Chemical changes in lipids and sulfur-containing substances during ripening of raw sausage

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This paper describes an attempt to elucidate gross changes in lipolysis and fat oxidation, and the relation of this to changes of sulfur containing materials and to correlate these with the development of aroma. Previous work (Nurmi and Niinivaara, 1964) has shown that Lactobacilli produce higher concentrations of peroxides than Micrococci. In addition to further decomposition of peroxides to carbonyl compounds, there is a possibility of reactions of these with amino acids, polyhydroxy compounds, and sulfhydryl substances (Watts and Greene, 1966; Mabrouk and Dugan, 1966; Wedemeyer and Dollar, 1963).

EXPERIMENTAL

The sausage was prepared according to the usual practice in this laboratory, using an Autotherm air conditioned cabin at a temperature of 20—22° C. When ^{14}C-labeled oleic or stearic acid was added, molten lard was used as a carrier, the mixing was made by hand, and the filling with a 300 ml wide-opened syringe.

The degree of lipolysis was determined by alkalititration of chloroform-methanol (2: 1) extracts of raw sausage at different stages of ripening. The proportions of individual fatty acids in the free fatty acid fraction, extracted from the total lipid with 5 % bicarbonate solution, was determined by gas chromatography. The methyl esters were prepared with sulfuric acid-methanol, and a six-feet polyethyleneglycol succinate column (15 % stationary phase) was used, the instruments being Perkin-Elmer 116 E and 800. The peak-areas were estimated by triangulation. The absolute contents of free fatty acids were obtained by converting the mass proportions to the molar ones and obtaining the appropriate factor to equalize the sum of molar proportions to the titration values.

The degree of oxidation of fatty material was evaluated by iodometric hydroperoxide titration in the presence of glacial acetic acid and by determination of the thiobarbituric acid (TBA) numbers at 532 and 455 nm

(Kärkkäinen and Antila, 1961). A map of the distribution of different carbonyl compounds was made by thin layer chromatography (Kieselgel Stahl, benzene, developed 5 times), and an attempt to evaluate the degree of oxidation of oleic acid, the most common of the fatty acids, was made, using uniformly labelled ^{14}C oleic acid, added during preparation of sausage.

Catalase activities were determined by the method of Mikola (1954).

Determinations of sulfhydryl and disulfide contents were made according to Hofmann and Hamm (1966), and hydrogen sulfide was determined by the method of Marbach and Doty (1956).

Volatile compounds were analyzed by programmed temperature gas chromatography (30—180°, 5°/min.) on a 6'EGSS (15 %) column (Perkin Elmer 800), after enrichment of the volatiles by ether-petane (1: 2) extraction, codistillation with highly purified capronic acid in fine vacuum from the fat, removal of acids with bicarbonate solution, separation of the main part of solvent by distillation on a spinning-cylinder column (30 plates), and a further concentration on a short precolumn, which was connected to the gas chromatograph before the analyzing column. Head space gas chromatograms were run from weighed samples equilibrated with the gas phase in 100 ml sealed flasks at 60° C, using a tight 5 ml syringe.

RESULTS AND DISCUSSION

a. *Lipolysis.* The quantities of the most abundant fatty acids showed generally a higher increase of unsaturated acids compared to sturated ones,

