming of the bacterial cultures during boiling hampered a careful timing.

3.3 Conclusion

In the headspace of bacterial surface cultures of *Bacillus cereus* strain ATCC 27522 and, to a lesser extent of strain ATCC 14737, several alkylpyrazines were detected. These pyrazines are shown in Table 3.1 with their flavour properties. Especially trimethylpyrazine (**143**) and 3-ethyl-2,5(6)-dimethylpyrazine (**144** and **145**) have the most interesting flavour properties, but they were formed in the lowest quantities.

Table 3.1. Pyrazines identified in the headspace of *Bacillus cereus* surface cultures with their flavour properties and retention indices (RI) [172]

Compound	Structure	Odour description	Odour threshold (ng/l, air)	RI (HP-5)	RI [172]
methylpyrazine	 **146**	nutty, green	> 2000	818	820
2,5-dimethylpyrazine	 **141**	nutty	1720	910	909
trimethylpyrazine	 **143**	roasted	50	1000	1000
3-ethyl-2,5-dimethylpyrazine	 **144**	earthy, roasted	0.011	1089	1083

Many experiments were conducted to evaluate the production of these Maillard flavour compounds by *Bacillus cereus* in small-scale solid-state fermentations. The production of pyrazines on plate count agar was very low, and the use of efficient precursors was necessary to increase the flavour production to quantifiable amounts. Supplementation with ornithine

and starch increased the production of pyrazines, of which 2,5(6)-dimethylpyrazine was quantitatively the most important one. The same pyrazines detected in the headspace of *Bacillus cereus* strains were also found in the headspace of sterile controls, especially of lysine-supplemented media. Therefore, the formation of these pyrazines does most probably not involve an enzymatic action of the *Bacillus cereus* strains. The metabolic activities of the microorganisms rather generate various precursors, which are converted to pyrazines by non-enzymatic chemical reactions, and which are increasingly formed upon supplementation with, among others, ornithine and starch.

2-Acetyl-1-pyrroline **1**, a flavour compound with extraordinary properties, was generally produced in very low amounts, which were difficult to detect. Only Likens-Nickerson extraction of sufficient amounts of bacterial culture allowed its detection. This simultaneous steam distillation - solvent extraction was shown, however, to induce thermal changes in the volatiles, and does not yield reliable results on flavour production. Careful choices of precursors showed that these particular *Bacillus cereus* strains catalyzed the formation of the rice flavour compound by the acetylation of 1-pyrroline, a degradation product of proline or ornithine. A clear biological catalysis in the formation of 2-acetyl-1-pyrroline from 1-pyrroline was demonstrated.

3.4 Materials and methods

3.4.1 Microorganisms

Bacillus cereus strains were bought from the DSMZ culture collection (*Deutsche Sammlung von Mikroorganismen und Zellkulturen*), or from ATCC (*American Type Culture Collection*) in the form of freeze-dried cultures. Equivalent strains are shown in Table 3.2.

Table 3.2. *Bacillus cereus* strains, equivalent from ATCC and DSM culture collections

ATCC	DSM
27522	-
14737	2896
10702*	487

* not tested here

3.4.2 Culture media

Standard Plate Count Agar (PCA) (*Oxoid Ltd.*) was used as the production medium, and sometimes also for growth and storage of the bacteria. For cultivation, proliferation and storage of the strains, the bacteria were grown as surface cultures on nutrient agar (NA) (*Oxoid Ltd*.) plates, and stored at 4 °C. The cultures were reinoculated periodically (at least every two months) in order to maintain their viability and activity. Nutrient broth (NB) (*Difco Laboratories*) was used to grow the bacteria before inoculation. Medium YMPG was composed to test the formation of Maillard flavour compounds by autoclaving the culture broth. The composition and pH of the culture media used are displayed in Table 3.3.

All culture broths were sterilized at 121 °C for 20 minutes and were allowed to cool down before manipulation.

Table 3.3. Composition (g/l) and pH of the culture media applied in the experiments

	Plate Count Agar (PCA)	Nutrient Agar (NA)	Nutrient Broth (NB)	YMPG
Glucose	1.0	-	-	1.0
Yeast extract	2.5	2.0	2.0	0.5
Malt extract	-	-	-	1.0
Lab-Lemco powder	-	1.0	1.0	-
Bacteriological peptone	-	5.0	5.0	0.5
Pancreatic digest of casein	5.0	-	-	-
Bacteriological agar	20.0	20.0	-	-
NaCl	-	5.0	5.0	-
pH	7.0 ± 0.2	7.4	7.4 ± 0.2	6.2

3.4.3 Formation of flavour compounds in autoclaved culture media

Plate count agar and YMPG culture media were prepared as described above. Aliquots (50 ml) of these media were heated at 1.2 bar for 20 minutes (121 °C), at 1.2 bar for 40 minutes, or at atmospheric pressure for 20 minutes (100 °C). After heating, the media were sampled by Solid Phase Microextraction (PDMS, 100 µm, roomtemperature) for 30 or 60 minutes. The liquid medium YMPG was also extracted with diethyl ether (two times 5 ml). To study the influence of the pH, the pH of the media was adjusted with dilute NaOH (4 %) prior to medium sterilization, to obtain pH values of 6, 8, 10 and 12. Absorption of 100 times diluted samples was measured using a Cary 50 UV Visible spectrophotometer (*Varian*).

3.4.4 Inoculation

Inoculation of solid sterile media with *Bacillus cereus* was performed with liquid 1-day old *B. cereus* cultures. One day before inoculation, 50 ml of nutrient broth was inoculated with an inoculation needle, and incubated for 24 h at 30 °C (rotating speed 200 rpm). Inoculation of

solid media was performed by spreading 0.5 or 0.1 ml of this liquid *B. cereus* culture over 50 or 10 ml of plate count agar, respectively.

3.4.5 Addition of supplements

Supplements L-proline **28**, L-ornithine **40** monohydrochloride, L-lysine **86**, L-glutamic acid, 4-aminobutanal diethyl acetal **152**, 1,3-dihydroxyacetone **118** dimer, starch (soluble), and D-glucose **85** were added before or after medium sterilization. When supplements were added to the medium after sterilization, they were sterilized in the form of concentrated solutions by filter sterilization. Starch was added without sterilization in solid form immediately after autoclaving to the warm agar.

3.4.6 Sampling of bacterial surface cultures

3.4.6.1 Dynamic headspace sampling

For cultivation in conical flasks, 50 ml of sterilized medium was poured into 250-ml conical flasks and the agar was allowed to solidify. The cultures were inoculated with 0.5 ml of *B. cereus* culture broth and were closed with aeration devices designed for headspace sampling. After two days of incubation at 30 °C, the flasks were placed at room temperature for dynamic headspace sampling. Purified air was swept over the surface cultures at a flow rate of 30 ml/min. Volatiles were adsorbed on Tenax TA [poly-bis(2,6-diphenyl)phenylether], packed into pyrex glass tubes of 21.5 cm length and 0.8 cm i.d. at its narrow part and 1.4 cm i.d. at the wide parts at both ends. The tubes were packed with 500 mg of Tenax TA 60/80 mesh (*Alltech*) between two glasswool plugs. Prior to headspace sampling, the Tenax tubes were conditioned at 250 °C for two hours under nitrogen flow. At regular time intervals, the Tenax tubes were desorbed by eluting with three volumes of 2.5 ml diethyl ether. Effluents

were collected in 10-ml conical glass tubes and concentrated until 500 µl under a gentle nitrogen flow.

3.4.6.2 Likens-Nickerson extraction

For the simultaneous steam distillation – solvent extraction (SDSE), specific Likens-Nickerson glassware was used (Figure 3.13). The culture medium with bacterial cultures from two petri dishes (40 ml) or from one conical flask (50 ml) was placed in a 500-ml round-bottom flask, and 300 ml of distilled water was added (W). On the other side of the distillation device, 20 ml of diethyl ether was placed in a peer-shaped 25-ml flask (S). To both flasks, boiling chips were added, and the internal standard collidine (as a dilute solution in water) was added to the aqueous sample. A condenser was placed (F) and both flasks were heated. From the moment the water boiled, steam and solvent were allowed to circulate for 1.5 h. After cooling down, the solvent with analytes was disconnected from the system, dried with $MgSO_4$, filtered and concentrated under a gentle nitrogen-flow.

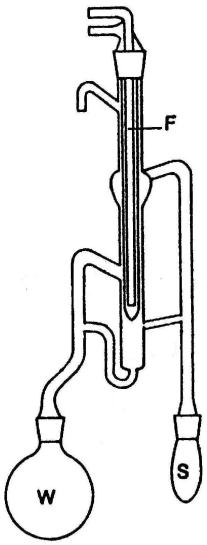


Figure 3.13. Schematic representation of Likens-Nickerson steam distillation – solvent extraction device

3.4.6.3 Solid Phase Microextraction

Solid Phase Microextraction makes use of a fused silica fiber, coated with a stationary phase. The extracted amount for a certain compound depends on the equilibrium of the distribution of that compound between the sample matrix, the sample headspace and the fiber coating (Figure 3.14).

For SPME sampling, the bacterial cultures were grown on 10 ml plate count agar in 22-ml glass vials, closed with a PTFE-silicone septum (*Supelco*). During extraction, the fiber was exposed to the headspace of the sample at room temperature during 30 minutes. Desorption of the absorbed/adsorbed compounds was performed in the injector of the GC at 250 °C for two minutes. A comparison was made for two different fibers (*Supelco*): 100 µm PDMS (polydimethylsiloxane) and DVB/Car/PDMS [divinylbenzene and carboxen (carbon molecular sieve) on polydimethylsiloxane]. Polydimethylsiloxane is a viscous liquid and the extraction process is mainly based on absorption, while divinylbenzene and carboxen are porous solids adsorbing the volatiles.

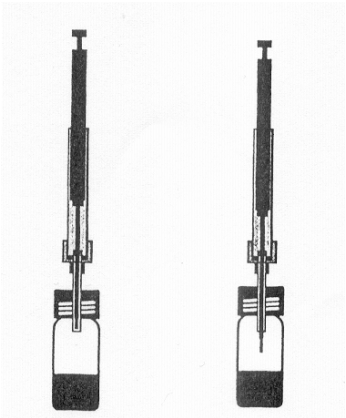


Figure 3.14. Schematic representation of headspace SPME-sampling

3.4.7 Analysis of samples

3.4.7.1 Quantification

Response factors towards the internal standard collidine (2,4,6-trimethylpyridine) were calculated for each analyte in scan as well as in SIM mode. For this purpose, exact amounts of standard solutions of methylpyrazine **146**, 2,5-dimethylpyrazine **141**, 2,6-dimethylpyrazine **142**, trimethylpyrazine **143**, 2,5(6)-dimethyl-3-ethylpyrazine (**144** and **145**), the internal standard collidine and 2-acetyl-1-pyrroline **1** were injected and detected three times in full scan and in SIM mode in order to obtain average values. This was repeated at different concentrations.

The equations used to calculate the amounts of flavour compounds recovered from the bacterial cultures are given below.

$$\text{Amount (x)} = \frac{PA(x)}{PA(IS)} \times \text{amount (IS)} \times RF(x)$$

with x = flavour compound to quantify
IS = internal standard collidine (2,4,6-trimethylpyridine)
PA = peak area

$$RF(x) = \frac{PA(IS) \times \text{purity}(x)}{PA(x) \times \text{purity}(IS)}$$

(when injecting equal amounts of x and IS)

Response factors for full scan and SIM mode are displayed in Table 3.4.

Table 3.4. Response factors for the quantified flavour compounds towards internal standard collidine (2,4,6-trimethylpyridine)

Flavour compound	Response Factor (scan mode)	Response Factor (SIM mode)
methylpyrazine	1.52	1.48
2,5-dimethylpyrazine	1.22	1.52
trimethylpyrazine	1.35	1.11
2,5(6)-dimethyl-3-ethylpyrazine	1.70	0.86
2-acetyl-1-pyrroline	2.78	7.00

In as many cases as possible, cultures were grown in triplicate. In this case, error bars are added in the graphs, representing twice the standard deviation. When duplicate cultures were sampled, this is specified in the graph captions.

3.4.7.2 GC-MS analysis

For the analysis of the extracts, a Hewlett-Packard 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector-Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) Injector (*Gerstel*), and a HP5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 µm) was used. Working conditions were: injector 250 °C; transfer line to MSD 260 °C; oven temperature start 35 °C, hold 5 min, programmed from 35 to 60 °C at 2 °C min^{-1} and from 60 to 250 °C at 20 °C min^{-1}, hold 5 min; carrier gas (He) 1 ml min^{-1}; splitless; ionization EI 70 eV; acquisition parameters in full scan mode: scanned m/z 40-200 (0-20 min), 40-400 (> 20 min). For SIM (Selected Ion Monitoring) mode the acquisition parameters are displayed in Table 3.5.

Table 3.5. Acquisition parameters for GC-MS analysis of headspace extracts in SIM mode

Compound	Time frame (min)	Selected ions (m/z)
methylpyrazine	1.0-11.0	94, 67, 53
2,5(6)-dimethylpyrazine	11.0-14.2	108, 81, 42
2-acetyl-1-pyrroline	14.2-17.0	83, 111, 69
collidine	17.0-19.0	121, 106, 79
trimethylpyrazine	19.0-19.5	122, 81, 42
3-ethyl-2,5(6)-dimethylpyrazine	19.5-32.0	136, 135, 108

3.4.8 Synthesis of 1-pyrroline

The synthesis of 1-pyrroline **19** was performed according to a method developed for the synthesis of 2,3,4,5-tetrahydropyridine.[173] In a dried 250-ml flask, 0.25 mol (33.3 g) of *N*-chlorosuccinimide was dissolved in 220 ml dry diethyl ether. Equimolar amounts of pyrrolidine **153** (17.8 g) dissolved in 10 ml diethyl ether were carefully added, while stirring in an ice bath. The combined reagents were allowed to react for 4 h at room temperature. After filtration and evaporation to one third of the volume, two equivalents of potassium hydroxide in methanol (30 g KOH in 120 ml methanol) were added and the mixture was refluxed for 3 h. The reaction mixture was poured in 100 ml of water, extracted with diethyl ether (two times 50 ml), washed with water (100 ml) and dried ($MgSO_4$). The yields of 1-pyrroline **19** were low (20 %, 3.4 g) since remainings of methanol had to be evaporated, leading to substantial losses of the volatile 1-pyrroline **19**.

4 MELANOIDINS AND THEIR ROLE IN FLAVOUR GENERATION AND FLAVOUR BINDING

4.1 Melanoidins and the search for their structural properties

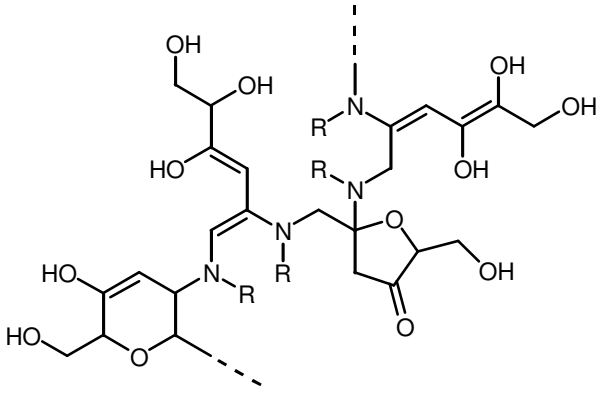
In the final stages of the Maillard reaction brown-coloured nitrogenous polymers are formed, known as melanoidins. Their formation is the result of a complex network of reactions, which are easily influenced by the available substrates and by the reaction conditions. As a result, the extremely complex and heterogeneous nature of melanoidins makes it very difficult to unravel their structures and chemical properties. Despite extensive studies, it has not been possible to isolate or completely characterize a single melanoidin, if possible at all. Still, melanoidins are important ingredients of the human diet, since they occur in many food products, such as coffee, malt, bread, breakfast cereals, roasted meat and soy sauce.[174]

Because of the complexity of real food systems, most studies on melanoidins have focused in the first place on model browning reactions of a reducing sugar with an amino compound. However, variations in the reaction conditions of the melanoidin preparation, such as water content, pH, temperature and time of heating, largely influence the resulting melanoidin structure and therefore complicate a comparative evaluation of the different research results.[175]

Three different analytical strategies can be distinguished in the vast number of experiments performed to gain more insight into the chemical structures of melanoidins. Direct spectroscopic analysis of complex melanoidin fractions has not yet succeeded in giving clear information on the structural domains of melanoidins, due to the structural diversity of the polymers and the very complex spectra thus obtained. Recently, some research has been devoted to the identification of low molecular weight coloured compounds as possible monomers of melanoidins, and to the polymerization routes that might lead to melanoidin-like

chromophores of high molecular weight. A third strategy comprises the chemical or thermal degradation of melanoidins, followed by the identification of the decomposition products formed. It is, however, difficult, to link the structure of the decomposition products directly with the original structure of the melanoidin, due to the inevitable formation of artefacts.

Kato and Tsuchida performed pyrolysis and oxidation experiments for structural investigations on melanoidins prepared from glucose or xylose with butylamine or ammonia in an aqueous system.[176] From these results, it was concluded that it is unlikely that the major repeating unit of these melanoidins consists of an aromatized ring structure, although it may be possible to form some aromatic structures in the melanoidins under more drastic reaction conditions. The hypothetical structure **154**, displayed in Scheme 4.1, showed the best fit with the results. However, the strong absorption of melanoidins in the visible region cannot be explained by such a structure.[174]



154

Scheme 4.1. Melanoidin backbone structure proposed by Kato and Tsuchida [176]

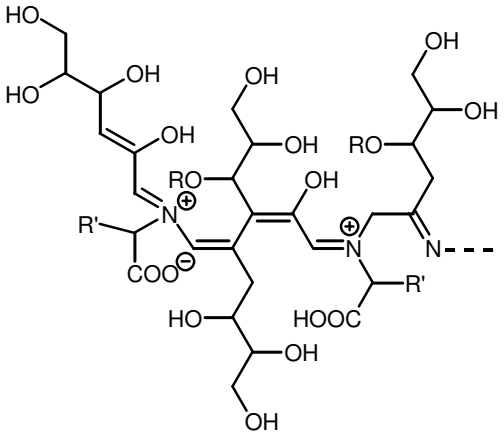
Experiments using ^{13}C- and ^{15}N-CP-MAS NMR of glucose/glycine melanoidins enriched with ^{13}C or ^{15}N showed that glycine was incorporated to a great extent into the melanoidins and indicated the presence of conjugated enamines.[177] The authors stated that pyridine and

pyrazine-type nitrogen atoms were not present in the melanoidins, but they were uncertain on the presence of pyrrole-type nitrogen. The content of furans and other heterocyclic compounds was assumed to be rather low. Another series of CP-MAS ^{15}N-NMR experiments of melanoidins prepared from xylose/glycine and glucose/glycine model reactions indicated the presence of nitrogen mainly in the secondary amide form with some nitrogen present as aliphatic amines and/or ammonium ions and some as part of pyrrole or indole structures.[178] CP-MAS ^{13}C-NMR experiments of high molecular weight xylose/glycine melanoidins showed an increase in aromaticity with increasing reaction temperature.[179]

Model melanoidins differ in composition depending on the ratio and type of reactants, and on the reaction conditions applied for their preparation.[175] Still, indications are found that the mechanism of melanoidin formation might display some specificity. For instance, changes in the molar ratio of glucose and glycine in the preparation of melanoidins and in the degree of decarboxylation of glycine were not reflected in the extinction coefficient of the resulting melanoidin. The composition of the melanoidins was insensitive to the composition of the reaction mixture. Four or five molecules of glucose were incorporated in the polymer for every four glycine molecules, resulting in an average empirical formula of $C_8H_{12}N_1O_5$.[175]

Cämmerer and Kroh confirmed that, under the same reaction conditions, the molecular composition of glucose/glycine melanoidins is only negligibly influenced by the molar ratio of the reactants.[180] However, pentoses showed a higher incorporation of sugar in the melanoidins than hexoses, and the elementary composition of the polymer was significantly influenced by the reaction conditions, such as temperature, time, pH and solvent. This makes it difficult to determine a fundamental melanoidin structure and makes it unlikely that a structure **154** as displayed in Scheme 4.1 has general validity. Therefore, a hypothetical melanoidin structure **155** was proposed, as is displayed in Scheme 4.2.[180] This structure contains carbohydrate and amino acid moieties in an equimolar ratio cross-linked via

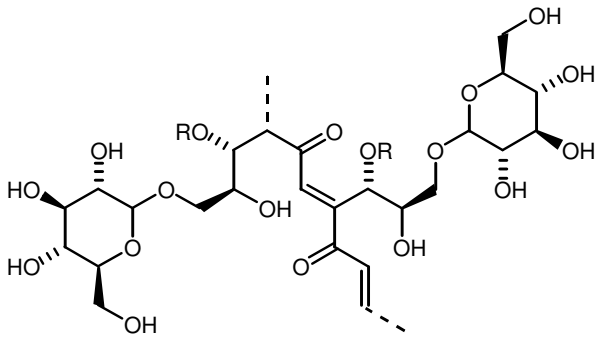
conjugated double bonds. The main and side chains contain many reactive centres and the structure of real melanoidins could be the result of various reactions (such as intramolecular cyclizations, decarboxylations, water eliminations) in the proposed basic framework, depending on the reaction conditions. Spectroscopic results (IR and NMR) supported this structure.



155

Scheme 4.2. Proposal for a general melanoidin structure from 3-deoxyhexosulose and amino acids (R = H or saccharides; R' = amino acid side chain) [180]

Acid hydrolysis experiments of different model melanoidins prepared under water-free reaction conditions, released considerable amounts of monomer carbohydrates.[181] The amount of carbohydrates released, increased with the polymerization degree of the carbohydrate starting material. Apparently, significant amounts of di- and oligosaccharides were incorporated in the melanoidin structure with an intact glycosidic bond, forming side chains to the melanoidin structure. These results are consistent with the melanoidin structure postulated in Scheme 4.2, by including subunits **156** as shown in Scheme 4.3.

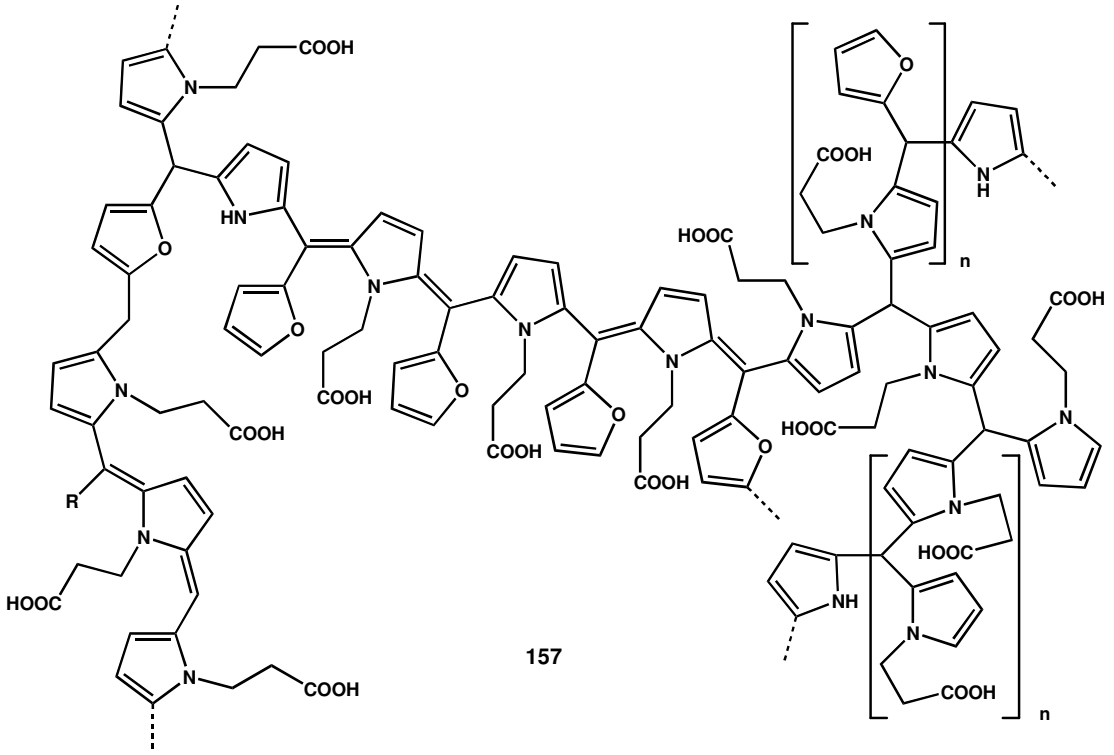


156

Scheme 4.3. Incorporation of 1,6-branched glucose side chains in melanoidins (R = H or saccharides) [181]

Yaylayan and Kaminsky isolated three polymers with a molecular weight between 10,000 and 20,000 Da from the non-dialyzable fraction of a glucose/glycine reaction mixture.[182] Only one of the polymers incorporated nitrogen (empirical formula $C_7H_{11}N_1O_4$), the other two could be classified as caramels, since they had the same empirical formula as glucose. The nitrogen-containing polymer was hypothesized to result from the Amadori compound, and pyrolysis experiments yielded pyrazines, pyrroles, pyridines, furans, etc. These results may support the melanoidin structure **155** proposed in Scheme 4.2.

