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IRRADIATION COMPOUNDS

Gas Chromatographic and Mass Spectral Identification of Some Volatile Components of Gamma-Irradiated Milk Fat

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Milk fat, sealed in cans under vacuum, was irradiated at 4.5 Mrad with cobalt-60. The small amount of oxygen present in the headspace of the cans was consumed, while H_2, CO, CO_2, and CH_4 were produced. The irradiated fat was analyzed for monocarbonyls by forming their 2,4-dinitrophenylhydrazone derivatives. The volatiles of the irradiated milk fat were obtained by vacuum-steam distillation, and the components in the distillate were extracted with ethyl ether and separated by gas-liquid chromatography (GLC). GLC in conjunction with rapid-scan mass spectrometry was used for identification. n-Alkanes and 1-alkenes from C_5 to C_{17}, some alkadienes, n-alkanals, short chain fatty acids, and certain γ- and δ-lactones were among the compounds identified. Possible mechanisms involved in the formation of these compounds are presented.

VARIOUS changes take place when milk fat is irradiated. In the presence of air or oxygen, hydroperoxides and secondary products of autoxidation are formed. The mechanism for the formation of these products is similar to that of autoxidation (*23, 30*). In the absence of air, hydroperoxides are not formed, but off-flavors are still produced along with carbonyl formation and the destruction of carotenoids (*7, 17*). Day and Papaioannou (*7*) reported that the flavor of irradiated milk fat was

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due to hydrolytic rancidity, oxidative rancidity, and the presence of components with a candle-like odor. It was suggested that carbonyls with medium to long carbon chains were responsible for the candle-like odor. Since gas-liquid chromatography (GLC) of volatiles from irradiated milk fat showed many components other than carbonyls, it was of interest to characterize these compounds to understand the effect of irradiation better.

Experimental Methods

Preparation and Irradiation of Milk Fat. The milk fat was obtained from raw sweet cream prepared from mixed herd milk in the Oregon State University creamery. The milk fat was washed free of phospholipid and clarified by centrifuging at 30,000 × G for 20 minutes. The clear milk fat was then sealed in C-enameled 307 × 409 (No. 2) cans under 29 inches of vacuum. The cans were irradiated with gamma rays from a 3600-curie cobalt-60 source at 0.4 Mrad per hour to a total dose of 4.5 Mrad. Although irradiation was carried out at room temperature, the temperature of the fat rose to approximately 40° C. during the irradiation period. The irradiated fat was stored at −18° C. until analyzed.

Analysis of Headspace Gases. The cans containing irradiated milk fat and

unirradiated control milk fat were warmed to 40° C., and the headspace gases were analyzed using a Fisher Model 25V Gas Partitioner. The GLC columns used were a 30 × $^1/_4$ inch o.d. aluminum column packed with 30% hexamethylphosphoramide (HMPA) on 30- to 60-mesh firebrick, and a 6$^1/_2$-foot × $^1/_4$-inch o.d. aluminum column containing 30- to 60-mesh 13X molecular sieve. A column temperature of 23.5° C. and a helium flow of 80 ml. per minute were employed.

Analysis of Steam Distillate. About 1 kg. of irradiated milk fat was steam-distilled at 40° C. and 1 to 2 mm. of Hg by the method of Day and Lillard (6). About 200 to 300 ml. of distillate was collected in the cold traps, and the distillate was then extracted with peroxide-free diethyl ether, and the extract was concentrated by a fractional distillation column.

The separation and identification of the components in the extract were achieved by GLC combined with fast-scan mass spectrometry of the eluted fractions. A Barber-Colman Series 5000 gas chromatograph equipped with a hydrogen flame detector, and temperature programmer was used. Initially the analyses were carried out on a 24-foot × $^1/_8$-inch o.d. stainless steel column with 20% diethylene glycol succinate (DEGS) on 80- to 100-mesh Celite 545. The column was operated isothermally at 63° C. for 14 minutes, then temperature-programmed at 2° C. per minute to 170° C. and held at 170° C. until the analysis was complete. The high boiling components were separated on a 10-foot × $^1/_8$-inch o.d. column containing the same DEGS packing and operated isothermally at 150° and 175° C. The extract was also analyzed on a nonpolar column, a 12-foot × $^1/_8$-inch o.d. column packed with 20% Apiezon M on 100- to 120-mesh Celite 545. A short,

