AN ABSTRACT OF THE THESIS OF

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Title FLAVOR CHEMISTRY OF BLUE CHEESE

Abstract approved _____
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Numerous attempts have been made to identify the flavor compounds in Blue cheese, however, duplication of Blue cheese flavor has not yet been accomplished. Therefore, it was desirable to make a qualitative and quantitative investigation of Blue cheese flavor compounds and to study the effect of certain microorganisms on Blue cheese flavor.

The aroma fraction of Blue cheese was isolated by centrifugation of the cheese and molecular distillation of the recovered fat. The volatiles were separated by gas chromatography on packed columns containing polar and nonpolar phases and by temperature programmed capillary column gas chromatography. Relative retention time data and fast scan mass spectral analysis of the capillary column effluent were used to identify compounds in the aroma fraction. Compounds positively identified were as follows: 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, 2-undecanone, 2-tridecanone, 2-propanol, 2-pentanol,

2-heptanol, 2-octanol, 2-nonanol, methyl butanoate, methyl hexanote, methyl octanoate, methyl decanoate, methyl dodecanoate, ethyl formate, ethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethanal, 3-methyl butanal, 2-methyl butanol, 3-methyl butanol, 1-pentanol, benzene, and toluene.

Tentatively identified compounds included acetone, delta-octalactone, delta-decalactone, methyl acetate, isopropyl hexanoate, 3-methylbutyl butanoate, pentyl hexanoate, ethyl-2-methylnonanoate, isopropyl decanoate, furfural, 2-methyl propanal, methanol, ethanol, 2-phenylethanol, cresyl methyl ether, dimethylcyclohexane, diacetyl, methyl mercaptan, and hydrogen sulfide.

A combination of liquid-liquid column chromatography and gas-liquid chromatography was utilized to quantitate the major free fatty acids in Blue and Roquefort cheese samples. The average concentration (mg acid/kg cheese) in three Blue cheese samples was as follows: 2:0, 826; 4:0, 1,448; 6:0, 909; 8:0, 771; 10:0, 1,318; 12:0, 1,588; 14:0, 5,856; 16:0, 12,789; 18:0, 4,243; 18:1, 12,455; 18:2, 1,072; 18:3, 987. Roquefort cheese was found to be proportionately more abundant in 8:0 and 10:0 acids and low in 4:0 acid compared to Blue cheese. No formic, propionic, or isovaleric acid was detected in any of the cheeses tested.

A quantitative procedure involving adsorption chromatography, liquid-liquid chromatography and absorption spectrophotometry was

used to isolate and measure the concentration of the C_3, C_5, C_7, C_9, and C_{11} methyl ketones in the fat of Blue and Roquefort cheese. The average methyl ketone concentration (micromoles ketone/10 g cheese fat) of five Blue cheese samples was as follows: acetone, 1.7; 2-pentanone, 5.9; 2-heptanone, 11.2; 2-nonanone, 9.3; 2-undecanone, 2.4. Considerable variation in ketone concentration was noted between samples, but no consistent differences were observed between Blue and Roquefort cheese. One Roquefort sample contained no acetone. The amount of ketone formed during cheese curing does not depend directly on the amount of available fatty acid precursor. There appears to be a selective conversion of the 8:0, and to a lesser extent the 6:0 and 10:0, fatty acids to methyl ketones by the Penicillium roqueforti spores.

The concentration of the C_5, C_7, and C_9 secondary alcohols was determined in the same cheeses used for ketone analysis. The previously measured ketones acted as internal standards and facilitated a semi-quantitative calculation of alcohol concentrations from peak areas of gas chromatograms. The average alcohol concentration (micromoles alcohol/10 g cheese fat) in five Blue cheese samples was as follows: 2-pentanol, 0.3; 2-heptanol, 2.1; 2-nonanol, 0.8. The alcohols were present in approximately the same ratios as their methyl ketone analogs, but at much lower concentrations.

A synthetic Blue cheese flavor was prepared using a blend

of butterfat, dry curd cottage cheese, cream, and salt as a base. The most typical flavor was obtained using the following compounds: the 2:0, 4:0, 6:0, and 8:0 fatty acids at two-thirds the average concentration found in cheese; twice the average concentration of the C_3, C_5, C_7, C_9, and C_{11} methyl ketones and C_5, C_7, and C_9 secondary alcohols found in cheese: 2.0 mg/kg of base of 2-phenylethanol; 1.5 mg/kg of base of ethyl butanoate; 6.0 mg/kg of base of both methyl hexanoate and methyl octanoate. Incorporation of higher acids caused a soapy flavor. The presence of 2-phenylethanol and the esters was judged as very important in duplicating Blue cheese flavor.

The mycelia of Penicillium roqueforti appear to be more active in the reduction of methyl ketones to secondary alcohols than the spores. Yeasts associated with Blue cheese are capable of reducing methyl ketones to secondary alcohols. Yeasts also may play a role in Blue cheese flavor by producing ethanol and other alcohols and certain esters.

FLAVOR CHEMISTRY OF BLUE CHEESE

by

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INTRODUCTION

The experimental approaches to flavor chemistry research problems vary widely, but most investigators share the same general goals. These are: 1) to qualitatively and quantitatively define flavor; 2) to establish the origin of the flavor compounds; 3) to determine how the compounds are affected during processing and storage of the food. The resulting knowledge enables fulfillment of the ultimate objective of flavor chemistry research, namely, to be able to control flavor in a food product.

In the case of Blue-vein type cheese, significant progress has been made in attaining the above goals. Certain of the more important flavor components have been identified and the origin of some of these compounds, the methyl ketones and fatty acids, has been established. However, it is still impossible to reproduce Blue cheese flavor using data available in the literature. Quantitative data are lacking on all flavor components in Blue cheese. In addition, it appeared from a review of the literature that a lack of qualitative data accounted in part for the inability to reproduce the desired flavor. It was, therefore, desirable to make a detailed qualitative and quantitative analysis of Blue cheese flavor.

There were four main objectives in this investigation. The

first was to further elucidate the qualitative composition of the aroma fraction of Blue cheese to determine if heretofore unidentified compounds were present. The second objective was to obtain more complete quantitative data on the major classes of flavor compounds, the fatty acids, methyl ketones, and secondary alcohols. The third objective was to use the data obtained to reproduce Blue cheese flavor and to evaluate the importance of certain flavor compounds which were identified. The final objective was to determine what influence various yeasts, bacteria, and molds associated with Blue cheese during curing have upon the formation of secondary alcohols, and in conjunction with this, to identify other flavor compounds produced by these microorganisms.

REVIEW OF LITERATURE

Because of its intense flavor, Blue cheese was one of the first cheeses to have investigations conducted on the identification and origin of compounds responsible for its unique flavor. The flavor compounds can arise from three sources. First, some of the flavor components that are normally in milk will be carried into the finished cheese. Second, added materials such as salt, have a pronounced effect on flavor. Third, and most important, are the flavor compounds produced by natural milk enzymes and the metabolism of microorganisms during the manufacture and curing of the cheese. A review concerning aspects of flavor and manufacture of Blue cheese was published by Bakalor (1962).

Flavor Compounds of Blue Cheese

Free Fatty Acids: Currie (1914) concluded that the "peppery" taste of Blue cheese was due to caproic, caprylic, and capric acids or their hydrolyzable salts. Thomasow (1947) found butyric, valeric, and caproic acid, and Coffman, Smith and Andrews (1960) identified fatty acids in dry Blue cheese by gas chromatography of their methyl esters. They were able to detect butyric, caproic, caprylic, capric, isovaleric, and heptanoic acids. Simonart and Mayaudon (1956a) identified formic and/or acetic, lactic, and succinic acids,

and in further work (Simonart and Mayaudon, 1956b) they isolated p-hydroxybenzoic, benzoic, and p-hydroxyphenylacetic acids. The flavor significance of the aliphatic and aromatic acids was not discussed.

Morris et al. (1955) found 0. 77 mg of butyric acid/g of cheese made from pasteurized-homogenized milk. The value for the fraction of caproic and higher acids (average molecular weight 228) was 24. 93 mg/g of cheese. Recently Morris et al. (1963) observed that the processing treatment given cheese milk affected the pH and fatty acid composition of the final cheese. Their data indicated that fatty acids were not the main flavor components. Anderson and Day (1965) quantitated the 2:0 - 18:3 fatty acids in domestic Blue and imported Roquefort cheese and observed that octanoic and decanoic acid were proportionately more abundant than butanoic in the Roquefort cheese.

Hydrolysis of Milk Triglycerides: Considerable hydrolysis of the milk triglycerides must occur to give the high concentrations of free fatty acids present in Blue cheese. There are three possible sources of lipase: 1) natural milk lipase, 2) Penicillium roqueforti lipase, and 3) lipase of other microorganisms present during ripening of the cheese.

Sufficient evidence now exists to support the view that all cows' milk contains a minimum of two lipolytic enzymes (Webb and Johnson, 1965). Tarassuk and Frankel (1957) found two lipases in milk, one

being present in the plasma, the second being associated with the fat globule membrane. Albrecht and Jaynes (1955) found two lipase systems in raw skimmilk with pH optima of 5.4 and 6.3. Schwartz, Gould and Harper (1956) found crude milk lipase to have three pH optima, 6.5-7.0, 7.9, and 8.5-9.0. Chandan and Shahani (1963) purified lipase from clarifier sediment and found it to hydrolyze milk fat from pH 5.0 to 10.0 with a maximum at pH 9.0-9.2. Harwalkar and Calbert (1961) determined the relative amounts of butyric, caproic, caprylic, capric, and lauric and higher acids released from milk fat by whole milk lipase. The fraction of lauric and higher acids was the major component released at all stages, but as lipolysis progressed it gradually decreased, and the butyric fraction increased. There was little change in the mole percent of the other fractions, but a slight degree of selectivity for the short chain acids was noticed upon extensive hydrolysis.

Penicillium roqueforti exhibits an active lipase system. Prokš, Doležálek and Pech (1956) indicated that strains of P. roqueforti isolated from Roquefort cheese differed mainly in the liberation of butyric, caproic, and caprylic acids. Morris and Jezeski (1953) characterized two lipases associated with P. roqueforti. One was obtained from the mycelia and exhibited a pH optimum for milkfat hydrolysis of 6.5-6.8. The other lipase was present in the growth medium and had a pH optimum of 7.0-7.2. Both preparations showed

one-third faster hydrolysis of tributyrin than milkfat. Imamura (1960a) separated lipolytic fractions from P. roqueforti with pH optima of 6. 5 and 7. 5 and found that the lipolytic activity of both was reduced by NaCl concentrations of greater than three percent. Shipe (1951) observed that tributyrin, tricaproin, tricaprylin, and tripropionin were hydrolyzed in the order of decreasing rate, respectively, by P. roqueforti lipase. Chandan, Carrancedo and Shahani (1961) reported that the intracellular and extracellular lipases of P. roqueforti were inhibited by concentrations of penicillin that did not reduce growth. Wilcox, Nelson and Wood (1955) found that two cultures of Geotrichum candidum released only butyric acid from milk fat and seven cultures released no detectable quantities of volatile fatty acids. Stadhouders and Mulder (1957) reported that cheeses showed higher acidity in the fat from outer surfaces than the interior. They concluded that surface organisms have a marked influence on fat hydrolysis, and therefore, the dimension and shape of the cheese affect its flavor. Coulter, Combs and George (1938) followed the pH changes during curing and observed the minimum (pH 4. 7) 24 hours after manufacturing. The pH increased gradually to 6. 5 at three months and then decreased to pH 5. 7 at nine months. It is obvious that the lipase systems must be active in the pH range occurring during curing and that curing conditions will affect the lipase activity.

Since fatty acids are known to be essential for typical Blue cheese flavor, several methods have been advocated to insure proper concentrations in finished cheeses. These include use of raw milk to insure activity of milk lipase, and homogenization to increase fat surface area. Parmelee and Nelson (1949) found that cheese flavor was improved by the addition of low molecular weight acids (butyric through lauric) to pasteurized-homogenized milk, but the cheese lacked fullness of flavor. Coulter and Combs (1939) stated that curing time could be reduced from 12 to five months by addition of steapsin to cheese milk, however, samples were criticized for being bitter. Peters and Nelson (1948) claimed an improved flavor when a cell-free lipase of Mycotorula (Candida) lipolytica was added to pasteurized-homogenized cheese milk. Kosikowski and Mocquot (1958) indicated that most of the added lipolytic agents caused undesirable, rancid, and bitter flavors.

Many of the investigations concerning addition of lipolytic agents to cheese have been aimed at duplicating the flavor of Roquefort cheese. Roquefort cheese is made from sheeps' milk, whereas domestic Blue cheese is made with cows' milk. The fatty acid composition of cows' milk and ewes' milk is different, thus if the triglyceride hydrolysis is nonselective, differences in the free fatty acid composition of Blue and Roquefort cheese would be expected. Sadini (1963) found the butyric:caproic plus caprylic fatty acid ratio was

lower for ewes' milk than for cows' milk. Hilditch (1956) stated that sheeps' milk is low in butyric and high in caprylic and capric acids compared to cows' milk. Benassi (1963), and Kuzdzal and Kuzdzal (1963) found more capric acid in ewes' milk than in cows' milk. Proks, Doležálek and Pech (1959b) reported that lipase in cows' milk is found mainly at the end of lactation, whereas sheeps' milk contains it throughout lactation. They postulated that this was one reason for a more full flavor of Roquefort cheese.

Production of Free Fatty Acids by Microorganisms: Some of the acids identified in Blue cheese do not occur in milk triglycerides or are present in very low concentrations, e.g., valeric (Thomasow, 1947), isovaleric and heptanoic (Coffman, Smith and Andrews, 1960) and acetic (Simmonart and Mayaudon, 1956a). Thus, they presumably arise from metabolism of microorganisms. Many microorganisms have the ability to carry out glycolysis, which provides a route for the formation of lactic and acetic acid. Yeasts, which grow on the cheese surface, can form acetic acid under aerobic conditions (Cook, 1958). Microorganisms in Blue cheese can also deaminate amino acids to yield ammonia and certain fatty acids (Foster et al., 1957).

Methyl Ketones: Starkel (1924) found that distillates of Roquefort cheese has an odor characteristic of 2-heptanone and 2-nonanone, and concluded that the flavor and aroma of the cheese was due to

these methyl ketones. Patton (1950) identified acetone, 2-pentanone, 2-heptanone, and 2-nonanone from steam distillates of Blue cheese. Utilizing vacuum distillation and paper chromatography of phenylhydrazone derivatives, Morgan and Anderson (1956) also found the C_3, C_5, C_7, and C_9 methyl ketones, plus 2-butanone and 2-undecanone. The 2-butanone was thought to have originated from valeric acid which resulted from microbial metabolism. Cheese of poor flavor contained no 2-heptanone or 2-nonanone. Bavisotto, Rock and Lesniewski (1960) identified 2-octanone, and Nawar and Fagerson (1962) identified 2-octanone and 2-hexanone in Blue cheese. Day and Anderson (1965) identified the C_3, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, and C_{13} methyl ketones in Blue cheese volatiles.

Schwartz and Parks (1963) quantitated the C_3, C_5, C_7, C_9, C_{11}, and C_{13+15} methyl ketones in domestic Blue cheese. They found no definite ratio between the quantities of ketones, however, 2-heptanone was the major ketone in all cheeses. Aged cheeses did not necessarily have a higher ketone content, and considerable variation in ketone concentration was observed among the samples. Similar conclusions were reached concerning Roquefort cheese (Schwartz, Parks and Boyd, 1963), however, one sample was found to contain more 2-nonanone than 2-heptanone.

It has been shown by several workers that certain mold cultures can convert fatty acids to methyl ketones of one less carbon number.

Starkel (1924) found that Aspergillus niger, A. fumigatis, and Penicillium roqueforti produced methyl ketones when grown several weeks with individual fatty acids as the carbon source. Stokoe (1928) found that P. palitans and P. glaucum produced methyl ketones from coconut oil. He also found something that was to be of significance in future work, i. e., that a nonspore forming fungus, Oidium lactis (Geotrichum candidum), did not form ketones.