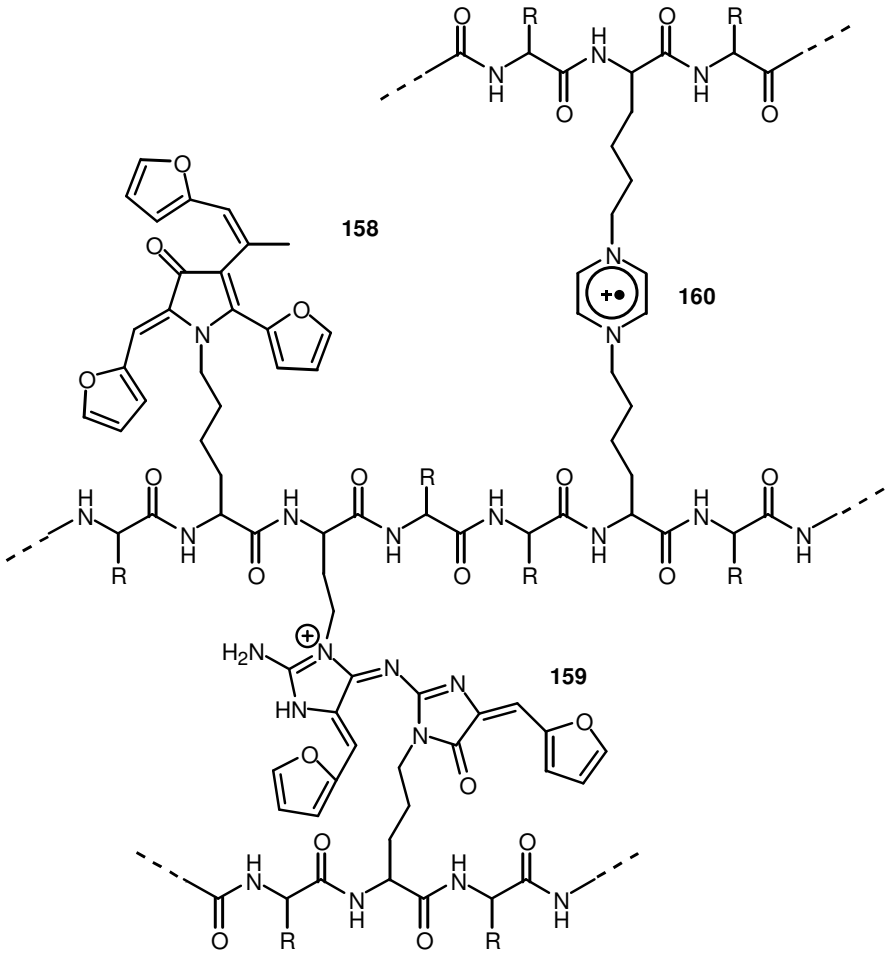
Tressl et al. proposed a different melanoidin structure resulting from the polycondensation of furans and pyrroles, which are important low molecular weight Maillard reaction products from hexoses and pentoses.[183] Model experiments with *N*-substituted pyrroles, furan-2-carbaldehydes and *N*-substituted pyrrole-2-carbaldehydes showed the extraordinary polycondensation activity of these compounds. Therefore, a complex macromolecular melanoidin structure **157** was proposed, integrating these compounds, as well as unsubstituted pyrroles and Strecker aldehydes (Scheme 4.4).



Scheme 4.4. Complex melanoidin structure resulting from the polycondensation of furans and pyrroles [183]

Hofmann postulated that several low molecular weight coloured Maillard reaction products with a restricted mesomeric system are incorporated as substructures into food melanoidins.[184] A red coloured compound **158** with a chromophoric system containing four condensed rings and an amino acid moiety was identified from the reaction of furan-2-carbaldehyde with amino acids.[185] By this reaction, the ε-amino group of a protein lysine side chain may form a cross-link between such a chromophoric, substituted 1*H*-pyrrol-3(2*H*)-one and the protein. In analogous research, Hofmann identified another chromophoric protein cross-link **159** from the reaction of two arginine side chains with glyoxal and furan-2-carbaldehyde.[186] In addition, a radical cross-link amino acid, the 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation, named 'CROSSPY' (depicted as **160**, incorporated in a protein, in Scheme 4.5), was identified in model melanoidins as well as in roasted coffee and bread crust as an important

intermediate in browning.[187] The identification of these types of compounds supports the hypothesis of the generation of melanoidin-type colorants by a cross-linking reaction between low molecular weight chromophores and colourless high molecular weight proteins.



Scheme 4.5. Structures of protein amino acid modifications isolated from browned model systems [185-187]

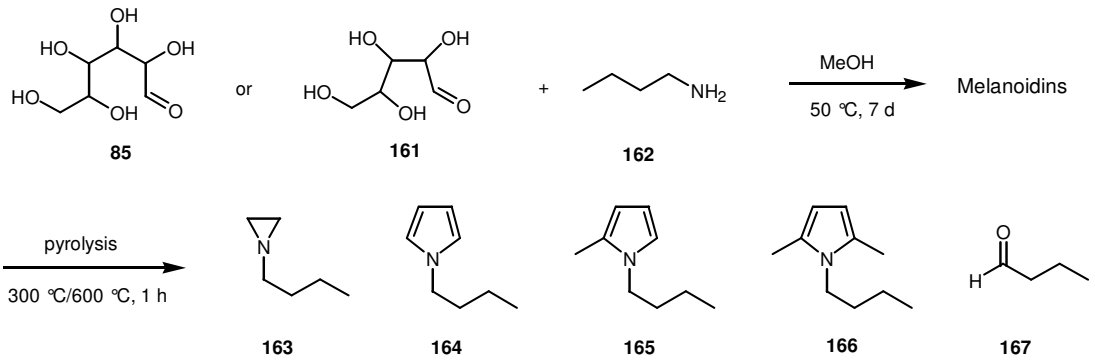
It is unlikely that only one polymeric material would result from a model system under specific conditions. Elimination and addition reactions of initial polymers may lead to the formation of more complex derived polymers. In addition, reactive sites on the polymer can interact with other compounds present in the Maillard mixture.[182]

Because of the great variations in melanoidin structure found with varying reaction conditions, and the diverse information found by different research groups, it can be assumed that, in real food systems, each of the structural proposals can be found and that different structures co-exist. Thus, it may not be possible to establish one fundamental melanoidin structure, or a universal repeating unit.

4.2 Characterization of model melanoidins by thermal degradation

4.2.1 Introduction

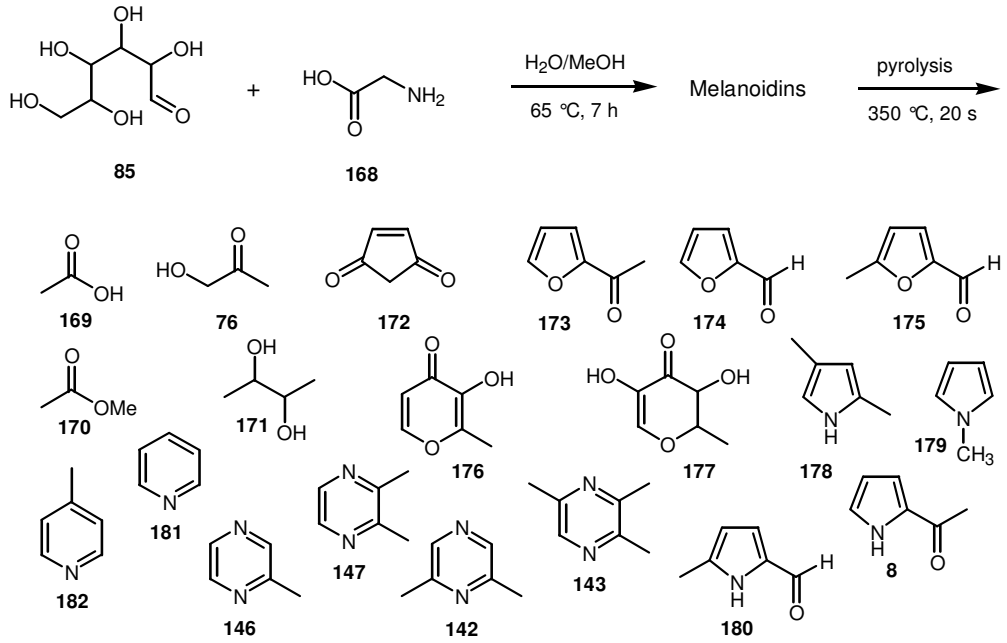
Pyrolysis of melanoidins, followed by identification of the decomposition products, is one of the analytical strategies applied to collect information on the structural domains of melanoidins. Pyrolysis of melanoidins (300 and 600 °C) prepared from D-glucose **85** or D-xylose **161** and butylamine **162** yielded 1-butylaziridine **163**, 1-butyl-1*H*-pyrroles (**164**, **165** and **166**) and butanal **167** (Scheme 4.6).[176]



Scheme 4.6. Volatiles identified after pyrolysis of glucose/butylamine and xylose/butylamine melanoidins [176]

Researchers of the same group pyrolyzed xylose/glycine and glucose/glycine melanoidins and identified acetic acid, methyl acetate, toluene, five furans, two pyrroles and two pyridines among the volatiles.[188] Non-dialyzable melanoidins prepared from D-glucose **85** and glycine

168 in a water/methanol system were reported to yield a variety of furans, pyrroles, pyrazines, pyridines, 2-cyclopenten-1,4-dione **172** and acetic acid **169** upon pyrolysis (Scheme 4.7).[182]



Scheme 4.7. Volatiles identified after pyrolysis of glucose/glycine melanoidins [182]

It is, however, difficult to link the structure of the decomposition products directly with the original structure of the melanoidin, since the thermal treatment induces chemical changes in the volatiles produced. In addition, it is possible that some of the pyrolysis products identified result from entrapped molecules or compounds associated with the melanoidins through hydrogen-bonding or charge-transfer interactions. Still, the spectrum of volatiles produced after thermal destruction of the macromolecular structure yields some relevant information on the structural elements of melanoidins.

During roasting or baking of many food products, melanoidins are thermally destructed by the heating process, and the volatiles thus produced take part in the development of the aroma. This is illustrated by the better organoleptic properties of bread prepared with extra-added melanoidins.[189] Since pyrolysis occurs through solid-phase interactions, thermal degradation of melanoidins can be a model for roasting and baking.[190] It was therefore considered

valuable to study and compare the production of flavour volatiles from heated melanoidins. For this purpose, heating at moderate temperatures (100-250 °C) has the highest relevance for food preparation. Pyrolysis experiments are usually conducted at 300-600 °C and involve more extensive chemical reactions, leading, for instance, to aromatization. The volatiles formed in this way are no longer representative for food flavour formation.

An extensive study was started of the generation of volatiles by thermal degradation of melanoidins, in order to distinguish, on the one hand, their role in food aroma formation and to collect information on the composition of the macromolecules by careful analysis of the volatiles produced, on the other hand.

4.2.2 Results and discussion

4.2.2.1 Choice of the model system

Because of the considerable impact of changes in reaction conditions during melanoidin preparation on the structure of the resulting melanoidin polymer, the choice of a standardized model system is crucial. As part of the European Research Programme COST Action 919 ('Melanoidins in Food and Health') a standardized protocol has been developed for the preparation of glucose/glycine melanoidins.[191] The purpose of this standard procedure was to allow a comparison between different laboratories and to facilitate the understanding of relationships between the structure of melanoidins and their functional properties. The model reaction between glucose and glycine is the most widely studied Maillard model system, because of the particular advantage of glycine that it displays the reactivity of the α-amino acid moiety without the complications of a reactive side chain.[175]

The first steps of the research were performed by Milda Keršienė, a Lithuanian PhD student, conducting cooperative research in our research group on Maillard reaction products.

Standard COST glucose/glycine melanoidins were thermally degraded at temperatures ranging from 100 °C to 300 °C. Among the volatile compounds furans, pyrazines, pyrroles and pyridines were detected. In general, the amount of volatiles produced increased with increasing temperature.[192]

In order to investigate whether these types of compounds are common to all kinds of melanoidins, and whether the generated volatiles include useful information on the composition of the model melanoidins, thermal degradation was used to compare different model melanoidins. Melanoidins were prepared from D-glucose with L-glutamic acid according to the same standard COST procedure. L-(+)-Ascorbic acid was reacted with glycine as a variation of the carbonyl compound in the Maillard reaction. Thus, the influence of the carbonyl compound and of the amino compound on the thermal degradation profile of the resulting melanoidins was investigated. Concerning melanoidins derived from L-(+)-ascorbic acid and amino compounds, only a few references are found.[193] Nonetheless, it is known that the non-enzymatic browning of juices and some food concentrates is partly due to the participation of L-(+)-ascorbic acid in the Maillard reaction.[194] The mechanisms and kinetics of the generation of L-(+)-ascorbic acid melanoidins prepared with glycine, lysine and glutamic acid have been investigated.[195]

4.2.2.2 Description of the model melanoidins

Melanoidins were obtained from model reactions of D-glucose/glycine (GlcGly), D-glucose/glutamic acid (GlcGlu) and L-(+)-ascorbic acid/glycine (ASAGly) in equimolar amounts in anhydrous medium at 125 °C. The water-soluble Maillard reaction products were fractionated by dialysis in a low (LMW < 12,000) and a high molecular weight fraction (HMW > 12,000) (Figure 4.1).

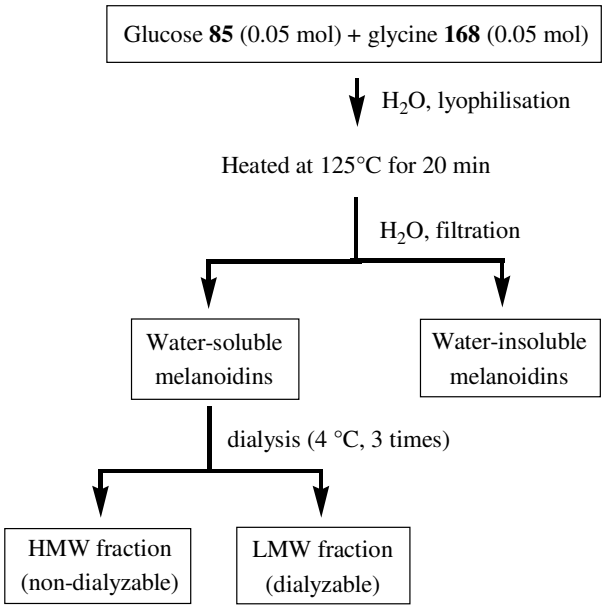


Figure 4.1. Preparation and fractionation of standard glucose/glycine melanoidins, according to the standard COST 919 procedure [191]

Water-insoluble melanoidins were obtained only from D-glucose/glycine melanoidins (39 % w/w) and in small amounts from L-(+)-ascorbic acid/glycine melanoidins (9 % w/w). This suggests a more extended polymerization reaction for D-glucose/glycine melanoidins, as compared to L-(+)-ascorbic acid/glycine and in particular D-glucose/glutamic acid melanoidins. Also the weight loss during melanoidin preparation (125 °C, 20 min) was significantly higher for D-glucose/glycine melanoidins (33 %), as compared to D-glucose/ glutamic acid melanoidins (16 %) and L-(+)-ascorbic acid/glycine melanoidins (17 %). Following the procedure proposed by COST Action 919, the water-soluble melanoidin fraction was characterized by measuring the UV-absorbance at specific wavelengths, as is displayed in Table 4.1. The complete UV-spectrum for the three different model melanoidins at the same concentrations is shown in Figure 4.2.

Table 4.1. UV-absorption at different wavelengths of solution A (5 g of reaction mixture in 250 ml of water $\times 10^{-2}$) of different model melanoidins

Wavelength (nm)	GlcGly	GlcGlu	ASAGly
280	0.68	1.03	4.29
360	0.30	0.35	0.34
420	0.08	0.10	0.16
460	0.05	0.05	0.09
520	0.02	0.02	0.04

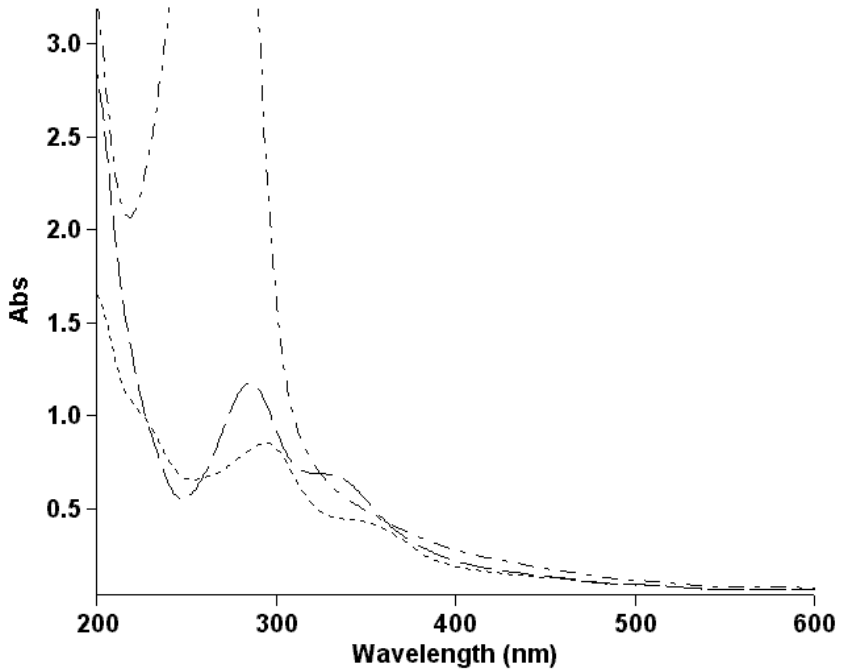


Figure 4.2. UV spectra of model melanoidins prepared from D-glucose/glycine (----), D-glucose/glutamic acid (— —) and L-(+)-ascorbic acid/glycine (— - —)

All three model melanoidins showed featureless end absorption, becoming more intense as the wavelength decreased. The shape of this UV spectrum has also previously been found to be characteristic of melanoidins.[184,196] The UV-absorption at intermediate wavelengths (200-300 nm) of L-(+)-ascorbic acid/glycine melanoidins was significantly higher than for the D-glucose-derived melanoidins.

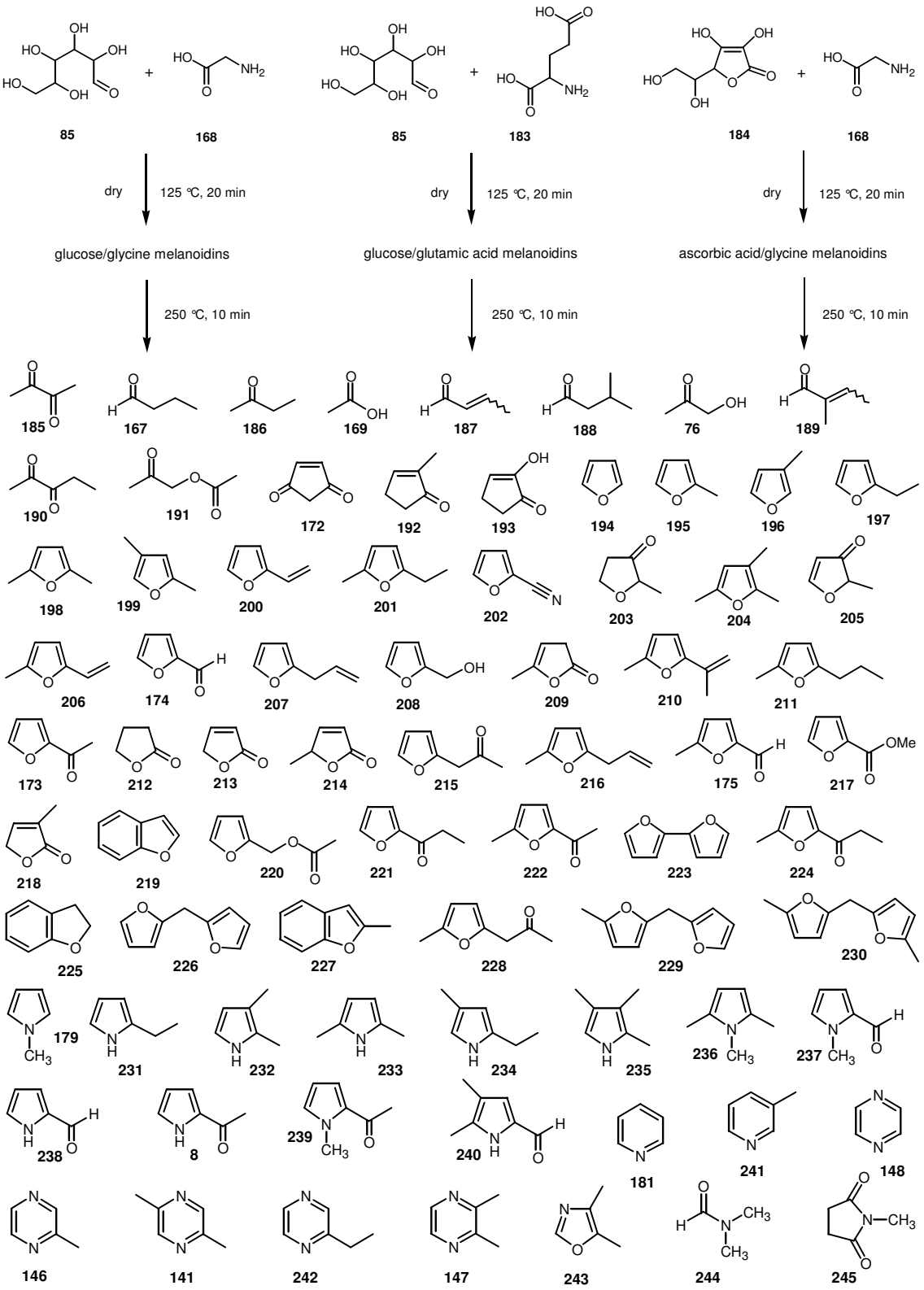
4.2.2.3 Analysis of volatiles from heated model melanoidins

For each melanoidin fraction, 50 mg were heated in a closed glass vial on a sand bath at a constant temperature of 200 °C or 250 °C (during 10 minutes). After cooling, the headspace was sampled by means of Solid Phase Microextraction (SPME) for five minutes followed by GC-MS analysis. Identification of the volatiles was based on the mass spectra (comparison with mass spectral libraries) and on comparisons with reference compounds. Retention indices on the non-polar HP5 capillary column were calculated, according to the Kovats system [197] and were compared with literature data, when available.

The generated volatiles belonged to six main groups based on their chemical structure: carbonyl compounds, furans, pyrroles, pyridines, pyrazines and oxazoles were found. In Scheme 4.8, the chemical structures of the compounds identified in the headspace of the different heated model melanoidins are depicted.

Table 4.2 displays all the volatiles identified in the headspace of the heated model melanoidins, with the percentage of the total GC peak area as a quantitative measure, and with the retention index (RI) to support the identification.

SPME is a solvent-free method for extracting analytes from a variety of matrices by partitioning them from a liquid or gaseous sample into an immobilized stationary phase. The sorption of a specific compound by the SPME-fiber is determined by the volatility of the compound, by its affinity for the fiber polymer, by several matrix effects and by experimental conditions. Therefore, no absolute quantitative results can be acquired using this technique. Careful control of experimental conditions, however, does enable reliable qualitative measurements and comparisons between samples.



Scheme 4.8. Structures of the volatiles identified in the headspace of heated glucose/glycine, glucose/glutamic acid and ascorbic acid/glycine HMW model melanoidins

Table 4.2. Volatiles identified from heated model HMW melanoidins (250 °C, 10 min) and starch, quantities expressed as percentage of total GC peak area. Kovats retention indices were calculated (RI calc) and compared with literature data (RI lit).

