Conversion of Triacylglycerols into Monoacylglycerols by *Penicillium roquefortii*

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This thesis is dedicated to all members of my families for their unfailing love and support from 7,000 miles away over the last three years.

Abstract

The synthesis and use of monoacylglycerols in food systems have been reviewed. The use of monoacylglycerols alone or in combination with free fatty acids as food preservatives has been discussed. Model systems have been devised to produce monoacylglycerols (MAGs) from butter and Shea oils with two strains of *Penicillium roquefortii*, FRR 2456 (isolated from a spoilt melon) and Wisbey PJ (a commercial dairy strain).

A semi-micro method was developed using Preparative Thin Layer Chromatography (PTLC) and Gas Chromatography - Mass Spectrometry (GC-MS) of the MAG trimethylsilyl ether derivatives to determine the identification, fatty acid composition and structural isomers of the individual MAG.

The main monoacylglycerols produced by spores and emerging mycelium were 1(3)-monoacyl-sn-glycerols (in suspension culture). Monopalmitin was the major MAG from butter and Shea oils. Monoacylglycerols produced by fungal mycelium (in solid-state culture) were mainly the 1(3)-monoacyl-sn-glycerols although approximately 30% were present as 2-monoacyl-sn-glycerols. Again the main MAG was monopalmitin. It suggested that *P. roquefortii* produced two lipases, one during germination with specificity to the sn-2 position in the original triacylglycerols (TAGs) and one 1,3-specific during growth of the fungal mycelium. In addition, flavour compounds, methyl ketones and γ-lactones, were found in solid-state culture. The composition of the MAGs formed by lipolysis using a commercial lipase (E.C.3.1.1.3) with 1,3-specificity gave the expected 2-isomers when butter oil was the substrate but gave 1(3)-monostearin rather than the expected 2-monoolein when Shea oil was the substrate. It suggested that acyl migration occurred due to the reactive nature of the original oleate group at the sn-2 position in the Shea oil TAGs. There were no significant differences with fungal strain or temperature of incubation (10 °C and 25 °C) on the composition of the MAGs.

The mechanism of formation of MAGs from butter and Shea oils has been discussed. It has been suggested that 1(3)-MAGs together with free fatty acids may be part of a natural antimicrobial system in high pH foods such as blue mould-ripened cheese where growth of foodborne pathogens such as *Listeria monocytogenes* can be a problem from time to time.

Advanced studies, seminars and conferences undertaken in connection with the research project

Studies and courses

1. Visits to the natural products research laboratory, Bush Boake Allen Ltd (London), to study GC/MS analysis techniques, 7 - 11 November 1994.

2. Attendance at a series of computer courses for staff held in Sheffield Hallam University in 1995 (The Internet, Introduction to Powerpoint, Excel).

3. Visit to Stilton cheese factory at Melton Mowbray on 17 April 1996.

4. Attendance at the combined studies course on Statistics, Mathematics and IT, given by Department of Biochemical Sciences at Sheffield Hallam University, Sheffield (January - June 1996). Gained level 1 and overall mark of 94 for this course.

5. Attendance at the advanced English courses in the TESOL centre at Sheffield Hallam University from September 1994 to December 1995. Obtained the certificate of Cambridge Advanced English in June 1996.

6. Attendance at the Mass Spectrometry element of Analytical Science III Unit course, given by Department of Chemistry at Sheffield Hallam University, Sheffield (February - June 1996).

7. Attendance at the workshop on Teaching and Learning in Higher Education, given by School of Education, Sheffield Hallam University, Sheffield in May 1996.

Conferences

1. Capillary Gas Chromatography 'a practical approach', seminar organised by Royal Society of Chemistry, London, 27 October 1994.

2. Planer Separations - Advances and New Applications, a joint meeting of Chromatographic Society & the Royal Society of Chemistry, London, 21 February 1995

3. Capillary Chromatography seminar, a seminar for practical chromatographers, organised by Thames Chromatography, York, 14 June 1995

4. Merck Chromatography workshops - sample preparation and application in planar chromatography, organised by Thames Chromatography, Atherston, 28 June 1995.

5. Hot Topics in Environmental Analysis, meeting organised by Environmental Research Centre of Sheffield Hallam University and Royal Society of Chemistry, Sheffield, 15 May 1996.

6. Lipid Chemistry and Analysis, Lipid Analysis Unit, workshop organised by Mylnefield Research Services Ltd & Scottish Crop Research Institute, Dundee, Scotland, 5-6 September 1996.

7. Lipids in Health and Nutrition, conference organised by Royal Society of Chemistry (Oils and Fats Group), Sheffield Hallam University, 9-10 September 1996.

8. The Environmental Consequences of Biofouling and Biofouling Control Measures, meeting organised by The Biodeterioration Society, Preston, 5 December 1996.

† 9. Microbiology for the Hospitality Industry, organised by Society for Applied Bacteriology, 14-15 Belgrave Square, London, 13 March 1997.

† 10. Winter Meeting, organised by Society for Applied Microbiology, 14-15 Belgrave Square, London, 21 January 1998.

† I was given a studentship by the Society for Applied Microbiology to attend these meetings

Publications

Liu, Q-T., Cooke, M. and Kinderlerer, J.L. (1995) Fatty acids composition in two samples of butter oil, poster presented at the conference of The Fats and Oils Industry Products & Applications, organised by Royal Society of Chemistry and Croda Universal, Hull, 13-14 September.

Liu, Q-T. and Kinderlerer, J.L. (1996) Structure and fatty acids composition of monoacylglycerols produced from Shea oil by *Penicillium roquefortii*, poster presented at the Symposium of the British Mycological Society, University of Sheffield, 10-12 April.

Liu, Q-T. and Kinderlerer, J.L. (1996) Structure of monoacylglycerols produced from Shea and butter oils by *Penicillium* spp., paper presented to the young authors meeting of Oils and Fats Group, Society of Chemistry and Industry, London, 16 July.

Liu, Q-T. and Kinderlerer, J.L. (1996) Synthesis and analysis of monoacylglycerols from shea and butter oils by *Penicillium roquefortii*, paper presented to the 96 International Workshop on the Impact of Development Genetics on Biotechnology, Shanghai, China, 3-5 October.

Liu, Q-T. (1997) Preparative Thin Layer Chromatography for the separation and subsequent derivatisation of acylglycerols, paper presented to the conference of Lipid Analysis, Old Problems - New Solutions, organised by SCI and RSC, Liverpool, 28-29 October.

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List of Abbreviations

°C	Degrees Centigrade
μg	Microgram
μl	Microlitre
μm	Micrometre
BSA	Bis(trimethylsilyl)-acetamide
BSTFA	N,N-bis(trimethylsilyl)-trifluoroacetamide
CAS	Chemical Abstracts Service
cm	Centimetre
CYA	Czapek Yeast Agar
d	Day(s)
DAGs	Diacylglycerols
DHA	Docosohexaenoic Acid
EEC	Europe
EPA	Eicosapentaenoic Acid
FAME	Fatty acid methyl ester
FAs	Fatty Acids
FAD	Flavin Adenine Dinucleotide
FDA	Food and Drug Administration
FFAP	Free Fatty Acid Phase
FID	Flame Ionisation Detector
F_r	Response Factors
g	Gram
G25N	25% Glycerol Nitrate
GB	Guo Biao
GC	Gas Chromatography
GC-MS	Gas Chromatography - Mass Spectrometry
h	Hour
HPLC	High Performance Liquid Chromatography
id	Internal diameter
IMS	Industrial Methylated Spirits
is	Internal standard
M	Molar
MAGs	Monoacylglycerols
MCFAs	Medium chain fatty acids
MEA	Malt Extract Agar
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Millilitre
mm	Millimetre
mmol	Millimole
Mol	Mole
M_w	Molecular weight
NAD(H)	Nicotinamide Adenine Dinucleotide (reduced form)
NC	Not Calculated
ND	Not Detected
nm	Nanometre
P. roquefortii	*Penicillium roquefortii*
PRC	Peoples' Republic of China
PTLC	Preparative Thin Layer Chromatography
SC-CO_2	Supercritical Carbon Dioxide
SD	Standard Deviation
T	Temperature
t	Time
TAGs	Triacylglycerols
TBDMS	t-butyldimethylsilyl
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
v/v	Volume / volume
in vacuo	Under vacuum
w/v	Weight / volume
wt	Weight

CHAPTER 1

Introduction

Section I Monoacylglycerols and Fatty Acids

Monoacylglycerols (MAGs, monoglycerides) are nonionic surfactants widely used as emulsifying agents in various food systems and account for about 75 % of the world production of food emulsifiers (Krog 1990). Monolaurin (glycerol monoester of lauric acid) is approved in the United States as a food emulsifier (21 CFR GRAS 182.4505). Some monoacylglycerols together with fatty acids have antimicrobial activity against Gram-positive bacteria (Kabara & Eklund 1991). For example monolaurin is used in Europe and the United States as a food preservative.

In addition to use as emulsifiers and food preservatives, monoacylglycerols are widely used in cosmetics and the pharmaceutical industry. For example, monopentadecanoylglycerol is used in shampoo or hair conditioners (Weiss 1990). Monoacylglycerols containing n-3-polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosohexaenoic acid (DHA) may help prevent cardiovascular disorders (Li & Ward 1993). Regioisomerically pure 1 (3)-monoacyl-rac-glycerols are starting materials for the synthesis of defined triacylglycerols, phospholipids, glycolipids, lipoproteins and in the preparation of numerous enzyme activators and inhibitors (Ahmed 1990). Furthermore, monoacylglycerols have been employed for the preparation of conjugates with various drugs (McNeill & Yamane 1991, Jacob *et al* 1990).

Food Legislation allowing monoacylglycerols to be used as food additives may vary in different countries. The applications of monoacylglycerols together with diacylglycerols and fatty acids as food additives in United Kingdom (UK), Europe (EEC), the United States of America (USA) and Peoples' Republic of China (PRC) are listed in Table 1.1.

Table 1.1 Monoacylglycerols used as food additives in UK, EEC, USA and PRC

[†] Butterworth Law of Food & Drugs (1997). The regulations implemented European parliament and Council Directive 95/2/EC (OJ No L61, 18.3.95, p1) on food additives other than colours and sweeteners which has to be read with Council Directive 89/107/EEC (OJ No L40, 11.2.89, p27).
[‡] From Food and Drug Administration (FDA) regulations (4-1-95 edition).
[*] From Catalogue of National Standards & Information Compilation of PRC (1995).
[a] Level 1: at a level not in excess of the amount reasonably required to produce its intended effect. [b] The additive does not exceed 200 parts per million of the combined weight of the oil or fat and the additive. [c] At a level not to exceed weight percent of dry ingredients used or finished materials.
NA: not mentioned, MAGs: monoacylglycerols, DAGs: diacylglycerols, FAs: fatty acids.

1.1 Antimicrobial effects of fatty acids and monoacylglycerols

There is considerable evidence in the literature to show that free fatty acids inhibit the growth of bacteria particularly Gram positive bacteria. The early work has been summarised in a classic review by Neiman (1954). Later work has been described by Kabara in a number of reviews (Kabara 1979, 1981, 1984a, 1984b, 1993, Kabara & Eklund 1991). Fatty acids range from 6 to over 20 carbon atoms in length. The lower homologues are somewhat soluble in water and as such would be expected to have an antimicrobial effect in an aqueous system. The higher homologues are only soluble in a hydrophobic system. If the long chain fatty acids have antimicrobial activity they would have to be dissolved in alcohols or in a hydrophobic carrier such as fat. There is general agreement that dodecanoic (lauric) acid ($C_{12:0}$) has considerable antibacterial activity. Kinderlerer *et al* (1996) in a study of the antilisterial properties of a homologous series of medium chain fatty acids showed that lauric acid was the most inhibitory of those studied ($C_{6:0}$, $C_{8:0}$, $C_{10:0}$ and $C_{14:0}$).

The use of monolaurin as a preservative in a food product (margarine) was pioneered by Moustafa and Agin (1980). From Table 1.1 monoacylglycerols and in particular monolaurin are permitted additives in foods in many countries. Monolaurin possesses broad spectrum antimicrobial activity in culture medium against Gram positive bacteria, moulds and yeasts, but not against Gram negative bacteria (Kabara 1979). The antimicrobial effects of monolaurin combined with lauric acid has been described by Wang and Johnson (1992) in culture medium. The use of monolaurin in extending the shelf-life of cottage cheese has been described (Bautista *et al* 1993) and in the destruction of biofilms on stainless steel (Oh & Marshall 1995).

Papageorgiou and Marth (1989) originally suggested that free fatty acids and their degradation products methyl ketones and secondary alcohols formed a preservation system in blue mould ripened cheeses. Work at Sheffield Hallam University has attempted to find the chemical reason

why *Listeria monocytogenes*, a serious foodborne pathogen, can grow in surface ripened cheeses but not in blue veined cheeses (Kinderlerer 1996). During ripening both cheeses undergo a secondary fermentation. White mould ripened cheeses such as Brie and Camembert are ripened by growth of *Penicillium camembertii* on the outside of the cheeses. Blue mould ripened cheeses such as Stilton and Bleu d'Auvergne are ripened by growth of *P. roquefortii* inside the blue veins (Kinderlerer 1995). When these mould-ripened cheeses are ready for consumption Kindererler *et al* (1996) demonstrated that in the soft ripened cheeses there were relatively no free fatty acids present. In Bleu d'Auvergne however there was high concentrations of free medium chain fatty acids particularly lauric and myristic acids in the blue veins. These authors demonstrated that there were high concentrations of blue conidia spores in the blue veins as well. They suggested that the conidia spores had considerable lipolytic activity which resulted in degradation of milk fat to give free fatty acids. It was suggested that the free medium chain fatty acids formed a natural preservation system which was absent in the white mould-ripened cheeses. However, neither Papageorgiou and Marth (1989) nor Kinderlerer *et al* (1996) suggested that monoacylglycerols were important in the natural preservation system.

Wang *et al* (1993) demonstrated that monoacylglycerols synthesised from coconut oil could be used to control *Listeria monocytogenes* in certain dairy products or in other foods that contain reduced fat. Their results indicated that monoacylglycerols ($MC_{10:0}$, $MC_{12:0}$ and coconut MAGs) could be used as preservatives in certain classes of minimally processed refrigerated foods, such as model Frankfurter sausage and seafood salad (Wang & Johnson 1997). The minimum inhibitory concentrations (MIC) for lauric acid, monolaurin and coconut monoacylglycerols against bacteria and fungi are summarised in Table 1.2.

Table 1.2 Minimum inhibitory concentrations (μg ml^{-1}) for lauric acid, monolaurin and coconut monoacylglycerols (MAGs) against bacteria and fungi in culture medium [†]

[†] NI (not inhibitory) = MIC > 1000 μg ml^{-1}.

1.2 Monoacylglycerols as food emulsifiers

Emulsifiers or emulsifying agents are chemical components which are capable of promoting emulsification or stabilisation of emulsions or foams by interfacial action. Other terms such as surfactants or surface active agents are also frequently used for these compounds. In general, these compounds possess amphiphilic properties.

The first use of mono- and diacylglycerols as emulsifiers in the margarine industry was in 1921 (cited Sumner 1954). However, it was not until 1929 that the first commercial application of monoacylglycerols as food emulsifiers was filed in the United States (Birnbaum 1955). By 1990, monoacylglycerols accounted for 75 % of the world production of food emulsifiers (Krog 1990). Monoacylglycerols are not toxic. According to 17th and 18th Reports of the Joint FAO/WHO

Expert Committee on Food Additives, there is no limit on the Acceptable Daily Intake (ADI) of mono- and diacylglycerols (WHO Technical Report 1974).

1.3 Production of monoacylglycerols

Monoacylglycerols can be produced by various chemical and enzymatic methods, such as controlled hydrolysis of triacylglycerols, esterification of fatty acids or transesterification of fatty acid esters with glycerol and, glycerolysis of triacylglycerol-rich fats and oils (Figure 1.1).

Figure 1.1 Different routes for production of monoacylglycerols. R_1, R_2 and R_3 are alkyl groups and may be the same or different.

1.3.1 Industrial synthesis

On an industrial scale, monoacylglycerols are manufactured by continuous chemical glycerolysis of fats and oils at high temperature (220 - 250 °C) with potassium or sodium hydroxide as catalysts (Scheme 3 in Fig. 1.1). The reaction is carried under a nitrogen gas atmosphere. There

are some disadvantages of this method, such as low yield, dark colour and burnt taste, etc. (Sonntag 1982).

Recently the glycerolysis of soyabean oil with reactions under a supercritical carbon dioxide (SC-CO_2) atmosphere with increased pressure has been reported (Temelli *et al* 1996). Yields of around 49.2 % were achieved at 250 °C, 20.7 MPa, at a glycerol to oil ratio of 25 and 4 % water after 4 h reaction. There are numerous advantages of undertaking the glycerolysis of fats and oils under a SC-CO_2 atmosphere, such as elimination of the alkali catalyst, production of a lighter colour, less odour and ease of separation of the CO_2 from the reaction products.

Regioisomerically or enantiomerically pure monoacylglycerols can be produced by esterification of protected glycerols with acid chlorides and followed by hydrolysis of the ketal (Scheme 2 in Fig 1.1) (Quinn *et al* 1967, Lok *et al* 1976, Kodali 1987, Eibl & Wooley 1986, Cockman *et al* 1990). Monoacylglycerols synthesised by this method have to be purified.

1.3.2 Enzymatic synthesis

Enzymes have useful properties to solve the problems for synthesis of organic molecules (Figure 1.2).

Figure 1.2 Properties of enzymes used for the organic synthesis. Adapted from Ader *et al* (1992).

Lipase-catalysed synthesis of monoacylglycerols has advantages over chemical synthesis as it requires lower reaction temperatures and mild reaction conditions, gives rise to higher yields and less unwanted materials and has greater selectivity so that the desired end products are produced (Gunstone 1996).

Microbial lipases used for biocatalysis are either native, immobilised or cell-bound. Many researchers have studied lipase-catalysed synthesis of monoacylglycerols using purified or immobilised lipases (Holmberg & Osterberg 1988, Sugihara *et al* 1991, Schuch & Mukherjee 1989, Goh *et al* 1993, Waldinger & Schneider 1996, Yang *et al* 1994, Arcos & Otero 1996). The major difficulty to the use of microbial lipases for the bulk manufacturing purposes is the cost of the enzymes. One of the techniques that allows the enzyme to be used without isolation, purification and immobilisation is as a naturally microbial cell-bound lipase. Legier & Comeau (1992) produced esters by cell-bound lipase from *P. cyclopium* in organic solvent and Long *et al* (1997) modified some vegetable oils by mycelium-bound lipase from *Aspergillus flavus*. However, a literature survey showed that little work had been carried out on production of monoacylglycerols by cell-bound lipases.

Lipases act at the oil/water interface for typical biphasic mixtures and microemulsions. Microemulsions can promote selectivity since association structures created by emulsifiers or solvents modify the properties of the interface thus influencing enzyme activity as well as reaction rates and equilibria (Mazur *et al* 1991). The concentration of substrate molecules at the interface (mol m^{-2}) determines the rate of lipolysis. The overall reaction rate of lipases cannot be described with the Michaelis-Menten model as this model is valid only in the case of one homogenous phase, i.e. for soluble enzymes and substrates (Jaeger *et al* 1994). A new model has been proposed to describe the kinetics of lipases. Because lipases have a peptide lid which blocks the active site, the first step is the physical adsorption of the enzyme at the lipid interface to activate

the enzyme, i.e. to open the lid. This is called the interfacial activation hypothesis. This hypothesis is based on conformational changes. For example, the closed-lid conformer of the lipase from *Rhizomucor miehei* is stabilised by hydrophobic interactions, while the open form is stabilised by hydrogen bonds (Martineller & Hult 1994). The second step is to form an enzyme-substrate complex which can be hydrolysed to give the product and regenerate the adsorbed enzyme. Only the second step may be described by an interfacial Michaelis-Menten model with the substrate concentrations expressed in [mol / surface] instead of [mol / volume] (Martineller & Hult 1994). The action of lipases at oil/water interface is outlined in Figure 1.3 (Verger 1980).

E_s*	active enzyme adsorbed in the interface
E_{is}*	inactive enzyme in the interface
S	substrate in the interface
E_s*S	enzyme-substrate complex in the interface
P	products in the interface
E*	active enzyme in the water phase
E	inactive enzyme in the water phase
S_w	substrate in the water phase
E*S_w	enzyme-substrate complex in water phase
P_w	products in water phase

Figure 1.3 Schematic graph showing action of lipases at oil/water interface. Adapted from Verger (1980).

In addition to mild reaction conditions, higher reaction rates and capacity for regulation, one of the most attractive features for enzyme-catalysed synthesis is that some of the enzymes have a wide range of substrate specificities depending on their source of origin and production conditions

(Iwai & Tsujisaka 1984, Stead 1986, Sonnet 1988). Substrate specificity refers to both positional specificity of acyl groups and the specificity of fatty acid structures. Lipases can vary from rigorously 1,3-specific to completely non-specific via weakly specific (Machida 1984). They are classified into 4 groups according to their different substrate specificity.

The first group of lipases shows no marked specificity with regard to position of the acyl group. These lipases catalyse the reaction randomly to yield 1,2-, 2,3- (α,α-) and 1,3- (α,β-) diacylglycerols. The end products of monoacylglycerols are normally mixtures of 1-, 3- (α-) and 2- (β-) isomers. Lipases from *Penicillium cyclopium* (Okumura *et al* 1976), *Candida cylindercea* (Benzonana & Esposito 1971) and *Psuedomonas fluorescens* (Sugiura *et al* 1977) belong to this group together with plant lipase from maize (Mukherjee & Hills 1994) and oat (Berner *et al* 1970).

The second group of lipases act preferentially on primary esters, i.e., the esters bonds at atoms C1 and C3 of glycerol. This type of lipolysis leads to the production of 2-monoacylglycerols (Bornscheuer 1995; Mazur *et al* 1991). Many lipases belong to this group, such as pancreatic lipase, fungal lipases from *Aspergillus niger* and *Rhizopus delemar* (Okumura *et al* 1976), milk lipases (Brokerhoff & Jensen 1974), bacterial lipases from *Pseudomonas fragi* (Pabai *et al* 1995) and plant lipases from rice and oilseed rape (Mukherjee & Hills 1994). In theory, 1,3-regioselective hydrolysis only yields β-monoacylglycerols. However, experimental data showed that α-monoacylglycerols were produced as well (Holmeberg & Osterberg 1988). It is widely accepted that the α-monoacylglycerols are formed *via* an intramolecular rearrangement, i.e. acyl migration (Brokaw *et al* 1955, McNiell *et al* 1991, Gunstone 1996). Boswinkel *et al* (1996) reported that a 1 to 6.5 mixture of α/β-monoacylglycerols appeared to rearrange to a mixture of 1 to 1 α/β-monoacylglycerols within 24 h in a two-phase reaction in the presence of hexane (20 %, w/w). However, the occurrence of α-monoacylglycerols can also be explained by assuming some degree of non-specific activity of the lipase.

The third group of lipases act preferentially at the sn-2 acyl position. The major products catalysed by lipases with sn-2 specificity were 1,3-diacylglycerols and 1(3)-monoacylglycerols. Although sn-2 catalysed hydrolysis is rare, some authors reported sn-2 specific lipases from microbial and plant origin (Krewson *et al* 1962, Sugihara *et al* 1991). For example, lipase from *Vernonia anthelmintica* catalysed specifically at the sn-2 position (Krewson *et al* 1962). A study using isopropyl esters also showed that the acid lipase cleaved fatty acids from the sn-2 position (Diez *et al* 1985). More recently a novel *Geotrichum candidum* lipase was reported to be specific to the sn-2 position at pH 5.6 (Sugihara *et al* 1991). It cleaved the 2-positioned ester bond about twice as fast as the 1(3)-positioned ester bond of triacylglycerol molecules.

The fourth group exhibits a pronounced fatty acid preference. Lipase B from *Geotrichum candidum* was specific to fatty acids with Δ9 double bonds (Marks *et al* 1968; Jensen 1974). Lipase from oil seed rape showed unusual selectivity against fatty acids containing cis-6 double bonds (Mukherjee & Hills 1994).

1.4 *Penicillium roquefortii* and lipases from *Penicillium* spp. for production of fatty acids and flavour compounds

1.4.1 *Penicillium roquefortii*

Penicillium roquefortii is added as a starter culture in blue mould-ripened cheeses such as Stilton and Danish blue and allowed to grow and sporulate in the veins. Lipases are produced by growth of this organism. These lipases hydrolyse the triacylglycerols to give free fatty acids which are then converted to methyl ketones. The production of free fatty acids, methyl ketones and secondary alcohols during ripening of blue cheeses results in the characteristic flavour of these cheeses (Kinsella & Hwang 1976).

Boysen *et al* (1996) demonstrated that *Penicillium roquefortii* could be divided into two varieties, one for cheese starter cultures, *P. roqueforti* var. *roqueforti*, and another ubiquitous patulin-producing variety, *P. roqueforti* var. *carneum*. Their investigation of the relationship between two previously described *Penicillium* varieties of the same *Penicillium* species involved both secondary metabolite profiling and DNA analysis. Based on these results they reclassified *P. roqueforti* into three species named *P. roqueforti*, *P. carneum* and *P. paneum*. *Penicillium roqueforti* isolates all produce Penicillium Roqueforti (PR) toxin, marcfortines and fumigaclavine A, while the *P. carneum* isolates produce patulin, penitrem A and mycophenolic acid and unidentified metabolites. *P. paneum* produces secondary metabolites in 5 chromophore families including the known mucotoxins patulin and botryodiploidin.

1.4.2 Lipases from *Penicillium* spp.

Work has been undertaken to investigate the lipase activity with respect to the production of free fatty acids as these acids are precursors for the formation of methyl ketones. The lipolytic activity of different strains of *Penicillium* spp. varies markedly (Lamberet & Lenoir 1972, Stepaniak & Habaj 1974, Farahat *et al* 1990). Lamberet and Lenoir divided 89 strains of *P. caseicolum* into 2 groups, one with high and one with low lipolytic activity, whilst Stepaniak & Habaj (1974) classified fungal strains into four categories with relative lipase activities of 100:63:44:14.

Production and properties of *Penicillium* lipases have been studied by some research groups (Oi *et al* 1967, Eitenmiller *et al* 1970, Iwai *et al* 1975, Lamberet & Menassa 1983, Lobyreva & Marchenkova 1983, Petrovic *et al* 1990). Two types of *P. crustosum* lipases were purified by Oi *et al* (1967). Both lipases had optimum pHs between 6 and 9 for production of free fatty acids. The specificity of lipase I was olive oil > tributyrin > methyl laurate > ethyl laurate > triacetin

>others tested. The specificity of lipase II was tributyrin > triacetin > olive oil > ethyl laurate > methyl laurate > others tested. Ca^{2+} and Mg^{2+} increased the activity of these enzymes.

In a study of lipases from a *P. roquefortii* strain isolated from a commercial blue cheese sample, Eitenmiller *et al* (1970) found that maximum lipolytic activity occurred with 5 % butter oil emulsion as substrate. The lipase was active at pHs between 7.5 and 9.0, and at temperatures between 25 °C and 45 °C. They suggested that pH and temperature optimum ranges may be affected by using different strains of *P. roquefortii*, substrates and assay conditions. Iwai *et al* (1975) purified two lipases from *P. cyclopium*. Lipase A showed optimum activity at 30 °C and pH 7 (active range 6.5 - 9.0), while lipase B was highly active at 30 °C and pH 6.0 (active range 4.0 - 6.5). Both lipases hydrolysed triacylglycerols composed of shorter chain fatty acids more rapidly than those composed of longer ones. The B-lipase showed specificity towards oleic acid ester. Menassa & Lamberet (1982) compared the properties of 2 extracellular lipases produced by the same strain of *P. roquefortii* under different culture conditions. They found one lipase with an optimum pH 6.0 and the other with an alkaline optimum pH. The acid lipase was further studied and the optimum activity was at pH 6.5 and 20 °C and 6.0 at 30 °C (Lamberet & Menassa 1983). The activity at pH 6.5 and 5 °C represented 37 % of the maximum value at pH 6.5 and 35 - 40 °C.

Russian researchers Lobyreva & Marchenkova (1983) studied the effect of different factors (amount of inoculum, aeration of the medium and duration of fungal cultivation) associated with the cultivation of *P. roquefortii* on the production of lipase. The optimum conditions of their lipase in terms of fatty acids production were at 43 °C and with pH between 5 - 8. Petrovic *et al* (1990) investigated the effects of various carbon sources on the production of extracellular lipases from one strain of *P. roquefortii* which had been isolated from a commercial sample of Roquefort cheese. They found the greatest lipase activity (10 U/ml) was obtained on the media of glucose with initial pH at 4.0.

Section II Extraction and Analysis of Acylglycerols and Fatty Acids

1.5 Extraction of acylglycerols with solvent mixtures

Acylglycerols are neutral and simple lipids. They exist as mono-, di- and triacylglycerols and are normally extracted with solvent mixtures. In order to extract acylglycerols from tissue, it is necessary to find solvents which will not only dissolve the acylglycerols readily but will overcome the interactions between acylglycerols and other compounds in the reaction mixtures (Christie 1989).

Dichloromethane (CH_2Cl_2, CAS number 75-09-2) may be used to extract methyl ketones and secondary alcohols which are the metabolites of medium chain acylglycerols and fatty acids (Kinderlerer 1987). This solvent has a low boiling point (41 °C), thus it is easily removed after extraction.

Most workers use the mixture of chloroform (CAS number 67-66-3) and methanol (CAS number 67-56-1) (2:1 v/v) with the endogenous water in tissues to extract lipids from these tissues and followed by Folch or modified Folch wash procedures (Folch *et al* 1957, Ways & Hanahan 1964). With this method, total lipids including acylglycerols, phospholipids and gangliosides can be extracted. However, there are many disadvantages for this method (Radin 1981). Methanol is toxic and can produce headaches if the room is inadequately ventilated. Chloroform is carcinogenic and storage of chloroform can lead to formation of phosgene and HCl. Some undesirable nonlipids such as sugars and amino acids can be dissolved by chloroform-methanol.

A mixture of hexane (n-hexane, CAS number 110543) and 2-propanol (isopropanol, isopropyl alcohol CAS number 67630) has most of the extraction properties of chloroform - methanol but

that it does not extract gangliosides quantitatively (Radin 1981). This solvent mixture has many other advantages over chloroform-methanol. The mixture is composed of a low-polarity and high-polarity solvent, the water miscible solvent can penetrate cell membranes and dissolve a wide range of lipids that differ considerably in their solubilisation. 2-Propanol is considered nontoxic (Radin 1981). With plant tissues, it is very important to extract first with a 100 fold excess (by weight) of 2-propanol in order to inactivate lipase activity (Nichols 1964, Christie 1989). The solvent mixture has a lower vapour pressure than chloroform-methanol, therefore, solutions of lipids are less prone to evaporation losses during storage or handling. Furthermore, it extracts almost no protein and little nonlipid material from tissues, it has a lower density thus permitting centrifugation as an alternative to filtration, and it absorbs far-ultraviolet light only slightly and will allow optical monitoring of column effluents (Radin 1981). The comparison of the two extraction methods is given in Table 1.3.

Table 1.3 Comparison of two different methods of solvent extraction for lipids †

† Data from Radin (1981).

It is possible to extract oils with one solvent. For example, hexane is commonly used to extract oil from oilseeds which is then removed by distillation (Hamilton 1993). However, the 1990 Amendments to the Clean Air Act listed hexane as a hazardous air pollutant in the USA, which has led to the search of alternative solvents.

The use of 2-propanol was proposed because it is considered non toxic. Additional information for evaluating 2-propanol as an alternative to hexane for the extraction of cottonseed, soybean and peanut oils have been summarised by Lusas (1997). The author suggested using 2-propanol for the extraction and then recovering the solvent. 2-Propanol has higher flash points and is heavier (6.83 vs. 5.60 lb./gal) than hexane, therefore, the major disadvantage of 2-propanol is the apparent greater energy requirements for removing solvent. However, extracts of crude oils are equal to or better than those obtained with hexane extraction (Lusas 1997).

Comparing 2-propanol to other alcohols, such as ethanol, oil is more soluble in 2-propanol than in ethanol (Johnson & Lusas 1983). The azeotrope of 2-propanol contains 12.3 % (w/w) water whilst the ethanol azeotrope contains 4 % water. This makes the performance of 2-propanol less sensitive to absorption of water from tissues being extracted. According to Lusas (1997), 2-propanol will not replace hexane but can be used to extract oils from some oilseeds.

1.6 Analysis of fatty acids and monoacylglycerols

Fatty acids and acylglycerols have been separated and analysed by various Chromatographic techniques, such as Thin Layer Chromatography (TLC), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC), etc. (Chaytor 1987, Hamilton *et al* 1987, Christie 1989, Shantha & Napolitano 1992, Gunstone *et al* 1994). Thin Layer Chromatography is a simple and inexpensive method to identify qualitatively lipid classes in a sample. Complete separation of mono-, di- and triacylglycerols and fatty acids can be achieved easily (Liu 1997). Thin layer chromatography has flexibility. Analytical TLC can be used to analyse qualitatively fatty acids and acylglycerols and to screen for different acylglycerols. Preparative TLC can be used to separate relatively large amount of acylglycerols (Christie 1996a, Liu 1997). Furthermore, silver ion TLC and silver ion HPLC with adsorbent of silica gel impregnated with sliver nitrate solutions is useful for separation of positional isomers of fatty acids (Christie

1996b). Polymorphic behaviour of diacylglycerols has been studied at Hamilton's group by IR spectra, Raman Spectroscopy and HPLC (Shannon *et al* 1992). These researchers were able to separate a low concentration (0.5 %) mixture of monoacylglycerols, 1,2- and 1,3- diacylglycerols and triacylglycerols by HPLC.

