Rate of Formation of Methyl Ketones during Blue Cheese Ripening

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Changes in the carbonyl content and methyl ketone composition of blue cheese were studied at progressive stages of ripening. The 2,4-dinitrophenylhydrazones (DNP) obtained from *n*-hexane extracts of the cheese were separated into various classes of carbonyl compounds on activated celite–Sea Sorb 43 columns. The methyl ketone fraction was regenerated with concentrated sulfuric acid, and the relative concentrations of the individual methyl ketones were determined by gas chromatography. At the beginning of the ripening period the concentration of the methyl ketone fraction was 1.86 μmol and this increased to 120.40 μmol per 10 g of dry cheese by the 70th day of the ripening period. The C7 and C9 methyl ketones were the major ketones present in the cheese at all stages of ripening, and generally accounted for more than 50% of the total methyl ketones.

Saturated *n*-methyl ketones (*n*-alkan-2-ones) have been identified as the principal group of compounds that give rise to the unique flavor of blue cheese (Anderson, 1966; Hawke, 1966). Starkel (1924) and Hammer and Bryant (1937) observed that methyl ketones of intermediate chain length were metabolic products of the mold *Penicillium roqueforti* which is the important mold for the ripening process in blue cheese. The homologous series of odd-carbon chain methyl ketones, C3 to C15 inclusive, and also some even-carbon chain methyl ketones, C4, C6, C8, and C10, were identified in blue cheese by Patton (1950), Morgan and Anderson (1956), Bavissoto *et al.* (1960), Nawar and Fagerson (1962), Schwartz *et al.* (1963) and Anderson (1966). Schwartz *et al.* (1963), Anderson and Day (1966), and Niki *et al.* (1966) quantified some of the above methyl ketones in blue cheese varieties. Although the quantities of the individual methyl ketones varied from sample to sample, the data of most investigators reveal that 2-heptanone and 2-nonanone were the principal methyl ketones occurring in blue cheeses. However, the analyses of Niki *et al.* (1966) revealed that 2-pentanone was one of the principal methyl ketones in some samples of blue cheese.

The mechanism of methyl ketone formation in blue cheese apparently involves the initial hydrolysis of the milk fat and the subsequent metabolism of the liberated fatty acids to methyl ketones by *P. roqueforti*. Gehrig and Knight (1961) reported that the methyl-ketone synthesizing activity is confined to the spores of *P. roqueforti*. Gehrig and Knight (1963) and Lawrence (1966, 1967) reported that β-oxidation of fatty acids and methyl ketone formation occur simultaneously in blue cheese. The β-keto-acyl CoA formed as an intermediate in the oxidation of a fatty acid apparently becomes deacylated into a β-keto acid, which is then decarboxylated into a methyl ketone or it undergoes further β-oxidation reactions.

The present study was undertaken to determine the rate of formation of methyl ketones in ripening blue cheese samples and to determine if all the methyl ketones were formed at approximately the same stage of ripening and at equivalent rates.

MATERIALS AND METHODS

Solvents. High purity *n*-hexane (Philips Petroleum) and reagent grade benzene were treated for removal of carbonyls by the method of Ellis *et al.* (1958). The 2,4-dinitrophenylhydrazine decomposition product in the distillate was removed with Sea Sorb 43 (Anderson, 1966).

Chloroform (ACS grade) was treated first by washing six times with half its volume of distilled water to remove alcohol contamination and dried overnight with anhydrous Na_2SO_4 crystals before purifying as above.

Ethylene chloride (1,2-dichloroethane) was glass-distilled and stored over anhydrous K_2CO_3. Nitromethane (analytical grade, Eastman Kodak) was used as received.

Absorbents. Celite (Johns-Manville analytical grade) was activated by heating at 150° C for at least 24 hr prior to use. The Sea Sorb 43 (Fisher Scientific Co.) was activated by heating at 150° C for at least 36 hr prior to use.

Two samples of 0.5 kg each of blue cheese were made by the procedure outlined by Kosikowski (1966). Two samples from each cheese were taken at specific intervals: 0, 10, 15, 25, 30, 50, 60, 70, 85, and 100 days of the ripening period.

