Autoxidation in Xylose/Lysine Model Systems

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The volatiles produced in xylose/lysine model systems added with an antioxidant (α-tocopherol, 2,6-di-*tert*-butyl-4-methylphenol, or rosemary extract) or a free radical initiator (α,α′-azobis-(isobutyronitrile), AIBN) were analyzed to investigate the effects of the presence of free radicals on the Maillard reaction. The pH was maintained constant at 4 or 6, by adding a base, and the data were compared by principal component analysis (PCA). The additives were more effective at pH 4 than pH 6. At pH 4, the model system added with AIBN is very well-discriminated by PCA from the models with the antioxidants and the reference model system, indicating that the volatiles are sensitive to compounds that can interfere in an opposite way with free radical formation.

Keywords: *Maillard reaction; xylose; lysine; flavor; antioxidant*

INTRODUCTION

Although the Maillard reaction has been known since 1912, its extraordinary complexity still leaves some uncertainty on the real mechanism of the formation of several of its typical products. For many years the classical Hodge scheme (Hodge, 1953) has been accepted by all researchers of the field, but very recently Tressl and co-workers (Tressl et al., 1998a,b) have demonstrated, by sophisticated labeling experiments, that the most abundant products, the melanoidins, are formed mostly not through the Amadori intermediate but by direct fragmentation of the early formed Schiff base.

It is generally agreed that most of the steps of the Maillard reaction are based on ionic mechanisms, but Namiki and co-workers showed by ESR that free radicals are formed in the very early stage of the sugar−amino acid interaction (Namiki, 1988) by observing clear ESR signals whose hyperfine structure disappears during heating. The spectral characteristics allowed to establish that they are *N*,*N*′-disubstituted pyrazine radical cations, and a possible pathway for their formation was proposed by Namiki and Hayashi (1983) and Hayashi and Namiki (1986). These intermediates would produce, besides other compounds, pyrazines that, owing to their high volatility and very low odor thresholds, are very important constituents of food aroma. The presence of radicals has been confirmed in the last years by other kinds of experiments (Cämmerer and Kroh, 1995; Roberts and Lloyd, 1997).

Recently we have started an investigation on the effects of the presence of free radicals on the formation of volatile compounds from the Maillard reaction and have published the results obtained (D'Agostina et al., 1998) by adding an antioxidant or a free radical initiator (pro-oxidant) to glucose/lysine model systems. α-Tocopherol, 2,6-di-*tert*-butyl-4-methylphenol (BHT), and rosemary extract (Chang et al., 1977) were chosen as antioxidants and α,α′-azobis(isobutyronitrile) (AIBN) as a free radical initiator.

Although glucose is the most abundant sugar in food, the high reactivity of pentoses in the Maillard reaction suggests that they may contribute in an important way to the formation of flavor and color during thermal processing. The most common pentoses are ribose and xylose; the first is encountered especially in meat and the second in cereals. Xylose was selected in our investigation, because it has been studied less than ribose by other groups (Farmer et al., 1989; Farmer and Mottram, 1990, 1992; Mottram and Whitfield, 1995).

The same additives added to glucose/lysine model systems were used also on xylose/lysine, and the same procedures were applied, but the heating time was reduced from 2 to 1 h, because it was verified that this time is sufficient to develop a reasonable amount of volatiles, with a reduced level of melanoidins, as the Maillard reaction is much faster with pentoses than with hexoses. Confirming the methodology applied in the preceding paper (D'Agostina et al., 1998), also in these experiments the pH was maintained constant at a value of 4 or 6, because this parameter affects strongly the Maillard reaction rate.

MATERIALS AND METHODS

Materials. D-(+)-Xylose, L-lysine hydrochloride, α-tocopherol, pyrazine, 2-methylpyrazine, 2(5*H*)-furanone, 3-methyl-1,2-pentanedione, and pentadecane were purchased from Aldrich; AIBN, tetradecane, dihydro-2(3*H*)-furanone, and Furaneol from Fluka; BHT and 3-hydroxybutan-2-one from Merck; 2-furanmethanol and 2-furancarboxaldehyde from Carlo Erba. Water was prepared with a Milli-Q (Millipore); dichloromethane was Ultra Resi-Analyzed (Baker, Deventer, The Netherlands). Rosemary extract was a gift of SOREMARTEC, Belgium.