More recently, Gehrig and Knight (1958) discovered that it is the P. roqueforti spore that produces ketones from fatty acid precursors and the vegetative cells are completely inactive in ketone formation. Gehrig and Knight (1963) found that when one micromole of labeled sodium octanoate was used as spore substrate, most of the molecule was recovered as CO_2 and no ketone was produced. When 20 micromoles was used as substrate, part of the molecule was converted to 2-heptanone and part was completely oxidized. They were unable to isolate intermediates of fatty acid oxidation, but concluded that their data indicated evidence for beta oxidation of fatty acids by spores of P. roqueforti. Some intermediates of fatty acid oxidation (beta-keto acids) have been detected in Blue cheese (Bassette and Harper, 1958), however, these are known to be present in normal milk fat (Parks et al., 1964). Girolami and Knight (1955) reported that little ketone formation resulted from decanoic or higher acids and that methyl ketones were themselves inhibitory to

P. roqueforti growth. They concluded that this may prevent excessive mold growth in curing cheeses.

Alcohols and Aldehydes: The neutral noncarbonyl volatiles from Blue cheese were studied by Jackson and Hussong (1958). They identified 2-pentanol, 2-heptanol,and 2-nonanol and postulated that these were reduction products of corresponding methyl ketone analogs. Ethanol (Bavisotto, Rock and Lesniewski, 1960), 2-propanol (Nawar and Fagerson, 1962), and acetylmethylcarbinol and diacetyl (Bassette and Harper, 1958) have been reported. Nawar and Fagerson (1962) also identified acetaldehyde, propionaldehyde, and isobutyraldehyde in Blue cheese volatiles.

Prescott and Dunn (1959) stated that small amounts of ethanol are produced by species of Penicillium, and acetaldehyde has been recovered from media fermented by species of Geotrichum and Penicillium. The breakdown of amino acids via transamination and decarboxylation is well known, and is probably widespread among fungi (Foster, 1949). These reactions lead to compounds such as isoamyl, active amyl, and isobutyl alcohol (fusel oils). These alcohols have not yet been reported in Blue cheese. However, their presence in Blue cheese would not be unexpected, especially in view of the fact yeasts are often present during ripening and these organisms are very active in fusel oil production.

The Role of Microorganisms other than *Penicillium roqueforti* in Blue Cheese Flavor

During curing of Blue cheese a slime consisting of yeasts, molds, and bacteria develops on the cheese surface. Investigations have been made to characterize the microflora of the slime and determine its importance in development of Blue cheese flavor. Hartley and Jezeski (1954) found five groups of microorganisms in the slime: *Bacterium linens*, *B. erythrogenes*, *Micrococcus sp.*, yeasts, and molds, predominately *P. roqueforti*. Kanauchi, Yoshioka and Hamamoto (1962) isolated *Torulopsis sphaerica*, *T. candida*, *Debaryomyces hansenii*, and *Candida pseudotropicalis* from cheese during ripening. Iya and Frazier (1949) found *Mycoderma sp.* on Brick cheeses which develop a slime similar to Blue cheese. Maxa and Jicinsky (1956) reported the principal yeast of Roquefort cheese to be *Torulopsis sphaerica*. They stated that it produces ethanol which is important for formation of esters. Lilly and Barnett (1951) stated that ethyl acetate and isobutyl acetate are among the esters formed by fungi. *P. digitatum* is reported to produce ethyl acetate in glucose media (Prescott and Dunn, 1959).

Morris, Combs and Coulter (1951) found that cheeses with normal surface slime had a slightly finer flavor than did cheeses with no slime. Purko, Nelson and Wood (1951) observed that yeasts

of Limburger cheese contributed to the growth of B. linens by decreasing acidity and secreting growth factors. Prokš, Doležálek and Pech (1959a) found T. sphaerica to have a slight stimulating effect on ketone production by P. roqueforti.

Proteolysis in Blue Cheese

The role of protein breakdown and its effect on Blue cheese flavor has received limited attention. Nishikawa (1958) showed the proteolytic activity of P. roqueforti to be greatest at 40° C and pH 5.5-6.0, and found that four percent or more NaCl slightly decreased proteolysis. Prokš, Doležálek and Pech (1959c) found variations in the proteolytic activity of different strains of P. roqueforti. They also identified 20 amino acids and amines in Roquefort cheese. Imamura and Kataoka (1961) stated that the volatile fatty acids released by P. roqueforti had an inhibitory effect on the protease and amino acid decarboxylase action of the mold. Imamura (1960b) found Streptococcus lactis, S. cremoris and Lactobacillus bulgaricus to have little proteolytic activity, but they stimulated the growth of P. roqueforti.

Bacterium linens readily degrades casein and is thought to be mainly responsible for the flavor of Limburger cheese (Foster et al., 1957). This organism is frequently found on Blue cheese (Hartley and Jezeski, 1954) and thus may influence cheese flavor. Szumski

and Cone (1962) studied the endoproteinases of yeasts isolated from the surface of Trappist cheese. They concluded that proper conditions existed during curing so that yeast endoenzymes could serve as ripening agents. Tsugo and Matsuoka (1962) could find little hydrogen sulfide or dimethyl disulfide in semi-soft white mold cheese after three weeks of ripening, but 0. 542 ppm methyl mercaptan was present.

Clemens (1954) could find no relationship between free amino acids, soluble N, and pH in ripening cheese. Moller-Madsen (1959) concluded that the characteristic properties of a given type of cheese were not to any important degree dependent on the amino acid distribution. Tuckey and Sahasrabudhe (1957) found no correlation between the presence of any single amino acid and characteristic flavor development in Limburger or Brick cheese.

EXPERIMENTAL

Isolation and Identification of Flavor Components from Blue Cheese Fat

Domestic Blue cheese obtained from a local plant was analyzed. The cheese was considered to be of good quality and typical in flavor. The technique of Libbey, Bills and Day (1963) was utilized for isolation of the neutral volatile flavor compounds. Fat from seven pounds of cheese was isolated by high speed centrifugation (30, 000 × G for 20 minutes). The fat fraction exhibited the typical Blue cheese aroma of the intact cheese.

The volatile compounds were removed from the fat by molecular distillation. The only modification from the technique of Libbey, Bills and Day (1963) was that one glass bead trap, instead of two, was used. Five hundred ml of fat was fed through the still at a rate of three ml/minute while maintaining two to four microns pressure and a temperature of 45° C.

After completion of the distillation, the trapped volatile components were subjected to gas chromatography by means of the direct injection technique of Libbey, Bills and Day (1963). The chromatographic conditions were: Barber Colman Model 20; beta ionization detector; column temperatures 70° C and 100° C; injector temperature, 165° C; detector temperature, 200° C; columns, 11 feet × 1/8

inch OD packed with 20% Apiezon M on 80/100 mesh acid-alkali washed Celite 545 and 20% diethylene glycol succinate (DEGS) on 80/100 mesh acid-alkali washed Celite 545; cell voltage 1250; column flow rate, 20 ml/minute; scavenge flow rate, 120 ml/minute; attenuation, x30. Comparison of relative retention times of knowns and unknowns was used for tentative identification of compounds.

The volatiles remaining in the glass bead trap were extracted with peroxide free, redistilled diethyl ether. After drying over anhydrous Na_2SO_4, the major portion of the ether was removed by fractional distillation. The fractionating column (1 × 60 cm packed with glass helices) was equipped with an automatic reflux head set to operate at a ratio of one second for collection and two seconds reflux. The residue remaining after removal of most of the ether was analyzed by gas chromatography using both DEGS and Apiezon columns at 70° and 100° C and Apiezon at 175° C. The operating parameters were as described previously.

A portion of the ethereal extract was analyzed by temperature programmed capillary gas chromatography in conjunction with mass spectral analysis. The chromatographic conditions were: 300 feet × 0.01 inch ID column coated with polypropylene glycol; column temperature, 73° C for eight minutes, then programmed at 25° per minute to 174° C and held isothermally until completion of the run. The above conditions were employed both for obtaining retention

times and for analysis of column effluent by mass spectrometry. Additional retention time data were obtained by temperature programming the capillary column from 30° to 50° C at 2.5° per minute and 25° per minute to 170° C.

Mass spectrometric studies were carried out as described by McFadden et al. (1963), McFadden and Teranishi (1963), and Teranishi et al. (1963). The only modification was that m/e 43 was monitored by gate two of the electron multiplier. This provided a concurrent strip chart recording of the gas chromatogram, and eliminated the need of plotting base peak intensity versus retention time to obtain a chromatogram as was done by McFadden et al. (1963). The other electron multiplier was used to scan m/e 12 to 250 in two to six seconds. The resulting spectra were recorded on a fast scan oscillographic recorder. The coincidence of relative retention times of knowns and unknowns on packed and capillary columns, plus mass spectral data provided a good means for identification of flavor compounds.

Quantitation of Free Fatty Acids

The method of quantitation used was essentially that of Bills and Day (1964). Four Blue-vein type cheeses were examined. Two of the samples were five pound wheels of domestic Blue cheese obtained from a local plant. The other two samples, one a domestic Blue and the other an imported Roquefort, were purchased as small

retail packages from a local market. All samples were stored at -30°F until analyzed.

Preliminary results indicated that samples from the edge of the wheels of cheese varied considerably in fatty acid content when compared to the interior of the wheel. To obtain reproducible results it was necessary to remove a wedge of cheese from the wheel and mix it thoroughly. A sample of the blended cheese was then used for analysis. In the case of the small retail packages, two packages were blended and a portion used for quantitation. Duplicate samples were analyzed in all cases.

Acetic Acid: The liquid-liquid partition column of Wiseman and Irwin (1957) was used to quantitate acetic acid. Fifteen g of cheese and 30 g of silicic acid were acidified to pH 1.9 with 50% H_2SO_4 and ground to a homogenous mixture in a mortar and pestle. Two g of the mixture were used as cap material for the column.

Butyric Acid: The liquid-liquid partition column devised by Keeney (1956) was used for quantitation of butyric acid and the total moles of higher fatty acids. Two grams of cap material prepared as just described were used. Ten ml fractions of column eluent were collected with an automatic fraction collector.

Fractions eluted from both columns were titrated with standardized isopropanolic KOH, using one drop of one-half percent phenolphthalein as an indicator. Carbon dioxide free air (prepared by passing

through 20% KOH) was used for agitation of the sample to prevent fading end points. Titration values of solvent blanks were used to correct acid containing fractions.

Higher Acids: A 50 g sample of cheese was ground with sufficient 50% H_2SO_4 to reduce the pH to 1.9. After adding the internal standards, 12.5 mg heptanoic acid and 125 mg heptadecanoic acid in five ml hexane, the cheese was analyzed as outlined by Bills and Day (1964). The free fatty acids were then converted to their methyl esters, extracted and concentrated as described by Bills, Khatri and Day (1963). The total moles of higher fatty acids determined by the Keeney column were distributed according to their ratios as determined by gas liquid chromatography (Bills and Day, 1964).

Quantitation of Methyl Ketones

Hexane and Benzene Purification: High purity hexane and reagent grade benzene were treated for removal of carbonyls by the method of Horstein and Crowe (1962) and then redistilled.

Ethylene Chloride Purification: Ethylene chloride was distilled and stored over anhydrous potassium carbonate.

Chloroform Purification: Chloroform was treated for removal of carbonyls by the method of Schwartz and Parks (1961).

Nitromethane Purification: Nitromethane was redistilled over boric acid.

Isolation of Methyl Ketones

Preparation of the Cheese: The quantitation procedure followed was similar to that described for fats and oils (Schwartz, Haller and Keeney, 1963) and adapted to analysis of cheese by Schwartz and Parks (1963). A wedge of cheese or two small retail packages were first diced and mixed to give a uniform blend. Ten g of the cheese and 15 g Celite 545 were thoroughly mixed in a mortar and pestle. The damp mixture was then put in a 1.8 cm ID chromatographic column which was plugged with glass wool at the bottom. Two hundred ml of hexane was passed through the column to extract the fat from the cheese-Celite mixture.

Formation of the 2, 4-Dinitrophenylhydrazones: The hexane-fat solution was passed over a ten g reaction column (Schwartz and Parks, 1961) to form the 2, 4-dinitrophenylhydrazones of the methyl ketones. The hexane was then removed under reduced pressure and the yield of fat was determined.

Removal of Fat from the DNP-Hydrazones: The fat was removed from the hydrazones using a modified procedure of Schwartz, Haller and Keeney (1963). Sea Sorb 43 (magnesium oxide unheated) and Celite 545 were used in a 1:2 ratio rather than the 1:1 ratio reported. Fourteen g of Sea Sorb 43 and 28 g Celite were slurried in hexane and poured into a chromatograph column (2.6 cm ID) plugged

with glass wool at the bottom.

After packing the column with air pressure, the fat-hydrazone mixture was dissolved in five ml of hexane and applied to the column. Two hundred ml of hexane was then passed through the column. The hexane wash was followed by 100 ml of a hexane-benzene mixture (1:1) and then 200 ml of benzene. The hydrazones were eluted from the column with 150 ml of a chloroform:nitromethane mixture (3:1). The chloroform-nitromethane solvent was removed from the eluent at reduced pressure with a rotatory evaporator. A trace of fat remained in the hydrazones, but it did not interfere with subsequent analysis.

Separation of DNP-Hydrazones into Classes: The method of Schwartz, Parks and Keeney (1960) was used to separate the methyl ketone derivatives from the other hydrazones. Using air pressure, a 2.5 cm ID column was packed with an ethylene chloride slurry of 15 g Sea Sorb 43 (heated to 400° C/48 hr) and 30 g of Celite 545 (heated to 150° C/24 hr). The hydrazone mixture from the previous step was taken up in four ml of ethylene chloride and applied to the column. The column was developed with ethylene chloride and the hydrazones of the methyl ketones were collected. Excellent separation of the ketone and aldehyde derivatives was achieved. The ketone derivatives were fat free at the completion of this step.

Separation of Ketone Derivatives into Individual Chain Lengths: The liquid-liquid partition column (method B) of Day, Bassette and Keeney (1960) was utilized to separate the methyl ketone hydrazone class into its individual members. Twenty-five g of packing were used for each column. The column eluate was monitored for absorption at 345 millimicrons with a Vanguard Automatic Ultraviolet Analyzer used in conjunction with an automatic fraction collector. A strip chart recording of absorption and fraction number was made concurrently, hence, by matching tube numbers and absorption peaks the individual members of the class could be pooled and collected.

Determination of Individual Methyl Ketone Concentrations: The concentrations of C_3, C_5, C_7, C_9, and C_{11} methyl ketone derivatives were determined by measuring their absorbance in chloroform at 363 millimicrons. The chain length of each member was tentatively assigned by its retention volume from the partition column, and was confirmed by the thin layer chromatography technique of Libbey and Day (1964). Each cheese was analyzed in duplicate.

The work of Langler (1963) showed that the recoveries from the columns used in this type of analysis were not as high as reported in the literature (Schwartz, Haller and Keeney, 1963). The percentage recovery of the individual ketones was determined using a standard mixture of the C_3, C_5, C_7, C_9, and C_{11} methyl ketones in hexane. Concentrations used were approximately those found in Blue

cheese. The mixture was run over the reaction column which was known to give quantitative conversion to hydrazones (Langler, 1963). The hydrazones were then added to three g of steam stripped milk fat and the sample analyzed as described for the cheese. Addition of fat after hydrazone formation reduced the possibility of residual ketones in the fat reacting to form derivatives. The average percentage recovery of each ketone as determined by duplicate analyses was used in the calculation of the ketone concentrations of the cheese samples.

Quantitation of Secondary Alcohols

Gas chromatography was used to quantitate 2-pentanol, 2-heptanol, and 2-nonanol in Blue cheese. In this study, chromatographic conditions were established so that the peak areas of the methyl ketones (quantitated previously) and the corresponding secondary alcohol, e.g., 2-heptanone and 2-heptanol, could be determined for Blue cheese fat. Under conditions used for cheese analysis, the recovery of the alcohol relative to the ketone was determined. Hence, by knowing that the ketone peak area represented a certain number of moles, the area of the alcohol peak could be related to the number of moles it represented.