Compound	starch	Glc-Gly	Glc-Glu	ASA-Gly	RI calc	RI lit
Carbonyl compounds						
2,3-butanedione **185**	2.63	10.70	3.60	5.61	602	<600[198]
butanal[a] **167**				4.83	605	596[199]
2-butanone[a] **186**		3.93	1.87	5.29	607	
acetic acid **169**	5.27				637	610[198]
2-butenal[a] **187**			0.25	2.37	651	
3-methylbutanal[b] **188**				0.18	655	650[199]
1-hydroxy-2-propanone[b] **76**	6.29				667	
2-methyl-2-butenal[a] **189**	0.45				702	
2,3-pentanedione **190**		3.28	2.07	2.40	702	696[198]
1-acetoxy-2-propanone[b] **191**	1.16		0.21		871	
2-cyclopenten-1,4-dione[a] **172**			0.12		885	
2-methyl-2-cyclopenten-1-one[a] **192**		0.20	0.05	0.19	908	
2-hydroxy-2-cyclopenten-1-one[a] **193**	1.20				930	
Total carbonyl compounds	*17.00*	*18.10*	*8.17*	*20.87*		
Furans						
furan[a] **194**		2.99	0.64	10.43	<600	
2-methylfuran **195**	5.92	24.09	18.59	10.93	612	604[200]
3-methylfuran[a] **196**				2.08	619	
2-ethylfuran[b] **197**		0.64	0.26	1.15	706	
2,5-dimethylfuran[b] **198**		19.96	10.79	2.59	711	

Table 4.2. continued

Compound	starch	Glc-Gly	Glc-Glu	ASA-Gly	RI calc	RI lit
2,4-dimethylfuran[a] **199**				1.24	716	
2-vinylfuran[a] **200**		0.85	0.42	0.99	724	
2-ethyl-5-methylfuran **201**			0.66	0.39	805	804[201]
2-furancarbonitrile[a] **202**		0.74			805	
2-methyldihydro-3(2*H*)-furanone[b] **203**		0.30	0.15		810	
2,3,5-trimethylfuran[a] **204**		0.59	0.56	0.47	817	
2-methyl-3(2*H*)-furanone **205**	0.84				820	819[202]
2-vinyl-5-methylfuran[a] **206**		2.42	2.46		831	
furfural **174**	15.73	0.45	9.64	0.50	832	830[200]
2-(2-propenyl)furan **207**			0.24	0.09	853	848[201]
2-furylmethanol **208**	46.67				860	864[201]
5-methyl-2(3*H*)-furanone[a] **209**			0.26		871	
2-methyl-5-isopropenylfuran[a] **210**			0.21		883	
2-methyl-5-propylfuran[a] **211**			0.10		888	
2-acetylfuran[b] **173**	2.29	0.31	0.61	1.19	912	
dihydro-2(3*H*)-furanone[a] **212**	3.31		1.65	1.04	920	
2(5*H*)-furanone[a] **213**				0.48	924	
5-methyl-2(5*H*)-furanone[a] **214**		0.11	0.19	0.14	946	
1-(2-furyl)-2-propanone **215**	0.51				955	952[201]
2-methyl-5-(2-propenyl)furan[a] **216**			3.65		960	
5-methylfurfural **175**	1.72	1.24	39.62		964	962[200]
methyl furan-2-carboxylate[a] **217**		0.07		0.12	977	
3-methyl-2(5*H*)-furanone[a] **218**				0.11	979	
benzofuran[a] **219**	0.63		0.08		995	

Table 4.2. continued

Compound	starch	Glc-Gly	Glc-Glu	ASA-Gly	RI calc	RI lit
2-furylmethyl acetate[a] **220**	0.58				1002	
1-(2-furyl)-1-propanone[a] **221**	0.27				1014	
2-acetyl-5-methylfuran[b] **222**	0.19	0.06	0.32		1037	
2-(2-furyl)furan[a] **223**	0.63				1040	
1-(5-methyl-2-furyl)-1-propanone[a] **224**	0.33				1066	
2,3-dihydrobenzofuran[a] **225**	0.50				1078	
2-(2-furylmethyl)furan[a] **226**	2.02				1084	
2-methylbenzofuran[b] **227**			0.39		1107	
1-(5-methyl-2-furyl)-2-propanone[a] **228**	0.33		0.05		1131	
2-(2-furylmethyl)-5-methylfuran[a] **229**	0.55		0.18		1183	
2-methyl-5-[(5-methyl-2-furyl)methyl]furan[a] **230**			0.12		1280	
Total furans	*83.00*	*54.84*	*91.83*	*33.92*		
Pyrroles						
1-methyl-1*H*-pyrrole[a] **179**		13.57		36.03	741	
2-ethyl-1*H*-pyrrole[a] **231**		0.17		0.51	816	
2,3-dimethyl-1*H*-pyrrole[a] **232**		1.89		2.10	837	
2,5-dimethyl-1*H*-pyrrole[a] **233**		7.48		1.30	869	
2-ethyl-4-methyl-1*H*-pyrrole[a] **234**				0.26	932	
2,3,4-trimethyl-1*H*-pyrrole[a] **235**		0.34			972	
1,2,5-trimethyl-1*H*-pyrrole[b] **236**		0.79			995	
1-methyl-1*H*-pyrrole-2-carbaldehyde[b] **237**		0.09		0.21	1005	
1*H*-pyrrole-2-carbaldehyde[a] **238**				1.19	1010	
2-acetyl-1*H*-pyrrole[a] **8**				0.19	1060	

Table 4.2. continued

Compound	starch	Glc-Gly	Glc-Glu	ASA-Gly	RI calc	RI lit
2-acetyl-1-methyl-1*H*-pyrrole[b] **239**		0.11		0.09	1075	
4,5-dimethyl-1*H*-pyrrole-2-carbaldehyde[a] **240**		0.11			1157	
Total pyrroles		*24.55*		*41.89*		
Pyridines						
pyridine **181**		1.63		0.65	747	757[201]
3-methylpyridine[a] **241**				0.07	866	
Total pyridines		*1.63*		*0.72*		
Pyrazines						
pyrazine **148**		0.03		0.16	733	745[201]
methylpyrazine **146**				0.66	823	820[172]
2,5-dimethylpyrazine[b] **141**		0.28			913	909[172]
ethylpyrazine[b] **242**		0.19		0.89	916	910[172]
2,3-dimethylpyrazine[b] **147**		0.13		0.13	919	916[172]
Total pyrazines		*0.64*		*1.84*		
4,5-dimethyloxazole[a] **243**		0.15		0.13	763	
N,*N*-dimethylformamide[a] **244**		0.09		0.27	788	
N-methylsuccinimide[a] **245**				0.37	1090	
Total GC peak area	**2.3×10^9**	**1.7×10^8**	**3.5×10^8**	**2.0×10^8**		

[a] Tentatively identified [b] Comparison with reference compounds

Furans and carbonyl compounds are compounds typically resulting from carbohydrate heating.[139] They represent an important group in the generated volatiles for the different types of melanoidins. Furans generally have very pleasant odours and largely determine the odour of processed food products. For example, furfural **174** has a freshly baked bread odour, 5-methylfurfural **175** a sweet caramel-like aroma, and 2-acetylfuran **173** a sweet balsamic odour. These furan-2-carbaldehydes are typical caramelization products. To be able to determine which volatiles result from polysaccharide-like structures, starch was heated and analysed in the same way as the melanoidins. The results are included in Table 4.2. From these data can be deduced that several carbonyl and furan compounds found in the headspace of heated melanoidins may be attributed to the presence of carbohydrate-like structures, but most compounds result from the degradation of a Maillard reaction products network. The most important compound produced from heated starch, namely 2-furylmethanol **208**, was not detected among the volatiles of heated melanoidins.

Pyrroles have been reported in various heated food products, especially in coffee.[203] They are generally less abundant in food products, and are formed only at elevated temperatures. The applied heating temperature of 250 °C is representative, e.g. for coffee roasting conditions. Alkyl- and acylpyrroles generally have unfavourable odours, but upon dilution, alkylpyrroles exhibit a sweet, slightly burnt-like aroma.[139] Tressl et al. studied the acid-catalyzed condensation reaction of *N*-substituted pyrroles and *N*-substituted pyrrole-2-carbaldehydes and suggested them to be part of the melanoidin macromolecular network.[183] However, according to Kato and Tsuchida, pyrroles are not originally present in melanoidins and are formed by the pyrolysis process.[176] It must be noted that these researchers applied very high pyrolysis temperatures (600 °C) and that the analysed melanoidins were prepared in an aqueous system; both factors complicate a comparison with the results obtained here.

Among the azaheterocycles, pyrazines exhibit the most agreeable odours. Alkylpyrazines are mostly associated with heated food systems; they have a roasted nutty flavour.[141] The most direct route to the formation of pyrazines results from the condensation of two molecules of α-aminoketone, generated by the Strecker degradation of α-amino acids and α-dicarbonyl compounds.

Pyridines significantly contribute to the organoleptic properties of some heat-processed food products. They generally have green, bitter, stringent, roasted or burnt properties although some have more pleasant characteristics.[204] Thermal decomposition of certain amino acids may result in pyridine formation.[205] Pyridines can be formed from the reaction of glycine with alkanals at 180 °C.[206]

4,5-Dimethyloxazole **243** can be formed from the reaction of glycine with 2,3-butanedione **185**, a common degradation product of glucose.[205] 2,3-Butanedione **185** is detected in the headspace of the three model melanoidins, and 4,5-dimethyloxazole **243** is indeed formed in both glycine-containing model systems.

To allow a more convenient comparison between the different model melanoidins prepared, a 'thermal degradation profile' of each melanoidin fraction was composed. For this purpose, the volatiles were grouped according to the predominant chemical functionality. The percentage of the total GC peak area for each class of compounds was depicted in bar graphs. In this way, the non-dialyzable melanoidins from the three different model systems were compared after heating at 200 °C (Figure 4.3) and 250 °C (Figure 4.4).

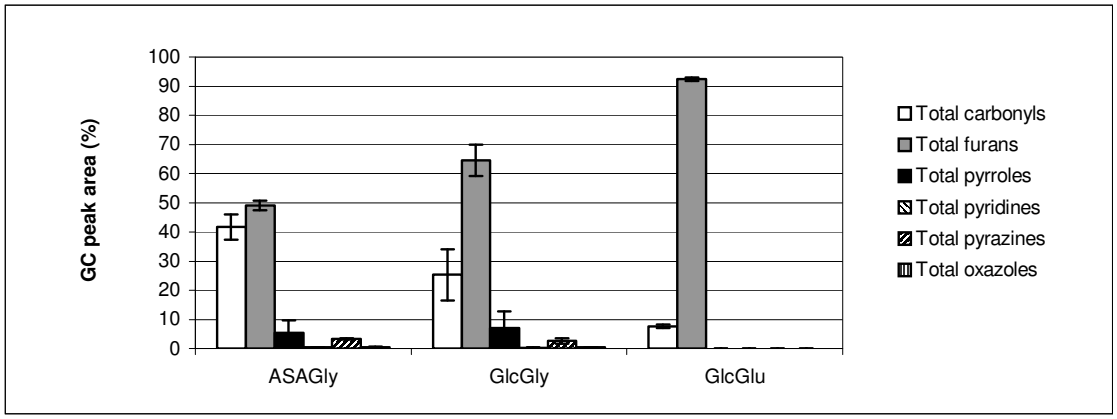


Figure 4.3. Thermal degradation profile (200 °C, 10 min) of HMW water-soluble melanoidins prepared from L-(+)-ascorbic acid/glycine (ASAGly), D-glucose/glycine (GlcGly), and D-glucose/glutamic acid (GlcGlu)

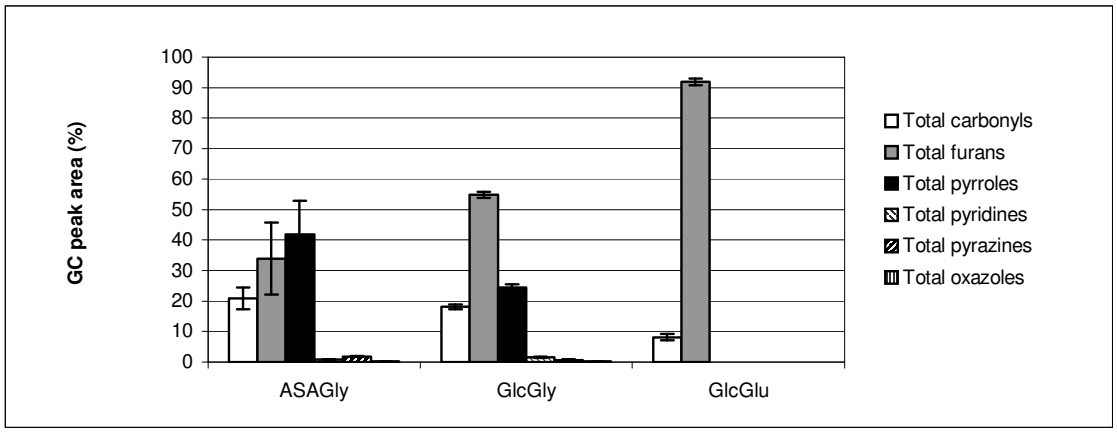


Figure 4.4. Thermal degradation profile (250 °C, 10 min) of HMW water-soluble melanoidins prepared from L-(+)-ascorbic acid/glycine (ASAGly), D-glucose/glycine (GlcGly), and D-glucose/glutamic acid (GlcGlu)

The error bars in the graphs represent twice the standard deviation of three replicated heating experiments of melanoidins prepared in the same experiment. Although the relative standard deviation (RSD) on absolute values of peak areas is sometimes high, for the amounts of single compounds relative to the total peak area, the average RSD was 32 %. Considering the nature of the experiments, this is acceptable, and will be taken into account for the conclusions of the

experiments. The reproducibility of the melanoidin preparation on the resulting thermal degradation profile was evaluated, by repeating the preparation of D-glucose/glycine melanoidins. Between the melanoidin preparations, an average RSD on the relative amounts of the individual volatile compounds of 48 % was found.

When comparing the thermal degradation profiles of HMW water-soluble melanoidins obtained from the different model systems D-glucose/glycine (GlcGly), L-(+)-ascorbic acid/glycine (ASAGly) and D-glucose/glutamic acid (GlcGlu) (Figure 4.3 and Figure 4.4), obvious differences can be observed in the composition of the headspace extracts. This allows the use of these thermal degradation profiles for the characterization of different kinds of model melanoidins.

The absolute amounts of carbonyl compounds detected are quite constant for the different melanoidins, and display little specificity. Furans are important volatiles for all types of melanoidins, but especially the azaheterocyclic compounds determine the difference. For these nitrogen-containing compounds, the production is substantially higher at 250 °C than at 200 °C. Therefore, the thermal degradation profiles at 250 °C are more useful to differentiate the model melanoidins.

The thermal degradation profile of standard HMW D-glucose/glycine melanoidins contained mainly furans. Pyrroles counted for 7 ± 6 % of the headspace profile at 200 °C, but amounted to 25 ± 7 % when the melanoidins were heated at 250 °C. Pyrazines, pyridines and 4,5-dimethyloxazole were found, but were quantitatively of minor importance in the headspace profile.

In the headspace of heated L-(+)-ascorbic acid/glycine melanoidins, besides furans and substantial amounts of carbonyl compounds, especially pyrroles were detected. The amount of pyrroles released after heating of the melanoidins increased strongly with the heating

temperature. An average of 42 ± 11 % of the volatiles detected after heating of L-(+)-ascorbic acid/glycine melanoidins at 250 °C were pyrroles. Thermal degradation of L-(+)-ascorbic acid as such, has been shown to yield mostly furan derivatives and α,β-unsaturated ketones with a five-membered ring.[195c] These are found among the furans and carbonyl compounds detected. *N*-Methylsuccinimide **245** has been reported in the literature as a pyrolysis product (500 °C) of polyglycine.[207] Intact glycine may be present in L-(+)-ascorbic acid/glycine melanoidins as a polypeptide substructure.

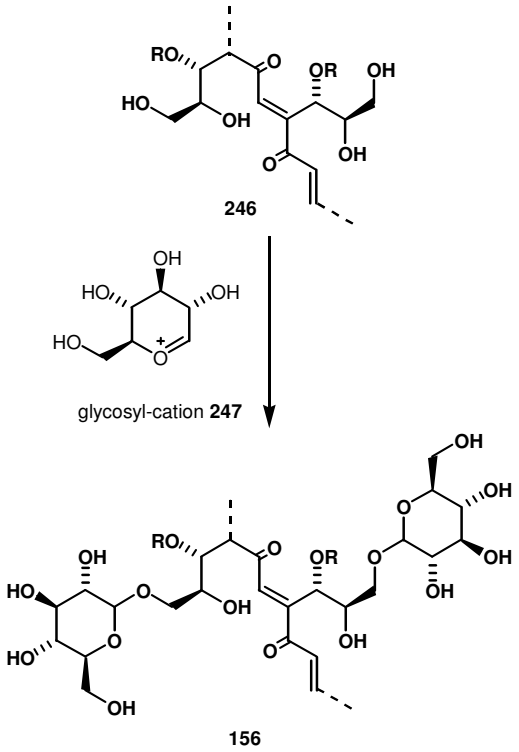
On the contrary, in the headspace of heated D-glucose/glutamic acid melanoidins, only furans and carbonyl compounds were found, and in very high amounts (as measured by the high total GC peak area, cf. Table 4.2). 2-Cyclopentene-1,4-dione **172** was quantitatively the most important compound among the carbonyl compounds detected. About 80 % (79 ± 2 %) of the headspace profile was composed of four major furan compounds: 2-methylfuran **195**, 2,5-dimethylfuran **198**, furfural **174** and 5-methylfurfural **175**.

It is known that glutamic acid has a low reactivity in the formation of volatile azaheterocyclic compounds. In a comparison of the reactivity of different amino acids in the formation of azaheterocyclic compounds upon heating, glutamic acid was shown to be the lowest contributor to flavour formation among the tested amino acids in the presence of labelled glycine.[205] In an investigation for the thermal degradation of amino acids, very little ammonia was released when glutamic acid was heated, in contrast to the easy deamination of most amino acids, among which the very similar aspartic acid.[208] Glycine, on the contrary, having no side chain, is more flexible than other amino acids, and therefore highly reactive. The lower reactivity and deamination capacity of glutamic acid in the Maillard reaction probably reduces its participation in the melanoidin formation, as compared to reactive sugar degradation compounds. While from heated D-glucose/glycine and L-(+)-ascorbic acid/

glycine melanoidins many nitrogen-containing heterocycles were produced, none were detected after heating of D-glucose/glutamic acid melanoidins under the same circumstances.

Among the furan compounds detected in the headspace of heated D-glucose/glutamic acid melanoidins some methylene-bridged furan derivatives were detected, such as 2-(2-furyl-methyl)-5-methylfuran **229** and 2-methyl-5-[(5-methyl-2-furyl)methyl]furan **230** (depicted in Scheme 4.8). The detection of these furanoid species indicates the presence of glycosidically linked sugar derivatives in the melanoidin skeleton.[182] These compounds were found among the volatiles of heated starch, and of heated D-glucose/glutamic acid melanoidins, but were not formed in detectable amounts from D-glucose/glycine, nor from L-(+)-ascorbic acid/ glycine melanoidins. Fragmentation of these bridged furans under thermal conditions may be an additional source of furan-2-carbaldehydes.[209]

Cämmerer et al. demonstrated the presence of considerable amounts of monomer carbohydrates in acid hydrolysates of model melanoidins, especially when the melanoidins were prepared under water-free conditions.[181] The presence of glucose-units in glucose/glycine melanoidins was explained by transglycosylation reactions. A free glucose molecule might react with the melanoidin structure via the formation of a glycosyl cation **247** to form a 1,6-branched glucose side chain (Scheme 4.9). In addition, di- and oligosaccharides can be incorporated as carbohydrate side chains in the melanoidins with an intact glycosidic bond. Investigations of maltose/glycine model systems indicated that about 20 % of the added maltose was incorporated into the melanoidin without degradation of the glycosidic bond, forming glucose side chains that could be split off by acid hydrolysis.[181]



Scheme 4.9. Schematic mechanism of 1,6-branched glucose side chain formation by transglycosylation, according to Cämmerer et al.[181]

Due to the low reactivity of glutamic acid as compared to glycine, the amounts of glucose incorporated in the resulting melanoidins are relatively higher, and apparently part of this glucose is incorporated with a glycosidic bond.

4.2.2.4 Thermal degradation of different fractions of glucose/glycine melanoidins

From the D-glucose/glycine model reaction, water-soluble melanoidins were separated by dialysis in HMW non-dialyzable melanoidins and LMW dialyzable compounds. In addition, a significant amount of water-insoluble melanoidins was isolated. The release of volatiles from these three different melanoidin fractions is considerably different, as is shown in Figure 4.5 (heating at 250 °C).

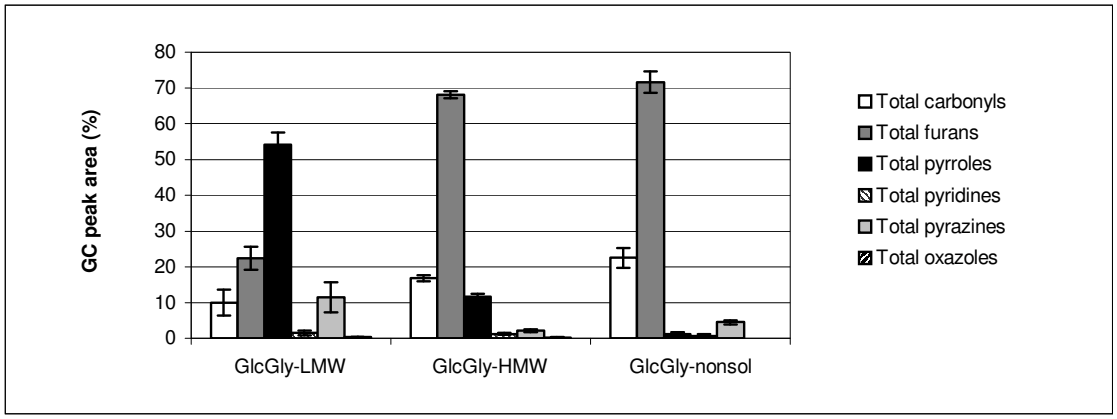
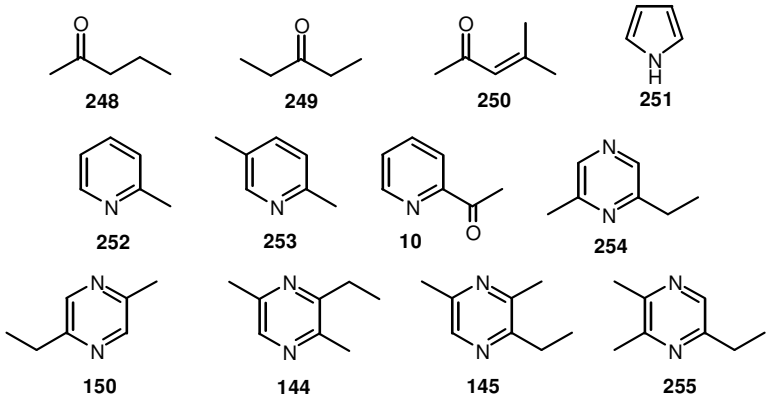


Figure 4.5. Thermal degradation profile (250 °C, 10 min) of the dialyzable (LMW), the non-dialyzable (HMW) and the water-insoluble (nonsol) fractions of D-glucose/glycine melanoidins

The majority of the volatiles produced from heating non-soluble D-glucose/glycine melanoidins were furans, as was the case for the non-dialyzable fraction. Very few azaheterocyclic compounds were formed upon heating. The graph shows that the low molecular weight fraction yielded significantly more nitrogen-containing compounds, especially pyrroles, than the other fractions. These dialyzable Maillard reaction products contained some non-reacted starting material (glucose and glycine). Heating at 250 °C of the dried LMW-fraction resulted in a progressing Maillard reaction with the formation of a very dark porous structure and a variety of volatiles. Analysis of the resulting volatile Maillard reaction products showed mainly pyrroles, furans and pyrazines. All the identified compounds are gathered in Table 4.3. Scheme 4.10 shows the structures of the volatiles detected in LMW and water-insoluble glucose/glycine Maillard reaction products, which were not identified in the HMW melanoidins discussed above.



Scheme 4.10. Volatiles identified after heating of non-soluble and LMW glucose/glycine model melanoidins that were not formed from HMW model melanoidins

Table 4.3. Volatiles identified from heated LMW and water-insoluble model glucose/glycine melanoidins (250 °C, 10 min), quantities expressed as percentage of total GC peak area. Kovats retention indices were calculated (RI calc) and compared with literature data (RI lit).

Compound	GlcGly LMW	GlcGly nonsol	RI calc	RI lit
Carbonyl compounds				
2,3-butanedione **185**	1.06	9.61	602	<600[198]
2-butanone[a] **186**	1.26	9.12	607	
2-butenal[a] **187**	0.06	1.07	651	
2-pentanone[a] **248**	0.12	0.71	693	
2,3-pentanedione **190**	0.36	0.57	702	696[198]
3-pentanone[a] **249**	0.13	0.63	703	
4-methyl-3-penten-2-one[a] **250**	0.65		802	
2-cyclopentene-1,4-dione[a] **172**	5.92	0.65	885	
2-methyl-2-cyclopenten-1-one[a] **192**	0.36	0.12	907	
Total carbonyl compounds	*9.92*	*22.47*		
Furans				
furan[a] **194**	0.31	3.22	<600	
2-methylfuran **195**	2.80	27.08	612	604[200]
2-ethylfuran[b] **196**	0.31	0.23	706	
2,5-dimethylfuran[b] **198**	3.56	27.11	711	
2-vinylfuran[a] **200**	3.11		724	
2-ethyl-5-methylfuran **201**		0.29	805	804[201]

Table 4.3. continued

Compound	GlcGly LMW	GlcGly nonsol	RI calc	RI lit
2-furancarbonitrile[a] **202**	0.57	0.76	806	
2-methyldihydro-3(2*H*)furanone[b] **203**	0.15		810	
2,3,5-trimethylfuran[a] **204**	0.04		817	
2-vinyl-5-methylfuran[a] **206**	2.11	0.22	831	
furfural **174**	1.54	2.57	832	830[200]
2-furylmethanol **208**	0.21		860	864[201]
2-acetylfuran[b] **173**	2.84	1.75	912	
dihydro-2(3*H*)-furanone[a] **212**	0.51		920	
5-methyl-2(5*H*)-furanone[a] **214**	0.17		924	
1-(2-furyl)-2-propanone **215**	0.22		955	952[201]
5-methylfurfural[a] **175**	2.51	7.86	963	
methyl 2-furylcarboxylate[a] **217**	0.79	0.26	977	
2-acetyl-5-methylfuran[a] **222**	0.52	0.23	1037	
Total furans	*22.27*	*71.59*		
Pyrroles				
1-methyl-1*H*-pyrrole[a] **179**	39.95	0.63	740	
1*H*-pyrrole[a] **251**	0.66		754	
2-ethyl-1*H*-pyrrole[a] **231**	1.16		817	
2,3-dimethyl-1*H*-pyrrole[a] **232**	2.46		836	
2,5-dimethyl-1*H*-pyrrole[a] **233**	3.26		870	
2,3,4-trimethyl-1*H*-pyrrole[a] **234**	0.93		972	
1,2,5-trimethyl-1*H*-pyrrole[b] **235**	0.63		995	
2-acetyl-1*H*-pyrrole[a] **8**	0.29		1060	
2-acetyl-1-methyl-1*H*-pyrrole[b] **239**	1.81		1074	
4,5-dimethyl-1*H*-pyrrole-2-carbaldehyde[a] **240**	2.63		1157	
Total pyrroles	*53.79*	*0.63*		
Pyridines				
pyridine **181**	0.69	1.19	740	757[201]
2-methylpyridine[a] **252**	0.06		820	
3-methylpyridine[a] **241**	0.11		858	
2,5-dimethylpyridine[a] **253**	0.37		923	
2-acetylpyridine[a] **10**	0.34		1033	1012[199]
Total pyridines	*1.57*	*1.19*		

Table 4.3. continued

Compound	GlcGly LMW	GlcGly nonsol	RI calc	RI lit
Pyrazines				
pyrazine **148**	0.02	0.56	732	745[201]
methylpyrazine **146**	0.98	2.53	822	820[172]
2,6-dimethylpyrazine[b] **142**	3.24	0.37	913	907[172]
ethylpyrazine[b] **242**	0.84	0.76	916	910[172]
2,3-dimethylpyrazine[b] **147**	0.36	0.11	919	916[172]
2-ethyl-6-methylpyrazine[a] **254**	1.08	0.07	999	
2-ethyl-5-methylpyrazine[a] **150**	2.71	0.09	1002	
3-ethyl-2,5-dimethylpyrazine[a] **144**	0.51		1080	1078[172]
2-ethyl-3,5-dimethylpyrazine[a] **145**	0.80		1085	1083[172]
2,3-dimethyl-5-ethylpyrazine[a] **255**	0.82		1087	1084[172]
Total pyrazines	*11.35*	*4.50*		
Oxazoles				
4,5-dimethyloxazole[a] **243**	0.36		761	
N,*N*-dimethylformamide[a] **244**	0.20		788	
Total GC peak area	**6.74×10^8**	**1.26×10^8**		

[a] Tentatively identified [b] Comparison with reference compounds

4.2.2.5 Acid hydrolysis of model melanoidins

Because of the significant amount of volatiles apparently resulting from carbohydrate-like structures, and following the reports on the presence of glycosidically linked sugar residues in melanoidins,[181] D-glucose/glycine melanoidins were subjected to an acid hydrolysis procedure in order to remove these glycosidically linked sugar moieties. The resulting fractions were then thermally degraded and analysed the same way as is described above.