Model Systems. Mixtures containing equimolar amounts of xylose and lysine (70 mL of 0.5 M water solution) and a variable amount of additive (0 or 60 mg, and in some case 120 and 180 mg) were heated under reflux for 1 h in a flask equipped with an autoclavable electrode (ATI Russel). During this time the pH was monitored and kept constant at the desired value by the addition of diluted sodium hydroxide (NaOH). At the end of the heating time, the pH was adjusted to 8.0. Tetradecane was added as a first internal standard, and the volatile compounds were recovered by continuous extraction with dichloromethane. The solvent was carefully concentrated to 1 mL and pentadecane was added as a second internal standard.

10.1021/jf990610m CCC: $19.00
Published on Web 02/03/2000

Table 1. Compounds Quantified in Xylose/Lysine Model Systems

compounds	K_i SPB-1701	standard K_i SPB-1701	label in the charts and tables
pyrazine	810	813	
3-hydroxybutan-2-one	837	831	
2-methylpyrazine	913	919	2-MP
2-furancarboxaldehyde	969	960	2-FA
2,5-dimethylpyrazine	1000	994	2,5-DMP
2,3-dimethylpyrazine	1011	1017	2,3-DMP
2-furanmethanol	1013	1007	2-FM
dihydro-2(3*H*)-furanone	1084	1086	DHF
2(5*H*)-furanone	1078	1092	
3-methyl-1,2-cyclo-pentanedione	1159	1157	3-MPCD
2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	1199	1200	Furaneol
2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	1279		DHMP

Analysis by GC and GC−MS. The volatiles were quantified by gas chromatography−flame ionization detection (GC−FID) on a DANI 86.10 gas chromatographer. Two internal standards, tetradecane and pentadecane, were used. Compound concentrations were calculated after determination of the correction factors and are expressed in "mg/model system". Peaks were identified by GC−mass spectrometry (GC−MS) on a Shimadzu QP-5000 by comparison with the NIST62 spectra library and commercial standards as far as possible. Ions were generated by EI at 70 eV. A capillary column SPB-1701 (30 m × 0.2 mm, film 1 μm) was used in both cases. Temperature program: 37 °C × 10 min, 4 °C/min to 200 °C, then held. Each experiment was repeated at least three times.

Principal Components Analysis. Principal components analysis (PCA) was performed with the program SYSTAT 1992 (SYSTAT, Inc.) using the default procedure with standardization of the variables prior to analysis. Factors were extracted using the default principal components method and then rotated using EQUAMAX, QUARTIMAX, and VARIMAX equations. The rotated factor patterns were very similar; the discussed results were derived with VARIMAX.

RESULTS AND DISCUSSION

It is well-known that the Maillard reaction is particularly sensitive to pH variations, from the point of view of both the reaction rate and the kind of volatiles that are formed. The methodology applied in this work was based on keeping the pH value constant at 4 or 6 by monitoring the pH and by adding a dilute base during heating (D'Agostina et al., 1998). Xylose/lysine model systems were heated for 1 h at 100 °C and then cooled, and the volatile compounds were extracted continuously with dichloromethane to avoid any progress of the Maillard reaction. Relevant Maillard compounds were quantified by the internal standard method after determination of all the necessary response factors. Table 1 reports a list of the compounds selected for quantification and their Kovats indexes (K_i).

Model systems with compounds which can interfere with free radical formation, i.e., either an antioxidant (α-tocopherol, BHT, and rosemary extract) or a free radical initiator, such as AIBN, were compared with standard model systems containing only the amino acid and the sugar. Table 2 shows the results of the model systems at pH 4 expressed as mean values and standard errors (obtained with Student's *t*-test). Pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2-furancarboxaldehyde, 2-furanmethanol, dihydro-2(3*H*)-furanone, 2(5*H*)-furanone, and Furaneol were selected for quantification; furaneol, 2(5*H*)-furanone, and pyrazine are particularly abundant.