The analysis was made using the entrainment and on-column trapping technique outlined by Morgan and Day (1965). The

chromatograph column used had a three inch U-shaped bend in it two inches from the injection port end. To initiate the procedure, the column was detached from the injection port and placed outside the chromatograph oven. The oven door was closed as far as possible to maintain oven temperature, and the carrier gas flow to the injection port was turned off by means of a valve in the supply line. The end of the column was attached to the purging apparatus (Figure 1) and the U-shaped portion immersed in a dry ice-methylcellosolve bath.

Five g of cheese fat, obtained by high speed centrifugation of the cheese, was placed in a 25 × 55 mm screw capped vial containing two g anhydrous Na_2SO_4. Caps with two 5/32 inch holes 7/16 inch apart were fitted with a 1/8 inch thick silicone rubber septum. The caps were then firmly tightened onto the vials so that the system was air tight.

The sealed vial was then raised onto the purging needles (Hamilton N-722, point style one, 22 gauge, two inch and one-half inch long), so that the long needle was immersed in the fat. A stop watch was started to time the length of purging. A water bath equipped with a magnetic stirrer was then raised under the sample so that the entire vial was immersed. The purge gas (N_2) was run through a six feet × one-fourth inch tubing containing molecular sieve 5A, prior to reaching the purging apparatus and was adjusted to give a

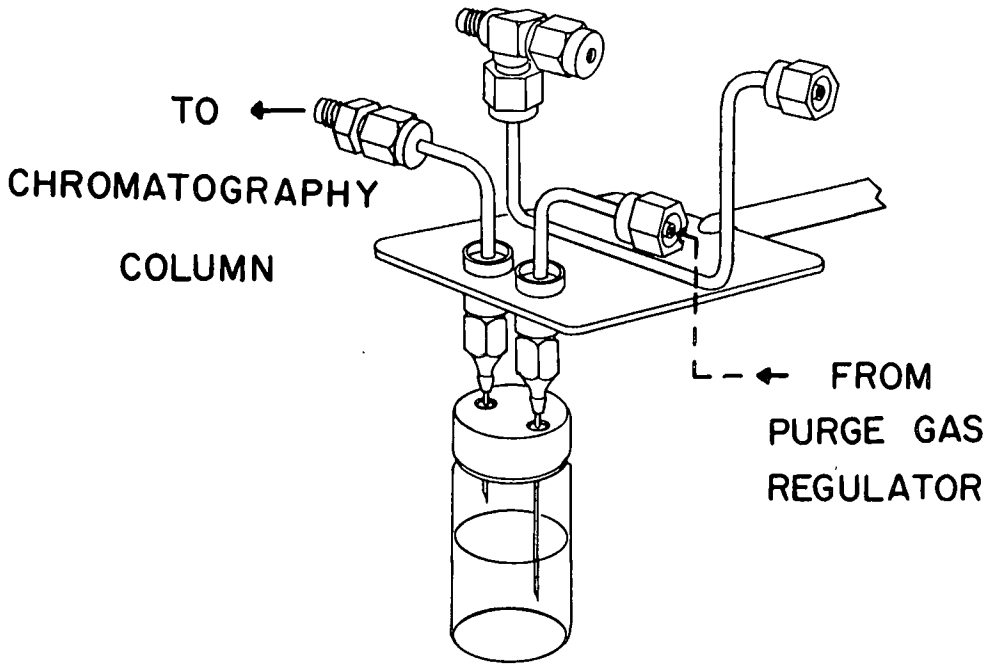

Figure 1. Apparatus used to purge cheese fat for quantitation of secondary alcohols by gas chromatography.

flow rate of nine ml/minute at the end of the chromatograph column. A hair dryer was used to heat the section of column from the dry ice bath to the purging apparatus. At the end of the purge time the sample bottle was removed, and the column detached from the purging apparatus and attached to the injection port in the chromatograph oven. The carrier gas was immediately turned on and the separation was allowed to progress under normal GLC conditions. Each cheese was analyzed in duplicate except for two samples which were in limited supply. Chromatograph conditions were: F and M model 810 equipped with hydrogen flame detector; detector temperature, 200° C; injection block temperature, 200° C; column, 12 feet × 1/8 inch OD packed with 20% Tris 1, 2, 3 (2, cyanoethoxypropane) on 60/80 mesh acid-alkali washed Celite 545. The conditions used for each analysis are given in Table 1.

Table 1. Conditions used in quantitating secondary alcohols in Blue cheese.

Compounds to be determined	GLC column temperature	Water bath temperature	Purge time
2-pentanone 2-pentanol	50° C	70° C	2 minutes
2-Heptanone 2-Heptanol	80° C	70° C	4 minutes
2-Nonanone 2-Nonanol	90° C	80° C	10 minutes

Recovery factors were determined using the same conditions as outlined for analysis of the cheese fat. Three different concentrations (5, 10, and 20 micromoles/10 g oil) containing equimolar quantities of the C_7 methyl ketone and secondary alcohol were prepared using paraffin oil as the diluent. A similar solution was prepared for the C_9 alcohol and ketone. Preliminary data indicated that a portion of the 2-pentanol was partitioning into the aqueous phase during centrifugation of the cheese. Therefore, determinations of the recovery factor for this alcohol were run using standards of a mixture of 60 parts paraffin oil and 40 parts saline solution (12% NaCl). The alcohol could then partition into an aqueous system similar to that of cheese. After thorough mixing, the standards were centrifuged and the oil layer used for chromatography. Partitioning into the aqueous phase was not a problem with the higher alcohols. All recoveries were done in duplicate.

After chromatography under the proper set of conditions, the peak areas of the ketone and alcohol were determined. By setting the area of the ketone peak equal to 1.00, a recovery factor could be calculated for the alcohol. This factor was then used in calculating the concentration of secondary alcohols in the cheese samples.

Compounding Synthetic Blue Cheese Flavor

A mixture of dry curd cottage cheese, butterfat, salt, and

pasteurized cream was used as base for evaluation of the synthetic flavor. Forty g of fat from melted sweet cream butter, 110 g of dry curd cottage cheese and 100 g of pasteurized cream were mixed in a Waring Blendor to make a thick slurry. Three and one-half percent salt was then added to the mixture. Solutions of the flavor compounds to be used were prepared using water or paraffin oil as diluents, depending on the solubility properties of each compound. The flavor compounds were added to the base while it was being mixed in a Waring Blendor. The samples were then placed in plastic cottage cheese containers and stored overnight at refrigeration temperature before evaluation.

Influence of Selected Microorganisms on Certain Blue Cheese Flavor Compounds

This study was undertaken to investigate the effect of certain microorganisms on the methyl ketones of Blue cheese. The reduction of 2-pentanone to 2-pentanol and acetone to 2-propanol was investigated. Microorganisms typical of those associated with Blue cheese during curing were selected for the investigation. Stock cultures were obtained as follows:

Penicillium roqueforti	- American	Type	Culture	Collection	10110
Torulopsis sphaerica	- "	"	"	"	2504
Mycoderma sp.	- "	"	"	"	6432
Geotrichum candidum	- "	"	"	"	12784
Bacterium linens	- "	"	"	"	9175
Streptococcus lactis	- Department stocks		C2-F		

Culture Media: The Penicillium, Geotrichum, Mycoderma, and Torulopsis organisms were grown in the fortified malt extract broth used by Jackson and Hussong (1958). The B. linens was propagated in the tryptone broth of Purko, Nelson and Wood (1951). The lactic broth described by Elliker, Anderson and Hannesson (1956) was used for growth of S. lactis. A synthetic medium (Meyers and Knight, 1958) was used for growth of P. roqueforti when sterile hyphal cells, i. e., hyphal cells that had not sporulated, were required. All media was dispensed in 50 ml quantities into 250 ml long neck culture flasks and sterilized at 121° C for 20 minutes.

The S. lactis stock culture was maintained in 11% solids non-fat reconstituted milk. All other stock cultures were maintained on agar slants of the appropriate media.

Preparation of Spore Suspension: The method of Gehrig and Knight (1963) was used to prepare P. roqueforti spore suspensions. The spores were stored at 4° C until used.

Addition of Substrate and Incubation of Cultures: Redistilled substrates (2-pentanone, 2-pentanol, acetone or 2-propanol) were added to the sterile broth with a ten microliter syringe. One ml of spore suspension or 18 hr broth culture was used to inoculate the broth containing the substrate.

The S. lactis cultures were incubated at 21° C. The other cultures were incubated at 25° C in a water bath equipped with a

shaker. All cultures were incubated 48 hr and then analyzed by gas chromatography for their effect on the substrate. Samples containing substrate only and organisms only were run as controls.

Incubation in Phosphate Buffer: Incubation in a pH 6.8 phosphate buffer (Umbriet, Burris and Stauffer, 1957) was used to study the individual action of the Penicillium spores and Penicillium hyphal cells. Samples were incubated with agitation for 12 hr at 25° C and then analyzed.

Gas Chromatographic Analysis: The incubated culture was transferred to a 125 ml erlenmyer flask equipped with a 24/40 ground glass joint. Fifty g of anhydrous Na_2SO_4 were added, and a standard taper ground glass joint with the top drawn out to accommodate a Barber Colman septum was used to stopper the flask. Rubber bands stretched between hooks on the stopper and the flask held the stopper tightly in place.

The sample was thoroughly mixed and a volume of nitrogen equal to the volume of headspace to be removed for gas chromatography was injected into the flask. The sample was again mixed and allowed to equilibrate for several minutes. A two ml ground glass syringe was used to inject headspace gases into the chromatograph. One-half ml samples were used for the Mycoderma and Torulopsis cultures, and two ml samples were used for all other cultures. All syringes and flasks were rinsed with diethyl ether and placed in an

80° C vacuum oven for 20 minutes prior to use. This removed impurities causing extraneous peaks in the chromatogram.

Chromatographic conditions were as follows: Aerograph Model 600 equipped with a hydrogen flame detector; column temperature, 70° C; injection block temperature, 125° C; 9 feet × 1/8 inch OD column packed with 15% Carbowax 1500 on 80/100 mesh acid-alkali washed Celite 545; flow rate 20 ml/minute. A 12 feet × 1/8 inch OD column packed with 20% 1, 2, 4-butanetriol on 80/100 mesh acid-alkali washed Celite 545 was also used under the same conditions. Matching of relative retention times of knowns and unknowns was considered tentative identification of peaks.

Mass Spectral Analysis: Fast scan mass spectrometry was used in conjunction with gas chromatography to positively identify the major peaks in the chromatograms. Headspace volatiles of selected cultures were analyzed by monitoring the gas chromatographic effluent with an Atlas-MAT CH-4 Nier-type mass spectrometer (nine inch, 60 degree sector, single focusing instrument). Operating conditions were:

Filament current	60 amps
Electron energy	30 ev
Accelerating voltage	3,000 v
Analyzer pressure	2×10^{-6} mm Hg
Multiplier voltage	1.85
Scan speed	m/e 25 to m/e 250 in 5 seconds

Spectra were recorded by a Honeywell 1508 Visicorder.

Chromatographic conditions were: Barber Colman Series 5000 equipped with a hydrogen flame detector; column temperature, 60° C; detector temperature, 185° C; 12 feet × 1/8 inch OD column packed with 20% 1, 2, 4-butanetriol on 80/100 mesh acid-alkali washed Celite 545; 1:1 split ratio of column effluent between GLC detector and EC-1 inlet of mass spectrometer.

Maximum ion intensity in the mass spectrometer did not correspond to maximum peak height on the GLC chromatogram. Therefore, in preliminary runs, m/e 43 was monitored to determine what point on the chromatograph peak corresponded to maximum ion intensity in the mass spectrometer. In subsequent runs, spectra were taken at the appropriate points on the chromatograms.

It was necessary to warm the culture samples to 60° C and use five ml of headspace to obtain sufficient sample to give mass spectra. It was necessary to lower the column temperature to 60° C to reduce column bleed and prevent excessive background signal in the spectra. As a result, compounds with long retention times were eluted as diffuse peaks of insufficient concentration to give spectra. To alleviate this problem, the headspace sample was injected (using a five ml Hamilton gas tight syringe) with the carrier gas pressure at 15 psi. Immediately after injection the pressure was raised to 60 psi and held there for the duration of the run. The rapid carrier gas flow rate sufficiently increased the concentration of compounds as they entered the mass spectrometer to give interpretable spectra.

RESULTS AND DISCUSSION

Identification of Compounds in Blue Cheese Volatiles

The volatiles from the cheese fat distillate were chromatographed on packed polar (DEGS) and nonpolar (Apiezon) columns at 70° and 100° C using the direct vapor injection method of Libbey, Bills and Day (1963). The chromatogram obtained at 100° C on Apiezon is shown in Figure 2. Tentative peak identifications are given in Table 2. Identification is based on the coincidence of relative retention times (t_R/t_R) of authentic compounds relative to diacetyl = 1.00.

The ether extract of the molecular still trap was concentrated and chromatographed on DEGS (70° and 100° C) and on Apiezon (70°, 100°, and 175° C). The chromatogram obtained on DEGS at 100° C is shown in Figure 3. Tentative peak identifications given in Table 3 were made by comparison of relative retention times of knowns and unknowns with diacetyl = 1.00.

In addition to analysis on packed columns, the ether extract was examined using capillary column temperature programmed gas chromatography in conjunction with mass spectrometry. A 300 feet × 0.01 inch ID capillary column coated with polypropylene glycol was operated isothermally for eight minutes and then temperature

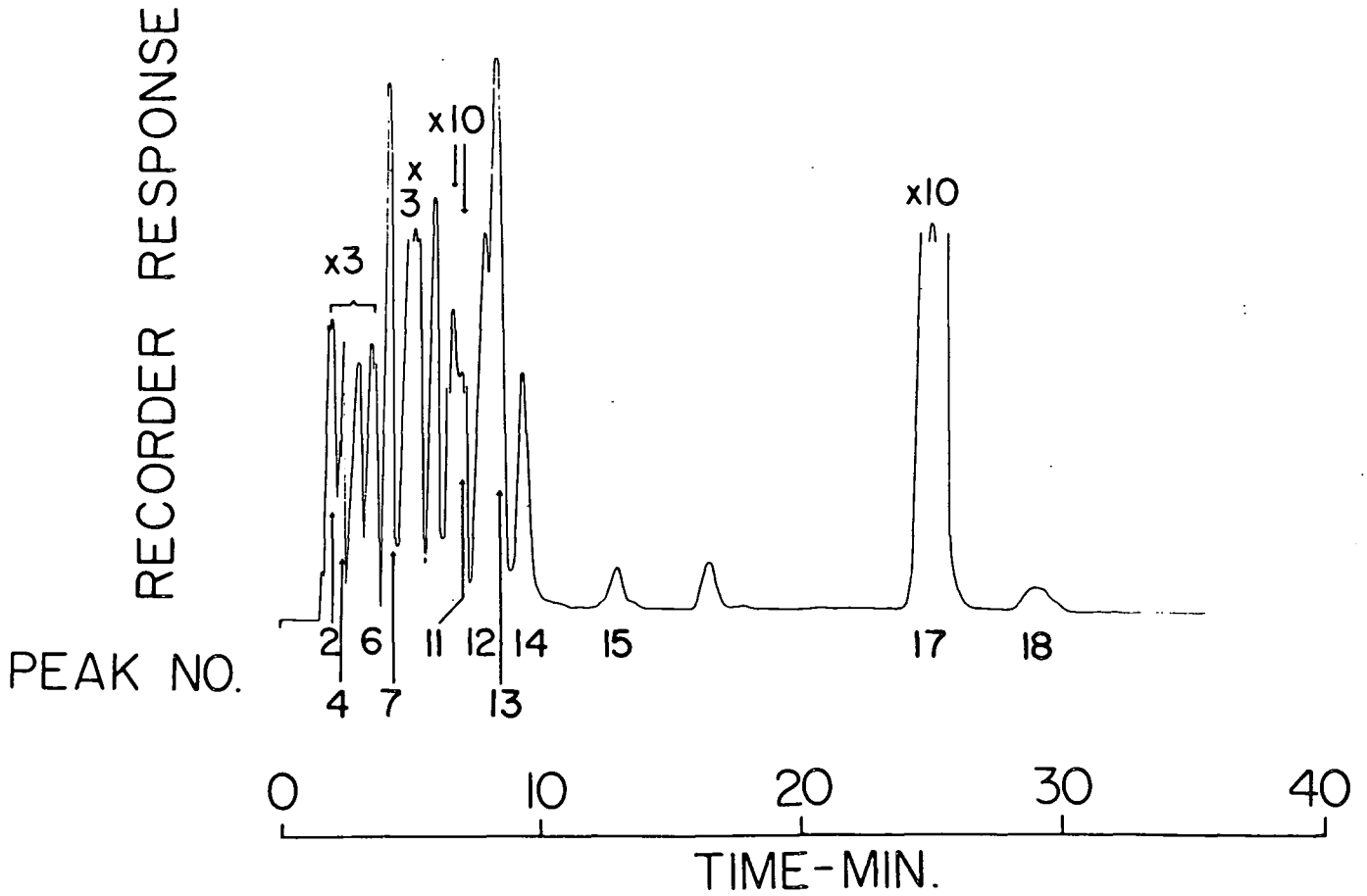

Figure 2. Gas chromatogram of Blue cheese volatiles obtained by the direct vapor injection method of Libbey, Bills and Day (1963). Apiezon M column at 100° C. See Table 2 for peak identifications.