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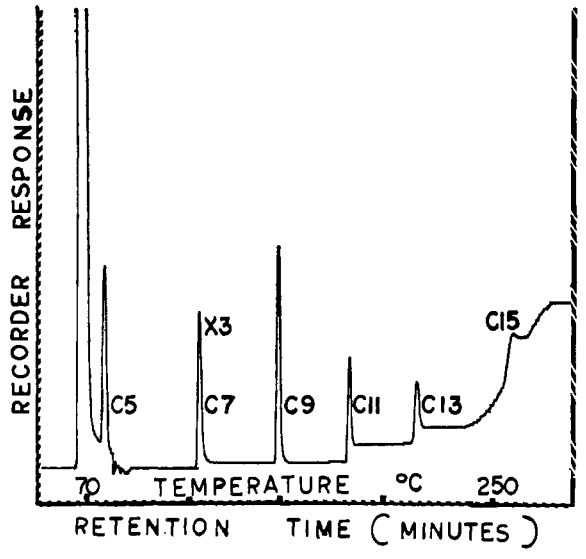

Figure 1. Gas chromatogram of methyl ketones regenerated from 2,4-dinitrophenylhydrazones obtained from blue cheese after 10 days of ripening

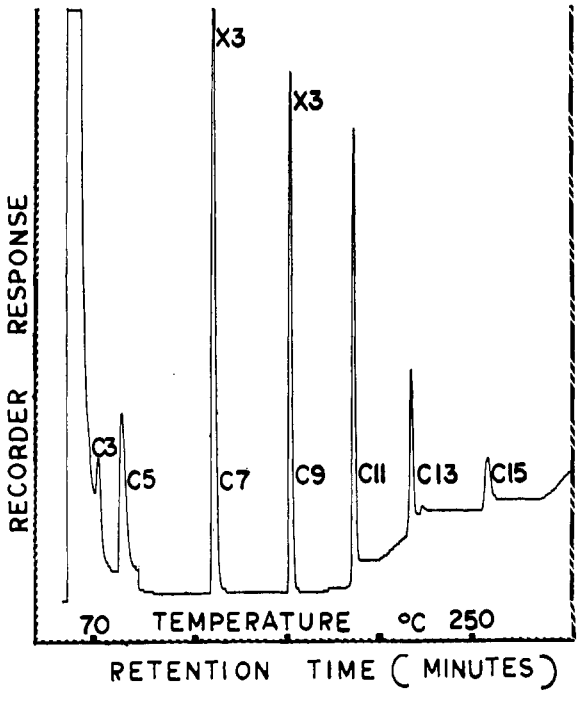

Figure 2. Gas chromatogram of methyl ketones regenerated from 2,4-dinitrophenylhydrazones obtained from blue cheese after 70 days of ripening

Table I. Concentrations of Total Carbonyl DNP Derivatives and Methyl Ketones in Blue Cheese at Progressive Stages of Ripening

Stage of Ripening (Days)	μmol/10 g Dry Cheese	
	Carbonyl DNP	Methyl Ketones
1	3.34	...
10	11.89	1.86
15	22.38	1.12
25	54.85	9.45
30	89.80	43.66
50	203.50	56.84
60	249.60	82.75
70	268.30	120.40
85	268.00	100.95
100	246.80	95.71

Each sample was immediately frozen and later freeze-dried at 0° C. The sampling procedure involved cutting through the cheese randomly and mixing small pieces of the cheese together to obtain representative samples of the ripening cheese. The cheeses made by the above procedure possessed the typical blue cheese quality and flavor.

The method of Schwartz and Parks (1961) was used to obtain the total concentration of carbonyl compounds in the blue cheese. The concentration of the total carbonyl DNP derivatives was calculated using the molar extinction coefficient, E = 22,500 (Schwartz *et al.*, 1963). The lipids in the effluent were removed by the procedure outlined by Schwartz and Parks (1961). The methyl ketone DNP derivatives were separated from the total monocarbonyl DNP derivatives by the method of Schwartz *et al.* (1962).