Pyrazine, 2-methylpyrazine, 2-furanmethanol, 2(5*H*)-furanone, and Furaneol are increased by the addition of BHT. With rosemary extract 2-methylpyrazine, 2,5-dimethylpyrazine, and 2-furancarboxaldehyde increase, while pyrazine decreases. α-Tocopherol produces a similar decreasing effect, but additionally 2-furanmethanol, Furaneol, dihydro-2(3*H*)-furanone, and 2(5*H*)-furanone decrease. With the pro-oxidant AIBN, pyrazine and 2-methylpyrazine decrease very much, while 2,5-dimethylpyrazine, dihydro-2(3*H*)-furanone, and 2(5*H*)-furanone increase.

As BHT appeared to be the most effective antioxidant, some model systems with higher concentrations of this additive (120 and 180 mg) were prepared. Most of the Maillard reaction products, such as 2-furanmethanol, dihydro-2(3*H*)-furanone, and 2(5*H*)-furanone, increase slightly, whereas pyrazine, 2-methylpyrazine, and 2,5-dimethylpyrazine are particularly sensitive to increasing amounts of BHT (Figure 1).

To find similarities and differences among the model systems, PCA was applied to these data. This technique finds the underlying factors (principal components) that influence a chemical system (Dillon and Goldstein, 1984; Malinowsky and Howery, 1980). These factors are linear combinations of a set of orthogonal vectors that are the eigenvectors of the variance−covariance matrix of the original data matrix. The procedure creates from the original ones a new set of variables which are called principal components (PC) and are orthogonal to each other. PC_1 accounts for the largest proportion of the variation in the original set; the other PCs account for smaller and smaller proportions of the variation. If the first two (three) eigenvalues obtained from the data matrix are large enough that they account for a substantial fraction of the total variance, the overall structure of the data set may be revealed by generating a two (three) dimensional plot of PC_1 vs PC_2 (vs PC_3).

Often the information contained in the data matrix contains redundancy (correlation between variables) that is easily identified comparing the weighted contributions (loadings) of the original variables in the PCs. In our study redundancy means that two or more

Table 2. Volatile Compounds Formed at pH 4 in the Presence of AIBN or an Antioxidant (60 mg)[a]

compounds	reference	BHT	tocopherol	rosmary extract	AIBN
pyrazine	1.34 ± 0.25	1.68 ± 0.27	0.291 ± 0.131	1.04 ± 0.131	0.405 ± 0.232
2-MP	0.076 ± 0.019	0.094 ± 0.009	0.095 ± 0.038	0.105 ± 0.045	0.038 ± 0.004
2,5-DMP	0.016 ± 0.002	0.015 ± 0.002	0.027 ± 0.008	0.030 ± 0.008	0.032 ± 0.008
2-FM	0.218 ± 0.049	0.402 ± 0.103	0.036 ± 0.014	0.216 ± 0.010	0.278 ± 0.070
2-FA	0.024 ± 0.006	0.039 ± 0.027	0.066 ± 0.014	0.044 ± 0.015	0.131 ± 0.053
Furaneol	13.7 ± 1.98	17.5 ± 1.490	8.74 ± 2.31	12.5 ± 1.53	[b]
DHF	0.036 ± 0.006	0.059 ± 0.029	0.015 ± 0.009	0.042 ± 0.003	0.164 ± 0.057
2(5*H*)-furanone	0.800 ± 0.101	1.03 ± 0.26	0.458 ± 0.134	0.884 ± 0.021	1.02 ± 0.25

[a] Amounts expressed as mg/model system. Means and standard errors ($P < 0.5\%$) were obtained at least in triplicate experiments. [b] In the chromatogram the peak of interest was covered by other compounds.