base-treated precolumn was used to remove the fatty acids present in the sample. The precolumn consisted of a 2-foot × $^1/_8$-inch o.d. stainless steel column containing 20% Apiezon M on 100- to 120-mesh Celite 545 plus 5% by weight of NaOH. The removal of fatty acids was necessary as they tailed and tended to obscure some of the smaller GLC peaks. The combined Apiezon M column and precolumn were operated at 75° C. for 6 minutes, then temperature-programmed at 2° C. per minute to 200° C. and held at that temperature until the analysis was completed. The high boiling components were more effectively resolved on a short 6-foot × $^1/_8$-inch o.d. column containing the 20% Apiezon M packing and operated isothermally at 220° C. GLC relative retention time (t_R/t_R) data were obtained for known compounds under the same conditions.

For the mass spectral analyses, the effluent from the GLC column was split with a Swagelok T-fitting at the end of the column; a portion (about $^1/_{20}$) of the effluent was directed to the flame-ionization detector and the rest to the mass spectrometer inlet. An Atlas-Mat CH-4 single focusing mass spectrometer equipped with the EC-1 gas inlet valve permitting the regulation of the amount of GLC effluent admitted to the ion source was used. The mass spectra were obtained by rapid magnetic scanning of the GLC effluent as each fraction was eluted. The spectra were recorded using a Honeywell Model 1508 Visicorder. The operating parameters for the mass spectrometer were:

Ionizing current	60 μa.
Electron energy	35 e.v.
Analyzer vacuum	2 × 10⁻⁷ mm. Hg
Analyzer vacuum while admitting GLC effluent	4 × 10⁻⁷ mm. Hg
Magnetic scanning speed	5 seconds for m/e 25 to 250

The concentration of several fractions was too low to obtain usable mass spectra with a single GLC run. These minor components were concentrated from several GLC runs by repeatedly trapping them on a 2-foot × $^1/_8$-inch

o.d. column packed with 20% Apiezon M on 100- to 120-mesh Celite 545 attached to the end of the GLC column with a Swagelok T-fitting. About one twentieth of the effluent went to the detector; the remainder was passed to the trapping column. The U-shaped trapping column was cooled by immersing it in a Dewar flask containing a methyl Cellosolve (2-methoxyethanol)–dry ice mixture. The trapping column was connected in front of the main column in the chromatograph oven and the trapped components were analyzed by GLC and mass spectrometry as usual.

Analysis of Monocarbonyls in Steam Distillate. A portion of the concentrated ether extract was mixed with 50 ml. of 5N HCl saturated with 2,4-dinitrophenylhydrazine (DNP-hydrazine) and stirred for 48 hours. The DNP-hydrazones were extracted with ethylene chloride (redistilled and stored over reagent grade anhydrous potassium carbonate). The solvent then was removed under vacuum, and the monocarbonyl derivatives were separated using the alumina column (25). The excess DNP-hydrazine reagent was removed on a Dowex 50W-X4 cation exchange resin column (26).

The monocarbonyls were resolved by liquid-liquid partition chromatography on a nitromethane–hexane–Celite column (Method B) of Day et al. (5) followed by paper chromatography (10). The carbon chain length of each band separated by partition chromatography was determined by comparing the threshold volumes of each band with those of known carbonyl derivatives, and also by the paper chromatographic techniques of Huelin (12) and Klein and DeJong (15). Cochromatography on paper also was used to confirm the carbon chain length of each band. The monocarbonyl derivatives from control milk fat were formed directly on the DNP-hydrazine–Celite–hexane reaction column (27).

