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Studies on the Ripening of Stilton Cheese: Lipolysis

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ABSTRACT

The levels of free fatty acids (FFA) in Stilton cheese showed very slight increases during the first 28 days, but they then increased rapidly up to the end of ripening. Some individual FFA, as mole percentages of total FFA, changed irregularly during ripening. Long-chain FFA were present at higher concentrations than were lower fatty acids. A relatively high level of carbonyl compounds, methyl ketones in particular, were present in Stilton during ripening. A good recovery of methyl ketones was obtained using a method based on reverse-phase high-performance liquid chromatography. The concentration of each methyl ketone did not depend on the level of available fatty acids. While the proportions of C_{13} and C_{15} ketones were relatively low compared with their precursor C_{14} and C_{16} fatty acids, the concentrations of C_7 and C_9 ketones were relatively high compared with their precursor C_8 and C_{10} fatty acids. With the exception of C_7 and C_9 ketones, which showed consistent and considerable increases up to the end of ripening ($C_7 + C_9$ accounted for 60% of total methyl ketones), the relative concentrations of methyl ketones fluctuated during ripening; this is probably the result of interconversions of the methyl ketones and/or their reduction to secondary alcohols.

INTRODUCTION

The lipid fraction of Blue cheeses contributes more to the development of characteristic flavour than any other component. Lipases that contribute to lipolysis in cheese arise principally from three sources: milk, microorganisms and some rennet preparations (Kinsella & Hwang, 1976*a*). The extent of lipolysis in Blue cheeses is governed mainly by the growth and lipolytic activity of *Penicillium roqueforti* and depends on the strain used, duration of ripening, amount of residual lipolytic activity of the milk, starter microflora and rennet, efficiency of homogenization of cheese milk, surface micro-organisms, pH, temperature, and NaCl concentration (Kinsella & Hwang, 1976*b*). Using gas–liquid chromatography, Anderson & Day (1965) found that $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ acids were the principal free fatty acids (FFA) in American Blue and Roquefort cheeses.

The homologous series of odd-carbon chain methyl ketones, C_3 to C_{15} inclusive, and some even-carbon chain methyl ketones have been identified and characterized as the principal compounds responsible for the unique flavour of Blue cheeses (Anderson & Day, 1966). According to Dartey & Kinsella (1971), heptan-2-one and nonan-2-one are the major ketones in Blue cheeses at all stages of ripening. Dartey & Kinsella (1973*a*, *b*) showed that while the major methyl ketones are presumably derived directly from the corresponding fatty acids by β-oxidation, the concentrations of heptan-2-one and nonan-2-one exceed the molar ratios of the corresponding $C_{8:0}$ and $C_{10:0}$ fatty acids in milk fat, indicating their possible derivation from longer-chain fatty acids.

Little work has been done on the ripening of Stilton cheese, which is one of the main sub-classes of Blue cheese. Therefore, this work was undertaken to study the type and extent of lipolysis in Stilton during ripening with regard to the accumulation of FFA, methyl ketones and other carbonyl compounds.

MATERIALS AND METHODS

Cheese samples

Samples of two lots of Stilton cheese, made from fresh milk (cheese A) or 24-h-old milk (cheese B), were obtained 4, 20, 28, 45, 55 and 70 days after manufacture (under commercial conditions) from St. Ivel Creameries, Melton Mowbray, UK. For comparative purposes, samples of Stilton and Danish Blue cheese (Danablu) were purchased at a local market.

Analytical techniques

FFA

The FFA were measured by an extraction method developed by Dr J. F. Connolly, The Agricultural Research Institute, Moorepark, Ireland. FFA were adsorbed on Amberlyst resin and converted into methyl esters, which were quantified by gas–liquid chromatography on a column (2 m × 2 mm I.D.) of 10% EGA on Gas-Chrom C2 (100–120 mesh) (Analab, North Haven, CT, USA).

Total monocarbonyl and total methyl ketones

The quantitative procedures for the derivatization of monocarbonyl compounds, in fats and oils (Schwartz *et al.*, 1963) and in cheeses (Schwartz & Parks, 1963), to dinitrophenylhydrazones (DNPHs) were used. The DNPH derivatives were separated, identified and quantified by high-performance liquid chromatography (HPLC).

HPLC solvent

Methanol (HPLC grade, Rathburn Chemicals, Walkerburn, Scotland) was mixed with distilled water in the proportions of 85 methanol:15 H_2O. The solvent was degassed before use by sonication for 30 min (Sonicleaner, DAWE Instruments Ltd, London, UK).

Preparation of standard methyl ketones

Standard crystalline methyl ketones, C_3 to C_9, and 10% solutions in acetone of methyl ketones C_{10} to C_{16} were obtained from Polyscience Corp. (Niles, IL, USA), kit 431, and prepared for calibration of HPLC as follows:

Each standard methyl ketone (2 mmol) was made up in 5 ml hexane and passed through a dinitrophenylhydrazine–Celite column as described by Schwartz & Parks (1963). The eluate (100 ml) was collected and evaporated to dryness. The residue was taken up in 25 ml HPLC-grade hexane.