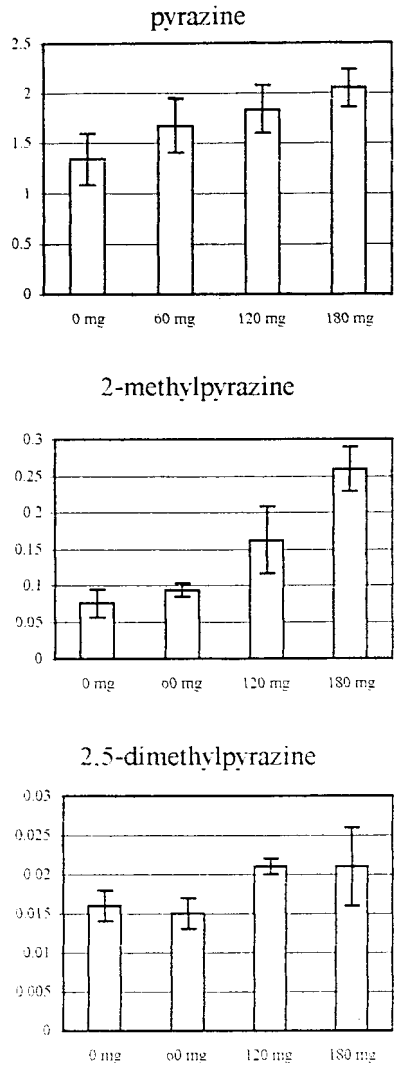

Figure 1. Xylose/lysine model systems at pH 4 added with variable amounts of BHT (amount expressed as mg/model system). Means and standard errors ($P < 0.5\%$) were obtained at least in triplicate experiments.

Table 3. Eigenvectors of PCs for the Volatiles Obtained from Model Systems at pH 4

descriptor	PC_1	PC_2	PC_3
pyrazine	−0.407	0.614	0.672
2-MP	−0.977	−0.121	0.099
2,5-DMP	0.189	−0.163	−0.960
2-FA	0.754	0.017	−0.630
2-FM	0.137	0.942	0.279
2(5*H*)-furanone	0.261	0.961	0.012
DHF	0.801	0.475	−0.364
% of total variance explained	35.048	35.078	28.428

volatile compounds are influenced in a similar way by the presence of the additives.

As for the application of PCA to the data at pH 4 (only model systems with 60 mg of additive), PC_1 explains 35.0% of the total variance of the system, PC_2 35.1%, and PC_3 28.4%. The three PCs together account for 98.5% of the original variance. Table 3 shows the eigenvectors for each PC. PC_1 is dominated by 2-methylpyrazine, dihydro-2(3*H*)-furanone, 2-furancarboxaldehyde, and in part pyrazine. 2-Furanmethanol, 2(5*H*)-furanone, and in part pyrazine dominate PC_2, whereas PC_3 is sensitive to 2,5-dimethylpyrazine, pyrazine, and 2-furancarboxaldehyde. Most compounds influence one PC, whereas pyrazine is distributed on all three PCs.

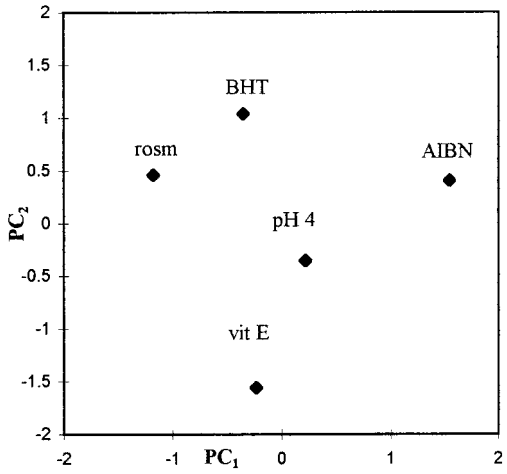

Figure 2. PCA of the volatiles obtained from model systems at pH 4: loading plot of PC_1 vs PC_2.

Figure 2 shows the score plot of PC_2 vs PC_1, useful to discuss the differences of the effects of the additives. The model system added with AIBN is very well-discriminated by PC_1 from the models with the antioxidants that are located together on the opposite side in respect to the reference model system that is in the center of the chart. This indicates that the volatiles are sensitive to compounds that can interfere in an opposite way with free radical formation. PC_2 (depending on 2(5*H*)-furanone, 2-furanmethanol, and pyrazine) is useful to separate the model systems containing the three antioxidants.