Acid-catalyzed hydrolysis of melanoidins was carried out using the optimal circumstances found by Cämmerer et al.,[181] namely 1 h reflux in a 1 N HCl solution. In addition, a 'soft' hydrolysis was performed, consisting of 1 h reaction at room temperature in a 1 N HCl

solution. It is expected that these hydrolytic conditions will result in other degradative actions, besides the cleavage of glycosidically linked sugars.

In first place, both hydrolytic procedures were applied to the non-soluble fraction of standard D-glucose/glycine melanoidins. The weight decrease after hydrolysis was noted as a measure for the breakdown of the melanoidin structure. Hydrolysis with 1 N HCl, at room temperature and under reflux conditions, degraded 11 % and 58 % of the non-soluble melanoidins into water-soluble compounds, respectively. This shows an important weight decrease of the non-soluble D-glucose/glycine melanoidins upon acid hydrolysis, even though these polymers usually show a very high resistance to many treatments (chemical and other) and are insoluble in common organic solvents.

After hydrolysis and subsequent neutralization, the residue was separated in a non-soluble and a soluble fraction by filtration, and the filtrate was separated by dialysis in HMW non-dialyzable and LMW dialyzable compounds. Each fraction was subjected to thermal degradation; the results for heating at 250 °C after acid hydrolysis type 1 (1 N HCl, room temperature) and type 2 (1 N HCl, reflux conditions) are shown in Figure 4.6 and Figure 4.7, respectively. Table 4.4 shows the total GC peak areas obtained after heating of the different fractions.

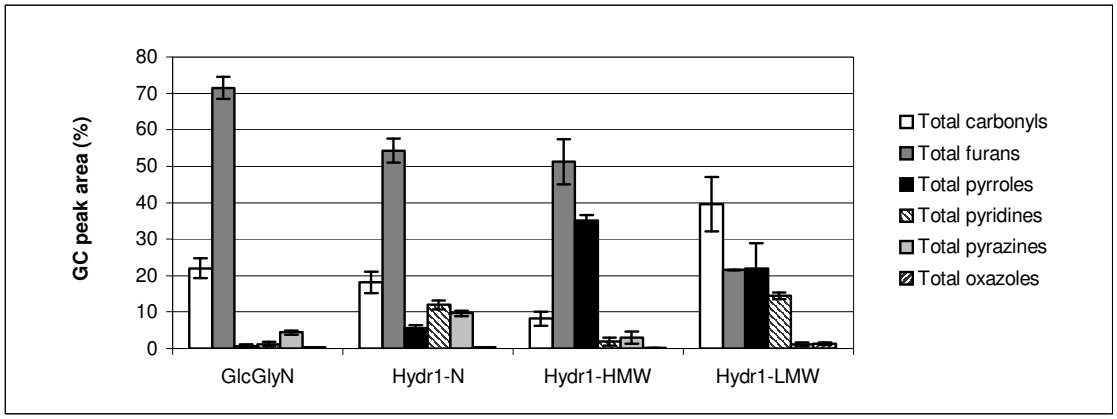


Figure 4.6. Thermal degradation profile (250 °C, 10 min) of different melanoidin fractions (N: non-soluble, HMW: High Molecular Weight, LMW: Low Molecular Weight), obtained from non-soluble D-glucose/glycine melanoidins, before (GlcGlyN) and after acid hydrolysis type 1 (Hydr 1 = 1 N HCl, 1 h, rt)

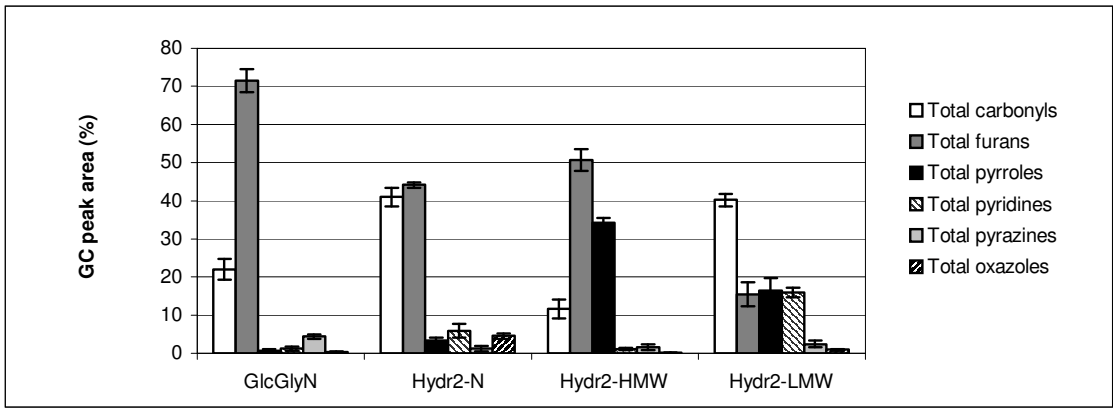


Figure 4.7. Thermal degradation profile (250 °C, 10 min) of different melanoidin fractions (N: non-soluble, HMW: High Molecular Weight, LMW: Low Molecular Weight), obtained from non-soluble D-glucose/glycine melanoidins, before (GlcGlyN) and after acid hydrolysis, type 2 (Hydr 2 = 1 N HCl, 1 h, reflux)

Table 4.4. Average and relative standard deviation (%) of the total GC peak area ($\times 10^8$) obtained after thermal degradation (250 °C, 10 min) of 50 mg of different melanoidin fractions obtained after acid hydrolysis of non-soluble D-glucose/glycine melanoidins

Type of hydrolysis	Melanoidin fraction	Total GC peak area ($\times 10^8$)	RSD (%)
before hydrolysis	non-sol	1.26	21.5
1 N HCl, 1 h, rt	non-sol	0.89	28.9
	HMW	4.28	22.2
	LMW[a]	2.76	33.7
1 N HCl, 1 h, reflux	non-sol	0.18	57.2
	HMW	1.58	56.3
	LMW[a]	2.90	16.1

[a]150 mg were heated, the exact amount of melanoidins is not known due to the presence of salt (NaCl resulting from neutralization)

The thermal degradation profile of the residual non-soluble fraction after hydrolysis showed a decreasing importance of the furans, and an increasing importance of azaheterocyclic compounds. Quantitatively few volatiles were released upon heating of the remaining non-soluble melanoidins (as measured by total GC peak area cf. Table 4.4), indicating a rigid structure remaining after hydrolysis. From the HMW melanoidins obtained by chemical hydrolysis of non-soluble melanoidins, especially furans and pyrroles were formed upon heating. In the LMW fraction, thermal degradation gave rise to the formation of comparable amounts of furans, pyrroles and pyridines, but a large amount of carbonyl compounds was formed. The results of both hydrolytic procedures are comparable.

One would expect that the presence of cleaved sugar residues would bring about mainly furan derivatives in the headspace of the heated LMW fraction. However, carbonyl compounds as well as furans, pyrroles and pyridines were formed. A twofold explanation for this can be given. On the one hand, it is known that the amount of cleaved sugars is rather limited. For HMW D-glucose/glycine melanoidins, Cämmerer et al. found that 2.9 mg glucose was

cleaved for every 100 mg of melanoidins.[181] This is a relatively low amount, which indicates that most of the sugar has been incorporated in the melanoidin skeleton after reaction, and is, as a consequence, visible in terms of furans upon heating. Since in our experiments, a weight decrease of 45 mg/100 mg HMW melanoidins was found under the same conditions, this also implies that a significant amount of other residues is cleaved off from the melanoidin skeleton. On the other hand, it has to be considered that the reaction conditions applied (acid solution, 1 h) are quite drastic, and that the cleaved sugar residues can react with other fragments in solution. When the melanoidins are prepared, glucose and glycine react under water-free conditions. Although a close contact between the reactants is pursued by a dissolution followed by a lyophilization, the question remains whether the contact between the reactants is sufficient. In any case, a reaction between sugar derivatives and nitrogen-containing melanoidin fragments in the acid solution during the hydrolysis procedure is probable. Reaction in solution of the different melanoidin degradation products, sugars as well as nitrogen-containing fragments, may be responsible for the formation of relatively more azaheterocyclic compounds upon heating, after hydrolytic degradation.

To examine which volatiles present in the thermal degradation profile of acid-treated melanoidins could result from glucose residues, an acid hydrolysis (1 N HCl, 1 h, rt) with subsequent thermal degradation of glucose was performed. Alcohols, ketones, and alkenes were detected after heating, especially butanol, cyclopentanol and cyclopentene derivatives. Therefore, only a neglectable amount of the volatiles detected upon heating of the melanoidin fractions after hydrolysis, results directly from glucose, and as a consequence the amount of free glucose in the mixture, obtained after acid hydrolysis of glucose melanoidins, is rather low.

Non-dialyzable D-glucose/glycine melanoidins were subjected to the same hydrolytic procedures. Thermal degradation of the non-dialyzable compounds left after hydrolysis of

HMW D-glucose/glycine melanoidins gave results similar to those obtained from hydrolysed non-soluble D-glucose/glycine melanoidins: relatively more azaheterocyclic compounds, especially pyridines, but less furans were formed upon heating. The stronger the hydrolytic procedure applied, the more obvious was this effect (Figure 4.8).

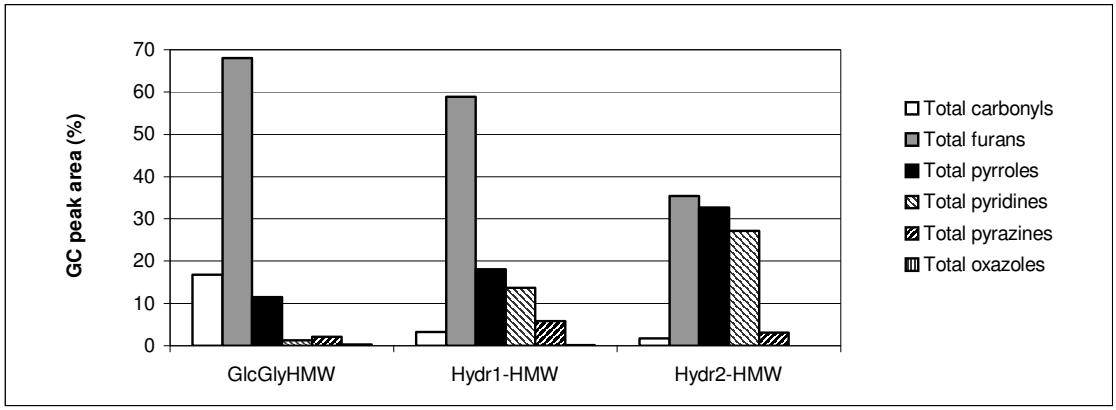


Figure 4.8. Thermal degradation profile (250 °C, 10 min) of different high molecular weight melanoidin fractions, obtained from HMW D-glucose/glycine melanoidins, before (GlcGlyHMW) and after acid hydrolysis, type 1 (Hydr 1 = 1 N HCl, 1 h, rt), and type 2 (Hydr 2 = 1 N HCl, 1 h, reflux)

Treatment of glucose/glycine non-dialyzable melanoidins with 1 N HCl for 1 h at reflux conditions resulted in 45 % weight decrease. The weight decrease of the non-dialyzable fraction was higher - 80 % and 90 % respectively - when L-(+)-ascorbic acid/glycine and D-glucose/glutamic acid melanoidins were hydrolysed (1 N HCl, 1 h, reflux conditions – data not shown). D-Glucose/glutamic acid melanoidins probably included more glucose residues with a glycosidic bond than D-glucose/glycine melanoidins, as was demonstrated by the presence of difurylmethanes after heating. This might explain in part the huge structural breakdown after hydrolysis. Apparently, L-(+)-ascorbic acid/glycine melanoidins are very susceptible to hydrolytic breakdown too, confirming that this acid hydrolysis has many more consequences than cleavage of glycosidic bonds alone. Furthermore, L-(+)-ascorbic acid/glycine melanoidins showed the highest weight decrease after thermal degradation, 21 %

on average. This confirms the low resistance of these L-(+)-ascorbic acid melanoidins. On the contrary,

D-glucose/glycine and D-glucose/glutamic acid melanoidins decreased only 9 and 5 % by weight, respectively, after heating at 250 °C for 10 min.

The HMW residue after acid hydrolysis of D-glucose/glutamic acid melanoidins still yielded only carbonyl compounds and furans upon heating, although much less than before hydrolysis (data not shown). For HMW L-(+)-ascorbic acid/glycine melanoidins, furans accounted for 88 % of the headspace profile after hydrolysis, indicating that in this case, mostly nitrogen-containing compounds are cleaved by the hydrolytic procedure (data not shown). The detection of *N*-methylsuccinimide upon heating of L-(+)-ascorbic acid/glycine non-dialyzable melanoidins (Table 4.2) was already an indication of the presence of glycine-polypeptide substructures. The susceptibility of these melanoidins to acid hydrolysis and the resulting loss of nitrogen-containing compounds might support this hypothesis, although many other compounds will also be degraded by the hydrolytic procedure.

4.2.2.6 Oxidative degradation of model melanoidins

When a general melanoidin structure is assumed, being built up mainly from sugar degradation products, probably branched via amino compounds, an oxidative degradation may as well induce several changes. Two oxidative agents were applied to non-soluble standard D-glucose/glycine melanoidins: potassium permanganate ($KMnO_4$) and sodium periodate ($NaIO_4$). Potassium permanganate is a strong oxidant with a very broad spectrum when used in aqueous solutions. In the melanoidin structure, primary alcohols and aldehydes can be converted to carboxylic acids, secondary alcohols to ketones; double bonds can be hydroxylated to form diols or become oxidatively cleaved. Ketones can be converted into ketoacids or acids, ethers to esters, amines to amides or imines.[210]

Sodium periodate is widely used for the oxidative cleavage of 1,2-diols to carbonyl compounds.[210] These vicinal diol structures are, among others, present in the sugar-containing side chains of the model melanoidins.

Oxidation of non-soluble D-glucose/glycine melanoidins with $KMnO_4$ yielded only soluble dialyzable yellow degradation fragments. After heating of this fraction mostly pyrroles were formed, in addition to some pyridines, some furans, 2-methyl-2-cyclopenten-1-one, pyrazine, and benzoxazole. Aside from this, aliphatic carbonyl compounds, such as 2- and 3-heptanone, 2- and 3-octanone, and 2- and 5-nonanone were detected. Also benzaldehyde, benzonitrile and acetophenone were formed; their presence indicates an aromatization process induced by the oxidative treatment. Benzylic oxidations and oxidations of aromatic rings are known reactions of $KMnO_4$.

After oxidation with $NaIO_4$ an important part of the reaction mixture remained non-soluble, consisting of 70 % by weight of the original melanoidin. Upon thermal degradation of this residue, very few volatiles were formed: mainly furans, some pyrazines, and 2-cyclopentene-1,4-dione. After heating of the dried filtrate, some alkyl and aryl iodides were produced, as detected by GC/MS.

These oxidative procedures induced significant changes in the melanoidin structure, but the impact of especially potassium permanganate treatment of melanoidins was too drastic to be able to extract useful data on the melanoidin composition from these results.

4.2.3 Conclusion

Thermal degradation of model melanoidins was a useful technique to study the differences in the composition of model melanoidins, when prepared from different starting products applying the same reaction conditions. Variation of the carbonyl compound, as well as of the amino acid in the Maillard reaction, influenced greatly the resulting melanoidin structure.

However, many generated flavour volatiles, mostly furan derivatives, were common to the different model melanoidins tested, which suggests the presence of conserved substructures, mainly composed of sugars and their degradation products. Heating of glucose/glycine melanoidins yielded a variety of furans, carbonyl compounds, pyrroles, pyridines and pyrazines. The reaction of glucose and glycine involved a fast polymerization, yielding a relatively high amount of water-insoluble melanoidins and a melanoidin network with moderate resistance to thermal and hydrolytic degradation. From glucose/glutamic acid melanoidins, no nitrogen-containing heterocycles were formed upon heating. The formation of methylene-bridged furan derivatives upon heating indicate the presence of glycosidically linked sugar residues. Melanoidins prepared from ascorbic acid and glycine yielded more azaheterocyclic compounds upon heating. The detection of *N*-methylsuccinimide and the cleavage of nitrogen-containing compounds after acid hydrolysis suggest the incorporation of nitrogen in, e.g., glycine-polypeptide substructures.

4.3 Characterization of real food melanoidins

4.3.1 Introduction

Studies on model systems have the advantage of their simplicity, and offer the possibility to study the influence of one or a limited amount of variables. However, the general validity of the conclusions and the relevance for complex real food systems remains unknown. Therefore, the conducted research was extended towards melanoidins isolated from three different food systems, namely bread crust, tomato puree and coffee. Isolation of water-soluble melanoidins and subsequent fractionation yielded different molecular weight fractions. Their composition was studied by thermal degradation, using the same procedure as described above for the model melanoidins. These results allow a comparison between the

Maillard reaction products of three different food products on the one hand, and with the results obtained from the thermal degradation of model melanoidins prepared from a single carbonyl compound with a single amino acid on the other hand.

4.3.2 Results and discussion

4.3.2.1 Isolation of melanoidins from real food systems

4.3.2.1.1 Bread crust melanoidins

Two types of melanoidins were obtained: melanoidins from a gluten/glucose model system and melanoidins from bread crust. The model system prepared from wheat gluten protein and glucose under dry reaction conditions was selected as an intermediate step in the evolution from a model system consisting of an amino acid and a sugar towards a real food system.

Gluten protein was prepared, mixed with the sugar and heated at 150 °C for 45 min.[211] After freeze-drying and grinding of the browned reaction mixture, 5.4 g of sample were obtained from 13 g of gluten (wet) and 2.6 g of glucose.

Solubilization of coloured material from gluten/glucose and bread crust is not possible by solvent or water extraction. Therefore, water-soluble melanoidins were obtained from gluten/glucose and bread crust by an enzymatic extraction procedure, as was developed for the extraction of melanoidins from bakery products.[212] Gluten/glucose Maillard reaction products and the bread crust samples, were incubated with Pronase in an aqueous buffer solution. At different time intervals, the absorbance of the liquid phase was compared with control samples to monitor the progress of the solubilization of the bread crust material. The results of the absorbance measurements at 360 nm are shown in Figure 4.9. The absorbance increased strongly during the first 2 days, but no further increase was noted when the samples

were incubated longer. A substantial amount of the bread crust material remained unsolubilized.

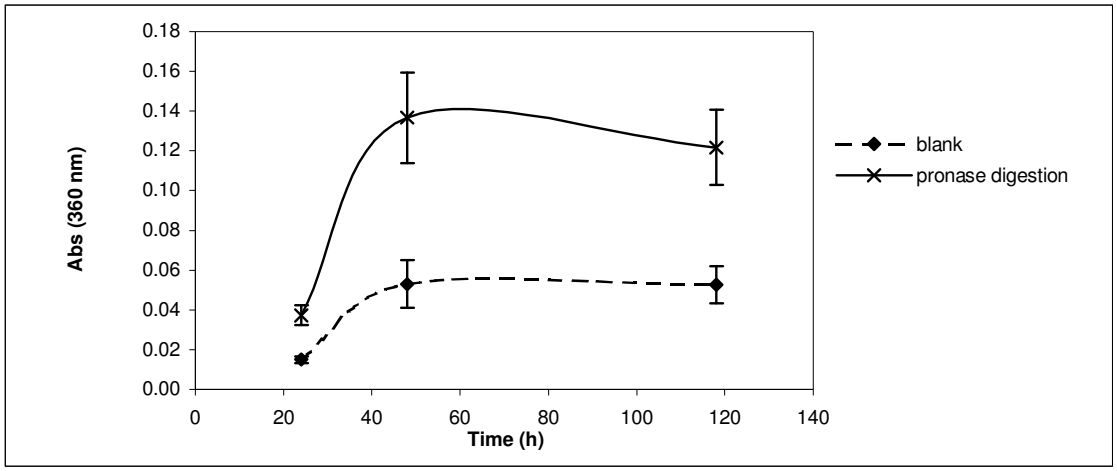


Figure 4.9. Time course of the enzymatic extraction of coloured compounds from bread crust material, as compared to bread crust samples in blank buffer solution

The extracts obtained after 120 h of digestion were then subjected to a trichloroacetic acid (TCA) fractionated precipitation, but upon centrifugation of the 20 %-TCA solution, only a small pellet of protein was removed (50 mg dry weight / g bread crust). This indicates that the enzymatic digestion procedure creates mostly soluble small peptides. In the following experiments, the supernatants were filtered immediately without TCA-precipitation, to exclude later interference of acid remaining in the samples. Ultrafiltration was used to separate the solubilized bread crust material in a high molecular weight fraction (HMW > 30,000), an intermediate molecular weight fraction (30,000 > IMW > 3,000) and a low molecular weight fraction (LMW < 3,000). Fractionation by ultrafiltration was preferred to gel filtration, since losses due to irreversible binding of browning products to the gel material have been reported.[213]

4.3.2.1.2 Tomato melanoidins

In an investigation for the effect of peeling and heating on the antioxidant activity of tomato, Graziani et al. isolated brown high molecular weight melanoidins with antioxidant activity from the water-soluble material of heated tomato purees.[214] Elementary analysis showed a $C_9H_{14}NO_5$ composition, similar to that of melanoidins obtained from different carbohydrate/amino acid model systems.[175] This is explained by the fact that the nitrogen-containing starting material of tomato (up to 1 g/ 100 g of fresh product) is mainly composed of free amino acids.[215]

Tomato purees were prepared using three different procedures. On the one hand, canned tomatoes were subjected to a relatively mild heat treatment of 8 h at 90 °C, and to a prolonged heat treatment of 40 h at 105 °C, both under reflux conditions. The described heat treatments are very severe as compared to common kitchen practice, although some recipes do require prolonged heating of tomato sauce. However, these procedures were necessary in order to collect sufficient melanoidin material. In addition, tomato melanoidins were obtained from a commercial sample of triple concentrated tomato puree, without additional heat treatment.

After removal of the carotenoids by extraction with dichloromethane and centrifugation, a brown (darker according to heat treatment) aqueous phase containing the melanoidins was obtained. The high molecular weight melanoidins were separated from the free sugars and amino acids by dialysis (MWCO 12,000 Da) and were freeze-dried. As is shown in Table 4.5, only a very small fraction of the dry weight obtained before dialysis, consisted of HMW melanoidins.

Table 4.5. Yield of melanoidins from different tomato samples before and after dialysis

	Yield before dialysis (mg/ml)	Yield HMW melanoidins (% of total yield)
Tomato puree, 8 h reflux	33.1	13.9
Tomato puree, 40 h reflux	67.5	6.1
Tomato puree, triple concentrated	73.6	2.3

In Figure 4.10, the absorbance values (360 nm) of the different tomato melanoidin fractions at the same concentration are shown. This graph demonstrates that the HMW fractions showed a considerably higher absorbance than the total extracts before dialysis. A longer heating period of the tomato puree resulted in a darker-coloured solution and the concentrated commercial tomato puree sample showed generally a lower absorbance than the heat-treated samples. A similar pattern was found by measuring the absorbance at 420 nm (data not shown).