Equipment

HPLC was performed using a Perkin-Elmer HPLC Series 2 instrument, fitted with a dual pump and equipped with a Rheodyne syringe loading injector, No. 7105, with a 1·1-ml loop. Separation was achieved using a Reverse Phase C column (4·6 mm I.D. × 250 mm length) of Spherisorb ODS, 5 μm particle size (Lab Data Control, Stone, Staffordshire, UK). The HPLC column was connected to a spectrophotometric detector (Perkin-Elmer LC-75 spectrophotometer) and a Perkin-Elmer 56 recorder.

Standard conditions for HPLC separation

The system was equilibrated by running 85% methanol (HPLC grade),

previously degassed, through the column at a rate of 1·5 ml/min. Samples (2 μl) were injected using a microsyringe (SGE Corp., North Melbourne, Australia). The eluate was monitored continuously at 345 nm. Methyl ketone DNPHs were identified by their retention times, established using authentic standards. The concentrations of identified methyl ketones were calculated from their peak areas, based on the peak areas of known molar concentrations of corresponding standards. The calculated concentrations were corrected for % recovery.

RESULTS AND DISCUSSION

FFA

The values for total FFA at different stages of ripening (Table 1; Fig. 1) showed little change up to 28 days but they continued to increase thereafter up to the end of the ripening period (70 days) at which stage they were still

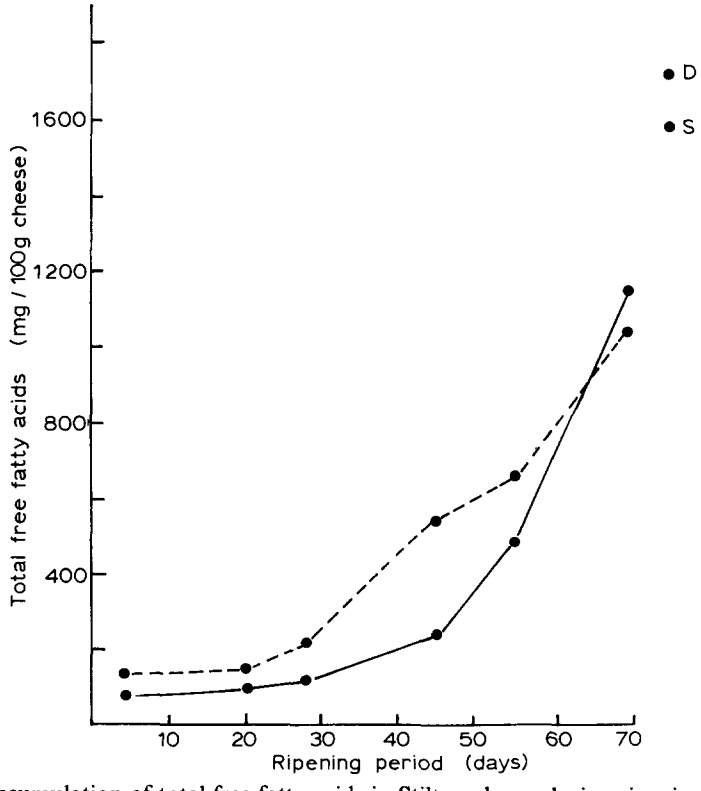

Fig. 1. Accumulation of total free fatty acids in Stilton cheese during ripening. Cheese A (——); Cheese B (- - - -) commercial Stilton: S; commercial Danablu: D.

TABLE 1
Concentration of Free Fatty Acids in Two Sets (*A* and *B*) of Stilton Cheese at Different Stages of Ripening

Age (Days)	*Cheese*	*FFA (mg/100 g cheese)*												
		C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	*Total*
4	A	—	—	2·6	2·9	2·5	5·7	17·8	2·7	12·3	19·5	—	—	66·0
	B	2·7	2·4	2·4	3·1	4·3	10·6	32·5	4·6	13·2	44·7	6·8	2·1	129·4
20	A	—	1·5	2·4	5·9	3·1	7·0	21·9	3·2	8·5	26·0	3·6	1·7	84·8
	B	6·3	3·0	3·4	4·1	5·6	14·5	44·6	5·6	20·0	61·9	9·2	2·2	162·4
28	A	—	1·9	3·5	3·5	3·3	6·7	27·0	3·2	18·5	28·0	3·8	3·1	102·5
	B	10·6	3·3	3·8	8·1	6·6	15·0	45·0	6·0	28·5	67·6	10·9	2·6	208·0
45	A	8·5	4·1	3·6	4·5	7·3	17·0	47·2	8·6	18·0	98·0	14·0	5·3	237·1
	B	15·8	11·3	7·7	11·0	15·5	40·0	112·2	17·9	44·9	217·0	35·0	10·0	538·3
55	A	27·9	13·9	9·3	11·9	14·1	33·7	98·6	15·5	34·0	172·2	35·3	8·3	474·7
	B	33·0	26·0	14·3	21·7	20·6	43·0	121·6	20·1	49·5	231·0	46·7	10·8	638·3
70	A	20·7	16·7	18·7	29·0	32·9	80·6	200·0	87·8	72·8	435·0	90·4	67·3	1 151·9
	B	13·0	24·0	20·6	36·0	32·0	59·4	183·4	95·5	64·8	340·0	90·6	23·7	983·0
Commercial samples	S[a]	35·0	24·4	17·9	57·1	79·9	207·0	465·5	47·2	194·5	544·0	105·4	19·7	1 797·6
	D[b]	107·0	53·3	27·1	49·5	60·6	158·0	388·6	60·3	138·3	703·0	118·5	26·3	1 890·5

