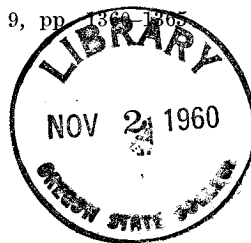


MILK LIPIDS SYMPOSIUM

AUTOXIDATION OF MILK LIPIDS¹

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Milk lipids are recognized as a major contributor in determining consumer acceptability of most dairy products. However, the reaction of these lipids with oxygen, resulting in flavor deterioration, creates serious problems in storage stability. Quality impairment due to autoxidation is widespread and manifests itself in many ways, depending upon the product involved. In certain products, particularly dry whole milk, autoxidation has been a major deterrent to the development of an acceptable product. In all products, considerable cost is added to provide protection against oxidative effects. Whatever is done to milk fat to improve the appeal of dairy products, whether it be by alteration of its physical properties or by more attractive prices through efficient production, we must be cognizant of the need to provide maximum storage stability. Current trends in marketing make this requirement more essential than ever.

No other chemical deterioration has been studied as extensively as autoxidation, nor has any other problem been investigated from so many different angles. Even so, controversy still prevails relative to the origin of the flavor compounds, the mechanism of the reactions, and the influence of environmental factors on the nature of the oxidation products. In a field as large and as controversial as autoxidation, brief treatment as desired here only can include some of the current thinking, with supporting evidence wherever possible. More thorough treatments on the autoxidation of dairy products are included in reviews by Brown and Thurston (2), Greenbank (13), and Pont (24), and on lipids in general by Morris (19), Holman (15), and Lea (17).

IDENTITY OF COMPOUNDS IN OXIDIZED MILK LIPIDS

One of the most fruitful approaches to the study of lipid oxidation deals with elucidation of hypoperoxide degradation products. Until recently, progress was hindered by lack of adequate tools to attack the problem. However, developments in chromatography and spectrophotometry over the past decade have provided many of the necessary implements and, as a result, the identification of compounds responsible for oxidized flavors is now being investi-

gated in many laboratories. Identification of the flavor compounds will be valuable in revealing differences in the sensory character of the flavor defects, in providing the necessary information for a direct chemical measurement of the flavor and in elucidating the reaction mechanisms of lipid oxidation which are by no means clear at the present time.

One of the first serious attempts to identify the flavor of oxidized milk lipids was made during World War II by van der Waarden (28) on cold storage butter. He observed that the flavor compounds were fat soluble and could be completely removed from the fat by degassing at a low pressure. The volatile compounds, isolated by this technique, were low in nitrogen, sulfur, and phosphorus, contained no peroxides, and imparted an oxidized flavor when added to fresh fat. From micro-qualitative studies of the volatiles and upon comparison of properties of the distillate with pure compounds, it was postulated that methyl ketones and aliphatic, unsaturated, unconjugated aldehydes were of importance to the flavor. Two other reports (16, 27) in the literature largely support the findings of van der Waarden and are the basis for the current belief, by many investigators, that unsaturated, unconjugated carbonyls are responsible for the oxidized flavor of fat. Keeney and Doan (16), in a study of oxidized fat, concluded that the bulk of the odor compounds was ketones. None of the ketones was methyl ketone and a large portion was unsaturated. Their conclusions were based upon the specificity of Girard's reagent for fractionation of aldehydes and ketones. The Girard's reagent has since been shown to be nonspecific (8) and it is likely that the compounds isolated by Keeney and Doan (16) were saturated and α,β -unsaturated aldehydes. Evidence for ketones in their carbonyl mixtures is not surprising in view of the heat treatment given the fat and the recent findings by Patton and Tharp (22) that ketones are produced by heat treatment.

Tamsma (27) also studied the volatile compounds from milk fat and reported on three classes of carbonyls contained in the Skellysolve soluble fraction. The unconjugated, unsaturated compounds were reported to be responsible for the flavor and along with the conjugated monene compounds, constituted the major portion of the volatile fraction. Conjugated dienes were present at trace levels and were reported to be ketonic. Tamsma's con-

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clusions were based largely on hydrogenation studies, in which it was claimed that the conditions of hydrogenation preferentially saturated double bonds without reducing the carbonyl groups. There was no direct evidence given to substantiate this claim. In fact, critical examination of the data would infer that the carbonyl group was reduced. Based on hydrogen uptake, Tamsma found the molar absorbance of the conjugated diene fraction to be 10,400 at 265 m μ , yet values in the literature are in the range of 26,000 and above (10, 23). This would suggest that the amount of hydrogen absorbed was substantially more than was necessary to saturate the double bonds. Therefore, an alternate conclusion is that hydrogenation eliminated oxidized flavor by reduction of the carbonyl group.