Among the different analytical methods, Gas Chromatography (GC) and Mass Spectrometry (MS) are the commonly used ones for analysis of fatty acids and acylglycerols because GC is sensitive and quantitative and MS is easily coupled with GC to identify unknown peaks by their fragmentation patterns.

1.6.1 Fatty acids

Fatty acids are classified into different groups, saturated fatty acids with even and odd carbon numbers, monounsaturated fatty acids, polyunsaturated fatty acids, branched chain fatty acids, cyclic acids and oxygenated fatty acids. Gas Chromatography is usually the common choice for analysis of fatty acids quantitatively (Christie 1989). The analysis is carried out with packed or capillary column, coupled to a flame ionisation detector (FID) (Jensen *et al* 1962, Timmen & Patton 1988, Shantha 1992).

Analysis of fatty acids normally involves two steps: the derivatisation (esterification or transesterification) of fatty acids and acylglycerols into fatty acid methyl esters (FAMEs) and the analysis of the FAMEs by GC. The goal of the derivatisation reactions is to reduce the polarity of a molecule by chemically derivatising hydroxyl groups, thereby increasing volatility and promoting thermal stability. Esterification and transesterification can be catalysed by either acid or base. The common reagents for acid-catalysed derivatisation are methanol containing 5 % hydrogen chloride, 1-2% sulphuric acid or 15% boron trifluoride (Christie 1996b). Different methods of derivatisation of fatty acids and acylglycerols into FAMEs have been used (Hitchcock & Hammond 1980, Badings & Jong 1983, Christie 1989). Some other derivatives may be

favoured for specific purposes: butyl esters for short-chain fatty acids (Iverson & Sheppard 1977, 1986, Jensen 1992, Ackman & Macpherson 1994), phenacyl esters for HPLC separation with UV detection (Christie 1996b) and picolinyl ester and related derivatives for enhancement of molecular ions in Mass Spectrometric identification (Anderegg 1988). The choice of derivatisation method is determined by the nature of the lipids and the sample preparation procedures directly affect the quantitative results in GC analysis (Al Makdessi *et al* 1985).

Although packed columns have been used for analysis of fatty acids in food products for a long time and are still acceptable for preliminary analysis or screening procedures, the greater efficiency of capillary columns offers excellent resolution capabilities for various sample types and small sample sizes. However, capillary columns need more careful laboratory practices, a higher detector response for sharp and rapidly eluting peaks and a more sensitive detector for small sample sizes (Shantha 1992). Several factors are normally considered for a good practical separation such as stationary phase, film thickness of stationary phase, column length and inner diameter, injection techniques and temperatures of oven, injector and detector, etc.. The stationary phase of the columns is the principle factor determining the nature of the separation that can be achieved as it interacts with injected samples.

Fatty acids can be separated on non-polar silicone liquid phases as well as polar polyester liquid phases. Non-polar phases, such as SE-30™, OV-1™, JXR™ or QF-1™, permit separation of fatty acid esters mainly on the basis of their molecular weights. Polar phases are much more suited to fatty acid analysis as they allow complete separation of esters of the same chain length but with different degrees of unsaturation (Christie 1989). Many polar columns are commercially available ranging from the highest polarity phases (SP 2340™, OV-275™) to low polarity phases (Carbowax 20M™) via medium polarity phases (FFAP, PEGA, BDS). Eddib *et al* (1986) designed a simple method for preparation of capillary columns coated with the highly polar cyanosilicone phases. Such columns are capable of resolving complex isomeric mixtures of

mono-unsaturated fatty acid methyl esters. The film thickness of the stationary phase is a primary factor in determining the retention of the sample components. A thick film thickness normally results in longer retention times and better peak resolution and is, therefore, recommended for volatile solutes such as short chain (C_2-C_8) fatty acids.

Resolution is a function of the square root of the column length. Therefore, longer columns have to be used in order to improve the resolution significantly if other chromatographic conditions remain unchanged. For a complex mixtures such as butter oil, relatively long columns (30 - 50 m) have to be used to obtain a complete separation (Badings & De Jong 1983). Column internal diameter also determines the column efficiency, retention time and column capacity. The smaller diameter would result in better peak resolution, however, would probably cause an overloading problem. In general, the use of columns of 0.25 mm i.d. is recommended (Shantha 1992).

Mass Spectrometry (MS) is widely used for the identification of individual fatty acid (Minnikin 1978, Jensen & Gross 1987, Christie 1989). Identification of fatty acid methyl esters by MS is mainly based on (i) the presence of an ion at m/z 74 ($[CH_2COOCH_3]^+$), the 'McLafferty ion', which is characteristic for a methyl ester, (ii) loss of an ion at m/z 32 (methanol) confirming that a methyl group is existing, and (iii) the molecular ions which are relatively abundant. Although there is no information on double bonds position, the retention times compared with standard samples and library search data can solve this problem (Christie 1996b).

1.6.2 Monoacylglycerols

Monoacylglycerols can be separated by GC or HPLC (Itabashi & Takagi 1986). These compounds are normally separated by use of short columns at relatively high temperature. Gas Chromatography has been used extensively for the separation of mono- and diacylglycerols in forms of the acetate (Khaled *et al* 1993), trimethylsilyl (TMS) (Wood *et al* 1965, Satouchi *et al* 1978, Evershed 1993), trialkysilyl other than TMS (Poole & Zlatkis 1979) or t-butyldimethylsilyl

(TBDMS) (Myher *et al* 1978, Evershed 1993) ether derivatives. TMS ether derivatives of mono- and diacylglycerols give much better spectra in GC-MS identification (Christie 1989). These derivatives are prepared easily and are volatile which allows them to be analysed by GC and GC-MS. The free hydroxyl groups existing in the monoacylglycerols can be converted to the TMS ethers by reaction with bis(trimethylsilyl)-acetamide (BSA) or N,N,-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) reagents at ambient temperature prior to analysis by GC with a non-polar capillary column (Christie 1989, Evershed 1993). Other researchers have used nicotinoyl derivatives for derivatisation of mono- and diacylglycerols with methyl branches and epoxy and cyclopropyl rings for GC-MS analysis (Zöllner & Schmid 1996).

Mono- and diacylglycerol TMS ethers can be separated on packed and capillary columns (Christie 1989). Wood *et al* (1965) were able to separate 1(3)- and 2-monoacylglycerols as TMS ether derivatives by GC. The 2-isomers eluted first within the same molecular weight and chain-length of monoacylglycerol TMS ethers. Non-polar and polar stationary phases can be used to obtain good separation, such as SE-30™ with 0.5 - 1.0 m packed column (Christie 1989) and SP-2330™ (Myher & Kuksis 1982) with relatively short capillary columns. Separation on non-polar columns depends largely on the molecular weight when monoacylglycerol TMS derivatives have the same chain-length. The elution temperature is generally higher than the analysis of FAMEs, therefore, oven temperature is normally needed to be programmed up to 250 or 300 °C. The column life for analysis of monoacylglycerol TMS ethers was found to be short, especially for polar columns (about 100 analyses) (Christie 1989). This is probably because relatively high oven temperatures are used.

It is normally advisable to use the response of flame ionisation detector for quantification of the main molecular species of monoacylglycerols TMS ethers, and to use GC-MS for identification of isomers within a single peak (Christie 1989). GC with high resolution capillary columns has great advantages for analysis of monoacylglycerols TMS derivatives, particularly coupled to the

relatively low cost quadrupole and ion trap MS system (Evershed 1989). Identification of monoacylglycerols TMS ether derivatives is mainly based on the ions at $(M-15)^+$ and $(M-90)^+$ which give the information of molecular weight of analysed component and the ions at m/z 73, 75, 129, 147, RCO^+ and $(RCO+74)^+$ which exist in every monoacylglycerol TMS ether. Ions at $(M-103)^+$ and m/z 205 appears to be absolutely characteristic for the 1(3)-isomers, while an ion at m/z 218 is highly favoured in the spectra of 2-isomers (Christie 1989). The ion at $(M-103)^+$ is formed by cleavage between carbons 2 and 3 or 1 and 2.

There is an opportunity to separate enantiomers of monoacylglycerols (1 and 3-isomers) by Chiral Chromatography. To effect the chromatographic resolution of enantiomers, it is necessary for the enantiomers to be derivatised with chiral moieties or to be retarded by the stationary phase of the GC or HPLC column. Enantiomeric compounds could be separated in a non-chiral environment after derivatisation with chiral reagent into diastereomers (Christie 1996c).

Christie (1996c) suggested that chiral diacylglycerol urethane derivatives could be prepared by reaction of the enantiomeric diacylglycerols with a single enantiomer of naphthylethyl isocyanate and then were separated by HPLC on silica gel columns. Alternatively, the diacylglycerols could be separated on HPLC columns containing stationary phase with chemically bonded chiral moieties. The author suggested that the choice of approach would depend on practical factors, including cost and availability or reagents and chemicals. Furthermore, Heidt *et al* (1996) demonstrated that R- and S- enantiomers of monoacylglycerols could be separated by GC with a chiral permethylated β-cyclodextrin phase.

1.6.3 Quantification of results

The quantification of fatty acids and monoacylglycerols can be achieved by adding internal or external standards which are not present in the original samples. The methods usually involve the establishment of a standard curve or simply an equality assumption between GC area percent and weight percent. The assumption of equality is defensible when the fatty acids or mono-acylglycerols to be analysed are similar. However, it is not acceptable with more complex samples like butter oil where there is a large range in the chain-length of the constituent fatty acids. If this is the case, the analysis of the short and long chain-length fatty acid esters will not be quantified (Kaufman & Polymeropoulos 1988, Liu & Xin 1989). Correction factors (response factors, F_r) have to be introduced (Scanlon & Willis 1985, Jacobsen *et al* 1994). The response factors are normally obtained from experiment (Koletzko *et al* 1988). A mixture of all major compounds of interest is analysed with internal standard. The response factors of the compounds of interest are determined relative to the internal standard. A recent report of Jacobsen *et al* (1994) suggested that statistical fitting analysis based on only a few experimental determinations enabled more accurate estimation of response factors for saturated fatty acids in butter oil.

Section III Aims

1.7 Aims of this study

Penicillium roquefortii and lipases from *Penicillium roquefortii* have been studied extensively with respect to their activity for release of free fatty acids and subsequent metabolites of methyl ketones (Lawrence 1966, Eitenmiller *et al* 1970, Kinsella & Hwang 1976, Hatton & Kinderlerer 1991). However, little work has been undertaken to evaluate their ability to produce the intermediate metabolites, monoacylglycerols. Although hydrolysis of triacylglycerols to monoacylglycerols by lipases of microbial origin has been investigated by several groups

(Mukherjee & Kiewitt 1984, 1986, Holmberg & Osterberg 1988, Mazur *et al* 1991), no literature was found on the study by cell-bound or commercial lipases from *P. roquefortii*. Furthermore, relatively little investigation has been undertaken on formation of monoacylglycerols from natural oils.

The aims of this study were to synthesise monoacylglycerols from triacylglycerols in butter oil and Shea oil (a cocoa butter substitute) by *P. roquefortii* and a commercial lipase from *P. roquefortii*, to set up methods for analysis of fatty acids and monoacylglycerols and to compare structures and composition of monoacylglycerols produced in different systems with the idea of using monoacylglycerols as natural antimicrobials in foods and dairy products.

Butter oil was selected because it is a milk fat (a cheese model) and also because of its heterogeneous properties with relatively large amount of medium chain fatty acids (MCFA, $C_{6:0}$ - $_{12:0}$ ~ 10 wt %) as well as long chain fatty acids (LCFA, $C_{16:0+}$ ~ 80 wt %). Shea oil contains large percentages of dipalmitoyl-oleoyl glycerol (POP), palmitoyl-oleoyl-stearoyl glycerol (POS) and distearoyl-oleoyl glycerol (SOS) (total POP, POS and SOS are 82.5 wt %). Shea oil was selected to compare with butter oil due to its simplicity in terms of fatty acids composition.

CHAPTER 2

MATERIALS AND METHODS

Section I Conversion of Triacylglycerols

2.1 Materials

2.1.1 Oils

Butter oil. Shea (stearin fraction) and palm oil (mid-fraction) were obtained as a gift from Unilever research (Colworth House, Bedford, UK). Samples of butter oil were obtained in January, 1993, February, 1995 and May, 1996. Shea oil and palm oil were obtained in April, 1995. Olive oil was purchased from Boots, Sheffield in July, 1996, and was BP grade. The oils were stored at 4 °C in sealed containers.

2.1.2 Chemicals

Standard fatty acids, fatty acid methyl esters, monoacylglyerols, N,O-bis(trimethylsilyl) acetamide (BSA) as pre-prepared reagent in sealed ampoules (5 ml), casein and casein enzymatic hydrolysate were obtained from Sigma Chemicals (Poole, UK), and were Sigma Grade. Casamino acids were obtained from DIFCO Laboratories (Detroit Michigan, USA). Agar (Bacteriological No. 1, L11), Malt Extract (L39), Yeast Extract (L21) and Peptone (L37) were obtained from Oxoid (Basinstoke, UK). *Penicillium roquefortii* lipase (E.C.3.1.1.3) was obtained from Fluka Chemicals (Gillingham, Dorset, England). Methanol, toluene and acetone were obtained from FSA Laboratory Supplies (Loughborough, UK), and were HPLC Grade. Dichloromethane, hexane, 2-propanol and diethyl ether were obtained from Romil Chemicals (Shepshed, Loughborough Leics., UK), and were double glass distilled. Sulphuric acid, potassium bicarbonate and sodium chloride were obtained from FSA Laboratory Supplies (UK)

Other chemical reagents were obtained from BDH Ltd (Merck House, Dorset, UK). All chemicals were Analar Grade.

2.1.3 Fungal strains

Penicillium roquefortii FRR 2456 was obtained as a gift from Dr. Ailsa D. Hocking, CSIRO, Australia in 1995. It had been isolated from a melon which had undergone fungal spoilage. *Penicillium roquefortii* Wisbey PJ (freeze dried spores) was obtained as a gift from Enolacto Limited (London, NW3 5AT) in 1991 and was a commercial dairy strain.

2.2 Maintenance and identification of fungal strains

2.2.1 Standard media

Two stock solutions were made. Solution A contained 30 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.05 g $CuSO_4 \cdot 5H_2O$ in 100 ml glass distilled water. Solution B contained 10 g K_2HPO_4 in 100 ml glass distilled water.

(a) Czapek Yeast Agar (CYA)

Czapek Yeast Agar contained 10 ml solution A, 10 ml solution B, 30 g sucrose, 3 g Yeast Extract (Oxoid, UK) and 15 g Agar (Bacteriological No. 1, Oxoid L11) in 1 litre glass distilled water.

(b) Malt Extract Agar (MEA)

Malt Extract Agar contained 20 g Malt Extract, 1.0 g peptone, 20 g glucose and 20 g Agar (Bacteriological No. 1, Oxoid L11) in 1 litre glass distilled water.

(c) 25% Glycerol Nitrate (G25N)

G25N contained 7.5 ml solution A, 3.7 g Yeast Extract, 250 g glycerol, 0.75 g KH_2PO_4 and 12 g Agar (Bacteriological No. 1, Oxoid L11) in 0.75 litre glass distilled water.

The ingredients in each medium were dissolved by heating to boiling and dispensed in Duran bottles (250 ml). The medium was sterilised by autoclaving at 121 °C for 15 min (Autoclave 280 EH, Harvard/LTE Ltd, Oldham, Lancs., UK). Medium (~ 20 ml) was poured into plastic Petri dishes (90 mm id.).

2.2.2 Stock cultures

Stock cultures were grown on Czapek Yeast Extract Agar (CYA) as described by Pitt and Hocking (1985). Slopes (10 ml) were prepared in Universal bottles (28 ml). Each slope was inoculated using a spore suspension prepared from a stock culture. A small amount of spores was removed with a sterile loop and added to 0.5 ml of sterile 0.002% Tween in a Bijoux bottle. Each slope was inoculated in the centre of the agar with a sterile needle which had been dipped into the spore suspension and the slopes were incubated for 7 days at 25 °C. Cultures showing heavy spore production were selected and stored at 4 °C for not more than 2 months.

2.2.3 Identification of *P. roquefortii* by growth rate

The method from Pitt (1988) was used to measure the growth rate of *P. roquefortii* FRR 2456 and Wisbey PJ. Cultures were grown at 25 °C for 7 days on three standard media (CYA, MEA and G25N) and also on CYA at 5 °C and 37 °C for 7 days.

Spore suspensions were prepared from stock cultures as described in section 2.2.2 and used to inoculate media in the Petri dishes. Petri dishes incubated at 25 °C were inoculated at three points. Cultures incubated at 5 °C and 37 °C were inoculated at two points per culture. The inoculation scheme on the three media is shown in Figure 2.1. Ten cultures of both strains of *P. roquefortii* were set up in 10 Petri dishes and the growth rates determined.

After 7 days growth, the diameters of the colonies were measured in millimetres from the reverse side of the Petri dish in two directions each at right angles to the other. For cultures grown at 5

°C and 37 °C, the micro-colonies were observed with a low power microscope (100×) (Olympus, Finlay House, Warwickshire, UK).

Figure 2.1 Inoculation scheme for growth of *Penicillium roquefortii* on three standard media, CYA, MEA and G25N (Data from Pitt 1988, redrawn).

Table 2.1 summarises the identification of *P. roquefortii* FRR 2456 and Wisbey PJ on standard medium, CYA, MEA and G25N. The growth rates were within the range quoted for *P. roquefortii* (Pitt 1988). Both strains grew on the three standard media at 25 °C and did not grow on CYA at 37 °C. Growth of *P. roquefortii* FRR 2456 on CYA at 5 °C could be observed under low power microscope (100×) but no growth of *P. roquefortii* Wisbey PJ was found on CYA at this temperature.

Good growth on CYA was observed for both strains of *P. roquefortii* at 25 °C, whilst the two strains grew poorly on MEA with low production of green conidia spores. Good sporulation was found when fungus was grown on G25N, however, less growth took place on G25N than on CYA. This is in agreement with the xenotolerance of *P. roquefortii* (Pitt 1988).

Table 2.1 Growth rates of *P. roquefortii* FRR 2456 and Wisbey PJ on three standard media †

T (°C)	Strain	Medium	Colony diam. (mm)	Mycelium (mm)	Ratio‡	Colony colour	Range of colony diam. (mm) (Pitt 1988)
25.0±0.3	FRR 2456	CYA	47 ± 1	7 ± 1	6.7	green	40 - 70
		MEA	43 ± 2	7 ± 2	6.1	beige	
		G25N	22 ± 1	13 ± 1	1.7	green	20 - 22
	Wisbey PJ	CYA	41 ± 2	6 ± 1	6.8	green	40 - 70
		MEA	40 ± 2	6 ± 1	6.7	beige	
		G25N	22 ± 2	12 ± 2	1.8	green	20 - 22
5.0 ± 0.2*	FRR 2456	CYA	4 ± 1	ND	-	white	2 - 5
	Wisbey PJ	CYA	ND	ND	-	no growth	
37.0±0.5*	FRR 2456	CYA	ND	ND	-	no growth	no growth
	Wisbey PJ	CYA	ND	ND	-	no growth	

† Results were the mean of 15 fungal colonies and 30 measurements ± standard deviation. ND: no colony detected.
‡ Ratio of colony diameter / mycelium.
* Colony morphology and growth rates were measured with low power microscope (100×).

2.3 Conversion of butter and Shea oils by *P. roquefortii* in solid-state cultures

2.3.1 Modified Czapek medium

Modified Czapek medium was based on Czapek Yeast Agar where 30 g/L sucrose was replaced with 40 g/L oil and 10 g/L organic nitrogen source.

(a) pH 4.5

Modified Czapek medium at pH 4.5 contained 10 ml solution A; 10 ml solution B; 10 g casein enzymatic hydrolysate, casein or casamino acids; 40 g butter or Shea oil and 20 g Agar (Bacteriological No. 1, Oxoid L11) in 1 litre glass distilled water.

(b) pH 7.0

Phosphate buffer (0.2 M, pH 7.0) was made up by combining 39.0 ml 0.2 M KH_2PO_4 and 61.0 ml 0.2 M K_2HPO_4 in 100 ml volumetric flask. The pH values were measured with Unicam 9460 Ion-Selective Meter (Unicam Ltd, Cambridge, UK). The electrode (Pye Unicam CE2) diameter

was 4 mm. Modified Czapek medium (pH 7.0) contained 10 ml solution A, 10 g casein enzymatic hydralysate, 40 g butter or Shea oil, 250 ml phosphate buffer (0.2 M, pH 7.0) to give a final concentration of buffer at 0.05 M and 20 g Agar (Bacteriological No. 1, Oxoid L11) in 1 litre distilled water.

Major ingredients in each medium (without oil) were dissolved by heating to boiling and 100 ml dispensed in each of 10 Duran bottles (100 ml). The medium and oils were sterilised separately by autoclaving at 121 °C for 15 min. Butter or Shea oil (4 g) was weighed aseptically into the medium in Duran bottles. The mixture was emulsified by shaking for 30 seconds. The final medium with oil (~ 20 ml) was poured immediately into sterile plastic Petri dishes (90 mm id.).

2.3.2 Spore suspensions for use in solid-state culture

Sterile Tween 80 solution (0.002%, w/v) (5 ml) with 5 glass balls (3.5 - 4.5 mm diam., BDH Ltd, UK) was added to the slope. The slope was agitated with a Rotamixer (Hook & Tucker Ltd) at full speed for 2 minutes and the suspension was poured back into the sterile Universal bottle (28 ml). Spores concentration was determined microscopically with a haemocytometer (improved Neubauer-type, Gallenkamp Ltd, Stockton-on-Tees) (Collins *et al* 1995) and the concentration was calculated as number of spores per ml suspension.

2.3.3 Effect of carbon and nitrogen sources on conversion of triacylglycerols

Penicillium roquefortii strains were grown on modified Czapek media at pH 4.5. Spore suspensions prepared as described in 2.3.2 were used to inoculate the medium. Petri dishes containing modified Czapek medium were inoculated with a 10 μl loop by drawing across the centre of the dish from left to right. The Petri dishes were incubated in an incubator (Vindon Scientific Ltd, Oldman, UK) at 25 °C for 7 days. The widths of the zone of spores and the zone of white mycelium were measured.

2.3.4 Conversion of butter and Shea oils on modified Czapek medium

Conversion of butter or Shea oil was carried out on modified Czapek medium with casein enzymatic hydrolysate (1% w/v). Inoculation technique was the same as described in 2.3.2. For the cultures with initial pH ~ 4.5, both strains of *P. roquefortii* were grown in five Petri dishes and the cultures were incubated in an incubator (Vindon Scientific Ltd, Oldman, UK) at 25 °C for 7 days. For cultures at pH 7.0, *P. roquefortii* was grown in 40 Petri dishes and the cultures were placed in incubators (Fisher Scientific, Loughborough, UK) at 10 °C for 20 days as well as at 25 °C for 7 days. The widths of the zone of green spores and white mycelium were measured in millimetres. Temperatures were recorded every 15 minutes with a Psion Organiser Digitron type S10 (Digitron Instrumentation, Hertford, Herts, UK) which was connected with Type K Thermocouples (RS Components Ltd, Northants, UK). Two thermocouples were placed in the incubator where the cultures were, one above and the other below the Petri dishes.

Growth of *P. roquefortii* FRR 2456 and Wisbey PJ on butter and Shea oils in solid-state culture is shown in Figure 2.2. Figure 2.3 shows growth rate of *P. roquefortii* on modified Czapek medium with butter and Shea oil as the carbon source and Casein Enzymatic Hydrolysate as the nitrogen source. In general, growth of *P. roquefortii* on butter oil was better than on Shea oil.

Figure 2.2 Growth of *P. roquefortii* (a), (c) FRR 2456 and (b), (d) Wisbey PJ on butter and Shea oils on modified Czapek medium in solid-state culture.

Figure 2.3 Growth of *P. roquefortii* on modified Czapek medium with (a) butter oil and (b) Shea oil (4 % w/v) as the main carbon source and Casein Enzymatic Hydrolysate (1% w/v) as the nitrogen source. Results were the mean of 20 measurements with error bars.
pHs refer to initial pH.

2.4 Conversion of butter and Shea oils by *P. roquefortii* in suspension culture

2.4.1 Spore suspensions for use in suspension culture

Modified Czapek medium (pH 7.0) with casein enzymatic hydrolysate (1%, w/v) (described in 2.3.1) was used to grow cultures for spore production. Butter or Shea oil was autoclaved separately at 121 °C for 15 min and was transferred into the medium aseptically with a sterile pipette. The medium was homogenised with a sterile head of a Polytron (CH-6014 Lucerne, Switzerland) at full speed for 2 minutes. Medium (35 ml) was poured aseptically into sterile Duran bottles (100 ml) to make slopes. Spore suspensions (~ 10 μl) prepared as described in 2.3.2 were inoculated onto the surface of the agar with an inoculation loop. The slopes were incubated at 10 °C for 21 days or 25 °C for 7 days.

Four slopes showing heavy spore production were selected. Sterile phosphate buffer (20 ml, pH 7.0, 0.05 M) containing 10 glass balls (3.5 - 4.5 mm diam., BDH) were added aseptically into each slope. The slope was agitated with Rotamixer at full speed for 2 minutes so that the liquid covered the surface of the slope. The suspension was filtered through two layers of buffered muslin in a sterile glass funnel into a sterile Erlenmeyer flask (250 ml). Filtrates containing spores (80 ml) were combined and filtered through a 0.45 μm cellulose nitrate overlaid by 0.8 μm cellulose nitrate in a Nalgene Unit (250 ml) (New York, USA) coupled to a vacuum pump (7 psi) (Millipore, Bedford, MA, USA). The membrane with the spores was carefully removed from the filtration unit and was re-suspended in 20 ml sterile phosphate buffer (pH 7.0, 0.05 M). The spore concentration was determined as described in 2.3.2, and the suspensions were stored at 4 °C for not more than 24 hours prior to use.

2.4.2 Preparation of oil emulsions

Gum arabic solution was filtered through 0.22 μm cellulose nitrate overlaid by 0.45 μm cellulose nitrate in a Nalgene unit (500 ml). Oils were autoclaved at 121 °C for 15 minutes and then weighed into the gum arabic solution. The Polytron head and all glass containers were autoclaved at 121 °C for 20 min.

Oils were warmed at 40 °C water bath for 1 hour. Emulsions were prepared by homogenising 30 gram autoclaved oils with 90 ml 2% gum arabic (membrane filtered). The mixture was emulsified with a sterile Polytron head at speed 2 for 2 minutes. The emulsion was then sonicated for 5 minutes in a Ultrasonicator (Bransonic 52, USA) at maximum current at ambient temperature.

2.4.3 Conversion of butter and Shea oils

Reaction mixtures were composed of butter or Shea oil emulsion (10 ml), phosphate buffer (pH 7.0, 0.1 M, 8 ml), $CaCl_2$ solution (0.01M, 2 ml) and spore suspension (100 - 300 μl). Spore suspensions were prepared as described in 2.4.1. Phosphate buffer (pH 7.0, 0.1 M) for conversion by *P. roquefortii* was filtered through 0.22 μm cellulose nitrate overlaid by 0.45 μm in a Nalgene unit (500 ml). Two types of controls were used, firstly, the mixtures without spores, and secondly, the mixtures with autoclaved spore suspensions (100 - 300 μl). Each sample was repeated in quadruplet.

Butter and Shea oils were degraded by *P. roquefortii* (FRR 2456 and Wisbey PJ) at pH 7.0, 10 °C and 25 °C. The 25 °C temperature was obtained in a heated shaking water bath (Tecator, Perstorp Analytical Ltd, Berkshire, England). The 10 °C temperature was obtained by use of the shaking water bath as well as a refrigerated cooler (Grant Instruments Ltd, Cambridge, UK). Reaction was carried out in Erlenmeyer flasks (250 ml) in the water bath. The shaking water bath was set at 72 strokes per mimute and the stroke length was 23.5 mm.

The reactions were started by adding the spore suspensions after equilibrating the mixtures at 10 °C or 25 °C for 30 min. Reactions were carried out at 10 °C for 5, 10, 15, 20 and 25 hours as well as at 25 °C for 0, 1, 2, 3 and 4 hours.

2.4.4 Effect of time on germination of the conidia spores of *P. roquefortii* FRR 2456 at 10 °C

Samples (4 ml) from butter and Shea oils by *P. roquefortii* FRR 2456, were removed aseptically at 5, 10, 15 and 25 hours. Spores and hyphae were separated from oil emulsions by holding the mixture at 40 °C water bath for 1 hour. Oil mixture with *P. roquefortii* was withdrawn aseptically into a Pasteur pipette filled with glass wool. The pipette was put in a test tube (10 ml) which was held at 40 °C water bath for a further 1 hour and then was cut in half with a ceramic scoring wafer (Restek, Thames Chromatography, Berks, UK). The glass wool was removed from the pipette with a pair of sterile tweezers and placed into a sterile Bijoux bottle (5 ml). Sterile distilled water (0.5 ml) was added into the Bijoux bottle which was stored at 4 °C for not more than 2 hours prior to observation of spores.

The change in appearance of the conidia spores with time at 10 °C was observed with a light microscope (Olympus CH) at a magnification of 400×. The microscope was equipped with a calibrated eyepiece scale (100 divisions = 1 mm).

Penicillium roquefortii spores changed from sub-globose (0 hour) to spherical swollen spores (5 hour) and finally to hyphae (after 15 hour) (Table 2.2). This table shows that the fungal strain may have utilised the oils and their degradation products as an energy source to grow in this reaction system. However, they undoubtedly used the food reserve in the spores.

Table 2.2 Germination of conidia spores of *Penicillium roquefortii* (FRR 2456) during conversion of butter oil in suspension culture at pH 7.0 and 10 °C †

Time (h)	Morphology	Size (μm)					
		Length			Width		
		Mean±SD	Max	Min	Mean±SD	Max	Min
0	spore sub-globose	5.5 ± 0.2	5.9	5.1	5.4 ± 0.2	5.9	4.3
5	spherical swollen spores	10.9 ± 0.6	11.4	9.5	9.2 ± 1.0	10.8	8.1
10	germinated spores	15.1 ± 2.9	18.7	10.8	9.1 ± 2.5	12.7	6.2
15	hyphae	NC	> 406.5	59.3	10.3 ± 0.7	11.1	9.5
25	hyphae	NC	> 542	139.6	9.8 ± 0.6	10.8	8.1

† Results were the mean of 50 measurements at 0 hour and 20 measurements at 5, 10, 15 and 25 hours. NC = not calculated.

2.4.5 Determination of free fatty acids by titration

Samples (4 ml) were removed with 10 ml pipette and put into 25 ml Erlenmeyer flasks. The reaction was stopped by adding 4 ml of 2-propanol. The mixtures were shaken and boiled for 5 minutes. Released fatty acids were determined by titration with 0.0555 M NaOH to the phenolphthalein end point. Titrations were made in triplicate. The following formula was used for calculation. Free fatty acids were calculated as μg lauric or oleic acid produced by 10^6 spores.

$$C = \frac{0.0555 \times (V - V_{ck}) \times \frac{20}{4}}{C_s \times v} M_w \times 10^{12} \qquad (2.1)$$

C = concentration of total released fatty acids (μg lauric or oleic acid / 10^6 spores)

V = volume of NaOH titrated for 4 ml reaction samples (ml)

V_{ck} = average volume of NaOH titrated for 4 ml controls (ml)

C_s = spores concentration per ml suspension (spores ml^{-1})

v = volume of spore suspension added into the reaction mixture (μl)

M_w = molecular weight of lauric or oleic acid

Time course for production of free fatty acids from butter and Shea oils by two strains of *P. roquefortii* at pH 7.0 and 25 °C is shown in Figure 2.4 and 2.5.

Figure 2.4 Time course for production of free fatty acids from butter oil by *P. roquefortii* (a) FRR 2456 and (b) Wisbey PJ in suspension cultures at pH 7.0 and 25 °C. Concentration of butter oil emulsion was 16.7 % (w/v).

Figure 2.5 Time course for production of free fatty acids from Shea oil by *P. roquefortii* (a) FRR 2456 and (b) Wisbey PJ in suspension cultures at pH 7.0 and 25 °C. Concentration of Shea oil emulsion was 16.7 % (w/v).

The highest concentration of free fatty acids (3000 - 3500 μg / 10^6 spores) was found for butter oil at 2 hours' reaction at 25 °C (Figure 2.4). It is observed from Figures 2.4 and 2.5 that (1) the fatty acid concentration decreased after 2 hours for butter oil, (2) more free fatty acids were produced from Shea oil than from butter oil and (3) the concentrations of free fatty acids from Shea oil were constant between one hour and four hours' of reaction.

2.5 Hydrolysis of oils by a commercial lipase from *P. roquefortii*

Butter and Shea oils were converted by a commercial lipase from *P. roquefortii* (E.C.3.1.1.3, Fluka Chemicals). Conversion was carried out at pH 7.0 in 250 ml Erlenmeyer flasks in the shaking water bath as described in 2.4.3 at 10 °C and 25 °C.

2.5.1 Preparation of oil emulsions

Oils were warmed at 40 °C water bath for 1 hour. The oil emulsions were prepared by homogenising 20 g oils with 60 ml 2% gum arabic solution using Polytron head at speed 2 for 2 min. The emulsions were then sonicated for 5 minutes in the Bransonic 52 Ultrasonicator at maximum current and ambient temperature.