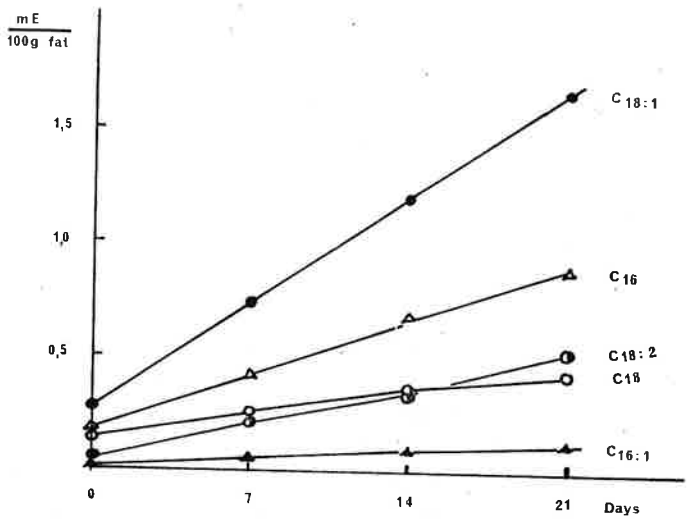

Fig. 1

as examplified in Fig. 1. Results obtained with ^{14}C-labelled stearic acid indicated that re-esterification of this saturated acid did not occur in the sausage. The lipolysis thus can be considered as acid-specific, in accordance with the work of Alford *et al.* (1964) on other micro-organisms. The ease of hydrolysis of ester bonds decreases in the order:

Control:	$C_{16:1}$	$C_{18:2}$	$C_{18:1}$	C_{16}	C_{18}
Lactobacilli:	$C_{16:1}$	$C_{18:2}$	$C_{18:1}$	C_{16}	C_{18}
Micrococci:	$C_{18:2}$	$C_{18:1}$	$C_{16:1}$	C_{16}	C_{18}
Lactobacilli + Micrococci	$C_{18:2}$	$C_{16:1}$	$C_{18:1}$	C_{16}	C_{18}

b. *Oxidative changes.* The peroxide numbers rose during the ripening from about 1.5 to 2. Sausages inoculated with micrococci contained less peroxide than the lactobacilli-inoculated or the control sausages. The highest values were produced by lactobacilli. Mixtures of lactobacilli and micrococci gave only slightly higher values than pure micrococci.

During the first week, the increase of peroxide numbers often coincided with a decrease of catalase activity, though not quite consistently. From the second week onwards, however, a positive correlation was found between the hydroperoxide content and the catalase activity. (Table 1.)

Table 1. *Correlation between catalase activity and peroxide number during ripening*

C = catalase activity, P = peroxide number

Type of sausage	*Correlation coefficient*	*Regression line*
Control	0.98	C = 410 P − 2036
Lactob.	0.74	C = 37 P + 68
Microc.	0.74	C = 28 P + 99
Lactob. + microc.	0.96	C = 43 P + 56

The TBA numbers had a similar trend, the changes being more distinct in the latter. During the first week the values increased in lactobacilli and lactobacilli + micrococci sausage. During the second week the changes were vice versa and during the third there was a further increase in the micrococci sausage and a further slight decrease in the lactobacilli sausage. In the control sausage there was a distinct decrease and in the lactobacilli + micrococci group a distinct increase (Fig. 2, Table 2).

Table 2. *End values (21 days) of peroxide and thiobarbituric acid numbers, and ratios of factors of increase for free fatty acids*

Sample	Perox. No.	TBA/g. fat 532 m	TBA/g. fat 455 m	$C_{16}=_1C_{18}$	$C_{18}=_1/C_{13}$	$C_{13}=_2/C_{18}$	$C_{16}=/C_{18}$	$C_{16}=_1/C_{18}$
Control	2.0	0.10	0.14	1.66	1.49	1.84	1.27	2.10
Lactobacilli	2.8	0.12	0.37	1.45	1.13	1.40	1.08	1.57
Micrococci	1.5	0.29	0.27	1.25	1.42	1.66	1.13	1.41
Lactobacilli+ micrococci	1.9	0.07	0.23	1.45	1.56	2.10	1.24	1.80