Unfortunately, refined chromatographic procedures were not available at the time the aforementioned studies were conducted. A careful examination of the data shows similarities to more recent findings. For instance, the fraction termed unsaturated, unconjugated by Tamsma appears similar to the saturated aldehydes found by Day and Lillard (5) and by Forss *et al.* (9). Similarly, the conjugated monene and diene compounds appear similar to 2-enals and 2,4-dienals (5, 9, 11).

Forss, Pont, and Stark (11) conducted a series of experiments in which they isolated and identified the compounds causing the copper-induced oxidized flavor of skimmilk. Ethanal, n-hexanal, the C_4 to C_{11} alk-2-enals, and the C_6 to C_{11} alk-2,4-dienals were identified. The most abundant compounds were the C_8 and C_9 2-enals and the C_7 and C_9 dienals. These compounds were isolated from 100 gal. of oxidized skimmilk. This quantity of skimmilk would contain approximately 75 g. of phospholipid (0.02%) and about 200 g. of fat (0.05%). Although it was not specified in their reports, phospholipid oxidation probably was responsible for a large proportion of the compounds.

van Duin (7) has studied the products of butter phospholipids when oxidized at pH 4.6 and 13° C. He presented tentative evidence for the C_2 to C_{18} alkanals, the C_5 to C_{11} alk-2-enals, the C_8 and C_9 alk-2,4-dienals, and small quantities of a number of different ketones. He subsequently found that the longer chain carbonyls, $>C_{12}$, were present in the phospholipids as acetals (plasmalogens) and were not products of oxidation. The flavors of the long chain aldehydes were described as pleasant and it is unlikely that they contribute to oxidized flavors. Their possible significance in natural flavors warrants further investigation.

Day and Lillard (5) identified the C_1 to C_{10} alkanals, the C_4 to C_{11} alk-2-enals, and obtained evidence for the odd-numbered C_3 to C_{15} methyl ketones in oxidized milk fat. The ketones were not attributed to oxidation since they were found in fresh fat. Since the above work was

published, we have also found evidence for traces of hepta-2,4-dienal in the fat. This was accomplished by refinement of our techniques through the use of the paper chromatography method of Gaddis and Ellis (12) in combination with column partition chromatography (4).

In a recent work, Forss *et al.* (9) have identified a number of the compounds that are responsible for the fishy flavor in butterfat. They employed a combination of procedures which included gas, paper and column chromatography. Two fractions with distinct flavors were obtained. One fraction exhibited an oily flavor and contained n-hexanal, n-heptanal, and 2-hexenal. The other flavor fraction was described as metallic and appeared to contain a single carbonyl compound in relatively small amounts. Compounds that were identified included the C_3 and C_5 to C_{10} alkanals, the C_3 and C_5 to C_6 alk-2-enals, hepta-2,4-dienal, a compound resembling octa-2,4-dienal, a compound with a mushroom flavor, and a compound that formed the 2,4-dinitrophenyllosazone of methylglyoxal. It is interesting that most of the compounds found by Forss in fishy flavored fat were also isolated from typical oxidized milk fat by Day and Lillard (5). Day and Lillard (5) found the complete series of alkanals from C_1 to C_{10} and the C_4 to C_{11} alk-2-enals whereas Forss *et al.* (9) found the C_3 and not the C_4 enal and they did not find the C_{11} , C_2 , and C_4 alkanals nor the C_{10} and C_{11} alk-2-enals. Also, Forss *et al.* (9) found the unidentified metallic and mushroom fractions. These unidentified fractions could have been missed by our techniques since they may occur in the dicarbonyl fraction. Likewise, the C_{10} and C_{11} enals could have been missed by Forss *et al.* (9), due to temperature limitations of their gas chromatography method.