2.5.2 Effect of pH on hydrolysis of triacylglycerols

Effect of pH for hydrolysis of triacylglycerols by a *P. roquefortii* lipase (E.C.3.1.1.3, Fluka Chemicals) was investigated by following the conversion of olive oil to free fatty acids. Phosphate buffer was added to maintain pH at 6.0, 6.5 and 7.0. Tris/HCl buffer was added to maintain pH at 7.5, 8.0 and 8.5. The pH values were measured with Unicam 9460 Ion-Selective Meter as described in 2.3.1 (b).

The reaction mixture contained 10 ml olive oil emulsion, 8 ml buffer solution (0.1 M), 2 ml $CaCl_2$ solution and 20 mg lyophilised lipase powder (Pabai *et al* 1995). Reaction was started by adding the lipase after 30 minutes equilibrium of the substrates at 37 °C shaking water bath

(Tecator). Reaction time was 30 minutes. Acetone : ethanol (1:1. v/v, 20 ml) was added to stop the reaction (Pabai *et al* 1995). The liberated fatty acids were titrated with NaOH (0.0555 M) to the phenolphthalein end point. Lipase activity was defined as μg oleic acid released by 1 mg lipase per minute at 37 °C.

$$C = \frac{0.0555 \times (V - V_{ck})}{t \times w_t} M_w \times 10^3 \qquad (2.2)$$

C = oleic acid concentration (μg min^{-1} mg^{-1} lipase)

t = reaction time (min)

Figure 2.6 Effect of pH on hydrolysis of olive oil by a commercial lipase from *P. roquefortii* at 37 °C. Substrate concentration was 16.7 % (w/v).

Figure 2.6 shows the effect of pH on conversion of olive oil at 37 °C. The optimum pH range was between pH 7.0 and 8.2. The lipase activity reduced dramatically between pH 8.0 and 8.5. This is in agreement with the report from Fluka Chemicals (UK) in which the lipase was reported as having optimum pH range between 6.0 and 8.0 at 37 °C when triolein was used as the substrate.

2.5.3 Hydrolysis of butter and Shea oils

Reaction conditions were the same as described in Section 2.4.3. Reaction mixtures were 10 ml oil emulsion, 8 ml phosphate buffer (pH 7.0, 0.1 M), 2 ml $CaCl_2$ solution (0.01 M) and 20 mg lyophilised lipase. Two types of controls were used. One was the mixture without lipase, the other was the mixture with lipase (20 mg) which had been autoclaved at 121 °C for 30 minutes and then dried in a thermostat oven (Griffin & George Ltd, Loughborough, UK) at 50 °C for 2 hours. Each sample was repeated in quadruplet.

Reactions were started by the addition of lipase after equilibrating the mixtures at 10 °C or 25 °C for 30 minutes. Reactions were carried out at 10 °C for 0, 5, 10, 15, 20 and 25 hours as well as at 25 °C for 0.5 hour.

2.5.4 Effect of time on hydrolysis of butter and Shea oils

The reaction was stopped by the addition of 20 ml 2-propanol. The reaction mixtures were shaken and boiled for 5 minutes. Released fatty acids from three samples were titrated with NaOH (0.0555 M) to the phenolphthalein end point. Concentration of free fatty acids was expressed as μg lauric acid and oleic acid produced by 1 mg lipase at 10 °C.

$$C = \frac{0.0555 \times (V - V_{ck})}{w_t} M_w \times 10^3 \qquad (2.3)$$

C = concentration of lauric or oleic acid produced by 1 mg lipase (μg mg^{-1})

w_t = weight of added lipase (mg)

Production of free fatty acids was inhibited after 10 hours of reaction with butter oil (Figure 2.7). This result is in agreement with the results from Pabai *et al* (1995). However, it is a first order reaction with Shea oil (Figure 2.8). It indicated that butter and Shea oils were different substrates in terms of production of fatty acids by the lipase from *P. roquefortii*. This difference

was due to different physical properties of the triacylglycerols and the different fatty acids profile of the two oils.

Figure 2.7 Time course for hydrolysis of butter and Shea oils by a commercial lipase from *P. roquefortii* (E.C.3.1.1.3) at pH 7.0 and 10 °C. Substrate concentration was 16.7 % (w/v).

Figure 2.8 Regression analysis of Ln fatty acids produced from Shea oil by lipase from *P. roquefortii* at pH 7.0 and 10 °C. Substrate concentration was 16.7 % (w/v).

Section II Extraction, Separation and Analysis of Mono-acylglycerols and Fatty Acids

2.6 Extraction of lipids

2.6.1 Extraction of lipids from solid-state cultures

(a) Extraction with dichloromethane for cultures grown at pH 4.5

After 7 days fermentation at 25 °C in Petri dishes, two clear bands of media adjacent to spores were cut from 5 Petri dishes. The agar medium was divided into three equivalent portions. Each portion was extracted three times with dichloromethane (5 ml). The extraction was carried out in cell-extractor (50 ml) cooled to 0 °C in ice water. The samples were filtered through filter paper (Whatman no. 1) into Erlenmeyer flasks (250 ml). Excess solvent was reduced to about 10 ml in a rotary evaporator (Heidolph VV2000 Series, Merck Ltd, Leicestershire, UK) at 50 °C. The evaporator was connected with a high vacuum pump (Edwards High Vacuum, Crawley, Sussex, England). Solvent was reduced to about 5 ml by blowing with nitrogen gas. The samples were stored in glass vials at -18 °C prior to further analysis.

(b) Extraction with hexane : 2-propanol (3:2, v/v)

This was used to extract total lipids from the cultures grown at pH 7.0 (Figure 2.9). Two bands of agar medium adjacent to spores were cut from 40 Petri dishes after 7 days growth at 25 °C or 20 days growth at 10 °C. Agar medium was also cut from Petri dishes without fungal growth as controls. Both media were weighed into 5 Erlenmeyer flasks (250 ml).

Step 1 Inactivation of lipase activity. 2-propanol (50 ml × 2) was added into each flask. The mixture was homogenised with Polytron head at full speed for 2 minutes, and then was filtered through filter paper (Whatman No. 1, Whatman). The residue was finally shaken overnight with

2-propanol (50 ml) in a rotary shaker (Model G2. New Brunswick Scientific Co. Inc. Edison. N.J. USA).

Figure 2.9 Flow chart for extraction of total lipids with hexane : 2-propanol (3:2. v/v). Methods from Christie (1989) & Radin (1981) with modification.

Step 2 Extraction 1. The filtrates collected from step 1 were combined (Filtrate 1 ~ 750 ml). Most solvent was removed under vacuum with a rotary evaporator at 50 - 60 °C. The volume of the residual filtrate (~ 40 ml) was measured and 1.5 fold of hexane (v/v) was added. The mixture was shaken for 2 minutes and poured into a separating funnel (100 ml). The upper hexane layer was collected. The lower layer was re-extracted with hexane : 2-propanol (3.2. v/v. 50 ml) and the combined hexane layers were stored over anhydrous sodium sulphate at 4 °C The lower aqueous layer was discarded.

Step 3 Extraction 2. Hexane : 2-propanol (3:2, v/v, 50 ml) was added into the flask (250 ml) with residue 1 collected from step 1. The mixture was homogenised with Polytron head at full speed for 2 minutes and suspended on a ceramic Büchner funnel with filter paper (Whatman No. 1). Pressure was applied to expel the wash. This procedure was repeated three times with 50 ml solvent mixture. The filtrates (Filtrate 2) were collected and stored over anhydrous sodium sulphate at 4 °C. The remaining residue (Residue 2) was discarded.

Step 4 Removing of solvent. The hexane layers obtained from Step 2 and the Filtrate 2 obtained from Step 3 were combined. Most solvent was removed under vacuum with a rotary evaporator at 50 °C. Excess solvent was removed under a stream of nitrogen. The extracted lipids were weighed and re-suspended in fresh hexane (50 ml). The lipid solution was stored in glass bottles with glass stoppers under nitrogen gas at - 18 °C until analysed.

2.6.2 Extraction of lipids from suspension cultures

Reaction samples (4 ml) were removed into glass flasks (25 ml) with 4 ml of 2-propanol over the time course at 10 °C and 25 °C. The mixtures were boiled for 5 minutes to inactivate the lipase activity. The samples were acidified by adding 0.4 ml 0.01 M hydrochloric acid. Lipids were extracted with hexane : 2-propanol (3:2, 6 ml) twice. The upper hexane layers were collected and combined. The final aqueous layer was discarded. Extracted lipids were stored over anhydrous sodium sulphate at -18 °C until analysed.

2.6.3 Soxhlet extraction of lipids from gum arabic and the commercial lipase

Gum arabic (10 g) and the lipase from *P. roquefortii* (0.5 g) were weighed into porous thimbles (33/80 mm, Ederol, Germany). The partially filled thimble was clamped and placed in a 250 ml glass beaker with 10 glass balls (0.4 mm diam.). Petroleum ether (80 ml) was added into the beaker. Gum arabic, lipase and blank control samples were refluxed in a Soxhlet extractor (SOXTHERM, Gerhardt, C. Gerhardt UK Ltd, Cheshire, UK) for 3 hours. The soxhlet extractor

was equipped with reflux condensers. Each sample was extracted in duplicate. The solvent in the beaker was reduced automatically to 2 - 5 ml by the extractor. Concentrated samples in petroleum ether were stored at 4 °C over anhydrous Na_2SO_4 (~ 1 g) in glass vials for not more than 24 hours prior to further analysis.

2.7 Analysis of lipids by Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC)

2.7.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was used for screening the production of acylglycerols, fatty acids and methyl ketones. Silica gel plates (60 Å, 20 × 5 cm (code 4861-620) or 20 × 20 cm, 0.25 mm thickness, with a fluorescent indicator) (Whatman Ltd, UK) were pre-heated at 105 °C for 50 minutes. Oils and acylglycerols were dissolved in dichloromethane, hexane or petroleum ether (1 - 2 %, w/v), and 2 - 5 µl samples were applied on the plates with a glass capillary (1 µl) in glass capillary tube (CAMAG, Merck Chromatography, UK). Mixture of stearic acid, mono-, di- and tristearin (~2%, w/v each) in dichloromethane (2 µl) were applied on the same plates as controls. The plates were developed with hexane : diethyl either : formic acid 80:20:2 (v/v/v) (Christie, 1989) in a sealed chamber (CAMAG, Merck, UK), and visualised with iodine vapour in a sealed tank or under UV light (254 nm). Methyl ketones were detected by spraying with 1% $KMnO_4$ containing 2% Na_2CO_3 (Dawson *et al* 1986). Carbonyl compounds such as methyl ketones gave a transient yellow spot against purple background.

2.7.2 Preparative Thin Layer Chromatography (PTLC)

Preparative Thin Layer Chromatography was used to separate acylglycerols and fatty acids. Relatively large volumes of samples were applied on the PTLC plates. Silica gel 60 F_{254} PTLC plates (20 × 20 cm with 4 × 20 cm concentration zone, 0.5 mm layer thickness and fluorescence

at 254 nm) (Merck Ltd, Dorset, UK) were used. The plates were activated by heating at 105 °C for 50 minutes. Lipids were dissolved in hexane or dichloromethane and 2 - 6 ml samples applied with a glass pipette (1 ml). The plates were developed with hexane : diethyl ether : formic acid 80:20:2 (v/v/v), and acylglycerols were visualised under UV light (254 nm) by exposure to iodine vapours for 20 s (Figure 3.2).

Figure 2.10 Application scheme of lipids on PTLC plate.

2.7.3 Extraction of acylglycerols and fatty acids from PTLC plates

Bands of acylglycerols or fatty acids were scraped from the PTLC plates with a scalpel and extracted with 2.5 ml of hexane and 2-propanol (3:2, v/v) 3×. The samples were centrifuged at 1,500 rpm for 2 minutes in a Lab Centrifuge (WIFUG 500E, Great Horton, Bradford, England) to remove the silica gel. The supernatants were collected in 5 ml pre-weighed reaction vessels. Solvent was removed with N_2 gas. Acylglycerols or fatty acids were weighed and stored at 4 °C under N_2 prior to GC and GC-MS analyses.

2.8 Determination of free fatty acids

Total free fatty acids in butter oil were determined as % dodecanoic acid (lauric acid) or cis-9-octadecenoic acid (oleic acid) following the method of Kellard *et al* (1985). Butter oil (10 g) was dissolved by boiling for 30 min in 50 ml industrial methylated spirits (IMS) and the

concentration of free fatty acids determined by titration with 0.01 M NaOH using phenolphthalein as the indicator.

2.9 Derivatisation of free fatty acids and acylglycerols

2.9.1 Esterification of fatty acids and acylglycerols to give fatty acid methyl esters (FAMEs)

Two methods of methylation were used to convert fatty acids into fatty acid methyl esters. The first method was methylation with 1% sulphuric acid in methanol (Christie, 1989) and was used for methylation of oils and standard fatty acids, and also for micro-methylation of acylglycerols of butter and Shea oils. Oils, standard fatty acids (~50 mg) or acylglycerols (0.2 ~ 5 mg) were dissolved in toluene (1 ml for oils and standard fatty acids, 0.5 ml for acylglycerols) in 5.0 ml reaction vessels (Supelco, Poole, Dorset, UK) and Pierce & Warners (Chester, UK). An internal standard (heptadecanoic acid, C17:0) was added into the vessel (18 ~ 20 mg for oils and standard fatty acids and 0.2 ~ 4.0 mg for acylglycerols). Sulphuric acid in methanol (1% v/v) (2 ml) was added and the mixture was left overnight at 50 °C in an incubator or a heating block (Rockford IL, USA). The mixture was poured into a pear-shaped flask (25 ml) to which 5% (w/w) NaCl was added. FAMEs were extracted with hexane (2 × 5 ml). The combined hexane layers were washed with distilled water (4 ml) containing 2% (w/v) potassium bicarbonate and dried over 2 g anhydrous Na_2SO_4 overnight at 4 °C. Excess solvent was removed by blowing with N_2 gas and samples were stored in glass vials under N_2 at 4 °C.

The second method was methylation with sulphuric acid : toluene : methanol 1:10:20 (v/v/v) (Hitchcock and Hammond, 1980) and was used for methylation of fatty acids in butter oil. Butter oil (~0.5 g), internal standard (C17:0, ~0.18 g) and methylating agent (H_2SO_4 : toluene : methanol 1:10:20 v/v/v, 80 ml) were refluxed for 1 hour in a round bottom flask (500 ml), fitted

with a condenser. Distilled water (100 ml) was added and the solution was extracted with diethyl ether (3 × 50 ml) in a 250 ml glass separating funnel. The upper organic layer was dried overnight at 4 °C with 6-7 g anhydrous Na_2SO_4. Excess solvent was removed with N_2 gas and samples were stored in glass vials under N_2 at 4 °C.

2.9.2 Derivatisation of monoacylglycerols to monoacylglycerol trimethylsilyl (TMS) ethers

This method was derived from Christie (1989). Standard monoacylglycerols (~ 5.0 mg) or monoacylglycerols extracted from PTLC plates (0.1 - 5.0 mg) were dissolved in 0.5 ml acetone in 5 ml reaction vessels. N,O-bis(trimethylsilyl) acetamide (BSA) (0.2 - 0.5 ml, Sigma) were added, and the vessels were sealed and shaken for 30 seconds and left at 30 °C for 15 minutes. Excess solvent was blown off under a stream of nitrogen. Distilled water (0.6 ml) was added and the TMS ethers were extracted with 2 × 3 ml hexane. The hexane layers were collected and dried over anhydrous Na_2SO_4 (~1 g) at 4 °C overnight. The sample was put in small glass vials (1.5 ml) and reduced to 0.2 - 0.5 ml with a stream of nitrogen and the samples were stored at -18 °C under N_2. The glass vials were put into 28 ml Universal bottles containing anhydrous silica gel.

2.10 Equipment and experimental conditions for GC and GC-MS analyses

2.10.1 Equipment

(a) Gas Chromatography (GC)

A Varian 3400 Gas Chromatograph equipped with a flame ionisation detector (FID) (Varian Assoicates, Walnut Creek, CA, USA) was coupled to a Pye Unicam PU 4810 Computing Integrator (Pye Unicam, Cambridge, UK) or a 486 computer with Varian Star Chromatography Workstation (Version 4.0) (Varian Associates, California, USA). Nitrogen was used as the carrier gas. Nitrogen cylinder was connected with an oxygen trap (Phase Separation Ltd,

Deeside, Clwyd, UK) to ensure the nitrogen introduced into GC column was oxygen free. The oxygen trap was regenerated with each new nitrogen cylinder.

(b) Gas Chromatography - Mass Spectrometry (GC-MS)

Gas Chromatography - Mass Spectrometry (GC-MS) was carried out on a Gas Chromatograph (Hewlett Packard 5890A) with a quadrupole Mass Spectrometer (VG Trio - 1) (Finnigan Mass Lab. Manchester, UK). Helium was used as the carrier gas. The ion source (electron impact, 70 ev) was held at 200 °C. The interface temperature was at 250 °C.

2.10.2 Experimental conditions

(a) Analysis of FAMEs

A 50 m × 0.25 mm i.d. free fatty acid phase (FFAP, Quadrex Scientific, Surrey, UK) fused silica capillary column bonded with Carbowax® PEG for acids as stationary phase was used to analyse fatty acid methyl esters by GC and GC-MS. The column film thickness was 0.25 μm. The injector was set at 220 °C.

GC analysis. Gas Chromatograph (Varian 3400) was programmed from 70 °C (with an initial delay of 4 minutes) to 200 °C at a rate of 5 °C min^{-1} and then held isothermal at 200 °C for 30 minutes. The detector was set at 300 °C. Gas flow rates were measured with Jour Digital GC Flowmeter (Jour Research, S-439 22 Onsala, Sweden). Carrier gas flow rate was 3 ml min^{-1}, H_2 flow rate was 30 ml min^{-1}, and air flow rate was 300 ml min^{-1}. A 1 μl sample was injected for split injection and between 0.2 and 0.5 μl sample was injected for splitless injection. The split ratio was 16:1. FID detector was run at 10^{-11} with an attenuation of ×4 or ×1 respectively.

GC-MS analysis. Gas Chromatograph (Hewlett Packard 5890A) was programmed from 30 °C to 70 °C at 10 °C min^{-1}, from 70 °C to 200 °C at 5 °C min^{-1} and then held isothermal at 200 °C for 30 minutes. A 1.0 μl sample was injected with splitless injection. The column head pressure was 7 psi.

(b) Analysis of monoacylglycerol TMS ethers

Two columns were cut from a 30 m × 0.22 mm id. with 0.25 μm film thickness BP1 fused silica capillary column (30QC2/BP1 0.25) with 100% dimethyl polysiloxane (SGE Ltd, Kiln Farm Milton Keynes, UK). One was 20 m, the other was 10 m. The injector was at 320 °C.

GC analysis. The temperature program was set up from 65 °C to 100 °C at 35 °C min^{-1}, from 100 °C to 240 °C at 20 °C min^{-1} and then from 240 to 300 °C at 12 °C min^{-1}. The detector was held at 370 °C. Carrier gas flow rate was 1.5 ml min^{-1}, hydrogen flow rate was 30 ml min^{-1}, and air flow rate was 300 ml min^{-1}. Split or splitless injection was used. The split ratio was 5:1. Samples (0.5 - 1.0 μl) were injected manually. The FID detector was run at 10^{-11} with attenuation of ×1.

GC-MS analysis. Temperature program was set up from 35 °C to 240 °C at 20 °C min^{-1}, from 240 °C to 300 °C at 12 °C min^{-1} and from 300 °C to 320 °C at 2.5 °C·min^{-1}. The head pressure was 7 psi. Splitless injection was used and samples (1.0 μl) were injected manually.

2.11 Determination of response factors (F_r)

Standard fatty acids (~50 mg, 3 - 8 mg for each acid) were weighed into a 5 ml reaction vessel with ~10 mg heptadecanoic acid ($C_{17:0}$). Samples were methylated in duplicate using the method which has been described in 2.9.1 (Christie 1989) and each sample was analysed in triplicate by GC. Response factors of fatty acids were determined with respect to the internal standard ($C_{17:0}$) by running a standard mixture of FAMEs.

Standard 1-monoacylglycerols (~20 mg, 2-5 mg for each monoacylglycerol) were weighed into a 5 ml reaction vessel with ~8 mg 1-monotetradecanoyl-glycerol (1-14:0). The mixtures were

silylated in duplicate and each sample was analysed in triplicate by GC. Response factors of monoacylglycerols were determined by running a standard mixtures of 1-monoacylglycerols with respect to 1-monotetradecanoate (1-14:0). The F_r values of 2-monoacylglycerols were assumed as the same values as the corresponding 1-monoacylglycerols.

Retention times (t_r), relative retention times (t_{rr}) and peak areas were recorded. The data was transferred into a Microsoft Excel 5.0 spread sheet.

Calculation of response factor for individual fatty acid or monoacylglycerol in internal standard method was based on the following formulae.

m_{is} = weight of internal standard (mg)

A_{is} = area integration of internal standard

m_i = apparent weight of individual fatty acid or monoacylglycerol (mg)

A = area integration of individual fatty acid or monoacylglycerol

m = true weight of individual fatty acid or monoacylglycerol (mg)

F_r = response factor of individual fatty acid or monoacylglycerol

$$\frac{A}{A_{is}} = \frac{m_i}{m_{is}} \tag{2.4}$$

$$F_r = \frac{m}{m_i}$$

i.e.

$$F_r = \frac{m \times A_{is}}{m_{is} \times A} \tag{2.5}$$

2.12 Analyses of fatty acids and monoacylglycerols by GC

2.12.1 Internal standard method

Qualitative analysis of major fatty acid compounds in oils and acylglycerols was achieved by (a) comparing the retention times and relative retention times of their methyl esters with the standard fatty acid methyl esters at the same GC conditions and (b) confirming the identification with GC-MS analysis. Minor fatty acid compounds were identified by GC-MS. Quantitative analysis of fatty acids was achieved by adding an internal standard (heptadecanoic acid, C17:0). With the Pye Unicam PU 4810 computing integrator, a fixed programme was set up to calculate the response factors which were used for the determination of fatty acid concentrations in oils and acylglycerols (g / 100 g oil). With the Varian Star Chromatography Workstation, retention times, relative retention times and peak areas were recorded. This data and calculated response factors were transferred into a Microsoft Excel 5.0 spread sheet. Fatty acids composition was calculated using the formulae 2.6, 2.7 and 2.8 (shown below).

Qualitative analysis of α- and β-monoacylglycerols was achieved by (a) comparing the retention times of their TMS derivatives with standard monoacylglycerol TMS ethers at the same GC conditions and (b) identification of their fragmentation patterns in GC-MS. Monoacylglycerols composition was expressed as a molar percentage (MAG mol %). Peak areas and response factors were transferred into a Microsoft Excel 5.0 spread sheet. Calculation of monoacylglycerols composition was based on formula 2.8.

m_o = weight of total oil (g)

n = amount of individual fatty acid or monoacylglycerol (mol)

M = molar mass of individual fatty acid or monoacylglycerol (g mol^{-1})

(1) Weight percentage of individual fatty acid with respect to total oils (g / 100 g oil)

$$= \frac{m}{m_o} \times 100\%$$

$$= \frac{m_{is}}{A_{is} \times m_o} AF_r \times 100\% \qquad (2.6)$$

(2) Weight percentage of individual fatty acid with respect to total fatty acids (wt %)

$$= \frac{m}{m_1 + m_2 + \ldots + m_n} \times 100\%$$

$$= \frac{\frac{m_{is}}{A_{is}} AF_r}{\frac{m_{is}}{A_{is}} A_1F_{r1} + \frac{m_{is}}{A_{is}} A_2F_{r2} + \ldots + \frac{m_{is}}{A_{is}} A_nF_{rn}} \times 100\%$$

$$= \frac{AF_r}{A_1F_{r1} + A_2F_{r2} + \ldots + A_nF_{rn}} \times 100\% \qquad (2.7)$$

(3) Molar percentage of individual fatty acid or monoacylglycerol (mol %)

$$= \frac{n}{n_1 + n_2 + \ldots + n_n} \times 100\%$$

$$= \frac{\frac{m}{M}}{\frac{m_1}{M_1} + \frac{m_2}{M_2} + \ldots + \frac{m_n}{M_n}} \times 100\%$$

$$= \frac{\frac{m_{is}}{A_{is}} \cdot \frac{AF_r}{M}}{\frac{m_{is}}{A_{is}} \cdot \frac{A_1F_{r1}}{M_1} + \frac{m_{is}}{A_{is}} \cdot \frac{A_2F_{r2}}{M_2} + \ldots + \frac{m_{is}}{A_{is}} \cdot \frac{A_nF_{rn}}{M_n}} \times 100\%$$

$$= \frac{\frac{AF_r}{M}}{\frac{A_1F_{r1}}{M_1} + \frac{A_2F_{r2}}{M_2} + \ldots + \frac{A_nF_{rn}}{M_n}} \times 100\% \qquad (2.8)$$

2.12.2 External standard method

(a) Standard curves

Methyl esters of tetradecanoic acid ($C_{14:0}$, 22.9 mg), hexadecanoic acid ($C_{16:0}$, 22.5 mg), heptadecanoic acid ($C_{17:0}$, 28.0 mg), octadecanoic acid ($C_{18:0}$, 21.0 mg) and cis-9-octadecenoic acid ($C_{18:1(9c)}$, 21.6 mg) were dissolved in dichloromethane in a 10 ml volumetric flask (2 mg / ml stock mixture). Heptadecanoic acid ($C_{17:0}$) was used as external standard. Subsequent standard solutions were made up by diluting the stock mixture into 1.67, 3.33, 5.56 and 11.11 times. The final concentration of standard solutions were 2 mg ml^{-1}, 1.2 mg ml^{-1}, 0.6 mg ml^{-1}, 0.36 mg ml^{-1} and 0.18 mg ml^{-1}. Each standard solution was analysed 3 - 6 times by GC. The area of each peak was recorded and area ratios of fatty acid methyl ester / $C_{17:0}$ were calculated. Standard curves were plotted as the area ratios against the concentration of each fatty acid methyl ester (Figures 2.11 - 2.14).

(b) Fatty acids composition in acylglycerols

Acylglycerols (~ 5 mg) produced from Shea oil by *P. roquefortii* FRR 2456 (in solid-state culture at pH 4.5 and 25 °C) were methylated as described in section 3.4.1 (a) but no internal standard was added. Heptadecanoic acid methyl ester (~ 5 mg / 10 ml) was added into the mixture of fatty acid methyl esters and the final volume was made up to 10 ml. Fatty acid methyl esters were analysed by GC for 6 times and the area ratio of each fatty acid methyl ester / $C_{17:0}$ was calculated. Concentration of each fatty acid was obtained by the standard curve with respect to the corresponding value of area ratio of fatty acid methyl ester / $C_{17:0}$.

Figure 2.11 Standard curve of tetradecanoic acid for external standard method. Heptadecanoic acid methyl ester was added as external standard.

Figure 2.12 Standard curve of hexadecanoic acid for external standard method. Heptadecanoic acid methyl ester was added as external standard.

Figure 2.13 Standard curve of octadecanoic acid for external standard method. Heptadecanoic acid methyl ester was added as external standard.

Figure 2.14 Standard curve of cis-9-octadecenoic acid for external standard method. Heptadecanoic acid methyl ester was added as external standard.

2.13 Identification of FAMEs and monoacylglycerol TMS ethers by GC-MS

2.13.1 FAMEs

The mass spectra of aliphatic saturated FAMEs and some of mono, di, and triunsaturated FAMEs were compared with the NBS (National Bureau Standards) library spectra as well as the spectra of standard methyl esters under the same GC-MS condition. Mono-branched chain FAMEs (iso- and anteiso-) were identified by comparison with the NBS library spectra and by their fragmentation patterns (Jensen & Gross 1987), especially the mass ions at $[M-29]^+$ and $[M-31]^+$. Other minor mono and diunsaturated FAMEs were identified according to NBS library comparison.

2.13.2 Monoacylglycerol TMS ethers

The mass spectra of α-monoacylglycerol TMS ethers were identified by comparing with the spectra of standard monoacylglycerols, the spectra from NBS library and by their fragmentation patterns (Christie 1989). The mass spectra of β-monoacylglycerol TMS ethers were identified by comparing with NBS library spectra and by their fragmentation patterns (Christie 1989).

2.14 Yields of monoacylglycerols and fatty acids

2.14.1 Yields of monoacylglycerols produced in solid-state culture

Yields of monoacylglycerols (Y) produced in solid-state cultures were determined using equation 2.9 as g MAGs / 100 g total extracted lipids from agar medium.

$$Y = \frac{50 \times wt_2}{wt_1 \times V} \times 100\ \% \qquad (2.9)$$

wt_1 = total extracted lipids (mg) which were dissolved in 50 ml fresh hexane

V = volume of sample applied on PTLC plates measured with a volumetric pipette

wt_2 = monoacylglycerols extracted from PTLC plates (mg)

2.14.2 Yields of monoacylglycerols and fatty acids produced in suspension culture

Yields of monoacylglycerols and fatty acids were measured for the samples converted from butter by the two strains of *P. roquefortii* at 10 °C for 25 h. Monoacylglycerols, fatty acids and triacylglycerols were extracted from the same PTLC plates and methylated with internal standard (C17:0) (method see 2.9.1). Fatty acids composition of monoacylglycerols, fatty acids and triacylglycerols were determined by GC and calculated to give total molar percent of fatty acids (method see 2.11). Yields of monoacylglycerols (Y_m) and fatty acids (Y_a) were expressed as molar percentage with respect to total triacylglycerols, monoacylglycerols and fatty acids (equation 2.10, 2.11).

$$Y_m = \frac{n_m}{n_m + n_a + n_t} \times 100\% \qquad (2.10)$$

$$Y_a = \frac{n_a}{n_m + n_a + n_t} \times 100\% \qquad (2.11)$$

n_m = amount of monoacylglycerols (mmol)

n_a = amount of fatty acids (mmol)

n_t = amount of triacylglycerols (mmol)

CHAPTER 3

RESULTS

Analysis of Fatty Acids and Monoacylglycerols in Standards and Oils

3.1 Analysis of fatty acids by GC

3.1.1 Standard fatty acid

Figure 3.1 Separation of standard fatty acids as methyl esters by GC. A FFAP fused silica capillary column (Quadrex, 50 m × 0.25 mm i.d., 0.25 μm film thickness) was programmed from 70 °C (with a initial stay of 4 min) to 200 °C at 5 °C min^{-1}, and then held at 200 °C for 30 min. Nitrogen was the carrier gas with flow rate of 3 ml min^{-1}. Injector was at 220 °C and detector (FID) was at 300 °C. Varian Star Chromatography Workstation 4.0 was used to record the chromatogram.

Figure 3.1 shows the separation of standard fatty acid methyl esters (FAMEs) with a FFAP capillary column. Fatty acid methyl esters (from medium chain $C_{6:0}$ to very long chain $C_{24:0}$) were separated completely under the experimental conditions which were used. Retention times, relative retention times and response factors of standard fatty acid methyl esters are given in Table 3.1. Good agreement was found for the relative retention times as determined by small standard deviations. Fatty acid methyl esters could be identified by relative retention times and retention times of samples which were compared with standards.

Table 3.1 Retention times (t_r), relative retention times (t_{rr}) and response factors (F_r) of standard FAMEs relative to heptadecanoic acid methyl ester (17:0) *

Fatty acid	CAS no[§]	t_r (min)	t_{rr}	F_{r1} [†]	F_{r2} [‡]
6:0	142-62-1	4.315 ± 0.049	0.148 ± 0.001	1.583 ± 0.101	1.447 ± 0.125
8:0	124-07-2	8.534 ± 0.071	0.293 ± 0.001	1.111 ± 0.012	1.157 ± 0.061
10:0	2082-76-0	13.708 ± 0.084	0.471 ± 0.001	1.140 ± 0.017	1.103 ± 0.042
11:0	112-37-8	16.201 ± 0.085	0.557 ± 0.001	1.020 ± 0.023	1.070 ± 0.061
12:0	143-07-7	18.618 ± 0.087	0.640 ± 0.001	1.228 ± 0.017	1.039 ± 0.032
13:0	638-53-9	20.852 ± 0.094	0.717 ± 0.001	1.001 ± 0.005	1.058 ± 0.047
14:0	544-63-8	23.051 ± 0.097	0.792 ± 0.001	1.036 ± 0.007	1.025 ± 0.020
14:1 (9c)	13147-06-3	23.798 ± 0.097	0.818 ± 0.000	0.999 ± 0.006	1.002 ± 0.012
15:0	1002-84-2	25.107 ± 0.098	0.863 ± 0.000	1.002 ± 0.003	1.003 ± 0.043
16:0	57-10-3	27.149 ± 0.105	0.933 ± 0.000	1.008 ± 0.001	1.022 ± 0.006
16:1 (9c)	373-49-9	27.607 ± 0.104	0.949 ± 0.000	1.063 ± 0.002	1.020 ± 0.012
17:0	506-12-7	29.095 ± 0.103	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000
18:0	57-11-4	30.962 ± 0.112	1.064 ± 0.000	1.178 ± 0.003	0.983 ± 0.021
18:1 (9c)	112-80-1	31.343 ± 0.125	1.077 ± 0.001	1.251 ± 0.020	1.239 ± 0.013
18:2 (9c,12c)	60-33-3	32.324 ± 0.147	1.111 ± 0.001	1.246 ± 0.014	1.158 ± 0.072
18:3 (6c,9c,12c)	506-26-3	33.038 ± 0.152	1.136 ± 0.001	1.001 ± 0.008	1.005 ± 0.072
18:3 (9c,12c,15c)	463-40-1	33.977 ± 0.161	1.168 ± 0.001	1.003 ± 0.004	1.029 ± 0.076
20:0	506-30-9	35.865 ± 0.182	1.233 ± 0.002	0.972 ± 0.020	0.945 ± 0.019
22:0	112-85-6	44.108 ± 0.246	1.516 ± 0.003	0.835 ± 0.024	0.869 ± 0.029
24:0	557-59-5	58.514 ± 0.392	2.011 ± 0.007	0.788 ± 0.043	0.823 ± 0.058

* Results were the mean of 8 methylation and 16 GC analyses ± standard deviation for t_r, t_{rr} and F_{r2}, and the mean of 3 methylation and 6 GC analyses for F_{r1}.

