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High Pressure Liquid Chromatographic Analysis of Methyl Ketones in Quick Ripened Blue Cheese

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Major professor

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HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF METHYL KETONES IN QUICK RIPENED BLUE CHEESE

By

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ABSTRACT

HIGH PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF METHYL KETONES IN QUICK RIPENED BLUE CHEESE

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The components of a high pressure liquid chromatographic (HPLC) system were assembled to separate and quantitate C_3 to C_{13} methyl ketones from Quick-ripened (QR) blue mold cheese and other cheese. The QR cheese were processed and cured during 7 to 15 days at 52° or 62°F and 95% RH. At the end of the ripening period portions of the cheese were packed and stored at 40°, 48°, or 62°F for an additional period of 4 to 15 days. The pH and moisture content of the samples were determined throughout the ripening and storage period.

Standard methyl ketones with 99^{+}% purity were derivatized with 2,4-dinitrophenylhydrazine dissolved in acetonitrile and separated on a μ Bondapak C^{18} column using acetonitrile:water (75:25 v/v) as the mobile phase. The separated components were detected by a U.V. monitor at 254 nm in the HPLC unit and were compared with their corresponding derivatives which had been isolated from Blue cheese. The

peak heights were used in quantitation. Percent recovery of individual methyl ketones and the slopes of calibration graphs were also used in quantitation.

Hexane extracts of the cheese samples were passed through a DNPH reaction column and after fat removal following several clean-up steps, the monocarbonyl derivatives were injected into the HPLC unit and analyzed for methyl ketones. HPLC and mass spectral analysis of 2,4-DNPH derivative of 2-undecanone obtained from the reaction column showed the possibility of geometric isomerization of methyl ketones.

Methyl ketone development in QR cheese followed a pattern similar to that observed in commercial Blue cheese ripened several months. The predominant methyl ketones were C_7 and C_9. White mold QR cheese contained the C_9 ketone predominantly whereas the C_7 ketone was most abundant in blue mold QR cheese. A large variation in the concentration of methyl ketones was observed in the samples. White mold QR cheese contained a lower concentration of methyl ketones compared with conventional blue mold cheese.

Brief storage at 48^{o}F after curing resulted in a greater formation of methyl ketones in the cheese. Cheese ripened at lower temperatures (52^{o}F) and stored two weeks at 40^{o}F was of superior flavor although total ketone content of such cheese was lower than in cheese ripened and stored at higher temperatures. Salting methods affected color and

methyl ketone formation in the samples. A darker color developed in cheese salted on days 1, 9, and 10 rather than 7, 8, and 9. Blue cheese salted on days 7, 8, 9 demonstrated a greater ketone concentration than cheese made by salting on days 1, 9, 10 and stored at 48^{o}F.

The cheese lost 9 - 13% moisture in the curing room, but negligible losses occurred in storage. All cheese showed the same changes in pH during ripening. Changes in pH were faster at higher temperature of curing.

Freeze drying of Blue cheese samples resulted in a significant loss of methyl ketones. The HPLC method was advantageous in this study for rapid analysis and minimal sample clean up.

Dedicated To
My Father
Dr. Morteza Dokhani

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INTRODUCTION

The consumption of blue mold cheeses has increased significantly in popularity since 1960. Much of this cheese is consumed directly, but increasing quantities and greater demands are for the food products which are flavored with blue mold cheeses such as French Roquefort, Italian Gorgonzola, English Stilton, Danish blue cheese, American blue cheese, etc., to provide salad dressings, mayonnaise, snack foods, bakery items and chip dips.

Because of the growing market for blue mold cheeses and their related food products, attempts have been directed toward more economical and efficient manufacturing and "quick ripening" of such cheeses. Considerable progress has been achieved in the elucidation of the flavor chemistry of Blue cheese. Other related research has been directed toward methods for the production of a quality Blue cheese flavor concentrate; and for the qualitative and quantitative redundant.

The purpose of this study was to develop a rapid technique for the quantitative analysis of methyl ketones by high pressure liquid chromatography. The improved procedure was then applied to the routine, rapid determination of these compounds during curing of quick ripened cheese.

REVIEW OF LITERATURE

The development of the unique peppery and sharp flavor of the cheese ripened with Penicillium roqueforti intrigued investigators for many years. More recently, the biosynthesis of the compounds responsible for Blue cheese flavor and the role of P. roqueforti in fermented cultures have been studied extensively. Different classes of flavor components in blue-veined cheese can arise from several sources. The important ones are developed by the concerted action of numerous enzymes of P. roqueforti involved in lipid and protein metabolism. The biosynthesis of such compounds during cheese ripening was recently reviewed by Kinsella and Hwang (1977).

The History, Chemistry, and Classes of Flavor Compounds in Blue Cheese

Free Fatty Acids

The "peppery and spicy" taste of Roquefort cheese, was initially studied by Currie (1914). He concluded that the short chain free fatty acids, caproic, caprylic, and capric and their readily hydrolyzable salts were responsible for the characteristic response of the tongue and taste receptors upon eating such cheese. Butyric, valeric, and

caprylic acids in Blue cheese were also reported by Thomasow (1947). Coffman et al. (1960) analyzed dry Blue cheese and detected butyric, caproic, caprylic, capric, isovaleric, and heptanoic acids by using gas chromatography. The presence of aromatic acids, p-hydroxyphenyl acetic, p-hydroxybenzoic, and benzoic acids was revealed in Roquefort cheese by Simonart and Mayaudon (1956b). Formic and/or acetic acids were identified by the same authors (1956a) from Roquefort cheese.

Several investigators have obtained quantitative data for free fatty acids in Blue cheese. Morris et al. (1955) separated and quantitated butyric and caproic acids in Blue cheese using partition chromatography. The concentration of acetic, butyric, caproic, and higher molecular weight free fatty acids (average molecular weight of 200) in Blue cheese was reported by Sjostrom and Willart (1959). Using a combination of liquid-liquid column chromatography and gas-liquid column chromatography, Anderson and Day (1966) and Anderson (1966) analyzed domestic and imported blue-veined type cheeses and quantitatively measured the major free fatty acids from acetic to linolenic. Similar concentrations of free fatty acids were obtained by Blakely (1970) in quick ripened (QR) loose curd blue-veined cheese. The lipid and fatty acid content of Blue cheese was also studied by Fujishima et al. (1971). Harte and Stine (1977) reported the same free fatty acid (butyric-linolenic)

patterns between QR and commercial Blue cheese. Their data are presented in TABLE 1. The data show the concentration of free fatty acids (FFA) in QR Blue Cheese after seven days ripening at 17°C and following storage at 4°C for two weeks. During storage the concentration of certain free fatty acids (butyric, capric, lauric, and myristic) increased significantly.

Table 1.--Free Fatty Acid Content of Quick Ripened and Commercial Blue Cheese.

	Free fatty acid (mg/kg)		
	Quick ripened		
Acid	Initial, after curing 7 days at 17°C	After 2 wk at 4°C	Commercial
Butyric	1,270	1,680	720
Caproic	490	490	60
Caprylic	420	360	70
Capric	820	2,240	870
Lauric	1,240	1,500	1,440
Myristic	4,090	4,660	5,700
Palmitic	12,500	12,500	12,700
Stearic	6,590	6,460	6,020
Oleic	13,900	13,000	13,800
Linoleic	790	750	610
Linolenic	710	650	630

Anderson (1966) showed a difference between the fatty acid content of Roquefort and imported Blue cheese. Roquefort lacked the strong pungent flavor and aroma of butyric acid and was more characteristic of caprylic and capric acid. These results were in accord with the composition of sheep's milk which is used in the manufacture of

Roquefort cheese. According to Hilditch (1956) sheep's milk is low in butyric and high in caprylic and capric acids compared to cow's milk. Similar results were obtained by Sadini (1963), Kuzdzal-Savoie and Kuzdzal (1963), and Benassi (1963).

Lipid Metabolism (lipolysis)

The processing of a quality Blue cheese is very much dependent upon the metabolism of the lipid substrate in the cheese. Triglycerides are progressively hydrolyzed to monoglycerides and free fatty acids. Triglycerides decrease from 96-98% of the lipids (about 35% of the cheese) in early stages of ripening to 75-80% of lipids (32% of cheese) at maturity (Kinsella and Hwang, 1976 and 1977).

The extent of lipolysis is governed by the lipase activity. The lipase systems involved in the hydrolysis of triglycerides in cheese may be from 3 possible sources (Anderson, 1966).

1. The lipase native to milk.
2. The lipase from P. roqueforti
3. The lipase of other microorganisms

The Lipase Native to Milk:

Cause a significant amount of fat hydrolysis, particularly, in the cheese from raw homogenized milk. Herrington and Krukovsky (1939) postulated the presence of at least two lipases in milk, based on formaldehyde sensitivity. This postulation was confirmed by Roahen and Sommer

(1940), and Peterson et al. (1943). Two general lipase systems, each composed of 2 lipase enzymes, were demonstrated by Albrecht and Janes (1955) in raw skim milk within the pH range of 5.0 and 6.6. Tarassuk and Frankel (1957) indicated two lipase systems native to milk, a plasma lipase associated with casein and a lipase absorbed on the fat globule membranes. Harper and Gaffney (1970) showed that lipase is an enzyme absorbed on various caseins and may form a multienzyme system in milk.

A pure and homogenous milk lipase with a single optimum pH of 9.0 to 9.2 was isolated and characterized by Chandan and Shahani (1963a and 1963b). The optimum temperature for this enzyme was 37°C. Schwartz et al. (1956) reported at least three pH optima: 6.5 to 7.0, 7.9, and 8.5 to 9.0 for characterization of milk lipase(s). Lipases with pH optima above 7.0 were reported to be less active in Blue cheese manufacturing (Bakalor, 1962).

Gould (1942) showed that milk lipase will hydrolyze butter fat and most natural fats, oils and hydrogenated oils. Kelly (1944 and 1945) demonstrated the ability of milk lipase in hydrolyzing tributyrin, tricaproin, triacetin, tripalmitin, ethyl oleate, and diacetin. Jenson (1964) reported that fats containing short chain acids in a mixture of triglycerides are attached preferentially by milk lipase.

Lipases of P. roqueforti:

Many researchers have examined the lipase of P. roqueforti and its importance in the ripening of cheese. Currie (1914) stated that "P. roqueforti produces a water soluble lipase which is the chief factor in the accomplishment of fat hydrolysis and free fatty acid accumulation in Roquefort cheese." Morris and Jezeski (1953) reported two lipase system in P. roqueforti, one with pH optima 7.0 to 7.8 on tributyrin and 6.5 to 6.8 on butter fat; the other lipase system, isolated from mycelial extract, had pH optima of 6.0 to 6.7 on tributyrin and 7.0 to 7.2 on butter fat. It was shown that the enzyme system was lipase and not an esterase. The effect of temperature, pH, type of substrate, and NaCl concentration on the activity of lipase system was demonstrated. Niki et al. (1966) studied an intra- and extra-cellular lipase system isolated from P. roqueforti in Blue cheese and reported two pH optima, 6.0 and 7.5, for both enzymes, indicating the possible presence of discrete enzymes in both preparations. Imamura and Kataoka (1963a and 1966) and Niki et al. (1966) showed that the extra-cellular lipase of P. roqueforti is more active than intra-cellular lipase.

Higher lipase activity was shown (Eitenmiller et al., 1970) by growing P. roqueforti in a medium containing "Casitone" broth rather than Czapek medium. Thibodeau and Macy (1942) and Morris and Jezeski (1953) had also obtained poor activity of the mold lipase on Czapek medium whose

only nitrogen source was nitrate. Imamura and Kataoka (1963b) indicated that lipase production by P. roqueforti was inhibited by lactose, glucose, and galactose. However, the addition of butterfat increased the production of lipase. In contrast, Eitenmiller et al. (1970) observed a low lipase production by adding 1% (v/v) butter oil or corn oil to the growth medium. Niki et al. (1966) found the greatest lipase production at neutral or alkaline pH and temperature at 20°C. In contrast, however, lipase production by P. roqueforti was reported by Eitenmiller et al. (1970) to be the greatest at lower pH (5.5) and a higher medium temperature (27°C).

The optimum temperature of lipase activity in P. roqueforti was reported to be 30 to 35°C by Shipe (1951), and Morris and Jezeski (1953). Eitenmiller et al. (1970) presented an optimum temperature of 37°C. Obviously, the variation in the optimum temperatures for the lipase activity may be due to the difference in growth condition, fungal strain and analytical method used. The lipase system of P. roqueforti has been shown to be active at low temperatures of cheese ripening such as 10°C (Kinsella and Hwang, 1977).

The ability of lipase to attack a mixture of triglycerides is of a practical importance from the standpoint of cheese ripening. Morris and Jezeski (1953) studied the lipase activity of P. roqueforti on different triglycerides

and found out that tributyrin and synthetic butterfat, composed of simple triglycerides with short chain fatty acids were hydrolyzed more rapidly by lipase than were natural milkfat or other substrate. Imamura and Kataoka (1963a) showed that extra-cellular lipase liberated caproic, capric, and caprylic acids selectively from butterfat. Shipe (1951) reported that the intra-cellular lipase of P. roqueforti hydrolyzed different triglycerides in the order of $C_4 > C_6 > C_8 > C_3$. Eitenmiller et al. (1970) presented similar results. Salvadori and Salvadori (1967) reported that lipase from different strains of P. roqueforti exhibited different substrate specificity. This was confirmed by Stepaniak and Habaj (1974). Knight et al. (1950) produced a white mutant of P. roqueforti by irradiating the mold with ultraviolet light and showed that lipolytic activity of the mold was not associated with the green color of the parent mold.

The effect of salt concentration on the lipolytic activity of P. roqueforti was studied by Morris and Jezeski (1953) for both lipase systems isolated from mycelia or growth medium. As the sodium chloride concentration in the reaction mixture was increased to 4.0%, there was a rapid decline in lipolytic activity. This finding was in agreement with the results obtained by Thibodeau and Macy (1942) and Poznanski et al. (1967). Similar inhibitory effects of sodium chloride were observed by Gould (1941) in raw homogenized milk and cream. Stadhouders (1956) reported that

the salt concentration in the range of 1-2% had more inhibitory effect on lipase than higher concentrations. Morris (1969) stated that a 6.0% salt concentration was inhibitory to P. roqueforti and no survival was noted at 10% salt concentration. Eitenmiller et al. (1970) studied the effect of different salts on the lipolytic activity of P. roqueforti. These authors found stimulation of lipase activity by adding 10^{-2}(M) $MnCl_2$ or 10^{-3}(M) $MgCl_2$ to the medium.

Homogenization of milk prior to Blue cheese manufacturing is a critical process in the hydrolysis of milk fat by lipase systems, particularly those from P. roqueforti. Morris et al. (1963) showed the importance of homogenization with progressive accumulation of free fatty acids during Blue cheese ripening. The effect of homogenization of milk on the manufacture of soft and white cheese was studied by Dozet et al. (1972). The triglycerides in non-homogenized raw milk are not readily available to lipase since an intact "membrane" surrounds the fat globule in discontinuous, multi-layers which inhibit attack and hydrolysis. On the other hand homogenization expands and alters the total surface of milk fat available for lipase attack (Kinsella and Hwang, 1976).

Lipases of Other Microorganisms Present During Ripening of Blue Cheese:

Although the hydrolysis of milk fat and accumulation of free fatty acids in Blue cheese is largely dependent upon

the lipase system of P. roqueforti, some investigators have suggested that other microorganisms may be involved in the release of fatty acids from triglycerides during cheese ripening.

Parmelee and Nelson (1947 and 1949) studied the lipolytic activity of organisms such as Candida lipolytica, Alcaligenes lipolyticus, Achromobacter lipolyticum, Psuedomonas fragi and Mycotorula lipolytica in the manufacture of Blue cheese. Only C. lipolytica and M. lipolytica improved the Blue cheese flavor. Stadhouders and Mulder (1957) showed that the bacteria which survived in the cheese had little lipolytic activity and Willart and Sjostrom (1959) reported that the starter organisms did not exert lipolytic effects. Stadhouders and Mulder (1960) suggested that if the microorganisms principally represented in raw milk (such as Alcaligenes, Achromobacter, Pseudomonas, and Serratia) were added to pasteurized milk, considerable amount of lipolysis would occur in the cheese made with that milk. Fryer and Reiter (1967) indicated a weak lipolytic activity in several strains of Streptococcus lactis. Khan et al. (1966) studied the effect of microbial lipases on milk fat and suggested that the microbial lipases released larger amounts of unsaturated fatty acids and smaller amounts of short chain fatty acids compared to milk lipase.

Methyl Ketones (2-Alkanones)

The occurence of aliphatic methyl ketone was first established by Williams (1858) in essential oils of rue. Watts (1886) found the same methyl ketone, 2-undecanone, in the essential oil of lime leaves. Heptan-2-one was shown to be present in Cinnamon oil by Walbaum and Huthing (1902). Haller and Lassieur (1910a and 1910b) identified several methyl ketones, 2-nonanone, 2-undecanone, and 2-tridecanone in the essence of Cocoanut. Undecan-2-one was reported by Jasperson and Jones (1947) in palm kernel, palm, peanut, cotton seed and sunflower seed oil. Many reports have confirmed a plant origin for methyl ketones (Forney and Markovetz, 1971).

Milk and dairy products have been studied for methyl ketones by many researchers. Stärkel (1924) reported the occurence of methyl ketones in Roquefort cheese and organoleptically showed that the characteristic flavor and aroma of this cheese was due to the presence of 2-heptanone and 2-nonanone produced by Penicillium roqueforti. Hammer and Bryant (1937) confirmed Stärkel's findings and indicated that 2-heptanone was the major methyl ketone in Blue cheese. By means of ether extraction and fractional distillation Patton (1950) isolated acetone, 2-pentanone, 2-heptanone, and 2-nonanone from Blue cheese, and stated that the distinct flavor of Blue cheese was due to the minute quantities of these ketones produced by the mold.

Morgan and Anderson (1956) identified odd-numbered methyl ketones, C_3-C_{11}, in French Roquefort, Italian Gorgonzola, Danish and Domestic Blue cheeses by chromatographic analysis of 2,4-dinitrophenylhydrazone derivatives of methyl ketones. The same technique was used by Harper and Bassett (1959) to analyze neutral and acidic carbonyl compounds in Blue Cheese.

Even-numbered methyl ketones are less important than odd-numbered ketones. Butanone-2 was isolated from Blue cheese by Morgan and Anderson (1956), and Harper and Bassett (1959). Bavisotto et al. (1960) reported the presence of 2-octanone in Blue cheese. Several even-numbered ketones, 2-butanone, 2-hexanone, and 2-octanone were identified by Nawar and Fagerson (1962). With combination of a gas chromatography and mass spectral analysis, the presence of methyl ketones: C_3, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11} and C_{13} was confirmed by Day and Anderson (1965) in blue vein cheeses.

Using chromatographic methods Schwartz and Parks (1963) quantitated the odd numbered methyl ketones from C_3 to C_{15} in domestic Blue cheese. Schwartz et al. (1963) made similar measurements for Roquefort cheese. Heptanone-2 was the major ketone in all samples except one which contained more 2-nonanone. Anderson and Day (1966) found the concentration of individual methyl ketones, C_3 to C_{11}, in domestic Blue and Roquefort cheese and showed a significant variation

among samples. These authors showed that 2-heptanone was the most abundant ketone in all samples. With the exception of one cheese, acetone was detected in all samples of Roquefort cheese. Several methyl ketones such as 2-pentanone, 2-heptanone, 2-nonanone, and 2-nonen (8,9)-one were reported by Svensen and Ottestad (1969) to be the major volatile compounds responsible for the flavor of Norwegian blue cheese (Normanna). Ney and Wirotama (1972) identified C_3, C_5, C_7, C_9 and C_{11} methyl ketones in a German blue cheese (Edelpilzkäse). Groux and Moinas (1974) reported 2-heptanone to be the ketone in greatest concentration in Roquefort and 2-nonanone in Camambert cheese. Blakely (1970) analyzed quick ripened (7 days ripened) Blue cheese and found much lower concentration of methyl ketones, C_3 to C_{13}, compared with normal ripened Blue cheese. The same author reported that the major ketone in Danish and quick ripened Blue cheese was 2-nonanone with a range of 34.4-53.3 micromoles per 10 gram fat for Danish and 8.5 to 15.2 micromoles per 10 g. fat for QR Blue cheese. In the same study, he obtained a large concentration of 2-undecanone in quick ripened cheeses made from filled milk containing hydrogenated coconut oil. Albert (1974) found much higher concentration of methyl ketones in several samples of quick ripened Blue cheese compared with Blakely's results. However, a lower content of methyl ketones was reported for commercial domestic Blue cheese by Albert.

Fatty Acid Metabolism and Methyl Ketone Formation

Dakin (1908a and 1908b) theorized the biochemical conversion of free fatty acids to methyl ketones. The ability of several mold cultures, Penicillium glaucum, P. roqueforti, Aspergillus niger, and A. fumigatus, to convert free fatty acids to methyl ketones was initially presented by Stärkel (1924). This investigator isolated methyl ketones, C_7, C_9 and C_{11} from oxidative decomposition products of cocoa fat and butter and compared them with ketones obtained by oxidation of pure fatty acids with H_2O_2. Stöke (1928) and Coppock et al. (1928) confirmed the conversion of individual fatty acids to their corresponding methyl ketones by pure cultures of a variety of fungi. Stöke (1928) reported that Penicillium palitans and P. glaucum produced methyl ketones when they were grown on Coconut oil. The same author suggested that the noted Penicillium species, as in the higher animals and man, oxidize a chain compound primarily at the β-carbon atom with the formation of a keto acid and subsequent decomposition of the keto acid to methyl ketone and carbon dioxide.

Mechanism of Methyl Ketone Formation by Fungi:

It was initially demonstrated that only spores of P. roqueforti were capable of producing methyl ketones from fatty acids (Girolami and Knight, 1955; and Gehrig and Knight, 1958, 1961, and 1963). These authors studied

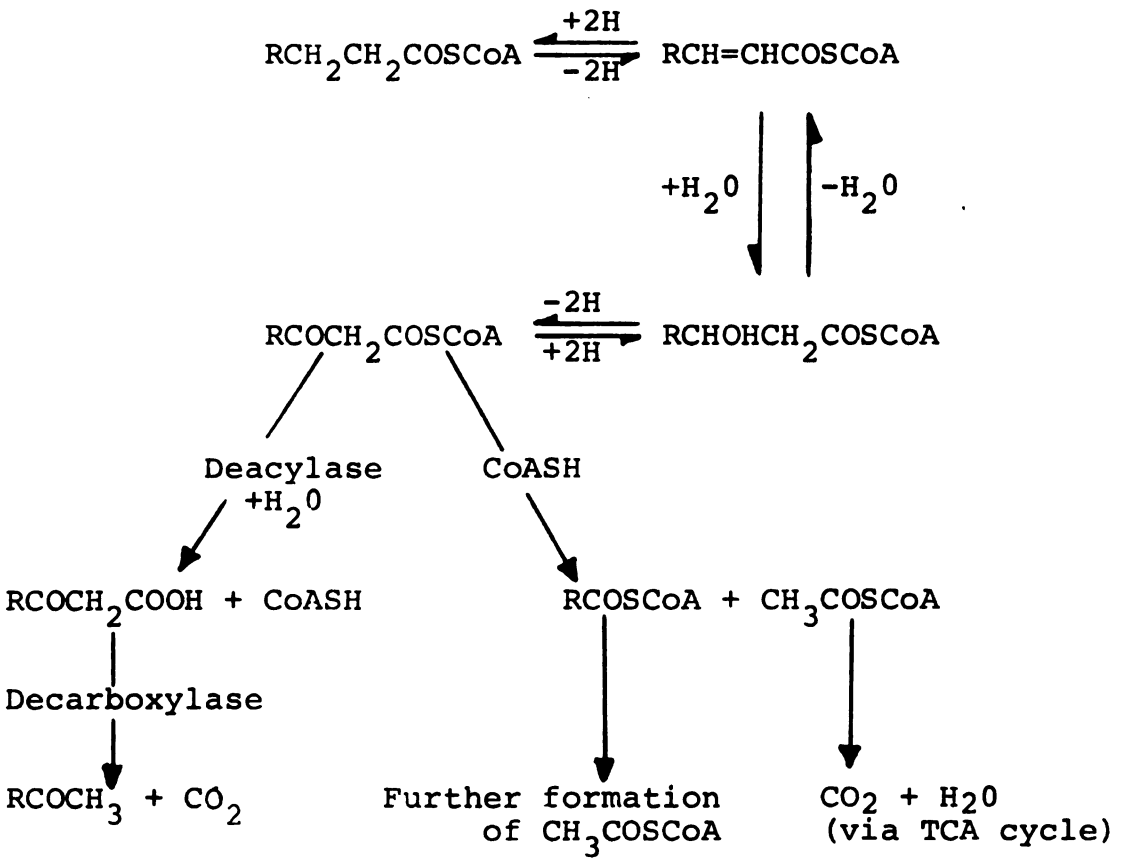
2-heptanone formation from octanoic acid by mold spores because of the importance of this compound in giving aroma and flavor to Blue cheese. The spores of various filamentous fungi such as Aspergillus and Penicillium genera were reported (Gehrig and Knight, 1961; and Franke et al., 1961) to be capable of producing methyl ketones from fatty acids. Gehrig and Knight (1963) concluded that the capacity of spores of P. roqueforti to form methyl ketones disappeared rapidly and progressively as the spores germinated. Lawrence (1965 and 1966) showed that the addition of those amino acids and sugars such as casamino acid, alanine, serine, proline, and glucose, xylose, galactose, and sucrose that stimulate germination of spores, markedly increased the slow rate of 2-heptanone formation from octanoic acid in a spore suspension of P. roqueforti. Hawke (1966) and Lawrence and Bailey (1969) suggested that the substances readily oxidizable by spores of P. roqueforti would stimulate germination and ketone production. Dartey and Kinsella (1973a and 1973b) showed that the addition of substances such as D-glucose and L-proline supressed the catabolism of fatty acids to carbon dioxide and increased the methyl ketone formation. Hwang et al. (1976) showed an increase in the rate of 2-undecanone formation by adding glucose to the resting spores of P. roqueforti. The changes in morphology and biochemical properties of the spores of P. roqueforti during germination have been studied by Fan and Kinsella (1976) and Fan et al. (1976).

In contrast with the data reported by Gehrig and Knight (1958, 1961 and 1963), Lawrence and Hawke (1968) reported the formation of methyl ketones by mycelia of P. roqueforti from low concentrations of fatty acids with less than 14 carbon atoms over a wide range of pH. Dwivedi and Kinsella (1974a and 1974b) confirmed the findings of Lawrence and Hawke by carefully obtaining mycelia cultures and demonstrating the ability of mycelia to oxidize fatty acid and produce methyl ketones. Fan et al. (1976) showed the relative rates of methyl ketone formation from fatty acids by spores of P. roqueforti at progressive stages of germination. They concluded that under short incubation conditions, mycelia were much more active; however, the resting spores showed very high activity during a long period of incubation. Lawrence and Hawke (1968), and Dwivedi and Kinsella (1974a) demonstrated the mycelia that had been cultured over 48 hours showed a superior ability to metabolize fatty acids to methyl ketones as compared to fresh mycelia.

