

Vitamin A Oxidation Products Responsible for Haylike Flavor Production in Nonfat Dry Milk

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Vitamin A (VA) fortification of nonfat dry milk is frequently responsible for haylike flavor development. Sensory analysis showed that volatile, steam-distilled oxidation products of VA palmitate had a strong, typical haylike flavor. Nineteen compounds were identified by GC/mass spectrometric analysis of steam-distilled products derived from thermal (100–110 and 150 °C) and photosensitized (at room temperature with rose bengal as a sensitizer) oxidation products of VA palmitate. The major compound from thermal oxidation included ionene, β -cyclocitral, β -ionone, geronic acid, 1,2-epoxy- β -ionone, *p*-methylacetophenone, and dihydroactinidiolide. Both ionene and *p*-methylacetophenone, which are probably intermolecular aldol condensation products and which have not been previously reported in the literature as flavor compounds, were formed by thermal oxidation of VA palmitate. It was found that both pure β -ionone and dihydroactinidiolide had a haylike flavor.

When butterfat is removed from milk in the production of skim milk, the fat-soluble vitamins, including vitamin A (VA), are also removed. Since the inception of mandatory VA fortification of skim milk and 2% fat milk, the frequent incidence of haylike flavor has been reported (Bauernfeind and Allen, 1963; Suyama et al., 1981; Thomas et al., 1965), especially when liquid milk was pouch packed or nonfat dry milk was stored. There have been many papers on VA oxidation in milk reporting the change in VA activity during storage (Anantkrishnan and Conochie, 1958; Bauernfeind et al., 1953; Olson et al., 1949; Sattar and deMan, 1975; Thompson and Erdody, 1974; Wilkinson and Conochie, 1958; Wodsak, 1953). Weckel and Chicoye (1954) examined haylike flavor produced in VA fortified milk and reported some preventive measures for haylike flavor development but did not identify the causative compounds. Much effort has been devoted to analysis of the compounds formed by photooxidation of carotenoids as these degradation products were isolated from tea and tobacco (Aisaka et al., 1978; Bricout et al., 1967; Buttery and Kamm, 1980; Dalle et al., 1969; Demole and Berthet, 1972; Ina et al., 1968; Isoe et al., 1969, 1972; Wahlberg et al., 1977). Only a few papers about volatile oxidation products of VA have been published (Isoe et al., 1972; Lerner et al., 1970).

The objectives of this report were to measure the heat stability of pure VA palmitate in air and to identify the volatile VA oxidative decomposition products which were reasonable for haylike flavor and to elucidate the effect of fluorescent light on the volatiles formed by oxidation of VA palmitate. For comparative purpose, the volatiles formed by photosensitized oxidation of VA palmitate were also investigated. Using gas-liquid chromatography/mass spectrometry, we were able to identify volatile compounds from their mass spectra and their respective retention times. The reference samples were either commercially available or synthesized. A scheme for the formation of volatiles by the oxidation of VA palmitate was discussed.

EXPERIMENTAL SECTION

Materials. Vitamin A (VA) palmitate (Sigma Chemicals, St. Louis, MO) was purified by recrystallization from acetone 3 times. All solvents used for extraction were glass

distilled, and the solvents used for high-performance liquid chromatography were UV spectroscopic grade.

Oxidation of VA Palmitate. Oxidation of VA palmitate was carried out under the following conditions. Method 1: VA palmitate (20 g) without solvent and sensitizer placed in a 500-mL three-neck distilling flask with condenser attached was oxidized for 5 h under an oxygen stream at a speed of 30 mL/min, at 100–110 °C. A 20-W fluorescent lamp (750 lm with emission peaks at 575 nm and minor at 550 and 435 nm) was placed at a distance of 1 cm from the reaction vessel. Method 2: The reaction conditions were the same as described in method 1, except that the oxidation was carried out in the dark. Method 3: Oxidation was carried out at 150 °C for 2 h in the dark. Other reaction conditions were the same as in method 1. Method 4: A solution of 10 g of VA palmitate in 200 mL of absolute ethanol was placed in a 500-mL round-bottom flask and then 0.2 g of rose bengal, as a sensitizer, was added to the solution. The photosensitized oxidation was carried out at room temperature for 40 h by illuminating with a 20-W fluorescent lamp at a distance of 1 cm from the reaction vessel with oxygen bubbling at a rate of 30–40 mL/min. After the reaction, the solvent was removed by using a rotary evaporator.