Table 2. Tentative identification of volatiles from Blue cheese obtained by the method of Libbey, Bills and Day (1963). See Figure 2 for gas chromatogram.

Peak No.	Compound	t_R/t_R [a] Blue cheese	t_R/t_R [a] Authentics
1	--	0.53	--
2	Methanol	0.55	0.56
3	--	0.63	--
4	Ethanol	0.64	0.64
5	--	0.82	--
6	n-propanol	0.95	0.98
	Diacetyl		1.00
7	Ethyl acetate	1.13	1.13
8	--	1.40	--
9	--	1.61	--
10	--	1.81	--
11	2-pentanone	1.90	1.90
12	2-pentanol	2.13	2.15
13	Methyl butanoate	2.24	2.18
14	3-methyl butanol	2.55	2.58
	2-methyl butanol		2.68
15	Ethyl butanoate	3.55	3.55
16	--	4.50	--
17	Methyl hexanoate	6.84	6.59
18	2-Heptanol	7.94	8.13

[a] Relative retention time with t_R/t_R of diacetyl = 1.00.

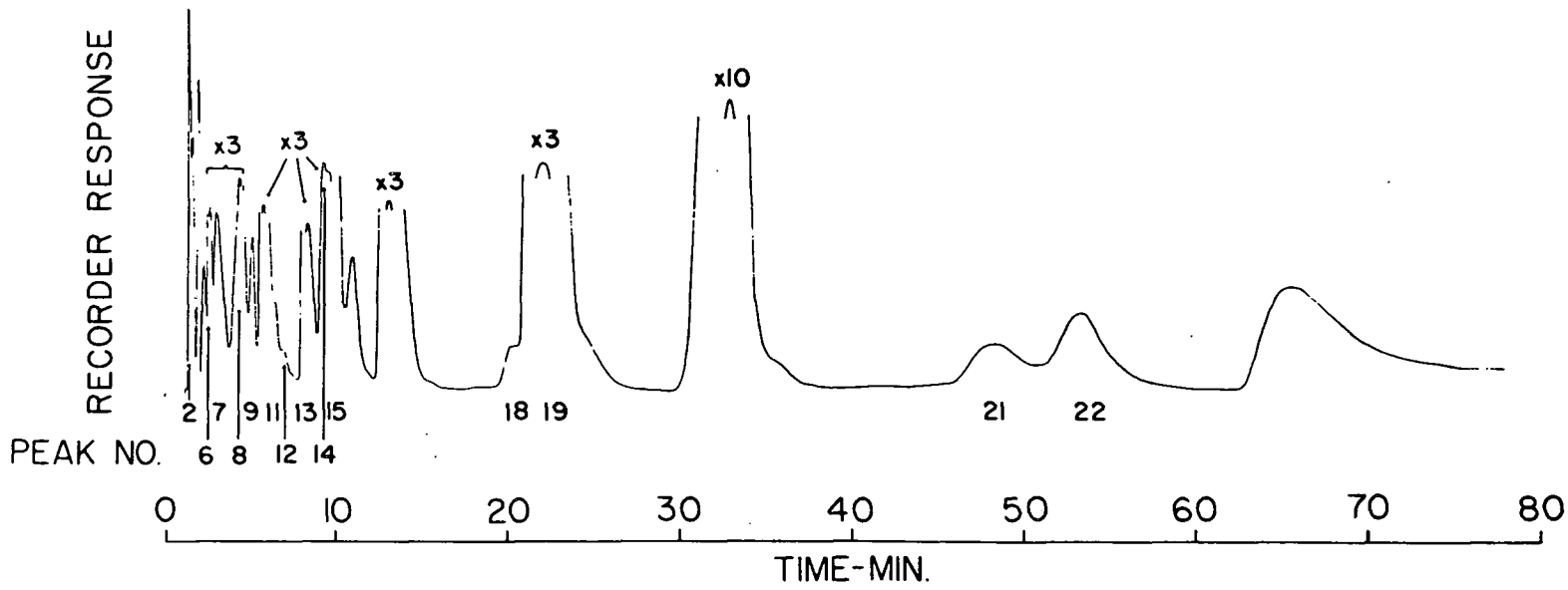

Figure 3. Gas chromatogram of ether extract of Blue cheese volatiles. DEGS column at 100° C. See Table 3 for peak identifications.

Table 3. Tentative identification of compounds in ether extract of Blue cheese volatiles. See Figure 3 for gas chromatogram.

Peak No.	Compound	t_R/t_R[a]	
		Blue cheese	Authentics
1	--	0.25	--
2	Ether	0.30	0.29
3	--	0.33	--
4	--	0.40	--
5	--	0.55	--
6	Ethyl formate	0.53	0.53
7	Ethyl acetate	0.62	0.63
8	2-Pentanone	0.89	0.92
9	Diacetyl	1.04	1.00
10	--	1.16	--
11	2-Pentanol	1.29	1.24
12	2-Hexanone	1.38	1.37
13	Methyl hexanoate	1.68	1.78
14	2-Heptanone	1.88	1.94
15	Ethyl hexanoate	1.93	2.02
16	--	2.21	--
17	--	2.63	--
18	Methyl octanoate	4.09	4.05
19	Ethyl octanoate 2-Nonanol	4.45	4.56 4.59
20	--	6.62	--
21	Methyl decanoate Ethyl decanoate	9.67	9.73 9.79
22	2-Undecanone	10.66	9.91
23	--	13.11	--

[a]Relative retention time with t_R/t_R of diacetyl = 1.00.

programmed at 25°/minute to 174° C. The chromatographic pattern is shown in Figure 4 and peak identifications are given in Table 4. Peak assignments were made by comparison of retention times of knowns and unknowns relative to ethyl acetate = 1.000. Table 4 also indicates whether or not the peak identity was confirmed by packed column GLC, whether the mass spectral identification was tentative or positive, and gives the reference used for the mass spectral identification. In some cases authentic compounds were not available for confirmation of peak identity by retention times, however, the mass spectra obtained were of such quality that positive identification could still be made. In other cases the mass spectra were weak and only tentative identification could be made.

Figure 4 shows the much superior separation achieved by capillary columns over that of packed columns. One-hundred distinct peaks are apparent in the chromatogram from the capillary column, whereas no more than 34 peaks were ever observed using packed columns. The separation achieved by the capillary column coupled with mass spectral analysis of the compounds as they eluted from the column provided an excellent means of identifying the compounds in the Blue cheese extract. Analysis by mass spectrometry and gas chromatography allows much more positive identification of a compound than does analysis by a single method.

The utility of mass spectrometry under the conditions just

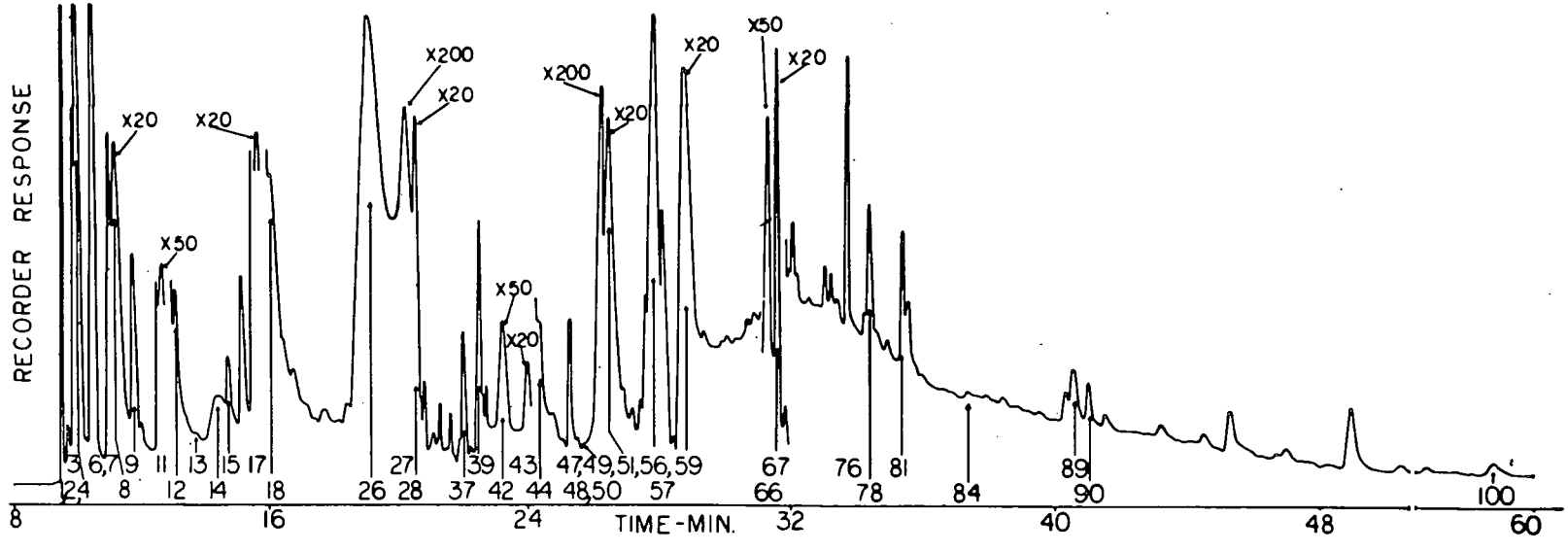

Figure 4. Gas chromatogram of ether extract of Blue cheese volatiles. PPG column operated isothermally at 73° C for eight minutes, then temperatures programmed at 25° per minute to 174° C. See Table 4 for peak identifications.

Table 4. Gas chromatographic and mass spectral identification of compounds in ether extract of Blue cheese volatiles. See Figure 4 for gas chromatogram.

Peak No.	Compound	t_R/t_R [a] Blue Cheese	t_R/t_R [a] Authentics	Confirmed by Packed Col. GC	Mass Spectral Identification	Ref.
1	Ethanal	0.867	0.867	Yes	Positive	(2)
2	Diethyl ether	0.888	0.883	Yes	Positive	(2)
3	Acetone	0.902	0.917	Yes	Tentative	(2)
4	Ethyl formate	0.906	0.924	Yes	Positive	(2)
7	Ethyl acetate	1.000	1.000	Yes	Positive	(2)
7	2-Propanol	1.004	1.007	Yes	Positive	(2)
8	3-Methylbutanal	1.007	...	Yes	Positive	(30)
9	Benzene	1.069	...	No	Positive	(2)
11	2-Pentanone	1.159	1.151	Yes	Positive	(2)
12	Methyl butanoate	1.198	1.200	Yes	Positive	(2)
13	Toluene	1.250	...	No	Positive	(2)
14	2-Pentanol	1.315	1.352	Yes	Positive	(27)
15	Dimethylcyclohexane	1.344	...	No	Tentative	(2)
17	Ethyl butanoate	1.414	1.414	Yes	Positive	(2)
18	2-Hexanone	1.464	1.467	Yes	Positive	(89)
25	2-Methylbutanol	1.678	...	No	Positive	(27)
26	3-Methylbutanol	1.735	1.730	No	Positive	(27)
26	1-Pentanol	1.735	...	No	Positive	(27)
27	2-Heptanone	1.842	1.834	Yes	Positive	(89)
28	Methyl hexanoate	1.875	1.879	Yes	Positive	(88)
37	Furfural	2.003	2.007	Yes	Tentative	(2)
39	Ethyl hexanoate	2.050	2.055	Yes	Positive	(88)
42	2-Heptanol	2.108	...	Yes	Positive	(58)
43	2-Octanone	2.183	...	Yes	Positive	(89)
44	Isopropyl hexanoate	2.217	...	No	Tentative	(88)
47	3-Methylbutyl butanoate	2.300	...	No	Tentative	(58)
48	2-Octanol	2.325	2.310	Yes	Positive	(30)
49	Cresyl methyl ether	2.342	...	No	Tentative	(2)
50	2-Nonanone	2.392	2.359	Yes	Positive	(3)
51	Methyl octanoate	2.410	2.376	Yes	Positive	(3)
56	Ethyl octanoate	2.528	2.510	Yes	Positive	(3)
57	2-Decanone	2.560	...	Yes	Positive	(58)
59	2-Nonanol	2.617	...	Yes	Positive	(58)
60	Pentyl hexanoate	2.621	...	Yes	Tentative	(58)
66	2-Undecanone	2.857	2.866	Yes	Positive	(89)
67	Methyl decanoate	2.892	2.886	Yes	Positive	(3)
76	Ethyl decanoate	3.078	3.103	Yes	Positive	(3)
77	2-Phenylethanol	3.128	...	No	Tentative	(58)
78	Ethyl-2-methylnonanoate	3.139	...	No	Tentative	(2)
81	Isopropyl decanoate	3.236	...	No	Tentative	(58)
84	δ-Octalactone	3.414	3.400	No	Tentative	(58)
89	2-Tridecanone	3.707	3.800	Yes	Positive	(58)
90	Methyl dodecanoate	3.750	3.880	No	Positive	(3)
100	δ-Decalactone	5.178	4.952	No	Tentative	(58)

[a] Relative retention time with t_R/t_R of ethyl acetate = 1.000.

mentioned is well demonstrated in this investigation. The technique of using mass spectrometry for identification of the chromatogram peaks is illustrated in Figure 5. Spectra A, B, C, and D represent the mass spectra of peaks 27, 28, 50, and 51 (Figure 4), respectively.

In spectrum A, the large peaks of m/e 43 and 58 ($OC_3H_6^+$, rearrangement ion for methyl ketones) point to a methyl ketone. The presence of the peak at m/e 114 corresponds to the parent ion for 2-heptanone. A comparison of the ratios of ion peaks 99, 85, 72, and 71 conform to the ratios of known 2-heptanone (Sharkey, Shultz and Friedel, 1956). Coincidence of retention times of peak 27 with authentic 2-heptanone (Table 4) and confirmation by packed column chromatography gives firm evidence for the identification of the compound.

Peak 28 in the chromatogram is not well separated from 27, and thus would be difficult to identify by GLC only. Spectrum B corresponds to peak 28 which still contains some 2-heptanone as evidenced by fragments at m/e 114, 85, 71, 58, and 43. However, the appearance of ion fragments at m/e 87 and 74 is indicative of a methyl ester. The ion peak at 130 corresponds to a molecular weight of methyl hexanoate. Further analysis shows that peak ratios at m/e 101, 99, 75, 59, and 55 correspond to published data (Sharkey, Shultz and Friedel, 1959) for methyl hexanoate. Retention time data further confirms the identification.