Accumulated information of many investigators have revealed that methyl ketone production from fatty acids by spores and mycelia, follows the classical fatty acid β-oxidation cycle. Gehrig and Knight (1963) used radioactive labeled fatty acid, (1-^{14}C) octanoate as substrate, and demonstrated that in small concentration (1 μM), sodium octanoate was oxidized completely by spores of

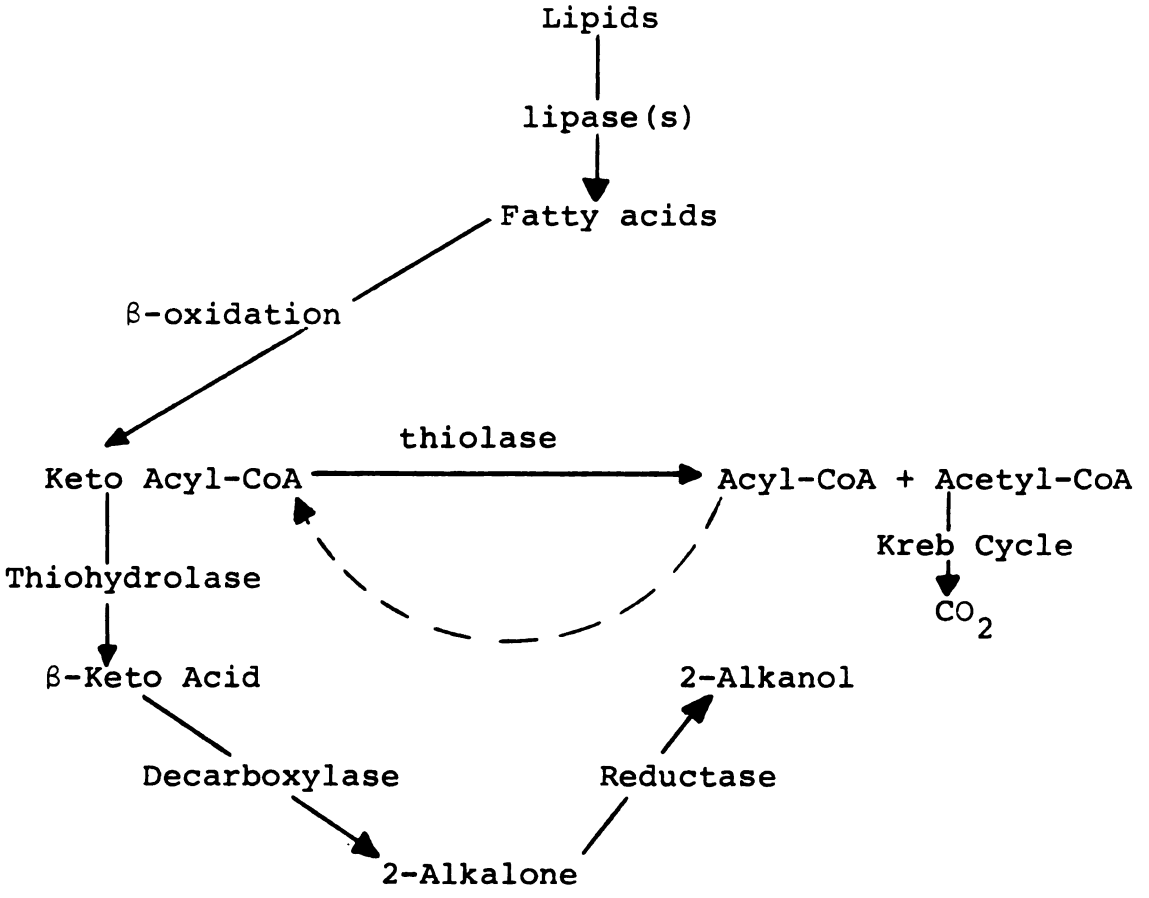
P. roqueforti; however, in larger concentrations (20 μM) it was partially oxidized to 2-heptanone and partly to carbon dioxide. The carbon dioxide was radioactive but 2-heptanone was not. Lawrence (1966) presented that 2-heptanone formed from (1-^{14}C) octanoate was not radioactive, whereas that from (2-^{14}C) octanoate was radioactive. On the other hand, by enzyme preparation from various fungi by Franke et al. (1961) had revealed the decarboxylation of β-keto acids to methyl ketones. Hawke (1966) outlined a schematic of the pathway of fatty acid oxidation for methyl ketone formation similar to the classical β-oxidation cycle of fatty acids as following:

Pathway of Fatty Acid Oxidation and Methyl Ketone Formation



This mechanism was reported for: (1) only methyl ketones of one less carbon atom than the fatty acids used as substrates, and (2) a simultaneous process for β-oxidation of fatty acids.

Kinsella and Hwang (1976 and 1977) presented a general outline of the fatty acid metabolism by the spores or mycelia of P. roqueforti as occurs in Blue cheese in the following:



These authors outlined the following steps similar to Hawke (1966):

1. Lipolysis of triglycerides to form free fatty acids.

2. Formation of the β-Keto Acyl-CoA by the dehydrogenation of the β-Hydroxy Acyl-CoA.
3. Deacylation of β-Keto Acyl-CoA and formation of β-Keto Acid and CoASH by a β-Keto Acyl-CoA deacylase (Thiohydrolase).
4. Rapid decarboxylation of β-Keto Acid to carbon dioxide and 2-Alkanones (methyl Ketones) by the β-Keto Acid decarboxylase.
5. Reduction of 2-Alkanones to their corresponding 2-Alkanols (secondary alcohols) by a reductase system.

In above steps the acyl group is from 4 to 16 carbons and the broken line denotes the possible inhibition of thiolase by acyl-CoA, thereby facilitating the deacylation-decarboxylation step (Kinsella and Hwang, 1977).

There have been many efforts to isolate the enzyme complex responsible for fatty acid oxidation from fungi, but they have failed due to difficulty in extracting and obtaining an active enzyme system from the mold. Franke *et al*. (1962) reported that β-decarboxylase was very active in several molds. Beta-keto Acyl decarboxylase enzyme was extracted from *P. roqueforti* by Hwang *et al*. (1976) and its activity was examined. They suggested the presence of 2 species of β-keto Acyl decarboxylase in the mold, one of which was heat stable and the other heat labile. It was demonstrated that this enzyme decarboxylates β-Keto-laurate

to 2-undecanone in the resting spores, germinating spores, and mycelia. The rate of methyl ketone formation in the mycelia was the greatest.

The major intermediates of the fatty acid β-oxidation cycle, β-keto acids, were isolated from Blue cheese and studied by Bassett and Harper (1958). The esters of these keto acids were reported to be formed in butterfat in the presence of moisture (Wong et al., 1958). Van der Ven et al. (1963) demonstrated that the formation of pyrazolones in butterfat during heating was due to the presence of nonvolatile β-keto acid esters. The same authors suggested that the six even number keto acids (C_6 - C_{16}) were the precursors of odd number methyl ketones (C_5 - C_{15}). The concentration of these keto esters as glycerides was only 0.03 percent. Schwartz and Parks (1963) reported that the existence of C_{13} or C_{15} ketones in Blue cheese was probably due to non-microbial breakdown of ketone precursors.

Studies have shown that β-Keto octanoyl-CoA is a preferential substrate for P. roqueforti in the deacylation step to produce 2-heptanone. However, methyl ketones of different chain length can be formed from longer chain fatty acids. Dartey and Kinsella (1973a and 1973b) used [U-^{14}C] lauric acid and [U-^{14}C] palmitate and showed the formation of a homologous series of odd number methyl ketones, C_3 - C_{15}, at successive cycles of β-oxidation.

Lawrence (1966) observed that the pH optimum for methyl ketone formation varied with the concentration and type of fatty acids. At a concentration of 1.0 mM octanoate, the pH optimum was between 5.5 and 6.0 and increased as the fatty acid concentration was increased. The yield of methyl ketone between pH 5.5 and 7.0 decreased progressively from a maximum of 75% from octanoic acid to zero from myristic acid. Hwang et al. (1976) reported an optimum pH, 6.5-7.0, for 2-undecanone formation from β-ketolaurate which corresponded to the optimum pH observed for methyl ketone production by Dartey and Kinsella (1973a).

Gehrig and Knight (1963) suggested an optimum temperature of 25°C for methyl ketone production. Lawrence (1966) indicated that the rate of methyl ketone formation reached a maximum at a temperature of 27°C and then rapidly decreased with further rise in temperature. Dartey and Kinsella (1973a and 1973b) reported a 20-fold increase in methyl ketone formation as the temperature of incubation was dropped from 37°C to 30°C.

The oxygen tension and carbon dioxide evolved in the reaction medium of P. roqueforti are important in the rate of methyl ketone production. Gehrig and Knight (1963) showed no ketone formation when the spores of the mold were incubated in 100% nitrogen or carbon dioxide. Lawrence (1966) demonstrated an increase in the rate of formation of 2-heptanone from octanoic acid in the presence of small amounts of carbon dioxide evolved in the reaction medium.

Lawrence and Hawke (1968) indicated that the removal of carbon dioxide as it was formed during incubation of the mold decreased the yield of methyl ketones up to 50%. Kinsella and Hwang (1976 and 1977) suggested that carbon dioxide may actually enhance methyl ketone formation by inhibiting the acetyl CoA via Kreb cycle and, therefore, causing a "back up" of the fatty acid oxidation pathway.

Langler and Day (1964) reported that there was no correlation between the relative amounts of ketones formed and their corresponding fatty acid precursors in milk fat. The results of Anderson and Day (1966) confirmed the above statement. It was found that the proportion of each methyl ketone produced in Blue cheese by P. roqueforti did not depend directly on the amount of available fatty acid precursor in the cheese. They showed that the proportion of acetone was relatively low compared with its precursor, butyric acid, and conversely the concentration of 2-heptanone was high relative to its precursor, octanoic acid.

High concentration of fatty acid precursors tend to decrease the complete fatty acid oxidation by P. roqueforti. This inhibitory effect of fatty acids was demonstrated by Lawrence (1966) on spores, and by Lawrence and Hawke (1968) on the mycelia of the mold. Spores are much more resistant to the inhibitory effects of fatty acids (Kinsella and Hwang, 1977). Optimum concentration of fatty acids for methyl ketone production is 1.0-5.0 mM for spores, and 1.0

mM for mycelia. The same authors suggested that the fatty acids may inhibit the thiolase enzyme of β-oxidation pathway, whereas they do not effect the ketoacyl-CoA deacylase (thiohydrolase) nor the β-keto acid decarboxylase. Therefore, in the presence of high concentration of fatty acids, the major products are methyl ketones. However at very low concentration of fatty acids, mycelia exhibit complete oxidation of fatty acids to carbon dioxide and water (Lawrence and Hawke, 1968). Thus, in Blue cheese manufacturing, strains of P. roqueforti with poor lipase activity produce much lower methyl ketones and strains with very active lipase system generate more ketones (Kinsella and Hwang, 1977).

Secondary Alcohols (2-Alkanols)

In addition to methyl ketones and free fatty acids, which appear to constitute the major flavor components of Blue cheese, secondary alcohols have also been identified. Stöke (1928) suggested the generation of secondary alcohols by P. roqueforti. It was reported that these alcohols are probably intermediate products of the mold oxidation process. Jackson and Hussong (1958) used paper and gas chromatography and isolated 2-pentanol, 2-heptanol, and 2-nonanol from Blue cheese. They suggested that secondary alcohols are formed by reduction of methyl ketones of the same chain length. The concentration of secondary alcohols in Blue cheese was measured by Anderson and Day (1966).

Blue cheese contains minute amounts of secondary alcohols compared to their corresponding methyl ketones. Svensen and Ottestad (1969) reported that 2-pentanol and 2-nonanol were among major flavor compounds in Norwegian blue cheese (Normanna). Considerable variation in concentration of these alcohols were found, in agreement with the results of Anderson and Day (1966). Nawar and Fagerson (1962) also demonstrated the presence of 2-pentanol, and 2-heptanol in Roquefort cheese. Ney and Wirotama (1972) identified C_5, C_7 and C_9 secondary alcohols in German blue cheese (Edelpilzkäse). Groux and Moinas (1974) observed significant amounts of secondary alcohols in Roquefort and Camembert cheese. Kinsella and Hwang (1977) suggested that when 2-alkanols are present in large concentrations in Blue cheese, they may impart a musty flavor in the cheese.

Alcohols Other than 2-Alkanols

Primary alcohols such as methanol, ethanol, 2-methyl butanol, 3-methyl butanol, 1-pentanol, and 2-phenyl ethanol were identified in Blue cheese by Anderson and Day (1966). It was suggested (Anderson, 1966; and Kinsella and Hwang, 1977) that these alcohols, and particularly methyl butanols, are probably formed by reduction of aldehydes derived from oxidative deamination of free amino acids present in Blue cheese. These alcohols may contribute to the fruity and nutty flavor of the cheese. Ney and Wirotama (1972) reported the presence of 1-butanol and

iso-pentanol-1 in German blue cheese (Edelpilzkäse). Acety methyl carbinol and diacetyl were isolated from Roquefort cheese by Csiszar et al. (1956). They suggested (as is well known for most fermented milk products) that the presence of diacetyl was due to the lactic starters metabolism. These compounds were also detected in domestic Blue cheese by Harper and Bassett (1959).

Unsaturated alcohols such as 1-octene-3-ol and 2-octene-1-ol are reported to be produced by penicillium species (kaminski et al., 1974). Groux and Monias (1974) identified 1-octene-3-ol in Camembert and Roquefort cheese. Trace amounts were identified in Roquefort cheese. These alcohols, which are characterized by a musty, mushroomy flavor at high concentrations may be formed by the oxidation of linoleic acid (Kinsella and Hwang, 1977). Linoleic acid was found to be abundant in the mycelia and spores of P. roqueforti (Fan and Kinsella, 1976; Shimp and Kinsella, 1977).

Aldehydes

Acetaldehyde was detected by Morgan and Anderson (1956) and Harper and Bassett (1959) in Blue cheese. Acetaldehyde, propionaldehyde, and isobutyraldehyde were reported in Blue cheese volatiles (Nawar and Fagerson, 1962). Anderson (1966) identified acetaldehyde, 2-methyl propanal, 3-methyl butanal, and furfural in Blue cheese. It was suggested (Anderson, 1966; Kinsella and Hwang,

1977) that the presence of these aldehydes may be due to the action of enzymes involved in the oxidative deamination of amino acids. Kinsella and Hwang (1977) presumed valine and leucine to be the precursors of 2-methyl propanal and 3-methyl butanal respectively. Ney and Wirotama (1972) reported the following aldehydes in German blue cheese (Edelpilzkäse): acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde, valeraldehyde, iso-valeraldehyde, and phenylacetaldehyde.

Esters

Anderson and Day (1966) initially reported the importance of methyl and ethyl esters in the overall composite flavor of Blue cheese. Prior to that, Bills and Day (1964) had suggested that excessive levels of ethyl butanoate and ethyl hexanoate were responsible for the fruity flavor defect of cheddar cheese. Ethyl esters of butanoate, hexanoate, octanoate, and decanoate were identified in German blue cheese by Ney and Wirotama (1972). Anderson (1966) identified different analogs of methyl, ethyl, and isopropyl esters. This author also tentatively identified two cyclic internal esters, deltaoctalactone, and deltadecalactone. Jolly and Kosikowski (1975) identified and quantitated five different lactones in Blue cheese containing added microbial lipase. The lactones are the result of internal esterification of hydroxy alkanoic acids in triglyceride molecules.

Other Classes (Miscellaneous) Flavor Components

Numerous organic compounds have been identified in Blue cheese which may directly flavor or function as part of the "background" cheese flavor. Significant quantities of organic acids such as acetic, lactic, pyruvic, malic, and isobutyric have been reported in Blue and Roquefort cheese (Kinsella and Hwang, 1977). Acids such as succinic, benzoic, hydroxybenzoic, p-hydroxybenzoic, and p-hydroxyphenyl acetic have been found in cheese (Simonart and Mayaudon, 1956a and 1956b; and Schormüller, 1968). Valeric (Thomasow, 1947), isovaleric and heptanoic (Coffman et al., 1960) acids have been reported in Blue cheese. Acidic carbonyl components such as beta-ketocaproic, beta keto-caprylic, beta-keto capric and alpha-acetolactic have been shown in Blue cheese (Harper and Bassett, 1959).

Free amino acids and peptides undoubtedly play an important role in the overall flavor of Blue cheese. Amino acids, depending on their composition, may contribute to the sweetish or bitter aftertaste in Blue cheese (Kinsella and Hwang, 1977). Glycine, alanine, proline, and threonine have a sweetish taste. The aliphatic hydrophobic amino acids and cystine are bitter (Ney, 1974). Volatile amines such as methyl amine, ethyl amine, n-butyl amine, isobutyl amine, iso-pentyl amine, di-methyl amine, di-ethyl amine, di-n-propylamine, and di-n-butyl amine were identified in German blue cheese (Edelpilzkäse) by Ney and Wirotama

(1972). Amines such as tyramine and tryptamine were detected in Roquefort, Stilton, and domestic Blue cheese (Voigt et al., 1974). The highest concentration, 1.1 mg/g, of tryptamine was reported in Blue cheese among different varieties of cheese tested. Sen (1969) found the concentration of tyramine in several varieties of blue-veined cheeses to be 27-520 micrograms per gram of cheese. The presence of tyramine in quick ripened Blue cheese was demonstrated by Kuehler (1974). This author reported 825 micrograms tyramine per gram of cheese after 39 days ripening. Commercial blue cheese, ripened for 90 days contained 40 micrograms tyramine per gram of cheese.

The presence of benzene, toluene, and cresyl methyl ether, identified by Anderson (1966) were unexpected components found in Blue cheese.

Protein Metabolism (Proteolysis)

Proteolysis (degradation of proteins) is necessary and important in Blue cheese ripening. This biochemical process develops a proper texture, and flavor background. Amino acids act as precursors of some flavor components, germination stimulants for spores of P. roqueforti, and enhancer for methyl ketone formation during Blue cheese ripening (Kinsella and Hwang, 1977).

Active proteolytic enzymes in P. roqueforti which degrade casein of milk have been found. The differences in proteolytic activity of some strains of P. roqueforti were

observed by Funder (1949). The lipolytic activity of the same strains had been shown to be different (Macy and Thibodeau, 1942). Porks *et al.* (1959) showed that the strains of *P. roqueforti*, used singly or in mixtures, differed in their lipolytic and proteolytic activities. Proteolytic activity was demonstrated by formation of different amounts of albumin, peptones and amino acids, or by different amino acids which were liberated. The proteolytic activity of white mutant of *P. roqueforti* produced by Knight *et al.* (1950) was shown to be independent of the green pigmentation of the original mold. Salvadori (1964) reported that proteolysis varied as much as five fold among 19 different strains of *P. roqueforti*. Niki *et al.* (1966) showed the strains with high protease activity, exhibited low lipolysis and vice versa. The same authors suggested that the strains of *P. roqueforti* with low proteolytic and high lipolytic activities produced a better quality Blue cheese in a shorter period of time. Modler *et al.* (1974a) observed different proteolytic activity among three strains of *P. roqueforti*. The bitterness of cheese has been attributed to the accumulation of peptones and peptides (Czulak, 1959). Niki *et al.* (1966) and Pelissier *et al.* (1974) related the bitterness with excessive protease activity.

The presence of an intra- and an extra-cellular proteases with an optimum pH of 5.5 was demonstrated by Niki *et al.* (1966). The intra-cellular protease, isolated

from mycellium contributes to the proteolysis throughout the ripening period, whereas the extra-cellular protease degrade casein during the early stages of ripening. Modler et al. (1974b) isolated and examined a pure extra-cellular protease. Gripon and Debest (1976) studied the proteolytic activity of eight strains of P. roqueforti. One or two exo-cellular proteases such as acid or alkaline carboxypeptidase and alkaline amino-peptidase were detected electrophoretically. The ability of different strains of P. roqueforti to hydrolyze proteins at acid, neutral, and alkaline pH was presented by the same authors. Gripon et al. (1977) showed the proteolytic activity of an acid protease from P. roqueforti and a neutral protease from P. caseicolum during curd ripening. Both enzymes exhibited high proteolytic activity and produced large amounts of pH 4.6-soluble nitrogen and non-protein nitrogen, but had little effect on production of free amino acids.

The intra-cellular protease isolated from mycellium had a range of pH optima, 5.5-7.0 (Niki et al., 1966). The extra-cellular protease exhibited a narrow range of pH optima, 5.0-6.0. Modler et al. (1974a) reported that pH optima of extracellular protease were different on different substrates. The optimum pH with 1% BSA was 3, whereas, with casein an optimum pH of 5.5 was obtained. The same authors demonstrated a wide range of pH, 3.0-6.0, for the maximum stability of extracellular protease. Modler et al.

(1974a and 1974b) showed that the optimum temperature for extracellular protease activity was 45 to 46°C. Two pH optima, 3 and 6, for casein hydrolysis were found by Gripon and Bergere (1972) using a protease isolated from Penicillium. The optimum temperature was found between 35 and 43°C. Below 25°C the activity of protease falls off. Therefore, ripening temperatures of 10-15°C minimize excessive peptide and amino acid accumulation in Blue cheese.

Addition of sodium chloride to the cheese also affects proteolysis. Gripon and Hermier (1974) reported the inhibition of extra-cellular protease by adding sodium chloride to the medium. Protease activity of Penicillium species was progressively decreased by sodium chloride concentration above 0.5% (Kikuchi and Takafugi, 1971). Protein breakdown during ripening of Blue cheese was suggested to be 20 to 30%. The protein degradation of quick ripened Blue cheese was studied by Kuehler (1974) during 11 or 39 days ripening.

Amino acids in several varieties of Blue cheese, Roquefort, Gorgonzola, and domestic Blue cheese were quantitated by Kosikowsky and Dahlberg (1954). The level of free amino acids in Camembert and Danish blue cheese (Danablue) was reported by Ismail and Hansen (1972). Danablue was characterized by a high level of free amino acids. This was in agreement with the results of Hanni (1967) who reported the presence of free amino acids up to 38% of total

nitrogen. Kuehler (1974) found the concentration of free amino acids in several quick ripened Blue cheese samples. The amino acid content increased to 28% of the total protein after ripening 39 days. This was compared with 16% after 11 days ripening.

The role of other microorganisms in hydrolyzing proteins of Blue cheese has been of little importance. Morris et al. (1951) concluded that the surface microflora of Minnesota blue cheese accelerated the ripening, particularly in the edged portions of the slimed cheeses. These microorganisms consisted primarily of yeasts and some molds followed by the growth of Cocci and rod forms of bacteria. Hartley and Jezeski (1954) reported five groups of bacteria in slimed Blue cheese. Bacterium linens and B. erythrogenes were predominant. Imamura (1960), and Knoop and Peters (1971) indicated that Streptococcus and Lactobacillus species may initially produce free amino acids in the cheese to stimulate germination of the spores of P. roqueforti. The proteolytic activity of Streptococcus lactis was studied by Gripon et al. (1977). Amino-peptidase activity was demonstrated in S. lactis, resulting in the accumulation of free amino acids. The same authors suggested a synergistic action between Penicillium proteases, resulting in the development of peptides, and exopeptidase, in liberating free amino acids from the

available peptides. Thus, microorganisms other than P. roqueforti may be helpful in the production of Blue cheese flavor components.

Blue Cheese Flavor Concentrates

The demand for the Blue cheese flavor in several foods such as salad dressings, processed cheese, snack foods, and baked goods has encouraged processors to provide typical Blue cheese flavor concentrates produced by either aerobic fermentation methods of chemical formulation.

The patent of Watts and Nelson (1963) described a fermentation method which consisted of incubating homogenized-sterilized whole milk inoculated with the spores of P. roqueforti. The mixture was shake-incubated at 21-25°C for 96 hours to produce the desired flavor components of blue mold cheeses. Knight (1963) patented a fermentation process involving the inoculation spores of P. roqueforti in milk containing lipolyzed milk fat or free fatty acids. Litchfield (1969) and Nelson (1970) have described the production of Blue cheese flavor by large scale submergion methods. The concentration of methyl ketones is ten fold larger in these cultures than is found in Blue cheese. Luksas (1973) patented a method of inoculating P. roqueforti in a medium containing 10% sodium caseinate and 5% butter fat. Agitation, aeration, and incubation for 48 hours at 25°C produced the desired level of flavor compounds. Dwivedi and Kinsella (1974a) studied

methyl ketone and total carbonyl production in lipolyzed milk fat by continuous production of P. roqueforti mycelia in a fermentation process. It was observed that octanoic and decanoic acids were easily metabolized by the mold to produce C_7 and C_9 methyl ketones. Sodium chloride lowered the concentration of total carbonyls. Based on their findings the same authors (1974b) designed a two stage, semi-continuous method of submerged fermentation by P. roqueforti. This system was suggested to be helpful in obtaining a desirable flavor balance to suit an individual food product. More recently, Jolly and Kosikowski (1975) demonstrated a submerged fermentation method with microbial lipase and P. roqueforti, using acid and sweet whey with butter fat or coconut oil. The freeze-dried powder of the liquid concentrate served as a good product with a ketone content almost five fold higher than Blue cheese.

Several investigators have attempted to formulate a synthetic Blue cheese flavor. Anderson (1966) suggested that an acceptable Blue cheese flavor could be obtained by blending selected fatty acids, methyl ketones, secondary alcohols, 2-phenyl ethanol, and methyl and ethyl esters in a cottage cheese and butter fat mixture. Moinas et al. (1974) prepared a Blue cheese flavor concentrate containing 0.8% 2-heptanone, 18% 2-nonanone, 14% 2-heptanol, 0.2% phenol, 63% propionic acid, 27% 1-octene-3-ol, and 2% methyl cinnamate. A combination of 38 flavor components

were used (Ney et al., 1975) to obtain a desirable Blue cheese flavor. Addition of 1-octene-3-ol to the mixture was reported to improve the synthetic Blue cheese flavor.

Accelerated (Quick) Ripening of Blue Cheese

A number of investigators have developed and described several methods for quick ripening or shortening the period during which desired texture, color, and flavor are obtained in Blue cheese. According to Mocquot and Béjambes (1960) French Roquefort blue cheese needs a ripening period of 3 months in Roquefort caves with a temperature of 43-46°F. Peters and Nelson (1948), and Babel (1953) reported roughly a 3 month period for Blue cheese, ripened at 50° or 50° - 55°F and 90-95% relative humidity. Lawrence (1966) suggested that in a typical Blue cheese, a period of 90 days is required for P. roqueforti to develop methyl ketones from fatty acids to a desired concentration. Jolly and Kosikowski (1975) manufactured Blue cheese cured 45-75 days at 5°C and demonstrated maximum methyl ketone and flavor development during this curing period. A method was developed by Kondrup and Hedrick (1963) and patented by Hedrick et al. (1968) to shorten the ripening period of Blue cheese from the initial 90 days to 10 days. The mold was incorporated into the milk at the time of rennet coagulation and the open granular curd rather than the normal hooped loaf was ripened at 62°F and over 95% relative humidity with the addition of up

to 4% salt. The curd was agitated daily until a desirable texture, color, and flavor was obtained. Blakely (1970) analyzed a 7-day ripened Blue cheese made by the same procedures and reported poor flavor development. A modified procedure of Hedrick et al. (1968) was used by Kuehler (1974), Harte (1974) and Albert (1974) to produce quick ripened Blue cheese with more desirable quality. These authors also used a white mutant of P. roqueforti (Knight et al., 1950) and obtained a satisfactory quick ripened cheese with a Blue cheese flavor. The manufacture of a Blue cheese made from soya milk and cured for two weeks was patented by Lundstedt and Yau-Yee Lo (1973). The soya milk was fortified with milkfat and nonfat milk solids and inoculated with P. roqueforti and Streptococcus diacetilactis. The latter organism aids the growth of the mold.