Table 4 reports the results obtained at pH 6. The first observation is that all additives have a limited effect in respect to pH 4, probably because the fast developing of the Maillard reaction reduces their potency. Pyrazine, 2-methylpyrazine, Furaneol, and DHMP are enhanced by the presence of BHT and α-tocopherol, whereas rosemary extract increases pyrazine, Furaneol, and DHMP and decreases 2,5-dimethylpyrazine. The only effect of AIBN is a decrease of 2,5-dimethylpyrazine and an increase of Furaneol and 3-MCPD.

Applying PCA, PC_1 explains only 27.9% of the system variance, PC_2 31.2%, and PC_3 32.1% (after rotation). The three PCs together account for 91.2% of the total variance (Table 5). PC_1 depends mainly on 2,5-dimethylpyrazine and 2,3-dimethylpyrazine; PC_2 on Furaneol, 2-methylpyrazine, and pyrazine; PC_3 on 3-MCPD, DHMP, pyrazine, and 3-hydroxybutan-2-one. The score plot (Figure 3a) shows that PC_1 separates well the reference system from the others, AIBN and BHT are well-separated by the combination of PC_1 and PC_2 that is dominated by the pyrazines, whereas the separation of α-tocopherol and rosemary extract from AIBN requires also PC_3 (Figure 3b). The small differences that were obtained at this pH make the interpretation difficult.

CONCLUSIONS

The Maillard reaction is a very complex network of competitive reactions, and the actual yield of each compound derives from a complicated combination of many reaction constants. Most of the Maillard reaction products are produced in very low yields, a fact particularly true in the case of volatiles. The pH was maintained by the addition of a base during the entire heating time, as some physical parameters, such as pH

Table 4. Volatile Compounds Formed at pH 6 in the Presence of AIBN or an Antioxidant (60 mg)[a]

compounds	reference	BHT	tocopherol	rosemary extract	AIBN
pyrazine	1.36 ± 0.54	1.99 ± 0.27	1.53 ± 0.15	1.70 ± 0.06	1.37 ± 0.15
2-MP	2.15 ± 0.19	3.04 ± 0.33	2.50 ± 0.13	2.10 ± 0.42	2.62 ± 0.06
2,5-DMP	0.106 ± 0.031	0.094 ± 0.024	0.078 ± 0.009	0.064 ± 0.015	0.084 ± 0.009
2,3-DMP	0.019 ± 0.007	0.018 ± 0.005	0.014 ± 0.002	0.013 ± 0.005	0.015 ± 0.002
3-hydroxybutan-2-one	0.435 ± 0.170	0.388 ± 0.042	0.460 ± 0.088	0.332 ± 0.059	0.423 ± 0.059
Furaneol	2.57 ± 0.83	10.8 ± 2.98	5.40 ± 0.28	7.76 ± 3.75	6.96 ± 1.69
DHMP	0.119 ± 0.010	0.136 ± 0.016	0.136 ± 0.014	0.137 ± 0.005	0.117 ± 0.021
3-MCPD	1.06 ± 0.398	1.08 ± 0.09	0.943 ± 0.069	0.773 ± 0.218	1.72 ± 0.45

[a] Amounts expressed as mg/model system. Means and standard errors ($P < 0.5\%$) were obtained at least in triplicate experiments.

Table 5. Eigenvectors of PCs for the Volatiles Obtained from Model Systems at pH 6

descriptor	PC_1	PC_2	PC_3
pyrazine	0.030	0.748	−0.663
2-MP	0.238	0.919	0.197
2,5-DMP	0.961	−0.037	0.269
2,3-DMP	0.980	0.075	0.071
3-hydroxybutan-2-one	0.360	−0.221	0.635
DHMP	−0.316	0.345	−0.804
Furaneol	−0.244	0.924	−0.251
3-MPCD	0.021	0.256	0.946
% of total variance explained	27.901	31.227	32.053

and temperature, have a much greater effect than the formation of free radicals. Moreover, it must be taken into account that the antioxidative efficacy depends on the reaction conditions and is not a linear function of the dose and that antioxidants can become pro-oxidants depending on the concentration (Pokorny, 1987).

