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Precursors of ethyldimethylpyrazine isomers and 2,3-diethyl-5-methylpyrazine formed in roasted beef

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Vorläufer der Ethyldimethylpyrazinisomere und des 2,3-Diethyl-5-methylpyrazins, die in gebratenem Rindfleisch entstehen

Zusammenfassung. Reaktionssysteme (pH 5,6), die in verschiedenen Kombinationen wasserlösliche Substanzen von Rindfleisch enthielten, wurden 7 min auf 180 °C erhitzt. Fehlte Alanin in einer Mischung, bestehend aus den freien Aminosäuren, Monosacchariden, Milchsäure, Carnosin und Kreatin, so war die Bildung von 2-Ethyl-3,6-dimethylpyrazin (I), 2-Ethyl-3,5-dimethylpyrazin (II), 2-Ethyl-5,6-dimethylpyrazin (III) und 2,3-Diethyl-5-methylpyrazin (IV) stark gehemmt. Ein Zusatz von Carnosin oder Milchsäure steigerte die Bildung der vier Pyrazine bei der Reaktion von Fruktose und Alanin. Das aktivste Modell wurde erhalten, wenn die Fruktose in der zuletzt genannten binären Mischung durch 2-Oxopropanal ersetzt wurde. Pyrazin I war bei allen Reaktionen das Hauptprodukt, doch auf der Basis seiner viel niedrigeren Geruchsschwelle war das Pyrazin II der wichtigste Geruchsstoff unter den drei Ethyldimethylpyrazinen.

Abstract. Reaction systems (pH 5.6) containing water-soluble substances of beef meat in various combinations were heated for 7 min at 180° C. Lack of alanine in a mixture consisting of free amino acids, monosaccharides, lactic acid, carnosine and creatine strongly inhibited the formation of 2-ethyl-3,6-dimethylpyrazine (I), 2-ethyl-3,5-dimethylpyrazine (II), 2-ethyl-5,6-dimethylpyrazine (III) and 2,3-diethyl-5-methylpyrazine (IV). Carnosine as well as lactic acid stimulated the formation of the four pyrazines in the reaction system fructose/alanine, but the most effective model was obtained when, in the latter binary mixture, fructose was replaced by 2-oxopropanal. In all reactions pyrazine I was the major product but, on the basis of its much lower odour threshold, pyrazine II was the most important odorant of the three ethyldimethylpyrazines.

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Introduction

Ethyldimethylpyrazine forms three positional isomers (I to III in Fig. 1) of which 2-ethyl-3,5-dimethylpyrazine (II) has been identified as a potent odorant of roasted beef [1, 2], roasted coffee [3], popcorn [4] and roasted sesame [5]. In these samples, 2,3-diethyl-5-methylpyrazine (IV in Fig. 1) was a further trialkylated pyrazine that strongly contributed to the roasty odour note [1–5].

Studies on the model systems reviewed by Maga [6], have shown that the formation of trialkylated pyrazines is closely related to non-enzymatic browning (Maillard reaction). Shibamoto and Bernhard [7] depicted a general scheme for the formation of alkylpyrazines and assumed that the breakdown of the monosaccharide in the presence of ammonia leads to α-amino carbonyl fragments that combine, with the formation of I and II. More recently, Arnoldi et al. [8] reacted fructose with eight amino acids at 120° C for 3 h. They found that the pattern of pyrazines formed depended on the amino acid used; e.g. leucine, phenylalanine and threonine were identified as precursors of I, alanine, aspartic acid and valine as those of II, and threonine as that of IV.

The aim of the following model experiments was to clarify the precursors of the pyrazines I to IV in beef meat. In the models, the pH was adjusted to 5.6, as meat

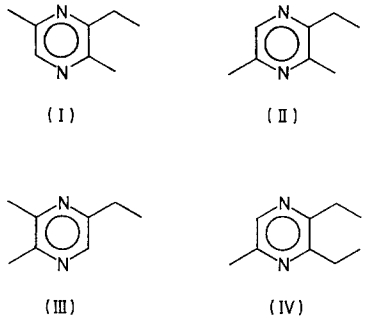

Fig. 1. Numerical key and structures of trialkylated pyrazines: *I*, 2-Ethyl-3,6-dimethylpyrazine; *II*, 2-ethyl-3,5-dimethylpyrazine; *III*, 2-ethyl-5,6-dimethylpyrazine; *IV*, 2,3-diethyl-5-methylpyrazine

is characterized by a pH ranging between 5.5 and 6.0 [9]. The reaction systems were heated at 180° C for 7 min, as these conditions are used for the roasting of beef.