Homogenization reduces the size of the fat globules in milk, thus increasing the area of the reactive surface exposed to lipolysis. Lane and Hammer (1937) observed a uniform, faster ripening and flavor development in the cheese made with homogenized milk compared with unhomogenized raw milk. Homogenized-pasteurized milk showed a rapid development of volatile acids and flavor characteristic of the cheese. A soft and brittle curd resulted in the cheese made with milk homogenized at 2000 Psi. Morris et al. (1955) reported a 9-11 months ripening period for Blue cheese made with unhomogenized raw milk. This period was reduced to 4-5 months by using raw or pasteurized

homogenized milk to obtain the "best flavor." Raw homogenized milk resulted in a higher accumulation of free fatty acids in Blue cheese compared with unhomogenized milk. However, the use of raw homogenized milk resulted in a less mold growth and smaller change in pH. Peters and Nelson (1961a) obtained similar trends, by using treatments such as 2000 Psi homogenization and 143°F/30 minutes pasteurization, while studying volatile free fatty acid changes during ripening. The quality of Blue cheese at different ripening temperatures was also studied by the same authors (1961b). An improved quality and faster ripening of Roquefort cheese was found by Shchedushnov (1961 and 1962) when a pasteurized mixture of homogenized cream and skim milk was used compared with unhomogenized controls. It was shown (Shchedushnov, 1961) that the breakdown of protein and fat and the formation of volatile acids increased in homogenized samples during ripening. The homogenization of milk had taken place at 50-100 atmospheres pressure. The highest quality cheese was obtained from milk homogenized at 50 atmospheres pressure. Lane and Hammer (1938) reported a normal Blue cheese ripening and a satisfactory growth of P. roqueforti when milk was homogenized two stage up to 2500 or 3500 Psi at 95°F. In both cases 500 Psi was used for the second stage. With 3500 Psi two-stage homogenization of milk, a rapid flavor development and less color was obtained. Babel (1953) suggested a mixture of homogenized cream, at 2500 Psi, with skim milk

for manufacturing American blue cheese. Kondrup and Hedrick (1963) used two stage homogenization, 1400 Psi on the first and 700 Psi on the second stage, of whole milk at 130^{o}F for manufacturing a 10 days - QR Blue cheese.

Many investigators have tried to accelerate the period of Blue cheese ripening and the flavor development by addition of enzymes, particularly lipases. Coulter and Combs (1939) tried to shorten the ripening period of Blue cheese by adding steapsin to the milk or curd. The same degree of flavor development was obtained after 5 months compared with 12 months ripened commercials. However, sensory evaluations indicated the cheese to be inferior to normal cheese due to a characteristic bitter flavor. Wojtkiewicz and Inikkoff (1934) attempted to accelerate the ripening of cheese by adding pepsin, trypsin, erepsin, and rennet individually or in various combinations. Faster ripening was obtained but cheese containing trypsin had a bitter flavor. Thibodeau and Macy (1942) produced a fine quality Blue cheese by using the enzymes from P. roqueforti in the form of mycellium homogenate. The ripening period was decreased to 5 months compared with a 10 month commercial cheese. The addition of lipolytic organisms such as Candida lipolytica to the pasteurized milk enhanced ripening and improved flavor (Parmelee and Nelsen, 1949). The same authors (1947) had indicated that not all lipolytic organisms, when added to the milk, caused an improvement in the flavor quality of Blue cheese.

Achromobacter lipolyticum and Alcaligenes lipolyticus were among the microorganisms which did not affect fat hydrolysis or flavor development. Peters and Nelson (1948) demonstrated that the addition of cell-free lipase from Mycotorula lipolytica to pasteurized-homogenized milk increased the hydrolysis of fat and intensified the desired flavor in Blue cheese. Lipolytic agents such as pancreatin, erepsin, steapsin, mulberry juice lipase, and chicken stomack lipase were reported (Kosikowski and Mocquot, 1958) to cause rancidity and bitter flavor in cheese. Nilson and Willart (1960 and 1961) reported an acceleration in flavor development of Blue cheese when lipases from Candida lipolytica and pancreatic substrate were added to milk, but bitterness resulted in the cheese. The rate of flavor development, particularly methyl ketones, was enhanced by adding microbial or animal lipase to the pasteurized milk (Jolly and Kosikowski, 1975). These results confirmed their findings (1973), showing that Blue cheese prepared with that method was ripened significantly faster than controls. The same authors (1975) added microbial lipases to a submerged fermentation of P. roqueforti and obtained a fast release of fatty acids, making them available in higher concentration for the mold, for subsequent conversion to methyl ketones in a Blue cheese flavor product.

Cheese Dehydration and Flavor Retention

Dehydrating natural cheese for preservation has been studied by many investigators. Cheese can be dried by techniques such as tray drying, spray drying, and freeze drying to produce cheese powder or products suitable for grating. The reduction in weight and bulk is an advantage of dehydration, as is storage stability at room temperature. Depending upon the mode of drying, dried cheese may contain between 3 and 18% moisture (Kosikowski, 1977). During dehydration or evaporation of foods, particularly heat-sensitive foods such as dairy products, loss of volatile components and changes in chemical constituents of food take place.

Dehydration of cheese was initially reported by Sanders (1943) to produce a cheese powder of 3% moisture or less for use by the Armed Forces. Cheese drying took place in two stages. First, partial drying of grated cheese at room temperature by blowing an air current above it to prevent lumping of particles, exudation of fat and loss of flavor. Second, the partially dried cheese was exposed to a temperature of 145^{o}F until the desired cheese flakes were obtained. Yakhontov (1956) dried a Soviet cheese in thin layers with air current at 38-42^{o}C during 6.0 hrs. The final moisture was 10% and the cheese required refrigeration at 10-15^{o}C 70-75% relative humidity. Olsanski and Schmidt (1960) reported dehydration of

Parmesan cheese by high quality by using dark infra-red lamps with filters for exclusion of visible light. Faster drying and a better keeping quality were obtained compared with bright infra-red lamps or drying with warm air current.

Spray drying of cheese slurry was reported for the first time by Bohác (1957) and Irvine et al. (1957). The latter authors spray dried a "low fat" Cheddar cheese emulsion which could be stored at 50°F for 6 months. According to Bullock et al. (1963) the addition of 5% Blue cheese to the cheese emulsion improved the flavor of the powder. The Cheddar cheese used for spray drying contained 20% fat. Slurries of cheddar and Blue cheeses (40% solids) were spray dried by Bradley and Stine (1962) in a commercial type horizontal, cocurrent dryer. The samples were homogenized at 2000 Psi before drying. The same authors (1963) reported a better flavor retention by drying larger particle cheese powder. These authors (1964) demonstrated foam spray drying of cheddar cheese by incorporating nitrogen gas into the slurries prior to atomization of the product into the spray-dryer. The larger particle diameter of cheese powders resulted in an improved flavor quality and better retention of volatile compounds. The incorporation of nitrogen enhanced the shelf life of the product. Hedrick (1968) reported that spray dried QR Blue cheese exhibited more intense flavor if an extended ripening period was employed.

Freeze drying was applied initially to milk and dairy products by Nickerson et al. (1952). The same flavor quality and shelf life were reported for freeze dried as did for spray dried whole milk and it was concluded that freeze drying of whole milk was not superior to spray drying. The application for freeze drying of liquid milk products was patented by Ogden (1967). Flosdorf and Hamilton (1957) claimed that a better freeze drying process for cottage cheese was achieved at a slower freezing rate. It was reported that better wettability and porosity were obtained if the cheese was slowly frozen over a 30 minute interval. Several varieties of cheeses such as Blue, Cream, Cheddar, Brick and Munster were lyophilized (freeze dried) by Meyer and Jokay (1959). The dried Blue or Cream cheese samples exhibited poor rehydration properties and had inferior shelf life. Jokay and Meyer (1959) developed a satisfactory processing technique for freeze drying of Cottage cheese. Schultz (1964) studied freeze drying of process cheese and natural cheeses such as Quarg, double Cream cheese, Edam, Camembert, Gouda, Emmental, Romadur, Filsit, and Danish Blue. Cheese of higher initial moisture content, such as quarg, had better reconstitutability.

The loss of volatile flavor component in a pure state during an evaporation or drying process is directly proportional to its vapor pressure. However, in aqueous solutions the loss of a volatile compound is dependent upon its vapor pressure, solubility, presence of salts and

sugars, and miscibility of the compound with other organic constituents. Volatility of a flavor compound results from high vapor pressure and low solubility in its solvent. Completely soluble substances in aqueous solutions such as acetic acid have a pattern of water-liquid equilibria of two miscible compounds during evaporation (Saravacos, 1968; and Hawrysh, 1970).

During spray drying of butter containing known amounts of volatile fatty acids it was observed (Boudreau et al., 1966) that the degree of retention of longer chain fatty acids was greater than short chain fatty acids. It is concluded (Boudreau et al., 1966; Saravacos, 1968; and Saravacos and Moyer, 1968) that generally, as the volatility of compound is increased, flavor retention in a dried product is decreased. Gas chromatographic studies by Bradley and Stine (1968) showed that the retention of volatile compounds in foam-spray dried Cheddar increased as the particle size of the powder increased. The retention of flavor compounds was greater in foam spray-dried than conventional spray dried samples. However, the retention of flavor compounds added to skim milk was found (Reineccius and Coulter, 1969) to be independent of the particle size of the resultant powders. Desai (1966) indicated that volatile acids and flavor components such as diacetyl were retained to a greater extent in freeze-dried sour cream than in foam spray powders.

Saravacos and Moyer (1968) observed that the retention of flavor components in vacuum dried fruit juices was affected by the temperature of drying, the vapor pressure of compound, the relative volatility and the composition of the juice. These findings were confirmed by the results of Hawrysh (1970) who studied the effect of freeze drying process on the retention of volatile monocarbonyl flavor compounds such as methyl ketones, saturated aldehydes, and unsaturated aldehydes (2-enals) by incorporating them into cream with 30.5% milk fat. During lyophilization, short chain aldehydes were retained to a greater concentration than short chain methyl ketones. Retention of both classes of aldehydes was greater than methyl ketones in freeze-dried samples. It was also suggested that the recovery of high molecular weight monocarbonyls from freeze-dried samples may be determined by factors such as boiling point, solubility in the lipid phase of the cream, molecular size, and properties of absorption and adsorption. Blakely (1970) observed that the loss of volatile fatty acids during freeze-drying of Blue cheese was related to their boiling points. The lower the boiling point, the greater was the loss of volatile acids during dehydration. The retention of fatty acids in freeze-dried Blue cheese was greater than spray dried samples. The same author showed that the retention of methyl ketones with less than 10 carbons was not related to the boiling point of the

component. Lower retention of methyl ketones was found in freeze dried than spray dried Blue cheese.

EXPERIMENTAL PROCEDURES

Production of Quick Ripened (QR) Cheese

Blue Mold Powder

Blue mold powder of P. roqueforti, Code #65001 was obtained from Midwest Mold Powder (Dairyland Food Laboratories, Inc., Waukesha, Wisconsin 53186) and used directly in the cheese.

Preparation of White Mold Powder

Spores of the white mutant of P. roqueforti were prepared by the method of Hussong and Hammer (1935). The mold was isolated from a slant obtained from Midwest Mold Company and transferred several times on acidified potato dextrose agar slants. Whole wheat bread (no additive added) was cut into cubes, approximately 0.5 inches and sterilized in wide mouth jars at 15 lbs pressure for about 30 minutes. After cooling to room temperature a suspension of spores in water was prepared from the slants and inoculated into the sterilized bread. The inoculated samples were incubated at 21°C for 12 days for spore formation. During incubation the bottles were shaken occasionally. After spore formation the bread pieces were dried in an oven at 38°—40°C for 36-48 hours (Pulay, 1959) and

pulverized in a sterile mortar and pestle. The dried mold was sieved through a 45 mesh sieve (United States Standard Testing sieve) and stored at 4°C for later use.

Manufacturing Procedure Used for Quick Ripened (QR) Cheese

QR Cheese using P. roqueforti or the white mutant of P. roqueforti was manufactured according to the method of Kondrup and Hedrick (1963), and Hedrick et al. (1968). Suggested modifications of Kuehler (1974), Harte (1974), and Albert (1974) were used with minor changes.

Cheeses were made from 20 gallon lots of milk. Mixed herd raw whole milk was obtained from Michigan Milk Producers Association. The milk was preheated to 135°F and homogenized in a two stage homogenizer (2000 Psi first stage, and 500 Psi second stage). The milk was then pasteurized at 143°F for 30 minutes, cooled to 88°F and transferred to a fifty gallon cheese vat. A frozen concentrated cheese starter (Hansen's Lab, Inc., Milwaukee, Wisconsin 53214) was added at the rate of 17 grams per batch. When the acidity increased 0.020-0.025%, 24 grams of mold powder and 10 ml single strength rennet were added to the milk with agitations. The coagulum was cut with 3/8" knives and allowed to idle for 10 minutes. The curd was stirred gently and heated to 104°F for approximately one hour. The drained curd was placed on a fine mesh stainless steel screen in the ripening room to a depth of 3 to 4 inches. The curing room was maintained at over 95 percent

relative humidity(RH) and temperatures of 52 ± 1°F or 62 ± 1°F for a period of 7 to 15 days. The cheese curd was stirred twice the first day and once each day during the ripening period to prevent matting. Salt was added to the curd in the curing room at a rate of 1.0 lb per 22 lbs of cheese. The sequence for adding salt was: ¼, ¼, and ½ of total salt on a varying three day schedule during ripening. Five different lots of cheese were prepared and cured (see Table 2), and then packed and stored at 40°, 48° or 62°F for a period 4 to 15 days after the initial curing period.

Table 2.--Process Variables Used in Preparation of Quick Ripened Blue Cheese.

Cheese lot	mold	Curing Temperature, °F	Salting, days	Curing period, days
1	white	52±1	1,6,9	11 or 15
2	white	52±1	6,7,8	11 or 15
3	blue	52±1	1,9,10	11 or 15
4	blue	52±1	7,8,9	11 or 15
5	blue	62±1	4,5,6	7

Commercial Blue Cheese

Commercial imported and domestic Blue cheeses were obtained from local markets in E. Lansing and were analyzed as obtained.

Freeze Drying of Commercial and Quick Ripened Blue Cheese

Quick ripened Blue cheese and commercial Blue cheese were freeze dried in a Virtis Repp Model 42

freeze-dryer. The freeze dryer was equipped with automatic condenser and platen temperature and absolute pressure controls. The samples were layered to a depth of 3/4" in 11 1/4 x 7 1/2 inches aluminum trays. Then the fresh cheese was frozen overnight to -10^{o}F and dried in the freeze dryer at a platen temperature of 100^{o}F. The condenser temperature was set at -80^{o}F. The vacuum of the chamber was 5 x 10^{-3} Torr (McLeod Gauge) during 24.0 hours drying period. After conclusion of the drying, the vacuum was released with nitrogen and the dried cheese was packed in polyethylene bags and stored at 4.4^{o}C.

ANALYTICAL PROCEDURES

pH

The pH of the cheese samples was measured with a Chemtrix digital pH meter, Type 60A, to ±0.01 pH unit.

Moisture

Moisture content of samples was determined according to the method of the Association of Official Analytical Chemists for cheese (AOAC, 1975).

Liquid Chromatographic Analysis of Methyl Ketones

Preparation and Purification of Solvents

Hexane:

Carbonyl-free hexane was prepared from redistilled hexane by the methods of Hornstein and Crow (1962), and Schwartz and Parks (1961). Forty five milliliters of concentrated sulfuric acid were added to 75.0 grams of celite 545 (Johns Manville) and blended to a homogeneous mixture. The acid-celite was packed in a chromatographic column, 25 x 500 mm, equipped with a 500 ml reservoir at the top and a stopcock (teflon) to control the flow rate. A piece

of glass wool was placed in the bottom of the column. Each portion of the acid-celite mixture was tapped and firmly packed with a glass rod. Anhydrous granular sodium sulfate was used to cover the lower and upper levels of the mixture in the column to a depth of about 10 cm. Redistilled hexane was passed over this column at a rate of 3-5 ml per minute.

The eluate from this column was passed over a 2,4 DNPH-reaction column (Schwartz and Parks, 1961). Five-tenths gram of recrystallized 2,4-dinitrophenylhydrazine (2,4-DNPH) was dissolved in 6.0 milliliters of 85% phosphoric acid by grinding in a mortar until a clear yellow solution was obtained. Four milliliters of deionized distilled water was added and the precipitated DNPH was redissolved by grinding. Ten grams celite (Johns Manville Analytical grade) was ground with the solution until a homogeneous bright yellow blend was obtained. The mixture was packed in a 25 x 500 mm chromatographic column containing 25 ml carbonyl-free hexane. Four equal portions of the mixture were added separately to the column and tapped firmly. A piece of glass wool was placed over the last portion to prevent disturbing the column materials at the upper level. The column impurities were flushed with 50 ml carbonyl-free benzene, followed by 50-100 ml carbonyl free hexane.

The eluate from the reaction column was mixed with Sea Sorb 43 (Adsorptive magnesia, iodine no. 80, Johns

Manville) as suggested by Blakely (1970). The Sea Sorb-hexane mixture was filtered through a fluted filter paper to remove the carbonyl derivatives from hexane. Carbonyl free hexanes were stored in brown bottles.

Benzene:

Carbonyl-free benzene was prepared by refluxing reagent grade benzene over 2,4-dinitrophenylhydrazine for 2.0 hours. Two grams of 2,4-DNPH per liter of solvent was used (Hawrysh, 1970). The benzene was redistilled and was stored in brown bottles.

Chloroform:

Reagent grade chloroform was refluxed over KOH pellets for 30 minutes and then redistilled. Alternatively, distilled in glass-chloroform (obtained from Burdick and Jackson Lab, Inc., Muskegon, Michigan) was used directly.

Nitromethane:

Nitromethane (Fisher Scientific Co.), or spectrophotometric grade nitromethane (Mallinckrodt, Inc., St. Louis, Missouri) was redistilled over boric acid. Purified solvent boiling between 100.7-101.5^{o}C was collected in brown bottles.

Acetonitrile:

High purity acetonitrile, 81-82^{o}C boiling range, was obtained from Burdick and Jackson Lab, Inc.

Ethanol:

Carbonyl free ethanol was prepared by distilling 95% ethanol over 2,4-dinitrophenylhydrazine, using one gram DNP per 1000 ml ethanol.

Ethylene Chloride:

Reagent grade 1,2-dichloroethane was obtained from Mallinckrodt, Inc. and redistilled over anhydrous potassium carbonate and stored in a glass bottle (Anderson, 1966).

Butanol-1:

Distilled in glass Butanol-1 was obtained from Burdick and Jackson, Inc.

Purification of 2,4-dinitrophenylhydrazine

The 2,4-dinitrophenylhydrazine reagent was obtained from Eastman Kodak, Inc., or Pharmaceuticals, Inc. and recrystallized (Vogel, 1962) to use in this study.

The 2,4-DNPH was added to butanol-1, at a concentration of 1:30 and heated on a steam bath with occasional shaking until most of the reagent was dissolved. The hot solution was filtered through a fluted filter paper and held overnight for recrystallization. Crystals were separated from the solvent by filtration through a Buchner funnel and dried at room temperature in a desiccator. Dried crystals were stored in a brown bottle at 4.4^{o}C.

Preparation of 2,4-DNPH Derivatives of Standard Methyl Ketones

Standard methyl ketones, 99% purity, were obtained from Analab, Inc. (North Haven, Conn. 06473), or Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin) and were used to form hydrazone derivatives according to Shriner and Fuson, and Curtin (1964). Four ml concentrated sulfuric acid were added to 0.8 grams of recrystallized 2,4-dinitrophenylhydrazine. Then 6.0 milliliters distilled water were added dropwise to the mixture with swirling until solution was complete. Twenty ml carbonyl-free ethanol were added to the mixture and this was called solution (A). A solution of standard methyl ketone (B) was prepared by adding 1.0 gram standard methyl ketone to 40 milliliters of 95% carbonyl free ethanol. Solution (A) and (B) were mixed in a beaker until precipitation occured. The 2,4-DNP-hydrazones were separated by filtration and dissolved in 60 ml of carbonyl-free ethanol and the mixture was heated on a steam bath until a complete solution was obtained. A few drops of distilled water was added until a cloud point was reached. Then the hot solution was filtered rapidly and allowed to stand overnight for recrystallization. If hydrazones were not dissolved in ethanol, ethyl acetate was added up to 2.0 milliliters to obtain complete solution. The recrystallized 2,4-DNP-hydrazones were filtered, using a Buchner funnel, dried at room temperature, and then stored in the freezer for later use.

Isolation of Monocarbonyls from Cheese Samples

Extraction of Fat:

Approximately 250 g of each sample of cheese was ground thoroughly using a mortar and pestle. Duplicate analysis were performed from these representative samples for methyl ketone determination. Ten grams of cheese sample were mixed with 15 grams celite 545, dried 24 hours at 150°C, and ground to a homogeneous blend. The mixture was packed into a 25 x 500 mm chromatographic column equipped with fritted glass filter and then tapped gently with a glass rod. The fat was extracted with 200 milliliters carbonyl-free hexane. A similar column was packed and extracted with 200 milliliters redistilled hexane into a 300 milliliters pre-weighed soxhlet flask to determine the fat recovery. The same flask was connected to a rotary evaporator and after removing the solvent from the fat, the flask was dried in a 100°C oven for 20 minutes, cooled in a desiccator, and re-weighed to calculate recovery.

Formation of 2,4-DNP-hydrazone Derivatives:

A 2,4-DNPH reaction column was prepared according to Schwartz and Parks (1961) to convert the carbonyl compounds present in fat extract to their corresponding 2,4-DNP-hydrazones. The hexane extract of the cheese sample was passed through this column. When the 200 ml hexane extract passed the column, 200-300 ml carbonyl free

hexane was added. The column was flushed using 1-3 psi nitrogen pressure until the eluate had the same spectral properties as before addition of the sample. The solvent was removed by rotary evaporator.

Removal of Fat from 2,4-DNP-hydrazones:

The fat containing hydrazones were dissolved in 5-10 ml carbonyl free hexane and the 2,4-DNP-hydrazones were isolated from fat as described by Schwartz et al. (1963) and Anderson (1966) with slight modification. Eighteen grams Sea Sorb 43 (adsorptive magnesia, iodine No. 80) were mixed with 36.0 grams celite 545, dried 24 hours at 150°C, in about 250 ml carbonyl free hexane. The slurry was poured into a 25 x 500 mm chromatographic column equipped with a coarse fritted glass disk and a connected 500 ml reservoir. The column was packed using 3-5 psi nitrogen pressure. The fat containing hydrazones was carefully applied to the column. When the 2,4-DNP-hydrazones were adsorbed on the column as a deep brown band, the fat was washed off the column by subsequent addition of 200 ml hexane, 100 ml hexane:benzene (1:1 v/v), and 200 ml benzene using 1-3 Psi nitrogen pressure. The hydrazones were eluted from the column by adding 250 ml chloroform-nitromethane (3:1 v/v). The eluate was evaporated in a rotary evaporator to remove the solvents from DNP-hydrazones.

Fractionation of 2,4-DNP-hydrazones on Weak Alumina:

The monocarbonyl derivatives were separated from ketoglycerides as described by Schwartz et al. (1963) on a weak alumina column (Schwartz and Parks, 1961). Alumina, grade F-20 (Aluminum Co. of America) was activated by heating 24 hours at 150°C and then partially deactivated by the addition of 6% (w/w) distilled water, mixing, and storing in a bottle for 24 hours to equilibrate. Five grams of partially deactivated alumina were packed in a 12 x 300 mm chromatographic column in hexane. The DNP-hydrazones described previous step were dissolved in 5 ml hexane and applied carefully to this column. The monocarbonyl derivatives were eluted by adding 50 ml hexane-benzene (1:1 v/v). The eluate was evaporated in the rotary evaporator.

Determination of Methyl Ketones by High Pressure Liquid Chromatography (HPLC)

HPLC Apparatus:

A high pressure liquid chromatographic system was used to isolate and quantitate the methyl ketones present in the monocarbonyl fraction of the cheese samples. A schematic of the HPLC system is shown in Figure 1.

HPLC Specification:

The components of the assembled HPLC system (Figure 1) were composed of the following:

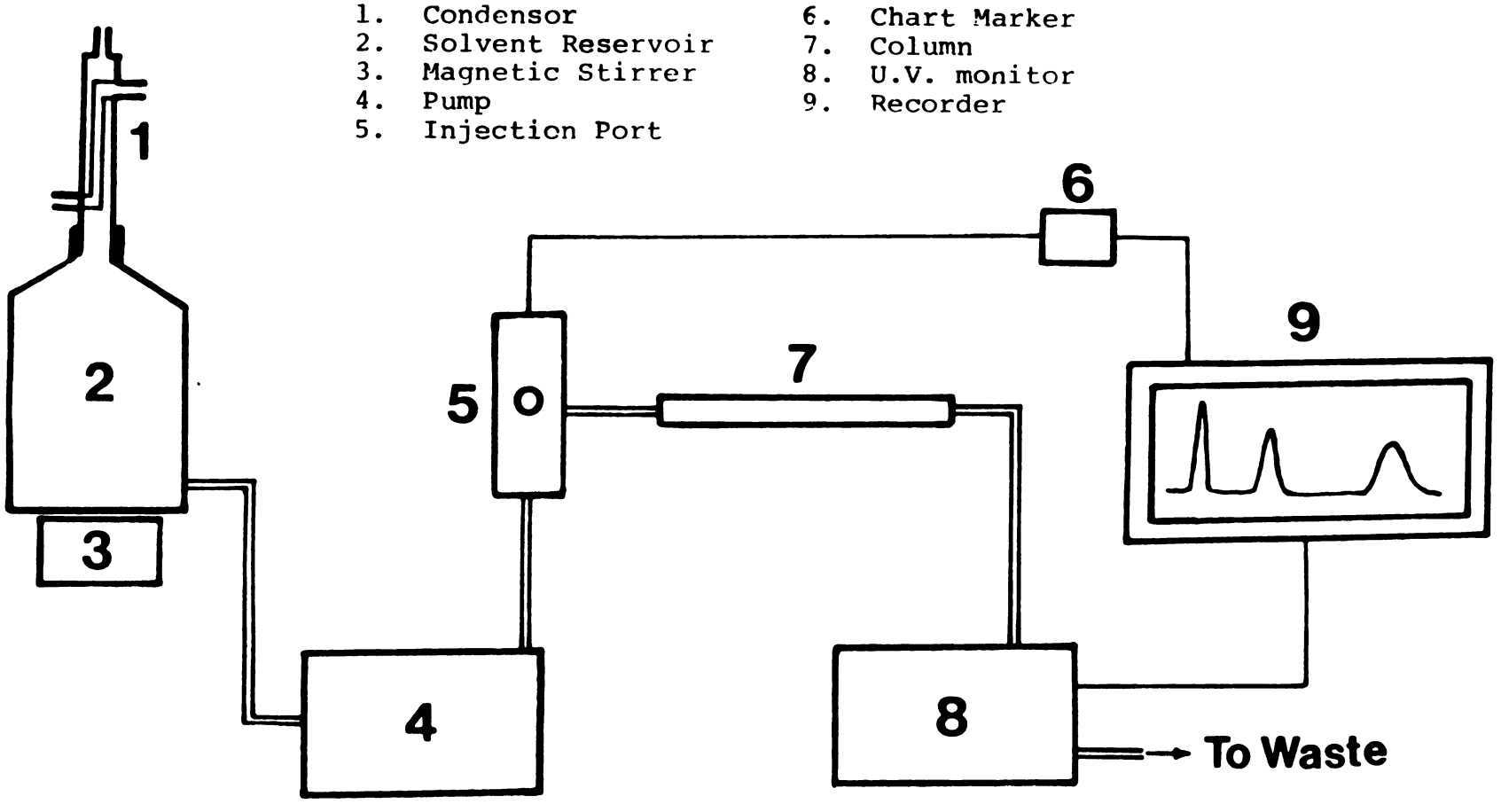


Figure 1. Schematic of high pressure liquid chromatographic (HPLC) system.

A. One liter solvent reservoir equipped with a condenser and a magnetic stirrer. The reservoir was placed about 5 inches above the pump to maintain a slightly positive pressure for the system.

B. C_{903} liquid chromatograph mini pump with motor, type NSI-33R (Waters Associates, Inc., Milford, Massachusetts) equipped with a 4000 Psi gauge pressure capable of a maximum flow rate of 2.7 ml per minute.

C. An injector port model U6K equipped with a chart marker (Waters Associates, Inc.).

D. A reversed-phase μBondapak C^{18} column with 3.9 (I.D.) x 300 mm dimensions (Waters Associates, Inc.).

E. A dual beam U.V. monitor Model 100 with 10.0 mm pathlength flow cell, 1.00 mm cell diameter, and 8 microliters cell volume (Pharmacia Fine Chemicals, Piscataway, New Jersey). The maximum sensitivity of the monitor was 0.01 Absorbance Units Full Scale (AUFS). The measurements were obtained at 254 nm.