[§] CAS no: Chemical Abstract Service number.

[†] Response factors of GC derived with Pye Unicam Computing Integrator. Values were determined in March, 1995.

[‡] Response factors of GC were obtained using the standard program in the Varian Star Chromatography Workstation. Values were determined in October 1995, May and October 1996, and April 1997.

Most of the response factors shown in Table 3.1 are approximately 1. The nearer the carbon numbers were to the internal standard (17:0), the closer the response factors were to 1. It seemed that there was discrimination of short and long chain fatty acid methyl esters (response factor ~1.5 for 6:0 and ~0.8 for 24:0) which were at the beginning and end of the GC Chromatogram. This was often observed in split injection ports together with other unpredictable variability (Jacobsen *et al* 1994). Response factors of cis-9-octadecenoic acid (oleic acid, 18:1 (9c)) were higher than other response factors, however, standard deviations were low and response factors were consistent.

3.1.2 Fatty acids in butter and Shea oils

Separation of fatty acids as methyl esters in butter and Shea oils by Gas Chromatography is shown in Figure 3.2.

(a) Butter oil

Butter oil is a very complex oil in terms of fatty acids composition. Over 30 fatty acids were detected with chain length from 6 to 24. These fatty acids could be classified into three groups, (1) aliphatic saturated fatty acids, (2) mono- and poly-unsaturated fatty acids and (3) branched chain saturated fatty acids.

More than 90 % butter oil was fatty acids with ~85 % major fatty acids and ~7 % minor fatty acids (Table 3.3). Palmitic acid (16:0) and oleic acid (18:1(9c)) were the most abundant fatty acids together with relatively large concentrations of medium chain fatty acids (MCFAs, 6:0 - 12:0).

The fatty acids composition in the 1993 sample and the 1995 sample was similar. Less oleic acid (18:1(9c)) and more palmitic acid (16:0) were found in the 1993 sample which had been stored at 4 °C for 2 years. Slightly more free fatty acids (0.29 g / 100 g oil) were found in 1993 sample than in 1995 sample and unsaturated fatty acids made up a lower proportion in 1993 sample.

Figure 3.2 Separation of fatty acids in butter and Shea oils by Gas Chromatography as methyl esters. A FFAP column (50 × 0.25 mm i.d., Quadrex) was used. GC conditions were given in the legend of Figure 3.1. Heptadecanoic acid (17:0, 18-20 mg) was added as an internal standard (is). Peak names and relative retention times are given in Tables 3.3 (butter oil) and 3.5 (Shea oil).

Comparing the two methods of methylation, fatty acids composition was similar by both methods. Method 1 was used as this eliminated the need to reflux the sample and smaller amount of samples could be analysed.

Weight percentage of major fatty acids in butter oil was compared with some published values (Table 3.2). There was difference between weight percentage and molar percentage of fatty acids composition. Short and medium chain fatty acids composition as molar percent increased as they had lower molecular mass. On the other hand, long chain fatty acids composition as molar percent decreased as they had higher molecular mass. The advantage of using molar percentage was that fatty acids composition in monoacylglycerols could be compared easily with the composition of monoacylglycerols (Chapter 4 and 5).

Table 3.2 Major fatty acids composition in butter oil (results in wt % and mol %)[†]

[†] Refers to the peaks in Figure 3.2 (a). Results were the mean of 3 methylation and 9 GC analyses ± standard deviation. Peaks were identified by GC-MS.
[‡] Published values were from: 1. Iverson & Sheppard 1986, 2. Jensen *et al* 1962, 3. Timmen 1988, 4. Badings & De Jong 1983 and 5. Cullinane *et al* 1984 (packed column).
[a] Samples were analysed from February to May 1995 and data was recorded by Pye Unicam Computing Integrator.
[b] Samples were analysed from June 1996 to July 1997 and data was recorded by Star Chromatography Workstation.

Table 3.3 Comparison of fatty acids composition in two samples of butter oil by two methods of methylation (results in g FA / 100 g butter oil)[1]

[1] Results were the mean of 3 methylation and 9 GC analyses ± standard deviation. Peaks were identified by GC-MS. Samples were analysed from February to May 1995. ni = not identified. Method 1. Christie 1989 (2.9.1); method 2. Hitchcock & Hammond 1980 (2.9.1). C7:0 and C9:0 were detected in GC-MS identification, but could not be detected in GC analysis with sensitivity of 4×10^{-11}. Composition of C17:0 was determined by running a sample without internal standard and compared the sample with the samples with internal standard (C17:0).
[a] Obtained from Unilever research (stock C) in January 1993 and stored at 4 °C not under nitrogen.
[b] Obtained from Unilever research (stock C) in February 1995 and stored at 4 °C not under nitrogen.
[c] Major fatty acids were 6:0, 8:0, 10:0, 12:0, 14:0, 14:1 (9c), 15:0, 15:0, 16:1 (9c), 18:0, 18:1 (9c), 18:1 (11c), 18:2 (9c,12c) and 18:3 (9c,12c,15c). [d] Minor fatty acids were the rest. [e] Medium chain fatty acids
[f] Total free fatty acids were determined by titration with 0.01 M sodium hydroxide.

With regard to the positional distribution of fatty acids in butter oil, hypercholesterolemic fatty acids (12:0, 14:0 and 16:0) are present in the sn-2 position in the major fraction of butter fat (Kermasha *et al* 1993). More short and medium chain fatty acids (C_4-C_{10}) are present at sn-1,3 positions of glycerol (Pabai *et al* 1995). Oleic acid is randomly distributed in sn-1, sn-2 and sn-3 positions at 21, 14 and 15 mol % respectively (Nawar 1985). Positional distribution of major fatty acids in butter oil is given in Table 3.4.

Table 3.4 Distribution of major fatty acids at sn-1(3) and sn-2 positions in butter oil triacylglycerols

† Data was derived from Pabai *et al* 1995. Random distribution was calculated using the formula described by Christie (1987). The percentage of each fatty acid was relative to the total fatty acids listed in the Table.
‡ Data from Table 3.2. The percentage of each fatty acid was relative to the total identified fatty acids in butter oil.
§ Calculation was based on $\frac{ab}{a+1}$. * Calculation was based on $\frac{b}{a+1}$.

(b) Shea oil

Shea oil is a less complex oil in terms of fatty acids composition compared to butter oil (Figure 3.2 (b)). Five major fatty acids were found in Shea oil. They were hexadecanoic acid (palmitic acid, 16:0), octadecanoic acid (stearic acid, 18:0), cis-9-octadecenoic acid (oleic acid, 18:1 (9c)), cis-9,12-octadecadienoic acid (linoleic acid, 18:2 (9c,12c)) and eicosanoic acid (arachidic acid, 20:0). The fatty acids composition in Shea oil is given in Table 3.5. Results of weight percentage was compared with record from Unilever research (personal communication). Stearic

acid (18:0) and oleic acid made up about 90 % of the fatty acids in Shea oil with stearic acid consisting of over 50 %. No medium chain fatty acids (6:0 - 14:0) were detected in Shea oil.

Table 3.5 Major fatty acids composition in Shea oil †

Peak no.	T_{rr}	Fatty acid	mol %	wt %	g FA / 100 g oil	g FA / 100 g oil ‡ (Unilever)
1	0.934	16:0	3.95±0.05	3.66±0.04	3.09±0.03	3.5
2	1.064	18:0	55.73±0.66	55.82±0.67	56.38±0.15	56.8
3	1.077	18:1(9c)	35.41±0.64	35.22±0.63	29.64±0.14	31.3
4	1.112	18:2(9c,12c)	3.03±0.02	2.99±0.02	3.16±0.14	2.8
6	1.235	20:0	1.62±0.05	1.78±0.05	1.62±0.02	2.0
total			99.74	99.73	94.36±0.37	96.4

† Refers to the peaks in Figure 3.2 (b). Results were the mean of 2 methylation and 6 GC analysis ± standard deviation. Peaks were identified by GC-MS.
‡ Recorded values from Unilever research.

Table 3.6 gives the distribution of fatty acids in Shea oil triacylglycerols. Results from Unilever Research where Shea oil was obtained for this study are compared to the results from Shukla (1995). The main triacylglycerols in Shea oil are SOS (S stands for all saturated fatty acids and O for oleic acid). These symmetrical triacylglycerols have oleic acid in the sn-2 position and saturated fatty acids in the sn-1,3 positions (Table 3.6). The composition of SOS is greater than 80 %.

Table 3.6 Composition of triacylglycerols in Shea oil (stearin fraction) *

S = all saturated fatty acids, O = oleic acid, Ln = linoeic acid.
† Unpublished data from Brian Jeffrey (1995), Unilever report. Results in weight percent.
‡ Data from reference paper (Shukla 1995). Results in molar percent.

3.2 Identification of FAMEs in butter oil by GC-MS

3.2.1 Total mass ion spectrum of FAMEs from butter oil

A total ion chromatogram of fatty acid methyl esters from butter oil is shown in Figure 3.3. Complete separation of FAMEs with the FFAP capillary column was achieved by GC-MS using helium as the carrier gas. Heptadecanoic acid (17:0, ~ 19 mg) was added as internal standard prior to methylation. Heptanoic acid methyl ester (7:0), nonanoic acid methyl ester (9:0) as well as some minor FAMEs could be identified by GC-MS with splitless injection (1 μl).

Figure 3.3 Total ion chromatogram of FAMEs in butter oil separated with FFAP capillary column (50 m × 0.25 mm i.d., Quadrex) by GC-MS. Helium was the carrier gas. The temperature was raised from 30 °C (with a initial stay of 1 min) to 70 °C at 10 °C min^{-1}, from 70 °C to 200 °C at 5 °C min^{-1}, and then stayed at 200 °C for 30 min. Heptadecanoic acid (17:0) was added as internal standard.

3.2.2 Identification of three groups of fatty acid methyl esters in butter oil

(a) Aliphatic straight chain saturated FAMEs

An example is given in Figure 3.4 to show the fragmentation of aliphatic saturated fatty acid methyl esters analysed by electron impact (EI) Mass Spectrometry. Table 3.7 summarises the characteristic ions of aliphatic saturated FAMEs. The most abundant (base) mass ion in aliphatic saturated FAMEs was m/z 74 ($C_3H_6O_2^{+\cdot}$). This ion was termed as the 'McLafferty rearrangement ion' and formed by cleavage of the β carbon from the carboxyl group of the parent molecule (Figure 3.5). Other characteristic ions were M^+, $(M-31)^+$ (loss of methoxyl radical), together with a series of ions with general formula $(CH_2)_nCOOCH_3^+$. The mass ion at $(M-43)^+$ was formed by loss of C_2, C_3 and C_4 as a result of a complex rearrangement (Christie 1989).

Figure 3.4 Mass spectra of (a) hexadecanoic acid methyl ester in butter oil and (b) standard hexadecanoic acid methyl ester. GC-MS conditions were given in the footnote of Figure 3.3.

Figure 3.5 McLafferty rearrangement of fatty acid methyl esters.

Table 3.7 Characteristic ions of aliphatic saturated FAMEs in butter oil †

Pk no.	t_r (min)	t_{rr} ‡	FAME	m/z 74*	M^+	$(M-31)^+$	$(M-43)^+$	$(CH_2)_nCOOCH_3^+$	library % ID
1	9.98	0.265	6:0	✓	×	99	87	101, 87, 73, 59...	91.8
2	12.40	0.329	7:0	✓	×	113	101	115, 101, 87, 73...	86.1
3	15.12	0.401	8:0	✓	×	127	115	129, 115, 101, 87...	94.5
4	17.88	0.474	9:0	✓	172	141	129	143, 129, 115, 101...	89.6
5	20.62	0.547	10:0	✓	186	155	143	157, 143, 129, 115...	91.3
6	25.62	0.679	12:0	✓	214	183	171	185, 171, 157, 143...	88.9
9	27.88	0.739	13:0	✓	228	197	185	199, 185, 171, 157...	85.2
11	30.18	0.800	14:0	✓	242	211	199	213, 199, 185, 171...	90.4
15	32.25	0.855	15:0	✓	256	225	213	227, 213, 199, 185...	91.0
17	34.84	0.924	16:0	✓	270	239	227	241, 227, 213, 199...	92.0
22	37.72	1.000	17:0	✓	284	253	241	255, 241, 227, 213...	89.1
25	41.03	1.088	18:0	✓	298	267	255	269, 255, 241, 227...	90.5
38	47.02	1.247	20:0	✓	326	295	283	297, 269, 255, 241...	90.0
41	61.34	1.626	24:0	✓	382	351	339	353, 325, 297, 269...	91.1

† Refers to the peaks in Figure 3.3.
* McLafferty rearrangement ion ($C_3H_6O_2^{+\cdot}$) and base ion for saturated FAMEs.
‡ Relative retention times were relative to heptadecanoic acid methyl ester (17:0) by GC-MS analysis (helium was carrier gas).

(b) Unsaturated FAMEs

Mass spectra of cis-9-octadecenoic acid methyl ester (methyl oleate) is illustrated in Figure 3.6. Characteristic ions for unsaturated FAMEs in butter oil are summarised in Table 3.8. Molecular ions (M^+) were distinctive for unsaturated FAMEs, even more pronounced for dienes and trienes. The base peaks in mono-, di- and trienoic unsaturated FAMEs were m/z 55, 67 and 79 respectively. These ions may have been formed by addition of a hydrogen atom to the

reactive aliphatic carbon adjacent to a double bond. Other characteristic ions were $(M-32)^+$ (loss of methanol), $(M-31)^+$ (loss of a methoxyl radical), $(M-74)^+$, $(M-116)^+$ and a series ions of $[M-74-(CH_2)_n]^+$ (Table 3.8). An ion at m/z 108 was a characteristic fragment of polyunsaturated FAMEs of (n-3) family (Figure 3.7), while an ion at m/z 150 was of diagnostic value for (n-6) family. These ions were believed to represent fragments from the terminal region of the molecule (Fellenberg *et al* 1987).

Figure 3.6 Mass ion spectra of (a) cis-9-octadecenoic acid methyl ester in butter oil and (b) standard cis-9-octadecenoic acid methyl ester. GC-MS conditions referred to Figure 3.3.

While there were no ions that served to indicate the location or stereochemistry of double bonds in positional isomers of FAMEs, the accurate molecular mass ion together with retention times

and relative retention times of GC were of considerable value to identify these FAMEs. Information from the NBS library was also very useful, especially when the percentage of identification was high (> 90%).

Figure 3.7 Mass spectra of (a) cis-9,12,15-octadecatrienoic acid methyl ester in butter oil and (b) standard cis-9,12,15-octadecatrienoic acid methyl ester. GC-MS conditions referred to Figure 3.3.

Table 3.8 Characteristic ions of mono- and polyunsaturated FAMEs in butter oil †

Pk no.	t_r (min)	t_{rr} ‡	Peak identity	M^+	$(M-31)^+$	$(M-32)^+$	$(M-74)^+$	$(M-116)^+$	$[M-74-(CH_2)_n]^+$	library %ID
12	30.93	0.820	14:1(9c)	240	209	208	166	124	152,138,124,96...	82.6
18	35.03	0.929	16:1(Δ7)	268	237	236	194	152	166,152,138,124...	90.4
19	35.42	0.939	16:1(9c)	268	237	236	194	152	166,152,138,124...	92.0
26	41.92	1.111	18:1(9c)	296	265	264	222	180	180,166,152,138...	93.7
27	42.02	1.114	18:1(11c)	296	265	264	222	180	180,166,152,138...	91.6
28	42.29	1.121	18:1(Δ8)	296	265	264	222	180	180,166,152,138...	90.3
29	42.84	1.136	18:2(Δ12,15)	294	263	262	220	178	178,164,150,136...	79.1
30	43.77	1.160	18:2(9c,12c)	294	263	262	220	178	178,164,150,136...	86.9
32	44.06	1.168	18:2(Δ10,13)	294	263	262	220	178	178,164,150,136...	92.1
36	48.32	1.281	18:2(Δ8,11)	294	×	262	220	178	178,164,150,136...	93.4
35	47.04	1.247	18:3(9c,12c,15c)	292	261	×	×	176	176,162,148,134...	87.6

† Refers to the peaks in Figure 3.3. ‡ relative to 17:0.

(c) Mono-branched chain saturated FAMEs

Both iso and anteiso hexadecanoic acid methyl esters (16:0) showed a great number of fragments in the region m/z 74. This represented $C_3H_6O_2^+$ as in aliphatic saturated fatty acids, which was formed by McLafferty rearrangement. In the anteiso branching, $(M-29)^+$ ion appeared with more abundance than the $(M-31)^+$ ion (Figure 3.8), which was a distinctive feature for anteiso branched chain fatty acids (Jensen & Gross 1987). However, in the iso branching, $(M-29)^+$ ions were in low abundance and the ratio of $(M-29)^+$ / $(M-31)^+$ were less than 1. (Table 3.9).

Figure 3.8 Mass spectra of (a) 14-methyl hexadecanoic acid methyl ester (anteiso) in butter oil and (b) 15-methyl hexadecanoic acid methyl ester (iso) in butter oil. GC-MS conditions referred to Figure 3.3.

Table 3.9 Characteristic ions of mono-branched chain saturated FAMEs in butter oil †

Pk no.	t_r (min)	t_{rr} ‡	Peak identity	m/z 74	M^+	$(M-29)^+$	$(M-31)^+$	ratio *	$[M-29-(CH_2)_n]^+$	library % ID
8	27.15	0.720	12:0(anteiso)	✓	228	199	197	> 1	185,171,157,143...	86.2
10	27.97	0.742	13:0(iso)	✓	242	213	211	< 1	199,185,171,157...	87.9
14	31.52	0.836	14:0(anteiso)	✓	256	227	225	> 1	213,199,185,171...	85.2
16	33.44	0.887	15:0(iso)	✓	270	241	239	< 1	227,213,199,185...	90.9
20	35.99	0.954	16:0(iso)	✓	284	255	253	< 1	241,227,213,199...	91.1
21	36.44	0.966	16:0(anteiso)	✓	284	255	253	> 1	241,227,213,199...	92.4
24	39.05	1.035	17:0(iso)	✓	298	269	267	< 1	255,241,227,213...	83.5
33	44.23	1.173	18:0(iso)	✓	312	283	281	< 1	269,255,227,213...	90.4

† Refers to the peaks in Figure 3.3. ‡ relative to 17:0. * ratio of $(M-29)^+$ / $(M-31)^+$.

3.3 Analysis of standard monoacylglycerols by GC and GC-MS

3.3.1 Analysis of standard monoacylglycerols by GC

Standard monoacylglycerols as trimethylsilyl (TMS) ethers were separated by High Temperature Gas Chromatography on a non-polar column (Figure 3.9). With a temperature program, complete separation was achieved within 15 minutes for the 20 m column and 10 minutes for the 10 m column.

Retention times, relative retention times and response factors of standard monoacylglycerol TMS ethers were determined with relevance to 1-monotetradecanoyl-rac-glycerol TMS ether (Table 3.10). The response factors were approximately 1. It demonstrated that there was linear response of flame-ionised detector between monoacylglycerol TMS ethers.

Figure 3.9 Separation of standard 1-monoacyl-rac-glycerol TMS ethers by GC on a BP1 fused silica capillary column (20 m × 0.22 mm i.d., SGE). Temperature was programmed from 65 °C to 100 °C at 35 °C min^{-1}, 100 °C to 240 °C at 20 °C min^{-1} and from 240 °C to 300 °C at 12 °C min^{-1}. Retention times, relative retention times and response factors are given in Table 3.8.

Table 3.10 Retention times (t_r), relative retention times (t_{rr}) and response factors (F_r) of monoacylglycerol trimethylsilyl ethers relative to 1-14:0 †

MAG	CAS no ‡	t_r (min)		t_{rr}		F_r
		10 m column	20 m column	10 m column	20 m column	
1-10:0	26402-22-2	5.446±0.004	7.616±0.082	0.787±0.001	0.792±0.002	0.937±0.048
1-12:0	40738-26-9	6.112±0.007	8.583±0.010	0.883±0.001	0.893±0.002	0.926±0.020
1-14:0	75685-84-6	6.920±0.013	9.613±0.017	1.000±0.000	1.000±0.000	1.000±0.000
1-16:0	19670-51-0	7.734±0.026	10.658±0.013	1.118±0.002	1.109±0.003	1.017±0.027
1-18:1 (9c)	111-03-5	8.706±0.019	11.725±0.061	1.228±0.001	1.220±0.004	0.936±0.015
1-18:0	22610-63-5	8.499±0.016	11.584±0.089	1.258±0.001	1.205±0.005	1.040±0.035

† Refers to the peaks in Figure 3.9. Monoacylglycerol TMS ethers were analysed by BP1 10 m or 20 m × 0.22 mm i.d. capillary column. Star Chromatography Workstation was used to record the data. Values of t_r and t_{rr} were the mean of 3 silylation and 9 GC analyses ± standard deviation for each column. F_r values were the mean of the 18 GC analyses ± standard deviation. *Fr* values of 2-isomers were assumed as the same values as their corresponding 1(3)-isomers.
‡ CAS no: Chemical Abstract Service number.

3.3.2 Mass spectra of standard monoacylglycerol TMS ethers

The mass spectra of standard 1(3)-hexadecanoyl-sn-glycerol TMS ether (1(3)-16:0) was compared with NBS library spectra (Figure 3.10). Molecular ions could not be seen in the TMS ether with electron impact ionisation. However ions equivalent to $(M-15)^+$ (loss of a methyl group) could be used to determine the molecular weight. The ions of m/z 73 $[(CH_3)_3Si]^+$ and m/z 75 $[HO{=}Si(CH_3)_2]^+$ were found in all monoacylglycerol TMS ethers. A profile of the TMS compounds in a complex mixture can be indicated by a mass chromatogram of m/z 73 (Evershed 1993). The presence of an ion at m/z 147 $[(CH_3)_2Si{=}OSi(CH_3)_3]^+$ demonstrates there were two TMS groups in the molecules. This ion was characteristic of monoacylglycerols. The ions of RCO^+ and $(RCO+74)^+$ were relatively abundant ions in both of the isomers. Characteristic ions at m/z 129 and 145 contained the parts of the glycerol backbones and TMS groups (Christie 1989).

Figure 3.10 Mass spectra of (1) standard 1(3)-monohexadecanoyl-sn-glycerol TMS ether and (b) 1(3)-hexadecanoyl-sn-glycerol TMS ether from NBS library. GC-MS conditions were the same as shown in the footnote of Figure 3.9.

Mass ions at $(M-103)^+$ was characteristic ions for 1(3)-monoacylglycerol TMS ethers. These ions were formed by cleavage between carbon 2 and 3. Small ions at m/z 205 were of further diagnostic value for the 1(3)-isomers.

3.4 Analysis of standard lipids by TLC

3.4.1 Separation of standards

(a) Standard acylglycerols and fatty acids

Standard mixtures of fatty acids and acylglycerols were separated on TLC plate (20 × 20 cm silica gel 60 Å, Whatman) (Figure 3.11).

Figure 3.11 One-dimensional Thin Layer Chromatography (TLC) analysis of standard mixtures of fatty acids and acylglycerols. TLC plates were developed in hexane : diethyl ether : formic acid (80:20:2, v/v/v) and visualised under iodine vapour. Lanes: 1. octanoic acid and trioctanoin (8:0); 2. decanoic acid, mono- and tridecanoin (10:0); 3. dodecanoic acid, mono-, di- and tridodecanoin (12:0); 4. tetradecanoic acid, mono- and tritetradecanoin (14:0); 5. hexadecanoic acid, mono- and trihexadecanoin (16:0); 6. cis-9-octadecenoic acid, cis-9-mono-, di- and trioctadecenoin (18:1(9c)); and 7. cis-9,12-octadecadienoic acid and cis-9,12-trioctadecadienoin (18:2(9c,12c). MAGs: monoacylglycerols, DAGs: diacylglycerols, TAGs: triacylglycerols, FAs: fatty acids.

Relatively non-polar triacylglycerols were nearest from solvent front, followed by free fatty acids, 1,3-diacylglycerols, 1,2-diacylglycerols and finally relative polar compounds, monoacylglycerols. Rf values of fatty acids and acylglycerols are given in Table 3.11.

(b) Standard methyl ketones

Standard mixtures of methyl ketones were separated on silica gel 60 Å TLC plate with different volumes of sample application (Figure 3.12). Rf values of methyl ketones were similar with the Rf values of some of the triacylglycerols (Table 3.11).

Figure 3.12 Separation of standard methyl ketones on silica gel 60 Å plate (20 × 5 cm, 0.25 mm layer thickness, Whatman). Mixtures of methyl ketones are 2-heptanone (18.3 mg), 2-octanone (23.4 mg), 2-decanone (21.2 mg) and 2-undecanone (22.7 mg) in 1 ml hexane. Lanes: 1. 5 µl application, 2. 3 µl application and 3. 2 µl application. Hexane-diethyl ether-formic acid (80:20:2 v/v/v) was the developing solvent. Plates were visualised by spraying 1% $KMnO_4$ in 2% Na_2CO_3.

3.4.2 Rf values of standard fatty acids, acylglycerols and methyl ketones

Separation of samples on TLC plates was measured with Rf value which was termed as distance moved by sample spot divided by distance moved by solvent front. Table 3.11 summarises Rf values of fatty acids, acylglycerols and methyl ketones. It demonstrated that Rf values of fatty acids and triacylglycerols varied slightly with the change of chain length.

Table 3.11 Rf values of fatty acids, acylglycerols and methyl ketones [†]

Lipid	Chain length	MAGs	1,2-DAGs	1,3-DAGs	FAs	TAGs	Methyl ketones
Fatty acids & acylglycerols [a]	8:0	NA	NA	NA	0.37	0.44	NA
	10:0	0.02	NA	NA	0.36	0.44	NA
	12:0	0.02	0.11	0.15	0.35	0.45	NA
	14:0	0.02	NA	NA	0.31	0.46	NA
	16:0	0.02	NA	NA	0.35	0.50	NA
	18:1 (9c)	0.02	0.13	0.17	0.36	0.51	NA
	18:2 (9c,12c)	NA	NA	NA	0.36	0.50	NA
Methyl ketones [b]		NA	NA	NA	NA	NA	0.56

[†] Results were the mean of three measurements. MAGs = monoacylglycerols; DAGs = diacylglycerols; FAs = fatty acids; TAGs = triacylglycerols.
NA = not added. ND = not detected.
[a] TLC separation was shown in Fig. 3.11.
[b] TLC separation was shown in Fig. 3.12.

CHAPTER 4

RESULTS

Separation, Identification, Structure and Fatty Acids Composition of Monoacylglycerols from Butter Oil

4.1 Separation of lipids derived from butter oil by TLC and PTLC

4.1.1 Effect of nitrogen sources on conversion of butter oil in solid-state culture

Figure 4.1 shows the separation of lipids produced in solid-state culture using butter oil as the main carbon source and a variety of organic nitrogen sources. The lipids were separated on TLC plates and details are given in the legend of Figure 4.1. Butter oil, trilaurin and mixtures of oleic acid and triolein were used as controls and separated under the same conditions. The separation pattern of mono-, di- and triacylglycerols and fatty acids was in agreement with the results from Christie (1989).

Monoacylglycerols were detected following conversion of butter oil by both spores and mycelium of *P. roquefortii* FRR 2456 and Wisbey PJ with casein or casein enzymatic hydrolysate as the nitrogen sources at pH 7.0 (Figure 4.1). The presence of Casein and Casein Enzymatic Hydrolysate promoted the production of monoacylglycerols (Figure 4.1 lanes 4-7, 10-13), while monoacylglycerols were hardly found when Casamino acids were added as the nitrogen source (Figure 4.1 lanes 8,9,14 & 15).

Figure 4.1 Separation of lipids produced from butter oil by *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C on silica gel 60 Å TLC plates (20 × 5 cm, 0.25 mm layer thickness, Whatman). Results were combined data of 5 TLC plates. Plates were eluted with hexane-diethyl ether-formic acid (80:20:2 v/v/v) and visualised with iodine vapour. Lanes: 1. butter oil, 2. trilaurin, 3. oleic acid and triolein, 4-9. FRR 2456, 10-15. Wisbey PJ, (4. mycelium & casein, 5. spores & casein, 6. mycelium & casein enzymatic hydrolysate, 7. spores & casein enzymatic hydrolysate, 8. mycelium & casamino acids, 9. spores & casamino acids, 10. mycelium & casein, 11. spores & casein, 12. mycelium & casein enzymatic hydrolysate, 13. spores & casein enzymatic hydrolysate, 14. mycelium & casamino acids, 15. spores & casamino acids). MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids, TAG: triacylglycerols.

4.1.2 Screening of lipids produced from butter oil at pH 7.0

Figure 4.2 shows the pattern of TLC separation of lipids derived from butter oil by the two strains of *P. roquefortii* at pH 7.0 in solid-state and suspension cultures. Rf values of the lipids are given in Table 4.1. Table 4.2 summarises all the degradation products derived from butter oil at 10 °C and 25 °C in the three reaction systems (two strains of *P. roquefortii* and the commercial lipase from *P. roquefortii*). Major results obtained from TLC separation are:

- Monoacylglycerols were produced by both strains of *P. roquefortii* and the commercial lipase from *P. roquefortii* after incubation in solid-state and suspension cultures. However, they were

not detected at 1 hour and 25 °C when butter oil was degraded by the two strains of *P. roquefortii* in suspension culture (Figure 4.2 and Table 4.2).

- Both structural isomers of diacylglycerols (1,2- and 1,3-) were detected in the original butter oil (Figure 4.2).

- In suspension culture more 1,3-diacylglycerols than the 1,2- (2,3-) isomers were derived from butter oil by both strains of *P. roquefortii* (Figure 4.2 and Table 4.2), however, 1,2- (2,3-) diacylglycerols were the main isomers when butter oil was hydrolysed by the commercial lipase (Table 4.2).

- In solid-state culture, on the other hand, more 1,2- (2,3-) diacylglycerols than the 1,3-diacylglycerols were found from butter oil by both strains of *P. roquefortii* (Figure 4.2 and Table 4.2).

- An extra band of diacylglycerols (DAG) was found after relatively long incubation and reaction time (7d at 25 °C or 20d at 10 °C in solid-state culture, 4h at 25 °C or 20h at 10 °C in suspension culture) (Figure 4.2 and Table 4.2). These diacylglycerols contained fatty acids with longer chains ($C \geq 14$).

- Methyl ketones were found with the two strains of *P. roquefortii* in solid-state and suspension cultures, however, they were not found with the commercial lipase from *P. roquefortii*. The mould has the ability to metabolise medium chain fatty acids and acylglycerols to give methyl ketones (Kinderlerer 1987, 1993).

- Lactones were detected in solid-state culture and in a few samples in suspension culture after 20 hours reaction when butter oil was hydrolysed by *P. roquefortii*. However, lactones were not found from butter oil with the commercial *P. roquefortii* lipase. Identification of lactones is discussed in Chapter 6.

Figure 4.2 Separation of lipids produced from butter oil by *P. roquefortii* at pH 7.0 on silica gel 60 Å TLC plate (20 × 5 cm, 0.25 mm layer thickness, Whatman). Plates were eluted with hexane-diethyl ether-formic acid (80:20:2 v/v/v) and visualised with iodine vapour followed by spraying 1% $KMnO_4$ in 2% Na_2CO_3. Plate 1: lipids produced in solid-state culture at 25 °C. Plate 2: lipids produced in suspension culture at 10 °C. Lanes: 1. butter oil, 2. *P. roquefortii* (FRR 2456) and 3. *P. roquefortii* (PJ).

Table 4.1 Rf values of lipids produced from butter oil by two strains of *P. roquefortii* at pH 7.0[†]

Lipid	**FRR 2456** solid-state cultures & 25 °C	**Wisbey PJ** suspension cultures & 10 °C
Monoacylglycerols	0.03 ± 0.01	0.02 ± 0.00
Lactones	0.12 ± 0.01	0.09 ± 0.00
1,2-diacylglycerols	0.15 ± 0.00	0.12 ± 0.01
1,3-diacylglycerols	0.18 ± 0.00	0.14 ± 0.00
diacylglycerols	0.22 ± 0.01	0.16 ± 0.01
Free fatty acids	0.34 ± 0.01	0.30 ± 0.00
Methyl ketones	0.55 ± 0.01	0.55 ± 0.01
Triacylglycerols	0.45 ± 0.01 - 0.61 ± 0.02	0.40 ± 0.01 - 0.56 ± 0.02

[†] Refers to separation in Figure 4.2.