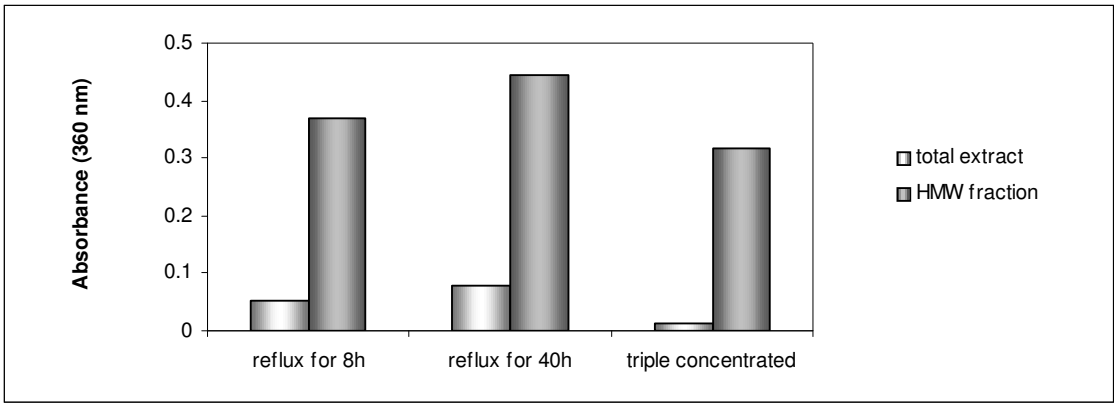


Figure 4.10. Absorbance at 360 nm of different melanoidin fractions isolated from different tomato puree samples (0.5 mg/ml)

These results confirm that in heated tomato the main contributors to the formation of water-soluble coloured material are, on a quantitative basis, the low molecular weight compounds (cf. Table 4.5). It has been shown that when melanoidins are formed in a glucose/protein model system, most of the coloured material is present in the HMW fraction, while in heated

glucose/amino acid solutions most of the coloured compounds are of low molecular weight.[213] In heated tomatoes about 90 % of the coloured material was eliminated during dialysis as LMW compounds. It should be noted, however, that the HMW fraction displays a visible absorption that is, at the same concentration, about tenfold higher than that of the total extract.

4.3.2.1.3 Coffee melanoidins

During coffee roasting, melanoidins are formed via the Maillard reaction, constituting about 23 % of roasted coffee.[139] In the present study, coffee melanoidins were isolated from roasted coffee beans according to a well-described protocol [216] and were fractionated by ultrafiltration into three different fractions: a high molecular weight fraction (HMW > 30,000), an intermediate molecular weight fraction (30,000 > IMW > 3,000) and a low molecular weight fraction (LMW < 3,000). The extracted coloured material was composed of 25 % HMW coffee melanoidins, 23 % IMW coffee melanoidins and 52 % LMW coffee brew material. The absorbance of the different fractions, at the same concentration, is shown in Figure 4.11. The higher molecular weight fractions clearly displayed the darkest colour and the highest UV absorbance, which is in agreement with previous studies.[216]

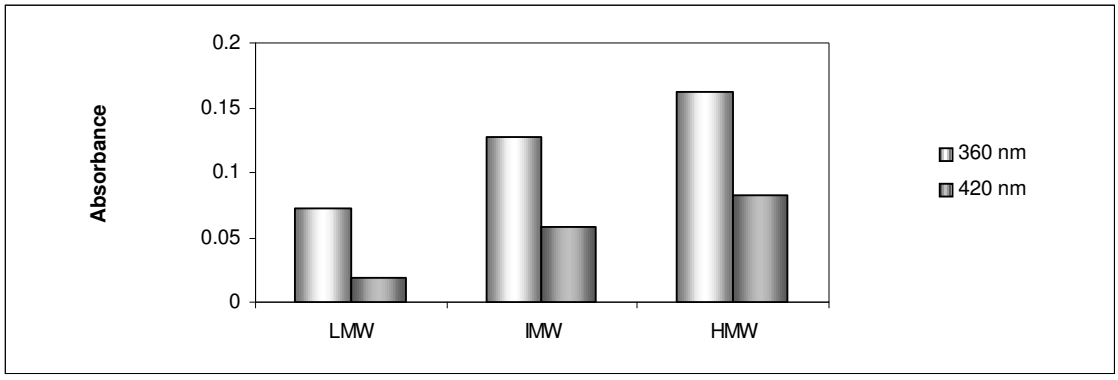


Figure 4.11. Absorbance at 360 and 420 nm of the different melanoidin fractions of coffee (0.05 mg/ml)

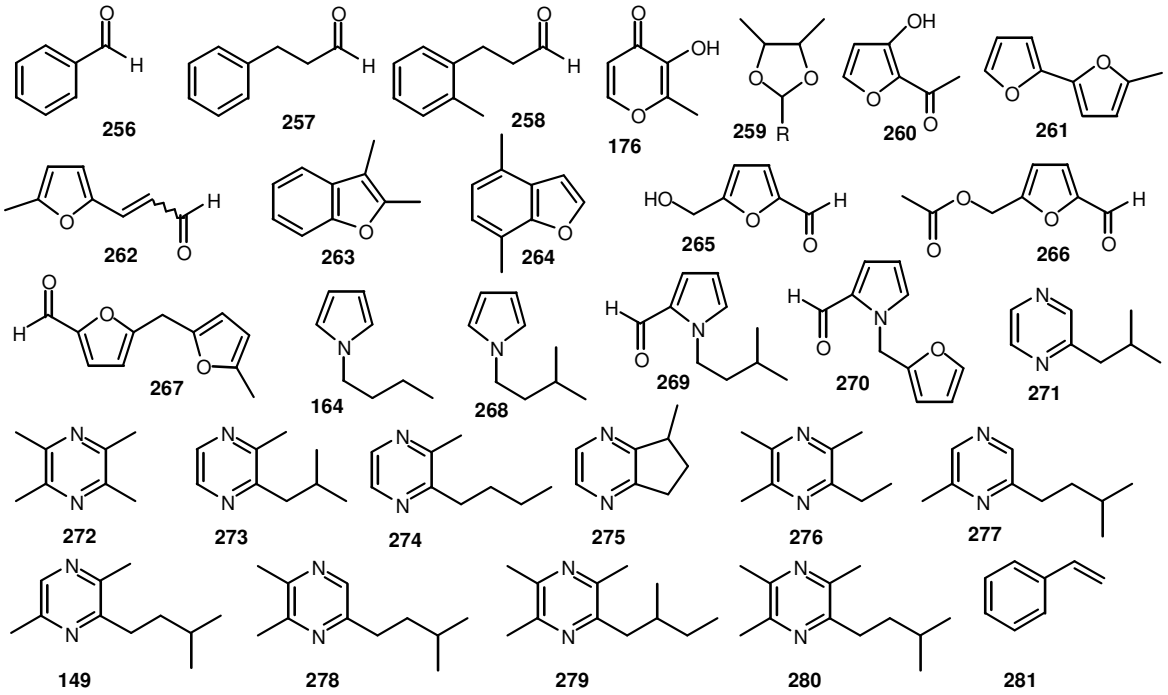
Coffee melanoidins have a significantly darker colour than the isolated tomato melanoidins, which are in fact only light brown. The absorbance of coffee melanoidins was measured at a tenfold lower concentration.

4.3.2.2 Thermal degradation of real food melanoidins

4.3.2.2.1 Bread crust melanoidins

Gluten/glucose melanoidins and the different melanoidin fractions obtained from bread crust were heated at 250 °C (10 min), and the produced volatiles were analysed by SPME-GC-MS. All analyses were performed in triplicate, except for LMW bread crust material, where very little product was available.

The headspace profile of the high molecular weight bread crust melanoidins consisted mainly of furans (74 % of the total GC peak area). Various 2-alkyl substituted 4,5-dimethyl-1,3-dioxolanes **259** (14 %) were detected, but the exact alkyl substituents could not be established for all compounds on the basis of mass spectrometry alone. The volatiles that were generated upon heating of the different bread crust melanoidin fractions, and that were not identified from heated model melanoidins, are depicted in Scheme 4.11. All identified volatiles in the headspace of heated gluten/glucose and bread crust melanoidins, with the corresponding percentage of the total GC peak area, are displayed in Table 4.6.



Scheme 4.11. Volatiles released after heating (250 °C, 10 min) of gluten/glucose and bread crust melanoidins, other than those identified from model melanoidins

Furfural **174**, maltol **176** and isomaltol **260**, which are important compounds in the headspace of heated HMW bread crust melanoidins, are typical caramelization products of sugars. Methylene-bridged furan derivatives (**228**, **229**, **230**) are indicators of glycosidically linked sugar residues. As opposed to these carbohydrate-derived flavour compounds, nitrogen-containing heterocycles were quantitatively of minor importance among the volatiles of heated HMW bread crust melanoidins.

Heating of IMW bread crust melanoidins yielded mostly the same compounds as the HMW fraction, but quantitatively more compounds were released from the IMW fraction. In addition, heating resulted in 25 % weight loss of IMW compounds, while 16 % weight loss was found for HMW bread crust melanoidins.

In case of the LMW-fraction (< 3,000), 55 % of the headspace profile consisted of pyrazines, in contrast with the low amounts of nitrogen-containing compounds detected after heating of the higher molecular weight fractions.

Application of an enzymatic digestion with Pronase should result in the cleavage of most peptide bonds. Therefore, it can be expected that the HMW fraction mainly consisted of carbohydrate-containing melanoidins, while the LMW fraction should be rich in small peptides and free amino acids, yielding mainly pyrazines upon heating. In fact, the higher molecular weight fractions yielded very few nitrogen-containing compounds upon heating, and thus, it can be concluded that few nitrogen-containing compounds are incorporated in the melanoidin backbone with other than peptide bonds, for instance as pyrroles, which could resist Pronase digestion. Carbohydrates and their degradation products are most likely the main constituents of the HMW melanoidin fractions isolated according to this procedure.

Melanoidins of high molecular weight, prepared from the gluten/glucose model system, also generated mainly furans upon heating, e.g. furfural **174** (41 %), and 5-methylfurfural **175** (34 %). No methylene-bridged furans were detected, but some pyrroles and pyrazines were identified.

Table 4.6. Volatiles from heated gluten/glucose and bread crust melanoidins (250 °C, 10 min), quantities expressed as percentage of total GC peak area. Kovats retention indices were calculated (RI calc) and compared with literature data (RI lit).

Compound	gltglc HMW	bread HMW	bread IMW	bread LMW	RI calc	RI lit
Carbonyl compounds						
2,3-pentanedione **190**			1.25		702	696[198]
2-cyclopenten-1,4-dione[a] **172**	3.36	0.77			885	
benzaldehyde **256**	2.23	0.73			961	961[200]
3-phenylpropanal **257**	0.28	0.39	0.65		1044	1043[200]
3-(2-methylphenyl)propanal[a] **258**	0.23				1103	
3-hydroxy-2-methyl-4*H*-pyran-4-one **176**	0.45	0.79			1113	1108[200]
Total carbonyl compounds	*6.56*	*2.68*	*1.91*			
1,3-Dioxolanes 259						
2,4,5-trimethyl-1,3-dioxolane[a]			5.19		730	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]		3.27	3.23		880	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]	0.30	3.78	5.15		979	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]		5.16			982	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]		1.00	1.50		1016	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]		0.51	0.56		1020	
2-alkyl-4,5-dimethyl-1,3-dioxolane[a]		0.68	0.50		1023	
Total 1,3-dioxolanes	*0.30*	*14.41*	*16.14*			
Furans						
2-methylfuran[b] **195**		2.45	2.75	5.31	612	

Table 4.6. continued

Compound	gltglc HMW	bread HMW	bread IMW	bread LMW	RI calc	RI lit
2,5-dimethylfuran[b] **198**		1.59	2.76	1.29	711	
2-vinylfuran[a] **200**		0.62	0.51	4.40	724	
2-vinyl-5-methylfuran[a] **206**		1.40	1.58	2.42	831	
furfural **174**	40.64	31.48	25.36		832	846[202]
2-furylmethanol **208**	1.90				860	864[201]
5-methyl-2(3*H*)-furanone[a] **209**	0.94	0.33	0.55		871	
2-acetylfuran[b] **173**	3.30	4.92	2.64	4.35	912	
2(5*H*)-furanone[a] **213**	1.32		0.66		924	
5-methyl-2(5*H*)-furanone[a] **214**			0.34		946	
2-methyl-5-(2-propenyl)furan[a] **215**			1.54		960	
5-methylfurfural **175**	33.56	24.40	28.82		964	962[200]
2-acetyl-3-hydroxyfuran[a] **260**	1.74	1.13	0.85		988	
benzofuran[a] **219**		0.24	0.50		995	
1-(2-furyl)-1-propanone[a] **220**	1.80	0.34	0.33		1014	
2-acetyl-5-methylfuran[b] **222**	0.22	0.19	0.38		1037	
2-(2-furyl)furan[a] **223**		0.19	0.30		1040	
2-(2-furylmethyl)furan[a] **226**		0.57	1.27		1084	
2-methylbenzofuran[b] **227**	0.29	0.69	1.81		1107	
1-(5-methyl-2-furyl)-2-propanone[a] **228**		0.12	0.26		1131	
2-(2-furyl)-5-methylfuran[a] **261**		0.28	0.46		1153	
2-(2-furylmethyl)-5-methylfuran[a] **229**	3.25	1.62	3.56		1183	
3-(5-methyl-2-furyl)-2-propenal[a] **262**	0.19				1192	

Table 4.6. continued

Compound	gltglc HMW	bread HMW	bread IMW	bread LMW	RI calc	RI lit
2,3-dimethylbenzofuran[a] **263**		0.07	0.25		1209	
4,7-dimethylbenzofuran[a] **264**		0.39	0.51		1214	
5-hydroxymethylfurfural[a] **265**			0.48		1239	
2-methyl-5-[(5-methyl-2-furyl)methyl]furan[a] **230**		0.80	2.17		1280	
5-acetoxymethylfurfural[a] **266**			0.17		>1300	
5-[(5-methyl-2-furyl)methyl]furfural[a] **267**			0.06		>1300	
Total furans	*89.15*	*73.84*	*80.87*	*17.77*		
Pyrroles						
1-butyl-1*H*-pyrrole[a] **164**				3.53	946	
1-(3-methylbutyl)-1*H*-pyrrole[a] **268**	0.56			3.85	1057	
1-(3-methylbutyl)-1*H*-pyrrole-2-carbaldehyde[a] **269**	0.18	0.23			1289	
1-(2-furylmethyl)-1*H*-pyrrole-2-carbaldehyde[a] **270**	0.32				>1300	
Total pyrroles	*1.06*	*0.23*		*7.38*		
Pyridines						
3-methylpyridine[a] **241**				2.29	866	
Pyrazines						
methylpyrazine **146**	0.88	0.93		3.73	823	820[172]
2,5-dimethylpyrazine[b] **141**				2.54	913	909[172]
2,3-dimethylpyrazine[b] **147**	0.84	2.55		4.39	919	916[172]

Table 4.6. continued

Compound	gltglc HMW	bread HMW	bread IMW	bread LMW	RI calc	RI lit
2-ethyl-5-methylpyrazine[a] **150**		0.65		2.65	1000	993[172]
2-(2-methylpropyl)pyrazine[a] **271**		0.18			1060	
3-ethyl-2,5-dimethylpyrazine[a] **143**	0.47	2.22		3.21	1087	
tetramethylpyrazine[a] **272**				19.18	1089	1090[172]
2-methyl-3-(2-methylpropyl)pyrazine[a] **273**		0.15		0.97	1135	
2-butyl-3-methylpyrazine[a] **274**		0.08			1137	
5-methyl-6,7-dihydro-5*H*-cyclopentapyrazine[a] **275**		0.16			1139	
2,3,5-trimethyl-6-ethylpyrazine[a] **276**				10.28	1162	
2-(3-methylbutyl)-6-methylpyrazine[a] **277**		0.60	0.30	1.68	1252	
2,5-dimethyl-3-(3-methylbutyl)pyrazine[a] **149**		0.23		1.72	>1300	1312[199]
2,3-dimethyl-5-(3-methylbutyl)pyrazine[a] **278**		0.61		1.84	>1300	
2,3,5-trimethyl-6-(2-methylbutyl)pyrazine[a] **279**				0.66	>1300	
2,3,5-trimethyl-6-(3-methylbutyl)pyrazine[a] **280**				2.19	>1300	
Total pyrazines	*2.19*	*8.37*	*0.30*	*55.04*		
styrene[a] **281**	0.74	0.47	0.79	17.52	891	893[199]
Total GC peak area	**3.36×10^9**	**5.36×10^9**	**8.18×10^9**	**2.83×10^9**		

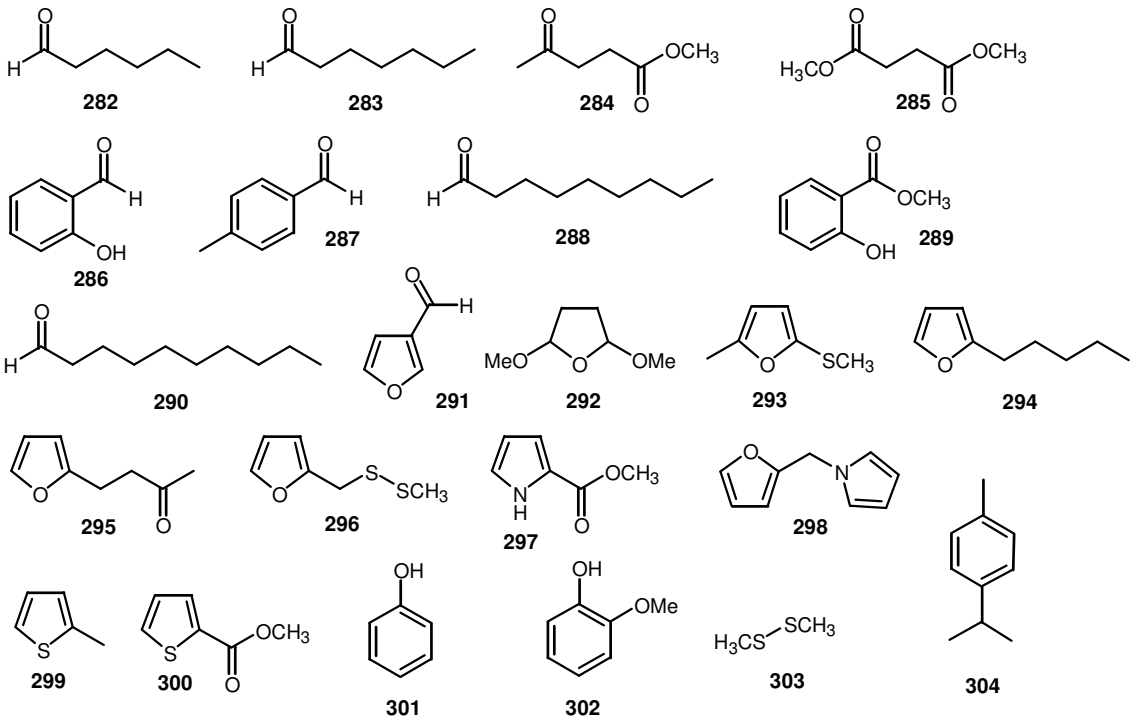
[a] Tentatively identified

[b] Comparison with standard reference compounds

4.3.2.2.2 Tomato melanoidins

High molecular weight melanoidins were recovered from three different heated tomato products: melanoidins isolated from tomato puree refluxed for 8 h, from tomato puree refluxed for 40 h and from triple concentrated tomato puree without additional heating. Since free sugars (glucose and fructose) and free amino acids are important constituents of tomato dry matter,[217] it can be expected that the formation of Maillard reaction products will show similarities with model melanoidins, prepared from a single sugar and amino acid, in particular with glucose/glutamic acid melanoidins.

Analysis of the volatiles released upon thermal degradation revealed the presence of mostly furans (70-80 % of the headspace profile) (Table 4.7). Compounds that are unique to tomato melanoidins among the sofar-tested melanoidins are depicted in Scheme 4.12.



Scheme 4.12. Volatiles identified after heating (250 °C, 10 min) of melanoidins prepared from heated tomato puree, which were not detected in model or bread crust melanoidins

Besides furans, the headspace profile of heated HMW tomato melanoidins consisted mainly of carbonyl compounds, a small amount (1-2 %) of pyrroles and methyl thiophene-2-carboxylate **300**. From the concentrated (non-heated) tomato paste 2-methylthiophene **299**, and methylpyrazine **146** were also generated. In general, the three tomato melanoidin preparations yielded a very similar spectrum of volatiles upon heating at 250 °C. The amount of volatiles released from the tomato melanoidins (as measured by the total GC peak area) increased with browning. After heating, the three differently prepared tomato melanoidins showed a very similar weight loss of 25 %.

2-Pentylfuran **294**, generated in this experiment, is an auto-oxidation product of linoleic acid.[218] Aliphatic aldehydes, such as decanal **290**, are also typical lipid oxidation products. In the preparation of the tomato melanoidins, dichloromethane extraction has been performed in order to remove the fatty fraction, but some lipids or their oxidation products may have been incorporated in the melanoidin skeleton. The formation of melanoidin-like coloured polymers from the reaction of proteins with lipid oxidation products has been shown.[219]

The results were compared with thermal degradation experiments of pectin (poly-D-α-galacturonic acid, commercial, from apple), since tomato pectin may be isolated together with the melanoidins, according to the procedure described. Heating of pectin released mainly furfural **174** (48 %), methyl furan-2-carboxylate **217** (21 %) and 5-methylfurfural **175** (15 %). Some compounds detected in the headspace of heated tomato melanoidins may therefore result from pectin-like fractions, but many other compounds cannot be ascribed to tomato pectin. It can be concluded that, during the preparation of tomato sauces, a relatively low amount of HMW light-coloured polymers is formed through the reaction of sugars and amino acids, but with fatty compounds and pectin-like fractions taking part in the reaction. Therefore, in a complex food system, many food constituents are included in the formation of the polymeric network, catalyzed by Maillard-type reactions.

Table 4.7. Volatiles from heated tomato melanoidins as compared with pectin (250 °C, 10 min), quantities expressed as percentage of total GC peak area. Kovats retention indices were calculated (RI calc) and compared with literature data (RI lit).