F. A two-pen recorder Model 281 (Linear Instruments Corporations, Costa Mesa, California).

Stainless steel tubings, 0.009 inches I.D., and 0.0625 inches O.D. were used to connect the pump, injection port, column, and the U.V. monitor. With these dimensions, a minimal dead volume was assured for the system. Teflon tubing was used to connect the solvent reservoir to the

pump. A 25.0 µl Pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, Louisiana) with a needle of 0.02 in 0.D. x 2.0 in. was used to make the injections into the HPLC system. The mobile phase, Acetonitrile:water, was degassed before applying to the system.

Preparation of Standard Curves:

A mixture of standard methyl ketone-2,4-DNP hydrazone in acetonitrile was prepared for HPLC analysis. Derivatives of 2-Propanone, 2-Pentanone, 2-hepanone, 2-nonanone, 2-Undecanone and 2-tridecanone were prepared at a concentration of 5000 µg ketone per 50 ml acetonitrile. The hydrazones mixture in acetonitrile was injected into the HPLC system at 3, 6, 10, and 15 microliter injection volume. The following HPLC conditions were used:

Temperature: ambient

U.V. monitor: 254 nm; 0.08-0.32 (AUFS) sensitivity range

Flow rate: 1.43 ml/min

Mobile phase: Acetonitrile-water (75:25 v/v)

Column: µBondapak C^{18} (3.9 x 300 mm)

Peak heights, chart units, were calculated on the base of 0.08 (AUFS) monitor sensitivity and were drawn vs concentration. The full-scale chart was 100 units which were equal to 10 inches. The slopes of standard curves were used in quantitation of methyl ketones. Duplicate analyses were performed for preparation of calibration graphs.

Recovery of Methyl Ketones:

The percent recovery of individual methyl ketones was determined as described in part by Anderson (1966), Blakely (1970), and this study. A mixture of accurately weighed standard methyl ketones: C_3, C_5, C_7, C_9, C_{11} and C_{13} were dissolved in 5 ml hexane. The mixture was passed over a 2,4-DNPH reaction column (Schwartz and Parks, 1961) to convert these ketones to their hydrazone derivatives. The concentrations used were approximately those found in Blue cheese. After the formation of hydrazones, 3.0 grams of fresh butter oil were added to the mixture to eliminate any hydrazone formation from fat. The remainder of the analysis was as described earlier for the cheese samples. The eluate from the alumina column was evaporated and the hydrazones were dissolved in acetonitrile and injected into HPLC. Duplicate analyses were performed for recovery determination. Two injections were made for each recovered sample. Percent recoveries were calculated as micromoles methyl ketone recovered divided by micromoles methyl ketone initially added.

Quantitation of Individual Ketones in Cheese Samples:

The monocarbonyls separated on weak alumina column were dissolved in small amount of pure acetonitrile and quantitatively transferred to volumetric flasks and made to mark with solvent. The injections into the HPLC system were

made at the same chromatographic conditions as for standard methyl ketones. The concentrations were in the range for calibration curves. Each sample was clarified before injection with a sample clarification kit (Waters Associates, Inc.) equipped with micropore solvent filters, 0.45 μm pore size. The retention times of methyl ketones were compared to their corresponding standard methyl ketone to identify each ketone. The peak heights were used in calculations of individual methyl ketone concentration (APPENDIX B). Duplicate analyses were performed in all samples, except those noted by asterisk (*) in results and discussion section.

Mass Spectral Analysis of 2-Undecanone-DNPH-Derivative

Preparation of the Sample:

A small quantity about 10 mg of pure, 99.0% 2-undecanone (C_{11}) was dissolved in 10 milliliters carbonyl free hexane and the mixture was passed through a 2,4-DNPH reaction column (Schwartz and Parks, 1961). After formation of 2,4 DNP-hydrazone of C_{11} methyl ketone, the solvent was removed and the derivative was dissolved in acetonitrile and the mixture was examined by injecting a 5 μl aliquot into the HPLC. Two fractions were present in the mixture.

The mixture of 2 fractions in acetonitrile was dried in rotary evaporator and then dissolved in

1,2-dichloroethane. This mixture was separated and collected individually by using a column of Celite 545-adsorptive magnesia (Schwartz et al., 1960; and Anderson, 1966). Fifteen grams adsorptive magnesia, heated to 400°C for 48 hours, and 30 grams of celite 545, heated to 150°C for 24 hours were slurried in 1,2-dichloroethane and packed in a 25 x 500 mm column equipped with a coarse fritted glass disk, using 3 - 5 Psi nitrogen pressure. The hydrazone derivatives of C_{11} methyl ketone were carefully applied to the column. The column was developed with 1,2-dichloroethane and the two separated fractions were collected in two flasks. The solvent was removed from each fraction and dissolved in acetonitrile. An aliquot of each fraction was injected into the HPLC and examined for single peak to confirm the presence of only one compound in each fraction. Finally each fraction was analyzed by mass spectrometry.

Mass Spectrometer Analysis:

The mass spectral analysis was accomplished in the Department of Biochemistry at Michigan State University. The measurements were done on a Varian MATCH5/DF which was connected to a PDP 11/or from Digital Equipment Corporation. The output routine was done with a PDP 11/40 interfaced to a Tektronic hard copy 4610 and a Tektronic scope 4010-1. The mass spectrometer parameters for both samples were: Electron Energy, 70 ev; Source Temperature, 190°C; direct

probe temperature linear heating scan from 20°C - 270°C best sample temperature, 210°C.

Separation of Methyl Ketones from Other Classes of Mono-Carbonyls in Blue Cheese

A sample of monocarbonyl from QR Blue cheese was prepared and dissolved in 1,2-dichloroethane. The adsorptive magnesia-celite 545 column (Schwartz et al., 1960; and Anderson, 1966) was used to separate methyl ketones from other classes of monocarbonyls such as aldehydes, if present in the cheese. The column was developed with 1,2-dichloroethane and separation was followed visually since different classes formed different colors on the adsorbent. The first eluted fraction with a gray color belonged to methyl ketones. This fraction was separated. The solvent was evaporated and methyl ketone hydrazones were dissolved in acetonitrile and analyzed in the HPLC unit.

RESULTS AND DISCUSSIONS

Liquid Chromatographic Determination of Methyl Ketones as their 2,4-Dinitrophenyl-hydrazone (2,4-DNPH) Derivatives

Extraction and Fractionation of Carbonyl Compounds in Cheese Fat

Generally, carbonyl compounds play a major role in the flavor of oxidized fats and oils. The majority of these constituents, over 95%, are non-volatile when the usual methods of distillation are used for their determination (Lillard and Day, 1961; and Lea and Swoboda, 1962). Therefore, extraction procedures with carbonyl-free solvents (Schwartz and Parks, 1961) were used to isolate the monocarbonyls responsible for Blue cheese flavor from cheese fat. Schwartz, Haller and Keeney (1963) extracted the carbonyl compounds of fats and oils, cheese fat and whole milk powder. The monocarbonyl fraction was separated into saturated aldehydes, methyl ketones, 2-enals and 2,4-dienals.

In this experiment the homogeneous mixture of cheese sample and dried celite were extracted with carbonyl-free hexane to obtain a near theoretical yield of fat. A quantitative yield of fat was not considered critical (Schwartz and Parks, 1963). The hexane-fat mixture was applied to

a 2,4-DNPH reaction column to convert all of the compounds with carbonyl groups to their 2,4-DNP-hydrazones. The derivatives prepared in such a way were reported to be acid free (Schwartz, Haller and Keeney, 1963), thereby eliminating the need for extraction or other manipulations. Also, extremely low concentrations of carbonyls can be derivatized into hydrazones in this column. On the other hand the same authors (1963) demonstrated a linear relationship between fat concentration in hexane and the yield of 2,4-DNP-hydrazones and reported that the reaction of carbonyls with 2,4-DNPH in this column was quantitative over a wide range of fat concentration. The hexane effluent from the 2,4-DNPH reaction column contains all of the original lipids and 2,4-DNP-hydrazone derivatives of carbonyl compounds which are soluble in the fat-hexane solution. Remaining on the column are those derivatives which are insoluble in the fat-hexane solution.

The fat-hydrazone mixture in hexane was applied to a Celite 545-adsorptive magnesia column and the fat was eluted from the hydrazones by the addition of a sequence of solvents: hexane, hexane-benzene and benzene. Schwartz, Haller and Keeney (1963) used a ratio of 1:1 (w/w) Celite 545 and SeaSorb (adsorptive-magnesia) and reported the use of 1.0 gram SeaSorb 43 per 5 to 10 μM hydrazone. However, Anderson (1966) and Blakely (1970) used a ratio of 2:1, 28.0 grams Celite 545 and 14.0 grams SeaSorb 43 and obtained

better results. The same ratio was used in this experiment, but because of overloading the column with hydrazones during extraction procedures of several samples, the amount of the adsorbent was increased to 36:18 grams. The adsorbed hydrazones on this column appeared as a deep brown color. After fat removal and complete elution of the hydrazones with chloroform-nitromethane mixture from the column, several blue, grey, and violet bands remained adsorbed on the column. Schwartz, Haller and Keeney (1963) reported that these bands which were retained on magnesia belong to all dicarbonyl bis (2,4-DNP-hydrazones). Therefore, the eluate from this column contained all monocarbonyls and ketoglycerides. The small amount of fat which was probably present in the eluate was removed from the samples in later steps.

The mixture of monocarbonyls and ketoglycerides in hexane was fractionated on a weak alumina column (Schwartz and Parks, 1961) to remove the ketoglycerides from the samples (Schwartz, Haller and Keeney, 1963). Several samples were re-chromatographed on this column to obtain a sharper fractionation and fractions of greater purity.

High Pressure Liquid Chromatographic (HPLC) Separation of Methyl Ketones

The Reliability of HPLC System:

The typical HPLC separations of 2,4-DNP-hydrazone derivatives of standard methyl ketones, C3 - C13, are shown

in Figures 2 and 3. These derivatives, which were prepared according to Shriner, Fuson and Curtin (1964) and stored in the freezer, were dissolved in acetonitrile at the time of analysis and injected into the HPLC. The data in Figure 2 represent the optimum separation conditions of standard methyl ketone derivatives: 2-propanone (3), 2-Pentanone (C_5), 2-heptanone (C_7), 2-nonanone (C_9), 2-undecanone (C_{11}) and 2-tridecanone. The time for separation was approximately 33 minutes. This time was sufficient to resolve all of the components present in the monocarbonyl fraction of Blue cheese samples (see Figure 4). Therefore, the resolving power of the HPLC system was determined by chromatographying the monocarbonyl fractions of Blue cheese samples (Figure 4) and comparing the separated peaks with standard peaks in Figure 2 which were obtained by injecting methyl ketone hydrazones into the HPLC system.

The chromatograms in Figure 3 demonstrate the rapid resolution by HPLC of six DNP-hydrazone derivatives of standard methyl ketones. The analysis time was approximately 6.0 minutes if 100% acetonitrile was used as the mobile phase (Figure 3-A). The separation time was longer when a mixture of acetonitrile and water was used; 12.0 minutes for acetonitrile-water (90:10 v/v); and 26.0 minutes for a ratio of 80:20 v/v (see Figure 3-B and C). The resolutions of methyl ketones in Figure 3 were obtained faster than that in Figure 2. However, a poorer separation and overlapping of peaks occurred for ketones present in the monocarbonyl

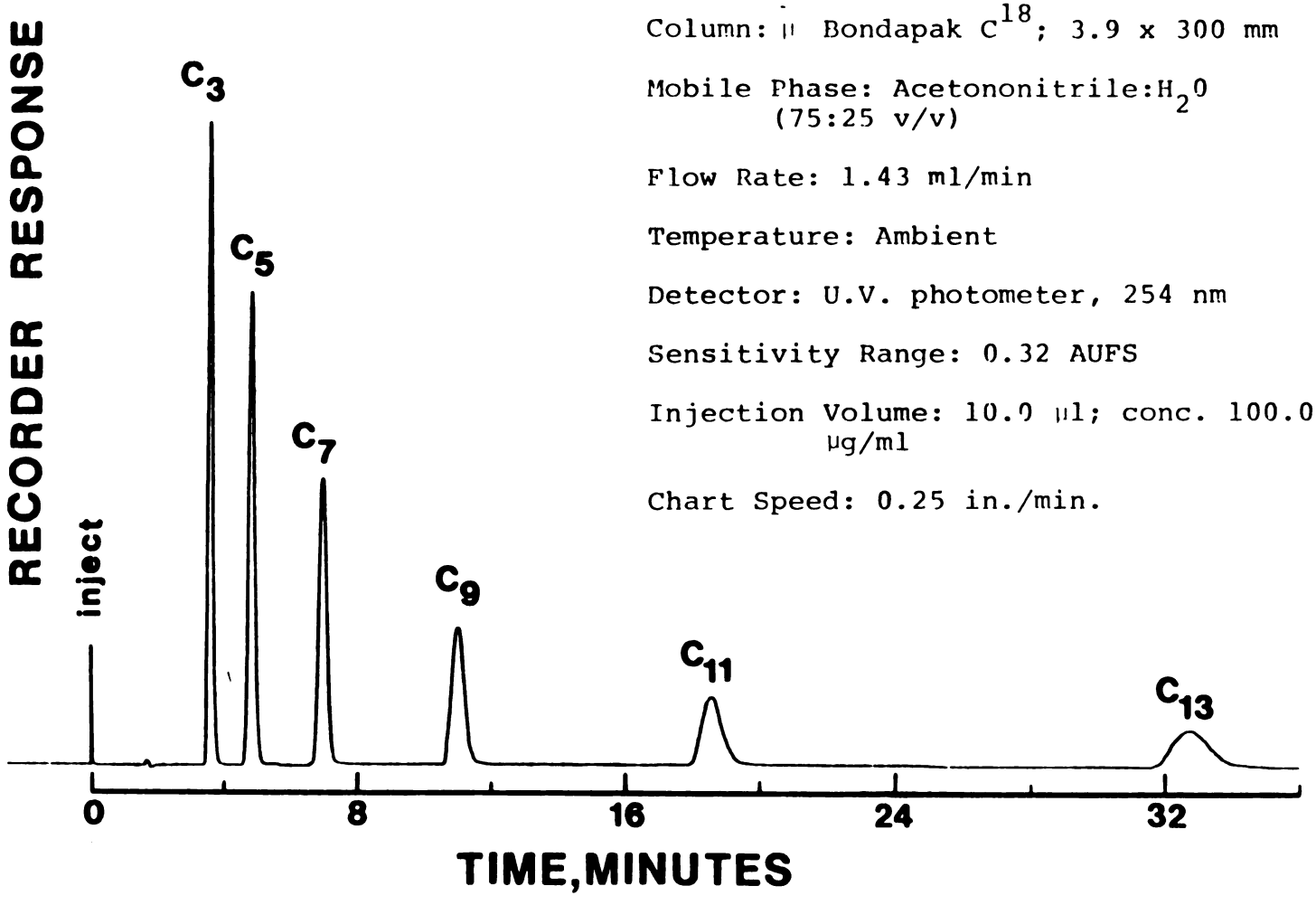


Figure 2. Typical HPLC separation of 2,4-dinitrophenylhydrazine derivatives of standard methyl ketones, 2-propanone (C_3), 2-pentanone (C_5), 2-heptanone (C_7), 2-nonanone (C_9), 2-undecanone (C_{11}), and 2-Tridecanone (C_{13}). The derivatives were prepared in solution and not "aged."

Figure 3. HPLC resolutions of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{13}, at different solvent ratio of acetonitrile:H_2O.

HPLC conditions

A) Column: μ Bondapak C^{18}; 3.9 x 300 mm.
Mobile Phase: Pure acetonitrile
Flow Rate: 1.08 ml/min
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.32 AUFS
Injection Volume: 5 μl
Concentration: 100 μg/ml per peak
Chart Speed: 0.25 in./min

B) Column: μ Bondapak C^{18}; 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (90:10 v/v)
Flow Rate: 1.10 ml/min
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.32 AUFS
Injection Volume: 5 μl
Concentration: 100 μg/ml per peak
Chart Speed: 0.25 in./min

C) Column: μ Bondapak C^{18}, 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (80:20 v/v)
Flow Rate: 1.20 ml/min
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.32 AUFS
Injection Volume: 5 μl
Concentration: 100 μg/ml per peak
Chart Speed: 0.25 in./min.

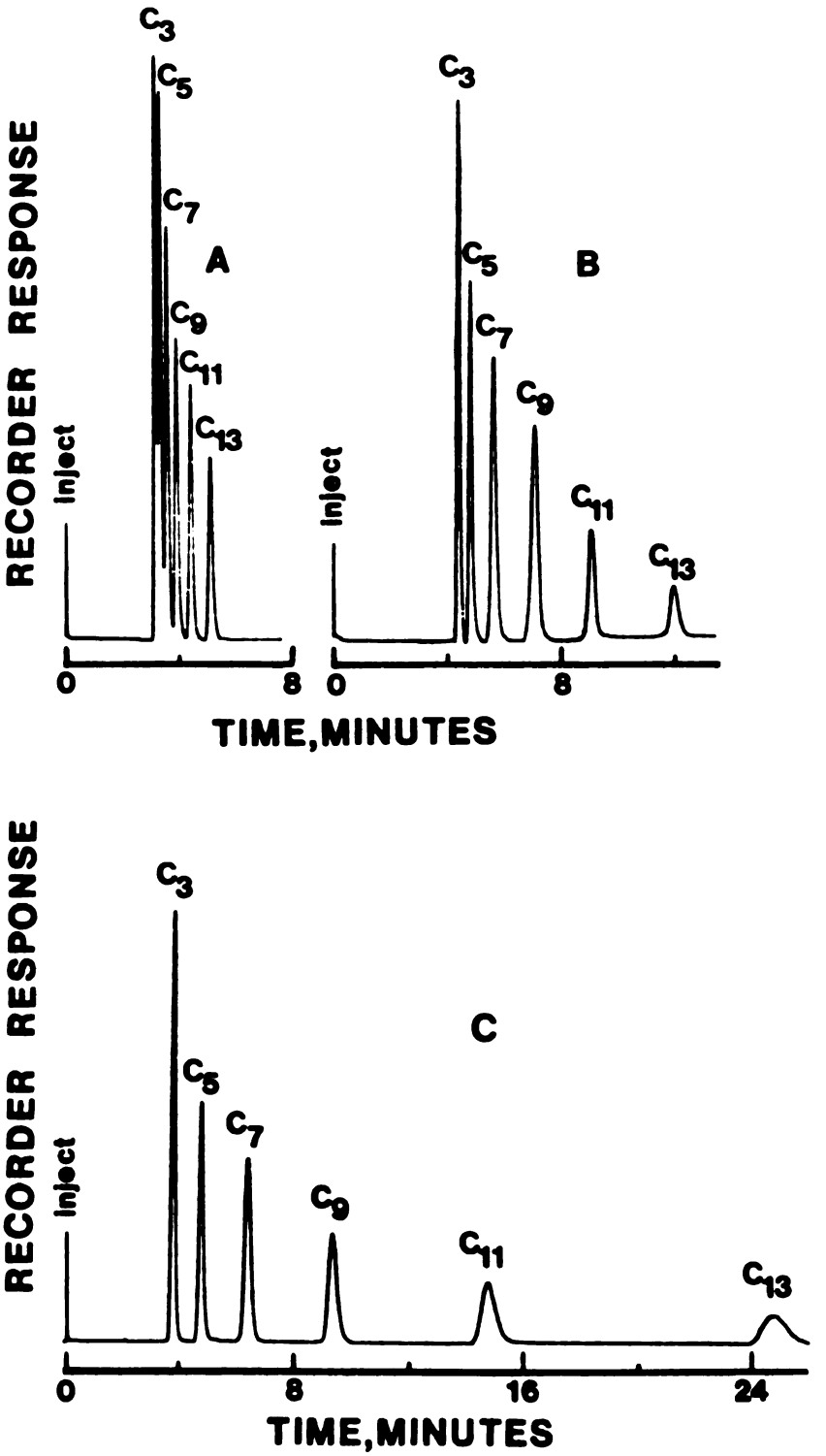


Figure 3. HPLC resolutions of 2,4-DNPH derivatives of standard methyl ketones, C_3 - C_{13}, at different solvent ratio of Acetonitrile:H_2O. A) 100:0; B) 90:10; and C) 80:20 (v/v). The derivatives were prepared in solution and not "aged."

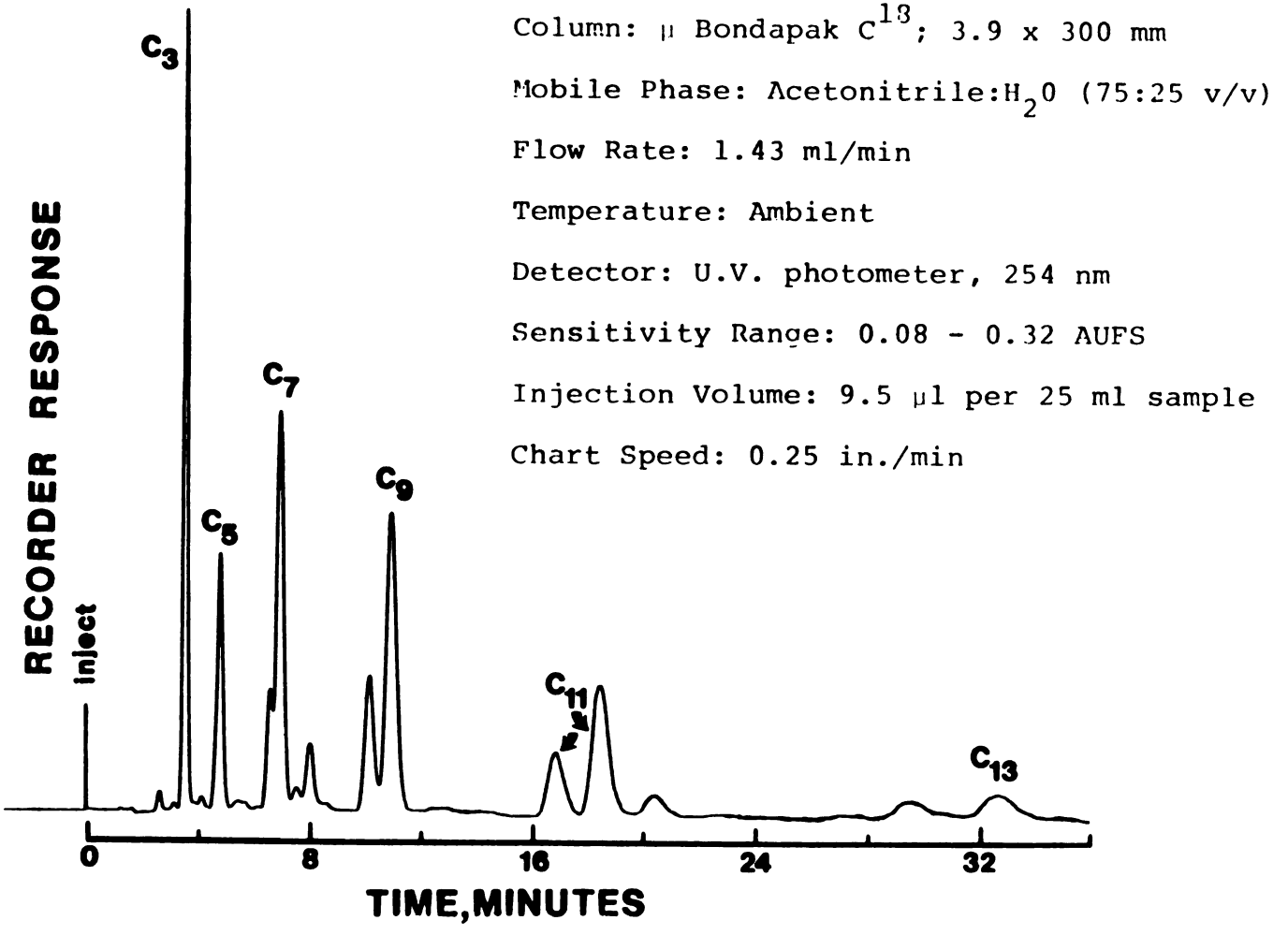


Figure 4. Typical HPLC separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{13}, present in monocarbonyl fraction of commercial blue cheese crumbles.

fraction of Blue cheese samples when faster analysis such as conditions in Figure 3 were used. The 33.0 minute separation of methyl ketones (Figure 2) was selected as optimal. Snyder and Kirkland (1974) showed that pure water exhibited the greatest polarity among solvents and if mixed with other polar solvents such as acetonitrile, an intermediate polarity would result. Therefore, water was used to increase the polarity of the solvent. Since a reversed-phase column (μ Bondapak C^{18}) was used in this study, the less polar solvent resulted in a faster elution of components from the column, particularly more polar compounds such as C_3 and C_5 methyl ketones, and some peak overlap and poorer resolution were obtained (Figure 3-A).

Peak height rather than peak area were used in this study for peak measurements. Peak height was measured as a distance from the baseline to the peak maximum. Snyder and Kirkland (1974) pointed out that the peak height method is almost always used for trace analysis. Peak height measurements are more accurate than peak area measurements because of lesser interference by neighboring and overlapping peaks. Snyder (1969) described the advantages of using peak heights rather than peak areas in measurements: a) relative insensitivity to small variations in solvent flow rate; b) insensitivity to marginal resolution; and c) easy calculations for quantitation. The disadvantage of sensitivity to changing sample retention volumes and band widths was reported by the same author.

Snyder and Kirkland (1974) state that "it is not necessary to work at the maximum absorption of a peak when using the U.V. photometric detector." The wavelength, 254 nm, is often satisfactory for compounds having UV absorption. The absorption spectra of several 2,4-DNPH derivatives of carbonyl compounds have been studied by Roberts and Green (1946). An absorption maximum of 360 nm was reported for the 2,4-DNPH derivative of acetone. The absorption maximum of 363 nm was presented (Day, 1965) for the whole class of 2,4-DNPH derivatives of methyl ketones in chloroform. In this study the absorbance of 2,4-DNPH derivatives of standard methyl ketones and ketones in mono-carbonyl fraction of Blue cheese samples were made at 254 nm. Papa and Turner (1972) and Selim (1977) used 254 nm and 336 nm respectively for the determination of several standard 2,4-DNPH derivatives of carbonyl compounds in their HPLC systems. These authors obtained excellent analyses of components, even at trace levels.

The typical retention times of separated methyl ketone derivatives by HPLC are presented in Table 3. These values were determined from double injection of samples into the HPLC system. The retention time of each peak was measured from the time of sample injection to the time the band maximum left the column (Snyder and Kirkland, 1974). Examples of typical chromatograms which were used in determining the retention times are shown in Figure 2 for

DNP-hydrazone of standard methyl ketones, and in Figure 4 for the hydrazones separated from the monocarbonyl fraction of Blue cheese. The 33 minutes HPLC analysis time was ended for each injection after the last component, C_{13} methyl ketone derivative, was eluted from the column. No ketone, nor any carbonyl compound was detected in the samples after C_{13} ketone was eluted from the column.

Table 3.--The retention time values for 2,4-DNP-hydrazones of standard methyl ketones and ketones present in monocarbonyl fraction of Blue cheese samples.

methyl ketone chain length	Retention time, minutes*	
	standards	Blue cheese
C_3	3.60	3.55
C_5	4.90	4.86
C_7	6.95	7.00
C_9	11.00	10.96
C_{11}	18.60	18.57
C_{13}	32.72	32.65

*Average values

The retention times obtained from standard methyl ketones were used to identify individual ketones in the Blue cheese samples (compare the values in Table 3). The slight difference in the retention times of the ketone of identical chain length in the standard mixture or monocarbonyl fraction may be explained as the variation in ambient

temperature of purity of samples. This small variation in retention times did not affect peak heights and therefore the quantitation of individual methyl ketones.