Table 4.2 Degradation products derived from butter oil by two strains of *P. roquefortii* and a commercial lipase from *P. roquefortii* †

Condition	Fungal strain	T (°C)	pH	time	MAG	1,2-(2,3-) DAG	1,3-DAG	DAG	FA	MK	LAC
Butter					-	+	+	-	-	-	-
Solid-state cultures	FRR 2456	10	7.0	20d	++	++	+	+	++	+	+
		25	4.5	7d	+	++	+	+	+	++	+
			7.0	7d	+	++	+	-	+	+	+
	Wisbey PJ	10	7.0	20d	+	++	+	-	+	+	+
		25	4.5	7d	+	++	+	+	++	++	+
			7.0	7d	+	++	+	-	+	+	-
Suspension cultures	FRR 2456	10	7.0	5h	+	+	+	-	+	ND	-
				10h	+	+	+	-	++	ND	-
				15h	+	+	++	-	++	ND	-
				20h	+	+	++	+	+	ND	-
				25h	+	+	++	+	+	ND	-
		25	7.0	1h	-	+	+	-	+	ND	-
				2h	+	+	+	-	++	ND	-
				3h	+	+	++	-	+	ND	-
				4h	+	+	++	-	+	ND	-
	Wisbey PJ	10	7.0	5h	+	+	+	-	+	+	-
				10h	+	+	+	+	+	+	-
				15h	+	+	+	+	+	+	-
				20h	+	+	++	+	+	-	
				25h	+	+	++	+	+	+	+
		25	7.0	1h	-	+	+	-	-	-	-
				2h	+	+	+	-	+	-	-
				3h	+	+	++	-	+	-	-
				4h	+	+	++	-	+	-	-
	Commercial lipase from *P. roqueforti*	10	7.0	5h	+	++	+	-	+	-	-
				10h	+	++	+	-	+	-	-
				15h	+	++	+	-	+	-	-
				20h	+	++	+	+	+	-	-
				25h	+	++	+	+	+	-	-
		25	7.0	0.5h	+	++	+	-	+	-	-

† Degradation products were separated by TLC with 5 μl sample application. +: present, ++: present with higher concentration, -: absent. ND: not determined. MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids, MK: methyl ketones, LAC: lactones.

4.1.3 Separation of Lipids by PTLC

Separation of lipids from butter oil by Preparative Thin Layer Chromatography is shown in Figure 4.3. Relatively large amount of sample (~ 8 % w/v, 2 ml) were applied. Rf values varied slightly. However, acylglycerols and fatty acids were separated completely to give the same pattern of separation as in TLC analysis (Figure 4.2). Again relatively more 1,3-diacylglycerols

than the 1,2- (2,3-) diacylglycerols were found from butter oil by *P. roquefortii* in suspension culture.

Figure 4.3 Preparative Thin Layer Chromatography (PTLC) separation of lipids produced from butter oil by *P. roquefortii* (FRR 2456) in suspension culture at pH 7.0, 10 °C for 25 hours. Silica gel 60 F_{254} plate (20 × 20 cm with 20 × 4 cm concentrating zone and 0.5 mm layer thickness, Merck) was activated at 105 °C for 50 min. Plates were eluted with hexane : diethyl ether : formic acid (80:20:2 by volume). MAG: monoacylglycerols, DAG: diacylglycerols, TAG: triacylglycerols, FA: fatty acids.

4.2 Total mass chromatogram of TMS ethers of monoacylglycerols

Figure 4.4 shows the total ion chromatogram of monoacylglycerol TMS ethers derived from butter oil in the three systems (two fungal strains and the commercial lipase). Identification of the structural isomers is given in section 4.3 and the structural isomers composition of monoacylglycerols is given in section 4.4.

Figure 4.4 Total ion chromatograms of monoacylglycerol TMS ethers produced from butter at pH 7.0 and 10 °C by (a) *P. roquefortii* FRR 2456 in solid-state culture (20 d), (b) *P. roquefortii* FRR 2456 in suspension cultures (25 h) and (c) a commercial lipase from *P. roquefortii* in shake flasks (25 h). Monoacylglycerol TMS ethers were analysed by GC-MS. GC was programmed from 35 °C (with a initial stay of 0.5 min) to 240 °C at 20 °C min^{-1}, from 240 °C to 300 °C at 12 °C min^{-1}. Ion source was at 200 °C, interface temperature was at 250 °C. Peak names of monoacylglycerols are given in Table 5.1. Other peaks were contaminants (silanes, FAMEs, etc)

The TMS ethers of monoacylglycerols were separated in 16 minutes by GC-MS (Figure 4.4). There was some small variation in retention time (Table 4.3) as 20 cm was cut from the injector and detector ends each time the column was installed. The same column was used in the Varian 3400 gas chromatograph for GC analysis and in the Hewlett Packard 5890 for GC-MS analysis.

4.3 Mass spectra of 1(3)- and 2-monoacylglycerol TMS ethers

Characteristic ions of 1(3)- and 2-monoacyl-sn-glycerol TMS ethers are summarised in Table 4.3. Mass spectra of homologous series of 1(3)- and 2-monoacylglycerol TMS ethers derived from butter oil are shown in Figure 4.6 - 4.14.

The most abundant ions at $(M-103)^+$ were found in all 1 or 3- monoacyl-sn-glycerol TMS ethers (Table 4.3, Figure 4.6-4.14). These ions were $[M - (CH_3)_3SiOCH_2]^+$ and were due to the cleavage between carbons 2 and 3, or 1 and 2. The ion at m/z 205 was characteristic for all 1(3)- isomers. This fragment was $[(CH_3)_3SiO]_2C_2H_3^+$ and was due to cleavage of TMS groups and part of the glycerol backbone of the 1(3)-isomers. The ion at m/z 218 was characteristic of the spectra of all 2- isomers (Table 4.3). This ion was $(M - RCOOH)^{+\cdot}$ and was due to cleavage of TMS groups and part of the glycerol backbone of the 2-isomers and was derived by a Mclaffertty rearrangement (Figure 4.5). Common ions at m/z 73, 75, 129 and 147 were found respectively for all monoacylglycerol TMS ethers and repeated $(M-15)^+$, RCO^+ and $(RCO+74)^+$.

Table 4.3 Identification of monoacylglycerol TMS derivatives (conversion at pH 7.0 & 10 °C)[†]

Reaction system	Peak identity		t_r (min)	m/z 205	m/z 218	$(M-103)^+$	$(M-15)^+$	RCO^+	$(RCO+74)^+$	Library ID %
(a) *P. roquefortii* FRR 2456, cultured in Petri dishes for 20 d	1(3)-isomer	10:0	10.59	+	-	287	375	155	229	87.2
		12:0	12.13	+	tr	315	403	183	257	86.5
		14:0	13.20	+	tr	343	431	211	285	90.1
		15:0	13.72	+	tr	357	445	225	299	90.3
		16:0	14.37	+	tr	371[a]	459	239	313	91.6
		18:0	15.45	+	tr	399	487	267	341	89.4
		18:1(9c)	15.25	+	tr	397	485	265	339	85.9
	2-isomer	10:0	10.50	-	+	-	375	155	229	85.4
		12:0	11.95	-	++	-	403	183	257	84.9
		14:0	13.00	-	++	-	431	211	285	87.1
		15:0	13.52	-	++	tr	445	225	299	84.7
		16:0	14.13	-	++	tr	459	239	313	89.9
		18:0	15.20	-	++	-	487	267	341	84.2
		18:1(9c)	15.02	-	++	tr	485	265	339	82.9
(b) *P. roquefortii* FRR 2456, cultured in shake flasks for 25 h	1(3)-isomer	10:0	10.77	+	-	287	375	155	229	85.9
		12:0	11.73	+	tr	315	403	183	257	90.3
		14:0	12.77	+	-	343	431	211	285	91.0
		16:0	13.85	+	tr	371	459	239	313	90.8
		18:0	14.87	+	tr	399	487	267	341	89.8
		18:1(9c)	14.73	+	-	397	485	265	339	85.9
	2-isomer	14:0	12.57	-	++	-	431	211	285	80.3
		16:0	13.62	-	+	tr	459	239	313	88.6
(c) lipase from *P. roquefortii* incubated in shake flasks for 25 h	1(3)-isomer	4:0	7.48	+	-	203	-	71[a]	145	86.2
		6:0	8.58	+	-	231	319	99[a]	173	84.5
		8:0	9.63	+	-	259[a]	347	127	201	87.2
		10:0	10.60	+	tr	287[a]	375	155	229	90.1
		12:0	11.55	+	-	315	403	183	257	92.2
		14:0	12.56	+	-	343[a]	431	211	285	95.4
		16:0	13.82	+	-	371	459	239	313	90.9
		18:0	14.87	+	tr	399	487	267	341	86.1
		18:1(9c)	14.73	+	tr	397	485	265	339	87.0
	2-isomer	4:0	7.63	-	+	-	-	71	145	88.4
		6:0	8.73	-	+	-	319	99	173	88.2
		8:0	9.77	-	+	-	347	127	201	88.0
		10:0	10.75	-	+++	-	375	155	229	80.4
		12:0	11.72	-	++	-	403	183	257	81.2
		14:0	12.72	-	++	-	431	211	285	82.3
		16:0	13.65	-	++	-	459	239	313	89.3
		18:0	14.67	-	+	tr	487	267	341	84.1
		18:1(9c)	14.52	-	++	-	485	265	339	90.7

[†] Refers to peaks in Figure 4.4. Other characteristic ions for both 1(3)- and 2-monoacyl-sn-glycerol TMS ethers were m/z 73, m/z 75, m/z 129 and m/z 147. tr = trace, abundance was less than 5% of the base ion. +: abundance was between 20% and 49% of the base ion. ++: abundance was between 50% and 90% of the base ion. +++: base ion. -: not detected.

[a] This is the most abundant ion (base ion) in the mass spectra.

Figure 4.5 Mechanism for formation of characteristic ions of 1(3)- and 2-monoacylglycerol TMS ethers in GC-MS analysis.

Figure 4.6 Mass spectra of (a) 1(3)- and (b) 2-monobutanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.7 Mass spectra of (a) 1(3)- and (b) 2-monohexanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.8 Mass spectra of (a) 1(3)- and (b) 2-monooctanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.9 Mass spectra of (a) 1(3)- and (b) 2-monodecanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.10 Mass spectra of (a) 1(3)- and (b) 2-monododecanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.11 Mass spectra of (a) 1(3)- and (b) 2-monotetradecanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.12 Mass spectra of (a) 1(3)- and (b) 2-monohexadecanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.13 Mass spectra of (a) 1(3)- and (b) 2-monooctadecanoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (c)). GC-MS conditions were shown in the footnote of Figure 4.4.

Figure 4.14 Mass spectra of (a) 1(3)- and (b) 2-cis-9-monooctadecenoyl-sn-glycerol TMS ethers from butter oil by a lipase from *P. roquefortii* at pH 7.0, 10 °C for 25 h (Peaks in Figure 4.4 (a)). GC-MS conditions were shown in the footnote of Figure 4.4.

4.4 Structural isomers of monoacylglycerols

4.4.1 Solid-state culture

A wide range of fatty acids were detected in MAGs ($C_{8:0}$ - $C_{20:0}$) derived from butter oil (Table 4.4). More 1 and 3 monoacyl-sn-glycerols than 2-monoacyl-sn-glycerols were produced by both strains of *P. roquefortii* at 10 °C and 25 °C (Table 4.4) at a ratio of approximately 65 : 35. 1 and 3 Monopalmityl-sn-glycerols (monopalmitin) were the main two isomers except for produced from *P. roquefortii* (Wisbey PJ) at 10 °C when it was 2-monooleinyl-sn-glycerol (2-monoolein) (Table 4.4).

4.4.2 Suspension culture

The composition and the molar ratio of the monoacylglycerols derived from butter oil is given in Table 4.5. Like the composition described in section 4.4.1, a wide range of fatty acids were detected in the monoacylglycerols ($C_{8:0}$ - $C_{20:0}$). Almost 90 % of the monoacylglycerols were 1 and 3 monoacyl-sn-glycerols and relatively low concentration of the 2-monoacyl-sn-glycerols were found. 1 and 3-Monopalmitin was the major 1(3)-isomer whilst 2-monopalmitin was the major 2-isomer. A decrease in the percent of the 1 and 3 monoacyl-sn-glycerols occurred at 10 °C from approximately 90 % at 5 hour to 83 % at 25 hour. The differences represented the change from conversion by fungal spores to conversion by fungal mycelium.

4.4.3 Lipolysis

The composition and the molar ratio of the monoacylglycerols derived from butter oil by lipolysis is given in Table 4.6. There was a considerable decrease in the proportion of the 1 and 3 monoacyl-sn-glycerols (approximately 30 %). The 2-monoacyl-sn-glycerols were the major products at 10 °C and 25 °C. 1(3)-Monopalmitin and 2-monopalmitin were the major

monoacylglycerols produced. Unlike sections 4.4.1 and 4.4.2, there were higher concentrations of short and medium chain length monoacylglycerols ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$).

Table 4.4 Structural isomers of monoacylglycerols produced from butter oil by two strains of *P. roquefortii* in solid-state culture at pH 7.0 *

temperature & time	Fungal strain	MAG	% composition (mol)		molar ratio †	
			sn-1(3)	sn-2	sn-1(3)	sn-2
10.0 ± 0.2 °C 20 days	FRR 2456	10:0	0.64 ± 0.09	0.37 ± 0.06	1.72 ± 0.20	1.00 ± 0.00
		12:0	0.56 ± 0.08	0.63 ± 0.08	1.42 ± 0.09	1.68 ± 0.13
		14:0	3.96 ± 0.19	3.20 ± 0.14	10.69 ± 0.34	8.66 ± 0.31
		16:0	26.71 ± 0.14	15.59 ± 0.15	73.59 ± 1.30	42.84 ± 0.85
		18:0	22.80 ± 0.68	6.39 ± 0.20	63.75 ± 0.89	17.69 ± 0.44
		18:1 (9c)	7.33 ± 0.14	7.05 ± 0.13	20.31 ± 0.64	19.52 ± 0.62
		Other [a]	2.31 ± 0.10	2.47 ± 0.07	6.29 ± 0.21	6.75 ± 0.17
		Totals	64.31	35.70	197.28	78.62
	Wisbey PJ	10:0	ND	ND	ND	ND
		12:0	ND	ND	ND	ND
		14:0	10.77 ± 1.41	8.24 ± 1.64	1.31 ± 0.16	1.00 ±0.00
		16:0	19.19 ± 0.80	11.06 ± 0.60	2.33 ± 0.09	1.34 ± 0.07
		18:0	6.26 ± 0.53	ND	0.76 ± 0.06	ND
		18:1 (9c)	30.81 ± 3.08	12.48 ± 0.65	3.74 ± 0.36	1.51 ± 0.07
		Other [a]	1.19 ± 0.21	ND	0.14 ± 0.02	ND
		Totals	68.22	31.78	8.28	3.86
24.9 ± 0.3 °C 7 days	FRR 2456	10:0	ND	ND	ND	ND
		12:0	1.19 ± 0.07	1.00 ± 0.07	1.19 ± 0.08	1.00 ± 0.00
		14:0	4.46 ± 0.19	3.70 ± 0.14	4.46 ± 0.20	3.70 ± 0.15
		16:0	25.69 ± 0.14	14.61 ± 0.15	25.69 ± 0.15	14.61 ± 0.16
		18:0	23.30 ± 0.68	6.89 ± 0.19	23.30 ± 0.70	6.89 ± 0.19
		18:1 (9c)	10.02 ± 0.14	4.35 ± 0.17	10.02 ± 0.15	4.35 ± 0.17
		Other [a]	2.51 ± 0.09	2.28 ± 0.07	2.51 ± 0.10	2.28 ± 0.08
		Totals	67.17	32.83	65.17	34.83
	Wisbey PJ	8:0	ND	0.36 ±0.10	ND	0.65 ± 0.18
		10:0	0.53 ± 0.04	0.55 ± 0.11	0.96 ± 0.07	1.00 ± 0.00
		12:0	1.27 ± 0.14	1.13 ± 0.15	2.31 ± 0.25	2.05 ± 0.27
		14:0	7.02 ± 0.10	5.79 ± 0.28	12.76 ± 0.18	10.53 ± 0.51
		16:0	24.29 ± 1.06	15.01 ± 0.34	44.16 ± 1.93	27.29 ± 0.62
		18:0	11.60 ± 0.11	ND	21.09 ± 0.20	ND
		18:1 (9c)	18.74 ± 0.31	9.84 ± 0.11	34.07 ± 0.56	17.89 ± 0.20
		Other [a]	2.10 ± 0.18	1.78 ± 0.13	3.82 ± 0.20	3.24 ± 0.24
		Totals	65.55	34.46	119.18	62.65

* Results are the mean of six GC analyses ± standard deviation. MAGs were derived from one fermentation and analysed as trimethylsilyl ethers by GC. ND: not detected.

† Calculated relative to 2-monodecanoylglycerol (2-10:0) for PJ at 25 °C and FRR 2456 at 10 °C and to 2-monotetradecanoylglycerol (2-14:0) for PJ at 10 °C. Calculated relative to 2-monododecanoylglycerol (2-12:0) for FRR 2456 at 25 °C.

‡ Other MAGs were 1(3)-15:0, 2-15:0, 1(3)-17:0, 2-17:0, 1(3)-20:0, 2-20:0 or some of them.

Table 4.5 Structural isomers of monoacylglycerols produced from butter oil by two strains of *P. roquefortii* in suspension culture at pH 7.0 *

temperature & time	Fungal strain	MAG	% composition (mol)		molar ratio †	
			sn-1(3)	sn-2	sn-1(3)	sn-2
9.9 ± 0.2 °C 5 hour	*P. roquefortii* spores (FRR 2456)	10:0	ND	ND	ND	ND
		12:0	7.14 ± 0.75	ND	1.00 ± 0.00	ND
		14:0	9.19 ± 0.56	0.80 ± 0.07	1.29 ± 0.08	0.11 ± 0.02
		16:0	38.37 ± 1.38	3.16 ± 0.43	5.37 ± 0.20	0.44 ± 0.06
		18:0	25.98 ± 1.07	1.93 ± 0.22	3.64 ± 0.15	0.27 ± 0.03
		18:1 (9c)	8.09 ± 0.62	3.20 ± 0.31	1.13 ± 0.09	0.45 ± 0.04
		Other [a]	0.93 ± 0.11	1.21 ± 0.14	0.13 ± 0.03	0.17 ± 0.02
		Totals	89.70	10.30	12.56	1.44
	P. roquefortii spores (Wisbey PJ)	10:0	1.70 ± 0.25	ND	1.00 ± 0.00	ND
		12:0	3.15 ± 0.46	ND	1.86 ± 0.27	ND
		14:0	14.86 ± 1.11	0.79 ± 0.15	8.76 ± 0.65	0.46 ± 0.09
		16:0	40.18 ± 1.92	2.39 ± 0.37	23.69 ± 1.11	1.41 ± 0.21
		18:0	15.48 ± 0.98	3.59 ± 0.55	9.13 ± 0.57	2.12 ± 0.32
		18:1 (9c)	11.64 ± 0.89	2.69 ± 0.23	6.86 ± 0.52	1.59 ± 0.13
		Other [a]	1.39 ± 0.22	2.16 ± 0.17	0.82 ± 0.13	1.27 ± 0.10
		Totals	88.39	11.61	52.12	6.84
9.8 ± 0.2 °C 25 hour	*P. roquefortii* mycelium (FRR 2456)	10:0	ND	ND	ND	ND
		12:0	7.13 ± 0.65	ND	1.00 ± 0.00	ND
		14:0	11.80 ± 0.72	2.09 ± 0.23	1.66 ± 0.10	0.29 ± 0.03
		16:0	36.92 ± 1.85	4.50 ± 0.15	5.18 ± 0.36	0.63 ± 0.03
		18:0	10.04 ± 0.53	3.89 ± 0.41	1.41 ± 0.13	0.55 ± 0.06
		18:1 (9c)	16.45 ± 0.71	5.19 ± 0.23	2.31 ± 0.09	0.73 ± 0.03
		Other [a]	0.87 ± 0.12	1.08 ± 0.49	0.12 ± 0.04	0.15 ± 0.07
		Totals	83.24	16.76	11.68	2.35
	P. roquefortii mycelium (Wisbey PJ)	10:0	3.22 ± 0.26	ND	1.00 ± 0.00	ND
		12:0	2.66 ± 0.19	ND	0.83 ± 0.06	ND
		14:0	12.88 ± 1.16	1.55 ± 0.35	4.00 ± 0.36	0.48 ± 0.11
		16:0	33.57 ± 0.65	7.78 ± 0.36	10.42 ± 0.20	2.41 ± 0.11
		18:0	7.32 ± 0.45	2.33 ± 0.48	2.27 ± 0.14	0.72 ± 0.15
		18:1 (9c)	22.57 ± 1.93	4.09 ± 0.23	7.01 ± 0.60	1.27 ± 0.07
		Other [a]	1.21 ± 0.70	0.82 ± 0.49	0.37 ± 0.22	0.25 ± 0.15
		Totals	83.43	16.56	25.90	5.14
25.0 ± 0.2 °C 4 hour	*P. roquefortii* (FRR 2456)	10:0	2.99 ± 0.56	ND	1.00 ± 0.00	ND
		12:0	3.07 ± 0.34	ND	1.03 ± 0.11	ND
		14:0	6.92 ± 1.06	ND	2.32 ± 0.35	ND
		16:0	30.86 ± 0.96	4.59 ± 0.99	10.34 ± 0.32	1.54 ± 0.33
		18:0	21.07 ± 0.71	3.23 ± 0.15	7.06 ± 0.24	1.08 ± 0.05
		18:1 (9c)	16.65 ± 0.91	ND	5.58 ± 0.30	ND
		Other [a]	5.65 ± 0.39	4.97 ± 0.09	1.89 ± 0.13	1.67 ± 0.03
		Totals	87.21	12.79	29.26	4.29
	P. roquefortii (Wisbey PJ)	10:0	2.99 ± 0.04	ND	1.00 ± 0.00	ND
		12:0	2.43 ± 0.13	ND	0.81 ± 0.04	ND
		14:0	6.95 ± 0.35	ND	2.33 ± 0.12	ND
		16:0	30.75 ± 0.69	7.56 ± 0.18	10.29 ± 0.23	2.53 ± 0.06
		18:0	19.69 ± 0.16	3.11 ± 0.08	6.59 ± 0.05	1.04 ± 0.03
		18:1 (9c)	19.57 ± 0.42	3.01 ± 0.08	6.55 ± 0.14	1.01 ± 0.04
		Other [a]	1.98 ± 0.01	1.98 ± 0.16	0.66 ± 0.05	0.66 ± 0.01
		Totals	84.35	15.66	28.23	5.24

* Results are the mean of six GC analyses ± standard deviation. MAGs were derived from one fermentation and analysed as trimethylsilyl ethers by GC. ND: not detected.

† Calculated relative to 1(3)-monododecanoylglycerol (1(3)-12:0) for FRR 2456 at 10 °C and to 1(3)-monodecanoylglycerol (1(3)-10:0) for the rest. [a] Other MAGs were 1(3)-15:0, 2-15:0, 1(3)-17:0, 2-17:0, 1(3)-18:2 (9c,12c), 1(3)-20:0, 2-20:0 or some of them.

Table 4.6 Structural isomers of monoacylglycerols produced from butter oil by a commercial lipase from *P. roquefortii* at pH 7.0 *

Temperature & time	MAG	% composition (mol)		molar ratio	
		1(3)-isomer	2-isomer	1(3)-isomer †	2-isomer †
9.9 ± 0.1 °C 25 hour	4:0	2.41 ± 0.37	0.92 ± 0.01	2.61 ± 0.41	1.00 ± 0.00
	6:0	1.81 ± 0.08	1.67 ± 0.06	1.96 ± 0.08	1.81 ± 0.07
	8:0	ND	1.97 ± 0.16	ND	2.13 ± 0.18
	10:0	0.97 ± 0.15	0.60 ± 0.05	1.05 ± 0.16	0.65 ± 0.05
	12:0	0.73 ± 0.22	1.78 ± 0.13	0.79 ± 0.24	1.93 ± 0.15
	14:0	2.40 ± 0.25	9.52 ± 0.60	2.60 ± 0.27	10.31 ± 0.65
	16:0	15.23 ± 0.97	26.92 ± 1.73	16.51 ± 1.05	29.18 ± 1.87
	18:0	4.20 ± 0.88	5.40 ± 0.27	4.55 ± 1.12	5.85 ± 0.29
	18:1 (9c)	8.20 ± 0.44	14.56 ± 0.71	8.88 ± 0.48	15.78 ± 0.77
	20:0	0.17 ± 0.01	0.55 ± 0.02	0.19 ± 0.01	0.60 ± 0.02
	Totals	36.12	63.89	39.14	69.24
25.0 ± 0.2 °C 0.5 hour	6:0	1.19 ± 0.06	ND	1.00 ± 0.05	ND
	8:0	1.12 ± 0.04	3.11 ± 0.23	0.94 ± 0.03	2.61 ± 0.19
	10:0	0.89 ± 0.11	3.77 ± 0.31	0.75 ± 0.09	3.17 ± 0.26
	12:0	1.22 ± 0.21	6.65 ± 0.42	1.02 ± 0.18	5.59 ± 0.35
	14:0	6.54 ± 0.82	23.76 ± 0.94	5.50 ± 0.69	19.98 ± 0.79
	16:0	14.18 ± 1.01	30.09 ± 1.85	11.93 ± 0.85	25.31 ± 1.56
	18:0	1.17 ± 0.21	0.52 ± 0.12	0.98 ± 0.18	0.44 ± 0.10
	18:1 (9c)	3.44 ± 0.42	1.57 ± 0.33	2.89 ± 0.35	1.32 ± 0.28
	20:0	0.20 ± 0.03	0.58 ± 0.05	0.17 ± 0.03	0.49 ± 0.04
	Totals	29.95	70.05	25.19	58.92

* Results are the mean of six GC analyses ± standard deviation. Monoacylglycerols were derived from one reaction, one extraction and analysed as trimethylsilyl ethers by GC. ND: not detected.
† Calculated relative to 2-monobutanoylglycerol (2-4:0) at 10 °C and to 1(3)-monohexanoylglycerol (1(3)-6:0) at 25 °C.

4.5 Fatty acids composition of monoacylglycerols and free fatty acids (as FAMEs)

4.5.1 Fatty acids composition of monoacylglycerols

The fatty acids composition of monoacylglycerols produced in solid-state culture, suspension culture and by lipolysis at 10 °C and 25 °C is given in Table 4.7. The composition of medium chain length fatty acids ($C_{6:0}$ - $C_{12:0}$) have decreased compared to the original butter for most of the experiments except for $C_{12:0}$ and $C_{14:0}$ in the monoacylglycerols derived by hydrolysis of butter oil with the commercial lipase.

Table 4.7 Fatty acid composition in monoacylglycerols produced from butter oil by *P. roquefortii* and a commercial lipase from *P. roquefortii* at pH 7.0 [†]

	Temperature (°C)	Reaction condition	C [a] (ml^{-1})	Fatty acid	6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1 (9c)	Other FAs [‡]
Butter oil			-	Mean	3.70	1.97	3.58	3.69	10.75	26.38	11.34	25.15	13.45
				SD	0.19	0.15	0.15	0.10	0.17	0.92	0.28	1.65	1.41
P. roquefortii (FRR 2456)	10.0 ± 0.2	solid-state culture (20 d)[f]	-	Mean	ND	ND	1.87	1.11	7.01	39.77	24.35	14.61	11.30
				SD	-	-	0.20	0.04	0.10	0.25	0.13	0.06	0.74
		suspension culture (25 h)[§]	3.79×10^5	Mean	ND	ND	0.97	2.22	9.88	27.54	21.17	20.30	17.93
				SD	-	-	0.03	0.07	0.20	0.76	1.23	0.55	0.77
		suspension culture (5 h) [§]	3.79×10^5	Mean	ND	ND	1.09	1.86	7.56	30.22	28.50	14.10	16.68
				SD	-	-	0.04	0.12	0.53	0.95	2.43	1.46	1.04
	25.0 ± 0.3	solid-state culture (7 d)[f]	-	Mean	ND	ND	ND	2.52	8.51	34.41	26.56	14.82	13.19
				SD	-	-	-	0.08	0.15	0.18	0.14	0.16	0.64
		suspension culture (4 h) [§]	3.60×10^5	Mean	ND	ND	1.16	2.41	8.20	29.97	21.69	17.48	19.09
				SD	-	-	0.47	0.19	0.62	0.80	0.75	0.81	0.92
P. roquefortii (Wisbey PJ)	9.8 ± 0.2	solid-state culture (20 d)[f]	-	Mean	ND	0.43	0.92	2.36	11.54	23.19	6.65	38.08	16.84
				SD	-	0.01	0.03	0.04	0.11	0.18	0.07	0.56	0.73
		suspension culture (25 h)[§]	5.48×10^5	Mean	1.95	ND	1.42	2.27	8.95	26.02	18.55	23.12	17.73
				SD	0.08	-	0.05	0.08	0.41	0.63	1.99	0.92	1.18
		suspension culture (5 h) [§]	5.48×10^5	Mean	ND	ND	1.35	2.11	7.20	24.19	26.42	20.46	18.27
				SD	-	-	0.13	0.12	0.46	0.86	1.29	0.67	0.86
	25.0 ± 0.2	solid-state culture (7 d)[f]	-	Mean	ND	0.33	1.02	2.89	10.71	34.63	13.05	23.99	13.39
				SD	-	0.02	0.01	0.02	0.05	0.15	1.54	1.50	0.18
		suspension culture (4 h) [§]	3.63×10^5	Mean	ND	ND	1.06	2.50	11.03	31.20	16.71	22.52	14.99
				SD	-	-	0.01	0.08	0.39	1.10	0.81	0.64	1.15
Lipase from *P. roquefortii* (E.C.3.1.1.3)	9.9 ± 0.1	suspension culture (5 h) [§]	1.01±0.02 mg	Mean	1.05	0.70	1.81	4.20	14.67	35.43	8.94	17.98	15.22
				SD	0.11	0.18	0.07	0.10	0.23	0.35	0.13	0.19	0.90
	25.0 ± 0.2	suspension culture (0.5h)[§]	1.03±0.02 mg	Mean	0.44	0.30	1.97	3.92	14.60	34.07	8.77	18.26	17.68
				SD	0.01	0.01	0.37	0.17	0.21	1.20	0.15	0.93	0.11

[†] Results are in composition % (mol) and are the mean of six GC analyses. MAGs were derived from one reaction, one extraction and fatty acids were analysed as their methyl esters by GC. SD: standard deviation. ND: not detected. [a] Concentration of spores or lipase per ml reaction mixtures. [‡] Other fatty acids were 13:0, 13:0 (iso), 14:1 (9c), 15:0, 16:1 (9c), 16:1 (7c), 16:1 (9c), 16:0 (iso), 16:0 (anteiso), 17:0, 17:0 (iso), 18:1 (11c), 18:1 (Δ5), 18:2 (9c,12c), 18:2 (12c,15c), 18:3 (6c,9c,12c), 18:3 (9c,12c,15c), 20:0, 22:0 and 24:0 or some of them. [f] Butter oil (4 %, w/v) was added as carbon source for fermentation. [§] Butter oil concentration is 16.7 % (w/v).

There was a tendency for increase of palmitic acid ($C_{16:0}$) in monoacylglycerols in the conversions catalysed by *P. roquefortii* and the commercial lipase from *P. roquefortii*. This is in agreement with the composition of monoacylglycerol structural isomers where 1(3)-monopalmitin was the main monoacylglycerol (Tables 4.4-4.6). Oleic acid ($C_{18:1(9c)}$) concentration decreased in almost all systems. However, an exceptional increase of oleic acid was found in monoacylglycerols produced by *P. roquefortii* Wisbey PJ in solid-state cultures at 10 °C.

4.5.2 Fatty acids composition in monoacylglycerols and free fatty acids in solid-state culture at pH 4.5

Fatty acids composition of monoacylglycerols and free fatty acids produced by the two strains of *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C is given in Table 4.8. Results were compared with the fatty acid composition of the original butter oil.

The results indicated that the medium chain fatty acids in the two strains were lowered compared with the concentration of these acids in the original butter oil. The composition of oleic acid was reduced in monoacylglycerols compared with the composition in butter oil, but there was an increase in the percent oleic acid in the free fatty acids. More palmitic acid was found in monoacylglycerols than in free fatty acids for both strains. Concentration of stearic acid in both monoacylglycerols and free fatty acids remained unchanged after conversion by *P. roquefortii* Wisbey PJ but the concentrations were slightly higher than in butter oil when the second strain of *P. roquefortii* FRR 2456 was used. The percent of γ-linolenic acid (18:3 (9c,12c,15c)) was significantly increased in monoacylglycerols with *P. roquefortii* FRR 2456.

Table 4.8 Fatty acids composition in monoacylglycerols and free fatty acids produced from butter oil by two strains of *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C (Results in wt %) †

Fatty acid	Unreacted butter oil (TAGs)	*P. roquefortii* (FRR 2456)		*P. roquefortii* (Wisbey PJ)	
		MAGs	FFAs	MAGs	FFAs
6:0	1.51±0.30	ND	ND	ND	ND
8:0	0.96±0.03	0.74±0.00	0.05±0.00	2.20±0.14	0.21±0.01
10:0	2.63±0.07	0.87±0.07	0.18±0.00	1.46±0.07	0.35±0.02
12:0	3.71±0.06	1.62±0.42	0.85±0.01	2.85±0.10	0.94±0.03
13:0	ND	0.64±0.00	0.06±0.00	0.36±0.01	ND
14:0	10.54±0.05	6.17±0.62	4.44±0.06	9.48±0.21	5.59±0.14
14:1 (9c)	0.83±0.01	0.74±0.19	0.23±0.02	0.72±0.01	0.28±0.02
15:0	1.13±0.01	1.90±0.30	0.79±0.04	1.54±0.07	1.01±0.04
16:0	26.86±0.09	28.48±0.92	25.41±1.60	38.30±0.21	25.85±0.42
16:1 (9c)	1.51±0.04	1.16±0.39	0.94±0.02	1.02±0.00	1.28±0.05
18:0	16.07±0.19	20.68±0.39	23.88±1.03	16.35±0.13	15.04±0.40
18:1 (9c)	29.87±0.21	17.16±1.18	37.46±0.33	21.48±0.15	44.29±0.46
18:2 (9c,12c)	2.95±0.02	2.16±0.46	2.94±0.55	0.93±0.08	3.20±0.08
18:3(6c,9c,12c)	0.11±0.01	13.56±0.16	1.83±0.50	0.19±0.04	0.30±0.01
18:3(9c,12c,15c)	0.66±0.01	0.87±0.21	0.38±0.10	2.40±0.02	0.65±0.01
20:0	0.21±0.03	1.14±0.00	0.50±0.06	0.36±0.01	0.82±0.09
22:0	ND	1.16±0.00	0.06±0.01	0.19±0.01	0.04±0.00
24:0	0.46±0.01	0.49±0.00	ND	0.18±0.01	0.08±0.01
Total 6:0 - 12:0	8.81±0.28	3.23±0.38	1.08±0.09	6.51±0.15	1.50±0.07
Total 14:0 +	91.20±0.75	96.77±1.19	98.92±1.78	93.49±0.59	98.50±0.69
Total	100.01	100.00	100.00	100.00	100.00

† Results are the mean of three GC analysis. MAGs and FAs were derived from one fermentation and one extraction. TAGs = triacylglycerols, MAGs = monoacylglycerols, FFAs = free fatty acid. ND = not detected.