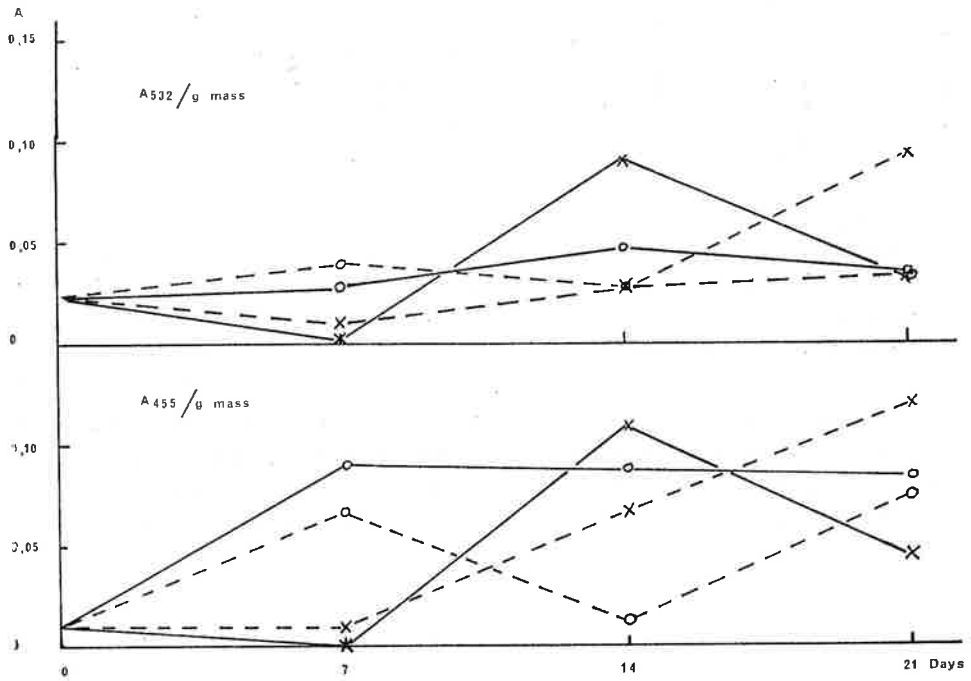

Fig. 2

As regards individual carbonyl compounds, a great variability from bath to bath was observed. Clear correlations to type of sausage could not be found for most of the components. Of the twelve carbonyl components observed by TLC (Fig. 3), only three showed indications of some connection to other variables. Spot N:o 2 seemed to be found somewhat more abundantly in lactobacilli sausage. Spots N:o 3 and 5 seemed to possibly correlate with the formation of aroma of lard.

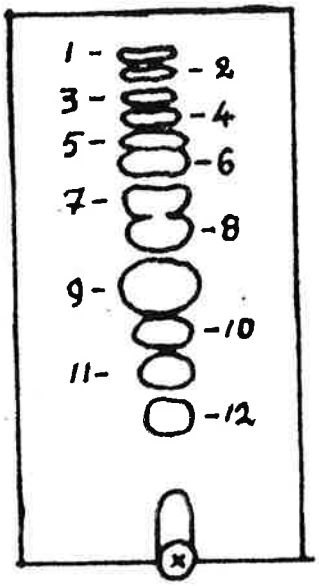

Fig. 3. TLC of dinitrophenylhydrazones of carbonyls obtained by steam distillation.

Attempts were made to map the formation of carbonyls from the most abundant fatty acid, oleic acid, using a uniform label with ^{14}C. Four active spots were detected by TLC and scintillation measurement. Two of these, which were very weak, had a very low R_f-value. The highest label was observed in the spot corresponding to N:o 12. With an average loss of 0.24 g of oleic acid/30 g of fat during 21 days of ripening, this activity corresponds to about 40 % of the activity associated with the loss of oleic acid. This compound was not identified, its IR spectrum is shown in Fig. 4. Spot N:o 5, corresponding to about 17 % of the loss of oleic acid, as estimated by relative activity, was identified by its IR spectrum as nonyl aldehyde.

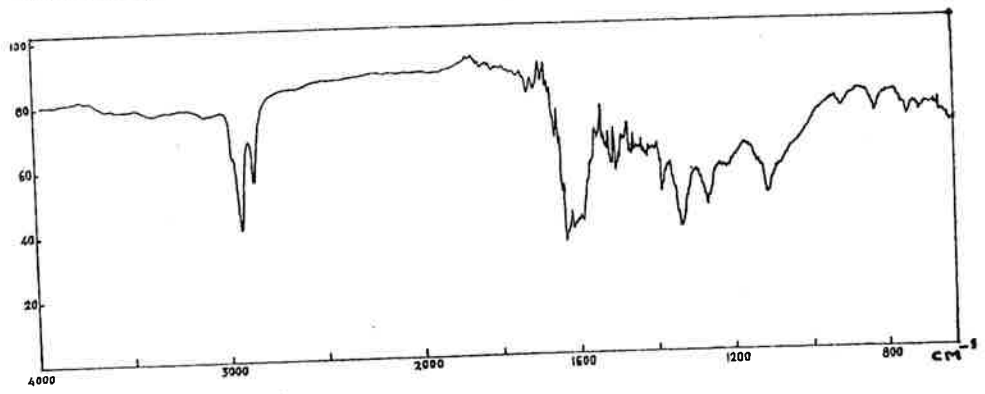

Fig. 4. IR-spectrum of the DNP:one containing the highest label when C oleic acid (U) is added to dry sausage. KBr pellet.