Materials and methods

Water-soluble fraction (WF). Fresh beef (bull, top round; 988 g) from a local market was trimmed of all excess fat and then freeze-dried. The material obtained (259 g) was defatted by extraction with diethyl ether in a Soxhlet apparatus for 3 h. The meat was soaked in water (1.5 l) and homogenized in a Waring Blendor. The homogenate was centrifuged for 20 min at 10,000 rpm and 4° C, and the resulting residue was extracted twice with water (600 ml each). After centrifugation all of the supernatants were combined and then freeze-dried to give WF.

Low molecular mass fraction (LMF). A solution of WF (from 988 g meat) in water (500 ml) was ultrafiltered (apparatus from Amicon, Witten, Germany) at first through a Diaflo membrane PM 10 and subsequently through a YM 1 membrane. The latter filtrate, containing the water-soluble substances with a relative molecular mass (M_r) $\leqq 1000$, was freeze-dried to give LMF. The yield to LMF amounted to 5.4 g/kg fresh meat.

Chemicals. All amino acids and monosaccharides listed in the tables were obtained commercially at the highest purity available. The following compounds were obtained from the sources given in brackets: 2,3-diethyl-5-methylpyrazine (Aldrich, Steinheim, Germany), 2-ethyl-5,6-dimethylpyrazine was a gift of Dr. I. Flament (Firmenich, Geneva, Switzerland).

Kieselguhr (Merck, Darmstadt, Germany) was treated with aqueous HCl and water and then dried [10]. The following compounds were synthesized according to the literature: [^{2}H]-2-ethyl-3,5-dimethylpyrazine (d-II) [2], [^{2}H]-2,3-diethyl-5-methylpyrazine (d-IV) [2]; unlabelled 2-ethyl-3,5-dimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine were synthesized from 2,6-dimethylpyrazine and 2,5-dimethylpyrazine, respectively, as reported [2] for the labelled 2-ethyl-3,5-dimethylpyrazine but by using unlabelled ethyl lithium.

Model experiments. The components of the reaction systems detailed in the tables were dissolved in 5 ml of Na/K-phosphate buffer (pH 5.6, 0.07 mol/l), the pH was checked and, if necessary, corrected to 5.6. The solution of the reactants was absorbed by kieselguhr (10 g), and this mixture was poured into the flask (Fig. 2) containing hydrogenated peanut oil (100 g), which was heated up to 180° C and stirred. After 7 min the flask was cooled at 10–15° C (water bath) for 10 min, and then a solution of definite amounts of the internal standards d-II and d-IV in diethyl ether (200 ml) was added. The mixture was filtered, and the solution of the volatiles in diethyl ether was distilled off from the non-volatile materials under high vacuum (5 mPa) in the apparatus recently described [11] and using conditions reported earlier [1]. The distillate was extracted with aqueous HCl (0.1 mol/l, 3 × 50 ml). The combined aqueous solutions were alkalified (pH 12) by addition of aqueous NaOH (20%, w/w), and the volatile basic substances were extracted with diethyl ether (3 × 100 ml). The extract was dried over anhydrous Na_2SO_4 and concentrated to 200 µl by distilling off the solvent on a Vigreux column (50 × 1 cm) and by microdistillation [12]. The pyrazines in the extract were quantified by mass chromatography.

