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Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
Preparation, characterisation and application of naturally derived polar lipids through lipolysis

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
in
Food Technology

at
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Abstract

Monoglycerides are lipid based emulsifiers extensively used for their broad technical function in the food industry. Commercial monoglycerides are generally manufactured through chemical synthesis; however, lipolysis of triglycerides by lipase enzyme provides a biochemical pathway by which monoglycerides may be produced. This is particularly appealing for consumers for whom all natural and clean labelled food products are a particular driver. Accordingly, rather than replacing monoglycerides from formulations with other types of emulsifiers (and that may lack the requisite functionality), an alternative approach may be to develop a non-chemical and more natural pathway to produce the emulsifier, thereby allowing the particular monoglyceride functionality to be retained within products. Therefore, this study was conducted to investigate the feasibility of using lipase enzyme as a processing tool to synthesise polar lipids, namely monoglycerides, in situ of the manufacture of whippable food emulsions, such as cream and ice cream.

This concept idea was initially proven viable through interfacial tension (IFT) measurements obtained using a straight-forward surface characterisation technique. *R. miehei* lipase was found to competitively bind at the interface of vegetable oils-water and that the adsorbed protein (sodium caseinate and whey protein isolate) or surfactant layer (lecithin and Tween 80) did not act as a barrier to lipase adsorption at the oil-water interface. IFT measurements were also able to demonstrate the progressive accumulation of polar lipids at the oil-water interface arising from lipolysis, and were additionally used to indicate how thermal treatment of the enzyme could be used to terminate activity.

In considering how the requisite functionality could be achieved for whippable emulsion formulations, emulsion droplet size, type of emulsifiers used as well as lipase concentration were shown to be key variables by which the extent and rate of lipolysis could be manipulated and controlled. The results showed that formulation (emulsifier types and oil content) and processing conditions (Microfluidizer® pressure and number of passes) had significant effects on the emulsion droplet size.

As part of controlling the extent of lipolysis, the conditions by which the reaction could be terminated were investigated by measuring the viability of *R. miehei* lipase against thermal treatment. Results showed that the *R. miehei* lipase was thermostable up to temperatures of 70 °C. Above this temperature, substantial reduction of the residual activity occurred. However, even elevated temperature of between 90 and 100 °C did not immediately inactivate the lipase, with heating for ~ 2 min required before activity was no longer detected. In terms of emulsion stability, the palm oil emulsion tested in this study was found to be thermostable up to 100 °C, thus allowing development of a thermalisation step that was able to inactivate the enzyme without compromising the stability of the emulsion.

The shear stability analyses on lipolysed O/W emulsion showed the lipolysed emulsions were susceptible to shear-induced aggregation, and that the degree of aggregation could be manipulated as a consequence of controlling the extent of lipolysis through either enzyme concentration or holding time. The drastic increase in the viscosity curve between the non-lipolysed and lipolysed emulsion suggested that the shear–induced partial coalescence was primarily due to the lipolysis reaction and was not as a result of the high fat content (30 %). The findings elucidate the ability of the generated polar lipids in the emulsion to displace the
existing sodium caseinate adsorbed layer, thus compromising emulsion stability upon shearing.

The quantification of synthesised polar lipids from the triglyceride component of fat droplets by the lipolysis reaction showed a mixture of fatty acids, di- and mono-glycerides being produced. Palmitic acid was observed to be the main liberated fatty acids. While, monoolein and monopalmitin were the most prominent monoglycerides, with measured concentrations of $3.755 \pm 0.895$ and $1.660 \pm 0.657$ mg / g fat respectively after 15 min with lipase concentration of 50 mg / g fat. The relative concentration of polar lipids produced was found to be dependent on the lipase concentration as well as time of lipolysis. Furthermore, up to 30 min of lipolysis (concentration 50 mg / g fat) were seen to have no observable effect on the droplet size distribution of the emulsion suggesting that quiescently stable emulsions could be produced. The results show the importance of controlling reaction conditions (both enzyme concentration and reaction time) in order to provide requisite functionality without excessively destabilising emulsions such that droplet structuring can occur under quiescent conditions.

The generation of monoglycerides at quantum satis levels able to impart critical functionality was demonstrated in whipped cream and ice cream. The addition of *R. miehei* lipase at very low concentration of 5 mg / g fat was able to produce a rigid and stable whipped cream with overrun exceeding 100 %. However, good stability of the whipped cream over time was achievable with concentration above 10 mg / g fat. Similarly, ice cream made with the addition of 5 mg / g fat exhibited good melt stability and firmness. The findings proved the feasibility of *in situ* production of polar lipids, namely monoglycerides and fatty acids, in replicating the functionality imparted by commercial monoglycerides in whippable emulsions.

Thus, the findings in this thesis offer an alternative biochemical pathway for the generation of polar lipids to that of commercially available monoglycerides, which are currently produced synthetically. The potential for using this approach as part of the processing step for food emulsion manufacture has also been demonstrated. The concept can be tailored for various emulsion based food products.
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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xviii</td>
</tr>
<tr>
<td>List of Equations</td>
<td>xix</td>
</tr>
</tbody>
</table>

## Chapter 1: General Introduction and Thesis Structure
1.1 Introduction                                                        1
1.2 Objectives                                                         4
1.3 Thesis Structure                                                   5

## Chapter 2: Literature Review
2.1 Emulsion                                                           8
   2.1.1 Emulsion formation                                             10
2.2 Emulsion stability                                                13
2.3 Destabilisation of emulsion                                       15
   2.3.1 Flocculation                                                  18
   2.3.2 Creaming                                                      19
   2.3.3 Coalescence                                                  20
2.4 Emulsion interfacial characteristic                               24
2.5 Emulsifier and their application                                  25
   2.5.1 Monoglycerides in food                                       33
   2.5.2 Legal aspect of emulsifiers                                  34
2.6 Lipid                                                             37
   2.6.1 Synthesis of monoglycerides                                  38
      2.6.1.1 Esterification                                           39
      2.6.1.2 Glycerolysis                                            38
      2.6.1.3 Enzymatic hydrolysis of triglycerides                   42
   2.6.2 Triglycerides                                                43
   2.6.3 Fatty acids                                                  46
2.7 Lipase                                                            47
   2.7.1 Microbial lipase                                             48
   2.7.2 Lipase specificity                                           50
   2.7.3 Lipase mediated reactions and synthesis of monoglycerides   52

## Chapter 3: Materials and Methodology
3.1 Materials                                                         60
   3.1.1 Lipase                                                       60
   3.1.2 Emulsifiers                                                  60
   3.1.3 Vegetable oils                                               60
Table of Contents

3.1.4 Chemicals .................................................................................................................... 61
3.2 Methodology ..................................................................................................................... 61
   3.2.1 Emulsification by microfluidization ................................................................. 61
   3.2.2 Determination of emulsion droplet size ....................................................... 62
   3.2.3 Determination of lipase activity ................................................................. 63
   3.2.4 Microscopic observation with confocal laser scanning microscope .......... 63
   3.2.5 Lipolysis of emulsion ................................................................................... 64

Chapter 4: Effect of Rhizomucor miehei lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface
4.1 Abstract ............................................................................................................................... 65
4.2 Introduction ......................................................................................................................... 66
4.3 Materials and methodology ............................................................................................. 69
   4.3.1 Materials ............................................................................................................. 69
   4.3.2 Methodology ........................................................................................................ 69
      4.3.2.1 Interfacial tension measurement by pendant-drop tensiometer ........ 69
      4.3.2.2 Statistical analyses ................................................................................ 71
4.4 Results and discussion ....................................................................................................... 71
   4.4.1 Effect of R. miehei lipase on the IFT of oil and water ........................................ 71
   4.4.2 Effect of different types of emulsifier on the adsorption of R. miehei lipase on the interface ................................................................. 75
      4.4.2.1 Protein emulsifiers-sodium caseinate and whey protein isolate ........ 75
      4.4.2.2 Low molecular weight surfactant – lecithin and Tween 80 .................. 78
   4.4.3 Effect of monopalmitin, monostearin and monoolein on the IFT of vegetable oil and aqueous phase containing protein emulsifiers and surfactants ......................... 84
4.5 Conclusion ........................................................................................................................ 90

Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions
5.1 Abstract ............................................................................................................................... 91
5.2 Introduction ......................................................................................................................... 92
5.3 Materials and methodology ............................................................................................. 95
   5.3.1 Materials ............................................................................................................. 95
   5.3.2 Methodology ........................................................................................................ 95
      5.3.2.1 Emulsification by microfluidization ................................................................. 95
      5.3.2.2 Lipolysis of emulsion ................................................................................ 95
      5.3.2.3 Determination of emulsion droplet size ....................................................... 95
      5.3.2.4 Determination of lipolysis rate ..................................................................... 95
      5.3.2.5 Microscopic observation with confocal laser scanning microscope .......... 96
      5.3.2.6 Experimental design ................................................................................ 96
      5.3.2.7 Statistical analyses ................................................................................... 98
5.4 Result and discussion ......................................................................................................... 98
   5.4.1 Effect of microfluidization pressure, number of passes, emulsifier types and oil content on emulsion droplet size distribution .................................................. 98
      5.4.1.1 Canola oil emulsion with protein emulsifiers i.e. sodium caseinate and whey protein isolate ................................................................. 98
      5.4.1.2 Canola oil emulsion with low molecular weight surfactants i.e. lecithin and Tween 80 ................................................................. 100
Table of Contents

5.4.1.3 Palm oil emulsion with protein emulsifier i.e. sodium caseinate and whey protein isolate ................................................................. 101
5.4.1.4 Microscopic observation of emulsion .......................................................... 103
5.4.1.5 Main effect and interaction plots ............................................................... 106
5.4.2 Effect of specific surface area and emulsifier types area on the lipolysis of O/W emulsion .............................................................. 116
5.4.3 Effect of enzyme loading on the lipolysis of O/W emulsion ...................... 122
5.4.4 Effect of oil mass and oil type on lipolysis rate of O/W emulsion .............. 123
5.4.5 Main effect of specific surface area, emulsifier type, oil mass and type, and lipase loading on V̇o and FFAmax during lipolysis of O/W emulsion ................................................................. 126
5.4.6 Kinetic of O/W emulsions lipolysis ................................................................. 130
5.5 Conclusion ........................................................................................................ 132

Chapter 6: Thermal stability of sodium caseinate stabilised O/W emulsion and Rhizomucor miehei lipase
6.1 Abstract ........................................................................................................... 134
6.2 Introduction ....................................................................................................... 134
6.3 Materials and methodology ............................................................................ 137
  6.3.1 Materials ..................................................................................................... 137
  6.3.2 Methodology ................................................................................................ 137
    6.3.2.1 Emulsification by microfluidization .......................................................... 137
    6.3.2.2 Lipolysis of emulsion ............................................................................. 137
    6.3.2.3 Heat stability studies on R. miehei lipase .................................................. 137
    6.3.2.4 Heat stability studies on O/W emulsion .................................................. 138
    6.3.2.5 Determination of lipase residual activity .................................................. 138
    6.3.2.6 Determination of emulsion droplet size .................................................. 138
    6.3.2.7 Statistical analyses ................................................................................ 138
  6.4 Results and discussion ..................................................................................... 139
    6.4.1 Influence of thermal treatment of R. miehei lipase activity ...................... 139
    6.4.2 Influence of thermal treatment of O/W emulsion stability ...................... 142
  6.5 Conclusion ....................................................................................................... 147

Chapter 7: Effect of lipolysis by Rhizomucor miehei lipase on the shear stability of sodium caseinate stabilised O/W emulsion
7.1 Abstract ............................................................................................................ 148
7.2 Introduction ....................................................................................................... 149
7.3 Materials and methodology ............................................................................ 152
  7.3.1 Materials ..................................................................................................... 152
  7.3.2 Methodology ................................................................................................ 152
    7.3.2.1 Emulsification by microfluidization .......................................................... 152
    7.3.2.2 Lipolysis of emulsion ............................................................................. 152
    7.3.2.3 Shear stability analysis ......................................................................... 152
    7.3.2.4 Determination of emulsion droplet size .................................................. 153
    7.3.2.5 Statistical analyses ................................................................................ 153
  7.4 Results and discussion ..................................................................................... 153
    7.4.1 Effect of shear rates on shearing stability of O/W emulsion ..................... 153
    7.4.2 Effect of lipolysis with R. miehei lipase on shearing stability of O/W emulsion ... 155
    7.4.3 Effect of aging on shearing stability of O/W emulsion ......................... 158
    7.4.4 Changes to emulsion droplets distribution profile during shearing .......... 159
  7.5 Conclusion ....................................................................................................... 162
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions
8.1 Abstract ............................................................................................................................... 164
8.2 Introduction ........................................................................................................................ 165
8.3 Materials and methodology .............................................................................................. 167
  8.3.1 Materials ..................................................................................................................... 167
  8.3.2 Methodology .............................................................................................................. 168
    8.3.2.1 Emulsification by microfluidization ....................................................................... 168
    8.3.2.2 Lipolysis of O/W emulsion ................................................................................... 168
    8.3.2.3 Determination of O/W emulsion droplet size ....................................................... 168
    8.3.2.4 Extraction of lipid from O/W emulsion ................................................................. 168
    8.3.2.5 Separation of lipid species by thin layer chromatography (TLC) ......................... 169
    8.3.2.6 Derivatization for fatty acid methyl ester (FAME) analysis ............................... 170
    8.3.2.7 Detection of FAMEs by gas chromatography (GC) ............................................. 171
    8.3.2.8 Conjugation of R. miehei lipase with carboxyl quantum dots ............................ 171
    8.3.2.9 Microscopic observation with confocal laser scanning microscope .................... 172
    8.3.2.10 Statistical analyses .............................................................................................. 172
8.4 Results and discussion ...................................................................................................... 173
  8.4.1 Lipolysis product quantification .................................................................................. 173
  8.4.2 Changes of O/W emulsion droplet size distribution as a result of lipolysis............... 180
  8.4.3 Microscopic observation of lipolysed O/W emulsion ................................................ 183
8.5 Conclusion ....................................................................................................................... 188

Chapter 9: Functionality of lipolysed O/W emulsion as imitation whipped cream and ice
cream
9.1 Abstract ............................................................................................................................... 189
9.2 Introduction ........................................................................................................................ 190
9.3 Materials and methodology .............................................................................................. 196
  9.3.1 Materials ..................................................................................................................... 196
  9.3.2 Methodology .............................................................................................................. 196
    9.3.2.1 Processing of emulsion mix for whipped cream .................................................. 196
    9.3.2.2 Whipping process and determination of overrun of whipped lipolysed emulsion ...... 200
    9.3.2.3 Processing of ice cream ....................................................................................... 201
    9.3.2.4 Physicochemical analyses .................................................................................... 203
      9.3.2.4.1 Visual test and piping stability of whipped cream ........................................... 203
      9.3.2.4.2 Determination of freeze-thaw stability of whipped cream ............................. 203
      9.3.2.4.3 Determination of serum drainage of whipped cream ................................. 204
      9.3.2.4.4 Determination of hardness of whipped emulsion ........................................... 205
      9.3.2.4.5 Determination of hardness and tackiness of ice cream ............................... 205
      9.3.2.4.6 Determination of emulsion droplet size for whipped cream and ice cream ....... 206
      9.3.2.4.7 Microscopic observation of whipped cream by confocal laser scanning microscope ........................................................................................................... 206
    9.3.2.5 Statistical analyses .............................................................................................. 207
9.4 Results and discussion ...................................................................................................... 207
  9.4.1 Physicochemical analyses of whipped cream made with lipolysed emulsion .......... 207
    9.4.1.1 Whipping process and overrun ........................................................................... 207
    9.4.1.2 Visual observation and piping stability of whipped cream ................................. 210
    9.4.1.3 Freeze thaw stability of whipped emulsion ......................................................... 218
    9.4.1.4 Serum drainage of the whipped emulsion ............................................................. 220
    9.4.1.5 Determination of hardness of whipped emulsion ................................................ 221
List of Figures

Figure 2.1: Various physical mechanisms of emulsion destabilisation, including creaming, sedimentation, flocculation, coalescence and phase inversion ........................................ 17

Figure 2.2: Different stages of the coalescence process; droplet contact, droplet crystallisation and oil mixing around the contact point .................................................... 23

Figure 2.3: Esterification of glycerol and fatty acid ............................................................. 39

Figure 2.4: Glycerolysis of triglycerides with glycerol ........................................................ 40

Figure 2.5: Hydrolysis of triglycerides by 1,3-selective lipase ............................................ 43

Figure 2.6: Triglyceride molecule ......................................................................................... 45

Figure 2.7: (1) The synthesis of 2-monoglyceride using selective hydrolysis with 1,3-specific lipase, (2) Esterification of glycerol and fatty acids and (3) glycerolysis of fats/oil with glycerol to produce 1(2)-monoglyceride ................................................................. 56

Figure 4.1: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase, with the addition of R. miehei lipase, inactivated R. miehei lipase or no enzyme into the system ........................................................................................................... 72

Figure 4.2: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing sodium caseinate, with or without the addition of R. miehei lipase into the system .................................................................................................................................................. 76

Figure 4.3: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing whey protein isolate, with or without the addition of R. miehei lipase into the system .................................................................................................................... 77

Figure 4.4: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing lecithin, with or without the addition of R. miehei lipase into the system ...... 80

Figure 4.5: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing Tween 80, with or without the addition of R. miehei lipase into the system .. 82

Figure 4.6: Main effects plot for interfacial tension (IFT) for oil type, presence of lipase and emulsifier type ................................................................................................................... 83

Figure 4.7: Interaction plot of all tested variables (oil type, presence of lipase and emulsifier type) for interfacial tension (IFT) ....................................................................................... 83

Figure 4.8: Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride ........................................................................................................... 85

Figure 4.9: Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride /sodium caseinate or whey protein isolate ......................... 87
Figure 4.10: Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride/lecithin or Tween 80 ......................................................... 89

Figure 5.1: Effect of processing and formulation on specific surface area of canola oil emulsion stabilised with 1 % (w/w) sodium caseinate or whey protein isolate ................. 99

Figure 5.2: Effect of processing and formulation on specific surface area of canola oil emulsion stabilised with 1 % (w/w) lecithin and Tween 80 .............................................. 101

Figure 5.3: Effect of processing and formulation on specific surface area of 30% palm oil emulsion stabilised with sodium caseinate and whey protein isolate (1 % w/w) ........ 102

Figure 5.4: Effect of Microfluidizer® processing on the microstructure of 30 % (w/w) canola oil emulsion stabilised with sodium caseinate (1 % w/w) ........................................ 104

Figure 5.5: Effect of Microfluidizer® processing on the microstructure of 30 % (w/w) palm oil emulsion stabilised with sodium caseinate (1 % w/w) ............................................. 105

Figure 5.6: Main effects plots of emulsifier type, oil content, Microfluidizer® pressure and number of passes on canola oil emulsion specific surface area ........................................ 107

Figure 5.7: Main effects plots of emulsifier type, Microfluidizer® pressure and number of passes on canola oil emulsion specific surface area ............................................. 108

Figure 5.8: Effect of specific surface area on initial velocity of lipolysis of canola oil (20 % w/w)-in-water emulsion .............................................................................................. 117

Figure 5.9: Effect of specific surface area on the extent of lipolysis (FFA_max) of canola oil (20 % w/w)-in-water emulsion ....................................................................................... 118

Figure 5.10: Effect of specific surface area on initial velocity of lipolysis of palm oil (30 % w/w)-in-water emulsion ............................................................................................... 121

Figure 5.11: Effect of specific surface area on the extent of lipolysis (FFA_max) of palm oil (30 % w/w)-in-water emulsion ....................................................................................... 122

Figure 5.12: Effect of lipase loading on the lipolysis of palm oil (30 % w/w)-in-water emulsion stabilised by sodium caseinate (1 % w/w) .............................................................. 123

Figure 5.13: Effect of oil mass and oil type on initial velocity of lipolysis of O/W emulsion ................................................................................................................................. 125

Figure 5.14: Effect of oil mass and oil type on the extent of lipolysis (FFA_max) of O/W emulsion ............................................................................................................................ 125

Figure 5.15: Main effect plot of specific surface area, emulsifier type, and lipase concentration on V_o and FFA_max for the lipolysis of O/W emulsion .............................................. 128

Figure 5.16: Main effect plot of oil mass and oil type on V_o and FFA_max for the lipolysis of O/W emulsion ............................................................ 129

Figure 5.17: Lineweaver-Burk double reciprocal plot of velocity as function of canola oil concentration (emulsion of canola oil-sodium caseinate) ........................................ 131
List of Figures

Figure 5.18: Lineweaver-Burk double reciprocal plot of velocity as function of canola oil concentration (emulsion of canola oil-lecithin) ................................................................. 131

Figure 6.1: Inactivation kinetics of *R. miehei* lipase at 50, 60, 70, 80, 90 and 100 °C ........ 139

Figure 6.2: Arrhenius plot of temperature dependence of the logarithm of $k_{\text{exp}}$ constant versus inverse temperature in K$^{-1}$ for the determination of energy of deactivation ........ 142

Figure 6.3: Particle size distribution of thermally treated palm oil (30 % w/w) emulsion 145

Figure 6.4: Particle size distribution of thermally treated lipolysed palm oil (30 % w/w) emulsion (*R. miehei* lipase 50 mg per g fat, 30 min) .......................................................... 147

Figure 7.1: Effect of shear rates on shearing stability of lipolysed O/W emulsion. Lipolysis time: 30 min, *R. miehei* lipase concentration: 50 mg / g fat (Insert: The shear curve of only 500, 800 and 1500 s$^{-1}$) ........................................................................................................ 155

Figure 7.2: Effect of *R. miehei* lipase concentration on shearing stability of lipolysed O/W emulsion. Lipolysis time: 30 min, Shearing rate: 100 s$^{-1}$ .............................................................. 157

Figure 7.3 Effect of lipolysis time on shearing stability of lipolysed O/W emulsion. *R. miehei* lipase concentration: 50 mg / g fat, Shearing rate: 100 s$^{-1}$ ................................................... 157

Figure 7.4: Effect of aging time on shearing stability of lipolysed O/W emulsion. Lipolysis time: 30 min, *R. miehei* lipase concentration: 50 mg / g fat, Shearing rate: 100 s$^{-1}$ ................... 158

Figure 7.5: The shear-induced aggregation curve of lipolysed O/W emulsion at shear rate 100 s$^{-1}$. Lipolysis time: 30 min, *R. miehei* lipase concentration = 50 mg per g of fat. Insert graph – The changes of lipolysed emulsions’ droplet distribution profile at different shearing time (A) 0s, B) 210 s, C) 1200 s, D) 1800 s and E) 3000 s ................................................................. 161

Figure 7.6: Specific surface area and D(4.3) of lipolysed palm oil (30 %) emulsion at different shearing time ..................................................................................................................... 162

Figure 8.1: Example of separated lipid spots on TLC plates when observe under UV light 173

Figure 8.2: Droplet size distribution of O/W emulsion lipolysed with *R. miehei* lipase (50 mg per g fat) at different lipolysis time .............................................................. 182

Figure 8.4 (a): Confocal laser scanning microscope image of O/W emulsion lipolysed with excess QDs conjugated *R. miehei* lipase .............................................................. 187

Figure 8.4 : (b) Confocal laser scanning microscope image of O/W emulsion lipolysed with limited QDs conjugated *R. miehei* lipase (c - h) Zoomed images of a single fat globules with attached QDs conjugated *R. miehei* lipase on the surface at different time (0, 1, 3, 5, 6 and 8 min) ................................................................. 187

Figure 9.1: Schematic diagram of the structure development taking place during the formation of whipped cream .......................................................... 192

Figure 9.2: Flow chart of emulsion mixes processing for whipping cream ........................ 199

Figure 9.3: Flow chart of ice cream processing ................................................................. 202
Figure 9.4: Paper ruler inserted to measure the cream shrinkage for determining the freeze-thaw stability ................................................................. 204

Figure 9.5: Determination of serum drainage set-up ......................................................... 205

Figure 9.6: Overrun of whipped O/W emulsion (30 % fat) lipolysed with different *R. miehei* lipase concentration (Lipolysis time - 15 min) ........................................ 208

Figure 9.7: Non-lipolysed emulsion remained in liquid form after aging process overnight at 4 °C ................................................................................................................. 211

Figure 9.8: Scoopable soft solid structure of lipolysed emulsion after aging overnight at 4 °C (Lipolysis condition: *R. miehei* lipase concentration 50 mg per g of fat, 15 min) .......... 211

Figure 9.9: Agglomerated fat globules forming after whipping of non-lipolysed emulsion .................................................................................................................. 212

Figure 9.10: Piping stability of whipped emulsion lipolysed with different lipase concentration at ambient conditions (21 °C) ................................................................. 214

Figure 9.11: The visual changes of whipped emulsion during the freeze-thaw stability test ......................................................................................................................... 219

Figure 9.12: Serum drainage of whipped emulsion lipolysed with different lipase concentration after 1 h at ambient temperature (21 °C) ............................................................... 221

Figure 9.13: Hardness of whipped emulsion represented as maximum force (N) recorded ................................................................................................................................. 222

Figure 9.14: Droplet size distribution of non-lipolysed emulsion in its original form and after whipping ............................................................................................................. 223

Figure 9.15: Droplet size distribution of whipped emulsion lipolysed with different lipase concentration ........................................................................................................ 225

Figure 9.16: Confocal micrographs of whipped cream from emulsion lipolysed with various *R. miehei* lipase concentration (black-air bubbles, red/yellow-fat, green-protein in serum phase), scale bar = 10 μm ................................................................. 226

Figure 9.17: Melt stability of ice cream samples at 21 °C .................................................. 228

Figure 9.18: Hardness of ice cream samples ...................................................................... 230

Figure 9.19: Tackiness of ice cream samples ..................................................................... 231

Figure 9.20: Droplet size distribution of ice cream ............................................................ 232
List of Tables

Table 2.1: Food emulsifiers, their legal numbers and typical uses in food .......................... 31
Table 2.2: Isolation of lipase from various microorganisms ............................................... 49
Table 2.3: Different types of lipase mediated reaction, substrates and source of lipase used by several authors .............................................................................................................. 59
Table 5.1: Factorial experiment factors and levels for investigating the effect of emulsifier type, oil content, Microfluidizer® pressure and number of passes on canola oil emulsion droplet size ......................................................................................................................... 96
Table 5.2: Factorial experiment factors and levels for investigating the effect of emulsifier type, Microfluidizer® pressure and number of passes on palm oil emulsion droplet size 97
Table 5.3: Variables used for investigating the effect of specific surface area, emulsifier type, oil content and lipase loading on lipolysis of O/W emulsion ................................................................. 97
Table 5.4: Analysis of variance for the effect of emulsifier type, oil content, Microfluidizer® pressure and number of passes on the specific surface area of canola oil emulsion ............. 106
Table 5.5: Analysis of variance for the effect of emulsifier type, oil content, Microfluidizer® pressure and number of passes on the specific surface area of palm oil emulsion .............. 108
Table 5.6: Combination of Microfluidizer® pressure and number of passes to obtain coarse, intermediate and fine emulsion used for lipolysis experiments for the analysis of effect of specific surface area and type of emulsifier ........................................................................................................... 114
Table 5.7: Combination of Microfluidizer® pressure and number of passes to obtain emulsion used for lipolysis experiments for the analysis of effect of lipase loading .................... 115
Table 5.8: Combination of Microfluidizer® pressure and number of passes to emulsion with similar specific surface area for lipolysis experiments for the analysis of effect of oil mass and type .................................................................................................................................................. 115
Table 5.9: Effect of specific surface area, emulsifier type, oil mass and type, and lipase concentration on $V_o$ and $FFA_{max}$ for the lipolysis of O/W emulsion ................................................................. 127
Table 6.1: Kinetic ($k_{exp}$ and $t_{1/2}$) parameters of $R. mhei$ lipase thermal inactivation ........... 140
Table 6.2: Mean volume weighted diameter ($D_{4,3}$) of thermally treated O/W emulsion 144
Table 6.3: Mean volume weighted diameter ($D_{4,3}$) of thermally treated lipolysed (50 mg per g fat, 30 min) O/W emulsion ................................................................................................................................. 148
Table 8.1: Fatty acids concentration from lipolysed O/W emulsion with 5 mg lipase per g of fat ............................................................................................................................................... 176
List of Tables

Table 8.2: Fatty acids concentration from lipolysed O/W emulsion with 25 mg lipase per g of fat ....................................................................................................................................... 177

Table 8.3: Fatty acids concentration from lipolysed O/W emulsion with 50 mg lipase per g of fat ....................................................................................................................................... 180

Table 8.4: Specific surface area, D(4,3) and D(3,2) of lipolysed O/W emulsion with R. miehei lipase (50 mg per g fat) at different time ........................................................................... 182

Table 9.1: Formulation of ice cream mix .................................................................................. 201

Table 9.2: The collapsed height of whipped emulsion and assessment on the cream shrinkage after the freeze-thaw test ........................................................................................................ 220
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degree celcius</td>
</tr>
<tr>
<td>cont.</td>
<td>continue</td>
</tr>
<tr>
<td>D(3,2)</td>
<td>particle volume/surface mean (also called the Sauter mean)</td>
</tr>
<tr>
<td>D(4,3)</td>
<td>particle mean diameter over volume</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester</td>
</tr>
<tr>
<td>FFA_{max}</td>
<td>extent of lipolysis</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic-lipophilic balance</td>
</tr>
<tr>
<td>IFT</td>
<td>Interfacial tension</td>
</tr>
<tr>
<td>k_{exp}</td>
<td>deactivation rate constant</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>μm</td>
<td>micrometre</td>
</tr>
<tr>
<td>μmol</td>
<td>micromole</td>
</tr>
<tr>
<td>MPa</td>
<td>megapascal</td>
</tr>
<tr>
<td>N</td>
<td>Newton</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>η_D/η_C</td>
<td>viscosity ratio dispersed over continuous phase</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>O/W</td>
<td>oil-in-water</td>
</tr>
<tr>
<td>Pa.s</td>
<td>Pascal second</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>[S]</td>
<td>substrate concentration</td>
</tr>
<tr>
<td>SMP</td>
<td>skim milk powder</td>
</tr>
<tr>
<td>sn</td>
<td>stereospecific numbering</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>t_{1/2}</td>
<td>half-life values</td>
</tr>
<tr>
<td>t_c</td>
<td>aggregation time</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>U</td>
<td>enzyme activity unit</td>
</tr>
<tr>
<td>US FDA</td>
<td>United State of America Food and Drug Administration</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>velocity</td>
</tr>
<tr>
<td>v_0</td>
<td>initial lipolysis velocity</td>
</tr>
<tr>
<td>wt %</td>
<td>weight percent</td>
</tr>
<tr>
<td>w/w</td>
<td>weight per weight</td>
</tr>
</tbody>
</table>
List of Equations

Equation 2.1- Terminal velocity of a creaming particle (Stoke’s law) ......................... 19
Equation 5.1- Michaelis-Menten model ............................................................................. 128
Equation 6.1- First order kinetic reaction equation ........................................................... 139
Chapter 1: General Introduction and Thesis Structure

1.1 Introduction

Monoglycerides are lipid based food additives that are widely used for their broad technical function in the food industry. Because of their amphiphilic nature (having both hydrophobic and hydrophilic regions), they are surface active and can provide stabilisation, structuring and controlled instability in many foods which contain mixtures of fat and water. For example, powdered dairy coffee cream or non-dairy coffee whiteners may benefit from the addition of monoglycerides which help to maintain finer fat droplets resulting in better powder dispersibility. Cream liqueurs that are stable in acidic conditions can be produced by substituting a protein emulsifier such as sodium caseinate with monoglycerides (Euston, 2008). Monoglycerides are also widely used in bakery products such as bread, cakes, pastry and icings. The addition of shortening containing monoglycerides produces cakes with greater aeration and are more resistant to collapse. Monoglycerides also help to slow the staling of baked goods (Orthoefer, 2008). In margarine and spreads, monoglycerides help reduce the interfacial tension (IFT) between fat and water, improving texture and imparting a less waxy mouthfeel (Young & Wassell, 2008). Monoglycerides can also be added to whippable emulsions to induce controlled destabilisation of fat globules, which eventually results in their partial coalescence. This has been widely applied in the manufacture of ice cream, leading to improvements in aeration and allowing the production of ice creams with higher overrun and with stable foam structures. Furthermore, the ice cream containing monoglycerides is drier on extrusion and thus easier to mould. Additional benefits include slower meltdown rate, smoother and creamier texture (Euston, 2008; Goff, 1997a; 1997b, 2008).
Commercial monoglycerides are produced either by direct esterification of glycerol with a fatty acid, or by glycerolysis, through the interesterification of triglycerides from fats and oils with glycerol. The latter process is more favoured by industry as it is more cost effective to work with fats and oils than fatty acids. This process is usually carried out at elevated temperatures of between 210 and 250 °C in the presence of an inorganic alkali catalyst, such as sodium or calcium hydroxide. The drawback of such harsh processing conditions, are low yield (35 - 50 %), dark colours and an odiferous product. The catalyst also requires neutralisation before being removed by filtration (Bornscheuer, 1995; H-Kittikun et al., 2008; Hasenhuettl, 2008; Senanayake & Shahidi, 2005). Most of the available monoglycerides on the market contain a mixture of both mono- and di-glyceride, unless these are labelled as pure preparation. Additional distillation of crude fractions of monoglycerides can be used to provide a product richer in monooester content (typically > 90 %). Structurally, monoglycerides contain 2 free hydroxyl groups, making them considerably more polar and surface active than diglycerides (Hasenhuettl, 2008), so distillation provide a mechanism to obtain a more functional product.

The manufacture of ‘clean’ label foods prepared using all natural ingredients and minimal processing (Ozturk & McClements, 2016) represents one the most challenging consumer drivers currently influencing food industry R&D. The use of alternative natural emulsifiers, such as lecithin derived from soybean oil, saponins from plant extracts and surface active protein or polysaccharide derivatives, can help meet this need. However, many of the functional properties of monoglycerides are difficult to fully replicate through the use of naturally derived analogues, and this has been found to be particularly true when considering whippable emulsions such as cream and ice cream, where the monoglycerides have such a significant role. However, rather than replacing monoglycerides from
formulations, an alternative approach may be to develop a non-chemical and more natural pathway to produce these materials, allowing their unique functionality to be retained within products. When considering this approach, it is worth noting that monoglycerides are produced as part of the digestion process involving the pancreatic lipolysis of triglycerides, and thus, in recent years researchers have begun to investigate the use of lipase enzymes as an alternative to the synthetic process of producing monoglycerides.

Lipase (triacylglycerol hydrolases EC.3.1.1.3) is an enzyme that catalyses the hydrolysis of fatty acids from triglyceride or diglyceride contained in fat. Various authors have utilised the reverse use of lipase to catalyse the esterification (Freitas et al., 2010; Zhao et al., 2011) and glycerolysis (Chetpattananondh et al., 2005; Fregolente et al., 2008; 2010; H-Kittikun et al., 2008; Kaewthong, 2004; Kaewthong et al., 2005; Yang et al., 2005) reactions to produce monoglycerides. These reactions may require the use of bioreactor, immobilisation of the lipase, a solvent-based system and a purification procedure such as distillation. While the use of lipase in the hydrolysis of fats and oils has been reported for the production of fatty acids (notably for use in producing flavour compounds) (de Renobales et al., 1992; Virto et al., 1991), it has seen very limited use in monoglycerides production. Mazur et al. (1991) successfully demonstrated 70% monoglycerides production by hydrolysing triglyceride in a biphasic system of hexane and alkyl alcohol. The process was applied to triglycerides from C8:0 to C14:0. Tsuzuki et al. (1992) demonstrated hydrolysis of glycerides by lipase in a homogeneous solvent system consisting of a buffer and tetrahydrofuran. Holmberg and Osterberg (1988) successfully obtained 80% monoglycerides yield by lipase hydrolysis in a micro-emulsion containing the anionic surfactant sodium bis (2-ethylhexyl) sulfosuccinate (AOT).
This lipase-catalysed monoglycerides production, as described above, is difficult to set up and can be unsuitable for ‘clean’ label and all natural food preparations due to the use of solvents and inorganic catalysts. An opportunity exists, therefore to explore alternative, convenient, food-safe processes for the production of monoglycerides. Therefore, the aim of this study was based on the key research question of whether lipase enzyme could be used as a processing tool, in order to generate polar lipids, namely monoglycerides, *in situ* of a food emulsion (and for which monoglycerides were known to impart critical functionality), thus enabling *quantum satis* levels of the emulsifier through control of reaction conditions (enzyme concentration, holding time, temperature) as part of the manufacturing process.

1.2 Objectives

The overall aim of this thesis was to develop an alternative to synthetic monoglycerides production by using lipase enzyme to generate monoglycerides *in situ* of a food emulsion.

The specific objectives of this thesis were:

1. To investigate the competitive adsorption of *R. miehei* lipase with other emulsifiers at the oil-water interface and to demonstrate synthesis and interfacial adsorption of surface active lipids arising from lipolysis;

2. To investigate factors affecting the degree of lipolysis of an oil-in-water (O/W) emulsion with *R. miehei* lipase;

3. To determine the effect of thermal treatment on the deactivation kinetics of *R. miehei* lipase as well as the thermal stability of an O/W emulsion with regard to changes in their droplet size;
4. To determine the influence of lipolysis on emulsion stability by investigating the shear rheological properties of emulsions undergoing varying degrees of lipolysis;

5. To characterise the lipolysed O/W emulsion in relation to the fatty acid, mono- and di-glyceride profiles liberated by the lipolysis of the O/W emulsion under controlled conditions;

6. To demonstrate the functionality of lipolysed O/W emulsions in model food systems requiring the use of emulsifiers (ice cream and whipped cream).

1.3 Thesis structure

In order to fulfil the objectives, the research in this thesis progressed from a proof of concept to an applied product. This thesis is presented in a series of chapters as below:

**Chapter 1** presents a brief introduction to the thesis as well as the objectives of the thesis.

**Chapter 2** presents a reviewed relevant literature on the background aspects related to the key points of the main framework of the thesis.

**Chapter 3** describes the source of materials and chemicals used as well as the general and standard methods carried out in most of the chapters. Specific chemicals or methods (or any modification thereof) used in any particular chapters were addressed exclusively in the respective chapters.

**Chapter 4** investigates the effect of *R. miehei* lipase on the interfacial tension (IFT) at the oil-water interface. This chapter provided a fundamental support feasibility study. By utilising a tensiometer, a basic water-oil IFT profile was observed. Changes to the IFT suggested the ability of *R. miehei* lipase to competitively adsorb on the protein-stabilised interface as well as the occurrence of lipolysis. It also served to demonstrate the absence of lipolysis as a consequence of using heat treated (inactivated) enzyme.
From the analysis of planar oil-water IFT in the previous chapter, lipolysis was then carried out in an O/W emulsion system in Chapter 5. The first part of this chapter examines the effect of homogenisation conditions using a Microfluidizer® and effect of formulation (emulsifier and oil content) on the lipid specific surface area. The data from this study was then used to formulate emulsions with certain specific surface areas to examine several factors that might influence the lipolysis of an O/W emulsion. Different rates of lipolysis were observed using the pH-stat method and were dependent on the manipulation of different factors. The polar lipids, namely monoglycerides, diglycerides as well as free fatty acids, that were generated were quantified using gas chromatography. These results showed the amount of mono- and di-glyceride that were produced.

Having demonstrated lipolysis of an O/W emulsion, the effects of thermal treatment on the deactivation kinetics of R. miehei lipase was investigated in Chapter 6. In any enzyme-catalysed reaction, the ability to switch off the reaction allows for the degree of reaction, in this case lipolysis, to be controlled. Furthermore, thermal treatment is common in food processing and may be used as part of the deactivation step. Finding showed when an O/W emulsion containing R. miehei lipase was heat treated up to 100 °C, not only did enzyme deactivation occur, the O/W emulsion remained stable through the thermalisation step.

Chapter 7 examines the shear-induced aggregation of a 30 % oil-in-water palm oil lipolysed emulsion as well as factors affecting the aggregation. This not only characterised the aggregation behaviour of the O/W emulsion, but also demonstrated the significant effect lipolysis has on aggregation behaviour.

Chapter 8 quantifies the generated polar lipid obtained from the lipolysis of O/W emulsion through chromatography analysis. Furthermore, analysis on the lipolysed emulsion droplet size was also observed followed by microscopic observation of the changes in the lipolysed O/W emulsion microstructure.
Chapter 9 moved the direction of the work from a proof of concept to application in pilot scale manufactured food products. The functionality of lipolyzed O/W emulsion in a real food system was demonstrated in ice cream and whipped cream compositions.

Chapter 10 summarised all the findings from the thesis as well as the implications of the findings. Recommendations for future research are included in this chapter.
Chapter 2: Literature Review

The aim of this literature review is to summarise the creation and stability of emulsion, and the importance of emulsifier on the aforementioned stability. Particular focus on monoglycerides as emulsifier is also discussed. Lipid chemistry is discussed as well mainly on triglycerides and the various chemical pathways in which monoglycerides can be produced. Furthermore, the understanding of lipase enzymes, their specificity and the reaction that it catalysed is covered.

2.1 Emulsion

The physical definition of emulsions is that they are colloidal two-phase systems, made by fine dispersion of one liquid in a second immiscible liquid (Dickinson, 1992; Gabriele et al., 2009; Krog, 2002; McClements, 2015). One of the two immiscible liquids (usually oil and water), will be dispersed as small spherical droplets in the other and these droplets usually have a diameter that measures between 0.1 and 100 μm (Dickinson, 1992; McClements, 2015). The droplets in an emulsion are referred to as the dispersed, discontinuous, or internal phase, while the surrounding liquid is identified as the continuous or external phase (McClements, 2015).

Emulsions are extensively used in various industrial fields including oil drilling (Yang et al., 2016), transport, cosmetics (Maes, 2016), pharmaceuticals (Leonardi et al., 2016) and foods (McClements, 2015). Interestingly, food emulsions are primarily significant because their physical and chemical properties directly contribute to quality aspects like texture, stability and shelf life in a complex way (Gabriele et al., 2009). Food emulsions can be structurally complex systems; not only do they contain oil and water, but other components as well such as gas cells, fat crystals, proteins, dissolved salts or carbohydrates, fibres, water-soluble polysaccharides,
starch granules, or gels (Garti, 2001). The process of converting two separate immiscible liquids into an emulsion, or reducing the size of the droplets in a pre-existing emulsion, can be done by applying shear and pressure. The most frequent method used is known as homogenisation (Garg et al., 2010; McClements, 2015).

Emulsions are typically categorised in accordance with the relative spatial distribution of the oil and aqueous phases. A system that consists of oil droplets suspended in an aqueous phase is called an oil-in-water or O/W emulsion. On the other hand, a system that consists of water droplets dispersed in an oil medium is called a water-in-oil or W/O emulsion (Clausse et al., 2005; McClements, 2015). On top of the conventional O/W or W/O emulsions available, there is also ‘multiple emulsions’, for example, oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) emulsions (Dagleish, 2004; Garti & Benichou, 2004; McClements, 2015).

Emulsions are dispersed multiphase systems that are thermodynamically unstable, but can be maintained in a kinetically stabilised state (Cerimedo et al., 2010). Oil-in-water (O/W) emulsions are the most commonly encountered food emulsion type. They exist in a variety of forms, ranging from liquids (e.g. milk, cream, dressings, beverages, and soups) to highly viscoelastic materials (e.g. mayonnaise, sauces, whipppable toppings and ice cream mixes). Their properties can be controlled by varying the surfactants used, the type of fat and as well as additional components present in the aqueous phase. Water-in-oil (W/O) food emulsions are usually semi-solid, plastic products. Examples of these types of emulsion include margarine, butter and low-calorie spreads. Because of their usually solid form, their stability is more dependent on the properties of the fat or oil and the surfactant used, rather than the properties of the aqueous
phase. Due to this limitation, there are fewer parameters which can be varied to control their structure and stability (Dalgleish, 2004; Krog, 2002; McClements, 2015).