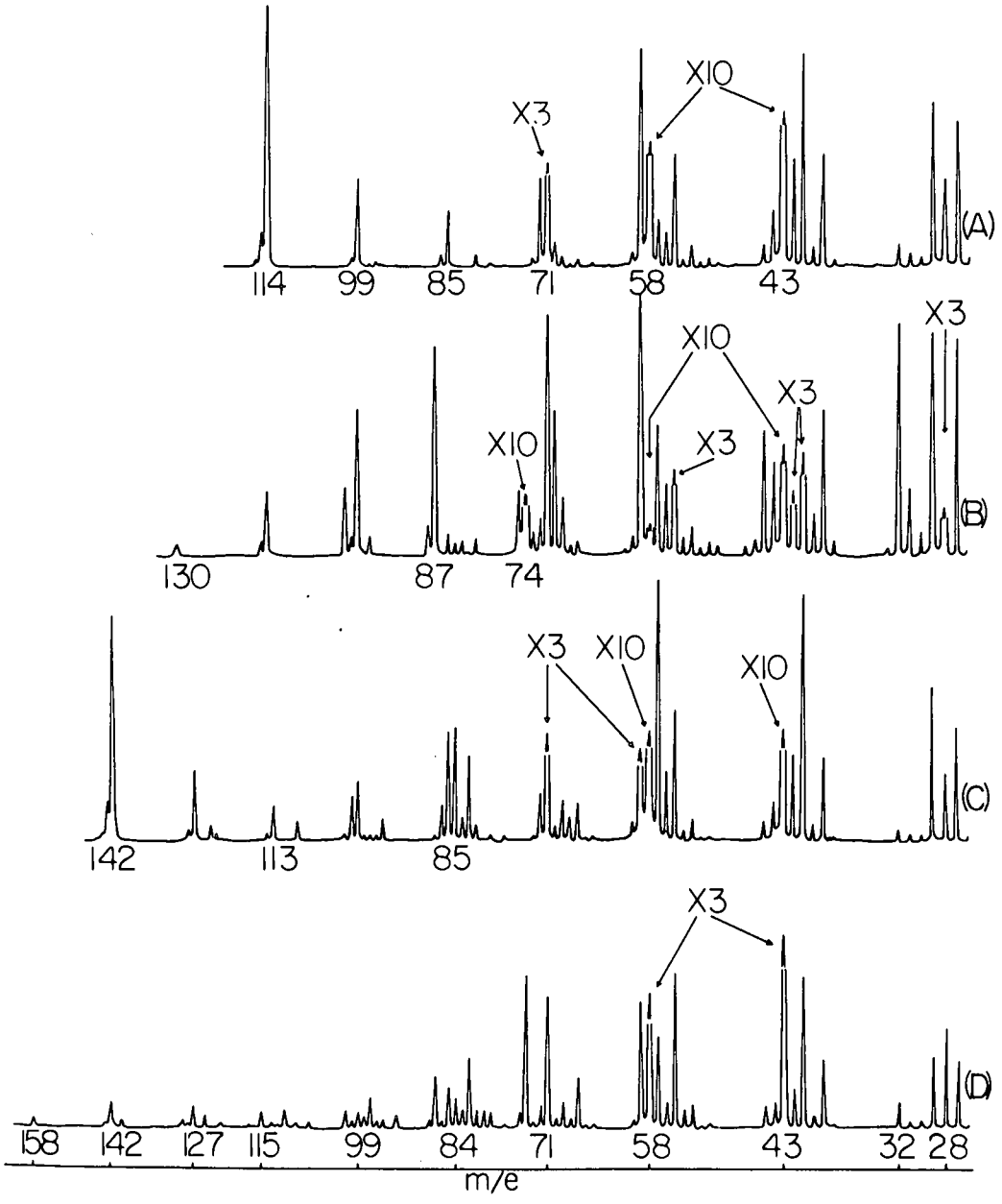

Figure 5. Mass spectral charts of chromatographic fractions: a) corresponds to chromatographic peak 27 in Figure 4; b) peak 28; c) peak 50; d) peak 51.

Table 5. Summary of compounds positively or tentatively identified in Blue cheese.

Compound	Cap. col. PPG Ext. T. P.	Packed columns: DEGS Vapor 70	DEGS Vapor 100	DEGS Ext. 70	DEGS Ext. 100	Apiezon Vapor 70	Apiezon Vapor 100	Apiezon Extract 70	Apiezon Extract 100	Apiezon Extract 175
Acetone	+	+	+	+				+		
2-Pentanone	+		+	+	+	+	+	+	+	
2-Hexanone	+		+						+	
2-Heptanone	+		+	+	+	+		+	+	+
2-Octanone	+			+						
2-Nonanone	+		+		+					+
2-Decanone	+									+
2-Undecanone	+				+					+
2-Tridecanone	+									+
2-Propanol	+		+							
2-Pentanol	+		+		+	+	+			
2-Heptanol	+						+			
2-Octanol	+									
2-Nonanol	+									+
Methanol[a]				+			+			
Ethanol[a]			+			+	+			
2-Methylbutanol	+					+		+		
3-Methylbutanol	+	+		+		+	+	+		
1-Pentanol	+			+		+				
2-Phenylethanol	+									
Methyl acetate[a]			+	+		+				
Methyl butanoate	+					+	+	+		
Methyl hexanoate	+	+	+	+	+			+		+
Methyl octanoate	+									+
Methyl decanoate	+				+					+
Methyl dodecanoate	+									
Ethyl formate	+	+	+		+	+			+	
Ethyl acetate	+	+			+	+	+	+	+	
Ethyl butanoate	+					+	+	+	+	
Ethyl hexanoate	+		+		+		+		+	+
Ethyl octanoate	+				+					+
Ethyl decanoate	+				+					+
Ethyl 2-methylnonanoate	+									
Isopropyl hexanoate	+									
Isopropyl decanoate	+									
Pentyl hexanoate	+									
3-Methylbutyl butanoate	+									

Table 5. Continued.

Compound	Cap. col. PPG Ext. T.P.	Packed columns: DEGS Vapor 70	DEGS Vapor 100	DEGS Ext. 70	DEGS Ext. 100	Apiezon Vapor 70	Apiezon Vapor 100	Apiezon Extract 70	Apiezon Extract 100	Apiezon Extract 175
Acetaldehyde	+			+		+		+		
2-Methyl propanal[a]						+				
3-Methylbutanal	+					+		+		
Furfural	+									
Hydrogen sulfide[a]								+		
Methyl mercaptan[a]						+		+		
Diacetyl[a]		+	+		+	+	+	+		
Diethyl ether	+			+	+			+	+	
Benzene	+									
Toluene	+									
Dimethylcyclohexane	+									
Cresyl methyl ether										
Delta-octalactone	+									
Delta-decalactone	+									

[a] Not analyzed by mass spectrometry

Observation of the spectra of chromatograph peaks 50 and 51 further demonstrates the advantage of using mass spectrometry for compound identification. Spectrum C corresponds to peak 50. Again the appearance of fragments at m/e 43 and 58 indicate a methyl ketone. The ion peak at m/e 142 points to 2-nonanone. This is corroborated by the ratios of peaks at m/e 127, 100, 99, 85, 82, and 71 plus chromatographic data on authentic 2-nonanone.

Spectrum D, which corresponds to peak 51, still contains some 2-nonanone, but new ion fragments are evident. Ion peaks at 74 and 58 point to a methyl ester. Fragment 158 is the parent ion for methyl octanoate. Ratios of peaks at 127, 115, 101, and 87 further point to methyl octanoate. Coincidence with the retention time of known methyl octanoate further confirms its identity.

The analysis as just described was used to identify all of the compounds listed in Table 4.

Table 5 summarizes the compounds tentatively or positively identified in Blue cheese. The columns used, the type of sample analyzed, and the temperature conditions are indicated in the table.

Origin and Significance of Compounds Identified in Blue Cheese

Fifty-one compounds were identified from the Blue cheese distillate. Most of the components identified fall into five main

classes: methyl ketones, secondary alcohols, primary alcohols, and methyl and ethyl esters of normal aliphatic acids.

Methyl Ketones: The presence of odd carbon numbered methyl ketones in Blue cheese is well documented, Patton (1950), and Morgan and Anderson (1956). The mode of formation of these compounds (beta oxidation of fatty acids) has been elucidated by Gehrig and Knight (1963).

The identification of even numbered methyl ketones in Blue cheese has been less common, however, Bavissoto, Rock and Lesniewski (1960) identified 2-octanone, and Morgan and Anderson (1956) isolated 2-butanone. The formation of large amounts of the odd carbon number ketones would be expected to be due to the readily available source of their precursors in milk fat, i. e., the even numbered fatty acids. The even numbered ketones (2-hexanone, 2-octanone, and 2-decanone were identified in this investigation) probably arise from the trace amounts of odd numbered fatty acids in milk fat. Herb et al. (1962) reported the percentage of saturated acids in the total acids of milk fat as being 0. 02, 0. 03, and 0. 03% for the C_5, C_7, and C_9 fatty acids, respectively. A relatively large amount of 2-octanone was observed in this study (see peak 43, Figure 8). The relative amounts of the individual ketones in Blue cheese will be discussed in a subsequent section.

Secondary Alcohols: The secondary alcohols appear to arise

by reduction of the corresponding methyl ketones (Jackson and Hussong, 1958). These workers isolated the C_5, C_7, and C_9 secondary alcohols from Blue cheese, however 2-octanol (Table 4) had not been reported prior to this thesis. Certain aspects concerning formation of secondary alcohols will be covered in a later section.

Primary Alcohols: Several primary alcohols were identified; methanol, ethanol, n-pentanol, 2-methyl and 3-methylbutanol, and 2-phenylethanol. The presence of ethanol and methanol appears to be of prime importance as evidenced by their occurrence in methyl and ethyl esters. Although ethanol is a common metabolic product, such is not the case for methanol. Even though methanol and methyl esters have been identified in various foods such as wine, Cheddar cheese and butter culture, the literature lacks explanations for the method of formation of methanol.

The 2-methyl and 3-methylbutanol and 2-phenylethanol undoubtedly are formed from amino acids via oxidative deamination and decarboxylation with subsequent reduction of the aldehyde analog. Fungi are known to carry out this reaction and it is one source for the "fusel oils" in yeast fermentations (Foster, 1949). The aldehyde analogs of the aforementioned alcohols would be expected to be present, however, only 3-methylbutanal was observed (Table 4). Foster (1949) states that only alcohol formation has been observed in fungi.

It is of interest to note the flavor properties of 2-phenylethanol.

It is a highly odorous compound and exhibits a yeasty type odor at high dilutions. Although its concentration appeared to be quite low in the extract, it may well be of significance in Blue cheese flavor. The unique flavor properties of such trace components may be at least partially responsible for the difference between typical Blue cheese flavor and synthetic flavors that are criticized for lacking "fullness of flavor".

The presence of n-pentanol in Blue cheese is not readily explained, however, Ingraham, Guymon and Crowell (1961) discuss a pathway for the formation of n-pentanol by mutant strains of Saccharomyces cerevisiae. The alcohol is thought to arise from intermediates in amino acid synthesis. A similar reaction could be initiated by molds or yeasts common to Blue cheese ripening.

Esters: Prior to this investigation esters had not been identified in Blue cheese. Their presence is not surprising however, in view of the large amounts of free fatty acids available for esterification. These compounds may play an important role in the overall flavor profile of Blue cheese. The importance of esters in cheese flavors is pointed out by the work of Bills et al. (1965). They found that excessive levels of ethyl butanoate and ethyl hexanoate were responsible for the fruity flavor defect in Cheddar cheese. Both methyl and ethyl esters appear to be common in dairy products as they have been found in Cheddar cheese by Day and Libbey (1964) and in heated

milk and butter culture by Lindsay (1965).

Miscellaneous Compounds: The delta-octalactone and delta-decalactone identified in the cheese extract are normal constituents of milk fat (Boldingh and Taylor, 1962). The identification of benzene, toluene, and cresyl methyl ether was unexpected and would not have been possible without mass spectrometry. The ethyl ether used for extraction was checked by gas chromatography and found to be pure. Therefore, it does not seem likely that the ether was the origin of the aromatic compounds. Wax used for coating the cheese is a possible source.

Quantitation of Free Fatty Acids in Blue Cheese

Table 6 gives the quantities of free fatty acids found in the cheeses analyzed. Samples A and B were domestic Blue cheeses obtained as five pound wheels, sample C was a domestic Blue purchased as retail packages, and sample D was an imported Roquefort purchased in retail packages. Results are given in mg acid/kg of cheese and represent the average of duplicate analyses. The average percent deviation of the duplicates from their mean was: 2:0, 3.3; 4:0, 4.0; 6:0, 0.8; 8:0, 1.8; 10:0, 0.9; 12:0, 1.0; 14:0, 1.3; 16:0, 1.7; 18:0, 1.4; 18:1, 1.4; 18:2, 2.2; 18:3, 2.7.

Table 6. Concentration of free fatty acids in Blue-vein type cheese.

Acid	Mg acid/Kg cheese[a] Cheese A(Blue)	B(Blue)	C(Blue)	D(Roquefort)
2:0	1,417	715	345	826
4:0	1,269	558	2,517	338
6:0	887	369	1,471	447
8:0	786	291	1,237	676
10:0	1,414	514	2,026	1,414
12:0	1,686	619	2,458	965
14:0	6,199	2,044	9,324	2,291
16:0	13,212	4,848	20,308	5,162
18:0	4,880	1,610	6,239	2,011
18:1	14,347	4,819	18,199	7,480
18:2	1,336	266	1,615	1,175
18:3	1,358	283	1,320	1,587
pH of cheese	5.6	5.5	5.3	5.6

[a] Average of duplicate analyses

Considerable variation in the fatty acid content of the cheeses is evident. This variation was reflected in the flavor and aroma of the cheeses. Sample B was mild in flavor and was criticized for not having typical Blue cheese flavor. The flavor of the Roquefort sample (D) was considerably different from that of the Blue cheese samples. It lacked the strong pungent flavor and aroma of butyric acid and was more characteristic of the 8:0 and 10:0 fatty acids. This observation is borne out by calculations of the relative mole

percentages of each acid. These percentage are given in Table 7. The Blue cheese samples contained relatively high percentages of butanoic acid compared to the Roquefort sample, i. e., 7. 2, 9. 0, and 9. 8 versus 3. 9%. The Roquefort sample contained more 10:0 acid (8. 3%) than the three Blue cheeses (4. 1, 4. 2, and 4. 1%). The same was true of the 8:0 acid. Hexanoic acid appears to be in about the same concentrations in all samples. These results might be expected in view of the composition of sheeps' milk. Hilditch (1956) states that sheeps' milk is low in butanoic acid and has high proportions of octanoic and decanoic acids, compared to cows' milk. Similar conclusions were reached by Sadini (1963), Kuzdzal and Kuzdzal (1963), and Benassi (1963). These differences in fatty acid composition may partially account for the different flavor of Roquefort and Blue cheese.

Table 7. Mole percentages of free fatty acids in Blue-vein type cheese.

Acid	Cheese			
	A (Blue)	B (Blue)	C (Blue)	D (Roquefort)
4:0	7. 2	9. 0	9. 8	3. 9
6:0	3. 8	4. 5	4. 4	3. 9
8:0	2. 6	2. 9	2. 9	4. 7
10:0	4. 1	4. 2	4. 1	8. 3
12:0	4. 2	4. 4	4. 2	4. 9
14:0	13. 6	12. 8	14. 0	10. 1
16:0	25. 7	26. 9	27. 2	20. 3
18:0	8. 6	8. 1	7. 5	7. 1
18:1	25. 4	24. 3	22. 2	26. 8
18:2	2. 4	1. 4	2. 0	4. 2
18:3	2. 4	1. 4	1. 6	5. 8

Isovaleric acid was identified in dry Blue cheese by Coffman, Smith and Andrews (1960). However, when using a beta ionization detector for greater sensitivity in gas chromatography analysis, there was no evidence for isovaleric acid in any of the cheeses analyzed in this investigation. No formic or propionic acids were detected in any of the samples.

It is important to note that the entire quantity of fatty acids does not exist as free acids in cheese. Considering the average pKa of the major acids to be 4. 8, and a pH range of 5. 3 to 5. 6 in the cheese, it is possible that between 75 and 85% of the acids exist as salts. Direct conformation to the Henderson-Hasselbalch equation is doubtful due to the complexity of the cheese system, however, the basic principle is important. The odor resulting from volatile short chain acids would be reduced considerably and the flavor properties of the long chain acids would be altered. The salts of long chain acids are soaps and since they possess definite flavor properties, it is probable that they lend a characteristic background flavor to Blue cheese.