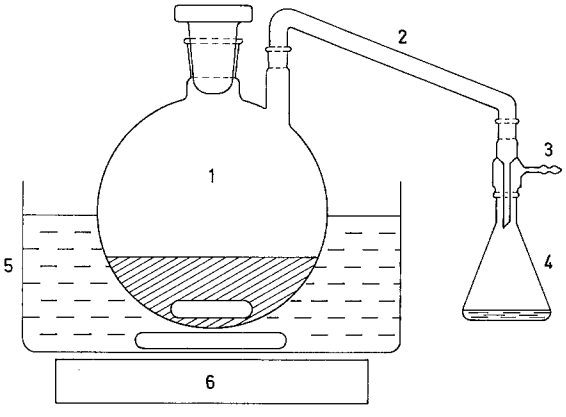

Fig. 2. Apparatus used for the model experiments: *1*, a two necked distillation flask (volume: 500 ml); *2*, bridge-shaped still head; *3*, receiver adapter; *4*, recipient vessel; *5*, oil-bath (180° C); *6*, magnetic stirrer

Mass chromatography. This was performed using an ion trap detector in combination with a gas chromatograph [13]. The ion trap detector was coupled with a DB-Wax capillary [2]. The injection of the samples and the temperature programme used for the high resolution gas chromatography (HRGC) were as recently described [1].

Results

Preliminary studies

A comparison of the odour thresholds of the pyrazines I, II and III indicated (Table 1) that they were very different. The lowest odour threshold value was found for the isomer II. It was lower by factors of 270 and 10,000, respectively, than those of the isomers I and III.

A method for the quantification of the four pyrazines formed in the reaction systems was developed. As reported in Materials and methods, the labelled internal standards d-II and d-IV were added to each model system. Then the fraction containing the volatile basic substances was isolated and separated by HRGC. As an example, Fig. 3 represents the HRGC profile of the volatile bases from experiment 3, Table 2. To extract the pyrazines I to IV from other components of the volatile basic fraction and to differentiate them from their internal standards, mass chromatograms were recorded for the ions presented in Fig. 4a–d. The amount of the pyrazines I, II, and III was calculated on the basis of the internal standard d–II and that of IV on the basis of d–IV.

Model experiments

Heating of the water-soluble fraction of beef meat resulted in the formation of the four pyrazines, among which I predominated (experiment 1, Table 2). No drastic change in the amounts of the pyrazines formed could

Table 1. Odour thresholds of ethyldimethylpyrazines [a]

Pyrazine	Threshold [b] (ng/l, air)
2-Ethyl-3,6-dimethyl (I)	2.45
2-Ethyl-3,5-dimethyl (II)	0.009
2-Ethyl-5,6-diemthyl (III)	> 95

[a] Odour threshold values were determined by an olfactometric high-resolution gas chromatography method [14]. The reference substance for the calculation of the odour threshold was (E)-2-decenal; odour threshold in air 2.7 ng/l [15]

[b] Mean value found by three assessors

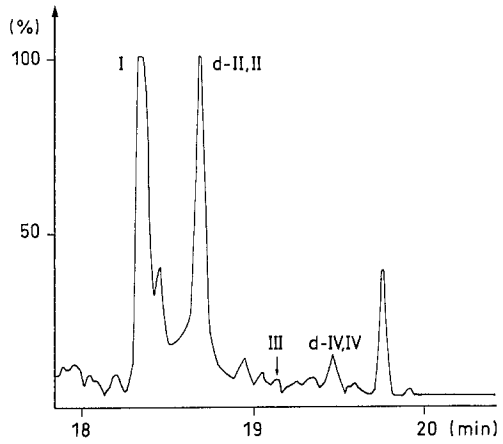

Fig. 3. Detail of the capillary gas chromatogram (total ion current) of the volatile bases isolated in experiment no. 3 (Table 2). The positions of pyrazines *I–IV* and their internal standards *d–II* and *d–IV* are shown

be observed when only the LMF was heated (experiment 2).

The free amino acids, the monosaccharides and the other substances listed in experiments 3–7 were components of the LMF [16, 17]. Therefore they were heated in various combinations to show which of them acted as precursors of the four pyrazines. A mixture consisting of glucose, fructose, their phosphates, ribose and 17 amino acids was heated in experiment 3. The four pyrazines were formed, but in much lower amounts than in experiments 1 and 2. The concentrations of the reactants in experiment 3 referred to a smaller amount of beef meat (200 g) than in experiments 1 and 2 (300 g), but this difference alone cannot explain the strong decrease in the yields of the pyrazines in experiment 3.