The volatiles formed by oxidation of VA palmitate were collected by steam distillation. The volatiles in the distillate were extracted with ethyl ether.

In another experiment, VA palmitate 10 mg was spread over a 9-cm diameter glass Petri dish and was heated at 80, 90, and 100 °C. At specified time intervals, samples were removed, extracted with *n*-hexane and applied to a high-performance liquid chromatograph.

High-Performance Liquid Chromatography (HPLC). Analysis of VA palmitate and its oxidation products was carried out on a Spectra Physics (Santa Clara, CA) Model SP 8700 HPLC attached to an SP 8400 UV-vis detector and an SP 4100 computing integrator. The column was a 10 × 0.3 cm Brownlee Laboratories (Santa Clara, CA) Silica Spheri-5 column. The mobile phase was a mixture of *n*-hexane-ethyl ether (80:20 v/v) and run at a flow rate of 1 mL/min. The detector wavelength was set at 325 nm.

For quantitation of VA palmitate by HPLC, an internal standard (acetone-dinitrophenylhydrazone) was used. The peak areas of VA palmitate and the internal standard were calculated by computer integrator.

Sensory Analysis. Four milligrams of the volatile oxidation products of VA palmitate oxidized by the me-

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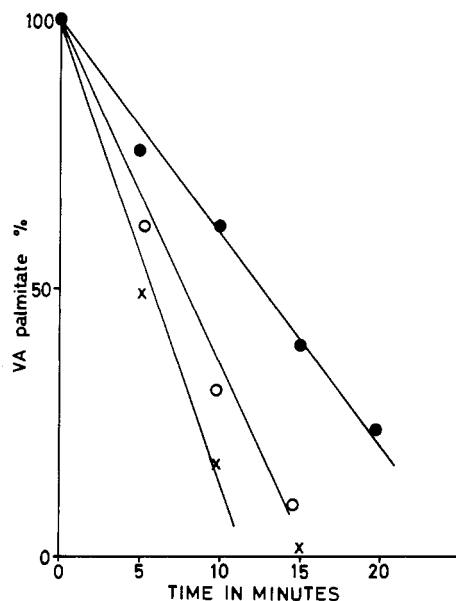


Figure 1. Relationships between quantity (percent) of VA palmitate and heating time. Vitamin A was determined by HPLC. (●) Heating at 80 °C; (○) heating at 90 °C; (X) heating at 100 °C.

thod described above was dissolved into 2 mL of ethyl alcohol and then mixed with 100 mL of skim milk which gave 4 mg of volatiles in 100 mL of fresh skim milk. From this original skim milk, diluted samples ranging from 10 μ g to 0.2 mg of volatiles per 100 mL of skim milk were prepared, and sensory evaluation was carried out by a group of four trained panelists. As a comparison, skim milk reconstituted from a commercially fortified skim milk powder exhibiting distinct haylike flavor was also tasted.

Gas-Liquid Chromatography (GC). Analytical GC was performed on a Hewlett-Packard (Palo Alto, CA) 5700A instrument with FID detector and a 10% SILAR 9-CP column (1.8 mm \times 6 ft), temperature programmed from 60 to 250 °C at 10 °C/min. The relative concentration of each volatile was calculated from GC peak areas.

GC/Mass Spectrometry (GC/MS). Two systems of instruments were used for GC/MS analysis: System 1 was a Finnigan (Sunny Vale, CA) 4001 operated at 70 eV, using the same column and GC operation conditions as described above. System 2 consisted of a Varian (Palo Alto, CA) MAT-111 operated at 70 eV, in which the original GC had been replaced by a Hewlett-Packard 5700A unit attached to a 5% OV-17 on Chromosorb W column (1.8 mm \times 6 ft) and temperature programmed from 80 to 250 °C at 5 °C/min. The second MS was connected in line to a Varian 620-L computer data system. The program used was developed in the Faculty of Pharmaceutical Sciences at the University of British Columbia.