2.1.1 Emulsion formation

Emulsion formation is generally achieved by applying shear, pressure and mechanical energy via vigorous agitation (Garg et al., 2010; Krog, 2002; Lizarraga et al., 2008; Mahungu & Artz, 2001). The most frequent method used to convert two separate immiscible liquids into an emulsion, or to decrease the size of the droplets in a pre-existing emulsion, is known as homogenisation (McClements, 2015). The type of mechanical mixers or homogenisers used differs based on the type of emulsion produced (Krog, 2002). In the food industry this process is typically done by means of mechanical devices which subject the liquids to powerful mechanical agitation, for example, colloid mills, high speed blenders, rotor-stator systems, high-pressure valve homogenisers, and colloid mills (McClements, 2015; Perrechil & Cunha, 2010).

Although immiscible phases can be homogenised by rupturing one phase into droplets and dispersed into the other, without additional stabilisation the suspended system can only be maintained for a limited period of time. The thermodynamically unstable system will eventually separate into its original phase with time, at equilibrium (Garti, 2001). The disruption of oil drops into finer droplets during emulsification is affected by the interfacial tension and the homogenisation force used to produce the emulsion. Conversely, the emulsification of oil-in-water emulsions practically always occurs under turbulent flow conditions which lower the contribution of interfacial tension to a negligible level compared to homogenisation energy. A simple guideline ratio of the relative contribution of energy density, interfacial tension, and
mass density are about 400:4:1. Accordingly, it is apparent that energy density is the crucial factor in influencing the droplet disruption (Faergemand & Krog, 2006).

During emulsion formation, also known as emulsification, there is a huge increase in surface area, up to several thousand-fold, which is dependent upon the number and size of the droplets. In order to generate and disperse these droplets, a considerable amount of energy or force must be supplied. Since emulsifiers lessen the surface tension, incorporating emulsifier should substantially reduce the amount of energy needed to form the emulsion (Mahungu & Artz, 2001). Initially, the emulsifier is dissolved in the aqueous or organic phase depending on the solubility of the emulsifier and on the type of emulsion followed by the rupture of the film in between the two immiscible phases to a level that droplets form. These droplets are mostly far too large in the beginning, and they are subsequently disrupted into smaller ones by sufficient agitation (Lizarraga et al., 2008; Mahungu & Artz, 2001). Sufficient emulsifier must also be available to adsorb at the aqueous/organic interface in order to form a stable emulsion and prevent fast re-flocculation or coalescence (Mahungu & Artz, 2001; Ozturk & McClements, 2016). The emulsifier therefore serves to control the interfacial viscosity between droplets, helps to dissipate the energy, adsorbs on the surface of the newly formed droplets, and alters its surface charge or nature (Garti, 2001; Ozturk & McClements, 2016).

W/O emulsions are prepared by adding the water phase to the oil phase while agitating with a low-energy propeller-type stirrer, resulting in a rather coarse distribution of water droplets ranging from 5 to 50 μm or more in size. O/W emulsions, such as dairy-based emulsions, are made by using high-energy homogenisation where oil or fat droplets are produced under
turbulent flow environment. This results in a dispersed phase with a particle size distribution ranging from 0.3 to 3 μm, typically around 0.5 μm on average (Krog, 2002). Emulsions produced from solid fat are often used when different textures and structures are required. Emulsions are always created at an elevated temperature by which all of the fat or oil is in a liquid form, to allow efficient emulsification, and crystallisation then occurs as the product is cooled to the temperature at which it is stored. Fats and oils which have the ability to crystallise in this way can be very significant in defining the functionality of the emulsion. This functional property is best typified by the involvement of partly crystalline fat in the mechanism of partial coalescence, which provides a significant contribution to the structure and properties of whipped emulsion products or in ice creams (Arboleya et al., 2009; Dalgleish, 2004). Physically, the properties of the emulsion droplets themselves can have a significant effect on the rheological properties of the emulsion as a whole (Boode & Walstra, 1993). Research has shown that processing parameters significantly influence the structural parameters of the emulsion such as particle size distribution, inter-droplet interactions, continuous phase rheology; and hence, have an effect on the emulsion rheological characteristics (Lizarraga et al., 2008).
2.2 Emulsion stability

McClements (2015), described the term ‘emulsion stability’ as the ability of an emulsion to resist any change in its properties over the time scale of observation. Emulsions are thermodynamically unstable systems and can undergo a number of different mechanisms of instability and various physical changes, with the progression of time (Garg et al., 2010; Krog, 2002; Lizarraga et al., 2008; Ozturk & McClements, 2016; Xiang et al., 2015). As discussed, oil and water are immiscible due to the surface energy (Gibbs free energy) of the oil–water interface. Due to the high interfacial tension between oil and water, any emulsion will tend to minimise the interfacial energy by reducing the interfacial area between oil and water to as minimum as possible. In the absence of surfactants, this is achieved by coalescence of the oil droplets, to give separated layers of oil and water (Dalgleish, 2004).

The kinetic stability of emulsions is affected by factors such as the adsorption of a stabilising interfacial layer (e.g. surfactants, biopolymers or particles) around the droplets of the dispersed phase, the particle size distribution of dispersed phase, and the ion concentration, pH and viscosity of the continuous phase (Krog, 2002). Other physical changes include those that involve the changes in primary droplet size, such as coalescence, ripening, inversion and breaking, which have subsidiary effects on the creaming behaviour, and those that involve the spatial rearrangement of the droplets with respect to each other and with respect to an external frame of reference, such as flocculation and creaming (Lizarraga et al., 2008; Ozturk & McClements, 2016). Droplet size and distribution are among the important parameters in characterising emulsions, affecting stability, creaming resistance, rheology, and chemical reactivity (Gabriele et al., 2009; Johns & Hollingsworth, 2007; Miyagawa et al., 2015). The surface charge (zeta
potential) of the droplet, in addition to the interfacial free energy interaction, was found to have effect on the stability too (Wiacek & Chibowski, 1999).

Emulsion stability can be imparted through interfacial (e.g. electrostatic, steric), and continuous phase stabilisation (immobilisation) of droplets (Garti, 2001). The notion of stability includes both retardation of sedimentation or creaming, reduction of coagulation and stabilisation of the interface with the aim of hindering coalescence linked to the viscoelastic properties of the interfacial layer (Sæther et al., 2004).

According to Stoke’s law, O/W emulsion creaming stability is achievable by a thickening effect, higher viscosity of the water phase by adding a stabiliser. It is also accomplished by generating smaller droplets with a homogenous size distribution and/or by a decreasing the density difference between the two phases, resulting from a complete and stable covering of the oil droplets (Pal, 1996; Tipvarakarnkoon et al., 2010).

Emulsion stability is also dependent on the conditions in which the emulsion was produced. This includes not only the components that makes up the emulsion system, but also the emulsifier concentration, the emulsion temperature on processing and storage, and the physical state (crystalline versus fluid) of the fat and/or emulsifiers (Mahungu & Artz, 2001). The stability of emulsions is contributed by various factors, among which the function of surface-active lipids or proteins is important (Krog, 2002; Lizarraga et al., 2008). The most important surface-active components in foods are proteins (e.g. as derived from milk, soy and egg), and low–molecular weight emulsifiers (lipids, phospholipids, surfactants, etc.). These various surface active
molecules and aggregates will rapidly adsorb on the newly formed oil–water interface during emulsion formation.

As indicated, the stability of an emulsion is inherently related to the properties of the adsorbed layer formed at the surface of emulsion droplets. The layer structure is a function of composition and concentration of the surfactant (emulsifier) present (Wiacek & Chibowski, 1999). Emulsion stability and rheological properties that contributes to texture are probably the most significant characteristics to be considered when a new product is formulated. Stability affects product processing (e.g. shear induced separation during pumping), unit operation design (stirring systems, pumps, etc.) and shelf life (potential phase separation before commercial limits). Rheological properties are necessary not only to design the proper unit operations (e.g. pumping systems) but distinctively crucial in determining the organoleptic characteristics perceived by the consumers (Gabriele et al., 2009).

2.3 Destabilisation of emulsion

Emulsions are metastable systems that have the tendency to destabilise due to separation of the dispersed phase through various possible mechanisms including flocculation, coalescence, creaming or sedimentation (Bengoechea et al., 2010; Cerime do et al., 2010; Krog, 2002; Ozturk & McClements, 2016). The various mechanisms by which an emulsion can be destabilised are depicted in Figure 2.1 (with the exception of Ostwald ripening). Sedimentation or creaming is determined by the density difference between droplets and the continuous phase, the size of the droplets, and the rheological properties of the continuous phase. In contrast flocculation is typified by reversible or irreversible droplet aggregation, countered for example by repulsion...
arising from the adsorbed layer while coalescence is linked to the viscoelastic properties of the interfacial layer (Sæther et al., 2004) and their ability to inhibit film rupture for droplets in contact.

For food emulsions such as milks and cream liqueurs, the ability to impart appropriate emulsion stability over the expected shelf life is a critical requirement in product design. Nevertheless, there are products in which controlled destabilisation, such as flocculation or partial coalescence, is favourable (Vanapalli & Coupland, 2001). The favourable destabilisation is particularly relevant in emulsions containing semi-solid dispersed phase, for example, in whippable emulsions such as ice cream, cream and whipped cream. This can be achieved by the addition of small molecule emulsifiers derived from lipids. The destabilisation process can involve several physical changes to the emulsion during manufacture and storage, such as crystallisation of the fat phase taking place at low temperature and partial desorption of interfacial protein, and which result in a decrease in emulsion stability under shear. This partial destabilisation of the emulsion plays a crucial role in contributing to other microstructural elements in the product, such as foam stabilisation and is essential to the perceived quality of the final product (Faergemand & Krog, 2006; Goff, 1997; Vanapalli & Coupland, 2001).
Figure 2.1: Various physical mechanisms of emulsion destabilisation, including creaming, sedimentation, flocculation, coalescence and phase inversion.

Legend: Blue – water phase, yellow – oil phase

2.3.1 Flocculation

The adherence of droplets to form aggregates or clusters is referred to as flocculation (Mahungu & Artz, 2001). Flocculation of aggregated particles may be either reversible or irreversible and can be a precursor to creaming and in some cases even coalescence. Controlled flocculation may also be a favourable phenomenon for some food products, such as cream cheese, where droplet aggregation leads to the formation of percolating droplet networks that can imparts particular material and sensory properties. Many emulsions that are stable in terms of coalescence are susceptible to flocculation (Krog, 2002; McClements, 2015).

Flocculation mainly occurs when the attractive forces, which are primarily long-range London–van der Waals forces and electrostatic forces, between the droplets exceed that of the repulsive forces, but without any corresponding collapse in the structural integrity of the interfacial film surrounding the droplets that would otherwise lead to coalescence (Mahungu & Artz, 2001). The propensity of flocculation can be influenced by a number of factors, such as the concentration of proteins present in relation to the concentration of emulsified oil or fat. For example, if insufficient protein is present to cover the entire surface of the fat/oil droplets during homogenisation, a so-called bridging flocculation may take place. The pH and ionic strength will also strongly affect the flocculation of dairy emulsions (Krog, 2002; McClements, 2015). One example that illustrate the flocculation state is the so-called ‘feathering’ of coffee cream in hot coffee, due to the flocculation of oil droplets caused by combination of the high temperature and a lower pH in the coffee than in the cream itself, which stimulates net particle attraction between the protein-stabilised oil droplets (Krog, 2002).
2.3.2 Creaming

Creaming and sedimentation are both forms of gravitational separation. Creaming is typified by the rise or upward movement of the dispersed particles to the surface of an emulsion and is due to the fact that they have a lower density than the surrounding liquid or serum phase, while sedimentation is attributed to the downward movement of droplets that have a higher density than the surrounding liquid (Dickinson & Golding, 1997; Krog, 2002; McClements, 2015). Creaming and sedimentation are influenced by changes in droplet concentration. Droplet concentration can increase preferentially in either the top or bottom portion of the emulsion depending upon the relative density of the two phases. Reducing the average droplet size and adding an emulsifier will substantially reduce the rate at which this occurs (Mahungu & Artz, 2001). The creaming rate of emulsion has been reported to be slower with the increase of oil volume fraction, due to the increase of weak flocculation of the oil droplet (Mollakhalili Meybodi et al., 2014). Furthermore, when the oil volume fraction is increased to near close packing of the droplets, the rate of creaming eventually decreased to zero as the droplets are ‘jammed’ (Coupland, 2014).

The terminal velocity of a creaming particle can be expressed by the Stokes’ equation:

\[ v_{\text{Stokes}} = \frac{2gr^2\Delta\rho}{9\eta} \]

Equation 2.1

where \( r \) is particle radius, \( \Delta\rho \) is the density contrast between the phases, \( \eta \) is the continuous phase viscosity, and \( g \) is the acceleration due to gravity (Coupland, 2005).

Although an emulsion can be destabilised by creaming, the process is providentially reversible. Among the factors that can contribute to reverse the process are the reduction of particle size,
reducing the density difference between the continuous and dispersed phase, and increasing the viscosity of the continuous phase. Reduction of particle size happens when the particle size distribution of milk fat globules, for examples, is reduced to less than 1 μm by homogenisation, creaming is practically eliminated due to Brownian motion which keeps the globules in suspension. Reducing the density difference between the continuous and dispersed phase is however not usually possible when working with food emulsions. Although the natural density difference between water and edible oils or fats may be decreased by the use of high density additives, however such additives are not permitted to be used in foods. Increasing the viscosity of the continuous phase can be achieved by adding hydrocolloids (gums) or carbohydrates. For example the stability of chocolate milk is due to high zero shear viscosity created by a network of milk proteins, hydrocolloids and cocoa particles formed at high temperatures (Krog, 2002).

2.3.3 Coalescence

Coalescence is an irreversible process in which two or more emulsion droplets merge to form larger droplets of greater volume but lower interfacial area. Coalescence occurs due to the thinning of the stabilising film surrounding the emulsion droplets resulting in the rupture of the protecting interfacial film separating the two adjacent emulsion droplets (Krog, 2002; van Aken, 2003). Coalescence can be promoted at high dispersed phase volume fractions (such as in the cream layer of an emulsion), by large droplet size, weak repulsion between droplets, too low interfacial tension and poor mechanical properties associated with the interfacial film on the surface of the droplet (Krog, 2002; Mahungu & Artz, 2001).
When coalescence occurs, the integrity of the interfacial film is lost and droplets in close contact merge, with the result of a reduction in the number of droplets. A strong, stable film on the surface of the droplet, due to addition of the correct concentration of the appropriate emulsifier, will minimise this type of destabilisation (Mahungu & Artz, 2001). Proteins are generally effective emulsion stabilisers and the surface shear rheology of adsorbed protein films is a significant factor influencing the coalescence kinetics of protein-stabilised O/W emulsions (Krog, 2002). When coalescence is able to proceed unhindered, total breakdown of the emulsion, resulting in total separation of the oil and water phase will occur (Krog, 2002; Mahungu & Artz, 2001).

One interesting variant of coalescence destabilisation is when the emulsion droplets comprise a certain amount of crystalline fat, thus rendering the droplets semi-solid. Solid fat droplets cannot fuse together as liquid oil droplets do, but form ‘clumps’ of aggregated fat globules. This is referred to as partial coalescence and is a favourable phenomenon in O/W emulsions which are aerated to a foam, as occurs in whipping cream and ice cream mix. Controlled destabilisation in the form of droplet flocculation (aggregation) and partial coalescence has been shown to improve both whippability and foam stability (Krog, 2002).

Partial coalescence is induced when solid fat crystals from one droplet penetrate into the liquid oil portion of another droplet (Boode & Walstra, 1993). Fat crystals form at the surface of the droplet they can penetrate another droplet on collision. The liquid oil in the second droplet then preferentially wets the crystalline fat and flows out to strengthen the link (Boode & Walstra, 1993).
The three stages of the process; droplet contact, droplet crystallisation and oil mixing around the contact point are illustrated in Figure 2.2. Initially, in illustration (a), the high temperature resulted in liquid emulsified oil and the droplets are spherical. The lipid phase becomes partly non-spherical during the partial crystallisation of the lipid phase due to the formation of crystal network. At this stage, when the droplets get in close contact and collide with each other, it allows the semi-solid crystal fat from one droplet to penetrate the surfactant coating of the second droplet. The penetration will cause the liquid oil inside the droplet to flow out to favourably wet the solid fat and reinforce the contact point (c). Partial coalesced droplets will be stable provided that there is a solid fat network to hold up the form, but rapidly coalesce when the solid fat is melted as shown in (d) as the merging droplets flowed into one larger droplet (Boode & Walstra, 1993; Vanapalli & Coupland, 2001).
Figure 2.2: Different stages of the coalescence process; droplet contact, droplet crystallisation, penetration of crystallised fat and oil mixing around the contact point

Source: (Vanapalli & Coupland, 2001)
2.4 Emulsion interfacial characteristics

An interface is a narrow section in-between two phases, which could be a gas and a liquid, a gas and a solid, two liquids, a liquid and a solid, or two solids (Walstra, 2003). The region of interface is so small that it barely makes up a significant fraction of the total volume of an emulsion when the droplet radius is less than about 1 μm. Nevertheless, it plays a major role in determining the bulk physicochemical and organoleptic properties of food emulsions, including their formation, stability, rheology, and flavour (McClements, 2015). Interfacial characteristics are often a major area of interest for food scientists as a contributing factor in the design of emulsion-based foods with specific functionalities (Dickinson, 1992; McClements, 2015).

For emulsions prepared by using a single type of emulsifier, the interfacial film of the emulsion will practically consist of this emulsifier only. Nonetheless, the concentration of emulsifier adsorbed at the droplet surfaces is dependent on several factors such as the initial emulsifier concentration, temperature, pH, ionic strength, and homogenisation conditions. However, it is important to note that many food emulsions contain a mixture of different surface-active components, rather than a single type of surface-active substance. In such conditions, the interfacial composition is determined by the concentrations of the range of surface-active substances present, their relative affinity for the interface, the method used to prepare the emulsion, the solution conditions, i.e, temperature, pH, and ionic strength, and the sequence in which the emulsifiers were added (McClements, 2015).

In complex food emulsions, there are two main kinds of molecules that have a strong propensity to adsorb at the surface of fat globules or at the air-water interface, namely, biopolymeric
species (e.g. proteins) and small molecule surfactants (e.g. surface-active lipids). Occasionally, these two types of molecules compete with each other for interfacial adsorption when they are both present in a food system. The presence of surface-active lipids usually yields a greater effect on interfacial tension than adsorbed proteins, and can accordingly dislocate them from the interface. The amount of proteins desorbed from the surface by emulsifiers depends highly on the available molar concentrations of both emulsifiers and proteins, as well as any potential interaction between the two species (Faergemand & Krog, 2006). Low-polar, oil soluble emulsifiers (monoglycerides) tend to form mixed lipid-protein interfacial films, while high-polar, water dispersible emulsifier (polysorbates) tend to dislocate most of the interfacially adsorbed proteins and govern the interfacial structure. A protein film yields high viscoelasticity, thus providing a strong barrier to fat globule coalescence. Mixed emulsifier-protein films are less coherent with reduced viscoelasticity, especially with emulsifiers that form liquid-condensed type of monolayer (e.g. unsaturated monoglycerides). Mixed emulsifier-protein films therefore generally promote destabilisation of emulsions, although the magnitude of this effect is dependent on the relative ratio between emulsifier and protein concentrations at the interface and on the type of emulsifier used (Krog, 2002).

2.5 Emulsifiers and their applications

The term ‘emulsifier’ in food systems is commonly used to define surface-active additives (usually derived from lipids) that adsorb to the surface of freshly formed droplets during homogenisation, forming a protective membrane and serve to lower surface tension between the two phases, thus enhancing emulsification and increasing emulsion stability (Garg et al., 2010; Huang, 2003; McClements, 2015; Nylander, 2004; Vaclavik & Christian, 2014b). However,
from a technical perspective, the term ‘emulsifier’ can be misleading, as these materials have considerably broader functionality than just emulsification. By reducing the interfacial tension between the two phases, thereby lowering Laplace’s pressure, thus greatly facilitating the formation of small droplets and reducing the amount of force required to overcome the surface energy to disperse one phase onto the other (Chan, 2014; Garti, 1999; Wiacek & Chibowski, 1999). They also contribute to the stabilisation of the dispersed droplets preventing the droplets from coming in contact with each other, by electrostatic or steric effects, to aggregate flocculate, coalesce, rupture and cause phase separation into two immiscible phases (Garg et al., 2010; Garti, 1999; Huang, 2003; McClements, 2015; Nylander, 2004; Vaclavik & Christian, 2014b). Terminology such as “additives that hinder precipitation of dispersed particles”, “decrease creaming rates of oil droplets or foams”, “prevent aggregation/desegregation of dispersed solid or liquid particles”, “prevent syneresis of gelled systems”, “condition or stabilise food systems”, and “retard coalescence of oil droplets” are frequently used. Technical functions such as foaming (de)stabilisation, crystal habit modification, control of wetting properties, dough conditioning and inhibition of starch retrogradation are some of the wider roles that these additives can be used for (Garti, 2001).

Emulsifiers can be classified as anionic emulsifiers, cationic emulsifiers, amphoteric emulsifiers, and nonionic emulsifiers (Hasenhüttl, 2008; Mahungu & Artz, 2001). The mechanism of emulsifier that enables them to stabilise emulsions is based on their ability to partition the interfacial region of un-mixable phases such as oil and water. This is attributable to the amphiphilic properties, specifically, the occurrence of polar and non-polar regions or both hydrophilic and hydrophobic moieties on the same molecule. The hydrophobic regions possess
affinity to the oil phase while the hydrophilic regions have an affinity to the water phase (Flack, 1996; Garg et al., 2010; Huang, 2003; Mahungu & Artz, 2001; McClements, 2015). The amphiphilic properties are often quantified in terms of their hydrophilic/lipophilic balance or HLB, ranging from zero to 20. Strongly lipophilic emulsifiers will give a low HLB value while a high HLB indicates a strongly hydrophilic emulsifier. Generally HLB is a useful tool in developing simple technical emulsions and as indicator of emulsifier’s solubility in either water or oil; however, it is much less practical in food systems because of the complex nature of food matrices (Chan, 2014; Fennema, 1996; Flack, 1996). Most of the common (additives type) emulsifiers used in the food industry are low molar mass surfactants (such as phospholipids or monoglycerides) and surface-active proteins (Aveyard et al., 2003; McClements, 2015; Nylander, 2004). This type of surfactants or emulsifiers provide stability to the resultant emulsion with respect to coalescence and flocculation (Ye et al., 2000), but as indicated, their functionality often extends beyond just emulsification.

When developing a new product, it is necessary to understand the relationship between macroscopic parameters related to perceived properties and material microstructure in order to comprehend the effects of relevant ingredients such as fats and emulsifiers. This aims to obtain a formulation with controlled characteristics by avoiding a long “trial and error” approach (Gabriele et al., 2009). Foods are very complex colloidal systems that may undergo changes during storage, resulting in deteriorating quality and changes in appearance or texture, and loss of flavour characteristics. Processed food is a considerable part of our daily diet, and industrial food production requires surface-active lipids (emulsifiers, surfactants) as processing
aids to facilitate uniform quality and ensure long shelf life of the finished products (Faergemand & Krog, 2006).

Due to the tremendous importance of emulsifiers in food and beverages, and the increasing demand in other industry sectors, such as in personal care products and cosmetics, the global emulsifier market is anticipated to hit 2.6 million metric tons in the year 2017. The emergence of new research development and technological advancements will help heighten the market growth in these coming years (Global Industry Analysts, 2016). The role of emulsifiers in emulsion formation and stabilisation has been extensively described in the literature of food colloids and food emulsions (Baer et al., 1997; Chan, 2014; Dalglish, 2004; Faergemand & Krog, 2006; Flack, 1996; Garti, 2001; Hasenhuettl, 2008; Huang, 2003; Krog, 2002; Krog & Vang Sparso, 2004; Mahungu & Artz, 2001; McClements, 2015; Rahmati et al., 2014; Vaclavik & Christian, 2014b; Wasan et al., 2004).

Various works have described the functional properties of emulsifiers in low fat food (Flack, 1996; Rahmati et al., 2014; Rahmati & Mazaheri Tehrani, 2014). Several authors have described emulsifiers properties in bakery products including bread, cakes and pastry (Chin et al., 2007; Gabriele et al., 2008; Gómez et al., 2004; Manohar & Rao, 1999; Rahmati & Mazaheri Tehrani, 2014; Rasper & Kamel, 1989; Ribotta et al., 2004; Tan et al., 2015; Zambrano et al., 2004), in beverages (Cheong et al., 2015; Mirhosseini & Tan, 2009; Qian et al., 2011; Singh et al., 2008a; Yadav et al., 2009) and in ice cream (Goff, 2008; Granger et al., 2005b; Lal et al., 2006; Patil & Jha, 2008; Rinaldi et al., 2014; Scholten, 2014; Silva & Bolini, 2006).
Food emulsifiers can also be categorised according to their nature of origin, namely whether naturally occurring surfactants or synthetically derived. Naturally occurring surfactants, include lecithins (from egg, soy and milk), and saponins (from a variety of plant sources). Synthetic surfactants permitted for food applications are mostly derived from fatty acids or triglycerides with various additional forms of chemical modification (Garti, 2001; Vaclavik & Christian, 2014b). Natural emulsifiers, such as phospholipids, have been consumed by humans since ancient times, while synthetic emulsifiers have been used more recently in the pharmaceutical, chemical, cosmetics, and food industries since about the 1930’s (Huang, 2003). Various common food emulsifiers, their legal number and typical applications in food are listed in Table 2.1.

Synthetic emulsifiers typically comprise polar lipids consisting of partial esters of fatty acids with chain lengths from C12 to C22, and various polyvalent alcohols (polyols) such as glycerols, polyglycerols, propylene glycol, sorbitol/sorbitan, and sucrose or their organic acid like lactic acid, or ethylene oxide derivatives and lactic acid esters of fatty acids. Partial esters may also be esterified with organic acids such as acetic, citric, diacetyl tartaric, or succinic. These types of emulsifiers can be synthetically derived by molecular distillation, ethoxylation, interesterification of fats and oils with glycerol or direct esterification of fatty acids with selected polyols (Flack, 1996; Hasenhuettl, 2008; Krog, 2002; McClements, 2008). Fats and oils are often used as a source for producing many amphiphilic molecules since they are inexpensive, easy to extract and easy to handle. Chemical reactions and/or enzymatic processes are often carried out on certain fats and /or oils, to obtain molecules with hydrophobic and hydrophilic groups attached to each other. Among the various polar lipids, monoglycerides are among the most commonly used as emulsifier in synthetic food grade emulsifiers in the industrial setting (Garti,
Chapter 2: Literature Review

2001; Golding & Sein, 2004; Hasenhuettl, 2008). Monoglycerides can be synthetically produced by direct esterification of edible fats or fatty acids (from vegetable or animal origin) with glycerol at elevated temperatures, or by interesterification of an oil with glycerol (Garti, 2001), and will be discussed in more detail in the following section.
### Table 2.1: Food emulsifiers, their legal numbers and typical uses in food

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>EU no. / INS no.</th>
<th>CFR no.</th>
<th>Acceptable daily intake (ADI) mg / kg body weight</th>
<th>Typical uses in food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>E322</td>
<td>184.1400</td>
<td>Not limited</td>
<td>O/W and W/O emulsions, bakery products, cereal, confectionery, cocoa products, ice cream and other dairy products, coffee whiteners, Oleomargarine, spreads, cheese products wetting and dispersion for vending products etc.</td>
</tr>
<tr>
<td>Mono- and di-glycerides (distilled monoglycerides)</td>
<td>E471</td>
<td>184.1505</td>
<td>Not limited</td>
<td>O/W and W/O emulsions, crumb-softening, cake and cream aeration, foam stabilising, amylose complexing, cereal, confectionery, ice cream and other dairy products, coffee whiteners, margarine, spreads, etc.</td>
</tr>
<tr>
<td>Acetic acid esters of monoglycerides (Acetem)</td>
<td>E472a</td>
<td>172.828</td>
<td>Not limited</td>
<td>Bakery products (cakes), dessert products, non-dairy cream, toppings, increases foam stability and stiffness, coating agent</td>
</tr>
<tr>
<td>Lactic acids ester of mono- and di-glycerides (Lactem)</td>
<td>E472b</td>
<td>172.852</td>
<td>Not limited</td>
<td>Bakery products (cakes), dessert products, non-dairy cream, toppings, Alpha-tending, dairy products, shortenings,</td>
</tr>
<tr>
<td>Citric acid ester of mono- and diglycerides (Citrem)</td>
<td>E472c</td>
<td>GRAS</td>
<td>Not limited</td>
<td>O/W and W/O emulsions, margarine, meat products</td>
</tr>
<tr>
<td>Diacetyl tartaric acid esters of monoglycerides</td>
<td>E472e</td>
<td>184.1101</td>
<td>0-50 GRAS</td>
<td>Bakery products dough-strengthening, extrusion aid, fat sparing, O/W emulsions, coffee whiteners, non-dairy creams, sauces</td>
</tr>
<tr>
<td>Succinic acid esters of monoglycerides</td>
<td></td>
<td>172.830</td>
<td>-</td>
<td>Bakery products (bread)</td>
</tr>
<tr>
<td>Ethoxylated mono- and diglycerides</td>
<td></td>
<td>172.834</td>
<td>-</td>
<td>Bakery products (bread)</td>
</tr>
</tbody>
</table>
Chapter 2: Literature Review

<table>
<thead>
<tr>
<th>Sodium and potassium salts of fatty acids</th>
<th>E470a</th>
<th>172.863</th>
<th>Co-emulsifier, Wetting and dispersion, O/W Emulsions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglycerol ester of fatty acids</td>
<td>E475</td>
<td>172.854</td>
<td>0-25 O/W and W/O emulsions, bakery products, dessert products, margarine, spreads cereal, confectionery products</td>
</tr>
<tr>
<td>Polyglycerol polyricinoleate</td>
<td>E476</td>
<td>-</td>
<td>- Chocolate, confectionery, cake margarine, low-fat spreads</td>
</tr>
<tr>
<td>Proplpylene glycol esters of fatty acids</td>
<td>E477</td>
<td>172.856</td>
<td>0-25 Cakes, dessert products, toppings, shortenings</td>
</tr>
<tr>
<td>Sucrose esters of fatty acids</td>
<td>E473</td>
<td>172.859</td>
<td>0-2.5 O/W emulsions, bakery products, dessert products, non-dairy creams, topping</td>
</tr>
<tr>
<td>Polyoxyethylene sorbitan mono stearate (Polysorbate 60)</td>
<td>E435</td>
<td>172.836</td>
<td>0-25 O/W emulsions, ice cream, bakery products, salad dressings, dairy products, wetting and dispersion for vending products</td>
</tr>
<tr>
<td>Polyoxyethylene sorbitan tristearate (Polysorbate 65)</td>
<td>E436</td>
<td>172.838</td>
<td>0-25 O/W emulsions, ice cream, bakery products, salad dressings, dairy products, wetting and dispersion for vending products</td>
</tr>
<tr>
<td>Polyoxyethylene sorbitan mono oleate (Polysorbate 80)</td>
<td>E433</td>
<td>172.840</td>
<td>0-25 O/W emulsions, ice cream, bakery products, salad dressings, dairy products, wetting and dispersion for vending products</td>
</tr>
</tbody>
</table>

Note:

a E number codes for food additives that have been assessed for use within the European Union
b INS number adapted for international use by the Codex Alimentarius Commission
c Regulation numbers in Title 21 of the U.S. Code of Federal Regulations (U.S Food and Drug Administration)
d Generally recognised as safe

2.5.1 Monoglycerides in food

Monoglycerides and their organic acid derivatives are among the most commonly used synthetic food grade emulsifiers in the global industrial setting (Bradić et al., 2010; Chan, 2014; Garti, 2001; Golding & Sein, 2004; Krog, 2002; Mahungu & Artz, 2001; Vaclavik & Christian, 2014b). The Codex Alimentarius Committee in their Toxicological evaluation of certain food additives with a review of general principles and specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives), WHO Technical Report Series, No. 539 in Geneva (1974), defined monoglycerides as “A mixture of mono- and di-glycerol esters of long chain, saturated and unsaturated fatty acids that occur in food fats; contain not less than 30 % of alpha-monoglycerides and may also contain other isomeric monoglycerides, as well as di- and triglycerides, free glycerol, free fatty acids, soap and moisture; usually manufactured by the glycerolysis of edible fats and oils, but may also be prepared by esterification of fatty acids with glycerol, with or without molecular distillation of the product.”

Monoglycerides are manufactured synthetically via direct process esterification of fatty acids with glycerol under alkaline conditions; or by catalytic interesterification of triglyceride with glycerol at elevated temperatures also known as glycerolysis (Garti, 2001; Golding & Sein, 2004; Hasenhuettl, 2008; Krog, 2002; Mahungu & Artz, 2001; Vaclavik & Christian, 2014a).

Monoglycerides are typically used alongside with a fat system and are usually present in combination with other emulsifiers. Monoglycerides are excellent emulsifiers given that they are partially soluble in both water (with the ability to form liquid crystalline phases) and fat, as well as being highly surface active due to the fact that they contain a hydrophilic hydroxyl
headgroup and fatty acid tailgroup. As in the case of other emulsifiers, Monoglycerides are found at the O/W interface with the polar groups which is the hydroxyl group of the molecule in the aqueous phase and the nonpolar group represent by the fatty acids component in the lipid phase. In this approach, monoglycerides act to reduce the interfacial tension and to stabilise emulsions (Griswold, 1962).

The major applications of monoglycerides in food include bakery products (Chan, 2014; Goldstein & Seetharaman, 2011; Koocheki et al., 2009; Madsen, 1987; Moonen & Bas, 2004; Russell, 1983; Sawa et al., 2009; Stampfli & Nersten, 1995), margarine and shortening (Garti, 2001; Madsen, 1987; Moonen & Bas, 2004; Vereecken et al., 2010), and frozen desserts and ice creams (Baer et al., 1997; Goff, 2002; Granger et al., 2005a; Granger et al., 2005b; Jensen et al., 1961; Moonen & Bas, 2004; Zhang & Goff, 2005). Garti (2001) stated that in ice cream, monoglycerides containing oleic acids are often used as emulsifier; while those derived from tallow, lard, cottonseed, peanut oils and soybean oils are normally used in cakes and icing shortenings.

2.5.2 Legal aspect of emulsifiers

Before a substance can be used as a legal food additive, two main criteria, technological efficacy and safety must be satisfied (Mahungu & Artz, 2001). Food emulsifiers must obviously pass certain requirements for non-toxicity, non-carcinogenic, and non-allergenic and has to go through a number of toxicological tests including short- and long-term feeding trials on several animal species together with studies on metabolism (Garti, 2001; Nylander, 2004). JEFCA recommended that food additives must obtained the appropriate approval by the governing
bodies of the different countries in which they are intended to be used and that legal control be based on a system of permitted or positive lists (Garti, 2001; Mahungu & Artz, 2001). All emulsifiers approved by local health authorities should be considered as safe food ingredients when used within their limits of the acceptable daily intake (ADI) values (Nylander, 2004).

Internationally, there are two sources of food standards that are widely known and can be used as guidance. The first one is the Codex Alimentarius standards under the patronage of the Food and Agricultural Organization/World Health Organization (FAO/WHO) on a worldwide basis European Economic Community (EEC) directives, applicable to the member states within the European Economic Community. Recommendations for the Codex Alimentarius Committee come from the Joint Expert FAO/WHO Committee on Food Additives (JEFCA) (Mahungu & Artz, 2001). The second one is in the United States by the department of the Food and Drug Administration (FDA) (Mahungu & Artz, 2001; Nylander, 2004). In the United States a list of the emulsifiers approved for use has been published in the Code of Federal Regulations (CFR), Part 172 of Title 21. The CFR covers food emulsifiers under two categories: those permitted only under specific conditions and those generally recognised as safe (CFR Title 21, 2010).

Only certain categories of emulsifiers can be used in food, some due to the health regulations and some because of their practical and limited application. For instance, ionic emulsifiers may have restricted application in the presence of acids or bases, since they are susceptible to chemical modifications and loss of surface activity. Hence, not many ionic emulsifiers are used by the food industry (Garti, 2001). Egg yolk phospholipid or lecithin is a type of ionic emulsifier however its effectiveness can be very limited due to its amphoteric properties (Boutte and
Skorgeson, 2007). Lactylates are anionic emulsifiers and may be labelled as calcium stearoyl lactylate (CSL), sodium stearoyl lactylate (SSL) or lactic esters of fatty acids (LEFA). Lactylates create negative charge around micelles and cause the micelles to repel each other thus minimizing coalescence. This is desirable in bakery products as it will result in higher volume and finer crumb texture (Boutte and Skorgeson, 2007; Fellows, 2009).

In terms of safety issues, monoglycerides are categorised as ‘generally recognised as safe’ or GRAS in short. These categories of products can be used in many food products, cosmetics and pharmaceuticals without any limitations (Garti, 2001). The Joint Expert FAO/WHO Committee on Food Additives (JECFA) considered that mono- and diglycerides differed little from food so that their use need not be limited (World Health Organization, 1974). No acute or chronic toxic effects have been observed with either lecithin or mono and diglycerides at normal dosage levels (JECFA, 1974). There was also no evidence of acute or chronic toxic effects with fatty acid salts (JECFA, 1970). Similarly, there were no observable acute and chronic toxicological effects from acetic, citric, lactic, tartaric, mixed tartaric, or acetic and diacetyltartaric acid esters of mono- and diglycerides (JECFA, 1974).

US FDA stated the food additive glycerides and polyglycerides of hydrogenated vegetable oils may be safely used in food in accordance with the following prescribed conditions:

(a) The additive is manufactured by heating a mixture of hydrogenated oils of vegetable origin and polyethylene glycol in the presence of an alkaline catalyst followed by neutralisation with any acid that is approved or is generally recognised as safe for this use to yield the finished product.
The additive consists of a mixture of mono-, di- and tri-glycerides and polyethylene glycol mono- and di-esters of fatty acids of hydrogenated vegetable oils and meets several specifications (CFR Title 21, 2010).

In New Zealand, the New Zealand Food Safety Authority is responsible in developing food safety standards and implementing the Food Standards Code. The code currently in use was developed by Food Standards Australia New Zealand, an independent bi-national authority responsible for setting food composition and labeling standards in New Zealand and Australia, with input from the New Zealand Food Safety Authority. According to the code, Part 1.3.1 in Food Additives, monoglycerides can be allowed to be present in processed foods as a result of use in accordance with GMP without any limitation except for a few categories of foods. These categories of food have limitation of the amount of monoglycerides allowable. These foods and their respective monoglycerides’ maximum permitted level include liquid milk to which phytosterols, phytostanols or their esters (2 g/kg), infant formula products (4000 mg / L), infant formula products for specific dietary use based on protein substitutes (5000 mg / L) and foods for infants (5000 mg / kg).

2.6 Lipids

Enzymatic synthesis of monoglycerides requires hydrolysis of lipids, and therefore familiarity with their composition and properties is important in appropriate use of this particular reaction pathway. Lipids is the term used for the commonly known fats (solid) and oils (liquid) in food and nutrition (Balcão & Malcata, 1998; Dupont, 2005; Gordon, 2003a;2003b). The most familiar physical characteristic of lipids is that they are immiscible with water; however, lipids can be soluble in organic solvents such as chloroform, ethyl ether, alcohols, and light petroleum. The
solubility of lipids are however dependent on their polarity thus determining their structural and functional characteristics. Among the many compounds of lipids, fatty acids, glycerides, phospholipids, sterols, glycolipids, and sphingolipids are included (Dupont, 2005; Gordon, 2003a; 2003b). Understanding the characteristics of lipids is also crucial to the formulation of food products as lipids can greatly influence the rheological properties and texture of food (Wright & Marangoni, 2005).

Storage lipids from plant sources mainly refined plant oils are widely used in the food industry with vast application including in frying oils, margarine, shortenings, mayonnaise and salad dressings, peanut butter and spreads, chocolate and confectionery products. This is long-established by the production of plant oils exceeding 50 million tonnes per year. The commercial edible plant oils include soybean oil, rapeseed oil, corn oil, palm and palm kernel oil, olive oil, sunflower oil, coconut oil and cocoa butter. Apart from that, lipids are also used as a feedstock for surfactants or emulsifiers in foods, hence, mixtures of mono- and diglycerides are the most frequent emulsifiers used in foods. Other lipid surfactants include acetylated monoglycerides, sucrose esters, sorbitan esters and polyoxyethylene sorbitan esters (Gordon, 2003b).

2.6.1 Synthesis of monoglycerides

2.6.1.1 Esterification

The reaction pathway of esterification of fatty acids with glycerol to produce monoglycerides is shown in Figure 2.3. The esterification of glycerol and fatty acid will yield a mixture of 1(3)-monoglyceride and 2-monoglyceride at 90% and 10% of the mixture respectively (Pouilloux et al., 1999). Due to the reversible nature of the reaction, it is necessary to have low water content
in the system to obtain high yield of monoglycerides. This can be achieved by removing the water through vacuum or molecular sieves (Yang et al., 2005b).

![Diagram of esterification of glycerol and fatty acid](image)

**Figure 2.3: Esterification of glycerol and fatty acid**

### 2.6.1.2 Glycerolysis

Glycerolysis is another pathway in which monoglycerides can be synthetically derived. The glycerolysis reaction is depicted in Figure 2.4. This is often used to produce monoglycerides as the glycerolysis offers the advantage of a higher yield. This is due to the fact that 1 mole of triglyceride can subsequently produce 3 moles of monoglycerides (Yang et al., 2005b). However, in real reaction systems, mixtures of monoglycerides and diglycerides are often obtained with some residues of unreacted triglycerides (10 %), residual glycerol (3 - 4 %) and free fatty acids (1 – 3 %). Often the use of excess glycerol rather than the theoretical 2 moles are needed (Chetpattananondh et al., 2005).
The industrial setting of glycerolysis process usually involves high temperature between 200-260°C and inorganic catalysts usually sodium hydroxide under a nitrogen gas atmosphere (Bradić et al., 2010; Garti, 2001; Nylander, 2004; Sonntag, 1982). The source of triglycerides used are often from hydrogenated vegetable oil such as soya bean, rapeseed and cottonseed oils, or animal fats such as lard and tallow (Krog, 2002; Mahungu & Artz, 2001). Often, apart from monoglycerides, derivatives of monoglycerides such as organic acid esters of monoglycerides are also used in food industries. These derivatives of monoglycerides can be produced by esterification of the monoglycerides with various organic acids, such as, lactic acid, citric acid, diacetyl tartaric acid, and or acetic acid. Due to the difference in type of organic acids, the resulting product often exhibit different attribute from those of the monoglycerides with regard to crystalline behaviour and surface activity (polarity) (Faergemand & Krog, 2006).

\[
\begin{align*}
\text{CH}_2\text{OCOR} & + 2 \text{CH}_2\text{OH} \rightarrow \text{CH}_2\text{OCOR} + \text{CH}_2\text{OH} \\
\text{CH}_2\text{OCOR} & \quad \quad \quad \quad \text{CH}_2\text{OH} \quad \quad \quad \quad \text{CH}_2\text{OCOR} \\
\text{CH}_2\text{OCOR} & \quad \quad \quad \quad \text{CH}_2\text{OH} \quad \quad \quad \quad \text{CH}_2\text{OH}
\end{align*}
\]

Triglyceride  Glycerol  1(3)-Monoglyceride  2-Monoglyceride

Source: Bornscheuer (1995)

**Figure 2.4: Glycerolysis of triglycerides with glycerol**
The catalysts require neutralisation when the reaction is finished to prevent the reaction to reverse, which can happen to an extent of about 30% and also to prevent the undesirable soapy taste, unstable colour and foaming of the final mixture (Corma et al., 1998). Although the addition of inorganic catalysts can help achieving high reaction rate at higher temperature, it has a shortcoming due to the non-selective nature of the catalysts, resulting numerous by-products being produced. This requires product clean-up from the mixtures obtained (Bradić et al., 2010). The monoglycerides percentage in the equilibrium blend gained relies on the glycerol-fat ratio in the reaction mixture and may vary from 10 to 60%. Majority of the commercial mono- and diglycerides usually contain 40 to 55% monoglycerides, 30 to 45% diglycerides, and 8 to 20% triglycerides (Faergemand & Krog, 2006; Krog, 2002).