4.5.3 Free fatty acids composition in suspension culture at pH 7.0

Table 4.9 shows that there was a reduction in the percent medium chain fatty acids $C_{6:0}$ - $C_{12:0}$ in the monoacylglycerols compared to the composition in the unreacted butter oil for both fungal strains. The percent composition of these acids after lipolysis was relatively unchanged compared with butter oil. The concentration of oleic acid increased in free fatty acids compared with the composition of this acid in butter oil. Changes in other fatty acids were slight.

Table 4.9 Free fatty acids produced by hydrolysis of butter oil with *P. roquefortii* spores or lipase at pH 7.0, 10 °C and 25 h (results in mol %) †

Fatty acid	Unreacted butter oil	*P. roquefortii* (FRR 2456) [a]	*P. roquefortii* (Wisbey PJ) [b]	Lipase from *P. roquefortii* [c]
6:0	3.70 ± 0.19	0.50 ± 0.05	ND	2.29 ± 0.04
8:0	1.97 ± 0.15	0.51 ± 0.08	0.53 ± 0.07	1.54 ± 0.05
10:0	3.58 ± 0.15	1.35 ± 0.18	1.48 ± 0.16	1.94 ± 0.85
12:0	3.69 ± 0.10	2.85 ± 0.32	3.19 ± 0.29	3.33 ± 0.53
14:0	10.75 ± 0.17	9.20 ± 0.83	9.65 ± 0.83	8.78 ± 1.33
16:0	26.38 ± 0.92	27.30 ± 1.03	26.10 ± 1.04	23.44 ± 1.90
18:0	11.34 ± 0.28	11.74 ± 0.55	12.49 ± 0.29	12.28 ± 0.99
18:1 (9c)	25.15 ± 1.65	30.31 ± 1.62	29.71 ± 2.23	30.48 ± 2.34
Other FAs [d]	13.45 ± 1.41	16.23 ± 0.18	16.85 ± 1.09	15.93 ± 1.43
Total 6:0 - 12:0	12.94 ± 0.23	5.21 ± 0.21	5.20 ± 0.30	9.10 ± 0.78
Total 14:0 +	87.07 ± 1.75	94.78 ± 1.66	94.80 ± 2.01	90.91 ± 2.19
Totals	100.01	99.99	100.00	100.01

† Results are the mean of six GC analyses ± standard deviation. Free fatty acids were derived from one reaction and one extraction. ND: not detected. Substrate concentration was 16.7 % (w/v).
[a] Concentration of spores was 3.79×10^5 per ml reaction mixture.
[b] Concentration of spores was 5.48×10^5 per ml reaction mixture.
[c] Concentration of lipase was 1.01 ± 0.02 mg per ml reaction mixture.
[d] Other fatty acids from butter oil were 13:0, 13:0 (iso), 14:1 (9c), 15:0, 16:1 (7c), 16:1 (9c), 16:0 (iso), 16:0 (anteiso), 17:0, 17:0 (iso), 18:1 (11c), 18:1 (Δ5), 18:2 (9c,12c), 18:2 (12c,15c), 18:3 (6c,9c,12c), 18:3 (9c,12c,15c), 20:0, 22:0 and 24:0 or some of them.

4.6 Yields of monoacylglycerols and free fatty acids

4.6.1 Yields of monoacylglycerols in solid-state culture

Yields of monoacylglycerols produced from butter oil in solid-state cultures were between 0.80 and 3.46 g / 100 g butter oil (Table 4.10). More monoacylglycerols were produced at 25 °C than at 10 °C. Comparing the two strains, *P. roquefortii* FRR 2456 produced more monoacylglycerols than Wisbey PJ.

Table 4.10 Yields of monoacylglycerols produced from butter oil by two strains of *P. roquefortii* in solid-state cultures at pH 7.0 (results in g / 100 g oil)

Strain	FRR 2456		Wisbey PJ	
Temperature (°C)	9.9 ± 0.2	25.0 ± 0.2	10.0 ± 0.2	25.1 ± 0.2
Agar medium (g)	98.20	102.12	95.54	101.15
Extracted oil (g)	3.73	3.69	3.80	4.83
Extracted oil (mg / ml hexane)	74.6	73.8	76.0	96.6
Volume (ml) †	3.0	2.0	2.0	3.0
Lipids (mg) ‡	223.8	147.6	152.0	289.8
Extracted monoacylglycerols (mg)	3.1	5.1	1.2	5.0
Yield (g / 100 g oil)	1.39	3.46	0.79	1.72

† Volume of samples applied on Preparative Thin Layer plates.
‡ Total lipids applied on Preparative Thin Layer plates (calculated from extracted oil and volume of applied sample).

4.6.2 Yields of monoacylglycerols and free fatty acids in suspension culture

Yields of monoacylglycerols from butter oil by *P. roquefortii* FRR 2456 in suspension cultures (Table 4.11) were less than the yields in solid-state cultures.

Table 4.11 Yields of monoacylglycerols and free fatty acids converted from butter by *P. roquefortii* FRR 2456 in suspension cultures at pH 7.0, 10 °C and 25 h †

Lipid	wt (mg)	wt % (g /100 g totals)	Mol (μmol)	mol % (mol / 100 mol totals)
MAGs	0.38 ± 0.02	0.19 ± 0.01	1.44 ± 0.06	0.18 ± 0.01
FAs	1.29 ± 0.10	0.62 ± 0.05	4.89 ± 0.38	0.60 ± 0.05
TAGs	205.51 ± 2.18	99.19 ± 0.06	812.42 ± 11.04	99.23 ± 0.06
Totals	207.19 ± 2.08	100.00	818.76 ± 10.67	100.00

† Results were the mean of six GC analyses ± standard deviation. Heptadecanoic acid (17:0) was added as internal standard.

CHAPTER 5

RESULTS

Separation, Identification, Structure and Fatty Acids Composition of Monoacylglycerols from Shea Oil

5.1 Separation of lipids derived from Shea oil by TLC and PTLC

5.1.1 Effect of mycelium and spores on conversion of Shea oil in solid state culture

Triacylglycerols were converted into monoacylglycerols in solid-state culture with Shea oil as the main carbon source and Casein Enzymatic Hydrolysate as an additional nitrogen source. Two strains of *P. roquefortii* (FRR 2456 & Wisbey PJ) were used. Separation of degradation products formed from Shea oil by mycelium and spores of *P. roquefortii* is given in Figure 5.1.

Monoacylglycerols and free fatty acids were produced by both mycelium and spores of *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C. Two structural isomers of diacylglycerols (1,2- (2,3-) and 1,3-) were separated on the TLC plates.

5.1.2 Screening of lipids produced from Shea oil at pH 7.0

Figure 5.2 shows the separation pattern of lipids produced from Shea oil by two strains of *P. roquefortii* in solid-state culture (plate 1) and suspension culture (plate 2) at pH 7.0. Table 5.1 summarises all the degradation products separated on TLC plates after conversion by the two strains of *P. roquefortii* and the commercial lipase from *P. roquefortii* in solid-state culture and suspension culture.

Figure 5.1 Separation of lipids produced from Shea oil by *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C on silica gel 60 Å plate (20 × 5 cm, 0.25 mm layer thickness, Whatman). Results are combination of 2 plates. Plates were eluted with hexane-diethyl ether-formic acid (80:20:2 v/v/v) and visualised with iodine vapour. Lanes: 1. Shea oil, 2. FRR 2456 (mycelium), 3. FRR 2456 (spores), 4. Wisbey PJ (mycelium), 5. Wisbey PJ (spores) and 6. standard mixture of oleic acid, mono-, di- and triolein. Concentration of samples were about 2 % (w/v) and 2 µl samples were applied on TLC plates. MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids and TAG: triacylglycerols.

Figure 5.2 Separation of lipids produced from Shea oil by *P. roquefortii* at (a) 25 °C (plate 1, solid-state culture) and (b) 10 °C (plate 2, suspension culture) on silica gel 60 Å plate (20 × 5 cm, Whatman). Solvent elution system and visualisation were given in the footnote of Figure 5.1. Lanes: 1. unreacted Shea oil, 2. *P. roquefortii* (FRR 2456) and 3. *P. roquefortii* (PJ). MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids and TAG: triacylglycerols.

Table 5.1 Degradation products from Shea oil by two strains of *P. roquefortii* and a commercial lipase from *P. roquefortii* †

Condition	Fungal strain	T (°C)	pH	Time	MAG	1,2-DAG	1,3-DAG	DAG	FA	MK	LAC
Shea					-	+	+	-	-	-	-
Solid state culture	FRR 2456	10	7.0	20d	+	+	+	-	+	-	+
		25	4.5	7d	+	++	++	-	++	-	-
			7.0	7d	++	++	+	-	+	-	+
	Wisbey PJ	10	7.0	20d	++	++	++	-	++	+	+
		25	4.5	7d	+	++	++	-	+	-	-
			7.0	7d	+	+	+	-	++	-	+
Suspension culture	FRR 2456	10	7.0	5h	-	+	+	-	-	-	-
				10h	-	+	+	-	+	-	-
				15h	-	+	+	-	+	-	-
				20h	+	+	+	-	+	-	-
				25h	+	+	+	-	+	-	-
		25	7.0	1h	-	+	+	-	+	ND	-
				2h	-	+	+	-	+	ND	-
				3h	-	+	+	-	+	ND	-
				4h	+	+	+	-	+	ND	-
	Wisbey PJ	10	7.0	5h	-	+	+	-	-	-	-
				10h	-	+	+	-	+	-	-
				15h	+	+	+	-	+	+	-
				20h	+	+	+	-	+	+	
				25h	+	+	+	-	+	+	-
		25	7.0	1h	-	+	+	-	+	ND	-
				2h	+	+	+	-	+	ND	-
				3h	+	+	+	-	+	ND	-
				4h	+	+	+	-	+	ND	-
	Commercial lipase from *P. roquefortii*	10	7.0	5h	-	+	+	-	+	ND	-
				10h	+	+	+	-	+	ND	-
				15h	+	+	+	-	+	ND	-
				20h	+	+	+	-	+	ND	-
				25h	+	+	+	-	+	ND	-
		25	7.0	0.5h	+	+	+	-	+	ND	-

† Degradation products were separated by TLC analysis with 5 μl sample application. Methyl ketones were visualised by spraying with 1 % $KMnO_4$ in 2 % Na_2CO_3. +: present, ++: present with higher concentration, -: absent. ND: not determined. MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids, MK: methyl ketones, LAC: lactones.

Major results from the TLC separation are:

1. Monoacylglycerols and free fatty acids were detected in both cultures by the two strains of *P. roquefortii* (Figure 5.2 and Table 5.1). Both monoacylglycerols and free fatty acids were found with relatively high concentration in solid-state culture where longer incubation time (7d at 25 °C

and 20d at 10 °C) was used. In suspension culture monoacylglycerols and free fatty acids could not be detected with short reaction time (5h at 10 °C and 1h at 25 °C) (Table 5.1).

2. Both structural isomers of diacylglycerols were detected in the original Shea oil (Figure 5.2). Nearly equal amount of 1,2- (2,3-) and 1,3-diacylglycerols were found in degradation products produced by the two strains of *P. roquefortii* and the commercial lipase from *P. roquefortii* (Table 5.1). The concentrations of diacylglycerols were higher in solid-state culture than in suspension culture.

3. Unlike the situation in butter oil (4.1.2), there was no extra bands of diacylglycerols produced from Shea oil under all reaction conditions (Table 5.1).

4. Lactones were found in four samples in solid-state culture when Shea oil was converted by the two strains of *P. roquefortii*, however, they were not detected in suspension culture at both temperatures (Table 5.1).

5. Methyl ketones were produced from Shea oil by the dairy strain of *P. roquefortii* (Wisbey PJ) at 10 °C (Table 5.1). However, methyl ketones were not found by the spoilage strain (FRR 2456) after spraying 1 % $KMnO_4$ in 2 % Na_2CO_3.

5.1.3 Separation of lipids from Shea oil by PTLC

Separation of lipids from Shea oil by Preparative Thin Layer Chromatography is shown in Figure 5.3. Relatively large volume of sample (2 ml, ~15 % w/v) was applied. Mono-, di- and tri-acylglycerols and fatty acids were separated completely to give the same pattern of separation as in TLC analysis (Figures 5.1 and 5.2). Rf values of each compound are given in Figure 5.3. Flavour compounds (methyl ketones and lactones) were not found in this sample even more samples were applied on the PTLC plates.

Figure 5.3 Preparative Thin Layer Chromatography (PTLC) separation of lipids produced from Shea oil by *P. roquefortii* (FRR 2456) in solid-state culture at pH 7.0, 25 °C for 7 days. Silica gel 60 F_{254} plate (20 × 20 cm with 20 × 4 cm concentrating zone, 0.5 mm layer thickness, Merck) was activated at 105 °C for 50 min. Plates were eluted with hexane : diethyl ether : formic acid (80:20:2 by volume) and visualised under 254 nm UV light. MAG: monoacylglycerols, DAG: diacylglycerols, FA: fatty acids and TAG: triacylglycerols.

5.2 Total mass chromatogram of monoacylglycerol TMS ethers

All monoacylglycerol samples derived from Shea oil were analysed by GC-MS. Figure 5.4 shows a total mass chromatogram of monoacylglycerol TMS ethers from Shea oil by *P. roquefortii* FRR 2456 in solid-state culture at pH 7.0 and 25 °C. The most abundant peak was 1 or 3-monooctadecanoyl-sn-glycerol 1(3)-18:0, followed by 1 or 3-monooctadecenoyl-sn-glycerol 1(3)-18:1 (9c), 2-monooctadecenoyl-sn-glycerol 2-18:1(9c) and 1 or 3-monohexadecanoyl-sn-glycerol 1(3)-16:0. Other monoacylglycerols are listed in Figure 5.4. Identification of pentonic acid, 3-deoxy-2,5-methoxyl-2-hydroxyl, γ-lactone is given in Chapter 6.

Figure 5.4 A total ion chromatogram of monoacylglycerol TMS ethers derived from Shea oil by *P. roquefortii* (FRR 2456) in solid-state culture at pH 7.0 and 25 °C. GC-MS conditions was given in the footnote to Figure 4.4.

5.3 Mass spectra of 1(3)- and 2-monoacylglycerol TMS ethers

Mass spectra of 1(3)- and 2-monohexadecanoyl-sn-glycerol (16:0) TMS ethers are shown in Figure 5.5. Identification of 1(3)- and 2-monoacylglycerol TMS ethers are summarised in Table 5.2. The library ID % of most of the monoacylglycerol TMS ethers were over 80 % (Table 5.2). Figures 5.6 and 5.7 outline the mechanism for formation of characteristic ions of 1(3)- and 2-monoacylglycerol TMS ethers.

The ion at $(M-103)^+$ was formed in all 1(3)-monoacyl-sn-glycerol TMS ethers. This ion was characteristic for 1(3)-isomers and derived from the cleavage between carbon 2 and 3, or 1 and 2

(Figure 5.6). The formula of this ion was $[M-(CH_3)_3SiOCH_2]^+$. The smaller ion at m/z 205 $[(CH_3)_3SiO]_2C_2H_3^+$ containing TMS group and parts of glycerol backbone was used, in addition, to identify 1(3)-monoacylglycerol TMS ethers. On the other hand, relatively large ion at m/z 218 (abundance between 20 % and 90 % of the most abundant ion) was characteristic of the spectra of 2-monoacyl-sn-glycerol (Table 5.2 & Figure 5.7). This radical cation was $(M-RCOOH)^{+\cdot}$ and was formed by McLafferty rearrangement (Figure 5.7). Hydrogen atom transferred to the carbonyl oxygen and the carbon (C2)-oxygen bond was cleaved, and finally formed one radical cation $(M-RCOOH)^+$ and a neutral molecule of fatty acid. The ion of $(RCO+74)^+$ was formed by McLafferty rearragement as well (Figure 5.7). The trimethylsilyl group was transferred to the carboxyl oxygen and the carbon (C2)-oxygen bond was cleaved to form a radical cation of $RCOOSi(CH_3)_3^+$, and then the ion $(RCO+74)^+$ was formed by losing a methyl group (Figure 5.7).

Figure 5.5 Mass spectra of 1(3)- and 2-monohexadecanoyl-sn-glycerol TMS ethers from Shea oil by *P. roquefortii* in solid-state culture at pH 7.0, 25 °C for 7 days (peak 5 in Figure 5.1) GC-MS conditions were the same as shown in the footnote of Figure 4.1

Table 5.2 Identification of monoacylglycerol TMS ethers by MS from Shea oil by two strains of *P. roquefortii* *

Strain & condition	Peak identity		t_r (min)	m/z 205	m/z 218	$(M-103)^+$	$(M-15)^+$	RCO^+	$(RCO+74)^+$	Library ID %
FRR 2456, solid-state culture (7d)	1(3)-isomer	10:0	11.40	+	-	287	375	155	229	88.3
		12:0	12.43	+	tr	315[a]	403	183	257	90.1
		16:0	14.57	+	-	371	459	239	313	81.4
		18:0	15.75	+	tr	399	487	267	341	91.0
		18:1(9c)	15.55	+	tr	397	485	265	339	81.8
		20:0	16.70	+	tr	427	515	295	369	90.5
	2-isomer	10:0	11.30	-	+	-	375	155	229	83.1
		12:0	12.31	-	++	-	403	183	257	84.9
		18:0	15.48	-	++	-	487	267	341	84.2
		18:1 (9c)	15.30	-	++	-	485	265	339	72.4
		20:0	16.51	-	++	-	515	295	369	88.2
Wisbey PJ, solid-state culture (7d)	1(3)-isomer	16:0	14.35	+	tr	371	459	239	313	91.3
		18:0	15.45	+	tr	399	487	267	341	83.2
		18:1 (9c)	15.30	+	-	397	485	265	339	88.8
		20:0	16.65	+	-	427	515	295	369	85.3
	2-isomer	18:1 (9c)	15.22	tr	++	-	-	265	339	87.7
		20:0	16.48	-	++	-	515	295	369	80.9
FRR 2456, suspension culture (4h)	1(3)-isomer	14:0	12.70	+	tr	343[a]	431	211	285	87.5
		16:0	13.75	+	tr	371[a]	459	239	313	85.3
		18:0	14.80	+	tr	399	487	267	341	84.6
		18:1 (9c)	14.66	+	-	397	485	265	339	82.1
		20:0	15.89	+	-	427	515	295	369	89.1
	2-isomer	14:0	12.56	-	++	-	431	211	285	80.5
		16:0	13.60	tr	++	tr	-	239	313	90.6
		18:0	14.60	-	++	tr	-	267	341	87.5
		20:0	15.71	-	++	-	515	295	369	79.8
Wisbey PJ, suspension culture (4h)	1(3)-isomer	14:0	12.70	+	-	343	-	211	285	77.4
		16:0	13.75	+	tr	371	459	239	313	78.9
		18:0	14.83	+	tr	399[a]	487	267	341	86.7
		18:1 (9c)	14.67	+	tr	397	485	265	339	87.1
		20:0	15.90	+	-	427	515	295	369	82.9
	2-isomer	16:0	13.61	-	++	-	459	239	313	91.2
		18:0	14.60	-	+++	-	471	267	341	89.3
		18:1 (9c)	14.48	-	++	-	485	265	339	88.9
		20:0	15.71	-	++	-	-	295	369	90.2

* Conversion was carried out at pH 7.0 and 25 °C. Ions at m/z 73, m/z 75, m/z 129 and m/z 147 existed in every 1(3)- or 2-monoacylglycerols. tr = trace, abundance was less than 5 % of the base ion. +: abundance was between 15 % and 49 % of the base ion. ++: abundance was between 50 % and 90 % of the base ion. +++: base ion. -: not detected. [a] This is the most abundant ion (base ion) in the mass spectra.

Other characteristic ions at m/z 73, 75, 129, 147, RCO^+, $(RCO+74)^+$ were of diagnostic value to identify both 1(3)- and 2-monoacylglycerol TMS ethers. The ions at m/z 73 $[(CH_3)_3Si]^+$ and m/z 75 $[HO{=}Si(CH_3)_2]^+$ indicated that they were TMS compounds. The presence of an ion at m/z

147 $[(CH_3)_2Si{=}OSi(CH_3)_3]^+$ indicated there were two TMS groups in the molecule. This was a distinctive ion for monoacylglycerol TMS ethers.

Figure 5.6 Cleavage of 1(3)-monoacyl-sn-glycerol TMS ethers to give characteristic ions.

Figure 5.7 Cleavage of 2-monoacyl-sn-glycerol TMS ethers to give characteristic ions.

Table 5.3 gives the formulas and the mass to charge ratios of molecular ions. Although molecular ions could not be seen with electron impact MS, the ions at $(M-15)^+$ helped to identify the molecular weight of the monoacylglycerol TMS ethers. These ions were formed by loss of a methyl group. Apparent molecular weight of monoacylglycerols TMS ethers derived from the ions at $(M-15)^+$ had the same value as calculated molecular weight of these compounds.

Table 5.3 Formulas and mass to charge ratios of molecular mass ions of monoacylglycerol TMS ethers from Shea oil (Conversion at pH 7.0 and 25 °C) *

Strain & conditions	Peak identity		t_r (min)	$(M-15)^{+\dagger}$	Formula	M^+ (apparent)	M^+ (calculated)
FRR 2456, solid-state culture, 7d	1(3)-isomer	10:0	11.40	375	$C_{19}H_{42}Si_2O_4$	390	390
		12:0	12.43	403	$C_{21}H_{46}Si_2O_4$	418	418
		16:0	14.57	459	$C_{25}H_{54}Si_2O_4$	474	474
		18:0	15.75	487	$C_{27}H_{58}Si_2O_4$	502	502
		18:1(9c)	15.55	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	16.70	515	$C_{29}H_{62}Si_2O_4$	530	530
	2-isomer	10:0	11.30	375	$C_{19}H_{42}Si_2O_4$	390	390
		12:0	12.31	403	$C_{21}H_{46}Si_2O_4$	418	418
		18:0	15.48	487	$C_{27}H_{58}Si_2O_4$	502	502
		18:1 (9c)	15.30	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	16.51	515	$C_{29}H_{62}Si_2O_4$	530	530
Wisbey PJ, solid-state culture, 7d	1(3)-isomer	16:0	14.35	459	$C_{25}H_{54}Si_2O_4$	474	474
		18:0	15.45	487	$C_{27}H_{58}Si_2O_4$	502	502
		18:1 (9c)	15.30	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	16.65	515	$C_{29}H_{62}Si_2O_4$	530	530
	2-isomer	18:1 (9c)	15.22	-	$C_{27}H_{56}Si_2O_4$	-	500
		20:0	16.48	515	$C_{29}H_{62}Si_2O_4$	530	530
FRR 2456, suspension culture, 4h	1(3)-isomer	14:0	12.70	431	$C_{23}H_{50}Si_2O_4$	446	446
		16:0	13.75	459	$C_{25}H_{54}Si_2O_4$	474	474
		18:0	14.80	487	$C_{27}H_{58}Si_2O_4$	502	502
		18:1 (9c)	14.66	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	15.89	515	$C_{29}H_{62}Si_2O_4$	530	530
	2-isomer	14:0	12.56	431	$C_{23}H_{50}Si_2O_4$	446	446
		16:0	13.60	-	$C_{25}H_{54}Si_2O_4$	-	474
		18:0	14.60	-	$C_{27}H_{58}Si_2O_4$	-	502
		20:0	15.71	515	$C_{29}H_{62}Si_2O_4$	530	530
Wisbey PJ, suspension culture, 4h	1(3)-isomer	14:0	12.70	-	$C_{23}H_{50}Si_2O_4$	-	446
		16:0	13.75	459	$C_{25}H_{54}Si_2O_4$	474	474
		18:0	14.83	487	$C_{27}H_{58}Si_2O_4$	502	502
		18:1 (9c)	14.67	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	15.90	515	$C_{29}H_{62}Si_2O_4$	530	530
	2-isomer	16:0	13.61	459	$C_{25}H_{54}Si_2O_4$	474	474
		18:0	14.60	471	$C_{27}H_{58}Si_2O_4$	486	486
		18:1 (9c)	14.48	485	$C_{27}H_{56}Si_2O_4$	500	500
		20:0	15.71	-	$C_{29}H_{62}Si_2O_4$	-	530

* Analysed by Electron Impact (EI) Mass Spectrometry (GC-MS).
† Ions observed in the mass spectra of monoacylglycerols TMS ethers with electron impact ion source.

5.4 Structural isomers of monoacylglycerols derived from Shea oil

5.4.1 Solid-state culture

The composition and the molar ratio of monoacylglycerols derived from Shea oil in solid-state culture is given in Table 5.4. 1 and 3-Monooctadecanoyl-sn-glycerols (monostearin, $C_{18:0}$) were the main isomers. Higher concentration of total 1(3)-isomers were found by *P. roquefortii* FRR 2456 than by *P. roquefortii* PJ, especially at 25 °C. Medium chain length monoacylglycerols ($C_{10:0}$ - $C_{14:0}$) were not detected after conversion of Shea oil by *P. roquefortii* PJ, however, these monoacylglycerols were found after conversion by *P. roquefortii* FRR 2456. These monoacylglycerols may have been synthesised by the mould during the long incubation time in solid-state culture.

5.4.2 Suspension culture

Table 5.5 shows the composition and molar ratio of monoacylglycerols produced from Shea oil in suspension culture. Monoacylglycerols ($C_{14:0}$ - $C_{20:0}$) were produced from shea oil by the two strains of *P. roquefortii* at pH 7.0 at 10 °C and 25 °C. Almost 90 % of the monoacylglycerols were 1 and 3-monoacylglycerols. 1 and 3-Monooctadecanoyl-sn-glycerols (1,3-18:0) were the major 1(3)- isomers whilst 2-monooctadecanoyl-sn-glycerol (2-18:0) was the major 2-isomer. Higher concentration of 1(3)-18:0 and lower concentration of 1(3)-18:1(9c) were found with *P. roquefortii* PJ than with *P. roquefortii* FRR 2456. Different from the situation in solid-state culture, it was expected that no medium chain fatty acids were found from Shea oil in suspension culture.

Table 5.4 Composition of monoacylglycerols produced from Shea oil by two strains of *P. roquefortii* in solid-state culture *

temperature & time	Fungal strain	MAG	% composition (mol)		molar ratio †	
			sn-1(3)	sn-2	sn-1(3)	sn-2
10.0±0.3 °C 20 days	FRR 2456	10:0	2.23 ± 0.63	1.20 ± 0.41	1.91 ± 0.59	1.00 ± 0.00
		12:0	2.43 ± 0.62	2.23 ± 0.44	2.12 ± 0.52	2.09 ± 0.96
		14:0	4.23 ± 0.67	3.97 ± 0.51	4.03 ± 1.07	3.08 ± 0.94
		16:0	12.66 ± 2.12	ND	10.22 ± 2.20	ND
		18:0	44.22 ± 2.64	2.68 ± 0.89	38.50 ± 3.74	2.56 ± 1.41
		18:1 (9c)	15.35 ± 1.58	ND	13.29 ± 3.56	ND
		Other [a]	7.20 ± 2.67	1.60 ± 0.59	6.61 ± 1.06	1.97 ± 0.75
		Totals	88.32	11.68	76.68	10.70
	Wisbey PJ	10:0	ND	ND	ND	ND
		12:0	ND	ND	ND	ND
		14:0	ND	ND	ND	ND
		16:0	8.62 ± 2.44	ND	1.00 ± 0.00	ND
		18:0	32.89 ± 1.37	ND	4.08 ± 1.14	ND
		18:1 (9c)	41.24 ± 1.43	15.52 ± 1.14	5.12 ± 1.44	1.91 ± 0.50
		Other [a]	1.72 ± 1.40	ND	0.23 ± 0.12	ND
		Totals	84.47	15.52	10.42	1.91
25.1±0.2 °C 7 days	FRR 2456	10:0	0.99 ± 0.14	1.21 ± 0.09	0.82 ± 0.08	1.00 ± 0.00
		12:0	0.94 ± 0.10	0.71 ± 0.10	0.78 ± 0.05	0.58 ± 0.05
		14:0	0.53 ± 0.06	0.29 ± 0.04	0.44 ± 0.03	0.24 ± 0.02
		16:0	6.09 ± 0.56	ND	5.09 ± 0.78	ND
		18:0	62.14 ± 3.53	6.20 ± 0.50	51.90 ± 3.88	5.14 ± 0.10
		18:1 (9c)	15.63 ± 2.83	ND	12.93 ± 2.25	ND
		Other [a]	3.80 ± 1.20	1.47 ± 0.45	3.15 ± 0.93	1.21 ± 0.33
		Totals	90.12	9.88	75.11	8.17
	Wisbey PJ	10:0	ND	ND	ND	ND
		12:0	ND	ND	ND	ND
		14:0	ND	ND	ND	ND
		16:0	12.22 ± 0.30	ND	1.00 ± 0.00	ND
		18:0	37.79 ± 0.26	ND	3.09 ± 0.09	ND
		18:1 (9c)	27.51 ± 0.18	20.46 ± 0.22	2.25 ± 0.06	1.68 ± 0.05
		Other [a]	1.41 ± 0.18	0.60 ± 0.02	0.12 ± 0.02	0.05 ± 0.00
		Totals	78.93	21.06	6.46	1.73

* Results were the mean of six GC analyses ± standard deviation. Conversion was carried out at pH 7.0. MAGs were derived from one fermentation, two extraction and analysed as trimethylsilyl ethers by GC. ND: not detected.

† Calculated relative to 2-monodecanoylglycerol (2-10:0) for FRR 2456 and to 1(3)-monohexadecanoyl-glycerol (1(3)-16:0) for Wisbey PJ.

[a] Other MAGs were 1(3)-15:0, 2-15:0, 1(3)-17:0, 2-17:0, 1(3)-20:0, 2-20:0 or some of them.

Table 5.5 Composition of monoacylglycerols produced from Shea oil by two strains of *P. roquefortii* in suspension culture *

temperature & time	Fungal strain	MAG	% composition (mol)		molar ratio †	
			sn-1(3)	sn-2	sn-1(3)	sn-2
9.9±0.2 °C 25 hour	FRR 2456	14:0	2.37 ± 0.36	ND	1.00 ± 0.00	ND
		16:0	17.35 ± 0.87	ND	7.32 ± 0.37	ND
		18:0	47.77 ± 1.49	7.82 ± 0.32	20.16 ± 0.63	3.30 ± 0.13
		18:1 (9c)	22.14 ± 1.03	ND	9.34 ± 0.43	ND
		Other [a]	1.45 ± 0.28	1.10 ± 0.22	0.62 ± 0.12	0.46 ± 0.09
		Totals	91.08	8.92	38.44	2.84
	Wisbey PJ	14:0	1.18 ± 0.32	ND	1.00 ± 0.27	ND
		16:0	11.02 ± 1.01	ND	9.30 ± 0.85	ND
		18:0	57.08 ± 2.53	13.67 ± 1.11	48.20 ± 2.14	11.54 ± 0.94
		18:1 (9c)	15.73 ± 0.92	ND	13.28 ± 0.78	ND
		Other [a]	1.32 ± 0.16	ND	1.11 ± 0.14	ND
		Totals	86.33	13.67	72.90	11.54
25.0±0.2 °C 4 hour	FRR 2456	14:0	4.75 ± 0.53	2.71 ± 0.25	1.75 ± 0.20	1.00 ± 0.00
		16:0	17.93 ± 1.23	5.62 ± 0.48	6.62 ± 0.45	2.07 ± 0.18
		18:0	43.10 ± 1.45	2.15 ± 0.21	15.90 ± 0.53	0.79 ± 0.08
		18:1 (9c)	16.89 ± 0.89	ND	6.23 ± 0.33	ND
		Other [a]	4.74 ± 0.67	2.11 ± 0.27	1.75 ± 0.25	0.78 ± 0.10
		Totals	87.41	12.59	32.25	4.65
	Wisbey PJ	14:0	8.43 ± 0.31	ND	1.00 ± 0.00	ND
		16:0	11.63 ± 0.13	2.50 ± 0.29	1.38 ± 0.02	0.30 ± 0.03
		18:0	50.63 ± 0.39	4.84 ± 0.92	6.01 ± 0.05	0.57 ± 0.11
		18:1 (9c)	11.44 ± 0.15	2.92 ± 0.41	1.36 ± 0.05	0.35 ± 0.05
		Other [a]	5.39 ± 0.54	2.22 ± 0.23	0.64 ± 0.03	0.26 ± 0.02
		Totals	87.51	12.48	10.39	1.48

* Results were the mean of six GC analyses ± standard deviation. Conversion was carried out at pH 7.0. MAGs were derived from one reaction, two extraction and analysed as trimethylsilyl ethers by GC. ND: not detected.
† Calculated relative to 1(3)-monotetradecanoylglycerol (1(3)-14:0) for FRR 2456 at 10 °C and Wisbey PJ at 10 and 25 °C and to 2-monotetradecanoylglycerol (2-14:0) for FRR 2456 at 25 °C.
[a] Other MAGs were 1(3)-15:0, 2-15:0, 1(3)-17:0, 2-17:0, 1(3)-20:0, 2-20:0 or some of them.