In xylose/lysine model systems, greater differences were encountered at pH 4 than pH 6, probably because at higher pH the Maillard reaction is much faster, giving rise to large amounts of intermediates that can be oxidized rapidly, and the presence of anti- or pro-oxidants is promptly neutralized. This was particularly true in the case of AIBN. Concentrating the discussion on pH 4, the fact that the model system with AIBN is very well-discriminated by the score plot of PCA from the models with the antioxidants, which are located together on the opposite side with respect to the reference model system, confirms that the volatiles are sensitive to compounds that can interfere with free radical formation.

Comparing BHT and AIBN, it can be said that they have an opposite effect on the formation of some Maillard reaction products, BHT being effective in increasing and AIBN in decreasing yields, for example, pyrazine and 2-methylpyrazine. We had already observed a similar behavior in glucose/lysine model systems (D'Agostina et al., 1998), at least at pH 6. These observations suggest that a free radical mechanism is in some way involved in the formation of these Maillard reaction products.

Some groups have shown by ESR experiments that primary (Namiki and Hayashi, 1983; Hayashi et al., 1986) and secondary (Roberts and Lloyd, 1997) amines, reacting with sugars, give rise to *N*,*N*-dialkylpyrazinium radical cations, an intermediate for the formation of pyrazines. Following this pathway, reagents that are able to decrease the formation of free radicals should deplete the yields of pyrazines. Our data, on the contrary, seem to indicate that an independent autoxidation of the already formed pyrazines or, better, of some intermediate can take place. The sensitive materials could be the C2 fragment necessary for the formation of pyrazine and 2-methylpyrazine.

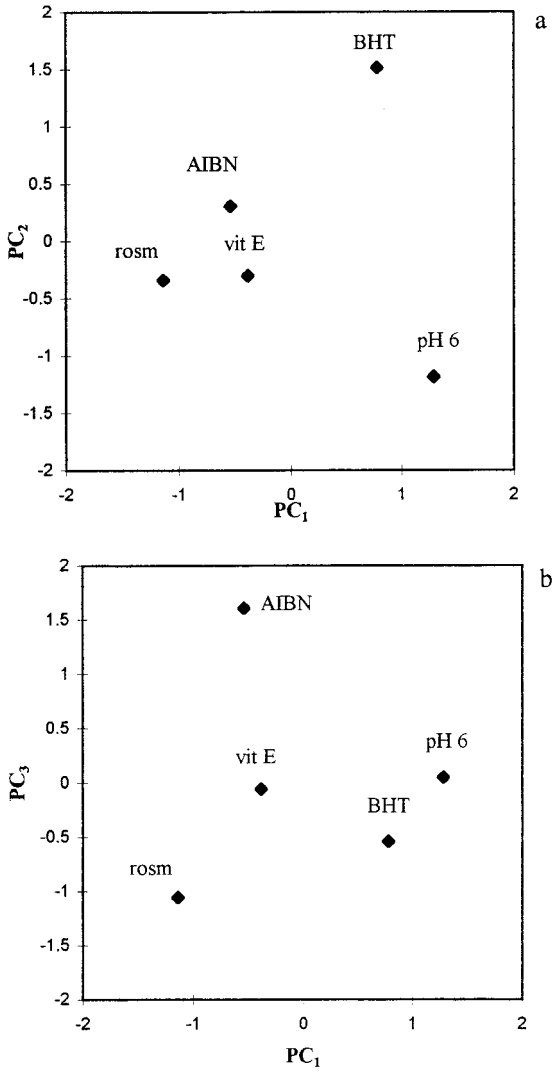

Figure 3. PCA of the volatiles obtained from model systems at pH 6: (a) loading plot of PC_1 vs PC_2 and (b) loading plot of PC_1 vs PC_3.

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Received for review June 7, 1999. Revised manuscript received November 23, 1999. Accepted November 29, 1999. This study was carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, Contract CT96-1080. "Optimisation of the Maillard reaction. A way to improve quality and safety of thermally processed food."

JF990610M