Due to the undesirable blends of by-product in the mixture obtained, purification of the monoglycerides are needed because they need to be highly pure for the use in the food industry since they have a better emulsifying properties rather than a mixture of acylglycerols (Bornscheuer, 1995). However, short-path distillation (molecular distillation) is not able to completely separate monoglycerides and free fatty acids (Shimada, 2005). Monoglycerides can be separated from the other components by further purification by repeated extraction or high-vacuum thin film molecular distillation process yielding relatively pure >92 alpha monoglyceride esters (Garcia et al., 1995; Krog, 2002; Mahungu & Artz, 2001; Nylander, 2004). The proportion of products after purification are typically 95% monoglycerides, 3 - 4% diglycerides, 0.5 - 1% free glycerol, and 0.5 - 1% free fatty acids (Krog, 2002).
Monoglycerides can be categorised as saturated or unsaturated depending on the nature of fatty acids chain in the triglycerides used. Saturated monoglycerides are formed from triglycerides or fatty acids with chain lengths of C16 (palmitic), C18 (stearic) and to a lesser extent lauric (C12), whereas unsaturated monoglycerides are mainly comprised of C18:1 (oleic) fatty acid triglycerides (Golding & Sein, 2004). These different fatty acids composing the triglycerides can also influenced the physical form of the monoglycerides produced of either solid or liquid. Unsaturated fatty acids mainly oleic acids will produce liquid monoglycerides and are more susceptible to oxidation and degradation, whereas saturated fatty acids will yield solid powders of monoglycerides that resembles a more waxy appearance. The melting points of the monoglycerides produced depends on the composition, origin and structure of fatty acids (Garti, 2001).

### 2.6.1.3 Enzymatic hydrolysis of triglycerides

Apart from the glycerolysis and direct esterification route, monoglycerides can also be produced from the hydrolysis of triglycerides. The established industrial process is known as the Colgate-Emery method, which often employed high temperature to about 250 °C and at 50 atm. Similar with the industrial glycerolysis, these harsh conditions often results in side reactions and requires further purification of the final products (Noureddini & Harmeyer, 1998). An alternative route for production of monoglycerides is the hydrolysis reaction catalysed by lipase enzymes, particularly by a 1,3-selective lipases as shown in Figure 2.6. One mole of triglycerides will produced 1 mole of 2-monoglycerides, which can turned into 1,3-monoglycerides due to acyl migration (Rodrigues & Fernandez-Lafuente, 2010a; 2010b; Yang et al., 2005b). Whilst, enzymatic hydrolysis is still rarely used for monoglyceride production it is becoming increasingly
recognised as a more natural pathway by which this material can be produced (Kaewthong, 2004; Yang et al., 2005b).

![Diagram of hydrolysis of triglycerides by 1,3-selective lipase]

**Figure 2.5: Hydrolysis of triglycerides by 1,3-selective lipase**

### 2.6.2 Triglycerides

Triester of glycerol and fatty acids also known as triglycerides or triacylglycerols constitute the bulk lipid mass and the main constituent of lipids from plant and animal origin, often representing more than 95% of the lipids (Balcão & Malcata, 1998; Gordon, 2003a; Lichtenstein, 2005; Macrae, 1985; Wright & Marangoni, 2005). Triglyceride is relatively a simple molecule formed by a trihydric alcohol; a glycerol moiety to which three fatty acids are linked through an ester bond. A wide range of different triglyceride species is possible due to the existence of large number of fatty acids in nature and the possibility of different positional arrangement of the fatty acids along the glycerol backbone (Gordon, 2003a; Kalo & Kemppinen, 2003; Lichtenstein,
2005; McClements, 2015; Sinanoglou et al., 2008; Taylor & MacGibbon, 2002; Wright & Marangoni, 2005). The typical structure of a triglyceride is depicted in Figure 2.5.

The prime fatty acids often possess an even number of carbon atoms (usually less than 24) and are non-branched, due to their natural biosynthetic pathway (Balcão & Malcata, 1998; McClements, 2015). These fatty acids account to approximately 95% of the molecular weight of a triglycerides molecule (Lichtenstein, 2005). The arrangement of these fatty acids and the position in which the fatty acids occupies within the triglycerides molecule will determine the physical properties of the lipids (Lichtenstein, 2005; Macrae, 1985); for instance, high melting point fatty acids will yield high melting triglycerides (Gordon, 2003a).

The glycerol molecule can be chiral if the primary hydroxyls are esterified with different fatty acids. Instead of the usual R/S of d/l principle for enantiomers, triglycerides enantiomers are referred by ‘stereospecific numbering’ (sn) according to the International Union of Pure and Applied Chemistry – International Union of Biochemistry (1967) commission (Kalo & Kemppinen, 2003). Thus, each of the carbons in the glycerol backbone allows for stereochemically different fatty acid bond position namely sn-1, sn-2, and sn-3 (Kalo & Kemppinen, 2003; Lichtenstein, 2005; Singh et al., 2009).
All triglycerides can be hydrolysed in the presence of catalysts; these include acids, bases, or enzymes belonging to the hydrolase class. Lipase enzyme is of particular importance as it can catalyse not only the hydrolysis of triglycerides (Gordon, 2003a); but also the synthesis of triglycerides as well (Chang & Wu, 2007; McNeill & Sonnet, 1995). The hydrolysis of triglycerides catalysed by lipase is dependent on the fatty acids composition as well as the distribution of the fatty acids. Complete hydrolysis of triglycerides will yield glycerol and 3 fatty acids (Gordon, 2003a). Hydrolysis of short chain fatty acids may result in distasteful rancid flavour, for example in the lipolysis of bovine milk fat, liberating unpleasant-tasting butyric and caproic acids (Kalo & Kemppinen, 2003). Intermediate products of triglycerides hydrolysis include monoglycerides and diglycerides, with one and two fatty acids, respectively. They rarely occur in large quantities in nature. However, they are often produced by processing to be added as emulsifier (Lichtenstein, 2005; Singh et al., 2009).
2.6.3 Fatty acids

Fatty acids are hydrocarbons with carbon chain greater than two and possess a carboxyl group at one end (Dupont, 2005). The structures of fatty acids are dependent on the carbon chain length, branched or linear, saturated or unsaturated, as well as position of double bonds. Most predominant fatty acids in plant and animal origin are straight-chain and possess an even number of carbon atoms due to their biosynthetic pathway (Balcão & Malcata, 1998; Rezanka & Sigler, 2009; Wright & Marangoni, 2005). Odd chain length of fatty acids are found from those from microbial origin (Hammond, 2003b). The carbon chain length may vary from 2 to 80, but the most common are from 12 up to 24. Short-chain fatty acids are for those with carbon chain length 2 to 6, medium-chain are from 8-10 and long-chain are from 12 and above (Leonard et al., 2004). The hydrocarbon chain of fatty acids is found to be hydrophobic while the carboxyl end is hydrophilic (Gordon, 2003a).

Although lipids contain ester of fatty acids, the free fatty acids themselves occur in relatively small amount in food. Edible plant oil contains only a minute amount of free fatty acids. In palm fruit which contain high concentration of lipid, free fatty acids may develop in bruised fruit due to the presence of lipase. In most edible oils, the amount of free fatty acids is below 5 % and is usually removed by refining process. This is due to the fact that free fatty acids will contribute to the undesirable effect on flavour (Gordon, 2003a). The normal chain lengths of fatty acids in triglycerides are usually between C14 and C22, with the major fatty acids being of the C16 and C18 types. Apart from chain-length, another different feature is the unsaturation of fatty acids by the presence of double bonds. Some fatty acids even possess up to six double bonds at specific position in the chain (Hammond, 2003a).
2.7 Lipase

As indicated, lipases (triacylglycerol hydrolase E.C. 3.1.1.3) are carboxylesterases that catalyse the reversible hydrolysis of triglycerides to corresponding glycerols and fatty acids; and therefore, also the synthesis of glycerol esters (Gandhi et al., 2000; Hou et al., 2009; Jaeger & Eggert, 2002; Noor et al., 2003; Schmid & Verger, 1998; Weete et al., 2008; Wu et al., 1996).

Lipases are naturally widely found in animals, plants and microbial sources. Although it is an esterase enzyme, lipases differ from other esterases which catalyse hydrolysis of a substrate in solution; instead, lipases act at the oil-water interface. Hence, lipase activity is greatly influenced by particular structural aspects of interfacial activation such as observed for emulsion systems, where high surface areas of substrates serve to optimise reaction conditions (Beisson et al., 2000; Noor et al., 2003; Tsujita et al., 1990; Weete et al., 2008). Lipases are increasingly being recognised in their ability to catalyse a range of reactions such as esterification, transesterification and amidation, as well as hydrolysis (Gandhi et al., 2000; Schmid & Verger, 1998; Wu et al., 1996).

Lipases are adaptable because of their unique chemoselectivity, regioselectivity and stereoselectivity. Further advantages includes their availability in commercially relevant large volumes, through large scale synthesis from microorganisms such as fungi and bacteria. These characteristics often make lipases the commonly used biocatalyst in lipid chemistry with various applications in the production of food additives, pharmaceutical products and chiral intermediates (Gandhi et al., 2000; Jaeger & Eggert, 2002).
2.7.1 Microbial lipases

Advancements in genetic engineering and microbiology techniques have enabled the production of lipase from recombinant bacteria and yeasts (Schmid & Verger, 1998). Microbial lipases have been found to be stable and able to catalyze a wide range of reactions due to the distinct stereoselectivity of the lipase produced from each of the different microbes (Gandhi et al., 2000; Hou et al., 2009). In addition, microbial lipases are considerably more economical than lipases from mammalian sources which are more difficult to isolate and purify. Microbial extracellular lipases can also possess improved thermostability and tolerance to other environmental factors, properties juxtaposing with animal and plant lipases (Tsujita et al., 1990). The numerous species of bacteria, yeast and molds producing lipases are shown in Table 2.2.
### Table 2.2: Isolation of lipase from various microorganisms

<table>
<thead>
<tr>
<th>Class</th>
<th>Lipase producing microorganisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td>sp</td>
<td>Dosanjh &amp; Kaur (2002); Kamijo et al. (2011); Haba et al. (2000); Nawani et al. (1998)</td>
</tr>
<tr>
<td>Bacillus</td>
<td>subtilis</td>
<td>Haba et al (2000); Olusesan et al. (2011); Rajakumara et al. (2008)</td>
</tr>
<tr>
<td>Chromobacterium</td>
<td>viscosum</td>
<td>Carvalho &amp; Cabral (2000); Kovac et al. (1996); Shu et al. (2010)</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>acnes</td>
<td>Weete et al. (2008)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>plantarum</td>
<td>Lopes et al. (2002)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>sp</td>
<td>Haba et al. (2000); Yu et al. (2007); Zhao &amp; Zheng (2011)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>aeruginosa</td>
<td>Baharum et al. (2010); Dalby (2011); Haba et al. (2000); Singh et al. (2008b)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>fluorescens</td>
<td>Dalby (2011); Fernandez-Lorente et al. (2011); Haba et al. (2000); Kojima et al. (2003)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>fragi</td>
<td>Haba et al. (2000)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>alcaligenes</td>
<td>Shu et al. (2010)</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>aureus</td>
<td>Weete et al. (2008)</td>
</tr>
<tr>
<td>Fungi and mould</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus</td>
<td>niger</td>
<td>Kamini et al. (1998); Lai et al. (2000); Okada &amp; Morrissey (2007); Shu et al. (2010); Weete et al. (2008)</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>oryzae</td>
<td>Carvalho &amp; Cabral (2000); Shu et al. (2010)</td>
</tr>
<tr>
<td>Geotrichium</td>
<td>candidum</td>
<td>Kovac et al. (1996); Shu et al. (2010)</td>
</tr>
<tr>
<td>Humicola</td>
<td>lanuginose</td>
<td>Carvalho &amp; Cabral (2000); Shu et al. (2010)</td>
</tr>
<tr>
<td>Penicillium</td>
<td>roqueforti</td>
<td>Shu et al. (2010)</td>
</tr>
<tr>
<td>Penicillium</td>
<td>camembertii</td>
<td>Carvalho &amp; Cabral (2000); Shimada (2005); Shu et al. (2010); Wilcox et al. (1955)</td>
</tr>
<tr>
<td>Rhizomucor</td>
<td>miehei</td>
<td>Alcantara et al. (1998); Carvalho &amp; Cabral (2000); Kraa, et al. (2008); Lai et al. (2000); Noel &amp; Combes (2003a); Shu et al. (2010); Weete et al. (2008)</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>aarhizus</td>
<td>Carvalho &amp; Cabral (2000); Kovac et al. (1996); Niu et al. (2006); Shu et al. (2010); Yang et al. (2005c)</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>delemar</td>
<td>Carvalho &amp; Cabral (2000)</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>javanicus</td>
<td>Lai et al. (2000); Okada &amp; Morrissey (2007)</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>oryzae</td>
<td>Shu et al. (2010); Weete et al. (2008)</td>
</tr>
<tr>
<td>Thermomyces</td>
<td>lanuginosus</td>
<td>Dizge et al. (2008); Shu et al. (2010)</td>
</tr>
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<td>Candida antarctica</td>
<td>Shu et al. (2010); Sun et al. (2007); Weete et al. (2008)</td>
</tr>
<tr>
<td>Candida</td>
<td>rugosa</td>
<td>Carvalho &amp; Cabral (2000); Haba et al. (2000); Kovac et al. (1996); Okada &amp; Morrissey (2007); Shu et al. (2010)</td>
</tr>
</tbody>
</table>
2.7.2 Lipase specificity

Lipases can be categorised according to their specificity either by positional specificity, substrates specificity either fatty acids or triglyceride, as well as stereo specificity (Hou et al., 2009; Malcata et al., 1992; Willis & Marangoni, 2008). Among these specificities, fatty acids and positional specificities are of particular significance for modification of lipids (Shimada, 2005).

Non-specific lipases show no specificity in relation with the position of acyl group as well as the type of fatty acids, and hydrolyse to glycerols and fatty acids all three ester bond of triglyceride, at the sn-1,2 and 3 position at the same velocity. These are lipases such as those produced from Corynebacterium acnes and Staphylococcus aureus (Weete et al., 2008), Candida antartica (Heldt-Hansen Hans et al., 1989), Geotriehum candidum and Penicillium cyclopiurn (Ergan & Trani, 1991) and Penicillium expansum (Hou et al., 2009)

Among the example of lipases that show substrates specificity for fatty acids are lipases from Candida rugosa which prefer C18 (cis-9) fatty acids and Aspergillus niger which prefer C10 and C12 or C18:1 (cis -9) (Sonnet, 1988). The fatty acid specificity of lipases has often been utilised in development of functional food with augmented lipids containing certain desired fatty acids mainly polyunsaturated fatty acids (PUFA) to improve the nutritional properties (Weete et al., 2008; Willis & Marangoni, 2008). Lipases generally act weakly on PUFA; it has been reported that Candida rugosa acts most weakly on PUFAs during hydrolysis of tuna oil containing 25 % docosahexaenoic acid (DHA) lipase in the presence of 50 % water, resulting in higher content of DHA in the remaining acylglycerols (Shimada, 2005).
Chapter 2: Literature Review

The most interesting specificity of lipases, of relevance to this study, is the positional specificity. This positional specificity can be either sn-1,3 specific or sn-2 specific (Malcata et al., 1992). The sn-1,3-specific lipases prefers to act on ester bonds of fatty acids at the terminal or outer positions on the glycerol backbone, leaving the fatty acids on the central position unaffected. The 2-specific type on the other hand favoured the release of fatty acids from the central position (Hou et al., 2009; Malcata et al., 1992; Millqvist et al., 1994; Stadler et al., 1995). The 1,3-specific lipases are derived from the steric hindrance conflict that deter the central fatty acid to bind with the lipases’ active site. As the central fatty acid is not susceptible to hydrolysis by the 1,3-specific lipases, products of the reactions often includes a mixture of diglycerides and monoglycerides (Stadler et al., 1995). Microbial lipases that possess 1,3-specificity have been observed from Rhizopus arrhizus (Hayes & Kleiman, 1993; Hou et al., 2009; Malcata et al., 1992; Tan & Yin, 2005), Aspergillus niger (Balcão & Malcata, 1998; Ghazali et al., 1995; Hou et al., 2009; Malcata et al., 1992; Stadler et al., 1995; Tüter et al., 1999), T. lanuginosa (Hou et al., 2009), Mucor miehei (Dourtoglou et al., 2001; Malcata et al., 1992; Schuch & Mukherjee, 1987; Sridhar & Lakshminarayana, 1992), Rhizomucor miehei (Ghazali et al., 1995; Hayes & Kleiman, 1993; Hou et al., 2009; Stadler et al., 1995; Xu et al., 1998), and Mucor javanicus (Balcão & Malcata, 1998; Stadler et al., 1995). Sn-2 specific lipase is rarely found however this type of specificity has been reported to be attributed with lipase III and IV from Geotrichum candidum (Malcata et al., 1992; Shimada, 2005).
2.7.3 Lipases mediated reactions and synthesis of monoglycerides

As described in section Error! Reference source not found.1 on the production of monoglycerides, these have been widely synthetically produced using harsh conventional chemical process including high temperature, high pressure and with the use of inorganic catalysts. This often results in high capital investment, high-energy consumption, low yield, and poor product quality; undesirable dark colour and burnt taste. In recent years, enzymatic syntheses of monoglycerides using lipases have garnered much attention as a substitute to the harsh manufacturing conditions and are slowly obtaining significance as lipases are now available at a reasonable price (Bornscheuer, 1995; Garcia et al., 1995; Jafari et al., 2008; Kaewthong et al., 2005; Noor et al., 2003). This is because, enzymatic splitting of fats by lipase is an environmentally friendly approach that offers a cleaner and milder bioprocess with advantages including biodegradability, ability to mimic natural pathways for controlled lipid-based reactions and exhibiting high selectivity thus producing specific yield (Bjorkling et al., 1991; Gandhi et al., 2000; Hou et al., 2009; Langone et al., 2002; Noureddini & Harmeier, 1998). In addition, the same monoglycerides/emulsifiers synthesis by the enzymatic reaction may be labelled as ‘natural’ (Bradić et al., 2010). Noureddini & Harmeier (1998) recounted that the operation of enzymatic glycerolysis under ambient conditions would require far less capital investment than conventional chemical methods.

The potential economical saving from using lipases are derived from the decrease in energy required due to lower temperature and eliminating the need of purification process due to the selectivity and specificity of lipases producing no unwanted by-products (Noureddini & Harmeier, 1998; Wang et al., 1988). Initially, the use of lipase induced a higher cost due to high
enzyme price. But in recent years, microbial lipases have been generated commercially with lower cost thus acquiring more interest in the feasibility of using lipase as lipid-splitting catalyst for a more energy saving process and for value-added products and heat sensitive lipids (Gandhi et al., 2000; Noureddini & Harmeier, 1998; Rathod & Pandit, 2010). Lipases also offer the advantage of operating under mild condition thus allowing sensitive substrates to be used (Bornscheuer, 1995; Millqvist et al., 1994). Other than that, lipases also enable a purification–free process because specificity in monoglycerides production avoids side product formation and is less polluting (Ferreira-Dias & Fonseca, 1995).

Lipase is activated at the interfacial region between oil and water typically found in an emulsion. The activation of lipases by interfaces is due to the more favourable alignment of substrate, a higher local concentration of substrate and a lower hydration state of substrate (Miller et al., 1991). Thus, lipase-catalysed reaction can be done in biphasic mixtures, compose of lipase-containing aqueous solution emulsified in a water-immiscible organic solvent. Some of the previous researchers configured their reaction with an inert organic solvent as the prime dispersing phase, while several authors abolished the use of solvent by allowing the substrates to act as the continuous phase. Solvent-free systems offer more advantages, being safer, reducing the solvent extraction cost, and increasing reactant concentration and subsequent yield (Dossat et al., 2002). In addition to that, solvent-free systems are preferable by the industry and several systems have been suggested (Baeza-Jiménez et al., 2014; Ferreira-Dias & Fonseca, 1995; Ghamgui et al., 2006; Hou et al., 2009; Lee & Lee, 2005; Shimada, 2005). This is due to the fact that a solvent-free system can evade the need for separation or purification of the products
and is safe from toxic and often highly flammable organic solvents. Moreover, the system can be easily applied in food processing (Yahya et al., 1998).

Naturally, lipase biological function is to catalyse the hydrolysis reaction of lipids. Nevertheless, as lipase can be derived from various sources, it can also display high specificity and stereoselectivity. Thus enabling lipase to be a very versatile enzyme that can mediate monoglycerides formation from a number of different lipid based reactions such as by enzymatic hydrolysis of oils (Holmberg & Osterberg, 1988); glycerolysis or alcoholysis (Chetpattananondh et al., 2005; Esmelindo et al., 2008; Ferreira-Dias et al., 2003; Ferreira-Dias & Fonseca, 1995; Garcia et al., 1995; Kaewthong, 2004; McNeill et al., 1990;1991; McNeill & Yamane, 1991), esterification (Bellot et al., 2001; Chang & Wu, 2007; Gandhi et al., 2000; Linder et al., 2005) and transesterification (Balcão & Malcata, 1998; Ghazali et al., 1995; Long et al., 2003; Marangoni & Rousseau, 2008; Rathod & Pandit, 2010; Richard et al., 2011; Suppes et al., 2004; Willis & Marangoni, 2008).

The yield of monoglycerides accounted in the aforementioned research can range from 30 – 90%. A noteworthy consideration that can be observed from the previous research is that the yield of monoglycerides tends to increase as the reaction temperature decreases. This may be due to melting point of monoglycerides that can be the highest among the other lipid component (fatty acid, diglyceride and triglyceride). Since lipases act very weakly on solid-state substrates, monoglycerides that solidify as the reaction temperature decreases inhibit lipase from hydrolysing it. Thus, a higher yield of monoglycerides can be anticipated (Hou et al., 2009).
Lipases activity is also affected by water molecules bound to the enzymes. Hou et al. (2009) reported the catalytic activity of *P. camembertii* lipase is greatest when sufficient water molecules have bound to the lipase. Hence, *P. camembertii* lipase is able to catalyse production of monoglycerides by esterification of free fatty acids with glycerol even at ordinary temperature with the yield reaching up to 90%. Normally, hydrolysis will take place in a system containing a large amount of water, and esterification occurs in a system with a small amount of water. Transesterification catalysed by lipase has also been reported to be effective in a mixture containing no water and with an immobilised enzyme (Shimada, 2005).

The enzymatic lipolysis reaction itself follows the pingpong bi-bi mechanism and takes place in three reversible steps. The first is deacylation of the triglyceride chain on the carbonyl carbon atom of the ester bond along with the formation of any acyl-enzyme complex. The acylated lipase is freed by nucleophilic attack by a water molecule subsequently producing free fatty acid moiety and diglyceride. The diglyceride will undergo the same steps until it became 2-monoglyceride (Balcão & Malcata, 1998; Millqvist et al., 1994; Rooney & Weatherley, 2001). 1,3-specific lipases have been used to produced 2-monoglyceride through lipolysis of triglycerides (van der Padt et al., 1992). The 1,3-specific lipases only attack on the outer position of the fatty acids attached to the glycerol, leaving the 2-position attached (Millqvist et al., 1994). A simplified diagram of lipase mediated lipid-based reaction pathway to synthesise monoglycerides is shown in Figure 2.7.
Figure 2.7: (1) The synthesis of 2-monoglyceride using selective hydrolysis with 1,3-specific lipase, (2) Esterification of glycerol and fatty acids and (3) glycerolysis of fats/oil with glycerol to produce 1(2)-monoglyceride. (By-products are not shown for clarity)

Source: Bornscheuer (1995); Macrae(1985); Rooney & Weatherley (2001); Willis & Marangoni(2008)
Chapter 2: Literature Review

The products of lipolysis include monoglycerides, diglycerides and free fatty acids. Fatty acids in particular can sometimes contribute to unpleasant flavours such as rancid and bitter. These are particularly pronounced in the lipolysis of milk fat, which contains short chain fatty acids namely butyric, caproic and caprylic that are predominantly responsible for these strong flavours. Accordingly, lipolysis in certain dairy products, most notably cheese, is important for flavour development, as exemplified in blue vein cheese which requires fatty acids produced by blue Penicillium mould lipase while lamb, kid and calf pregastric lipases are used in the manufacture of Parmigiano and Romano to produce short-chain fatty acids which impart a piquant flavour (Deeth & Hubert, 2002).

Willis & Marangoni (2008) reported that glycerolysis can be performed by non-specific lipases to give a wide range of reaction products. van der Padt (1992) have previously described how enzymatic glycerolysis by lipase are done by a mixture of glycerol, a trace of water and triglycerides that were emulsified before addition of lipase. The initial reaction temperature was 45 °C which will then lower after a while to allow precipitation of monoglycerides. This method yields monoglycerides concentration larger than 90 % (w/w).

Lipase-catalysed esterification reactions have been shown with long-chain fatty acids. However, it is not shown to synthesise short-chain monoglycerides due to the relatively high acidity of the short chain fatty acids (Lee et al., 2004). Freitas et al (2010) have studied the synthesis of monoglycerides in a medium solely composed of substrates by direct lipase-catalysed esterification of glycerol with fatty acids, without any solvent or surfactant. Numerous authors have explored the usefulness of lipase in catalysing glycerolysis, esterification,
transesterification and hydrolysis for monoglycerides production. Table 2.3 listed several authors and the different types of lipase mediated reaction including the source of lipase and substrate used to synthesised monoglycerides.

In conclusion, the understanding of enzymatic lipolysis reaction in the synthesis of polar lipids can be manipulated as part of generating monoglycerides in situ as part of food emulsion processing. Thus, the work in this thesis intends to apply this biochemical pathway to impart the desired functionality in food emulsion products whilst eliminating the need for chemically derived monoglycerides.
Table 2.3: Different types of lipase mediated reaction, substrates and source of lipase used by several authors

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Substrates</th>
<th>Lipase source</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterification</td>
<td>Glycerol and fatty acids</td>
<td><em>Penicillium camembertii</em></td>
<td>Freitas et al. (2010)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Glycerol and oleic acid</td>
<td><em>Rhizomucor miehei</em></td>
<td>Bellot et al. (2001)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Glycerol and oleic acid</td>
<td><em>Candida sp.99-125</em></td>
<td>Zhao et al. (2011)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Glycerol and palmitic acid or myristic acid</td>
<td><em>Pseudomonas</em> sp. LP7315</td>
<td>Sakiyama et al. (2001)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Glycerol and oleic acid</td>
<td><em>Staphylococcus simulans</em></td>
<td>Ghamgui et al. (2006)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Glycerol and fatty acids</td>
<td><em>Mucor miehei</em></td>
<td>Langone et al. (2002)</td>
</tr>
<tr>
<td>Glycerolysis</td>
<td>Refined olive residue oil and glycerol</td>
<td><em>Candida rugosa</em></td>
<td>Ferreira-Dias et al. (2003)</td>
</tr>
<tr>
<td>Glycerolysis</td>
<td>Commercial olive oil (Arisco, Brazil) and glycerol</td>
<td>Amano AY30 (<em>Candida rugosa</em>)</td>
<td>Esmelindro et al. (2008)</td>
</tr>
<tr>
<td>Glycerolysis</td>
<td>Glycerin and triolein</td>
<td><em>Candida rugosa</em></td>
<td>Cetina et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>and olive oil</td>
<td><em>Chromobacterium viscosum</em></td>
<td></td>
</tr>
<tr>
<td>Glycerolysis</td>
<td>Glycerol and oleic acid</td>
<td><em>Mucor miehei</em></td>
<td>Ergan et al. (1990)</td>
</tr>
<tr>
<td>Esterification</td>
<td>Tripalmitin and ethanol</td>
<td><em>Rhizopus arrhizus</em></td>
<td>Millqvist et al. (1994)</td>
</tr>
<tr>
<td>Trans-esterification</td>
<td>High oleic sunflower oil and butanol</td>
<td>Lipozyme® <em>Rhizomucor miehei</em></td>
<td>Dossat et al. (2002)</td>
</tr>
<tr>
<td>Trans-esterification</td>
<td>Triolein and lauric acid</td>
<td><em>Candida cylindracea</em></td>
<td>Mustranta et al. (1993)</td>
</tr>
<tr>
<td>Trans-esterification</td>
<td>Lauric acid and different alcohols</td>
<td><em>Aspergillus niger</em></td>
<td></td>
</tr>
<tr>
<td>Trans-esterification</td>
<td>Trilaurin or dilaurin and lauric acid</td>
<td><em>Pseudomonas fluoscens</em></td>
<td>Miller et al. (1991)</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>Olive oil</td>
<td><em>Candida rugosa</em></td>
<td>Wang et al. (1988)</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>Tributyrin or castor oil</td>
<td><em>T. lanuginosus</em></td>
<td>Rathod &amp; Pandit (2010)</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>Soybean oil</td>
<td><em>Rhizomucor miehei</em></td>
<td>Rodrigues &amp; Ayub (2011)</td>
</tr>
</tbody>
</table>
Chapter 3: Materials and Methodology

3.1 Materials

3.1.1 Lipase

Lipase (liquid form) obtained from *R. miehei* with ≥20,000 U / g activity was purchased from Sigma-Aldrich NZ Ltd., Auckland, New Zealand. Lipase from *R. miehei* was chosen as it has found usage in various applications due to its high activity and good stability (Rodrigues and Fernandez-Lafuente 2010a; 2010b). Furthermore, the procurement of lipase of microbial source, in this case the fungi *R. miehei* has made it more cost effective (as described in detail in subsection 2.7.1) together with the desirable 1,3-position specificity needed for the production of 2-monoglyceride (subsection 2.7.2).

3.1.2 Emulsifiers

Sodium caseinate (Alanate 180) was supplied by Fonterra Co-operative Group Ltd., Auckland, New Zealand while whey protein isolate (Alacen 895) was supplied by Fonterra Ltd. Whareroa, Hawera, New Zealand. Tween 80 was from BDH Laboratory Supplies Ltd, England and lecithin refined (Alfa Aesar) sourced from soybean with partial water solubility was obtained from Global Science, Auckland, New Zealand.

3.1.3 Vegetable oil

‘Essente’ brand canola oil imported by Marsanta Foods, Wellington, New Zealand and ‘Simply’ brand pure soybean oil imported by Sucrogen Foods, Auckland, New Zealand were purchased from Davis Trading Company, Palmerston North, New Zealand. Olive oil used was ‘Pams’ brand (Pams Product Ltd, Auckland, New Zealand). ‘Pioneer’ brand solid palm oil
from Bakels Edible Oils, New Zealand Ltd was purchased from Bidvest Palmerston North, New Zealand. The oils were used without any further purification.

3.1.4 Chemicals

All chemicals used except for chromatography analysis were of analytical grade obtained from either ThermoFisher Scientific New Zealand Ltd or Sigma-Aldrich Co. LLC (MO, USA).

3.2 Methodology

The methods described in this chapter will be general and standard methods used in most of the chapters. Specific methods or any other modification will be described in the respective chapters separately.

3.2.1 Emulsification by microfluidization

Emulsifiers and surfactant solutions (1 wt %) were prepared by dispersing appropriate quantities of sodium caseinate, whey protein isolate, lecithin or Tween 80 in RO water and mixed for 2 h at 20 °C using a magnetic stirrer to ensure complete dissolution. Vegetable oils were then added to the solutions to form final emulsion containing 10 wt % oil (or other wt % as defined within the chapters). Emulsion mixtures were then homogenised using a conventional homogeniser (LabServ homogeniser D500, Biolab Ltd, New Zealand) for 5 min to produce a coarse temporary O/W emulsion, followed immediately with homogenisation in the Microfluidizer® (M-110P Microfluidizer®, Microfluidics Corporation, MA, USA). Microfluidization is a high-pressure homogenisation system that is efficient in producing O/W emulsion with very small particle sizes with narrow distribution. The interaction chamber of the Microfluidizer® has a fixed geometry and is present in the form of a confined capillary tube. The Microfluidizer® divides the liquid into two or more microstreams that are projected at right angles where disruption of the droplets occurs due to liquid-liquid and,
most of all, liquid-solid shear forces. The microstreams have very high velocities and undergo a sudden pressure drop when they collide (Forster, 1993; Lin et al., 1995). The Microfluidizer® has a pressure range up to 30000 psi with the process temperature increases approximately 1.7 °C per 1000 psi applied. Specific pressure used will be defined in the relevant chapters.

3.2.2 Determination of emulsion droplet size

The determination of the average droplet diameter was carried out using a laser diffraction technique (Malvern Mastersizer 2000, Malvern Instrument Ltd, Malvern, Worcestershire, UK). The laser diffraction technique is based on the Mie theory to calculate the droplet size distribution, whereby it predicts the scattering of light by spherical particles. The main principle is that particles passing through a monochromatic light beam, generated by a laser (helium/neon, λ = 632.8 nm) will diffract the light at an angle directly related to their size (Malvern Instrument Ltd, 1997). Mastersizer measures the angular diffraction of light from the emulsion droplets and fits the diffraction to well-known theoretical models. The angular diffraction of the light is the inverse function of the droplet size, whereby as the droplet size decreases, the observed scattering angle increases logarithmically. In addition to the diffraction angle, the intensity of the light beam is also taken into account as it is also dependent on droplet size and can diminish in relation to the droplet’s cross-sectional area. Larger droplets will tend to diffract light at narrow angles with high intensity, while smaller droplets diffract at wider angle but with low intensity. The average droplet size was determined by using the presentation code 2NAD. The pump speed was set at 1800 rpm. The absorbance value of the emulsion particles was 0.001. Samples were introduced into the recirculating water in the Hydro MU measuring cell unit until an obscuration rate of 11 -
12 \% was obtained as indicated by the instrument. The specific surface area as well as the D (4,3) data obtained were used in this study.

3.2.3 Determination of lipase activity

The determination of lipase activity was carried out using titrimetry method by pH-stat (Radiometer Analytical SAS, Villeurbanne Cedex, France). An initial 40 ml of emulsion was placed in the mechanically stirred reaction cell and temperature was maintained at 25 °C. Lipase was then added to start the lipolysis process on the emulsion at concentration of 25 mg per g of lipid in the emulsion (or varied according to specific treatments defined in some chapters). The released of free fatty by lipolysis was then automatically titrated with 0.05 M sodium hydroxide (NaOH). The volume of NaOH titrated to maintain equilibrium was used to calculate the concentration of free fatty acids release during lipolysis based on the equation below:

\[
\text{Free fatty acids (\(\mu\text{mol ml}^{-1}\))} = \frac{V_{\text{NaOH}} - V_{\text{NaOH (blank)}} \times 0.05 \times 10^{-3}}{V_{\text{emulsion}}}
\]

3.2.4 Microscopic observation with confocal laser scanning microscope

Microscopic observation of emulsion droplets was carried out using confocal laser scanning microscopy (Leica SP5 DM6000B, Leica Microsystem, Heidelberg, Germany). Fluorescent protein dye Fast Green FCF (Merck, Darmstadt, Germany) was used to stain the protein matrix of the emulsion, whilst Nile Red (Sigma Aldrich, St. Louis, MO, USA) was used to stain the lipid droplets. For the analysis of whipped emulsion, the dyes were added to the emulsion mix prior to aging, before being subsequently whipped the next day. A small scoop of the aerated emulsion was then transferred onto a concave glass microscope slide. A cover
slip was carefully placed over the sample avoiding trapping any air bubbles and to minimise disruption to the sample structure. The samples were then scanned with a 40x or 63x oil immersion objectives using laser with excitation wavelength of 561 nm and 633 nm for Nile Red and Fast Green FCF respectively. The images were captured by two channels of photomultiplier tube (PMT), one for each wavelength used. The images from both PMT channel was then overlapped and processed in Leica Application Suite Advanced Fluorescence (LAS AF) software platform.

3.2.5 Lipolysis of emulsion

Emulsions were lipolysed by adding *R. miehei* lipase into 40 ml of prepared O/W emulsion in a 100 ml beaker. Concentrations of lipase used (25 and 50 mg / g fat) were calculated against grams of fat in the emulsion (some chapters had different lipase concentration due to the different experimental treatment investigated). The mixture was then gently swirled from time to time to allow the lipolysis reaction to take place at 21 °C. Unless otherwise mentioned, lipolysis was carried out for 30 min and the mixture was then heated in a 100 °C water bath for 2 min to terminate the lipolysis reaction. Emulsions were immediately cooled in ice water prior to further analysis.
Chapter 4: Effect of Rhizomucor miehei lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

4.1 Abstract

Monoglyceride emulsifiers impart a wide range of functional properties in food products, including emulsification, foaming, crystal habit modification, starch complexation and even antimicrobial properties. Whilst commercial monoglycerides are synthetically derived, this does not represent the only pathway by which these molecules may be produced. Biologically, lipolysis of triglycerides, which occurs during all mammalian digestion of lipids, results in hydrolysis of fats and oils to fatty acids and 2-monoglycerides component.

Therefore, by utilising lipase enzymes as a processing aid (using Rhizomucor miehei in this specific study), monoglycerides fractions may be potentially derived in situ in food emulsions as a consequence of lipolysis, thereby providing a non-synthetic means of delivering emulsifier functionality in products such as ice cream, whipping cream and spreads. This chapter aims to investigate the feasibility of this approach by using surface characterisation techniques to determine the occurrence of lipolysis at the oil-water interface. The inclusion of lipase into the aqueous phase was seen to result in decreasing of interfacial tension (IFT) compared to systems not containing the lipase. This decrease of IFT was attributed to the triglyceride hydrolysis resulting in the production of 2-monoglycerides and fatty acids, both of which are surface active. Triglyceride hydrolysis was found to occur for oil-water interfaces comprising a range of protein emulsifiers i.e. sodium caseinate and whey protein isolate as well as low molecular weight emulsifiers i.e. lecithin. However, droplets stabilised with Tween 80, are much more unstable with the presence of lipase suggesting possible interaction between the two. All monoglycerides (monopalmitin, monostearin and
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

monoolein) tested in this study were able to competitively adsorb on with protein emulsifier, based on the reduction of IFT obtained. However, the IFT results for monoglycerides with low molecular weight emulsifier; lecithin and Tween 80 were not conclusive due to the same stabilising mechanism of the emulsifier group. Hence, it was not certain the reduction of IFTs was due to the adsorption or desorption of monoglycerides, lecithin or Tween 80. In conclusion, this chapter showed that the adsorbed protein or surfactant layer does not act as a barrier to lipase adsorption at the oil-water interface.

4.2 Introduction

Many emulsion-based food products make use of protein emulsifiers such as milk protein concentrates/caseinates, whey protein isolate, or low molecular weight emulsifiers such as monoglycerides, phospholipids, polysorbate/Tweens surfactants; to provide stability or functionality to the emulsion systems in question (Golding & Sein, 2004; Rodríguez Patino et al., 2001b). In many formulations, a combination of these emulsifiers and/or surfactants might be used. Combinations of emulsifiers can be used to provide greater stability to the emulsion by the synergistic effect of the emulsifiers working together, for example, addition of smaller molecule emulsifiers may be able to provide supplementary interfacial coverage when limited coverage is provided by protein (Rodríguez Patino et al., 2001a). For example, polysorbates emulsifiers when used in low levels help to stabilise protein-stabilised emulsions by forming complex with the protein and causing it to be more hydrophobic (Norn, 2014). Additionally, combinations of emulsifiers can be used to actively promote destabilisation, most notably in products such as ice cream and whipping cream, where partial coalescence of the emulsion occurs as a result of competitive adsorption between proteins and small molecules surfactants (Mackie et al., 1999; Rodríguez Patino et al., 2001a). In the case of ice cream the practice of adding monoglycerides to the formulation
causes displacement of protein from the stable interface; weakening the protein film and thus enabling solid fat crystals from one droplet to penetrate into the liquid oil portion of another droplet resulting in partial coalescence (Boode & Walstra, 1993; Rodríguez Patino et al., 2001b).

Monoglycerides are probably the most commonly used synthetic food grade emulsifiers in the global industrial setting (Bradić et al., 2010; Garti, 2001; Golding & Sein, 2004; Krog, 2002; Mahungu & Artz, 2001). Commercial monoglycerides are manufactured synthetically via direct process esterification or by catalytic interesterification, also known as glycerolysis, which often employs very harsh processing conditions (i.e. high temperature, alkaline catalysts) (Garti, 2001; Golding & Sein, 2004; Krog, 2002; Mahungu & Artz, 2001). However, this does not represent the only pathway by which monoglycerides can be produced, for example the natural biological digestion of fat in the small intestine by pancreatic lipase enzyme, hydrolysing triglycerides to component fatty acids and monoglycerides. The use of lipase enzymes to synthesise monoglycerides without the use of the harsh manufacturing conditions has consequently gained much interest (Bornscheuer, 1995; Garcia et al., 1995; Jafari et al., 2008; Kaewthong et al., 2005; Noor et al., 2003).

A number of commercially available lipases (triacylglycerol hydrolase E.C. 3.1.1.3) are available, which catalyse the hydrolysis of triglycerides to their corresponding glycerols and fatty acids (Gandhi et al., 2000; Hou et al., 2009; Jaeger & Eggert, 2002; Noor et al., 2003; Schmid & Verger, 1998; Weete et al., 2008; Wu et al., 1996). As lipase is a water-soluble enzyme that acts on water-insoluble substrates, it is therefore crucial that the lipase is able to adsorb to the oil-water interface for the catalytic reaction to occur (Fainerman et al., 2006). Lipase mediated reactions and the factors affecting the efficacy of the lipolysis
reaction have been reviewed in detailed in Chapter 2 (subsection 2.7). In brief, lipolysis can be greatly influenced by interfacial properties, such as relative surfaces area, and the composition of the interface, as well as other factors typical of enzymatic reactions such as concentrations of enzyme and substrate, temperature and pH (Beisson et al., 2000; Chu et al., 2009; Miller et al., 1991; Noor et al., 2003; Tsujita & Okuda, 1990; Weete et al., 2008). Due to the importance of interfacial properties on lipase catalysis, a pendant drop technique was initially used to determine the progression of lipolysis by providing a direct measurement of the IFT at the oil-water interface. The pendant drop technique developed by Stauffer (1965), on the basis of the Young-Laplace equation (Laplace, 1806; Young, 1805) was used to calculate the interfacial loci by depicting the shape of a liquid drop as related to the surface tension of the interface and by the gravitational force. This technique has previously been utilised to observe the effect of lipolytic activity of lipase on oil/water interface, primarily in the study of fat lipolysis that occurs in the stomach and small intestine. Accordingly, the choice of lipase used in these previous works were either gastric/pancreatic or phospholipases, and the condition of the pendant drop experiment were set up to provide a simulation of the dietary fat digestion processes, such as with the presence of bile salt and low pH (Chu et al., 2009; Mun et al., 2007; Nury et al., 1987; Reis, et al., 2008c).

The main objective of the research is to investigate the potential of using lipolysis to provide in situ synthesis of monoglycerides as part of the manufacture of emulsions-based foods such as ice cream and whipping cream. In developing understanding towards this objective, this chapter aims to investigate the kinetics of lipolysis at the oil-water interface using the pedant drop technique. Adsorption and activity of R. miehei lipase an sn-1,3-specific enzyme, at the oil-water interface was studied. The additional inclusion of protein emulsifiers (sodium caseinate and whey protein isolate) and low molecular weight emulsifiers (lecithin)
and surfactant (Tween 80) and their influence on the adsorption of lipase on the interface of vegetable oils was also considered. Accordingly, IFT measurements provide a means by which the rate of synthesis of polar lipids arising from triglyceride lipolysis can be measured.

4.3 Materials and methodology

4.3.1 Materials

The source of *R. miehei* lipase, sodium caseinate, whey protein isolate, lecithin and Tween 80 have been described in Chapter 3, subsection 3.1.1. Pure fractions of monoglycerides were supplied by Nu-Chek Prep Inc., MN, USA. The monoglycerides were dispersed by heating in oil at 60°C according to procedure by Britten & Giroux (1991).