At the present time, it appears that the majority of the volatile monocarbonyl compounds occurring in oxidized milk lipids belong to three classes; namely, the alkanals, alk-2-enals, and alk-2,4-dienals. There are probably traces of other compounds and these uncommon ones may well have an influence on oxidized flavor. However, recent studies under different conditions of oxidation show that practically all of the monocarbonyls will fit into the above classes (5, 9). The generally accepted free radical mechanism of lipid oxidation will explain the formation of most of the carbonyls, provided the unsaturated fatty ester precursors are present in the system.

STUDIES ON THE SENSORY CHARACTER OF OXIDIZED FLAVOR

Since the volatile monocarbonyls are largely responsible for oxidized flavors of dairy products, qualitative and quantitative variations of the flavor mixture must account for differences in flavor character. For example, qualitative differences appear responsible for the specific flavors of oxidized skimmilk as compared to

those of milk fat. Alk-2-enals and alk-2,4-dienals predominated in skimmilk (11); whereas, alkanals and alk-2-enals are the major flavor compounds in oxidized milk fat (5). Differences in the qualitative composition of oxidized flavors are influenced by the composition and susceptibility of the substrate. The phospholipids of fluid milk contain a higher percentage of polyethenoic acids than do the triglycerides (26). Furthermore, the phospholipids being oriented at the fat-serum interface are conceivably more susceptible to oxidative attack. Thus, in fluid milk it is believed that the phospholipids are preferentially oxidized. There is a wealth of circumstantial evidence to support this view. It is questionable, however, if the flavor comes entirely from phospholipid oxidation. The intimate association of phospholipids and triglycerides at the fat-serum interface in milk should afford conditions for oxidation of both. It is unlikely, therefore, that the triglycerides are completely free from oxidative attack, especially in the presence of the peroxides and the free radicals resulting from phospholipid oxidation. Oxidation of the more susceptible and highly reactive phospholipids would predominate, nevertheless.

The influence of quantitative changes in the volatile carbonyl mixture on the flavor character of oxidized fats has been largely ignored in the past. This problem is of importance when attempting to relate objective to sensory methods in flavor studies. If the percentage composition of the carbonyl mixture varies, correlation of individual compounds with flavor intensity may be impractical, unless the flavor is due to a single compound. However, if the percentage composition remains constant regardless of flavor intensity, a single compound should give high correlation with sensory measurements. In a study of 16 milk fat samples covering a wide range of oxidation (peroxide values 0 to 64), Day and Lillard (6) observed considerable variation in the percentage composition of the volatile monocarbonyl mixtures. Certain carbonyls always predominated but their percentage of the mixture was not constant. Furthermore, the carbonyls at highest concentration did not give the highest correlation with oxidized flavor intensity. The mean percentage of heptanal for 16 samples was 18.8% but its correlation with flavor intensity was 0.426. In contrast, the mean percentage of non-2-enal was 4.18% but its correlation with flavor was .942. It is significant that Holm *et al.* (14) also found a high correlation between the α,β -unsaturated carbonyls and the oily flavor of butters.

The oxidized flavor intensities of the 16 milk fats were ascertained by determining the absolute flavor threshold (FT) of the fat in whole milk media. By knowing the FT of the oxidized fat and the amount of each monocarbonyl in the fat, it was possible to calculate the concentration of carbonyl compounds re-

quired to give a perceptible oxidized flavor in whole milk. It was found that at the FT of the fat, the carbonyls were present in concentrations of parts per billion. In most cases, the individual compounds were at marginal or subthreshold levels. This was based on published flavor thresholds for carbonyl compounds (18). There is insufficient sensory data to draw conclusions but it appears likely that when the oxidized flavor of milk is just perceptible, the flavor may be due to the additive effects of compounds, with each compound occurring at subthreshold levels, but the combined concentrations equaling or exceeding the threshold of any one in the mixture. Compounds such as the C₇, C₈, and C₉ enals, which are very similar in sensory character, may act in such a way.

The same phenomenon may explain observed differences in flavors of oxidizing milk fats. A case in point is the tallowy (5) and fishy (9) flavors of oxidized milk fats. It is notable that the volatile monocarbonyls from both fats are practically identical. The most striking difference in the data from the two fats is the percentage composition of the carbonyl mixtures. A comparison of the data is given in Table 1.