An adaptation of the method of Bassette and Day (1960) was used to regenerate the methyl ketones from their DNP derivatives. The ethylene chloride solvent was evaporated and the methyl ketone DNP residue was dissolved in chloroform and transferred into a 50-ml distillation flask with a sidearm. The chloroform was evaporated off with a stream of nitrogen gas. A coil condenser system with cold water running through it was fitted to the flask. A delivery tube fitted to the condenser was also fitted to a 25-ml receiving flask containing 10 ml of *n*-pentane in an ice bath kept at 0° C. Six milliliters of concentrated H_2SO_4 was added to the methyl ketone DNP mixture through the sidearm. Fifteen milliliters of water was added into the distillation flask and the sidearm was tightly closed. The flask was swirled gently and the content was distilled over a steam bath at 100° C for 30 min. The receiving flask was removed immediately and stoppered. It was shaken gently for 20 sec to dissolve any methyl ketones in the vapor phase in the flask. The top

Table II. Concentrations of Individual Methyl Ketones in Blue Cheese at Progressive Stages of Ripening

Methyl Ketones	Stage of Ripening (Days)								
	10	15	25	30	50	60	70	85	100
	(μmol[a]/10 g dry cheese)								
C_3	...	0.04	0.01	...	...	2.88	2.95	1.01	...
C_5	0.25	0.08	0.51	1.51	3.75	2.07	6.60	5.10	3.87
C_7	0.62	0.30	3.29	16.50	17.51	37.60	52.45	44.20	38.55
C_9	0.44	0.27	3.33	15.70	18.15	25.80	40.90	36.90	34.71
C_{11}	0.39	0.18	0.22	5.58	7.75	7.05	10.35	11.34	12.03
C_{13}	0.12	...	1.46	4.37	6.50	4.40	4.60	2.40	6.55
C_{15}	0.04	0.25	0.50	...	3.18	2.95	2.55	...	...

[a] Each result was an average of four determinations.

Table III. Recovery of 2,4-Dinitrophenylhydrazones of Methyl Ketones from Celite–Sea Sorb 43 Columns and Efficiency of Regeneration of Methyl Ketones

Methyl Ketone	Recovery (%)	Efficiency of Regeneration (%)
C3	96	95
C3	100	90
C7	98	80
C9	100	75
C11	100	69
C13	98	56
C15	101	55

n-pentane layer was transferred with a Pasteur pipette into a vial. The bottom water layer was reextracted with 5 ml of *n*-pentane, and the *n*-pentane layer was transferred into the vial. A half gram of activated Sea Sorb 43 was shaken with the *n*-pentane fraction to remove color contamination of the solution. The methyl ketones were separated and quantified using a 6 ft U-shaped glass column packed with 15% Apiezon L on 60/80 mesh Gas Chrom P in a Barber–Colman Model 5000 gas chromatograph. The initial temperature of 70° C was held for 3 min after injection of a sample, and thereafter it was programed at 15° C per min temperature rise until attaining a final column temperature of 250° C. The carrier gas flow rate was initially maintained at 25 ml/min for 3 min and then increased to 50 ml/min for the remainder of the analysis. The detector response was calibrated using both individual methyl ketones and standard mixture of methyl ketone solutions.

The efficiency of the analytical procedures was determined by subjecting standard solutions of a mixture of methyl ketones to the various analytical steps involved in the cheese analysis. The final results of the quantitative data reflected corrections made for the individual methyl ketone concentrations.

RESULTS

The concentrations of the total carbonyl DNP derivatives and total methyl ketones in 10 g of the freeze-dried blue cheese at various stages of ripening are shown in Table I. The total carbonyl accumulation occurred in the third week of ripening, and maximum concentration of the carbonyl compounds was found in the sample analyzed on day 70 of the ripening period. A decline in concentration occurred after the 85th day of ripening. The concentration of methyl ketones, which was very low initially, 1.86 μmol, increased during the ripening period to 120.40 μmol per 10 g of dry cheese in the sample analyzed on day 70. A decline in methyl ketone concentration occurred in the cheese samples analyzed on days 85 and 100 of the ripening period.

