Stability of Thiols in an Aqueous Process Flavoring

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The flavor stability of an aqueous solution of a savory model process flavoring based on ribose and cysteine was investigated during accelerated storage at 50 °C. Of the three sulfur-containing flavor-impact components investigated, 2-methyl-3-furanthiol was found to be the least stable (59% decrease/24 h), and it was followed by 2-furfurylthiol (28% decrease/24 h), 2-mercapto-3-butanone (14% decrease/24 h), and 2,5-dimethyl-4-hydroxy-3(2H)-furanone (max. 10% decrease/24 h). Both cysteine and ribose were found to affect the stability of various flavor compounds. A mechanism for the instability of 2-methyl-3-furanthiol is proposed, and was confirmed by H−D exchange experiments.

Keywords: *Process flavor stability; 2-methyl-3-furanthiol; 2,5-dimethyl-4-hydroxy-3(2H)-furanone*

INTRODUCTION

Process flavorings, which are generally prepared by thermal treatment of an aqueous solution of reducing sugars and amino acids, show considerable flavor instability in aqueous solution. To overcome this instability, process flavors are generally spray-dried. The resulting powder has sufficient flavor stability, if temperature and humidity do not get too high. The stability in water is so limited however, that a great deal of effort in planning is required to prevent significant flavor loss before the product is spray-dried. Especially thiols, which are essential for a good meaty character of savory process flavors, are particularly unstable. This urged us to look in more detail at flavor instability of a model savory process flavor in aqueous solution.

Thermal treatment of a solution of ribose and cysteine results in the formation of several specific character-impact components with an overall meaty impression (*1*−*3*). The use of cysteine in combination with a pentose is responsible for the formation of very potent sulfur-containing meat flavor compounds such as 2-methyl-3-furanthiol (MFT), 3-mercapto-2-pentanone (3MP), 2-mercapto-3-butanone (MB), and 2-furfurylthiol (FFT) (*1*, *2*). Some of these compounds were similar to those identified in beef (*4*), and they were recently quantified in several types of heated meat (*5*). In the study described here, the ribose/cysteine-based process flavor reported by Hofmann (*1*, *2*) was used as a model for a meaty process flavor. As thiols are known for their reactivity/instability, such as oxidation and involvement in nucleophilic or radical reactions, we assumed that the degradation of these compounds is a likely cause of the limited shelf life or flavor instability of savory process flavors.

The interaction of thiol and disulfide flavor compounds with food components has been studied by Mottram et al. (*6*). The authors indicated that these compounds undergo redox reactions with proteins. Hofmann et al. (*7*) and Guth et al. (*8*) reported model studies on the oxidative stability of odor-active thiols and disulfides, respectively. Eisenreich et al. (*9*, *10*) reported studies on antioxidative activities of volatile sulfur-containing heterocyclic compounds, confirming their reactivity in free radical oxidation reactions.

The study described in this paper was designed to determine the (in)stability of the most important character-impact components in an aqueous solution of the model process flavor described by Hofmann and Schieberle (*1*, *2*), and to investigate why some of the character-impact components are unstable.

MATERIALS AND METHODS

Materials. 2-Methyl-3-furanthiol and 2-mercapto-3-butanone (10% solution in triacetin) were obtained from Oxford Chemicals (Cleveland, U.K.). 2-Furfurylthiol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, maltol, and D(−)-ribose were obtained from Aldrich (Steinheim, Germany), and L-cysteine, 2,5-dimethyl-3-methylfuranthiol, and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone were obtained from Quest International (Naarden, The Netherlands).

Analytical Instruments. A Fisons 8000 series gas chromatograph type 8130, equipped with a HP-5 capillary column (50 m × 0.32 mm; 1.05 μm film), a flame ionization detector (FID), and a Fisons AS 800 autosampler, were used for GC analysis. The GC was run with an injector temperature of 225 °C and detector temperature of 250 °C. The oven temperature was programmed from 65 °C to 120 °C at 3 °C/min and from 120 to 250 °C at 40 °C/min. Chrom-Card for Windows (Fisons Instruments) was used for data handling. HRGC mass spectrometry was performed using a Finnigan MAT TSQ 70 mass spectrometer in the electron impact mode at 70 eV. Cysteine and ribose were analyzed by capillary electrophoresis using a Hewlett-Packard 3D CE apparatus.