Alford *et al.* (1966) stated that catalase-positive microbes reduce the peroxide-number of rancid pork-fat. The micrococci used were catalase-positive, the lactobacilli negative. During the first week of ripening, the peroxide as well as the TBA numbers were of the direction expected from the quoted reference. At the same time the catalase activity decreased. If the then observed positive correlation between -OOH and catalase is not a coin-

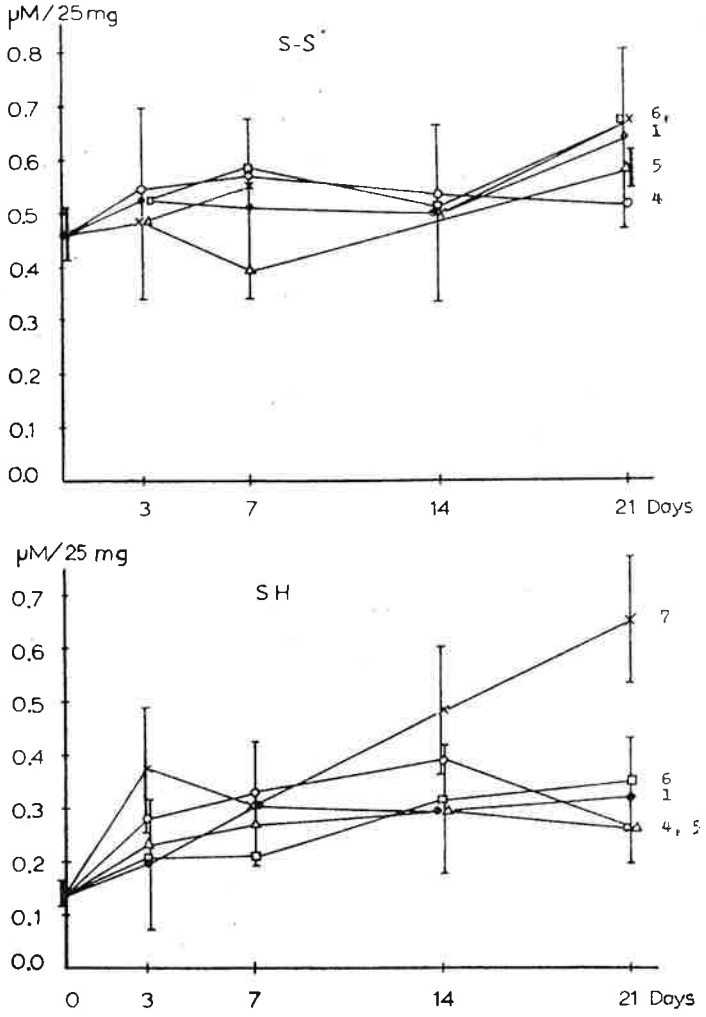

Fig. 5. Variation of S-S and — SH content of dry sausage during ripening. 1 = control, 4 = lactobacilli + micrococci, 5, 6, same, with various amounts of spices, 7 = lactobacilli alone.

cidence, it seems to obviate the idea that catalase, because it lowers the H_2O_2 content also should decrease the oxidation of unsaturated acids. The question is, however, more complicated, because the slower formation of carbonyl compounds, which should accompany the slower formation of hydroperoxides, is observed only at the start of ripening, while high carbonyl concentrations prevailed toward the end of the process. According to Tappel (1953), hematin compounds can catalyze the decomposition of —OOH to carbonyls. If the catalase would behave in this manner, it could explain the positive correlations found between catalase activity, —OOH numbers and carbonyl content. It is also known that catalase can utilize other peroxides than H_2O_2 as oxygen donors, if some oxidizable substrate, other than peroxide, is present. It seems that the properties of the catalase/s present in dry sausage have not yet been investigated in this respect.