The vegetable oils used in the lipolysis reactions were commercial canola oil, soybean oil and olive oil. ‘Essente’ brand canola oil was imported by Marsanta Foods, Wellington, New Zealand. ‘Simply’ brand pure soybean oil was imported by Sucrogen Foods, Auckland, New Zealand. Both oils were purchased from Davis Trading Company, Palmerston North, New Zealand. Olive oil used was Pams brand (Pams Product Ltd, Mt Roskill, Auckland, New Zealand). No food additives or antifoaming agents were listed on the label. The oils were used without any further purification. Solid fats were not investigated at this stage due to limitations regarding temperature control of the tensiometer, such that these fats could be analysed in their molten state.

4.3.2 Methodology

4.3.2.1 Interfacial tension measurement by pendant-drop tensiometer

IFT measurement was carried out via pendant drop analysis using a KSV CAM optical tensiometer (KSV Instrument Ltd, Helsinki, Finland). The equipment was equipped with LED
background light source and enhanced high-speed video digitisation to capture the drop shape throughout the desired duration. In this study, the duration was set for a maximum of 1 h with frame interval set at 60 s. Calibration was carried out by a stainless steel ball with 4 mm diameter. This method strictly requires clean materials and equipment as well as a transparent vessel to produce reliable results. The camera, sample stage and syringe clamp can be adjusted and tilted to give the correct position in the light path and the sharpness of the drop shape image on the CCD camera. Prior to analysis, the measurement for IFT of air-water at room temperature was carried out to ensure cleanliness and contaminant-free interface, with a value of 72.5 ± 1 mN m⁻¹. Preparation of 1% emulsifier solutions (sodium caseinate, whey protein isolate, lecithin or Tween 80) were carried out as described in Chapter 3 (subsection 3.2.1). The desired aqueous phase of either water or emulsifier solution was carefully inserted into a 1 ml Hamilton adjustable precision syringe without creating any air bubbles. The liquid drop was formed at the end of the stainless steel needle tip immersed in a surrounding oil phase in a clear transparent cuvette.

For the measurement with lipase, 5 mg of *R. miehei* lipase was mixed with the 1 ml of desired aqueous phase before carefully inserting it into the syringe. Inactivation of lipase was carried out by heating the *R. miehei* lipase solution at 100°C for 10 min to ensure complete deactivation. Experiments analysing commercial monoglyceride fractions were carried out using 0.3 mg of each monoglyceride per ml of oil. The measurement was carried out until the specified time period of 1 h or until the drop detached from the syringe. The captured image was then analysed by CAM2008 software for curve fitting image analysis to determine the IFT. The fitting method used was the Young-Laplace equation which relates the pressure difference across a curved interface (Lin et al., 1995; Lin & Hwang, 1994;
Stauffer, 1965). All experiments were performed at 20 °C. All analyses were carried out with at least three replications.

4.3.2.2 Statistical analyses

Analysis of variance, main and interaction effect plots were derived using MINITAB 16 (Minitab Inc., State College, PA, USA) to determine the effects of type of vegetable oil and emulsifiers as well as *R. miehei* lipase catalysed reaction on the IFT of oil and water.

4.4 Results and discussion

4.4.1 Effect of *R. miehei* lipase on the IFT of oil and water

The pendant drop method has been developed to enable the measurement of liquid-gas or liquid-liquid IFT. The IFT was calculated from the suspended liquid droplet known as pendant drop by a combination of surface tension and gravitational effects. The balance between the surface tension as well as the gravitational force can be analysed from the shape of the pendant drop and the curve-fitting of the drop edge to the Young-Laplace equation. This method is capable of capturing the profile of the drop by the high definition CCD camera thus it is possible to study the IFT in dynamic systems in a time dependent approach (Hoorfar & Neumann, 2011; Lin & Hwang, 1994). Pendant drop techniques have a number of advantages over alternative methods such as the Wilhelmy plate, for example, requiring small amounts of sample, ease of handling, and eliminating the dependency on drop volume or drop size (Hoorfar & Neumann, 2011).

Initially, the IFT of water and vegetable oils were measured in the absence of lipase or emulsifiers. The IFT means were found to be quite constant averaging at 27.08 ± 0.24, 28.08
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

± 0.21 and 29.95 ± 0.18 mN m⁻¹ respectively for canola, olive and soybean oil as shown in Figure 4.1. This is expected since the pendant drop of the water phase is not stabilised by any emulsifier or surfactant against the bulk oil phase. No time dependent changes were observed for at the oil-water interface.

On addition of *R. miehei* lipase into the water phase, the IFT was found to decrease for each of the vegetable oils during the 1 h measurement. The decreases from the initial IFT values at the start point to the end point for canola, olive and soybean oil were as follow: from 14.29 to 4.95, from 12.33 to 3.75, and from 11.78 to 4.88 mN m⁻¹ respectively. The reduction in IFT was considered indicative that lipolysis of triglyceride into fatty acids and monoglycerides was taking place, with these polar and amphiphilic lipid fractions partitioning at the interface.

![Figure 4.1: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase, with the addition of *R. miehei* lipase, inactivated *R. miehei* lipase or no enzyme into the system](image-url)
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

*R. miehei* lipase used in this study possesses sn-1,3-specificity and accordingly acts on the sn-1 or 3 position on the glycerol, producing a monoglyceride in the centre position or sn-2 position and two free fatty acids (Hou et al., 2009; Stadler et al., 1995). From the lowering of the IFT value, it is deduced that the addition of lipase into the systems resulted in the lipolysis of the triglycerides of all three of the vegetable oils used. In this case, the lipolysis product is the ester of the centre hydroxyl group of glycerol which is the sn-2-monoglycerides. This species represents a non-ionic, polar low molecular weight emulsifier. Xu et al. (1998) and Reis et al. (2008c) had found the sn-2 monoglyceride species to be stable towards further degradation and acyl migration, as the acyl group migration to sn-1,3 monoglyceride is favoured by a more acidic condition.

The study by Reis et al. (2008a) on the hydrolysis of a short chain triglyceride namely tricaprylin (C8) by *R. miehei* lipase, showed that among the hydrolysis products; i.e. caprylic acid, monocaprylin, and dicaprylin, the monocaprylin had the highest capability in lowering the IFT as being the most surface active. A further study made comparison of monoglycerides with their corresponding fatty acid species with the same hydrocarbon length and found that monocaprylin possesses a higher surface activity compared to caprylic acid, as with monopalmitin with palmitic acid and monodocosahexaenoic. Mixtures of fatty acids and their respective monoglycerides also resulted in IFT values similar to those obtained with only the monoglycerides. This suggested that the interface is mainly occupied with the monoglycerides (Reis et al., 2008c), although the adsorption of polar fatty acids cannot be ignored. Furthermore, the use of sn-1,3 regiospecific lipase was found to produce 2-monoglycerides which ultimately displaced the lipase from the interface and caused the lipolysis to cease (Reis et al., 2008a). Whilst the IFT measurements clearly demonstrate the adsorption of surface active species at the oil-water interface.
interface, the specific composition of adsorbed species cannot be directly determined using this technique.

Indeed, since many enzymes possess amphiphilic structures, it might be argued that the decrease in IFT might simply be due to the adsorption of the lipase enzyme, rather than as a consequence of the generation of polar lipids arising from lipolysis. In order to deduce that the reduction in the IFT was due to the production of surface active lipids a similar measurement was carried out but using thermally inactivated lipase. Data from Figure 4.1 show the mean IFT obtained for canola, olive and soybean oil with the inactivated *Rhizomucor miehei* lipase to be $13.87 \pm 0.46$, $15.28 \pm 0.59$ and $15.69 \pm 0.33$ mN m$^{-1}$ respectively. These findings show that whilst the presence of the enzyme protein does results in reduction of the IFT when compare to the pure system, it remained in equilibrium during the 1 h period and did not decrease to the values observed when the active enzyme was used. The decrease in the IFT in the system with active *Rhizomucor miehei* lipase compared to the pure water/oil system was due to combination of initial enzymatic adsorption (which at the start of the experiment showed a similar IFT to that of the inactivated enzyme) followed by the dynamic formation and adsorption of polar lipids at the interface arising from lipolysis (Brockman, 2002; Chu et al., 2009). It was also interesting to note that the dynamics of lipolysis appeared similar across all three samples, although the final IFT of the olive oil sample after 1 h reaction time was observed to be $\sim 1.25$ mN m$^{-1}$ lower than that observed for canola or soybean oil. It is difficult to say with certainty whether this is statistically significant, but may reflect differences in the population of fatty acids and monoglycerides (i.e. in terms of chain length and degree of unsaturation) being produced as a consequence of lipolysis.
4.4.2 Effect of different types of emulsifier on the adsorption of *R. miehei* lipase at the interface

4.4.2.1 Protein emulsifiers -sodium caseinate and whey protein isolate

Food emulsions cannot be prepared in the absence of emulsifiers. Sodium caseinate and whey protein isolate are both milk proteins that are commonly used to stabilised food emulsions (Schokker & Dalgleish, 1998; Ye, 2011). The inclusion of these protein emulsifiers into the pendant drop system was intended to create interfacial layers more typical of those encountered in emulsion systems. Caseins possess good emulsifying and foaming properties and are able to adsorb on the interfaces of either air-water or oil-water. Thus, they are widely used in food formulations for the manufacturing of long-term stable emulsions such as for ice cream, cream liqueurs, whipped toppings, coffee whiteners. Their properties are derived from the structures of four casein proteins from bovine milk which are β-casein, α_{s1}-casein, α_{s2}-casein, and κ-casein (Rodríguez Patino et al., 2001a).

From Figure 4.2, it was observed that the presence of sodium caseinate immediately lowered the IFT in comparison with oil-water, suggesting rapid protein adsorption at the interface. Progressive decreasing IFT over the course of the experiment indicated ongoing adsorption of protein. Final IFT values with drops containing sodium caseinate were 11.57, 7.24 and 8.91 mN m$^{-1}$ for canola, olive and soybean oil respectively. However, these values do not necessarily represent equilibrium, and it was particularly noted that wide variations in final IFT according to oil type were observed. The variations in IFT might have arisen due to variances in the triglyceride content of the vegetable oils. The major different was both olive oil and soybean oil contain about 14 – 15 % of saturated palmitic and stearic fatty acids, compared to ~6 % of those in canola oil (Boskou, 2011; Przybylski, 2011; Przybylski et al., 2005; Wang, 2011). Effective triglyceride contact with caseins during the arrangement at
the interface can be compromised by additional spatial constraints by the double bonds of the fatty acids (Figueiredo et al., 2008).

![Graph showing interfacial tension over time for different oil-water systems](image)

**Figure 4.2: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing sodium caseinate, with or without the addition of *R. miehei* lipase into the system**

Final IFT values with drops stabilised with whey protein isolate were 9.71, 9.45 and 11.17 mN m⁻¹ for canola, olive and soybean oil respectively as shown in Figure 4.3. The starting IFT was again lower than that observed for the pure oil-water interface, but did not get reduced as much as was seen for the caseinate oil-water system.

Whey proteins are a second example of a protein emulsifier commonly used in the preparation of food emulsions. According to Drusch et al. (2012), the surface activity of whey protein isolate is lower than that of sodium caseinate, due to the fact that the major protein
in whey protein is β-lactoglobulin, which has a slower relative surface adsorption. The β-lactoglobulin structure is representative of a globular protein, which being compact and highly internally structured, results in a much slower unfolding and adsorption at the interface compared to the more disordered and open caseinate (Reis et al., 2008b). This can be seen by the starting IFT for systems with the more flexible sodium caseinate which are much lower between 12.02 – 15.53 mN m⁻¹, compared to a slightly higher initial reading of 20.15 - 21.22 mN m⁻¹ for the more rigid whey protein isolate stabilised systems.

![Figure 4.3: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing whey protein isolate, with or without the addition of *R. miehei* lipase into the system](image)

Interestingly, when *R. miehei* lipase was added into the system, a greater reduction for the starting IFT with both protein emulsifiers can be observed from both figures (Figure 4.2 and 4.3), and also when compared to enzyme adsorption in the absence of protein (Figure 4.1). The IFTs were found to decrease (3.14, 4.24 and 3.74 mN m⁻¹) for the system containing...
sodium caseinate and *R. miehei* lipase. Whilst, for whey protein isolate plus *R. miehei* lipase were 6.06, 5.18 and 5.58 mN m\(^{-1}\) for canola, olive and soybean oil respectively. As it has been described in the previous section, the sn-1,3-specific *R. miehei* lipase was able to adsorb at the interface, thus enabling the catalytic reaction of the lipase enzyme of the triglyceride substrate subsequently producing sn-2 monoglycerides. Even with the presence of protein emulsifiers such as sodium caseinate and whey protein isolate, lipase was still able to competitively adsorb at the interface (Reis et al., 2008b).

It has been postulated that the production of sn-2 monoglycerides by lipase can be a self-limiting process. Orogenic displacement of the lipase enzymes from the interface through as a consequence of the synthesis and adsorption of highly surface active polar lipids arising from lipid hydrolysis may render the interface impervious to subsequent lipase adsorption (Reis et al., 2009a; 2009b). This is in agreement with Reis et al. (2008a) who demonstrated using a gastro-intestinal model that the presence of sn-2 monoglycerides hindered the lipolysis process at the oil-water interface with the production of sn-2 monopalmitin found to ultimately inhibit lipolysis. The authors attributed this to a combination of exclusion of the substrate and the enzyme from the interface.

**4.4.2.2 Low molecular weight surfactants – lecithin and Tween 80**

In addition to protein emulsifiers, the impact of low molecular weight surfactant lecithin and Tween 80 adsorption on lipolysis was also explored in this study. Lecithins are natural small molecules comprising a number of phospholipid fractions (depending on source material) such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol (Ghosh & Bhattacharyya, 1997), whilst the Tweens are a class of high HLB synthetic emulsifiers for
which the polysorbate headgroup provides greater hydrophilicity, and are accordingly soluble in water.

Figure 4.4 showed the IFT of canola, olive and soybean oil with lecithin containing aqueous phase, with and without the presence of \textit{R. miehei} lipase. The endpoint IFT for drops stabilised with lecithin were 12.26, 10.91 and 10.75 mN m\(^{-1}\) for canola, olive and soybean oil respectively, whilst with the addition of \textit{R. miehei} lipase were 4.61, 5.85 and 4.69 mN m\(^{-1}\) respectively. The IFT values were clearly lower with the presence of \textit{R. miehei} lipase and the curves were significantly different from those without the enzyme for all the three types of oil used. However, it was interesting to note that the drops containing both lecithin and \textit{R. miehei} lipase appeared to be rather unstable and often detached early in the measurement, obtaining readings only during 1000 s as observed in the graph. This might suggest some form of interaction between the lecithin and \textit{R. miehei} lipase. Chu et al. (2009) reported that the presence of lecithin will enhance the rate of lipolysis. It was found that the higher the molar ratio of lecithin in the systems, the faster the lipolysis rate is. This is because lecithin does not possess a relatively large headgroup that can sterically hamper the adsorption of lipase on the interface. However, prior work conversely showed that hydrolysis of triglyceride in emulsion by pancreatic lipase was decreased with the increase of lecithin concentration (Klein et al., 1967). This is in agreement with Pieroni et al. (1979) who described that the addition of lecithin resulted in lipid-lipid interaction by forming an external phase on the emulsified triglyceride droplets and inhibit the lipase-substrate attachment causing decrease in lipolysis.

Brockman (2002) additionally reported that the decrease of lipolysis is due to the inhibition of lipase adsorption in phosphatidylcholine-rich surfaces. Nevertheless, these previous
researches focused more on the lipolysis of lipid-water interface by pancreatic lipase, thus the experiment conditions are representative of the stomach and duodenal environments (Chu et al., 2009). Another factor is, that lipid based emulsifiers like lecithin and Tween 80 have the possibility of reacting with lipase just as well as any other oil or fat substances. In fact, previous work has showed the possibility of using lipase in soy lecithin modification by transesterification of fatty acids in phosphatidylcholine and phosphatidylethanolamine (Aura et al., 1995; Ghosh & Bhattacharyya, 1997).

Figure 4.4: Interfacial tension of canola oil, soybean oil and olive oil with aqueous phase containing lecithin, with or without the addition of *R. miehei* lipase into the system

Figure 4.5 showed the IFT of canola, soybean and olive oil with Tween 80 containing aqueous phase, with and without the presence of *R. miehei* lipase. The IFT values obtained showed that the Tween 80 rapidly and substantially lowered IFT. The end IFT obtained with Tween 80 were 7.13, 6.47 and 9.13 mN m\(^{-1}\) for canola, olive and soybean oil respectively.
Interestingly, the curves obtained with the addition of \textit{R. miehei} lipase were not clearly distinguishable and the end IFT for canola and soybean oil was even higher than without \textit{R. miehei} lipase at 9.12 and 8.16 mN m$^{-1}$. Mun et al. (2007) reported that when pancreatic lipase was added to an oil-water interface covered with Tween 20, less lipid hydrolysis and droplet coalescence was observed compared to the interface covered with lecithin. Although they used a different Tween species, it can be postulated that the physicochemical mechanism of the process is that the Tween 20 or 80 in this case, is more surface active than the lipase molecule itself thus it becomes a barrier for the lipase to bind to the interface (Gargouri et al., 1983). This is true because low molecular weight surfactants have been reported as being more surface active than proteins (Mackie, 2004).

Inhibition of pancreatic lipase in a corn oil emulsion by Tweens was also reported by Minard et al. (1953). Gargouri and co-workers investigation on a larger range of various ionic and non-ionic surfactant including Tween 80, also showed the inhibition on lipase activity (Gargouri et al., 1983). This is similar to Christiansen et al. (2010) who showed that Tween 80 was able to inhibit the lipolysis of olive oil (dependant on concentration). Apart from being more surface active than protein molecules, the structure of Tween 80 itself could contribute to inhibition of lipolysis. This could be due to the fact that Tween surfactant contains large polyoxyethylene headgroups and these rigid headgroups will sterically act as a wall hindering the adsorption of lipase to the interface (Chu et al., 2009). Furthermore, another possible factor is the susceptibility of Tween 80 as a lipase substrate. This is because Tween 80 structure consists of polyoxyethylene sorbitan attached to fatty acids and previous work by Plou et al. (1998) has investigated the feasibility of using Tween 80 as lipase substrate for lipolytic activity assay.
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

Based on the IFT experiments carried out, it can be inferred that there is no significance in the type of vegetable oil used, but the effects mainly lie with the presence of *R. miehei* lipase and type of emulsifier present. This is based on the main effects plot (Figure 4.6) and interaction plot (Figure 4.7) derived from the data. The presence of emulsifiers namely sodium caseinate, whey protein isolate, lecithin and Tween 80 significantly decreased the IFT. The addition of *R. miehei* lipase provides a significantly lower reading of IFT regardless of the presence of other emulsifiers in the systems; except for Tween 80. The mean IFT for interaction between Tween 80 and *R. miehei* lipase suggests that the Tween 80 alone is better in lowering the IFT than with the presence of *R. miehei* lipase.
Figure 4.6: Main effects plot for interfacial tension (IFT) for oil type, presence of lipase and emulsifier type

Figure 4.7: Interaction plot of all tested variables (oil type, presence of lipase and emulsifier type) for interfacial tension (IFT)
4.4.3 Effect of monopalmitin, monostearin and monoolein on the interfacial tension of vegetable oil and aqueous phase containing protein emulsifiers and surfactants

Among the lipolysis products, monoglycerides are the most surface active molecules being soluble in the oil phase, and with the ability to form assemblies in water phase, compared to diglycerides and fatty acids (Fainerman et al., 2001). Although the later might display a certain degree of polarity and preference to the interface, the portioning is often weighted more to either the bulk oil phase or water phase depending on fatty acid chain length. It has been demonstrated that among the lipid species produced during fat digestion, the monoglycerides are the most surface active at expelling lipase or proteins from the interface (Xu et al., 1998). Three types of monoglycerides namely monopalmitin, monostearin and monoolein were used in this experiment to observe the different carbon chain length and saturation of the monoglycerides on IFT. This experiment was carried out to investigate the behaviour of different monoglycerides (saturated, unsaturated and polyunsaturated) on the interface, especially with the presence of other emulsifiers, to represent the different monoglycerides produced from the lipolysis of oil’s triglycerides.

Figure 4.8 shows the IFT obtained with the three monoglycerides. The end IFT obtained were 3.93, 4.319 and 6.092 mN m$^{-1}$ for monopalmitin, monostearin and monoolein respectively. It can be observed that the shorter carbon chain monopalmitin (C16) has greater affinity towards reducing the IFT compared to monostearin (C18). Unsaturated monoolein (C18:1) was less effective compared to the same carbon chain length monostearin (C18). The effect of fatty acyl chain length and degree of saturation play a crucial role in deeming the behaviour of certain monoglycerides as it will affect the molecular packing of the lipids at the interface (Chu et al., 2009). Reis et al. (2008a) found that shorter acyl chain monoglyceride like monocaprylin (C8) has stronger affinity for the interface compared to
monopalmitin (C16). However, when comparing to a longer acyl chain monoglyceride like monoarachidin (C20), the medium chain monopalmitin was more surface active as the latter is a more balanced amphiphile between the two, and the long hydrocarbon chain fatty acids of monoarachidin will tend to display strong partition in the bulk oil phase (Reis et al., 2008c; Zwierzykowski et al., 1976). The unsaturated monoglyceride like monoolein is more rigid and has been found to be less effective in displacing protein compared to monopalmitin that can display a tighter packing at the interface (Golding & Sein, 2004; Rodríguez Patino et al., 2001a).

Figure 4.8: Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride

The IFT obtained with these monoglycerides in a system containing protein emulsifiers namely sodium caseinate and whey protein isolate is shown in Figure 4.9. For aqueous phase containing sodium caseinate, the end IFT was found to be 4.031, 4.377 and 5.013 mN m$^{-1}$,
whilst with whey protein isolate it was 4.355, 5.665 and 5.535 mN m\(^{-1}\) for monopalmitin, monostearin and monoolein respectively. Curves obtained with sodium caseinate and monoglycerides showed a lower initial IFT than those obtained with whey protein isolate and monoglycerides. The nature of the sodium caseinate and whey protein isolate structures determines the rate in which the adsorption of the protein molecules take place at the interface. As discussed earlier, the disordered nature of sodium caseinate is considered to be more surface active than the rigid whey protein isolate. This is due to the ease of the amphiphilic caseins to adsorb as a monolayer while globular protein like whey protein isolate produce a highly viscoelastic interface via non covalent intermolecular interactions and covalent disulfide cross-linking (Dickinson, 1999; Golding & Sein, 2004; Rahman & Sherman, 1982; Rodríguez Patino et al., 1999). The presence of monoglycerides produce a slightly lower IFT when compared to the system containing \(R.\) miehei lipase as described in the previous section. This is because with \(R.\) miehei containing systems, the initial stability of the interface was majority provided by the protein emulsifiers followed by the polar lipolysis products which subsequently decrease the IFT. In Figure 4.9, the monoglycerides and proteins emulsifiers adsorb at the interface at the start of the measurement. It is known that low molecular weight emulsifiers like monoglycerides can displace protein molecules from the interface. This was found to be similar for both sn-1,3 and sn-2 monoglycerides (Britten & Giroux, 1991; Reis et al., 2008a; 2008c). The end IFT obtain was in the range with the end IFT value in systems containing \(R.\) miehei lipase, suggesting that the reduction of IFT was due to lipolysis products such as monoglycerides.
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

Figure 4.9: Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride/sodium caseinate or whey protein isolate

The dispersion of monoolein and caseinate does not provide good stabilisation and resistance to coalescence as compared to monopalmitin and is more successful at displacing protein (Golding & Sein, 2004). Rodríguez Patino et al. (2001a) research on air-water interface has found that caseinate can be displaced from the interface either by monopalmitin or monoolein and the monoglyceride-caseinate system can form a heterogeneous mixed film at the interface. On another similar study, they also found the same behaviour of whey protein isolate displacement by monoglycerides (Rodríguez Patino et al., 2001b). However, the type of protein play an important role in determining the type of interaction between the protein-monoglyceride. It was suggested that globular protein like whey protein isolate possess stronger protein-monoglyceride interactions compared to disordered protein like caseinate (Reis et al., 2008a; Rodríguez Patino et al., 2001b).
cross-linking of globular protein in particular β-lactoglobulin was more difficult to displace from the interface which will then impact on the rate of lipid digestion (Sandra et al., 2008).

Figure 4.10 shows the IFT obtained in systems containing lecithin or Tween 80. For aqueous phase containing Tween 80, the end IFT was found to be 5.071, 5.324 and 6.957 mN m⁻¹, whilst with lecithin it was 6.051, 7.341 and 7.804 mN m⁻¹ for monopalmitin, monostearin and monoolein respectively. The IFT curves obtained from Figure 4.10 were different than those obtained in the previous section with protein emulsifiers. Lecithin, Tween 80 and monoglycerides are considered as low molecular weight surfactants, thus their stabilising mechanisms are completely different and incompatible than those of proteins. Often, these low molecular weight emulsifiers have been paired and compared with protein; lecithin and proteins (Courthaudon et al., 1991; Fang & Dalgleish, 1995; McSweeney et al., 2008), Tween emulsifiers and protein (Dickinson et al., 1999; Rodríguez Patino et al., 2003; Wang et al., 2008), monoglycerides and protein (Boots et al., 2002; Carrera Sánchez & Rodríguez Patino, 2004; Doxastakis & Sherman, 1984; McSweeney et al., 2008; Rahman & Sherman, 1982; Rodríguez Patino et al., 2001b), but not with other low molecular weight emulsifiers. When any of the low molecular weight emulsifiers and proteins are present in the same system, the displacement of the protein often takes place. Protein emulsifiers stabilise the emulsion by forming an immobile but strong viscoelastic layer on the interface. On the other hand, the low molecular weight surfactants employ the Gibbs–Marangoni mechanism during which the surfactants have high level of mobility. Due to the low molecular weight, these surfactants also tend to be more surface active (Golding & Sein, 2004; Walstra, 2003).
Chapter 4: Effect of *Rhizomucor miehei* lipase-catalysed reactions on the interfacial tension (IFT) at the oil-water interface

Individually, proteins emulsifiers and surfactants work well in stabilising an interface, however adding a surfactant to a protein-stabilised interface results in reducing rather than enhancing stability due to the displacement of proteins from the interface which will weakens the protein film network (Golding & Sein, 2004; Mackie et al., 1999). This is why the production of monoglycerides, which are low molecular weight surfactant tend to displace the protein emulsifier from the surface. In this case it is difficult to pinpoint the decrease of IFT was due to which emulsifiers in the case of lecithin, Tween 80 and monoglycerides and which emulsifiers dominantly resides on the interface. It may be which emulsifiers occupy the interface first and the stability was as a result of synergistic Gibbs–Marangoni stabilisation of both emulsifiers. The shorter chain monoglyceride, in this study, monopalmitin was shown to be more surface active and is capable to decrease the IFTs even with the presence of other emulsifiers.

**Figure 4.10:** Interfacial tension of canola oil with aqueous phase stabilised by three different types of monoglyceride/lecithin or Tween 80

![Graph showing interfacial tension over time for different emulsifiers](image-url)
4.5 Conclusion

Results showed that *R. miehei* lipase adsorption and subsequent hydrolysis of the lipid had varying effects on the IFT depending on the initial composition of the interfacial layer. The presence of monoglycerides produced via hydrolysis were attributed as reducing to minimum values the IFT. *R. miehei* lipase was found to competitively bind at the interface of vegetable oils namely canola, soybean and olive oil drops in an aqueous phase containing protein emulsifiers i.e. sodium caseinate and whey protein isolate. Lipases have the ability to displace the protein emulsifiers allowing the lipolysis to take place thus producing monoglycerides. However, in systems containing low molecular weight surfactants namely lecithin and Tween 80, *R. miehei* lipase showed a slower competitive adsorption towards the surface. It was also postulated that in systems containing lipid based surfactant such as lecithin and Tween 80, a certain degree of lipolysis upon the surfactant itself might be possible.

In conclusion, *R. miehei* lipase adsorption is dependent on the interfacial composition. Although the interfacial composition of food emulsions is far more complex in nature, the findings have provided some interesting insights on the feasibility and potential of naturally derived monoglycerides by *R. miehei* lipase and the ability of the lipase to co-adsorb on an oil-water interface.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

5.1 Abstract

This chapter aims to investigate the factors affecting the lipolysis of oil-in-water (O/W) emulsions. These include droplet specific surface area, oil mass, emulsifier type and lipase loading. The first factor investigated was lipid droplets’ specific surface area which was determined by the efficiency of the processing conditions i.e. microfluidization pressure and number of passes, the type of emulsifier used (sodium caseinate, whey protein isolate, lecithin and Tween 80) and oil mass. Furthermore, specific surface areas were found to decrease with increasing oil phase volumes. Droplet size data enabled the classification of emulsions as coarse, intermediate or fine, dependent on relative surface area. It was observed that except for emulsion stabilised with lecithin, all other emulsions’ initial rate of lipolysis \( (v_0) \) increased with higher specific surface area. Although the initial rate of lipolysis did not change for the emulsions stabilised with lecithin, the extent of lipolysis \( (\text{FFA}_{\text{max}}) \) was influenced by the specific surface area. The differences displayed showed that emulsions with the same range of specific surface area do not behave similarly when subjected to lipolysis. Hence, the interfacial composition appears to play a crucial role as well. The nature and characteristic of the residing emulsifiers influenced the ability of the lipase to adsorb on the interface (although no interfacial composition was seen to be entirely inhibitory of enzyme adsorption). It was also found that the initial velocity was not influenced by the oil mass. However, palm oil emulsion showed a faster initial rate of lipolysis compared to the canola emulsion counterpart; but this trend was the opposite with the \( \text{FFA}_{\text{max}} \). The effect of enzyme loading clearly demonstrates the increase of lipolysis rate with higher lipase concentration. Thus, it can be concluded that higher specific surface area will increase
lipolysis rate, however, the most predominantly important factor is the type of emulsifier used to stabilise the interface before lipolysis.

5.2 Introduction

O/W emulsions can exhibit a variety of attributes and physicochemical characteristics based on their microstructure, composition of surfactants used and the components present in the aqueous phase (Dalgleish, 2004; Krog, 2002; Maindarkar et al., 2012; McClements, 2004). Generally, emulsions may have droplet diameters ranging between 0.1 and 100 μm. In a food emulsion, the choice of emulsifier often determines the stability and characteristics of the emulsion. Various types of emulsifier are used in the food industrial settings, which include amphiphilic proteins, low molecular weight surfactants, polymers, particles or combinations thereof (Garg et al., 2010; Kuhn & Cunha, 2012; McClements, 2004) (reviewed in details in Chapter 2 – subsection 2.5). The emulsification process (i.e. the formation of small droplets and their stabilisation by an adsorbing layer) is critical in defining emulsified product properties, such as stability, appearance, taste, mouthfeel, odour and shelf life (Gabriele et al., 2009; Maindarkar et al., 2012). The advancement of homogenisers in recent times allows for higher homogenisation pressures. Increased high-pressure homogenisation disrupts the oil into much smaller droplets (being also dependent on the material properties of the dispersed and continuous phases, and the nature of the surfactant used). It is known that droplet size distribution influences emulsion properties such as rheology, stability, texture and appearance. In this chapter, the effect of high-pressure homogenisation on droplet size distribution was investigated. The mechanism of emulsification has been described in Chapter 2 – subsection 2.1.1, the droplet size distribution obtained after emulsification is as a result of drop deformation and breakage, and also droplet re-coalescence (Maindarkar et al., 2012; Tcholakova et al., 2003). For many products the ability
to produce finer emulsions is seen as advantageous, on the basis of imparting greatly prolonged stability against creaming (and thus shelf life), improved appearance, faster delivery system and flavour release, and enhanced mouthfeel (Gabriele et al., 2009; Lee et al., 2013; Maindarkar et al., 2012; McClements, 2011). Thus, the first part of this chapter will investigate the effect of processing (Microfluidization condition) as well as formulation (oil mass and type of emulsifier) on the specific surface area which is directly related to the droplet size of O/W emulsion produced.

However, as much as the droplet size of emulsions is crucial on the quality and characteristic of the emulsions, it is also a potentially important mechanism by which the lipolysis rate of an emulsion can be controlled. The decrease of the droplet size will increase the surface area of the oil droplets, thus increasing the adsorption site available for the lipase on the interface. The study on the effect of droplet size and surface area on the degree of lipolysis has been the research topic of numerous authors (Armand et al., 1992; Christiansen et al., 2010; Helbig et al., 2012; Li et al., 2011). However, almost all of these studies focused on the digestibility of emulsions, whereby the emulsions had undergone tremendous changes from their original form consumed as they travel from the mouth, stomach to intestine and were influenced by the varying conditions present within the GI-tract.

In this regard, lipolysis of emulsions in relation to the digestibility of emulsified lipids is recognized as an area of increasing research interest (Armand et al., 1992; Christiansen et al., 2010; Helbig et al., 2012; Li et al., 2011). Digestive lipases are water-soluble enzymes that acts on water-insoluble substrates. The active trypsin-like catalytic site of the lipase is protected from the aqueous phase by a ‘lid’ domain. Upon adsorption on the interface, the lid is displaced to enable the hydrophobic active site to reach the lipid substrate (Fainerman
et al., 2006; Peters & Bywater, 2001). Therefore, the characteristics and composition of the emulsions may affect the binding of lipase thus influencing the lipolysis rate of the emulsion. These include factors such as lipid droplet size, emulsifier type, concentration and nature of oil phase and enzyme loading.

Notably, the composition of the interfacial layer may be expected to play a crucial role in determining the rate and extent of lipolysis, acting as the primary barrier for lipase accessibility to the lipid component. The ability of the lipase to either displace or co-adsorb with pre-existing interfacial layers may therefore influence the rate of the lipolysis process. The ability of lipase to displace these emulsifiers depends on the structure and physicochemical properties of the emulsifiers molecules themselves. This is because different types of emulsifiers, for example proteins and low molecular weight surfactant stabilise the interface by different adsorption kinetics as well as imparting highly variable interfacial assemblies and material properties (Mun et al., 2007; Wooster et al., 2008).

This chapter seeks to study the lipolysis of emulsions as a potential process in food industry applications. Therefore, whilst lipolysis is carried out without mimicking the gastro-intestinal conditions such as low pH, presence of bile and simulated gastric fluid, due consideration needs to be made instead to the manufacturing conditions under which the emulsion lipids are being hydrolysed. In this chapter, emulsions with different droplet size will be subjected to lipolysis by *Rhizomucor miehei* lipase. Other factors including type of emulsifier, oil mass, and lipase loading which was also investigated to obtain insight on the lipolysis capability of *R. miehei* lipase on oil-in-water emulsions.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

5.3 Materials and methodology

5.3.1 Materials

Vegetable oil, emulsifiers and *R. miehei* lipase enzyme used have been described in Chapter 3 (subsection 3.1).

5.3.2 Methodology

5.3.2.1 Emulsification by microfluidization

Emulsification process by Microfluidizer® have been described in Chapter 3 (subsection 3.2.1). Different concentration of canola oil (10, 20 and 30 %) and palm oil (30 %) were used. For the study of effect of Microfluidization processing conditions, the emulsions were produced with 5000, 10000, 15000 and 20000 psi pressure and at different number of recirculation (1-4 times).

5.3.2.2 Lipolysis of emulsion

Lipolysis of emulsion was carried out according to the method described in Chapter 3 (subsection 3.2.5).

5.3.2.3 Determination of emulsion droplet size

The determination of the average droplet diameter was carried out using laser diffraction procedures described in Chapter 3 (subsection 3.2.2).

5.3.2.4 Determination of lipolysis rate

The determination of lipolysis rate was carried out by using the titrimetry procedure described in Chapter 3 (subsection 3.2.3).
5.3.2.5 Microscopic observation with confocal laser scanning microscope

Microscopic observation of emulsion droplets was carried out using confocal laser scanning microscopy as described in Chapter 3 (subsection 3.2.4).

5.3.2.6 Experimental design

For the first part of this chapter, a factorial experimental design was carried out to study the influence of Microfluidizer® pressure, number of passes, type of emulsifier and canola oil content on the emulsion droplet profile. The levels for each factor were as in Table 5.1. For palm oil emulsion, the oil content was fixed at 30% and only protein emulsifiers were used (Table 5.2).

Table 5.1: Factorial experiment factors and levels for investigating the effect of emulsifier type, canola oil content, Microfluidizer® pressure and number of passes on canola oil emulsion droplet size

<table>
<thead>
<tr>
<th>Factors</th>
<th>No of levels</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsifier type (1% w/w)</td>
<td>4</td>
<td>Sodium caseinate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lecithin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween 80</td>
</tr>
<tr>
<td>Canola oil content (%)</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Microfluidizer® pressure (psi)</td>
<td>4</td>
<td>5000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20000</td>
</tr>
<tr>
<td>Number of passes</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Table 5.2: Factorial experiment factors and levels for investigating the effect of emulsifier type, Microfluidizer® pressure and number of passes on 30% palm oil-in-water emulsion droplet size

<table>
<thead>
<tr>
<th>Factors</th>
<th>No of levels</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsifier type (1 % w/w)</td>
<td>2</td>
<td>Sodium caseinate, Whey protein isolate</td>
</tr>
<tr>
<td>Microfluidizer® pressure (psi)</td>
<td>4</td>
<td>5000, 10000, 15000, 20000</td>
</tr>
<tr>
<td>Number of passes</td>
<td>4</td>
<td>1, 2, 3, 4</td>
</tr>
</tbody>
</table>

For the second part of the chapter on lipolysis study, results obtained from the first part were used to produce O/W emulsions with the desired specific surface area and droplet size. The specific surface areas were divided into coarse, intermediate and fine based on the range obtained from the first part. The experimental design is shown in Table 5.3. The experiments were not carried out in a factorial manner, however, selective combination were employed depending on which factor was being investigated.

Table 5.3: Variables used for investigating the effect of specific surface area, emulsifier type, oil content and lipase loading on lipolysis of O/W emulsion

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific surface area</td>
<td>Coarse, intermediate, fine</td>
</tr>
<tr>
<td>Emulsifier type (1 % w/w)</td>
<td>Sodium caseinate, whey protein isolate, lecithin, Tween 80</td>
</tr>
<tr>
<td>Lipase loading</td>
<td>2.5, 5, 12.5, 25, 50, 100, 150 mg per g fat</td>
</tr>
<tr>
<td>Oil type &amp; mass</td>
<td>Canola oil (10, 20 and 30 %)</td>
</tr>
<tr>
<td></td>
<td>Palm oil (30 %)</td>
</tr>
</tbody>
</table>

Note: no pH adjustment, temperature 21°C
5.3.2.6 Statistical analyses

Analysis of variance, main and interaction effect plot was derived using MINITAB 16 (Minitab Inc., State College, PA, USA) to determine the effects of the analysed factors.

5.4 Result and Discussion

5.4.1 Effect of Microfluidization pressure, number of passes, emulsifier type and oil content on emulsion droplet size distribution

5.4.1.1 Canola oil emulsion with protein emulsifiers i.e. sodium caseinate and whey protein isolate

It can be seen that increasing Microfluidizer® pressure caused the specific surface area of O/W emulsion stabilised with 1 % (w/w) sodium caseinate and whey protein isolate to be increased (Figure 5.1). For all measurements obtained with sodium caseinate, it can be observed that the smallest specific surface area was obtained during the first pass regardless of the pressure used. In most cases, the specific surface area of the third and fourth passes was found to be almost identical, suggesting that by the third passes, the surface of the droplets have been completely stabilised by the emulsifier used, in this case sodium caseinate. Increasing the oil content from 10 % to 30 % showed decrease in the specific surface area. The smallest specific surface area was obtained with emulsion made from the lowest pressure of 5000 psi, ranging from 11.45 ± 1.2 to 23.8 ± 0.99 m⁻¹, while the largest was 53.75 ± 5.59 m⁻¹ from 10 % oil at 20000 psi, third passes. The highest pressure at 20000 psi produced a significantly larger specific surface area except for oil content at 30 %. At the highest oil content of 30 %, the specific surface area was found to be smaller than those emulsions with 10 or 20 % oil content.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Figure 5.1: Effect of processing and formulation on specific surface area of canola oil emulsion stabilised with 1% (w/w) sodium caseinate or whey protein isolate

Similar trend was observed for whey protein isolate stabilised emulsion, whereby the first passes resulted in a much lower specific surface area. However, the case with third and fourth passes was slightly different compared with sodium caseinate, in which the specific surface area still increased between the third and fourth passes. The greatest specific surface area obtained was 59.85 ± 3.18 m\(^{-1}\) from 10% oil at 20000 psi, fourth passes. Interestingly, it could be clearly seen how the oil content affected the specific surface area of the emulsion stabilised by whey protein isolate. Here, the specific surface area decreased as the oil content was increased from 10 to 30% for the same pressure and number of passes.
5.4.1.2 Canola oil emulsion with low molecular weight surfactants i.e. lecithin and Tween 80

The effect of Microfluidizer® pressure was also carried out for emulsions stabilised with either 1 % (w/w) lecithin or Tween 80. Figure 5.2 shows that for emulsions stabilised with lecithin, the oil content does not influence the emulsion droplets, but these are majorly influenced by the pressure and number of passes. Contrary from the emulsion made from protein emulsifier discussed previously, increasing the oil content did not appear to be a limiting factor as well as homogenising above the third pass. For each of the oil contents used, the largest specific surface area was obtained by employing the highest pressure of 20000 psi and the highest number of passes of four resulting in 49.7 ± 2.97, 54.1 ± 2.12 and 47 ± 0.28 m⁻¹ for 10 %, 20 % and 30 % oil content respectively. For the Tween 80 stabilised emulsions, increases in Microfluidizer® pressure resulted in a larger specific surface area, however, the effect was not as pronounced as those obtained from sodium caseinate, whey protein isolate or lecithin. Interestingly, the emulsion made with 30 % oil resulted in a smaller specific surface area ranging from 16.2 ± 3.25 to only 20.6 ± 0.28 m⁻¹ for all pressure and number of passes. Whilst at 10 % and 20 % oil contents the specific surface area ranged widely from 22.6 ± 1.27 to 44.95 ± 4.31 and 27.9 ± 1.41 to 47.1 ± 3.81 m⁻¹ respectively.
5.4.1.3 Palm oil emulsion with protein emulsifier i.e. sodium caseinate and whey protein isolate

Experiments were additionally carried out using palm oil as solid fat source to produce the emulsion. The palm oil was used at only 30% oil content and 1% (w/w) protein emulsifiers namely sodium caseinate and whey protein isolate were used. Figure 5.3 shows the specific surface area obtained for palm oil emulsion. It was shown that the largest specific surface area for emulsions with sodium caseinate was at $34.9 \pm 0.7 \text{ m}^{-1}$ obtained with pressure 10000 psi and four times passes, whilst the largest for emulsions with whey protein isolate was at $23.25 \pm 0.69 \text{ m}^{-1}$ obtained with pressure 20000 psi during the fourth pass.
Figure 5.3: Effect of processing and formulation on specific surface area of 30% palm oil emulsion stabilised with sodium caseinate and whey protein isolate (1% w/w)
5.4.1.4 Microscopic observation of emulsion

The microscopic observation on the effect of processing was carried out using 30% canola oil and palm oil emulsion stabilised by sodium caseinate. Figure 5.4 shows the images obtained with canola oil emulsion. It can be observed that the first pass through the Microfluidizer® produced emulsion with irregular mixes of large and small lipid droplets regardless the pressure employed. The lipid droplets eventually became finer and uniform as the number of passes increased.