[a] S = Stilton.
[b] D = Danablu.

much lower than those for the commercial samples of Stilton and Danablu. The total FFA increased to 14- and 8-fold their initial values after 70 days of ripening for cheeses A and B, respectively.

The concentrations of all individual FFA increased as ripening progressed with increases ranging between 4- and 20-fold at the end of ripening (Table 1; Fig. 2). Some of the individual FFA (C_4 and C_6) were considerably lower in the commercial Stilton sample than in the experimental cheeses after the normal ripening period. Butyric acid was not detected in cheese A until it was 45 days old, but it increased rapidly thereafter to reach a value of 81 mg/100 g cheese at 70 days. Trace amounts of butyric acid were found in the 4-day-old cheese B and its concentration increased gradually throughout the ripening period to reach a value of 43 mg/100 g cheese at 70 days. While caproic acid (C_6), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were evident only after 20 days in cheese A, they were detected in appreciable quantities in cheese B after 4 days of ripening.

The FFA were classified into three groups according to chain length and degree of saturation. The levels of the three classes as mole percentages of total FFA (Fig. 3) showed irregular changes throughout ripening. Generally, the levels of long-chain, saturated fatty acids (C_{12}, C_{14}, C_{16} and C_{18}) and the unsaturated fatty acids ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$) were considerably higher than those for the shorter-chain acids (C_4, C_6, C_8 and C_{10}) at all stages of ripening. The initial values of short-chain acids in cheese B were considerably higher than in cheese A; the mole percentages of the short-chain FFA were 8 and 16% of the total FFA for 4-day-old cheeses A and B, respectively.

The present results show that FFA accumulate to high levels in Stilton during ripening. This has been demonstrated for other Blue cheeses by several workers (Anderson & Day, 1965; Woo *et al.*, 1984). It has been reported that FFA, which are important flavour compounds in Blue cheeses (Badings & Neeter, 1980; Law, 1981), accumulate to relatively greater proportions in Blue cheeses than in most other types of cheese (Woo *et al.*, 1984). Although the milk used for the experimental cheeses was pasteurized, the initial values of total FFA were fairly high in both sets of cheese, particularly in cheese B, and were higher than those observed with other types of cheese at the same stage of ripening. A considerable concentration of FFA was also reported by Morris *et al.* (1963) in American Blue cheese during the early stages of ripening. This may reflect the activity of residual indigenous milk lipase during the early stages of ripening. The values for total FFA increased only slightly up to the 28th day of ripening; this may be due to the utilization of FFA by the mould during the early stages of ripening or only very slight lipolysis may have occurred until after sporulation of the mould. The latter is more likely to be correct since it has