Others. ^1H NMR were determined on a Varian T-60, with tetramethylsilane as an internal standard in deuteriochloroform as a solvent.

Preparative TLC of volatile oxidation products from method 3 was run on an aluminum sheet precoated with silica gel 60 F₂₄₅ (E. Merck, West Germany) by using a mixture of *n*-hexane-ethyl ether (90:10 v/v) as a solvent system. The bands separated were extracted with ethyl ether, and then these extracts were applied to GC/MS system 2.

RESULTS AND DISCUSSION

Stability of Pure VA Palmitate. Figure 1 shows the relationships between heating time and quantity of VA palmitate after heating in air at 80, 90, and 100 °C in the

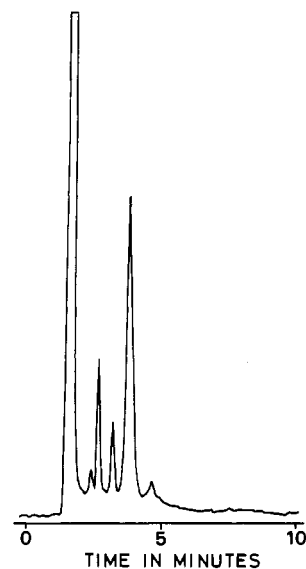


Figure 2. Typical HPLC pattern of oxidized VA palmitate heated at 80 °C for 15 min.

dark. The loss of VA palmitate could be described by zero-order kinetics, which is indicative of an autoxidation mechanism. The degradation rate of VA palmitate was observed to be a function of the temperature.

When unheated, purified VA palmitate was injected into the HPLC, only one main peak (retention time, t_R , of 1.68 min) appeared. Figure 2 shows a HPLC pattern of VA palmitate heated at 80 °C for 15 min. When compared to the pattern of the unheated sample, the VA palmitate peak decreased by about 60% and two other major peaks ($t_R = 2.49$ and 3.80 min) and several minor peaks appeared. It was observed that almost all of the peaks produced on heating in air decreased when a *n*-hexane solution of the heated VA palmitate was vigorously shaken with aqueous 5% sodium bisulfite as a reducing agent. It was assumed that the compounds corresponding to the major peaks were hydroperoxides. However, in this experiment, further identification of these oxidation products was not attempted. In addition, after heating VA palmitate at 100 °C, for 30 min, all peaks, including VA palmitate itself, disappeared while the haylike flavor increased. It is suggested that the additional peaks produced after heating at 80 °C for 15 min are important intermediates in the formation of volatile haylike flavor compounds as a result of degradation of VA palmitate.

Sensory Analysis. The steam-distilled volatiles which were obtained by oxidation method 2 had a strong haylike flavor. When diluted with fresh unfortified skim milk to a concentration of 100 μ g/100 mL of milk, the flavor of this sample was typical of reconstituted commercially fortified skim milk powder exhibiting haylike flavor. The flavor threshold values for the panel ranged from 10 to 50 μ g/100 mL of skim milk.

GC of Volatile Oxidation Products of VA Palmitate. GC patterns of major volatile compounds produced by oxidizing VA palmitate as described in methods 2 and 4 under Experimental Section are shown in Figure 3. GC patterns of major volatiles produced by thermal oxidation methods (methods 1-3) are similar. However, there are differences between the GC patterns of the volatiles from photosensitized oxidation (method 4) and those from thermal oxidation, and the flavor of volatiles obtained from photosensitized oxidation is milder than that of thermal oxidation.