Microscopic images of emulsion made with 30% palm oil stabilised with sodium caseinate are shown in Figure 5.5. The lipid droplets from the first pass possess a more ‘coarse’ look than the canola oil counterpart. This is apparent by the presence of large lipid droplets within the sample. Increasing the number of passes as well as the pressure resulted in finer lipid droplets profiles. However, an interesting observation is for samples made with four number of passes, some lipid coalescence phenomenon might have occurred resulting in the appearance of some large lipid droplets in the population.
Figure 5.4: Effect of Microfluidizer® processing on the microstructure of 30 % (w/w) canola oil emulsion stabilised with sodium caseinate (1 % w/w) (Scale bar = 5μm)

Note:
5k = 5000 psi, 10k = 1000 psi, 15k = 15000 psi and 20k = 20000 psi of Microfluidizer® pressure.
1x = 1 pass, 2x = 2 passes, 3x = 3 passes and 4x = 4 passes through the Microfluidizer®
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Note:
5k = 5000 psi, 10k = 1000 psi, 15k = 15000 psi and 20k = 20000 psi of Microfluidizer® pressure.
1x = 1 pass, 2x = 2 passes, 3x = 3 passes and 4x = 4 passes through the Microfluidizer®

Figure 5.5: Effect of Microfluidizer® processing on the microstructure of 30 % (w/w) palm oil emulsion stabilised with sodium caseinate (1 % w/w) (Scale bar= 5μm)
5.4.1.5 Main effect and interaction plots

All factors (emulsifier type, oil content, Microfluidizer® pressure and number of passes) investigated in this study were found to be significant (p<0.05) on the specific surface area of canola oil emulsion (Table 5.4 and Figure 5.6). All the quadratic terms were also found to be significant. Based on the main effects figure, it can be clearly observed that the specific surface area increases with the increase of Microfluidizer® pressure and number of passes. However, higher canola oil content generally reduced the specific surface area. Among the emulsifier used, sodium caseinate emulsion had the largest specific surface area followed by Tween 80, whey protein isolate and lecithin.

Table 5.4: Analysis of variance for the effect of emulsifier type, oil content, Microfluidizer® pressure and number of passes on the specific surface area of canola oil emulsion

<table>
<thead>
<tr>
<th>Effect</th>
<th>Df</th>
<th>MS</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emulsifier type (ET)</td>
<td>3</td>
<td>791.44</td>
<td>0.000</td>
</tr>
<tr>
<td>Oil content (OC)</td>
<td>2</td>
<td>9677.38</td>
<td>0.000</td>
</tr>
<tr>
<td>Microfluidizer® pressure (MP)</td>
<td>3</td>
<td>4873.36</td>
<td>0.000</td>
</tr>
<tr>
<td>No of passes (NP)</td>
<td>3</td>
<td>4873.36</td>
<td>0.000</td>
</tr>
<tr>
<td>Two-way interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET x OC</td>
<td>6</td>
<td>987.15</td>
<td>0.000</td>
</tr>
<tr>
<td>ET x MP</td>
<td>9</td>
<td>421.41</td>
<td>0.000</td>
</tr>
<tr>
<td>ET x NP</td>
<td>9</td>
<td>88.09</td>
<td>0.000</td>
</tr>
<tr>
<td>OC x MP</td>
<td>6</td>
<td>375.44</td>
<td>0.000</td>
</tr>
<tr>
<td>OC x NP</td>
<td>6</td>
<td>53.99</td>
<td>0.000</td>
</tr>
<tr>
<td>MP x NP</td>
<td>9</td>
<td>87.83</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Figure 5.6: Main effects plots of emulsifier type, oil content, Microfluidizer® pressure and number of passes on canola oil emulsion specific surface area

For palm oil emulsions, all factors (emulsifier type, Microfluidizer® pressure and number of passes) were also found to be significant (p<0.05) on the specific surface area (Table 5.5 and Figure 5.7). However, for two way interactions for palm oil emulsion, only the model for emulsion type versus Microfluidizer® pressure was significant. Compared to canola oil emulsion, Microfluidizer® pressure only had uptrend effect on specific surface area up to 15000 psi only. Palm oil emulsion stabilised with sodium caseinate had higher specific surface area compared to whey protein isolate.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Table 5.5: Analysis of variance for the effect of emulsifier type, oil content, Microfluidizer® pressure and number of passes on the specific surface area of palm oil emulsion

<table>
<thead>
<tr>
<th>Effect</th>
<th>Df</th>
<th>MS</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emulsifier type (ET)</td>
<td>1</td>
<td>909.023</td>
<td>0.000</td>
</tr>
<tr>
<td>Microfluidizer® pressure (MP)</td>
<td>3</td>
<td>94.002</td>
<td>0.002</td>
</tr>
<tr>
<td>No of passes (NP)</td>
<td>3</td>
<td>306.021</td>
<td>0.000</td>
</tr>
<tr>
<td>Two-way interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET x MP</td>
<td>3</td>
<td>58.638</td>
<td>0.021</td>
</tr>
<tr>
<td>ET x NP</td>
<td>3</td>
<td>10.053</td>
<td>0.598</td>
</tr>
<tr>
<td>MP x NP</td>
<td>9</td>
<td>6.013</td>
<td>0.936</td>
</tr>
</tbody>
</table>

Figure 5.7: Main effects plots of emulsifier type, Microfluidizer® pressure and number of passes on canola oil emulsion specific surface area

Based on the results obtained, it can be seen that the Microfluidizer® is effective in producing a stable emulsion with controllable droplet size. The emulsification process is a complex process affected by the mechanical energy or pressure, the hydrodynamic of the mixing devices that provides the break up and re-coalescence of droplets as well as type and concentration of emulsifier used to coat the newly formed interface (Lee & Norton, 2013; Lee et al., 2013; Niknafs et al., 2011). The high shear energy contributed is significantly higher
to overcome the Laplace pressure gradient thus enabling the droplet to break up and the specific surface area increases. This new interface can then be stabilised by emulsifiers provided that the adsorption of emulsifiers occur before droplet collision (Floury et al., 2003; Santana et al., 2013).

In this chapter, two types of protein emulsifiers were used namely sodium caseinate and whey protein isolate, and two types of low molecular weight emulsifiers namely lecithin and Tween 80. The results also showed that all the emulsifiers (sodium caseinate, whey protein isolate, lecithin and Tween 80) used in this study were stable under the high homogenisation pressure used and were effective in stabilising the emulsion produced. Although some differences can be seen from the droplet size and distribution profiles of each emulsifier, this maybe because different types of emulsifiers have different adsorption kinetics as well as stabilising mechanisms (Wooster et al., 2008). The protein emulsifiers like globular proteins stabilise an interface via unfolding and forming an immobile viscoelastic layer on the interface; whilst low molecular weight emulsifiers employ the Gibbs–Marangoni mechanism during which the surfactants have high level of mobility (Floury et al., 2003; Santana et al., 2013). The other reason of lecithin and Tween 80 droplets profiles having a slightly lower droplet sizes from the protein emulsifiers is maybe because the rate of adsorption of lecithin and Tween 80 is quicker thus it stabilises the emulsion faster during the droplets break up. This in agreement with Lee et al. (2013) description of Tween 20 producing the largest droplet, due to the fact that they are faster to stabilise an interface compare to proteins that require molecular configuration to adsorb themselves on the interface.
The increase in homogenisation pressure has resulted in decrease in the droplet size as well as producing a narrower droplet distribution in all emulsion tested in this study. The reduction in particle size in relation to increasing the pressure is in agreement with several previous studies (Donsi et al., 2011; Kuhn & Cunha, 2012; Lee et al., 2009; Perrechil & Cunha, 2010; Qian & McClements, 2011). Creating small particles are crucial, but forming a uniform and narrow droplet distribution is also important and is among the advantages of high pressure homogenizer such as Microfluidizer®. Jafari et al. (2007) found that Microfluidizer® resulted in a smaller emulsion droplet size and narrower distribution compared to other different emulsifying devices. The increase in number of passes also contributes in decreasing the droplet size similar to several studies (Floury et al., 2000; Kuhn & Cunha, 2012; Lee & Norton, 2013; Perrier-Cornet et al., 2005). As shown in our result, not only increasing the number of passes produced smaller droplets sizes but also decrease the width of the droplet distribution considerably. This is because the increase in number of passes allows more energy input and longer emulsifying time to be exerted on the emulsion, which lead to further breakups of the remaining larger droplets (Jafari et al., 2007).

However, choosing the right pressure and number of passes for homogenisation is a balancing act. This is because utilising the utmost highest pressure and increasing number of passes might not be as effective. Some previous studies have reported that under severe homogenising conditions, emulsion droplet size will start to increase and the widening of droplet distribution will occur. This is because the higher energy input will increase the frequency of droplet collision thus promoting re-coalescences of the droplets (Kuhn & Cunha, 2012). It is therefore important to identify which pressure and how many number of passes have the maximum efficacy to produce stable small droplets emulsion with narrow distribution for that particular emulsion mix (Floury et al., 2004; Santana et al., 2013). In this
study, it was found that the effect of recirculation diminishes after the third passes as there were little to no significant reduction in droplet size for most of the emulsion produced. Several authors have described the condition in which higher pressure and number of passes decreased the emulsion droplet size as ‘over-processing’ (Desrumaux & Marcand, 2002; Jafari et al., 2007; Santana et al., 2013). Desrumaux & Marcand (2002) for example found the phenomenon of ‘over-processing’ is rather complicated as their sunflower oil emulsion stabilised with whey protein start to increase in droplet size above 100 MPa (= 14500 psi), but decrease when the pressure was increased between 200 – 250 MPa. Kuhn & Cunha (2012) found the monomodal distribution of their flaxseed oil and whey protein isolate emulsion became bimodal with increased pressure above 80 MPa (= 11600 psi), indicating droplet re-coalescence had occurred. Another study used pressure much higher than in this paper, above 300 MPa (= 43500 psi) that caused broadening of droplet size distribution (Floury et al., 2000).

Apart from the high pressure itself causing re-coalescence, other internal factors might include the direct effect of pressure on emulsifiers especially with protein emulsifiers as well as increase in the processing temperature. There is a possibility that at very high pressure, the protein structure can undergo denaturation causing a reduction of their emulsifying properties (Bouaouina et al., 2006; Floury et al., 2000; Kuhn & Cunha, 2012; Santana et al., 2013). On the contrary, some authors have reported that high pressure can in fact enhance the interfacial properties of protein. Floury et al. (2002) found that high pressure around 150-200 MPa causes globular protein to unfold and increase the strength of hydrophobic effect and protein-protein interaction that increases the stability of emulsion towards creaming. Hayes & Kelly (2003) also found that high pressure homogenisation up to 200 MPa does not induced whey protein denaturation. Bouaouina et al. (2006) showed that high
pressure treatment did not affect the conformation of the proteins, however it only caused
dissociation of large protein aggregates by uncovering the hydrophobic groups, thus
improving protein solubility and therefore improving the foaming ability and foam stability.
These findings showed that as long as the homogenisation pressure does not exceed a
certain threshold, the high pressure might have no to positive effect on the protein
emulsifier. The pressure used in this study was maximum at 20000 psi equivalent to 137.8
MPa, thus it is safe to conclude that these pressure do not affect the protein emulsifiers
negatively.

Another factor with increasing the pressure is the subsequent temperature increase, as
homogenisation process will incur greater dissipation of mechanical energy in the form of
heat (Desrumaux & Marcand, 2002; Kuhn & Cunha, 2012; Santana et al., 2013). Jafari et al.
(2007) and Desrumaux & Marcand (2002) reported that emulsion temperature exiting
Microfluidizer® increase linearly with the pressure and number of passes. Hayes & Kelly
(2003) described the increase of temperature is partially due to adiabatic heating while
majority is because of the high turbulence, shear and cavitation forces which change into
thermal energy. Perrier-cornet et al. (2005) described temperatures between 40 – 70 °C can
promote the decrease of droplet size. The temperature of the emulsion they produced after
3 and 4 passes can rise higher than 50°C. However, the decreasing effect on droplet size
diminished above 80 °C. The droplet size produced was reported to be dependent on
viscosity ratio (\(\eta_D/\eta_C\)) of the dispersed (\(\eta_D\)) and continuous phase (\(\eta_C\)). Because fluid viscosity
can decrease with increasing temperature, consequently this will also cause reduction in the
interfacial tension and Laplace pressure, thus facilitating the production of smaller droplets
(Floury et al., 2000; McClements, 2004). However, excessive rise in temperature beyond the
denaturation temperature of proteins will result in adverse effects of losing their interfacial stabilising capabilities (Floury et al., 2003).

However, the effectiveness of homogenisation process can be obscured by the emulsion composition such as the oil-emulsifiers ratio. If not enough amount of emulsifiers are available in the systems, the homogenisation pressure does not have effect on the droplet size due to the fact that there is insufficient emulsifier to adsorb on the newly formed interface thus making the homogenisation process futile. In condition where there are sufficient or excess emulsifier, the emulsion droplet size will then be predominantly determined by the homogenising conditions (Floury et al., 2003; Santana et al., 2013; Wooster et al., 2008). In this study with emulsions stabilised by Tween 80, at higher oil mass there is probably insufficient amount of Tween 80 to adequately cover the expanded surface area thus resulting in a significant decrease of the emulsion droplet size. In this case, the impact of homogenisation pressure was of less relevance due to insufficient emulsifier concentration. Higher oil content producing larger droplets had also been reported by several authors; Floury et al. (2000), Mohan & Narsimhan (1997) and Pandolfe (1995).

**Processing condition for the lipolysis study**

Results from the first part of this chapter were used to produce O/W emulsions of variable droplet size in order to determine the effect of relative emulsion surface area on rate and extent of lipolysis. In order to analyse the effects of specific surface area, coarse, intermediate and fine droplet size emulsions were produced. The range used to determine the classifications were obtained by analysing data from work described in previous section. The homogenisation conditions and their respective emulsions’ specific surface area produced to represent the levels are shown in Table 5.6. For the investigation of the effect
of lipase loading, one type of emulsion with similar oil type and mass, emulsifiers and processing condition was used as shown in Table 5.7. Next, the investigation of effect of oil type and mass were carried out by choosing processing conditions that produced similar specific surface area as shown in Table 5.8. These factors are to explore mechanisms by which lipolysis can be manipulated and controlled.

### Table 5.6: Combination of Microfluidizer® pressure and number of passes to obtain coarse, intermediate and fine emulsion used for lipolysis experiments for the analysis of effect of specific surface area and type of emulsifier

<table>
<thead>
<tr>
<th>Oil</th>
<th>Emulsifier 1% (w/w)</th>
<th>Emulsion size</th>
<th>Microfluidizer® pressure</th>
<th>No of passes</th>
<th>Specific surface area (m⁻¹)</th>
<th>D(4,3)</th>
<th>D(3,2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% canola oil</td>
<td>Sodium caseinate</td>
<td>Coarse</td>
<td>5000</td>
<td>1</td>
<td>18.9</td>
<td>0.837</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>15000</td>
<td>1</td>
<td>33.7</td>
<td>0.411</td>
<td>0.194</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>15000</td>
<td>3</td>
<td>48.7</td>
<td>0.209</td>
<td>0.150</td>
</tr>
<tr>
<td></td>
<td>Whey protein isolate</td>
<td>Coarse</td>
<td>5000</td>
<td>2</td>
<td>21.9</td>
<td>0.516</td>
<td>0.296</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>15000</td>
<td>2</td>
<td>33.6</td>
<td>0.388</td>
<td>0.195</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>20000</td>
<td>3</td>
<td>44.7</td>
<td>0.284</td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>Lecithin</td>
<td>Coarse</td>
<td>10000</td>
<td>3</td>
<td>19.4</td>
<td>0.568</td>
<td>0.337</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>15000</td>
<td>3</td>
<td>37.5</td>
<td>0.307</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>15000</td>
<td>4</td>
<td>45.6</td>
<td>0.232</td>
<td>0.143</td>
</tr>
<tr>
<td></td>
<td>Tween 80</td>
<td>Coarse</td>
<td>5000</td>
<td>1</td>
<td>27.9</td>
<td>0.513</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>5000</td>
<td>4</td>
<td>32.8</td>
<td>0.329</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>20000</td>
<td>3</td>
<td>45.7</td>
<td>0.237</td>
<td>0.143</td>
</tr>
<tr>
<td>30% palm oil</td>
<td>Sodium caseinate</td>
<td>Coarse</td>
<td>10000</td>
<td>1</td>
<td>17.2</td>
<td>0.668</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>20000</td>
<td>2</td>
<td>23.2</td>
<td>0.468</td>
<td>0.271</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>15000</td>
<td>3</td>
<td>34.9</td>
<td>0.312</td>
<td>0.183</td>
</tr>
<tr>
<td></td>
<td>Whey protein isolate</td>
<td>Coarse</td>
<td>15000</td>
<td>2</td>
<td>17.8</td>
<td>0.524</td>
<td>0.346</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>20000</td>
<td>4</td>
<td>23.3</td>
<td>0.489</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

Note: Coarse, intermediate and fine emulsions were categorized by comparing emulsions with no statistical significant difference (p<0.05). The highest and lowest range chosen was representative of the specific surface area range obtained within the experimental condition in this study.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Table 5.7: Combination of Microfluidizer® pressure and number of passes to obtain emulsion used for lipolysis experiments for the analysis of the effect of lipase loading

<table>
<thead>
<tr>
<th>Oil</th>
<th>Emulsifier 1 % (w/w)</th>
<th>Specific surface area (m⁻¹)</th>
<th>Microfluidizer® pressure</th>
<th>No of passes</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Palm oil</td>
<td>Sodium caseinate</td>
<td>~22</td>
<td>20000</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5.8: Combination of Microfluidizer® pressure and number of passes to obtain emulsions with similar specific surface area for lipolysis experiments for the analysis of effect of oil mass and type

<table>
<thead>
<tr>
<th>Oil type</th>
<th>Oil mass (%)</th>
<th>Emulsifier 1 % (w/w)</th>
<th>Specific surface area (m⁻¹)</th>
<th>Microfluidizer® pressure</th>
<th>No of passes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil</td>
<td>10</td>
<td>Sodium caseinate</td>
<td>33</td>
<td>20000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey protein isolate</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lecithin</td>
<td>23.9</td>
<td>5000</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween 80</td>
<td>44.05</td>
<td>20000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Sodium caseinate</td>
<td>31.15</td>
<td>10000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey protein isolate</td>
<td>16.4</td>
<td>5000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lecithin</td>
<td>23.2</td>
<td>10000</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween 80</td>
<td>44.7</td>
<td>15000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Sodium caseinate</td>
<td>32.95</td>
<td>15000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey protein isolate</td>
<td>18.25</td>
<td>20000</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lecithin</td>
<td>23.4</td>
<td>10000</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween 80</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Palm oil</td>
<td>30</td>
<td>Sodium caseinate</td>
<td>31.1</td>
<td>15000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey protein isolate</td>
<td>17.8</td>
<td>15000</td>
<td>2</td>
</tr>
</tbody>
</table>

Note:
- n.a = not available, due to no similar specific surface area was obtained for across all the oil % used
5.4.2 Effect of specific surface area and emulsifier type on the lipolysis of O/W emulsion

Emulsions with three different levels of specific surface area (coarse, intermediate and fine) were used. The initial reaction velocity ($v_0$) was obtained by plotting free fatty acids liberated against time. The tangent to the initial linear part of the curve was used to calculate initial velocity ($v_0$). The initial velocity described the initial rate of lipolysis as μmol of free fatty acids liberated per ml per min. In this study, we used up until 300 seconds of measurement as the initial linear portion of the reaction. The effect of surface area on the initial velocity of the lipolysis of canola oil-water emulsions stabilised by different emulsifiers is shown in Figure 5.8. The results obtained showed that the largest specific surface area displayed higher initial velocity except for the emulsion stabilised with lecithin. Sodium caseinate stabilised emulsions had the highest initial velocity for all the emulsion size (coarse, intermediate, fine) at $0.244 \pm 0.015$, $0.463 \pm 0.029$, $0.730 \pm 0.064$ μmol ml$^{-1}$ min$^{-1}$ respectively.

Due to the fact that lipase is an interfacial enzyme, the start of the catalytic reaction requires the adsorption of lipase at the lipid droplet interface, thus the importance of specific surface area. The increased in specific surface area resulted in an increase to the available binding sites for lipase adsorption. Accordingly this resulted in a higher lipolysis rate. The results found in this chapter demonstrate the said principle, and is in agreement with works done by other researchers (Armand et al., 1992; 1999; Borel et al., 1994; Golding & Wooster, 2010; Helbig et al., 2012; Li et al., 2011; McClements & Li, 2010; Pafumi et al., 2002; Reis et al., 2008b).

In addition to that, the extent of lipolysis ($FFA_{max}$) as the maximum amount of free fatty acids generated after the measurement duration of 60 min was measured. The effect of specific
surface area on $\text{FFA}_{\max}$ of canola oil emulsion is shown in Figure 5.9. It can be observed that the increased in specific surface area resulted in the increased in the $\text{FFA}_{\max}$. This is because the larger binding sites also means more lipolytic products can be produced and accumulated on the interface before the interface become saturated and halt the lipolysis reaction by the surface active lipid fractions produced (Li et al., 2011; Reis et al., 2008b).

Figure 5.8: Effect of specific surface area on initial velocity of lipolysis of canola oil (20 % w/w)-in-water emulsion
Figure 5.9: Effect of specific surface area on the extent of lipolysis (FFA\text{max}) of canola oil (20% w/w)-in-water emulsion

Based on Figure 5.8 and 5.9, it can be observed that emulsions stabilised by different emulsifiers had different initial velocity and FFA\text{max}. The differences were detected even if the emulsion had specific surface area within the same range. For example, emulsions produced with intermediate specific surface area of between 32 - 34 m^{-1} had initial velocity of 0.463 ± 0.029, 0.355 ± 0.007, 0.334 ± 0.0207 and 0.455 ± 0.0814 μmol ml^{-1} min^{-1} for sodium caseinate, whey protein isolate, lecithin and Tween 80 respectively. While the FFA\text{max} were 4.502 ± 0.278, 4.817 ± 0.298, 3.879 ± 0.240 and 3.550 ± 0.636 μmol ml^{-1} for the same emulsion. Despite the fact that the interfaces were covered by either protein emulsifiers or low molecular weight surfactants, the R. miehei lipase used in this study was able to successfully lipolyse the emulsion. This is in agreement with Sandra et al. (2008) that lipase is able to adsorb on emulsion interface regardless of what type of stabilising emulsifier is on the surface.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

The initial velocity with whey protein isolate covered interface was found to be lower than sodium caseinate. This may be due to the fact that caseins are easier to displace since they have flexible random coil structure compared to the more rigid globular protein (Singh et al., 2009; Wilde & Chu, 2011). In addition, the whey protein can also form covalent cross link network on the interface making them harder to displace (Hur et al., 2009). The resultant FFA_{max} were also observed to be slightly higher for protein (sodium caseinate and whey protein isolate) stabilised emulsion compared to lecithin and Tween 80. One study by Hur et al. (2009) found that the extent of lipolysis increased in the following order: non-ionic surfactant (Tween 20) < phospholipid (lecithin) < proteins (caseinate and whey protein isolate). The higher lipolysis extent with protein-stabilised emulsion, may also be due to the surface activity of the proteins compared to low molecular weight emulsifiers.

The lipolysis reaction products (fatty acids, monoglycerides and diglycerides) can also compete for the interface. The polar lipid fractions produced by lipolysis especially monoglycerides are more surface active compared to proteins, and hence may inhibit the lipolysis process by inhibiting adsorption of lipase (Mun et al., 2007). Reis et al. (2008d) found that the lipolysis of emulsion droplets coated with monoglycerides were lower than those coated with proteins. In a study of in vitro lipolysis of emulsion by pancreatic lipase (Mun et al., 2007), low molecular weight emulsifier Tween 20 and lecithin inhibit the adsorption of pancreatic lipase more than protein emulsifier casein and whey protein. This is in agreement with Christiansen et al. (2010) who showed that Tween 80 was able to inhibit the lipolysis of olive oil (dependant on concentration). Furthermore, Tween surfactant contains large rigid polyoxyethylene headgroups that will sterically act as a wall hindering the adsorption of lipase to the interface (Chu et al., 2009). Moreover, another possible factor is the susceptibility of Tween 80 as a lipase substrate. This is because Tween 80 structure
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

consists of polyoxyethylene sorbitan attached to fatty acids and previous work by Plou et al. (1998) has investigated the feasibility of using Tween 80 as lipase substrate for lipolytic activity assay. These could explain the high error bar obtained with Tween 80 (Figure 5.8 and Figure 5.9), as similar unstability was also obtained previously from the IFTs data in Chapter 4 (subsection 4.4.2.2).

The effect of surface area on the initial velocity and extent of lipolysis of palm oil-water emulsion stabilised by protein emulsifiers are shown in Figure 5.10 and Figure 5.11. The specific surface area affected the initial velocity and FFA$_{\text{max}}$ of palm oil emulsion similar to what had been observed with canola oil emulsion. However, when comparing palm oil emulsion stabilised by sodium caseinate and whey protein isolate, they display a slightly different trend compared to the canola oil counterpart. Comparing emulsions with the same range of specific surface area, no significant difference in the initial velocity of palm oil emulsion stabilised by the sodium caseinate and whey protein was found. This was unlike the previously observed canola oil emulsion stabilised by the same emulsifier. The FFA$_{\text{max}}$ for palm oil-whey protein isolate emulsion were relatively slightly higher than palm oil-sodium caseinate. This suggests that the type of lipid in the emulsion might influence the initial adsorption of the lipase on the interface.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Figure 5.10: Effect of specific surface area on initial velocity of lipolysis of palm oil (30 % w/w)-in-water emulsion

![Graph showing the effect of specific surface area on initial velocity of lipolysis of palm oil (30 % w/w)-in-water emulsion.

- **Palm oil - sodium caseinate**
- **Palm oil - whey protein isolate**

Figure 5.10: Effect of specific surface area on initial velocity of lipolysis of palm oil (30 % w/w)-in-water emulsion.
5.4.3 Effect of enzyme loading on the lipolysis of O/W emulsion

The effect of enzyme loading was investigated using 30 % palm oil emulsions stabilised by sodium caseinate. Figure 5.12 clearly demonstrated that the increase in *R. miehei* lipase concentration from 2.5 mg to 150 mg per g of fat in the emulsion resulted in increase of the lipolysis rate. This is in agreement with several authors that reported the increase of hydrolysis rate as lipase enzyme increase (Li et al., 2011; Rathod & Pandit, 2010; Rooney & Weatherley, 2001; Serri et al., 2008). This is expected because of the increase in available lipase on the oil-water interface thus increasing the catalytic conversion of the droplets’ triglyceride into fatty acids and monoglycerides (Reis et al., 2009b). Conversely, there will be a point in which increasing the lipase concentration will not cause any more changes in the lipolysis rate as the enzyme had saturated the interface.
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

5.4.4 Effect of oil mass and oil type on lipolysis rate of O/W emulsion

The effect of oil mass and oil type on the initial velocity ($v_0$) and extent of lipolysis (FFA$_{max}$) of palm O/W emulsion are shown in Figure 5.13 and 5.14. Each group of emulsion stabilised by each different emulsifier is produced to the closest specific surface area possible. The average specific surface area is provided in the parentheses in the legend on each figure. This is because the lipolysis of emulsions not only influenced by the droplet size, but lipid composition as well (Helbig et al., 2012). However, when analysing a certain factor on the lipolysis of emulsions, it is crucial that the emulsion have similar droplet size (Li et al., 2011). It was observed that the initial velocity was not influenced by the oil mass. All oil mass tested (10, 20 and 30 %) displayed similar initial velocity. This showed that the availability of lipase binding site is the critical factor for the initial lipolysis. However, differences occur between two different oil types; canola oil and palm oil. Despite possessing similar specific surface
area range, the initial velocity of palm oil emulsion was higher than the canola oil counterpart. Palm oil-sodium caseinate had the initial velocity of $0.623 \pm 0.023 \, \mu\text{mol ml}^{-1}\text{min}^{-1}$, while canola oil-sodium caseinate had $0.485 \pm 0.008 \, \mu\text{mol ml}^{-1}\text{min}^{-1}$. While for palm oil-whey protein isolate was $0.446 \pm 0.010$ and canola oil-whey protein isolate $0.219 \pm 0.033 \, \mu\text{mol ml}^{-1}\text{min}^{-1}$. Interestingly, this trend does not continue for the extent of lipolysis ($\text{FFA}_{\text{max}}$). Palm oil emulsion had lesser $\text{FFA}_{\text{max}}$ than the canola oil emulsion. The $\text{FFA}_{\text{max}}$ for palm oil-sodium caseinate was $5.683 \pm 0.100$ compared to a relatively higher $6.391 \pm 0.101 \, \mu\text{mol ml}^{-1}$ for canola oil-sodium caseinate, while the $\text{FFA}_{\text{max}}$ for palm oil-whey protein isolate was $3.943 \pm 0.147$ and for canola oil-whey protein isolate was $5.329 \pm 0.110 \, \mu\text{mol ml}^{-1}$. The differences in initial velocity and $\text{FFA}_{\text{max}}$ are because of the nature and physical properties of canola oil and palm oil used. This is due to the different types of triglyceride with different chain length, degree of saturation as well as solid fat content and crystallisation. Refined edible oils contains up to 95 % triglycerides, which possess different properties depending on the fatty acids esterified, this includes the chain length, esterified position on the glycerol backbone, and presence of double bond. The triglycerides lipolysed will determine the resulting physico-properties of polar lipids liberated namely fatty acids, di- and mono-glycerides (Singh et al., 2009).
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Note: Average specific surface area (m$^{-1}$) are provided in the parentheses

Figure 5.13: Effect of oil mass and oil type on initial velocity of lipolysis of O/W emulsion

Note: Average specific surface area (m$^{-1}$) are provided in the parentheses

Figure 5.14: Effect of oil mass and oil type on the extent of lipolysis (FFA$_{max}$) of O/W emulsion

Note: Average specific surface area (m$^{-1}$) are provided in the parentheses
Golding & Wooster (2010) raised an important question on whether the behaviour observed can be solely attributed to the degree of saturation regardless whether the fat is solid or liquid. The canola oil used in this study was liquid whilst the palm oil was solid at room temperature, hence the differences in the FFA$_{\text{max}}$ obtained. Findings have found that solid fat can suppress lipid digestion (Seimon et al., 2009). Emulsions containing high degree of unsaturation (C18:1 and C18:2 triglycerides) had been found to be easily lipolysed compared to the saturated triglyceride of the same chain length (C18:0) (Golding & Wooster, 2010; McClements & Li, 2010). Another interesting angle that the oil type can influence the lipolysis rate is by the species of lipolysis product produce. Li et al. (2011) compared the lipolysis extent of β-lactoglobulin stabilised emulsion prepared with either corn oil or medium chain triglycerides (MCT) and found that the rate of lipolysis for MCT emulsion was higher than corn oil emulsion. Rather than because the lipase have more affinity for MCT lipid, they reasoned the higher lipolysis extent to be due to the FFA produced from MCT having higher dispersibility in the aqueous phase. This in turn allows the FFA to migrate rapidly to the continuous phase thus freeing the interface for more lipolytic reaction. The longer chain fatty acids on the other hand tend to accumulate on the interface thus inhibiting further lipolysis (Li et al., 2011). This showed that the proper choice of lipid in an emulsion system is crucial as the rate of lipolysis depends on it too (McClements & Li, 2010).

### 5.4.5 Main effect of specific surface area, emulsifier type, oil mass and type, and lipase loading on Vo and FFA$_{\text{max}}$ during lipolysis of O/W emulsion

The effects of specific surface area, emulsifier type, oil mass, oil type and lipase concentration on Vo and FFA$_{\text{max}}$ for the lipolysis of O/W emulsion have been compiled as in Table 5.9. The main effect plot for each of these factors is shown in Figure 5.15 and 5.16. Both specific surface area and lipase concentration were found to have significant effect.
(p<0.05) on both $V_o$ and $FFA_{\text{max}}$ of the lipolysis reaction. This supports the discussion in the previous subsection on the importance of the lipase binding site made available by the specific surface area. Upon adsorption, the kinetics of lipolysis reaction gain momentum, as the extent of lipolysis, $FFA_{\text{max}}$ was significantly (p<0.05) affected by the specific surface area, emulsifier type, oil mass, and lipase concentration. The factor that was found to be insignificant to both $V_o$ and $FFA_{\text{max}}$ was the oil type. This shows that the rate of lipolysis was not affected whether liquid or solid source of fat were used as long as it is in emulsified state. However, due to the limited number of oil mass for palm oil (only 30% was used), the comparison on the effect of oil type might not have been representative of the tested conditions.

Table 5.9: Effect of specific surface area, emulsifier type, oil mass and type, and lipase concentration on $V_o$ and $FFA_{\text{max}}$ for the lipolysis of O/W emulsion

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>$V_o$</th>
<th>P</th>
<th>$FFA_{\text{max}}$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS</td>
<td>P</td>
<td>MS</td>
<td>P</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>2</td>
<td>0.17518</td>
<td>0.000*</td>
<td>10.441</td>
<td>0.000*</td>
</tr>
<tr>
<td>Emulsifier type</td>
<td>3</td>
<td>0.03111</td>
<td>0.174</td>
<td>2.6841</td>
<td>0.048*</td>
</tr>
<tr>
<td>Oil mass</td>
<td>2</td>
<td>0.00249</td>
<td>0.831</td>
<td>5.7595</td>
<td>0.001*</td>
</tr>
<tr>
<td>Oil type</td>
<td>1</td>
<td>0.03231</td>
<td>0.306</td>
<td>2.1929</td>
<td>0.128</td>
</tr>
<tr>
<td>Lipase concentration</td>
<td>6</td>
<td>0.22670</td>
<td>0.000*</td>
<td>15.1803</td>
<td>0.000*</td>
</tr>
</tbody>
</table>
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Figure 5.15: Main effect plot of specific surface area, emulsifier type, and lipase concentration on $V_o$ and $FFA_{max}$ for the lipolysis of O/W emulsion
Figure 5.16: Main effect plot of oil mass and oil type on $V_o$ and $FFA_{max}$ for the lipolysis of O/W emulsion.
5.4.6 Kinetics of O/W emulsion lipolysis

The Michaelis-Menten model (Equation 5.1) was used to describe the kinetics parameters of the lipolysis reaction:

\[ v_o = \frac{v_{\text{max}}[S]}{(K_m[S])} \]  

Equation 5.1

Where \( v \) is the reaction rate or velocity, \([S]\) is the substrate concentration and \( K_m \) is the Michaelis constant.

The double reciprocal Lineweaver-Burk plot was used to determine the Michaelis-Menten constant, \( K_m \) and maximum velocity, \( V_{\text{max}} \). Figure 5.17 shows the Lineweaver-Burk plot for canola oil-sodium caseinate, with derived \( K_m \) of 0.2 g ml\(^{-1}\) and \( V_{\text{max}} \) 0.463273 μmole min\(^{-1}\). Figure 5.18 shows the Lineweaver-Burk plot for canola oil-lecithin emulsion, with derived \( K_m \) of 0.0220 g ml\(^{-1}\) and \( V_{\text{max}} \) 0.3632 μmole min\(^{-1}\). In this chapter, only canola oil emulsion stabilised with sodium caseinate and lecithin were able to be analysed for their kinetics \( K_m \) and \( V_{\text{max}} \).

Although emulsions made with the other emulsifiers were also run at various substrate concentration (oil mass), however, only emulsions with the same range of specific surface area across all the substrate concentration used was chosen. Numerous works often described the lipolysis reaction by the Michaelis-Menten equation for esterase, however lipase is a bit more complicated since it catalyses an insoluble substrate, and therefore depends on interfacial activation. As results have shown, the processing of emulsions under the same homogenising conditions did not produce the same droplets criteria when the formulation (oil mass and emulsifier type) was varied. The \( K_m \) or \( V_{\text{max}} \) attribution could be meaningless if the droplet size is disregarded (Verger, 1997; Verger & De Haas, 1976).
Chapter 5: Effect of lipid specific surface area, oil mass and type, emulsifier type and lipase loading on the lipolysis of oil-in-water (O/W) emulsions

Figure 5.17: Lineweaver-Burk double reciprocal plot of velocity as function of canola oil concentration (emulsion of canola oil-sodium caseinate)

\[ y = 0.0313x + 1.9383 \]

Figure 5.18: Lineweaver-Burk double reciprocal plot of velocity as function of canola oil concentration (emulsion of canola oil-lecithin)

\[ y = 0.063x + 2.7377 \]
5.5 Conclusions

The results showed that formulation (emulsifier type and oil content) and processing conditions (Microfluidizer® pressure and number of passes) had significant effects on the emulsion droplet size. The emulsifiers chosen would behave differently whether it’s a protein emulsifier or low molecular weight emulsifier. Both exhibit different adsorption mechanism and rely on the homogenisation conditions as well. High pressure might cause configurational changes to the emulsifiers’ molecule resulting in changes in adsorption efficiency. The higher the oil content the less available emulsifiers would be thus causing a limiting interfacial stabilisation by the emulsifiers. This would cause less droplet coverage during the droplets break up and will result in an increase in droplets size. Increasing the Microfluidization pressure and number of passes generally produce smaller droplets; however there is a limit at which going over will result in over-processing. A good combination of high pressure and number of passes is needed for a dynamic balance of droplets break up and coalescence in the interacting chambers.

Emulsion specific surface area (droplet size) determines the availability of the lipase binding site hence influence the rate of lipolysis of emulsion. However, it can also be affected by what type of emulsifier is residing on the interface. The results showed higher fatty acids liberated with protein emulsifiers compared to the low molecular weight surfactants, which is explained by the differences in the structure and surface activity of the two molecules. Utilising either liquid oil or solid oil making an emulsion will have effect on the initial velocity and FFA$_{\text{max}}$ of them. Apart from that, oil phase volume does not seem to affect the initial velocity of the reaction, but does influence the end FFA$_{\text{max}}$. Increasing the lipase concentration also increased the lipolysis rate and extent. Besides the numerous previous researches on digestibility of emulsion by lipase, our results showed R. miehei lipase is viable
in lipolyzing O/W emulsion under ambient conditions. There is no significant inhibition of the lipolysis process, even with different emulsifier utilised. It was either slower or faster depending on the specific surface area, oil mass and enzyme concentration. It can therefore be proposed that the detection of liberated fatty acids also suggests the production of other polar lipids species i.e. monoglycerides. The emulsion droplet size, type of emulsifier used as well as lipase concentration are important factors that provide means by which the extent and rate of lipolysis can be manipulated. It is important to tailor the approach in food emulsions as the ability to control the lipolysis provide appropriate functionality to the properties of the food system. The subsequent chapters investigate how far the reaction needs to proceed in order to achieve functionality, but the parameters studied in this particular chapter provide a way of achieving an appropriate end point.
Chapter 6: Thermal stability of sodium caseinate stabilised O/W emulsion and *Rhizomucor miehei* lipase

6.1 Abstract

The viability of *R. miehei* lipase against thermal treatment was investigated as a measurement of the residual activity of the enzyme as part of controlling extent of lipolysis in emulsion based formulations. The effects of enzyme thermalisation on the thermostability of palm oil emulsions were also observed. The results showed that the *R. miehei* lipase was thermostable up to temperatures of 70 °C. Above this temperature, substantial reduction of the residual activity occurred. However, even elevated temperature being between 90 and 100 °C did not immediately inactivate the lipase, with heating for ~ 2 min required before activity was no longer detected. In terms of emulsion stability, the palm oil emulsion tested in this study was found to be thermostable up to 100 °C. However, when the emulsion was heated at a prolonged time (more than 15 min), significant (p<0.05) changes to the emulsion droplet size was observed. Similar thermostability was also observed for lipolysed emulsions that were able to withstand heating up to 100 °C for 7 min before significant (p<0.05) changes to droplet size were recorded. Findings allowed development of a thermalisation step that was able to inactivate the enzyme without compromising the stability of the emulsion.

6.2 Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are interfacially active enzymes that have widespread industrial applications based on their ability to catalyse organic and synthetic esterification and transesterification reactions of lipids (Harwood, 1989). Unlike many other enzymes, the use of lipases is also defined by their ability to react on a variety of immiscible substrates and react in non-aqueous organic solvents (Schmid and Verger, 1998). The
mechanisms of lipase mediated reactions and various factors affecting their reaction rate, as well as typical use in lipid modification, have been reviewed in detail in Chapter 2 (subsection 2.7). Examples of usage include modification of low cost vegetable oils to cocoa butter equivalents, production of glyceride emulsifiers, the regio-selective acylation of glycols, application in biotechnology such as detergent additives and biodiesel (Dijkstra, 2007; Harwood, 1989; Hasan et al., 2006; Pastor et al., 1995; Schmid and Verger, 1998) and development of fatty acids for flavour applications.

Lipases occur naturally in animals and plants; however, natural and recombinant microorganisms are more commonly used to produce cheaper lipases in bulk quantities (Noel and Combes, 2003a; 2003b; Schmid and Verger, 1998), such as lipase from R. miehei. Lipase from R. miehei is commercially available in both soluble and immobilised form, and has found usage in various applications due to its high activity and good stability (Rodrigues and Fernandez-Lafuente 2010a; 2010b). Various authors have described the use of R. miehei lipase in biodiesel production (Al-Zuhair, 2005; 2007; Demirkol et al., 2006; Ling et al., 2007; Nelson et al., 1996; Selmi and Thomas, 1998), synthesis of esters (Perraud and Laboret, 1995; Shieh and Chang, 2001; Yadav and Trivedi, 2003), synthesis of propylene glycol non-ionic detergent (Basu and Bhattacharyya, 1998; Ghosh and Bhattacharyya, 1998; Liu and Shaw, 1995) and also as biosensors (Persson et al., 2001).

Because lipase is an interfacial enzyme, various interfacial factors are able to influence the measurement of lipase activity, such as stirring of the reaction mixture, charge surface densities, surface viscosity, presence of emulsifiers and the oil-water interfacial structure (Reis et al., 2008a; 2009b; Schmid and Verger, 1998; Verger, 1997). Therefore, according to Schmid and Verger (1998), lipase activity measurements from different commercial
manufacturers are not easily comparable, and thus valid comparisons of enzymatically treated samples can only be achieved under stringently similar parameters, preferably within the same laboratories.

This chapter aims to investigate the thermostability of the *R. miehei* enzyme. Thermostable enzymes can be useful because of higher reactivity and process yield across a broader range of conditions; however, this post-reaction inactivation may require elevated temperatures to be achieved. For food applications this is important because the ability to control the deactivation of the enzyme is crucial in terms of ensuring the requisite functionality can be achieved, and once achieved the reaction can be terminated. Thermal treatment is commonly employed in food manufacturing as part of ensuring product safety and the thermal treatment of emulsion based foods via pasteurisation is seen as providing an adaptable mechanism by which the lipase could be inactivated. A particular consequence of modifying processing conditions is whether emulsion stability might be compromised as a consequence of both lipolysis and subsequent heat treatment. Accordingly, the thermostability of palm oil emulsions stabilised by sodium caseinate was also investigated in this chapter, in regards to changes in the droplet size. Emulsion stability can be determined as having no palpable change in the droplet distribution size (Dickinson, 2001). The emulsion used will correspond as the reaction medium in which *R. miehei* lipase will be used in. It is therefore important that the heat treatment does not produce significant changes to the emulsion but is sufficient to inactivate the lipase enzyme.
6.3 Materials and methodology

6.3.1 Materials

Details of palm oil, sodium caseinate and *R. miehei* lipase used have been described in Chapter 3 (subsection 3.1).

6.3.2 Methodology

6.3.2.1 Emulsification by microfluidization

O/W emulsions with 30 % wt palm oil stabilised by 1 % wt sodium caseinate were prepared by microfluidization according to the procedures described in Chapter 3 (subsection 3.2.1).

6.3.2.2 Lipolysis of emulsion

Lipolysis of emulsion was carried out according to the method described in Chapter 3 (subsection 3.2.5). Lipase concentration used was 50 mg per g fat with 30 min lipolysis time.