Quantitation of Methyl Ketones in Blue Cheese Fat

The recoveries of the individual methyl ketones from the standard mixture at the completion of analysis is given in Table 8. The average percentage recovery was used in the calculation of the ketone concentrations of the cheese. Results of the quantitative

analysis of the C_3, C_5, C_7, C_9, and C_{11} methyl ketones in seven Blue-vein type cheese samples are presented in Table 9. The data are given as the average micromoles of ketone/10 g cheese fat as determined by duplicate analyses. The average percent deviation of the duplicates from their mean was: acetone, 15.2; 2-pentanone, 6.3; 2-heptanone, 4.0; 2-nonanone, 7.0; 2-undecanone, 9.5.

Table 8. Percentage recovery of individual methyl ketones at completion of analytical procedure.

Methyl ketone chain length	Percentage recovery		
	A	B	Average
3	62.5	67.4	64.9
5	81.1	83.7	82.4
7	90.3	92.9	91.6
9	93.4	95.8	94.6
11	96.4	98.6	97.5

Table 9. Concentration of methyl ketones in fat extracted from Blue-vein type cheese.

Methyl ketone chain length	Micromoles/10 g of extracted fat[b]						
	Cheese[a]						
	A	B	C	D	E	F	G
3	1.7	1.5	2.5	1.0	1.6	1.3	0.0
5	6.3	2.6	9.0	2.6	7.0	6.2	1.5
7	10.5	5.2	23.3	5.4	11.8	17.0	5.5
9	5.8	4.9	23.0	4.8	10.3	15.4	3.5
11	1.1	1.1	6.5	1.0	2.5	1.1	0.5

[a]Samples A-E were domestic Blue cheese
[a]Sample F and G were imported Roquefort cheese
[b]Average of duplicate analyses

The results show a large variation in the total quantity of ketone in the cheeses. Heptanone-2 was the most abundant ketone in all the cheeses. Schwartz and Parks (1963) made the same observations on three cheese samples. The high ketone content of sample C was reflected in a distinct ketone flavor and aroma of the cheese. It is of interest to note that although flavor variations were observed, all the cheeses were judged as acceptable. This points out the importance of "flavor balance", i.e., the importance of the relative amounts of flavor compounds in a food, rather than the total amounts.

Some interesting observations can be made by comparing the mole percentages of the methyl ketones and free fatty acids from the same cheese sample. Since the fatty acids are known to be the precursors of the methyl ketones, it would be expected that the relative amount of each ketone formed would be nearly equal to the relative amount of fatty acid present. The free fatty acids found in cheeses examined were present in approximately the same ratio as they are in milk triglycerides. However, the methyl ketones were not present in the same ratio as their precursors. Data illustrating this point are presented in Table 10. It can be seen in the first three samples that butanoic acid constitutes a high percentage of the acids while acetone represents a relatively small portion of the ketones present. Similarly, the amount of 12:0 acid is high compared to

Table 10. Comparison of the mole percentages of the methyl ketones and free fatty acids found in Blue-vein type cheese.

Cheese[a]	Acid chain length	Mole % acid in milk fat[b]	Mole % acid in cheese	Ketone chain length	Mole % ketone in cheese
A	4	44.3	32.8	3	6.8
	6	12.2	17.4	5	24.8
	8	9.0	12.0	7	41.3
	10	13.2	18.7	9	22.8
	12	21.4	19.2	11	4.2
B	4	44.3	36.0	3	10.1
	6	12.2	18.1	5	16.8
	8	9.0	11.5	7	33.8
	10	13.2	16.9	9	32.2
	12	21.4	17.6	11	7.2
E	4	44.3	38.7	3	4.7
	6	12.2	17.2	5	21.2
	8	9.0	11.6	7	35.7
	10	13.2	15.9	9	31.2
	12	21.4	16.6	11	7.1
F	4	44.3	15.1	3	3.2
	6	12.2	15.2	5	15.1
	8	9.0	18.5	7	41.4
	10	13.2	32.3	9	37.4
	12	21.4	19.0	11	2.8

[a]Samples A-E were domestic Blue cheese.

[a]Sample F was an imported Roquefort cheese.

[b]Calculated from data compiled by Jack and Smith (1956).

that of the C_{11} ketone. Conversely, the 6:0, 8:0, and 10:0 acids represent a smaller percentage of the fatty acids while the C_5, C_7, and C_9 ketones constitute a high percentage of the total ketones. This is especially true of the 8:0 acid and the C_7 ketone. Similar observations can be made concerning Sample F, a Roquefort cheese. It is interesting to note that the percentage of decanoic acid in the Roquefort cheese was nearly double that of Blue cheese, but the concentration of 2-nonanone in the Roquefort was only slightly increased over that of the Blue cheese.

It would appear that the amount of ketone formed by P. roqueforti spores during cheese curing is not directly related to the amount of fatty acid available. Instead, there seems to be a selective conversion of certain fatty acids to ketones which is related to the chain length of the acid. Octanoic acid appears to be converted to 2-heptanone most readily. Hexanoic and decanoic acids are transformed to 2-pentanone and 2-nonanone respectively, at a slightly reduced rate, while butanoic and dodecanoic acids are converted to ketones least readily. No data on the amount of ketone produced from various fatty acids under controlled conditions are available in the literature. However, Girolami and Knight (1955) found that oxygen uptake during incubation of P. roqueforti with fatty acid substrates increased as the fatty acid chain length went from four to nine and then decreased as the carbon number increased further.

Quantitation of Secondary Alcohols in Blue Cheese Fat

The concentration of the secondary alcohols, 2-pentanol, 2-heptanol, and 2-nonanol in Blue cheese was determined by a semi-quantitative gas chromatographic method. The previously quantitated methyl ketones acted as internal standards and facilitated the use of chromatogram peak areas for calculation of the alcohol concentrations.

Recorder response was found to be linear to concentration in the ranges being investigated. However, the alcohols and the corresponding ketones were recovered from cheese fat at different rates. Therefore, standard solutions containing the ketone and the corresponding alcohol were carried through the analytical procedure to determine recovery factors. Three concentrations (5, 10, and 20 micromoles/10 g of oil) of each of the C_5, C_7, and C_9 ketone-alcohol standards were analyzed. Each concentration was run in duplicate. The peak area of the ketone was set equal to 1.00 and the average recovery factor for each alcohol was calculated. The factors are given in Table 11.

Results of the cheese analyses are given in Table 12. Representative chromatograms (sample E) are shown in Figure 6. The data represent the average concentration of 2-pentanol, 2-heptanol, and 2-nonanol in micromoles/10 g cheese fat. The average percent

deviation of the duplicates from their mean was: 2-pentanol, 4. 7; 2-heptanol, 5. 0; 2-nonanol, 9. 7. The data show that the alcohols are present in much lower concentrations than the ketones (see Table 9).

Table 11. Recovery factors for correcting alcohol peak area when using methyl ketones as internal standards for quantitation of secondary alcohols.

Alcohol	Recovery factor
2-Pentanol	1. 60
2-Heptanol	3. 77
2-Nonanol	1. 52

Table 12. Concentration of secondary alcohols in fat of Blue-vein type cheese.

Secondary alcohol chain length	Micromoles/10 g of fat[a]						
	Cheese[b]						
	A	B	C[c]	D	E	F[c]	G
5	0. 2	0. 1	0. 8	0. 5	0. 1	0. 2	0. 1
7	1. 6	2. 2	2. 9	2. 9	0. 9	1. 0	1. 3
9	0. 8	0. 8	1. 1	0. 6	0. 6	---[d]	---[d]

[a]Average of duplicate analyses

[b]Samples A-E were domestic Blue cheese

[b]Samples F and G were imported Roquefort cheese

[c]Single analysis due to limited sample

[d]Peak area could not be measured

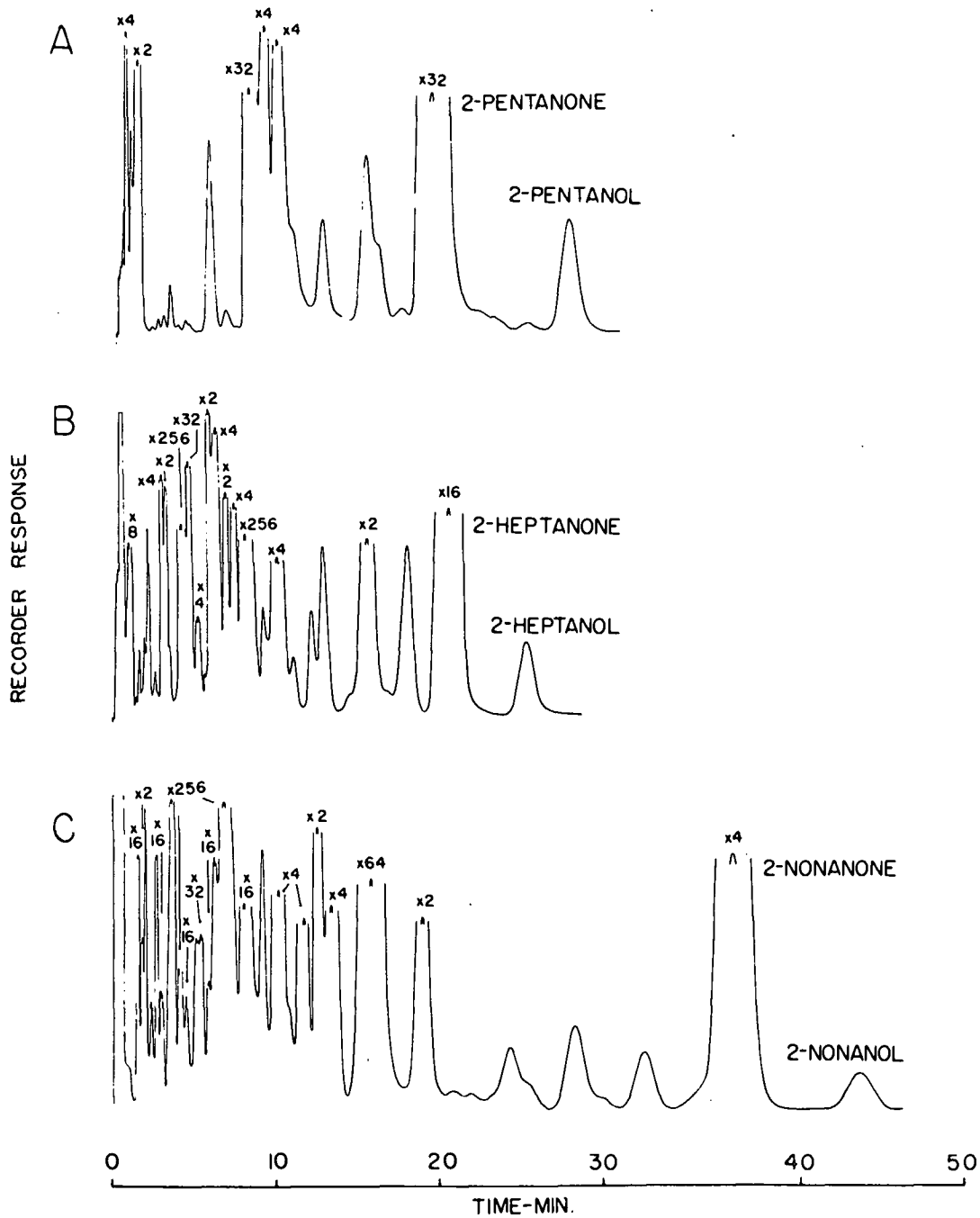

Figure 6. Gas chromatograms of Blue cheese fat showing the C_5, C_7, and C_9 methyl ketone and secondary alcohol peaks.

A comparison of the relative percentages of the C_5, C_7, and C_9 methyl ketones and the relative percentages of the C_5, C_7, and C_9 secondary alcohols is given in Table 13. The C_7 alcohol is predominant in the alcohol fraction just as the C_7 ketone is predominant in the ketone series. The relative percentage of alcohols is similar to that of the ketones, however 2-pentanol is lower than expected. Although the C_5 standards were allowed to partition into a water phase similar to that of cheese (12% NaCl), the exact ratio of partition during centrifugation of the cheese cannot be determined. A greater partitioning of 2-pentanol into the aqueous phase of the cheese than into the aqueous phase of the standards would account for the low concentration of 2-pentanol found in the cheese. Although the partitioning of the C_7 and C_9 alcohols and ketones during centrifugation cannot be determined exactly, it is of minor significance because of their water insolubility.

Another source of error which is inherent to quantitation by gas chromatography is the enlargement of the peak area of a supposedly single compound by other components with the same retention time. Initially, conditions were set up so that the compounds to be studied were well separated from other components in the cheese being used. However, for any given cheese it cannot be unequivocally assumed that the same peaks are devoid of contaminating compounds. If beta-ketoacids were decarboxylated by heat during

Table 13. Comparison of the mole percentages of the methyl ketones and secondary alcohols in Blue cheese.

Cheese[a]	Chain length	Mole % ketone	Mole % alcohol
A	5	27.9	9.0
	7	46.4	60.9
	9	25.6	30.1
B	5	20.3	3.5
	7	40.8	70.9
	9	38.9	25.6
C	5	16.2	16.0
	7	42.1	61.2
	9	41.7	22.8
D	5	20.2	12.9
	7	42.3	72.2
	9	37.5	14.9
E	5	24.1	7.7
	7	40.6	54.4
	9	35.4	37.9

[a]All samples were domestic Blue cheese.

purging the fat, methyl ketones would be produced which would enlarge the ketone peak area. This should not be a problem, however, in view of the work of Schwartz, Parks and Boyd (1963). They found that no methyl ketones were produced in Blue cheese fat that was heated to 100° C for 40 hr under nitrogen. They concluded that all the beta-ketoacids had been converted to methyl ketones by microbial action.

It will be noted that no value is given for 2-nonanol in the Roquefort cheeses, Samples F and G, Table 12. There appeared to be a small amount of 2-nonanol present in both samples, however, a large peak in the chromatogram prevented measurement of the alcohol peak area. The interfering peak is evident in Figure 7. There was no interfering peak in any of the domestic Blue cheese samples, a representative chromatogram of which is shown in Figure 6. It appears that the interfering peak was unique to the Roquefort cheese samples. No attempt was made to identify the compound. It is possible that this compound, along with others, may be involved in flavor differences between Blue and Roquefort cheese.