Results and Discussion

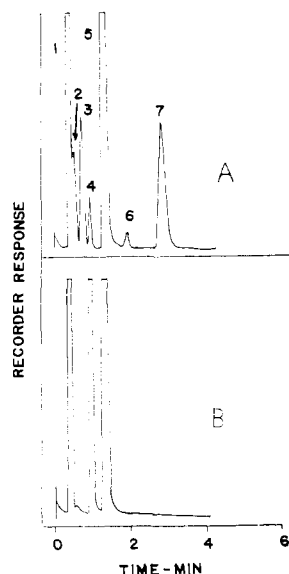**Headspace Gases.** Figure 1, depicting the gas chromatograms obtained for

Figure 1. Analysis of headspace gases

A. Irradiated milk fat (4.5 Mrad)
B. Control milk fat
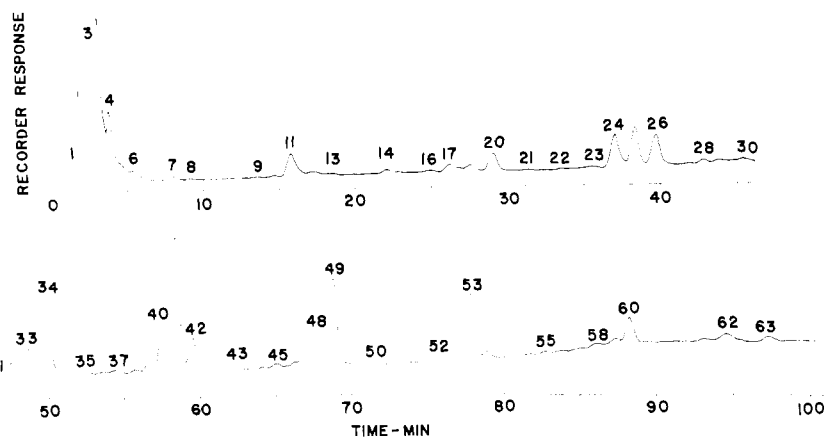(1) N₂ + O₂ composite, (2) CO₂, (3) H₂, (4) O₂, (5) N₂, (6) CH₄, and (7) CO

Figure 2. Gas chromatogram of 4.5 Mrad milk fat steam volatiles

2-foot × $^1/_8$-inch o.d. base precolumn + 12-foot × $^1/_8$-inch o.d. main column packed with 20% Apiezon M on 100- to 120-mesh Celite 545; isothermal at 75° C. for 6 minutes, then temperature-programmed at 2° C. per minute to 200° C.

the headspace gases by Fisher Gas Partitioner, shows that some air was still left in the headspace of the control. Irradiation of the canned milk fat caused oxygen consumption, which is suggestive of oxidative reactions. It is known that irradiation of fats in the presence of oxygen accelerates the oxidation process (23). Figure 1 also shows that CO_2, CO. H_2, and CH_4 were produced.

Possible mechanisms for the formation of H_2 have been discussed in the literature (20, 31). Methane can be formed by end group scission of fatty acid chains (19). The production of CO_2 and CO has been reported in irradiated free fatty acids (4, 28). In fats, these gases may be formed by a free radical mechanism (19).

The headspace of irradiated milk fat probably contains other compounds (especially hydrocarbons) which were not identified by the techniques employed—for example, the irradiation of fatty acids leads to the formation of trace quantities of short-chain hydrocarbons (4, 28). Merritt et al. (22) reported finding the C_3 to C_7 n-alkanes and 1-alkenes in irradiated methyl oleate. Desrosier and Rosenstock (8) have reported that irradiation of oxygen-free fats produces primarily gaseous hydrogen, small amounts of methane, and still smaller traces of alkanes up to butane. In the light of these reports, it can be safely stated that the headspace of irradiated milk fat may also contain other short chain hydrocarbons.

GLC and Mass Spectral Identification of Components in Steam Distillate. Figure 2 is a gas chromatogram showing the separation of the volatile components in the steam distillate of irradiated milk fat. This chromatogram does not show peaks for the fatty acids, because it was obtained on the Apiezon M column with the base-treated precolumn. The identities of the GLC fractions with the supporting data are given in Table I. The t_R/t_R data along with mass spectral identification constitute sufficient evidence for the positive identification of many components. Further confirmation of the identifications was obtained by the t_R/t_R data and mass spectral analyses using the DEGS column. Because of the excessive bleed from the DEGS column at high temperatures, the mass spectral identification in some cases was only tentative. The high boiling components were analyzed by GLC and mass spectrometry on short Apiezon M and DEGS columns. The presence of fatty acids was also confirmed by the disappearance of peaks when the base-treated precolumn was used. Table II lists the high boiling compounds identified by GLC and mass spectral analysis.