Detection of "Pre-Peaks" related to individual methyl ketones:

During HPLC analysis, it was observed that 2,4-DNP-hydrazone derivatives of methyl ketones such as C_7, C_9, C_{11} and C_{13} demonstrated "Pre-peaks" which appeared just before the major component was eluted from the column, and were detected at longer retention time than 7.0 minutes (see Figures 5 and 6). Comparing these Figures with Figure 2, it was found that this phenomenon happened if the standard methyl ketones were "aged." A mixture of standard methyl ketones, C_3 - C_{13}, was passed through the DNPH-reaction column and after formation of hydrazones the same procedures were followed as did for the Blue cheese sample. The HPLC separation of the last eluate, derivatives mixture in acetonitrile, is shown in Figure 6. The pre-peaks of C_7, C_9, C_{11} and C_{13} methyl ketone derivatives were detected after 6.5, 10.5, 17.0 and 30.0 minutes respectively. However, the retention times of the major peaks were comparable with the corresponding values in Table 3. Both major and related pre-peak of individual standard methyl ketones were similar to those obtained from Blue cheese samples (compare Figures 4 and 6).

In another trial a mixture of 2,4-DNPH derivatives of standard methyl ketones, C_3 - C_{13}, was dissolved in 5 ml

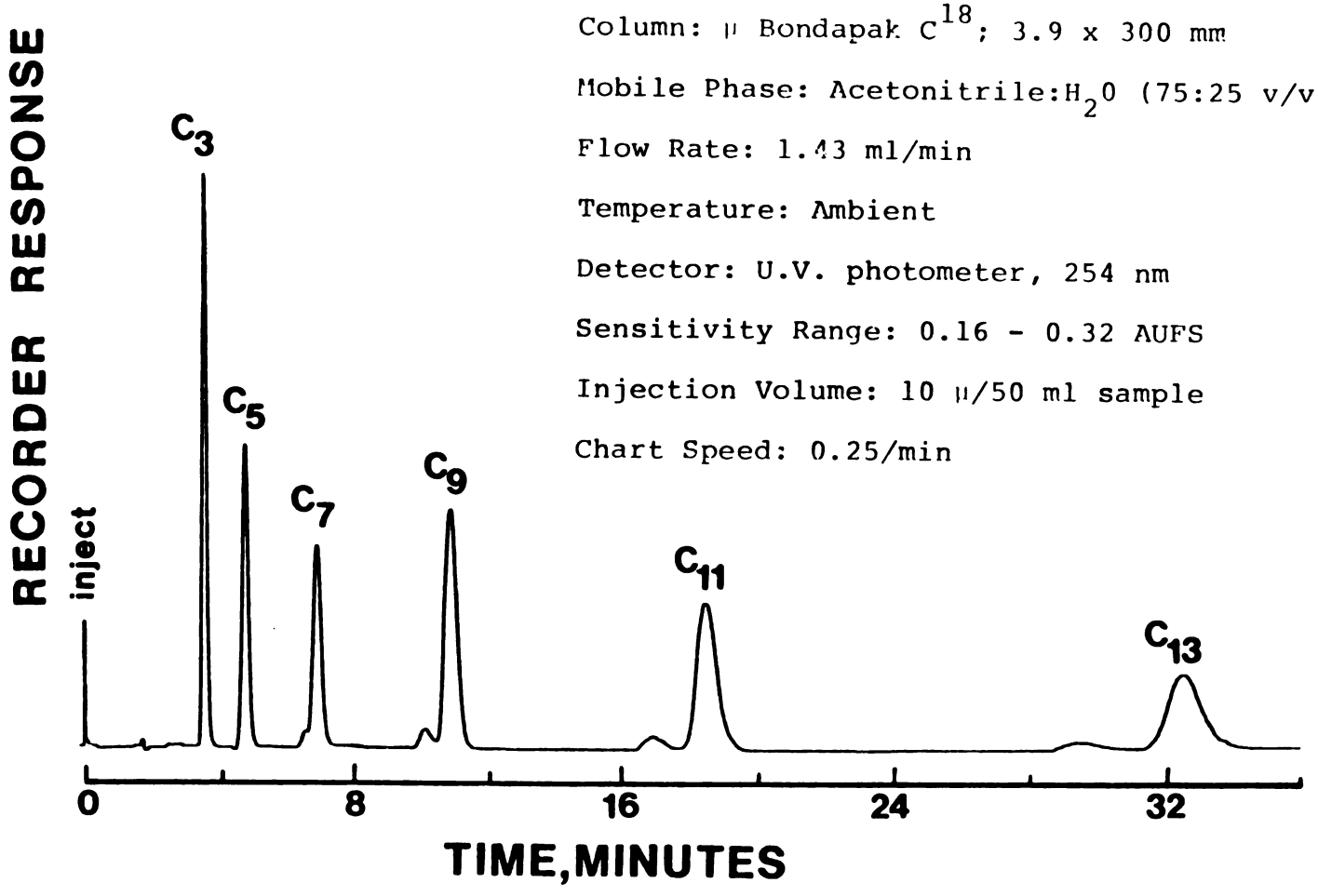


Figure 5. HPLC separation of 2,4-DNPH derivatives of standard methyl ketones, C_3 - C_{13}. The derivatives were prepared in solution and "aged" by passing through a celite-magnesia, and alumina column.

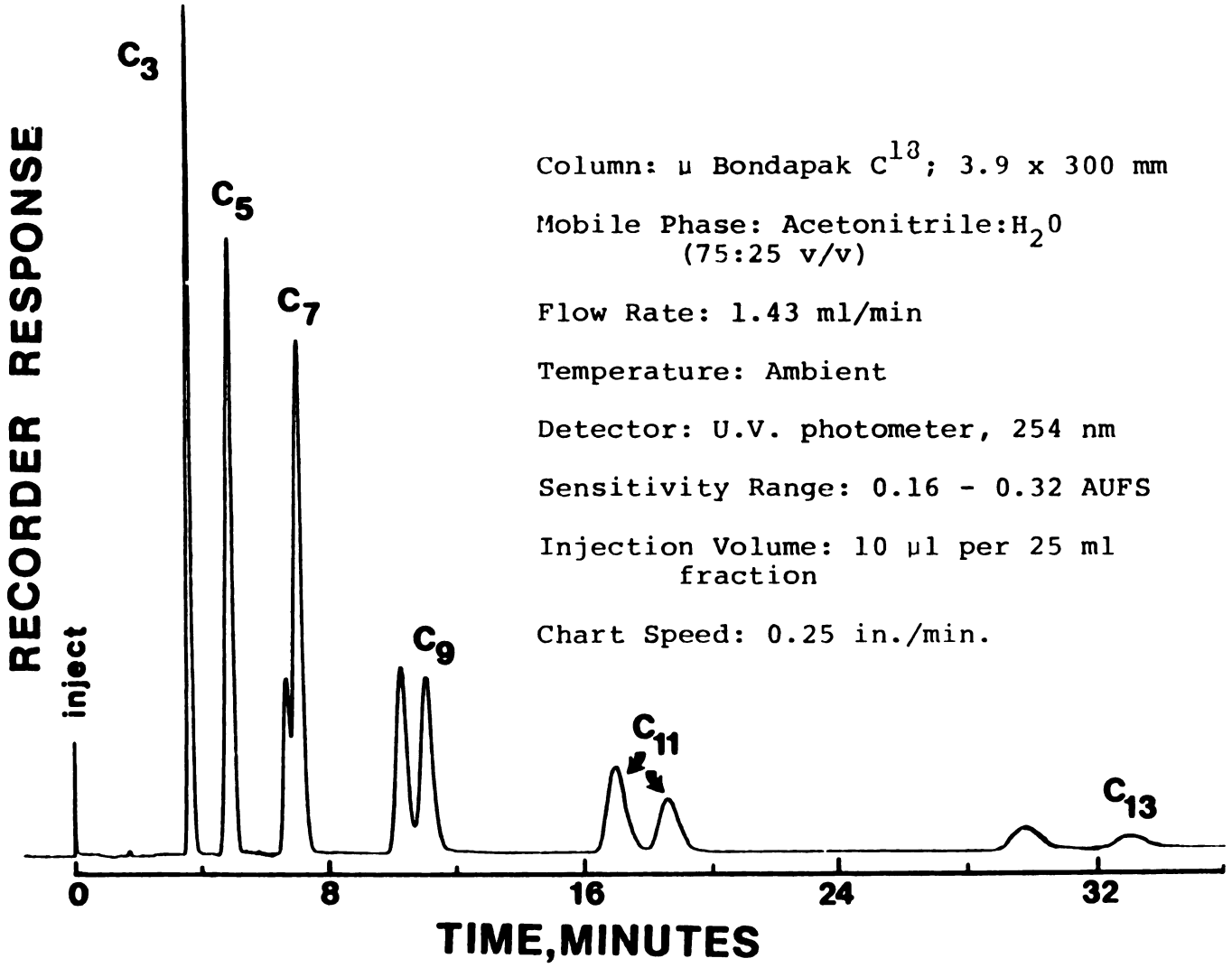


Figure 6. HPLC separation of 2,4 DNPH derivatives of standard methyl ketones, C_3 - C_{13}. The derivatives were prepared in a 2,4-DNPH reaction column and then passed through a celite-magnesia, and alumina column.

acetonitrile and the same above procedures, excluding the DNPH-reaction column, were applied to "age" the standards. The resulting HPLC separation is presented in Figure 5. The same pre-peaks were formed, but at slower rates. The smaller pre-peaks in Figure 5 (as compared to Figure 6) demonstrate the possibility of a significant conversion of original methyl ketone derivatives to their corresponding prepeaks components during derivatization in the DNPH-reaction column.

In a further examination of pre-peaks, the HPLC mobile phase concentration was changed to acetonitrile: water (70:30 v/v), and a 60 cm μ Bondapak C^{18} column was used instead of 30 cm. The pre-peak for C_5 methyl ketone derivative was also detected as well as longer chain methyl ketones; however, the total HPLC analysis time was considerably longer; more than 60.0 minutes. The retention time of C_{13} methyl ketone was over 60 minutes and hardly eluted from the column. The same results were obtained when the mobile phase concentration was changed to 65:35 (v/v) acetonitrile-water and the column was reduced in length to 30 cm. In this case the retention time for the first eluted peak, C_3 methyl ketone DNP-hydrazone, was 4.5 minutes and for C_{13} methyl ketone was over 60 minutes. These observations revealed the possibility of pre-peak detection for C_3 methyl ketone DNP-hydrazone, if the polarity of the mobile phase was changed to a lower concentration of acetonitrile and higher concentration of

water compared to 65:35 (v/v) acetonitrile-water. In contrast, when 100% acetonitrile was used as the mobile phase the pre-peaks were combined with their corresponding major peaks as shown in the HPLC chromatogram in Figure 3-A. This suggested similar characteristics between a pre-peak and its corresponding major peak of methyl ketones.

HPLC and mass spectrometric analysis of 2,4-DNPH derivative of C_{11} methyl ketone (2-undecanone)

Pre-peaks were further analyzed by preparing an individual methyl ketone DNPH-derivative and examining by both HPLC unit and then mass spectrometry. The derivative was obtained by applying 2-undecanone (99^{+}% pure) to the DNPH-reaction column. The C_{11} methyl ketone-DNP-hydrazone was then injected into the HPLC (Figure 7 - $inject_1$). Two peaks, C_{11} and its corresponding Pre-peak (C_{11}*) obtained which were qualitatively similar to the peaks for C_{11} methyl ketone in Figures 4, 5, and 6, shown with arrows. The slight difference in the retention time of peaks in Figure 7 with the other chromatograms was due to the difference in flow rates. This may also be related to a higher concentration of the samples in Figure 7 (Snyder and Kirkland, 1974). The mixture of C_{11} + C_{11}* was carefully fractionated on a Celite-adsorptive magnesia column which was developed by dichloroethane as described in the analytical procedures. The appearance of the two bands fractionated on the column was very similar. The color of the

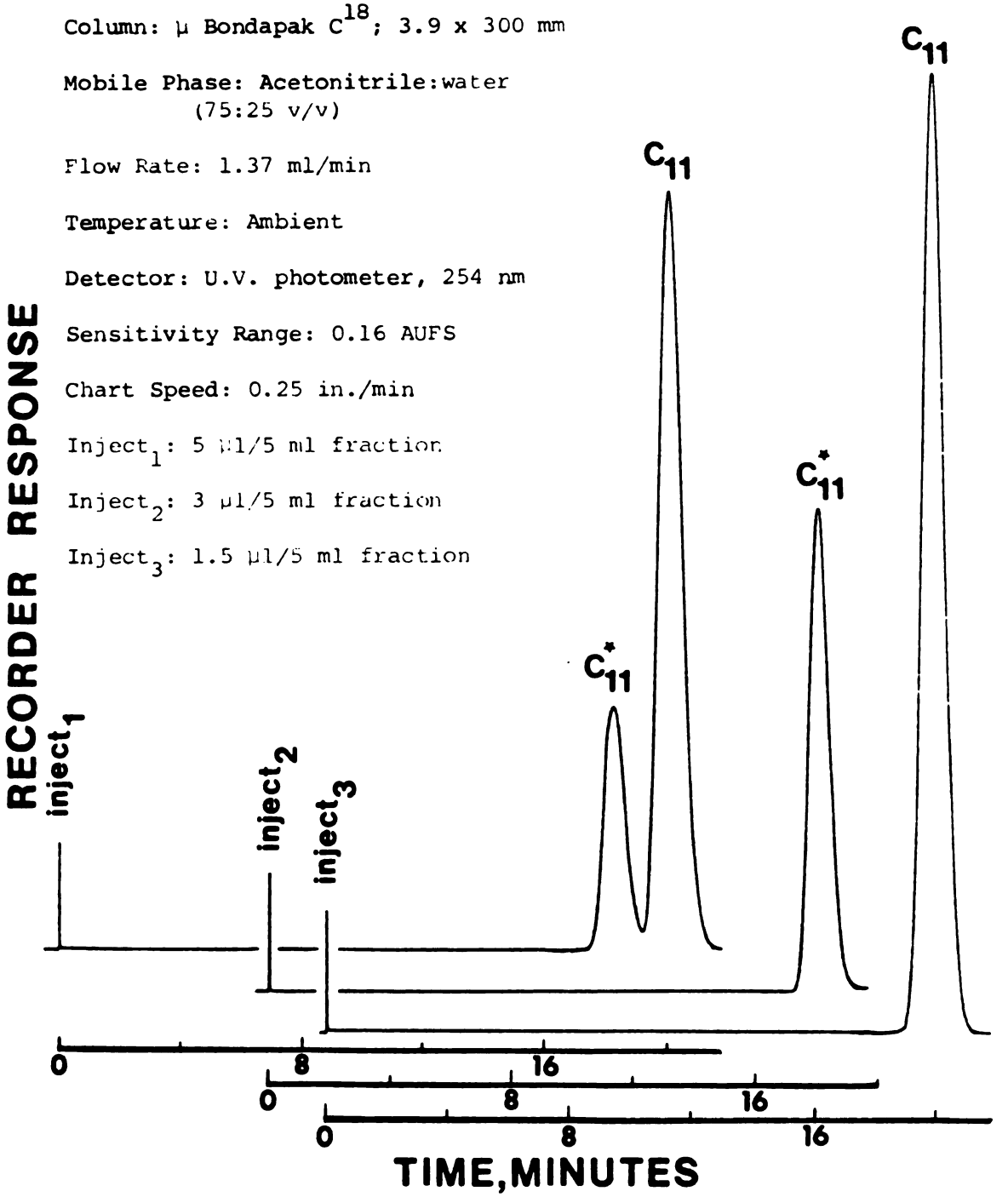


Figure 7. Injection 1: HPLC separation of 2,4-DNPH derivatives of standard 2-undecanone (C_{11}). The derivative was prepared in 2,4-DNPH reaction column.

Injection 2 and 3: HPLC detection of Pre-peak (C_{11}*) and original (C_{11}) peaks. C_{11}* or C_{11} were prepared by fractionating the mixture (C_{11} + C_{11}*) through a celite-magnesia column.

2 bands was reddish brown. The first fraction eluted from this column was examined by HPLC and the chromatogram C_{11} (Figure 7 - $inject_3$) was obtained. This was identical to C_{11} in the mixture (compare $inject_1$ and $inject_3$ in Figure 7). When the second fraction from Celite-adsorptive magnesia column (C_{11}*) was separated and injected into the HPLC unit, a faster elution was observed (Figure 7 - $inject_2$). This opposite elution pattern for the 2 peaks, C_{11} and C_{11}*, in conventional or HPLC fractionation demonstrated the difference between a normal and a reversed-phase μ Bondapak C^{18} column.

The two separated components, C_{11} or C_{11}*, were also analyzed on the mass spectrometer and the results are shown in Figure 8. Fragment ions of m/e above 350 were of very low intensity. The basepeak of 100% intensity in both spectra were shown to be at mass 41.0. According to Kleipool and Heins (1964) and Hawrysh (1970) the appearance of ion fragments at mass 178 and 238 are indicative of a methyl ketone 2,4-DNPH derivative. Kleipool and Heins (1964) studied the mass spectrometric identification of several DNPH derivatives of carbonyl compounds and concluded that aldehydes give strong peaks at mass 224, methyl ketones at mass 238, and ethyl ketones at mass 252. Both spectra in Figure 8 were similar and no peaks were observed at m/e 224 or 252. However, peaks are present at mass 178 and 238. A comparison of intensity of C_{11} and C_{11}* for peaks higher than 20% of basepeak is shown in Table 4.

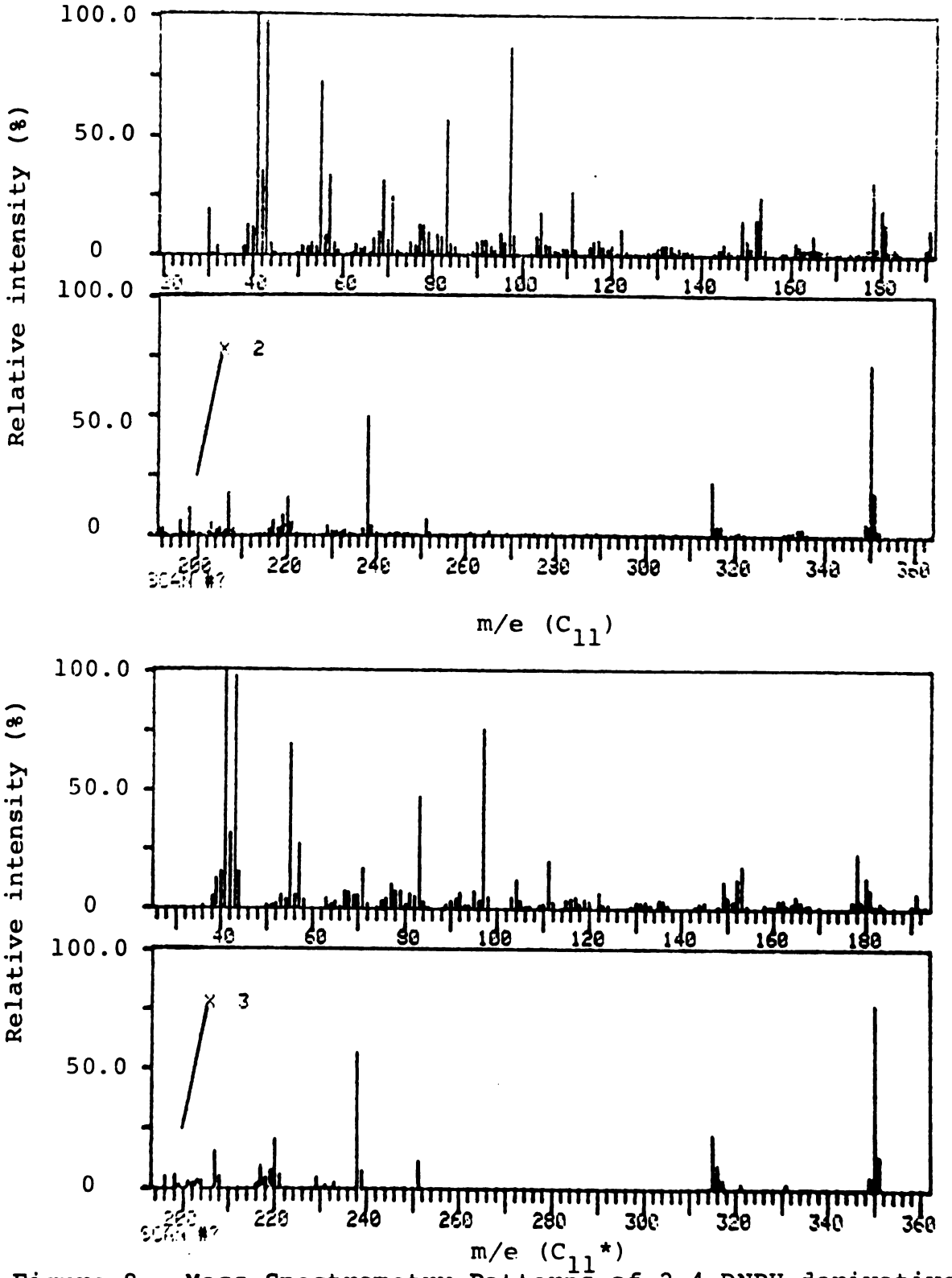


Figure 8. Mass Spectrometry Patterns of 2,4-DNPH derivative of standard 2-undecanone. The derivatives were obtained by treating the methyl ketone with the DNPH-reaction column. Top scan: C_{11}, M.W. = 350. Bottom scan, C_{11}*, M.W. = 350.

Table 4.--Intensity comparison of C_{11} and C_{11}* for peaks higher than 20% of basepeak.

m/e	% intensity of basepeak, (m/e 41.0), Δ		
	C_{11}*	C_{11}	ΔT^a
41.0	100	100	0
55.0	72	74	2
83.0	49	57	8
97.0	80	87	7
111.0	20	26	6
178.0	23	32	9
207.0	16	20	4
220.0	11	18	4
238.0	58	50	8
315.0	24	23	1
350.0	75	77	2

[a]Differences in intensities of C_{11} and C_{11}* at different fragment ions.

Since the instrumentation error, ΔT, was less than 10%, the identity of the 2 spectra can be confirmed. The total molecular weight of either DNPH-C_{11} or DNPH-C_{11}* was presented to be at mass 350 (Figure 8) which is comparable with the theoretical molecular wt. of 2-undecanone-DNPH-derivative, 350.44 (see Appendix A).

From the above findings, it can be presumed that pre-peak C_{11}*-DNPH derivative may be a geometric cis or

trans isomer of 2-undecanone-DNPH derivative. Similar to this, the pre-peaks shown in other methyl ketones such as C_7, C_9, and C_{13} (Figure 6) may exhibit the same molecular weights as their major peaks and thus cis or trans isomers. Titlar (1960) studied the isomerization of the 2,4-DNPH derivatives of acetophenone (phenyl methyl ketone) and prepared the two cis and trans isomers of this compound. This author reported that the rate of isomerization of such compounds in aqueous acidic solutions was eight times greater than their bromination rate. Therefore, it is likely that during reaction of methyl ketones, C_3 - C_{13}, with 2,4-dinitrophenylhydrazine in the reaction column and presence of phosphoric acid, geometric isomerization would take place and pre-peaks result which are detectable by HPLC (see Figure 6). This did not happen when derivization was performed in solution by the method of Shriner, Fuson and Curtin (1965), and the mixture of derivatives in acetonitrile was injected without "aging" (see Figure 2). However, if this mixture was "aged," slight formation of pre-peaks were demonstrated (see Figure 5). The same derivatives did not exhibit any pre-peaks as long as they were in the crystalline form and stored in the freezer.

HPLC separation of methyl ketones from other classes of monocarbonyls

To ascertain that the pre-peaks, described in the previous section, were not of aldehyde or any other classes

of carbonyl compounds, methyl ketones were separated from other classes of monocarbonyls in QR Blue cheese (Schwartz and Parks, 1961). The typical HPLC separation of these ketones is presented in Figure 9. Similar patterns of methyl ketones were observed and the retention time of each resolved peak was the same as was demonstrated for the separated ketones in the monocarbonyl fraction of Blue cheese or the standard ketones (compare Figure 9 with Figures 4 and 6). Several narrow and small aldehyde bands remained in the Celite-adsorptive magnesia column during fractionation of ketones from other classes of monocarbonyls (Schwartz and Parks, 1961). Therefore it can be stated that classes of monocarbonyls such as saturated aldehydes, 2-enals, and 2,4-dienals, if present in QR Blue cheese are in small concentration and are retained on the columns during extraction of other monocarbonyl compounds from Blue cheese. Kinsella and Hwang (1977) suggested that very small quantities of alkanals, alkenals, and alkdienals occur in Blue cheese. Blakely (1970) used several long procedures in addition to extraction of monocarbonyls to separate the trace amounts of these aldehydes from ketones in Blue cheese. HPLC analysis of monocarbonyl fraction in this study was much faster and made possible the analysis of cheese samples every day as they were obtained from the curing room.

Separation of several standard aliphatic and aromatic carbonyl compounds derivatized with

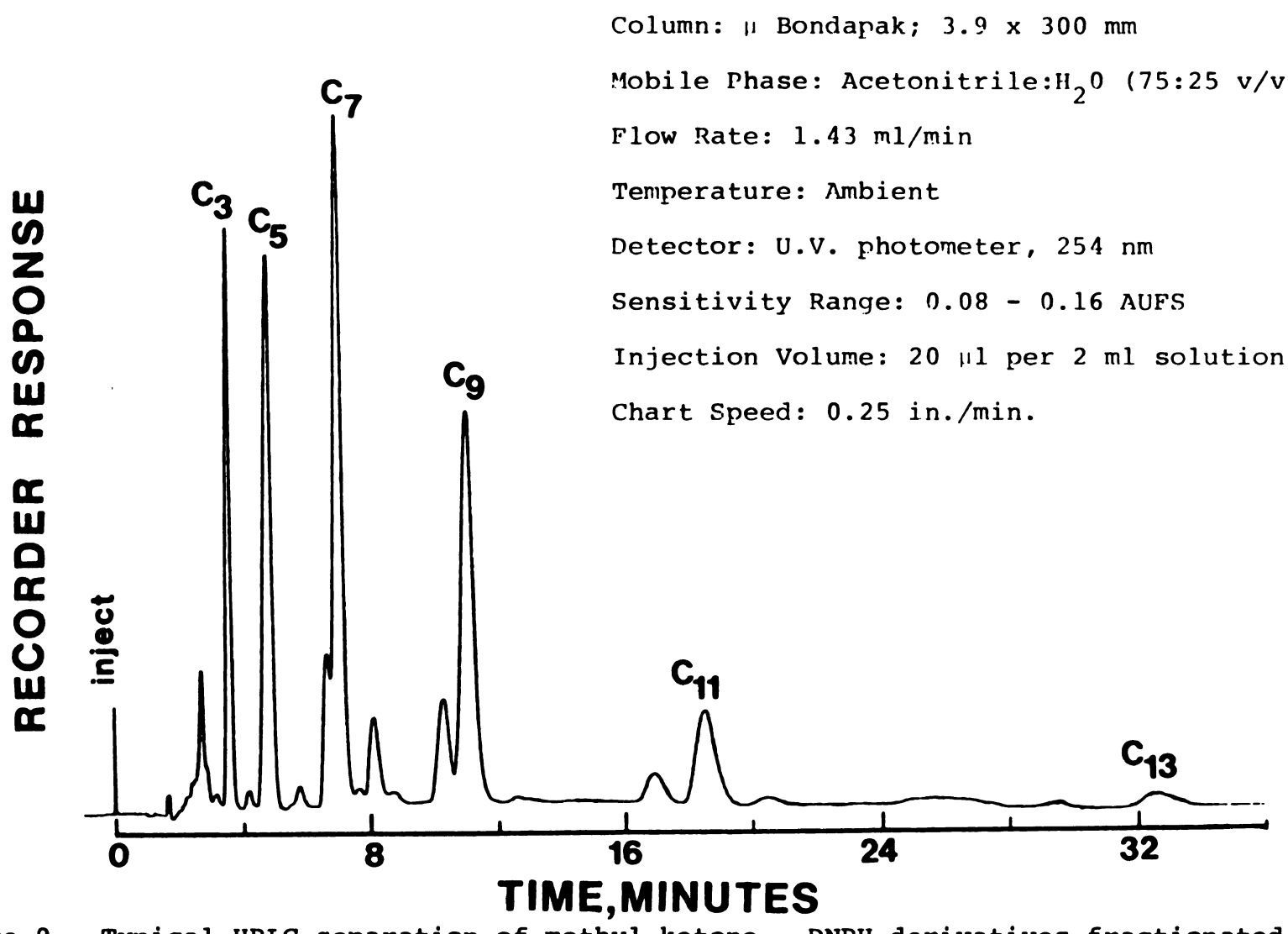


Figure 9. Typical HPLC separation of methyl ketone - DNPH-derivatives fractionated from other classes of monocarbonyls, present in quick ripened cheese.