The major volatile compounds were identified by GC/MS using system 1, and the quantitative data and

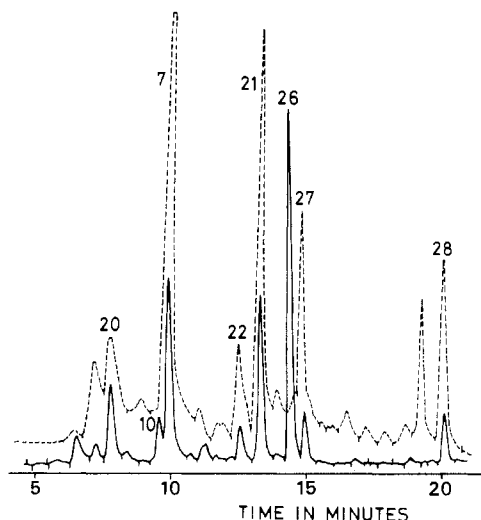


Figure 3. GC patterns of volatile compounds produced by thermal oxidation of VA palmitate as described by method 2 (—) and by photosensitized oxidation as described by method 4 (---).

Table I. Comparative Amounts of Volatile Oxidation Products of VA Palmitate Oxidized under Various Conditions As Determined by GC

peak no.	compounds	method of oxidation ^a			
		method 1	method 2	method 3	method 4
7	β -cyclocitral	149	132	156	288
10	ionene	19	20	38	
21	β -ionone	100 ^b	100 ^b	100 ^b	100 ^b
26	geronic acid	152	178	202	
27	1,2-epoxy- β -ionone	41	31	20	53
28	dihydroactinidiolide	30	27	29	49

^a Experimental details were given in the text. ^b The peak area of β -ionone was assigned a value of 100.

constituents produced by interaction among the various oxidation products are given in Table I. The relative concentrations of volatiles formed by each of the thermal oxidation methods are similar and the predominant volatiles include ionene, β -cyclocitral, β -ionone, geronic acid, dihydroactinidiolide, and unknown compounds ($M^+ = 184$, peak no. 20, and $M^+ = 156$, peak no. 22). In photosensitized oxidation, β -cyclocitral, β -ionone, 1,2-epoxy- β -ionone, and dihydroactinidiolide were produced in larger quantities and ionene and geronic acid were undetectable. The most remarkable effect of increasing temperature on volatiles was the significant reduction in the amount of β -ionone and increase in the amount of ionene and geronic acid. Most of the volatiles, however, were produced in greater amounts when VA palmitate was heated at higher temperatures (150 °C in method 3 rather than 100–110 °C in method 2).

GC/MS of Volatiles after Separation by TLC. In order to obtain more information about volatile degradation products of VA palmitate, the volatiles produced from oxidation method 3 were separated by preparative TLC into three fractions as shown in Figure 4. These fractions were the least polar fraction (fraction I), slightly polar fraction (fraction II), and strongly polar fraction (fraction III). Before GC/MS analysis, aromagrams were taken by sniffing the volatiles to detect which fractions were more responsible for haylike flavor. Fraction I had a very strong, medicinal haylike flavor. Fraction II had a mild, typical haylike flavor, whereas fraction III had a mild, sweet tealike aroma. Figures 5, 6, and 7 show the GC patterns

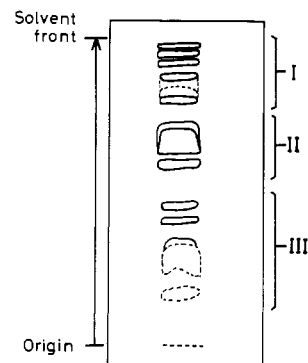


Figure 4. TLC separation of volatile compounds of VA palmitate oxidized by method 3. Solvent: *n*-hexane-ethyl ether (90:10 v/v). Visualization: sprayed with 10% H_2SO_4 and heated at 100 °C for 5 min. Fractions I–III were applied to GC/MS.