Figure 3 illustrates the type of mass spectral data obtained in this study. Spectra A, B, and C of Figure 3 cor-

Table I. GLC and Mass Spectral Identification of Some Components in the Steam Distillate of Gamma-Irradiated Milk Fat

GLC Peak No.	Identity	t_R/t_R[a,b] Irradiated Fat	t_R/t_R Authentic Compound	Confirmed Column GLC[c]	Mass Spectral Identification	Ref. for M.S.
1	1-Pentene	0.042	...	Yes	Positive	(2)
1	n-Pentane	0.042	...	Yes	Positive	(2)
3	Diethyl ether (solvent)	0.050	0.050	Yes	Positive	(2)
4	Ethyl acetate	0.093	0.112	Yes	Positive	(2)
6	Chloroform	0.135	0.152	...	Positive	(2)
..	Benzene	...	0.230	Yes	Positive	(1)
..	n-Pentanal	...	0.217	Yes	Positive	(1, 11)
7	1-Heptene	0.204	0.248	Yes	Positive	(1, 2)
8	n-Heptane	0.232	0.272	Yes	Positive	(1, 2)
10	2-Hexanone	0.369	0.416	...	Tentative	(2)
11	n-Hexanal	0.396	0.411	Yes	Positive	(2, 11)
12	1-Octene	0.435	0.451	Yes	Positive	(1)
13	n-Octane	0.468	0.484	Yes	Positive	(1)
14	4-Heptanone	0.556	0.589	...	Tentative	(2)
16	2-Heptanone	0.619	0.652	...	Positive	(2)
17	n-Heptanal	0.659	0.661	Yes	Positive	(11)
18	Methyl hexanoate	0.675	0.692	...	Tentative	(2)
19	1-Nonene	0.694	0.703	Yes	Positive	(1)
20	n-Nonane	0.730	0.738	Yes	Positive	(1)
23	2-Methylnonane	0.889	...	...	Tentative	(1)
24	n-Octanal	0.931	0.931	Yes	Positive	(11)
24	1,?-Decadiene	0.931	...	...	Tentative	...
25	1-Decene	0.964	0.967	Yes	Positive	(1)
26	n-Decane	1.000	1.000	Yes	Positive	(1)
30	o-Dichlorobenzene	1.145	...	...	Positive	(2)
31	2-Methyldecane	1.175	...	...	Tentative	(1)
32	n-Nonanal	1.194	1.197	Yes	Positive	(1, 11)
32	1,?-Undecadiene	1.194	...	...	Tentative	...
33	1-Undecene	1.224	1.224	Yes	Positive	(1)
34	n-Undecane	1.256	1.256	Yes	Positive	(1)
35	Branched undecene	1.319	...	...	Tentative	...
37	Branched undecane	1.373	...	...	Tentative	...
39	2-Methylundecene	1.427	...	...	Tentative	...
40	n-Decanal	1.450	1.455	Yes	Positive	(11)
40	1,?-Dodecadiene	1.450	...	...	Tentative	...
41	1-Dodecene	1.472	1.478	Yes	Positive	(1)
42	n-Dodecane	1.500	1.506	Yes	Positive	(1)
43	γ-Octalactone[d]	1.569	1.615	...	...	...
46	δ-Octalactone[d]	1.667	1.732	...	...	...
47	n-Undecanal	1.690	1.701	Yes	Positive	(11)
48	1-Tridecene	1.704	1.711	Yes	Positive	(1)
49	n-Tridecane	1.732	1.740	Yes	Positive	(1)
53	n-Dodecanal	1.958	1.931	Yes	Tentative	(11)
53	1-Tetradecene	1.958	1.931	Yes	Positive	(1)
54	n-Tetradecane	1.988	...	Yes	Positive	(1)
59	1-Pentadecene	2.198	2.148	Yes	Positive	(1)
60	n-Pentadecane	2.222	2.173	Yes	Positive	(1)