Compound	8h, reflux	40h, reflux	triple conc	pectin (apple)	RI calc	RI lit
Carbonyl compounds						
acetic acid **169**	2.76	2.12			637	610[198]
2,3-pentanedione **190**	0.39		0.43		702	696[198]
hexanal **282**	1.78		1.99		804	800[200]
1-acetoxy-2-propanone[b] **191**				2.39	875	
2-cyclopenten-1,4-dione[a] **172**	1.57	1.64	0.72	0.43	885	
heptanal **283**			0.90		904	899[200]
2-methyl-2-cyclopenten-1-one[a] **192**	0.33	0.41	0.60		908	
benzaldehyde **256**	8.06	3.41	2.50		961	961[200]
methyl 4-oxopentanoate[a] **284**	11.20	8.15	2.73	1.95	990	
dimethyl succinate[a] **285**				1.77	1036	
2-hydroxybenzaldehyde[a] **286**	0.65				1043	
3-phenylpropanal **258**	0.35	0.89	0.59		1044	1043[200]
4-methylbenzaldehyde[a] **287**	0.14	1.15	0.46		1081	
nonanal **288**	1.16		2.04	0.05	1106	1102[200]
3-hydroxy-2-methyl-4*H*-pyran-4-one **176**		0.80	0.80		1113	1108[200]
methyl salicylate **289**	0.35	0.48			1195	1190[200]
decanal **290**	0.78		2.57		1207	1204[200]
Total carbonyl compounds	*29.53*	*19.05*	*16.31*	*6.59*		

Table 4.7. continued

Compound	8h, reflux	40h, reflux	triple conc	pectin (apple)	RI calc	RI lit
Furans						
2-vinylfuran[a] **200**	0.67		0.24		724	
furan-3-carbaldehyde[a] **291**	0.33	0.67	0.44		817	
furfural **174**	35.33	37.16	28.35	47.51	832	830[200]
2-(2-propenyl)furan **207**				0.12	853	848[201]
2-furylmethanol **208**	1.32	2.13	2.60	0.73	860	864[201]
5-methyl-2(3*H*)-furanone[a] **209**	0.29	0.44	0.40		871	
2,5-dimethoxytetrahydrofuran[a] **292**	1.41	1.70	2.60	0.56	833	
2-acetylfuran[b] **173**	1.26	1.67	3.13	1.49	912	
2-(5*H*)-furanone[a] **213**				2.01	924	
5-methyl-2(5*H*)-furanone[a] **214**	1.16	0.65	0.77	0.38	946	
5-methylfurfural **175**	17.06	26.54	31.22	14.81	964	962[200]
2-methyl-5-methylthiofuran[a] **293**	0.74	0.77	1.02		975	
methyl furan-2-carboxylate[a] **217**				21.43	977	
2-pentylfuran **294**	3.73	1.24	2.40		994	996[201]
benzofuran[a] **219**	1.13	1.44	0.68	1.48	995	
2-furylmethyl acetate[a] **220**			1.01		1002	
1-(2-furyl)-1-propanone[a] **221**					1014	
2-acetyl-5-methylfuran[b] **222**			0.34		1037	
2-(2-furyl)furan[a] **223**	0.17	0.47			1040	
1-(2-furyl)-3-butanone[a] **295**	0.73	0.45	1.15	0.47	1076	
2,3-dihydrobenzofuran[a] **225**	0.29	0.45	0.25	0.10	1078	

Table 4.7. continued

Compound	8h, reflux	40h, reflux	triple conc	pectin (apple)	RI calc	RI lit
2-(2-furylmethyl)furan[a] **226**	0.60		2.16	0.70	1084	
2-methylbenzofuran[b] **227**	0.48	1.44	0.66	0.58	1107	
2-(2-furylmethyl)-5-methylfuran[a] **229**	0.07	0.25	0.42	0.13	1183	
2-[(methyldithio)methyl]furan[a] **296**			0.12		1211	
Total furans	*66.78*	*77.46*	*79.95*	*92.50*		
Pyrroles						
1-methyl-1*H*-pyrrole-2-carbaldehyde[b] **237**	0.83	0.71	0.70		1005	
1*H*-pyrrole-2-carbaldehyde[a] **238**	0.43		0.42		1010	
2-acetyl-1*H*-pyrrole[a] **8**		0.82	0.74		1060	
methyl 1*H*-pyrrole-2-carboxylate[a] **297**		0.10			1121	
1-(2-furylmethyl)-1*H*-pyrrole[a] **298**	0.12		0.36		1183	1182[201]
Total pyrroles	*1.38*	*1.63*	*2.22*			
Pyrazines						
methylpyrazine **146**			0.18		823	826[200]
Thiophenes						
2-methylthiophene **299** (or 3-methylthiophene)[a]			0.45		773	
methyl thiophene-2-carboxylate[a] **300**	0.50	0.22			1111	
Total thiophenes	*0.50*	*0.22*	*0.45*			

Table 4.7. continued

Compound	8h, reflux	40h, reflux	triple conc	pectin (apple)	RI calc	RI lit
Phenols						
phenol **301**		0.28			988	980[199]
2-methoxyphenol **302**	0.10	0.27			1090	1092[220]
Total phenols	*0.10*	*0.55*				
dimethyl disulfide **303**	1.28	0.56			743	751[201]
p-cymene **304**	0.42	0.52	0.89	0.92	1024	1026[200]
Total GC peak area	**6.00×10^9**	**6.20×10^9**	**4.60×10^9**	**5.21×10^9**		

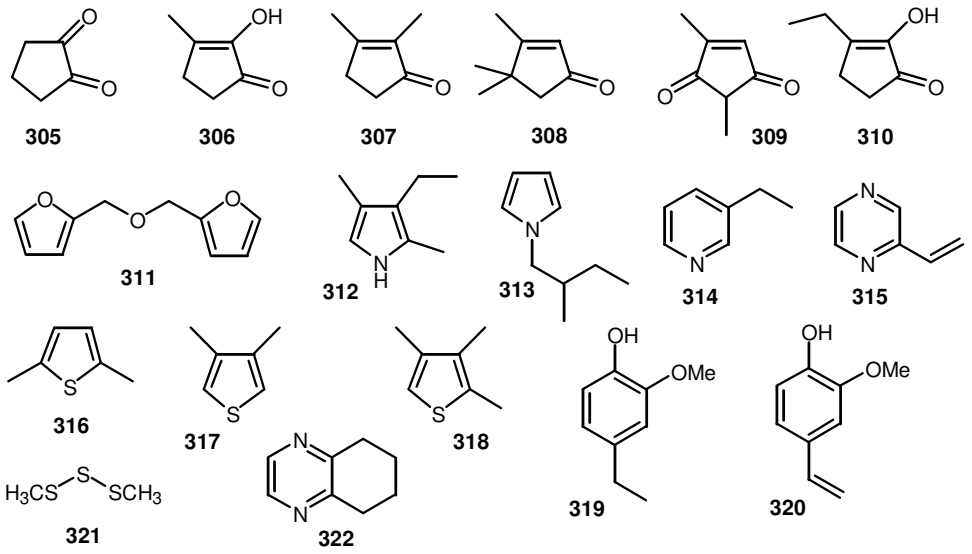
[a] Tentatively identified

[b] Comparison with standard reference compounds

4.3.2.2.3 Coffee melanoidins

Heating at 250 °C of coffee melanoidins and low molecular weight coffee brew compounds resulted in a weight loss of 16 % for the HMW melanoidins, of 23 % for the IMW fraction, and of 7 % for the LMW compounds. Heating of HMW coffee melanoidins yielded a large variety of volatiles, among which furan compounds dominated. The lower molecular weight fractions yielded a larger proportion of nitrogen-containing compounds, especially pyridines. Also sulfur-containing compounds were found in the headspace of heated coffee melanoidins, e.g. 3,4-dimethylthiophene **317** and 2-methyl-5-methylthiofuran **293**. The headspace profiles of the heated IMW and HMW coffee melanoidins were quite similar.

Compounds that were not found in the headspace extracts of melanoidins described above are depicted in Scheme 4.13; all volatiles are gathered in Table 4.8.



Scheme 4.13. Volatiles identified after heating (250 °C, 10 min) of coffee melanoidins, which were not detected in the melanoidin fractions described above

Most of the carbohydrates in coffee are insoluble polysaccharides, while monosaccharides hardly occur. Therefore, the reactive carbohydrate fraction in coffee is relatively small, and

typical sugar caramelization products such as furan-2-carbaldehydes do not predominate among the volatiles of heated coffee melanoidins, as was the case for bread crust and tomato melanoidins. Maltol **176**, which can be formed from disaccharides or Amadori compounds,[221] has been identified in roasted coffee aroma and was detected among the volatiles of heated coffee melanoidins.

Sulfur-containing furan-type compounds, such as 2-[(methyldithio)methyl]furan **296**, play an important role in the flavour of roasted coffee. 2-Furfurylthiol, the best-known sulfur-containing coffee flavour compound, was not detected upon heating of coffee melanoidin fractions. Other sulfur compounds produced, such as dimethyl disulfide **303** and thiophenes (**316**, **317**, **318**) may result from the degradation of incorporated sulfur-containing amino acid residues.

Pyridines and pyrroles can be formed from the thermal decomposition of amino acids, and from the interaction of amino acids with sugars or aliphatic aldehydes. Pyridine **181** and 3-methylpyridine **241**, however, are also known degradation products of trigonelline (*N*-methylnicotinic acid), a known coffee constituent.

Essential constituents of coffee flavour are phenols, such as guaiacol **302**, 4-ethylguaiacol **319** and 4-vinylguaiacol **320**. They are formed from the decarboxylation of phenolic carboxylic acids, which are apparently incorporated in the melanoidin structure. The presence of significant amounts of phenolic compounds in coffee melanoidins and their contribution to the antioxidant activity of coffee has been shown before.[216] Among the degradation products resulting from Curie point pyrolysis (600 °C) of coffee melanoidins, one third of the identified products were phenols.[222] However, at these high temperatures, aromatization is induced, and only few compounds could be identified as chlorogenic acid degradation products.

The detection of a whole range of compounds, many resulting not only from carbohydrate - amino acid interactions, indicate that other coffee constituents, such as chlorogenic acids and trigonellin, are also involved in the browning reactions and are incorporated in the HMW structures formed.

The LMW fraction of the coffee brew yielded considerably more nitrogen-containing compounds upon heating as compared to the higher molecular fractions. Pyridine **181** accounted for 28 % of the headspace profile. In Figure 4.12, a comparison is made of the importance of the different functional groups in the headspace of heated coffee melanoidin fractions. This graph shows that lower molecular fractions yielded especially nitrogen-containing compounds, while from the higher molecular fractions, considerably more furans and carbonyl compounds were released.

These results lead to the conclusion that carbohydrate degradation products probably possess the highest polymerization capacity. Amino acids catalyze the conversion of carbohydrates to reactive degradation products, but seem to be incorporated to a lesser extent in the higher molecular weight melanoidin structures.

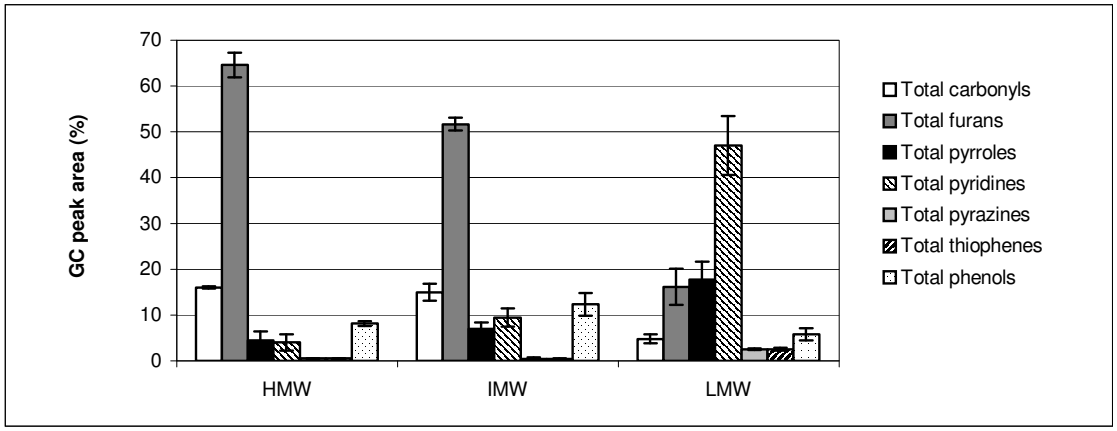


Figure 4.12. Thermal degradation profiles of different coffee melanoidin fractions (250 °C, 10 min)

Table 4.8. Volatiles from heated coffee melanoidins (250 °C, 10 min), quantities expressed as percentage of total GC peak area. Kovats retention indices were calculated (RI calc) and compared with literature data (RI lit).

Compound	coffee HMW	coffee IMW	coffee LMW	RI calc	RI lit
Carbonyl compounds					
acetic acid **169**	4.62	3.42		637	610[198]
2,3-pentanedione **190**	1.70	1.34		702	696[198]
1-acetoxy-2-propanone[b] **191**	3.19	3.43	0.89	877	
2-cyclopentene-1,4-dione[a] **172**			0.48	885	
2-methyl-2-cyclopenten-1-one[a] **192**	1.87	1.50	1.39	908	
1,2-cyclopentanedione[a] **305**	0.97	1.03		932	
2-hydroxy-3-methyl-2-cyclopenten-1-one[a] **306**	0.93	1.55		1031	
2,3-dimethyl-2-cyclopenten-1-one[a] **307**			0.61	1040	
3-phenylpropanal **257**	0.51	0.44		1044	1043[200]
3,4,4-trimethyl-2-cyclopenten-1-one[a] **308**			0.67	1062	
2,4-dimethylcyclopent-4-ene-1,3-dione[a] **309**	0.87	0.59	0.73	1102	
3-hydroxy-2-methyl-4*H*-pyran-4-one **176**	1.05	1.66		1113	1108[200]
3-ethyl-2-hydroxy-2-cyclopenten-1-one[a] **310**	0.31			1120	
Total carbonyl compounds	*16.01*	*14.98*	*4.77*		
Furans					
2-methylfuran[b] **195**	2.97	3.07		612	
2,5-dimethylfuran[b] **198**	1.72	1.39		711	
2-vinylfuran[a] **200**	0.76	0.63		724	
2-vinyl-5-methylfuran[a] **206**	0.56	0.73	0.81	831	

Table 4.8. continued

Compound	coffee HMW	coffee IMW	coffee LMW	RI calc	RI lit
furfural **174**	7.50	5.24	1.85	832	830[200]
2-(2-propenyl)furan **207**	0.29	0.28	0.27	853	848[201]
2-furylmethanol **208**	22.54	20.07	3.13	860	864[201]
2-acetylfuran[b] **173**	1.86	1.81	0.90	912	
dihydro-2(3*H*)-furanone[a] **212**	1.68	1.49	1.50	920	
2-(5*H*)-furanone[a] **213**	0.88	1.65		924	
2-methyl-5-isopropenylfuran[a] **210**			0.34	936	
5-methyl-2-(5*H*)-furanone[a] **214**	0.32	0.35		946	
1-(2-furyl)-2-propanone **215**			0.55	955	952[201]
5-methylfurfural **175**	6.24	3.31	1.15	964	962[200]
2-methyl-5-methylthiofuran[a] **293**	1.34	0.89		975	
methyl furan-2-carboxylate[a] **217**	0.61	0.44	0.72	977	
2-furylmethyl acetate[a] **220**	4.51	3.45	3.07	1002	
1-(2-furyl)-1-propanone[a] **221**	0.86		0.63	1014	
2,3-dihydrobenzofuran[a] **225**	0.61	0.47	0.28	1078	
2-(2-furylmethyl)furan[a] **226**	7.11	4.59	0.52	1084	
2-(2-furylmethyl)-5-methylfuran[a] **229**	1.63	1.37		1183	
2-[(methyldithio)methyl]furan[a] **296**	0.26	0.21	0.44	1211	
2-[(2-furylmethoxy)methyl]furan[a] **311**	0.30	0.23		>1300	
Total furans	*64.55*	*51.68*	*16.16*		

Table 4.8. continued

Compound	coffee HMW	coffee IMW	coffee LMW	RI calc	RI lit
Pyrroles					
1-methyl-1*H*-pyrrole[a] **190**	1.04	1.10	4.08	741	
1*H*-pyrrole **251**			5.26	767	765[201]
2-ethyl-1*H*-pyrrole[a] **231**	0.86	0.94	0.91	816	
2,3-dimethyl-1*H*-pyrrole[b] **232**			2.36	837	
2,5-dimethyl-1*H*-pyrrole[b] **233**			1.49	869	
2-ethyl-4-methyl-1*H*-pyrrole[a] **234**			0.94	932	
1-butyl-1*H*-pyrrole[a] **164**	0.42	0.87	0.34	946	
3-ethyl-2,4-dimethyl-1*H*-pyrrole[a] **312**			0.78	991	
1-methyl-1*H*-pyrrole-2-carbaldehyde[b] **237**	0.66		0.66	1005	
1-(2-methylbutyl)-1*H*-pyrrole[a] **313**	0.25	0.86		1053	
1-(3-methylbutyl)-1*H*-pyrrole[a] **268**	0.94	2.20	0.54	1057	
2-acetyl-1*H*-pyrrole[a] **8**		0.66		1060	
1-(2-furylmethyl)-1*H*-pyrrole **298**	0.30	0.38	0.34	1183	1182[201]
Total pyrroles	*4.48*	*7.01*	*17.70*		
Pyridines					
pyridine **181**	3.66	8.13	40.06	747	757[201]
2-methylpyridine[a] **252**			0.26	820	
3-methylpyridine[a] **241**	0.36	0.79	2.39	866	
3-ethylpyridine[b] **314**		0.57	4.32	960	
Total pyridines	*4.02*	*9.48*	*47.02*		

Table 4.8. continued

Compound	coffee HMW	coffee IMW	coffee LMW	RI calc	RI lit
Pyrazines					
pyrazine **148**		0.23		733	745[201]
methylpyrazine **146**	0.19	0.51	1.19	823	820[172]
2,6-dimethylpyrazine[b] **142**			0.62	913	907[172]
2-vinylpyrazine **315**			0.57	930	927[172]
Total pyrazines	*0.19*	*0.74*	*2.38*		
Thiophenes					
2,5-dimethylthiophene[a] **316**			1.04	877	
3,4-dimethylthiophene[a] **317**	0.50	0.46	0.94	904	
2,3,4-trimethylthiophene[a] **318**			0.53	1012	
Total thiophenes	*0.50*	*0.46*	*2.52*		
Phenols					
phenol **301**	3.99	7.20	1.78	988	
2-methoxyphenol **302**	2.44	3.08	3.31	1090	1092[220]
4-ethyl-2-methoxyphenol **319**	0.97	1.18	0.46	1281	1283[220]
2-methoxy-4-vinylphenol **320**	0.77	0.89	0.23	>1300	1320[220]
Total phenols	*8.17*	*12.34*	*5.78*		
dimethyl disulfide **303**	0.98			743	751[201]
styrene[a] **281**	1.10	1.70	1.44	891	893[199]

Table 4.8. continued

Compound	**coffee HMW**	**coffee IMW**	**coffee LMW**	**RI calc**	**RI lit**
dimethyl trisulfide **321**		1.22	2.04	967	970[220]
5,6,7,8-tetrahydroquinoxaline[a] **322**		0.39	0.19	1205	
Total GC peak area	**5.70×10^9**	**5.61×10^9**	**6.81×10^9**		

[a] Tentatively identified

[b] Comparison with standard reference compounds

4.3.3 Conclusion

Melanoidins were isolated from three basic food systems, i.e. bread, coffee and tomato sauce. Thermal degradation experiments showed significant differences in the generated volatiles of these food melanoidins and clearly demonstrated that important food flavour compounds result from the thermal degradation of the melanoidin network. In addition, it was shown that the formation of these Maillard polymers does not solely include carbohydrates and proteins. In the case of tomato melanoidins, lipid oxidation products were incorporated in the melanoidin structure, while phenolic compounds constituted an important fraction of coffee melanoidins.

4.4 Comparison of model melanoidins and food-derived melanoidins based on generated volatiles

The results obtained from the thermal degradation of standard D-glucose/glycine, D-glucose/glutamic acid and L-(+)-ascorbic acid/glycine melanoidins were compared with the results obtained from glucose/gluten melanoidins, as well as from melanoidins isolated from bread crust, tomato and coffee. For each type of melanoidins, and for each molecular weight fraction, a whole range of compounds was released upon heating, comprising on the one hand quite universal and on the other hand very specific volatiles.

A comparison was made between the relative importances of the different chemical classes of compounds in the headspace profile of the different melanoidin fractions. These data were subjected to principal components analysis to get an insight in the variability of the data. Principal components analysis is used to perform a dimension reduction on a multivariable data set. The original variables are transformed into a new set of variables, named principal components (PC's), in such a way that the first principal components account for the largest

proportion of variation in the original data set. Thus, it is possible to visualize the data in a two-dimensional plot of PC1 vs. PC2, covering as much information as possible.

The IMW fraction of bread and coffee melanoidins have been omitted for the construction of the graph for reasons of clarity and because of the high similarity with the HMW fraction. The resulting principal components analysis biplot is shown in Figure 4.13, depicting the various melanoidin fractions in the plane of the two first principal components.

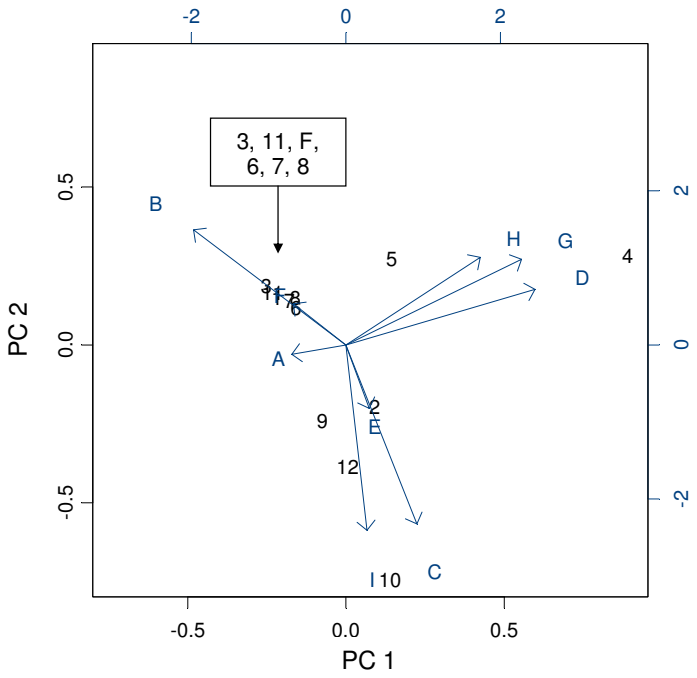


Figure 4.13. Principal components analysis biplot, depicting the different melanoidin fractions in the plane of the two first principal components (explaining together 59 % of variance)

1	glucose/gluten HMW	A	carbonyl compounds
2	bread crust LMW	B	furans
3	bread crust HMW	C	pyrroles
4	coffee LMW	D	pyridines
5	coffee HMW	E	pyrazines
6	tomato, reflux 8 h, HMW	F	1,3-dioxolanes
7	tomato, reflux 40 h, HMW	G	thiophenes
8	tomato, triple concentrated	H	phenols
9	glucose/glycine LMW	I	oxazoles
10	glucose/glycine HMW		
11	glucose/glutamic acid HMW		
12	ascorbic acid/glycine HMW		

Melanoidins with a high negative value of PC 1 (explaining 31.5 % of variance) released high amounts of furans, 1,3-dioxolanes and carbonyl compounds upon heating, and low amounts of especially pyridines, thiophenes and phenols. Melanoidins with a high positive value of PC 2 (explaining 27.5 % of variance) yielded especially high amounts of furans, phenols and thiophenes, and low amounts of pyrroles and pyrazines.

In Figure 4.13 can be seen that the HMW melanoidins derived from bread crust and tomato are not differentiated from each other based on the flavour generation profile, and are located on the left-hand upper side of the graph, indicating the generation of mainly furan and 1,3-dioxolane compounds upon heating. Coffee melanoidins are differentiated from the others by the generation of phenolic compounds, pyridines and thiophenes upon heating. The LMW fractions (bread, coffee) are clearly differentiated from the HMW melanoidins. Model melanoidins prepared from either glucose or ascorbic acid with glycine are differentiated from food-derived melanoidins by the yields of pyrroles, pyrazines and oxazoles. As determined by the volatiles produced upon heating, the variability between glucose/glycine model melanoidins and food-derived melanoidins, such as bread crust and tomato melanoidins, is high. Model melanoidins prepared from D-glucose and glutamic acid showed a much higher similarity to food-derived melanoidins. This is probably due to the low reactivity of the amino acid that mainly catalyzed the conversion of the sugar in reactive degradation products that are able to polymerize. This situation seems to describe more adequately what happens in real food systems, indicating that glucose/glycine melanoidins are perhaps not the best model system to study the properties of food-derived melanoidins.

4.5 Food melanoidins and their interaction with flavour compounds

4.5.1 Introduction

In addition to their obvious contribution to food colour, melanoidins display a wide range of functional properties that have been the subject of many studies. One of the most interesting properties of melanoidins is a clearly demonstrated antioxidant activity, by which melanoidins protect food against lipid oxidation.[223,224]

Furthermore, macromolecular melanoidins are capable of binding various food constituents by a combination of chemical and physical interactions. Binding of nutritionally important metals by Maillard reaction products decreases the bioavailability of calcium and other metal ions.[225] This metal chelating ability explains in part the antioxidant activity of melanoidins.[226,227] Potentially hazardous compounds, such as heterocyclic amines that are known mutagenic food constituents, can also be bound by melanoidins, which exert in this way a health-promoting effect.[228] Interactions of melanoidins with flavour compounds can influence the release of these flavour compounds from a food matrix to the headspace, and thus have an important impact on food quality in terms of aroma perception. Flavour release is a research area that has emerged in recent years and that is of important interest to the food industry.[229] Various studies on the interactions of food matrix constituents with flavour compounds have indicated that carbohydrates and proteins can bind, adsorb, entrap, complex, or encapsulate flavour compounds.[230] These processes are mainly reversible and therefore allow the release of flavour compounds in the oral cavity during the eating process. Lipids generally show a significant retention of especially hydrophobic flavour compounds, such as long-chain aldehydes. The reaction of carbonyl-containing flavour compounds with amino groups or thiols in proteins may lead to covalent bonds.[231] In this way, the headspace flavour profile of the food product is selectively altered, which influences the overall food aroma.

Due to the presence of a variety of functional groups, melanoidins certainly have the potential to covalently bind specific flavour compounds.

Hofmann et al. investigated the influence of coffee melanoidins on coffee flavour volatiles.[232,233] It was found that the predominant coffee flavour volatiles, such as acetaldehyde, 2-methylpropanal, 3-methylbutanal, 2,3-butanedione and 2,3-pentanedione were not influenced by the presence of coffee melanoidins. However, the roasty-sulfury aroma of an aqueous coffee melanoidin solution was decreased by a significant reduction of the headspace concentrations of thiols. Spectroscopic measurements indicated that these thiols are covalently bound to the coffee melanoidins via Maillard-derived pyrazinium compounds formed as oxidation products of 1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations (named CROSSPY **160** – cf. Scheme 4.5). Thus, coffee melanoidins were shown to be important activators of the aroma staling of freshly prepared coffee brew.

In previous flavour release studies, performed in cooperation with Prof. Obretenov (Higher Institute of Food and Flavour Industry, Plovdiv, Bulgaria) and cooperating PhD student Milda Keršienė (Kaunas University of Technology, Kaunas, Lithuania), Solid Phase Microextraction was used for the study of the release of 3-methylbutyl acetate by model melanoidins in aqueous solutions. The results demonstrated that melanoidins prepared from D-glucose or L-(+)-ascorbic acid with glycine exerted mainly a solvating effect and increased the release of 3-methylbutyl acetate in the headspace. Melanoidins prepared from L-(+)-ascorbic acid and glutamic acid or lysine, on the contrary, showed a clear retention of 3-methylbutyl acetate. This was explained by the formation of basic or acidic melanoidins from lysine or glutamic acid, respectively, that are able to form dipole-dipole or dipole-ion ($-NH_3^+$, $-COO^-$) interactions with the 3-methylbutyl acetate ester function.

Quite some research has been devoted to the flavour retention capacity of coffee melanoidins.[234,235] Other food-derived melanoidins, however, have not yet been investigated

for their interaction with flavour compounds. Therefore, some flavour release studies were performed in order to assess the flavour retaining capacity of bread crust and tomato melanoidins.