TABLE 1
Per cent composition of carbonyls from tallowy and fishy flavored milk fats

Carbon-yl	Tal-lowy ^a	Fishy ^b	Car-bon-yl	Tal-lowy ^a	Fishy ^b
C ₇ +C ₈ al	32.47	C ₈ enal	?
C ₈ al	5.04	?	C ₉ enal	0.42
C ₉ al	1.41	C ₁₀ enal	1.46	3
C ₁₀ al	9.96	4	C ₇ dienal		8
C ₁₁ al	9.23	25	C ₈ enal	0.38	4
C ₁₂ al	18.80	20	C ₉ enal	1.91	3
C ₁₃ al	3.28	1	C ₁₀ enal	0.66	2
C ₁₄ al	2.70	5	C ₁₁ enal	4.18	5
C ₁₅ al	1.82	1	C ₁₂ enal	1.23
C ₁₆ one	4.01	C ₁₃ enal	1.08

^a Mean of 16 samples (6).

^b Taken from Forss *et al.* (9).

STUDIES OF DICARBONYL COMPOUNDS IN OXIDIZING MILK LIPIDS

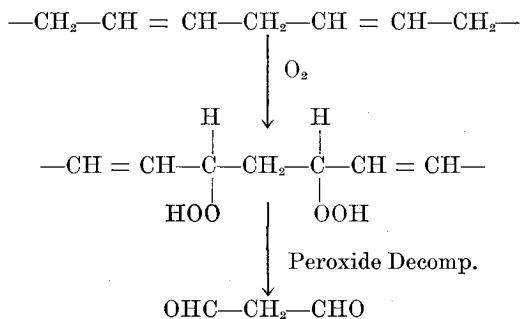
The dicarbonyl compounds of oxidized milk lipids have received limited attention in the studies of lipid oxidation. Most investigators have eliminated these compounds as possible contributors to flavor defects but this is open to question. These compounds comprise a significant portion of the volatiles from oxidized fats and even though they may not contribute directly to the flavor, their reactivity in browning type reaction makes them worthy of consideration. Such reactions undoubtedly occur in oxidizing dairy products where proteins are present. Therefore, possibilities for both flavor and physical defects in dairy products

through lipid browning reactions deserve consideration.

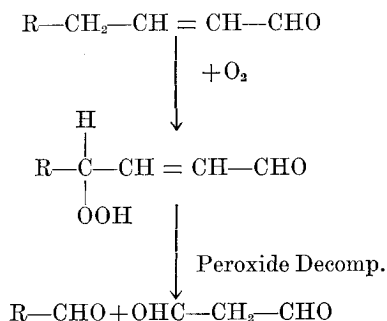
The main reason that dicarbonyls have not been studied more extensively is the difficulty in handling them, either in the free or derivative form. The most common reagent used is 2,4-dinitrophenylhydrazine and mixtures of the derivatives are troublesome to separate because of solubility problems. The use of gas chromatography for separating the free compounds, and the development of suitable paper and column chromatography procedures for the derivatives, will be valuable adjuncts in elucidating the nature of these compounds. Forss (9) has found evidence for a \bar{C}_8 - α -dicarbonyl in oxidized fat by gas chromatography and we have recently fractionated the dicarbonyl mixture of oxidized milk fat into six fractions. The identity of these fractions has not been determined and the mixture obviously is complex.

Most of the work on dicarbonyls has been concerned with the nature of the reactants in the Kreis and TBA tests. Patton and Kurtz (20) obtained evidence that malonaldehyde was the compound responsible for the reaction in oxidized fats. More recently, Sinnhuber *et al.* (25) isolated a TBA pigment from oxidized salmon oil which had properties identical to the TBA pigment of malonaldehyde. The unknown TBA pigment was homogenous and they concluded that malonaldehyde was the compound responsible for the TBA reaction.

The origin of malonaldehyde in oxidizing lipids poses some interesting questions relative to the mechanism of the oxidation reaction. Bernheim *et al.* (1) reported that oxidized oleic acid gave a negative TBA test, linoleic acid only a slight reaction; whereas, linolenic acid gave a strong test. Patton and Kurtz (21) have reported that oxidized 2-enals give the reaction. An explanation of how malonaldehyde can be formed through generally accepted oxidation schemes is still forthcoming. One can only speculate on possible mechanisms. In polyethenoic acids, containing the pentadiene system, diperoxide formation at the double bonds, with a simultaneous shift in double bonds, followed by cleavage of the peroxides could give malonaldehyde.