2,4-dinitrophenylhydrazine was also demonstrated by Papa and Turner (1972) and Selim (1977), utilizing HPLC systems. Selim (1977), at the same time with this study, used a reversed-phase μ Bondapak C^{18} column and acetonitrile:water as the mobile phase in a HPLC unit and separated the traces of low molecular weight aldehydes and ketones. The same author (1977) suggested that the reversed-phase liquid chromatography was more advantageous than adsorption chromatography (Papa and Turner, 1972) in separating non-polar derivatives of carbonyl compounds.

Quantitation of individual methyl ketones from C_3 to C_{13}

Preparation of standard curve:

The linear-calibration graphs for six standard methyl ketone-2,4-DNPH derivatives are shown in Figure 10. These curves were presented as micromoles of hydrazones vs. the peak height units based on 0.08 AUFS detector sensitivity. The peak height units were expressed as the chart units. A full scale chart unit (100 divisions) were equal to 10.0 inches. The standard curves were obtained by injecting different volumes of known concentration of hydrazone mixture in pure acetonitrile into the HPLC. Duplicate analysis were performed and the slopes of these curves were calculated (Table 5) utilizing a programmed calculator. The curves were drawn from 5 points including (0.0). Correlation coefficients and intercepts were

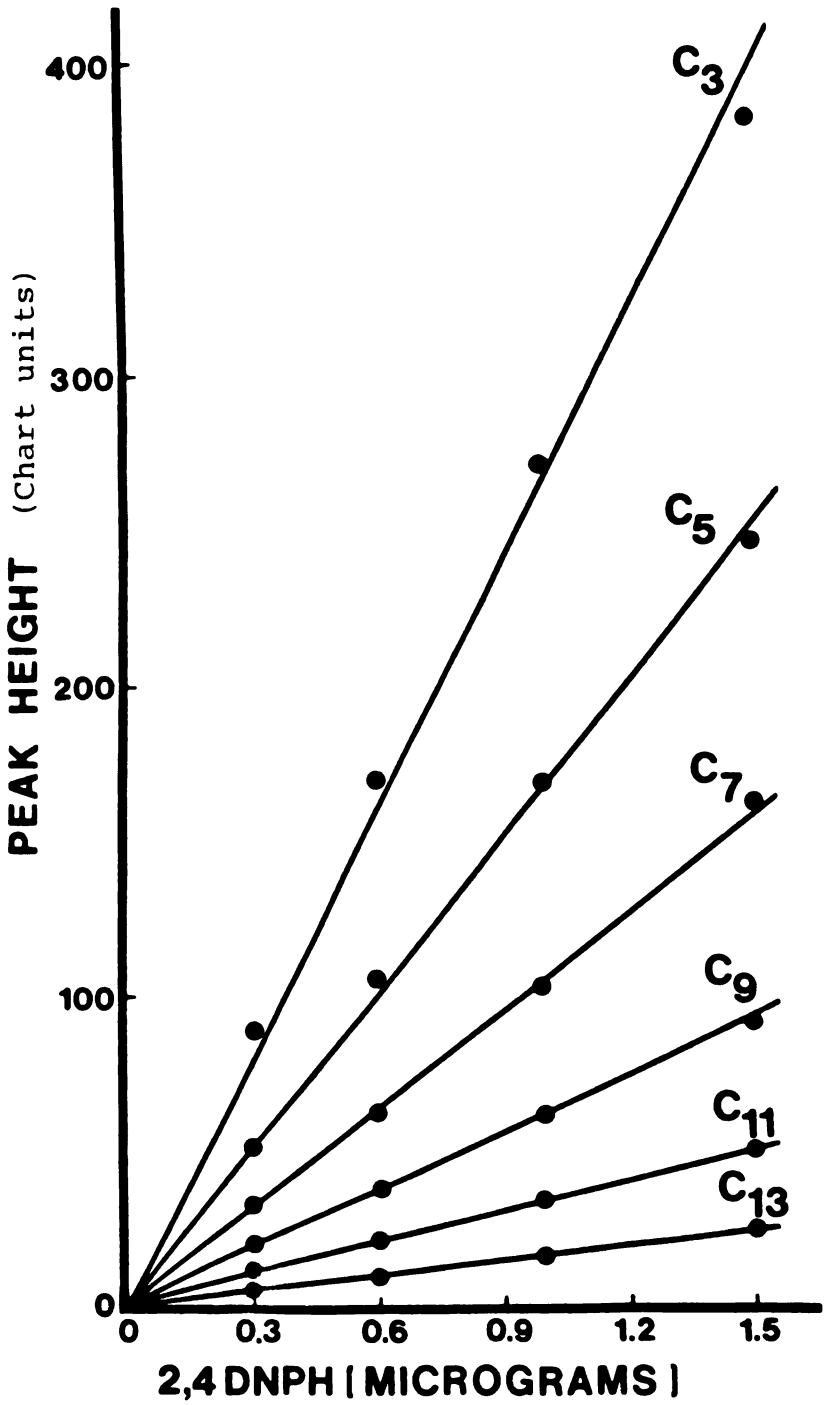


Figure 10. Calibration graphs, concentration vs peak height units, for 2,4-DNPH derivatives of methyl ketones, C_3 - C_{13}, based on a detector attenuation of 0.08 AUFS. Peak height: 100 chart units equal to 10 inches.

reported. The linearity of the standard curves were excellent in using the corresponding slopes in the quantitation of methyl ketones (Appendix B). The concentration of the methyl ketone in the samples was kept within the range found in the calibration curves.

Table 5.--The values for slopes, correlation coefficient, and intercepts of standard curves of 2,4-DNP-hydrazone derivatives of C_3 - C_{13} methyl ketones.

Methyl ketone chain length	Slope*	Correlation coefficient	Intercepts
C_3	254.02	0.9985	9.1282
C_5	162.85	0.9993	4.4972
C_7	97.22	0.9993	2.5481
C_9	60.10	0.9993	1.8504
C_{11}	33.07	0.9994	0.8939
C_{13}	15.82	0.9998	0.2034

*Average of duplicates

Recovery of methyl ketones, C_3 - C_{13}:

The percent recoveries of standard methyl ketones, C_3, C_5, C_7, C_9, C_{11} and C_{13} are presented in Table 6. A carefully weighed mixture of standard methyl ketones (99^+%) in carbonyl-free hexane was passed through a reaction column (Schwartz and Parks, 1961), and after formation of derivatives fresh butter oil was added to the mix to duplicate the extraction conditions when cheese was

analyzed. The fat added in this manner did not contribute any hydrazones to the samples. Recovered methyl ketones were analyzed as previously described. The high pressure liquid chromatogram obtained from recovered components was similar to the pattern in Figure 6. For the determination of peak heights of individual methyl ketones, the corresponding pre- and major peak of each methyl ketone were combined and percent recovery was calculated from the following equation:

$$\% \text{ Recovery} = \frac{\mu\text{moles methyl ketone recovered}^{(1)}}{\mu\text{moles methyl ketone initially added}^{(2)}} \times 100$$

and,

(1) μmoles methyl ketone recovered =

$$\frac{\text{Peak Height (chart units)}}{\text{injection volume (μl)}} \times$$

$$\frac{\text{Detector Attenuation (AUFS)}}{\text{0.08 (AUFS)}} \times$$

$$\frac{\text{x Dilution Factor (ml) x 1000}}{\text{Slope of Std. curve } (\frac{\text{chart unit}}{\mu g}) \text{ x M.W. of hydrazone}}$$

(2) μmoles methyl ketone initially added =

$$\frac{\mu g \text{ methyl ketone initially added}}{\text{M.W. of corresponding methyl ketone}}$$

The molecular weights of methyl ketones and their hydrazone derivatives are presented in Appendix A.

It was shown (Table 6) that the percent recoveries of methyl ketones are decreased as the chain length of the ketones are lowered. Similar relationships between ketone

Table 6.--Percent recoveries of standard, C_3 - C_{13}, methyl ketones.

Methyl ketone chain length	% Recovery		
	A	B	Averages*
C_3	77.97	77.01	77.49
C_5	81.05	79.33	80.19
C_7	90.71	88.57	89.64
C_9	94.92	92.88	93.90
C_{11}	96.09	95.80	95.94
C_{13}	98.50	99.03	98.76

*The average recoveries were used in quantitation procedures of methyl ketones in the cheese samples.

chain length and recoveries were observed by Anderson (1966), Allen and Parks (1969), Arnold and Lindsay (1969), Blakely (1970). The recovery values of Anderson (1966) were quite similar to the values in Table 6. However, this author reported 64.9% recovery for acetone. This may be explained as a greater retention of acetone in several additional chromatographic steps that this author used. Blakely (1970) used similar procedures as Anderson and found a 63.8% recovery for acetone. However, 20-30% lower recoveries for longer chain methyl ketones were obtained by Blakely.

Methyl ketones in commercial imported or domestic Blue cheese

The concentration of individual methyl ketones in Blue cheese samples were calculated (Appendix B) as micromoles (μM) methyl ketone per 10 grams fat in cheese. Several investigators, Schwartz and Parks (1963), Anderson (1966), Blakely (1970), and Albert (1974) reported their results in the same manner. Table 7 represents the methyl ketone concentration in several commercial Blue cheese samples. It was demonstrated that 2-heptanone and 2-nonanone were the major ketones in the samples. However, in one brand of Blue cheese crumbles (domestic C_1), domestic C_2, and Gorgonzola$_I$ larger concentration of 2-propanone (C_3) was shown. The minimum ketone content was observed in Gorgonzola$_{II}$ with a total of 3.20 μM and the largest concentration was found in Blue cheese crumbles (domestic C_2) with a total of 180.1 μM per 10 grams fat. The methyl ketone, C_{13}, was not detected in the majority of Blue cheese samples.

Significant variation of methyl ketone concentrations which were found among individual ketones in the same brand or among different varieties of Blue cheese samples (Table 7), confirmed the results of Anderson (1966) and Blakely (1970). These authors reported a large variation in the concentration of methyl ketones in Blue cheese samples. The analysis of Roquefort cheese for methyl

Table 7.--Methyl ketone concentration of domestic and imported commercial Blue cheese.

Blue cheese sample (a)	μM methyl ketones/10 g. fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
Domestic A	2.83	11.13	19.73	6.53	1.93	-	42.15
Domestic B	2.85	4.70	11.08	5.20	1.70	-	25.53
Domestic C_1	39.23	12.24	20.88	9.85	1.94	-	84.14
Domestic C_2	59.69	24.24	34.07	40.01	15.08	6.99	180.1
Danish	4.49	7.84	13.25	14.17	3.53	0.85	44.13
Norwegian	0.38	1.31	20.71	34.12	7.52	0.77	64.91
$Gorgonzola_I$	16.24	13.22	12.29	10.03	1.93	-	53.71
$Gorgonzola_{II}$	0.28	0.47	1.81	0.64	Trace	-	3.2
Stilton	1.54	0.83	1.89	0.93	0.21	-	5.4
$Roquefort_I$	0.29	0.99	6.22	6.06	0.37	-	13.93
$Roquefort_{II}$	0.43	0.21	6.06	7.59	0.67	-	15.01

(a) Domestics A and B: Different brands.

Domestic C_1 and C_2: Blue cheese crumbles, same brand, purchased at different periods.

Samples I and II: Purchased at different local markets.

ketones (see Table 7 and Figure 11-B) showed that C_7 and C_9 methyl ketones are the dominant and the most responsible ketone flavor in this cheese. Ketones other than C_7 and C_9 were found at concentrations less than 1.0 μM per 10 grams fat. Stärkel (1924) initially demonstrated that C_7 and C_9 methyl ketone were the major flavor components in Roquefort cheese. Many other investigators confirmed Stärkel's findings. In Figure 11-A, the high pressure liquid chromatogram of Wisconsin blue cheese demonstrates several other peak components, at approximately 3.0 minutes retention time which can be related to acetaldehyde. The first peak resolved after 2.5 minutes of injection in the same chromatogram was free hydrazine present in this sample. The peaks which were detected between C_3 and C_5 or C_5 and C_7 ketones could be representative of 2-butanone (C_4) and 2-hexanone (C_6). The retention times of these peaks were at 3.8 and 5.5 minutes after the injection. The presence of these ketones in the cheese samples were confirmed by injecting a mixture of corresponding standard carbonyl-DNPH-derivatives (99^+%) into the HPLC and comparing the retention times. The detection of trace even-numbered methyl ketones such as 2-butanone, 2-hexanone, and 2-octanone in Blue cheese has been reported by Bavisotto *et al*. (1960), Newar and Fagerson (1962), Dolézálek and Brabcová (1964), and Anderson (1966). The peak related to a DNPH-derivative of a carbonyl compound (Figure 11), eluted after 8.0 minutes, was present in all chromatograms. This compound could not

Figure 11. Typical HPLC separation of 2,4-DNPH-derivatives of methyl ketones, C_3 - C_{11}, in commercial: A) Wisconsin Blue; and B) French Roquefort cheeses.

HPLC conditions

A) Column: μ Bondapak C^{18}; 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (75:25 v/v)
Flow Rate: 1.43 ml/min
Temperature: Ambient
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.16 AUFS
Injection Volume: 15 μl per 20 ml sample
Chart Speed: 0.25 in./min

B) Column: μ Bondapak C^{18}, 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (75:25 v/v)
Flow Rate: 1.43 ml/min
Temperature: Ambient
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.16 AUFS
Injection Volume: 20 μl per 25 ml sample
Chart Speed: 0.25 in./min

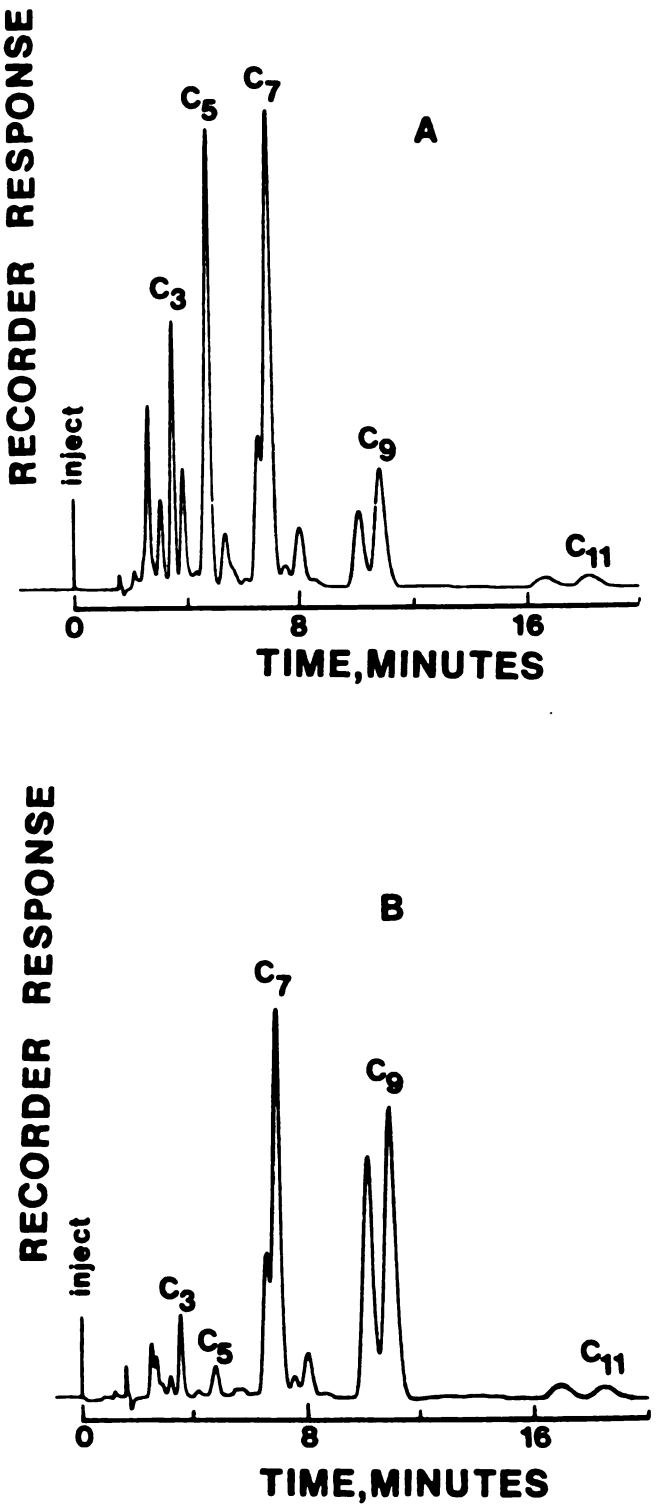


Figure 11. Typical HPLC separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{11}, in commercial: A) Wisconsin blue; and B) French Roquefort cheeses.

be identified. The high pressure liquid chromatograms of Danish and Norwegian blue cheeses are shown in Figure 12. Both samples contained small concentrations of C_{13} methyl ketone (see also Table 7). Like Roquefort (Figure 11), the Norwegian blue cheese exhibited a very low concentration of acetone, 0.28 μM per 10 grams fat (Table 7 and Figure 12); however, the concentration of C_7 and C_9 methyl ketones were several fold greater in Roquefort cheese. Both Roquefort samples, obtained at different local markets, showed the same patterns of methyl ketones, but the samples of Gorgonzal$_{I}$ and $_{II}$ (Table 7) resulted in a 15 fold difference in concentration. This may be explained due to a different handling and storage conditions of such cheeses at the time of shipping.

In this study, it seemed from the majority of high pressure liquid chromatograms such as those in Figure 4, 11, and 12 that the monocarbonyl fraction used for injection contained methyl ketones as the largest proportion. Very similar patterns were observed between these chromatograms and the one in Figure 9. One may conclude that if monocarbonyls other than ketones were present, their concentrations were so small that they were retained during the conventional column chromatographic procedures.

Sensory evaluation of commercial Blue cheese

The scores for sensory evaluation of several commercial cheese samples are presented in Table 8. Each

Figure 12. Typical HPLC separation of 2,4 DNPH derivatives of methyl ketones, C_3 - C_{13}, in: A) Danish; and B) Norwegian commercial Blue cheeses

HPLC conditions for both A and B

Column: μ Bondapak C^{18}; 3.9 x 300 mm

Mobile Phase: Acetonitrile:H_2O (75:25 v/v)

Flow Rate: 1.43 ml/min

Temperature: Ambient

Detector: U.V. photometer, 254 nm

Sensitivity Range: 0.08 - 0.16 AUFS

Injection Volume: 15 μl per 20 ml sample

Chart Speed: 0.25 in./min

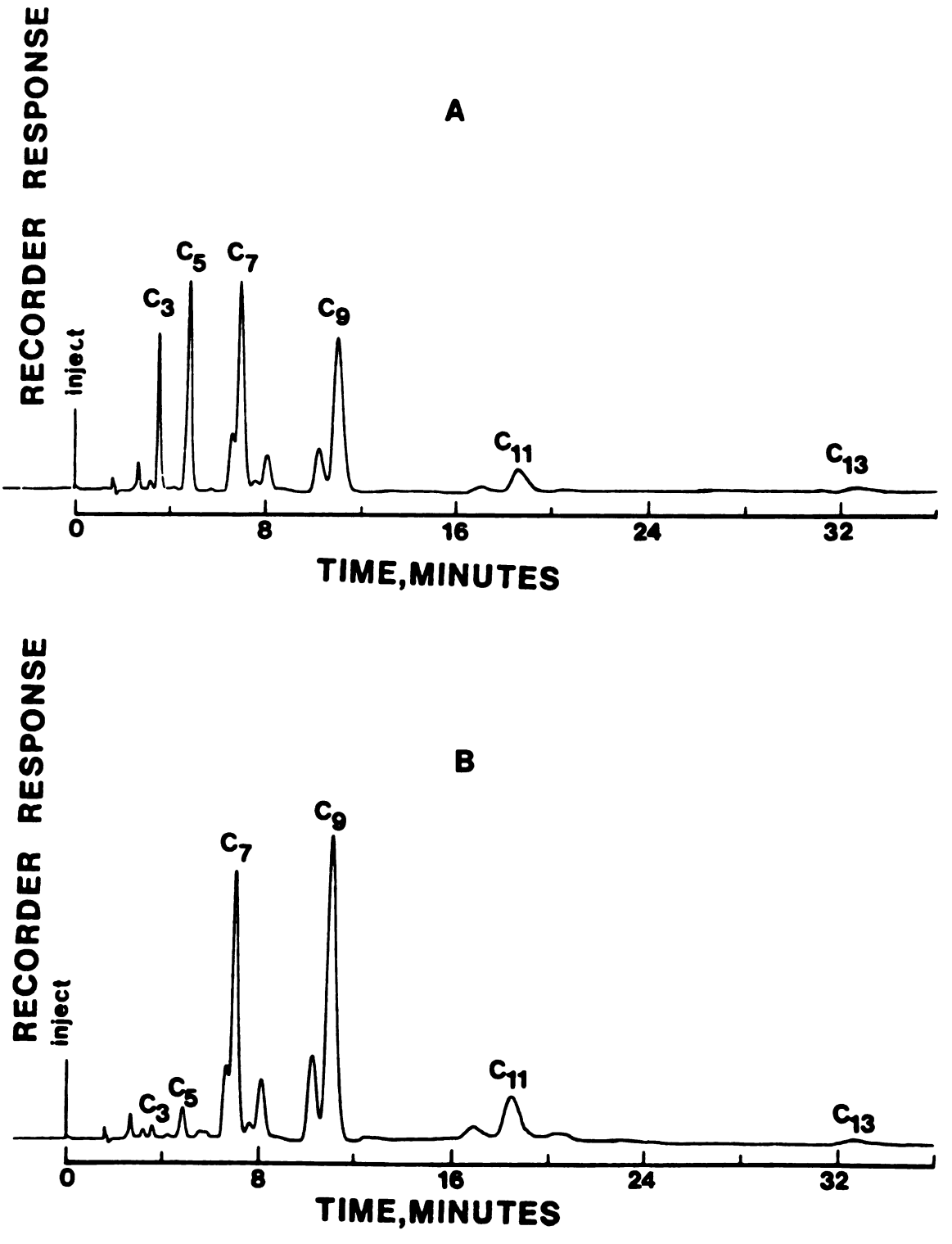


Figure 12. Typical HPLC separation of 2,4 DNPH derivatives of methyl ketones, C_3 - C_{13}, in A) Danish; and B) Norwegian commercial Blue cheeses.

Table 8.--Sensory evaluation of commercial imported and domestic Blue cheese.

Cheese sample (a)	Average scores		
	Color (15)	Flavor (20)	Texture (10)
Domestic A	13.0	17.5	8.5
Domestic C_1	12.0	18.5	8.5
Danish	13.5	18.0	9.0
Norwegian	13.5	18.5	8.5
$Gorgonzola_I$	15.0	19.5	10.0
Stilton	11.0	15.0	9.0
$Roquefort_I$	12.5	19.0	9.5

(a) Description of samples were the same as in Table 7.

sample was evaluated for color, flavor, and texture by a panel of two experienced judges. The scores are the average values reported by judges and were based on a maximum score of 20 for flavor, 15 for color, and 10 for texture. The flavor was considered to be the most important quality factor for incorporating the cheese samples into the other food products. Among the commercial cheeses, $Gorgonzola_I$ received the maximum score for color and texture, and a score of 19.5 for the flavor. The lowest score was given to Stilton for color and flavor. The very low methyl ketone concentration, particularly C_7 and C_9 ketones (Table 7) substantiate this poor flavor evaluation. An "ammonia" flavor was judged to be predominant in the Stilton cheese.

Kuehler (1974), Harte (1974), and Albert (1974) used the same method of sensory evaluation and reported a cheese was judged unacceptable if a total score of 30 was given. Based on this scoring all of the commercial samples in Table 8 were acceptable.

Methyl ketones in quick ripened (QR) cheese incorporated with white mutant of P. roqueforti

Utilizing a white mutant of P. roqueforti, two batches of cheese were prepared using this mold and salted on days 1, 6, 9 or 6, 7, and 8 during 15 days quick ripening. The white mutant of P. roqueforti was developed by Knight et al. (1950) by the ultraviolet irradiation of the blue mold spores. The purpose of using a white mutant in this study was to produce a white cheese with Blue cheese flavor. Kuehler (1974), Harte (1974), and Albert (1974) used the same white mutant of P. roqueforti to produce quick ripened cheese. They obtained a satisfactory white cheese with Blue cheese flavor.

Tables 9 and 10 represent the methyl ketone formation during the initial ripening of cheese in the curing room, maintained at 52°F (11°C) and over 95% RH. The every other day analysis of this cheese showed that during 15 days ripening of cheese only small concentrations of methyl ketones were formed. On the 15th day, a total of 10.18 μM per 10 grams fat was produced in the cheese salted on days 1, 6, and 9 (Table 9) and a total of 7.84 μM was presented

Table 9.--Methyl ketone concentration of quick ripened cheese, using white mutant of P. roqueforti, at 52 ± 1°F (11°C) and over 95% RH, salted on days 1, 6, and 9 during 15 days in the curing room.

Time (days)	μM methyl ketone/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
0	0.82	Tr.[a]	Tr.	Tr.	-	-	0.82
1	0.26	0.31	1.93	0.91	Tr.	-	3.41
3	2.34	1.21	5.15	5.64	0.57	-	14.91
5	1.08	4.51	0.80	3.03	0.64	-	10.06
7	1.43	1.47	0.60	4.80	1.96	0.21	10.47
9	0.92	0.82	0.40	4.44	1.45	Tr.	8.03
11	0.66	0.66	0.70	5.43	1.61	Tr.	9.06
13	0.56	1.62	0.65	5.43	1.84	Tr.	10.10
15	0.50	1.33	0.75	5.64	1.96	Tr.	10.18

(a) Tr. = Trace

Table 10.--Methyl ketone concentration of quick ripened cheese, using white mutant of P. roqueforti, at 52 ± 1°F (11°C) and over 95% RH, salted on days 6, 7, and 8 during 15 days in the curing room.

Time (days)	μM methyl ketones/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
0	0.83	Tr.[a]	Tr.	Tr.	-	-	0.82
1	0.26	0.31	1.93	0.91	Tr.	-	3.41
3	1.87	1.02	5.72	7.15	0.68	-	16.44
5	0.79	14.98	0.80	2.67	0.66	-	19.86
7	1.25	1.27	0.57	4.59	1.31	Tr.	8.99
9	1.09	1.62	0.67	5.40	1.98	0.31	11.07
11	0.87	0.99	0.67	4.86	1.98	0.41	9.78
13	0.52	1.41	0.62	5.74	2.30	0.62	11.21
15	0.47	0.71	0.28	4.62	1.76	Tr.	7.84

(a) Tr. = Trace

in the sample salted on days 6, 7, and 8 (Table 10). The major ketone in both lots (Tables 9 and 10) was 2-nonanone. However, on the fifth day of ripening, C_5 methyl ketone was present in greatest concentration, particular in the cheese which had not yet been salted (Table 10). The concentration of C_5 methyl ketone decreased after the fifth day of ripening. The initial analysis of the curd revealed a very low concentration, 0.82 μM/10 g fat, of 2-propanone and trace amounts of C_5, C_7, and C_9 methyl ketone. No C_{11} and C_{13} ketone were detected. The C_{13} methyl ketone was not detected until the 7th day of ripening. A large variation in the concentration of total methyl ketones was observed and almost similar in both lots during the initial ripening period (Tables 9 and 10).