5.4.3 Lipolysis

The composition and molar ratio of the monoacylglycerols derived from Shea oil by a commercial lipase from *P. roquefortii* is given in Table 5.6. Medium chain monoacylglycerols were not found in the hydrolysed samples. Although 1(3)-isomers were the main monoacylglycerols, there was a decrease in the proportion of total 1(3)-monoacyl-sn-glycerols (about 65 mol % at both 10 °C and 25 °C) compared to the total 1(3)-monoacyl-sn-glycerols produced by *P. roquefortii* in solid-state and suspension cultures shown in Tables 5.4 and 5.5.

Table 5.6 Composition of monoacylglycerols produced from Shea oil by a commercial lipase from *P. roquefortii* in shake flasks *

Temperature & time	MAG	% composition (M)		Molar ratio	
		sn-1(3)	sn-2	sn-1(3) †	sn-2 †
9.9±0.1 °C 25 hour	16:0	4.35 ± 0.07	ND	1.00 ± 0.00	ND
	18:0	51.28 ± 1.14	14.50 ± 0.32	11.79 ± 1.01	3.33 ± 0.25
	18:1 (9c)	8.66 ± 0.20	17.90 ± 0.44	1.99 ± 0.02	4.11 ± 0.43
	20:0	2.21 ± 0.18	1.10 ± 0.09	0.51 ± 0.06	0.25 ± 0.03
	Totals	66.50	33.50	15.29	4.24
24.9±0.2 0.5 hour	16:0	2.36 ± 0.18	ND	1.00 ± 0.00	ND
	18:0	47.77 ± 0.36	5.99 ± 0.29	20.24 ± 0.35	2.59 ± 0.09
	18:1 (9c)	13.94 ± 1.18	27.81 ± 0.84	6.03 ± 0.44	11.78 ± 0.56
	20:0	ND	2.17 ± 0.14	ND	0.94 ± 0.05
	Totals	64.07	35.97	27.27	15.31

* Results were the mean of six GC analyses ± standard deviation. Conversion was carried out at pH 7.0. MAGs were derived from one reaction, one extraction and analysed as trimethylsilyl ethers by GC. Contaminants (~ 40 mol % at 9.9±0.1 °C and ~ 10 mol % at 24.9±0.2 °C) were detected and identified by GC-MS as alcohols (C_8), alkanes (C_{18}), bis(2-methylpropyl)- benzenedicarboxylic acid, fatty acid TMS esters and fatty acid methyl esters. ND: not detected.
† Calculated relative to 1(3)-monohexadecanoylglycerol (1(3)-16:0).

5.5 Fatty acids composition in monoacylglycerols and free fatty acids

5.5.1 Fatty acids composition of monoacylglycerols produced at pH 7.0

The fatty acids composition of monoacylglycerols produced in solid-state culture, suspension culture and by lipolysis at 10 °C and 25 °C is given in Table 5.7. Medium chain fatty acids were found in monoacylglycerols produced by *P. roquefortii*, especially by the spoilage strain FRR 2456 in solid-state culture, however, they were not detected in monoacylglycerols produced by the commercial lipase. Palmitic acid ($C_{16:0}$) accumulated in monoacylglycerols compared to its composition in Shea oil. Stearic acid was the major fatty acid in monoacylglycerols derived from Shea oil except for *P. roquefortii* Wisbey PJ in solid-state culture. The concentration of oleic acid decreased dramatically in monoacylglycerols when degraded by *P. roquefortii* FRR 2456 at 10 °C and 25 °C but increased slightly when degraded by *P. roquefortii* PJ in solid-state culture.

Table 5.7 Fatty acids composition in monoacylglycerols produced from Shea oil by *P. roquefortii* and a commercial lipase from *P. roquefortii* at pH 7.0 *

	Temperature (°C)	Reaction condition	C [a] (ml^{-1})	Fatty acid	10:0	12:0	14:0	16:0	18:0	18:1 (9c)	Other FAs [†]
Shea oil			-	Mean	ND	ND	ND	3.22	55.11	36.74	4.92
				SD	-	-	-	0.42	1.26	0.98	0.08
P. roquefortii (FRR 2456)	10.0 ± 0.2	solid-state culture (20 d) [‡]	-	Mean	3.37	4.69	6.48	15.47	42.64	17.38	9.97
				SD	0.19	0.14	0.06	0.34	0.71	0.31	1.21
		suspension culture (25 h) [§]	4.40×10^5	Mean	ND	ND	1.90	17.81	39.84	20.55	19.91
				SD	-	-	0.19	1.09	1.26	1.39	2.29
	25.1 ± 0.2	solid-state culture (7 d) [‡]	-	Mean	3.63	2.64	2.39	9.47	46.04	16.84	18.99
				SD	0.16	0.04	0.03	0.15	0.20	0.30	0.24
		suspension culture (4 h) [§]	3.79×10^5	Mean	ND	0.92	2.73	18.48	46.10	17.85	13.93
				SD	-	0.20	0.51	0.97	2.04	1.53	2.07
P. roquefortii (Wisbey PJ)	10.1 ± 0.1	solid-state culture (20 d) [‡]	-	Mean	ND	ND	ND	6.81	37.12	51.38	4.68
				SD	-	-	-	0.15	0.22	0.35	0.41
		suspension culture (25 h) [§]	3.94×10^5	Mean	ND	ND	1.23	10.41	59.67	17.15	11.55
				SD	-	-	0.10	0.59	1.18	1.60	0.66
	25.0 ± 0.2	solid-state culture (7 d) [‡]	-	Mean	0.47	0.96	2.26	10.67	35.66	44.15	5.82
				SD	0.01	0.03	0.06	0.42	0.55	0.34	0.95
		suspension culture (4 h) [§]	3.51×10^5	Mean	ND	ND	6.23	19.35	38.37	20.99	15.06
				SD	-	-	0.72	0.94	0.91	1.68	1.65
lipase from *P. roquefortii* (E.C.3.1.1.3)	9.9 ± 0.1	Shaking water bath (5 h) [§]	1.02 ± 0.01 mg lipase	Mean	ND	ND	ND	6.72	46.24	29.77	17.27
				SD	-	-	-	0.53	2.22	1.25	0.38
	24.9 ± 0.2	Shaking water bath (0.5 h) [§]	1.02 ± 0.01 mg lipase	Mean	ND	ND	ND	11.49	31.05	44.81	12.65
				SD	-	-	-	0.81	0.21	2.10	1.18

* Results were in % composition (mol) and were the mean of six GC analyses. MAGs were derived from one reaction and two extractions. SD: standard deviation. ND: not detected. tr: trace. Fatty acids were analysed as their methyl esters by GC and GC-MS. [a] Concentration of spores or lipase per ml reaction mixtures. [†] Other fatty acids were 13:0, 15:0, 16:1 (9c), 16:0 (iso), 16:0, 17:0, 17:0 (iso), 18:1 (11c), 18:2 (9c,12c), 18:2 (12c,15c), 18:3 (6c,9c,12c), 18:3 (9c,12c,15c), 20:0, 22:0 and 24:0 or some of them. [‡] Shea oil (4 %, w/v) was added as carbon source for fermentation. [§] Shea oil concentration was 16.7 %, (w/v).

5.5.2 Fatty acids composition in monoacylglycerols and free fatty acids in solid-state culture at pH 4.5

Fatty acids composition of monoacylglycerols and free fatty acids produced by two stains of *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C are given in Table 5.8. Results were compared with the fatty acids composition of the original Shea oil.

Table 5.8 Fatty acid composition in monoacylglycerols and free fatty acids produced from Shea oil by *P. roquefortii* in solid-state culture at pH 4.5 and 25 °C †

Fatty acid	Original Shea oil (TAGs)	*P. roquefortii* (FRR 2456)		*P. roquefortii* (Wisbey PJ) ‡	
		MAGs	FFAs	MAGs	FFAs
8:0	ND	ND	0.10±0.00	ND	ND
10:0	ND	3.26±0.22	0.29±0.01	ND	ND
12:0	ND	1.49±0.17	0.92±0.02	ND	ND
13:0	ND	0.98±0.15	ND	ND	ND
14:0	ND	2.48±0.20	6.07±0.05	0.81±0.00	0.51±0.02
14:1 (9c)	ND	0.41±0.00	0.32±0.01	ND	ND
15:0	ND	0.91±0.20	1.09±0.02	ND	ND
16:0	3.27±0.03	12.70±0.40	27.37±0.31	12.79±0.06	13.35±0.03
16:1 (9c)	ND	0.63±0.02	1.63±0.05	ND	ND
18:0	59.75±0.16	39.14±0.54	14.01±0.07	63.96±0.00	52.85±0.68
18:1 (9c)	31.41±0.15	29.67±0.86	43.35±0.26	22.49±0.20	33.27±1.63
18:2 (9c,12c)	3.35±0.15	2.68±0.13	3.52±0.06	ND	ND
18:3(6c,9c,12c)	ND	1.32±0.20	0.29±0.01	ND	ND
18:3(9c,12c,15c)	0.50±0.01	0.48±0.08	0.80±0.03	ND	ND
20:0	1.72±0.02	0.09±0.01	0.11±0.01	ND	ND
22:0	ND	0.09±0.02	0.02±0.00	ND	ND
24:0	ND	ND	0.12±0.03	ND	ND
Total MCFAs	ND	4.75±0.29	1.31±0.09	ND	ND
Total LCFAs	100.00	95.25±0.86	98.69±0.57	100.05	99.98
Total	100.00	100.00	100.00	100.05	99.98

† Results were the mean of three GC analyses. Monoacylglycerols and free fatty acids were derived from one fermentation and one extraction. TAGs = triacylglycerols, MAGs = monoacylglycerols, FFAs = free fatty acids. ND = not detected.
‡ Determined by external standard method according to standard curve of 14:0, 16:0, 18:0 and 18:1(9c)

More medium chain fatty acids were detected with *P. roquefortii* FRR 2456 than with *P. roquefortii* Wisbey PJ. The composition of oleic acid decreased in monoacylglycerols compared with the composition of the acid in Shea oil, but there was an increase in the concentration of

oleic acid in the free fatty acids fraction. Palmitic acid accumulated in monoacylglycerols and free fatty acids. Concentration of stearic acid in monoacylglycerols and free fatty acids remained relatively unchanged after conversion with *P. roquefortii* Wisbey PJ but the concentrations were reduced compared to them found in Shea oil after degradation by *P. roquefortii* FRR 2456.

5.5.3 Free fatty acids composition in suspension culture at pH 7.0

The composition of free fatty acids produced from Shea oil by *P. roquefortii* and the commercial lipase from *P. roquefortii* in suspension culture is given in Table 5.9. More palmitic acid was found after conversion by the two strains of *P. roquefortii* and the commercial lipase. Concentrations of stearic and oleic acid decreased compared to their concentration in unreacted Shea oil. Medium chain fatty acids were not found after lipolysis but were detected with the fungal strain FRR 2456. This again indicated that these MCFAs were synthesised by this fungus.

Table 5.9 Free fatty acids produced by hydrolysis of Shea oil with *P. roquefortii* spores or lipase at pH 7.0 (results in mol %) [†]

Fatty acid	**Unreacted Shea oil**	***P. roquefortii* (FRR 2456)** [a]	**Lipase from *P. roquefortii*** [b]	
		10.1 ± 0.1 °C	**9.9 ± 0.1 °C**	**24.9 ± 0.2 °C**
10:0	ND	5.98 ± 0.66	ND	ND
12:0	ND	2.16 ± 0.20	ND	ND
14:0	ND	3.03 ± 0.37	ND	ND
16:0	3.22 ± 0.42	12.18 ± 1.59	12.09 ± 0.21	10.10 ± 0.10
18:0	55.11 ± 1.26	46.72 ± 2.39	51.22 ± 1.01	51.64 ± 1.72
18:1 (9c)	36.74 ± 0.98	23.99 ± 1.25	26.46 ± 1.24	29.64 ± 1.42
Other FAs [c]	4.92 ± 0.08	5.94 ± 1.06	10.23 ± 0.67	8.62 ± 1.81
Total 6:0 - 12:0	ND	8.14 ± 0.75	ND	ND
Total 14:0 +	100.01	91.85 ± 2.09	100.00	100.00
Totals	100.01	99.99	100.00	100.00

[†] Results were the mean of six GC analyses ± standard deviation. Free fatty acids were derived from one reaction, two extraction and analysed as methyl esters. ND: not detected. Substrate concentration was 16.7 % (w/v).

[a] Concentration of spores was 4.40×10^5 per ml reaction mixture. Reaction time was 25 hours.

[b] Concentration of lipase was 1.01 ± 0.02 mg per ml reaction mixture. Reaction time was 25 hours at 10 °C and 0.5 hour at 25 °C.

[c] Other fatty acids from Shea oil were 13:0, 15:0, 16:1 (9c), 16:0 (iso), 17:0, 17:0 (iso), 18:2 (9c,12c), 20:0 and 22:0 or some of them.

5.6 Yields of monoacylglycerols in solid-state culture

Yields of monoacylglycerols produced from Shea oil by *P. roquefortii* in solid-state culture were between 0.30 and 1.80 g / 100 g Shea oil (Table 5.10). More monoacylglycerols were produced at 25 °C than at 10 °C. *P. roquefortii* FRR 2456 produced more monoacylglycerols than *P. roquefortii* Wisbey PJ. Comparing the results with butter oil (Table 4.10), less mono-acylglycerols were produced from Shea oil than from butter oil by the two strain of *P. roquefortii* at 10 °C and 25 °C. It indicates that butter oil is a better substrate for the two strains of *P. roquefortii* in terms of production of monoacylglycerols.

Table 5.10 Yields of monoacylglycerols produced from Shea oil by two strains of *P. roquefortii* in solid-state culture at pH 7.0 (results in g / 100 g oil)

Strain	**FRR 2456**		**Wisbey PJ**	
Temperature (°C)	9.9 ± 0.2	25.0 ± 0.2	10.0 ± 0.2	25.1 ± 0.2
Agar medium (g)	96.80	71.70	103.59	104.34
Extracted oil (g)	3.12	2.78	4.19	5.00
Extracted oil (mg / ml hexane)	62.4	55.6	83.8	100.0
Volume (ml) †	3.0	2.0	2.0	3.0
Lipids (mg) ‡	187.2	111.2	167.6	300.0
Extracted monoacylglycerols	1.2	2.0	0.5	1.2
Yield (g / 100 g oil)	0.64	1.80	0.30	0.40

† Volume of samples applied on Preparative Thin Layer plates.
‡ Total lipids applied on Preparative Thin Layer plates (calculated from extracted oil and volume of applied sample).

6.1 Thin Layer Chromatography of standard lactones

Standard lactones (γ-hexalactone 5.1 mg ml^{-1}, δ-hexalactone 9.2 mg ml^{-1}, δ-decalactone 6.3 mg ml^{-1}) were dissolved in hexane and were analysed on the same TLC plate with standard monoacylglycerols (1:10:0 6.3 mg ml^{-1}, 1-14:0 5.6 mg ml^{-1}, 1-16:0 5.0 mg ml^{-1}, 1-18:0 4.9 mg ml^{-1}). Figure 6.1 shows the separation of standard lactones and monoacylglycerols. The spot of lactones was near the spot of monoacylglycerols. Rf value of standard monoacylglycerols was 0.03 which was in agreement with the values of monoacylglycerols (see section 3.4, 4.1 and 5.1). Rf value of standard lactones was 0.18 which was in the range of the diacylglycerols (see section 3.4, 4.1 and 5.1).

Figure 6.1 Separation of standard lactones and monoacylglycerols on Silica gel 60 Å TLC plate (20 × 5 cm, 0.25 mm layer thickness, Whatman). Plates were eluted with hexane-diethyl ether-formic acid (80:20:2, v/v/v) and visualised in iodine vapour. Sample application was 5 μl. Lanes: 1. standard monoacylglycerols, 2. standard lactones, 3. standard monoacylglycerols + standard lactones.

CHAPTER 6

RESULTS

Lactones Produced from Butter and Shea Oils by *Penicillium roquefortii*

Lactones are normally formed from γ- and δ- hydroxy acid after losing water spontaneously (Grosch 1982). This reaction is called intramolecular esterification.

Lactones are important food flavours existing in almost all classes of foods, such as milk products, nut products, fruits, vegetables, beverages, meat products and bread systems etc. It is believed that lactones can be formed from heated butter or from oils by micro-organisms and enzymes (Maga 1976). Flavour development during the ripening of blue cheese is due to hydrolysis of milk triacylglycerols by *Penicillium roquefortii* lipases and subsequent metabolism of free fatty acids into methyl ketones via β-oxidation by the mould (Kinsella & Hwang 1976, Kinderlerer 1993). In this investigation, γ-lactones with hydroxyl groups were detected when some samples of monoacylglycerols (which had been synthesised from butter and Shea oils) were derivatised and analysed by GC-MS. In order to probe the possibility for existence of lactones in fungal degradation products, standard and sample lactones were analysed by TLC and identified by GC-MS.

6.2 Identification of standard lactones by GC-MS

6.2.1 Total mass chromatogram of standard lactones

Mixtures of standard γ- and δ-lactones were analysed by GC-MS. Experimental conditions of GC-MS were the same as analysis of monoacylglycerol TMS ethers (see section 2.10). Figure 6.2 shows the total mass chromatogram of lactones. These lactones (C_6 - C_{14}) were separated within 10 minutes with a 20 m non-polar BP1 column.

Figure 6.2 Separation of standard γ- and δ-lactones by GC-MS on a BP1 fused silica capillary column (20 m × 0.22 mm i.d., 0.25 μm film thickness, SGE). GC-MS conditions were the same as the analysis of monoacylglycerol TMS ethers (see section 2.10).

6.2.2 Mass spectra of standard γ- and δ-lactones

Mass spectra of γ-hexalactone, δ-octalactone and δ-decalactone are given in Figure 6.3 - 6.5. Molecular ions could be seen in the spectra of standard lactones. The most abundant ions were

the ions at $(M-R)^+$ (R = alkyl group). These ions were formed by cleavage of alkyl groups from cyclic backbones. Other characteristic ions are listed in Table 6.1.

Figure 6.3 Mass spectra of γ-hexalactone. (a) standard spectra, (b) library spectra.

Figure 6.4 Mass spectra of δ-octalactone. (a) standard spectra, (b) library spectra.

Figure 6.5 Mass spectra of δ-decalactone. (a) standard spectra, (b) library spectra.

Table 6.1 Identification of standard γ- and δ-lactones by GC-MS *

Tr	Lactone	M^+	$(M-R)^{+\,\dagger}$	$(M-OH-1)^+$	$CH(CH_2)_nCH_3^+$	$(CH_2)_nCH_3^+$	ID %
4.53	γ-hexa-	114	85	-	42,56,70...	43,57,71...	83.2
5.90	δ-octa-	-	99	124	42,56,70,84...	43,57,71,85...	86.8
6.72	δ-nona-	156	99	138	42,56,70,84...	43,57,71,85...	76.8
7.60	δ-deca-	170	99	152	42,56,70,84...	43,57,71,85...	81.2
8.78	δ-dodeca-	198	99	180	42,56,70,84...	43,57,71,85...	80.5
9.88	δ-tetradeca-	326	99	-	42,56,70,84...	43,57,71,85...	78.3

* A 20 m non-polar BP1 capillary column was used for analysis. GC-MS conditions were the same as the analysis of monoacylglycerol TMS ethers (see section 2.10). R = alkyl group.
† These are the most abundant peaks of the mass spectra.

6.3 Identification of lactones in degradation products by GC-MS

6.3.1 Mass chromatogram of lactones in degradation products

Pentonic acid, 3-deoxy-2,5-methoxyl-2-hydroxyl-, γ-lactones and hexonic acid, 3-deoxy-2,5,6-trihydroxyl-, γ-lactone were detected when monoacylglycerol TMS ethers from butter and Shea oils were analysed by GC-MS. Figures 6.6 and 6.7 show the total ion chromatogram of these hydroxy lactones in the samples of monoacylglycerol TMS ethers. Retention times of the lactones were between 9 and 10 min.

Figure 6.6 A total ion chromatogram of hydroxy γ-lactones in samples of monoacylglycerols as TMS ethers from Shea oil by *P. roquefortii* (Wisbey PJ) in solid-state culture at pH 7.0, 10 °C and 20 d. GC-MS conditions were the same as described in 2.10. Peak: 1. ni; 2. erythro-pentonic acid, 3-deoxy-2,5-dihydroxyl-2-methoxyl-, γ-lactone (82.3% ID); 3. threo-pentonic acid, 2,5-dihydroxyl-2-methoxyl, γ-lactone (78.4% ID); 4. ribo-hexonic acid, 3-deoxy-2,5,6-trihydoxyl, γ-lactone (78.3% ID); 5. 3,6,9,12-tetraoxa-2,13-disilatetradecane, 2,2,13,13-tetrametyl- (82.9% ID); 6. ni; 7. 2-16:0 (90.4% ID); 8. 1(3)16:0 (81.1% ID); 9. 2-18:1(9c) (86.7% ID); 10. 1(3)-18:1(9c) (87.1% ID); 11. 1(3)-18:0 (78.4% ID). Peaks 7-11 are monoacylglycerols. ni = not identified.

Figure 6.7 A total ion chromatogram of hydroxy lactones in samples of monoacylglycerols as TMS ethers from butter oil by *P. roquefortii*, FRR 2456 in solid-state cultures at pH 7.0, 25 °C and 7d. GC-MS conditions were the same as described in 2.10. Peak 1. pentonic acid, 5-deoxy-2,3-dihydroxyl, γ-lactone (80.1% ID); 2. 4-hydroxy-butanoic acid (86.8% ID); 3. hexanoic acid (73.5% ID); 4. ni; 5. ni; 6. D-threo-pentonic acid, 3-deoxy-2,5-dihydroxyl-2-methoxyl, γ-lactone (82.1% ID); 7. 1(3)-12:0; 8. 2-16:0; 9. 1(3)-16:0; 10. 2-18:1(9c); 11. 1(3)-18:1 (9c); 12. 1(3)-18:0; 13. 2-10:0 and 14. 1(3)-20:0. Peaks 7-14 are monoacylglycerols.
ni = not identified.

6.3.2 Mass spectra of TMS ethers of hydroxyl lactone

Mass spectra of pentonic acid 3-deoxy-2,5-dihydroxyl-2-methoxyl-, γ-lactone TMS ethers are given in Figure 6.8. Molecular ion was not seen in the sample spectra, however, relatively high intensity of ion fragment at m/z 348 was found in pentonic acid 3-deoxy-2,5-dihydroxyl-2-methoxyl, γ-lactone TMS ethers. This ion was due to loss of two methyl groups from the molecular cation, whilst an ion at m/z 349 was correspond to the loss of an ethyl group from the molecular cation. Formulas of other characteristic ions are listed in Table 6.2.

Figure 6.8 Mass spectra of pentonic acid, 3-deoxy-2,5-dihydroxyl-2-methoxyl, γ-lactone TMS ethers from butter oil by *P. roquefortii* FRR 2456 in solid-state cultures at pH 7.0, 25 °C and 7 d. (a) sample spectra, (b) library spectra. GC-MS conditions were the same as described in section 2.10.

Table 6.2 Characteristic ions for identification of TMS ethers of pentonic acid, 3-dexoy-2,5-dihydroxyl-2-methoxyl-, γ-lactone

m/z	Formula
348	$[M - (CH_3)_2]^+$
349	$[M - CH_2CH_3]^+$
318	$[M - (CH_3)_4]^+$
245	$[M - CH_2OSi(CH_3)_3 - (CH_3)_2]^+$
147	$[(CH_3)_2Si{=}OSi(CH_3)_3]^+$
75	$[HO{=}Si(CH_3)_2]^+$
73	$[(CH_3)_3Si]^+$

The pentonic acid γ-lactone was formed from pentonic acid through intramolecular esterification and then silylised into its trimethylsilyl ether (Figure 6.9). Pentonic acid could be formed in the oils by *Penicillium* mould or from the low concentration of lactose (0.02 %) present in casein enzymatic hydrolysate (Quest personal communication 1992) which was used as a nitrogen source in the medium in solid-state cultures (see section 2.3.1).

corresponded to the hydroxy lactones. It was possible that some silica gel containing the hydroxy lactones was scraped from the preparative TLC plates with monoacylglycerols resulting in identification of the lactones in some of the monoacylglycerol samples (Figure 6.6 & 6.7).

Table 6.3 Lactones identified by GC-MS in degradation products from butter and Shea oils by *P. roquefortii* in solid-state culture at pH 7.0 [†]

Substrate	Date of GC-MS analysis	Fungal strain & reaction conditions	Detected lactone
Shea oil	1 May, 96	By FRR 2456 at 10 °C and incubated for 20 d	Pentonic acid, 3-deoxy-2,5-dihydroxyl-2methoxyl-, γ-lactone (D-threo-)
	1 July, 96	By Wisbey PJ at 10 °C and incubated for 20 d	1. Pentonic acid, 3-deoxy-2,5-dihydroxyl-2methoxyl-, γ-lactone (D-erythro- and threo-) 2. Hexonic acid, 3-deoxy-2,5,6-trihydroxyl-, γ-lactone (ribo)
Butter oil	30 April, 96	By FRR 2456 at 25 °C and incubated for 7 d	1. Pentonic acid, 3-deoxy-2,5-dihydroxyl-2methoxyl-, γ-lactone (D-threo), 2. Pentonic acid, 5-deoxy-2,3-dihydroxyl, γ-lactone
	12 August, 96	By Wisbey PJ at 10 °C and incubated for 20 d	Pentonic acid, 3-deoxy-2,5-dihydroxyl-2methoxyl-, γ-lactone

[†] Lactones were present in the samples of monoacylglycerols when analysed by the GC-MS as their TMS ethers.

6.5 Sources of lactones and pathways for formation of lactones

The presence of lactones in food was first reported in the mid 1950s. With improved separation and identification techniques, these flavour compounds were found with steadily growing in various food classes, such as fruits and vegetables, bread systems, nut products, vegetable oils, beverages, meat products and milk products (Maga 1976). Lactones occur universally in milk fat, for example, the sweet flavoured γ-dodecanolactone and γ-dodec-cis-6-enolactone occur at much higher levels in milk from grain fed than from pasture fed cows (Urbach 1997). The

Figure 6.9 Formation of (a) pentonic acid, 3-deoxy-2,5-dihydroxyl-2-methoxyl, γ-lactone and (b) hexonic acid, 3-deoxy-2,5,6-trihydroxy, γ-lactone, and their TMS ethers from precursor acids. BSA: bis(trimethylsilyl)-acetamide.

6.4 Lactones produced from butter and Shea oils by *P. roquefortii*

Among the 12 samples of degradation products from Shea oil by *P. roquefortii* and the commercial lipase from *P. roquefortii*, lactones were identified with GC-MS analysis in two samples which had undergone degradation by *P. roquefortii* FRR 2456 and Wisbey PJ in solid-state culture (Table 6.3). Similarly 2 out of 12 degradation products from butter oil contained lactones were identified (Table 6.3). Preparative Thin Layer Chromatographic separation of the degradation products showed that there was an additional band between monoacylglycerols and 1,2-diacylglycerols (Figure 4.3). Comparing the Rf values of TLC separation of standard lactones (Figure 6.1) to the value of the sample band (Figure 4.3), it seemed that it was the band

much higher levels in milk from grain fed than from pasture fed cows (Urbach 1997). The pathways for formation of lactones involve thermal oxidation of fatty acids, reactions catalysed by micro-organisms and enzymes and, carbohydrates-amino acids degradation.

6.5.1 Thermal oxidation

The pathway for formation of γ- and δ-lactones by thermal oxidation of higher fatty acids is given in Figure 6.10 (Watanabe & Sato 1971). Free lactones are absent in freshly secreted milk fat, however, the formation of lactones can be induced by heating or storage. This observation implied that the lactone precursors were present in bound form (Dimick *et al* 1969). The level of saturated aliphatic γ- and δ-lactones with chain length from 6 to 16 carbons increased in butter oil after heating (Maga 1976). Recently Lee *et al* (1991) isolated and identified 6 δ-lactones with chain length 6 - 14 from heated butter up to 200 °C. Abdel-Mageed & Fadel (1995) studied the volatiles in freeze-stored butter and found that the lactones reached its maximum after storage of 3 months at -18 °C.

6.5.2 Catalysis by micro-organisms and enzymes

One of the most important formation pathways of lactones is from hydroxy fatty acids by micro-organisms and enzymes. Enzyme technologies and micro-organisms cultivated in submerged culture may yield complex volatiles including lactones (Belin *et al* 1992). Other than catalysis of hydrolysis, esterification and transesterification reactions, lipases from microbial origin have the ability to catalyse intramolecular esterification (lactonization) to form lactones (Christen & López-Munguía 1994). Microbial biosynthetic pathways for the production of natural flavours is important economically. For example, the bioproduction of γ-decalactone decreased in price from US$20,000/kg in the early 1980s down to US$1,200/kg in 1995 because of a move to microbial production (Feron *et al* 1996). In the early days, Boldingh and Taylor (1962) postulated a pathway for formation of lactones from triacylglycerols containing a hydroxy fatty acid (Figure

only two possible optically active forms. The finding demonstrated that these hydroxy fatty acids were of biological origin and thus were specific to animal lipid metabolism. Therefore, it would then suggest the involvement of acetate in the formation of δ-hydroxy fatty acids.

Figure 6.10 Pathway for formation of γ- and δ-lactones by thermal oxidation (Watanabe & Sato 1971).

Figure 6.11 Formation of γ- and δ-lactones by pyrolysis of a triacylglycerol containing a hydroxy fatty acid. From Boldingh & Taylor (1962), redrawn.

No long before, the biochemical pathways for lipid degradation involved many enzyme-catalysed reactions, ranging from the hydrolysis of triacylglycerols to the catabolism and oxidation of single-chain fatty acids. The first step for the liberation of free fatty acids from triacylglycerols is catalysed by lipases. Due to the high toxicity of fatty acids towards micro-organisms, these fatty acids are converted to Coenzyme A (CoA) esters before being catabolised by β-oxidation system (Figure 6.12). Stanley and Tubbs (1975) detected the accumulation of intermediates during the β-oxidation of palmitate by intact mitochondria. This finding indicated that it should be possible to exploit microbial β-oxidation pathways to obtain an important group of natural compounds. It has already been used for the production of lactones from precursor fatty acids with side-chain hydroxyl groups (Feron *et al* 1996).

Figure 6.12 β-oxidation cycle. Adapted from Feron (1996). FAD: flavin adenine dinucleotide. NAD(H): nicotinamide adenine dinucleotide (reduced form). R = $CH_3(CH_2)_n$-.

In eukaryotic organisms such as yeast and fungi, fatty acids are catabolised in a specific subcellular compartment: the peroxisome. Higher fungi including *P. roquefortii* have the ability to produce lactones from natural substrates including sugars, fats and oils (Abraham & Berger 1994, Chalier & Crouzet 1992). A peach odour mixture of lactones was formed by hydrolysis of soybean oil by an exogenous lipase and *P. roquefortii* spores (Chalier & Crouzet 1992). The lactones were identified by GC-MS as 4-hexanolide, cis-6-dodecen-4-olide and 4-dodecanolide. The latter two compounds were the major compounds responsible for the peach odour. It is generally assumed that cheese lactones are formed by hydrolysis of the hydroxy fatty acids of milk fat, followed by a lactonisation induced by heating milk fat in the presence of water (Adda *et al* 1982). Fatty acids are not only flavour compounds themselves, but also precursors of flavours of milk fat, such as methyl ketones, lactones, alcohols and esters (Molimard *et al* 1997). Another line of work with *Penicillium* species involved synthesis of new macrocyclic lactones (Barrow

1997), methylenolactocin (de Azevedo *et al* 1992) and tetracyclic sesquiterpene lactones (Massias *et al* 1990) by fermentation. Barrow (1997) suggested that the new macrocyclic lactones were ATP citrate lyase inhibitors and were structurally related to both curvularin and the resorcyclic macrocyclic lactones, but possessed a modified carbon skeleton. (-)-Methylenolactocin is believed to be a densely functionalised and isomerisation-prone antitumor antibiotic (de Azevedo *et al* 1992). The authors illustrated a novel and potentially general approach to synthesis the enantiopure γ-butyrolactones - (-)-methylenolactocin.

Although the β-oxidation cycle leading to the formation of methyl ketones by fungi has been well-investigated (Kellard *et al* 1985, Kinderlerer 1987, 1993), all the metabolic steps leading to the formation of lactones ring by micro-organisms are not known (Feron *et al* 1996). Interest in the biotechnological production of lactones has not been converted into industrial processes due to some limitations:

- the lack of fundamental research on the various microbial metabolic pathways,
- the lack of cheap precursors available in large quantity (except for castor oil),
- the toxicity of the precursors and products,
- the lack of adequate research into alternative technologies, such as solid-state fermentation and immobilised cells, for the production of the desired compounds (Feron *et al* 1996).