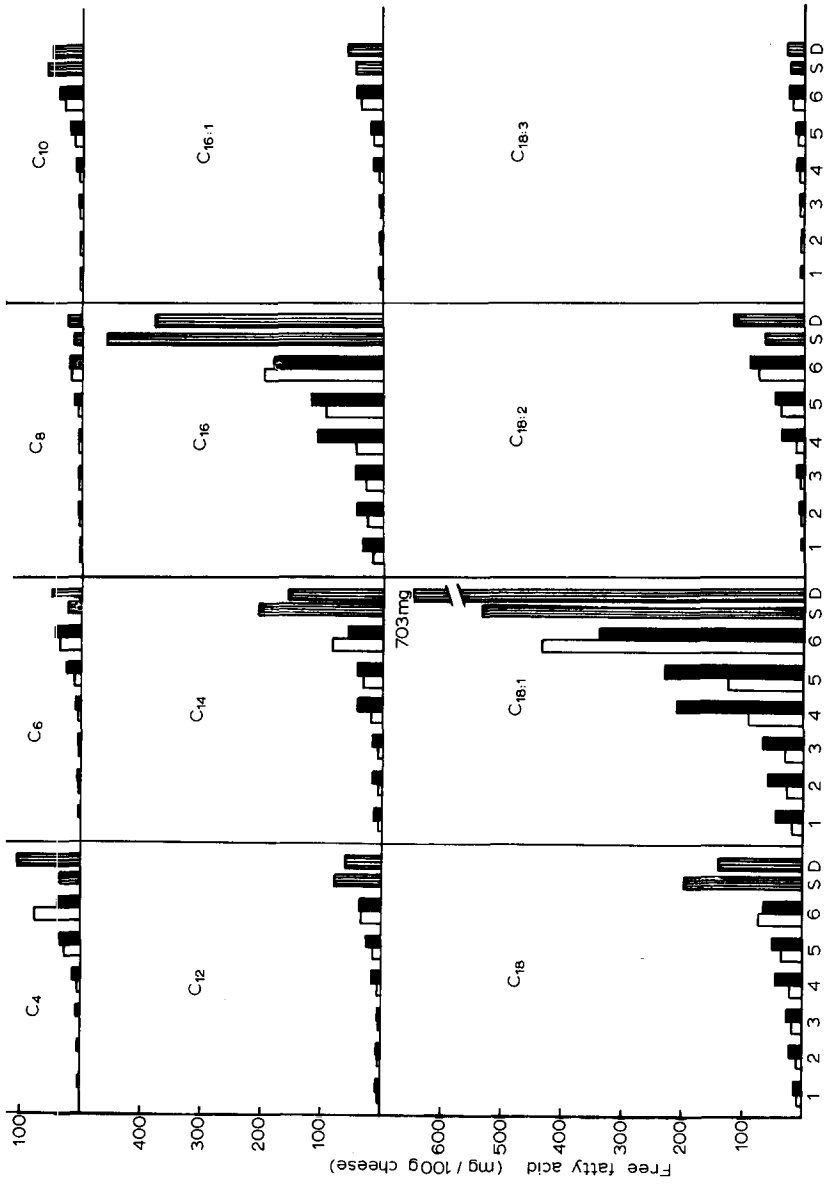

Fig. 2. Free fatty acid profiles in Stilton cheese at several stages during ripening. Cheese A (□), cheese B (■). 1. 4d; 2. 20d; 3. 28d; 4. 45d; 5.55d; 6. 70d; ▥ commercial samples of Stilton (S) and Danablu (D).

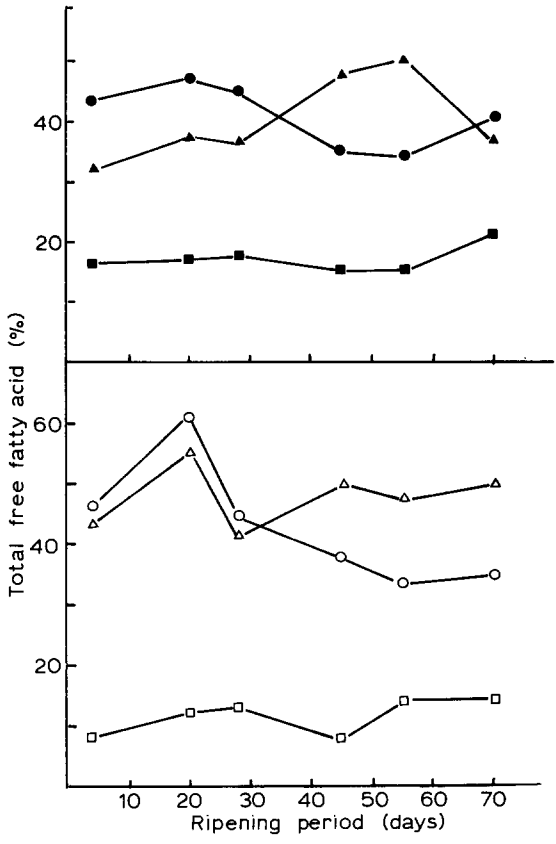

Fig. 3. Distribution of free fatty acids according to chain length and degree of saturation in Stilton cheese at different stages of ripening. Cheese: A □, B ■ (short-chain FFA); A ○, B ● (long-chain saturated FFA); A △, B ▲ (long-chain unsaturated FFA).

been shown (Thibodeau & Macy, 1942; Kinsella & Hwang, 1976*a*) that lipases are released by the mould only after sporulation; the mould sporulated in the cheese at about the 28th day of ripening after which the concentration of total FFA increased considerably.

The irregular changes in individual fatty acids, as mole percentages of the total FFA, probably demonstrate some interconversions to other flavour compounds, e.g. lower fatty acids and methyl ketones, or the utilization of some fatty acids by *P. roqueforti* to supply the energy requirements for active growth (Kinsella & Hwang, 1976*b*; Godinho & Fox, 1981). The present data show that butyric acid and some other lower fatty acids were present at greater quantities in Danablu than in Stilton, and this may be expected to contribute significantly to the stronger, sharper flavour of Danablu compared with Stilton. When calculated as mole percent of the total FFA in

Stilton, the saturated and unsaturated higher fatty acids (C_{12}–$C_{18:3}$) were very much more abundant than the lower fatty acids. The salts of the long-chain acids, which possess mild soapy flavours (Anderson & Day, 1965), probably contribute significantly to the characteristic background flavour of Stilton cheese.