[a] t_R/t_R = relative retention time calculated on basis t_R/t_R of n-decane = 1.000.
[b] (1) 2-foot × $^1/_8$-inch o.d. stainless steel precolumn packed with 20% Apiezon M on 100–120-mesh Celite 545 coated with 5% by weight of NaOH (2) 12-foot × $^1/_8$-inch o.d. stainless steel main column packed with 20% Apiezon M on 100–120-mesh Celite 545. GLC conditions: temperature 70° C. for 6 minutes then programmed at 2° per minute to 220° C. and held until end of analysis. Carrier gas (He) flow 20 ml. per minute.
[c] 24-foot × $^1/_8$-inch o.d. stainless steel column packed with 20% diethylene glycol succinate (DEGS) on 80–100-mesh Celite 545.
[d] Only tentative identification on basis of t_R/t_R and odor characteristics of peaks.

Table II. High Boiling Compounds Identified in Steam Distillate from Gamma-Irradiated Milk Fat

Compound	t_R/t_R Confirmed[a] on Apiezon M column	DEGS column	Mass Spectral Identification	Ref. for M.S.
n-Hexadecane	Yes	Yes	Positive	(1)
1-Hexadecene	Yes	Yes	Positive	(1)
1,?-Hexadecadiene	...	...	Tentative	...
n-Heptadecane	Yes	Yes	Positive	(1)
1-Heptadecene	Yes	Yes	Positive	(1)
1,-?Heptadecadiene	...	...	Tentative	...
γ-Decalactone	Yes	Yes	Positive	(21)
δ-Decalactone	Yes	Yes	Positive	(21)
δ-Undecalactone	Yes	Yes	...	...
δ-Dodecalactone	Yes	...	...	...
Butyric acid	Yes	Yes	Positive	(2)
Hexanoic acid	Yes	Yes	Positive	(2)
Octanoic acid	Yes	Yes	Positive	(2)
Decanoic acid	Yes	Yes	...	...

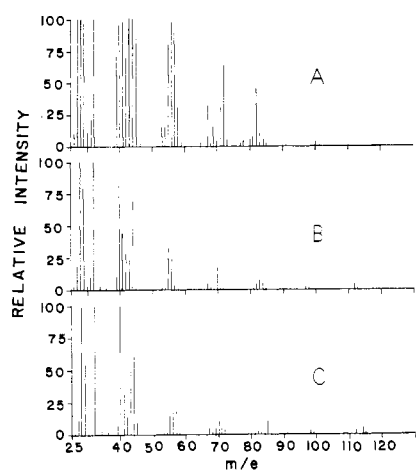[a] t_R/t_R were confirmed by cochromatographic techniques.

Figure 3. Mass spectral charts for chromatographic fractions

A. Chromatographic peak 11 in Figure 2
B. Peak 12
C. Peak 13

respond, respectively, to GLC peaks 11, 12, and 13 of Figure 2. Structures were assigned to the GLC fractions by noting the parent ion (molecular weight) and studying the fragmentation pattern in comparison to the mass spectra of known compounds as reported in the literature. In spectrum A the ions at m/e 28, 29, 32, 40, and 44 are mainly due to background, and are caused by traces of air present in the mass spectrometer and also by impurities in the helium carrier gas. The compound representing spectrum A has a parent ion at m/e 100 and a base peak at m/e 43. The fragment ions at m/e 82 $(P - 18)$, 72 $(P - 28)$, and 56 $(P - 44)$ suggest that the compound may be an aldehyde (29). This evidence indicates that spectrum A represents hexanal. Confirmation that compound A is hexanal was obtained by comparing the spectrum A with those published in the ASTM tables (2) and in the study by Gilpin and McLafferty (11). The mass spectral evidence, along with retention data (Table I), is sufficient for the positive identification of spectrum A as that of hexanal. Similarly, mass spectra B and C represent 1-octene and n-octane, respectively.

In the mass spectral examination of aldehydes, a marked tendency for aldehydes of six carbons or more to cyclize to aromatic compounds was noted if the inlet of the mass spectrometer was at about 205° C. or higher. The appearance of a fragment at m/e 78 in the hexanal is being used in our laboratory as a test compound to indicate this type of thermal breakdown in the GLC inlet system to the mass spectrometer.