c. *Sulfur compounds.* Some typical curves for the changes in disulfide and mercaptan groups are given in Fig. 5. The mercaptan content has in some cases a statistically significant rise (the only micrococci sausage investigated in this respect, and some lactobacilli sausages). The disulfide content rises in most cases. The increase observed is probably not associated with a change of the titrability of protein S-S and —SH groups, as the method used is claimed to make all the groups available for titration (Hofmann and Hamm, 1966). Thus it can be concluded that a net synthesis of S-S and -SH compounds from other sulfur-containing materials usually occurs, even if the weight loss during ripening were 20 %. Sometimes the increase of the

Table 3. *Change of disulfide and mercaptan during ripening.*

Type of sausage	*Increase of S-S + SH as % of initial value, corrected for weight loss.*
Control	34
Lactobacilli	110
»	19
»	39
Lactobacilli + micrococci	13
»	51
»	3
»	44
»	44
Micrococci (low activity)	52
Mixture of a few Lb and Mc strains	20
Mixture of lyophilized Lb ans Mc + lipolytic strains	39

S-S + -SH material exceeds 100 % (Table 3). In a few experimental series no net production of -SH could be detected by amperometry.

The source of sulfur for this synthesis is not known to us; one possiblity could be a demethylation process of methionine, another, a reduction of mucoid sulfate material. The chemical identity of the mercaptans and disulfides formed is not yet confirmed. It can however be claimed that hydrogen sulfide is not responsible for more than a negligible fraction of the observed increases in -SH, because the H_2S determinations showed that its content is about 10^{-4} times that of other -SH compounds on a molar basis, at the end of ripening. Generally, the concentration of H_2S at 21 days was found to be two to three times higher than in the mass, the end concentration being 3 -6 $\times$ 10^{-5} μM/25 mg.

d. *Relations between S-S and -SH groups and other characteristics.* Because of the well-known cross-linking of protein molecules by disulfide bridges, some relation between disulfide values and the consistency of sausage would be expected. The experiments show, however, that no correlation between the S-S/-SH ratios and consistency can be detected. Also the correlation between this and the total disulfide content was negligible. This could be interpreted by assuming that other disulfides of lower molecular weight are formed.

On basis of the work of Wedemeyer and Dollar (1963), who showed that S-containing amino acids are co-oxidized in an autoxidizing lipid system according to the pattern

$$2\ \text{R-SH} \longrightarrow \text{R-S-S-R} \longrightarrow \text{R-S-S-R} \downarrow \text{O}$$

it was considered of interest to compare the peroxide values with the ratio S-S/-SH and with the total -SH + S-S content during ripening. The ratio S-S/-SH correlated very weakly to -OOH values, but there is a negative correlation if the peroxide value at 3 days is compared with the S-S + -SH content at 7 days, peroxide at 7 days with S-S + SH at 14 days, etc. This seems to indicate that a high peroxide content leads to a subsequent conversion of mercaptans via disulfides to further oxidation products, not detectable by the amperometric method used. Assuming that mercaptans and disulfides are continuously produced by bacterial action, the S-S and SH content should rise again in the sausage when its peroxide content has been sufficiently loweved, as is actually observed (Fig. 6).

Comparisons of -SH and S-S groups with organoleptic evaluations seem to indicate that an abundant formation of mercaptan and disulfide, especially at the beginning of the ripening process, mostly leads to inferior results, correlating to a specific off-flavor which is often found in sausage of the lactobacilli-type, but sometimes also in controls. The comparisons are com-