6.3.2.3 Heat stability studies on *R. miehei* lipase

*R. miehei* lipase (1 ml) was filled in a glass tube suspended into a beaker filled with water. The beaker was heated in a thermostat controlled water bath. The temperature of the heated lipase was observed with a thermometer inserted into the glass tube. The glass tubes were sealed with aluminium foil to minimise heat escape. The lipase was heated at different temperatures (50, 60, 70, 80, 90 and 100 °C). After heating, samples were rapidly cooled in an ice water bath and residual activities were determined. Heating time were varied from 1 to 30 min.
6.3.2.4 Heat stability studies on O/W emulsion

O/W emulsion (1 ml) was placed in a glass tube suspended into a beaker filled with water. The beaker was heated in a thermostat controlled water bath. The temperature of the heated emulsions was observed with a thermometer inserted into the glass tube. The glass tubes were sealed with aluminium foil to minimise heat escape. The emulsions were heated at different temperatures (60, 70, 80, 90 and 100 °C). After heat treatment, samples were rapidly cooled in an ice water bath and particle size analyses were carried out.

6.3.2.5 Determination of lipase residual activity

The determination of lipase residual activity was carried out using titrimetry method by Titralab pH-stat procedure described in Chapter 3 (subsection 3.2.3). The residual activity was determined by assuming the lipase activity without any heat treatment as 100 % activity.

6.3.2.6 Determination of emulsion droplet size

The determination of the average droplet diameter was carried out using laser diffraction procedures described in Chapter 3 (subsection 3.2.2).

6.3.2.7 Statistical analyses

All analysis were carried out at least in triplicates and results presented as mean and standard deviation of the measurements. Analysis of variance was performed using MINITAB 17 (Minitab Inc., State College, PA, USA) and Tukey post-hoc comparison was employed to determine the significant difference (p<0.05) between the results.
6.4 Results and discussion

6.4.1 Influence of thermal treatment of \textit{R. miehei} lipase activity

\textit{R. miehei} lipase enzyme was incubated at different temperatures ranging from 50 – 100 °C, after which lipase activity was determined. The degree of lipase activity was presented as residual activity compared to non-thermally treated lipase showing 100 % activity. Figure 6.1 represents the residual activities of \textit{R. miehei} lipase at different incubation temperatures and times. \textit{R. miehei} lipase used in this study was shown to be fully stable at temperature 60 °C and below. Crooks et al. (1995) demonstrated \textit{R. miehei} lipase maximum hydrolytic activity with a synthetic lipase substrate, \(\rho\)-nitrophenylbutyrate (pNPC4) at 40 °C. However, substantial reduction of the residual activity could be observed at temperatures of 70 °C and above. Interestingly, at temperature as high as 90 and 100 °C, the \textit{R. miehei} lipase was not inactivated instantly. This can be seen by the enzymes residual activity curve halting around the 3 min mark.

![Figure 6.1: Inactivation kinetics of \textit{R. miehei} lipase at 50, 60, 70, 80, 90 and 100 °C](image_url)
The residual activities plot allowed the determination of half-life values ($t_{1/2}$), which could be defined as the time needed to reduce the enzyme activity to half of the initial value. Furthermore, using the first order kinetic reaction equation (Equation 6.1), the deactivation rate constant ($k_{exp}$) was then calculated as shown in Table 6.1.

\[
\ln [A]_t = -kt + \ln [A]_0 \quad \text{Equation 6.1}
\]

Where $A_0$ is the initial activity, $A_t$ is the activity at time $t$ and $k$ is the rate constant.

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>$k_{exp} \times 10^{-3}$ (min$^{-1}$)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 $^\circ$C</td>
<td>6.76</td>
<td>102.557</td>
</tr>
<tr>
<td>60 $^\circ$C</td>
<td>11.94</td>
<td>58.04</td>
</tr>
<tr>
<td>70 $^\circ$C</td>
<td>117.5</td>
<td>5.899</td>
</tr>
<tr>
<td>80 $^\circ$C</td>
<td>203.25</td>
<td>3.41</td>
</tr>
<tr>
<td>90 $^\circ$C</td>
<td>627.28</td>
<td>1.105</td>
</tr>
<tr>
<td>100 $^\circ$C</td>
<td>864.27</td>
<td>0.802</td>
</tr>
</tbody>
</table>

Using the Arrhenius equation, the logarithm of $k_{exp}$ versus the inverse temperature in Kelvin was plotted (Figure 6.2), this in turn allowed the calculation of the deactivation energy calculated which was found to be 105.6 kJ mol$^{-1}$. This value was found to be within the same range of the findings by Weemaes et al. (1998) with 131 and 117 kJ mol$^{-1}$ for plum and pear polyphenoloxidase deactivation energy respectively, at atmospheric pressure. However, some other enzymes have been reported to possess a higher deactivation energy such as 241 kJ mol$^{-1}$ for amylglucosidase from Aspergillus niger (Zanin and Moraes, 1998). Nevertheless, findings by Noel and Combes (2003a) on powdered R. miehei lipase indicate
the enzyme to have a higher deactivation energy of 304 kJ mol\(^{-1}\) when compared to the \textit{R. miehei} lipase used in this study. Prazeres et al. (1992) reported deactivation energy in the range of 86.2 to 186.3 for \textit{Chromobacterium viscosum} lipase at different pH.

The thermal deactivation of lipase has been attributed due to the unfolding of the protein structure (Klibanov, 1983); however, Noel and Combes (2003a; 2003b) work on \textit{R. miehei} lipase and Fu et al. (2010) studies on \textit{Yarrowia lipolytica} lipase suggested that the deactivation was mainly due to the formation of aggregates. Fu et al. (2010) described the deactivation of lipase in three stages; 1) changes to the protein tertiary structure resulting in minor activity loss, 2) aggregation of the protein tertiary and secondary structure resulting in most of the activity loss and 3) inactivated lipase further unfolded and completely denatured. Thus, at a temperature of 60 \(^{\circ}\)C and below, the tryptophan residue environment of lipase enzymes was not completely altered, hence the stability shown. However, as the temperature increased, conformational changes to the lipase structure, as well as potential intermolecular associations would be expected to result in the observed decrease in residual activity. This consideration would benefit from further investigation in the future on conformational changes by fluorescence spectroscopy, or the use of SDS-PAGE to determine whether polymerisation was taking place, in order to better understand the mechanisms influencing enzyme activity.
Chapter 6: Thermal stability of sodium caseinate stabilised O/W emulsion and *Rhizomucor miehei* lipase

6.4.2 Influence of thermal treatment of O/W emulsion stability

Palm oil-in-water emulsions (30 % w/w) were subjected to different temperatures for varying periods of time in order to observe the influence of thermal treatment on emulsion stability. Results of the mean volume weighted diameter ($D_{4,3}$) of the thermally treated emulsions are shown in Table 6.2. It was observed that the palm oil emulsion used in this study was relatively stable to thermal treatment. For up to 10 min of heating time, there were no significant differences in droplet size ($p<0.05$) for all the emulsions, regardless of the heating temperature. Changes in the mean droplet size were only found when the emulsions were exposed to heat at a prolonged time (30 min) and at elevated temperatures (90 and 100 °C). The increased in the mean droplet size of these emulsions was due to the manifestation of a smaller population of large droplets as it can be seen from the particle distribution curve in Figure 6.3. The emulsion used in this study was stabilised by sodium...
caseinate, and was stable across the temperature range analysed except for prolonged heating at 30 min. Sodium caseinate is known to be quite heat stable without major changes to its physicochemical characteristics (Guo et al., 1989), so these findings are in line with expectations.
Table 6.2: Mean volume weighted diameter (D 4,3) of thermally treated O/W emulsion

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>No treatment</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.412 ± 0.062</td>
<td>0.409 ± 0.004</td>
<td>0.399 ± 0.012</td>
<td>0.413 ± 0.035</td>
<td>0.392 ± 0.009</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.386 ± 0.014</td>
<td>0.395 ± 0.004</td>
<td>0.405 ± 0.010</td>
<td>0.412 ± 0.025</td>
<td>0.399 ± 0.007</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.402 ± 0.010</td>
<td>0.392 ± 0.007</td>
<td>0.411 ± 0.006</td>
<td>0.415 ± 0.005</td>
<td>0.426 ± 0.050</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.41 ± 0.007</td>
<td>0.395 ± 0.024</td>
<td>0.408 ± 0.003</td>
<td>0.419 ± 0.017</td>
<td>0.442 ± 0.055</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.387 ± 0.018</td>
<td>0.406 ± 0.012</td>
<td>0.412 ± 0.005</td>
<td>0.415 ± 0.020</td>
<td>0.448 ± 0.035</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.395 ± 0.025</td>
<td>0.403 ± 0.009</td>
<td>0.412 ± 0.004</td>
<td>0.426 ± 0.032</td>
<td>0.466 ± 0.053</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.398 ± 0.023</td>
<td>0.412 ± 0.006</td>
<td>0.415 ± 0.006</td>
<td>0.433 ± 0.030</td>
<td>0.475 ± 0.047</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.406 ± 0.021</td>
<td>0.417 ± 0.012</td>
<td>0.411 ± 0.010</td>
<td>0.432 ± 0.012</td>
<td>0.477 ± 0.011</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.411 ± 0.012</td>
<td>0.415 ± 0.003</td>
<td>0.413 ± 0.011</td>
<td>0.446 ± 0.012</td>
<td>0.481 ± 0.018</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.403 ± 0.018</td>
<td>0.415 ± 0.004</td>
<td>0.418 ± 0.051</td>
<td>0.462 ± 0.022</td>
<td>0.488 ± 0.013</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.419 ± 0.029</td>
<td>0.446 ± 0.014</td>
<td>0.483 ± 0.016</td>
<td>0.633 ± 0.026</td>
<td>0.845 ± 0.140</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.381 ± 0.026</td>
<td>0.421 ± 0.051</td>
<td>0.448 ± 0.063</td>
<td>0.518 ± 0.024</td>
<td>0.647 ± 0.020</td>
<td>1.263 ± 0.086</td>
</tr>
</tbody>
</table>

Note:
Means within the same column followed by the same superscript lowercase letters are not significantly different at p<0.05
Means within the same row followed by the same superscript uppercase letters are not significantly different at p<0.05
The effect of thermal treatment on emulsion stability was also studied on lipolysed O/W emulsions. These emulsions were subjected to lipolysis with 50 mg of *R. miehei* lipase per g of fat for 30 min at room temperature (21°C). Table 6.3 shows the mean volume weighted diameter (D 4,3) of the thermally treated lipolysed emulsions. It can be observed that although the lipolysed emulsions had a marginally larger initial droplet diameter compared to the non-lipolysed emulsion, the thermal behaviour was comparable to that of the non-lipolysed emulsion, with no significant differences in droplet size (p<0.05) for all the emulsions heated for up to 10 min at 80 and 90 °C. However, when subjected to heating at 100 °C, a slight difference can be observed compared to the non-lipolysed emulsions, whereby the emulsion displayed an increase of droplet size at 8 min of heating time. After the 8 min time, there were significant
(p<0.05) increases to the droplet size up to 30 min of heating. Figure 6.4 depicted a much larger second population produced for lipolysed emulsion heated at 100 °C for 30 min. As described in the previous section, the non-lipolysed O/W emulsions were stable across all temperatures tested up to 15 min of heating, because sodium caseinate used to stabilise the emulsion was heat stable. However, in the lipolysed emulsion, the sodium caseinate interface will have been at least partially displaced by polar lipolysis products. Thus, this may promote coalescence of the oil that is now in molten state.

### Table 6.3: Mean volume weighted diameter (D 4,3) of thermally treated lipolysed (50 mg per g fat, 30 min) O/W emulsion

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
<th>No treatment</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>±0.013</td>
<td>±0.030</td>
<td>±0.019</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.636</td>
<td>0.666</td>
<td>0.642</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.637</td>
<td>0.680</td>
<td>0.650</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.638</td>
<td>0.681</td>
<td>0.653</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.638</td>
<td>0.680</td>
<td>0.665</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.645</td>
<td>0.683</td>
<td>0.675</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.652 ± 0.017</td>
<td>0.649</td>
<td>0.690</td>
<td>0.707</td>
</tr>
<tr>
<td>7</td>
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<td>0.652 ± 0.017</td>
<td>0.652</td>
<td>0.694</td>
<td>0.807</td>
</tr>
<tr>
<td>8</td>
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<td>0.655</td>
<td>0.709</td>
<td>0.925</td>
</tr>
<tr>
<td>9</td>
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<tr>
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<tr>
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<tr>
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<td>0.652 ± 0.017</td>
<td>0.707</td>
<td>1.153</td>
<td>2.829</td>
</tr>
</tbody>
</table>

Note:
Means within the same column followed by the same superscript lowercase letters are not significantly different at p<0.05
Means within the same row followed by the same superscript uppercase letters are not significantly different at p<0.05
6.5 Conclusion

Findings from this chapter demonstrate the possibility to include a thermalisation procedure to halt the lipolysis process in an O/W emulsion without severe disruption to the emulsion’s stability, even with the lipolysed emulsions. Although the \textit{R. miehei} lipase was found to need higher temperature (90 and 100 °C) for fast deactivation, the emulsion showed little changes to its droplet size profile under conditions appropriate for inactivation. Thus, thermal treatment can be used to halt the lipolysis process in an O/W emulsion.
Chapter 7: Effect of lipolysis by *Rhizomucor miehei* lipase on the shear stability of sodium caseinate stabilised O/W emulsion

7.1 Abstract

Shear-induced partial coalescence in partially crystalline oil-in-water food emulsions provides an essential contribution to the development of structure in food products such as ice cream and whipped topping. Emulsions used in these formulations are comprised of mixed interfacial layers, in which low molecular weight emulsifiers are added in order to displace a portion of the protein stabilised droplet surface in order to facilitate the controlled destabilisation of the interfacial film prior to achieving partial coalescence. In this study, protein stabilised palm-oil emulsions were subjected to lipolysis by *R. miehei* lipase to generate sufficient polar lipids to cause partial displacement of the interfacial protein film. The shear-induced aggregation of these lipolysed emulsions showed that they are shear sensitive. The lipolysis of the emulsion, as influenced by lipase concentration and holding time, was assumed to enable adsorption of polar lipids at the interface, as there was profound shear-induced aggregation of lipolysed emulsion compared to no aggregation found with the non-lipolysed emulsion. Increasing the extent of lipolysis was also observed to reduce the onset time of partial coalescence. Droplet size measurement showed that the distribution of lipolysed emulsions transitioning from a monomodal distribution to a bimodal distribution as a consequence of shear, with the upper modal distribution appearing as a consequence of the partial coalescence of fat globules. These findings proved that the lipolysis of emulsion generates polar lipolysis products capable of influencing the shear stability of emulsions in a manner analogous to that of synthetic surfactants.
7.2 Introduction

Partial coalescence in partially crystalline oil-in-water food emulsion is an example of emulsion destabilisation. Following the term ‘destabilisation’, one will think it must be avoided as it will affect the food negatively, and thus to be prevented in foods. This is quite true in products like sauces, dressings, creamers and flavoured milks. However, in products like ice cream and whipped topping, destabilisation through partial coalescence is necessary to produce a desirable structure and related material and sensory characteristics (Fredrick et al., 2010; Goff, 1997; Méndez-Velasco & Goff, 2012; Zhang & Goff, 2005).

Partial coalescence is a rather complex process (Boode & Walstra, 1993; Boode et al., 1993; Walstra, 2003), with the mechanism influenced by a range of factors such as: solid fat content, interfacial composition i.e surfactant or emulsifier used, fat globule shape and size and pre-heat treatment (Fuller et al., 2015a; Granger et al., 2005; Relkin & Sourdet, 2005; Rousseau, 2000). Droplets structural dynamics are an important aspect in the preparation of emulsions with an appropriate propensity towards partial coalescence. For example, fat droplets in an emulsion are usually spherical and smooth after homogenisation. However, for whippable emulsions comprising solid fat and emulsifiers, upon cooling of emulsion, the surface of the droplets will start to become rough owing to fat crystallisation generating structural irregularities at the surface of droplets (Thivilliers-Arvis et al., 2010). Crystals may accordingly protrude at the interface and these crystals are generally considered a critical component in the mechanism of partial coalescence (Boode & Walstra, 1993; Boode et al., 1993). When fat globules collide or approach one another under shear, these protruding fat crystals may pierce the interfacial film of any neighbouring fat globules they come into contact with, allowing liquid oil to flow between them and bridges the connection. Due to the presence of a mixture of solid and liquid oil, complete coalescence is avoided hence
partial coalescence, in which the structural integrity of the initial droplet can be partially retained.

Oil-in-water food emulsions are commonly stabilised by protein emulsifiers, such as casein or whey protein, which provide both steric and electrostatic stabilisation of the fat globules (Euston, 2008). The viscoelastic interfacial films of protein network do not make the fat globules readily susceptible to partial coalescence (Fuller et al., 2015a). Therefore, for emulsions where partial coalescence is a requirement, the interfacial stability of the adsorbed protein layer can be appropriately compromised by the addition of low molecular weight emulsifiers. Many types of low molecular weight emulsifiers are used in the food industry to promote control destabilisation such as monoglycerides or their derivatives and polyoxyethelene derivatives of glycol or glycol esters, also known as Tween (Barford et al., 1991; Euston, 2008). In Chapter 4 (subsection 4.4.2), low molecular weight emulsifiers and protein emulsifiers resulted in different IFTs due to their different stabilising mechanisms, which have been discussed in detail. The orogenic displacement resulted in inhomogeneous interfacial network with low molecular weight emulsifiers adsorbing onto the interface and forming small patches in-between the protein network (noting that this mechanism can be promoted by addition of both oil and water soluble emulsifiers). As the concentration of the emulsifiers increases, the patches region expands as well, constricting the protein network to a smaller area. This will make the fat globules more susceptible to partial coalescence, notable when an external force such as during shear is introduced (Fuller et al., 2015a; Fuller et al., 2015b; Granger et al., 2005; McClements, 2004; Munk et al., 2014; Rousseau, 2000).

One of the most common low molecular weight emulsifiers used in the formulation of whippable emulsions are monoglycerides, which are produced through transesterification
of hydrogenated oils or natural fats with glycerol. The reaction usually produces a mixture of mono- and di-esters of fatty acids which are then further purified by extraction or molecular distillation (Garti, 1999), to produce materials with a mono-ester content typically > 90 %. Monoglycerides are considered GRAS (generally recognised as safe) but are still a product of chemical synthesis. With consumers increasingly showing interest towards food produced with all natural ingredients and process, there is motivation for the application of non-synthetic derived additives in foods. Applying this particular consideration to the use of emulsifiers, research and development has been carried out on the utilisation of lipase to catalyse fat modification reaction to produce monoglycerides. The use of \textit{R. miehei} lipase enzyme to catalyse the lipolysis of the oils triglyceride in an oil-in-water emulsion has been investigated in previous chapters into producing polar lipids. The polar lipids especially interfacial monoglycerides generated \textit{in situ} in lieu of commercial monoglyceride, but with equivalent functionality in the design of emulsions with controlled instability towards partial coalescence.

This chapter focusses on demonstrating that the reaction is able to successfully synthesise polar lipids at the oil-water interface, thus manipulating the stability of the emulsion system. The susceptibility of the lipolysed emulsion towards shear-induced aggregation was observed using the technique described by Fuller et al. (2015b). This technique employs a fixed shear rate to induce the emulsion to a jamming transition with a characteristic aggregation time ($t_c$), which provides an indication of the relative stability of the system towards aggregation. Furthermore, the cone and plate geometry allows homogenous shear rate to be applied to the emulsion throughout the measurement (Mezger, 2006).
7.3 Materials and methodology

7.3.1 Materials

Source of palm oil, sodium caseinate and *R. miehei* lipase used have been detailed in Chapter 3 (subsection 3.1).

7.3.2 Methodology

7.3.2.1 Emulsification by microfluidization

A 30 % wt palm oil in water emulsion stabilised by 1 % wt sodium caseinate was prepared by microfluidization procedure described in Chapter 3 (subsection 3.2.1).

7.3.2.2 Lipolysis of emulsion

Emulsions were lipolysed through addition of *R. miehei* lipase of varying concentration into 40 ml of emulsion. The concentration of lipase used was calculated relative to grams of fat in the emulsion. The mixture was then gently swirled from time to time to allow the lipolysis reaction to take place. Mechanical stirring by magnetic stirrer or vortex was avoided as so not to introduce any mechanical shearing to the sample. Unless otherwise mentioned, lipolysis was carried out for 30 min and the mixture was then heated at 100 °C in water-bath for 2 min to terminate the lipolysis reaction. Emulsions were immediately cooled in ice water and ready for analysis. Emulsion samples that required aging were kept in a refrigerator at 4 °C for the desired time before analysis.

7.3.2.3 Shear stability analysis

The stability of lipolysed emulsion under shear was analysed according to the method by Fuller et al. (2015b). The shear stability was analysed versus time at a constant shear rate using a Paar Physica MCR 301 stress-controlled rheometer (Anton Paar GmbH, Germany) with a truncated cone-and-plate geometry (50 mm diameter, 2° angle, 47 μm gap). All
measurements were carried out at 100 s\(^{-1}\) constant shear rate except during the
determination of effect of shear rates where shear rates of 100 –1500 s\(^{-1}\) were employed.
The plate temperature was held at 5 °C by a Peltier plate system. The lipolysed emulsion was
introduced onto the centre of the plate with a pipette and the holding time of emulsion on
the plate was 5 min. A solvent trap was included to prevent evaporation.

7.3.2.4 Determination of emulsion droplet size

The determination of the average droplet diameter was carried out using laser diffraction
procedures described in Chapter 3 (subsection 3.2.2).

7.3.2.5 Statistical analyses

All analyses were carried out at least in triplicates and results were presented as mean and
standard deviation of the measurements. Analysis of variance was performed using MINITAB
17 (Minitab Inc., State College, PA, USA) and Tukey post-hoc comparison was employ to
determine the significant difference (p<0.05) between the results.

7.4 Results and discussion

7.4.1 Effect of shear rates on shearing stability of O/W emulsion

By employing the rheological method by Fuller et al. (2015b), constant shear rates were
applied to enable the emulsion to undergo shears-induce jamming transition, where the
kinesis of the particles will be limited when they are forced into a small region of space by
an externally applied shear stress causing the particles to exhibit solid-like behaviour (Liu &
Nagel, 1998).

Figure 7.1 demonstrated the effect of the shear rates on the shear aggregation of the
lipolysed emulsion. The most obvious trend observed was that increasing shear rates
resulted in a decrease of the peak aggregation viscosity. This is not unexpected as the measurement was carried out using constant shear, thus the faster rates causes lower viscosity. The term aggregation time or induction time, $t_c$ has been used to described the onset of rapid aggregation viewed as the sudden increased in viscosity on the rheological curve (Fuller et al., 2015b; Guery et al., 2006). Shear rates of 500, 800 and 1500 s$^{-1}$ actually exhibited an immediate $t_c$, showed by the direct increase in the viscosity. The lowest shear rates 100 s$^{-1}$ however had a slower $t_c$, but the peak viscosity was much higher than the other shear rates used. This indicates that the lipolysed emulsions are capable of undergoing partial coalescence upon shearing (Fuller et al., 2015b). This slower $t_c$ with shear rates maybe due to the fact that a longer time was needed to overcome the repulsive energy barrier to allow the lipolysed emulsion to aggregate (Prasad et al., 2003). On the other hand, the hydrodynamic force generated by the larger shear rates, overcame the energy much sooner because of the increase frequency of fat globules collision.

The increase in shear stress was considered indicative of fat agglomeration (Walstra, 2003).

Overall, the rheological profiled observed with lipolysed emulsions follows the 2-step process of aggregation proposed by Fuller et al. (2015b) to describe the viscosity increase (step 1) and time delay (step 2) before either the stabilisation of viscosity or breakdown of the emulsion (which can lead to expulsion of sample from the geometry). However, one observation that is different from the previous findings was that in this study, instead of achieving a steady state after the jamming transition, the viscosity of these emulsion seems to increase for a second time. Fuller et al. (2015b) observation of emulsion made with 35 % hydrogenated palm kernel oil and 0.5 % Tween 20, showed a similar profile up to the drop in viscosity after the first viscosity increase curve. However, the Tween stabilised emulsion did not demonstrate a second increase in the viscosity even after shearing for several hours.
Chapter 7: Effect of lipolysis by *Rhizomucor miehei* lipase on the shear stability of sodium caseinate stabilised O/W emulsion

### Figure 7.1: Effect of shear rates on shearing stability of lipolysed O/W emulsion.

- Lipolysis time: 30 min, *R. miehei* lipase concentration: 50 mg/g fat
- (Insert: The shear curve of only 500, 800 and 1500 s⁻¹)

#### 7.4.2 Effect of lipolysis with *R. miehei* lipase on shearing stability of O/W emulsion

The effect of lipolysis (lipase concentration and lipolysis reaction time) on the shear-induced aggregation of emulsions is shown in Figure 7.2 and 7.3. It can be observed that the extent of lipolysis had a significant effect on the rheological curve of the emulsion. Increasing both lipase concentration (Figure 7.2) and reaction time (Figure 7.3) resulted in increases to the peak viscosity during shearing. The lipolysis time had effect on the aggregation time $t_c$, as the longer lipolysis time had undergo immediate jamming transition. These results clearly demonstrate the influence of the interfacial composition of the shear-induced aggregation of the lipolysed emulsion. This also provides an indirect indication that the lipolysis of O/W emulsions by *R. miehei* lipase was successful in hydrolysing the triglyceride into polar lipids.
(i.e. monoglycerides and fatty acids). As mentioned in the introduction to this chapter, monoglycerides, as well as other types of low molecular weight emulsifier such as Tween 20 are often added into a protein stabilised emulsion to promote destabilisation process. The addition of these emulsifiers promotes an orogenic protein displacement (Mackie, 2004; Mackie et al., 1999), which reduces the stability of fat globules upon shearing causing partial coalescence (Hotrum et al., 2005; Ihara et al., 2010; Pugnaloni et al., 2004; Zhang & Goff, 2005). Therefore, it is the presence of polar lipolysis products on the emulsion interface that promotes the partial coalescence of the fat globules as depicted by the aggregation peak.

No increase in the viscosity was observed for the non-lipolysed emulsion. This showed that although the emulsion was made with high amount of fat; 30 % palm oil, it does not display aggregation behaviour upon shearing at 100 s\(^{-1}\) for 1 h, indicating that the sodium caseinate interfacial layer was able to provide sufficient mechanical stability to the interface to prevent partial coalescence. As the lipolysis products increased, it is assumed that more protein was being displaced from the interface. These new polar lipolysis products-rich interfaces tend to be more susceptible to the shear-induced partial coalescence as shown by the decrease of aggregation time. This was also demonstrated by Fuller et al. (2015a) whereby a Tween-20 dominated interface resulted in higher partial coalescence compared to a mixed sodium caseinate-Tween 20 interface. Some other works have proposed that the degree of protein displacement and partial coalescence can be influenced by the either saturated or unsaturated monoglyceride (Davies et al., 2000; Granger et al., 2005). However, the exact composition of the droplet interface is hard to decide as the lipolysis is very dynamic and will produce a mixture of mono- and di-glycerides as well as fatty acids. All these lipid species are somewhat polar compared to its native triglyceride thus may all have the affinity for the interface.
Chapter 7: Effect of lipolysis by *Rhizomucor miehei* lipase on the shear stability of sodium caseinate stabilised O/W emulsion

**Figure 7.2** Effect of *R. miehei* lipase concentration on shearing stability of lipolysed O/W emulsion. Lipolysis time: 30 min, Shearing rate: 100 s\(^{-1}\)

**Figure 7.3** Effect of lipolysis time on shearing stability of lipolysed O/W emulsion. *R. miehei* lipase concentration: 50 mg /g fat, Shearing rate: 100 s\(^{-1}\)
7.4.3 Effect of aging on shearing stability of O/W emulsion

All the previous shearing analysis presented before was carried out at 5 °C, whereby it was assumed that the palm oil had undergone crystallisation to certain extent. However, effect of aging on the shear stability of lipolysed emulsion was carried out to demonstrate if the degree of crystallisation influences the shearing-induced aggregation. It was assumed that the longer aging time resulted in more crystallisation the fat globules. Figure 7.4 depicted a very different trend from the previously discussed rheology profile. Aging the emulsion for 1 and 4 h resulted in a faster \( t_c \). However, the jamming transition was not observed for emulsion that was aged for 8 h and overnight. Both of the emulsions also had a higher initial viscosity, and the shearing tend to thin down the emulsion.

![Figure 7.4: Effect of aging time on shearing stability of lipolysed O/W emulsion. Lipolysis time: 30 min, \textit{R. miehei} lipase concentration: 50 mg/g fat, Shearing rate: 100 s\(^{-1}\)](image)

It has been reported that during aging, changes between the interfacial protein and emulsifiers continue to happen which leads to subsequent destabilisation of the fat globules.
(Gelin et al., 1994; Krog & Larsson, 1992; Zhang & Goff, 2004). As crystallisation of fat proceeds during aging, the fat molecules tend to assemble together forming crystal structures, primarily as a consequence of van de Waals attraction (Fredrick et al., 2010). A study by Davies et al. (2000) described thickening and a very high apparent viscosity after cooling of emulsion added with 3.5 % monooleate. Inevitably, fat crystals are crucial in destabilisation of emulsion upon shearing. Previous authors (Boode & Walstra, 1993; Boode et al., 1993; Fredrick et al., 2010) had illustrated the role of protruding fat crystals from the fat globules surfaces in terms of piercing the interface of another fat droplet in the path before the droplets partially coalesce together. However, it is the liquid oil that flows between the droplets what glues it together (Boode & Walstra, 1993). Hence, a completely solid fat will caused the shear to have no effect on the droplets (Davies et al., 2000). Boode et al. (1993) has reported the increase of partial coalescence when solid fat content was increased. However, they also reported that increasing the solid fat content after a certain point resulted in decreasing the partial coalescence. In an oil-in-water emulsion made with vegetable oil, the triglyceride is strongly heterogeneous; hence the fat crystals in the fat droplet will exist in a wide range of temperature. However, as the time in which the emulsion was subjected to cold temperature is lengthen, the content of the crystalline fat increase as well as their protruding distance (Fredrick et al., 2010). The solid fat content was not monitored in this study but it would have been useful to have measured it as a consequence of aging time.

7.4.4 Changes to O/W emulsion droplet distribution profiles during shearing

The changes to the emulsion droplet distribution profile during shear-induced aggregation are shown in Figure 7.5. The laser light scattering data provided the droplet distribution profile shown in the insert Figure 7.5 – A and B. The lipolysed emulsion prior to shearing
Chapter 7: Effect of lipolysis by *Rhizomucor miehei* lipase on the shear stability of sodium caseinate stabilised O/W emulsion

(Figure 7.5 – Insert A) had a narrow monomodal distribution similar to that of the non-lipolysed emulsion (data not shown); with D(4,3) of 0.69 ± 0.04 μm (Figure 7.6). At 210 s of shearing, even though no obvious increase of emulsion viscosity was observed (Figure 7.5 – Insert B), the emulsion droplet distribution was already found to have become bimodal with D(4, 3) increasing to 13.04 ± 4.34 μm (Figure 7.6). As shearing continues, a multimodal distribution was observed, encompassing a broad range of particles size. This eventually transitions back to a predominantly monomodal distribution with D(4,3) of 81.73 ± 19.94 μm, which represents a significant increase in particle size relative to the starting emulsion. The steady shearing of the emulsion was able to control the jamming transition of the emulsion and it did not undergo breakdown or being expelled from under the gap as the findings by Fuller et al. (2015b). Therefore, perhaps, the second viscosity increase could signal that the emulsion might go through another round of fat aggregation, until phase inversion could occur and the curve will halt.
Figure 7.5: The shear-induced aggregation curve of lipolysed O/W emulsion at shear rate 100 s\(^{-1}\). Lipolysis time: 30 min, \textit{R. miehei} lipase concentration = 50 mg per g of fat. Insert graph – The changes of lipolysed emulsions’ droplet distribution profile at different shearing time (A) 0s, B) 210 s, C) 1200 s, D) 1800 s and E) 3000 s.
Chapter 7: Effect of lipolysis by *Rhizomucor miehei* lipase on the shear stability of sodium caseinate stabilised O/W emulsion

7.5 Conclusion

The shear stability analyses on lipolysed O/W emulsions showed the lipolysed emulsions are susceptible to shear-induced aggregation, and that the degree of aggregation could be manipulated as a consequence of controlling the extent of lipolysis through either enzyme concentration or holding time. The drastic increase in the viscosity curve between the non-lipolysed and lipolysed emulsion suggested that the shear –induced partial coalescence was primarily due to the lipolysis reaction and was not as a result of the high fat content (30 %). The findings elucidate the ability of the generated polar lipids in the emulsion to displace the existing sodium caseinate upon shearing. The external mechanical force provided during shearing helps
in creating a dynamic environment in the emulsion internal structure, whereby after the displacement of the protein emulsifiers, partial coalescence of fat globules occurred when they are induced to come into close contact with each other. This shows that the lipolysed emulsions can potentially be used in the formulation of whippable emulsions and emulsion foams, where such structures are a requisite component of product design. Chapter 9 specifically investigates this possibility by investigating the functionality and properties of lipolysed emulsions as used in the manufacture of a non-dairy whipping cream and ice cream.
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

8.1 Abstract

The incorporation of *R. miehei* lipase of an O/W emulsion has been shown to synthesise polar lipids from the triglyceride component of fat droplets. The quantification of these lipolysis products showed a mixture of fatty acids, di- and mono-glycerides being produced. For the palm oil emulsions used in this study, palmitic acid was observed to be the highest concentration of liberated fatty acids across all the lipase concentrations (5, 25, 50 mg / g fat) and lipolysis time (15, 30, 45 and 30 min) tested. Monoolein and monopalmitin were the most prominent monoglycerides, with measured concentrations of 3.755 ± 0.895 and 1.660 ± 0.657 mg /g fat respectively after 15 min with lipase 50 mg / g fat. The relative concentration of polar lipids produced was found to be dependent on the lipase concentration as well as time of lipolysis. In terms of the effects of polar lipid synthesis on the stability of emulsions, reaction times of up to 30 min of lipolysis were seen to have no observable effect on the droplet size distribution of the emulsion. However, the appearance of a second modal distribution after 45 and 60 min lipolysis time, suggested excessive displacement of the sodium caseinate from the interface by the polar lipid fractions leading to aggregation of fat droplets. Significant difference (p<0.05) of the specific surface area, D(4,3) and D(3,2) of the lipolysed emulsions were only observed between the 0 and 60 min suggesting that quiescently stable emulsions could be produced for lipolysis times below 60 min, and lipase concentration below 50 mg / g fat. However, it should be noted that micrographs of the lipolysed emulsion depicted some aggregation of the fat globules into smaller network at 45 and 60 min lipolysis. In conclusion, the addition of *R. miehei* lipase hydrolyses palm oil emulsions into predominantly long chain fatty acids, di- and mono-glycerides (with a relatively high proportion of monounsaturated monoglycerides
produced). Subsequent emulsion stability was found to be influenced as a concentration of both lipase concentration and holding time.

8.2 Introduction

Previous chapters (Chapter 4 and 5) have shown that O/W emulsions stabilised by protein can be successfully lipolysed in situ by addition of R. miehei. Furthermore, these lipolysed emulsions displayed a propensity towards shear-induced aggregation (as shown in Chapter 7), supporting the suggestion that polar lipids, assumed to be predominantly monoglycerides and fatty acids, were adsorbing at the oil-water interface as a consequence of droplet hydrolysis. These generated low molecular weight emulsifiers were assumed to displace the existing protein emulsifiers in a manner analogous to that imparted by the addition of commercial monoglycerides as used in the manufacture of aerated emulsions such as ice cream or whipped topping (Barford et al., 1991; Goff, 1997; 2008). Monoglycerides are one of the most widely used emulsifiers in the food industry. Usually, commercial monoglycerides comprise predominantly long chain monoglycerides and are sold as saturated or unsaturated or combinations of the two, and primarily comprising chain lengths of 16 or 18 carbon atoms (Davies et al., 2001). The differences in the chain length and saturation can influence the properties (e.g. melting temperature) and functionality of monoglyceride emulsifiers. For example, low temperature as well as the presence of monoglycerides during ice cream preparation will induce crystallisation of the oil phase. Monoglycerides crystallisation has been reported to induce reduction of IFT and protein displacement from the interface (Gelin et al., 1997). The fatty acids part in monoglycerides represent the lipophilic part of the emulsifier, hence the chain length will influence the hydrophilic-lipophilic balance (HLB) of the monoglycerides. The extent of destabilisation during shearing is reported to be higher with shorter chain and unsaturated monoglycerides (Davies et al., 2001; Pelan et al., 1997).
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

The majority of the commercial monoglycerides are produced synthetically through thermal glycerolysis (interesterification), with temperatures reaching 200 – 250 °C and with the additional application of several metallic catalysts (Ferreira-Dias & Fonseca, 1995; Monteiro et al., 2003). However, the use of lipase enzyme has been increasingly explored as an alternative approach in the manufacture of monoglycerides, with the advantages of specificity of the monoglycerides produced as well as mild reaction conditions (as discussed in Chapter 2 (subsection 2.6.3)). Typically, lipases are used to catalyse the esterification of fatty acids with triglycerides or transesterification of fatty esters with glycerol, with variations in reaction conditions such as immobilized or not, temperatures and solvent system allowing control over yields and composition of lipids produced (Bornscheuer, 1995; Ferreira-Dias & Fonseca, 1995; Fregolente et al., 2010; Monteiro et al., 2003; Rodrigues & Ayub, 2011). Most notably in the context of this work, sn-1,3-specific lipases have also been used to hydrolyse triglycerides into fatty acids and monoglycerides in various types of bioreactors or solvent system (Balcão & Malcata, 1998; Holmberg & Osterberg, 1988; Mukherjee, 1990; van der Padt et al., 1992).

This chapter aims to quantify the generated polar lipids obtained by in situ lipolysis of O/W emulsions. Furthermore, as the oil droplets in the emulsion are being lipolysed, quantification of the changes to the emulsion droplet size will be followed in parallel. It has been shown for example, during the digestion of emulsified lipids, 1,3-specific pancreatic lipase hydrolysed the lipid droplets into free fatty acids, mono-, di- and tri-glycerides in different proportions resulting in the lipid droplets to become smaller (Tarvainen et al., 2010). In addition, it has been mentioned earlier, the displacement of the existing protein emulsifier will occur when another low molecular weight emulsifier is added into the mix.
This has been known to promote partial coalescence of the fat globules that will change the droplet size distribution of the emulsion.

To observe the effect of *R. miehei* lipase on the fat globules in an O/W emulsion, quantum dots (QDs) were used. QDs are nanometer inorganic semiconductors with sizes usually less than 10 nm in diameter. The main advantage of QDs is that they are stable light emitters and fluoresce at sharp and distinct wavelengths, with great brightness level. They are additionally very stable against photobleaching (Hermanson, 2013; Sonesson et al., 2007). The interaction of QDs with various biological molecules has led to increasing use of these materials in analysis for biological and medical applications, as it allows longer observation time of various molecules under fluorescence microscopy. This has been found to be particularly useful for time dependent observations, such as for following cellular activities, biochemical reactions, molecule dispersion and mobility (Fayi & Warren, 2011; Sonesson et al., 2007). In this chapter, QDs with a carboxylate surface coating that could be conjugated to amine-containing molecule via carbodiimide coupling were used. This was used to enable the conjugation of *R. miehei* lipase to QDs, thus allowing the visualisation and tracking of the lipase in the O/W emulsion under fluorescence.

### 8.3 Materials and methodology

#### 8.3.1 Materials

Details for *R. miehei* lipase, emulsifier and oil used in the emulsion have been described in Chapter 3 (subsection 3.1). All chemical reagents and standards for quantification analysis were of chromatography grade. Pure lipid standards of different fatty acids, monoglycerides and diglycerides from Nu-Chek Prep (MN, USA) were purchased through Purescience Ltd., Poriroa, New Zealand. The cadmium telluride (CdTe) core type quantum dots (QDs) with
carboxylate functionalised was from Sigma Aldrich, MO, USA. The QDs emission wavelength was 520 nm.

8.3.2 Methodology

8.3.2.1 Emulsification by microfluidization

Canola oil-in-water emulsions stabilised by 1 % sodium caseinate were prepared following the emulsification by microfluidization method described in Chapter 3 (subsection 3.2.1). A coarser droplet size emulsion was produced with < 5000 psi pressure for lipolysis with photoactivated quantum dots for fluorescence observation with CLSM.

8.3.2.2 Lipolysis of emulsion

Emulsions were lipolysed according to the procedure described in Chapter 3 (subsection 3.2.5). Concentrations of lipase used (5, 25 and 50 mg / g fat) were calculated against grams of fat in the emulsion.

8.3.2.3 Determination of O/W emulsion droplet size

The determination of the average droplet diameter was carried out using procedures described in Chapter 3 (subsection 3.2.2).

8.3.2.4 Extraction of lipid from O/W emulsion

Extraction of the lipid component from the O/W emulsion was carried out using a 2 step method by Bligh and Dyer (1959). New or solvent-cleaned glass tubes were used to avoid contamination. To each 1 ml of sample, 3.75 ml of 1:2 (v/v) chloroform:methanol was added before being vortexed for 10 - 15 min. An internal standard of 50 μL margaric acid (C17:0, 20 mg L⁻¹) was included at this stage. After that, 1.25 ml chloroform was added and vortexed
for 1 min. Then, 1.25 ml water was added and vortexed again for another 1 min. The mixture was then centrifuged at 1000 rpm using a bench-top centrifuge (Thermo Scientific Heraeus\textsuperscript* Multifuge\textsuperscript* 1S-R, Thermo Electron LED GmbH, Osterode, Germany) for 5 min at room temperature to give a two-phase separation. The bottom phase was recovered by inserting Pasteur pipette through the upper phase with gentle positive-pressure. The remaining upper phase was added with 1.88 ml of chloroform, vortexed and centrifuged again. The lower phase was collected once more and combined with the first collection. The mixture was then gently dried under a stream of nitrogen to allow evaporation of the solvent. The extracted lipid was then redissolve in 200 μl 2:1 chloroform:methanol.

8.3.2.5 Separation of lipid species by thin layer chromatography (TLC)

The separation of extracted lipid was carried out using thin layer chromatography (TLC) technique (Hendrikse et al., 1994; Thomas et al., 1965). Commercial TLC plates (Silica gel G 60, Merck, Ref. 5721), 20 x 20 cm were pre-run in a Latch-lid™ TLC developing chambers (Sigma-Aldrich, MO, USA) containing chloroform/methanol (50:50, v/v) up to 1 cm migration from the top of the plates to remove any contaminant from the silica gel. The plates were air dried under a fume hood. Impregnation was carried out by dipping the silica gel plates upside down (silica part facing the solution) in a solution of 2.3 % boric acid in ethanol solution. The plates were then air dried for a few minutes and activated by heating to 100 °C in an oven for 15 min. Concentrated solutions of lipids in chloroform/methanol mixtures (2:1, v/v) were then applied as fine spots using a Hamilton micro-syringes (10 μl) about 1.5 cm from the bottom of the plate and separated from each other by 1 cm. Nitrogen flow was used to assist the evaporation of solvent from the lipid spots. Re-spotting was done if necessary to concentrate the lipid spots. Pure lipid standards (fatty acids, monoglycerides, diglycerides and triglycerides) were also spotted onto the plates as guide. Separation of the
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

Lipid was done by placing the plate in the developing chamber containing solvent mixture chloroform/acetone (96:4, v/v) and the solvent was allowed to ascend to about 2 cm of the top of the plates. Plates were then removed and allowed to dry under the fume hood. Primuline solution (5 mg primuline powder with 50 % dye content (Sigma Aldrich, MO, USA) in 100 ml of acetone/water (80:20, v/v)) was sprayed with an all-glass atomiser (Preval spray unit, Sigma-Aldrich, MO, USA) onto the plate. Lipid component spots can be viewed on the plate under ultraviolet light (340 nm) (Spectroline, USA). Lipids appeared as bright yellow spots on the plates. Based on the standards spot, the lipid fraction was identified and circled with a pencil. The spots were then scraped with a 13 mm steel blade scraper and the silica carefully inserted into glass tubes. The lipids were recovered from the silica by eluting in two washes with 3 ml diethyl ether. The ether phase was then washed rapidly with 2 ml of water to remove traces of boric acid before being evaporated under nitrogen stream. The remaining extract was then redissolved in minimum amount of chloroform in amber glass vials with Teflon lined screw top closure for gas chromatography analysis.