4.5.2 Results and discussion

4.5.2.1 Interaction of bread crust and tomato melanoidins with 3-methylbutyl acetate

To enable a comparison with the results obtained from model melanoidins, in first instance, the interaction of bread crust and tomato melanoidins with 3-methylbutyl acetate was studied. 3-Methylbutyl acetate is a very common fruity flavour compound that is used as a flavouring agent in different food products.[237]

Bread crust and tomato melanoidins were freshly prepared, were separated by dialysis (MWCO 12,000 Da) in a high and low molecular weight fraction, and were stored at –20 °C before use. Aqueous solutions of 100 and 1000 mg/l melanoidins were prepared, and the flavour compound was added as a concentrated solution in ethanol to improve the solubility. After 30 minutes of equilibration, the headspace of the flavour - melanoidin mixtures was sampled by headspace Solid Phase Microextraction (DVB/Car/PDMS). A very short sampling time of one minute was applied, since this was shown to represent equilibrium headspace concentrations.[236]

The headspace 3-methylbutyl acetate concentration of aqueous solutions was compared with melanoidin-containing aqueous solutions. Each measurement was performed in triplicate. The results for the interaction of bread crust material with 3-methylbutyl acetate are shown in Figure 4.14. The addition of HMW bread crust melanoidins to 3-methylbutyl acetate-containing aqueous solutions had a solvating effect, leading to an increased concentration in

the headspace. The highest concentrations of dialyzable LMW bread crust material showed a tendency towards flavour retention, which was, however, only significant at the lowest flavour concentration. The reproducibility of the experiments was very high.

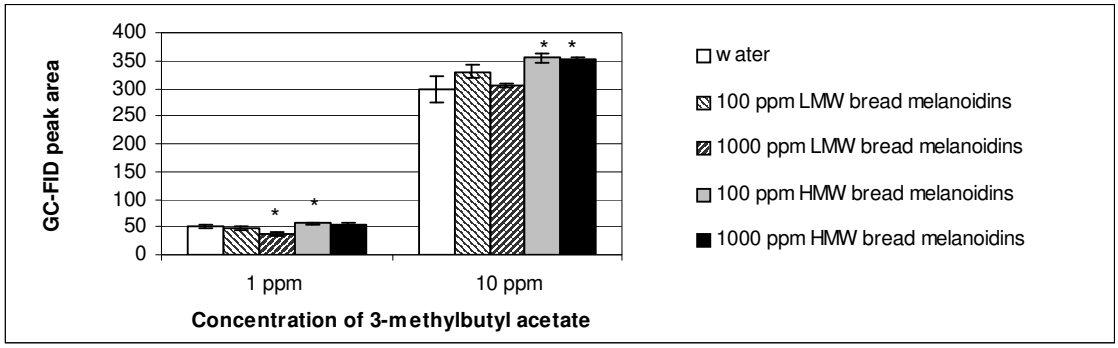


Figure 4.14. Influence of bread crust dialyzable (LMW) and non-dialyzable (HMW) melanoidins on the 3-methylbutyl acetate headspace concentration of aqueous solutions, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

Similar experiments were performed with melanoidins isolated from heated tomato puree (heated at reflux for 40 h). In this case, a significant flavour retaining capacity was shown for HMW tomato melanoidins with the lowest 3-methylbutyl acetate concentration, and for the highest concentration of dialyzable tomato melanoidins with 3-methylbutyl acetate in both applied flavour concentrations (Figure 4.15).

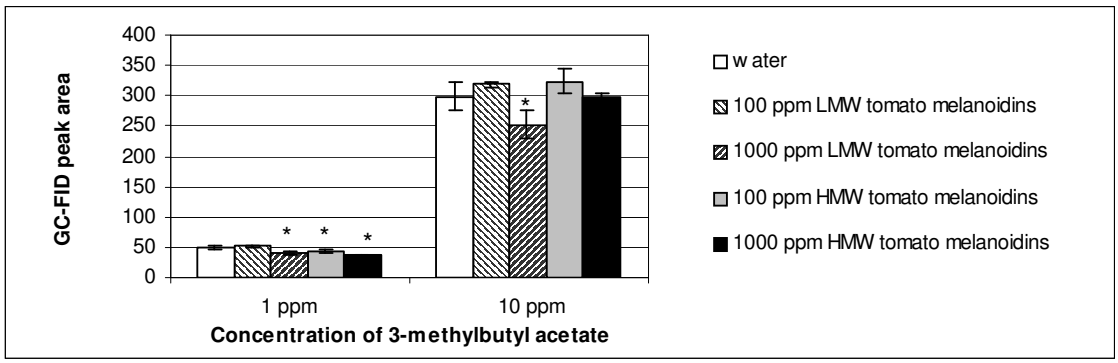


Figure 4.15. Influence of tomato puree dialyzable (LMW) and non-dialyzable (HMW) melanoidins on the 3-methylbutyl acetate headspace concentration of aqueous solutions, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

As compared to model L-(+)-ascorbic acid/lysine and L-(+)-ascorbic acid/glutamic acid melanoidins, the retention of 3-methylbutyl acetate by bread crust and tomato melanoidins is low. It can be expected that melanoidins formed in a real food system from the interaction of carbohydrates with proteins and peptides have a much lower charge density than model melanoidins prepared from a carbonyl compound and a single polar amino acid. In tomatoes, relatively high amounts of free amino acids, such as glutamic acid, are present. In addition, part of the tomato pectin fraction might also be included in the isolated tomato melanoidins. This polygalacturonic acid (with a variable number of methyl ester groups) may participate in the formation of a more polar melanoidin polymer. This might explain the low but significant flavour retention of 3-methylbutyl acetate by tomato melanoidins.

In a study for the calcium complexation by melanoidins, model melanoidins formed moderately stable complexes with Ca^{2+}, while pigments from toasted bread and coffee showed no measurable calcium-binding ability.[225] A difference in charge density between model melanoidins and food-derived melanoidins was concluded.

Assessment of the interaction of 3-methylbutyl acetate with coffee melanoidins showed no effect on the headspace flavour concentration (data not shown). This result is in agreement with previous experiments by other research groups, which showed no interaction between coffee non-volatiles and esters.[234]

4.5.2.2 Interaction of bread crust and tomato melanoidins with model flavour compounds of different functionalities

3-Methylbutanal was selected as a second flavour compound under study, since this flavour compound is one of the flavour volatiles of bread and tomato, and allows the evaluation of the reactivity of an aldehyde functionality. The effect of the presence of bread crust and tomato melanoidins on the headspace concentration of 3-methylbutanal in aqueous solutions is shown

in Figure 4.16. Bread crust melanoidins had no effect on the headspace concentration of 3-methylbutanal, but tomato melanoidins showed a significant flavour retaining capacity in all cases, except for the highest concentration of both 3-methylbutanal and tomato melanoidins, where the retention was not significant ($\alpha = 0.05$). It was reported that coffee melanoidins showed no interaction with aldehydes.[234] These results were confirmed in our experiments with 3-methylbutanal (data not shown).

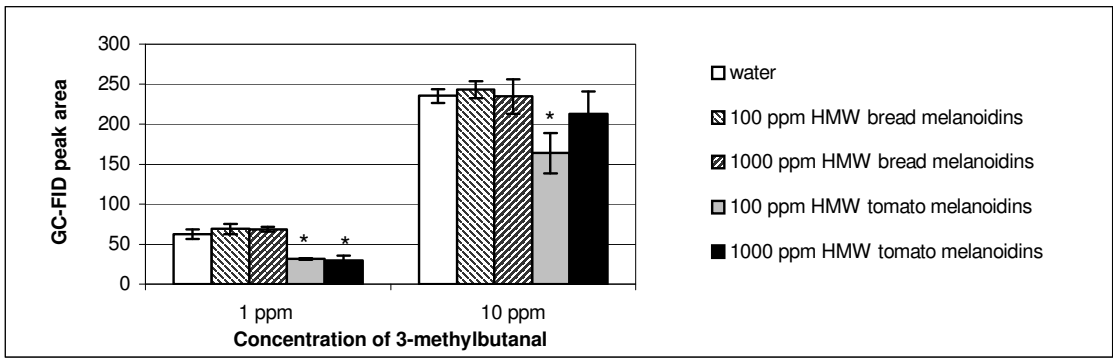


Figure 4.16. Influence of bread crust and tomato puree non-dialyzable (HMW) melanoidins on the 3-methylbutanal headspace concentration of aqueous solutions, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

Two other flavour compounds that are important in bread as well as in tomato aroma are 2-acetylfuran and 2-acetylpyridine.[237] These compounds are, however, less potent flavour compounds and were applied in higher concentrations of 1000 ppm in order to obtain a sufficient sensitivity by one minute of headspace sampling. The results of the SPME analyses are shown in Figure 4.17 and Figure 4.18. A clear decrease in reproducibility is found for these flavour compounds at the applied concentrations. In all cases, no significant effect of flavour retention or release could be concluded.

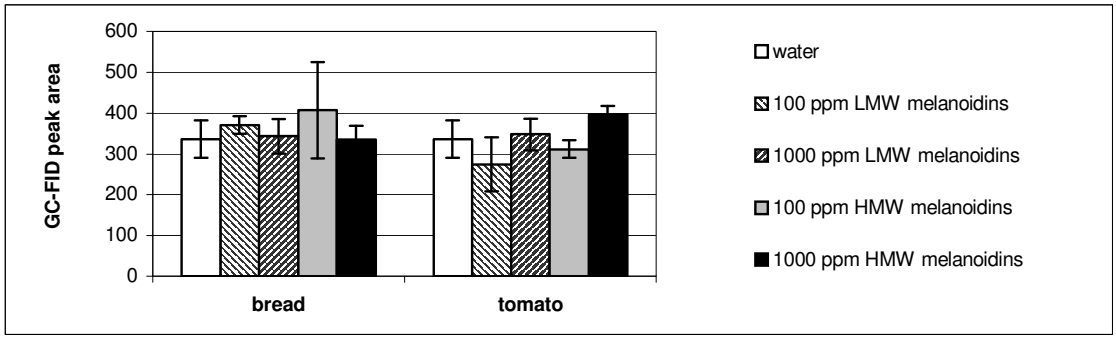


Figure 4.17. Influence of bread crust and tomato puree dialyzable (LMW) and non-dialyzable (HMW) melanoidins on the headspace concentration of aqueous solutions containing 1000 ppm 2-acetylfuran, as measured by SPME.

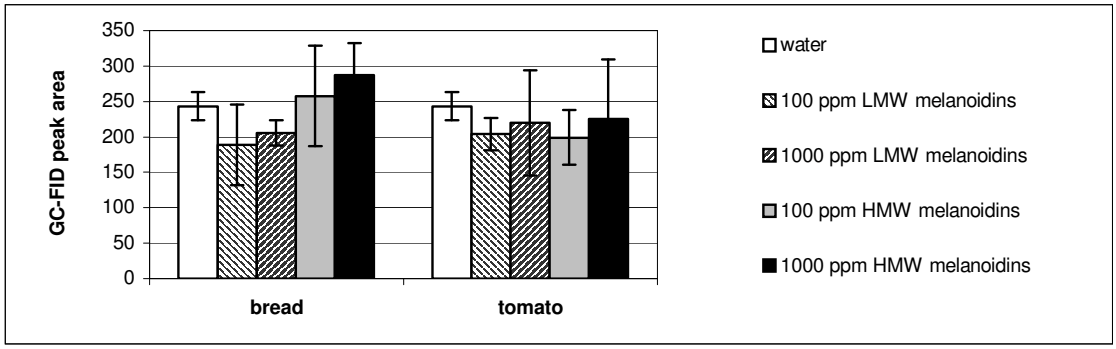


Figure 4.18. Influence of bread crust and tomato puree dialyzable (LMW) and non-dialyzable (HMW) melanoidins on the headspace concentration of aqueous solutions containing 1000 ppm 2-acetylpyridine, as measured by SPME.

Evaluation of the interaction of melanoidins from bread crust and tomato with 3-methylbutanol showed that bread crust melanoidins displayed a flavour retaining capacity for the alcohol, which was, however, only significant at the highest melanoidin concentration (1000 ppm). No significant interaction with tomato melanoidins was shown (Figure 4.19).

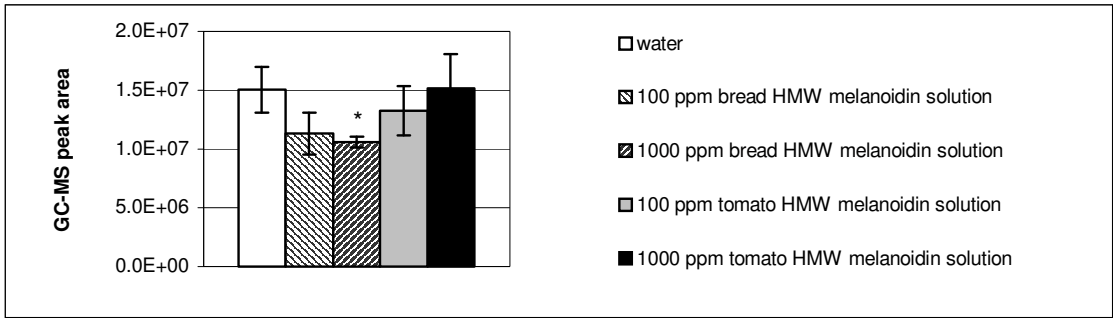


Figure 4.19. Influence of bread crust and tomato puree non-dialyzable (HMW) melanoidins on the headspace concentration of aqueous solutions containing 100 ppm 3-methylbutanol, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

The same conclusions could be drawn from studies of the interaction of bread crust and tomato melanoidins with limonene. Bread crust melanoidins showed a significant retention of the hydrocarbon terpene limonene at the highest melanoidin concentration, but tomato melanoidins demonstrated no significant retention or release (Figure 4.20).

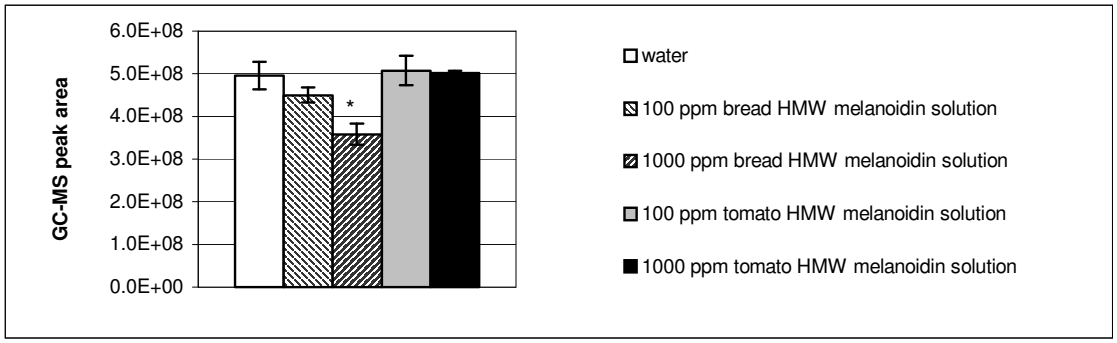


Figure 4.20. Influence of bread crust and tomato puree non-dialyzable (HMW) melanoidins on the headspace concentration of aqueous solutions containing 10 ppm limonene, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

6-Acetyl-1,2,3,4-tetrahydropyridine **3** (6-ATHP) is an important constituent of bread crust flavour.[69] The interaction of bread crust melanoidins with 6-ATHP-containing Maillard reaction mixtures resulting from the model reaction of proline with 1,3-dihydroxyacetone, as

described in Chapter 2, was investigated. As compared to blank aqueous solutions, the presence of bread crust melanoidins caused a significant retention of 6-ATHP **3** at concentrations of 1000 ppm. 6-Acetyl-5-methyl-2,3-dihydro-1*H*-pyrrolizine **119**, the main side product of the reaction, was significantly released from the melanoidin-containing aqueous solutions. In this specific case, the interaction with a flavour mixture is tested. Therefore, an additional competition effect influences the results.

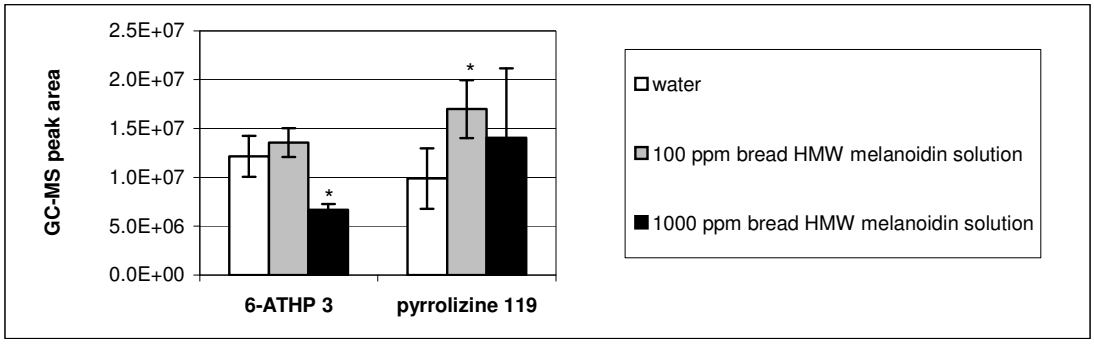


Figure 4.21. Influence of bread crust non-dialyzable (HMW) melanoidins on the headspace concentration of aqueous solutions containing 6-ATHP **3** and 6-acetyl-5-methyl-2,3-dihydro-1*H*-pyrrolizine **119** in proline/1,3-dihydroxyacetone Maillard reaction mixtures, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

4.5.2.3 Interaction of coffee melanoidins with 2-furfurylthiol

A striking retention of thiol compounds by coffee melanoidins has been reported in the literature.[232,234] In order to confirm these reports, using Solid Phase Microextraction as sampling technique, the influence of coffee melanoidins on the headspace concentration of 2-furfurylthiol was investigated (Figure 4.22). When using a mass selective detector, the chromatographic peak of 20 ppm 2-furfurylthiol decreased below detectable levels in the presence of HMW coffee melanoidins. Comparison of different molecular weight fractions of coffee melanoidins indicated that the flavour binding capacity was the highest for the HMW

(> 30,000) fraction, and the lowest for the LMW (< 3,000) coffee brew constituents. These results are not in agreement with the results of Hofmann et al.,[232] who found the lowest molecular weight fraction to have the highest retention capacity. However, in this case fractionation was performed by gel filtration, and four fractions were isolated with molecular weight ranges that are different from the ultrafiltration ranges applied here.

When the concentration of 2-furfurylthiol was elevated to 100 ppm, the presence of all molecular weight fractions of coffee melanoidins had no significant effect on the 2-furfurylthiol headspace concentration, indicating a saturation effect of limited binding sites.

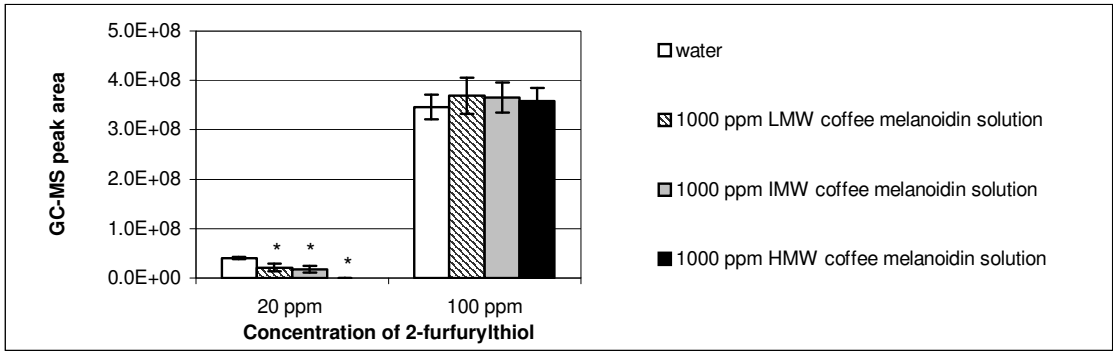


Figure 4.22. Comparison of the influence of different molecular weight fractions of coffee on the headspace flavour concentrations of 2-furfurylthiol, as measured by SPME - * indicates significantly different values ($\alpha = 0.05$).

The retention of 2-furfurylthiol by coffee melanoidins was shown to involve covalent interactions with 'CROSSPY' radical cations.[233] These radical cations have been identified in coffee and in bread crust melanoidins.[187] However, bread crust melanoidins showed absolutely no retention of 2-furfurylthiol, when administered in concentrations of 1, 10 and 20 ppm. Bread crust and tomato melanoidins had no significant influence on the headspace concentrations of 2-furfurylthiol in aqueous solutions (data not shown).

4.5.3 Conclusion

The interaction of food-derived melanoidins with food-relevant flavour compounds was investigated, using SPME as the sampling technique. The results, reported in literature, on the retention of thiols by coffee melanoidins, and the lack of interaction of coffee melanoidins with other flavour compounds, were confirmed. Concerning the interaction of bread crust melanoidins with model flavour compounds, a significant retention of 3-methylbutanol, limonene and 6-acetyl-1,2,3,4-tetrahydropyridine was found, while the headspace concentration of the other tested flavour compounds was not significantly influenced by the presence of bread crust melanoidins. Melanoidins isolated from heated tomato puree, showed no interaction with 3-methylbutanol, limonene, 2-acetylpyridine, 2-acetylfuran and 2-furfurylthiol, but significantly retained 3-methylbutanal and 3-methylbutyl acetate in solution. These results suggest that melanoidins isolated from tomato are more polar in nature than bread crust melanoidins, which can be explained by the relatively high amounts of free amino acids in tomato.

The gathered results confirm that the melanoidins isolated from three different food systems show significant differences, in flavour generation as well as in flavour retention. Different functionalities are present and, therefore, each food system has to be investigated separately to elucidate its chemical and functional properties.

4.6 Materials and methods

4.6.1 Preparation of model melanoidins

In a 300-ml Christ filter bottle, 0.05 mol of D-glucose **85** (9.00 g) or L-(+)-ascorbic acid **184** (8.80 g), and 0.05 mol of glycine **168** (3.75 g) or glutamic acid **183** (7.35 g) were dissolved in 20 ml of distilled water. The solution was frozen in a bath of liquid nitrogen. Subsequently it was freeze-dried (*Christ Alpha 1.4*) until all the water was removed (i.e. constant weight). The carbonyl compound - amino acid mixture was placed in an oven (*Memmert*), which was equipped with a fan and had been preheated to and stabilized at 125 °C. The mixture was heated for exactly 2 h without covering. After heating, the filter bottle was allowed to cool down to room temperature in a desiccator. The solid was transferred to a mortar and carefully ground to a fine powder. Five grams of the ground material were added to 200 ml of distilled water and the solution was stirred for 12 h to dissolve as much material as possible. This suspension was filtered through Whatman No. 4 filter paper and the filtrate, which contained the water-soluble melanoidins, was collected. The residue on the filter paper was washed with two times 20 ml of distilled water. The combined filtrate and washings were made up to 250 ml with distilled water. This mixture is called solution A. The residue obtained, the so-called water-insoluble fraction of the melanoidins, was freeze-dried and stored at -20^{o} C until further use.

Dialysis tubing with a flat width of 33 mm was prepared according to the manufacturer's instructions. This cellulose membrane retains > 90 % cytochrome c (MW 12,400) in solution over a 10 h period. Fifty ml of the soluble melanoidin solution A was brought in 21 cm of dialysis tubing and was dialyzed against 1 l of distilled water for 24 h at 4 °C with four changes of the surrounding water. At the end of the dialysis, the contents of the dialysis tubing with the high molecular weight fraction (HMW) or so-called non-dialyzable

melanoidins, were transferred to a 500-ml round-bottom flask, were frozen in a liquid nitrogen bath and freeze-dried until all the water was removed. When the low molecular weight fraction (LMW) of the melanoidins was needed, the dialysate resulting from the first change of water was collected and freeze-dried.

4.6.2 Acid hydrolysis of model melanoidins

Acid hydrolysis was performed according to the optimal conditions found by Cämmerer et al.[181] Non-soluble melanoidins were suspended or HMW melanoidins were dissolved in acid solution to a concentration of 20 mg/ml. The mixture was heated in an oil bath, and stirred for 1 h. After rapid cooling in an ice bath, 1 ml of distilled water was added for every ml of solution. The mixture was neutralized using a 2 N NaOH solution and filtered. The water-insoluble residue on the filter was washed with distilled water and dried. The filtrate was subjected to dialysis, as described higher, leading to a high molecular weight (HMW) and a low molecular weight (LMW) fraction. For the acid hydrolysis, two combinations of concentration and temperature were used: (1) 1 N HCl, 1 h, room temperature (Hydr 1); (2) 1 N HCl, 1 h, reflux (Hydr 2).

4.6.3 Oxidation of model melanoidins

Oxidative degradation of melanoidins was done according to the procedure of Kato and Tsuchida.[176] Five gram of non-soluble D-glucose/glycine melanoidins were suspended in 2 N K_2CO_3 (80 ml) and 3 % $KMnO_4$ (800 ml) was gradually added, while stirring at 30 °C. The reaction mixture was heated at 60 °C until decolourization and filtered. For the oxidation with sodium periodate, 5 g of non-soluble D-glucose/glycine melanoidins were suspended in water (80 ml), 0.5 M $NaIO_4$ (200 ml) was added and the mixture was allowed to react for 4 h at room temperature.

4.6.4 Isolation of food melanoidins

4.6.4.1 Isolation of gluten/glucose and bread crust melanoidins

Gluten was prepared by extensive washing under tap water of dough obtained from wheat flour containing 9.5 % proteins (*Barilla*). Glucose (2.6 g) was added to the gluten (13 g) and mixed. The mixture was heated in an oven (*Memmert*) at 150 °C for 45 min. The resulting brown cake was freeze-dried and ground in a blender. Bread crust was separated with a kitchen knife from a 1-kg bread, type 'San Sebastiano', which is characterized by a thick and dark crust. The crust samples were freeze-dried, and ground in a kitchen blender.