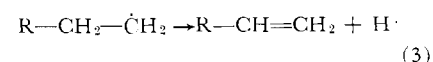Some of the GLC components could not be identified by the mass spectral techniques described above. For example, when authentic C_{10}, C_{11}, and

C_{12} γ- and δ-lactones were analyzed using the Apiezon M column (without base precolumn), only δ-decalactone gave a mass spectrum comparable to that reported by McFadden $et\ al.$ (21). Earlier, however, γ- and δ-decalactones were positively identified using the DEGS column, and strong mass spectra of authentic lactones were obtained using GLC and mass spectrometry. No suitable explanation can be given for these observations. The lactones have been reported to be present in heated milk fat $(3, 13)$. The δ-octa-, δ-deca-, and δ-dodecalactones were present in the control milk fat. However, no γ-lactones could be identified in the control, and irradiation of the fat increased the concentration of δ-lactones. The compounds identified in the control milk fat steam distillate are shown in Table III. The monocarbonyls present are shown in Table IV.

The major constituents of the steam distillate from irradiated milk fat are saturated and unsaturated hydrocarbons. The complete series of n-alkanes and 1-alkenes from C_5 to C_{17} in chain length, and some alkadienes were identified. The position of the double bonds in alkadienes could not be positively ascertained from the mass spectra. Evidence was obtained for several branched-chain hydrocarbons, but their identification was not achieved. The finding of a series of saturated and unsaturated hydrocarbons in irradiated

milk fat is not surprising as Merritt $et\ al.$ (22) reported the presence of the C_1 to C_7 n-alkanes and 1-alkenes in irradiated methyl oleate and beef. Burton (4) and Sheppard and Burton (28) reported that fatty acids when irradiated lose carbon dioxide to yield a hydrocarbon with one less carbon in the alkyl chain. A similar mechanism may also be responsible for hydrocarbon formation in irradiated lipid. The alkyl free radical obtained from fat could form hydrocarbons:

$$R—CH_2—C—O\cdot \rightarrow R—\dot{C}H_2 + CO_2$$
$$\underset{O}{|}$$
$$\qquad\qquad\qquad (1)$$

$$R—CH_2 + XH \rightarrow R—CH_3 + X\cdot \qquad (2)$$
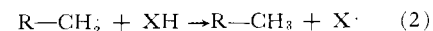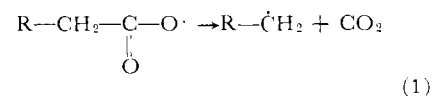
1-Alkenes may be formed as follows:

$$R—CH_2—\dot{C}H_2 \rightarrow R—CH{=}CH_2 + H\cdot$$
$$\qquad\qquad\qquad (3)$$

The short-chain hydrocarbons could result from chain scission reactions. The branched-chain compounds could be formed also as a result of irradiation. Two alkyl free radicals, or an alkyl and a methyl free radical, may combine to form a branched-chain compound.

A series of saturated aldehydes from C_5 through C_{12} was identified by GLC and mass spectrometry. However, the steam distillate from irradiated milk fat contains many more monocarbonyls as determined by column chromatography of their DNP-hydrazone derivatives

Table III. Components Identified in Steam Distillate of Control Milk Fat

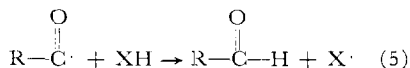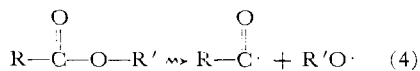| | t_R/t_R Confirmed[a] on | | | |
| | Apiezon M | DEGS | Mass Spectral | Ref. for |
Compound	column	column	Identification	M.S.
Ethyl ether (solvent)	Yes	Yes	Positive	(2)
Ethyl acetate	Yes	Yes	Positive	(2)
Chloroform	Yes	Yes	Positive	(2)
Benzene	Yes	Yes	Positive	(1)
Toluene	Yes	. . .	Positive	(1)
Ethylbenzene	. . .	. . .	Tentative	(1)
2-Heptanone	Yes	Yes	Tentative	(2)
Propylbenzene	. . .	. . .	Tentative	(1)
o-Dichlorobenzene	Yes	. . .	Positive	(2)
δ-Octalactone	Yes	Yes	. . .	. . .
δ-Decalactone	Yes	Yes	. . .	. . .
δ-Dodecalactone	Yes	Yes	. . .	. . .
Butyric acid[b]	Yes	Yes	. . .	. . .
Hexanoic acid[b]	Yes	Yes	. . .	. . .
Octanoic acid[b]	Yes	Yes	. . .	. . .
Decanoic acid[b]	Yes	Yes	. . .	. . .