On the 11th and 15th days of ripening period the cheese samples were packed in beakers and covered with plastic wraps and transferred from the curing room to a refrigerator at 40°F (4.4°C) for a period of two weeks. The weekly analysis of methyl ketones are shown in Table 11. Single analysis were performed because of solvent deficiency at the time of analysis. The major and dominant ketone during this storage period was C_9 methyl ketone. The total ketone concentration of 11th day cheese was increased from 9.06 to 19.95 μM/10 g fat during two weeks refrigeration when salted on days 1, 6, and 9 of initial ripening (compare Tables 9 and 11). The same increase in concentration was from 9.78 to 17.49 when the white cheese

Table 11.--Methyl ketone concentration of the 11th and 15th days quick ripened cheese, using white mutant of P. roqueforti, at 52 ± 1°F (11°C) and over 95% RH, salted on days 1, 6, 9 or 6, 7, 8, plus two weeks stored at 40 ± 1°F (4.4°C).

Methyl ketone chain length	μM methyl ketone/10 g fat*			
	11th day			
	1st week		2nd week	
	1,6,9	6,7,8	1,6,9	6,7,8
C_3	2.31	1.30	3.17	2.02
C_5	1.02	0.35	2.72	0.87
C_7	2.84	2.04	3.97	2.28
C_9	4.50	4.06	8.05	8.25
C_{11}	1.44	2.23	2.04	4.07
C_{13}	Tr.	Tr.	Tr.	Tr.
Total	12.11	9.98	19.95	17.49
	15th day			
C_3	1.26	0.29	1.30	0.73
C_5	0.95	0.78	0.44	1.45
C_7	2.23	1.86	2.04	2.91
C_9	5.91	5.72	8.63	7.85
C_{11}	2.77	3.30	3.34	4.08
C_{13}	Tr.	Tr.	Tr.	Tr.
Total	13.12	11.95	15.75	17.02

*Single analysis

was salted on days 6, 7, and 9 (compare Tables 10 and 11). Likewise, the concentration of methyl ketones of the cheese of 15th day of ripening increased during refrigeration. The typical HPLC separation of methyl ketones in the white cheese is shown in Figure 13. The slight decrease in the retention times compared to standard ketone (see Figure 2 and Table 3) was due to an increase in flow rate of mobile phase from 1.43 to 1.48 ml/min. The other chromatographic conditions were the same as standards. However, the quantitation of ketones was carried out at the standard separation conditions.

The total methyl ketone concentration at the end of second week storage (Table 11) was lower than the values reported by Albert (1974) for the QR cheese with white mutant of P. roqueforti. This author reported that the white mutant of the mold produced a desirable QR cheese, although low methyl ketone concentration was obtained compared with commercial Blue cheeses. Nelson (1970) obtained ketone concentrations 7 to 15 times in a submerged culture of white mutant of P. roqueforti grown in homogenized milk plus lipolyzed milk fat.

Sensory evaluation of quick ripened white cheese

The average scores which were given by the judges for the color, flavor, and texture of this type of cheese are presented in Table 12. The scores for color and flavor were low, representing a poor quality cheese. However, the

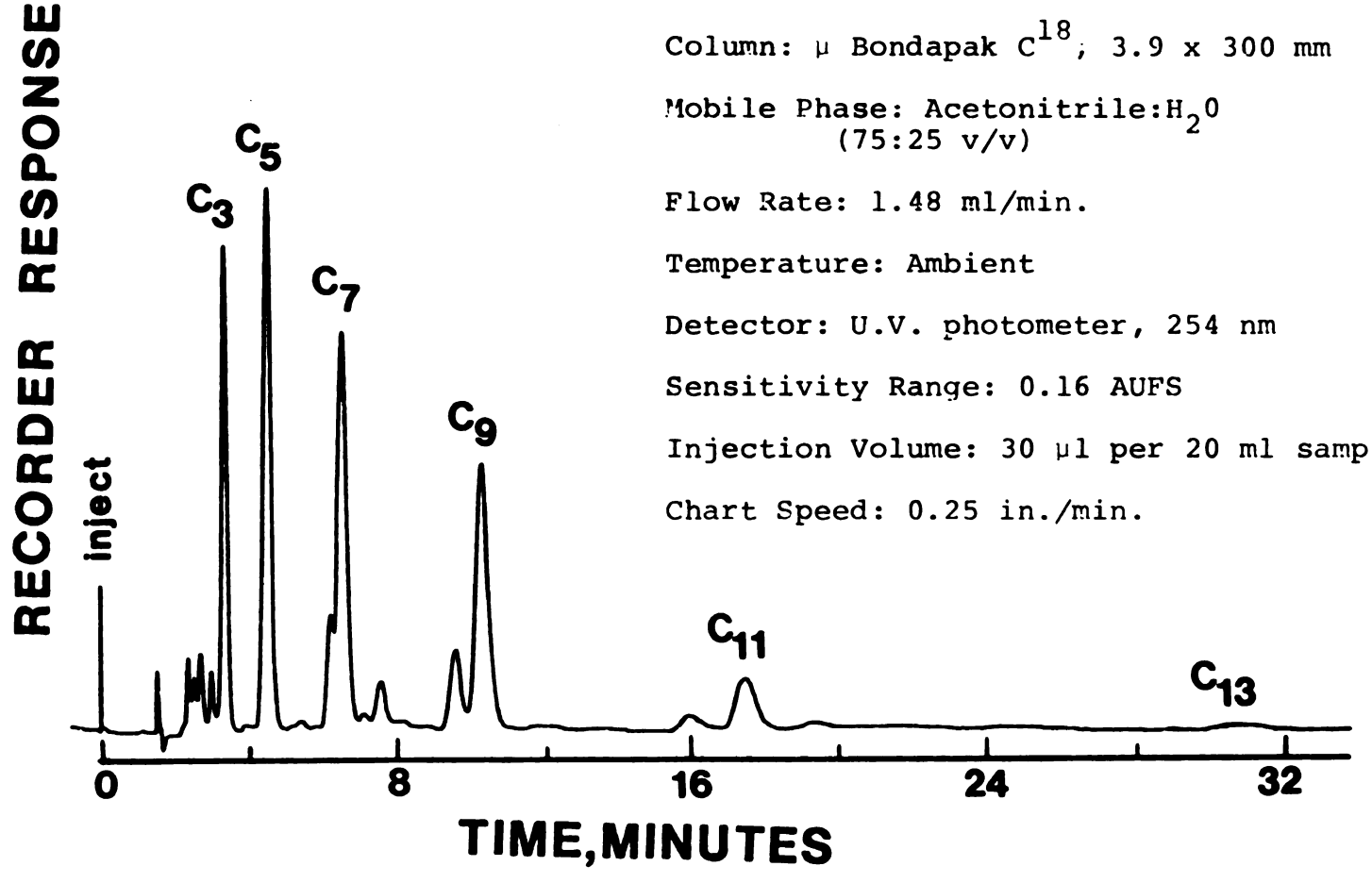


Figure 13. Typical HPLC Separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{13}, in QR cheese, incorporated with white mutant of P. roqueforti.

total score for the three quality factors were acceptable. Both batches of cheese samples, 1, 6, 9 and 6, 7, 8 days salted, turned to a yellowish color on the 5th day of ripening at 52°F (see Figure 16) and this color remained in the cheese throughout the initial ripening or refrigeration periods. Kuehler (1974), Harte (1974), and Albert (1974) obtained higher scores for color of the same cheese. However, they had incorporated a decolorizing agent in the milk at the time of rennet coagulation to produce a whiter color. In this experiment no decolorizer was used during cheese processing, and therefore, the development of the yellow color was probably more evident in the cheese. The QR cheese samples were criticized for possessing a mild Blue cheese flavor, even after a brief storage at 40°F.

Table 12.--Sensory evaluation of QR cheese, using white mutant of P. roqueforti at 52 ± 1°F (11°C) and over 95% RH, salted on days 1, 6, 9 or 6, 7, 8, during 15 days ripening plus two weeks storage at 40 ± 1°F (4.4°C).

Cheese sample(a)	Average scores					
	Color (15)		Flavor (20)		Texture (10)	
	1,6,9	6,7,8	1,6,9	6,7,8	1,6,9	6,7,8
A	9.0	9.0	13.0	12.0	9.0	9.0
B	9.0	9.0	15.0	14.5	9.0	9.0
C	9.0	9.0	14.5	14.0	8.5	8.5

(a) A: 15th day ripened cheese
B: 11th day ripened cheese, plus 2 weeks at 40 ± 1°F
C: 15th day ripened cheese, plus 2 weeks at 40 ± 1°F

Methyl ketones in Blue cheese quick ripened at 52°F (11°C)

The development of methyl ketones in 2 batches of Blue cheese, salted on days 1, 9, 10 or 7, 8, and 9 is presented in Tables 13 and 14. During 15 days in the curing room small concentration of ketones was resulted. Like white cheese (see Tables 9 and 10) the total ketones in Blue cheese samples (Tables 13 and 14) exhibited a maximum concentration between the 3rd and 5th days of ripening. A maximum C_9 methyl ketone development of 14.26 μM per 10 g fat (Table 13) was demonstrated in the cheese salted on days 1, 9, and 10 on the 5th day of ripening. This value for 7, 8, and 9 days salted cheese (Table 14) was 12.62 μM per 10 g fat. The rapid increase in C_5 methyl ketone concentration occurred on the 7th day of ripening. The ketone concentrations decreased rapidly after 24-48 hrs following the noted ripening days. The decrease in methyl ketone concentration may be explained by the ready reduction of ketones to secondary alcohols (Jackson and Hussong, 1958; Kinsella and Hwang, 1977). This may happen, particularly, when the mold is still in the lag phase (Fan et al., 1976; and Fan and Kinsella, 1976) of its activity and thus not enough ketone has been produced from free fatty acids. Comparing Tables 13 and 14, it was shown that the sequence of salting days had none or very slight effect on methyl ketone production during which the cheese samples were in the curing room. The significant effect of salt treatment of methyl

Table 13.--Methyl ketone concentration of Blue cheese quick ripened at 52 ± 1°F and over 95% RH, salted on days 1, 9, 10, during 15 days in the curing room.

Time (days)	μM methyl ketones/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
0	0.75	Tr.	Tr.	Tr.	-	-	0.75
1	1.68	0.21	1.90	2.23	-	-	6.02
3	1.20	0.19	3.11	6.88	Tr.	-	11.38
5	2.50	0.90	2.74	14.26	Tr.	-	20.40
7	1.09	6.31	0.21	2.66	Tr.	-	10.27
9	0.60	6.15	0.16	0.52	0.19	-	7.62
11	0.54	0.94	0.31	1.47	0.49	Tr.	3.75
13	0.45	0.53	0.33	1.91	0.49	Tr.	3.71
15	0.29	0.29	0.33	2.31	0.73	Tr.	3.95

Table 14.--Methyl ketone concentration of Blue cheese quick ripened at 52 ± 1°F and over 95% RH, salted on days 7, 8, 9 during 15 days in the curing room.

Time (days)	μM methyl ketones/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
0	0.75	Tr.	Tr.	Tr.	-	-	0.75
1	1.68	0.21	1.90	2.23	-	-	6.02
3	0.74	0.14	2.97	5.96	Tr.	-	9.77
5	2.21	2.39	2.60	12.62	Tr.	-	19.82
7	0.90	6.65	0.15	1.87	Tr.	-	9.57
9	0.38	2.04	0.30	0.88	0.11	-	3.71
11	0.59	0.84	0.39	1.90	0.41	-	4.13
13	0.32	0.45	0.43	3.08	0.83	0.26	5.37
15	0.29	1.08	0.31	2.89	0.99	0.39	5.95

ketone accumulation was observed during storage of the same cheese at lower temperatures, particularly 48°F (Tables 15 and 16).

Tables 15 and 16 represent the methyl ketone concentration of the 11th or 15th day ripened cheese during two weeks storage at lower temperatures, 40°F (4.4°C) or 48°F (8.9°C), than the ripening temperature. It seemed from Table 16 that higher temperature of storage, 48°F, favored methyl ketone production in the cheese, salted on days 7, 8, and 9. This batch of cheese showed a rapid increase in methyl ketones, particularly, C_3, C_5, C_7 and C_9 ketones during the first week of storage. The degree of methyl ketone formation in cheese salted on days 1, 9, 10 was very slow at this temperature of storage. However, the same cheese stored at 40°F produced more ketones than cheese salted on days 7, 8, and 9 (see Table 15). The 15th day cheese, salted on days 7, 8, 9 exhibited more ketones than the same cheese salted on days 1, 9, and 10. Total ketone concentration of (7, 8, 9) cheese at 48°F after 2 weeks storage was 149.1 μM per 10 g fat (Table 16). This value for the same cheese at 40°F was 35.89 μM per 10 g fat (Table 15). Comparing the 11th and 15th day QR cheese stored two weeks at 40°F in the same table, similar values for total ketone concentration was obtained for 7, 8, and 9 days salted cheese. However, the 11th day cheese, salted on days 1, 9, 10 exhibited a higher concentration than the

Table 15.--Methyl ketone concentration of the 11th and 15th days Blue cheese quick ripened at 52 ± 1°F and over 95% RH, salted on days 1, 9, 10 or 7, 8, and 9 plus two weeks stored at 40 ± 1°F.

methyl ketone chain length	μM methyl ketones/10 g fat*			
	11th day			
	1st week		2nd week	
	1,9,10	7,8,9	1,9,10	7,8,9
C_3	6.13	0.56	6.21	10.81
C_5	3.53	1.20	7.44	7.88
C_7	4.35	0.95	16.49	12.34
C_9	4.58	4.67	6.59	3.80
C_{11}	0.97	0.83	0.71	1.06
C_{13}	0.19	Tr.	-	-
Total	19.75	8.21	37.44	35.89
	15th day			
C_3	1.99	0.97	3.07	5.32
C_5	2.21	1.44	3.37	9.29
C_7	4.26	2.75	4.95	13.71
C_9	2.77	2.77	3.30	5.61
C_{11}	0.66	0.66	0.79	0.71
C_{13}	Tr.	-	Tr.	Tr.
Total	11.89	8.59	15.48	34.64

*Single analysis.

Table 16.--Methyl ketone concentration of the 11th day Blue cheese quick ripened at 52 ± 1°F and over 95% RH, salted on days 1, 9, 10 or 7, 8, and 9, plus two weeks stored at 48 ± 1°F.

methyl ketone chain length	μM methyl ketones/10 g fat*			
	1st week		2nd week	
	1,9,10	7,8,9	1,9,10	7,8,9
C_3	0.68	41.91	0.98	37.63
C_5	1.50	36.08	3.33	40.11
C_7	1.48	44.29	6.69	47.62
C_9	4.25	23.69	4.18	23.08
C_{11}	2.35	1.56	2.04	0.68
C_{13}	0.33	Tr.	-	-
Total	10.59	147.5	17.22	149.1

*Single analysis.

15th day cheese when stored for two weeks at 40°F. The 15th day cheese samples stored for two weeks at 48°F were not analyzed, because they developed off-flavors during the first week of storage at this temperature. Therefore, it seemed that 11 days ripening period for the cheese QR at 52°F was preferred to 15 days if a brief storage at 48°F was necessary to develop more ketones in the cheese.

The values obtained for methyl ketone in QR Blue cheese stored at 48°F were comparable with findings of Albert (1974). However, more acetone was found in the cheese analyzed in this study (Tables 15 and 16). The

relatively low concentration of total methyl ketones in cheese stored at 40°F (Table 15) was similar to the data obtained by Blakely (1970) in 7 days QR Blue cheeses. Blakely and Albert reported that C_9 methyl ketone was the major ketone in their sample, however, despite of white cheese in this study (Tables 9, 10 and 11), almost all quick ripened Blue cheeses exhibited a dominant C_7 methyl ketone after the storage period. The dominancy of C_7 methyl ketone in Blue cheese has been confirmed by several investigators such as Hammer and Bryant (1937), Schwartz and Parks (1963), and Anderson (1966). Dartey and Kinsella (1971) reported that C_7 and C_9 methyl ketones were the major ketones present in the cheese at all stages of ripening. These authors showed that generally, both C_7 and C_9 accounted for more than 50% of the total methyl ketones. Figure 14 represents the typical methyl ketone separation in QR Blue cheese. The chromatogram showed a clean separation of methyl ketones among other monocarbonyls which were probably in trace amounts.

Sensory evaluation of Blue cheese quick ripened at 52°F

The subjective analyses of several samples of Blue cheese quick ripened at 52°F are presented in Table 17. Generally, most of these samples were judged to have a "mild" and "quite clean" flavor. Such cheeses scored between 15 and 18 for flavor. These cheese samples contained a total ketone concentration of 35 to 45 μM per

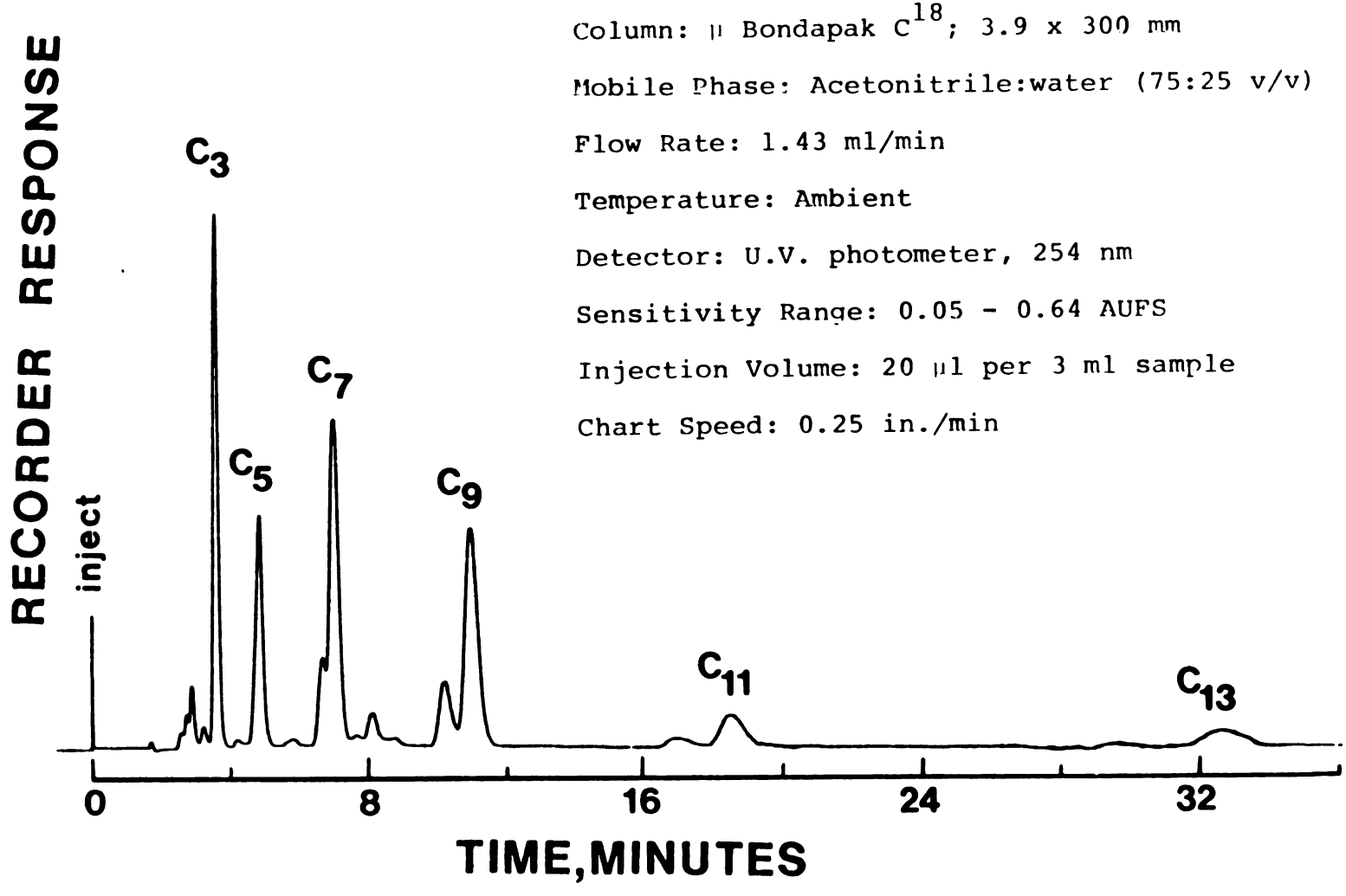


Figure 14. Typical HPLC separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{13}, in QR Blue cheese.

Table 17.--Sensory evaluation of Blue cheese quick ripened at 52 ± 1°F and over 95% RH, salted on days 1, 9, 10 or 7, 8, and 9 during 15 days ripening, plus two weeks stored at 40° or 48°F.

Cheese Sample(a)	Average scores					
	Color (15)		Flavor (20)		Texture (10)	
	1,9,10	7,8,9	1,9,10	7,8,9	1,9,10	7,8,9
A	11.0	14.0	12.0	12.0	7.0	8.0
B	15.0	13.0	13.5	15.0	8.0	9.0
C	12.0	13.0	17.5	15.0	8.5	8.5
D	13.0	13.5	16.5	19.5	9.0	9.0
E	11.5	13.5	18.0	16.5	8.5	9.0
F	12.5	14.0	16.0	18.0	8.5	9.0
G	13.5	12.5	18.0	16.0	9.0	8.5
H	13.5	13.0	18.0	18.5	9.0	9.0

(a) A: 11th day ripened cheese

B: 15th day ripened cheese

C: 11 days ripened plus one week stored at 40°F

D: 11 days ripened plus one week stored at 48°F

E: 11 days ripened plus two weeks stored at 40°F

F: 11 days ripened plus two weeks stored at 48°F

G: 15 days ripened plus one week stored at 40°F

H: 15 days ripened plus two weeks stored at 40°F

10 g fat (Table 15). The highest score was given to the 11th day ripened cheese, salted on days 7, 8, and 9, plus one week storage at 48°F (see Table 17, Sample D). This cheese exhibited an excellent ketone flavor. An intense blue-green color was demonstrated in cheese A, salted on days 1, 9, and 10. This cheese received the lowest score for color. The color of this cheese improved during two weeks storage at lower temperatures. The method of salting caused a difference in color of the cheese at the end of initial curing day, 11th or 15th day (see Table 17 and also Figure 16). Higher scores were given to the samples which were salted on days 7, 8, and 9. An increase in mold growth was noted by judges for those cheeses salted on days 1, 9, and 10. Several samples with high flavor scores such as cheese F in Table 17 were criticized for an atypical aftertaste. Texture was improved after 11 days ripening and, therefore, higher scores were obtained.

Methyl ketones in Blue cheese quick ripened at 62°F (16.7°C)

Table 18 presents the methyl ketones of QR Blue cheese during 7 days in the curing room. Since the rate of mold growth and methyl ketone formation at this ripening temperature seemed greater than other lots ripened at lower temperature, daily analysis of methyl ketones was performed to observe the changes in ketone concentration. Hedrick et al. (1968) reported that 7 days were sufficient to ripen a Blue cheese at 62°F using their method, which was similar

Table 18.--Methyl ketone concentration of Blue cheese quick ripened at 62 ± 1°F and over 95% RH, salted on days 4, 5, and 6 during 7 days in the curing room.

Time, (days)	μM methyl ketone/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
0	0.87	Tr.	Tr.	Tr.	-	-	0.87
1	1.31	0.43	3.14	1.58	0.12	-	6.58
2	0.96	0.29	3.15	3.49	0.10	-	7.99
3	1.31	5.05	1.67	6.65	0.26	-	14.95
4	1.13	8.37	0.31	0.51	0.39	-	10.71
5	0.67	4.11	0.26	0.64	0.26	-	5.94
6	0.38	0.91	0.45	2.25	0.99	0.21	5.19
7	0.81	1.46	1.33	5.67	2.30	0.29	11.85

to the method used to manufacture the cheese in this study. Blakely (1970) confirmed this ripening period and quantitated methyl ketones in a 7-days quick ripened Blue cheese. This author reported low methyl ketone concentration in several batches of QR Blue cheese. The predominant ketone in the sample was C_9 methyl ketone. The total methyl ketone concentration that this author reported in 3 samples of QR Blue cheese was 19.0 - 27.0 μM per 10 grams fat. However, Albert (1974) reported a greater concentration of methyl ketones in Blue cheese quick ripened by this method.

Methyl ketone accumulation in Blue cheese during seven days ripening was neither uniform nor cumulative

(Table 18). This was also shown for other lots of cheese, quick ripened at 52°F (see Tables 9, 10, 13, and 14) during 15 days ripening. The largest concentration of total ketones which exhibited in Blue cheeses ripened at 52°F on the 5th day of ripening (Tables 13 and 14), occured on the 3rd day of ripening for this lot of cheese (Table 18). Like other lots of cheese, large concentrations of C_5 and C_9 methyl ketones were demonstrated during 7 days ripening at 62°F.

The 7th day ripened cheese (Table 18) was packed and stored at different temperatures, 40°, 48°, or 62°F. Tables 19 and 20 represent the methyl ketone content of the cheese samples at those storage conditions. In all samples, C_7 methyl ketone was dominant, however, C_9 methyl ketone was also considered to be the major ketone. Only, trace amount of C_{13} methyl ketone was found in the cheese ripened at 62°F. The large concentrations of methyl ketones, 117.3 µM per 10 g fat, on the third day of storage at 62°F (Table 19) started to decline afterwards. It was necessary to refrigerate this cheese sample on the 4th day of storage to lower temperatures for longer shelf life. The sudden increase in the concentration of methyl ketones from 11.85 µM/10 g fat on the 7th day of ripening (Table 18) to 65.57-117.3 µM per 10 g fat during storage at 62°F (Table 19) demonstrates that the enzymes responsible for methyl ketone production are not necessarily

dependent on mold growth. And, when they are produced in such levels by the mold, they may generate methyl ketones at a large concentration in a short period of storing Blue cheese at 62°F (compare Tables 18 and 19).

Table 19.--Methyl ketone concentration of the 7th day Blue cheese quick ripened at 62 ± 1°F and over 95% RH, plus 4 day storage at 62 ± 1°F.

Time (days)	μM methyl ketone/10 g fat						
	C_3	C_5	C_7	C_9	C_{11}	C_{13}	Total
1	7.37	15.23	20.78	18.68	3.51	Tr.	65.57
2	13.54	25.98	32.45	24.53	3.90	Tr.	98.40
3	13.00	24.97	37.52	35.55	6.24	Tr.	117.3
4	8.29	20.48	25.35	19.16	3.51	Tr.	76.79

The large accumulation of methyl ketones in quick ripened cheese, stored two weeks at lower temperatures, 40° or 48°F is also shown in Table 20. Cheese stored for two weeks at 48°F exhibited the maximum methyl ketone content, 184.8 μM per 10 g fat. This value was similar to the obtained for commercial Blue cheese crumbles, 180.1 μM per 10 g fat (see Table 7). Overall, C_7 and C_9 methyl ketones were predominant during the storage period. Figure 15 represents a profile for C_7 and C_9 ketone production. The development of these two important flavor components was revealed significantly during the brief storage after 7 days initial curing of the cheese (B, C, and D).