The pyrazines were not formed when only alanine was lacking in the reaction system (experiment 4) containing the monosaccharides and the free amino acids of beef meat. Addition of a mixture of carnosine, glutamine, creatine and lactic acid to the free amino acids and monosaccharides enhanced the formation of the pyrazines (experiment 5). Their yields reached approximately the levels which were found after heating of the WF (experiment 1) and the LMF (experiment 2).

A comparison of experiments 6 and 7 indicated that only alanine was required from the free amino acids and that the mixture of carnosine, glutamine, creatine and lactic acid was active in stimulating the production of the pyrazines.

In the experiments 8–11 (Table 3), alanine was heated with the major monosaccharides of beef meat [16]. The amounts of each of the four pyrazines formed were not very different, indicating that the fraction of monosaccharides investigated was involved in the formation of the pyrazines without preference for one of the hexoses.

To study the stimulation of the pyrazine formation (cf. experiment 7, Table 2) in more detail, fructose was reacted both with alanine and with the nitrogen-containing substances reported in experiment 7. The results (experiments 12–15, Table 4) show that only the reaction system containing alanine, yielded the pyrazines. However, in combination with alanine, carnosine especially (experiment 16) enhanced the formation of the pyrazines. However, the enhancement of the concentration of the nitrogen source was not the reason for the effect of carnosine, because the addition of lactic acid in experiment 18 also stimulated the formation of the pyrazines. After addition of glutamine (experiment 17), isomer I was higher than with alanine alone (experiment 12), whereas ammonium formate (experiment 19) did not affect the production of the pyrazines by the reaction system fructose/alanine.

There are indications [18, 19] that the retro aldol scission of 1- and 3-deoxyosones, which are formed from

Table 2. Water-soluble components of beef as precursors of pyrazines I to IV

Experiment no.	Reaction system [a]	Amount of pyrazines (μg) [b]			
		I	II	III	IV
1	Water-soluble fraction (WF) of beef meat	43	15	5.5	7.4
2	Low-molecular-mass water-soluble fraction (LMF) of beef meat	57	23	10	4.4
3	Monosaccharides + amino acids	11	3.1	0.6	2.6
4	Reaction system no. 3 without alanine	< 0.1	< 0.1	< 0.1	< 0.1
5	Monosaccharides + amino acids + carnosine + glutamine + creatine + lactic acid	78	14	2.4	5.2
6	Monosaccharides + carnosine + glutamine + creatine + lactic acid	4.9	1.5	0.1	< 0.1
7	Reaction system no. 6 + alanine	80	6.9	1.1	5.3

[a] Reactants: WF and LMF from beef meat (300 g); monosaccharides: glucose (214 mg, 1.19 mmol), fructose (57 mg, 0.32 mmol), ribose (20 mg, 0.13 mmol), glucose 6-phosphate monosodium salt (429 mg, 1.52 mmol), fructose 6-phosphate monosodium salt (118 mg, 0.42 mmol); amino acids: alanine (63 mg, 0.71 mmol), arginine (15 mg, 0.09 mmol), aspartic acid (1.5 mg, 0.01 mmol), cysteine (7.4 mg, 0.06 mmol), glutamic acid (20.6 mg, 0.15 mmol), glycine (19.2 mg, 0.26 mmol), histidine (9.7 mg, 0.06 mmol), isoleucine (8.9 mg, 0.07 mmol), leucine (16 mg, 0.12 mmol), lysine·HCl (13.9 mg, 0.08 mmol), methionine (4.6 mg, 0.03 mmol), proline (7.8 mg, 0.07 mmol), phenylalanine (10.3 mg, 0.06 mmol), serine (7.2 mg, 0.07 mmol), threonine (6.3 mg, 0.05 mmol), tyrosine (9.5 mg, 0.05 mmol), valine (16 mg, 0.14 mmol); further reactants: glutamine (140 mg, 0.96 mmol), carnosine (600 mg, 2.65 mmol), creatine·H_2O (570 mg, 3.82 mmol), lactic acid (90%, 1 g, 10 mmol)