The headspace entrainment technique used in this study would provide an excellent means of obtaining comparative chromatograms of cheese fat samples. Since the fat samples are purged and the volatiles immediately trapped on the GLC column, the method gives

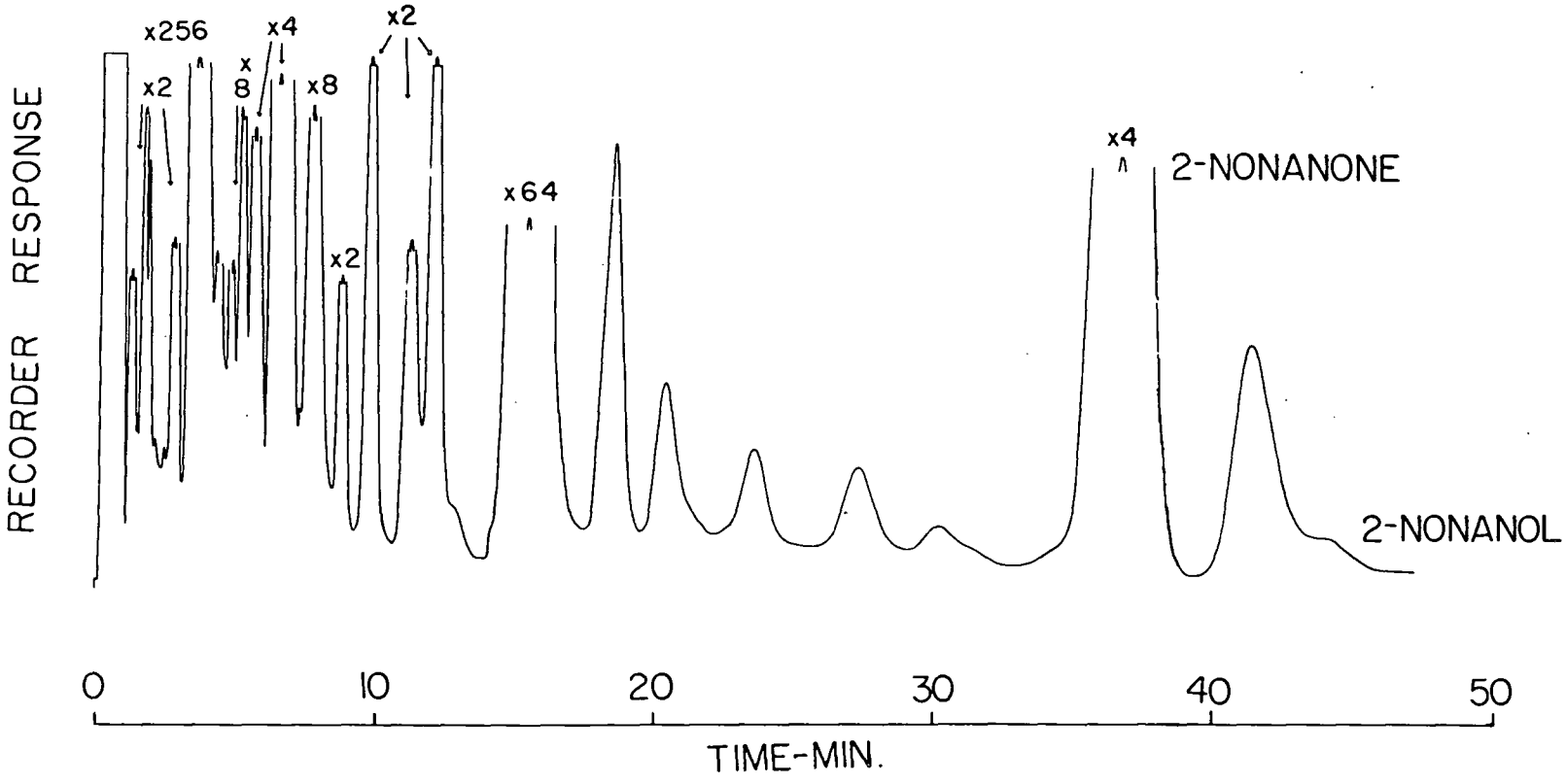

Figure 7. Gas chromatogram of Roquefort cheese fat showing the C_9 methyl ketone and secondary alcohol peaks.

a semi-quantitative profile of the flavor compounds. This technique can also be used with mass spectrometry to provide positive identification of components. The identification of all the flavor compounds that are responsible for Roquefort cheese flavor could be of significant value. Use of cows' milk and a flavor supplement to produce a domestic Blue-vein type cheese with the flavor and aroma of imported Roquefort might enlarge the market for Blue-vein type cheese in the U.S.A. and be of economic value to the dairy industry.

Evaluation of Synthetic Blue Cheese Flavor

The qualitative and quantitative data obtained thus far provided a starting point for making a synthetic Blue cheese flavor. Selected compounds were added to a base of dry curd cottage cheese, butterfat, cream, and salt in an attempt to duplicate Blue cheese flavor. The first mixture contained methyl ketones, secondary alcohols, and the 2:0-14:0 fatty acids in the same quantities as the average concentrations found in cheeses A, B, and C (Table 6) and A, B, and E (Table 9 and 12). The sample was not at all typical in flavor, and exhibited a very strong, bitter, soapy flavor. The soapiness was especially noticeable as an after-taste and was quite objectionable.

The next mixture was prepared using only the 2:0-10:0 fatty acids. This reduced the soapy taste considerably, however it was still evident as an off flavor. It was found that the bitter flavor and

soapiness was reduced by lowering the total free fatty acid concentration to two-thirds that found in the cheese samples. The reduction in acids also made the ketone flavor more predominant, which was judged desirable for the samples. Further samples were prepared using only the 2:0-8:0 fatty acids at two-thirds the concentration found in cheese in an attempt to alleviate the problem of soapiness. Elimination of the 10:0 acid from the mixture reduced the amount of soapiness to a point where it was no longer considered a defect. Normal Blue cheese has a certain amount of soapy character, however, it is at such a level and balanced with other flavor components so that it is not objectionable.

Although the samples initially prepared with acids, alcohols, and ketones were similar to Blue cheese in flavor and aroma, they were lacking certain characteristics which are typical of Blue cheese. It was found that the addition of 2-phenylethanol, ethyl butanoate, methyl hexanoate, and methyl octanoate gave a very desirable character to the samples. The sharpness of the acids was reduced and the overall flavor and aroma of the samples was much more typical of Blue cheese when the esters and 2-phenylethanol were present.

The pH of the synthetic mixtures was relatively low (pH 4. 7) compared to Blue cheese. Therefore, samples were prepared in which the pH was raised to pH 5. 6 with NaOH. It was thought that the samples might become soapy at a higher pH, but no increase in

soapy flavor was observed. Little or no flavor difference was evident between the samples at pH 4.7 and those at pH 5.6.

The mixture judged to be most typical of Blue cheese is given in Table 14. The 2:0-8:0 fatty acids were used at two-thirds the concentrations found in Blue cheese. The C_3-C_{11} methyl ketones and the C_5-C_9 secondary alcohols were used at twice the concentrations found in the cheese samples. The amount of esters and 2-phenylethanol used was estimated from their relative peak heights in gas chromatograms. These estimated amounts were then altered as dictated by the flavor of the samples.

It can be seen that the concentration of the compounds in the synthetic mixture and the natural cheese were not the same. In the case of the acids, there was also a qualitative difference since the 2:0-18:3 acids are present in cheese, but only the 2:0-8:0 acids were used in the synthetic mixture. When the 10:0, 12:0, and 14:0 fatty acids were added to the mixture, a pronounced soapy flavor resulted even though they were present in the same concentrations as in cheese. This soapy flavor probably results from the fatty acids being distributed differently in the synthetic mixture than in cheese. When fatty acids are released by lipolysis in cheese, they may remain closely associated with the fat globule, where, even though they may be in the salt form, their flavor properties are such that they do not exert a soapy flavor as they do when added directly to

Table 14. Compounds used in synthetic Blue cheese flavor.

Compounds	Concentration-mg/kg	
	Added to mixture	Found in cheese
Acetic acid	550	826
Butanoic acid	964	1448
Hexanoic acid	606	909
Octanoic acid	514	771
Acetone	6.2	3.1
2-Pentanone	30.3	15.2
2-Heptanone	69.5	34.8
2-Nonanone	66.3	33.1
2-Undecanone	17.0	8.5
2-Pentanol	0.9	0.4
2-Heptanol	12.1	6.1
2-Nonanol	7.0	3.5
2-phenylethanol	2.0	---
Ethyl butanoate	1.5	---
Methyl hexanoate	6.0	---
Methyl octanoate	6.0	---

fat. Although the concentration of compounds in a food can be determined, knowledge of the distribution and state in which these compounds exist in a complex system such as cheese is a major problem in reproducing natural flavors.

The mixture given in Table 14 closely resembles a typical Blue cheese flavor and aroma, however, it did not duplicate the flavor and aroma of a high quality Blue cheese. Several reasons for the differences are obvious.

1. No proteolysis occurred in the synthetic mixture. As a result no significant amount of free amino acids, sulfur compounds or other proteolysis products which have flavor properties were present in the synthetic mixture.

2. The solids content of the synthetic base was lower than in cheese in order to facilitate blending and addition of the flavorings.

3. The complete complement of compounds identified in Blue cheese was not used in the mixture. The compounds used were selected because of their high concentration in cheese or in the case of 2-phenylethanol, because of its obvious Blue cheese like character. The addition of other esters and selected aldehydes and alcohols would probably help to make the flavor of the synthetic mixture more like natural Blue cheese.

Influence of Selected Microorganisms on Certain Blue Cheese Flavor Compounds and Identification of Volatiles from Culture Headspace

It is well established that methyl ketones are of considerable importance in the flavor of Blue cheese. Hence, a change in the concentration of the ketones, e. g., by reduction to their secondary alcohol analogs, could have an effect on the flavor of a cheese. It was therefore of interest to determine what influence certain microorganisms associated with cheese curing would have on a representative methyl ketone. Pentanone-2 was selected for this purpose.

The ketone concentration of Blue cheese could be affected by several classes of organisms:

1) the Penicillium mold, both spores and mycelia;

2) organisms growing on the cheese surface--bacteria, yeasts, and other molds;

3) lactic starter organisms used in the cheese manufacture.

Representative members of each type were tested for their ability to reduce 2-pentanone to 2-pentanol and oxidize 2-pentanol to 2-pentanone. In conjunction with this work, the major components appearing in the chromatograms of cultures were identified by gas chromatographic and mass spectrometric analyses.

Effect of Growing Penicillium roqueforti Cultures: Figure 8 shows the chromatograms of headspace from P. roqueforti cultures

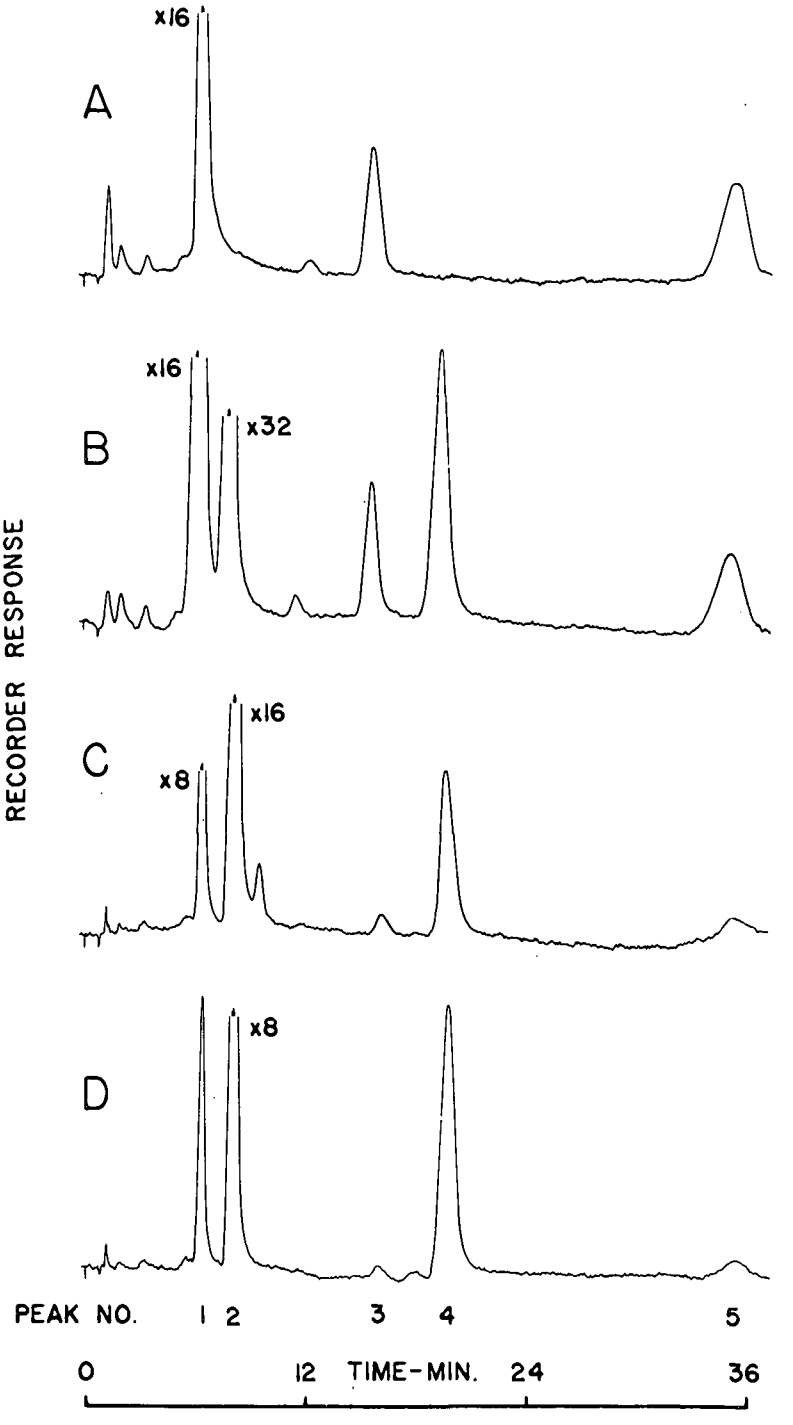

Figure 8. Headspace chromatograms of Penicillium roqueforti cultures. A) P. roqueforti only; B) contained added hexanoic acid; C) contained added 2-pentanone; D) contained added 2-pentanol. See Table 15 for peak identifications.

containing various substrates. Chromatogram A represents the control of Penicillium with no added substrate. Peak number one is ethanol, three is isobutanol, and five is a mixture of 2-methyl and 3-methyl butanol. Chromatogram B is of a culture to which hexanoic acid had been added and it is evident that two new compounds are present; peak number two is 2-pentanone and three is 2-pentanol. Chromatogram C is of a culture containing added 2-pentanone. Although the 2-pentanone was redistilled, the peak following 2-pentanone (chromatogram C, peak 2) was a contaminant which could not be removed. The appearance of peak number 4 in the GLC pattern indicates that a portion of the added 2-pentanone was reduced to 2-pentanol. Chromatogram D is the pattern obtained from a culture containing added 2-pentanol, and it can be seen that 2-pentanone was produced. Peak identities for Figure 8 are given in Table 15.

Table 15. Compounds identified in headspace of Penicillium roqueforti cultures. See Figure 8 for chromatogram.

Peak No.	Compounds			
	Chromatogram			
	A	B	C	D
1	Ethanol	Ethanol	Ethanol	Ethanol
2		2-pentanone	Added 2-pentanone	2-pentanone
3	Isobutanol	Isobutanol	Isobutanol	Isobutanol
4		2-pentanol	2-pentanol	Added 2-pentanol
5	2 and 3 methyl butanol	2 and 3 methyl butanol	2 and 3 methyl butanol	2 and 3 methyl butanol

Effect of Penicillium roqueforti Spores and Mycelia in Phosphate Buffer: Figure 9 shows the individual effect of the P. roqueforti spores and nonsporulating hyphal cells on 2-pentanone-2-pentanol interconversions. Chromatogram A represents the headspace of mycelia after incubation in a phosphate buffer containing added 2-pentanone, and it is evident that 2-pentanol (peak number 2) was produced. Pattern B shows that incubation of the mycelia with added 2-pentanol resulted in formation of 2-pentanone. The peak immediately before 2-pentanol (chromatogram B, peak 2) was a contaminant in the alcohol. When spores were incubated in phosphate buffer with 2-pentanone only a trace of 2-pentanol was formed (chromatogram C). Incubation of spores with 2-pentanol resulted in production of 2-pentanone as shown in chromatogram D.

Effect of Growing Mycoderma Cultures: Figure 10 shows the chromatograms of headspace from a Mycoderma sp. culture. Identification of the peaks is given in Table 16. Pattern A is of the control and no 2-pentanone or 2-pentanol is evident. Peak number one is ethyl acetate, two is ethanol, four is isobutanol, and five is a mixture of 2-methyl and 3-methyl butanol. Chromatogram B represents a culture containing added hexanoic acid and no ketone or alcohol was produced. In pattern C it can be seen that a portion of the added 2-pentanone (peak 3) was reduced to 2-pentanol (peak 5). It is evident from chromatogram D that a portion of the added 2-pentanol

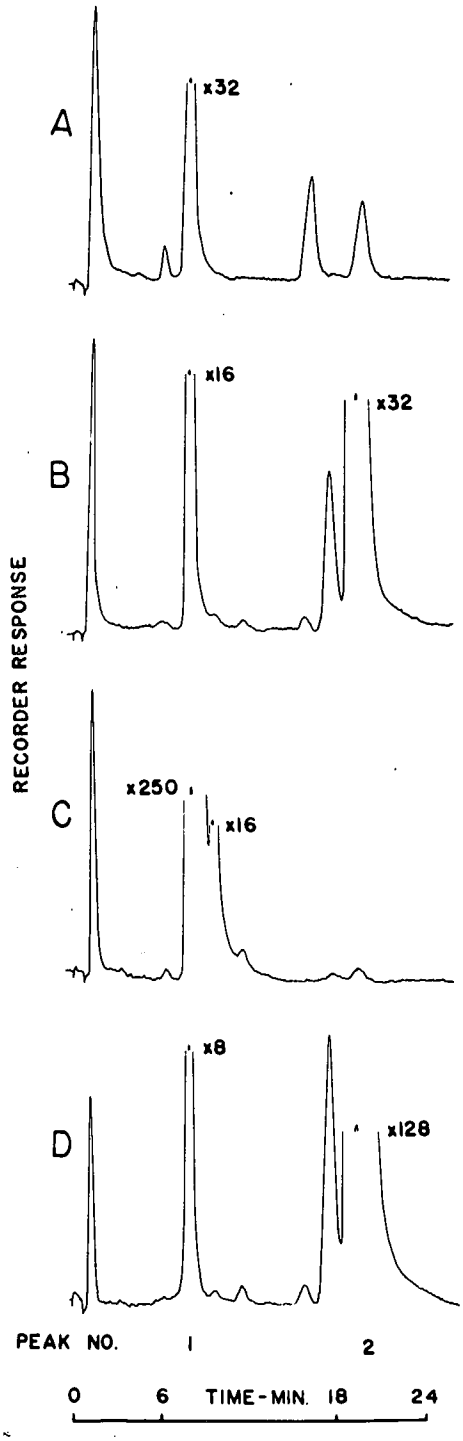

Figure 9. Headspace chromatograms of Penicillium roqueforti incubated in phosphate buffer. A) mycelia + 2-pentanone; B) mycelia + 2-pentanol; C) spores + 2-pentanone; D) spores + 2-pentanol.