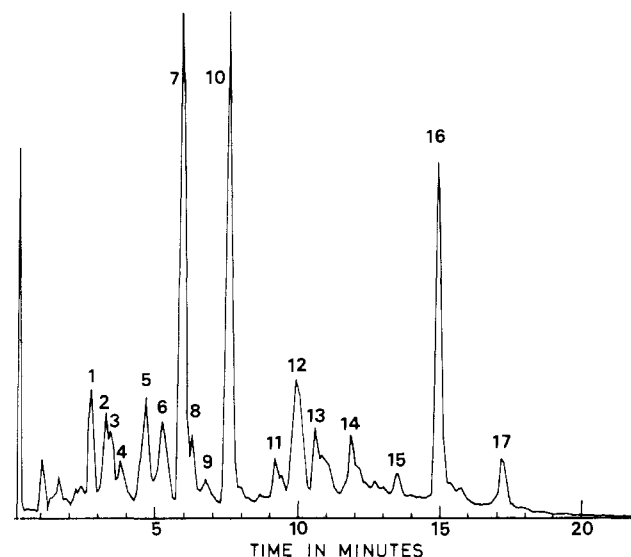


Figure 5. GC pattern of volatile compounds in fraction I from preliminary TLC separation of VA palmitate oxidized by method 3. The GC/MS system used included a 5% OV-17 column (system 2).

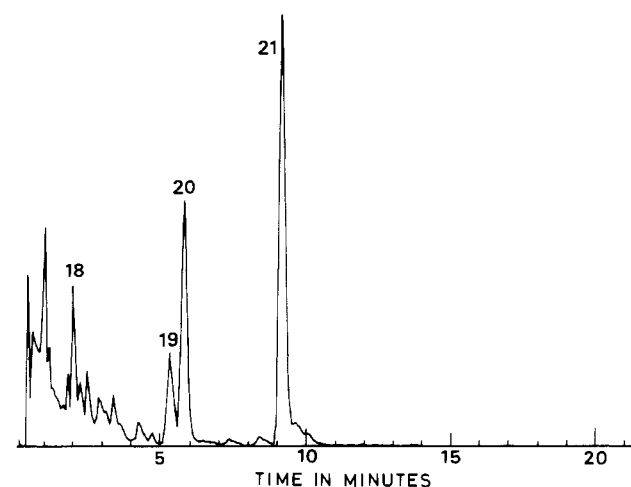


Figure 6. GC pattern of volatile compounds in fraction II from preliminary TLC separation of VA palmitate oxidized by method 3. GS/MS system 2 was used.

of fractions I, II, and III, respectively. The components identified are presented in Table II and their peak numbers correspond to the numbers in Figures 3 and 5–7. In this paper most identifications were done by mass spectral analysis of GC/MS patterns. The obtained mass spectra

Table II. Volatile Compounds Identified from Thermal Oxidation of VA Palmitate by Method 3^a

peak no.	compounds	M ⁺	formula	identification ^b
1	2,2,6-trimethylcyclohexanone	140	C ₉ H ₁₆ O	MS, <i>t_R</i> , <i>s</i>
2	3,5,5-trimethyl-2-cyclohexenone	138	C ₉ H ₁₄ O	MS, T
3	safranal	150	C ₁₀ H ₁₄ O	MS, <i>t_R</i>
4	unknown (methyl ester)	?		
5	unknown	168	C ₁₀ H ₁₆ O ₂ ^c	MS, T
6	3,5,5-trimethylcyclohexanone	140	C ₉ H ₁₆ O	MS, T
7	β-cyclocitral	152	C ₁₀ H ₁₆ O	MS, <i>t_R</i> , <i>s</i>
8	unknown	166	C ₁₀ H ₁₄ O ₂ ^c	MS, T
9	unknown (methyl ester)	?		
10	ionene	174	C ₁₃ H ₁₈	MS, <i>t_R</i> , <i>s</i>
11	unknown	208	C ₁₃ H ₂₀ O ₂ ^c	MS, T
12	2,6-bis(1,1-dimethylethyl)-4-methylphenol	220	C ₁₅ H ₂₄ O	MS, <i>t_R</i> , <i>s</i>
13	unknown	218	C ₁₅ H ₂₂ O ^c	MS, T
14	myristic acid methyl ester	242	C ₁₅ H ₃₀ O ₂	MS, <i>t_R</i> , <i>s</i>
15	unknown	218	C ₁₅ H ₂₂ O ^c	MS, T
16	palmitic acid methyl ester	270	C ₁₇ H ₃₄ O ₂	MS, <i>t_R</i> , <i>s</i>
17	stearic acid methyl ester	298	C ₁₉ H ₃₈ O ₂	MS, <i>t_R</i> , <i>s</i>
18	unknown	126	?	MS, T
19	<i>p</i> -methylacetophenone	134	C ₉ H ₁₀ O	MS, <i>t_R</i> , <i>s</i>
20	unknown	184	C ₁₀ H ₁₆ O ₃	MS, T
21	β-ionone	192	C ₁₃ H ₂₀ O	MS, <i>t_R</i> , <i>s</i>
22	unknown	156	C ₁₀ H ₁₂ O ^c	MS, T
23	unknown	154	C ₉ H ₁₄ O ^c	MS, T
24	unknown	?		
25	unknown	?		
26	geronic acid	172	C ₉ H ₁₆ O ₃	MS, T, ¹ H NMR
27	1,2-epoxy-β-ionone	208	C ₁₃ H ₂₀ O ₂	MS, T
28	dihydroactinidiolide	180	C ₁₁ H ₁₆ O ₂	MS, <i>t_R</i> , <i>s</i>