[b] Amount formed after 7 min at 180° C; mean values of duplicates

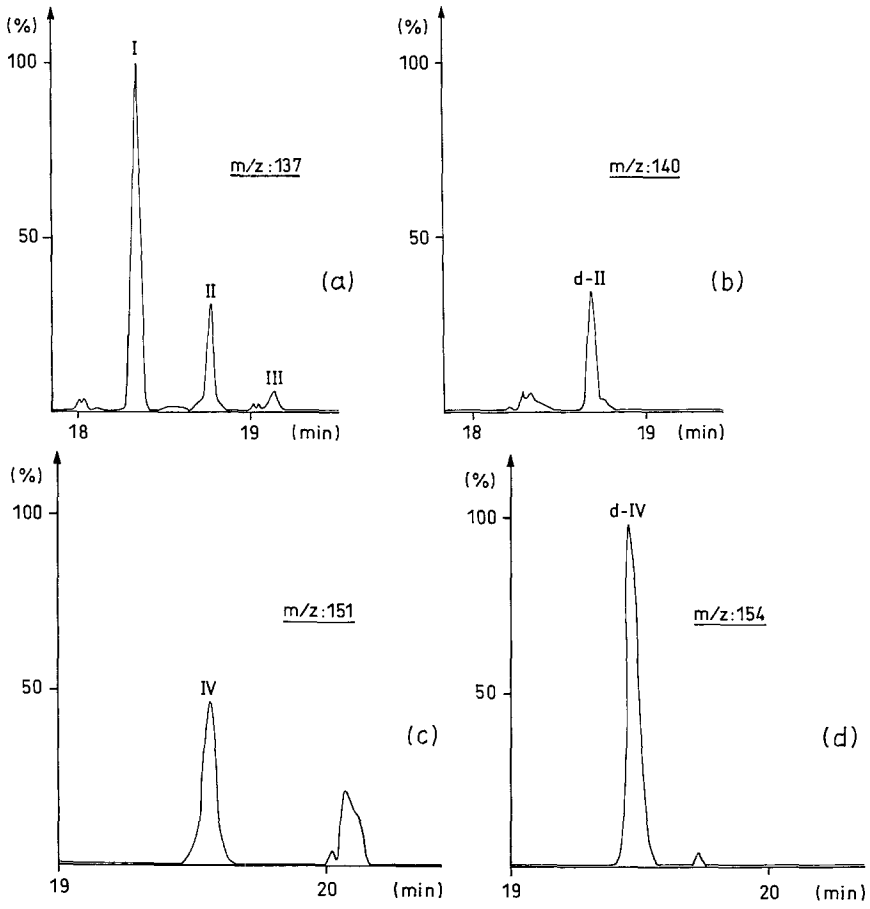

Fig. 4 a–d. Mass chromatograms of the volatile bases (Fig. 3) recorded at (**a**) m/z 137, (**b**) m/z 140, (**c**) m/z 151 and (**d**) m/z 154

Table 3. Alanine and different monosaccharides as precursors of the pyrazines I to IV

Experiment no.	Reaction system [a]: alanine plus	Amount of pyrazines (μg) [b]			
		I	II	III	IV
8	Fructose	49	3.9	0.5	8.8
9	Glucose	26	2.1	0.4	5.3
10	Fructose 6-phosphate	28	6.3	2.1	7.9
11	Glucose 6-phosphate	25	3.0	1.6	4.4

[a] Amount of the reactants: 2 mmol each
[b] Amount formed after 7 min at 180° C; mean values of duplicates

Table 4. Fructose and different nitrogen sources as precursors of the pyrazines I to IV

Experiment no.	Reaction system [a]: fructose plus	Amount of pyrazines (μg) [b]			
		I	II	III	IV
12	Alanine	49	3.9	0.5	8.8
13	Carnosine	> 0.15	< 0.15	< 0.15	< 0.15
14	Creatine	< 0.1	< 0.1	< 0.1	< 0.1
15	Glutamine	< 0.1	< 0.1	< 0.1	< 0.1
16	Alanine + carnosine	174	8.3	0.5	32
17	Alanine + glutamine	77	4.9	0.6	12
18	Alanine + lactic acid [c]	172	17	1.5	30
19	Alanine + ammonium formate	40	3.6	0.4	15