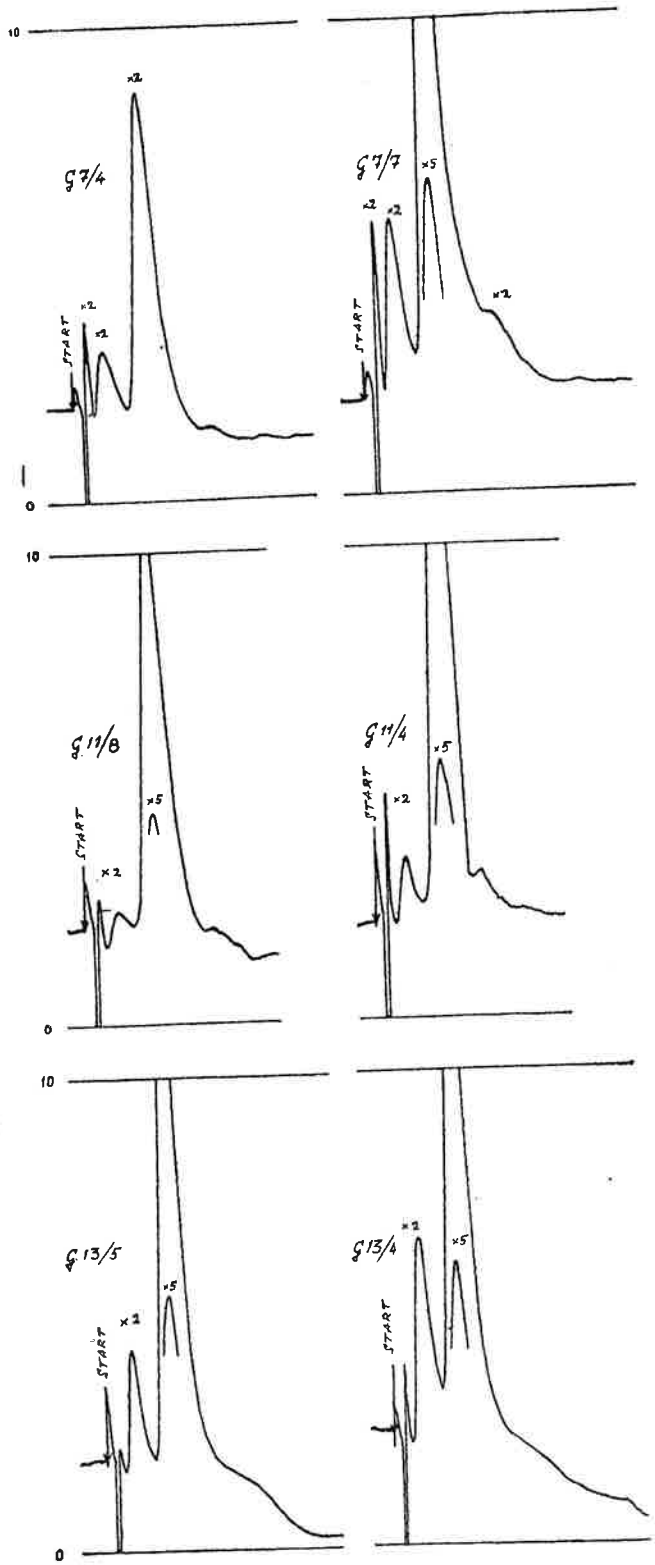

Fig. 7. Head-space gas chromatogr ams of dry sausages. Left: normal flavor. Right: specific off-flavor.

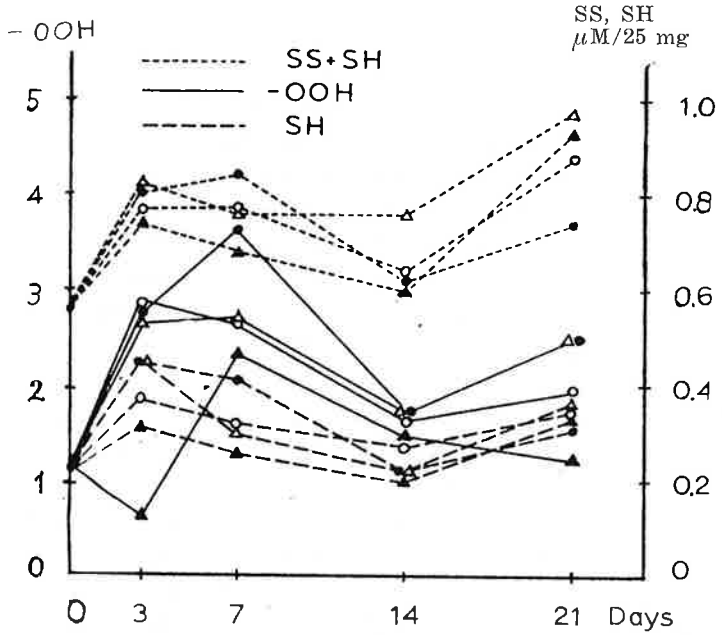

Fig. 6. The course of mercaptan + disulfide and peroxide contents in dry sausage during ripening.