Preparation of the Aqueous Model Process Flavoring. A solution of 180 mmol D-ribose and 60 mmol L-cysteine in 1800 mL of phosphate buffer (0.5 M, pH 5.0) was heated in a 2-L autoclave from room temperature to 130 °C in 10 min and was kept at 130 °C for 20 min, followed by rapid cooling with tap water.

Storage and Analysis of the Aqueous Model Process Flavoring. Portions of 100 mL were stored in the dark at 50 °C in closed bottles under both air and argon atmosphere. At

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Table 1. Decrease in Concentration (% per day) of Five Character-Impact Components of an Aqueous Model Process Flavor (Entry 1) and Reconstituted Models (Entries 2−5) During Storage at 50 °C: Influence of Ribose and Cysteine

	decrease in concentration (% per day)				
compound	**1**[a] model process flavoring	**2**[b] no addition	**3**[b] + 250 mM ribose	**4**[b] + 150 mM cysteine	**5**[b] + 150 mM cysteine + 250 mM ribose
2-mercapto-3-butanone (MB)	14	> 90	> 90	26	25
2-methyl-3-furanthiol (MFT)	59	> 90	> 90	69	42
2-furfurylthiol (FFT)	28	> 90	> 90	39	43
4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDF)	< 10	< 10	< 10	77	66
3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone)	n.d.[c]	< 10	< 10	22	< 10

[a] According to the procedure of Hofmann and Schieberle (*2*), a phosphate-buffered (0.5 M, pH 5.0) solution of D-ribose (100 mM) and L-cysteine (33 mM) was heated in an autoclave from room temperature to 130 °C in 10 min and was kept at 130 °C for 20 min. Character-impact components were formed in the order of concentrations of around 5 μM. [b] 50 μM of each character-impact component was added to an aqueous phosphate-buffered solution (0.5 M; pH 5.0). [c] Not determined.

time intervals of 0, 4, 8, and 24 h, after addition of maltol (50 μg) as the internal standard, the aqueous reaction mixtures were extracted with dichloromethane/diethyl ether 7:3 v/v (20 mL). The extract was dried (Na_2SO_4), carefully concentrated to a volume of 2 mL at room temperature and a pressure of 200 mbar using a rotary evaporator, and finally concentrated under a stream of argon to 1.0 mL and subjected to HRGC analysis.

Storage and Analysis of Reconstituted Model Process Flavorings. A stock solution in ethanol of 2-methyl-3-furanthiol, 2-mercapto-3-butanone, 2-furfurylthiol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone was added to an aqueous phosphate buffered solution (0.5 M; pH 5.0) to obtain a final concentration of 50 μM of each of these components, which is a concentration approximately 10-fold higher than that found in the aqueous model of Hofmann and Schieberle (*2*). Either ribose or cysteine, or both, were added in various concentrations, typically in the range of 25−250 mM. Volumes of 5 mL of these solutions were stored in the dark at 50 °C in closed glass containers (15-mL) under air atmosphere. At time intervals of 0, 4, 8, and 24 h, after addition of maltol (500 μg) as the internal standard, the aqueous solutions were extracted with dichloromethane/diethyl ether 7:3 v/v (2.0 mL). The extract was dried (Na_2SO_4) and analyzed by HRGC.

Quantitative Analysis. Internal standardization was used for quantitative measurements. Method validation was established with three replicate measurements (for extraction and analysis parameters see above) using model solutions of analytes and internal standard (maltol) in phosphate buffer (0.5 M; pH 5.0). Recoveries of the analytes were determined to be ≥ 70%, and the relative standard deviation was ≤ 10%. Reconstituted model process flavor samples were analyzed in duplicate after 0, 4, 8, and 24 h. All plots of relative area against storage time showed a linear decrease, indicating zero-order or pseudo-zero-order kinetics for the compounds investigated. In all storage experiments described in this report, zero-order models were used on the basis of visual assessment and the coefficient of correlation (*R*) obtained from regression analysis. The relative decrease in the starting concentration of the analytes (% per day) was calculated by linear regression, using the least-squares method.