Carbonyl compounds

The concentrations of total monocarbonyl DNPH derivatives and total methyl ketone DNPHs/1 g dry cheese at various stages of ripening are shown in Fig. 4. There was little change in total carbonyls or methyl ketones during the first 20 days, but thereafter both increased rapidly,

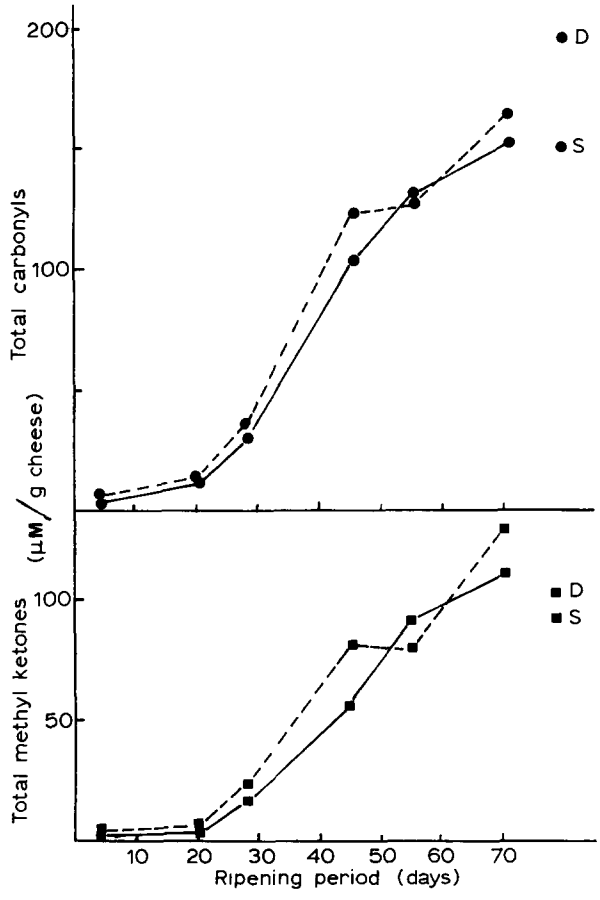

Fig. 4. Accumulation of total carbonyls (●) and total methyl ketones (■) in Stilton cheese during ripening. Cheese A ——; cheese B – – – –; commercial Stilton: S; commercial Danablu: D.

TABLE 2
Concentrations of Individual Methyl Ketones in Stilton Cheese at Different Stages of Ripening

Age of cheese (days)		*Propan-2-one*	*Pentan-2-one*	*Ketone*					
				Heptan-2-one	*Nónan-2-one*	*Undecan-2-one*	*Tridecan-2-one*	*Heptadecan-2-one*	*Total*
				(μmol/10 g dry cheese)					
4	A	—	0·16	0·61	1·39	0·26	—	—	2·45
	B	—	0·22	0·87	1·60	0·31	—	—	3·00
20	A	—	0·56	1·52	2·08	0·78	—	—	4·94
	B	0·11	0·67	1·60	3·12	1·02	—	0·10	6·02
28	A	0·9	2·52	5·54	4·34	1·93	0·98	—	16·21
	B	1·15	2·24	8·80	7·30	2·08	1·66	0·23	23·46
45	A	2·11	4·14	20·60	22·44	7·39	3·30	0·92	58·79
	B	2·20	6·03	28·54	25·52	11·83	4·93	4·93	83·98
55	A	1·86	7·89	30·16	33·35	12·76	5·80	1·74	92·00
	B	2·35	4·41	26·17	27·30	8·56	6·01	4·41	74·55
70	A	6·60	7·92	31·56	38·40	12·06	7·50	6·40	110·44
	B	10·88	13·96	42·75	37·12	11·20	5·76	8·64	130·04
Commercial samples	S[a]	0·44	5·18	28·49	22·64	13·69	17·76	1·88	90·08
	D[b]	0·37	4·14	37·00	31·08	9·99	19·24	0·92	102·74

[a] S = Stilton.
[b] D = Danablu.

especially between the 28th and 45th days, i.e. the most active phase of mould growth. The levels of total carbonyls and total methyl ketones, which were generally higher in cheese B than in cheese A, increased only slightly in cheese B between the 45th and 55th day of ripening, but increased thereafter to reach a value higher than that of cheese A at the end of ripening, i.e. 110 and 130 mole/g dry cheese A and B, respectively. While the commercial Stilton sample had a slightly lower concentration of total methyl ketones than the 70-day experimental cheeses, the Danablu had about the same concentration as the 70-day-old cheese B.

The concentrations of the individual methyl ketones in the cheeses at different stages of ripening are shown in Table 2 and representative high-performance liquid chromatograms of DNPH derivatives of methyl ketone isolated from cheeses of different ages are shown in Fig. 5(*a*) and 5(*b*).

The methods used in this study (Schwartz *et al.*, 1963) give nearly quantitative conversion of carbonyls into DNPHs, which are subsequently separated on Celite–magnesia columns (Keen *et al.*, 1976). The present study confirmed that >90% of the methyl ketones in a mixture were converted to DNPH derivatives on the reaction column and good recovery (Table 3) of the 2,4-dinitrophenylhydrazones of the methyl ketones was obtained. The chromatograms and data presented demonstrate the applicability and sensitivity of detection; for example, peak 2 in the 4-day cheeses (Fig. 5(a)) corresponds to 2 ppm. While methyl ketone derivatives prepared by direct reaction in acidic solutions of 2,4-dinitrophenylhydrazine showed instability when identified by HPLC, those separated on reaction columns gave clean separation of individual methyl ketone DNPHs. Schwartz *et al.* (1963) demonstrated that the column procedure permits conversion of extremely low concentrations of carbonyls into 2,4-dinitrophenylhydrazones, which are stable throughout the subsequent fractionation; this cannot be achieved accurately when reaction of 2,4-DNPH and carbonyls occurs in solution. Tichivangana (1984) reported that the 2,4-DNPH derivatives of carbonyls in oxidized meat are effectively separated by reverse-phase HPLC. Satisfactory resolution of the DNPH derivatives of all the methyl ketones likely to be produced by β-oxidation of fatty acids was obtained using isocratic elution with methanol–water (85:15) at room temperature.