The average concentration of the individual methyl ketones in blue cheese at progressive stages of ripening is presented in Table II, and representative gas chromatograms of the methyl ketones isolated from the blue cheese on days 10 and 70 of the ripening period, respectively, are shown in Figures 1 and 2. The results showed that the homologous series of methyl ketones of odd-carbon chain length, C3 to C15, occurred in the cheese sampled on days 25, 60, and 70 of the ripening period. The methyl ketones, C3, C13, and C15, which were generally present in very low concentrations, were not detected in some of the samples. Acetone, which conceivably was present, was not detected by these methods and probably remained with the *n*-alkanal fractions during fractionation. The results also show a general fluctuation in the relative concentrations of the methyl ketones in the course of the ripening, with the exception of 2-heptanone and 2-nonanone, which consistently increased up to day 70 and then declined gradually.

The concentrations of the individual methyl ketones in each sample determined from the peaks on the gas chromatograms showed deviations of less than 5% from the means of four determinations reported in Table II. The recovery of 2,4-dinitrophenylhydrazones of the methyl ketones from the celite–Sea Sorb 43 columns and the efficiency of regeneration of the methyl ketones from their DNP are presented in Table III. The results showed good recovery of the methyl ketones as their DNP. The efficiency of the regeneration mechanism was found to decrease generally with the increase in carbon chain length because the longer the carbon chain length, the less volatile were the ketones. The concentrations of the two principal methyl ketones, 2-heptanone and 2-nonanone, accounted for over 50% of the total concentrations of the methyl ketones up to day 50 of the sampling. The concentrations increased to over 75% from day 60 onward, indicating that the two methyl ketones are the major components of the flavor of blue cheese.

DISCUSSION

Carbonyl compounds are produced from the milk fatty acids by the metabolic activity of the mold *P. roqueforti*. The milk fat is hydrolyzed by the lipases of the mold and the free fatty acids are subsequently metabolized into methyl ketones and other carbonyl compounds (Lawrence, 1964). Some short chain carbonyl compounds could also arise, to a limited extent, from the metabolic activity of the mold on the β-keto acids released from the milk ketoglycerides (Parks *et al.*, 1964).

Examination of the data in this investigation showed that when the concentrations of the total carbonyl compounds were plotted against time, a smooth sigmoid curve could be realized. However, the total concentrations of the methyl ketones would not produce a similar type curve. These observations showed that whereas the total carbonyl compounds were being biosynthesized uniformly, the methyl ketones were not so produced. The data on the concentrations of the individual methyl ketones in blue cheese at progressive stages of ripening clearly showed that no definite ratios or correlations existed between the relative concentrations of the component methyl ketones in the cheese. The fluctuations in the concentrations of the methyl ketones during ripening, as shown in this study, were conceivably the result of the interconversions of the methyl ketones and their corresponding secondary alcohols. Franke *et al.* (1962) discovered that methyl ketones were reduced to their corresponding secondary alcohols under anaerobic conditions by certain molds. The reaction was found to be reversible under aerobic conditions. Day and Anderson (1965) demonstrated this phenomenon of interconversion in blue cheese. This reaction could be the determining factor in producing the different type curve for the plot of the total concentrations of the methyl ketones with time from that of the total carbonyl DNP derivatives. This reaction also offers a possible explanation for the nonuniform distribution of the individual methyl ketones in blue cheese during the ripening period.

The flavor of the cheese was typical of good commercial blue cheese by the 60th day of ripening, and it remained good until the final sampling period. This corresponded to the accumulation of the C7 and C9 alkan-2-ones.

The preferential formation of these two ketones has not been explained. It is not known if they are formed from fatty acids of specific chain length. It is conceivable that the corresponding β-keto acid derivatives dissociate from the oxidative enzymes very easily or these specific β-keto acids may tend to accumulate for some metabolic reason. These speculations are being studied further.

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Correction

In the March/April, 1971, issue of J. AGR. FOOD CHEM. the correct pages, authors, and titles are as follows: page 360, Kossmann, Geissbühler, and Boyd, "Specific Determination of Chlorphenamidine [*N'*-(4-Chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine] and of Some Potential Metabolites in Plant Materials by Thin-Layer and Flame Ionization Gas Chromatography"; page 365, Geissbühler, Kossmann, Baunok, and Boyd, "Determination of Total Residues of Chlorphenamidine [*N'*-(4-Chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine] in Plant and Soil Material by Colorimetry and Thin-Layer and Electron Capture Gas Chromatography."