6.5.3 Carbohydrates - amino acids degradation pathways

In addition to being produced by thermal and oxidative reactions, micro-organisms and enzymes, lactones can be formed by carbohydrates-amino acids degradation pathways, such as lactose-casein browning system, N-formyl-L-lysine-D-lactose browning system, roasting alkyl-α-amino acids with D-glucose and carbohydrate reduction-levulinic acid-angelica lactone formation system, etc. (Maga 1976). Ferretti and Flanagan (1971) browned a mixture of lactose and casein

at 75 °C for 11 days at a relative humidity of 75 %. 4-Methyl-2-butenoic acid γ-lactone, 3,4-dimethyl-2-butenoic acid γ-lactone and 2,3-dimethyl-2-butenoic acid γ-lactone were found. The authors postulated that 4-methyl-2-butenoic acid γ-lactone was resulted from the hydrolytic cleavage rearrangement of furfuryl alcohol via the intermediate levulinic acid although levulinic acid was not identified in the study.

6.5.4 Pathways for formation of hydroxy lactones in this study

In this study the precursors of the hydroxy lactones have monosaccharide structures (Figure 6.9), it is likely that the precursors were formed by carbohydrate-amino acid degradation system. However, no carbon source other than butter and Shea oils was used in the solid-state cultures where the hydroxy lactones were detected (section 2.3.1) although 0.02 % lactose was present in the casein enzymatic hydrolysate (Quest personal communication 1992). Therefore, the precursors might have been formed from the two commercial oils or casein enzymatic hydrolysate with catalysis of fungal enzymes. Clear pathways may be studied in a later investigation using simple starting substrates to undergo fungal degradation.

CHAPTER 7

DISCUSSION

The fatty acids composition and structural isomers of monoacylglycerols produced by whole cell systems from butter and Shea oils were compared to the monoacylglycerols produced by a commercial lipase from *P. roquefortii* from the same substrates. In the three systems (two strains of *P. roquefortii* and the commercial lipase) there were differences in the fatty acids composition and the composition of the structural isomers of monoacylglycerols. These differences must reflect differences in the specificities of the individual lipase enzymes involved in the hydrolysis of the two substrates.

7.1 Butter oil suspension culture

7.1.1 Monolaurin ($C_{12:0}$)

Figure 7.1 (a) gives the molar percentage composition of monolaurin produced from butter oil by spores and emerging mycelium at 10 °C and mycelium at 25 °C for both strains of *P. roquefortii* and the commercial lipase. The two fungal strains produced only the 1- and 3- isomers suggesting that the lipase present in spores and mycelium was 2-specific. Strain FRR 2456 gave higher yields of monolaurin than the dairy strain (Wisbey PJ). The commercial lipase, on the other hand, gave mainly the 2-isomer in agreement with the 1,3-specificity reported by the manufacturer of this enzyme. The analysis of the position of lauric acid ($C_{12:0}$) in the original butter oil triacylglycerols is derived from the data given by Pabai *et al* (1995) (Table 3.4).

7.1.2 Monopalmitin ($C_{16:0}$)

Figure 7.1 (b) gives the molar composition of monopalmitin in the monoacylglycerols derived from butter oil by spores and mycelium at 10 °C and mycelium at 25 °C for both strains of *P. roquefortii* and the commercial lipase. The main isomer was 1,3-monopalmitin although low concentrations of the 2-isomer were produced. These results confirm the mainly 2-specificity of the endogenous lipase. The reverse situation was demonstrated for the specificity of the commercial lipase where the main action was 1,3-specific. The analysis of the position of palmitic acid (C16:0) in the original butter oil triacylglycerols is derived from the data given by Pabai *et al* (1995) (Table 3.4).

7.1.3 Monoolein ($C_{18:1(9c)}$)

Figure 7.1 (c) gives the molar composition of monoolein in the monoacylglycerols derived from butter oil by spores and mycelium at 10 °C and mycelium at 25 °C for both strains *of P. roquefortii* and the commercial lipase. The pattern is similar to that described in Figures 7.1 (a) and 7.1 (b) although there was a slight increase in the concentration of the 2-isomer with the two strains of *P. roquefortii*. With the commercial lipase again 1,3-specificity was observed at 10 °C in agreement with the specificity shown in Figures 7.1 (a) and 7.1 (b). Considerable 2-specificity was observed with the commercial lipase at 25 °C. The higher concentrations of 1- and 3-isomers at 25 °C could be caused by the rearrangement of the acyl group (acyl migration) from the sn-2 positions to the sn-1(3) positions.

Figure 7.1 Structural isomers of monoacylglycerols produced from butter oil by two strains of *P. roquefortii* and a commercial lipase from *P. roquefortii* in suspension culture at pH 7.0. (a) monolaurin, (b) monopalmitin and (c) monoolein.
† Ratio of 1(3)-MAG : 2-MAG. Data derived from Tables 4.5 & 4.6. ‡ Ratio of fatty acid in sn-1(3) positions : fatty acid in sn-2 positions in butter oil. Data derived from Pabai *et al* (1995)

7.1.4 Overall

Figure 7.2 shows the overall pattern for the position of the acyl group in the monoacylglycerols derived from butter oil. It can be seen that the two strains of *P. roquefortii* have the acyl group esterified to the sn-1 or sn-3 position of the glycerol backbone irrespective of the temperature of incubation. This indicated the 2- specificity of the endogenous enzyme. The monoacylglycerols derived from butter oil after hydrolysis by the commercial lipase have the acyl group esterified to the sn-2 position of the glycerol backbone confirming the mainly 1,3- specificity of this enzyme. There was considerable proportion of the acyl group at the sn-1 or sn-3 position of the glycerol backbone suggesting either 2- specificity of the enzyme or acyl migration from the sn-2 to the sn-1 or 3 position in the glycerol molecule under the reaction conditions used for the commercial lipase action.

Figure 7.2 Structural isomers of all monoacylglycerols produced from butter oil by two strains of *P. roquefortii* and a commercial lipase from *P. roquefortii* in suspension culture at pH 7.0.
† Ratio of total 1(3)-isomers : total 2-isomers in monoacylglycerols. Data derived from Table 4.4 and 4.5.
‡ Ratio of total fatty acids in sn-1(3) positions : total fatty acids in sn-2 positions in butter oil. Data derived from Pabai *et al* (1995).

7.2 Butter oil solid-state culture

7.2.1 Monolaurin

Figure 7.3 (a) gives the molar percent composition of monolaurin produced by the growing the mycelium of two strains of *P. roquefortii* at 10 °C and 25 °C respectively. There has been a reduction in the concentration of lauric acid ($C_{12:0}$) in the monoacylglycerols compared to the concentration in the original butter. Roughly equal concentrations of the 2- and the 1,3-monolaurin were found. Monolaurin was not produced by the dairy strain (Wisbey PJ) at 10 °C. The reduction in the concentration of monolaurin together with the evidence of the presence of methyl ketones shown in Figure 4.2 suggested that the medium chain monoacylglycerols were converted to methyl ketones by these moulds.

7.2.2 Monopalmitin

Figure 7.3 (b) shows that more 1,3-monopalmitin than 2-monopalmitin was formed from butter oil by both strains of *P. roquefortii* at both incubation temperatures. The ratios demonstrated that approximately a third of the monopalmitin was acylated at the sn-2 position. The incubation temperature had relatively little effect on the production of monopalmitin by the two strains of *P. roquefortii*. In addition, the differences between the two strains were not significant.

7.2.3 Monoolein

Figure 7.3 (c) demonstrates that 1- and 3-monoolein were the main reaction products particularly by the dairy strain (Wisbey PJ). Considerably less monoolein was formed by the spoilage strain (FRR 2456) than the dairy strain (Wisbey PJ) at both incubation temperatures. This indicates that, in addition to positional specificity, the spoilage strain (FRR 2456) shows some degree of specificity towards oleic acid. However, the dairy strain is not specific to oleic acid.

Figure 7.3 Structural isomers of monoacylglycerols produced from butter oil by mycelium-bound lipase from two strains of *P. roquefortii* (FRR 2456 and Wisbey PJ) in solid-state culture at pH 7.0, (a) monolaurin, (b) monopalmitin and (c) monoolein.
† Ratio of 1(3)-MAG : 2-MAG. Data derived from Table 4.4.
‡ Ratio of fatty acid in sn-1(3) positions : fatty acid in sn-2 positions in butter oil. Data derived from Pabai *et al* (1995).

7.2.4 Overall

Figure 7.4 shows the percent composition of the structural isomers for all the monoacylglycerols produced by both fungal strains in solid-state culture. The major reaction products are the 1- or 3-monoacylglycerols, unlike the monoacylglycerols produced by the hydrolysis in suspension culture, a significant proportion of the monoacylglycerols were present as 2-isomers. This suggested that a different lipase which is 1(3)-specific was produced with longer incubation time (7 days at 25 °C and 20 days at 10 °C) in the solid-state culture.

Figure 7.4 Structural isomers of all monoacylglycerols produced from butter oil by mycelium-bound lipase from two strains of *P. roquefortii* (FRR 2456 and Wisbey PJ) in solid-state culture
† Ratio of 1(3)-isomers : 2-isomers. Data derived from Table 4.4.
‡ Ratio of total fatty acids in sn-1(3) positions : total fatty acids in sn-2 positions in butter oil. Data derived from Pabai *et al* (1995).

7.3 Shea oil suspension culture

7.3.1 Saturated monoacylglycerols

Figures 7.5 (a) and 7.5 (b) give the molar percent composition of monopalmitin and monostearin produced from Shea oil by two strains of *P. roquefortii* and the commercial lipase. In all cases the main end product of the reaction was 1- or 3-monoacylglycerols. This result suggests that the lipase always cleaves at the sn-2 position of the glycerol backbone and either the 1 or 3 position when oleic acid occupies the sn-2 position of the original triacylglycerol in all experiments. With stearic acid ($C_{18:0}$) there was between 10-15 % of the 2-isomer. Oleic acid is present at the sn-2 position in Shea oil. It appears that the fungal lipases preferentially cleave oleic acid irrespective of their specificity for other acyl groups (see section on butter oil 7.2.3).

7.3.2 Unsaturated monoacylglycerols (monoolein $C_{18:1}$)

Figure 7.5 (c) shows the molar percent of monoacylglycerols derived from Shea oil where the acyl group is oleate. Results from two fungal strains show oleate solely in the 1 or 3 position. With the commercial lipase more oleate was seen at the expected 2 position (this enzyme having 1,3 specificity) than the 1 or 3 position but there was a significant concentration of the 1- or 3-isomer.

7.4 Shea oil solid-state culture

7.4.1 Saturated monoacylglycerols

Figures 7.6 (a) and 7.6 (b) give the molar percent composition of monopalmitin and monostearin produced from Shea oil by the two strains of *P. roquefortii*. In all cases the main end product was the 1- or 3-monoacylglycerol. Small concentrations of 2-monostearin were formed.

Figure 7.5 Structural isomers of monoacylglycerols produced from Shea oil by two strains of *P. roquefortii* (FRR 2456 and Wisbey PJ) and a commercial lipase from *P. roquefortii* in suspension culture at pH 7.0, (a) monopalmitin, (b) monostearin and (c) monoolein.
The composition of monoacylglycerols was derived from Table 5.5 and 5.6. Fatty acids distribution in original Shea oil was derived from Table 5.7.

Figure 7.6 Structural isomers of monoacylglycerols produced from Shea oil by two strains of *P. roquefortii* (FRR 2456 and Wisbey PJ) in solid-state culture at pH 7.0, (a) monopalmitin, (b) monostearin and (c) monoolein.
The composition of monoacylglycerols was derived from Table 5.4. Fatty acids distribution in original Shea oil was derived from Table 5.7.

7.4.2 Unsaturated monoacylglycerols (monoolein $C_{18:1}$)

Figures 7.6 (c) gives the molar percent composition of monoolein produced from Shea oil by two strains of *P. roquefortii*. Almost all the monoolein was found at the unexpected sn-1 or sn-3 position of the glycerol backbone except for the dairy strain (Wisbey PJ) where the 2-moloolein was found as well.

7.5 Structures of monoacylglycerols produced from butter and Shea oils

Table 7.1 summarises the main structures of the monoacylglycerols produced from butter and Shea oils. Major conclusions are:

1. The commercial lipase cleaved acyl groups from the triacylglycerols in butter oil at the sn-1 and sn-3 positions to give the expected 2-monoacylglycerols as the main products. Small amounts of 1(3)-monoacylglycerols may have been produced by acyl migration from the sn-2 position to the sn-1(3) positions.

2. In suspension culture the two *P. roquefortii strains* (spores and emerging mycelium) cleaved the acyl groups from the triacylglycerols mainly at the sn-2 position to give 1- and 3-monoacylglycerols in butter oil.

3. In solid-state culture the growing fungal mycelium cleaved the triacylglycerols at the sn-2 (and 3 or 1) position to give the 1,3-monoacylglycerol as the main end product. There were significant amounts of 2-monoacylglycerols produced as well. This suggests that another lipase was produced during fungal growth with 1,3-specificity on butter oil as the sole carbon source.

4. There was no significant effect of temperature on the ratios of the reaction products in butter oil.

5. The situation in Shea oil was more complex. Almost all the original triacylglycerols have the structure SOS (S stands for saturated fatty acids and O for oleic acid). Table 7.1 shows that 1(3)-monoolein was produced. This compound must originate by acyl migration from the original 2 position on the glycerol backbone. Monopalmitin and monostearin occurred mainly at the expected 1 or 3 position.

6. With Shea oil as a substrate the specificity of lipases was partly lost. This may be due to the reactivity of the oleate group. This fatty acid is liquid whilst stearic and palmitic acids are solids at room temperature. If 1,3 specificity of an enzyme was expressed then monoolein would be the main end product.

7. There was no significant effect of strains on production of monoacylglycerols by *P. roquefortii*. The only difference observed was that the dairy strain (Wisbey PJ) produced more monoolein (mainly 1,3-isomers) than the spoilage strain (FRR 2456) (Figure 7.3 c and 7.6 c).

Table 7.1 Structures of monoacylglycerols formed from butter and Shea oils †

Experiment	Substrate	Main end product (isomer)	Bonds split	Minor end product (isomer)
Lipase †	Butter	2-	sn-1,3	1(3)-
P. roquefortii suspension culture	Butter	1(3)-	sn-2	2-
P. roquefortii solid-state culture	Butter	1(3)-	sn-2 then sn-1,3	2-
Lipase suspension culture	Shea (SOS) ‡	16:0 1(3)- 18:0 1(3)- 18:1 2-	sn-1,3	16:0 2- 18:0 2- 18:1 1(3)-
P. roquefortii suspension culture	Shea (SOS)	16:0 1(3)- 18:0 1(3)- 18:1 1(3)-	sn-2	16:0 2- 18:0 2-
P. roquefortii solid-state culture	Shea (SOS)	16:0 1(3)- 18:0 1(3)- 18:1 1(3)- 18:1 2- as well (dairy strain Wisbey PJ)	sn-2 then sn-1,3 for Wisbey PJ	2-

† Data was derived from Tables 4.4, 4.5, 4.6, 5.5, 5.6 and 5.7.
‡ S = saturated fatty acids, O = oleic acid.

Acyl- migration is an intramolecular rearrangement which has been identified as an unavoidable consequence for most of the lipase-catalysed reactions. This type of rearrangement can be influenced by reaction time, temperature and the addition of a specific solvent (Boswinkel *et al* 1996, Millquist *et al* 1994, Janssen *et al* 1993). Boswinkel *et al* (1996) studied the kinetics of the rearrangement of 2- to 1(3)-monoacylglycerols with different chain length at 30 °C. These authors found that the equilibrium constants of the rearrangement reactions were relatively unaffected by fatty acid chain length. Equilibrium was reached after a prolonged reaction time (50 hours) and the ratio of 1(3)- to 2-monoacylglycerols varied between 4.6 and 8.5. In this study, reactions were carried out for a relatively short time (up to 25 hours at 10 °C and 4 hours at 25 °C) in suspension culture. Therefore, acyl- migration was not significant and 2-monoacylglycerols were the major isomers produced from butter oil by the commercial lipase (Figures 7.1 & 7.2).

Three research groups have used 1,3-specific lipases to catalyse the hydrolysis of triacylglycerols. The data is summarised in Table 7.2. Most commercial microbial lipases are 1,3-specific but some fungal lipases are 2-specific. For example, Sugihara *et al* (1991) reported that monoolein and diolein were produced by a novel sn-2 specific *Geotrichum candidum* lipase.

Table 7.2 Formation of monoacylglycerols by lipase-catalysed hydrolysis †

Source	Substrate	Bond split	pH	T (°C)	Main products	Methods of obtaining interface	References
Rhizopus ‡ *arrhizus*	castor oil	sn-1,3	7.5	am-bient	monoricinolein, ricinoleic acid	stirred tank reactor	Flenker & Spener 1990
Rhizopus ‡ *delemar*	palm oil	sn-1,3	7.0	35	1(3) & 2-MAG, acyl migration was reported	anionic surfactant	Holmberg & Osterberg 1988
Lipolase from Novo Nordisk	tricaprylin	sn-1,3	7.0	25	2-MAG, 1,2(2,3)-DAG	biphasic system modified by solvent	Mazur *et al* 1991
Geotrichum ‡ *candidum* (lipase IV)	triolein	sn-2	5.6	30	1,2(2,3)-DO : 1,3-DO (1:1.1), 1(3)-MO : 2-MO (3.6:1)	continuous stir at 500 rpm	Sugihara *et al* 1991

† MAG: monoacylglycerol, DAG: diacylglycerol, DO: diolein, MO: monoolein.
‡ Fungal species.

When butter oil was used as the substrate, the extracellular lipases produced by growth of *P. roquefortii* spores and emerging mycelium (suspension culture) were obviously 2-specific. However, two lipases were produced in the whole cell system (solid-state culture), the first was 2-specific and the second had the same specificity as the commercial *P. roquefortii* lipase (1,3-specific). This can be illustrated with the fungal growth cycle (Figure 7.7).

Figure 7.7 Growth cycle of *P. roquefortii* and the sampling systems in suspension and solid-state culture.

Degradation products were sampled in two stages of fungal growth in this investigation (Figure 7.7). Mainly 1(3)-monoacylglycerols were produced by the two strains of *P. roquefortii* at stage 1. In solid state culture (Figure 7.7), 1(3)-monoacylglycerols were the main isomers produced but there were 2-isomers produced as well. The ratios of 1(3)- : 2-isomers were about 2:1.

7.6 Pathways for conversion of butter oil

7.6.1 Hydrolysis by a commercial lipase from *P. roquefortii*

The action of the commercial *P. roquefortii* lipase on triacylglycerols in butter oil can be illustrated in Figure 7.8 (a) (Route 1). The lipase cleaved first the acyl group at sn-1 or sn-3 position to give 1,2 or 2,3-diacylglycerols and then the other external acyl group to leave the 2-monoacylglycerols as the main end products. Evidence of TLC analysis (Table 4.2) demonstrated that 1,2 (2,3)-diacylglycerols were the main intermediate products. The acyl group

of the 2-isomers can migrate to the sn-1 or sn-3 position of the glycerol backbone resulting in the production of small amount of 1(3)-monoacylglycerols.

Figure 7.8 Pathways for conversion of butter oil by *P. roquefortii* and a commercial lipase from *P. roquefortii*, (a) commercial *P. roquefortii* lipase in suspension culture, (b) two strains of *P. roquefortii* in suspension culture and (c) two strains of *P. roquefortii* in solid-state culture. TAG = triacylglycerols, DAG = diacylglycerols, MAG = monoacylglycerols, FAs = fatty acids. R_1, R_2 and R_3 refer to alkyl groups and may be the same or different.

7.6.2 Two strains of *P. roquefortii* in suspension culture

Pathways for conversion of triacylglycerols into monoacylglycerols in butter oil by the two strains of *P. roquefortii* in suspension culture are given in Figure 7.8 (b). The lipases from the

two strains of *P. roquefortii* cleaved acyl groups at the sn-2 position. This is confirmed by the TLC analysis where 1,3-diacylglycerols were the predominant intermediate products (Figure 4.2 and Table 4.2). 1,2 (2,3)-Diacylglycerols were detected as well possibly due to the existence of these diacylglycerols in original butter oil. The 1,3-diacylglycerols were then converted into 1- or 3- monoacylglycerols and fatty acids. 2-Monoacylglycerols could be produced by acyl migration and the ratios of 1(3)- to 2-monoacylglycerols are between 5.0 and 8.7 (Figure 7.2). These ratios are in agreement with the results of Boswinkel *et al* (1996) which has been described in section 7.5.

7.6.3 Two strains of *P. roquefortii* in solid-state culture

Possible pathways for conversion of butter oil into monoacylglycerols by the two strains of *P. roquefortii* in solid-state culture are given in Figure 7.8 (c). It was suspected that two lipases were produced by the two strains of *P. roquefortii* since relatively large proportion of 2-monoacylglycerols were found in solid-state culture compared to the monoacylglycerols produced in suspension culture (Figure 7.2 and 7.4). Firstly, the 2-specific lipase which was found in suspension culture hydrolysed triacylglycerols in butter oil into 1,3-diacylglycerols (Figure 7.8 c), and then the 1,3-diacylglycerols were converted into 2-monoacylglycerols. Acyl-migration from 2-isomers to 1(3)-isomers may have occurred. Secondly, with long incubation time, another lipase may have been produced. This lipase cleaved butter triacylglycerols at the sn-1 or sn-3 position to give 1,2- or 2,3-diacylglycerols (Figure 7.8 c), and then the 1,2- or 2,3-diacylglycerols were converted into 1 or 3-monoacylglycerols followed by possible acyl-migration. Iwai *et al* (1975) reported the presence of two lipases in *P. cyclopium*, a species closely related to *P. roquefortii*. These lipases have different pH optimum with respect to hydrolysis of olive oil. Lipase A is reactive between pH 6.5 and 9.0 and lipase B is reactive between pH 4.0 and 6.5. Eitenmiller *et al* (1970) purified two lipases from a *P. roquefortii* strain which would support the evidence in this thesis that two lipases were produced by *P. roquefortii*

with different specificity for triacylglycerols. The presence of two enzymes led to the production of α-and β-monoacylglycerols.

7.7 Pathways for conversion of Shea oil

Shea oil was selected in this investigation because of the limited numbers of long chain fatty acids in the triacylglycerols where most of the oleic acid occupies the sn-2 position of the triacylglycerol together with the absence of medium chain length fatty acids. In theory, Shea oil would be a good substrate for the determination of substrate specificity of lipases. A 1,3-specific lipase will hydrolyse the acyl groups from the positions sn-1 and sn-3 of the glycerol molecule where most stearic acid occupies, leaving oleic acid at the sn-2 position of the monoacylglycerols (Goh *et al* 1993). The proportion of 2-monoolein would be expected to increase dramatically in monoacylglycerols if the lipase is 1,3-specific.

A low concentration of monoolein and a high concentration of monostearin were found when Shea oil was hydrolysed by the 1,3-specific lipase (Figure 7.5). The reason for the unexpected low concentration of monoolein may be due to the greater reactivity of oleic acid. This acid is liquid at ambient temperature which may affect the stability of the acylglycerol allowing intramolecular rearrangement to occur. Results in TLC separation have confirmed that the concentrations of 1,2- (2,3-) and 1,3-diacylglycerols were similar when Shea oil was hydrolysed by the lipase (Table 5.1 & Figure 5.2). The oleate group could migrate from the sn-2 position to the sn-1(3) positions in the intermediate products, diacylglycerols as well as in the monoacylglycerols (Figure 7.9). The mixtures of diacylglycerols would be hydrolysed to give mixtures of monoacylglycerols (Figure 7.9).

Figure 7.9 Pathways for conversion of Shea oil by the commercial lipase at pH 7.0. S = saturated fatty acids (most of them are stearic acid), O = oleic acid, TAG = triacylglycerols, DAG = diacylglycerols, MAG = monoacylglycerols, FAs = fatty acids.

7.8 Monoacylglycerols as natural antimicrobials

The structures of monoacylglycerols determine the antimicrobial properties of these compounds.

Monoacylglycerols are amphiphilic. The hydroxyl group are hydrophilic whilst the positions with acyl groups are hydrophobic. These types of structures have antimicrobial activity at the aqueous / oil interface (Kabara & Eklund 1991).

Monoacylglycerols are more inhibitory against Gram positive bacteria than the corresponding fatty acids (Kabara & Eklund 1991). Unpublished work (Burgess, Sheffield Hallam University)

would contradict this. Burgess has demonstrated that lauric acid was more inhibitory than monolaurin in culture medium against *Listeria monocytogenes*. In a survey of the microbiological literature on the antimicrobial properties of monolaurin in culture medium and cheese, the research was carried out using the racemic mixtures of monoacylglycerols (Wang & Johnson 1992, Bautista *et al* 1993, Oh & Marshall 1995). There are at least three different chemical isomers in monoacylglycerols (1, 2 and 3-isomers). No work has yet been undertaken to establish the antimicrobial properties of the different isomers.

In a study on antilisterial properties of medium chain fatty acids in blue and white mould-ripened cheeses, Kinderlerer *et al* (1996) found that MCFAs existed in the blue veins where many conidia spores were present. These authors suggested that the localised MCFAs in the veins of blue mould-ripened cheese could act as natural preservatives and inhibit growth of listerias. In this study, monoacylglycerols (mainly 1(3)-isomers) were produced from butter oil (a cheese model) by both spores and mycelium by two strains of *P. roquefortii* at 10 °C and 25 °C. These results together with the findings of Kinderlerer *et al* (1996) demonstrate that the monoacylglycerols together with MCFAs produced by *P. roquefortii* during the ripening of blue cheese could form a natural preservation system and inhibit the growth of foodborne pathogens such as *Listeria monocytogenes*.

CHAPTER 8

CONCLUSIONS

1. A PTLC-GC-MS system was developed for the analysis and identification of small samples of acylglycerols and fatty acids (Liu 1997).

2. Monoacylglycerols were produced by conversion of butter and Shea oils by two strains of *P. roquefortii* and a commercial lipase from *P. roquefortii* at pH 7.0 and two temperatures (10 °C and 25 °C).

3. Butter oil was a better substrate than Shea oil for production of monoacylglycerols This reflected the considerable differences of physical properties and fatty acids composition in butter and Shea oils.

4. The commercial lipase cleaved acyl groups from the triacylglycerols in butter oil at the sn-1 and sn-3 positions to give the expected 2-monoacylglycerols as the main products. Small amounts of 1(3)-monoacylglycerols may have been produced by acyl migration from the sn-2 position to the sn-1(3) positions.

5. In suspension culture the two *P. roquefortii strains* (spores and emerging mycelium) cleaved the acyl groups from the triacylglycerols mainly at the sn-2 position to give 1- and 3-monoacylglycerols in butter oil.

6. In solid-state culture the growing fungal mycelium cleaved the triacylglycerols at the sn-2 (and 3 or 1) position to give the 1,3-monoacylglycerols as the main end products. There were significant amounts of 2-monoacylglycerols produced as well. This suggests that another lipase was produced by the mature mycelium of *P. roquefortii* with 1,3-specificity on butter oil as the sole carbon source.

7. The situation in Shea oil was more complex. Almost all the original triacylglycerols have the structure SOS (S stands for stearic acid and O for oleic acid). Unexpectedly 1(3)-monoolein was produced. This compound must originate by acyl migration from the original sn-2 position on the glycerol backbone. Monopalmitin and monostearin occurred mainly at the expected sn-1 or sn-3 position.

8. With Shea oil as a substrate the specificity of lipases was partly lost. This may be due to the reactivity of the oleate group. This fatty acid is liquid whilst stearic and palmitic acids are solids at ambient temperature. If the 1,3 specificity of an enzyme was expressed then monoolein would be the main end product.

9. There was no significant effect of temperature on the ratios of 1(3)- and 2- isomers of mono-acylglycerols in butter oil.

10. The effect of strains on production of monoacylglycerols *by P. roquefortii* was not significant. The only difference observed was that the dairy strain (Wisbey PJ) produced more monoolein (mainly 1,3-isomers) than the spoilage strain (FRR 2456) in solid-sate culture (Figure 7.3 c and 7.6 c).

11. Flavour compounds such as lactones and methyl ketones were derived from both oils by *P. roquefortii* but not by the commercial *P. roquefortii* lipase.

12. The evidence for the local production of free fatty acids in the blue region of blue cheese (Kinderlerer *et al* 1996) and the work reported in this thesis for the production of 1(3)-monoacyl-sn-glycerols by spores and emerging mycelium may lead to increasing research of natural antimicrobial systems in foods.

13. Further work could be undertaken by microbiologists to test which isomers of the mono-acylglycerols have antimicrobial activity. Chiral Chromatography coupled with GC or

HPLC could be utilised to separate the enantiomers of 1- and 3-monoacylglycerols. A comparison of the monoacylglycerols structures in blue and white regions of blue mould-ripened cheeses could be carried out to see if spore metabolism in the blue region resulted in the fermentation of 1(3)-monoacylglycerols. This would confirm the work reported in this thesis which was undertaken in a model system (butter oil) in a real food fermentation.

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Synthesis and analysis of monoacylglycerols produced from Shea and butter oils by *Penicillium roquefortii*

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Monoacylglycerols (MAGs) are used as emulsifying agents in various food systems and account for about 75% of the world production of food emulsifiers.[1] Monoacylglycerols have anti-microbial activity against Gram-positive bacteria.[2] For example monolaurin is used in Europe and the United States as a food preservative.

A spoilage strain of *Penicillium roquefortii*, originally isolated from melon, provided by Dr. Ailsa Hocking, was used in this work. This strain will be identified by random amplified polymorphic DNA analysis.[3] Degradation of butter and shea oil (a cocoa butter substitute) was studied in Petri dish culture at two temperatures (10 °C and 25 °C). The MAGs were isolated by Preparative Thin Layer Chromatography and the fatty acid composition was determined by Gas Chromatography (GC) and Gas Chromatography - Mass Spectrometry (GC-MS). The composition and chemical structure of the MAGs were determined by high temperature GC and GC-MS analysis of the trimethylsilyl ethers.

The MAGs from both oils contained significantly less oleic acid (cis-9-18:1) than the substrates. Higher concentrations of the 1- isomers than the 2- isomers were found in the MAGs produced from each oil. Unexpectedly, medium chain fatty acids (MCFAs) (6:0 - 12:0) were found in the MAGs derived from shea oil which were not found in the substrate. It is not known if they were degradation products from the substrate or if they had been synthesised by the *Penicillium* mould. Medium chain fatty acids were preferentially used at 10 °C when butter oil was used as a substrate. The reduction of MCFAs in butter oil could be due to a partial beta oxidation leading to production of methyl ketones.[4]

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Preparative Thin Layer Chromatography for the separation and subsequent derivatisation of acylglycerols

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Thin Layer Chromatography (TLC) has been a reliable, well-documented and widely used separation technique based on differential dynamic sorption/dissorption equilibrium of solute molecules since 1980's. The aim of this study was to use simple and inexpensive preparative TLC technique to separate acylglycerols which had been produced by fungal catalysis. The final aim was to find which monoacylglycerols were formed from milk fat with the idea of using the compounds as natural antimicrobials in foods.

Acylglycerols were extracted from medium by solvent mixture of hexane and 2-propanol. Production of acylglycerol species were screened by analytical TLC. Relatively large volume of acylglycerols in solvent (2 - 6 ml, 10 % w/v) were separated by preparative TLC technique. The band of monoacylglycerols were isolated from the preparative TLC plate and the structural isomers of monoacylglycerols were analysed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) as their trimethylsilyl (TMS) ethers.

Complete separation of acylglycerols and fatty acids were achieved by preparative TLC. The Rf values of mono-, di- and triacylglycerols and fatty acids were 0.048, 0.157, 0.202, 0.546 and 0.699 respectively. Followed by derivatisation and GC and GC-MS techniques, acylglycerols composition and structures could be determined. This study demonstrated that with simple and inexpensive preparative TLC technique, the structures of complex biosynthetic compounds could be analysed easily.

Appendix 2 Coshh Regulations

LFM - COSHH FORM COSHH REF NO:

ASSESSMENT OF HEALTH RISK ASSOCIATED WITH PROPOSED PROCEDURE

Personnel Involved: QIN TAO LIU	
Title of Experiment/Procedure: Extraction of lipids from agar medium Aim: To analyse acylglycerols	
Brief Description of Procedure: Extraction of lipids with solvent, and then analysis with GC	
Substances used: 2-propanol (isopropanol), hexane	Hazards identified: flamable & harmful substance
Information sources: BDH	
Is there a less hazardous substance? If so, why not use it? NO.	
Control Measures to be adopted: Use gloves, eye protection. Keep away from sources of ignition. Use in well ventilated area.	
Required checks and their frequency, on the adequacy and maintenance of control measures during the course of the experiment: Check control measures periodically during procedure	
Disposal procedures during and at end of experiment: Store in waste bottles	

Name of Assessor:	Name of Supervisor: (for students only)	Head of School, or Nominee
Qintao Liu		
Status of Assessor: Research student		
Date: 15/4/96	Date 15/4/96	Date
Signed	Signed Judith Kuhlen	Signed

DW02.COSHH2

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EMERGENCY PROCEDURES If any of the substances or procedures identified overleaf is likely to pose a special hazard in an emergency, then identify below the action to be taken.
Spillage/uncontrolled release: Clean with plenty of water and mop. Flush to drains with large quantities of water.
Fire: Get out with CO_2 extinguisher
If personnel are affected (fume, contamination, etc) treatment to be adopted: Standard First Aid treatment Seek medical services if necessary.

COMPLACENCE WITH THE ABOVE PRECAUTIONARY MEASURES WILL ENSURE HAZARD ASSOCIATED RISKS ARE MINIMISED

Anyone other than the assessor involved in this procedure should sign the statement below.

I have read this document and understand it.

(Signed) ..... (Date) .....

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