The results (Table 2) showed that an homologous series of methyl ketones of odd-carbon chain length, C_3 to C_{15}, was present in the cheeses after the 28th day of ripening although the methyl ketones C_3, C_{13} and C_{15}, which were generally present at low concentrations, were present only in trace amounts until the 45th day of ripening. Acetone was not detected by the method used and probably remained with the *n*-alkanal fraction during fractionation of the methyl ketone DNPHs from the mixture of total DNPH. Hawke (1966) showed that the short-chain members of each class of

carbonyl DNPHs, e.g. acetone, move more slowly than the higher members of the homologous series during elution from a Celite–magnesia fractionation column and are incompletely separated from the class of compounds that follows in order of elution (higher methyl ketones elute first, followed by aldehydes).

The results also show a general fluctuation in the relative concentrations of methyl ketones during the course of ripening, the only exception being heptan-2-one (C_7) and nonan-2-one (C_9), which increased consistently

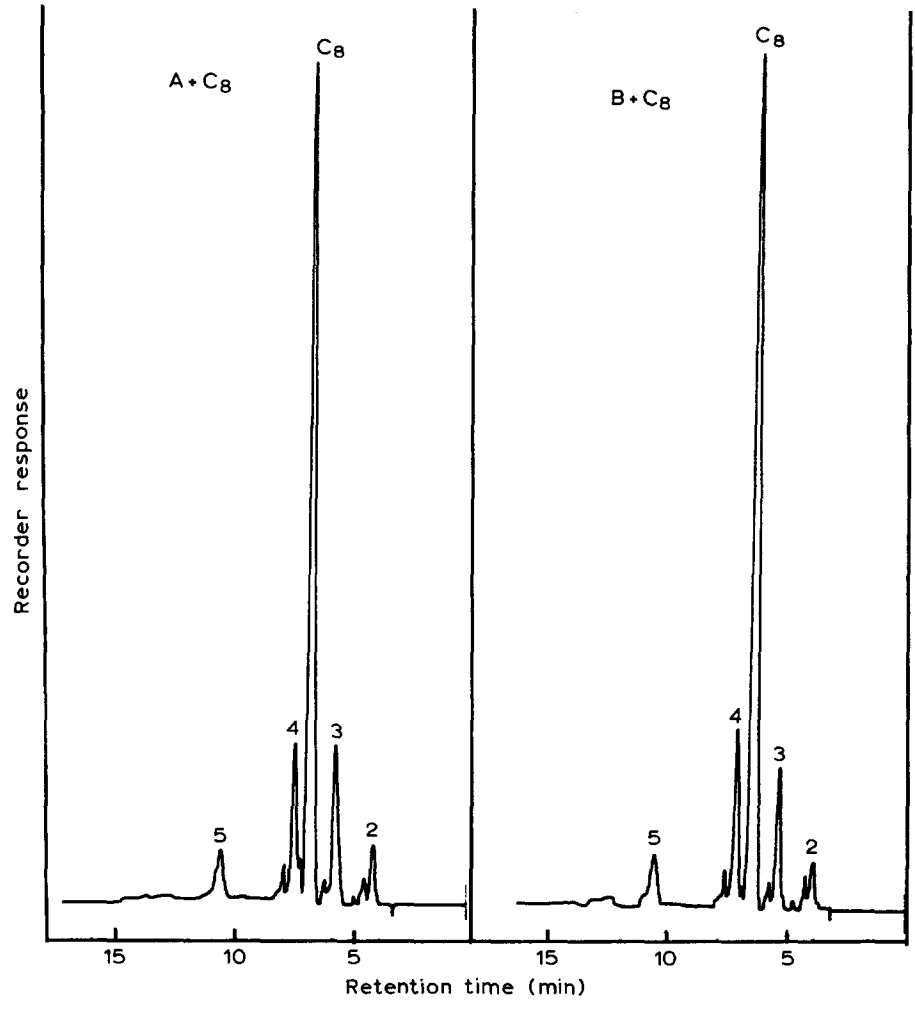

Fig. 5(a). High-performance liquid chromatograms of methyl ketones isolated from Stilton cheeses A and B after 4 days of ripening. The peaks were identified as: (2) pentan-2-one; (3) heptan-2-one; (4) nonan-2-one; (5) undecan-2-one. Prior to extraction of the 4-day-old cheeses, 50 μmols octan-2-one (C_8)/g cheese were added to check on methyl ketone recovery.