[a] By cochromatography.
[b] Confirmed by their reaction with base precolumn.

Table IV. Monocarbonyls in Gamma-Irradiated Milk Fat

Sample	n-Alkanals	Alk-2-ones	Alk-2-enals
Irradiated sample (4.5 Mrad)	C_1, C_2, C_3, C_4 iso-C_4, C_5, iso-C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}	C_3, C_6-(trace) C_8(trace), C_9, C_{15}	C_5(trace), C_6(trace) C_9, C_{12}
Control	C_9, C_{10}, C_{12} C_{16}	C_3, C_6(trace) C_7(trace) C_{13}, C_{15}	

(Table IV). These results confirm earlier reports (7, 17) that mono-carbonyls are formed upon irradiation of fats under vacuum. Day and Papaioannou (7) obtained evidence for alk-2-enals and alk-2,4-dienals in irradiated milk fat. In the present study, traces of the C_5, C_6, C_9, and C_{12} alk-2-enals but no alk-2,4-dienals were identified. Qualitatively, the methyl ketones did not show a significant change upon irradiation (Table IV); however they are present in much larger quantities in irradiated milk fat. The origin of methyl ketones and long chain aldehydes has been discussed by Day and Papaioannou (7). They suggested that hydrolytic cleavage of ester and enol-ether linkages were responsible for these compounds. Work in our laboratory (14) has demonstrated that hydrolysis as well as cleavage of ester linkages occurs with irradiation. Langler and Day (18) have suggested that methyl ketones are formed in heated milk fat as a result of hydrolysis. Also, long chain aldehydes occur in milk fat as bound aldehydes (9, 24). Thus, irradiation-induced hydrolysis of ester and enol-ether linkages is probably a likely mechanism accounting for the production of ketones and long chain aldehydes in irradiated milk fat. The formation of short chain aldehydes may also be explained by a similar mechanism.

$$R—\overset{O}{\overset{\|}{C}}—O—R' \rightsquigarrow R—\overset{O}{\overset{\|}{C}}\cdot + R'O\cdot \quad (4)$$

$$R—\overset{O}{\overset{\|}{C}}\cdot + XH \rightarrow R—\overset{O}{\overset{\|}{C}}—H + X\cdot \quad (5)$$

The finding of C_6 and C_8 methyl ketones is difficult to explain. The presence of these and many other even- and odd-numbered saturated and unsaturated ketones, and the probable mechanism of their formation in irradiated fats have been reported by Kohn (16).

Compounds such as ethyl acetate, benzene, chloroform, and dichloro-benzene were also detected in the control sample (Table III). Chloroform has been reported in milk fat by Wong and Patton (32). It was suggested that this compound arises from pesticide residues in milk. Dichlorobenzene, which has not been reported previously in milk, could also have its origin in pesticides.

Four components listed in Table I (peak numbers 31, 35, 37, and 39) had a characteristic candle-like odor. These compounds were present in relatively small quantities (Figure 2). The exact nature of these components could not be determined. Work is in progress in our laboratory to characterize and identify these components further.

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ANALYTICAL METHOD

Determination of the Pungent Constituents of *Piper nigrum*

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SINCE spice oleoresins have come into the market in several forms, the need for good analytical methods for determination of the active constituents has grown. This holds especially true for pepper. For piperine (1-piperoylpiperidine), the following methods have been described in the literature:

A. Kjeldahl determination of nitrogen (1, 6, 7).
B. Polarographic determination (6, 11).
C. Iodometric procedure (5).
D. Spectrophotometric method (ultraviolet-absorption) (6, 7).
E. Colorimetric method using chromotropic acid (2, 7, 13).

F. Colorimetric method using nitric acid (10).
G. Colorimetric method using sulfuric acid and an aromatic aldehyde (8).
H. Colorimetric method using phosphoric acid (9).
I. Method presented in this paper.
Figure 1 shows the part of the piperine