Deuterium Exchange Experiment with 2-Methyl-3-furanthiol and bis(3-Furanyl) Disulfide. ^{1}H NMR was used to follow the deuterium exchange of 2-methyl-3-furanthiol and bis(3-furanyl) disulfide at room temperature in CH_3OD solution (0.01 mM each) containing DCl during several days. ^{1}H NMR measurements were performed on a JEOL 400 EX spectrometer operating at a proton frequency of 399,65 MHz. TMS was used as internal reference.

RESULTS AND DISCUSSION

Flavor Stability of the Aqueous Model Process Flavoring. The decrease in concentration (% per day) of important flavor compounds during accelerated storage at 50 °C of an aqueous model process flavoring is shown in Table 1 (entry **1**). 2-Methyl-3-furanthiol (MFT) was found to be the most unstable component (59% decrease per day), followed by 2-furfurylthiol (28% decrease per day), and 2-mercapto-3-butanone (14% decrease per day). The furanones 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDF) and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) were relatively stable. Furthermore, an interesting observation was the fact that there was almost no difference between storage of the aqueous model process flavoring under air or argon atmosphere (data not presented here), probably due to an anti-oxidative effect of the matrix. The anti-oxidative properties of the matrix component cysteine, as well as of Maillard reaction products, are well-known.

Entry **2** (Table 1) shows the results obtained from a storage experiment of a reconstituted mixture of flavor compounds in aqueous solution, using concentrations which are approximately 10-fold higher than in the original process flavor. The concentrations were increased to allow more facile analysis of the components of interest. All three thiols were almost decomposed after 1 day of storage at 50 °C, whereas the furanones were relatively stable.

We anticipated that the presence of residual cysteine and/or ribose could have an effect on the stability of some of the character-impact components of the model process flavor. Residual cysteine and ribose concentrations were determined in a freshly prepared model process flavor, and were found to be 15 mM and 25 mM, respectively. The effect of additional cysteine and/or ribose on the stability of the flavor-impact components was therefore investigated in the reconstituted mixture (Table 1, entries **3**−**5**).

Accelerated storage of the reconstituted mixture in the presence of ribose (entry **3**) or cysteine (entry **4**) revealed that ribose addition has no effect on stability (compare entry **3** with entry **2**). However, a stabilizing effect through the addition of cysteine was clearly present (entry **4**). A comparison of entry **4** with entry **5** shows that when both ribose and cysteine are present (entry **5**), an even larger stabilizing effect on MFT and the furanones than with cysteine alone can be observed.

Because of the stabilizing effect of cysteine, the stability of the three thiols in an reconstituted aqueous mixture (50 μM each) was investigated at various cysteine concentrations. Figure 1 shows the decrease in concentrations (% per day) of 3-mercapto-2-butanone (MB), 2-furfurylthiol (FFT), and 2-methyl-3-furanthiol

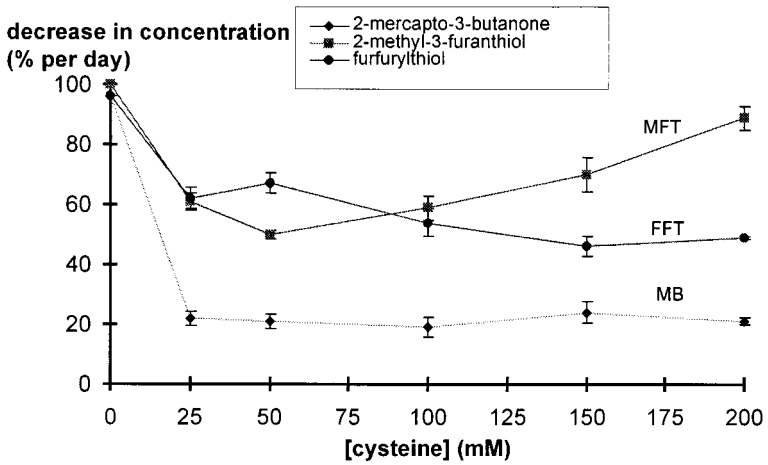

Figure 1. Decrease in concentration (% per day) of thiols in a reconstituted model process flavor (0.5 M phosphate buffer; pH 5) as a function of cysteine concentration.

(MFT) as a function of cysteine concentration. Interestingly, the decrease in concentration (% per day) of MFT shows a clear minimum at a cysteine concentration of around 50 mM, which is different from what is observed for MB and FFT.