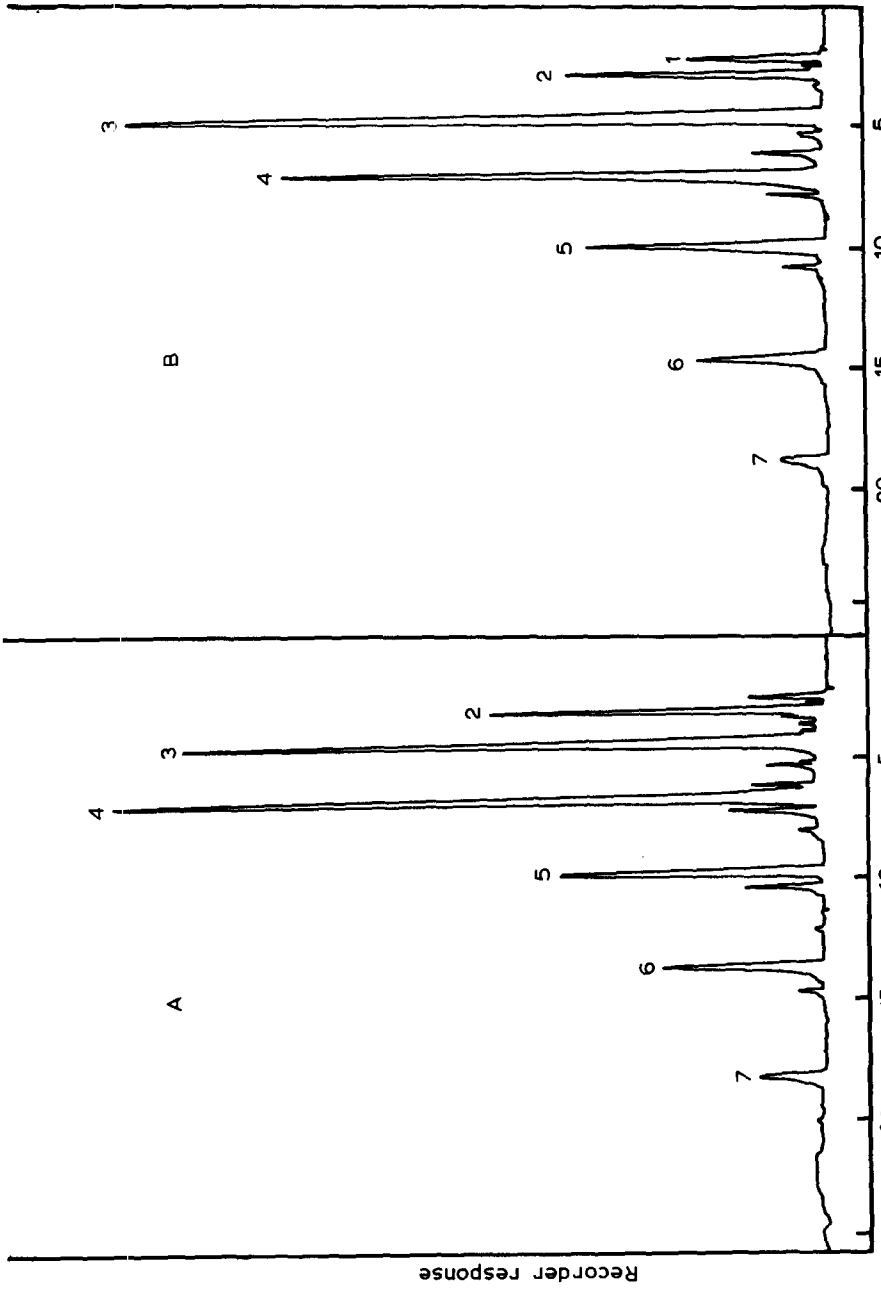

Fig. 5(b). High-performance liquid chromatograms of methyl ketones isolated from Stilton cheeses A and B after 70 days of ripening. The peaks were identified as: (1) propan-2-one; (2) pentan-2-one; (3) heptan-2-one; (4) nonan-2-one; (5) undecan-2-one; (6) tridecan-2-one; (7) heptadecan-2-one.

TABLE 3
Recovery of the 2,4-Dinitrophenylhydrazones of Methyl Ketones from Celite–Seasorb Columns

Methyl ketones	(%) *Recovery*[a]
C_3	90
C_5	95
C_7	98
C_8[b]	102
C_9	95
C_{11}	100
C_{13}	94
C_{15}	92

[a] Average of duplicates.
[b] Known concentrations (50 μmol/g cheese) of C_8 were added to 4-day-old cheeses.

throughout ripening. C_7 and C_9 were the principal methyl ketones in the cheeses and accounted for $>60\%$ of the total concentration of methyl ketones throughout ripening, suggesting that these two methyl ketones are probably the major components of Stilton cheese flavour. According to several observers (*cf.* Kinsella & Hwang, 1976*a*), the homologous series of methyl ketones, particularly heptan-2-one and nonan-2-one, is responsible for the typical flavour of Blue cheese types, e.g. Stilton, Roquefort, Danablu and Gorgonzola.