Diperoxides have not been reported as initial oxidation products in unconjugated fatty acids and since TBA reactants are detected at very early stages of oxidation the above scheme is questionable. In α,β -unsaturated aldehydes, the carbon alpha to the double bond is labile and may be susceptible to oxidative attack. If so, malonaldehyde could arise through the following mechanism.



Quantitative studies of the TBA reactant in fats have shown that the only part of the malonaldehyde exists free in the fat. Patton and Kurtz (20) could remove only part of it by water extraction. Sinnhuber *et al.* (25) removed 2.2% by nitrogen ebullition of salmon oil and Day and Lillard (6) have been able to remove approximately 10% by vacuum steam distillation. Consequently, it can be assumed that most of the malonaldehyde is produced during the reaction by breakdown of a precursor, by dissociation of dimers, or by other mechanisms. It is of particular interest that in running the TBA test, conditions are conducive for peroxide decomposition. It is also notable that Day and Lillard (6) have found the TBA and peroxide tests to be highly correlated, .962. While evidence is still inadequate to justify conclusions, it would appear that the TBA test is an indirect measure of the free malonaldehyde plus the peroxides when used on intact fats. A similar situation may not hold for foods such as whole milk. Peroxide decomposition may enable the highly reactive malonaldehyde to combine with available amino groups to give a Schiff's base. The conditions employed in the TBA test could reverse the reaction and enable measurement of the bound malonaldehyde, provided an Amadori rearrangement has not occurred.

AREAS FOR CONTINUING RESEARCH

If nothing else, this presentation should indicate the need for more research on lipid oxidation. The oxidation reaction in dairy products is very complex and it is influenced by a multitude of factors. The identification of lipid peroxide decomposition products is far

from complete. The effect of metals, light, antioxidants, vitamins, and other factors on the types of oxidation products formed needs more thorough study. In addition, the reaction of secondary products of lipid oxidation with other constituents in dairy products deserves consideration. Browning reactions involving products of lipid oxidation have been reported in dried eggs (17). The reaction appears similar to heat induced browning except that the carbonyl apparently is derived through lipid oxidation. This type of reaction has been largely ignored in dairy products, but it is conceivable that it may be of significance in flavor deterioration.

There is still controversy on the subject of spontaneous oxidation in fluid milks. Whether the cause of the reaction is enzymatic, metal catalyzed, or both is currently controversial. In any case, it is known that the ration of the dairy cow has some influence on the incidence of oxidized flavors in the milk. The incidence of spontaneous oxidation is highest when the animals are on hay and concentrated rations. The lipids in hay and concentrates should oxidize quite readily. Consequently, it is conceivable that dry feeds could contain appreciable amounts of lipid peroxides. The question arises: Is it possible for lipid peroxides of the ration to be consumed by the cow and synthesized into milk lipids? The low Eh of the rumen would be conducive to peroxide reduction, but is the rumen 100% efficient? Apparently, it is not in the case of long chain unsaturated fatty acid synthesis. If this possibility is ruled out, is it possible for lipid peroxides to form in the fatty tissue of the cow and subsequently mobilize for milk fat synthesis? Peroxides have been detected in the depot fat of chickens (3) and swine and rats (17) where their diets contained either oxidized fats or unsaturated fatty acids and were complicated by lack of vitamin E. Theoretically, it would take only one hydroperoxide in the milk lipids to initiate the oxidation reaction. If there is a remote possibility that lipid peroxides can be synthesized, their effect on spontaneous oxidation of fluid milk is obvious.

Many terms have been used by different workers to describe oxidized flavors in dairy products. Such terms as stale, tallowy, oily, metallic, fishy, and cardboard are quite common. Many of these terms may be justified, but it makes communication among investigators practically impossible. Standardization of flavor terminology is urgently needed. Rather than describe a flavor as tallowy it would be more desirable to associate it with a specific compound. Continued research on the identification and the sensory evaluation of compounds responsible for oxidized flavors should give the necessary reference compounds for this purpose.

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