8.3.2.6 Derivatisation for fatty acid methyl ester (FAME) analysis

Derivatisation of free fatty acids extract to FAME was carried out by mixing 2 mg of extracted free fatty acids with 40 μL methyl-tert-butylether before adding 20 μL tri methyl sulfonium hydroxide (TMSH). The mixture was allowed to shake for 15 min at 75 °C (Molkentin & Giesemann, 2007). The glycerides extracted were derivatised using the direct transmethylation of glycerides for fatty acids analysis method of Weston et al. (2008) adapted by Harmanescu (2012). About 0.3 - 0.5 g lipid extract is weighed into a 10 mL Teflon tubes and added with 2.5 mL of boron trifluoride (50 % w/w) in methanol solution. The tubes were then heated at 70 °C in a water bath for 30 min. Once the tubes were cooled down, 1 mL of 10 % sodium chloride solution was added. 2 mL of hexane was then added followed
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

by 1 g sodium sulfate and 1 g activated carbon. The mixture was then centrifuged for 5 min at 25000 rpm. The supernatant was collected in vials for gas chromatography analysis.

8.3.2.7 Detection of FAME by gas chromatography (GC)

Detection of FAME was carried out on an Agilent 7890 GC (Agilent Technologies, CA, USA) (Zhu et al., 2013) equipped with a flame ionisation detector and a split/splitless injector. A 30 m Supelcowax™ 10 capillary column (0.35 mm diameter × 0.50 μm thickness) (Supelco Park, PA, USA) was fitted to the GC. Helium was used as the carrier gas at 20 cm s⁻¹, at a pressure of 76 kPa at the head of the column. Both the injector and the detector were set at 260 °C. The column was held at initial temperature of 180 °C for 5 min, followed by increase to 210 °C at a rate of 1 °C min⁻¹ and then maintained at 210 °C for 25 min. The split ratio was 20:1. The FAME peaks were identified and quantified by Agilent 5975 GC/MS system.

8.3.2.8 Conjugation of *R. miehei* lipase with carboxyl quantum dots (QD)

The CdTe QDs were conjugated to *R. miehei* lipase via carbodiimide coupling (Hermanson, 2013). QDs were mixed with 10 mM borate buffer, pH 7.4 to form 1 μM solution of QDs. *R. miehei* lipase was then added to the mixture and stirred well. The added *R. miehei* lipase was at a 40-fold molar excess of lipase to QDs. A solution of N-ethyl-N’-dimethylaminopropyl-carbodiimide (EDC) was freshly prepared in water at a concentration of 10 mg ml⁻¹. Immediately, 57 μL of the EDC solution was added to the lipase/QDs mixture. The mixture was stirred gently for 2 h at room temperature to allow conjugation. After that, the mixture was filtered through a 0.2 μm PES (polyethersulfone) syringe filter (Whatman Cat. 6876-2502) to remove any large aggregates or precipitation. Excess *R. miehei* lipase was then removed by using a 15 mL ultrafiltration unit (Amicon Ultra 15, Millipore Cat. UFC910008). The filled ultrafiltration unit was centrifuged at 4500 rpm for 20 min for at least
5 buffer exchange using 50 mM borate buffer, pH 8.3. The QDs conjugate solution was kept at 4 °C until further analysis. pH-stat analysis was carried out to confirm that conjugation of *R. miehei* lipase with QDs did not hinder the lipolytic activity of the enzyme.

### 8.3.2.9 Microscopic observation with confocal laser scanning microscope

Microscopic observation of emulsion droplets was carried out using confocal laser scanning microscope described in Chapter 3, subsection 3.2.4. The lipolysed emulsions were scanned with a 40x oil immersion to observe the changes caused by lipolysis using laser with excitation wavelength of 561 nm and 633 nm for Nile Red and Fast Green FCF respectively. A 63x oil immersion objectives was used for QDs visualisation and only Nile Red was added to the emulsion. The excitation wavelengths used were 561 nm and 520 nm for Nile Red and QDs respectively. The images were captured by two channels of PMT, one for each wavelength used.

### 8.3.2.10 Statistical analyses

All analyses were carried out at least in triplicates and results are presented as mean and standard deviation of the measurements. Analysis of variance was performed using MINITAB 17 (Minitab Inc., State College, PA, USA) and Tukey post-hoc comparison was employed to determine the significant difference (p<0.05) between the emulsion lipolysed with different lipase concentrations, where possible.
8.4 Results and discussion

8.4.1 Lipolysis product quantification

Quantification of lipolysis products in O/W emulsion was carried out using GC - FAME analysis after subsequent separation of the lipid component by TLC. The initial TLC separation provided a good qualitative assessment of the lipolysis product as it can be seen from Figure 8.1. From the TLC plates, it can be generally observed, the production of monoglycerides, diglycerides and fatty acids as the emulsion was subjected to lipolysis.

Figure 8.1: Example of separated lipid spots on TLC plates when observe under UV light

The separated lipid components were then subjected to FAME analysis using gas chromatography. Table 8.1, 8.2 and 8.3 showed the fatty acids profile based on each lipid component extracted from the TLC plates; with each figure representing different lipase concentration (5, 25, and 50 mg per g of fat) used to lipolysed the emulsion. Results in Chapter 5 (subsection 5.4.3) indicated that lipolysis on O/W emulsion was successful at various lipase concentration from 2.5 – 150 mg per g of fat. However, for specific
quantification of the lipolysis products, only three concentrations were used for GC analysis. It can be observed that increasing the lipolysis time (15, 30, 45 and 60 min) as well as lipase concentration (5, 25 and 50 mg / g fat) resulted in an increased concentration of the amount of the polar lipids produced. The predominant fatty acid liberated was palmitic acid, followed by stearic acid and oleic acid. This is expected, as the most predominant fatty acid in palm oil is palmitic acid (C16:0) (41 – 68 %). Oleic acid (C18:1), however, represents the second most abundant molar fatty acids fraction in palm oil, with around 36 - 44 %, followed by either stearic (C18:0) or linoleic acid (C18:2) (Agilent Technologies Inc., 2011; Basiron, 2005; Tan & Nehdi, 2012; Tan & Che Man, 2010; Zambiazi et al., 2007). The lower than expected liberation of oleic acid may be explained when considering relative amounts of monoglycerides generated. Furthermore, it can be observed that other types of fatty acid were also cleaved from the triglycerides. These other types of fatty acids were assumed to be primarily the short chain myristic acid (C14:0) based on the location of the peaks on the chromatogram. The diglycerides produced were measured as the fatty acids equivalent and from the results, it can be observed that the fatty acids attached to the diglycerides extracted were mostly palmitic acids followed by oleic and stearic acids. Oleic acids were found to be higher than stearic acids in diglycerides, this maybe due to the configuration of these fatty acids in the palm oil triglyceride.

The fact that monoolein was the most predominant monoglyceride liberated, followed by monopalmitin indicates that oleic acid has high occupancy in the sn-2 position of the triglycerides in palm oil, which agrees with prior analysis of palm oil which determined the dominant triglycerides species as POO, PPO, POP, POL, PPP and PLP (P = palmitic acid, O= oleic acid, L = linoleic acid) (Agilent Technologies Inc., 2011; Tan & Nehdi, 2012; Tan & Che Man, 2010). The high relative value of monoolein is therefore consistent with the understanding that *R. miehei* cleaves triglycerides at the 1 and 3 positions to release 1, 3
fatty acids and an sn-2 monoglyceride. On this basis, it is perhaps unsurprising that, in comparison, monostearin was found to be almost non-detectable across all treatments. Although triglyceride species with stearic acid as the central fatty acid are not common, there are some reports of triglycerides species such as PSO, SSO and PSS (S = stearic acid) found in palm oil. However, the percentage of these triglycerides is still negligible (Agilent Technologies Inc., 2011; Tan & Nehdi, 2012).
Table 8.1: Fatty acids concentration from lipolysed O/W emulsion with 5 mg lipase per g of fat

<table>
<thead>
<tr>
<th>Lipid fraction (mg per g of fat)</th>
<th>Lipolysis time with 5 mg lipase per g of fat</th>
<th>Lipolysis time with 5 mg lipase per g of fat</th>
<th>Lipolysis time with 5 mg lipase per g of fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
<td>45 min</td>
</tr>
<tr>
<td><strong>Fatty acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.887 ± 0.119 B</td>
<td>1.653 ± 0.399 B</td>
<td>1.557 ± 0.323 Bab</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.682 ± 0.108 BCD</td>
<td>1.416 ± 0.450 B</td>
<td>1.408 ± 0.299 BCD</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.296 ± 0.026 CDEa</td>
<td>0.571 ± 0.263 CDEa</td>
<td>0.329 ± 0.040 DEab</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.021 ± 0.006 E</td>
<td>0.038 ± 0.011 Fab</td>
<td>0.033 ± 0.015 Fab</td>
</tr>
<tr>
<td>Others</td>
<td>0.034 ± 0.015 E</td>
<td>0.067 ± 0.011 Fab</td>
<td>0.091 ± 0.018 Ebc</td>
</tr>
<tr>
<td><strong>Total fatty acids</strong></td>
<td>1.814 ± 0.053 A</td>
<td>2.999 ± 0.078 Ab</td>
<td>3.418 ± 0.152 Ab</td>
</tr>
<tr>
<td><strong>Diglyceride</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.363 ± 0.042 CDEa</td>
<td>0.528 ± 0.066 CDEa</td>
<td>0.553 ± 0.095 DEab</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.226 ± 0.088 DEa</td>
<td>0.230 ± 0.022 Ea</td>
<td>0.272 ± 0.068 Ea</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.276 ± 0.146 DEa</td>
<td>0.334 ± 0.180 DEa</td>
<td>0.310 ± 0.154 EA</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.009 ± 0.008 E</td>
<td>0.043 ± 0.036 Fab</td>
<td>0.068 ± 0.019 Eb</td>
</tr>
<tr>
<td>Others</td>
<td>0.007 ± 0.006 E</td>
<td>0.050 ± 0.012 Eb</td>
<td>0.059 ± 0.016 Eb</td>
</tr>
<tr>
<td><strong>Total diglycerides</strong></td>
<td>0.882 ± 0.099 B</td>
<td>1.184 ± 0.132 B</td>
<td>1.261 ± 0.119 BCD</td>
</tr>
<tr>
<td></td>
<td>(fatty acid equivalent)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monopalmitic</td>
<td>0.187 ± 0.066 DEa</td>
<td>0.299 ± 0.089 Fab</td>
<td>0.557 ± 0.205 DEbc</td>
</tr>
<tr>
<td>Monostearic</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.004 ± 0.006 E</td>
</tr>
<tr>
<td>Monoleic</td>
<td>0.579 ± 0.414 BCDa</td>
<td>0.667 ± 0.416 CDEa</td>
<td>0.931 ± 0.356 CDA</td>
</tr>
<tr>
<td>Monolinoleic</td>
<td>0.027 ± 0.024 E</td>
<td>0.033 ± 0.025 E</td>
<td>0.076 ± 0.053 E</td>
</tr>
<tr>
<td><strong>Total monoglycerides</strong></td>
<td>0.793 ± 0.485 BCa</td>
<td>1.000 ± 0.447 BCDA</td>
<td>1.568 ± 0.537 Ba</td>
</tr>
</tbody>
</table>

Note:
- Mean within the same column followed by the same uppercase superscript letters are not significantly different (p<0.05)
- Mean within the same row followed by the same lowercase superscript letters are not significantly different (p<0.05)
- n.d. = not detected
## Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

Table 8.2: Fatty acids concentration from lipolysed O/W emulsion with 25 mg lipase per g of fat

<table>
<thead>
<tr>
<th>Lipid fraction (mg per g of fat)</th>
<th>Lipolysis time with 25 mg lipase per g of fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td><strong>Fatty acid</strong></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>3.958 ± 0.851 BCa</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.072 ± 0.940 Ba</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>1.008 ± 0.233 DEFGa</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.215 ± 0.002 FGa</td>
</tr>
<tr>
<td>Others</td>
<td>0.479 ± 0.106 EFGa</td>
</tr>
<tr>
<td><strong>Diglyceride</strong></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>1.382 ± 0.126 DEa</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.521 ± 0.045 EFGa</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>1.059 ± 0.199 DEFGa</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.032 ± 0.031 Ga</td>
</tr>
<tr>
<td>Others</td>
<td>n.d. -</td>
</tr>
<tr>
<td><strong>Total diglycerides</strong></td>
<td>2.994 ± 0.400 Ca</td>
</tr>
<tr>
<td><strong>Monoglyceride</strong></td>
<td></td>
</tr>
<tr>
<td>Monopalmitic</td>
<td>0.574 ± 0.102 EFGa</td>
</tr>
<tr>
<td>Monostearic</td>
<td>n.d. -</td>
</tr>
<tr>
<td>Monooleic</td>
<td>1.248 ± 0.056 DEFa</td>
</tr>
<tr>
<td>Monolinoleic</td>
<td>0.041 ± 0.010 Ga</td>
</tr>
<tr>
<td>Others</td>
<td>n.d. -</td>
</tr>
<tr>
<td><strong>Total monoglycerides</strong></td>
<td>1.863 ± 0.168 Da</td>
</tr>
</tbody>
</table>

Note:
- Mean within the same column followed by the same uppercase superscript letters are not significantly different (p<0.05)
- Mean within the same row followed by the same lowercase superscript letters are not significantly different (p<0.05)
- n.d.= not detected
Another interesting finding was the extent by which acyl migration of the generated 2-mono- 
glycerides into 1,3-mono- 
glycerides had occurred. Based on the TLC separated spots, both types of mono- 
glyceride species were detected, proving that the acyl migration had taken place. 
This may be due to the fact that the continuous phase of the emulsion is water 
since acylation can be suppressed in alcohol (Compton et al., 2007). Individual quantification 
of 1,3-mono- 
glyceride and 2-mono- 
glyceride was not carried out, due to the location of the TLC spots being too close together and sometimes overlapped. Purportedly, the acylation to
1,3-monoglyceride should make the molecule prone to further lipolysis, resulting in complete hydrolysis to free fatty acids. However, monoglycerides were still detected at significant concentration compared with diglycerides. Another possible reason is that this is a consequence of the lipolysis being carried out at 21 °C, and thus not all 2-monoglyceride had undergone acylation at this temperature on the understanding that acylation increases with temperature up to 80 °C (Compton et al., 2007; Yamane, 2004).

Tarvainen et al. (2010) reported findings on the digestion of rapeseed oil with 1,3-specific lipase, showing a mixture of 1,3- and 2-monoglyceride being produced. Their results showed that 35 % of monoolein, 25 % of monolinolein and 15 % monolinolenin were still the 1,3-configuration. Furthermore, the acidic condition used in this digestion model has been reported to increase acylation reaction.

The results shown in Table 8.3, were produced with 50 mg of lipase per g of palm oil used in the emulsion. This represents the highest amount of lipase concentration used within this study. It can be observed that the total diglycerides and monoglycerides concentration produced after just 15 min of lipolysis were 10.373 ± 3.904 and 5.578 ± 1.586 mg per g of fat, with the total of both at ~16 mg g⁻¹. If this were to be translated into a 1 kg of food emulsion containing 30 % of fat/oil, the mono- and di-glycerides will account to about 0.48 %. Juxtaposing this to existing food products utilising mono and diglycerides in their formulation, the usual concentration of these emulsifiers added is about 0.2 - 0.3 % in baked goods, 0.4 - 0.6 % in margarine and spreads and 0.15 % in ice cream (Moonen & Bas, 2015). Considering that the amount of mono- and di-glycerides produced with 50 mg lipase /g fat is on the higher side, the concentration of lipase can be lowered as to produce a different concentration range of the polar lipids. Lower concentration of lipase was also tested (25
mg/g fat (Table 8.2) and 5 mg/g fat (Table 8.1)) to quantify the generated mono- and di-glycerides. These will be further demonstrated in Chapter 9, whereby functionality of the lipolysed emulsion was still achievable with lower lipase concentration. Furthermore, the generated fatty acids, although not as polar as the monoglycerides, still have an affinity for the interface (Armand et al., 1996; Singh et al., 2009). Thus, if the fatty acids and monoglycerides were to be combined together, the amount of polar lipids generated will be much higher. Accordingly, using lesser amount of lipase is reasonable to generate sufficient polar lipids in the emulsion. However, another important factor to consider is the off flavour that might be contributed by the free fatty acids. As palm oil predominantly contains long chain fatty acids, the strong off flavour from the shorter chain fatty acids can be somewhat evaded. Free fatty acids below 2% have been reported to not usually cause flavour change (Hammond, 2003c).

8.4.2 Changes of O/W emulsion droplet size distribution as a result of lipolysis

Changes to O/W emulsion droplet size distributions during lipolysis are presented in Figure 8.2. Monomodal distribution was observed for up to 30 min of lipolysis time, after which the distribution became bimodal (at 45 and 60 min lipolysis). This result represented O/W emulsion lipolysed with 50 mg R. miehei lipase per g of fat, which resulted in a higher amount of polar lipids produced as it has been detailed in the previous section (subsection 8.4.1). However, significant (p<0.05) increase of specific surface was only obtained between 0 and 60 min of lipolysis time at 17.567 ± 2.397 and 11.900 ± 0.849 m⁻¹ respectively. Accordingly, the decreased in D(4,3) and D(3,2) was significantly different (p<0.05) between 0 and 60 min lipolysis time (as shown in Table 8.4). This shows that significant changes to the emulsion droplet size only occur after extended period of lipolysis (60 min). Polar lipids such as monoglycerides have been added to food emulsion initially stabilised by a protein film, to induced control destabilisation which resulted in partial coalescence of the oil globules.
(Barford et al., 1991; Euston, 2008). As indicated in Chapter 4 (subsection 4.4.3) and Chapter 5 (subsection 5.4.2), because proteins and monoglycerides stabilise interfaces with different mechanisms, orogenic displacement of the existing proteins network by the monoglycerides can cause the fat globules to be susceptible to partial coalescence. However, no significant changes to the lipolysed emulsion droplet size up to 30 min of lipolysis were observed, suggesting that partial coalescence did not happen immediately. From the results, displacement of protein from the interface might not cause instantaneous partial coalescence of the emulsion, however, the weakening of the protein interface will result in partial coalescence once the emulsion is subjected to aging or shear (Fuller et al., 2015; Granger et al., 2005; McClements, 2004). This instability may be related to increasing concentration of monoolein adsorbing at the interface. Monoolein had been reported to produce emulsions that are both quiescently stable and shear sensitive (when shear stress surpasses a certain value) (Davies et al. 2000; 2001).
Figure 8.2: Droplet size distribution of O/W emulsion lipolysed with *R. miehei* lipase (50 mg per g fat) at different lipolysis time

Table 8.4: Specific surface area, D(4,3) and D(3,2) of lipolysed O/W emulsion with *R. miehei* lipase (50 mg per g fat) at different time

<table>
<thead>
<tr>
<th>Lipolysis time</th>
<th>Specific surface area (m^{-1})</th>
<th>D(4,3)</th>
<th>D(3,2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>17.567 ± 2.397 a</td>
<td>0.598 ± 0.059 a</td>
<td>0.346 ± 0.051 a</td>
</tr>
<tr>
<td>15 min</td>
<td>15.267 ± 1.537 ab</td>
<td>0.653 ± 0.055 a</td>
<td>0.396 ± 0.043 a</td>
</tr>
<tr>
<td>30 min</td>
<td>14.400 ± 1.217 ab</td>
<td>0.678 ± 0.052 a</td>
<td>0.419 ± 0.036 a</td>
</tr>
<tr>
<td>45 min</td>
<td>13.767 ± 1.305 ab</td>
<td>3.012 ± 0.556 ab</td>
<td>0.689 ± 0.068 b</td>
</tr>
<tr>
<td>60 min</td>
<td>11.900 ± 0.849 b</td>
<td>6.011 ± 4.022 b</td>
<td>0.739 ± 0.155 b</td>
</tr>
</tbody>
</table>

Note:
- Mean within the same column followed by the same superscript letters are not significantly different (p<0.05)
8.4.3 Microscopic observation of lipolysed O/W emulsion

Microscopic observation of the lipolysed O/W emulsion was carried out using confocal microscope, for which the fat globules appear as yellow/orange, and the green is the protein rich aqueous phase. The micrographs shown in Figure 8.3 depict O/W emulsions lipolysed with *R. miehei* lipase at 50 mg/g fat at different lipolysis times. Larger droplets were visible at a lipolysis time of 30 min; however, increasing lipolysis reaction time to 45 and 60 min resulted in droplets that were increasingly connected together. The droplets did not appear to aggregate into localised clumps, but rather formed a network of partially coalesced droplets. This may be why the droplet size distribution shown in the previous section (Figure 8.2) had bimodal distribution for 45 and 60 min lipolysis time. As the emulsion was introduced into the recirculating water in the measuring cell unit of the Mastersizer, the weaker coalesced fat network may be separated. The stronger coalesced fat droplets resulted in the second population of larger droplets.
Figure 8.3: Images of O/W emulsion fat globules when subjected to lipolysis with *R. miehei* lipase (50 mg per g fat) at different lipolysis time (Scale = 15 μm)
Chapter 8: Characterisation and quantification of lipolysis on O/W emulsions

To further observe the effect of lipolysis on the properties of O/W emulsions, *R. miehei* lipase was conjugated with QDs nanocrystals which can fluoresce at discrete wavelengths proportional to their sizes. The main advantage of QDs are that they can resist photobleaching thus this allow images to be captured over extended periods of time but without loss of fluorescence as compared to conventional dye (Hermanson, 2013). Conventional fluorophores will tend to photobleach within seconds whereas QDs have been reported to be stable for several minutes (Sonesson, 2007; Sonesson et al., 2007). Figure 8.4 (a) depicted the effect of *R. miehei* lipase on the fat globules (blue). The O/W emulsion was reacted with QDs conjugated *R. miehei* lipase, and the confocal image showed the lipase dominating the fat globules interface. Due to a rich lipase environment, the surface of the fat globules can be observed to be covered by a layer of lipase. Furthermore, certain lipase concentrated spots, depict changes to the original smooth spherical fat globules to a rougher jagged surface. The experiment was then carried out by lipolyzing the O/W with a limited QDs conjugated *R. miehei* lipase concentration. Figure 8.4(b) depicts the images taken for this emulsion. It can be observed that a thinner layer of lipase attached to the surface of the emulsion compared to Figure 8.4 (a). Moreover, a few concentrated spots of lipase can be observed as well. Figure 8.4 (c-h) focussed on the changes to a single fat globule from Figure 8.4 (a) with a dominant spot of lipase attached. The *R. miehei* lipase was found to attach on the surface of the fat globules, and works its way into the globule, causing changes to the structure of the globules. Sonesson et al. (2007) had analysed the mobility of single lipase molecule conjugated to QD on a trimyristin substrate surface, and found that the trajectories of the lipase molecule became restricted in certain regions before migrating to other regions. This is maybe why the lipase in this appears to remain adsorbed to the globules it had attached to rather than desorbing and move to another droplet, and may also account for the lack of displacement as a consequence of preferential fatty acid or monoglyceride
adsorption (depending on whether lipolysis is occurring within the timeframe of the observation).

The findings suggest that at lower concentration, not only polar lipids were found to be produced that can encourage partial coalescence, but also changes to the fat globules surface might contribute to that as well. Although, only lipase rich domains on the interface were found to trigger significant changes to the fat globules, it can be particularly beneficial because not all the fat globules were hydrolysed. This can maintain the overall stability of the emulsion but it remains susceptible to aggregation when provided with shear. For example, in ice cream, to achieve the desirable texture and creaminess, less than 20% of the fat should be partially coalesced (Hasenhuettl, 2008).

Figure 8.4 (a): Confocal laser scanning microscope image of O/W emulsion lipolysed with excess QDs conjugated *R. miehei* lipase (Scale bar = 5 μm)
Figure 8.4: (b) Confocal laser scanning microscope image of O/W emulsion lipolysed with limited QDs conjugated *R. miehei* lipase

(c - h) Zoomed images of a single fat globules with attached QDs conjugated *R. miehei* lipase on the surface at different time (0, 1, 3, 5, 6 and 8 min) (Scale bar = 5 μm)
8.5 Conclusion

Measurement of lipid fractions produced during the \textit{in situ} lipolysis of palm O/W emulsions using GC-FAME was able to quantify the synthesis of polar lipids as a consequence of lipase concentration and reaction time. Fatty acids were present in highest concentrations followed by monoglycerides. It was also observed that the \textit{R. miehei} lipase enzyme used in this work, favoured the splitting of unsaturated fatty acids from the triglyceride rather than the saturated fatty acid, resulting in higher monoolein (unsaturated) fractions being produced as the monoglyceride component. Palmitic acid was found to be the predominant fatty acid fraction produced through lipolysis.

By observing the droplet size distribution of the lipolysed emulsion, significant changes were found only when the lipolysis was carried out for extended periods of time (45 and 60 min). Complementary microscopic imaging showed increasing incidence of droplet aggregation. Structural changes were attributed to the accumulation of increasing concentrations of polar lipids, notably monoolein, at the oil-water interface, thereby enhancing the propensity towards quiescent aggregation. Findings show the importance of controlling reaction conditions (both enzyme concentration and reaction time) in order to provide requisite functionality (e.g. whipping properties) without excessively destabilising emulsions such that droplet structuring can occur under quiescent conditions. Subsequent Chapter 9 will demonstrate examples of the required functionality in two types of O/W emulsion food namely whipped cream and ice cream.
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

9.1 Abstract

Aerated food emulsions such as whipped cream and ice cream are complex food systems that require controlled destabilisation of the emulsion state to develop their requisite structure and texture. The stability of these aerated forms is owed partly to the partial coalescence of fat globules in the presence of small molecule emulsifiers such as monoglycerides. As an alternative to the use of synthetically manufactured monoglycerides, *R. miehei* lipase enzyme was used to generate the monoglycerides *in situ* during the manufacture of model whipped and ice cream compositions. This chapter aims to demonstrate the functionality of the lipolysed emulsion as used to create appropriate aerated structures in whipped cream and ice cream based on the synthesis of polar lipids at the oil-water interface (as demonstrated in previous chapters). O/W emulsions stabilised initially by a protein emulsifier i.e. sodium caseinate underwent lipolysis at varying enzyme concentrations before being aerated. Emulsions lipolysed with *R. miehei* lipase concentration above 0.25 mg / g fat all produced overruns of greater than 150 %. The lipolysed whipped emulsions were also observed to produce stable foams of similar consistency to that of commercial whipped cream. Stable foams with clean distinct sharp edges with no broken piping definition were obtained with 10, 15, 20, 25 and 50 mg lipase / g fat that remained stable with little to no syneresis after 1 h. The freeze-thaw stability of the emulsions was found to show very little cream shrinkage at lipase concentrations of 20, 25 and 50 mg / g fat. However, reducing the concentration to 5 and 2.5 mg / g fat resulted in very unstable foams that lost their structure after the freeze-thaw cycling. A similar trend was observed for serum drainage, whereby lipase at 2.5 and 5 mg / g fat had the largest volume of serum drainage. Foam firmness of the whipped emulsion decreased significantly
below a critical concentration of *R. miehei* lipase of 10 mg / g fat. In terms of microstructure, the droplet distribution of the lipolysed whipped emulsions produced a bimodal distribution, which was attributed to the partial coalescence of fat globules. Confocal micrographs likewise indicated a structure of agglomerated fat providing connectivity to fat droplet coated air bubbles, consistent for structures expected for whipped creams. Lipolysis was shown to have a positive influence on the structure and properties of ice cream. At fat content of 5 %, ice cream that had undergone lipolysis with 7.5 and 15 mg *R. miehei* lipase / g fat showed significantly slower rate of melt than without lipolysis. The melting rate was also comparable to ice cream made with the addition of 0.3 % commercial monoglycerides. Hardness analysis on the ice cream showed that ice cream made with low fat (5 %) in the absence of emulsifiers was the firmest. The presence of 0.3 % monoglycerides in low fat (5 %) ice cream was found to significantly decrease the hardness of ice cream, with a similar reduction in firmness observed for ice cream that had been lipolysed with *R. miehei* lipase at 7.5 mg / g fat. In conclusion, *in situ* lipolysis using *R. miehei* lipase enzyme was shown to successfully generate functional polar lipids that were effective at promoting partial coalescence and foam stabilisation in whipped cream and ice cream, with corresponding delivery of favourable product attributes.

### 9.2 Introduction

Whipped cream and ice cream are examples of emulsion-based food foams that are widely consumed for their creamy perception (Sajedi et al., 2014). Both whipped cream and ice cream can be produced from either dairy or non-dairy based fats with the requisite that lipid type must be appreciable solid fat content at the temperature of aeration. Whipped cream typically contains 30 to 40 % fat (Allen et al., 2006; Bazmi & Relkin, 2009), noting that even at these fat contents whipped cream cannot be made with unsaturated oils, as these have
an antifoaming effect during the whipping process (Leser & Michel, 1999). The generation of the whipped cream structure from its initial liquid O/W emulsion to a viscoelastic solid is a product of shear destabilisation, where semi-solid fat globules in the cream during whipping undergo partial coalescence (Allen et al., 2006; Stanley et al., 1996). The partially coalesced fat is an integral part in providing mechanical rigidity to the foam. The incorporated air bubbles during whipping are also stabilised by the adsorption of fat globules to the bubble interface, forming a structure of fat-coated bubbles trapped within an agglomerated interconnected network of fat (Bazmi & Relkin, 2009; Boode & Walstra, 1993). In addition to providing physical stability to the foam, this structure provides sought after texture and mouthfeel associated with whipped cream (Camacho et al., 2001).

The development of whipped cream structure occurs over three phases. Firstly, a large volume of air bubbles around 40 - 60 % volume is incorporated by the whisking action and (milk) protein is predominant in stabilising the newly formed air/serum interface (Anderson et al., 1987; Camacho et al., 2001; Hotrum et al., 2005). Figure 9.1 depicts the structure development taking place during the formation of whipped cream. Secondly, depending on the shearing parameters, the incorporated air bubbles will gradually be further comminuted into smaller ones and a narrow air bubbles size distribution is reached. Fat globules will adsorb onto the air/serum interface forming a densely packed coverage (Bazmi & Relkin, 2009; Camacho et al., 2001), and this aspect of the process is also believed to facilitate partial coalescence of fat droplets. The partial coalescence of fat globules eventually leads to formation of a network trapping the air bubbles and serum phase in an irregular grid-like matrix that contribute to the rigidity of the whipped cream (Hotrum et al., 2005). The aggregation of the fat globules is greatly influenced by crystallisation of the fat phase (Dickinson, 1992), hence the need for cooling to about 4 °C prior to whipping (Moran, 1994).
Also of consequence to the whipping process is the composition of the oil-water interface (as determined by the addition of emulsifiers).

Fat globules (containing fat crystals) dispersed in O/W emulsion typically milk cream

Large air bubbles introduced into the cream upon shear, fat globules start to partially coalesced with each other

Continued shearing produced smaller air bubbles and facilitate the formation of partially coalesced fat globules network trapping the air bubbles

Reference: Anderson et al. (1987); Bazmi & Relkin (2009); Camacho et al. (2001); Hotrum et al. (2005)

Figure 9.1: Schematic diagram of the structure development taking place during the formation of whipped cream
Various parameters have been used to describe the endpoint of the whipping process such as maximum overrun, index of globule clumping and maximum stiffness; however, after the endpoint, excessive whipping will often cause the foam to collapse due to rupture of the air bubbles as the fat globules agglomerate into large clumps. This will result in phase inversion of the emulsion as it starts to churn like in butter production (Allen et al., 2006; Hotrum et al., 2005).

Ice cream is another example of an aerated food emulsion, but is more structurally complex because of the additional presence of ice crystals. Furthermore, the unfrozen serum phase may contain a variety of other ingredients compared to whipped cream, such as sugars and stabilisers (notably high molecular weight polysaccharides) (Goff, 1997a). Ice cream can contain varying amounts of fat dependent on whether the ice cream is premium, standard or low fat. Standard ice cream typically contains a minimum of 10 % fat, with about 40 - 50 % air by volume, 10 % milk solids, 15 % sugars and 60 - 65 % water by weight (Stanley et al., 1996; Thomas, 1981). Due to the lower fat content in ice cream compared to whipped cream, the extent of partial coalescence in ice cream tends to be lower (Goff, 1997a). Nevertheless, ice cream can contain low amount of fat because the emulsion mix will be whipped concurrently with the cryo-concentration of the aqueous phase during freezing (Bazmi & Relkin, 2009). Similar to whipped cream, the fat in ice cream undergoes crystallisation when the emulsion is cooled to 4 °C after pasteurisation and homogenisation. The mix is cooled typically for 8 h or overnight in a process called aging (Goff, 1997a), allowing equilibrium of both the solid fat content and emulsifier adsorption (when included in the formulation) to be reached.
Development of good whipped cream and ice cream structure benefits from the controlled destabilisation of fat during processing which in both cases results in partial coalescence and adsorption of fat droplets to the air bubble interface. Partial coalescence is a result of intrinsic instability of an emulsion. When the emulsion is subjected to shear, the interfacial protein-emulsifier barrier will rupture (this can be greatly facilitated by the presence of crystalline fat at the droplet surface that enables penetration of the interface). Because the fat globules are a mixture of liquid and crystalline fat form, not all liquid fat will flow during the coalescence hence the incomplete coalescence called partial coalescence (Leser & Michel, 1999). Partial coalescence will also result in increase of the serum viscosity resulting in decrease in air bubble size and movement (Anderson et al., 1987), with the growth in size of the individual fat globules (Shim et al., 2003) that is crucial in forming a continuous network that still retains some globular shape and has the ability to build structure during the whipping operation (Stanley et al., 1996).

To achieve partial coalescence, it is a common practice to add low molecular weight emulsifiers, such as monoglycerides, into the emulsion mix. The presence of monoglycerides is not to aid emulsification (in terms of enhancing emulsion stability), but rather to promote protein displacement at the oil-water interface, thereby reducing the interfacial stability of fat globules, and enabling partial coalescence of the fat globules under shear (Euston et al., 1995; Goff, 1997b; Hotrum et al., 2005; Ihara et al., 2010; Stanley et al., 1996). The desorption of proteins from the oil/water interface by different types of monoglyceride has been demonstrated by various authors (Barfod, 1995; Boots et al., 2002; Carrera Sánchez & Rodríguez Patino, 2004; Davies et al., 2000; 2001; Rahman & Sherman, 1982; Reis et al., 2009a). Thus, the addition of emulsifiers improves the whipping process with increased overrun, reduction of whipping time and good product consistency (Stanley et al., 1996). In
ice cream production, the ice cream mix with added monoglycerides will have partially coalesced fat globules that are crucial in forming a semi continuous matrix of fat structure. This is favourable because the ice cream produced will have a drier appearance, will be easier to handle, mould or extruded into various shapes, will have a smoother texture, creaminess and good melt resistance (Goff, 1997a; Pelan et al., 1997; Segall & Goff, 1999; Zhang & Goff, 2005).

The incorporation of monoglycerides into whipped cream and ice cream has been widely reported to decrease the amount of adsorbed protein (Davies et al., 2000; Pelan et al., 1997) due to the orogenic mechanism suggested by Gunning et al. (1999) and Mackie et al. (1999). The adsorption of the low molecular weight emulsifier happens at the gaps in the protein stabilised interface; this will lead to the reduction of surface tension of the interface and compression of the protein network. Thus, the protein network will eventually weakened and desorb from the interface.

Commercial monoglyceride emulsifiers are usually a mixture of saturated and unsaturated monoglycerides (Davies et al., 2001). They are produced synthetically through interesterification of fats and oils with glycerol at high temperature of 180 – 230 °C with the presence of alkaline catalyst (Young et al., 1994). The synthetic process may not appeal to consumers who want a ‘clean’ label and all natural products. Furthermore fats and oils of unknown animal origin maybe an issue for certain religious or ethnic concerns (Goff, 1997a).

In lieu of this concern, we have previously demonstrated in previous chapters, the ability of employing lipase enzyme to generate monoglycerides in situ of an emulsion. This chapter aims to demonstrate that the functionality of enzymatically synthesised monoglycerides as part of in situ processing is able to deliver appropriate microstructures and product
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

properties when applied to aerated food emulsions (for both whipped cream and a more complex frozen ice cream).

9.3 Materials and methodology

9.3.1 Materials

Details for the *R. miehei* lipase, sodium caseinate and palm oil used have been described in Chapter 3 (subsection 3.1). Skim milk powder (SMP) was obtained from Fonterra (Fonterra Co-operative Group, New Zealand). Vanilla extract was purchased from the local supermarket. Sucrose used was commercially available table sugar from the supermarket. Guar gum was from Hawkins Watts Ltd (Auckland, New Zealand). Commercial monoglycerides Palsgaard® DMG 0093 (Palsgaard Asia Pacific Pte Ltd, Singapore) was used. The monoglycerides powder is the distillation product of vegetable fatty acids.

9.3.2 Methodology

9.3.2.1 Processing of emulsion mix for whipped cream

The process flow chart for the emulsion mix to be used as whipping cream is shown in Figure 9.2. Sodium caseinate, used as the protein emulsifier, was dispersed in RO water to achieve a final concentration of 1 wt %. The mixture was mixed using an overhead stirrer for at least 2 h at 21 °C to ensure complete dissolution. Palm oil was melted in a stainless steel bucket immersed into a steam jacketed pan. Palm oil used was weighed to contribute 30 wt % towards the final emulsion. After ensuring all the solid palm oil had melted by visual inspection, the sodium caseinate solution was then mixed together into the melted palm oil. This mixture was then warmed to about 60 °C in a steam jacketed pan. This was to ensure all the palm oil used had melted and did not solidify back upon mixing with the emulsifier solution. The following steps as described below were carried out:
i) Pre-homogenisation

The warmed mixture was then homogenised using a conventional homogeniser (LabServ homogeniser D500, Biolab Ltd, New Zealand) for 1 min to produce a coarse temporary O/W emulsion.

ii) Homogenisation

The coarse emulsion was then homogenised using a two-stage Rannie homogeniser (Albertslund, Denmark) operating at first-stage pressure of 10 MPa and a second-stage pressure of 3.5 MPa. The emulsion was homogenised twice for effective homogenisation.

iii) Addition of skim milk powder (SMP)

Skimmed milk powder was added to the homogenised emulsion and stirred using an overhead stirrer for 2 h at 21 °C, to produce an SMP concentration of 6 wt %. The SMP was added as a source of non-fat milk solids as well as contribute to light dairy flavour. The lactose content also helps in controlling sucrose crystallisation.

iv) Lipolysis of emulsion mix

The emulsion mix was then treated using *R. miehei* lipase enzyme at different concentrations (0.05, 0.25, 1.25, 2.5, 5, 10, 15, 20, 25 and 50 mg / g fat) and allowed to lipolyse for 15 min at 21 °C.

v) Pasteurisation

The lipolysed emulsion mixes were then passed through a plate heat exchanger pasteuriser (Ultra high temperature pasteuriser plant, In-house built, Pilot Plant, IFNHH, Massey University, Palmerston North, New Zealand) at 110 °C and with a
holding time of 10 s. The output temperature was set at 18 °C to quickly cool down the emulsion temperature. The heating of the lipolyzed emulsion mixes aimed not only to pasteurise the emulsion but also to ensure inactivation of the lipase enzyme, hence the higher temperature. The emulsion mix was then collected into plastic containers in a laminar flow cabinet (Air Care Technology Ltd, Australia).

vi) Aging of emulsion mix

The pasteurised emulsions were then kept in a 4 °C chiller for overnight (>12 h) to age the emulsion.
Figure 9.2: Flow chart of emulsion mixes processing for whipping cream

1. Dispersing of sodium caseinate into solution
2. Melting palm oil
3. Warming the mixture to 60 °C
4. Pre homogenisation - hand held homogeniser
5. Homogenisation - 2 stage homogeniser
6. Addition of SMP
7. Lipolysis with *R. miehei* lipase at 21 °C for 15 min
8. Pasteurisation at 110 °C for 10 s
9. Aging at 4 °C overnight >12 h
10. Emulsion mix ready for whipping process
9.3.2.2 Whipping process and determination of overrun of whipped lipolysed emulsion

Emulsions that have been aged overnight were taken out from the chiller immediately prior to whipping. The aged emulsions (1000 g) were aerated using an electric stand mixer (Kenwood Titanium Chef KM010) with a standard wire whisk attachment. The stainless steel bowl of the mixer was chilled before use. The overrun of the emulsions samples were determined as volume fraction of air incorporated after whipping (Allen et al., 2008). A small amount of the aged emulsion mix was transferred into a tared 50 ml plastic cup using a spatula, excess mix was levelled off before being weighed. The mixer set to run at speed level 4. Every minute of the whipping process, a small amount of sample was carefully removed from the centre of the mixing bowl into the same tared 50 ml plastic cup used earlier. The whipped emulsion mix was then returned to the mixing bowl after each weighing. Any entrapped air and artificial bubbles were avoided to ensure only the sample occupied the volume area of the cup. Excess whipped emulsion was levelled off before being weighed.

The whipping process was stopped when the weight of the whipped emulsion no longer decreased or has started to increase instead. The overrun of the whipped emulsion was determined as:

\[
\% \text{ Overrun} = \frac{\text{weight of aged emulsion} - \text{weight of whipped emulsion}}{\text{weight of whipped emulsion}} \times 100
\]
9.3.2.3 Processing of ice cream

The formulations of processed ice cream are as described in Table 9.1. Apart from SMP, guar gum and sugar were added into the homogenised emulsion. Vanilla flavour was added after the aging process. The processing of ice cream premix was similar to the emulsion processing for whipping cream but with slight modification as shown in Figure 9.3.

The aged premix was processed using a continuous ice cream freezer (Tetra Pak KF 80, Denmark). The machine freezes, mixes and whips the ice cream mix to incorporate air automatically. Overrun was set at 100 %. Ice cream collected was filled into plastic containers and kept in the freezer at -18 °C for at least 2 days before analysis to allow the samples to harden.

### Table 9.1: Formulation of ice cream mix

<table>
<thead>
<tr>
<th>%</th>
<th>Full fat control</th>
<th>Low fat with emulsifier</th>
<th>Sample -Low fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm oil</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>SMP</td>
<td>12</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Sucrose</td>
<td>12</td>
<td>13.7</td>
<td>14</td>
</tr>
<tr>
<td>Guar gum</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Monoglyceride</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Vanilla flavour</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Water</td>
<td>65.75</td>
<td>65.75</td>
<td>65.75</td>
</tr>
</tbody>
</table>
Figure 9.3: Flow chart of ice cream processing

- Dispersing of sodium caseinate into solution
- Melting of palm oil
- Warming the mixture to about 60 °C
- Pre homogenisation - hand held homogeniser
- Homogenisation - 2 stage homogeniser
- Addition of SMP, guar gum and sugar (2 h)
- Lipolysis with *R. miehei* lipase (lipase concentrations - 2.5, 7.5 and 15 mg / g fat), lipolysis time - 15 min, temperature - 21 °C or addition of 0.3 % monoglyceride
- Pasteurisation at 110 °C for 10 s
- Aging at 4 °C overnight
- Addition of vanilla flavour
- Ice cream processing
9.3.2.4 Physicochemical analyses

9.3.2.4.1 Visual test and piping stability of whipped cream

Emulsions were photographed before and after the aging process. This included both lipolysed and non-lipolysed emulsions. The non-lipolysed emulsion was also whipped and photographed to observe any physical changes to the consistency. The piping stability test was carried out by whipping each emulsion at their maximum overrun values obtained from the overrun test. The whipped emulsion was filled into a piping bag equipped with a star shape nozzle used for cake decoration. By using the star shape nozzle, the piped whipped emulsion will possess distinct ripple and edges, and this will allow observation on how well the piped cream stand before collapsing or any noticeable change that may happen. The piped samples were assessed visually at 0 and 1 h after piping in a temperature-controlled room at 21 °C and description of any visible changes were noted.