With the exception of undecan-2-one (C_{11}) and tridecan-2-one (C_{13}), the levels of all methyl ketones were lower in the commercial Stilton sample than in the 70-day-old experimental cheeses (Table 2). No consistent differences between the commercial Stilton and Danablu were noted. Only very low concentrations of any ketone above C_{15} were found in the present study. Since high molecular weight ketones were not isolated or identified by others (Morgan & Anderson, 1956; Anderson & Day, 1966; Dartey & Kinsella, 1971; King & Clegg, 1979) it is probable that few or no additional ketones were present in the experimental cheese. Kinsella & Hwang (1976*a*) demonstrated that there is negligible oxidation of the longer-chain fatty acids, $>C_{14}$, to their corresponding ketones at the pH prevailing during the ripening of Blue cheeses.

P. roqueforti produces methyl ketones with one or less carbon atom during β-oxidation of precursor fatty acids (Gehrig & Knight, 1963; Lawrence & Hawke, 1968; Kinsella & Hwang, 1976*a*, *b*). However, the present results showed that the quantity of each ketone produced did not depend directly on the concentration of available fatty acid precursor. The

proportions of C_{13} and C_{15} methyl ketones were relatively low compared with their precursor, C_{14} and C_{16}, fatty acids; conversely, the concentrations of C_7 and C_9 methyl ketones were very high relative to their precursor, C_8 and C_{10}, fatty acids. This is illustrated in Fig. 6 by a plot of the average mole percent of the individual methyl ketones and their fatty acid precursors in Stilton after the normal ripening period. Comparable results for Roquefort and American Blue cheese were presented by Anderson & Day (1966). Dartey & Kinsella (1973*a*, *b*) showed that, while the major methyl ketones are presumably derived directly from the corresponding fatty acids by β-oxidation, the concentrations of C_7 and C_9 methyl ketones exceed the molar ratios of the corresponding C_8 and C_{10} fatty acids, indicating their possible derivation from longer-chain fatty acids. They also reported that the generation of the homologous series of methyl ketones, C_3 to C_{15}, inclusive, from palmitic acid (C_{16}) was significant. The use of uniformly labelled radioactive fatty acids by those workers demonstrated that several methyl

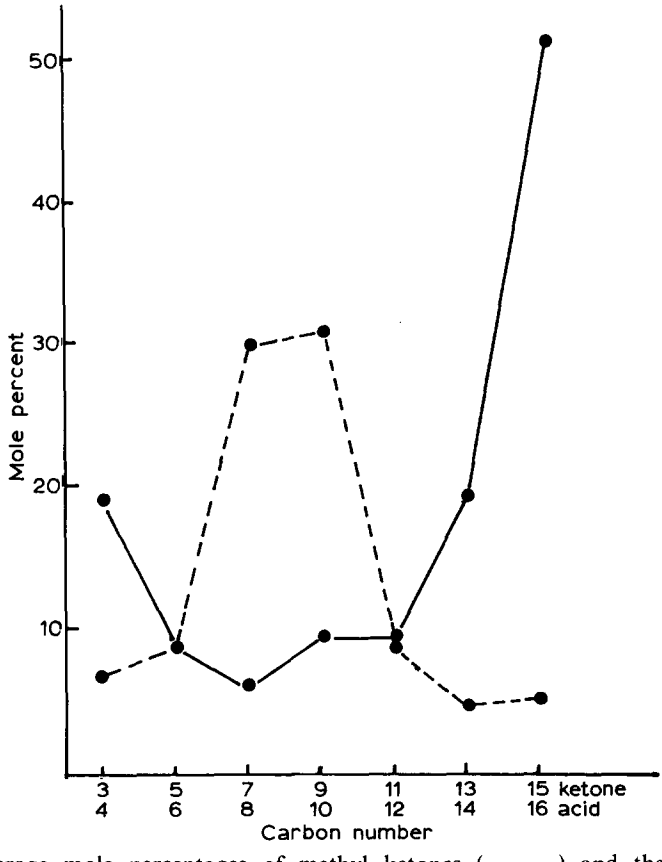

Fig. 6. Average mole percentages of methyl ketones (– – – –) and their fatty acid precursors (——) in Stilton cheese after the normal period of ripening (70 days).

ketones can be formed via β-oxidation from lauric and myristic acids. Therefore, it is logical to assume that some of the major methyl ketones in Stilton, i.e. heptan-2-one and nonan-2-one, may originate from longer-chain fatty acids through β-oxidation as well as from the corresponding fatty acids with one more carbon atom. Thus, the long-chain fatty acids of milk fat may be a significant source of the methyl ketones present in mould-ripened cheese (Anderson & Day, 1966; Dartey & Kinsella, 1971).

While the total concentration of carbonyls increased progressively with time, the levels of individual methyl ketones at progressive stages of ripening clearly showed that no definite ratios or correlation existed between the relative concentrations of the methyl ketones in the cheese (Table 2). The fluctuations in the concentrations of the methyl ketones were conceivably the result of the interconversions of the methyl ketones and their reduction to the corresponding secondary alcohols.

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