^a Experimental details were given in the text. ^b MS = interpretation of mass spectra. *t_R* = retention time from GC coinjections of standards. *s* = direct comparison of mass spectra with standards. ¹H NMR = nuclear magnetic resonance identification. T = tentative identification. ^c The most reasonable formula postulated by M⁺ values and the structure of VA palmitate.

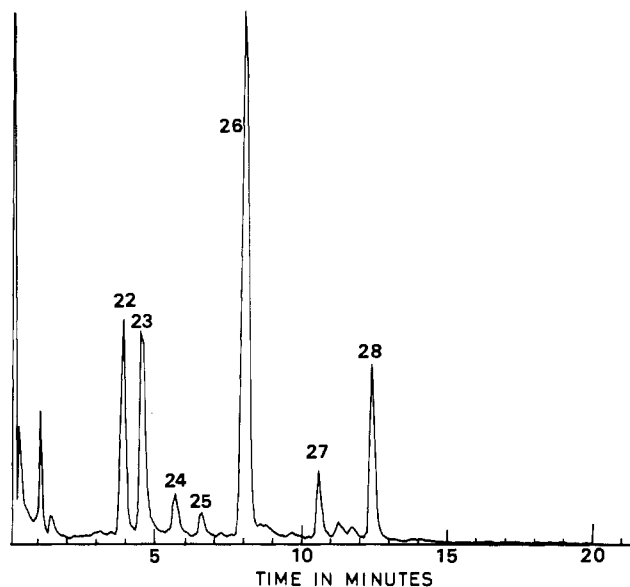


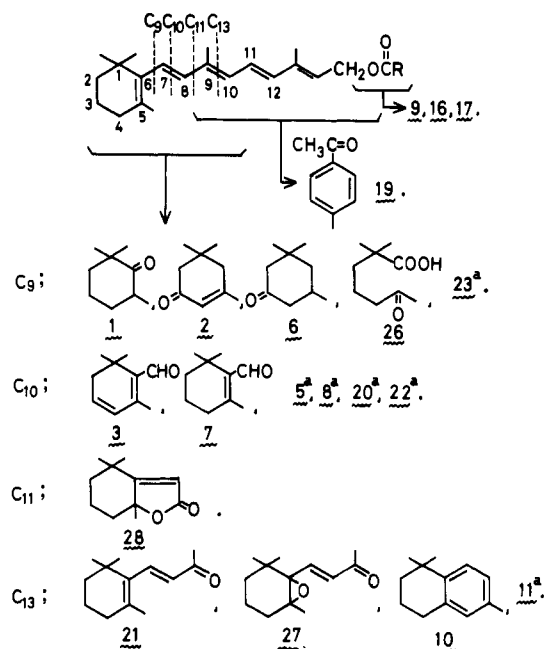
Figure 7. GC pattern of volatile compounds in fraction III from preliminary TLC separation of VA palmitate oxidized by method 3. GS/MS system 2 was used.

were compared to the file and literature spectra. Whenever possible the retention indices, comparison with the authentic products, and NMR data were also used for more accurate identification.