9.3.2.4.2 Determination of freeze-thaw stability of whipped cream

The determination of freeze-thaw stability was carried out following Lundin (2013) with slight modifications. The whipped samples were carefully transferred into plastic containers avoiding any trapped air and were then gently tapped on the table to ensure maximum filling of the container. The surface was then levelled off with a spatula. Sample were then frozen at -18 °C for at least 24 h. The plastic containers were then taken out of the freezer and placed in a temperature-controlled room at 21 °C. The samples were then allowed to thaw for 3 h. To measure the height of the cream shrinkage, we used a paper ruler with scale in millimetres that was inserted into the edge of the bottle (Figure 9.4). Visual observation of the cream after shrinkage was also noted.
9.3.2.4.3 Determination of serum drainage of whipped cream

The rate of serum drainage was determined by placing 20 g of whipped emulsion on a glass funnel at ambient temperature (21°C). The glass funnel was suspended over a tared conical flask to collect the drained serum from the whipped emulsion as shown in Figure 9.5. The conical flask were weighed after 1 h to measure the serum collected, as this will be an index of stability of the whipped emulsion (Padiernos et al., 2009).

The percentage of serum drainage was calculated as follow:

\[
\% \text{ serum drainage} = \left( \frac{\text{weight of collected serum}}{\text{weight of whipped emulsion}} \right) \times 100
\]
9.3.2.4.4 Determination of hardness of whipped emulsion

The determination of hardness was investigated using a TA. XT Plus texture analyser (Stable Micro Systems, Surrey, UK). Whipped emulsions were carefully transferred into a polypropylene plastic container (volume 70 ml, diameter 40 mm) using a spatula. The container was filled only three quarter full avoiding any void gap or air pockets. This was to ensure that the force measurement recorded by the texture analyser was not influenced by the empty spaces in the container. An acrylic disk probe with a diameter of 38 mm was used to a penetration depth of 25 mm at a speed of 2 mm s\(^{-1}\). The system was loaded with a 5 kg load cell. The force (N) recorded was analysed and charted by the Exponent software. The experiment was carried out at 21°C.

9.3.2.4.5 Determination of hardness and tackiness of ice cream

The determination of hardness and tackiness was carried out using a TA. XT Plus texture analyser (Stable Micro Systems, Surrey, UK). Ice cream for texture analysis was packed in a rectangular cardboard box. A hacksaw was used to cut off a cube of the ice cream measuring 4.5 cm x 5 cm x 5 cm. The ice cream was then returned back to the freezer for 2 h. Each
sample was immediately analysed after taken out from the freezer to avoid melting of the ice cream. Samples were transferred onto the centre of the texture analyser stage. An acrylic cylindrical probe with a diameter of 13 mm and 40 mm height was used to a penetration depth of 30 mm at a speed of 2 mm s\(^{-1}\). The system was load with a 5 kg load cell. Hardness of ice cream was indicated by the peak positive force recorded to achieve the indentation by the plunger, while tackiness was expressed as the peak negative force obtained during the plunger withdrawal. The force (N) recorded was analysed and charted by the Exponent software. The experiment was carried out at 21°C.

**9.3.2.4.6 Determination of emulsion droplet size for whipped cream and ice cream**

The determination of the average droplet diameter was carried out using procedures described in Chapter 3, subsection 3.2.2. Whipped emulsion and ice cream samples were diluted with water (1:10) before being introduced into the recirculating water in the Hydro MU measuring cell unit until an obscuration rate of 11 - 12% was obtained as indicated by the instrument.

**9.3.2.4.7 Microscopic observation of whipped cream by confocal laser scanning microscope**

Microscopic observation of emulsion droplets was carried out using confocal laser scanning microscope described in Chapter 3, subsection 3.2.4. For the analysis of whipped emulsion, the dyes were added to the emulsion mix prior to aging, before being subsequently whipped the next day. A small scoop of the aerated emulsion was then transferred onto a concave glass microscope slide. A cover slip was carefully placed over the sample avoiding trapping any air bubbles and to minimise disruption to the sample structure.
9.3.2.5 Statistical analyses

All analyses were carried out with at least in triplicates. Analysis of variance (ANOVA) with post hoc Tukey test was performed using MINITAB 16 (Minitab Inc., State College, PA, USA) to determine the differences between mean of the physicochemical analyses of whipped cream and ice cream, where possible. All statistical analyses were considered significant when p<0.05.

9.4 Results and discussion

9.4.1 Physicochemical analyses of whipped cream made with lipolysed emulsion

9.4.1.1 Whipping process and overrun

The whipping of the lipolysed emulsion mixes tested in this study all resulted in a stable aerated foam of the emulsion similar to what can be achievable when milk cream is whipped. The overrun of the emulsion are shown in Figure 9.6. It can be seen from Figure 9.6 that lowering the lipase concentration down to 0.05 mg of \( R. miehei \) lipase per g of fat produced a whippable emulsion with a maximum of 141 % overrun, although a longer whipping time (10 min) was required to generate this overrun. Increasing enzyme content to 0.25 and 1.25 mg \( R. miehei \) lipase per g of fat produced foams with 176 and 182 % respectively after 4 min whipping. Utilising \( R. miehei \) lipase at concentrations higher than 1.25 mg / g fat resulted in higher overruns of between 254 – 348 %. However, it was noted that overrun did not increase linearly with enzyme concentration; reaching a maximum of 350 % for 10 mg of lipase / g fat instead, and dropping back to 250 % for 50 mg lipase / g fat.

A good whipping cream should be easy to whip and can retain the incorporated air (Kováčová et al., 2010). Thus, overrun is often used to gauge the effectiveness of the cream’s internal structure to hold the newly formed foam (Jakubczyk & Niranjan, 2006; Kováčová et
The maximum or close to maximum overrun are usually considered the endpoint of the whipping process and relate to maximum stability and stiffness of the whipped cream (van Aken, 2001; Walstra et al., 2006; Zhao et al., 2009).

**Figure 9.6: Overrun of whipped O/W emulsion (30 % fat) lipolysed with different \textit{R. miehei} lipase concentration (Lipolysis time - 15 min)**

The stability of the foam is reported to be as a result of coalesced fat droplets arranging evenly on the serum/air interface thus encapsulating the air bubbles (Zhao et al., 2009). Whipping introduces a large amount of air into the emulsion as large bubbles, with ongoing shearing causing the bubbles to be comminuted into smaller bubbles. However, in the early stages of whipping these newly formed air bubbles will not be able to stay inside the emulsion due to the lack of mechanical immobilisation of the foam. Halting the whipping process too soon will cause the air bubbles to rise onto the surface and burst into the air (van Aken, 2001). The whipping process also provides the shearing energy on the emulsion causing the aggregation of the fat globules which provides the reinforcing scaffold to the
foam. These air bubbles and network of fat globules ultimately provide the stiff, stable structure of whipped cream (Ihara et al., 2010; Stanley et al., 1996; van Aken, 2001). The extent of agglomeration likewise has an impact on cream properties. Too little agglomeration and the structural rigidity will be insufficient to provide stand-up to the foam. However, excessive agglomeration can lead to the formation of compact butter grains, such that the fat network is destroyed. Excessive fat agglomeration can also have an antifoaming effect on the stability of the bubbles as well.

To confirm the crucial role of lipolysis in producing whippable emulsions, an attempt was made to whip a protein stabilised emulsion that had not undergone lipolysis. The whipped emulsion failed to achieve a satisfactory overrun (Figure 9.6 – 0 mg lipase / g fat). The maximum overrun achieved was just below 20 % after 11 min of whipping time. All emulsions that showed good overrun had undergone similar processing and shear condition apart from the different lipolysis conditions. The creation of appropriate whipped structures is considered a consequence of polar lipid synthesis (sn-2 monoglycerides and fatty acids) arising from lipolysis of the palm oil triglycerides core of the emulsion droplets, and adsorption of these surface active moieties at the oil-water interface. This is in agreement with various authors suggesting that apart from the shear induced partial coalescence of the fat droplets, the presence of small molecules emulsifiers such as mono- and di-glyceride helps weaken the adsorbed protein network to encourage partial coalescence (Bos et al., 1997; Goff, 1997b; Rodríguez Niño & Rodríguez Patino, 1998). Pelan et al. (1997) reported that the addition of monoglycerides improved air cell stability during the whipping process due to the adsorption of the protein-displaced fat droplets on the air bubble interface.
9.4.1.2 Visual observation and piping stability of the whipped emulsion

Visual images from Figure 9.7 show the appearance of non lipolysed emulsion after extended aging for > 12 h at 4 °C. It can be seen that the emulsion maintained its liquid characteristic similar to before the aging process. However, from Figure 9.8, it can be observed that the consistency of the most highly lipolysed emulsion (50 mg lipase/ g of fat) had changed from liquid to almost solid. The aged emulsion had a scoopable soft solid like consistency, however as the concentration of lipase used decreased, the viscosity of the emulsion reduces as well. The different physical characteristics of the aged emulsion between the non-lipolysed and lipolysed emulsion showed that there are changes happening on their internal structure. The greater the extent of lipolysis, the greater the displacement of protein from the interface and at elevated lipase concentrations this was clearly sufficient to cause quiescent partial coalescence of the fat globules during the aging process. The aging of emulsion usually at 4 °C have been reported to change the state of both emulsified fat droplet as well as the interfacial layer of lipid and protein emulsifiers (Krog & Larsson, 1992). Zhang & Goff’s (2004) Transmission Electron Microscope images of ice cream mix found that the release of casein was initiated during the aging aged at 4 °C overnight. Davies et al. (2000) reported an extensive destabilisation and thickening after cooling of emulsions containing 3.5 % monooleate. The emulsion displayed a cream-cheese-like consistency and a very high apparent viscosity.
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

Figure 9.7: Non-lipolysed emulsion remained in liquid form after aging process overnight at 4 °C

Figure 9.8: Scoopable soft solid structure of lipolysed emulsion after aging overnight at 4 °C (Lipolysis condition: R. miehei lipase concentration 50 mg per g of fat, 15 min)
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

It has been mentioned in the previous subsection, that when the non lipolysed emulsion was whipped, it failed to achieve a satisfactory overrun and no stable aerated structure was obtained although the initial emulsion did contain high amount of fat (30%). Figure 9.9 depicts the visual images of the sample where the bulk of the emulsion remained liquid, with some temporary bubbles and distinct clumped or agglomerated fat started to appear. Whipping cream typically contains a fat content between 30 - 40% (Kováčová et al., 2010; Sajedi et al., 2014). Whilst a publication by Smith et al. (2000a) indicated that a cream with at least 30% fat content could be aerated to achieve a stable foam structure. According to van Lent et al. (2008), whipping of cream is a destabilisation process to encourage the partial coalescence of fat globules. This will not be successful if the interfacial layer is too strong and the emulsion is too stable (Leser & Michel, 1999). Davies et al. (2001) work on protein stabilised emulsion found that emulsion with no added monoglycerides was quiescently stable for about a month and was not affected by shearing.

![Figure 9.9: Agglomerated fat globules forming after whipping of non-lipolysed emulsion](image)

When the lipolysed emulsion was whipped, a stable aerated structure was achieved similar to that expected for a whipped dairy cream. The piping stability of the whipped emulsion is shown in Figure 9.10. Emulsions were whipped to about their maximum overrun based on
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

the results in the previous section (Figure 9.6). From Figure 9.10, it can be seen that stable foams with clean distinct sharp edges with no broken piping definition were obtained with 10, 15, 20, 25 and 50 mg \textit{R. miehei} lipase per g of fat. Foam remained stable with no visual syneresis or floating out of the cream after 1 h. Only some drying of the outer layer of the cream was observed. These very stable creams might suggest that the lipolysis may have been taken a bit too far as to produce too much mono- and di-glycerides needed. On, the other hand, whipped emulsions produced with \textit{R. miehei} lipase at 5, 2.5 and 1.25 mg per g of fat had stable foam but with softer edges. These foams however had syneresis and floated out after 1 h. Foam from 0.25 mg lipase / g fat was very soft and collapsed quickly after piping.
Table 9.1: Piping stability of whipped emulsion lipolysed with different lipase concentration at ambient conditions (21 °C)

<table>
<thead>
<tr>
<th>Lipase Concentration (per g of fat)</th>
<th>0 h</th>
<th>1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 9.10: Piping stability of whipped emulsion lipolysed with different lipase concentration at ambient conditions (21 °C)
Continued from previous page

<table>
<thead>
<tr>
<th>R. miehei lipase concentration (per g of fat)</th>
<th>0 h</th>
<th>1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mg</td>
<td><img src="image1.png" alt="Image of whipped cream and ice cream" /></td>
<td><img src="image2.png" alt="Image of whipped cream and ice cream" /></td>
</tr>
<tr>
<td>10 mg</td>
<td><img src="image3.png" alt="Image of whipped cream and ice cream" /></td>
<td><img src="image4.png" alt="Image of whipped cream and ice cream" /></td>
</tr>
<tr>
<td>5 mg</td>
<td><img src="image5.png" alt="Image of whipped cream and ice cream" /></td>
<td><img src="image6.png" alt="Image of whipped cream and ice cream" /></td>
</tr>
</tbody>
</table>

Figure 9.10 (continued)
Although lower concentrations of *R. miehei* lipase concentration down to 0.05 mg / g fat did produce whippable emulsions with over 100 % of overrun, the piping stability test showed a noticeable lack of stability after certain period of time. However, enzyme concentrations above this level were able to produce a whippable emulsion with good stability. It has been
reported that a good stable whipped emulsion is achieved by the adsorption of partially coalesced fat droplets on the air bubbles interface forming a network around the air bubbles, hence acting as a physical barrier require for good stability (Boode & Walstra, 1993; Goff, 1997b).

During the initial stage of whipping, the oil-water interfaces are covered by protein which will eventually change into an intermingled layer of proteins and fat globules. Subsequent whipping will then promote partial coalescence of the fat globules and further adsorption of fat on the air bubbles (Hotrum et al., 2005; Needs, 1991). Because of the shearing force and temperature during whipping, the partially coalesced fat will release some liquid fat which acts as a glue on the air bubble interface and also between the fat droplets. This is very important to achieve a stable whipped cream (Hotrum et al., 2005). However, the temperature during whipping is usually kept low so that the bulk of the crystallise fat remains crysrystallised. As discussed earlier in Chapter 7 (subsection 7.4.4), the crystallised fat is crucial for the rigidity and stable structure of the whipped emulsion. The crystallise fat also hinder the complete coalescence of the fat into a single globule. At the end of whipping, the partially coalesced fat globules had form a network of protective layer on the air bubbles, bridging and immobilising the air bubbles and forming pockets of trapped serum phase providing the stiff texture of the whipped emulsions (Allen et al., 2006; Goff, 1997b; Hotrum et al., 2005; Needs, 1991; van Aken, 2001).
9.4.1.3 Freeze thaw stability of whipped emulsions

Freezing of the whipped emulsion causes changes in the serum phase as it turns into ice, the ionic strength and solute concentration also changes. This phenomenon named cyro-concentration greatly affects the structure and stability of the whipped emulsion (Dickinson, 1992). The growth of ice crystals during freezing as well as crystallisation of the fat globules might result in triggering physical damage to the air bubbles (Krog & Larsson, 1992) especially visible by the collapsed of the whipped emulsion structure upon thawing.

Figure 9.11 shows the selected images of whipped emulsions tested for freeze-thaw stability to depict samples with little or a lot of shrinkage. Upon taking out the emulsion from the freezer, the frozen whipped emulsion still filled up the plastic container up to the brim. After the whipped emulsions were allowed to thaw, different levels of shrinkage can be observed. Table 9.2 shows the shrinkage height of the thawed cream. It can be observed that emulsion lipolysed with *R. miehei* lipase concentration of 20, 25 and 50 mg per g of fat showed very little cream shrinkage and the collapse heights recorded were not significantly different (p<0.05) between the higher levels of lipase. Medium amount of shrinkage was observed for emulsion lipolysed with 15 mg *R. miehei* lipase / g fat, followed by a lot of shrinkage with 10 mg lipase / g fat. Reducing the concentration of lipase to 5 and 2.5 mg / g fat resulted in very unstable foam that lost its properties after the freeze-thaw experiment. The overall trend observed was that high level of shrinkage occurred when lower concentrations of *R. miehei* lipase were used. This proves that the level of lipolysis determines how much of the partially coalesced fat is holding the air bubbles structure in the whipped emulsion. The weaker structure found with the lower lipase level, suggests little network of the partially coalesce fat is holding the air bubbles. The collapsing of the aerated foam can be attributed to several factors including the drainage of serum phase and the fusing of smaller air bubbles to larger
bubbles (Smith et al., 1999) due to the lack of sufficient fat globules or network protecting the air interface.
Table 9.2: The collapsed height of whipped emulsion and assessment on the cream shrinkage after the freeze-thaw test

<table>
<thead>
<tr>
<th>R. miehei lipase concentration per g of fat (mg)</th>
<th>Collapsed height</th>
<th>SD</th>
<th>Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.57</td>
<td>± 0.12^a</td>
<td>There was little palpable shrinkage in the creams.</td>
</tr>
<tr>
<td>25</td>
<td>0.70</td>
<td>± 0.1^ab</td>
<td>There was little palpable shrinkage in the creams</td>
</tr>
<tr>
<td>20</td>
<td>0.63</td>
<td>± 0.06^a</td>
<td>There was little palpable shrinkage in the creams</td>
</tr>
<tr>
<td>15</td>
<td>0.93</td>
<td>± 0.06^b</td>
<td>There was medium amount shrinkage in the cream.</td>
</tr>
<tr>
<td>10</td>
<td>1.97</td>
<td>± 0.12^c</td>
<td>There was a lot of shrinkage in the cream.</td>
</tr>
<tr>
<td>5</td>
<td>2.70</td>
<td>± 0.17^d</td>
<td>The creams had lost its foam properties. A lot of serum drainage.</td>
</tr>
<tr>
<td>2.5</td>
<td>3.47</td>
<td>± 0.06^e</td>
<td>Completely liquid state, no foam properties left.</td>
</tr>
</tbody>
</table>

Note:
- Means with different superscript letters within the same column are significantly different (p<0.05)
- SD = Standard deviation

9.4.1.4 Serum drainage of the whipped emulsion

Measurement of serum drainage has been used to assess the stability of aerated emulsion (Smith et al., 1999; Stanley et al., 1996). Figure 9.12 shows the serum drainage of whipped emulsions produced an hour after whipping. It can be clearly observed that emulsions lipolysed with lower concentration of R. miehei lipase concentrations at 2.5 and 5 mg per g of fat had the largest percentage of serum drainage. There were no significant differences (p<0.05) found for whipped emulsions lipolysed with R. miehei lipase concentration < 10 mg per g of fat. Kováčová et al. (2010) described that a good whipped cream should have good stability and should not be prone syneresis. Allen et al. (2006) also stated that a fully whipped dairy cream can be stable from any serum drainage for approximately 90 min. In addition, Stanley et al. (1996) described weak stability and pronounce serum drainage for whipped
cream without any added emulsifiers compared to whipped cream containing mono and diglycerides emulsifiers.

Note:
- Different letters above the bars indicate statistical significance between the means serum drainage (%) (p<0.05)

**Figure 9.12:** Serum drainage of whipped emulsion lipolysed with different lipase concentration after 1 h at ambient temperature (21 °C)

9.4.1.5 Determination of hardness of whipped emulsion

Obtaining desirable textural characteristics is one of the goals for food production (Stanley, Goff, & Smith, 1996). Firmness represents the force necessary to attain a given deformation (N). Figure 9.13 shows the maximum force recorded for each of the samples. Because of the instability and collapsing of the aerated emulsion obtained with lower *R. miehei* lipase concentration < 10 mg per g of fat, the measurement of hardness was not possible with those samples. The hardness of the whipped emulsion decreased as the lipase concentration
decreased. This corresponds to the results in the previous section, where the higher lipase concentration resulted in a sturdier and rigid foam formation. Texture of an aerated emulsion is influenced by the incorporation of air bubbles into the system (Jakubczyk & Niranjan, 2006) as well as the fat/water interface, air/water interface and proteins (Zhao et al., 2009).

Note:
- Different letters above the bars indicate statistical significance between the means (p<0.05)

Figure 9.13: Hardness of whipped emulsion represented as maximum force (N) recorded
9.4.1.6 Droplet size distribution of whipped emulsion

The droplet size distribution of a non-lipolyzed emulsion showed a monomodal distribution (Figure 9.14). When this emulsion was whipped, it failed to achieve any stable aerated foam structure as described in previous section. Instead the whipped emulsion had granules of agglomerated fat and this can be seen in the droplet distribution, where a second smaller peak was observed. However this peak falls at a very large size range of 100 μm. When lipolyzed emulsions were whipped, all samples produced bimodal droplet distribution as can be seen in Figure 9.15. This is because the droplet size profile will shift to a larger range when a cream is whipped due to the effect of mechanical shear and the introduction of air bubbles into the system (Zhao et al., 2009). The appearance of a bimodal distribution was similar to (Goff, 1997a) findings which represents the partial coalescence of fat globules.

![Figure 9.14: Droplet size distribution of non-lipolyzed emulsion in its original form and after whipping](image-url)
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

9.4.1.7 Microscopic observation of whipped emulsion microstructure

Figure 9.16 shows the microscopic observation of whipped cream obtained in this study from lipolysed emulsion. It can be observed that the extent of fat coalescence was very much influenced by the lipase concentration. Higher concentration of lipase resulted in higher degree of fat coalescence. van Aken (2001) investigated the light microscopic observation of whipped cream and found the presence of individual fat globules as well as aggregated fat globules. The aggregated fat globules were clumped by partial coalescence and are irreversibly since dilution did not break the aggregates into individual globules.

Furthermore, these images also clearly revealed how the air bubbles (in black) were stabilised by the partially coalesced fat globules. High concentration of *R. miehei* lipase (25 and 50 mg per g of fat) proved to be excessive in a way that the fat globules have coalesced
too much. The adsorption adhering of fat globules on the air bubbles interface has been widely reported by various authors (Barfod, 1995; Smith et al., 2000b; Widlak et al., 2001). Partially coalesced fat is an irreversible agglomeration of the fat globule, glued together by the liquid fat and maintain its structure owing to the crystallised portion of the fat (Goff, 1997a).
Chapter 9: Functionality of lipolysed O/W emulsion in imitation whipped cream and ice cream

Figure 9.16: Confocal micrographs of whipped cream from emulsion lipolysed with various *R. miehei* lipase concentration (black-air bubbles, red/yellow-fat, green-protein in serum phase), scale bar = 10 μm
9.4.2 Physicochemical analyses of ice cream made with lipolysed emulsion

9.4.2.1 Determination of melt stability

Figure 9.17 shows the melt stability test carried out on ice cream produced with different formulations. It can be observed that ice cream with lower fat content at 5% without any added emulsifier had the fastest melting rate. This is similar to ice cream with 5% and *R. miehei* lipase 2.5 mg / g fat. Ice cream made with a higher fat content of 10% but without any emulsifier had better melting rate. Further improvements in melt stability were observed for the 5% fat with 0.3% added monoglycerides formulation. Interestingly, the melting rate became much slower for ice cream made with 5% fat with *R. miehei* lipase 15 mg / g fat and the slowest with *R. miehei* lipase 7.5 mg / g fat. Hotrum et al. (2005) reported that destabilisation of ice cream mix upon shearing was influenced by the presence of low molecular weight emulsifiers. This is in agreement with various other authors (Goff, 2002; Pelan et al., 1997). It was also reported the whipping time was significantly reduced when higher concentration of low molecular weight emulsifiers was used. Pelan et al. (1997) reported poor melting resistance with ice cream without emulsifiers due to the lack of fat network holding the aerated emulsion system. The same authors also demonstrated improved melting resistance with ice cream with the addition of monoglycerides. This was believed to be due to the semi-coalescence fat that holds the structure once the ice has melted, thus retarding drainage of the liquid phase from within the foam structure. This was confirmed with the analysis of the collected serum containing hardly any fat.

Méndez-Velasco & Goff (2012) demonstrated the reduction of ice cream stability as the percentage of palm kernel oil use was decreased. This is because the reduction causes a decrease in the network of partially coalesced fat holding the ice cream structure together. The D(4,3) was highly influencing the percentage of mass loss of ice cream samples. This is
apparent because the larger aggregates by the partially coalesced fat protect the lamellae between the air bubbles more efficiently than individual fat globules. The mechanisms of protection by the fat network have been comprehensively explained by Koxholt et al. (1999; 2001). The agglomerated fat globules grew in sizes thereby increasing their effectiveness to block and hinder the serum drainage. Furthermore, these fats form a network by forming loose bridges between the air bubbles which exhibit a mousse-like foam structure.

It has been discussed that the partial coalescence of the fat globules and the formation of a continuous fat network stabilises the air bubbles introduced during whipping (Allen et al., 2006; Goff, 2008). Furthermore, in ice cream, this will contribute to the ‘dryness’ of the yielded ice cream at extrusion (Goff, 2008). Granger et al. (2005) found that the presence of partially unsaturated mono- and di-glyceride causes a significant delay in the melting rate.

Figure 9.17: Melt stability of ice cream samples at 21°C
Fat aggregation was reported to contribute to the development of the complex structural network surrounding the air bubbles of an ice cream hence resulting in resistance to melt down rate. Abd El-Rahman et al. (1997) also found better melt resistance in ice cream made with emulsifiers compared to without emulsifiers regardless of the milk fat fraction used i.e cream, anhydrous milk fat, low melting milk fat fraction, and a very high melting milk fat fraction.

### 9.4.2.2 Determination of hardness and tackiness of ice cream

Figure 9.18 shows the firmness of ice cream, as indicated by the peak positive force recorded to achieve the indentation by the plunger. It can be observed that ice cream made with low fat (5 %) and no emulsifier was the hardest followed by 5 % fat and *R. miehei* lipase 2.5 mg / g fat with the recorded force at 49.6 and 47 N respectively. The addition of 0.3 % monoglycerides in 5 % fat ice cream had significantly lowered the hardness of the ice cream. High fat ice cream (10 %) with no emulsifier was also found to be less hard. This is in agreement with the finding of Guinard et al. (1996) and El-Nagar et al. (2002) that demonstrated the presence of higher fat content in ice cream inversely affect the hardness.

The presence of 0.3 % monoglycerides in low fat (5 %) ice cream was found to also decrease the hardness of ice cream. Ice cream that was lipolysed with *R. miehei* lipase 7.5 mg / g fat was found to have the lowest hardness.

The tackiness of ice cream expressed as the peak negative force obtained during the plunger withdrawal is shown in Figure 9.19. The results depict that the ice cream made with low fat (5 %) and without any added emulsifier was the least adhesive or sticky compared to high fat (10 %) ice cream or ice cream made with the addition of monoglycerides or lipase. El-Nagar et al. (2002) produced high fat yoghurt ice cream that also had lower tackiness.
compared to the low fat version. The hardness and tackiness of ice cream is very much influenced by its internal structure. Lowering the fat content in ice cream will increase the growth of ice crystal, thus making the ice cream less sticky. Abd El-Rahman et al. (1997) reported a harder textured ice cream made with emulsifier compared to without. Ice cream containing emulsifier also had a drier appearance with a smoother texture. This may be due to the increased in partially coalesced fat in ice cream containing emulsifiers (Gelin et al., 1994).

Note:
- Different letters above the bars indicate statistical significance between the means (p<0.05)

**Figure 9.18: Hardness of ice cream samples**
9.4.2.3 Determination of droplet size distribution of ice cream

The droplet size distribution of the ice cream produced displayed a main peak distribution (Figure 9.20). However, all samples except ice cream made without emulsifiers and with R. miehei lipase 2.5 mg / g fat, had a second minor peak. Granger et al. (2005) also reported a two distinct population of droplet size distribution for their ice cream formulations with one major peak and another minor peak. It has been reported that the presence of the second minor peak is due to the partially coalesced fat droplets that was produced upon shearing during the processing of ice cream (Gelin et al., 1994; 1996; Goff, 1997b). The absence of the second peak with low fat ice cream made without emulsifier further reinforce the importance of the emulsifier in promoting the partial coalescence of fat. Lower concentration of R. miehei lipase (2.5 mg / g fat) was not able promote the destabilisation
of the fat droplets like the higher concentration of *R. miehei* lipase (7.5 and 15 mg / g fat). In an ice cream mix containing monoglycerides, Gelin et al. (1996) described the location of monoglycerides as being at the periphery of fat globules. These monoglycerides have effectively displaced protein and is on the primary layer on the interface. It is well known that emulsifiers in ice cream, such as mono- and di-glycerides (MDG), displace proteins from fat–serum interfaces during the freezing and aeration process resulting in promoting the partial coalescence of fat globules (Goff, 1997b; 2002; 2008) in ice cream.

![Droplet size distribution of ice cream](image)

**Figure 9.20: Droplet size distribution of ice cream**

Zhang & Goff (2004) investigated TEM images of ice cream made with the addition of mono- and di-glycerides. The images showed discontinued protein network and connection of fat globules forming a network of its own. These fat-fat connections were found to be also lacking of protein. These fat globules were found to attach on the air bubbles surfaces. Pelan
et al. (1997) reported the presence of large air bubbles (>100 μm) in ice cream without emulsifiers. The large air bubbles are due to smaller air bubbles coalesced together because of low stability and lack of fat globules on the interface. Presence of monoglycerides in the ice cream however leads to a smaller air cell with improved stability. The desorption of the protein by the monoglycerides cause the fat globules to be more hydrophobic and more inclined to adsorb on the air bubble interface.

9.5 Conclusion

In this chapter, the addition of *R. miehei* lipase at very low concentration of 5 mg / g fat was able to produce a rigid and stable whipped cream with overrun exceeding 100 %. However, good stability of the whipped cream over time was achievable with concentration above 10 mg / g fat. Similarly, ice cream made with the addition of 5 mg / g fat exhibited good melt stability and firmness. Commercially, ice cream and whipped cream used synthetic monoglycerides to promote partial coalescence of the fat phase in their emulsion base. Interestingly, the findings in this chapter pointed to the successful of the *in situ* produced polar lipids namely monoglycerides in contributing to the functionality as what commercial monoglycerides can achieve. This will offer an alternative to the commercially available monoglycerides that are produced synthetically. It was also shown that the incorporation of *R. miehei* lipase in the O/W emulsion needs not to be at high concentration to impart the desired functionality.
Chapter 10: Summary and Recommendation for Future Works

10.1 Summary

The research presented in this thesis has sought to answer the main research question of whether lipase enzyme can be used as a processing tool, in order to generate polar lipids, specifically monoglycerides, in situ of a food emulsion. This is in line with consumers’ demand for ‘clean’ label food containing all natural ingredients. Currently, commercial monoglycerides are produced synthetically either through glycerolysis or esterification, both of which involve harsh processing conditions, such as very high temperatures and the use of inorganic catalysts.

The first objective of this thesis was to investigate the competitive adsorption of R. miehei lipase with other emulsifiers on an oil-water interface, as described in Chapter 4. The IFT data were obtained using a tensiometer. Different types of vegetable oil and four types of emulsifiers namely sodium caseinate, whey protein isolate, lecithin and Tween 80 were used. These initial findings gave a fundamental insight on the feasibility of the main research idea. The IFT data obtained suggested that the R. miehei lipase was able to competitively adsorb on the oil-water interface although the adsorption of lipase was found to be dependent on the interfacial composition. Significant decreases in IFT with protein emulsifiers i.e. sodium caseinate and whey protein isolate suggested the lipase was able to displace these proteins. However, the displacement of low molecular weight emulsifiers i.e. lecithin and Tween 80, though achievable, was not as pronounced as their protein counterparts. Since various food emulsifiers are used in food emulsions, it is crucial that R. miehei lipase be able to adsorb on the interface for its catalytic reaction to take place. Although interfacial composition of food emulsions can be more complex in reality, the results obtained in this chapter showed the capability of lipase to co-adsorb on the oil-water interface, thus warranting more detailed
investigation. The IFT also provided an indication as to the time required for saturation of the interface in relation to the rate of polar lipid synthesis. The technique was also useful in demonstrating how thermally inactivated enzyme was still able to be adsorbed at the interface, but did not lead to any further IFT reduction due to the loss of hydrolytic activity.

The second objective was to investigate factors affecting the lipolysis of an oil-in-water (O/W) emulsion with *R. miehei* lipase. Canola oil and palm oil were used to produce the emulsions (Chapter 5). Palm oil was introduced to observe the behaviour of an emulsion made with a solid fat, thus in line with lipid composition required for aeratable emulsions. The first part of the chapter investigated the effect of Microfluidization and formulation on the lipid specific surface area of the emulsion. The specific surface area was deemed crucial since it determined the region available for lipase adsorption (thus providing a means for controlling rate and extent of lipolysis). Furthermore, maintaining similar specific surface area was going to be crucial when investigating the effects of lipase loading, oil type and mass, and emulsifier type on the lipolysis rate. The findings in this chapter showed that formulation (emulsifier type and oil content) and processing conditions (Microfluidizer® pressure and number of passes) had significant effects on the emulsion droplet size. The results showed that while Microfluidization was effective in producing stable emulsions with small droplet size, subjecting the emulsion to a more severe homogenisation (higher pressure and number of passes) did not necessarily produce a finer emulsion. After a certain pressure and number of passes are reached, ‘over-processing’ of the emulsion occurred in which droplet size was not reduced but instead started to increase due to re-coalescing. This is because high pressure might cause change to the molecular configuration of the emulsifier resulting in changes in adsorption efficiency. Thus, an ideal combination of pressure and number of passes is needed for a dynamic balance between the break-up of droplets and their re-coalescence in the
interacting chambers. Nevertheless, the effectiveness of Microfluidization can be disguised by the emulsion formulation that is type of emulsifier, and types and mass of oil used.

Several different factors affecting lipolysis were then investigated in this chapter. The O/W emulsions were prepared to the desired specific surface area based on the results from the microfluidization trials. Generally, a larger specific surface area resulted in a higher lipolysis rate across all emulsions tested. Because lipase is an interfacial enzyme, the specific surface area corresponds to the binding sites available for lipase. The results also showed that a higher lipolysis rate was obtained with protein emulsifiers compared to that obtained with low molecular weight surfactant, which corresponds to the IFT data in Chapter 4. Lipolysis substrate (being either liquid or solid oil) affected lipolysis as did the lipase concentration. The results of this chapter demonstrated that lipolysis can be controlled by manipulating the composition of the emulsion and that no significant inhibition of lipolysis occurred with the different emulsifiers used.

Having demonstrated lipolysis of an O/W emulsion, it was important to be able to terminate the enzymatic reaction under controlled conditions, in order to be able synthesise appropriate levels of polar lipids for emulsion functionality. Thermal deactivation of the *R. miehei* lipase was investigated, as this could be incorporated as part of existing thermal processing treatments (e.g. pasteurisation, sterilisation) already widely used in food emulsion manufacture (Chapter 6). The findings showed that *R. miehei* lipase had significant decrease in its lipolytic activity from temperatures of 70 °C and above. Half-lives ($t_{1/2}$) of the enzyme at 90 °C and 100 °C were found to be 1.1 and 0.8 min respectively. Results showed that the enzyme did not deactivate instantly even at high temperature. However, results for the 100 °C $t_{1/2}$ might actually be considerably lower than the 0.8 min due to limited number
of data points (where data collection was done every minute). The thermal stability of an O/W emulsion itself under these conditions was also examined by evaluating the changes in emulsion droplet size. A palm oil (30 %) emulsion stabilised with sodium caseinate was used. D(4,3) of the emulsion showed significant changes to droplet size only after prolonged heating times of 15 and 30 min at temperatures above 70 °C. In fact, D(4,3) of emulsion heated at 100 °C showed an incremental change only after 15 min. This showed that the emulsion was able to maintain stability through heat treatment without significant changes to its droplet profile when heated for a shorter period of time of less than 15 min. Similar thermostability was also observed for lipolysed emulsions that were able to withstand heating up to 100 °C for 7 min before significant (p<0.05) changes to droplet size were recorded.

Chapter 7 examined the shear-induced aggregation of the lipolysed emulsion as well as the different factors affecting the process. Palm oil (30 %) emulsion stabilised with sodium caseinate was again used. The lipolysis of O/W emulsion was assumed to produce monoglycerides and fatty acids. It was predicted that these polar lipids would displace the existing sodium caseinate, subsequently promoting partial coalescence of the fat globules. Therefore, lipolysed O/W emulsion was subjected to shear to induce the partial coalescence in the partially crystalline O/W emulsion. The most interesting finding was that non-lipolysed O/W emulsion failed to aggregate upon shear being applied compared to the lipolysed emulsion, and that the propensity towards aggregation could be manipulated through control of reaction conditions, such as enzyme concentration and reaction time. In this chapter, the ability to tailor the functionality of the lipolysed emulsion was demonstrated. The shear-induced partial coalescence of the lipolysed sample can accordingly be related to the same partial coalescence obtained in products like ice cream and whipped topping,
where commercial low molecular weight emulsifiers such as monoglycerides are often added to achieve similar results.

Quantification of the polar lipids generated by lipolysing 30 % palm oil-in-water emulsion was carried out in Chapter 8. This provides a value to how much of each component; fatty acids, monoglycerides and diglycerides were being produced. Taking the example of an O/W emulsion lipolysed with 50 mg \textit{R. miehei} lipase / g fat for 15 min, it was calculated that the total monoglycerides and diglycerides produced were 10.373 ± 3.904 and 5.578 ± 1.586 mg per g of fat, with the total being ~16 mg / g fat. The values would correspond to ~0.48 % of total emulsion. This is within the range of 0.1 - 0.6 % of monoglycerides usually added to food.

Finally, the application of \textit{R. miehei} lipase in a commercially relevant food emulsion products was carried out, using whipped cream and ice cream as case studies (Chapter 9). Interestingly, non-lipolysed O/W emulsion failed to produce whipped cream compared to the lipolysed emulsion. Different concentrations of lipase were used to determine the threshold at which the functionality of the generated polar lipids could still be achieved. Emulsions lipolysed with \textit{R. miehei} lipase at concentrations of more than 1.25 mg / g fat, all produced whipped cream with more than 100 % overrun. However, stability of the whipped cream was found to be enhanced for samples lipolysed with lipase concentration of more than 10 mg / g fat. Lipolysis also had a positive effect on the structure and properties of ice cream comparable to ice cream made with commercial monoglycerides. Results from this chapter suggest that utilising lipase at around 10 mg / g fat is adequate to provide functionality in the emulsion tested. Comparing this with the quantification data obtained in Chapter 8, the total monoglycerides and diglycerides would account to ~0.1 % (w/w). Nevertheless, significant
functionality was still achieved. One reason is that the generated fatty acids, although not as polar as their monoglycerides counterparts, also exhibit surface activity (Armand et al., 1996; Singh et al., 2009). Thus, if the fatty acids, and monoglycerides were to combine together, the amount of polar lipid generated would be much higher. Another interesting finding was the changes to the surface of the fat globules after lipolysis. Micrographs of fat globules lipolysed by *R. miehei* lipase showed change from a smooth to a jagged and rough surface. These fat crystals protuberances might have been enhanced as a consequence of lipolysis, thus contributing to partial coalescence.

### 10.2 Recommendations for future work

Although the work of this thesis demonstrated the successful application of lipase to generate polar lipids *in situ* of a food emulsion, several knowledge gaps and opportunities were recognised and warrant further investigation:

1. In this work, the successful adsorption of *R. miehei* lipase was initially associated with the decrease in the IFT value, and subsequently by pH-stat and quantification of the lipolysis product using GC analysis. However, the amount of protein emulsifiers on the surface as well as the rate and amount of displacement was not quantified. Thus, the quantification of surface loading of the O/W emulsion may give valuable insight on the displacement of the protein emulsifiers as a result of lipolysis.

2. Quantification of lipolysed O/W emulsion had shown both 1-monoglyceride and 2-monoglyceride were detected. However, because *R. miehei* lipase is an sn-1,3-specific lipase, the 1-monoglyceride detected was as the result of acyl migration from 2-monoglyceride. It might be interesting to further investigate what factors
influence the rate of acyl migration and what difference it makes to the interfacial composition.

3. Successful application of *R. miehei* lipase in a food product was shown in whipped cream and ice cream. However, real food systems are actually very complex in their formulations and their processing. Hence, process optimisation could be used to implement the lipolysis process in a food product. Furthermore, the thesis began (in Chapter 4 and 5) by examining the effects of different types of emulsifiers i.e. sodium caseinate, whey protein isolate, lecithin and Tween 80. However, as work progressed in this thesis, only O/W emulsion stabilised with sodium caseinate was used. Further investigation of the shear aggregation and functionality of lipolysed emulsions using the other types of emulsifiers should be carried out. Effect of factors such as changes in pH, ionic strength and temperature on the displacement of the emulsifiers should be analysed. Furthermore, this technology might be used in the manufacture of other emulsion based foods using emulsifiers, e.g. spreads.

4. The work in this thesis was concerned with the fatty acids generated from the lipolysis reaction. It is well-known that fatty acids, especially shorter chain fatty acids, may result in off-flavour. This is why the oil of choice in this thesis was palm oil, which predominantly contains longer chain fatty acids. Although the functionality of *R. miehei* lipase was successfully demonstrated in whipped cream and ice cream, it will be interesting to ascertain the sensory profile of the resulting products. Sensory evaluation should give information on the impact of the fatty acids generated, as well as whether the attributes such as creaminess and texture are affected. This is particularly important when applying this approach to alternative fat types with
different triglyceride profiles. A wider screening of lipases might also be useful. While the work carried out in this thesis was achievable with *R. miehei* lipase used, there may be opportunities to improve yield, or manipulate the polar lipid fractions generated through the use of other lipases.

### 10.3 Concluding remarks

The work in this thesis has proven that *R. miehei* lipase can competitively adsorb to a protein-stabilised interface. The adsorption of lipase catalysed the hydrolysis of the triglyceride in the oil droplets into polar lipids, namely monoglycerides and fatty acids. By manipulating factors such as lipid specific surface area, emulsifier type, oil mass and lipase concentration, the extent of lipolysis can be controlled. Lipolysed O/W emulsions were also showed to be sensitive to shear-induced aggregation, as a result of protein emulsifier displacement by the generated polar lipid. Therefore, when used in food products such as whipped cream and ice cream, the use of *R. miehei* lipase can be substituted for commercial synthetic monoglycerides to achieve the desired controlled destabilisation of the emulsion.
References


Advances in Colloid and Interface Science, 100-102, 503-546.

Baer, R. J., Wolkow, M. D., & Kasperson, K. M. (1997). Effect of emulsifiers on the body and 

enrichment of γ-linolenic acid from evening primrose oil in a solvent-free system. 
Journal of the American Oil Chemists’ Society, 91(7), 1147-1153.

gene expression of organic solvent-tolerant lipase from Pseudomonas aeruginosa 

Advances, 16(2), 309-341.

Amsterdam: Elsevier Science B.V.

fat interaction in ice cream mix during ageing I: quantitative analyses. Lipid / Fett, 
93(1), 24-29.

Basiron, Y. (2005). Palm oil In Shahidi, F. (Ed.), Bailey’s Industrial Oil and Fat Products, Sixth 

deactivation energy of free and immobilized invertase. Brazilian Journal of Chemical 
Engineering 17: 867-872.

compounds by lipase-catalyzed hydrolysis and esterification reactions. Journal of 
Surfactants and Detergents 1(3): 343-344.

parameters of whipped dairy emulsions containing various fatty acid compositions. 
Journal of Dairy Science, 92(8), 3566-3574.


thermodynamic modeling to enhance selectivity during monoglyceride synthesis by 
lipase-catalyzed esterification. Enzyme and Microbial Technology, 28(4-5), 362-369.

and pH as factors influencing droplet size distribution and linear viscoelasticity of 
O/W emulsions stabilised by soy and gluten proteins. Food Hydrocolloids, 24(8), 783-
791.

Trends in Biotechnology, 9(10), 360-363.
References


References


Lundin, J. (2013). *Investigation of how different fat systems and other ingredients affect the properties of whipping creams based on vegetable fat*. Linnaeus University, Sweeden.


References


kinetics of a non-regioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS 8. *New Biotechnology, 28*(6), 738-745.


References


References