plicated by the great variability of the S-S/-SH ratio in sausages of different types, and significant differences between the types of bacterial inoculations are not detected. Also, the variability of the starting material is great, S being 0.8, and $S_{av.}$ 0.36 for an average of 1.8. Therefore it seemed pertinent to compare the direction and amount of change of the S-S/-SH ratio in the sausage with respect to the starting value of the particular mass used, with the organoleptic evaluations. The average Δ (S-S/SH) for sausages (n = 43) with a quality lower than normal is — 0.45 (the minus sign indicating a decrease of the ratio). For sausages in the group having normal or better than normal quality (n = 60), the average Δ (S-S/SH) is + 0.08. This leads to a value for t = 2.68, with DF = 101. At this number of degrees of freedom, $t_{crit.}$, 1 % is 2.630, so the difference in Δ (S-S/SH) is statistically regarded as very significant.

In an attempt to reveal the cause of the specific off-flavor referred to above, a chromatographic search was made for compounds associated with this flavor. Thin layer chromatography of the carbonyl material (as dinitrophenylhydrazones) did not reveal any spot consistently associated with the occurrence of this off-flavor. Nor did liquid chromatograms of the difficultly volatile fractions disclose material with the appropriate odor. In the gas chromatograms of volatile fractions collected by co-distillation, only rapidly

eluting components, following immediately upon the solvent peak show differences which could be connected to this type of flavor. Therefore, headspace chromatograms were run from sausages with this fault and with a high aroma quality, of the same age. The differences in the concentration of the easily volatile constituents parallel the degree of off-flavor (Fig. 7). These gas chromatographic peaks are considerably reduced if the vapor is treated with concentrated lead or mercuric acetate solution in water. If these solutions are afterwards treated with sulfuric acid, the peaks in question can be detected in large quantities, so they presumably are mercaptans or sulfides. It is interesting to compare this finding with the correlation between organoleptic properties and the S-S and -SH values found by titration.

Considering the interplay of lactobacilli and micrococciduring sausage ripening, we have the experimental indications that lactobacilli, which usually produce higher concentrations of peroxide than micrOcci also cause a faulty color in the sausage, when a mixture of micrococci and lactobacilli, which leads to lower concentrations of hydroperoxides, also has a favorable effect on color and flavor. Therefore it can be stated that a too oxidative milieu should be avoided. It could be thought to cause lard flavors due to carbonyl compounds formed by oxidation of unsaturated fatty acids. On the other hand we have the relations between the sulfur containing materials and flavor. Now, the disulfide to mercaptan ratio reflects to some extent the redox-potential prevailing in sausage. If one dares to generalize from this, it could be said that too a reductive milieu is deleterious to the ripening process. These factors are opposing: strongly oxidative and strongly reductive conditions chould be avoided. If this reasoning holds, it could be useful to develop a buffering system for the redox-potential within the sausage. As the normal potential for mercaptan groups is about 100 mV, it thus seems that the potential should be buffered at a somewhat more oxidizing level than this value. In this connection it is of interest to remember the composition of certain mixtures which are used as additives for color improvement in sausage. Some of these contain ascorbic acid and some sulfur-containing amino acid, e.g. cysteine. The addition of this material to the mass could be looked upon as an attempt to stabilize the redox-potential. When such additions are made, there is, however, a danger that the mass can become too reducing. Besides, it seems very possible that sulfur-containing substances can be converted to ill-smelling mercaptans during the ripening process, especially if there is not sufficiently of oxidizing material present to convert disulfides further to sulfinic acids or sulfones.

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