The melanoidins from bread crust and gluten/glucose were extracted in an aqueous environment using an enzymatic digestion with Pronase E, from *Streptomyces griseus*. To monitor the enzymatic digestion, 250-mg samples were dissolved in 3 ml TRIS - HCl buffer (20 mM, pH 8) in triplicate. One series of three samples served as the control, while to another series of three samples the enzyme was added. Pronase was added to the samples up to a concentration of 0.1 mg/ml. In a separate flask, aliquots of bread crust (or of the gluten/glucose reaction mixture) were treated for digestion in the same way. The samples were carefully mixed (vortex) and incubated at 37 °C, while shaking (75 rpm).

The digestion was monitored by spectrophotometric measurements. For this purpose, samples were centrifuged (4000 g/min, 4 °C, 10 min), after which 40 µl of supernatant was added to 1 ml of distilled water before measuring the absorbance at 360 nm and 420 nm after 24 h, 48 h and 118 h. After this time, the samples were centrifuged and the supernatants collected. Precipitation with trichloroacetic acid (TCA) was performed by adding TCA to the sample up to a concentration of 9.5 %, 13 %, 17.5 % and 20 %, followed by centrifugation.

To fractionate the resulting melanoidin solution, ultrafiltration was performed with a stirred ultrafiltration cell (*Amicon*). Thus, three fractions were obtained: a high molecular weight

fraction (HMW > 30,000), an intermediate molecular weight fraction (30,000 > IMW > 3,000) and a low molecular weight fraction (LMW < 3,000).

4.6.4.2 Isolation of tomato melanoidins

In a round-bottom flask, 350 g of canned tomatoes (passata, *AnnaLisa*) were placed in a preheated water bath, and allowed to reflux with stirring for 8 h (or 40 h). The sample was allowed to cool down and was extracted with five times 250 ml of dichloromethane. Upon centrifugation (4000 g/min, 10 min, 4 °C), the brownish aqueous phase containing the water-soluble melanoidins was collected. The HMW melanoidins were separated by dialysis (*Medicell International*, MWCO 12,000-14,000 Da) during three days (4 °C, with stirring). The water was removed by lyophilization. Fifty grams of triple concentrated tomato puree (*Oro di Parma*), dissolved in 200 ml of water, were treated in the same way as the heated tomato purees.

4.6.4.3 Isolation of coffee melanoidins

Standard roasted coffee (100 g) was ground to a fine powder in a blender. A first solid-liquid extraction was carried out by adding 300 ml of hot water (75 °C) and stirring for 5 min. The resulting mixture was filtered and the residue on the filter was extracted again with 300 ml of hot water (75 °C). After filtration, both filtrates were combined and defatted by extraction with dichloromethane (two times 200 ml). The melanoidin solution was concentrated by lyophilization and fractionated in three fractions by ultrafiltration: a HMW fraction (> 30,000), an IMW fraction (30,000 > MW > 3,000) and a LMW fraction (< 3,000).

4.6.5 Thermal degradation experiments and analysis of volatiles

Silanized 4-ml SPME-vials (*Supelco*) filled with 50 mg of melanoidins were covered with PTFE-silicone septa and open top polypropylene (*Supelco*) closures and heated on a sand bath to maintain a constant temperature (± 5 °C) during 10 minutes. After cooling down of the vials to room temperature, the SPME fiber (DVB/Carboxen/PDMS, *Supelco*) was exposed to the headspace of the heated melanoidins during five minutes. Volatile compounds were desorbed in the injection port of the GC at 250 °C for two minutes.

For the analysis of the SPME-extracts a Hewlett-Packard 6890 GC Plus coupled with a HP 5973 MSD (Mass Selective Detector-Quadrupole type), equipped with a CIS-4 PTV (Programmed Temperature Vaporization) Injector (*Gerstel*), and a HP5-MS capillary column (30 m × 0.25 mm i.d.; coating thickness 0.25 µm) was used. Working conditions were: injector 250 °C, transfer line to MSD: 250 °C, oven temperature: start 40 °C, hold 2 min; programmed from 40 to 120 °C at 4 °C min^{-1} and from 120 to 240 °C at 30 °C min^{-1}, hold 2 min; carrier gas (He) 1.2 ml min^{-1}; splitless; ionization EI 70 eV; acquisition parameters: scanned m/z 40-200 (0-10 min), 40-300 (10-20 min), 40-400 (> 20 min). Substances were identified by comparison of their mass spectra and retention times with those of reference substances and by comparison with the Wiley (6^{th}) and the NIST Mass Spectral Library (Version 1.6d, 1998). When only MS data were available, identities were considered to be tentative.

4.6.6 Spectrophotometric measurements

Absorbances of model melanoidin solutions were recorded with a Cary 50 UV Visible Spectrophotometer (*Varian*). For the measurement of the aborbances of food melanoidin solutions of specific concentrations, as indicated in the respective tables, a UV VIS 2100 (*Shimadzu*) was used.

4.6.7 Flavour release studies

Bread crust and tomato melanoidins were isolated as described above from commercial samples. They were fractionated by dialysis (*Sigma*, MWCO ± 12,000 Da) in a HMW non-dialyzable, and a LMW dialyzable fraction.

To study the release of model flavour compounds, solutions of the different melanoidins in water were prepared at different concentrations, namely 100 and 1000 mg/l. Stock solutions of the flavour compounds were prepared in ethanol, and adequate amounts of these solutions were pipetted into the melanoidin-containing solutions to final concentrations of 1 and 10 ppm (or in some cases 100 and 1000 ppm, in which case higher concentrated stock solutions were prepared). One millitre of a flavoured solution was placed in a 4-ml silanized vial and stirred. After equilibration for 30 minutes, a headspace SPME extract was taken using a DVB/Car/PDMS fiber during one minute at 25 °C. The SPME fiber was desorbed during 2 min at 250 °C in the chromatographic inlet of the GC. Gas chromatographic analyses were performed with a HP 6890 GC Plus, equipped with a split/splitless injector, an FID detector and an EC-5 column (30 m × 0.25 mm i.d.; coating thickness 0.25 µm). Operating conditions were: injector 250 °C; detector 300 °C (make-up gas He 10 ml/min); oven temperature start 35 °C, hold 5 min, programmed from 35 °C to 60 °C at 5 °C/min, from 60 °C to 200 °C at 20 °C/min, hold 2 min; carrier gas He 0.8 ml/min; splitless injection. In a later stage, flavour release studies were performed with a MPS-2 autosampler (*Gerstel*) to increase the reproducibility. In this case, 5-ml samples were prepared in 20-ml vials. The mixture was equilibrated at 30 °C for 30 minutes and sampled by SPME for exactly one minute. GC separation was performed on a HP 6890 GC Plus coupled with a HP 5973 MSD with a HP5 column using the same conditions as described above.

4.6.8 Statistical analysis

To calculate the statistical significance of flavour release or flavour retention, a two-way Student's t-test was applied, assuming equal variances ($\alpha = 0.05$). For the principal components analysis, the statistical package SPlus 6.1 was used, and standard parameters were applied.

5 SUMMARY

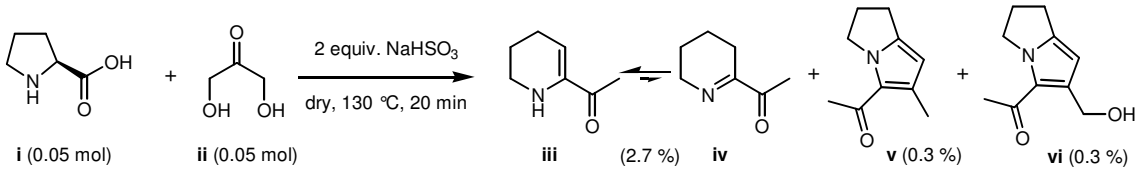
During the cooking and processing of food products, a complex network of reactions takes place, initiated by the condensation reaction of a reducing carbohydrate with a compound possessing a free amino group. This network of reactions is known as the Maillard reaction and has important implications for food quality. The development of flavour and colour is essential for the desirable organoleptic properties of food. In addition, a wide range of reaction products is formed during the Maillard reaction, with important implications for the nutritional value of food products.

The aim of this thesis was to study in detail the formation in a model system of 6-acetyl-1,2,3,4-tetrahydropyridine, one of the most important Maillard flavour compounds, to evaluate a microbial fermentation process for the production of the rice flavour compound 2-acetyl-1-pyrroline, and to assess the role of melanoidins in the development of the flavour properties of heated food products.

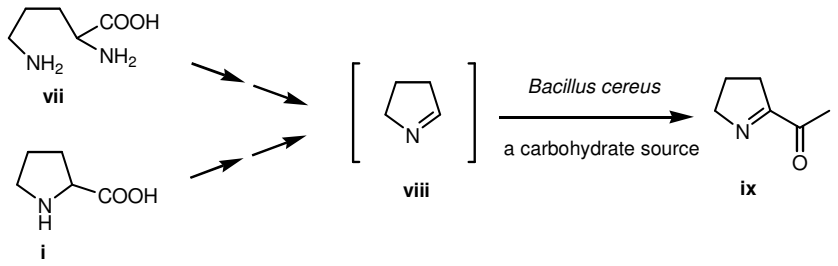
Two Maillard flavour compounds of extraordinary importance are 2-acetyl-1-pyrroline and 6-acetyl-1,2,3,4-tetrahydropyridine. Both are very potent flavour compounds, displaying an extremely low odour threshold, and they are key flavour compounds of a wide variety of food products, in particular of cooked rice and bread crust, respectively. An overview of their ubiquitous occurrence and fascinating chemistry of formation was given.

The model reaction of L-proline (**i**) and 1,3-dihydroxyacetone (**ii**) for the production of 6-acetyl-1,2,3,4-tetrahydropyridine (**iii**) led to its identification as bread flavour compound, but the reaction outcome remained poorly defined in the literature. Therefore, a detailed study of this reaction was undertaken. Optimal reaction conditions were determined: by heating of a dry mixture of L-proline and 1,3-dihydroxyacetone at 130 °C in the presence of two equivalents of sodium bisulfite a maximal yield of 2.7 % 6-acetyl-1,2,3,4-tetrahydropyridine

was accomplished. The significant influence of sodium bisulfite is most probably due to a combination of its reductive activity, providing necessary reagents for the reaction, and of its stabilizing effect on 6-acetyl-1,2,3,4-tetrahydropyridine, improving the recovery of this unstable compound from the reaction mixture. Side reactions yielded 2,3-dihydro-1*H*-pyrrolizines, among which 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**v**) was quantitatively the most important, and 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**vi**) was described as a new compound.



Reports are found in the literature on the production of 2-acetyl-1-pyrroline by specific *Bacillus cereus* strains. This fermentation was evaluated for the possibility to produce a 'natural' 2-acetyl-1-pyrroline flavour with potential applications in food. The influence of different precursors on the production of 2-acetyl-1-pyrroline and pyrazines by *Bacillus cereus* was investigated. Especially the addition of ornithine was shown to significantly increase the amounts of pyrazines produced by *Bacillus cereus* strains. 2,5-Dimethylpyrazine (or 2,6-dimethylpyrazine) was quantitatively the most important pyrazine. The best flavour-producing strain was in all cases *Bacillus cereus* ATCC 27522. Yields remained, however, rather low and the pyrazines were also detected in sterile controls. An enzyme-catalyzed pyrazine formation was concluded to be unlikely. The production of 2-acetyl-1-pyrroline (**ix**) was enhanced by the addition of heat-treated 4-aminobutanal diethyl acetal and in particular by the addition of 1-pyrroline (**viii**). This indicates that the formation of the rice flavour compound by these bacteria proceeds via the acetylation of 1-pyrroline, which is a degradation product of proline (**i**) or ornithine (**vii**). A clear biological catalysis was shown.



Besides a wide variety of low molecular weight flavour compounds, the Maillard reaction results in brown high molecular weight melanoidins. Melanoidins are important dietary components, but little is known on their structure and chemical properties. The flavour profile of heated food products is influenced by the presence of melanoidins. On the one hand, thermal destruction of melanoidins contributes to flavour formation. On the other hand, melanoidins can selectively bind flavour compounds and thereby influence the food flavour properties. The volatiles produced from the thermal degradation (250 °C) of model melanoidins, prepared according to a standard protocol, were systematically studied. Careful interpretation of the structure of these compounds gave information on the structural entities present in the melanoidin structure. Thermal degradation of glucose/glycine melanoidins was compared with glucose/glutamic acid and ascorbic acid/glycine melanoidins. Significant differences in the generation of volatiles were found. Melanoidins prepared from glucose and glycine and from ascorbic acid and glycine yielded a wide variety of furans, carbonyl compounds, pyrazines, pyrroles and pyridines. From glucose/glutamic acid melanoidins, on the contrary, no nitrogen-containing compounds were formed: only furans and carbonyl compounds were detected. This indicates a low participation of this amino acid in the melanoidin network, which is due to the low reactivity of glutamic acid as compared to glycine. In addition, the formation of relatively high amounts of methylene-bridged furans upon heating of glucose/glutamic acid melanoidins indicated the presence of glycosidically linked sugar derivatives, which was confirmed by the susceptibility of these melanoidins to

hydrolytic degradation, although other structures were degraded as well by this procedure. Ascorbic acid/glycine melanoidins yielded relatively more nitrogen-containing flavour compounds upon heating as compared to glucose/glycine melanoidins. Indications were found of the presence of glycine-polypeptide substructures.

In continuation of this research for the characterization of melanoidins by thermal degradation at temperatures relevant for food preparation, melanoidins were isolated from three food systems, namely bread crust, tomato sauce and coffee. Heating of these food melanoidins showed the participation of other dietary compounds in the melanoidin formation in food, such as lipid oxidation products in the case of tomato puree, and phenolic compounds in coffee melanoidins. Principal components analysis applied to the generated functionalities upon heating of the different melanoidins showed similarities between melanoidins isolated from bread crust, tomato sauce and glucose/glutamic acid model systems, while coffee melanoidins, model melanoidins prepared with glycine and LMW fractions were significantly differentiated from the others.

In the last part of this work, the flavour binding capacity of food-derived melanoidins was investigated, because of its significant influence on food flavour and its perception. The very strong binding of thiols by coffee melanoidins was confirmed using solid phase microextraction as the sampling technique. Bread crust melanoidins showed a significant retention of 3-methylbutanol, limonene and 6-acetyl-1,2,3,4-tetrahydropyridine, while tomato melanoidins significantly retained 3-methylbutanal and 3-methylbutyl acetate in aqueous solution.

These findings illustrate that the melanoidins isolated from three different food systems and prepared from model reactions all show significant differences in stucture and resulting flavour generation. Universal as well as very specific volatiles were formed. Also the flavour-

retaining capacity of the various melanoidins prepared was significantly different, indicating the presence of totally different functionalities.

The obtained results support the hypothesis of a melanoidin structure composed of a framework of carbohydrate and amino acid (or protein) moieties in varying ratios, with side chains and many reactive centres allowing the participation of other dietary compounds in the melanoidin formation.

6 SAMENVATTING

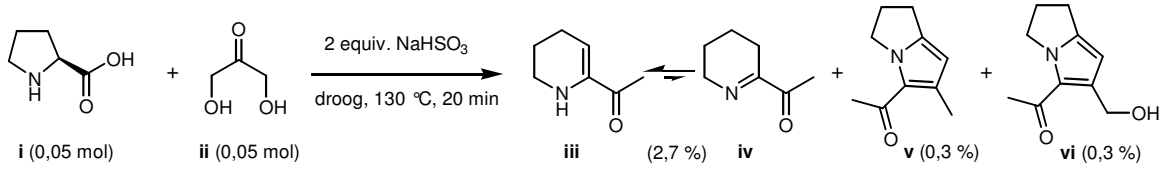
Bij de verhitting en de bereiding van voedingswaren vindt een complexe opeenvolging van reacties plaats, ingezet door de condensatiereactie van een reducerend koolhydraat met een verbinding die over een vrije aminogroep beschikt. Dit netwerk van reacties staat bekend als de Maillard-reactie en heeft belangrijke implicaties voor de kwaliteit van levensmiddelen. De ontwikkeling van kleur en aroma is essentieel voor de vorming van de gewenste organoleptische eigenschappen van levensmiddelen. Daarbij komt nog dat door de Maillard-reactie een heel scala van reactieproducten gevormd wordt met belangrijke gevolgen voor de nutritionele waarde van voedsel.

Het doel van deze thesis was om in detail de vorming van 6-acetyl-1,2,3,4-tetrahydropyridine, een van de belangrijkste Maillard-aromaverbindingen, in een modelsysteem te onderzoeken, om een microbieel fermentatieproces voor de vorming van de rijstaromaverbinding 2-acetyl-1-pyrroline te evalueren, en om de rol van melanoïdinen in de ontwikkeling van het aromaprofiel van verhitte levensmiddelen te bestuderen.

Twee Maillard-aromaverbindingen van uitzonderlijk belang zijn 2-acetyl-1-pyrroline en 6-acetyl-1,2,3,4-tetrahydropyridine. Beide zijn zeer sterk aromatische verbindingen met een bijzonder lage drempelwaarde, en ze leveren een onvervangbare bijdrage tot het aroma van uiteenlopende levensmiddelen, voornamelijk van gekookte rijst en gebakken brood, respectievelijk. Een overzicht van hun alomtegenwoordigheid en fascinerende vormings-chemie werd weergegeven.

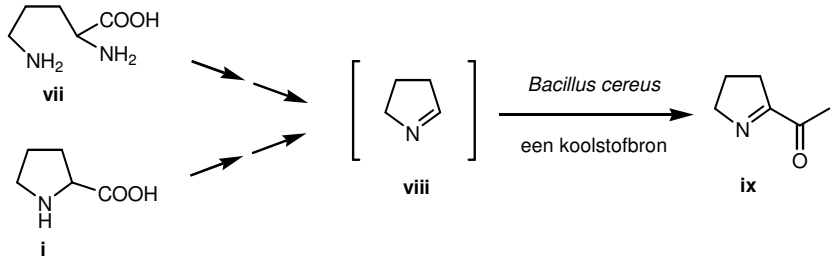
De modelreactie van L-proline en 1,3-dihydroxyaceton voor de vorming van 6-acetyl-1,2,3,4-tetrahydropyridine leidde tot de identificatie ervan als broodaromaverbinding, maar deze reactie bleef in de literatuur gebrekkig beschreven. Daarom werd een gedetailleerde studie van deze reactie uitgevoerd. Optimale reactieomstandigheden werden bepaald: door de

verhitting van een droog mengsel van L-proline (**i**) en 1,3-dihydroxyaceton (**ii**) bij 130 °C in aanwezigheid van twee equivalenten natriumbisulfiet werd een maximaal rendement van 2,7 % aan 6-acetyl-1,2,3,4-tetrahydropyridine (**iii**) gerealiseerd. De opmerkelijke invloed van natriumbisulfiet is hoogstwaarschijnlijk het gevolg van een combinatie van het reducerend vermogen, waardoor essentiële reagentia voor de reactie beschikbaar worden, en van het stabiliserend effect op 6-acetyl-1,2,3,4-tetrahydropyridine, waardoor de isolatie van deze onstabiele verbinding uit het reactiemengsel verbeterd wordt. Als bijproducten van de reactie werden verschillende 2,3-dihydro-1*H*-pyrrolizinen beschreven, waarvan 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine (**v**) kwantitatief de belangrijkste was, en 5-acetyl-6-hydroxymethyl-2,3-dihydro-1*H*-pyrrolizine (**vi**) als een nieuwe verbinding werd beschreven.



In de literatuur werd de vorming van 2-acetyl-1-pyrroline door specifieke *Bacillus cereus*-stammen beschreven. Deze fermentatie werd geëvalueerd met betrekking tot de mogelijkheid om 2-acetyl-1-pyrroline als 'natuurlijke' aromaverbinding te produceren voor eventueel gebruik in voedingswaren. De invloed van verschillende precursoren op de vorming van 2-acetyl-1-pyrroline en pyrazinen door *Bacillus cereus* werd bestudeerd. In het bijzonder de toediening van ornithine zorgde voor een significante verhoging van de productie van pyrazinen door *Bacillus cereus*-stammen. 2,5-Dimethylpyrazine (of 2,6-dimethylpyrazine) was kwantitatief het belangrijkste pyrazine. Van de onderzochte stammen bleek *Bacillus cereus* ATCC 27522 de beste aromaproducerende stam te zijn. De rendementen bleven echter laag, en de pyrazinen werden eveneens gedetecteerd in steriele voedingsbodems. Een enzymatisch-gekatalyseerde pyrazinevorming werd als onwaarschijnlijk geconcludeerd. De vorming van 2-acetyl-1-pyrroline (**ix**) werd verhoogd door de additie van hittebehandeld

4-aminobutanal diethylacetal en in het bijzonder door de additie van 1-pyrroline (**viii**). Dit toont aan dat de vorming van de rijstaromacomponent door de bacteriën gebeurt door een acetylering van 1-pyrroline, een afbraakproduct van proline (**i**) of ornithine (**vii**). Een duidelijke biologische katalyse werd aangetoond.



Naast tot een grote verscheidenheid aan laagmoleculaire aromaverbindingen, leidt de Maillard-reactie eveneens tot de vorming van bruingekleurde melanoïdinen van hoog moleculair gewicht. Melanoïdinen zijn belangrijke voedingscomponenten, maar over hun structuur en chemische eigenschappen is nog weinig bekend. Hun aanwezigheid in voeding beïnvloedt echter het aromaprofiel van levensmiddelen. Aan de ene kant draagt de thermische afbraak van melanoïdinen bij tot de vorming van aroma's in voeding. Aan de andere kant kunnen melanoïdinen bepaalde aromacomponenten selectief binden en daardoor het waargenomen aromaprofiel van voedingswaren beïnvloeden. De vluchtige verbindingen gevormd door thermische degradatie (250 °C) van modelmelanoïdinen, die bereid werden volgens een gestandaardiseerde procedure, werden systematisch bestudeerd. Zorgvuldige interpretatie van de structuur van deze verbindingen gaf informatie over de structurele kenmerken van het melanoïdine-skelet. Thermische degradatie werd uitgevoerd voor glucose/glycine melanoïdinen in vergelijking met glucose/glutaminezuur melanoïdinen en ascorbinezuur/glycine melanoïdinen. Significante verschillen in de generering van vluchtige verbindingen werden vastgesteld. Melanoïdinen bereid uit glucose en glycine en uit ascorbinezuur en glycine leidden bij de verhitting tot de vorming van furanen,

carbonylverbindingen, pyrazinen, pyrrolen en pyridinen, terwijl uit glucose/glutaminezuur melanoïdinen geen stikstofhoudende verbindingen werden gevormd: enkel furanen en carbonylverbindingen werden gedetecteerd. Dit toont een beperkte inbouw van dit aminozuur in het melanoïdine-netwerk aan, wat te wijten is aan de lage reactiviteit van glutaminezuur in vergelijking met glycine. De relatief hoge vorming van difuranen, die door middel van een methyleenbrug met elkaar verbonden zijn, wees bovendien op de aanwezigheid van relatief hoge hoeveelheden glycosidisch-verbonden suikereenheden, wat bevestigd werd door de hoge vatbaarheid van glucose/glutaminezuur melanoïdinen voor hydrolytische afbraak, hoewel daarbij ook andere structuren werden afgebroken. Uit melanoïdinen bereid uit ascorbinezuur en glycine werden relatief meer stikstofhoudende aromaverbindingen gevormd, in vergelijking met glucose/glycine melanoïdinen. Er werden bovendien aanwijzigingen gevonden voor de aanwezigheid van glycine-polypeptide substructuren.

In vervolg van dit onderzoek naar de karakterisering van melanoïdinen door thermische afbraak bij temperaturen relevant voor de bereiding van voedsel, werden melanoïdinen geïsoleerd uit levensmiddelen, namelijk uit broodkorst, tomatensaus en koffie. Verhitting van deze melanoïdinen toonde de tussenkomst van andere voedingsbestanddelen in de melanoïdinevorming aan, zoals van vetoxidatieproducten in het geval van tomatensaus en van fenolische verbindingen in het geval van koffiemelanoïdinen. Principale componentenanalyse toegepast op de gegenereerde functionele groepen bij de verhitting van verschillende melanoïdinen toonde de gelijkaardigheid aan van melanoïdinen uit broodkorst, tomatensaus en glucose/glutaminezuur-modelsystemen, terwijl koffiemelanoïdinen, modelmelanoïdinen bereid met glycine en de laagmoleculaire fracties duidelijk gedifferentieerd waren van de rest.

In een laatste onderdeel van dit werk werden de aromabindingseigenschappen van voedingsmelanoïdinen bestudeerd omwille van de significante invloed op het aroma van levensmiddelen. De zeer sterke binding van thiolen door koffiemelanoïdinen werd bevestigd,

door gebruik te maken van ‘solid phase micro-extraction’ (SPME) als bemonsteringstechniek. Broodkorstmelanoïdinen vertoonden een significante retentie van 3-methylbutanol, limoneen en van 6-acetyl-1,2,3,4-tetrahydropyridine, terwijl tomaatmelanoïdinen 3-methylbutanal en 3-methylbutylacetaat significant weerhielden in waterige oplossingen.

Deze bevindingen illustreren dat melanoïdinen, geïsoleerd uit drie verschillende levensmiddelen en bereid via modelreacties, alle significante verschillen vertonen in aromavorming. Zowel universele als heel specifieke vluchtige verbindingen werden gevormd bij verhitting. Ook de aromabindende eigenschappen van de verschillende onderzochte melanoïdinen waren sterk verschillend, duidend op de aanwezigheid van verschillende functionele groepen.

De bekomen resultaten ondersteunen de hypothese van een melanoïdinestructuur, samengesteld uit koolhydraten en aminozuren (of eiwitten) in verschillende verhoudingen, met zijketens en reactieve centra die de inbouw van andere voedingsbestanddelen in het melanoïdine-netwerk toelaten.

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