The optimum stability of MFT in the presence of 50 mM cysteine suggests that the observed stabilization of MFT by additional ribose (Table 1, entries **4** and **5**) was caused by a decrease in cysteine concentration due to the formation of thiazolidinecarboxylic acid derived from cysteine and ribose. The actual concentration of cysteine after addition of ribose (250 mM) will be lower than 150 mM, as a result of the formation of this thiazolidinecarboxylic acid.

The results shown in Figure 1 also suggest that the mechanism of degradation of MFT in the presence of cysteine is slightly different from that of MB and FFT. Apart from an oxidative degradation pathway via (mixed) disulfide formation, another pathway leading to nonvolatile or polar degradation products by reaction of MFT with, e.g., cysteine may be possible, which would account for the lower stability of MFT at higher cysteine concentrations.

Evidence for the exceptional reactivity of MFT came from another storage experiment, which involved storage of a mixture of the three thiols MB, FFT, and MFT in phosphate-buffered solution (0.5 M; pH 5.0) in the absence of cysteine. The amount of MB and FFT at the beginning was equal to the total amount of both thiols and (mixed) disulfides at the end of storage. This was absolutely not the case for MFT. After storage a large amount of MFT was not detected, neither as thiol nor as (mixed) disulfide, using GC-analysis. When the same experiment was performed with 2,5-dimethylfuranthiol (DMFT) instead of MFT, the total amount of DMFT at the beginning was equal to the total amount of both DMFT and (mixed) disulfides at the end of storage (data not presented).

We think that this can be explained by the greater ability of MFT to undergo oligomerization/polymerization. It is known that MFT easily undergoes polymerization, when stored neat at ambient temperature, as some of the commercial samples already contain considerable amounts of polymeric material. This led us to propose the hypothetical mechanism for the polymerization of MFT as shown in Figure 2, which can explain the above-mentioned observations. Protonation at the 2-position leads to an electrophilic species, which can

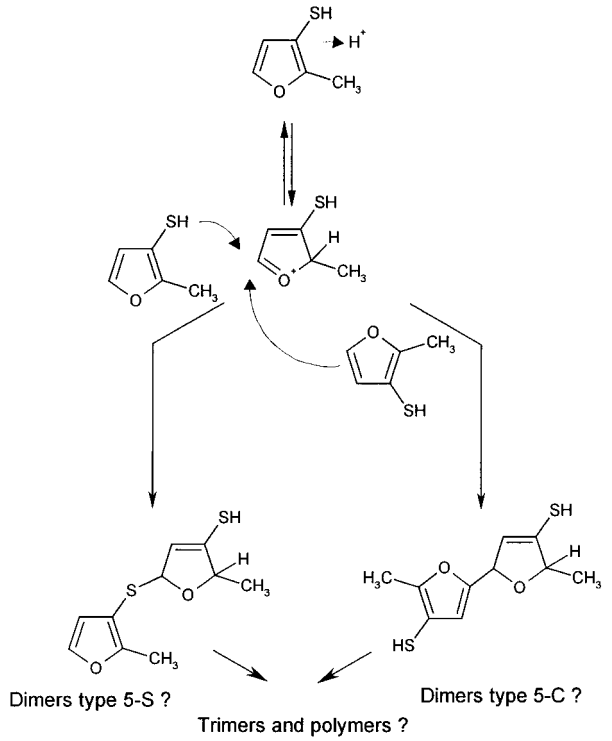

Figure 2. Proposed mechanism for polymerization of 2-methyl-3-furanthiol.

easily react with a nucleophile. As both the thiol group and the furan ring of MFT are good nuclophiles, protonation would catalyze dimerization/polymerization. Apart from the reaction of MFT with itself, other thiols (e.g., cysteine) could react as well, when present in higher concentrations. This mechanism can explain the different effect of the cysteine concentration on the stability of MFT in comparison to that on other thiols such as MB and FFT (Figure 1). In the case of DMFT, polymerization at the 5-position would be more difficult because of steric hindrance caused by the 5-methyl group.

If the left-hand part of the mechanism is of practical importance, coupling with other thiols would be expected as well. Because no loss of the other thiols was observed in the reconstituted mixture experiments , we must conclude that this part of the proposed mechanism is not significant.