[a,b] See footnotes [a] and [b] in Table 3
[c] Amount: 10 mmol

Table 5. Reaction system 2-oxopropanal and alanine

Experiment no.	Amount of each reactant	Amount of pyrazines (μg) [a]			
		I	II	III	IV
20	2 mmol	256	27	2.6	18
21	4 mmol	837	45	1.8	54

[a] Amount formed after 7 min at 180° C

glucose or fructose on the Maillard route, yields amongst others 2-oxopropanal, the precursor of methylpyrazines [19]. Actually, there was a drastic increase in the pyrazines, when, in the reaction system fructose/alanine (experiment 12, Table 4), fructose was replaced by 2-oxopropanal (experiment 20, Table 5). Isomer I was preferentially formed and accounted for 84% of the pyrazines. This isomer and pyrazine IV increased by 3.3- and 3.0-fold, respectively, when the concentrations of the reactants were doubled (experiment 21). Also the concentration of pyrazine II was higher in experiment 21 than in experiment 20, but the increase amounted only to 70%.

Discussion

The results indicate that the precursors of pyrazines II and IV, which contributed to the flavour of roasted beef, occurred in the water-soluble fraction of beef meat and were identified as glucose, fructose, their 6-phosphates

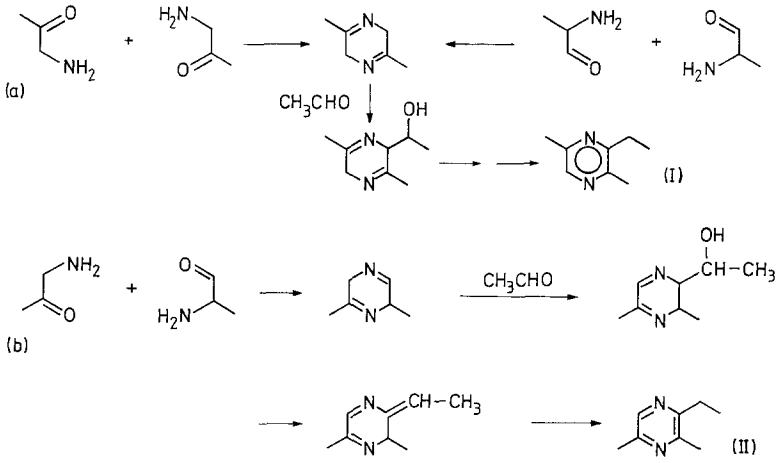

Fig. 5 a, b. Proposed reaction routes to (**a**) 2-ethyl-3,6-dimethylpyrazine and (**b**) 2-ethyl-3,5-dimethylpyrazine

and alanine. Furthermore, in all reaction systems containing a monosaccharide and alanine, pyrazine I was the major product. Nevertheless, isomer I did not belong to the potent odorants of roasted beef, because its odour threshold was more then 250 times higher than that of the isomer II.

The highest yields of I and II were obtained after heating a mixture of alanine and 2-oxopropanal, which is generated in the Maillard reaction of glucose and fructose [18, 19]. To explain the formation of I and II we suggest, as first step, the Strecker degradation of alanine by 2-oxopropanal yielding aminoacetone, 2-aminopropanal and acetaldehyde. According to the mechanism proposed in the literature [7, 19], pyrazine I can be generated by both the condensation of aminoacetone and that of 2-aminopropanal. As shown in Fig. 5a, 2,5-dimethyldihydropyrazine is formed as an intermediate. Its reaction with acetaldehyde and dehydration affords pyrazine I. The mechanism proposed for I can also explain the generation of II, when the condensation of aminoacetone with 2-aminopropanal is expected initially (Fig. 5b).

In the model reactions, pyrazine I was always formed as the major and II as the minor product. This difference might be the consequence of a preferential formation of one of the two amino compounds in the Strecker degradation, e.g. more aminoacetone is produced from 2-oxopropanal, because the aldehyde group of the latter is more reactive than the keto group.

Bemis-Young et al. [20] found pyrazine IV after heating of glucose/glycine model systems and proposed its formation by a combination of 2-aminopropanal and 2-hydroxy-3-amino-4-hexanone. This reaction can explain the formation of IV in the reaction system glucose (fructose)/alanine but not in that consisting of 2-oxopropanal and alanine.

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