Table II shows that aldehydes and ketones are the most important volatile compounds in oxidized VA palmitate. These volatile compounds may formally be produced by cleavages of the 6-7, 7-8, or 9-10 bonds of VA palmitate through the formation of peroxide as an intermediate (Scheme I).

Isoe et al. (1972) showed that photosensitized oxidation of β-carotene as well as VA acetate yielded dihydro-

Scheme I



^a The most reasonable formula postulated by M⁺ values and the structure of VA palmitate.

actinidiolide, β-ionone, 2-hydroxy-2,6,6-trimethylcyclohexanone, and deoxyanthoxine. Lerner et al. (1970) showed the formation of dihydroactinidiolide via peroxide from retinal. Some of the compounds listed in Table II, β-ionone, β-cyclocitral, dihydroactinidiolide, 2,6,6-trimethylcyclohexanone, and 6-hydroxy-2,2,6-trimethylcyclohexanone, are found in tobacco (Wahlberg et al., 1977), tea (Aisaka et al., 1978; Bricout et al., 1967), and photosensitized oxidation products of β-carotene (Dalle et al., 1969; Isoe et al., 1969). In addition, β-ionone is also

easily oxidized by photosensitized oxidation to form dihydroactinidiolide and other various oxidation products (Mousseron-Canet et al., 1967). Considering these facts, dihydroactinidiolide may also be formed from a C₁₃ precursor such as β -ionone or epoxy- β -ionone. The concentration of β -cyclocitral in the volatiles of photosensitized oxidation is higher than in that of thermal oxidation and may arise not only by cleavage of the 7-8 bond of VA palmitate but also from β -ionone.

Both ionene and β -methylacetophenone are unique compounds because they were not formed by photosensitized oxidation of VA palmitate and were not found as flavor components of food, tea, tobacco, or β -carotene oxidation. Ionene may be formed by intermolecular aldol condensation reaction of methyl-6-hydroxy-4-methylhexa-2,4-dien-1-one which is formed by oxidative cleavage of the 8-9 bond of VA palmitate.

Some methyl esters of palmitic, myristic, and stearic acid are probably formed by a series of the reactions during the thermal oxidation, i.e., decomposition of VA palmitate (including small amounts of VA myristate and stearate as contaminants) to fatty acids and then esterification with methanol. Methanol is probably also formed from VA palmitate during thermal oxidation. On the other hand, palmitic acid was separated from the nonvolatile fraction of VA palmitate oxidation products as crystals. The mechanisms of hydrolysis of VA palmitate without water in the reaction system cannot be explained. It is reasonable to assume that water was formed by oxidation. Among the unidentified compounds, there are some quantitatively important ones such as peaks no. 8, 11, 13, 15, 20, 22, 23, and 24 with some minor unidentified ones. Identification of these compounds, for which no reference spectra could be found in the literature, was not feasible by mass spectral analysis.

In this paper, we could not clearly identify the specific compounds which gave haylike flavor or off-flavor to skim milk. However, β -ionone and dihydroactinidiolide both have a mild, typical haylike odor in the pure state and are the main oxidation products of VA palmitate. They give pleasant odors to tea and tobacco (Aisaka et al., 1978; Bricout et al., 1967; Wahlberg et al., 1977) but may give an unpleasant haylike flavor to milk.

There were large quantities of nonvolatile oxidation products which included oxidized and polymerized VA palmitate but did not contribute to the objectionable haylike flavor in milk. However, the presence of these compounds may produce additional flavor compounds on further oxidation.

It should be noted that almost all compounds were carbonyl compounds; thus, they may react with amino

groups of skim milk to give various condensation products.

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Registry No. 1, 2408-37-9; 2, 78-59-1; 3, 116-26-7; 6, 873-94-9; 7, 432-25-7; 10, 475-03-6; 12, 128-37-0; 14, 124-10-7; 16, 112-39-0; 17, 112-61-8; 19, 122-00-9; 21, 79-77-6; 26, 461-11-0; 27, 23267-57-4; 28, 17092-92-1; VA, 68-26-8; VA palmitate, 79-81-2.

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