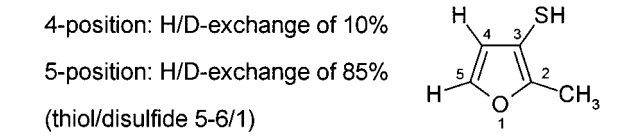

Figure 3. Deuterium exchange experiment with 2-methyl-3-furanthiol in CH_3OD/DCl during 64 days at room temperature.

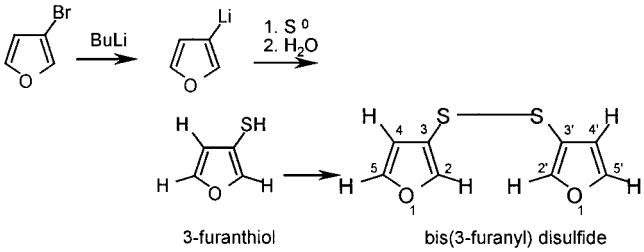

Figure 4. Synthesis of bis(3-furanyl) disulfide.

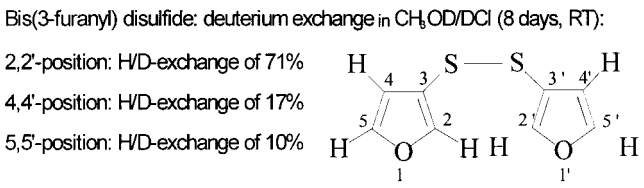

Figure 5. Deuterium exchange experiment with bis(3-furanyl) disulfide.

Deuterium Exchange Experiment with MFT and bis(3−Furanyl) Disulfide. To confirm our hypothesis of the degradation mechanism of MFT in aqueous solution, a deuterium exchange experiment with MFT was performed. Using ^1H NMR, 85% of H−D exchange was observed at the 5-position after several days of storage of MFT in CH_3OD/DCl solution at room temperature, whereas at the 4-position only 10% of the H atoms were exchanged (Figure 3). This is in agreement with the preference of electrophilic substitution at the position next to the heteroatom of aromatic heterocycles. However, except for the formation of the corresponding disulfide, no evidence for the formation of dimers was found, as was expected according to the mechanism in Figure 2. An explanation for this could be that in the model NMR experiment a methanolic solution had to be used instead of an aqueous phosphate-buffered (0.5 M; pH 5) solution, because of the low solubility of MFT in water.

To obtain deuterium exchange data for the 2-positon of 3-furanthiols, bis(3-furanyl) disulfide was synthesized according to Hofmann (*1*). The synthesis route is shown in Figure 4. Deuterium exchange experiments with this compound could provide evidence for the proposed mechanism of the degradation of MFT (see Figure 2). The disulfide was used for the deuterium exchange experiment instead of the 3-furanthiol, because some difficulties were encountered during the isolation of 3-furanthiol. The purity of the isolated thiol was not satisfactory (^1H NMR), possibly caused by the inherent instability of 3-furanthiol.

However, bis(3-furanyl) disulfide could be isolated in a pure form. The high exchange rate at the 2- and 2′-positions was remarkable when compared to that of the other positions (Figure 5). The preference for exchange at the 2- and 2′-positions is the result of the directing and activating effect of the S-atom linked to C-3 and C-3′. These results suggest that 2-protonation is facile for MFT, and is possibly a crucial step in the degradation of MFT as shown in Figure 2. Addition of nucleophiles to the 5-position of protonated MFT will most likely proceed via an ionic mechanism. Nevertheless, other mechanisms (e.g., radical mechanism) could play a role as well in the degradation of MFT.

CONCLUSIONS

The data presented indicate that in meatlike process flavors based on cysteine and ribose, 2-methyl-3-furanthiol is one of the least stable character-impact components. The instability is not due to disulfide formation, but appears to result from electrophilic coupling reactions. H−D exchange experiments showed that H-5 exchanges much faster than any other proton in MFT, suggesting that the reactivity of the 5-position is responsible for the rapid decomposition of MFT. This is in agreement with the lower stability of MFT in comparison with that of 2,5-dimethyl-3-furanthiol. Residual cysteine stabilizes MFT and other thiols.

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