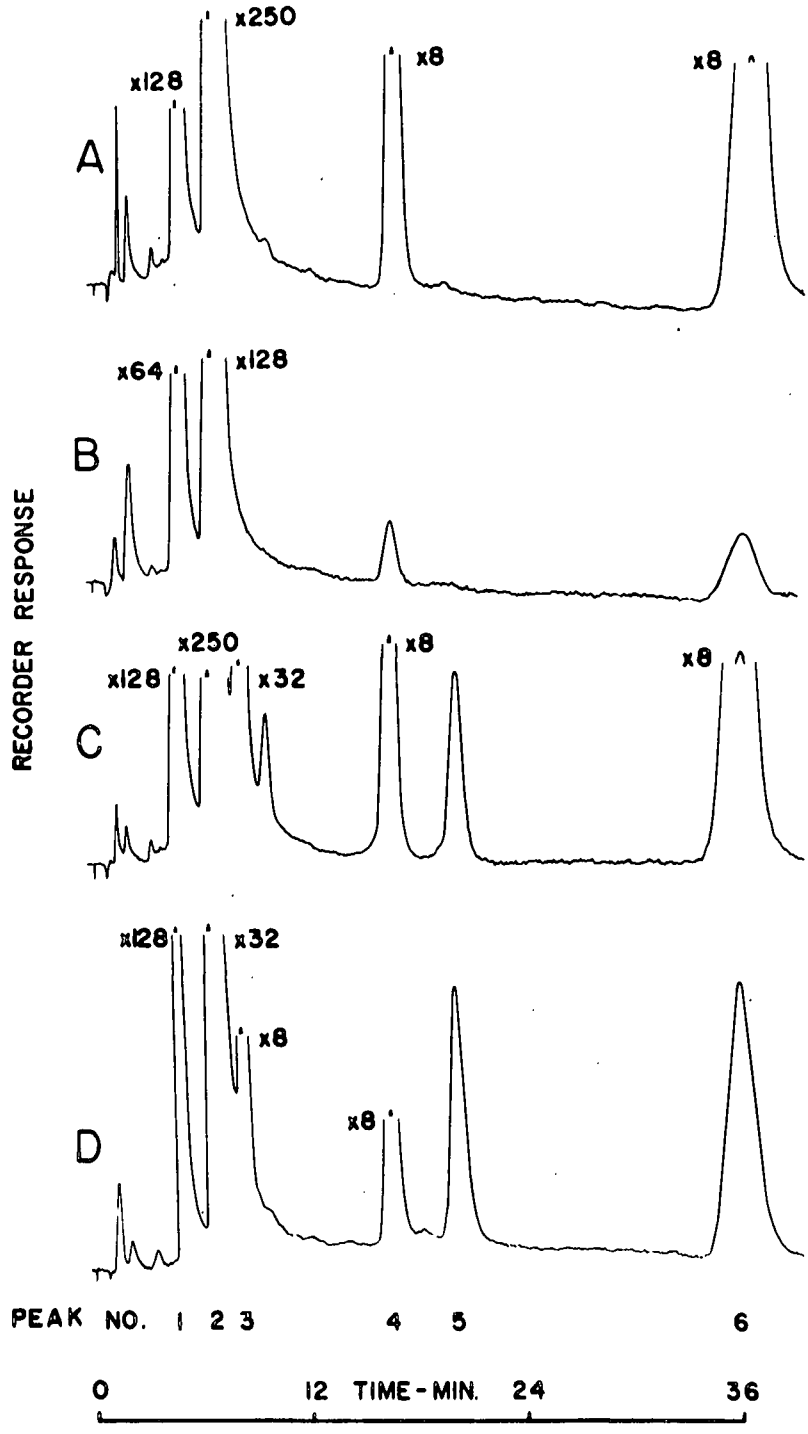

Figure 10. Headspace chromatograms of Mycoderma sp. cultures. A) Mycoderma only; B) contained added hexanoic acid; C) contained added 2-pentanone; D) contained added 2-pentanol. See Table 16 for peak identifications.

was converted to 2-pentanone.

Table 16. Compounds identified in headspace of Mycoderma sp. cultures. See Figure 10 for chromatogram.

Peak No.	Compounds Chromatogram A	B	C	D
1	Ethyl acetate	Ethyl acetate	Ethyl acetate	Ethyl acetate
2	Ethanol	Ethanol	Ethanol	Ethanol
3			Added 2-pentanone	2-pentanone
4	Isobutanol	Isobutanol	Isobutanol	Isobutanol
5			2-Pentanol	Added 2-pentanol
6	2 and 3-methyl butanol	2 and 3-methyl butanol	2 and 3-methyl butanol	2 and 3-methyl butanol

In each trial one sample of media containing 2-pentanone and one containing 2-pentanol was incubated and analyzed under the same conditions as the cultures. No oxidation of the alcohol or reduction of the ketone was detected in any case.

The interconversion of acetone and 2-propanol was also determined. Chromatographic analysis was done on a column of 20% 1, 2, 4-butanetriol on 80/100 mesh acid-alkali washed Celite 545. This column gave good separation of ethanol and 2-propanol as well as 2-methyl and 3-methyl butanol for subsequent mass spectral

identification. However, it was necessary to use the 15% Carbowax 1500 on 80/100 mesh acid-alkali washed Celite column to check for conversion of 2-propanol to acetone in the Mycoderma and Torulopsis cultures. This was because the acetone peak was covered by ethyl acetate on the butanetriol column.

A summary of the compounds identified in culture headspace and the ketone-alcohol interconversions observed is given in Table 17. The Mycoderma, Torulopsis, Geotrichum, and Penicillium cultures actively interconverted 2-pentanone and 2-pentanol. The B. linens culture converted 2-pentanol to 2-pentanone but had no effect on 2-pentanone, acetone or isopropanol. S. lactis had no effect on either the ketone or the alcohol. Penicillium spores converted 2-pentanol to 2-pentanone but only slightly reduced 2-pentanone to 2-pentanol. The mycelia actively interconverted the ketone and alcohol. It appears that the mycelia are most active in reducing the methyl ketone to the secondary alcohol, after the ketone has been produced by the spore. Girolami and Knight (1955) found that the methyl ketones produced by P. roqueforti were themselves inhibitory to mold growth. It is possible that the reduction of methyl ketones to secondary alcohols acts as a detoxication mechanism by reducing the ketone concentration.

It is of interest to note that chromatograms of the Bacterium linens culture were essentially devoid of any peaks other than added

Table 17. Compounds identified and ketone-alcohol interconversions observed with certain microorganisms associated with Blue cheese.

Compound identity	Growing culture						In phosphate buffer	
	Mycoderma	Torulopsis sphaerica	Geotrichum candidum	Bactium linens	Streptococcus lactis	Penicillium roqueforti	Penicillium roqueforti	
							Spores	Mycelia
Ethyl Acetate	+	+	-	-	-	-		
Ethanol	+	+	+	-	-	+		
Isobutanol	+	+	+	-	-	+		
2-Methyl butanol	+	+	+	-	-	+		
3-Methyl butanol	+	+	+	-	-	+		
Conversion observed								
2-pentanone → 2-pentanol	+	+	+	-	-	+	+ (weak)	+
2-pentanol → 2-pentanone	+	+	+	+	-	+	+	+
Acetone → 2-propanol	+	+	+	-	-	+		
2-propanol → acetone	+	+	+	-	-	+		

substrates. The organism is known to be proteolytic and to play a predominant role in curing surface ripened cheeses (Foster et al. 1957). The culture itself had a very strong aroma and probably contained H_2S, which would not have been detected with the flame ionization detector, along with other compounds which were not eluted from the GLC column. Higher molecular weight compounds were not detected because headspace samples were used.

Yeasts are known to indirectly affect the ripening of Limburger cheese by stimulating the growth of Bacterium linens (Purko, Nelson and Wood, 1951). A similar stimulation of Penicillium roqueforti was noted by Prokš, Doležálek and Pech (1959a). Results of the present study indicate that yeasts may affect flavor of Blue cheese in several ways. The reduction of methyl ketones to secondary alcohols could affect the flavor by reducing the ketone concentration and by producing a new class of compounds. Blue cheese samples can be found that are overpowering in the distinct flavor of methyl ketones. A partial explanation of this might be that conditions during curing were such that sufficient reduction of the ketones to alcohols did not occur. The concentration of secondary alcohols in a cheese might be influenced by the amount of yeast growth on the cheese since the yeasts appear to actively reduce methyl ketones to secondary alcohols. The pH of the curing cheese might also affect the ketone-alcohol equilibrium by influencing the enzymes

responsible for the reduction.

The growth of yeasts on Blue cheese can have a more direct influence on flavor by production of flavor compounds such as ethanol, isobutanol, 2-methyl and 3-methylbutanol, and ethyl acetate. Ethanol is especially important due to its part in ester formation. The aldehyde analogs of the aforementioned alcohols, i. e., ethanal, isobutanal, and 2-methyl and 3-methylbutanal, have very distinct flavor and aroma properties and may be important in Blue cheese flavor. It should be mentioned that although the formation of alcohols by transamination and decarboxylation of amino acids is well documented, Ingraham and Guymon (1960) have found other modes of alcohol formation. Their data indicated that a portion of the alcohols are formed from carbohydrate and are intermediates in amino acid synthesis. They state that it is likely that over two-thirds of the higher alcohols formed in grape juice media are formed from the carbohydrate.

Significance of Flavor Chemistry Investigations on Blue Cheese

Blue cheese is the most important of the Blue-vein type cheeses consumed in the United States. Kondrup et al. (1964) state that production for 1961 was approximately 16 million pounds and more than four million pounds were imported. Under present manufacturing conditions Blue cheese is aged at least 90 days. This

inventory of ripening cheese represents an economic liability to the manufacturer. The primary reason for aging cheese is the development of flavor, therefore, any method whereby the aging time can be shortened or the flavor development controlled is of considerable economic advantage to the manufacturer. The first step in realizing "controlled flavor development" is to know what compounds are responsible for flavor and their levels of concentration in a food. A step in this direction has been made with Blue cheese.

There are several specific ways in which the knowledge of Blue cheese flavor could be used. First, it may be possible by use of flavor supplements, to make a Blue-vein type cheese from cows' milk which has the flavor of Roquefort cheese. Development of such a cheese could lead to increased utilization of dairy products in this country.

Research has been conducted on methods of reducing labor and decreasing the time required for Blue cheese manufacture. Graham and Rowland (1959) and Janzen (1964) have developed a process whereby the draining, hooping and inoculation of Blue cheese curd is done mechanically. In an effort to reduce curing times Kondrup et al. (1964) has developed a quick-ripened loose curd Blue cheese. Curing time for this method is ten days rather than the normal three months.

In the quick-ripen method, pasteurized milk is used and normal

manufacturing procedures are followed until after the curd is cut. The whey is then drained and instead of placing the curd in hoops it is placed on a fine mesh stainless steel screen at a depth of three to four inches. The trays are put in ripening rooms where salt is added and the curd is stirred periodically. At the end of ten days the cheese is packaged in polyethylene bags and stored at 45° F. The authors indicated that the quick-ripened cheese was milder in flavor than normal Blue cheese. The addition of selected flavor compounds to a cheese made by a process similar to the quick-ripen method might lead to the production of a high quality Blue-vein type cheese at a much reduced cost. Compounds such as the methyl and ethyl esters, which would be formed during the long aging period of normal cheese, might be of value in making a quick-ripened cheese with the same flavor as normally cured Blue cheese.

SUMMARY AND CONCLUSIONS

Gas liquid chromatography with packed and capillary columns was used to separate the volatile neutral flavor components from Blue cheese fat. The flavor compounds were identified by comparison of retention times with authentic compounds and by mass spectral analysis.

The major free fatty acids ranging from acetic to linolenic were quantitatively measured in four Blue-vein type cheeses. A combination of liquid-liquid column chromatography and gas-liquid chromatography was used for the analyses.

The concentration of odd carbon number methyl ketones, ranging from acetone to 2-undecanone, was determined for seven Blue-vein type cheeses. Four of the samples were the same ones analyzed for fatty acids. The method consisted of extracting the cheese fat, formation of the 2, 4-dinitrophenylhydrazones, removal of the fat, isolation of the methyl ketone hydrazones, separation of the methyl ketone derivatives into individual members, and measuring the concentration of the hydrazone by absorption spectrophotometry. A combination of adsorption and liquid-liquid chromatography was used in the procedure.

The concentration of the C_5, C_7, and C_9 secondary alcohols in Blue cheese fat was determined in the same cheeses used for

ketone analysis. The previously measured methyl ketones acted as internal standards and facilitated a semi-quantitative calculation of alcohol concentration from peak areas of gas chromatograms.

Certain microbiological aspects of Blue cheese curing were investigated. Gas chromatography was used to determine the effect of selected microorganisms on a class of Blue cheese flavor compounds, the methyl ketones.

The findings of the investigation were the following:

1. A total of 31 compounds were positively identified from the neutral volatile fraction of Blue cheese fat. Nineteen compounds were tentatively identified. The main classes of compounds were methyl ketones, secondary alcohols, methyl esters, and ethyl esters. The methyl and ethyl esters had not been previously identified in Blue cheese.

2. The concentration of free fatty acids in Blue cheese was found to vary considerably. The relative composition of the free fatty acids appear to be nearly the same as they are in the milk triglycerides. Roquefort cheese contains more octanoic and decanoic acid and less butyric acid than Blue cheese. This difference in fatty acid composition appears to be one of the reasons for the flavor difference between Roquefort and Blue cheese.

3. A large variation in the concentration of methyl ketones in Blue cheese was observed. No distinct difference in methyl

ketone concentrations were evident between Blue and Roquefort cheese. The amount of individual methyl ketones formed during curing is not directly related to the available concentration of their individual precursors. Heptanone-2 was the predominant ketone in all cheeses, yet its precursor, octanoic acid, represents a small portion of the total free fatty acids.

4. The concentrations of 2-pentanol, 2-heptanol, and 2-nonanol in Blue cheese exhibited considerable variation and were present in much lower concentrations than their methyl ketone analogs. The secondary alcohols appeared to be present in the same ratio as the methyl ketones.

5. An acceptable Blue cheese flavor does not depend entirely on the total amount of flavor compounds present, but rather the relative amounts of each compound present. A reasonable duplication of Blue cheese flavor and aroma can be obtained by a blend of selected fatty acids, methyl ketones, secondary alcohols, 2-phenylethanol and methyl and ethyl esters in a cottage cheese and butterfat base. The known compounds could not be used in the same concentrations as found in cheese. The long chain fatty acids (10:0 and higher) were not used and the total acid concentration was reduced by one-third.

6. The mycelia of Penicillium roqueforti appear to be more active in the reduction of methyl ketones to secondary alcohols than the spores. Yeasts associated with Blue cheese are capable

of reducing methyl ketones to secondary alcohols. Yeasts also may play a role in Blue cheese flavor by producing ethanol and other alcohols, and certain esters.

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