Table 20.--Methyl ketone concentration of the 7th day Blue cheese quick ripened at 62 ± 1°F and over 95% RH, plus two weeks stored at 40 ± 1° or 48 ± 1°F.

methyl ketone chain length	μM methyl ketones/10 g fat			
	1st week		2nd week	
	40°	48°	40°	48°
C_3	19.96	23.52	19.82	29.28
C_5	29.04	32.78	29.35	45.91
C_7	40.99	44.79	54.08	64.23
C_9	38.92	44.32	37.73	39.52
C_{11}	7.80	7.32	5.86	5.86
C_{13}	Tr.	Tr.	Tr.	Tr.
Total	136.7	152.7	146.8	184.8

Sensory evaluation of Blue cheese quick ripened at 62°F

The scores for color, flavor, and texture of the cheese ripened at 62°F are listed in Table 21. The scores for color were low, particularly those samples which were stored at higher temperature, 62°F (see Samples A, B, C, D, and E in Table 21). The color was slightly improved during two weeks storage at lower temperatures, 40° or 48°F and higher scores were given. The color was judged to be brownish green and less pleasant than the color of samples ripened at 52°F (see Figure 16). The scores for flavor (Table 21) were high, especially for the cheese stored at lower temperatures (Samples F, G, H, and I in Table 21). An

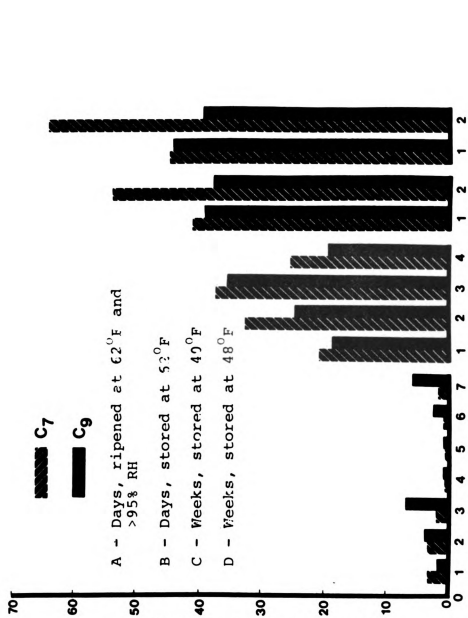


Figure 15. Typical concentrations of 2-Heptanone and 2-Nonanone in Blue cheese quick ripened at 62°F and stored at 40°, 48°, and 62°F.

Table 21.--Sensory evaluation of Blue cheese quick ripened at 62 ± 1°F and over 95% RH during 7 days plus 4-15 days storage at 40 ± 1°, 48 ± 1°, or 62 ± 1°F.

Cheese Samples (a)	Average scores		
	Color (15)	Flavor (20)	Texture (10)
A	10.5	15.0	7.5
B	10.0	15.5	7.5
C	10.0	16.5	8.0
D	10.0	17.5	8.0
E	10.0	15.5	8.0
F	11.0	19.0	9.0
G	11.0	18.0	8.0
H	11.5	19.0	9.0
I	11.5	18.0	8.5

(a) A: 7th day Blue cheese

B: C, D, E: cheese samples stored at 62°F for 1, 2, 3, and 4 days respectively

F, G: first week stored at 40° or 48°F respectively

H, I: second week stored at 40° or 48°F respectively

atypical aftertaste was noted for cheese E. The texture of the cheese was improved during the two weeks of storage, particularly at 40°F.

Changes in pH During Quick Ripening of Cheese

The pH of the cheese samples were measured in duplicate every day of quick ripening and during the storage

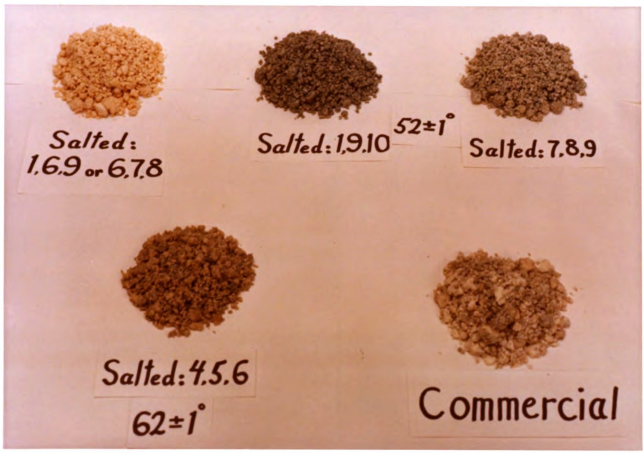


Figure 16. Commercial Blue cheese crumbles and quick ripened cheeses incorporated with white or blue mold powder of P. roqueforti.

period. In each analysis a homogeneous sample was used. Morris and Jezeski (1953) showed the effect of pH on the enzyme activity of P. roqueforti, and therefore, the importance of pH during ripening of Blue cheese.

The change in pH during ripening of 3 batches of cheese is presented in Figure 17. Almost similar patterns were shown for all batches. Blakely (1970), Kuehler (1974), and Harte (1974) showed the same pH changes during quick ripening of Blue cheese. The range of pH maxima was 6.70 - 7.00. Each batch showed a slightly different pH maximum: 6.75 for the cheese ripened at 62°F; 7.0 for white cheese QR at 52°F; and 6.70 for the Blue cheese QR at 52°. The maximum acidity in QR cheese was not obtained until approximately 24.0 to 72.0 hrs after the cheeses were manufactured. The pH values at this stage of ripening were 4.7 - 4.8. Both cheese samples QR at 52°F remained at this low pH for a period of 48.0 hrs, 1st to 3rd days of ripening period. For the white cheese (C), and 2nd to 4th days of this period for the Blue cheese (A). Remaining at the minimum pH was during a shorter period, 24 hrs, for the cheese ripened at higher temperature (B).

The initial pH in Sample A, 6.25, which was higher than other batches may be explained as an insufficient lactic acid production at the time of manufacturing by the streptococcus starter. However, this cheese showed the same pH pattern, decrease and increase in pH, at a longer period of time, approximately 24.0 hrs, than other batches.

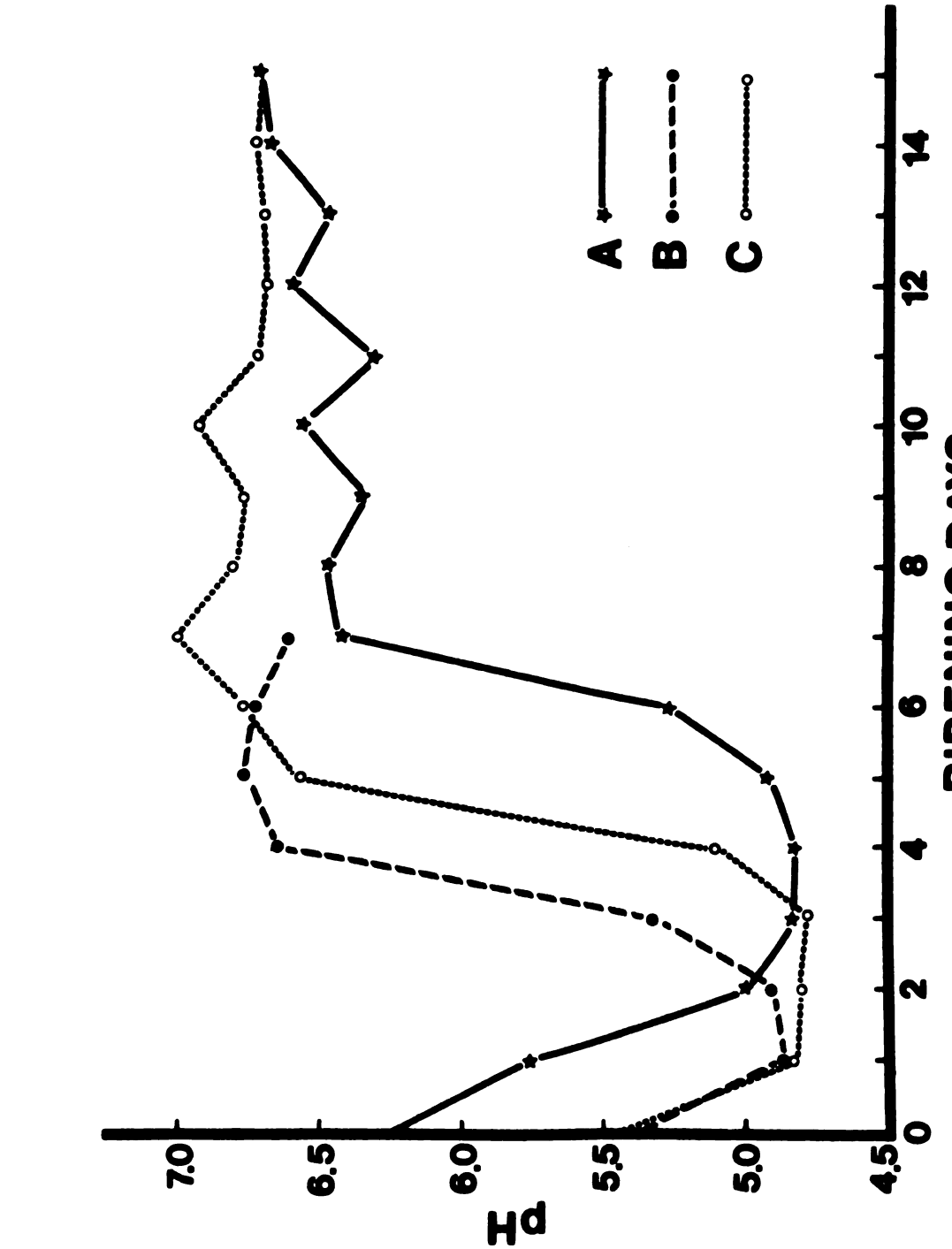


Figure 17. Changes in pH during quick ripening of cheese inoculated with Blue or white mutant of P. roqueforti. A (*——*), Blue cheese ripened at 52°F; B (●----●), Blue cheese ripened at 62°F; and C (○·····○), white cheese ripened at 52°F.

The maximum acidity in QR cheese was due to the lactic acid production by Streptococcus type starter organisms. Coulter et al. (1938), and Foster et al. (1961) reported that acid production reached its maximum, pH, in Blue cheeses 24 hours after manufacture. Utilizing 2 - 4% starter a minimum pH of 4.7 was obtained 24.0 hrs after manufacture. The slow or fast acid production by adding different concentration of starter culture was demonstrated by Thibodeau and Macy (1942) and Morris (1964).

The sudden rise in pH (Figure 17) was associated with heat production and the first visible signs of mold growth. The cheese curd was bitter at this stage of ripening. This bitterness which was probably due to the accumulation of bitter peptides or amino acids, was not present in the cheese 2-3 days after bitterness was first noted. Generally, unsalted cheese samples reached the stage of bitterness faster than cheese salted on the 1st days of ripening. The first visible sign of the mold growth was reached 24.0 hrs faster in unsalted cheese. The increase in pH from its minimum during QR of cheese was probably due to proteolysis by P. roqueforti. A progressive proteolysis associated with the increase in pH during ripening of Blue cheese was demonstrated by Lane and Hammer (1938), Morris et al. (1951). This relationship was also shown by Kuehler (1974) in QR Blue cheese. A continuing increase in tyrosine was reported (Morris et al., 1963) to be associated with rapid increase in pH after 4 weeks.

Kuehler (1974) reported the slight increase of tyrosine in QR cheese from day 7 through day 39 of quick ripening.

The pH values were stabilized after the sudden rise and slightly decreased (see Figure 17 B and C) because of increasing levels of free fatty acids released by the lipase system of the mold. Morris et al. (1963) and Morris (1964) reported that the slight decrease in pH during 4-9 months of Blue cheese ripening was attributed to the accumulation of free fatty acids during lipolysis by P. roqueforti. The pH values of QR cheese and several commercial samples are shown in Appendix D.

Changes in Moisture During Ripening of QR Blue Cheese

The percentage of moisture during QR of Blue cheeses was determined in duplicate according to the method of AOAC to observe moisture loss of the samples during the time they were in the curing room. The results are shown in Table 22. During 7 or 15 days ripening of cheese at 62^{O} or 52^{O}F respectively, 8 - 13% moisture loss occurred in the cheese. Although the values for the % moisture varied, the rate of moisture losses were almost similar with slightly faster moisture loss in the cheese ripened at higher temperature. The difference in the moisture level of these lots were difficult to explain. However, one explanation for the cheese with higher moisture content may be the failure of the starter culture during manufacturing to develop a sufficient acidity, and therefore retaining more

Table 22.--Moisture content of Blue cheese quick ripened at 52° or 62°F and over 95% RH, salted on days 1, 9, 10 and 7, 8, 9 or 4, 5, and 6 during 7 or 15 days ripening.

Days at 52°F	% Moisture		Days at 62°F	% Moisture
	1,9,10	7,8,9		4,5,6
0	57.25	57.25	0	52.54
1	57.28	57.28	1	48.78
3	55.40	55.40	2	48.52
5	55.51	54.62	3	48.09
7	54.68	52.98	4	45.07
9	51.73	49.15	5	42.21
11	49.67	49.0	6	40.45
13	49.24	49.0	7	39.75
15	49.20	49.25		

water in the curd (Morris, 1969). The higher initial pH of Blue cheese ripened at 52°F (see Figure 17) may be a reason for the high percentage of moisture in this batch (Table 22).

Table 23 contains data for the moisture content of QR cheese during storage and also the percentage moisture of several commercial Blue cheese as they were obtained. No loss of moisture was observed during the storage of cheese samples at 40° or 48°F. The percent moisture of commercial samples were in the range of 40 - 47% which is comparable with the values obtained for QR cheese samples,

Table 23.--Moisture content of quick ripened cheese, during storage period, and commercial Blue cheeses.

Cheese sample[(a)]	% Moisture
QR_1 (white)	45.60
QR_2 (white)	45.03
QR_3 (Blue)	48.65
QR_4 (Blue)	48.25
QR_5 (Blue)	40.50
$Domestic_I$	46.36
$Domestic_{II}$	44.10
Gorgonzola	40.24
Roquefort	39.23
Stilton	46.37

(a) QR_1 and QR_2: salted on days 1, 6, 9 and 6, 7, 8 respectively and stored 2 weeks at 40°F

QR_3 and QR_4: salted on days 1, 9, 10 and 7, 8, 9 respectively and stored 2 weeks at 40°F

QR_5 : ripened at 62°F and stored one week at 40°F

$Domestic_I$ and $_{II}$: Blue cheese crumbles, the same brand, purchased at different times

40 - 48%. Harte (1974) reported a 42.5% moisture content in a commercial Blue cheese and a range of 39.0 to 49% moisture in QR Blue or white cheese. A moisture content of 45% was noted in the white cheese after quick ripening and storage (Table 23).

Retention of Methyl Ketones During Freeze Drying of Blue Cheese

Several lots of Blue cheese, quick ripened or commercial, were freeze dried and then according to the procedures in Appendix C were rehydrated to their original moisture content for HPLC analysis. The results of methyl ketone concentrations were compared with the values obtained for the same sample before freeze drying and the percent retention of methyl ketones in Blue cheese were calculated.

Table 24 represents the percentage retention of methyl ketones in several Blue cheeses. The results were comparable with those reported by Blakely (1970) except for C_{11} methyl ketone. This author showed less than 10% retention for C_3 to C_9 methyl ketones in Blue cheese, utilizing the same freeze drying methods. Hawrysh (1970) confirmed Blakely's results by adding standard methyl ketones to cream following freeze drying. The rate of methyl ketone losses was the same as Blakely's. In this study, C_{11} methyl ketone retention was negligible in two samples (see Table 24). Since C_{13} methyl ketone was not defected in the samples, its percent retention was not determined. Blakely (1970) reported an average percent retention of 24.0 and 70.0 for C_{11} and C_{13} methyl ketones respectively during freeze drying of Blue cheese.

Relatively small concentrations of methyl ketones were retained during the freeze drying operations (see Table 24) in Blue cheese. The loss of ketones increases

Table 24.--Percent retention of methyl ketones in freeze-dried commercial or quick ripened Blue cheese.

Methyl ketone chain length	% Retention		
	A	B	C
C_3	6.24	2.74	0.75
C_5	6.13	9.27	1.41
C_7	9.12	12.50	5.77
C_9	13.38	12.00	11.10
C_{11}	∿0	5.68	∿0

A: Blue cheese QR at 52°F during 11 days plus 2 weeks at 40°F

B and C: Commercial Blue cheese crumbles.

as molecular weight decreases. However, according to Blakely (1970) and Hawrysh (1970) the retention of these components in overall does not appear to be related to the boiling points. The HPLC separations of methyl ketones in sample B (Table 24) before and after freeze drying are presented in Figure 18. A separated component shown as asterisk (Figure 18-B) with a retention time similar to acetaldehyde was present in freeze dried samples. This may be a decomposition product of longer chain methyl ketones such as C_{11}, but no data are available to substantiate this. This compound may also have developed from ethanol present in Blue cheese, during freeze drying. A large concentration of this component in dried cheese was detected by HPLC.

Figure 18. Typical HPLC separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{11}, in: A) commercial Blue cheese crumbles; and B) freeze-dried commercial Blue cheese crumbles.

HPLC conditions

A) Column: μ Bondapak C^{18}, 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (75:25 v/v)
Flow Rate: 1.43 ml/min
Temperature: Ambient
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.08 - 0.32 AUFS
Injection Volume: 15 μl per 20 ml sample
Chart Speed: 0.25 in./min.

B) Column: μ Bondapak C^{18}, 3.9 x 300 mm
Mobile Phase: Acetonitrile:H_2O (75:25 v/v)
Flow Rate: 1.43 ml/min
Temperature: Ambient
Detector: U.V. photometer, 254 nm
Sensitivity Range: 0.08 - 0.16 AUFS
Injection Volume: 18 μl per 5 ml sample
Chart Speed: 0.25 in./min.

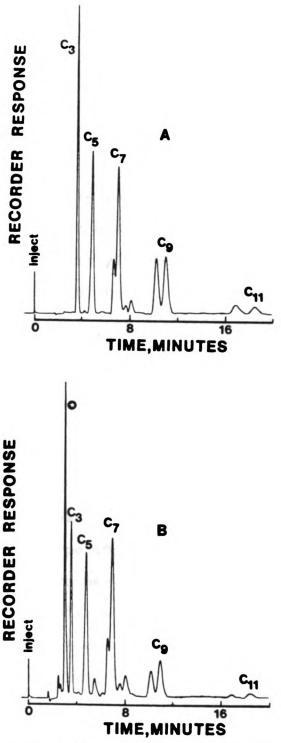


Figure 18. Typical HPLC separation of 2,4-DNPH derivatives of methyl ketones, C_3 - C_{11}, in: A) commercial Blue cheese crumbles; and B) freeze-dried commercial Blue cheese crumbles.

SUMMARY AND CONCLUSIONS

A rapid technique was developed, utilizing a high pressure liquid chromatographic system to separate and quantitate C_3 to C_{13} methyl ketones in quick ripened (QR) or commercial blue-type cheese. Quick ripened cheese using white or blue mold powder of Penicillium roqueforti were manufactured in 5 different lots, varying process parameters such as curing temperatures and salting method. On the days 7th, 11th, or 15th of curing period, the cheese samples were stored for 4 to 15 days at different temperatures, 40°, 48°, or 62°F. The pH values and the moisture content of the samples were determined throughout the ripening and storage periods.

The 2,4-dinitrophenylhydrazone derivatives of standard methyl ketones were prepared and compared with their corresponding derivatives which had been isolated from Blue cheese. At the chromatographic conditions used in this study, the DNPH derivatives of ketones such as C_7, C_9, C_{11}, and C_{13} exhibited several related "Pre-peaks." The pre-peaks were formed during derivatization of ketones in the DNPH-reaction column. The mass spectral analysis of 2-undecanone-DNP-hydrazone and its corresponding pre-peak

showed identical molecular weights for the two fractions, indicating possible geometric isomerization of ketones during derivatization. Therefore, the combined pre- and major peaks of each individual methyl ketones were used in quantitation.

Methyl ketone development in QR samples exhibited a pattern similar to that observed in commercial Blue cheese ripened several months. The predominant methyl ketones were C_7 and C_9. QR cheese made with a white mutant of P. roqueforti contained the C_9 ketone predominantly, whereas the C_7 ketone was most abundant in blue mold QR cheese. Several commercial samples were contained appreciable concentrations of 2-propanone. Generally, a large variation in the concentration of methyl ketones was observed among individual components or total methyl ketones among cheese samples. The total ketone production of cheese during ripening was not cumulative due to metabolism or conversion to other compounds.

The cheese prepared with the white mutant of P. roqueforti contained a lower concentration of methyl ketones compared with conventional blue mold cheese. The maximum ketone concentration was found in commercial, crumbled Blue cheese and in Blue cheese quick ripened at 62°F and stored at 48°F for two weeks. All samples showed small increases in methyl ketones during curing. However, after even brief storage at low temperatures (particularly 40° or

48°F), a much larger concentrations methyl ketones were formed in the cheese.

Generally, cheese ripened at lower temperatures such as 52°F and stored 2 weeks at 40°F was of superior flavor. Higher flavor scores were accorded such cheeses despite the fact that they contained lower ketone concentrations than samples prepared at higher temperatures of ripening and storage. Salting methods affected color and methyl ketone formation in the samples. A darker color was shown on samples salted on days 1, 9, and 10 rather than 7, 8, and 9. A brownish green color was obtained in cheese ripened at 62°F. Cheese samples stored at high temperatures such as 62°F exhibited atypical aftertastes, however, they showed a high concentration of methyl ketones. Blue cheese samples, salted on days 7, 8, and 9 showed a greater ketone concentration than (1, 9, 10) days salted cheese when they were stored at 48°F.

The moisture content of Blue cheeses at the time of manufacture was 52-57% and decreased to 39% at the end of ripening or storage period. The moisture loss from cheese samples was faster at higher temperature of ripening.

The pH of the QR samples decreased to a minimum of approximately 4.8, 24 - 48 hours after manufacture. The pH increased to 6.4 - 7.0 between the 3rd and 6th days of ripening and then slightly decreased during the storage period of cheese.

Freeze drying of Blue cheese samples resulted in a significant loss of methyl ketones.

APPENDICES

APPENDIX A

APPENDIX A

Table A.--The molecular weight of methyl ketones[a] or their 2,4-Dinitrophenylhydrazone derivatives.

Methyl ketone chain length	M.W. of non-derivatized	M.W. of derivatized[b]
C_3	58.8	238.94
C_5	86.1	266.24
C_7	114.2	294.34
C_9	142.2	322.34
C_{11}	170.3	350.44
C_{13}	198.4	378.54

(a) Values taken from Handbook of Chemistry and Physics, 1975-1976.

(b) The molecular weight of derivatives were calculated as following:

M.W. derivative =

= (M.W. methyl ketone + M.W. 2,4-dinitrophenylhydrazine)- M.W. H_2O

where

M.W. 2,4-dinitrophenylhydrazine = 198.14

M.W. H_2O = 18.0

APPENDIX B

APPENDIX B

Calculation of individual methyl ketone concentration:

Micromoles (μM) methyl ketone per 10 grams extracted fat =

$$\frac{\frac{\text{Peak height (chart units)}}{\text{injected volume (μl)}} \times \frac{\text{Detector Attenuation used}}{0.08} \times \text{Dilution factor (ml)} \times 1000 \times 10}{\text{Slope of std curve } (\frac{\text{chart unit}}{\mu g}) \times \text{Recovery} \times \text{M.W. derivative} \times \text{grams fat recovered}}$$

Above equation was developed as following:

(A) μg 2,4-DNPH of methyl ketone =

$$= \frac{\frac{\text{Peak height units}}{\text{injected vol. (μl)}} \times \frac{\text{Attenuation}}{0.08} \times \left[\text{Dilution Factor (ml)} \times 1000\right]\mu l}{\text{Slope } (\frac{\text{peak units}}{\mu g \text{ 2,4-DNPH}}) \times \text{Recovery}}$$

(B): μM methyl ketone = $\frac{\mu g \text{ 2,4-DNPH obtained (A)}}{\text{M.W. 2,4-DNPH (Appendix A)}}$

(C): μM methyl ketone/10 g fat =

$$\frac{\mu M \text{ methyl ketone (B)} \times 10 \text{ grams fat}}{\text{grams fat recovered from 10 g cheese}}$$

Therefore, the result of A, B, and C is the above equation.

APPENDIX C

APPENDIX C

Reconstitution of dried cheese to original moisture content:

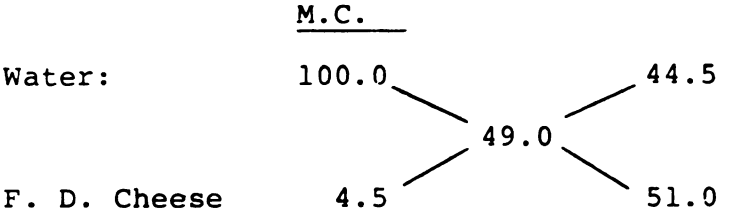
Table C.--Moisture content of fresh and freeze dried Blue cheese.

Sample[(a)]	% moisture	
	Fresh	Freeze dried
A	49.00	4.50
B	46.34	3.75
C	44.10	3.81

(a) The same samples as in Table 24.

Pearson square method was used to calculate the amount of water which was added to the dried cheese for rehydration.

Example for cheese A.



Therefore, for 10 g F. D. Cheese:

$\frac{44.5}{51.0} \times 10 = \underline{8.72}$ grams H_2O should be added to F.D. cheese to obtain 49% moisture cheese.

Proof:

10 g Freeze dried cheese + 8.72 g H_2O = 18.72 rehydrated cheese

10 g Freeze dried cheese x 0.045 = 0.45 g H_2O in Freeze dried cheese

18.72 x 0.49 = 9.17 g H_2O in rehydrated cheese

9.17 - 0.45 = $\underline{8.72}$ g H_2O added

APPENDIX D

APPENDIX D

Table D_1.--pH values of QR cheese, using blue or white mold powder of P. roqueforti, cured at 52° or 62°F and over 95% RH, salted on 3 days schedule during 7 to 15 days ripening.

Days	pH values[a]				
	Blue mold, 52°F		Blue mold, 62°F	White mold, 52°F	
	1,9,10	7,8,9	4,5,6	1,6,9	6,7,8
0	6.25	6.25	5.38	5.43	5.43
1	5.75	5.75	4.86	4.82	4.82
2	4.99	5.01	4.90	4.80	4.78
3	4.87	4.91	5.37	4.78	4.82
4	4.86	4.69	6.65	5.12	5.58
5	4.90	4.87	6.75	6.58	6.52
6	5.27	5.51	6.73	6.76	6.75
7	6.43	6.28	6.60	7.05	6.95
8	6.47	6.56	-	6.80	6.59
9	6.34	6.34	-	6.76	6.66
10	6.55	6.45	-	6.93	6.82
11	6.30	6.47	-	6.70	6.69
12	6.60	6.56	-	6.69	6.70
13	6.46	6.56	-	6.70	6.77
14	6.66	6.70	-	6.73	6.73
15	6.70	6.72	-	6.70	6.70

(a) Duplicate analysis

Appendix D Continued.

Table D_2.--pH values of commercial Blue cheese or QR cheese during two weeks storage at 40°, 48°, or 62°F.

Sample	pH
Gorgonzola	5.34
Stilton	6.47
Roquefort	6.39
Domestic (crumbles)	5.94
QR_1 (4 days at 62°F)	6.01
QR_2 (2 weeks at 40°F)	6.22
QR_3 (2 weeks at 48°F)	6.20
QR_4 (1 week at 48°F)	5.99
QR_5 (1 week at 48°F)	6.47
QR_6 (2 weeks at 40°F)	5.77
QR_7 (2 weeks at 40°F)	5.64
QR_8 (2 weeks at 40°F)	6.15
QR_9 (2 weeks at 40°F)	6.00
QR_{10} (1 week at 40°F)	6.48

$QR_{1, 2, 3}$: Blue cheese QR at 62°F and stored at 62°, 40°, and 48°F respectively.

$QR_{4, 5, 6, 7}$: Blue cheese QR at 52°F for 11 days, salted on days 7, 8, 9; 1, 9, 10; 7, 8, 9; or 1, 9, 10 respectively.

$QR_{8, 9}$: Blue cheese QR at 52°F for 15 days, salted on days 7, 8, 9 or 1, 9, 10 respectively.

QR_{10}: QR cheese using white mutant of P. roqueforti salted on days 6, 7, 8.

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